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LEVELS OF THIAMINE AND ITS ESTERS IN BEE COLLECTED POLLEN USING LIQUID CHROMATOGRAPHY AND ROBOTICS

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SUMMARY

The levels of thiamine (B_1) and its mono- and diphosphate forms were determined in fresh pollen collected from free-flying colonies of honey bees (Apr.-Oct. 1985) located at Beltsville, MD. The entire analytical process was performed by a laboratory robotic system.

The seasonal thiamine levels (μ moles/100 g) in bee-collected pollen varied greatly depending on the floral source and time of year. All three forms of this vitamin are present in the pollen samples but thiamine is the principle form of the vitamin. The second most abundant form was the diphosphate followed by the monophosphate form.

INTRODUCTION

Pollen, the high protein and vitamin diet of honey bees, is a rich source of B-vitamins (HAYDAK and VIVINO, 1943 ; KOCK and SCHWARZ, 1956 ; and LUNDEN, 1954). VIVINO and PALMER (1944) reported that of the B-vitamins, pantothenic acid, nicotinic acid, thiamine and riboflavin plus ascorbic acid were present in the greatest amounts.

The levels of thiamine (B_1) in bee-collected pollen have been reported by several investigators (VIVINO and PALMER, 1944 ; HAGEDORN and BURGER, 1968). Although the precise function of thiamine in honey bees is not known, it may have a specific metabolic function in the oxidation of carbohydrates. It forms part of an enzyme system which is necessary for the decarboxylation of pyruvic acid, an intermediate compound of carbohydrate metabolism. In biological samples, vitamin B_1 activity is due not only to thiamine but also to the mono, di and triphosphate derivatives of thiamine. Thiamine and its mono-

and diphosphate forms are normally the most prevalent forms. Pollen apparently supplies the honey bees requirement for this vitamin since there is no real evidence that it can be synthesized by insects. Thiamine is relatively stable in acid pH and pollen provides an acid environment (HERBERT and SHIMANUKI, 1978). In stored pollen the vitamin has been shown to exist for up to 4 years (HAGEDORN and BURGER, 1968).

The present investigation was conducted to determine the forms of thiamine and to quantify their amounts in pollen samples collected from free-flying colonies of honey bees (Apr.-Oct. 1985) located at Beltsville, MD. The entire analytical process was performed by a laboratory robotic system programmed to perform the necessary extraction steps, the injection into the sample purification system, the collection of the effluent and the injection into the final analytical system.

MATERIALS AND METHODS

Front-mounted pollen traps were placed on hives of free-flying colonies of honey bees located at Beltsville, Maryland. Fresh pollen was collected weekly from April to October 1985. All samples were frozen at − 10 °C until the determinations were made.

The vitamins were extracted from bee pollen with a procedure similar to that employed for vitamin B_6 (VANDERSLICE *et al.*, 1980). Ten ml of 5 % (w/v) sulfosalicylic acid (SSA) and 10 ml of hexane were added to a sample tube containing 1 g of pollen and a known amount of the internal standard amprolium (VANDERSLICE and HUANG, 1986). The mixture was mixed for one minute, centrifuged at 2400 g for 10 minutes, the water layer removed and filtered through a 0.45 μ disposable filter, and 0.5 ml of the filtrate was injected into a sample clean-up system.

The sole purpose of the sample cleanup system is to remove SSA from the filtrate. This is because SSA fluoresces strongly and interferes with the quantitative analysis. The cleanup system is a simple high performance liquid chromatograph (HPLC) system consisting of pump, injection valve, commercial anion exchange column (packed with 200-400 Bio-Rad AG2-X8 anion exchange resin) and ultraviolet detector (Hitachi Model 100-10 Spectrophotometer). The vitamers begin to elute after 1.5 min and 4.3 ml of effluent is collected. This effluent contains all of the vitamers plus the internal standard, amprolium. A 0.1 ml aliquot is then injected into the final analytical HPLC system which is described elsewhere (VANDERSLICE and HUANG, 1986). This is a reverse phase chromatographic system which separates the various vitamers from one another and from the internal standard. Upon elution from the column, the different species are oxidized to form thiochrome derivatives which are monitored by the fluorescence detector. The relative areas under the observed peaks are integrated and the concentration is automatically determined from the known amount of internal standard orginally added to the sample.

