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Determination of organic and inorganic anions in insect haemolymph and midgut contents by ion chromatography

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Abstract

A simple and rapid ion-exchange chromatographic method with conductimetric detection for the determination of carboxylic acids (succinic, malic and citric) and inorganic anions (chloride, phosphate and sulphate) in haemolymph and midgut content of Lepidoptera larvae is provided. The mobile phase is 0.975 m*M* phthalic acid at pH 4.1. The procedure of sample extractions was simple. Comparison of the results with whose reported in the bibliography showed that the values were similar. The ion-exchange chromatographic–conductimetric detection method permits the analysis of various organic and inorganic anions in small biological samples. © 1997 Elsevier Science B.V.

Keywords: *Manduca sexta*; *Heliothis virescens*; Carboxylic acids; Inorganic anions

1. Introduction

Knowledge of the concentration of the organic and inorganic anions present in biological samples, such as insect haemolymph and midgut contents, is important for comprehension of maintenance of the fluid and electrolyte homeostasis, as well as in understanding some insecticide resistance mechanisms in insects [1–3].

In spite of the growing number of studies conducted with lepidopteran moths to elucidate most of their physiological aspects, only a small number has given information about the composition of haemolymph organic and inorganic anions [1,4–6] and there are none describing the anion composition of the midgut content.

The procedures used to date, in studies of the anion composition of insect haemolymph, required a complex mixture of specific methods, coupled to specific methods of sample treatment, as can be exemplified in the work of Pannabecker et al. [1] with fifth instar larvae of *Lymantria dispar*. In this study, chloride concentrations were determined by silver chloride titration with a chloridometer, as have been done before in *Spodoptera exigua* adults [4].

Analysis of carboxylic acid has proved difficult. The use of paper chromatography [7,8], thin-layer chromatography [9] and isotachophoresis [10] have met with limited success. Gas chromatography has provided best separations with a high degree of sensitivity, and have been used widely in the analysis of biological samples [5]. However, there are some problems preventing the simultaneous analysis of organic acids under these conditions.

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Ion-exclusion chromatography for organic acid analysis, or HPLC on Aminex HPX-87 H cation-exchange resins [5], have allowed separation of organic acids from insect haemolymph, although no quantitative data have been obtained. Moreover these authors did not analyse the inorganic anions.

A common feature to all the above-mentioned methods is the need for large quantities of biological sample; this caused Bindokas and Adams [11] (on *Heliothis virescens*) and Pannabecker et al. [1] to fail in the analysis of the contents in larvae of the earliest stages or when the budworms were starved.

Advances in ion-exchange chromatography techniques allowed the determination of both carboxylic acids and inorganic anions from wines [12], using a simple method which does not require derivatization or extraction of the sample.

Within the economically important Lepidoptera, most crop damage is caused by larval stages. A more complete knowledge of the physiology and pharmacology of these insects may offer new insights for the development of pest control strategies, as a prelude to more detailed studies on the mechanism of action of δ-endotoxins from *Bacillus thuringiensis* (*B.t.*).

In the present study, one ion chromatography column and a conductimetric detector were used to separate and detect inorganic and organic anions present in haemolymph and midgut content samples of *Manduca sexta* larvae and two strains of *Heliothis virescens*, one resistant and other susceptible to *B.t.* δ-endotoxin. This method allowed us to analyse Cl^-, SO_4^{2-} and $H_2PO_4^-$, malate, succinate and citrate in the same sample with economy of effort and money.

2. Experimental

2.1. Insects

Newly emerged third instar larvae of two *Heliothis virescens* strains (Lepidoptera, Noctuidae), provided from F. Gould Laboratory (Entomology Department, North Carolina State University), were used in our experiments: (1) a parental δ-endotoxin-susceptible strain (CPN) and (2) a δ-endotoxin-resistant strain ($CP73_3$). After some generations of selective pressure, the resistant strain presented cross-resistance to CryIA(b), CryIA(c) and CryIIA [13].

