Received: 12 July 2023 | Revised: 1 December 2023 | Accepted: 13 December 2023

DOI: 10.1002/ecs2.4796

ECOSPHERE
AN ESA OPEN ACCESS JOURNAL

ARTICLE

Methods, Tools, and Technologies

Assessing the analytical reliability of traditional and novel sampling methods for the study of flower rewards quality

E. Pioltelli[1,2] | **L. Guzzetti**[1,2] | **A. Copetta**[3] | **M. Labra**[1,2] | **A. Galimberti**[1,2] | **W. Guidi Nissim**[1,2] | **P. Biella**[1]

[1]Department of Biotechnology and Biosciences, University of Milan-Bicocca, Milan, Italy

[2]NBFC, National Biodiversity Future Center, Palermo, Italy

[3]C.R.E.A. Research Centre Vegetable and Ornamental Crops, Sanremo, Italy

Correspondence
W. Guidi Nissim
Email: werther.guidinissim@unimib.it

Funding information
European Union – NextGenerationEU National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment Line 1.4: H43C22000530001; European Union – NextGenerationEU National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment Line 1.5: H43C220005500001

Handling Editor: T'ai H. Roulston

Abstract

Pollinator insects are declining worldwide also due to the alteration of their diet which plays a pivotal role in influencing their health status. Despite interspecific and intraspecific diversity in the diet, pollinators completely or partly rely on pollen and nectar as food sources. A precise characterization of the chemical composition of these flower resources represents a key step in the definition of pollinators' nutritional ecology. However, pollen and nectar represent challenging sources to collect and analyze, especially due to their small amounts per flower, and the application of suitable sampling and analysis tools is a pivotal step to perform dedicated studies and comparisons. Here, we compared a recently proposed tool based on a portable vacuum cleaner for floral pollen collection (E-PoSa, i.e., Electronic Pollen Sampler) with traditional pollen sampling methods (i.e., anther collection and anther sieving) together with the evaluation of different nectar sampling techniques (i.e., centrifugation, microcapillaries, washing, and microrinsing) by looking at the differences in their quantitative recovery as well as their chemical profiling. Pollen and nectar were collected from three model flower species each. Our results demonstrated that different collection methods introduce biases in the nutritional profiling of floral rewards and specifically: (1) underestimation of the pollen protein and lipid content in the anther collection method; (2) reduction in the volume of recovered nectar by centrifugation; (3) overestimation of the glucose content in the nectar collected by flower washing and underestimation of the glucose content by microrinsing; and (4) relevant biases in the phytochemical profiles of pollen and nectar by analyzing the whole anthers and the nectar collected by washing the entire flower. Differences in methods were not directly related to the different productivity of pollen and nectar across species. The final goal of the study is to propose standardized, comparable, and easily accessible strategies for the study of flower resources that ultimately impact on pollinators'

E. Pioltelli and L. Guzzetti contributed equally to this study.

nutritional ecology. Acknowledging the potential influences of the sampling techniques and moving toward shared field protocols will advance the comprehension of species interactions, foraging patterns, and pollinators' nutritional needs.

KEYWORDS
HRMS, nutritional ecology, phytochemicals, plant–pollinator interaction, pollen and nectar, pollinator diet

INTRODUCTION

Insect pollinators are declining worldwide due to multiple global issues (Cardoso et al., 2020), such as climate change (Vasiliev & Greenwood, 2021), exposure to pesticides (Goulson et al., 2015), and habitat loss (Potts et al., 2016). However, another main threat is represented by the reduction of food resources available with consequent impoverishment of the pollinator diet, such as the reduction in terms of quantity and quality of foraged pollen and nectar (Hülsmann et al., 2015; Jones & Rader, 2022; Vaudo et al., 2015), with implications also for plant–insect interactions (Jamieson et al., 2017). Land-use changes related to the expansion of urban areas and agricultural intensification significantly reduce the extension and connectivity of suitable habitats and trophic resources for pollinators (Lau et al., 2023; Wenzel et al., 2020). Moreover, progressive (e.g., due to climate change events) or sudden (e.g., due to local scale transformation of habitats) changes in environmental conditions such as temperature, light exposure, and precipitation influence plant physiology, thus altering the chemical composition of their floral resources (Biella et al., 2022; Pioltelli et al., 2024; Russell & McFrederick, 2022).

Pollen and nectar display relevant differences in their chemical composition (Palmer-Young et al., 2019), with implications for both the nutritional and ecological perspectives. Different compounds found in pollen and nectar can mediate ecological interactions, such as pollinators' attraction to flowers (Galen et al., 2011; Junker et al., 2010), the inhibition of microbial activities (Junker & Tholl, 2013), and chemical defense, preventing excessive pollen harvesting (Vanderplanck et al., 2020). Regarding the macronutrient composition, nectar is rich in free sugars and provides ready-to-use energy (Nicolson et al., 2018), thus representing the main carbohydrate source for adult bees (Pamminger et al., 2019), whereas pollen has a high protein content, and at least in bees, it represents the main food source for larvae (Nicolson, 2011). Multiple evidence about the importance of adequate nutrition for pollinator conservation has fostered a growing interest in the investigation of the nutritional landscape for a better understanding of the relationships existing between pollinating insects and floral resources (Jamieson et al., 2017; Leonhardt et al., 2022; Vaudo et al., 2016, 2018; Venjakob et al., 2022). In this context, nutritional analyses of pollen and nectar are of paramount importance. However, these studies are usually challenging due to the scarcity of nectar and pollen whose sampling is often subjected to contamination by other floral parts (e.g., anthers and petal parts). Indeed, many flowers produce a low volume of nectar (<1 μL; Power et al., 2018) and a low amount of pollen (<1 mg; Jeannerod et al., 2022).

To improve the sampling efficiency and to face the critical issues of the retrieval of an adequate quantity of pollen and nectar for achieving reliable nutritional analyses, researchers have developed many sampling approaches for the sampling of floral rewards (Pioltelli, Guzzetti, et al., 2023). This wide panel of techniques for pollen and nectar sampling results in a great heterogeneity among the different studies, hampering the comparison among the results obtained, as also claimed by Power et al. (2018) and Morrant et al. (2009). Such limitations are of great concern since these kind of data carry significant implications for the comprehension of the overall health status of pollinators. Indeed, besides macronutrients (i.e., proteins, sugars, and lipids) which have a primary role in the development, sustenance, and metabolism regulation of pollinators (Di Pasquale et al., 2013; Nicolson, 2011; Vaudo et al., 2016), pollen and nectar also represent a source of micronutrients. They contain vitamins, minerals, phytosterols, and free amino acids that play crucial roles in many biological processes (Lau et al., 2023), from larval development (Vanderplanck et al., 2014) to learning performance (Palmer-Young et al., 2019). Another class of relevant compounds occurring in flower resources is that of phytochemicals (e.g., flavonoids, phenolic acids, terpenes, and alkaloids), which are even more considered for the role they play for pollinators at the physiological level (Ardalani et al., 2021; Koch et al., 2019; Mao et al., 2013; Niño et al., 2022). Pollen and nectar contain secondary compounds able to influence pollinators' health through reduction, prevention, or by increasing

tolerance to infections (Koch et al., 2017; Richardson et al., 2015) and can also play an important role in coping with oxidative stress (Berenbaum & Calla, 2021), thus acting as nutraceuticals in pollinator diet (Ardalani et al., 2021; Stevenson et al., 2022). Despite the potential beneficial effects on pollinators' health, they can display some toxicity or act as a deterrent, often in a dose-dependent manner (Vanderplanck et al., 2020). Furthermore, the chemistry of floral rewards contributes to the definition of plant–pollinator interactions and the collection specificity of plant taxa (Ruedenauer et al., 2020; Woodcock et al., 2014). These issues require the sampling effort to be performed with the assurance of both precise sampling of the matrices and in enough quantities to be chemically characterized. With such a variety of methods for the sampling of floral resources, each one with different benefits and drawbacks (Pioltelli, Guzzetti, et al., 2023), the need to standardize the collection effort is becoming even more urgent.

Therefore, the aims of the present study are (1) to compare different protocols for the sampling of flower resources to assess their reliability in terms of recovery and nutritional profiling and (2) to understand the magnitude of the biases generated by nonspecific sampling and how to cope with these issues.

