Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC fingerprinting†

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Small organic molecules found on the skin of amphibians may help impart resistance to pathogens, such as the lethal fungus *Batrachochytrium dendrobatidis*. The study of these compounds has traditionally required euthanasia of the amphibian, followed by chemical extraction of excised skin. As an alternative method, we report the development and assessment of a non-lethal technique using foam-tipped swabs and HPLC analysis to directly isolate and characterize small molecules found on the skin of amphibians. This protocol was field-tested on Bioko Island, Equatorial Guinea with forty-seven frogs (representing 14 native species). Multiple species (particularly *Afrixalus paradorsalis* and *Didynamipus sjostedti*) carried sets of species-specific compounds (*i.e.*, a chromatographic fingerprint). A principal coordinate analysis (PCO) of the commonly occurring compounds detected across all species revealed a significant relationship between chromatographic profile and species for all swab samples.

1 Introduction

The mucous layer on amphibian skin is a rich source of bioactive compounds that have been implicated as part of the animal's defense against pathogens such as the fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus.[1–3] Examples of these protective compounds include alkaloids and antimicrobial peptides (AMPs) of amphibian origin, as well as small molecules produced by symbiotic, cutaneous bacteria.[4,5] Specifically, the bacterially-produced, antifungal compounds violacein and indole-3-carboxaldehyde have been detected on amphibian skin and at concentrations inhibitory to *Bd*.[4,6,7] The diversity of cutaneous, antifungal bacteria known to live on amphibians[8,9] suggests that these examples are but a small fraction of the total secondary metabolite pool that plays a role in amphibian defenses against pathogens.

AMPs and alkaloids found in the mucous layer on amphibian skin have been successfully isolated by bathing or rinsing a specimen in a collection buffer that extracts the compound of interest.[10,11] However, less water-soluble compounds are not amenable to this aqueous washing method, and have traditionally been isolated *via* the chemical extraction of excised skins from sacrificed amphibians.[4,6,12]

Unfortunately, this lethal method is not suitable for the fast and efficient sampling of a large number of specimens in the field, requires extensive handling (and potential contamination) of the tissue prior to chemical extraction, precludes the potential for time-resolved studies of amphibian cutaneous natural products, and is less ideal for those species facing extinction.

Well-known practices exist for the non-lethal collection of *microbial* samples from the surface of animals using swabs.[13] Less common are methods for similarly and unobtrusively collecting *chemical* samples from skin surfaces, although the idea has been explored.[10,14–19] Generally in these chemical examples, a collection vehicle is used to adsorb the target analytes from the surface, with subsequent chemical extraction, chromatographic analysis, and/or detection.

Inspired by the above biological and chemical methods, we present herein the development of a swab-based protocol for collecting and analyzing the diversity of small molecules found on amphibian skin. Once developed, we field-tested this technique for collecting cutaneous natural products from frogs on Bioko Island, Equatorial Guinea. Bioko is species-rich in comparison to other islands, making it an ideal location for a field-test of our newly-developed swabbing methodology. These preliminary results not only demonstrate the use of this new swabbing methodology but also show promise that chromatographic fingerprints can be used to identify frog species, irrespective of sampling location.

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2 Methods and materials

Ethics statement

This work was conducted under appropriate permits from Universidad Nacional de Guinea Ecuatorial and approved by the Institutional Animal Care and Use Committee (IACUC) at Drexel University. All amphibians were collected and handled following Drexel IACUC Protocol #18748 and #20182.

Materials

Six swab types representing different tip materials were tested: polyurethane foam swabs from Fisher Scientific (cat no. 14-960-3J); Dryswab™ Standard polyester (Dacron) and Dryswab™ Fine Tip (rayon) swabs, both from Medical Wire & Equipment, Wiltshire, England; and cotton, PurFlock® (nylon), and HydraFlock® (nylon) swabs, all from Puritan Medical Products, Guilford, Maine. For actual metabolite collection, polyurethane swabs were pretreated with methanol as described below. HPLC-grade solvents (methanol, water, and acetonitrile) were obtained from Fisher Scientific. Formic acid (>96%, ACS Reagent), violacein, and quinine hydrochloride were obtained from Sigma-Aldrich. 2,4-Diacetylphloroglucinol was purchased from Chem-Impex International. Phenazine-1-carboxylic acid was purchased from Princeton Biomolecular Research. All chemicals were used as received.

