

THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF INSECT HEMOLYMPH SUGARS BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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Abstract—1. A procedure is described for the analysis of sugars in insect hemolymph by high performance thin-layer chromatography.

2. Densitometric scanning of spots after plate developments shows linear responses with most sugars in an analytical range of 125 ng–2.0 µg; detection limits range from 30–60 ng.

3. Quantitative measurements of trehalose, glucose and fructose can be made on hemolymph sample volumes of less than one microliter, allowing blood sugar analysis of individual insects.

4. Hemolymph sugar determinations on six species of insects agree well with values reported in the literature, but mean values for a species often showed higher variability because samples were taken from individuals.

INTRODUCTION

The analysis of carbohydrate levels in insect hemolymph has formed the basis of numerous physiological and metabolic studies (Beenackers *et al.*, 1984; Florikin and Jeuniaux, 1974; Wyatt, 1967). The carbohydrates of major interest include the non-reducing disaccharide trehalose, the principal blood sugar in most insects, and the monosaccharide glucose, which is typically found at relatively low levels. Other saccharides such as fructose, sucrose, maltose, cellobiose, ribose and fructomaltose, have been identified in hemolymph. These sugars are not common, however, and usually reflect some degree of specialization in the diet (Binder *et al.*, 1984; Maurizio, 1965; Wyatt, 1967).

The analysis of blood sugar composition and sugar levels in insects has presented problems due to small sample volumes, small amounts of sugar and assay specificity. Various analytical techniques have been used; some providing direct quantification of hemolymph sugars, others providing a measure of total sugars but only direct estimates of sugars such as trehalose. Techniques which have been utilized for the analysis of insect hemolymph sugars and/or tissue extracts include spectrophotometric assays with reagents such as anthrone (Downer, 1979; Singh, 1986; Teo *et al.*, 1987; Van Handel, 1965, 1978), enzymatic determinations utilizing trehalase and glucose oxidase (Dahlman, 1973), as well as standard chromatographic techniques including paper chromatography (Caldes and Prescott, 1975; Maurizio, 1965), thin layer chromatography (TLC) (Mack *et al.*, 1979; Thomas and Nation, 1984) and gas chromatography.

Gas chromatography is presently one of the more commonly utilized techniques for hemolymph sugar determination and quantification (Binder *et al.*, 1984; Moreau, 1984; Steele and Hall, 1985). However, the use of the GC requires relatively clean samples, derivatization of the sugars to be analysed

(i.e. formation of trimethylsilyl or other derivatives) and considerable time for sample analysis. Some researchers have combined methods to improve analytical accuracy. Kramer *et al.* (1978) developed a method for the separation of trehalose from hemolymph by gel-permeation chromatography, followed by quantification with an anthrone assay. The method provided accurate determination of trehalose but required considerable processing time (up to 5 hr) for each sample. Boctor (1974) and Hayes and Keeley (1985) used a similar type of approach, combining thin layer chromatography with anthrone determinations. Sugars were separated by TLC, eluted from the chromatograms and analysed with an anthrone reagent.

Recent improvements in high performance thin layer chromatography (HPTLC) systems, however, allow for accurate quantitative determinations of sugars utilizing densitometric scanners after chromatogram development and visualization. The use of a thin-layer chromatography system offers several advantages, including ease of sample preparation and the simultaneous analysis of multiple samples. In addition the use of HPTLC can provide a high degree of analytical precision and decreases in the time required for analysis (Lee *et al.*, 1979).

Bounias (1980, 1981) has analysed hemolymph carbohydrates using a quantitative TLC system on 10 × 15 cm plates and densitometric scanning. The method provides for determinations of hemolymph sugars in a nanomole range using N-(1-Naphthyl)-ethylenediamine dihydrochloride as a visualizing reagent. In this paper, an HPTLC technique that provides better sugar separations with a shortened analysis time is described. The detection reagent provides a similar sensitivity range using a reflectance/absorbance scanner and better linearity over an analytical assay range of approximately 0.15 to 6.0 nmol (50 ng–2.0 µg) for insect hemolymph sugars such as trehalose and glucose.