MATERIALS AND METHODS

Study species

The target flower species were selected based on their taxonomy to account for a wider set of families characterized by different floral morphologies and different amounts of pollen and nectar produced. For the collection of anthers and pollen grains, a panel of three species was selected: *Tropaeolum majus* L. (Fam.: Tropaeolaceae), *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae), and *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae) (Figure 1A–C). Nectar sampling was performed on three nectariferous species: *Agapanthus praecox* FM. Leight (Fam.: Amaryllidaceae), *Russelia equisetiformis* Schletcht. & Cam. (Fam.: Scrophulariaceae) and *Salvia greggii* A. Grey var. "Purple Queen" (Fam.: Lamiaceae) (Figure 1D–F). For each species, pollen and nectar were collected from the flowers beard by the same plant, to minimize variations due to the genetics of the individuals. Sampled flowers were covered with nylon mesh 24 h before the sampling to avoid possible depletion of the resources by pollinator visits. The study took place at the C.R.E.A Institute (Council for Agricultural Research and Economics) of Sanremo, Italy, where the studied species were cultivated in greenhouses.

FIGURE 1 Images of the flowers of all the studied species. (A) *Tropaeolum majus* L. (Fam.: Tropaeolaceae); (B) *Hippeastrum vittatum* Herb. (Fam.: Amaryllidaceae); (C) *Alstroemeria aurea* Graham (Fam.: Alstroemeriaceae); (D) *Agapanthus praecox* FM. Leight (Fam.: Amaryllidaceae); (E) *Russelia equisetiformis* Schletcht. & Cam. (Fam.: Scrophulariaceae); (F) *Salvia greggii* A. Grey var. "Purple Queen" (Fam.: Lamiaceae). Photo credit: Andrea Copetta.

Pollen sampling

Three pollen collection approaches were adopted: (1) anthers were collected by carefully removing them from the flowers using forceps; (2) pollen grains obtained by dehisced anthers through multiple steps of sieving starting with a mesh of 100-μm size to a final mesh of 50 μm in order to isolate pollen grains from other floral parts and used as the control group (hereafter mesh); and (3) E-PoSa (Electronic Pollen Sampler) (Pioltelli, Guzzetti, et al., 2023), by vacuuming the pollen from each flower for 30 s (details on the tool are reported in the text of Appendix S1 and Appendix S1: Figure S1). For each sampling approach, we collected pollen from 20 flowers per species to gain enough material for all the subsequent analyses. All the collected samples were dried in an oven at 30°C for 12 h.

Nectar sampling

Nectar was sampled by following four different protocols: (1) nectar was sampled by washing the flowers in 15-mL tubes containing 2-mL H_2O for 30 s per flower—hereafter wash; (2) nectar was sampled by adding 2 to 20 μL of H_2O (depending on the nectar viscosity) and then recovered by using glass syringes (Hamilton, USA)—hereafter microrinsing; (3) nectar was sampled by using commercially available glass capillary (Merck, Germany)—hereafter capillary; and (4) nectar was sampled by centrifuging flowers in a 50-mL tube endowed with a wire mesh (20-μm diameter) and then recovered with a pipette at the bottom of the tube—hereafter centrifuge. Nectar collection was performed between 25 and 26 July 2021 in an hour range between 10 am and 12 am for all the species to minimize as much as possible biases due to the daytime. Samples were stored in a 1:1 v/v nectar/EtOH ratio to avoid microbial-mediated degradation of the occurring compounds and stored at −80°C up to the analyses.

Chemical characterization of pollen samples

Prior to the nutritional analyses, all the pollen samples were freeze-dried to normalize each one to the dry mass. All the nutritional analyses were performed on ~1 mg for at least three biological replicates per method per species.

Macronutrients (i.e., proteins, lipids, and sugars) were extracted by following the protocol proposed by Vaudo et al. (2016) with a minor modification to the extraction of lipids, which was carried out with two subsequent extraction cycles. The secondary compounds were extracted by a hydroalcoholic solvent made of EtOH/H_2O 1/1 v/v in a drug/solvent ratio of 1:1000 w/v for three cycles of extraction in an ultrasound bath (37 Hz, 30°C). Samples were dried under gaseous nitrogen and resuspended in 1-mL ultrapure Milli-Q H_2O. The total nutrient composition of samples was evaluated by using different colorimetric assays. In particular, the Bradford method was used for protein quantification and the vanillin assay for the detection of lipids. The content of free sugars was carried out by using a commercial enzymatic kit (Megazyme, Ireland) as reported in Pioltelli et al. (2024). The total phenol and flavonoid contents of pollen were evaluated following the protocols described in Pioltelli et al. (2024), while the total antioxidant activity (intended as the ABTS radical scavenging capacity) was estimated as in Pioltelli, Sartirana, et al. (2023). The hydroalcoholic extracts were 10-fold-diluted and analyzed through RP-LC-HRMS (see Appendix S1 for details).

Chemical characterization of nectar samples

Nectars were dried under nitrogen and resuspended in 1-mL ultrapure Milli-Q H_2O. The solution was filtered using hydrophilic polytetrafluoroethylene (PTFE) filters with 0.22-μm pore size (Macherey-Nagel, Germany) to obtain a clean solution. Samples were analyzed for the free sugars content and phytochemicals profile as reported for pollen samples.

Statistical analyses

To test whether the sampling method affects the volume of nectar retrieved, we used a regression approach based on a linear model (LM) with the sampling method in interaction with the species as a fixed effect. Concerning the chemical composition of pollen, we set a series of generalized linear models (GLMs) with a binomial or quasi-binomial distribution of the dependent variable depending on the overdispersion parameter (if higher than 1 a quasi-binomial distributed models were run to avoid type I errors), while a Gamma distribution was used for analyzing data about nectar sugar composition. The sampling method in interaction with the species was treated as a fixed effect. When the interaction terms did not result significant, GLMs were set with method and species as independent fixed effects, and a backward stepwise model selection based on Akaike information criterion (AIC) was used to remove variables that did not improve the model fit. The analyses were carried out in R (version 4.3.1). The *p* values for multiple comparisons

were adjusted by using the Tukey post hoc test for multiple comparisons. Packages exploited were "interactions" (Long, 2022), "ggplot2" (Wickham, 2016), "MuMIn" (Bartoń, 2022), and "multcmp" (Hothorn et al., 2008).

For the high-resolution mass spectrometry (HRMS) data, we used MS-DIAL software version 4.9 for peak picking, deconvolution, noise level setting, and identification of metabolites. The identified peaks were aligned on a quality control sample, also to allow the monitoring of the instrument's response. Deconvoluted chromatograms were normalized on the total ion current (TIC) and analyzed through a principal components analysis (PCA) to account for the effect of the sampling method followed by PERMANOVA to account for statistical significance ($\alpha = 5\%$) by exploiting the "vegan" package (Oksanen et al., 2022). Significant metabolites responsible for the clusterization among the experimental groups were more deeply characterized by data dependent acquisition (DDA) setting as ion intensity threshold a value of 5×10^4. The chemical identity of these phytochemicals was disclosed mainly by literature comparison (Appendix S1: Tables S2–S4 and S6–S8).