Eggs of *M. sexta* (Lepidoptera, Sphingidae) were purchased from Carolina Biological Supply Co. After hatching the larvae were fed on artificial diet. Newly emerged third instar larvae were used in the study.

Both species were reared in the laboratory at 25±1°C, 60±10% relative humidity and 16 h photophase. Animals were fed on artificial diet in individual 25-ml cups.

2.2. Sample treatment

Larvae starved from 15 to 20 h were selected and cold-anesthetized. A cut at the first abdominal segment, behind the first abdominal prolegs was performed and the intestinal duct was excised free, the Malpighian tubules were then removed and the midgut content only was kept at −20°C. At the same time, the tip of the second abdominal proleg was cut with scissors and haemolymph samples were collected in capillary tubes coated with heparin, while applying gentle pressure to the abdomen, and maintained at −20°C.

A pooled sample of 0.3 g was obtained from the midgut contents and haemolymph of 15–20 larvae.

For compositional analysis, midgut contents and haemolymph samples (100 mg) were first homogenized and then centrifuged at 4°C and 8000 rpm for 20 min to remove solid particles and the hemocytes, respectively, obtaining a precipitate and approximately 50 μl supernatant. Precipitate was washed two times with 50 μl water. Unless otherwise stated, all analyses were conducted with cell-free haemolymph. Then approximately 150 μl of each sample were separated with chloroform (1:1) for 30 min under continuous agitation. Later, samples were centrifuged at 20°C and 8000 rpm for 20 min. The organic phase obtained was washed with 100 μl of water and added to supernatant (approximately 150 μl). The resulting volume (250 μl) was heated at 60°C for 5 min.

Prior to chromatographic injection, any interference as well as any other substance that could bind irreversibly to the column and damage it, must be eliminated by means of solid-phase extraction. The midgut content or haemolymph final samples (250

μl) were passed through 200-mg solid-phase extraction cartridges (C_{18}) (Analytichem International, Varian Division) conditioned with 2 ml methanol and 2 ml water, with addition of 250 μl of water. Subsequently, an aliquot of 20 μl was injected onto the chromatographic system after filtering through a 0.45-μm filter (Micro Separations). The number of replicates was 5, and each one was injected three times.

2.3. Apparatus

We employed a Shimadzu ion chromatograph consisting of a basic module (HIC-6A) equipped with a manual valve injector (20 μl sample loop) and a simple-piston pump (LP-6A), a temperature-controlled oven for column and detector (CTO-6AS), an ionic conductivity detector (CDD-6A) and a recorder–integrator (Chromatopac C-R6A) for signal processing. pH measurements were performed using a Crison digilab 517 pH meter.

The sensitivity of this technique allowed us to quantitate chloride, sulphate, phosphate, succinate, malate and citrate in a single haemolymph and midgut injection.

2.4. Stationary phase

A Shimpack IC-AI column (100×4.6 mm I.D.) filled with quaternary ammonium polymethacrylate (particle size, 12.5 μm; mass, 0.92 g dry resin) was used. This was a low-capacity organic polymer column (0.050 mequiv./g), capable of supporting maximum pressures of 25 kg/cm^2, with an operating temperature of up to 50°C, a wide operating pH range (2–12) and an efficiency of 2150 plates for chloride.

2.5. Reactives

The different reactives used were of analytical quality (Merck, Darmstadt, Germany). Ultrapure water (Milli-Q, Millipore, Molsheim, France) was employed.

2.6. Mobile phase

The eluents most frequently cited in the literature are aromatic organic acids, and the most important factor for separation is the charge of the anion. In general, the greater the charge, the more rapid is the elution.

Different eluents (*para*-hydroxybenzoic acid, KOH, dicarboxylic acid, phthalic acid and potassium hydrogenophthalate) were tried, as were several chromatographic conditions and pH values. The final eluent used to detect and quantify inorganic and organic anions in biological samples was 0.975 m*M* phthalic acid adjusted to pH 4.1 with 0.1 *M* Tris [12], with a background conductivity of 125 μS/cm and a flow-rate of 1.5 ml/min at 40°C.

