IECD 2022

MDPI

Proceeding Paper

Diplodomica III. Chemical Ecology of Defensive Secretions from Neotropical Archipelago. Isolation of 3,4-Dimethoxyphenol from Ejected Secretion of Endemic Cuban Millipedes (Spirobolida, Rhinocricidae, *Rhinocricus*). Study Case *Rhinocricus duvernoyi* Karch 1881, La Palma Population †

Juan Enrique Tacoronte Morales [1,*], Joseph Cruel Sigüenza [2], Carla Bernal Villavicencio [3], Gerardo Nuñez [4] and María Elizabeth Canchingre [5]

[1] Chemical Engineering Coordination, Faculty of Science & Technology, Campus New Horizons, Technical University of Esmeraldas, Esmeraldas, Ecuador
[2] Chemical Engineering Coordination, Faculty of Science & Technology, Campus New Horizons, Technical University of Esmeraldas, Esmeraldas, Ecuador; josephcruel1983@gmail.com
[3] Chemical Engineering Coordination, Faculty of Science & Technology, Campus New Horizons, Technical University of Esmeraldas, Esmeraldas, Ecuador; carla_patita@hotmail.com
[4] Faculty of Biology, Pontifical University of Ecuador, 12 de Octubre y Roca, Quito, Ecuador; dga@puce.edu.ec
[5] Chemical Engineering Coordination, Faculty of Science & Technology, Campus New Horizons, Technical University of Esmeraldas, Esmeraldas, Ecuador; elizabeth.canchingre@utelvt.edu.ec
* Correspondence: juan.tacaronte.morales@utelvt.edu.ec; Tel.: +593-991702915
† Presented at the 2nd International Electronic Conference on Diversity (IECD 2022)—New Insights into the Biodiversity of Plants, Animals and Microbes, 1–15 March 2022; Available online: https://iecd2022.sciforum.net/.

Academic Editor: Matthieu Chauvat

Published: 14 March 2022

Abstract: Millipedes (Arthropoda, Diplopoda), are terrestrial invertebrates distributed in all geographical areas of the planet. These organisms respond to any disturbance (predators) by ejecting a repugnatorial secretion, with purgent "phenolic" odor and variable composition. Specifically, for species of the orders Spirobolida and Spirostreptida, they secrete p-benzoquinones. These defensive secretions constitute an eco-sustainable source of biologically active secondary metabolites with potential biocidal action. The Cuban archipelago mega-edaphofauna is characterized by endemic millipedes of the gen. *Rhinocricus* (*R. duvernoyi* and *R. maximus*). The objective of the present communication is to report the majority composition of this secretion in individuals of the species *Rhinocricus duvernoyi* Karsch 1881 that inhabit the western zone of Cuba in ecogeographic formations of mogotes (karst), in La Palma. The collected secretion, after chemical analysis (TLC, FTIR, CG-MS, RMN), revealed the existence of a majority component, a new metabolite, of phenolic nature, (3,4-dimethoxyphenol) for the order Spirobolida, Family Rhinocricidae and Genus *Rhinocricus*, belonging to a population of the endemic millipede *Rhinocricus duvernoyi* Karch 1881, in the western ecogeographical zone of the Cuban archipelago La Palma (Pinar del Río).

Keywords: Diplopoda; *Rhinocricus* sp defensive secretion; dimethoxyphenol

1. Introduction

The Millipedes (Arthropoda, Diplopoda), are very ancient, geologically dating back to Cambric period, 560 Ma, terrestrial invertebrates, comprising 14,000 species distributed

in all geographical areas of the planet [1]. These organisms respond, physiologically, to a predator attack and mechanical disturbances by ejecting a brown repugnatorial secretion, with a typical phenolic odor and repellent action that can cause serious epidermal or mucosal irritation to the attacker; being sometimes victims of these attacks the human being itself that invades the habitat of these invertebrates.

The chemical composition of these ejected secretions, generally of poly-component nature, varies according to the taxonomic order of taxa. Species of the orders Julida, Spirobolida and Spirostreptida secrete poly-substituted, or not, p-benzoquinones; Polydesmida species discharge hydrogen cyanide and nitroalkanes, Glomerida and Polyzoniida eject alkaloids as well as terpenoids such as ß-pinene and limonene [2–12]. These defensive secretions constitute an eco-sustainable source of biologically active secondary metabolites, benzenoid type, with potential broad-spectrum of microbiocidal action [13–17].