RESULTS

Pollen nutrient composition

The estimate of the nutrient composition of pollen is shown in Figure 2, and the output of the statistical analyses is reported in Table 1. The total protein content was found to be significantly different among the species considered and based on the adopted sampling method. Specifically, pollen of *A. aurea* showed significantly lower protein content compared with *T. majus* ($\beta = 1.78$; $p < 0.001$) and *H. vittatum* ($\beta = 1.75$; $p < 0.001$) (Figure 2). In all the species, the protein content was significantly lower in dry anthers compared with mesh ($\beta = 0.36$; $p = 0.004$) (Figure 2), while no significant differences emerged in the comparison between the total protein content in pollen collected with E-PoSa and the mesh ($\beta = 0.12$; $p = 0.524$). Concerning the lipid content, we found that the use of the E-PoSa did not introduce significant variations compared with the mesh group in none of the three species considered, while a significantly lower

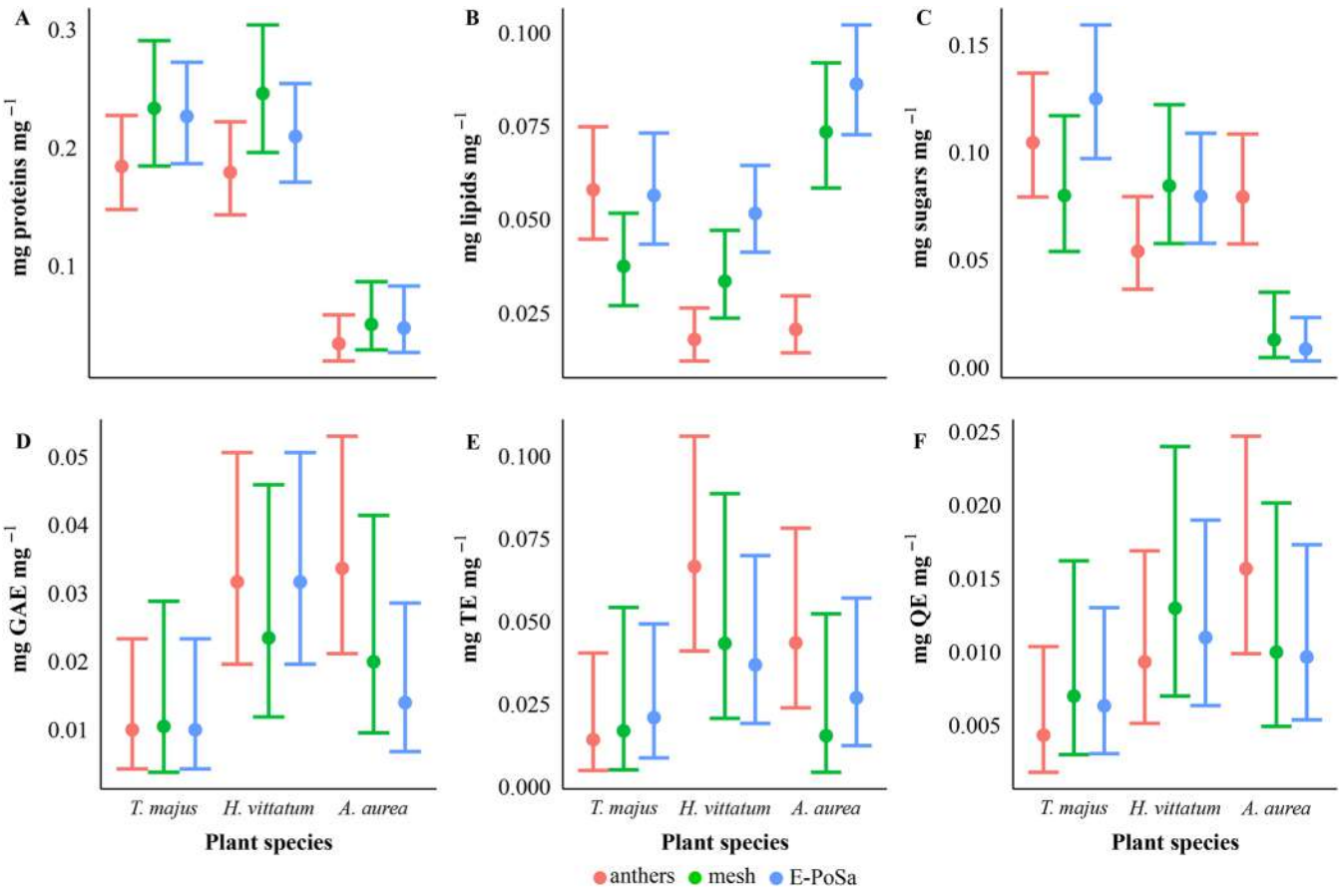

FIGURE 2 Quantified percentages of the pollen nutrient composition of the three studied species obtained through the different sampling methods. Data are expressed as (A) milligram of proteins, (B) lipids, (C) milligram of total free sugars, (D) gallic acid equivalent (GAE), (E) Trolox equivalent (TE), and (F) quercetin equivalent (QE) per milligram of pollen and are reported as the mean regression coefficient of the model ± SE. E-PoSa, Electronic Pollen Sampler.

TABLE 1 The output of the statistical analyses conducted on pollen chemistry and nectar retrieval and sugar composition.

Floral resource	Response	Final model covariates	F/χ^2	p
Pollen	Proteins	Method	11.09	0.04
		Species	217.78	<0.001
	Lipids	Method × species	50.01	<0.001
	Free sugars	Method × species	48.58	<0.001
	TPC	Species	15.54	<0.001
	TEAC	Species	10.61	0.005
	TFC	Species	9.60	0.008
Nectar	μL retrieved	Method × species	495.4	<0.001
	Sucrose	Method × species	13.12	0.04
	Glucose	Method	8.38	0.04
		Species	210.66	<0.001
	Fructose	Species	222.80	<0.001
	Free sugars	Method × species	13.32	0.04

Note: For each nutrient, the covariates included in the final model are indicated.
Abbreviations: TEAC, Trolox equivalent antioxidant capacity; TFC, total flavonoid content; TPC, total phenolic content.

amount of lipids occurred in the anthers of *A. aurea* compared with the mesh control group ($\beta = 1.32$; $p < 0.01$). Concerning the total sugar content of the pollen, anthers showed significant differences compared with mesh samples in all three species, while the pollen collected with E-PoSa was found to be comparable with the mesh samples in the three species. Conversely, no significant differences among the three sampling methods were found concerning the total phenol content, total antioxidant activity, and total flavonoid content. These latter chemical categories were found to be influenced only by the species, with *T. majus* exhibiting significantly lower values compared with the other species.

Pollen phytochemical composition

The results obtained from the HRMS analyses of the hydroalcoholic extracts are reported in Figure 3. Results showed that in two out of the three species (i.e., *H. vittatum* and *A. aurea*), the collection of anthers led to an estimate of the phytochemical profile significantly different from what obtained by extracting mesh or E-PoSa derived pollens (Figure 3; Appendix S1: Table S1). In particular, the phytochemical profile of *H. vittatum* anthers showed the occurrence of many glycosylated phenolic acids (hydroxybenzoic acid, vanillic acid, sinapic acid, and 3-hydroxy-3-hydroxyphenyl-propionic acid) not findable in E-PoSa and mesh-sampled pollen. Anthers also showed a different set of flavonoids compared with E-PoSa and mesh samples. A similar pattern was observed in *A. aurea*, where the E-PoSa and mesh-pollen collection led to the detection of different flavonoid glycosides compared with the anthers. Generally, anthers were characterized by higher diversity and quantity of phytochemicals. A detailed characterization of the discriminating compounds is reported in Appendix S1: Tables S2–S4. In *T. majus*, however, no significant differences in the phytochemical profile were found based on the collection method. These results indicate that the E-PoSa pollen sampling led to a much more accurate definition of the phytochemistry of pollen compared with anther sampling, which frequently may result in impaired metabolic profiles.

Nectar retrieval and sugar composition

As shown in Figure 4 and as reported in Appendix S1: Table S1, the volume of nectar recovered in *S. pratensis* was significantly lower in the centrifuge method compared with both microrinsing ($\beta = 2.4$, $p = 0.036$) and microcapillary ($\beta = 5.48$, $p < 0.001$). A similar trend was observed for *R. equisetiformis* with centrifuge recovery significantly lower than that obtained through microrinsing ($\beta = 7.46$, $p < 0.001$) and capillary ($\beta = 5.93$, $p < 0.001$), while for *A. praecox*, no significant differences among the three methods were observed. It was not possible to estimate the amount of nectar recovered by the wash method, because the nectar is directly suspended in a fixed volume of water.

