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Note

Comparison of methanol and perchloric acid extraction procedures for analysis of nucleotides by isotachophoresis

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During the past decade, isotachophoresis has become increasingly popular for the determination of nucleotide spectra owing to its high sensitivity and reproducibility. At present, this is one of the more important applications of the technique in view of the number of publications in the field. In a review article in 1980 [1], Holloway and Luestorff cited 57 references on the subject. Gower and Woledge [2] and Woledge and Reilly [3] reported the determination of nucleotides in frog muscles by isotachophoresis. Oerlemans et al. [4] introduced a screening method of inborn errors based on nucleotide profiling in human muscles. Aomine and co-workers [5, 6] studied cardiac and skeletal muscles in different species. Perez et al. [7] investigated the ATP/ADP ratio in different rat tissues. Eriksson [8] and Eriksson and Straath [9] analysed nucleotides in rat liver and Talbot [10, 11] measured nucleotides in red blood cells. We have previously reported on the analysis of first-cleavage-stage embryos of *Nassarius reticulatus* (trefoils) [12, 13].

An important step in nucleotide analysis of biological tissues is the extraction procedure. Extraction procedures must fulfil certain criteria: enzymatic activity should be suppressed quickly and efficiently, and the components of interest should be extracted with high and reproducible recovery.

Numerous extraction procedures are described in the literature, all of which

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use methanol, perchloric acid (PCA) or both. From the literature cited, there is not a consensus as to the best extraction procedure. Methanol, methanol—EDTA, methanol—perchloric acid and perchloric acid were among the extraction media reported. For this variety of samples, it is unlikely that there is one best method. Therefore, for our specific case, an evaluation of some of the extraction procedures was carried out with respect to recovery, quality of separation, detection limit, specificity and possible interferences.

EXPERIMENTAL

Isotachophoresis

Capillary isotachophoresis was carried out in equipment for column-coupling, developed by Verheggen et al. [14]. This enables the analysis of samples with a relatively high ionic strength, without adversely affecting the analysis time or the detection limit [14]. For nucleotides in general, a pH of ca. 4 is most commonly used [1]. Our operational conditions are listed in Table I. All chemicals were of analytical-reagent grade and were purchased from either Merck (Darmstadt, F.R.G.) or Sigma (St. Louis, MO, U.S.A.). The detector signals were recorded with a potentiometric recorder (Kipp, Delft, The Netherlands). The detectors were: a.c. conductivity and UV absorption at 254 or 280 nm. For identification purposes, the zones were characterized by their relative step-height from the conductivity detector; UV absorption at 254 nm; ratio of absorption at 254 and 280 nm; and relative position in the isotachopherogram. Response factors for individual nucleotides were calculated from standard calibration curves, which were sufficiently linear in the range of interest (0.1—1 nmol). Zone lengths were determined from either the differential recording of the conductivity signal, or from the width at half-height of the UV

TABLE I

OPERATIONAL CONDITIONS FOR DETERMINATION OF NUCLEOTIDES BY ISOTACHOPHORESIS USING COLUMN-COUPLING

PVA = poly(vinylalcohol), Mowiol (Hoechst, Frankfurt, F.R.G.). CTAB = cetyltrimethylammonium bromide (Merck). PVA decreases the electro-osmotic flow, whereas CTAB reverses its direction. Both effects are favourable in anionic analyses [15].

Parameter	Preseparation compartment	Detection compartment
Leading electrolyte		
Anion	0.01 *M* Chloride	0.01 *M* Chloride
Counter-ion	β-Alanine	β-Alanine
pH	3.9	3.9
Additive	0.05% PVA	0.05% PVA
	0.02 m*M* CTAB	0.02 m*M* CTAB
Terminating electrolyte		
Anion	0.005 *M* Capronate	0.005 *M* Capronate
Counter-ion	Sodium	Sodium
Capillary diameter	0.8 mm	0 2 mm
Driving current	350 μA	25 μA
Injection volume	2 3—5 μl	
Total analysis time	15—20 min	

signal at 254 nm. There was good agreement whenever both methods could be applied.