METHODS AND MATERIALS

Apparatus and Reagents

Merck pre-coated silica gel 60 plates for HPTLC (10 × 10 cm) have been used for all chromatographic work. The plates are prewashed in methanol (Baker HPLC grade) and then dried with a hair dryer before use. After the prewash, the plates are pretreated by spraying with a 0.1 M sodium bisulphite solution, dried and then sprayed with a citrate buffer (1:10 dilution of Sigma citrate buffer: water, pH 4.8) to improve sugar separations (Ghebregzabher *et al.*, 1976; Pruden *et al.*, 1975). The plates are placed in a drying oven at 100°C for 1 hr. Following the final heat activation, the plates are transferred to a desiccator where they are stored until use. Pretreated plates may be stored for up to 8 weeks before use.

Sample solutions are applied using a Camag[®] Nanomat 1 applicator and Drummond[®] microcaps. Samples are spotted in either a 1.0 or 0.5 µl volume, depending on the desired sensitivity range. Smaller sample volumes provide better sensitivity and quantitative measurements in the 30–500 ng range. After spotting, the plates are dried for 1 min with a hair dryer before development. Plates are developed 3 times in the same direction with an 85:15 mixture of acetonitrile and water (Gauch *et al.*, 1979). Covered Camag Twin Trough Chambers for 10 cm × 10 cm HPTLC plates are used for development with 3–4 ml of fresh developing reagent required for each run. The solvent is allowed to run up 7 cm from the bottom of the plate (6.5 cm from the origin); average elution time per run is approximately 10 min. Plates are dried for 1 min between each run with a hair dryer; incomplete drying may cause diffuse spots, streaking and a variable solvent front.

After final development and drying, the plates are dipped into a ceric sulfate/sulfuric acid solution and then heated for 15 min at 110°C in a drying oven to char the sugars for visualization (Pruden *et al.*, 1975). The dipping reagent is made by diluting 1 part of 0.100 N ceric sulfate in 2 N sulfuric acid (Ricca Chemical Corp., Arlington, Texas) into 10 parts of 15% sulfuric acid. Sugars (tri- and disaccharides and hexoses) appear as light to dark brown spots, depending on concentration; pentoses appear as yellowish brown spots. The background is white unless the plates are overheated. We have also found that gentle brushing of the plates with a camel hair brush, after the final drying and before dipping, helps to remove small particles of lint and dust that char during the visualization heating. This provides a cleaner background and better quantification.

Quantitative measurements are made by absorbance scanning using a CAMAG TLC Scanner II, interfaced to an Sp-4270 integrator. The plates are scanned at a wavelength of 440 nm, using a slit length of 3 mm and slit width of 0.2 mm. Slit length and width were selected to reduce signal-to-noise ratios and maximize sensitivity (Poole *et al.*, 1985). Instrument sensitivity and span settings were 160–170 and 12–15, respectively. Integrator parameters are set according to the CAMAG TLC program with a peak width of 1 and peak threshold value of 1000. All scans are made in the direction of chromatographic development and peak areas are used for quantification. A series of mixed standards at four or five concentrations is run with each plate. Values obtained from standards are used for the construction of a standard curve for each sugar.

Sample collection

Haemolymph samples are collected from individual insects in 1 µl microcaps by clipping an antenna, puncturing an intersegmental membrane, or in some cases, puncturing the cuticle on the metathorax. Before sample collection, the insects are immobilized by slow cooling, a procedure which also helps to reduce possible hypertrehalosemic or hyperglycemic effects from handling (Downer, 1979; Matthews and Downer, 1973). Hemolymph samples are mixed into

5–15 µl of cold 70% ethanol for deproteinization and lipid removal and then centrifuged at 12,000 RPM (Fisher Microcentrifuge) for 5 min. The supernatant is used directly for analysis and spotted onto plates as previously described. If samples cannot be analysed within a few hours, 5–10 µl of the supernatant is removed to a second vial, dried under nitrogen and frozen until the analysis can be made. In most cases the major problem in holding samples is solvent loss; adjustments for this problem can be made by the addition of an internal standard such as sucrose.