Preparation of foam swabs

Polyurethane foam swabs were pretreated to remove methanol-soluble impurities prior to use in small molecule collection in the field. Accordingly, each swab was placed in a beaker containing enough methanol to cover the foam tip. The swabs were manually stirred in this wash methanol for one minute, then the methanol was discarded. This washing procedure was repeated once. The swabs were then inverted, placed in a short Erlenmeyer flask (to preclude any contact with the swab tips), and left to air-dry overnight in a closed fume hood.

LCMS analysis of small molecule release efficiency from swabs

Swab tips representing six different tip materials were loaded with a standard solution (10–15 μL in 30 : 70 acetonitrile : water) of representative natural product standards: quinine (as HCl salt), violacein, phenazine-1-carboxylic acid, and 2,4-diacetyl-phloroglucinol. Under the LC conditions used, the retention times of these compounds are 9.61, 14.53, 15.98 and 17.43 min, respectively. Standard solutions were applied using a microliter pipette. The swab tips were cut off, placed in 2 mL micro-centrifuge tubes, and allowed to air-dry overnight. Once dry, the tips were extracted with methanol (0.5 mL) as described below. The methanolic extracts were evaporated to dryness *in vacuo*, reconstituted in 50 μL 30 : 70 acetonitrile : water, and analyzed by LCMS to determine the percent recovery of each compound. Separation and analysis was accomplished with either an Agilent 1290 Infinity liquid chromatograph [equipped with an Agilent Eclipse Plus C18 column (1.8 μm, 2.1 × 100 mm, 50 °C) and an Agilent 6530 Accurate-Mass Q-TOF operating in negative mode]

or the Shimadzu LC-20 liquid chromatograph described below. Authentic standards were used to generate a calibration curve and thus quantify concentrations recovered from swab samples.

Comparing swab-based small molecule collection to conventional skin extractions

Thirteen frogs were acquired from Carolina Biological Supply Company (1 *Xenopus laevis*, 4 *Hyla cinerea*, and 8 *Rana spheno-cephala*) and used to compare conventional skin extractions to swabs for the isolation of natural products. Frogs were stored in lab aquariums, separated by species, and acclimated at room temperature for 24 hours after being acquired. Each of the 13 specimens were swabbed using washed, polyurethane foam-tipped swabs. Frogs were swabbed across all ventral and dorsal surfaces 3–5 times depending on body size (including legs, feet/webbing, and pelvic "drink patch") with a total time of swabbing lasting 20–30 seconds per individual.[13] Swab tips were immediately placed in a dry 1.5 mL sterile tube, the excess swab stalk cut away by scissors, and the tube immediately capped and frozen. Swabbed frogs were isolated in order to pair swab samples with excised skin samples from the same specimen. All frogs were then euthanized individually *via* immersion in an aqueous solution of tricaine methanesulfonate (250 mg L^{-1}) following American Veterinary Medical Association guidelines for amphibian euthanasia, also included in the relevant IACUC protocols for this study. All skin was excised from the dorsal and ventral surfaces of the torso above the hind legs and below the front legs, removed in one unbroken "sheet", and placed immediately into separate sterile 15 mL Falcon tubes. Tissue sizes ranged in area from approximately 2 cm^2 (*Hyla cinerea*) to 54 cm^2 (*Xenopus laevis*). Bench surfaces and instruments were sterilized between each excision. To extract compounds, each skin was vortexed four times with 5 mL HPLC-grade methanol. The methanol extracts were combined and filtered using 13 mm syringe filters (0.2 μm PTFE membrane, VWR) to remove any insoluble environmental material. [Before use, syringes (1 mL HSW Norm-Ject® disposable syringe) and filters were pre-washed by taking up 1.0 mL of methanol in the syringe and slowly passing it through the filter.] Filtered extracts were evaporated to dryness *in vacuo*, reconstituted with 250 μL methanol (containing 1 ppm naphthalene as internal standard), and analyzed by HPLC (see below).