3. Results and discussion

3.1. Qualitative analysis

A standard solution (carboxylic acids and inorganic anions) was prepared for qualitative purpose. The elution order was as follows: injection peak (t_R=0.8 min), phosphate (t_R=2.0 min), succinate (t_R=2.5 min), chloride (t_R=3.4 min), malate (t_R=4 min), citrate (t_R=7.1 min) and sulphate (t_R=12.5 min). All the anions tested were detected in midgut content (Fig. 1) and haemolymph (Fig. 2). We detected two unknown peaks called 6 and 8 (Figs. 1 and 2) in all samples at t_R=5.5 min and t_R=10 min, respectively. We have been unable to identify peaks 6 and 8 since they do not correspond with other organic and inorganic standards tried in the system (namely acetate, lactate, tartrate, oxalate, fumarate, nitrate and nitrite).

3.2. Quantitative analysis

3.2.1. Calibration

In a previous work [12] we found that the standard addition method produced an advanced and a disproportionate increase in the system peak, which led to partial overlapping with the sulphate peak and a deformation giving faulty integration of this anion. In the same work we found that the method of calibration lines provided similar results without the interferences mentioned. Therefore we chose the calibration line method for quantification. Five standards were used for the calibration. Values of range

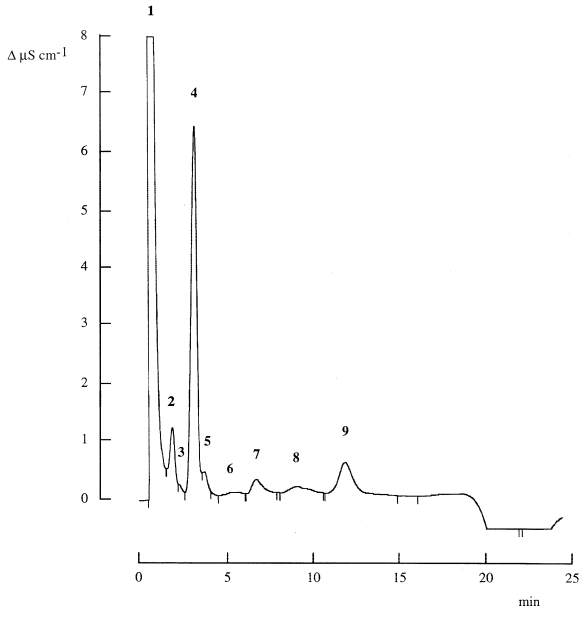

Fig. 1. Chromatogram of midgut content sample from CPN strain *H. virescens*. 1, injection peak; 2, phosphate; 3, succinate; 4, chloride; 5, malate; 6, unknown; 7, citrate; 8, unknown; 9, sulphate. Column, Shimpack IC-AI; mobile phase, 0.975 m*M* phthalic acid (pH 4.1); flow-rate, 1.5 ml/min; column temperature, 40°C; Conductivity detector. Injection automatic volume, 20 μl. Positive polarity.

