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CHEMICAL COMPOSITION OF FLORAL NECTAR FROM A RARE NORTH AMERICAN TERRESTRIAL ORCHID, *PLATANTHERA INTEGRILABIA* (CORRELL) LUER

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Abstract

Orchid flowers employ a bewildering array of clever strategies aimed at maintaining pollinator interest. These include visual and olfactory cues (*e.g.*, colours, patterns, chemical fragrances *etc.*) and a reward that consists of sugar-rich nectar, oils, and/or waxes. Information on orchid nectar has been scanty especially concerning the types of sugars present and their percentages. The present paper reports the chemical compounds present in a rare North American terrestrial orchid, *Platanthera integrilabia* (Correll) Luer, for the first time. In August of 2023, during peak flowering, nectar samples from two different natural populations were collected in Kentucky and subsequently analyzed in the laboratory using gas chromatography mass spectrometry (GC/MS) and high-performance liquid chromatography (HPLC). Three sugars were detected: sucrose, fructose, and glucose. The ratio of sucrose to fructose to glucose was 45.1:4.6:1.0, respectively. For sucrose to hexose, the ratio was 8:1, and for fructose to glucose the ratio was 4.6:1.0. Using GC/MS, the presence of other compounds, namely ribitol and gluconic acid was detected. An assessment of amino acids by HPLC-DAD demonstrated the presence of glutamic acid, glycine, and leucine. The morphology of *P. integrilabia* floral parts, coupled with lower sugar content, and low sucrose/hexose ratios recorded by the present study, clearly point to Lepidoptera pollination especially by hawk moths (Sphingidae), and to a lesser extent, larger butterflies (Hesperiidae, Papilionidae).

Introduction

ORCHIDS DIFFER from many other angiosperms by having complicated life-history strategies, one involving sophisticated floral mechanisms to facilitate cross-pollination. This is exemplified by the column- a unique structure in the center of the flower that houses both the androecium and the gynoecium. Within the androecium is a single fertile anther composed of 2-6 compact bundles of pollen (= pollinia) attached to a secondary structure, usually a short stalk with a sticky attachment point (= viscidium). Collectively, these structures are termed as the pollinarium (Christenhusz *et al*., 2018; Dressler, 1981). This structure, which essentially serves as a *Pollen package* carried intact by the pollinator, is nestled within the column at the apex, out of reach of most visiting insects except those uniquely capable of contacting, removing, and transporting it to the stigma of another flower.

To ensure that the pollinarium is delivered to another individual of the same species, orchid flowers employ a bewildering array of clever strategies aimed at maintaining pollinator interest. These include visual and olfactory cues (*e.g.*, colours, patterns, chemical fragrances *etc.*) and a reward that consists of sugar-rich nectar, oils, and/or waxes (Ackerman, 1986; Brzosko and Mirski, 2021; Dressler, 1981; Tremblay *et al*., 2005). A surprising number of orchid species (30-40%) deceive their pollinators by offering little or no reward, but those that do generally have greater reproductive success, especially for species that offer nectar (Brzosko and Mirski, 2021). The primary sugars present in flower nectar are sucrose, glucose, and fructose, and to a lesser extent, mannose, xylose, and maltose (Brzosko and Mirski, 2021). Because different pollinator preference is influenced by sugar composition as well as sugar concentration, knowing more about the specific kinds of carbohydrates found in nectar, their ratios, and their role(s) on pollinator behavior, is of considerable interest.