Swabbing of Bioko frogs

Frog specimens were collected at night by hand from four different locations on Bioko Island. A total of 47 specimens were collected for bacterial and small molecule swabbing across all sites. Except in instances where a voucher specimen was taken, all individuals were swabbed and released in good condition to the site of collection within 24 hours of capture. Medical-grade nitrile gloves were used during each capture to avoid contamination. After capture, each frog was placed individually in a sterile plastic bag that was sealed until the individual was swabbed. Frogs were stored at ambient temperature overnight, and processed in order of collection the following morning. All swabbing took place in a controlled environment

(Moka Wildlife Center) to reduce potential contaminants from the field. Each of the 47 specimens was swabbed for both bacteria (unpublished results) and cutaneous compounds using two separate, sterile swabs. For small molecule collection, pre-treated polyurethane-tipped swabs were used as described above. The type of swab used first for each specimen (bacteria vs. small molecule) was alternated between each individual from any given collection event to avoid potential swab-bias on small-bodied species. All swabs were kept frozen until extraction. Swab samples were pre-labeled with only the specimen field ID number to ensure that lab analyses were blind to any information relating to species and/or collection site. Frog cataloging information and a description of the sampling sites may be found in the ESI.†

General procedure for extraction of small molecules from swabs

For extraction, 1.0 mL of methanol was added to each swab in its centrifuge tube. The tubes were capped and vortexed for 5 seconds, allowed to sit for 10 minutes, and then vortexed a second time. The swab tip was then removed using forceps, taking care to squeeze out any methanol adsorbed in the porous swab on the inside wall of the centrifuge tube. This methanolic extract was then slowly filtered into another centrifuge tube using 13 mm syringe filters (0.2 μm PTFE membrane), as described above. Filtered extracts were evaporated in vacuo using a DNA120 SpeedVac with the heating function turned off. Dried swab extracts were reconstituted in 100 μL of methanol containing 1 ppm naphthalene (as internal standard), vortexed, and analyzed by HPLC.

HPLC analysis of extracted small molecules

The reconstituted skin and swab extracts were analyzed by reversed-phase, high performance liquid chromatography (HPLC, 25 μL injection) using a Shimadzu LC-20 liquid chromatograph equipped with an ACE C18 column (3 μm, 150 × 4.6 mm), a Shimadzu SPD-M20A diode array detector, and an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer (operating in positive electrospray ionization mode). Compounds were separated with a binary mobile phase flowing at 0.5 mL min⁻¹ consisting of acidified water (0.1% formic acid, v/v; solvent A) and acidified acetonitrile (0.1% formic acid, v/v; solvent B). The gradient was as follows: 10% B (2 min hold) ramped to a final mobile phase concentration of 100% B over 18 minutes (5 min hold). Compounds that eluted from samples were characterized by retention time and, where applicable, characteristic UV-Vis chromophores (λ_{max}) and positively-charged ions. Total Wavelength Chromatograms (TWC, 200–700 nm) of field samples were compared against the TWC of extractions of unused, washed swabs (controls). The TWC was used rather than the mass spectrometer's Total Ion Chromatogram (TIC) because peaks were more reproducibly present in the TWC upon reinjection. Methanol "blank" injections were inserted into the HPLC queue after every four swab samples to ensure that there was no inter-sample contamination.

Statistical analysis

All statistical analyses utilized R version 2.11.1. Variation in detected compounds (presence/absence) among different amphibian species was assessed with Principal Coordinate Analysis (PCO) using the dsvdis() function in the labdsv package with Steinhaus index. Variation in detected compounds (presence/absence) among different amphibian species was tested for significance using the adonis() function in the vegan package. The adonis() function is an analysis of variance that can be used with distance matrices. This function uses a permutation approach that generates a distribution against which observed distances can be compared. The method allows a partitioning of sources of variation (amphibian species). Here, the number of permutations was set at 1000 with all other arguments at default values as set in the function.

3 Results

Selection and preparation of swabs

A primary concern for developing this protocol was the selection of the swab tip material. An ideal swab should not only be effective in removing the metabolite-rich mucus from an amphibian specimen's skin but also able to release the collected compounds efficiently upon chemical extraction. In addition, the swab should be relatively clean of chemical contaminants that would be extracted along with the desired compounds and interfere with detection. It was quickly recognized that traditional cotton swabs failed due to poor extraction of swab-bound compounds. In total, six commercially available swabs with varying tip materials were tested (cotton, rayon, Dacron (polyester), polyurethane foam, and two flocked types of nylon: HydraFlock® and PurFlock®).

