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SUGAR CONTENT OF THE NECTARY EXUDATE OF *EPIPACTIS ATROPURPUREA* RAFIN

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ABSTRACT

In order to study the sugar content of the nectary of the spontaneous orchid *Epipactis atropurpurea* Rafin., a procedure for the analysis of complex mixtures of carbohydrates was developed. These were analysed as the corresponding trimethylsilyl derivatives by means of capillary gas chromatography. Application of capillary columns allowed the analysis of components in the picomole range, leading to the identification of small amounts of sorbitol, maltose, cellobiose, melibiose, and gentiobiose together with the main components fructose, glucose, and sucrose. Surprisingly, a high content of raffinose was also found.

Key-Words : *Epipactis* - Gas chromatography - Nectaries - Orchids - Sugars.

INTRODUCTION

Floral and extrafloral nectaries have been described in a great number of plant families, but the chemical composition of the exudates has been little studied in detail (MAURIZIO, 1975).

The Orchidaceae, maybe one of the largest families of angiosperms, have been subject to some attention because of the great number of genera possessing floral nectaries. The nectar secretion of orchids was first described by DARWIN (1862) who related it with pollination mechanisms. The identification of the sugars contained in

orchid nectars has been attempted by several authors mainly by means of paper chromatography (PERCIVAL, 1961) or thin layer chromatography (ARDITTI *et al.*, 1971). BASKIN and BLISS (1969) for the first time used gas chromatography in the study of the sugar content of extrafloral exudates of tropical orchids. In the thirty species studied they were able to identify fructose, glucose, sucrose, and in some instances, raffinose as the corresponding trimethylsilyl ethers. For the preparation of these derivatives they adopted a modification of the original procedure of SWEELEY (SWEELEY *et al.*, 1963) in order to avoid the inconvenience of the tailing caused by the presence of pyridine in the solvent peak. However, this procedure led to the appearance of a significant number of extraneous peaks in the chromatogram, the unequivocal assignment of the peaks in unknown samples thus becoming difficult. This multiplicity of peaks obtained in the gas chromatography of carbohydrates trimethylsilyl ethers is a common event and a serious inconvenience for the analysis of complex mixtures by this method. Anomers of different sugars may appear in the chromatogram as superimposed peaks which makes their direct identification impossible (REID *et al.*, 1970). The use of a derivatization method affording single peaks for pure sugars, together with the highly efficient capillary columns may very well be an incomparable tool for the analysis of complex naturally occurring mixtures of carbohydrates.

We wish now to report the results obtained by the application of capillary gas chromatography to the study of the sugar content of the nectar of *Epipactis atropurpurea* Rafin. This is a spontaneous orchid in Portugal, frequently visited by insects. We thought that the particular interest of insects in this flower could be somehow related to the composition of the exudate contained in a highly differentiated cup-like nectary of large dimensions occupying practically two thirds of the lip and possessing a notable secretion capacity. If it would be so, the knowledge of the chemical composition of the nectar would be of considerable interest (POUVREAU, 1974).

MATERIALS AND METHODS

1. Collection and storage of the nectars

The exudates from the nectaries were collected by means of a glass capillary tube from flowers of about thirty plants until a volume of 3 ml was attained. After collection the nectar was immediately frozen and stored at − 25 °C for 2-4 weeks until use.

2. Gas chromatography

The analyses were carried out with a Pye Unicam 204 instrument, equipped with a flame ionization detector and a OV-101 wall coated open tubular column (WCOT) 25 m × 0.25 mm i.d. (approx. 42.000 theoretical plates). Operation conditions were as follows : carrier gas, nitrogen at a flow rate of 2 ml/min; split ratio : 1/65; injector and detector temperature 300°; temperature programme : 190° isothermal for 3 min, heating rate 6°/min, held isothermal when 280° were attained. Alternatively, a SE-52 WCOT column was used.

3. Derivatization of the samples

10 μl of the nectar mixture were measured by means of a high precision microsyringe into a 1 ml Teflon-lined screw-cap derivatization vial and evaporated to dryness under a light stream of nitrogen at reduced pressure. The residue was dried at 0.1 mm Hg over P_2O_5 for 3 h at 25°. After that time, the residue was dissolved in 20 μl of dry dimethylformamide and 40 μl of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) were added. The solution was vigorously shaken for 30 s, heated at 65° for 15 min, and used directly for gas chromatography. The volume injected was 0.1 μl. For the preparation of standards 0.1 mg of each sugar was used under the same derivatization procedure.