Information on orchid nectar has been scanty especially concerning the types of sugars present and their percentages. Nevertheless, Brzosko and Mirski (2021) analyzed nectar data from 110 orchid species belonging to 36 genera using literature published between 1984-2021, calculating sugar ratios in 43 cases. They reported that generalist orchids (*i.e.*, those that rely on many different kinds of pollinators) prefer hexose-rich nectar dominated by glucose over fructose. In orchids pollinated by birds, moths and wasps, the sucrose/hexose ratios were lower as compared to those catering to bees and butterflies. In Lepidoptera-pollinated species, sugars are less concentrated (more diluted), presumably to facilitate

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uptake by their long tubular proboscis. Interestingly, orchids with the longest nectar spurs (>5 cm) were pollinated by a fewer number (1.7) of species, whereas orchids with shorter spurs had double the number (3.3-3.7). Thus, the longer the nectar spur, the more likely, the orchid is a specialist, targeting one or two pollinator species. As interesting as this may be, Brzosko and Mirski (2021) revealed a sizable information gap on a global level with respect to orchid nectar and pollination, as less than 0.5% of all known orchid species were included in their 2021 study. Considering that many aspects of orchid biology have been thoroughly studied dating back to Darwin (1877), this paucity of information involving nectar is startling (Brzosko and Mirski, 2021). Moreover, little is also known about orchid pollination and pollinators, exemplified by the genus *Platanthera* Rich. containing *ca*. 200 species worldwide (Janes *et al*., 2024). For rare specialist orchids faced with extinction, identifying the factors ensuring orchid pollination success becomes an urgent conservation priority.

The white fringeless orchid, *Platanthera integrilabia* (Correll) Luer (Figs. 1-2), is a U.S. Federally threatened terrestrial species native to semi-shaded wetlands along the Cumberland Plateau in the SE United States, primarily in three states (Georgia, Kentucky, and Tennessee). The floral characteristics exhibited by this species include a long (41-55 mm) nectar spur, uniform white colour, and sweet-smelling fragrance that intensify towards nightfall- all suggestive of hawk moth (Sphingidae) pollination. In a previous study, Zettler *et al.* (1996) documented three diurnal Lepidoptera pollinators of this orchid in Tennessee consisting of a skipper (Hesperiidae) and two swallowtail butterflies (Papilionidae). More recently, Littlefield (unpubl. data) observed that *P. integrilabia* was pollinated by a diurnal hawk moth, *Hyles lineata*, in Kentucky. Other insects, including butterflies and two Hymenoptera species were observed seeking nectar, but did not carry pollinia. Because *P. integrilabia* is threatened with extinction and its pollination ecology remains unresolved, the present study examined the chemical constituents of floral nectar so as to augment what little is known about this important topic. In particular, we wanted to document the types of sugars and their ratios to determine if this orchid might be targeting nocturnal or diurnal pollinators, especially Lepidoptera. In addition to sugars, we also wanted to know what other chemical compounds may be present (*e.g.*,

Fig. 1. A population of *Platanthera integrilabia* in Kentucky during peak flowering during August 2022. This population lies beneath a powerline in an open, sun-exposed area actively managed to remove encroachment by hardwoods.

Fig. 2. Inflorescence of *Platanthera integrilabia* in Kentucky with seven open flowers, prior to pollination. Note the long nectar spurs and white uniform coloration.

amino acids) and offer insight into their potential role(s).

Material and Methods

Field Sites

Platanthera integrilabia grows in acid seeps (wetland sites) in Kentucky and throughout the Cumberland Plateau region of the United States (Fig. 3). Wetlands supporting this species consist of a surrounding overstory and thin midstory of mixed hardwood trees (*e.g.*, *Acer rubrum*, *Nyssa sylvatica,* and *Quercus alba*) and pines (*Pinus echinata*), and a shrub layer consisting of *Ilex verticillata*/*opaca*, and *Alnus serrulata*. The understory typically consists of an herbaceous layer of native ferns, sedges and forbs that may, at times, compete with *P. integrilabia* for space and light. These wetland sites also have a shallow water table, with standing or flowing surface moisture linked to rain events. During seasonal dormancy and/or dry periods, soil moisture is retained by a dense carpet of sphagnum moss overlying the mineral soil where the orchid's delicate root systems are embedded. While the majority of populations occur in sites shaded by a canopy of trees, some Kentucky populations exist in open sites such as areas with overhead powerlines (Fig. 1) where utility companies and state and federal agencies control the growth of larger woody vegetation that would otherwise dominate the overstory.