Sample preparation

In order to design an optimal strategy for nucleotide analysis, in principle, all variables have to be tested separately and in combination. Some procedural steps are only meaningful in connection with the material that has to be analysed. In our investigations, for instance, a lot of effort has been spent on investigating the influence of homogenization (either by pottering or sonication or both). For our system, this was necessary because embryonic material contains many yolk granules, which are of a very rigid structure and do not easily disintegrate, a fact that might hamper extraction. This question has only limited significance, however, and will not be discussed in this paper.

Methanol extraction. For methanol—EDTA and methanol extraction, 500 trefoils were collected on ice, culture medium (seawater) was removed with a microcapillary and 5 μl of 50% methanol (0°C) with or without 1.25 m*M* EDTA (disodium salt) were added. The trefoils were then homogenized by pottering with a pestle, which was subsequently washed with 20 μl of 50% methanol with or without 1.25 m*M* EDTA, at 0°C. The contents of the pottering vessel were mixed with an automatic pipette and left to stand on ice for 15 min. The contents were then transferred to a microvessel (Eppendorf) and centrifuged for 5 min at 4°C and 39 000 *g* in a Sorvall centrifuge. From the supernatant, 20 μl were taken off and stored in a microvessel at —80°C after quick freezing on dry ice. Prior to analysis, the sample was mixed with an equal volume of distilled water.

Perchloric acid extraction. For PCA extraction, 1000 trefoils were collected on ice and subsequently homogenized via three cycles: freezing, thawing and vortexing. After addition of 42 μl of 0.5 *M* PCA (0°C) with a Hamilton syringe, the sample was left to stand in melting ice for 15 min. The sample was then centrifuged for 5—7 min at 4°C (39 000 *g*). A fixed volume of supernatant (35 μl) was transferred to a microvessel with a Hamilton syringe. After neutralization by addition in quick succession of 5 μl of 0.5 *M* imidazole and 5 μl of 3.5 *M* potassium hydroxide, the resulting potassium perchlorate precipitates were removed by centrifugation (39 000 *g*, 4°C). The pH of the samples should be 7.0—7.5, which was verified with pH-indicating paper using a small aliquot of supernatant. The supernatant was stored in two separate amounts of 20 μl at —80°C. The samples were analysed without dilution. Nucleotide concentrations were calculated from the respective zone lengths (taking into account the equivalent number of trefoils analysed, ca. 50) and the trefoil volume (3.2 nl) [13] and are expressed in nmol/μl of biological material.

RESULTS AND DISCUSSION

When comparing extraction procedures for nucleotides, the primary criteria are recovery and variance in quantitative data. A number of secondary criteria, however, are also relevant: amount of sample needed, number of procedural steps involved, susceptibility to interference and, finally, the ionic strength of the extract to be analysed. The latter can be a problem in conventional iso-

tachophoresis equipment, a difficulty largely overcome by column-coupling [14]. The selectivity of the procedure is also important: components other than nucleotides co-migrating in the operational system will affect the resolving power of the ions of interest. In the operational system used, organic acids with a pK of $\leqslant$ 4 will generally co-migrate. Another possible source of error in nucleotide analysis is decomposition of triphosphates into diphosphates and finally into monophosphates. Enzymatic activity and the effect of temperature are important in this respect. As the parameter for judging stability we have used the total triphosphate/total diphosphate ratio, which should be high.