The Cuban neotropical archipelago is characterized by a mega-edaphofauna where the endemic millipeds of the gen. *Rhinocricus* (*R. duvernoyi* and *R. maximus*) have the highest biomass index [18]. These millipedes can ejaculate their secretions up to distances of 50 cm. The objective of the present communication is to report the major chemical composition of this secretion in individuals of the species *Rhinocricus duvernoyi* Karsch 1881 that inhabit the western zone of Cuba in ecogeographic formations of *mogotes* (karst), in La Palma (λ = 83°33′15″ W, φ = 22°45′24″ N).

2. Materials and Methods

All reagents used were supplied by MERCK, Darmstadt, Germany, and were used without prior purification.

2.1. FTIR Spectroscopy

The infrared spectra were recorded on a PHILIPS ANALYTICAL FTIR PU-9600 spectrophotometer, Germany; the samples were prepared in potassium bromide (KBr) tablets at 25 °C. Alternatively, the spectra were recorded in a JASCO-Canvas 4600, Japan system in CsBr tablets at 25 °C.

2.2. NMR Spectroscopy

NMR spectra were recorded on a BRUKER AC-250 instrument, Germany, at 25 °C. The protonic chemical (δ) shifts are given in ppm, using tetramethyl silane as internal reference (TMS, δ = 0.0) and as a solvent $CDCl_3$. The chemical shifts (δ) for ^{13}C refer to the central peak of the $CDCl_3$ solvent at 77.03 ppm.

2.3. Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

A Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) with 5973 quadrupole detection system (GC-MS) was used. The separations were carried out through a capillary column of Ultra 2 type (J & W Scientific, Folsom, CA, USA), 12 m long and 0.22 mm of internal diameter. As carrier gas, He was used at a flow of 1 mL/min. Temperature ramp: 60 °C with increments of 10 °C/min up to 300 °C (isothermal 5 min). Run time 30 min. Injection volume 2 μL at a temperature of 280 °C, in split mode (1:10 ratio). The ionization source was IE at 70 eV operating at 230 °C. Acquisition mode: Full Scan. Range of m/e 40–700.

The following databases were used for structural characterization: Nist98 (National Institute of Standards and Technology, Gaithersburg, MD, USA), PMW_TOX2 (Wiley Library and Pfleger Maurer Weber (PMW), National Metrology Institute of Japan (NMIJ, Tokyo, Japan), and National Institute of Advanced Industrial Science and Technology (AIST, Tokyo, Japan). In addition, the databases that report the chemical composition of the invertebrate defensive secretions (https://www.pherobase.com, accessed 16 October 2021) and their GC-MS (libraries for the rapid identification of metabolites in complex biological samples, Max-Planck Institute of Plant Molecular Physiology, Potsdam,

Germany) were considered. Using the reported sources and m/e data from the GC-MS, the most likely structures and their fragmentation mechanisms are postulated. Identification of the components was based on comparison of GC retention data and mass spectra. Each component was quantified using n-hexadecane as internal standard.

2.4. HPLC with PDA Detection

Detection using a diode array: 190–505 nm, KNAUER equipment, SMART LINE, Konik Column: Extrasil ODS2 5 μm, 250 × 4 mm, at 25 °C. Mobile Phase: methanol gradient (Lichrosolv, Merck): 100–60% in 10 min, 60–50% in 5 min maintained 50% for 2 min, 50–100% in 9 min.

Defensive secretion (650 μL/ind.) was collected from five male individuals in vivo and subsequently they were released. The ejected secretion was collected on Whatman-4 filter paper, stored at −10 °C, extracted from the filter paper with diethyl ether and concentrated with nitrogen flow to dryness (6 mg) and then was stored at −10 °C.

1 mg of the dried extract was weighed, dissolved in 1 mL of methanol (1000 μg/mL) and injected with 10 μL of methanol in the HPLC system described *vide supra* [19].