Nectar Collection

Nectar was collected from *Platanthera integrilabia* during peak flowering (25 August, 2023) from two open wetland habitats beneath powerlines in Laurel and Pulaski counties in Kentucky. The nectar was collected with an unused graduated Drummond® PCR pipette (Drummond® #5-000-1001-X10; Fig. 4) and transferred to a clean 0.5 ml Eppendorf™ Safe-Lock Tube (Eppendorf™ #022363611). All samples were kept cool in the field using an insulated container sheltered from direct sunlight and promptly stored under refrigeration (*ca.* 4°C) prior to shipping 24-48 hr later. Samples were promptly shipped in an insulated container from Kentucky to Illinois College via overnight priority mail. Upon arrival, the samples were stored at or below 0°C until chemical analysis was carried out.

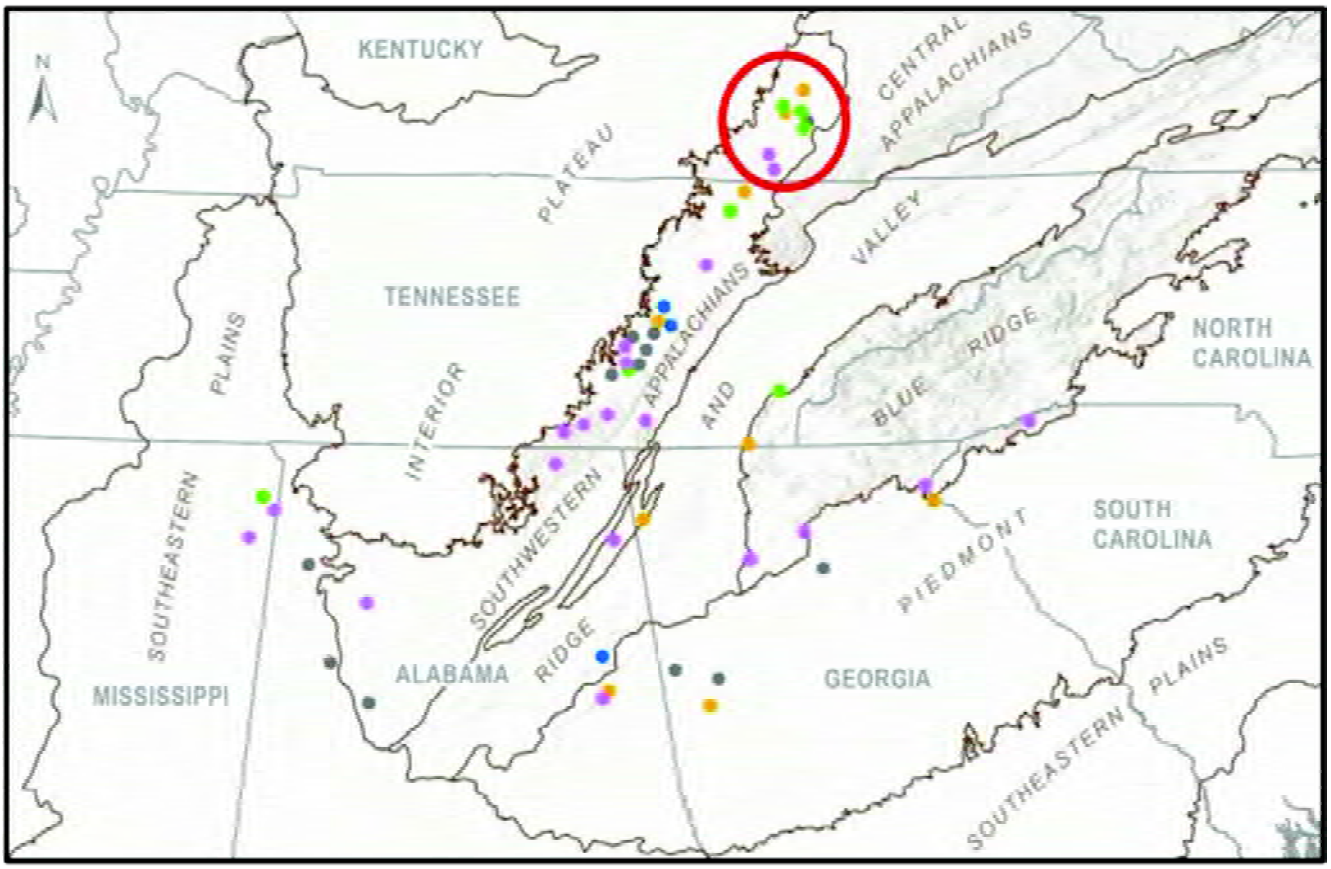

Fig. 3. Distribution of *Platanthera integrilabia* in the SouthEastern United States. Populations in Kentucky are indicated within the red circle (U.S. Fish & Wildlife Service, 2021).

Fig. 4. Nectar is collected from the nectar spur of a *Platanthera integrilabia* flower using a microcapillary pipette.

GC-MS Nectar Analysis

Identification of ribitol and gluconic acid was achieved by gas chromatography and mass spectrometry (GC-MS) after trimethyl silyl (TMS) derivatization of nectar samples (Pais and Chaves Das Neves, 1980). Water was removed from the nectar samples by placing an opened Eppendorf™ tube containing the sample in a vacuum chamber with Drierite® under high vacuum at ambient temperature for 17 hr. The residue that remained was dissolved with anhydrous N,N-dimethylformamide (10 µL), the solution was vortexed for 10 sec, and then transferred to a 250 µL glass insert with polymer feet (Agilent #5181-1270) situated in a 2 mL A-Line screw top vial (Agilent 5190-9589) with a 12 mm cap (Agilent #5182-0720). N,O-(bistrimethylsilyl) trifluoroacetamide (BSTFA) (20 µL) was added via micro-syringe, the capped vial was vortexed for 10 sec, and then heated at 65°C for 15 min.

