A simple, rapid and highly sensitive method of separation and quantification of uric acid, hypoxanthine, and xanthine by HPLC

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Summary. Uric acid, hypoxanthine, and xanthine can be analyzed in ng quantities from a variety of insect materials in less than 10 min from sample collection to quantification of all 3 purines.

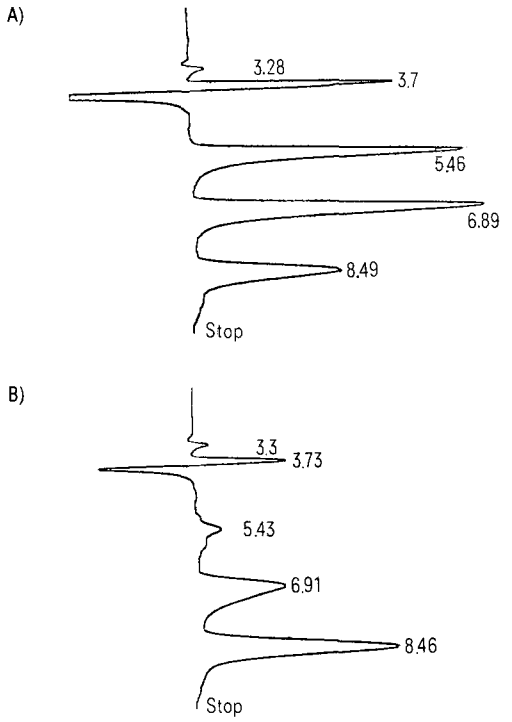
Identification and quantification of purine waste products, especially uric acid, have been the subjects of numerous investigations. Since uric acid and its related purines are of great importance clinically and with respect to the nutritional and excretory biology of many terrestrial animals, the accurate analysis of these compounds, especially of trace amounts, is of extreme interest to biologists and biochemists. The fact that numerous questions remain unanswered regarding uric acid metabolism in insects[2] underscores the need for ever more microscopic methods of analysis[3].

The need for rapid and highly specific methods leads to selection of chromatographic techniques. TLC has been used frequently for separation of purines, but quantification of TLC separations is cumbersome. Recently, several authors developed schemes of purine separation by high performance liquid chromatography (HPLC)[4,5]. These authors used normal phase HPLC and reversed phase HPLC to separate numerous purines with a solvent gradient system. Pachla and Kissinger[6] reported a method of analyzing uric acid with HPLC and an amperometric detector. While this method is even more sensitive than UV-detection, it is also more specific and unapplicable to a variety of other purines such as xanthine, guanine, and hypoxanthine. Brown et al.[7] described a reversed phase HPLC method for analyzing xanthine, hypoxanthine and uric acid with UV-detection. The method reported here incorporates a different paired ion chromatographic (PIC) reagent than the 1-heptane sulfonic acid used by Brown et al.[7] and a different solvent system that improves the peak separation. The present method was found to be sensitive down to 500 pg of each purine and because of the use of a precolumn, it required no deproteinization or other sample clean-up.

This paper presents a simplified HPLC technique that incorporates an isocratic (single solvent) rather than a gradient solvent system. The technique takes advantage of the resolving capabilities of paired ion chromatography reagents to separate rapidly and completely the purines of interest resulting in symmetrical, fairly narrow peaks that are easily quantified. The instrument used was a Beckman Model 332® HPLC. A single pump was used, and separation of samples was achieved with a 4.6×250 mm column packed with Ultrasil™ ODS (C-18) (10 µm) and a 10 µm ODS precolumn. Samples were detected at 254 nm, and quantification by integration of the area under peaks was performed by a Beckman CR1-A® recording integrator. Also tests were performed with detection at 280 and 292 nm. Several solvent systems were tried. The best for rapid, baseline separation of peaks was 900:100:25, water:methanol:tetrabutylammonium phosphate (PIC™Reagent A from Waters Associates, Milford, Mass.). The PIC reagent formed a 0.005 M solution of the paired ion reagent.

After being weighed, material to be analyzed was placed in a micro-tissue grinder with 100 µl of 0.5% Li_2CO_3/water (W/V) solution and thoroughly ground and mixed. A 20 µl aliquot of this solution was injected into the HPLC. Hundreds of injections with samples that were not deproteinated had negligible effect on column efficiency proving that the simple preparation method with no sample clean-up does not threaten column life. This simplification of sample preparation is a result of both the small sample sizes and use of the precolumn.

The chromatogram illustrated in figure A represents the separation of hypoxanthine, xanthine and uric acid, each present in 27 ng quantities. Repeated tests with standards gave the following recoveries (±SD): xanthine 93.2(±3.09)%; hypoxanthine 107.0(±1.38)%; urid acid 84.8 (±6.45)%. The other chromatogram (fig. B) illustrates the purines present in a single egg, weighing 105 µg, from a tobacco budworm, *Heliothis virescens* (F.). This method was used with several other insect materials including single samples of fecal material from the hemipteran, *Geocoris punctipes* (Say), from the corn earworm, *Heliothis zea* (Boddie), and from *H. virescens*; single eggs from *H. virescens* and the hemipteran *Lygus hesperus* Knight were successfully analyzed; and 1 µl of hemolymph and about 1 µg of fat body from *H. zea* were analyzed. In the case of the eggs, sub-samples were tested after treatment



A Chromatogram of pure hypoxanthine (5.46), xanthine (6.89) and uric acid (8.49) separated on a 4.6×250 mm Ultrasil ODS(10 µm) column with 1.0 ml/min. 900:100:25 H_2O:MeOH; PIC-A detected at 254 nm. *B* Chromatogram of a single egg of a tobacco budworm *(Heliothis virescens)* ground and suspended in 0.5% Li_2CO_3/H_2O solution. The 3 purines described above are present respectively at 5.43, 6.91 and 8.46 min in concentrations of 2.7 ng, 8 ng, and 3.8 ng per egg.

with uricase to determine unequivocally that the peak for uric acid was authentic.