3. Results and Discussion

The chemical-structural and compositional analysis was first oriented to search for *intelligent signals* (FTIR, NMR-^{1}H) from the crude extract itself. 2 mg of dry extract were subjected to FTIR analysis. In the registered spectrum, a broad band was observed at 3357 cm^{-1} corresponding to OH valence vibration and another one in the zone of in-plane bends at 1244 cm^{-1} (σ CO), being both signals typical of phenols. Characteristic bands of aromatics were also observed in the range 3050–3100 cm^{-1} (νCsp2-H) and 1450–1665 cm^{-1}. The appearance of signals between 2928–2857 cm^{-1} and the signal at 1103 cm^{-1}, corresponding to Csp3-H and C-O-C valence vibration, respectively, suggest the potential existence of -OCH_3 molecular fragments that may be attached to the aromatic nucleus. The absence of signals in the region of the CO valence vibrations (1670 cm^{-1}, carbonyl) is of great significance because it rules out the possibility of the presence of quinones as major metabolites in the secretion. Substituted quinones have been described as repellent components in repugnatorial secretions for several species of the order Spirobolida and family Rhinocricidae. ^{1}H NMR analysis of the crude secretion shows characteristic aromatic signals given by a multiplet at 7.65–6.30 ppm and two singlets at 3.780 ppm and 4.020 ppm attributable to -OCH_3 fragments that corroborate what was previously considered via FTIR. The HPLC-PDA profile, is represented in Figure 1, and revealed that the defensive secretion shows a major chromatographic peak at a retention time (Rt) = 15.68 min., as shown in Figure 1A, with an absorption maximum at =254 nm, characteristic of aromatic derivative as depicted in Figure 1B (band B).

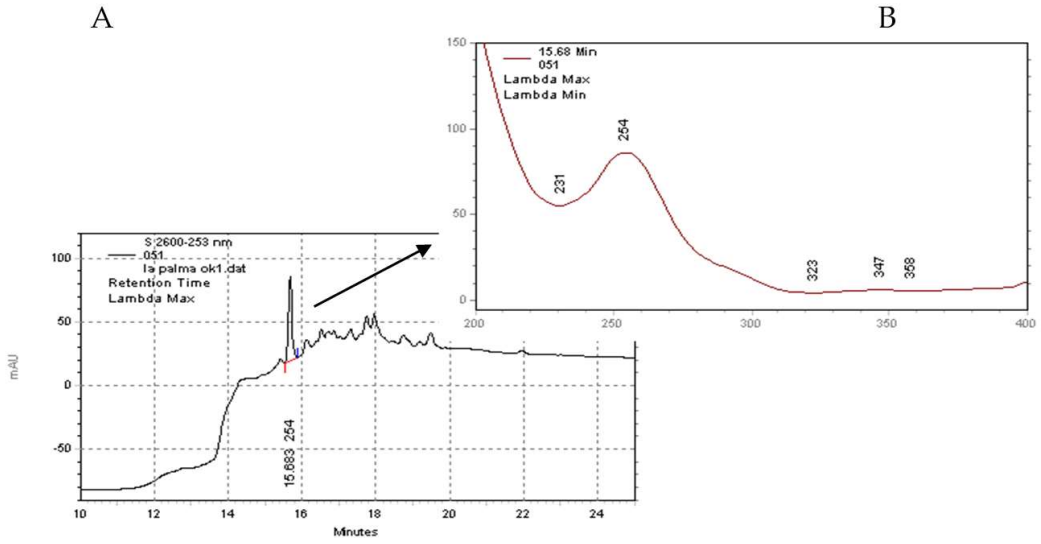

Figure 1. (**A**): HPLC-PSD profile of defensive secretion. (**B**): Ultraviolet spectrum (200–400 nm) of the major metabolite.

The analysis of volatile components by gas chromatography coupled to mass spectrometry, (GC-MS), using a simple protocol: 1 mg of dry extract was dissolved in 1 mL of chloroform (1000 μg/mL), diluted with chloroform to 50 μg/mL and injected 2 μL at 280 °C. All the results derived from GC-MS are represented in Figure 2A–C. According to the GC profile depicted in Figure 2A, a single major chromatographic peak (100%) is detected at a retention time (Rt) = 3, 246 min. The mass spectrum, as shown in Figure 2B, coincided (>95% matching) with that corresponding to 3,4-dimethoxyphenol, according to the spectral database of reference substances (NIST) [20]. The analysis of the mass spectrum and the structural fragment-*m*/*z* correlations corroborated the proposed structure and the fragmentation mechanism, as depicted in Figure 2C.