The derivatized samples were analyzed on an Agilent 6890/5973 coupled GC-MS. Sample (1 µL) was introduced using a 10:1 split injection at 230°C. A 30 m DB-5MS + 10m Duraguard Agilent J & W GC column (Agilent # 122-5532G) with 0.25 mm ID and a film thickness of 0.25 µm was used with helium carrier gas at constant flow with an average velocity of 37 cm/sec. The GC oven was programmed with an initial temperature of 70°C held for 4 min followed by a temperature ramp at a rate of 10°C/min to a final temperature of 325°C. The final temperature was maintained for 16 min. The injector and MS transfer line temperature were maintained at 230°C. The ion source temperature was 220°C with EI ionization at 70 eV and the quadrupole scan range set to m/z 50 to 600 with a scan rate of 2.7 scan/sec. The GC/MS data were processed using Agilent Chemstation version 3.02. Compounds were identified by comparison with NIST mass spectral library Version 2.0.

HPLC-RID Nectar Analysis

Relative mass concentration of fructose, glucose, and sucrose was determined by high performance liquid chromatography with a refractive index detector (HPLC-RID). Nectar and reference samples were diluted with Type I grade water. Aliquots were transferred to a 250 µL glass insert with polymer feet (Agilent #5181-1270) situated in a 2 mL A-Line screw top vial (Agilent 5190-9589) sealed with a 12 mm cap (Agilent #5182-0720). Samples were analyzed on an Agilent 1220 Infinity HPLC. Separation of the carbohydrates was achieved on an Agilent ZORBAX® Carbohydrate Analysis column (4.6 × 150 mm) (Agilent #843300-908) held at 30°C with an isocratic mobile phase (80% acetonitrile and 20% water by volume), 1.5 mL / min flow rate, and 15-min run time. Peaks were detected with an Agilent 1260 Infinity II Refractive Index Detector (RID) maintained at 35°C. Peak identification and concentration determination was achieved by comparison with prepared reference solutions of fructose, glucose, and sucrose. Integration of individual peaks was achieved by Agilent Open Labs ChemStation software.