Confirmation procedures

The analysis procedures have been confirmed, first with regard to sugar identification and quantification and secondly with regard to possible interference from other compounds extracted with the hemolymph sugars. Accuracy of sugar determinations for hemolymph analysis was examined in two ways: (1) from qualitative and quantitative comparisons made with a different solvent system and visualization reagent and (2) through the use of high pressure liquid chromatography to examine both hemolymph sugars and sugars separated by the HPTLC technique.

The alternate development solvent used to examine sugar separations consisted of ethyl acetate:acetic acid:methanol:water (60:15:15:10) (Ghebregzabher *et al.*, 1976). Plates were pretreated and spotted, as described, and then developed a single time using this solvent system. Development is slower with this system, requiring 30 min for the single run. Sugars were visualized using both ceric sulfate and the reagent described by Bounias (1980), a 6.5 mM solution of N-(1-naphthyl)ethylenediamine dihydrochloride in methanol with 3% H₂SO₄. This latter reagent was sprayed onto the plates after development and final drying; the plates were then heated at 100°C for 10 min. Comparisons between the two systems were made on the hemolymph sugars from the honey bee (*Apis mellifera*).

Evaluations of the technique were also made using high pressure liquid chromatography. Hemolymph sugars from the American cockroach (*Periplaneta americana*) were separated on thin layer plates, one end section of the plate was cut off and developed to determine the location of trehalose. Using the stained section as a guide, silica in corresponding areas of the original plate was scraped off, the sugar (trehalose) extracted with 50% ethanol and the extract analysed by HPLC. The analysis was made on a Waters HPLC with a refractive index detector using a propylamine column and an 80:20 acetonitrile:water mixture as a carried solvent. The column was run at a pressure of 2500 psi and a flow rate of 1.2 ml/min (Palmer, 1979).

In addition, an analysis of trehalose levels in the hemolymph of *P. americana* and the corn earworm (*Heliothis zea*) was made using both the HPTLC system and the HPLC. The lower sensitivity of the HPLC made it necessary to pool hemolymph samples from several insects; thus, the comparisons were made on the basis of pooled samples and not individual insects. Sample collection and analysis procedures were the same as described above. A total of 25 µl of hemolymph was obtained from both the cockroaches and the earworm adults and used for the analysis procedures. (A 4 × dilution of the pooled sample was used for the HPTLC analysis).

Reagent specificity was examined with regard to possible amino acid interference, using a 10.2 µg/µl solution of casein hydrolysate in 0.05N HCl and a 5 µg/µl solution of tryptophan. Hemolymph samples from adult corn earworm moths were spotted on each of two plates along with sugar standards, casein hydrolysate standards and tryptophan standards. After development, one of the plates was sprayed with a ninhydrin visualization reagent for amino acid detection, the other with the ceric sulfate reagent.

Possible interference, as well as recovery, was also examined by a spike-over analysis in which duplicate 1 µl hemolymph samples were collected from each of five male