4. Reduction of the sugar content of the nectars

A solution of 10 μl of nectar in 100 μl of water was treated with sodium borohydride according to a known method (SWEELEY *et al.*, 1963) and the product of the reaction silylated as described.

Reduction with BH_3-THF : 10 μl of nectar were evaporated and the residue treated with 200 μl of a 1 M solution of diborane in THF, at room temperature for 30 min. The mixture was cooled in an ice bath and excess diborane was destroyed by careful addition of methanol. When hydrogen evolution stopped, a drop of water was added and the mixture treated by the above procedure.

5. Detection of components soluble in organic solvents

100 μl of nectar were dissolved in an equal amount of water and the solution was extracted three times with 100 μl of ether. The ethereal fractions were collected and the ether evaporated under a stream of nitrogen. When evaporation was complete 5 μl of ether were added to the flask and 2 μl of the obtained solution were directly injected in the chromatograph. In that case the temperature was linear programmed from 60° to 280° at a heating rate of 6°/min. An identical procedure was carried out with ethyl acetate as solvent.

6. Thin layer chromatography

For tlc analysis a solution of the nectar in water (1 : 10 v/v) was used. 5 μl of that solution were applicated on a plate coated with Silica Gel G impregnated with a 0.02 M solution of boric acid and eluted with a mixture of methyl ethyl ketone : acetic acid : methanol (12 : 3 : 3). The eluted plate was dried and again eluted in the same solvent. The spots were visualised with a diphenylamine reagent (JEFFREY *et al.*, 1969) and identified by comparison of the corresponding R_f values with those of standards. By this method, the sugars (R_f) identified were glucose (0.79, no distinction between α and β anomers), fructose (0.69), sucrose (0.55), gentiobiose (0.45, traces), and raffinose (0.27).

RESULTS AND DISCUSSION

In order to eliminate any possible individual variation, the experiments were performed in samples taken from a total volume of 3 ml of nectar obtained from a significant number of plants. Extraction of the nectar with diethyl ether or ethyl acetate and analysis of the extracts by gas chromatography did not show the presence of significant materials. This fact, together with dry residue determinations, led to the conclusion that the nectar collected from the flowers of *Epipactis atropurpurea* Rafin. is an aqueous solution, where total sugars are present in a concentration of 20 % (w/w). Derivatization studies carried out with pure standards showed that it was possible to obtain a single peak for each sugar when dimethylformamide was used as a solvent and BSTFA as a silylating agent. Under the conditions used, silylation was complete and appeared to be quantitative. No tailing was observed for the solvent

peak in the chromatogram. Analysis of mixtures of anomers of known composition in the presence of sorbitol as an internal standard showed no significant alteration of the relative proportions of each component. Furthermore, ascorbic acid which appears to give rise to two peaks under the classical conditions of derivatization (PIERCE, 1977), only gives rise to one peak under our conditions.

By the use of capillary columns and an appropriate choice of the temperature program, it is possible to accomplish total separation of the sugars in a short time with minimal amounts of sample. Thus, it was possible to obtain a clean chromatogram, free of significant extraneous peaks, where direct correlations with the peaks afforded by pure standards could unequivocally be made (Fig. 1). Fructose,

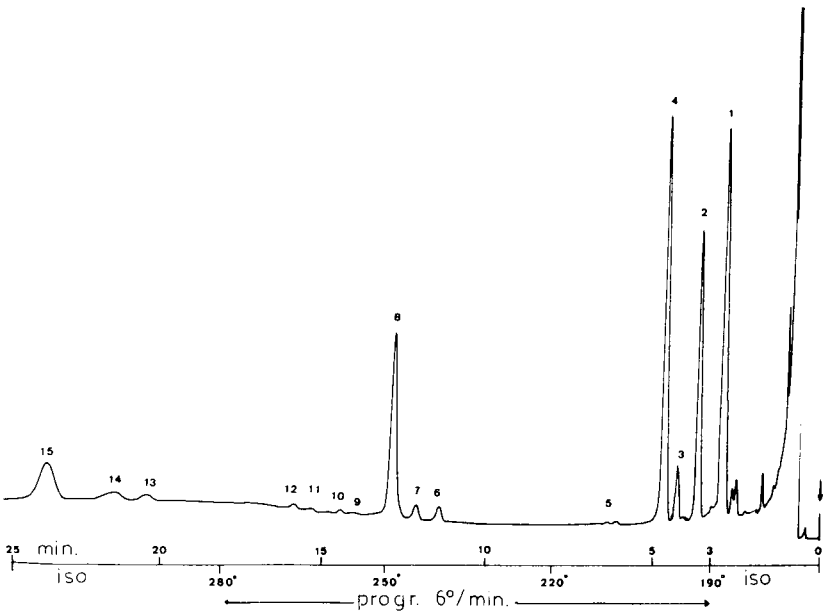

FIG. 1. — *Capillary gas chromatography of the carbohydrates in floral exudate of* Epipactis atropurpurea *Rafin. Injected volume 1 µl. Attenuation 1/64. Column OV-101, 25 m × 0.25 mm.*