HPLC-DAD Analysis

Amino acid analysis (Henderson *et al*., 2022, n.d.) by high performance liquid chromatography coupled with diode array detection (HPLC-DAD) enabled the detection of glutamic acid, glycine, and leucine. Nectar and reference samples were diluted with 0.1 N HCl. Aliquots were transferred to a 250 µL glass insert with polymer feet (Agilent #5181-1270) situated in a 2 mL A-Line screw top vial (Agilent 5190-9589) sealed with a 12 mm cap (Agilent #5182-0720). An Agilent 1220 Infinity HPLC was used for the derivatization, separation, and analysis of samples. The HPLC autosampler was used to derivatize samples prior to injection by mixing them with a solution of o-phthalaldehyde (OPA) and 3-mercaptopropionic acid (3-MPA).

An Agilent ZORBAX® Eclipse-AAA column (4.6 × 150 mm) (Agilent #964300-902) separated the derivatized primary amino acids. Two solvent systems were prepared and employed in the mobile phase of the separation. Solvent A was a pH 7.8 buffered aqueous solution of 40 mM Na_2HPO_4 and Solvent B consisted of 45% CH_3CN 45% CH_3OH, and 10% H_2O by volume. The gradient mobile phase started with 0% B, 100% A for 1.9 min; it was ramped to 57% B, 43% A over 16.2

min; ramped to 100% B, 0% A over 0.5 min; maintained at 100% B, 0% A for 3.7 min; ramped to 0% B, 100% A over 0.9 min; and maintained at 0% B, 100% A for the final 2.8 min. The column was kept at 40°C for the duration of the separation with a 1 mL / min flow rate. Peaks were detected by an Agilent 1220 Infinity Diode Array Detector at a wavelength of 338 nm. Peak identification was achieved by comparison with a reference solution (Aldrich AA-S-18).

Results and Discussion

Nectar collected from *Platanthera integrilabia* in Kentucky and subsequently analyzed by GC/MS and HPLC, revealed the presence of three sugars: sucrose, fructose, and glucose (Fig. 5). Overall, the ratio of sucrose (S) to fructose (F) to glucose (G) was 45.1:4.6:1.0. For sucrose to hexose (S: F + G) the ratio was 8:1, respectively. For fructose to glucose (F: G) the ratio was 4.6:1.0, respectively. Using GC/MS, the presence of other compounds, namely ribitol and gluconic acid was also detected (Fig. 6). An assessment of amino acids by HPLC-DAD demonstrated the presence of glutamic acid, glycine, and leucine (Fig. 7). To our knowledge, this is the first report documenting chemical compounds in floral nectar in this species and one of the few such reports on the North American continent. In South Florida, Chandler *et al*. (2022) applied the same techniques to the moth-pollinated ghost orchid, *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, which also revealed the presence of these three sugars. In addition, they detected three acids (lactic, malic, threonic) as well as 4-hydroxyl benzyl alcohol. Although they did not include information on the ratios of the three sugars, presence of sugars in *D. lindenii*, together with our findings for *P. integrilabia*, supports the assertion that these two species provide their pollinators with a sugar reward typical of many other orchid species worldwide (Brzosko and Mirski, 2021), and that other compounds are present in nectar in addition to sugars.

Zettler *et al.* (1996) sampled floral nectar from 109 flowers of *P. integrilabia* in Tennessee during a 24 hr period. Using a Bellingham and Stanley pocket refractometer, they revealed a mean sugar content of 18.9% (range = 10-23%), but the identity of these sugars was not reported. They did, however, record nectar volume in each spur and documented a mean of 4.4 µl (range = 0.8-19.9 µl). Interestingly, fluctuations in nectar volume and sugar concentration over the 24 hr period were not significantly different. Compared to butterfly-pollinated orchids that generally have a higher sugar content (26%) as reported by Brzosko and Mirski (2021), nectar in *P. integrilabia* is more diluted paralleling values