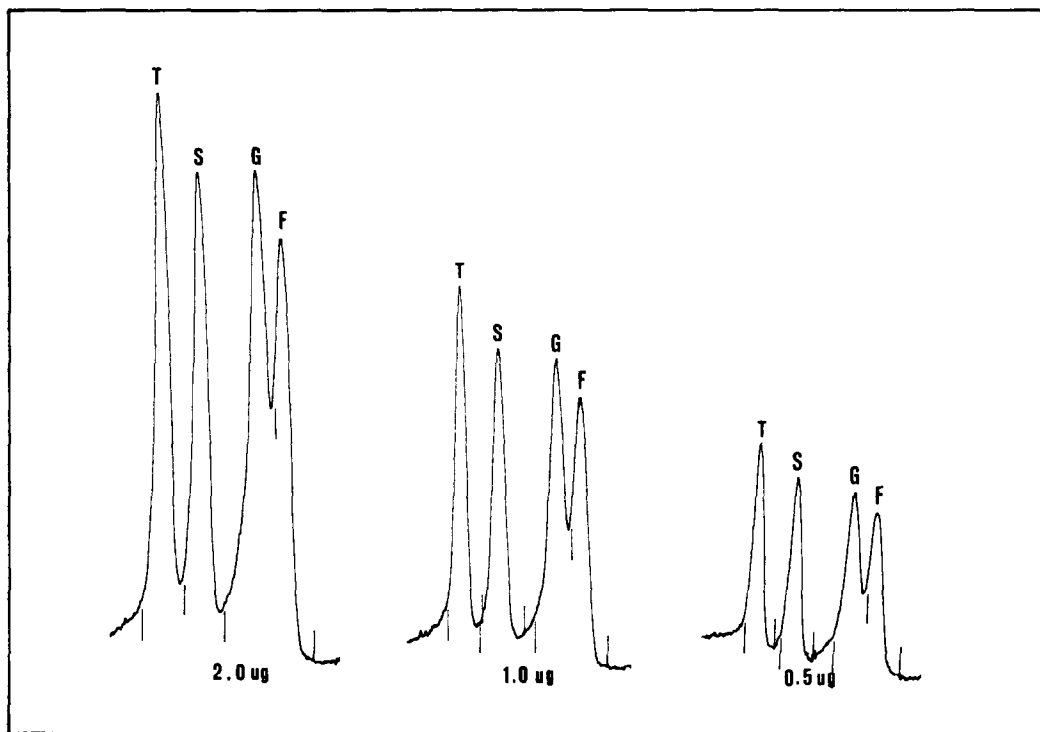


Fig. 1. Scanner recordings of sugar chromatograms on silica gel 60 HPTLC plates after visualization. Three concentrations (2.0, 1.0 and 0.5 μg) of a standard mixture of trehalose (T), sucrose (S), glucose (G) and fructose (F) are shown. Peak areas for different sugars are similar.

American cockroaches. One of samples was placed in 10 μl of 70% ethanol, the other in 10 μl of ethanol to which 5 μg of trehalose and glucose had been added. Both samples were carried through normal analysis procedures, as described.

RESULTS AND DISCUSSION

Scan results are shown in Fig. 1 for a standard mixture of trehalose, sucrose, glucose and fructose at three different concentrations (2.0, 1.0 and 0.5 μg). Peak areas increase linearly with concentration over a sample range of 125 ng–2 μg as shown in Fig. 2 for

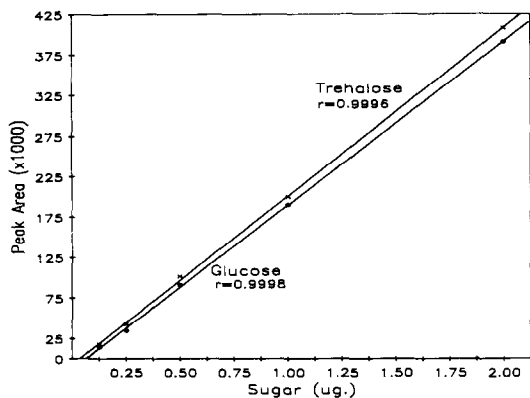


Fig. 2. Standard curves for trehalose and glucose calculated from scan data obtained with the CAMAG TLC Scanner II. The plates were developed 3 \times with acetonitrile:water (85:15) and the sugars visualized with the ceric sulfate reagent.

trehalose and glucose. Minimal detection levels for most sugars fall in the range of 30–60 ng, depending upon the sample volume. One half microliter samples provide for greater sensitivity and better standard curves in a concentration range of 50–500 ng; the minimal detection level for one half microliter samples is approximately 30 ng. One microliter samples provide more consistent results in the range of 250 ng–2 μg . Peak areas should not be used for the determination of sugar concentrations much above 2 μg ; peak areas in this region are not linear when the standard curves are established from standards under 2 μg . However, reasonable standard curves can be constructed from peak area determinations in the range of 2.0–8.0 μg s. Separate standards should be run for higher level determinations with the realization that some precision will be lost.