Concerning the analyses of free sugars, the fructose content varied significantly only according to the species considered (Table 1, Figure 5). The content of sucrose and the total sugar content showed a significant relationship

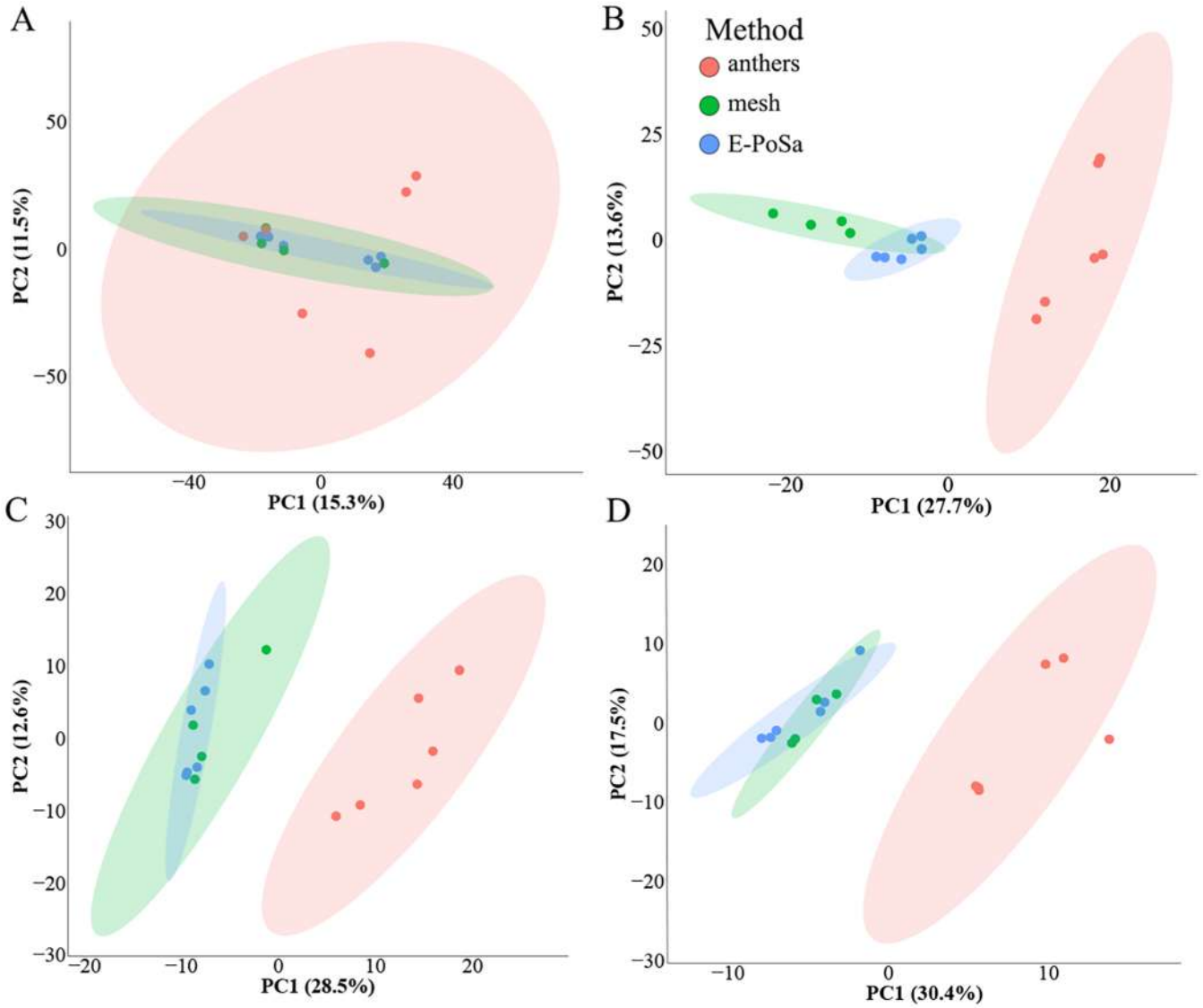

FIGURE 3 Output of the principal components (PC) analyses performed on the high-resolution mass spectrometry (HRMS) chromatographic traces of hydroalcoholic extracts for (A) *T. majus*, negative ionization mode; (B, C) *H. vittatum*, negative and positive ionizing modes; and (D) *A. aurea*, negative ionizing mode. In red are reported anthers, in green pollen sampled with mesh, and in blue pollen collected with E-PoSa (Electronic Pollen Sampler**)**.

with the interaction term between sampling method and species (Table 1) since the sugar content among the analyzed methods varied differently depending on the species (Figure 5). Despite not producing results always significant at the statistical level, we found variations in the direction of the relationships that justify the significance of the interaction term (e.g., see Figure 5 for the variations in the total sugars and sucrose content of nectars sampled by microcapillaries that are lower compared with the other methods in *A. praecox* and *R. equisetiformis*, but higher in *S. greggii*). The only sugar that showed significant variation among the sampling method and irrespective of the species was glucose, which showed lower concentration in samples collected by microrinsing than in wash ($\beta = 0.48$, $p = 0.03$).

Nectar phytochemical composition

As shown in Figure 6 and Appendix S1: Table S6, in two out of the three species analyzed (i.e., *S. greggii* and *A. praecox*), the ordination analyses performed on nectar metabolomic data showed that the microcapillary and flower centrifugation are the most indicated methods to avoid sample contamination for nectar collection. The microcapillary-sampled nectar from *R. equisetiformis* displayed higher phytochemical similarity with the one obtained through microrinsing than centrifugation. Generally, sampling nectar by washing resulted in phytochemical profiles contaminated by the occurrence of typically pollen-originating compounds

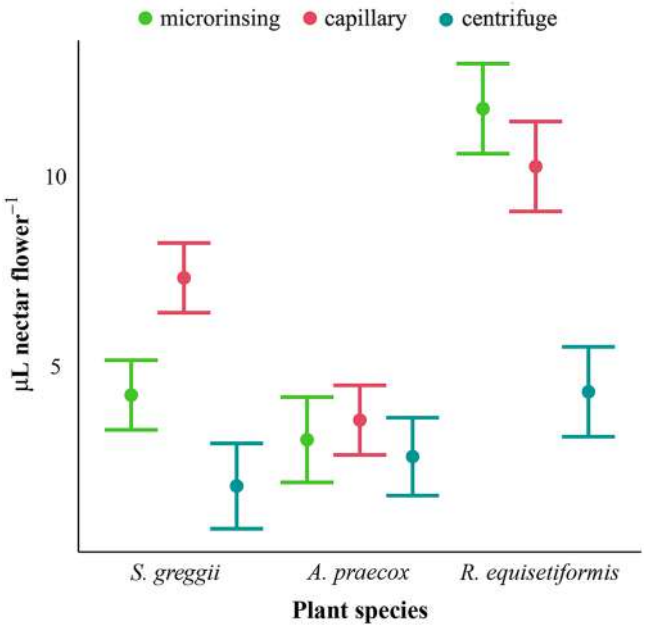

FIGURE 4 Quantified microliters of nectar retrieved per flower of the different species by comparing the different sampling methods. Data are reported as the mean regression coefficient of the model ± SE.

(see Appendix S1: Tables S6–S8 for their identification), responsible for consistent biases from the actual phytochemical composition of the nectar, while methods such as centrifuge and microrinsing appeared to be more accurate even though not always perfectly overlapping with the results obtained by the sampling through microcapillary tubes.

DISCUSSION

The most important result of this comparative study is that the different sampling methods of flower rewards cause important differences in the amount of recovered nectar and in the pollen and nectar nutritional profiling at both the macronutrient and phytochemical levels.

Pollen sampling and biases

The characterization of pollen nutritional aspects from wild plants requires precise analytical tools to define as better as possible its role in the nutritional balance of pollinators (Lau et al., 2022). It is well known that the recovery of pollen grains from wild plants is a difficult task to perform since the anthers of entomogamous species usually produce low amounts of pollen (e.g., Jeannerod et al., 2022; Palmer-Young et al., 2019). However, obtaining a significant amount of pollen with no contaminants originating from other floral parts may be very time-consuming, thus reducing the efficiency and the feasibility of nutritional ecology studies.