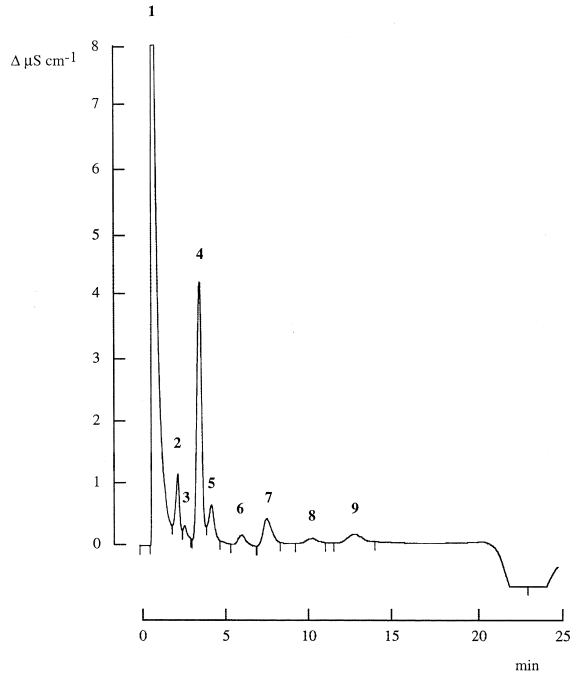

Fig. 2. Chromatogram of haemolymph sample from CPN strain *H. virescens*. 1, injection peak; 2, phosphate; 3, succinate; 4, chloride; 5, malate; 6, unknown; 7, citrate; 8, unknown; 9, sulphate. Chromatographic conditions as in Fig. 1.

concentrations, linear correlation coefficients, sensitivity and intercept, as well as the detection limit for each individual anion calculated as previously described [12], are shown in Table 1.

Table 2 shows anion concentrations of midgut contents and haemolymph samples from *M. sexta* and *H. virescens* strains. It can be appreciated that the concentrations of both organic and inorganic anions are generally above the detection limits. The exception is succinic acid, which could be detected only in haemolymph samples of *M. sexta* and *H. virescens*, and all the organic acids in the gut contents of *M. sexta*.

An overlapping occurs between chloride and malate ions, so to determine small portions of malate when great quantities of chloride are present could be a problem. In our experiments, 3 μg/ml is the minimal concentration detected. This phenomenon is also true for succinate ions, with 5 μg/ml as the minimal values detected in our assays. These concentrations correspond to 30 and 50 μg/g fresh weight, respectively.

3.2.2. Recovery results

To carry out recovery assays for chloride, sulphate, phosphate, succinate, malate and citrate anions, prior to chloroform treatment, a known quantity of aqueous solution of all anions was added to blanks as well as to biological samples. The subsequent treatment was the same as described in Section 2.2. Table 3 shows the results obtained when the indicated amounts of each ion were introduced into the system as blank and as *M. sexta* haemolymph samples. In all cases recovery was high for all ionic species tested.

3.2.3. Midgut contents

Chloride was the dominant inorganic anion identified in our analysis, and accounted for nearly 74% in *M. sexta* and 57 and 59% of the total molar concentration of inorganic anions in CPN and $CP73_3$

Table 1
Analytical parameters for organic and inorganic anions by ion-exchange chromatography and detection limits

Sample ion	Range concentration (μg/ml)	Linear correlation coefficient	Sensitivity (μV s mg^{-1} l)	Intercept (μV s)	Detection limit (μmol/g fresh mass)
Phosphate	9–316	0.9998	132±8	400±600	0.6
Succinate	5–30	0.9994	240±5	−1000±400	0.4
Chloride	8–240	0.9996	1970±19	700±700	0.07
Malate	3–60	0.9993	350±8	−1600±500	0.2
Citrate	10–200	0.9996	270±11	−2000±600	0.12
Sulphate	4–80	0.9999	100±20	−300±900	0.2

Peak evaluation was with areas. Injected volume, 20 μl.

Table 2
Anion concentrations in μmol/g fresh mass±S.D. of haemolymph and midgut content of third instar *M. sexta* and *H. virescens* larvae