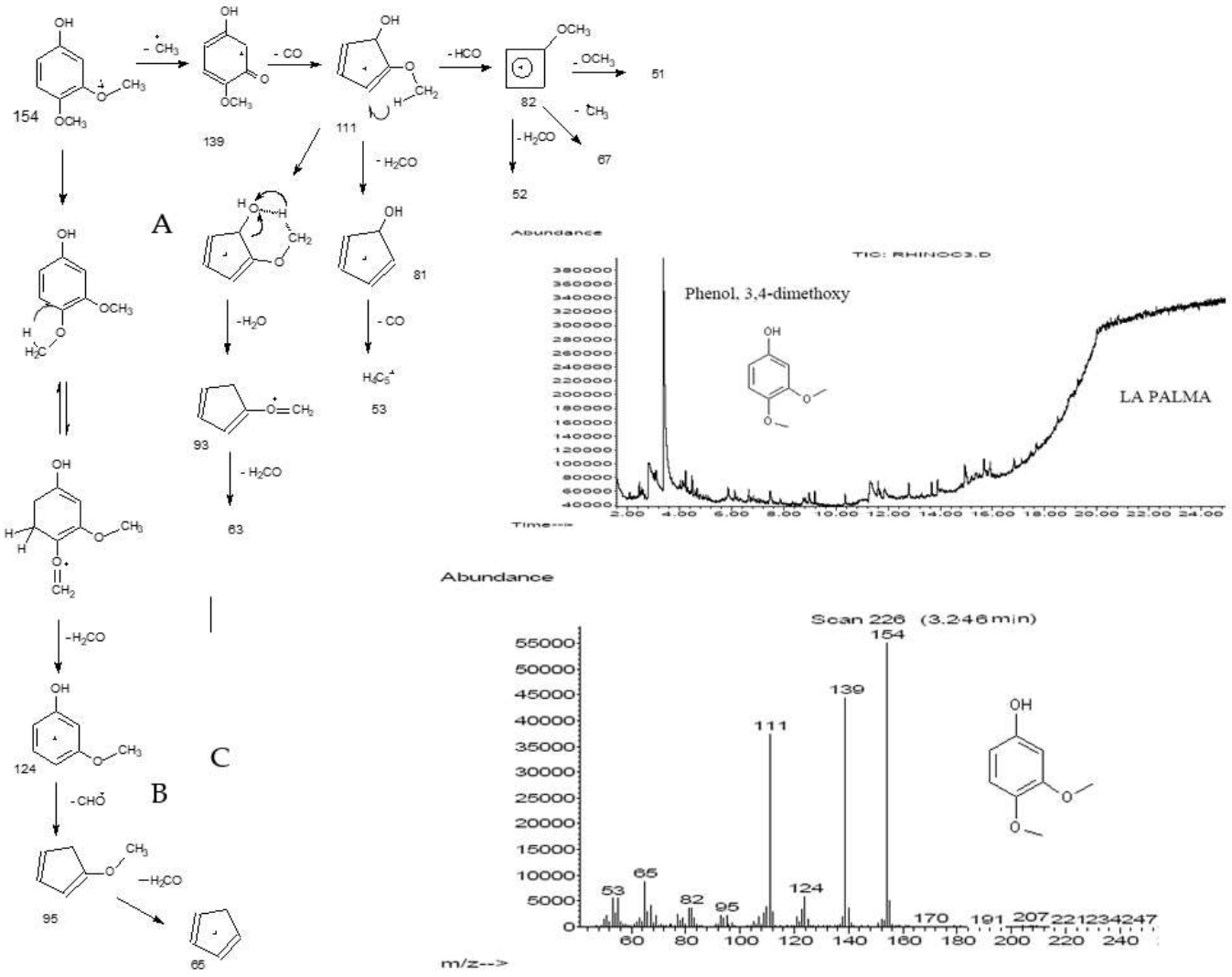

Figure 2. CG-MS profile of crude defensive secretion. (**A**): GC profile, (**B**): mass spectrum, (**C**): Proposed structure and fragmentation mechanism.