The Laboratory robotic system used in this study is shown in Fig. 1. The robotic system consists of an arm that can move in three dimensions and has two interchangeable multipurpose hands which performs such acts as capping, mixing, spinning, injecting and a module which provides communication between the robotic system and other instruments used in the analytical procedure. This robot can be programmed to perform analyses continuously over a twenty-four hour period. The operator need only to prepare the samples and reagents and add internal standard to the weighed sample in the sample tubes.

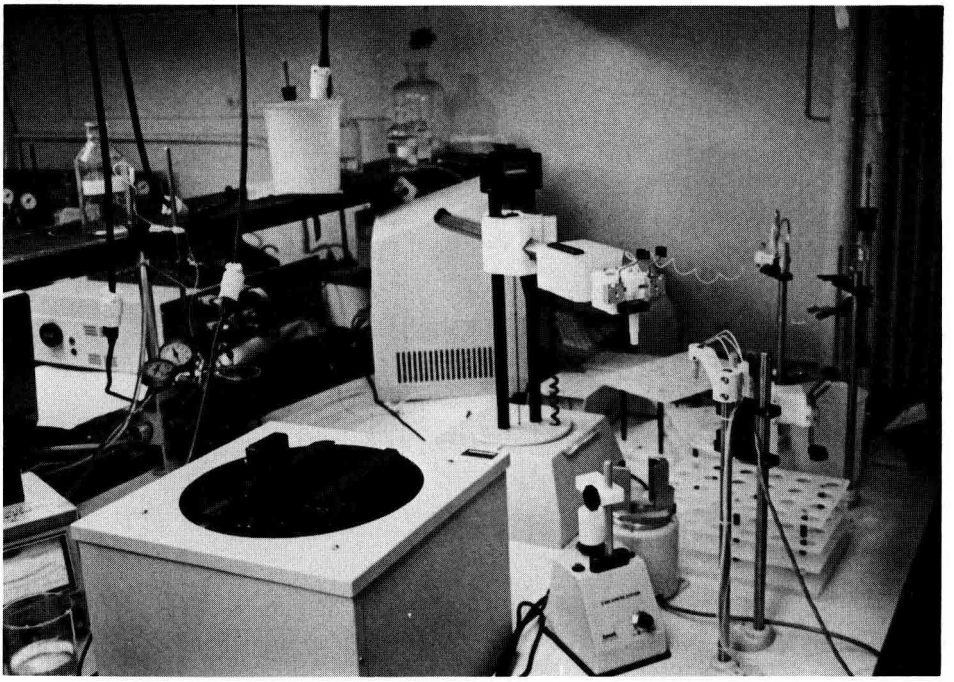

FIG. 1. — *Overall view of the three dimensional robot arm*

RESULTS

A typical chromatogram for a mixture of standards and for an extract from bee pollen is shown in Fig. 2. The elution times are the same for both standard mixture and pollen extract. Also, no peaks which interfere with the determination of thiamine, thiamine monophosphate and thiamine diphosphate are present which indicates that the extraction and cleanup column are yielding « clean » samples for analysis.

The seasonal thiamine levels (μ moles/100 g) in bee collected pollen are presented in Fig. 3. All three forms of this vitamin are present in the pollen samples but thiamine is the principle form of the vitamin. The second most abundant form was the diphosphate followed by the monophosphate form. Pollen samples contained the greatest amounts ot the vitamin in early spring (12 Apr. collection), late May — early Jun (31 May and 7 Jun collection) and early fall (4 Oct. collection). The level apparently depends on the time of year and floral source. For the most part, the variation in the level of total vitamin is due to the variation in thiamine content as the levels of the monophosphate and diphosphate forms remain relatively constant throughout the season. To be