We tested the ability of various swab materials to release adsorbed small molecules by using a set of structurally-diverse natural products: violacein, phenazine-1-carboxylic acid (tubermycin B), and 2,4-diacetylphloroglucinol (2,4-DAPG). These compounds have a variety of functional groups and a range of polarities, and (as a practical matter) they are examples of the antifungal, bacterially-produced small molecules that are the primary target of this sampling methodology. Compared to the other swabs tested, the polyurethane foam-tipped swabs proved to be preferable for releasing all three of the compounds (Table 1). In addition, methanol proved superior to other tested extractions using water or ethyl acetate. Finally, we investigated the ability of the polyurethane swabs to release the basic alkaloid quinine (applied as its HCl salt) in order to determine if methanol would successfully extract a cationic natural product. The observed percent recovery of quinine was 63.8 ± 3.7% (average ± standard deviation).

Although the polyurethane swab tips were preferred for releasing adsorbed small molecules, control extractions showed that they unfortunately released a number of impurities during methanol extraction. Although these chemical interferences have not been identified, we suspect that they may be derived from such sources as the adhesive used to bind the foam tip to the swab's polypropylene handle and any plasticizers contained

Table 1 Percent recovery ± standard deviation of common secondary metabolites extracted from the various investigated swab materials. All tests were run in triplicate

		Violacein	Phenazine-1-carboxylic acid	2,4-DAPG	Quinine
Swab type	Cotton	2.1 ± 1.8	6.6 ± 1.0	16.8 ± 5.9	n.t.
	Rayon	4.0 ± 1.6	17.8 ± 0.6	50.0 ± 1.4	n.t
	Dacron	6.9 ± 6.7	4.3 ± 0.4	25.7^a	n.t
	Polyurethane foam	12.7 ± 4.5	61.2 ± 4.1	36.2 ± 0.9	63.8 ± 3.7
	PurFlock	0.4 ± 0.1	3.1 ± 0.3	22.0 ± 2.6	n.t.
	HydraFlock	0.4 ± 0.2	10.3 ± 0.3	24.3 ± 0.3	n.t.

^{a} One replicate of 2,4-DAPG/Dacron (59% recovery) was not included in the average percent recovery as it was deemed an outlier and only the average of two trials is reported. n.t., not tested.

within the foam or handle. When swab extracts were analyzed by HPLC with UV-Vis detection, these impurities gave signals that were at least two orders of magnitude larger than those of the compounds of interest. This complication was avoided by prewashing the swabs prior to use for natural product collection. The polyurethane foam-tipped swabs were washed twice with methanol before use, essentially subjecting them to a "pre-extraction" that removed methanol-soluble impurities before the swabs were used (Fig. 1). The pretreatment procedure did not visibly appear to alter the swab's material properties or utility in collecting compounds from amphibians in the field.

Comparison of swab protocol to conventional skin extractions

The swabbing protocol was compared against a conventional skin-extraction procedure for small molecule isolation and detection. Thirteen frogs (1 *Xenopus laevis*, 4 *Hyla cinerea*, and 8 *Rana sphenocephala*) were swabbed with pretreated polyurethane foam swabs to collect their secondary small molecule-rich mucous. After swabbing, the specimens were euthanized and subsequently skinned. Excised skins were immediately extracted with methanol.

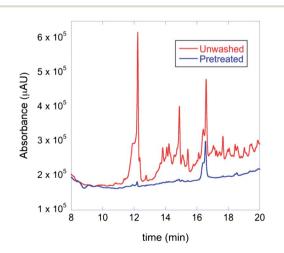Extracts from the new swab and conventional skin methods were analyzed by HPLC (with both UV-Vis and MS detection) to

Fig. 1 HPLC chromatogram (220 nm) of unused, "control" polyurethane swabs. Pretreatment of the swabs removes many swab-derived contaminants.

compare the methods and chromatographic profiles. Naphthalene (1 ppm) was included as an internal standard in all HPLC injections. It was chosen because it is highly nonpolar (with a late retention time that does not obscure the detection of slightly more polar natural products), has a distinct UV-Vis chromophore, and can be readily removed from samples *in vacuo.* The inclusion of naphthalene as an internal standard allowed us to quantify the precision of the HPLC analysis. Over 26 injections, the naphthalene peak area varied no more than 11%, and its retention time was consistent within 0.02 min.