1 - fructose; 2 - α-glucose; 3 - sorbitol; 4 - β-glucose; 5 - reagent impurity; 6 and 7 - unidentified; 8 - sucrose; 9 - α-maltose; 10 - β-maltose; 11 - α-cellobiose; 12 - β-cellobiose; 13 - melibiose; 14 - gentiobiose; 15 - raffinose.

α-glucose, β-glucose, sucrose, and raffinose were identified as the main components, together with significant amounts of sorbitol, melibiose, and gentiobiose. Cellobiose and maltose were also present in minor quantities (Tabl. 1).

These results were reproduced in two different columns and were partially confirmed by thin layer chromatography. Reduction of the nectar contents either with sodium borohydride or diborane in THF led to the disappearance of the peaks

TABL. 1. — *Sugar composition of the nectar of* Epipactis atropurpurea *Rafin. as determined by gas chromatography.*

Sugar	Retention time relative to sorbitol	Composition %
Fructose	0.66	26.0
α-Glucose	0.86	13.5
Sorbitol	1.00	3.0
β-Glucose	1.08	19.0
Sucrose	2.94	16.0
α-Maltose	3.16	0.1
β-Maltose	3.23	0.3
α-Cellobiose	3.49	0.1
β-Cellobiose	3.59	0.3
Melibiose	4.61	1.7
Gentiobiose	4.48	3.0
Raffinose	5.32	13.4

corresponding to the monosaccharides. Instead, only sorbitol and mannitol were found.

Quantitative results were obtained from the chromatogram after calculation of the molar response factors (MRF). These were calculated relative to sucrose from the analysis of a 1×10^{-3} M solution of each sugar in dimethylformamide and the values obtained were introduced as correcting factors in the calculation of the peak areas. The results are presented in Table 1. Owing to the high sensitivity of capillary gas chromatography, minor components could easily be analysed by working at low values of attenuation of the electromer response.

From Table 1 it can be seen that sugar anomers are found in about the same relative proportions that correspond to the ones found in aqueous solutions at mutarotation equilibrium. The most important feature is undoubtedly the unexpected high content of the nectar in raffinose. Although this trisaccharide has been previously found in some orchid nectars (PERCIVAL, 1961; BASKIN and BLISS, 1969) it could not be detected in the nectar of *Epipactis atropurpurea* by means of paper chromatography (PERCIVAL, 1961). This author, after having studied the qualitative compositions of about 900 species of 101 families of angiosperms, came to the conclusion that although maltose, melibiose, raffinose, and a fourth " unknown " oligosaccharide (which we now assume to be gentiobiose) can be found in the nectars of a significant number of species, in no case they occurred together in the same nectar. This is not found to be the case in our samples. An explanation for this lies possibly in the extraordinary analytical potentialities of capillary gas chromatography. Its low limit of detection, allowing the work in the picomole scale, together with its ability to effect separation of sugar anomers, makes this method the means of choice for the rational approach to the problems related with chemical composition of floral exudates. In the case of orchids, the question of the

physiological and biochemical role of the nectaries is still open. We feel that a systematic study of the chemical composition of nectars by means of accurate analytical methods and its possible relation with environmental conditions (RAW, 1953), could bring a new insight into the subject, especially regarding the specificity of the pollinating agents and its relation with nectar composition.

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RÉSUMÉ

TENEUR EN SUCRES DE L'EXSUDAT DES NECTAIRES D'*EPIPACTIS ATROPURPUREA* RAFIN.

L'exsudation florale et extraflorale a été décrite chez un grand nombre de familles botaniques. De ce point de vue la famille des Orchidaceae est particulièrement intéressante, étant donné le rapport entre l'exsudation des nectaires et les mécanismes de pollinisation.