The separation of sugars commonly found in biological samples is shown in Table 1. R_f values are shown for separations using pretreated plates and two different solvent systems, acetonitrile and water and the ethyl acetate–acetic acid–methanol–water system. Good separations and distinct spots were obtained for each of the sugars listed using the acetonitrile/water solvent system. This system is not suitable for the separation of cellobiose and maltose, however. Scanner resolution is good for most spots, although peak overlap is common with spot R_f values less than 0.05 (see Fig. 1). The ethyl acetate based system provides adequate separation of glucose and trehalose, but not glucose and fructose. Resolution of glucose and fructose with this system is not acceptable for accurate quantitative analysis.

Table 1. HPTLC of sugars on silica gel 60 pretreated with 0.1 M NaHSO₃ and citrate buffer

Sample	<i>R_f</i>	
	(1)	(2)
Ribose	0.65	0.54
Fructose	0.51	0.43
Glucose	0.46	0.42
Galactose	0.38	0.36
Sucrose	0.30	0.30
Maltose	0.24	0.26
Trehalose	0.20	0.23
Melezitose	0.15	0.18

Mobile phase: (1) acetonitrile-water (85:15 v/v), developed 3 ×; (2) ethyl acetate-acetic acid-methanol-water (60:15:15:10 v/v).

The results of the qualitative and quantitative comparison of hemolymph sugars using different visualization reagents are shown in Table 2. Sugar determinations by the two methods were similar, with similar levels of variability within and between samples. More consistent results with respect to colour development were often obtained with the ceric sulfate, probably due in part to the fact that ceric sulfate plates are dipped, whereas the N-(1-naphthyl)ethylenediamine dihydrochloride is applied by spray application. Attempts at the application of NEDD by dipping were not successful and led to background colorization.

The separation of trehalose was confirmed by high pressure liquid chromatography as further verification of the technique. Following the initial HPTLC separation of the sugars in a pooled hemolymph sample from cockroaches, extraction of the silica gel for trehalose and analysis by HPLC, only a single peak corresponding to the trehalose standard was observed. The additional analysis of trehalose levels in pooled hemolymph samples from American cockroaches and from corn earworm adults by both HPLC and HPTLC provided further confirmation of the procedures. The results of these analyses are shown in Table 3; each value represents the mean of three replicate samples for the HPLC and four to five samples for the HPTLC data.

The analysis of reagent specificity and possible interference by amino acids indicated that amino acids present in hemolymph should not interfere with the determination of sugars. Insect hemolymph samples and casein hydrolysate standards run on TLC plates with an acetonitrile/water solvent system and sprayed with Ninhydrin show the presence of amino acids. However, no colour development occurred with the casein hydrolysate standards at levels up to 10 µg/µl when visualized with the ceric sulfate reagent. Insects are characterized by high levels of

Table 2. Comparative determinations of hemolymph sugar levels in honey bees BY HPTLC using different visualization reagents for sugar detection

Sugar	Ceric sulphate	NEDD*
	µg/µl (±SE)	µg/µl (±SE)
Trehalose	10.44 (±2.38)	9.80 (±2.24)
Glucose	4.13 (±0.54)	2.80 (±0.38)
Fructose	5.48 (±0.86)	5.20 (±0.70)

*NEDD = N-(1-naphthyl)ethylenediamine dihydrochloride.

†n = 6 bees, with individual samples run from 2 to 4 × each.