The total number of small molecules detected by both methods was essentially the same and varied little according to species of frog. The average number of compounds detected in a single swab extract was 50 ± 7 (mean ± standard deviation), while each skin extract showed on average 51 ± 11 compounds. Many, although not all, of the same chromatographic features were detected by both swabbing or skin extraction methods. Comparing the two chromatographic profiles collected for a given frog (see ESI†), we observed that 21 ± 6% of the compounds detected in skin extracts were similarly found in the swab extracts. For example, a compound eluting at 8.99 min with a characteristic UV chromophore (279 nm) and parent ion (*m/z* 205) was detected on a *H. cinerea* specimen by both swabbing and skin extraction methods; this compound was not detected in controls. This compound was later identified as tryptophan by comparison to an authentic standard.

Field test of swabbing protocol

Swabs from 47 frogs representing 14 species sampled on Bioko Island were processed to extract isolated cutaneous compounds and subsequently analyzed by HPLC. Each of the swab extracts showed between one and 28 distinct chemical compounds derived from the frog specimen. In total, 124 distinct compounds were detected from the 47 swab extracts analyzed. Of these, 66 compounds were found on three or more frogs. For this initial investigation, our analysis only noted the presence or absence of a given feature, and the identities of most compounds remain unknown at this time. The 66 commonly detected compounds (with UV-Vis and mass spectral characterization) are tabulated in Table 2; a complete table of detected compounds appears in the ESI.†

Interestingly, we found that certain frogs carried specific sets of compounds, observed as a chromatographic fingerprint. All of the *Afrixalus paradorsalis* specimens ($N = 5$) carried compounds with retention times of 12.18, 12.25, 13.94, 14.11, 14.50, 14.63, and 15.13 min (Fig. 2). Although some of these features are shared by other species, no species consistently carried this exact cluster of seven features other than *A. paradorsalis*. Additionally, each of these key *A. paradorsalis* compounds were found to have a distinct UV-Vis chromophore consisting of two λ_{max} absorbances at 288 and 280 nm with a shoulder at 272 nm (ESI†). This common chromophore bears striking resemblance to that of the characteristic indole chromophore[20] and could suggest some structural similarity between these species-specific compounds.

Similarly, all *Didynamipus sjostedti* specimens ($N = 5$) carried compounds that elute at 10.25, 10.69, 10.82, 11.16, 11.32, 11.39, 11.60, and 11.99 min (Fig. 3). Although the quantity of each detected compound varied from frog to frog, no other species consistently carried all eight of these compounds. Moreover, these eight compounds all bear a UV-Vis chromophore (λ_{max}) at approximately 300 nm (ESI†). In sum, certain species appear to have these chromatographic fingerprints. Although not all species carried clear fingerprints, the *A. paradorsalis* and *D. sjostedti* results prompted us to pursue more quantitative analyses of the relationships between features observed by HPLC and other specimen factors.

Principal coordinate analysis (PCO) and adonis

A PCO using the 66 compounds occurring three times or more showed different distribution patterns in different species of amphibians (Fig. 4). Only those frogs belonging to a species that was sampled more than once were included in the analysis. There was a significant variation in cutaneous compounds among amphibian species (adonis analysis: $R^2 = 0.78$; $F_{10,42} = 11.23$; $P < 0.001$). The cutaneous compounds from *Afrixalus paradorsalis* (Apar) and *Didynamipus sjostedti* (Dsjo) had a different factor score on the first and second principal coordinate axes compared to other amphibian species. The cutaneous compounds from *Afrixalus paradorsalis* (Apar), *Arthroleptis*
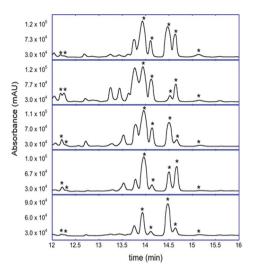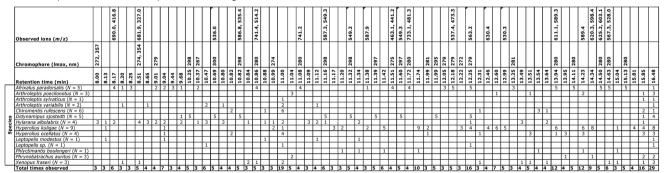

Fig. 2 Comparisons of total wavelength chromatograms of small molecule extracts from all *A. paradorsalis* specimens. Fingerprint compounds in each are starred (*).

variabilis (Avar), *Chiromentis rufescens* (Cruf), *Didynamipus sjostedti* (Dsjo), *Hylarana albolabris* (Halb), and *Xenopus fraseri* (Xfra) were separated from the cutaneous compounds in the other four amphibian species along the second principal coordinate axis. The variation explained by PCO1 and PCO2 is 15.7% and 14.9% respectively.