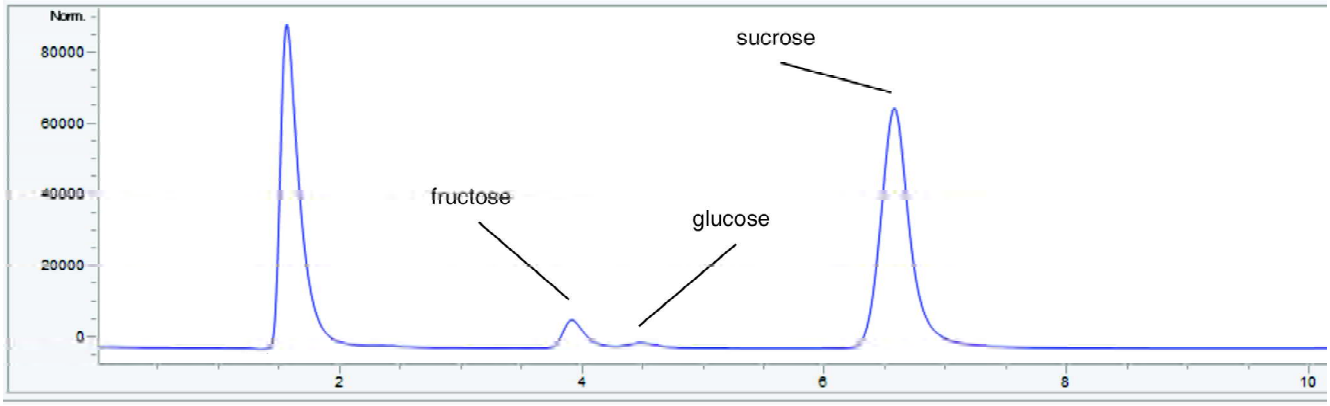

Fig. 5. Results of HPLC-RID providing the relative mass concentration of fructose, glucose, and sucrose.

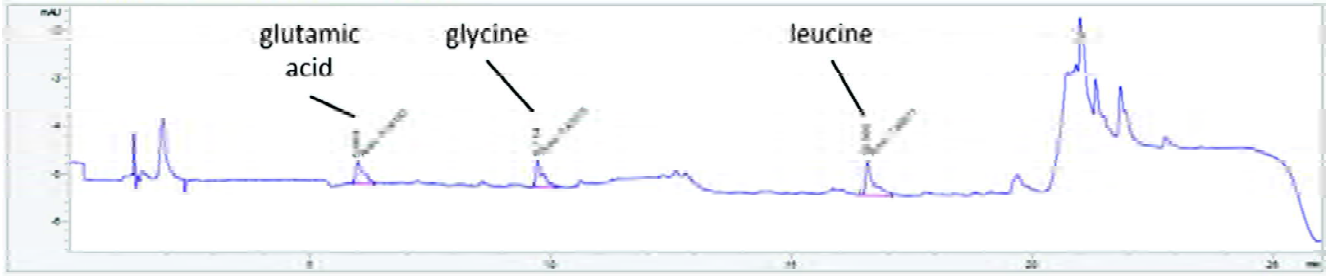

Fig. 6. Results of HPLC-DAD demonstrating the detection of glutamic acid, glycine, and leucine.