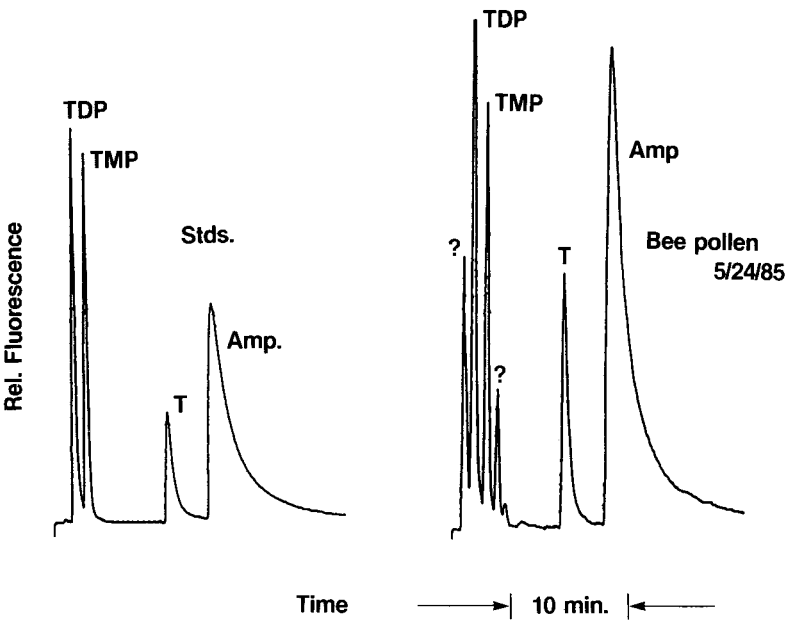

FIG. 2. — *Typical chromagrams for a mixture of standards and for a sample of pollen*

TDP = Thiamine diphosphate ; TMP = Thiamine monophosphate ; Th = Thiamine ; AMP = Amprolium.

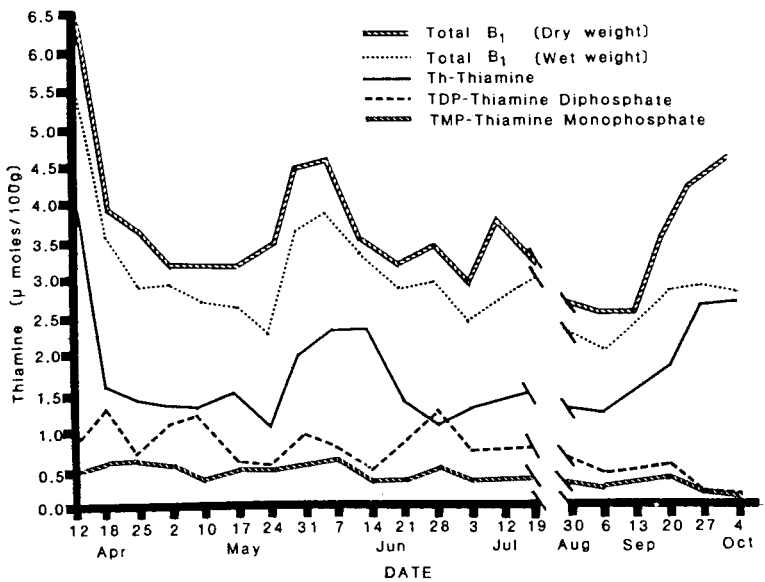

FIG. 3. — *Thiamine levels of fresh bee-collected pollen (April-October 1985)*

certain that the variation was not due to moisture variation the pollen samples were vacuum dried at 80 °C overnight and the total vitamin values on a dry weight basis are also shown in Fig. 3.

As noted in Fig. 3, thiamine values are not presented between 19 July and 30 Aug. Pollen samples were collected during this period but because of excessive levels of starch these samples were difficult to filter during the extraction process. HERBERT *et al.* (1985) identified corn *(Zea mays)* as the predominant pollen source in Beltsville, MD. during this period. TODD and BRETHERICK (1942) noted that while most pollens contain less than 3 % starch, the levels in corn pollen often exceeded 20 %.

DISCUSSION

It was of interest how the levels of total thiamine as determined by chromatographic methods would compare with the levels determined by the classical method (VIVINO and PALMER, 1944). These authors reported the average values of total vitamin B_1 in air dried pollen collected from four periods during the season. Only total vitamin B_1 was reported since individual forms of the vitamin can not be identified by this method. The agreement between the values for total thiamine by the two different methods is remarkably good with the possible exception of their (VIVINO and PALMER, 1944) early May collection which was lower than ours. In the older methodologies for the analysis of vitamin B_1, the phosphate forms are enzymatically converted to thiamine and the total amount of vitamin B_1, not the individual vitamers, is then determined. With modern high performance liquid chromatography, the various forms of vitamin B_1 are first extracted from the sample, separated on a column, and the amount of each determined by the response of a fluorometric detector as the individual forms elute from the column. The total vitamin B_1 is then determined by addition of the vitamer concentrations.

Because of the large numbers of analyses that have to be performed in biological research, laboratory robotic systems are increasingly favored in vitamin research (HIGGS *et al.*, 1986). Robots can be programmed to perform practically all of the manual operations performed in the laboratory from extraction to analytical procedures. The use of a robotic system to completely automate the procedure from extraction to analysis should open new horizons in areas of bee nutrition, toxicololy and physiology.