Results from the chemical analyses performed on the three target species show that pollen collected with the E-PoSa did not show any significant difference in terms of total nutrient composition compared with the mesh-sampled pollen in most of the studied species, suggesting that this newly proposed tool is able to provide for pollen grains free of contaminants (Pioltelli, Guzzetti, et al., 2023). The inadvertent or deliberate inclusion of other floral tissues like anthers may result in some biases in the total nutrients profiling of samples. These contaminations from other plant portions are even more evident when HRMS analyses are performed, as we found that the chemical composition of anthers samples is clearly biased compared with the other experimental groups. For example, in *H. vittatum* and *A. aurea*, many phenolic acids not occurring in pure pollen grains were detected. This may result in an improper evaluation of pollinators' nutrition due to a bias from the flawed selection of the matrix and not from the effective production of toxic or health-promoting compounds in pollen (i.e., phenolic acids). The pollen grains sampled with the E-PoSa display a chemical profile similar to the mesh sampled ones, indicating a generally accurate sampling of the matrix of interest. However, the E-PoSa method is associated with a significantly higher recovery of pollen per floral unit compared with the mesh, thus suggesting being a low time-consuming and high-yielding sampling procedure for floral pollen, also in those species characterized by a low pollen yield per flower, such as entomogamous meadow species (Pioltelli, Guzzetti, et al., 2023).

Nectar sampling and relative biases

Nectar sampling suffers from relevant biases in the definition of the amino acid profile if the sampling doesn't exclude contamination from other floral parts such as pollen and, to a lower extent, petals (Power et al., 2018). In this study, we found that also the sugar composition of nectars is affected by the sampling method depending on flower morphologies (in the present study, three different floral morphologies were considered: tubular corolla in the case of *R. equisetiformis*, zygomorphic symmetry for *S. greggii* variety, and radial shape for *A. praecox*). In all the species considered, the glucose content was significantly lower in the nectar collected using microrinsing than wash. Both the methods however did not differ significantly compared with microcapillary

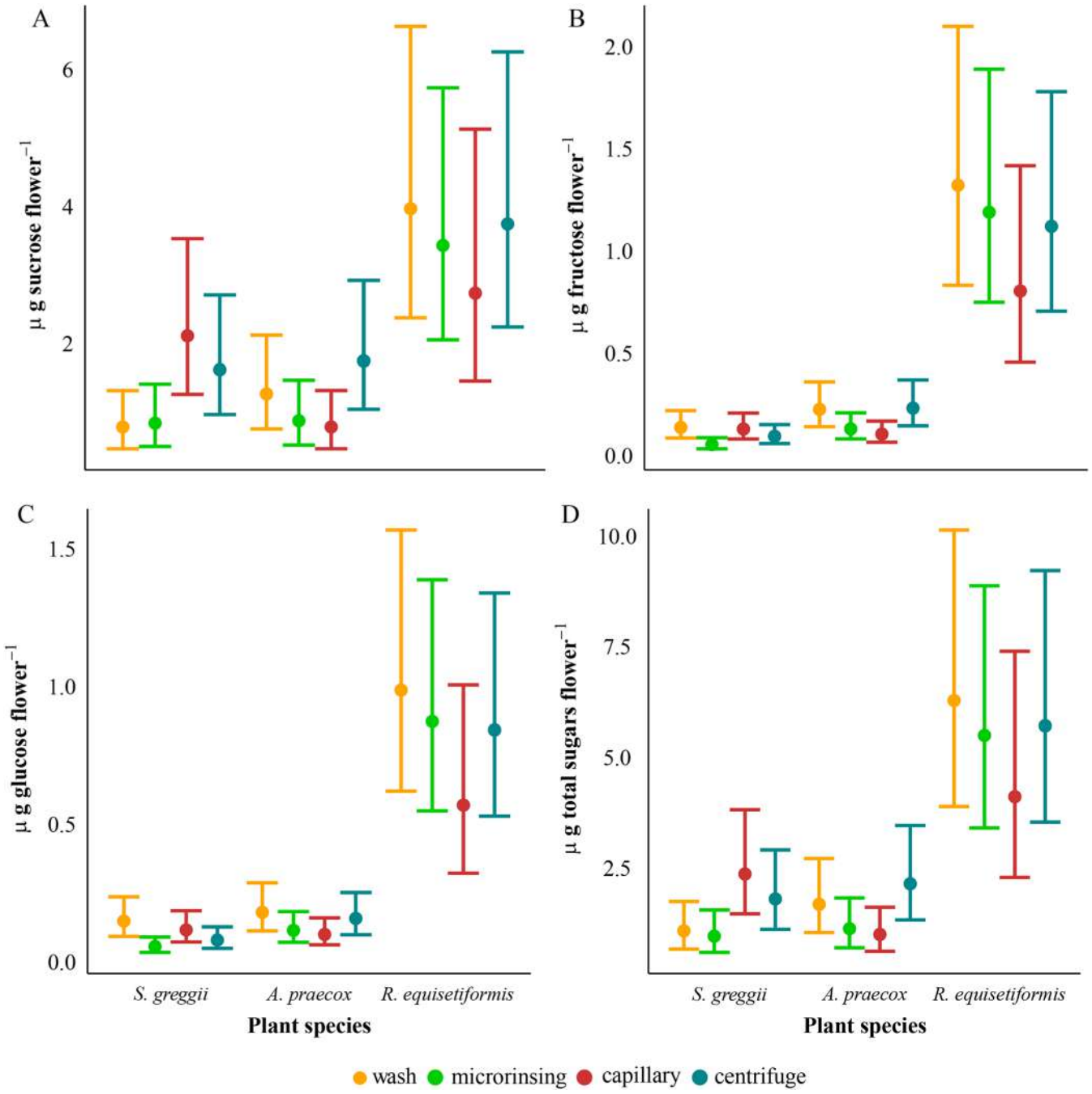

FIGURE 5 Quantity (in milligrams) of sugars recovered per flower in the three studied species based on the four studied sampling methods. Data are expressed as micrograms of (A) sucrose, (B) glucose, (C) fructose, and (D) total free sugars per flowers and are reported as the mean regression coefficient of the model ± SE.

and centrifuge, suggesting both possible contaminations of other floral tissues deriving from the wash method and incomplete retrieval through the microrinsing method. A factor that could influence the results is nectar productivity. For instance, a significantly higher content of sugars was found in *R. equisetiformis*, which flowers also yielded the highest amount of nectar among the investigated species. This is explainable by the fact that this species is known to be visited by vertebrates, such as hummingbirds (Vasconcellos & Freitas, 2007), justifying the need for a higher nectar production compared with insect-visited species such as *S. greggii* and *A. praecox*. However, variations in the sugar content among the sampling methods are not dependent on the volume of nectar produced. For instance, the yield of nectar obtained in *A. praecox* and *S. greggii* was similar; however,