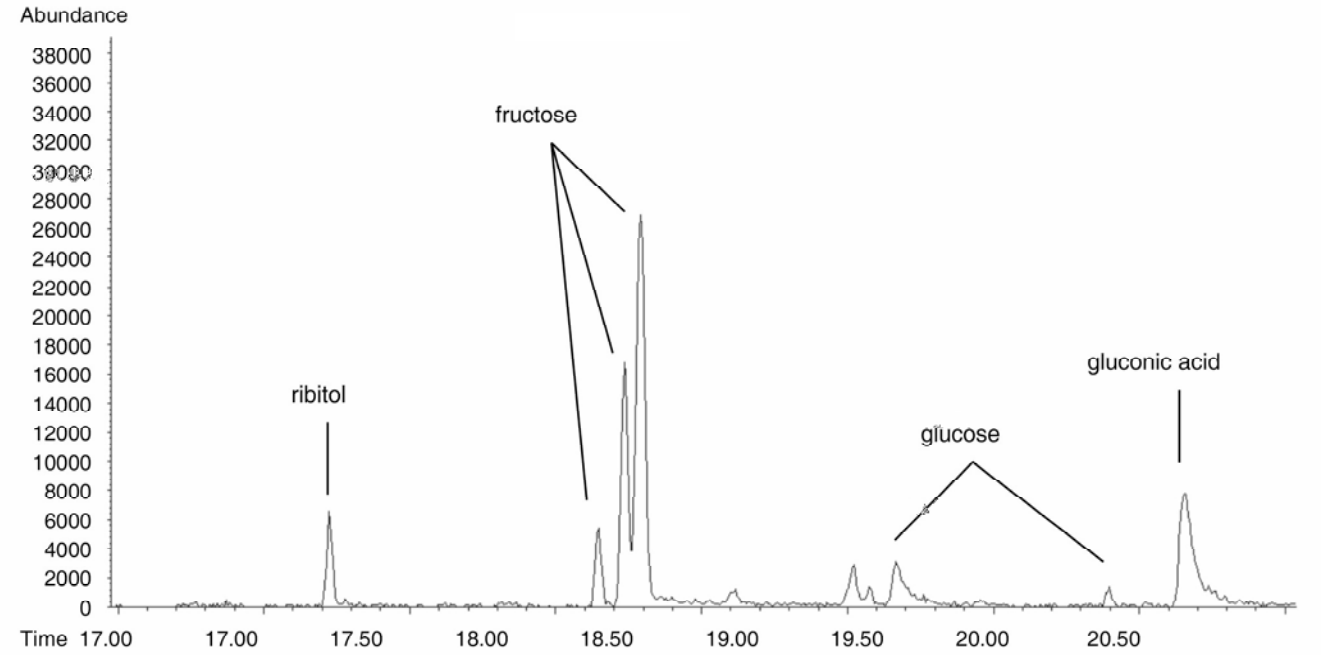

Fig. 7. Results of GS-MS identification of ribitol and gluconic acid.

found in moth-pollinated orchids. Moreover, the nectar spur length of this orchid was relatively long (3.5-5 cm; Fig. 1; GoOrchids, 2024; Zettler and Fairey, 1990) as compared to other related *Platanthera* species pollinated by butterflies and even bees in the region (*e.g.*, *P. blephariglottis* = 1.5-5 cm, *P. ciliaris* = 2.0-3.5 cm, *P. cristata* = 0.4-1.0 cm, *P. grandiflora* = 1.5-3.5 cm). The only North American *Platanthera* species with nectar spurs of comparable length to *P. integrilabia* are the prairie fringed orchids of the Midwest (*P. leucophaea* = 2.8-4.7 cm, *P. praeclara* = 3.6-6.4 cm; GoOrchids, 2024), both of which have white flowers and are pollinated by hawk moths (GoOrchids, 2024; Pollack, 2009; Sheviak and Bowles, 1986). Although we did not observe pollinia on butterflies that visited *P. integrilabia* inflorescences in Kentucky, Zettler *et al*. (1996) did document butterfly pollination in Tennessee but reported that pollinia removal was infrequent despite numerous flower visits. They attributed the phenomenon to the width of their compound eyes being narrower (42-45 mm) than the distance between the two viscidia (55 mm). According to P. Catling (in Zettler, 1999; pers. com.), it is not uncommon for visiting insects to receive only one of the two pollen sacs per flower visit which increases the likelihood that pollen from one individual will sire numerous offsprings on two different plants. Taken together, the morphology of *P. integrilabia* floral parts, coupled with lower sugar content, and low sucrose/hexose ratios recorded by the present study, clearly point to Lepidoptera pollination especially by hawk moths (Sphingidae), and to a lesser extent, the larger butterflies (Hesperiidae, Papilionidae).

A Dark Side to Nectar Chemistry?