4 Discussion

The results above demonstrate the use of polyurethane foam-tipped swabs to gently collect small molecules from the mucous layer on amphibian skins. This mucus contains a variety of compounds including polar antimicrobial peptides and less-polar small molecules of amphibian, bacterial, and perhaps fungal origin. Given the nature of the reversed-phase chromatographic analysis used, highly polar compounds such as peptides and proteins are not retained by the C18 column, elute

Table 2 Summary of 66 compounds detected 3 or more times, indicating the number of times a given compound was detected on a member of a given species. $N =$ the number of frogs of a given species that were sampled. Where possible, each compound was characterized by retention time, chromophore and observed positive ions (highest and/or most intense m/z)
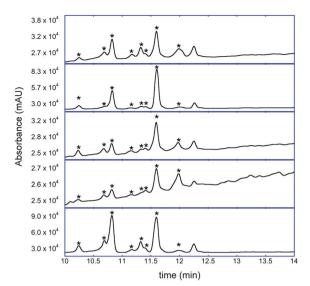
Fig. 3 Comparisons of total wavelength chromatograms of small molecule extracts from all *D. sjostedti* specimens. Fingerprint compounds in each are starred (*).

with the solvent front, and are thus undetected by this method. We therefore hypothesize that the compounds detected here are small, secondary metabolites from the frogs' skins. These compounds are largely unknowns at the present time; however, further and ongoing analysis of key compounds using tools such as MS/MS will assist in chemical identification.
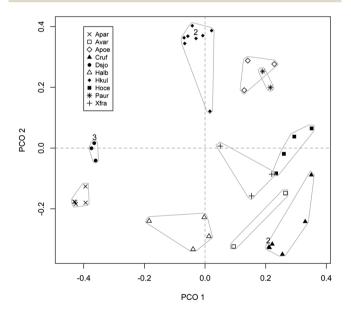

Fig. 4 Principal coordinate analysis plot of cutaneous metabolites from 43 amphibians across 10 different species from Bioko Island. *Afrixalus paradorsalis* (Apar), *Arthroleptis variabilis* (Avar), *Arthroleptis poecilonotus* (Apoe), *Chiromentis rufescens* (Cruf), *Didynamipus sjostedti* (Dsjo), *Hylarana albolabris* (Halb), *Hyperolius kuligae** (Hkul), *Hyperolius ocellatus** (Hoce), *Phrynobatrachus auritus* (Paur), and *Xenopus fraseri* (Xfra). Numbers placed above multiple samples with overlapping points. Polygons has been added to emphasize members of the same species. Asterisks (*) denote species whose samples were collected at two different sites.

A head-to-head comparison of this swabbing methodology to the conventional, lethal skin extraction procedure shows that the swab protocol allows the detection of a statistically similar number of small molecules, although only a small number (21%) are commonly detected by both methods. We believe this is a result of the different small molecule "pools" from which the two methods (*i.e.*, total skin *versus* swab/mucous) sample and actually demonstrates the ability of the swab to target only those compounds found on the exterior of living amphibian's skin. By its very nature, chemical extractions of excised skin can introduce a number of compounds from the internal side of the excised skin and pigments directly extracted from the tissue. For example, in all 13 skin extracts collected from our "head-to-head" control experiment, the amino acid tryptophan was detected (identified by its retention time, chromophore, and parent ion compared to an authentic standard). It is perhaps unsurprising that a common amino acid would be detected in a chemical extraction of a whole tissue. However, only two of the swab samples from the same study (tree frogs with total skin areas <4 cm^2) showed the presence of tryptophan. This suggests that tryptophan is only found on the *external* skin surface for those two frogs and that the more traditional skin extractions might have led to an over-estimation of the ubiquity of tryptophan. In other, unpublished studies, we have detected compounds in whole skin extracts bearing the characteristic Soret absorbance of the heme prosthetic group[21] (suggesting blood-derived components). Furthermore, we note that skin extracts often take on a bright yellow or green color (potentially skin pigments). Both of these latter observations again suggest the strength of a collection method targeting only those compounds on the external skin surface. Finally, the euthanasia procedure itself also risks introducing contaminants, as evidenced by the detection of tricaine methanesulfonate as the major chemical component in all of the traditional skin extractions. Thus, a swab-based protocol focusing exclusively on targeting the external, cutaneous mucous avoids many likely complications and therefore has potential as a complementary method for cutaneous small molecule collection.