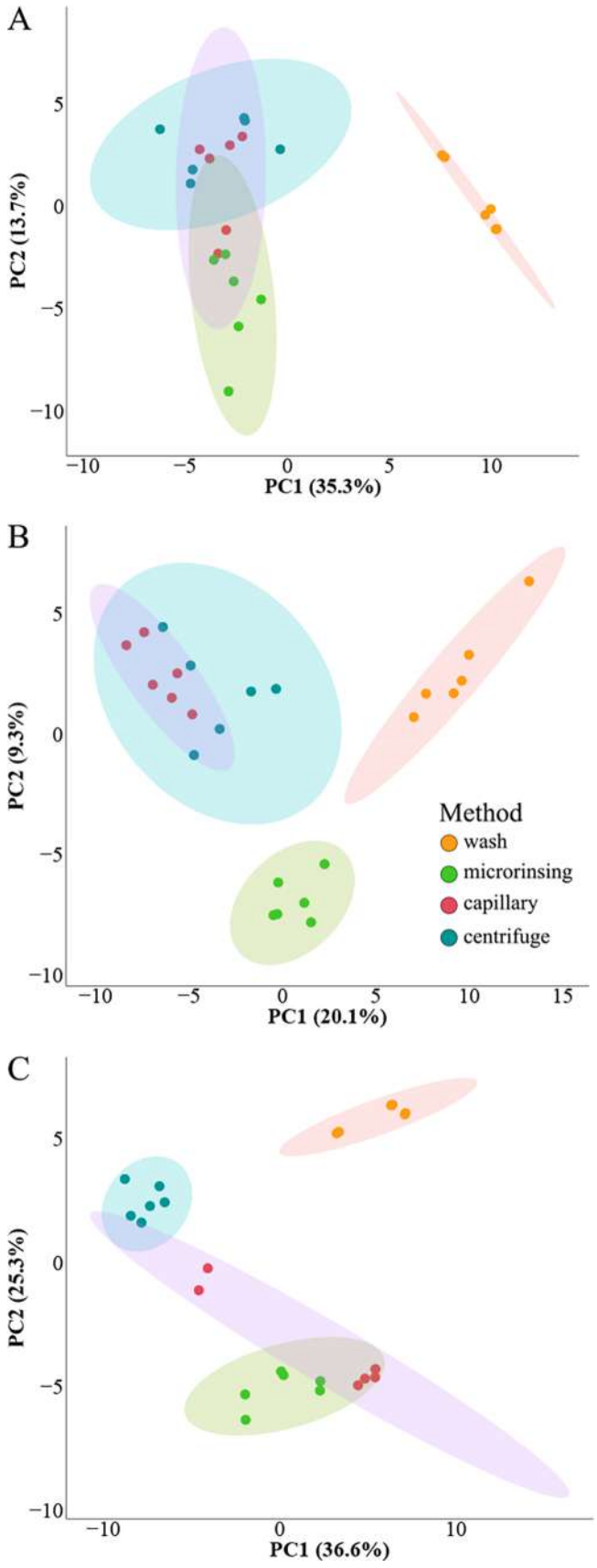

FIGURE 6 Output of the principal components (PC) analyses performed on the high-resolution mass spectrometry (HRMS) chromatographic traces of nectars sampled from (A) *S. greggii*, (B) *A. praecox* and (C) *R. equisetiformis*.

different effects of the sampling methods on the estimated sugar content per flower were observed. This phenomenon may be explained by differences in the floral morphologies. For example, the recovery of nectar by capillary yields more on *S. greggii* flowers that display bilateral symmetry, while those of *A. praecox* are characterized by radial symmetry. This indicates that the recovery of nectar (and consequently the amount of estimated sugar produced per flower) could be more effective by microcapillaries when bilateral floral symmetries occur, while in the case of radial-shaped flowers, this sampling method may partly underestimate the nectar yield. Overall, an important recommendation for the sampling of nectar is to consider the floral morphology to define the most suitable method, prioritizing—whenever possible—the sampling through microcapillaries for an optimal nectar recovery, especially if large nectar volumes allow it (Biella et al., 2021). When the amount of nectar produced by a flower is too low to be sampled by glass microcapillaries and/or centrifugation, it is worth considering that the microrinsing method may account for an alternative recovery method (Biella et al., 2019).

Concerning the putative contamination of nectar due to pollen, three aspects should be underlined. Firstly, the analysis of the secondary compounds showed that the wash method strongly separated in the ordination multivariate space from centrifugation, glass microcapillaries, and microrinsing. Secondly, the microrinsing and wash method revealed the presence of rutin, a well-known secondary compound strongly associated with the presence of pollen (Rocchetti et al., 2019). Thirdly, many other compounds belonging to the classes of flavonoids, triterpenoids, and polyamines are found exclusively or in significantly higher concentration in wash nectar samples suggesting an evident extra-nectar origin. These observations are relevant if considering that in recent years several studies have clarified the role of secondary metabolites in the definition of the nutritional value of flower rewards (Palmer-Young et al., 2019; Stevenson et al., 2017). Rutin, for instance, has been proven to act as a protective agent against the negative impacts of common insecticides (Riveros & Gronenberg, 2022). Therefore, the identification of such kinds of compounds when not occurring within nectar or their over-quantification may result in a great bias for the definition of the nutritional features and value of flower rewards. Furthermore, the biases in the estimation of the sugar content of nectar could produce misleading interpretations in research dealing with plant–pollinator interactions as multiple studies observed species-specific preferences for nectar sugars (Kelber, 2003; Romeis & Wäckers, 2000; Woodcock et al., 2014).

CONCLUSIONS

The comparison carried out in this work highlights two main points that remain open to further research insights. The first one concerns the need to develop shared protocols and adequate technologies for the analysis of floral resources. A more extensive comparison project able to cover flowers with diverse morphologies and belonging to phylogenetically distant plant families could help to provide more accurate and standard indications for floral rewards nutritional research.

The second point refers to the heterogeneity of nectar and pollen chemistry data, possibly due to the sampling methods which for such sensitive biological samples (i.e., flower resources) can lead to analytical mischaracterization. With the results obtained in the present study, we suggest that the collection methods of floral resources that minimize the contamination from other flower parts should be preferred, to avoid biased data and interpretations on the nutritional value of flower rewards.

AUTHOR CONTRIBUTIONS

E. Pioltelli, L. Guzzetti, and W. Guidi Nissim conceived the ideas and designed the methodology. E. Pioltelli, L. Guzzetti, and A. Copetta collected the data. E. Pioltelli and L. Guzzetti analyzed the data. E. Pioltelli, L. Guzzetti, A. Galimberti, W. Guidi Nissim, and P. Biella led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

ACKNOWLEDGMENTS

Funder: The research was funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree No. 3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU. Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP, H43C22000530001 Project title "National Biodiversity Future Center - NBFC and within the MUSA—Multilayered Urban Sustainability Action project (Spoke 1), supported by the European Union—NextGenerationEU, under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment Line 1.5: "Strengthening of research structures and creation of R&D" "innovation ecosystems", set up of "territorial leaders in R&D". Project code ECS00000037, CUP, H43C220005500001. The authors are grateful to Barbara Ruffoni, Paglo Mussano, and the team of the CREA of Sanremo for their support. Special thanks go to Fausto Ramazzotti and Luca Tonietti for their help during the sampling activity. The authors thank Maura Brioschi for her support with the mass spectrometry analysis. P. Biella was supported by the Italian Ministry of University and Research with resources from PONRI FSE REACT-EU 2014–2020 – "Azione IV.4 – Dottorati e contratti di ricercar su tematiche dell'innovazione, Azione IV.6 Contratti di ricercar su tematiche Green."

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Pioltelli, 2024) are available from Figshare: https://doi.org/10.6084/m9.figshare.25140092.v1.

ORCID

A. Galimberti https://orcid.org/0000-0003-3140-3024
W. Guidi Nissim https://orcid.org/0000-0001-6738-0122

REFERENCES

Ardalani, H., N. H. Vidkjær, P. Kryger, O. Fiehn, and I. S. Fomsgaard. 2021. "Metabolomics Unveils the Influence of Dietary Phytochemicals on Residual Pesticide Concentrations in Honey Bees." *Environment International* 152: 106503. https://doi.org/10.1016/j.envint.2021.106503.

Bartoń, K. 2022. "MuMIn: Multi-Model Inference." R Package Version 1.47.1. https://CRAN.R-project.org/package=MuMIn.

Berenbaum, M. R., and B. Calla. 2021. "Honey as a Functional Food for *Apis mellifera*." *Annual Review of Entomology* 66(1): 185–208. https://doi.org/10.1146/annurev-ento-040320-074933.

Biella, P., A. Akter, A. J. Muñoz-Pajares, G. Federici, A. Galimberti, J. Jersáková, M. Labra, F. Mangili, N. Tommasi, and L. Mangili. 2021. "Investigating Pollination Strategies in Disturbed Habitats: The Case of the Narrow-Endemic Toadflax *Linaria tonzigii* (Plantaginaceae) on Mountain Screes." *Plant Ecology* 222: 511–523. https://doi.org/10.1007/s11258-021-01123-7.

Biella, P., A. Akter, J. Ollerton, S. Tarrant, Š. Janeček, J. Jersáková, and J. Klecka. 2019. "Experimental Loss of Generalist Plants Reveals Alterations in Plant-Pollinator Interactions and a Constrained Flexibility of Foraging." *Scientific Reports* 9(1): 7376. https://doi.org/10.1038/s41598-019-43553-4.

Biella, P., N. Tommasi, L. Guzzetti, E. Pioltelli, M. Labra, and A. Galimberti. 2022. "City Climate and Landscape Structure Shape Pollinators, Nectar and Transported Pollen along a Gradient of Urbanization." *Journal of Applied Ecology* 59(6): 1586–95. https://doi.org/10.1111/1365-2664.14168.