Abiotic factors present in orchid habitats (*e.g.*, soil chemistry, sunlight) may influence nectar traits and these traits may differ amongst populations of the same orchid species (Brzosko and Mirski, 2021; Gardener and Gillman, 2002; Gijbels *et al*., 2014). Wasserthal (1997), for example, noted a lower nectar concentration for an orchid (*Angraecum sororium*) that grew beneath a tree canopy in Madagascar than would be expected given the lower light levels available to generate sugars from photosynthesis. Sadler *et al*. (2011) proposed that some of the carbon compounds present in orchid floral fragrances may originate from mycorrhizal fungi (mycotrophy), not just photosynthetic pathways. Similarly, Chandler *et al.* (2022) went a step further by suggesting that mycotrophy may contribute to carbon compounds (*e.g.*, sugars) found in orchid nectar. Given that *P. integrilabia* associates with mycorrhizal fungi (*Tulasnella inquilina*) into maturity throughout its range (Currah *et al*., 1997), we cannot deny the likelihood that some of the compounds present in nectar originated from mycotrophy, at least in part, especially for individuals that grow in more shaded habitats.

Growing in shade may also influence pollinators, and therefore, pollination. For instance, sun-exposed

habitats may provide pollinators with more sugar-rich nectar linked to higher rates of photosynthesis, thereby catering more to diurnal pollinators such as bees and butterflies. Indeed, orchid flowers that target bees and butterflies generally have higher sugar concentrations (40% and 26%, respectively) as compared to moth-pollinated orchids (Brzosko and Mirski, 2021). To minimize competition between related species (*e.g*., *Platanthera ciliaris*) and to maximize cross-pollination success, *P. integrilabia* may have adapted to more shaded habitats on the Cumberland Plateau by targeting different pollinators, perhaps evolving into a pollinator specialist species. This concept seems reasonable given that no other orchid species native to the region has flowers better suited for hawk moth pollination (*e.g*., white colouration, long nectar spurs, and strong, sweet-smelling evening fragrance; Faegri and van der Pijl, 1979). Moreover, lower nectar sugar concentrations would reduce the demand for more sugar generated from photosynthesis which may be preferable to moths because it is more diluted, expediting their feeding. New research is being planned to compare nectar from *P. integrilabia* in more open areas versus shady habitats so as to determine if, and to what extent, nectar composition may differ. Such knowledge could assist land managers, for example, in making informed decisions regarding the removal (thinning) of encroaching vegetation, thereby opening up orchid habitats to more sunlight. Furthermore, if future research demonstrates that *P. integrilabia,* is a pollinator specialist, knowing more about the ecology and life histories of these insect species (larvae and mature stages) will be vital to the long-term conservation of this orchid in the natural setting.

Why Amino Acids?

While sugars provide energy for pollinator flight muscles and other metabolic functions, amino acids are known to influence nectar taste and they may, in fact, be more important than their nutritive value (Gardener and Gillman, 2002; Zhang and Gao, 2017). Gardner and Gillman (2002) reported that plants pollinated by butterflies have nectar with a higher concentration of amino acids as compared to those pollinated by birds or flies. While it is not known if moth nectar has a similar amino acid load to those species which are pollinated by butterflies, the presence of three amino acids (glutamic acid, glycine, and leucine) revealed herein argue in support of further studies, in this direction. Establishing a link between these three amino acids and taste, for example, may shed more light on how orchids like *P. integrilabia* maintain pollinator interest.

Conclusion

Platanthera integrilabia nectar is composed of a mixture of different sugars and other chemical compounds (amino acids, ribitol, gluconic acid) that cater to lepidoptera pollinators. Why these other non-sugar chemical compounds are present remains to be determined. Future studies are underway to document and quantify additional chemicals that may be present in *P. integrilabia* nectar for orchids inhabiting shaded vs. more open sites, as well the nectar from co-habiting species (*e.g*., *P. ciliaris*). In addition, remote camera traps are being used to record insect pollinators of this orchid throughout its range to ascertain if there are any links between these insects and nectar chemistry. To conclude, the present information will be useful for enhancing conservation management practices in Kentucky and throughout the region.

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