In summary, vitamin B_1 levels in bee pollen as determined by the automated liquid chromatographic method are in good agreement with the levels obtained by the classical method lending credence to the idea that both

procedures are reliable. The chromatographic procedures does, however, yield information on the different forms of the vitamin present which will be helpful in future studies on the bio-availability of the different forms.

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RÉSUMÉ

UTILISATION DE LA CHROMATOGRAPHIE LIQUIDE ET DE LA ROBOTIQUE POUR DÉTERMINER LES TENEURS EN THIAMINE ET SES ESTERS DU POLLEN RÉCOLTÉ PAR LES ABEILLES

Les teneurs en thiamine (B_1) et ses formes mono et diphosphate ont été déterminées dans du pollen frais prélevé dans des colonies d'abeilles en plein air, à Beltsville, Maryland. Le processus d'analyse a été entièrement éxécuté par le système robotique du laboratoire (Fig. 1) qui se compose d'un bras qui peut bouger dans les trois dimensions et qui possède deux mains interchangeables multifonctionnelles, capables de recouvrir, de mélanger, de tourner, d'injecter, et d'un module qui assure la communication entre le système robotique et les autres instruments utilisés dans le procédé d'analyse.

Les vitamines ont été extraites du pollen par un procédé semblable à celui employé pour la vitamine B_6. Dix ml à 5 % (w/v) d'acide sulfosalycilique et 10 ml d'hexane ont été ajoutés dans un tube échantillon contenant 1 g de pollen et un volume connu de standard interne, « amprolium ». La figure 2 présente un chromatogramme type pour les mélanges de standards et pour un extrait de pollen.

La figure 3 présente les teneurs saisonnières en thiamine (μ moles/10 g) du pollen récolté par les abeilles. Les trois formes de cette vitamine sont présentes dans les échantillons de pollen mais la thiamine est la forme la plus courante de la vitamine ; vient ensuite le diphosphate, suivi du monophosphate. Les échantillons de pollen contenant la plus grande quantité de vitamine sont ceux du printemps (récoltes du 12 avril, 31 mai et du 7 juin) et ceux du début de l'automne (récolte du 4 octobre). La teneur dépend apparemment de la saison et de l'origine florale.

ZUSAMMENFASSUNG

BESTIMMUNG DES THIAMIN-SPIEGELS UND SEINER ESTER IN BIENEN-GESAMMELTEM POLLEN DURCH AUTOMATISIERTE FLÜSSIG-CHROMATOGRAPHIE

Der Gehalt an Thiamin (B_1) und seiner Mono- und Diphosphatverbindungen wurde in frischem Pollen bestimmt, der von frei fliegenden Völkern in Beltsville, Maryland, gesammelt worden war. Der gesamte Analysengang wurde durch ein Laboratoriums-Robotersystem ausgeführt (Fig. 1), bestehend aus einem in drei Ebenen beweglichen Arm und zwei austauschbaren Mehrzweckhänden, die solche Vorgänge wie Verschließen, Mischen, Drehen und Injizieren ausführen können, und einem Modul, das die Kommunikation zwischen dem Robotsystem und den anderen in der Analysenprozedur benützten Instrumenten herstellt.

Die Vitamine wurden aus dem Bienenpollen nach einem ähnlichen Verfahren extrahiert, wie es für Vitamin B_6 benutzt wurde. Zehn ml von 5 % (w/v) Sulfosalizylsäure und 10 ml Hexan wurden einem Probenglas zugesetzt, das 1 g Pollen und eine bekannte Menge eines internen Standard-Amproliums enthielt. Ein typisches Chromatogramm einer Mischung aus Standard und einem Extrakt aus Bienenpollen ist in Fig. 2 dargestellt.

Die saisonalen Schwankungen der Thiaminmengen (μ mol/100 g) in bienengesammeltem Pollen sind in Fig. 3 dargestellt. Alle drei Formen dieses Vitamins sind in den Pollenproben vorhanden, aber Thiamin ist die wichtigste Form des Vitamins. Die zweithäufigste Form war das Diphosphat, gefolgt von dem Monophosphat. Die Pollenproben enthielten die höchsten Vitaminmengen im Frühjahr (Proben vom 12. April, 31. Mai und 7. Juni) und im Frühherbst (Probe vom 4. Oktober). Die Menge hängt offensichtlich von der Jahreszeit und der Blütenart ab.

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