Cardoso, P., P. S. Barton, K. Birkhofer, F. Chichorro, C. Deacon, T. Fartmann, C. S. Fukushima, et al. 2020. "Scientists' Warning to Humanity on Insect Extinctions." *Biological Conservation* 242: 108426. https://doi.org/10.1016/j.biocon.2020.108426.

Di Pasquale, G., M. Salignon, Y. Le Conte, L. P. Belzunces, A. Decourtye, A. Kretzschmar, S. Suchail, J. L. Brunet, and C. Alaux. 2013. "Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter?" *PLoS One* 8: e72016. https://doi.org/10.1371/journal.pone.0072016.

Galen, C., R. Kaczorowski, S. L. Todd, J. Geib, and R. A. Raguso. 2011. "Dosage-Dependent Impacts of a Floral Volatile Compound on Pollinators, Larcenists, and the Potential for Floral Evolution in the Alpine Skypilot *Polemonium viscosum*." *The American Naturalist* 177(2): 258–272. https://doi.org/10.1086/657993.

Goulson, D., E. Nicholls, C. Botías, and E. L. Rotheray. 2015. "Bee Declines Driven by Combined Stress from Parasites, Pesticides, and Lack of Flowers." *Science* 347(6229): 1255957. https://doi.org/10.1126/science.1255957.

Hothorn, T., F. Bretz, and P. Westfall. 2008. "Simultaneous Inference in General Parametric Models." *Biometrical Journal* 50(3): 346–363. https://doi.org/10.1002/bimj.200810425.

Hülsmann, M., H. von Wehrden, A. M. Klein, and S. D. Leonhardt. 2015. "Plant Diversity and Composition Compensate for Negative Effects of Urbanization on Foraging Bumble Bees." *Apidologie* 46(6): 760–770. https://doi.org/10.1007/s13592-015-0366-x.

Jamieson, M. A., L. A. Burkle, J. S. Manson, J. B. Runyon, A. M. Trowbridge, and J. Zientek. 2017. "Global Change Effects on Plant–Insect Interactions: The Role of Phytochemistry." *Current Opinion in Insect Science* 23: 70–80. https://doi.org/10.1016/j.cois.2017.07.009.

Jeannerod, L., A. Carlier, B. Schatz, C. Daise, A. Richel, Y. Agnan, M. Baude, and A. L. Jacquemart. 2022. "Some Bee-Pollinated Plants Provide Nutritionally Incomplete Pollen Amino Acid Resources to Their Pollinators." *PLoS One* 17(8): e0269992. https://doi.org/10.1371/journal.pone.0269992.

Jones, J., and R. Rader. 2022. "Pollinator Nutrition and Its Role in Merging the Dual Objectives of Pollinator Health and Optimal Crop Production." *Philosophical Transactions of the Royal Society B: Biological Sciences* 377(1853): 20210170. https://doi.org/10.1098/rstb.2021.0170.

Junker, R. R., R. Bleil, C. C. Daehler, and N. Blüthgen. 2010. "Intra-Floral Resource Partitioning between Endemic and Invasive Flower Visitors: Consequences for Pollinator Effectiveness." *Ecological Entomology* 35(6): 760–67. https://doi.org/10.1111/j.1365-2311.2010.01237.x.

Junker, R. R., and D. Tholl. 2013. "Volatile Organic Compound Mediated Interactions at the Plant-Microbe Interface." *Journal of Chemical Ecology* 39(7): 810–825. https://doi.org/10.1007/s10886-013-0325-9.

Kelber, A. 2003. "Sugar Preferences and Feeding Strategies in the Hawkmoth *Macroglossum stellatarum*." *Journal of Comparative Physiology A* 189(9): 661–66. https://doi.org/10.1007/s00359-003-0440-0.

Koch, H., M. J. Brown, and P. C. Stevenson. 2017. "The Role of Disease in Bee Foraging Ecology." *Current Opinion in Insect Science* 21: 60–67. https://doi.org/10.1016/j.cois.2017.05.008.

Koch, H., J. Woodward, M. K. Langat, M. J. F. Brown, and P. C. Stevenson. 2019. "Flagellum Removal by a Nectar Metabolite Inhibits Infectivity of a Bumblebee Parasite." *Current Biology* 29(20): 3494–3500.e5. https://doi.org/10.1016/j.cub.2019.08.037.

Lau, P., P. Lesne, R. J. Grebenok, J. Rangel, and S. T. Behmer. 2022. "Assessing Pollen Nutrient Content: A Unifying Approach for the Study of Bee Nutritional Ecology." *Philosophical Transactions of the Royal Society B: Biological Sciences* 377(1853): 20210510. https://doi.org/10.1098/rstb.2021.0510.

Lau, P. W., I. L. Esquivel, K. A. Parys, K. L. J. Hung, and P. Chakrabarti. 2023. "The Nutritional Landscape in Agroecosystems: A Review on How Resources and Management Practices Can Shape Pollinator Health in Agricultural Environments." *Annals of the Entomological Society of America* 116(5): 261–275. https://doi.org/10.1093/aesa/saad023.

Leonhardt, S. D., B. Peters, and A. Keller. 2022. "Do Amino and Fatty Acid Profiles of Pollen Provisions Correlate with Bacterial Microbiomes in the Mason Bee *Osmia bicornis*?" *Philosophical Transactions of the Royal Society B: Biological Sciences* 377(1853): 20210171. https://doi.org/10.1098/rstb.2021.0171.

Long, J. A. 2022. "*Interactions*: Comprehensive, User-Friendly Toolkit for Probing Interactions." R Package Version 1.1.6. https://cran.r-project.org/package=interactions.

Mao, W., M. A. Schuler, and M. R. Berenbaum. 2013. "Honey Constituents Up-Regulate Detoxification and Immunity Genes in the Western Honey Bee *Apis mellifera*." *Proceedings of the National Academy of Sciences of the United States of America* 110(22): 8842–46. https://doi.org/10.1073/pnas.1303884110.

Morrant, D., S. R. Schumann, and S. Petit. 2009. "Field Methods for Sampling and Storing Nectar from Flowers with Low Nectar Volumes." *Annals of Botany* 103(3): 533–542. https://doi.org/10.1093/aob/mcn241.

Nicolson, S. W. 2011. "Bee Food: The Chemistry and Nutritional Value of Nectar, Pollen and Mixtures of the Tw2." *African Zoology* 46(2): 197–204. https://doi.org/10.1080/15627020.2011.11407495.

Nicolson, S. W., S. Da Silva Das, H. H. Neves, and C. W. W. Pirk. 2018. "Digestibility and Nutritional Value of Fresh and Stored Pollen for Honey Bees (*Apis mellifera scutellata*)." *Journal of Insect Physiology* 107: 302–8. https://doi.org/10.1016/j.jinsphys.2017.12.008.

Niño, E. L., S. Yokota, W. H. O. Stacy, and S. H. Arathi. 2022. "Dietary Phytochemicals Alter Hypopharyngeal Gland Size in Honey Bee (*Apis mellifera* L.) Workers." *Heliyon* 8(9): e10452. https://doi.org/10.1016/j.heliyon.2022.e10452.

Oksanen, J., G. Simpson, F. Blanchet, R. Kindt, P. Legendre, P. Minchin, R. O'Hara, et al. 2022. "Vegan: Community Ecology Package." R Package Version 2.6-4. https://CRAN.R-project.org/package=vegan.

Palmer-Young, E. C., I. W. Farrell, L. S. Adler, N. J. Milano, P. A. Egan, R. R. Junker, R. E. Irwin, and P. C. Stevenson. 2019. "Chemistry of Floral Rewards: Intra- and Interspecific Variability of Nectar and Pollen Secondary Metabolites across Taxa." *Ecological Monographs* 89(1): e01335. https://doi.org/10.1002/ecm.1335.

Pamminger, T., R. Becker, S. Himmelreich, C. W. Schneider, and M. Bergtold. 2019. "Pollen Report: Quantitative Review of Pollen Crude Protein Concentrations Offered by Bee Pollinated Flowers in Agricultural and Non-Agricultural Landscapes." *PeerJ* 7: e7394. https://doi.org/10.7717/peerj.7394.