Table 3. Comparative analysis of trehalose levels in adult insect hemolymph samples by HPTLC and HPLC

	HPTLC	HPLC
Cockroach (<i>Periplaneta americana</i>)	10.68 (±0.24)*	9.63 (±0.11)
Corn earworm (<i>Heliothis zea</i>)	22.85 (±0.47)	22.10 (±0.58)
Corn earworm (Pooled sample, not adjusted for dilution)†	4.68 (±0.34)	4.65 (±0.14)

* µg trehalose/µl hemolymph or sample (±SE).

†Samples were collected from several moths, deproteinized in alcohol, centrifuged and the supernatants combined. No effort was made to account for dilution differences. An anthrone analysis of this sample gave a value for total sugars of 5.24 (±0.05) µg/µl.

free amino acids in the hemolymph; concentrations of total amino acids range from 6 to almost 22 mg/ml (Chen, 1985). The 5–15-fold dilution of hemolymph samples in ethanol before analysis reduces the amino acid concentration in samples to levels considerably below those tested with the casein hydrolysate. Thus, interference from amino acids is not expected, although tryptophan offers a possible exception. Visualization of tryptophan occurred at levels of both 5 and 1 µg/µl, following the use of ceric sulfate, but problems are not expected for two reasons. First the level of free tryptophan in insects is generally low (Chen, 1985) and secondly the *R_f* of tryptophan exceeds that of the hexoses and disaccharides examined and therefore should not cause problems with either qualitative or quantitative determinations of hemolymph sugars.

The analysis of hemolymph samples from some insects, however, produces a small, faint triangular spot, light brown in color, which runs above fructose. An identification of the spot has not been made, but it does not appear to be either a sugar or amino acid, as no color development occurred with the NEDD or ninhydrin visualization reagents. In most analyses this spot should not interfere with sugar determinations.

The results of the spike-over analysis further support the lack of interference from hemolymph components and indicate good recovery of hemolymph sugars. The recovery data presented in Table 4 represent the mean values for trehalose and glucose levels in the diluted samples of spiked and unspiked hemolymph. The difference between the two samples shows an approximate 1.7% overestimation of trehalose. Glucose levels in cockroach hemolymph were low and could not be accurately determined in the samples as the peak areas were too small. However, an estimated range of between 50 and 75 ng/µl of diluted sample was used for calculations. These numbers represent the lower detection limit for 1 µl

Table 4. Sugar recovery from spike-over analysis of hemolymph using male cockroaches (*Periplaneta americana*)

Sample	*Mean sugar levels (±SE)	
	Trehalose (µg/µl)	Glucose (µg/µl)
Hemolymph	0.916 (±0.126)	<0.075
"Spiked" Hemolymph	1.379 (±0.123)	0.521 (±0.029)
Difference	0.463 (±0.034)	(0.446 to 0.471)
Expected difference	0.455	0.455
Estimator error	1.7%	2.0–3.5%

*N = 5 (levels in diluted samples); SE = standard error.

Table 5. Carbohydrate levels in insect hemolymph determined by HPTLC

Insect		Mean sugar levels $\mu\text{g}/\mu\text{l}$ (\pm SD)		
		Trehalose	Glucose	Fructose
Orthoptera				
<i>Periplaneta americana</i>	*A m -10	9.79 \pm 1.49	0.60-0.75	
	A m -6	13.94 \pm 4.23	<1.0	
	A f -8	15.36 \pm 7.06	<0.60	
<i>Parcoblatta pennsylvanica</i>				
Coleoptera				
<i>Tenebrio molitor</i>	A m -4	11.84 \pm 2.22		
	A f -4	15.53 \pm 1.58		
Lepidoptera				
[†] <i>Trichoplusia ni</i>	A m -8	28.12 \pm 7.90		
	A f -13	25.59 \pm 7.89		
<i>Heliothis zea</i>	A -6	10.48 \pm 5.00		
Hymenoptera				
<i>Apis mellifera</i>	A f -8	20.23 \pm 4.19	15.89 \pm 5.56	10.76 \pm 6.50

*A = adult, f = female, m = male, no. = sample size.