The model structure reveals the presence of methoxyl-, and hydroxyl-, substituents as represented in Figure 3. This type of derivatives possesses an intense molecular ion with little tendency to protonation which constitutes the base peak of the mass spectrum. This is appreciated at *m*/*z* 154. The Ar-O-CH_3 function is demonstrated by the following fragmentation series: loss of methyl radical followed by the elimination of a neutral molecule, CO. The fragment ions corroborating this model are: *m*/*z* 139, 111, being ambiguous the stereochemistry. A characteristic fragmentation pattern is the elimination of H_2CO (methanal) which is defined by fragment ions *m*/*z* 124, 81, 65, and 52. Another possible fragmentation is the elimination of the $^{\cdot}OCH_3$ radical (*m*/*z* 65, 51). The phenolic nature is corroborated by the formation of fragment ions *m*/*z* 95, 81, 65, 53, generated by the classical fragmentation mechanism for phenols. The Ar-fragment is corroborated by the presence of the ionic series *m*/*z* 51–53, 65–67.

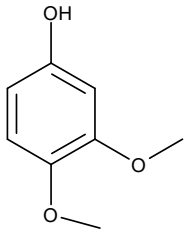

Figure 3. 3,4-dimethoxyphenol, a major metabolite in the defensive secretion of millipede *Rhinocricus duvernoyi* millipedes collected in La Palma population, karst region of the western zone of Cuba archipelago.

4. Conclusions

A new benzenoid metabolite of phenolic nature (3,4-dimethoxyphenol) was detected, and characterized, for the order Spirobolida, family Rhinocricidae and genus *Rhinocricus*, belonging to a population of the endemic millipede *Rhinocricus duvernoyi* Karch 1881, in the karst area of La Palma (Pinar del Río), in the western region of the Cuban archipelago.

Author Contributions: Individual contributions are as follows: J.E.T.M. conceptualization and methodology, funding acquisition, investigation and spectral data analysis; J.C.S., writing—original draft preparation; G.N., taxonomical considerations and investigation, C.B.V., writing and preparation, review and editing; M.E.C., final editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: Authors deeply thank Juan Antonio Mesa (CSIC-Barcelona, Spain) for facilitating spectral data acquisition. This study is a contribution of the Technical University of Esmeraldas "Luis Vargas Torres", Republic of Ecuador in collaboration with Pontifical University of Ecuador, Campus Quito, which logistically supported the preliminary structural studies.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships and do not have any potential conflict of interest. The Universities had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Hoffman, R. Practical keys to the orders and families of millipedes of the Neotropical region (Myriapoda: Diploda). *Amazonia* **1996**, *14*, 1–35.
2. Sierwald, P.; Bond, J. Current status of the myriapod Class Diplopoda (millipedes): Taxonomic diversity and phylogeny. *Annu. Rev. Entomol.* **2007**, *52*, 401–420. https://doi.org/10.1146/annurev.ento.52.111805.090210.
3. Bueno-Villegas, J.; Petra Sierwald, P.; Bond, J. Diplopoda. In *Biodiversidad, Taxonomía y Biogeografía de Artrópodos de Mexico*; Universidad Nacional Autónoma de México, Instituto de Biología: Mexico City, Mexico, 2004; Chapter 22, pp. 569–578.
4. Eisner, T.; Alsop, D.; Hicks, K.; Meinwald, J. Defensive secretions of millipedes. In *Handbook of Experimental Pharmacology*; Arthropod Venoms; Sergio, B., Ed.; Springer: Berlin/Heidelberg, Germany, 1978; Volume 48, pp. 41–72.
5. Arab, A.; Zacarin, G.; Fontanetti, C.; Camargo-Mathias, M.; Dos-Santos, M.; Cabrera, A. Composition of the defensive secretion of the neotropical millipede *Rhinocricus padbergi* Verhoeff 1938 (Diplopoda: Spirobolida: Rhinocricidae). *Entomotropica* **2003**, *18*, 79–82.
6. Deml, R.; Huth, A. Benzoquinones and hydroquinones in defensive secretions of tropical millipedes. *Naturwissenschaften* **2000**, *87*, 80–82. https://doi.org/10.1007/s001140050014.
7. Sekulic, T.; Vujisic, L.; Curcic, B.; Mandic, B.; Antic, D.; Trifunovic, S.; Godovac, D.; Vajs, V.; Tomic, V.; Makarov, S. Quinones and non-quinones from the defensive secretion of *Unciger transsilvanicus* (Verhoeff, 1899) (Diplopoda, Julida, Julidae), from Serbia. *Arch. Biol. Sci.* **2014**, *66*, 385–390. https://doi.org/10.2298/ABS1401385S.
8. Shear, W. The chemical defenses of millipedes (diplopoda): Biochemistry, physiology and ecology. *Biochem. Syst. Ecol.* **2015**, *61*, 78–117. https://doi.org/10.1016/j.bse.2015.04.033.
9. Attygal, A.; Xu, S.; Meinwald, J. Defensive secretion of the millipede *Floridobolus penneri*. *J. Nat. Prod*. **1993**, *56*, 1700–1706. https://doi.org/10.1021/np50100a007.
10. Kuwahara, Y. 2-Nitroethenylbenzenes as natural products in millipede defense secretions. *Naturwissenschaften* **2002**, *89*, 308–310. https://doi.org/10.1007/s00114-002-0328-9.
11. Williams, L.; Singh, P. Biology and Biological Action of the defensive Secretions of Tropical Millipide. *Naturwissenschaften* **1997**, *84*, 143–144. https://doi.org/10.1007/s001140050364.