Pioltelli, E. 2024. "Dataset on Pollen and Nectar." Figshare. Dataset. https://doi.org/10.6084/m9.figshare.25140092.v1.

Pioltelli, E., L. Guzzetti, M. O. Larbi, R. Celano, A. L. Piccinelli, A. Galimberti, P. Biella, and M. Labra. 2024. "Land Use Influences the Nutrient Concentration and Composition of Pollen and Nectar Rewards of Wildflowers in Human-Dominated Landscapes." *Science of the Total Environment* 29(908): 168130. https://doi.org/10.1016/j.scitotenv.2023.168130.

Pioltelli, E., L. Guzzetti, L. Tonietti, A. Copetta, P. Biella, L. Campone, and A. Galimberti. 2023. "E-PoSa: A Novel and

Effective Tool for Sampling Pollen Directly from Flowers." *Methods in Ecology and Evolution* 1–9: 51–59. https://doi.org/10.1111/2041-210X.14241.

Pioltelli, E., C. Sartirana, A. Copetta, M. Brioschi, M. Labra, and L. Guzzetti. 2023. "*Vigna unguiculata* L. Walp. Leaves as a Source of Phytochemicals of Dietary Interest: Optimization of Ultrasound-Assisted Extraction and Assessment of Traditional Consumer Habits." *Chemistry & Biodiversity* 20: e202300797. https://doi.org/10.1002/cbdv.202300797.

Potts, S. G., V. Imperatriz-Fonseca, H. T. Ngo, M. A. Aizen, J. C. Biesmeijer, T. T. D. Breeze, L. V. Dicks, et al. 2016. "Safeguarding Pollinators and Their Values to Human Well-Being." *Nature* 540: 220–29. https://doi.org/10.1038/nature20588.

Power, E. F., D. Stabler, A. M. Borland, J. Barnes, and G. A. Wright. 2018. "Analysis of Nectar from Low-Volume Flowers: A Comparison of Collection Methods for Free Amino Acids." *Methods in Ecology and Evolution* 9(3): 734–743. https://doi.org/10.1111/2041-210X.12928.

Richardson, L. L., L. S. Adler, A. S. Leonard, J. Andicoechea, K. H. Regan, W. Z. E. Anthony, J. S. Manson, and R. E. Irwin. 2015. "Secondary Metabolites in Floral Nectar Reduce Parasite Infections in Bumblebees." *Proceedings of the Royal Society B: Biological Sciences* 282(1803): 20142471. https://doi.org/10.1098/rspb.2014.2471.

Riveros, A. J., and W. Gronenberg. 2022. "The Flavonoid Rutin Protects the Bumble Bee Bombus Impatiens against Cognitive Impairment by Imidacloprid and Fipronil." *Journal of Experimental Biology* 225(17): jeb244526. https://doi.org/10.1242/jeb.244526.

Rocchetti, G., S. Castiglioni, G. Maldarizzi, P. Carloni, and L. Lucini. 2019. "UHPLC-ESI-QTOF-MS Phenolic Profiling and Antioxidant Capacity of Bee Pollen from Different Botanical Origin." *International Journal of Food Science & Technology* 54(2): 335–346. https://doi.org/10.1111/ijfs.13941.

Romeis, J., and F. L. Wäckers. 2000. "Feeding Responses by Female Pieris Brassicae Butterflies to Carbohydrates and Amino Acids." *Physiological Entomology* 25(3): 247–253. https://doi.org/10.1046/j.1365-3032.2000.00188.x.

Ruedenauer, F. A., D. Raubenheimer, D. Kessner-Beierlein, N. Grund-Mueller, L. Noack, J. Spaethe, and S. D. Leonhardt. 2020. "Best be(e) on Low Fat: Linking Nutrient Perception, Regulation and Fitness." *Ecology Letters* 23(3): 545–554. https://doi.org/10.1111/ele.13454.

Russell, K. A., and Q. S. McFrederick. 2022. "Elevated Temperature May Affect Nectar Microbes, Nectar Sugars, and Bumble Bee Foraging Preference." *Microbial Ecology* 84(2): 473–482. https://doi.org/10.1007/s00248-021-01881-x.

Stevenson, P. C., H. Koch, S. W. Nicolson, and M. J. F. Brown. 2022. "Natural Processes Influencing Pollinator Health." *Philosophical Transactions of the Royal Society B: Biological Sciences* 377(1853): 20210154. https://doi.org/10.1098/rstb.2021.0154.

Stevenson, P. C., S. W. Nicolson, and G. A. Wright. 2017. "Plant Secondary Metabolites in Nectar: Impacts on Pollinators and Ecological Functions." *Functional Ecology* 31(1): 65–75. https://doi.org/10.1111/1365-2435.12761.

Vanderplanck, M., H. Gilles, D. Nonclercq, P. Duez, and P. Gerbaux. 2020. "Asteraceae Paradox: Chemical and Mechanical Protection of Taraxacum Pollen." *Insects* 11(5): 304. https://doi.org/10.3390/insects11050304.

Vanderplanck, M., R. Moerman, P. Rasmont, G. Lognay, B. Wathelet, R. Wattiez, and D. Michez. 2014. "How Does Pollen Chemistry Impact Development and Feeding Behaviour of Polylectic Bees?" *PLoS One* 9(1): e86209. https://doi.org/10.1371/journal.pone.0086209.

Vasconcellos, N. C., and L. Freitas. 2007. "Floral Resources for Hummingbirds in the Arboretum of the Rio de Janeiro Botanical Garden, Brazil." *Davidsonia* 18: 3–15.

Vasiliev, D., and S. Greenwood. 2021. "The Role of Climate Change in Pollinator Decline across the Northern Hemisphere Is Underestimated." *Science of the Total Environment* 775: 145788. https://doi.org/10.1016/j.scitotenv.2021.145788.

Vaudo, A. D., L. M. Farrell, H. M. Patch, C. M. Grozinger, and J. F. Tooker. 2018. "Consistent Pollen Nutritional Intake Drives Bumble Bee (*Bombus impatiens*) Colony Growth and Reproduction across Different Habitats." *Ecology and Evolution* 8(11): 5765–76. https://doi.org/10.1002/ece3.4115.

Vaudo, A. D., H. M. Patch, D. A. Mortensen, J. F. Tooker, and C. M. Grozinger. 2016. "Macronutrient Ratios in Pollen Shape Bumble Bee (*Bombus impatiens*) Foraging Strategies and Floral Preferences." *Proceedings of the National Academy of Sciences of the United States of America* 113(28): E4035–E4042. https://doi.org/10.1073/pnas.1606101113.

Vaudo, A. D., J. F. Tooker, C. M. Grozinger, and H. M. Patch. 2015. "Bee Nutrition and Floral Resource Restoration." *Current Opinion in Insect Science* 10: 133–141. https://doi.org/10.1016/j.cois.2015.05.008.

Venjakob, C., F. A. Ruedenauer, A. M. Klein, and S. D. Leonhardt. 2022. "Variation in Nectar Quality across 34 Grassland Plant Species." *Plant Biology* 24(1): 134–144. https://doi.org/10.1111/plb.13343.

Wenzel, A., I. Grass, V. V. Belavadi, and T. Tscharntke. 2020. "How Urbanization Is Driving Pollinator Diversity and Pollination – A Systematic Review." *Biological Conservation* 241: 108321. https://doi.org/10.1016/j.biocon.2019.108321.

Wickham, H. 2016. *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag. https://ggplot2.tidyverse.org.

Woodcock, T. S., B. M. H. Larson, P. G. Kevan, D. W. Inouye, and K. Lunau. 2014. "Flies and Flowers II: Floral Attractants and Rewards." *Journal of Pollination Ecology* 12: 63–94. https://doi.org/10.26786/1920-7603(2014)5.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Pioltelli, E., L. Guzzetti, A. Copetta, M. Labra, A. Galimberti, W. Guidi Nissim, and P. Biella. 2024. "Assessing the Analytical Reliability of Traditional and Novel Sampling Methods for the Study of Flower Rewards Quality." *Ecosphere* 15(3): e4796. https://doi.org/10.1002/ecs2.4796