[†]Data from D. Davidson.

samples and an upper estimate of glucose based on peak height. The use of this range gave an estimated error for glucose recovery between 2.0% and 3.5%. Glucose levels in the hemolymph of the American cockroach are generally low and have been reported to vary from 0.55 $\mu\text{g}/\mu\text{l}$ (Bedford, 1977) to as high as 7.2 $\mu\text{g}/\mu\text{l}$ (Treherne, 1960). By comparison, conversion of the 50-75 ng levels used for the above calculations to glucose concentrations in the hemolymph gives a range from 0.55 $\mu\text{g}/\mu\text{l}$ to 0.83 $\mu\text{g}/\mu\text{l}$.

The results of hemolymph sugar determinations for six species in four different insect orders are shown in Table 5. The concentration levels for different sugars are consistent with the values that have been reported in the literature. Levels of trehalose in the American cockroach, *P. americana*, have been reported to vary from 6.5 $\mu\text{g}/\mu\text{l}$ (18.9 $\mu\text{M}/\text{ml}$) (Bedford, 1977) to 14-18 $\mu\text{g}/\mu\text{l}$ (40.9-52.6 $\mu\text{M}/\text{ml}$) (King *et al.*, 1986; Treherne, 1960; Van Handel, 1978). This variability is partly a function of diurnal cycles and when (time of day) the insects are sampled (Fell and Tignor, unpublished data), as well as other factors such as age, sex, reproductive condition etc. The two sets of values presented in Table 5, for trehalose levels in the hemolymph of the American cockroach, came from groups of males sampled at different times of the day. However, we have consistently found high degrees of variability between individual insects sampled from the same colonies at the same time. Variability in hemolymph sugars between individuals has often not been apparent due to the use of pooled samples (Dahlman, 1973; King *et al.*, 1986; Mullins, 1985). Our data suggest that the variation between individuals with regard to the concentration of sugars in the hemolymph may be considerably greater than has been reported previously. An indication of this variability can be found in the standard deviation data presented in Table 5 for the cockroach, *Parcoblatta pennsylvanica*, and the honey bee, *Apis mellifera*.

The data on hemolymph sugar concentrations in the honey bee are also in general agreement with that reported previously. Wyatt (1967) reported levels of trehalose, glucose and fructose in honey bee hemolymph of 6-12 $\mu\text{g}/\mu\text{l}$, 11-14 $\mu\text{g}/\mu\text{l}$ and 8-10 $\mu\text{g}/\mu\text{l}$, respectively. Arslan *et al.* (1986) reported means of 9.43 $\mu\text{g}/\mu\text{l}$, 6.00 $\mu\text{g}/\mu\text{l}$ and 6.44 $\mu\text{g}/\mu\text{l}$ for trehalose, glucose and fructose levels in the

hemolymph of 2-week-old worker honey bees collected in August. The values reported in Table 5 are slightly higher but may result from the fact that the bees were collected in June, a period characterized by nectar flows and active foraging. Bounias (1981) has reported levels in foraging worker honey bees collected during the autumn as high as 33.1 $\mu\text{g}/\mu\text{l}$ trehalose, 24.3 $\mu\text{g}/\mu\text{l}$ glucose and 15.1 $\mu\text{g}/\mu\text{l}$ fructose.

In conclusion the method described in this paper provides a means for the rapid qualitative and quantitative analysis of sugars in insect hemolymph or tissue extracts. The techniques are simple, require minimum sample preparation and handling, and hemolymph sample volumes of 1 μl or less. The use of small samples not only allows for the analysis of individual insects, but also opens the possibility for repeated sampling of the same individual. The method provides a high degree of sensitivity and reproducibility with a strong linear response between peak area and sugar concentration over an analytical range of approximately 50 ng-2.0 μg . Furthermore, the values obtained from the analysis of hemolymph samples from various insects agree well with the concentration levels reported in the literature by other researchers.

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