12. Rodriguez, J.; Jones, T.; Sierwlad, P.; Marek, P.; Shear, W.; Brewer, M.; Kocot, K.; Bond, J. Step-wise evolution of complex chemical defenses in millipedes: A phylogenomic approach. *Sci. Rep*. **2018**, *8*, 3209. https://doi.org/10.1038/s41598-018-19996-6.
13. Ilić, B.; Unković, N.; Ćirić, A.; Glamočlija, J.; Ljaljević Grbić, M.; Raspotnig, G.; Bodner, M.; Vukojević, J.; Makarov, S. Phenol-based millipede defence: Antimicrobial activity of secretions from the Balkan endemic millipede *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida). *Sci. Nat.* **2019**, *106*, 37. https://doi.org/10.1007/s00114-019-1631-z.
14. Taira, J.; Tamashiro, M.; Naka, K.; Gakiya, S.; Taira, K. Initial defensive secretory compounds emitted from the live millipede and the induction of apoptotic cell death. *Sci. Rep.* **2021**, *11*, 8222. https://doi.org/10.1038/s41598-021-87390-w.
15. Pesewu, G.; Billah, M.; Kwang, D.; Adofo, C.; Olutaiwo, M. Antibacterial activities of millipede extracts against selected bacterial pathogens. *J. Microbiol. Antimicrob. Agents* **2015**, *1*, 30–35.
16. Roncadori, R.; Duffey, S.; Blum, M. Antifungal Activity of Defensive Secretions of Certain Millipedes. *Mycologia* **1985**, *77*, 185–191. https://doi.org/10.1080/00275514.1985.12025084.
17. Lana, E.; Carazza, F.; Takahashi, J. Antibacterial Evaluation of 1,4-Benzoquinone derivatives. *J. Agric. Food Chem*. **2006**, *54*, 2053–2056. https://doi.org/10.1021/jf052407z.
18. Tacoronte, J.; Chervas, T.; Prieto, D.; Rodríguez, C.; Díaz, M.; Gonzáles, V. Una Bezoquinona natural aislada de la secreción defensiva de un milípedo cubano endémico, Rhinocricus duvernoyi Karch, del Valle de Yumurí. *Rev. Cienc. Químicas-CNIC* **2005**, *36*, 115–116.
19. Dickschat, J. Capturing volatile natural products by mass spectrometry. *Nat. Prod. Rep*. **2014**, *6*, 838–861. https://doi.org/10.1039/c3np70080a.
20. Available online: http://chemdata.nist.gov/mass-spc/Srch_v1.7/index.html (accessed on 25 October 2021).