	Manduca sexta		*Heliothis virescens*				
	Midgut content	Haemolymph	CPN		$CP73_3$		*n*
			Midgut content	Haemolymph	Midgut content	Haemolymph	
Inorganic anions							
$H_2PO_4^-$	9.8±1.1	4.7±1.8	11.9±0.7	23±5	10.57±0.04	19±3	5
Cl^-	37±5	40.4±0.4	21.1±1.4	30±9	21.66±0.09	28±3	5
SO_4^{2-}	2.7±0.2	1.24±0.01	4.0±0.6	2.7±1.2	3.9±0.5	1.0±0.3	5
Total	49.5	46.3	37.0	55.7	36.1	48.0	
Organic anions							
Succinate	—[a]	0.83±0.06	—[a]	0.68±0.15	—[a]	0.62±0.18	5
Malate	—[a]	1.6±0.7	0.33±0.03	3.1±0.7	0.26±0.01	3.14±0.10	5
Citrate	—[a]	7±2	2.13±0.09	7.6±1.1	1.8±0.6	8±3	5
Total	—	9.4	2.5	11.4	2.1	11.8	

[a]Below detection limit.

H. virescens strains, respectively. In order of its relative importance, phosphate was the second inorganic anion studied in all samples. Our method allowed us to detect specific variations in ion content, as has been previously described for other insect samples [1,11]. Both *H. virescens* strains showed higher levels of phosphate (32 and 29%, respectively) than *M. sexta* (19%). This trend is maintained for the third inorganic anion studied, sulphate.

The succinate contents in all midgut samples was below the detection limit, whereas malate and citrate could be detected in both *H. virescens* strains without any differences between them, citrate being the more important component. None of the organic anions could be detected in *M. sexta* samples.

Table 3
Recoveries of pattern and samples of haemolymph

Sample ion	Added (μmol/ml)	Blank (%)	Samples (%)
Phosphate	12.2	104	105
Succinate	0.98	98	97
Chloride	3.01	106	103
Malate	1.02	99	95
Citrate	2.5	98	99
Sulphate	2.4	97	100

One of the aims of this study was to determine ionic composition of the midgut contents, since this is important for understanding the mechanisms of action of *B.t.* δ-endotoxins. These toxic proteins are activated when the midgut conditions are adequate. This leads, through a series of steps, to the formation of a pore in the membrane followed by equilibration of ions and small molecules across the membrane, ending by osmotic influx of water and eventually lysis of the cells [14–24]. Therefore, the results presented here should help in the understand of the mechanisms of resistance to *B.t.*, which would be linked to toxin activation and processing prior to binding to toxin receptors [13].

3.2.4. Haemolymph

As in the midgut content samples, chloride was the predominant inorganic anion accounting for 87% in *M. sexta* and 53 and 58% in CPN and $CP73_3$ *H. virescens* strains, respectively. Phosphate accounted for 10% in *M. sexta* and 41 and 39% in *H. virescens*, whereas the sulphate content was much lower, having values of only 2–4% in both species.

The different proportions of the anions follow the patterns described in the literature for the same or related species [1,4,11], although it must be kept in mind that some differences may arise due to the different methodologies used.

The content of organic anions is approximately 20% of the total anion content in the samples. Here citrate is the predominant anion in all samples, and there are no differences between species. The values for succinate are, also, very similar, whereas there is a strong difference in the contents of malate: the samples from *H. virescens* strains presenting twice as much as those from *M. sexta*.

There are few reports on the organic anion composition of insect haemolymph [5,6]. In one case [6], determination was done in mosquitoes using an enzymatic method, which makes it very difficult to compare with the results obtained here. On the other hand, Womersley et al. [5] used cation-exchange HPLC for separation of tricarboxylic acid cycle acids and other related organic acids in *Bombyx mori* haemolymph, but no quantification of the results was made.

4. Conclusion

The method described has allowed us to detect specific differences in the anion composition of samples from both species studied.

The method has been used to analyse midgut content and haemolymph samples of insect larvae, and showed that with the prior treatment of the sample, as described, no loss of efficiency of the column was seen, even after several hundred injections.

To investigate the physiology of the organisms, where organic and inorganic anions may be involved, this determination by ion chromatography using a non-suppressed conductimetric detection appears to be suitable, especially for small samples of insect gut contents and haemolymph. Lower detection limits could be obtained by the use of suppressed mode and a greater injected volume.

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