With the swab protocol developed and assessed, a field test on Bioko Island not only demonstrated its practical utility but also gave impactful results. The chromatographic profile of small molecules detected on each Bioko frog was information-rich and showed, in certain cases, that a frog's profile could potentially serve as a fingerprint of its species. Profiles such as these are often used as fingerprints for the authentication of complex mixtures, especially traditional Chinese medicines.[22] Most notably, all swabs collected from specimens of *Afrixalus paradorsalis* (*N* = 5) and *Didynamipus sjostedti* (*N* = 5) revealed the presence of groups of chemical compounds unique to each of these species (Fig. 2 and 3).

The species-specific profiles of cutaneous small molecules is even more striking when the sampling sites are considered, and we note two major observations. For one, multiple different species found to be co-habitating in the exact same environment carried statistically *different* sets of cutaneous metabolites. Additionally, members of the same species sampled from geographically separated sites carried statistically *similar* sets of cutaneous metabolites.

All of the sampled *A. paradorsalis* ($N = 5$) specimens were found to be cohabitating in a small concrete basin of standing water (*ca.* 1.5 m wide × 3 m long × 1 m deep) along with sampled specimens of *Hylarana albolabris* ($N = 4$), *Hyperolius kuligae* ($N = 4$), and *Chiromantis rufescens* ($N = 6$) from a single sampling site in Risule (ESI†). However, despite their cohabitation, none of the *H. albolabris*, *H. kuligae*, or *C. rufescens* specimens carry the chromatographic fingerprint found on *A. paradorsalis* (Fig. 5). One might expect chemical compounds found on the skin of amphibians sharing the same confined pool of water, as was the case here, to be similar. However, all four species sampled from this water basin in Risule showed statistically different profiles of chemical compounds (Fig. 4), suggesting that the detected compounds are not simply derived from the aquatic or terrestrial environment that the frogs share.

Complementing these results, we observed that members of the same species that were sampled in geographically distinct locations also carried statistically similar sets of cutaneous compounds. For example, *H. kuligae* ($N = 9$) was sampled from two different sites (Moka and Risule) that are approximately 12 km apart, yet all members of this species cluster together when their chromatographic fingerprints are subjected to PCO analysis. Similarly, the sampled *H. ocellatus* ($N = 4$) were sampled at two sites (Risule and Arena Blanca) that are 8 km apart. (see ESI.†)

Taken together, these results further suggest the intriguing possibility that the reported swabbing procedure could be used as a useful fingerprint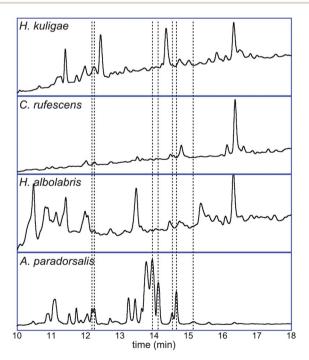ing method to differentiate between species without the interference of species co-habitation or environmental contamination. For example, *H. kuligae* and *H. ocellatus* are difficult to distinguish by eye, and the PCO analysis of their cutaneous metabolites discriminated one from the other before DNA analysis conclusively identified their species (unpublished data). However, more efforts are needed to explore the broad applicability of species fingerprinting, and this work is underway.

The results reported above demonstrate the use of this simple swabbing technique to target and collect cutaneous natural products from amphibians, providing an informative and useful "snapshot" of the diversity of the cutaneous small molecules carried on their skin. We are currently investigating potential correlations of the cutaneous metabolite pool with other factors including seasonal changes, animal health, and cutaneous microbial diversity. Additional ongoing work will also focus on chemically identifying those compounds that contribute most to species-specific and other correlations.

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Fig. 5 Comparisons of total wavelength chromatograms from cohabitating frog species. Four frog species at Risule (*H. kuligae, C. rufescens, H. albolabris,* and *A. paradorsalis*) were found to be cohabitating in a cement basin of standing water. Dotted lines indicate the retention times of characteristic compounds found only in *A. paradorsalis*. (Chromatogram intensities not normalized.)

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