La composition du nectar a été étudiée par des méthodes chromatographiques, sur papier ou en couche mince, mais elles n'ont pu fournir que des résultats partiels. La méthode de chromatographie gazeuse ne peut pas être appliquée directement à l'analyse des sucres. Il faut dérivatiser, avant l'analyse, les sucres sous forme d'éthers TMS. Un problème sérieux qui se pose dans l'analyse de mélanges complexes d'éthers TMS est celui de la multiplicité des pics obtenus généralement en chromatographie gazeuse. Cet inconvénient peut être surmonté en utilisant du N, O-bis (triméthylsilyl) trifluoroacetamide (BSTFA) comme agent de silylation. Ainsi on a pu établir une corrélation univoque entre les pics produits par le chromatogramme du nectar et ceux produits par les substances témoins pures. L'utilisation de colonnes capillaires permet à la fois une détection extrêmement fine de l'ordre des picomoles et une très haute résolution, ce qui conduit à une très bonne séparation des anomères des sucres dans un temps d'analyse restreint.

Cette méthode a été utilisée pour la détermination de la teneur en sucres de l'exsudation des nectaires floraux de l'orchidée spontanée au Portugal *Epipactis atropurpurea* Rafin. Dans la séquence nous avons identifié le fructose (26,0 %), l'α-glucose (13,5 %), le sorbitol (3,0 %), le β-glucose (19,0 %), le sucrose (16,0 %), le maltose (0,4 %), l'α et β-cellobiose (0,4 %), le mélobiose (1,7 %), le gentobiose (3,0 %) et le raffinose (13,4 %). La teneur élevée de l'exsudat en raffinose est à noter. La chromatographie en couche mince n'a pu confirmer que partiellement ces résultats.

Nous pensons que le développement d'une méthode analytique très sensible permettra une étude systématique de la composition chimique des nectars et de ses relations possibles avec les conditions du milieu, particulièrement la spécificité des agents pollinisateurs.

ZUSAMMENFASSUNG

DER ZUCKERGEHALT DES NEKTARS VON *EPIPACTIS ATROPURPUREA* RAFIN.

Florale und extraflorale Absonderungen wurden bei einer grossen Zahl von Pflanzenfamilien beschrieben. Unter diesen haben die Orchidaceae ein erhebliches Interesse gefunden. Die Zusammensetzung

ihres Nektars wurde mit dem Bestäubungsmechanismus in Beziehung gebracht. Zur Untersuchung der Nektarzusammensetzung wurden Methoden wie Papierchromatographie oder Dünnschichtchromatographie angewandt, aber sie haben nur Teilresultate erbracht.

Die Gaschromatographie kann für die Analyse von Zuckern nicht in direkter Form angewandt werden. Die Zucker müssen vor der Analyse erst umgewandelt werden, gewöhnlich in TMS-Äther. Die Vielzahl von Peaks, die man allgemein bei der Gaschromatographie von Kohlehydrat-TMS-Äthern erhält, führt zu ernsten Schwierigkeiten bei der Analyse komplexer Mischungen.

Diese Schwierigkeit kann überwunden werden durch Verwendung von Bis-(trimethylsilyl) trifluoracetamid (BSTFA) als Silylierungsmittel. Unter den vorliegenden Bedingungen sind im Chromatogramm keine Extrapeaks entstanden und es können eindeutig direkte Korrelationen mit den Peaks von reinen Standardlösungen errechnet werden. Die Anwendung von Kapillarsäulen verbindet die Vorteile einer extrem tiefen Nachweisgrenze (es werden dadurch Arbeiten im Pikomolbereich möglich) mit einer hohen Auflösungsleistung, die zu einer guten Trennung der Zuckeranomere und einer kurzen Analysenzeit führt.

Diese Methode wurde für die Untersuchung des Zuckergehalts des Nektars von *Epipactis atropurpurea* Rafin. benutzt, einer häufig von Insekten besuchten und in Portugal heimischen Orchidee. Folgende Zucker konnten identifiziert werden : Fruktose (26,0 %), α-Glukose (13,5 %), Sorbitol (3,0 %), β-Glukose (19 %), Sucrose (16,0 %), Maltose (0,4 %), α-und β-Cellobiose (0,4 %), Melobiose (1,7 %), Gentiobiose (3,0 %) und Raffinose (13,4 %). Besonders beachtenswert ist der hohe Raffinosegehalt in der Absonderung.

Die Dünnschichtchromatographie konnte diese Ergebnisse nur zum Teil bestätigen. Da man jetzt also über eine genügend empfindliche analytische Methode verfügt, kann man erwarten, dass sich die Tür zu einer systematischen Untersuchung der chemischen Zusammensetzung der Nektare und ihrer Beziehungen zur Umwelt öffnet, besonders in Hinblick auf die Spezifität der Bestäuber.

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