The results of these tests confirm that this method could be used to quantify less than 1.0 ng of each of the purines in a variety of biological materials, including individual insect eggs that weighed about 100 µg. If uric acid is the sole compound of interest, 10 pg quantities can be measured using 292 nm rather than 254. However, this study revealed that for the 3 purines studied here, 254 nm is the best compromise wavelength. This method is used in our laboratory to quantify fecal matter production and protein utilization in entomophagous insects, but the extreme sensitivity and the speed and the simplicity of this procedure would recommend it for study of these purines in such minute samples as individual insect organs or even smaller tissue samples.

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Simple procedure to isolate coelomocyte - free oocytes from coelomic fluid of *Perinereis cultrifera* Grübe (Annelida; Polychaeta)

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Summary. Based on a 1-g sedimentation through a Ficoll cushion, a simple and rapid method is described to obtain the complete separation of oocytes and coelomocytes from the coelomic fluid of *Perinereis cultrifera.* Light and electron microscope observations have shown that the 2 cell populations are obtained without any cross-contamination and are perfectly suitable for further biological and biochemical investigations.

In the study of oogenesis in Nereids, the functional relationships between coelomocytes and oocytes contained in the coelomic fluid have become an important issue.

Biochemical approaches to this problem require the separation of well-defined, pure populations of cells. Up to now, the common method to separate coelomocytes from oocytes was based on differential low-speed centrifugation. This approach never leads to a complete separation of the 2 cell types and the oocyte pellet always contains a small percentage of coelomocytes. Although this contamination does not bring large error in determining chemical composition of cellular material[2,3], it may become critical in screening the enzymatic properties of each cell population. This is particulary important to determine enzymatic markers related to the various cell types.

In the present work, we describe a simple and rapid method to isolate large quantities of oocytes completely devoid of coelomocytes from the coelomic fluid of *Perinereis cultrifera.*

Materials and methods. Perinereis cultrifera was collected from the beaches of 'Luc-sur-Mer' (Calvados, France) and the animals were kept in the laboratory in sea-water at 12 °C. The diameter of oocytes from each animals was determined by measuring several oocytes obtained by coelomic puncture[4]. Ficoll solution (Ficoll-400 Pharmacia Fine Chemicals, Sweden) was freshly prepared or kept at 4 °C for no longer than 2 weeks. Electron-microscopic observations were carried out as previously described[5].

Results. The method is based on a 1-g sedimentation of the coelomic contents through a cushion of Ficoll solution (see fig. 1) at 20 °C. The oocytes sediment while coelomocytes do not.

For oocytes larger than 130 µm in diameter, the procedure is as follows: the coelomic fluid from 1 animal is collected by puncture, diluted to 2 ml with filtered sea water and then layered above 5 ml of a 5% (w/v) Ficoll solution in sea water, in a conical tube (2 cm by 8 cm). The oocytes are allowed to settle for 5-10 min; the coelomocytes stay at the top.

In the case of smaller oocytes (less than 130 µm in diameter), the same procedure is applied but 0.1 M sodium phosphate pH 7.4 buffer containing 0.35 M NaCl is used instead of sea water. This phosphate buffer allows dissociation of the coelomocytes which aggregate when the coelomic fluid is taken out of the animal. The concentration of the Ficoll solution is 2.5% (w/v) in phosphate buffer.

After separation, the coelomocyte-containing supernatant (2 ml) is removed with a pasteur-pipette and the remaining medium is discarded, leaving the coelomocyte-free oocyte pellet. Ficoll is removed from the cell suspensions by low-speed centrifugation washings in sea water or phosphate buffer for large and small oocytes, respectively. Figure 2,a shows the content of the coelomic fluid after puncture. As revealed on figure 2,b and c, the 2 cell populations obtained after separation are devoid of contaminants.

Discussion and conclusion. The above described procedure provides a simple and rapid preparation of coelomocytes and oocytes from the coelomic fluid of *Perinereis cultrifera.*

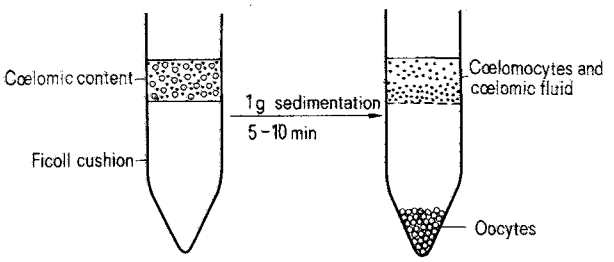

Figure 1. Schematic description of the procedure.