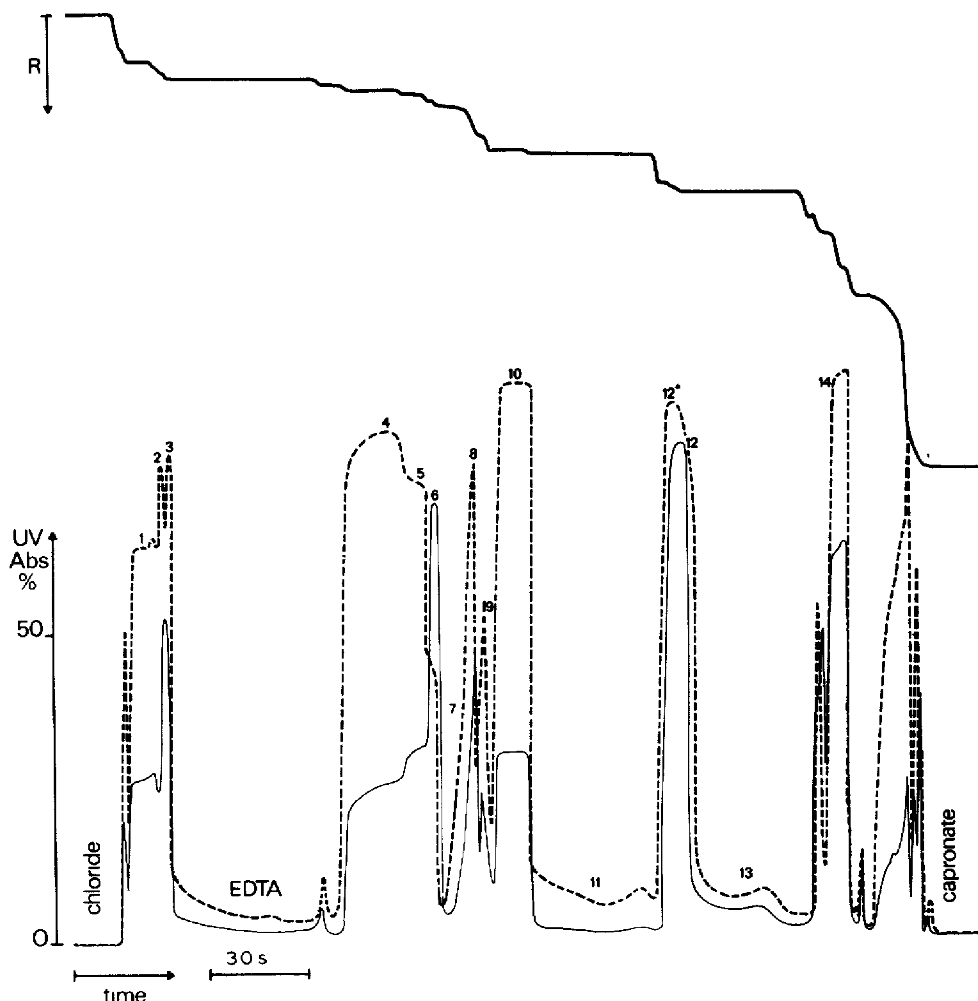

Fig. 1. Analysis of nucleotides extracted from the first cleavage stage of *Nassarius reticulatus* with methanol—EDTA. Detection was a c. conductivity (R) and UV absorption (%) at 254 and 280 nm (dashed line). Peaks: 1 = UTP, 2 = ITP; 3 = GTP; 4 = ATP; 5 = UDP; 6 = CTP; 7 = phosphate; 8 = GDP; 9 = citrate; 10 = ADP; 11 = lactate; 12 = CDP; 13 = glucose 6-phosphate; 14 = GMP. In this run, an additional zone for EDTA is seen, whereas zone 12* (CDP) also contains an impurity which represents an EDTA—metal complex.

A representative isotachopherogram, obtained after analysis of a methanol—EDTA extract, is given in Fig. 1. EDTA evidently increases the sample load as it tends to form mixed zones with adjacently migrating nucleotides. Furthermore, EDTA gives rise to an additional zone of relative step-height 0.380 with a high UV absorption. As this zone is also found in blank extracts, it probably represents an EDTA—metal complex, which migrates in between the nucleotides. The CDP zone appears to be mixed up with this zone. UTP, ITP, GTP, ATP, UDP, CTP, ADP and lactate were identified. Phosphate and GDP form an incompletely resolved mixed zone and, consequently, GDP had to be estimated by the area of the UV profile. It has been suggested [3] that EDTA be used to compensate for variation in injection volume. However, our experiments have shown that the length of the EDTA zone was not correlated with the length of any nucleotide zone. This means that, in our method of injection [13], the variance of injection can be neglected with respect to the biological variance.

The analysis of methanol extracts without EDTA (Fig. 2) is characterized by a higher resolution, especially of the ATP zone, as the EDTA zone is absent.

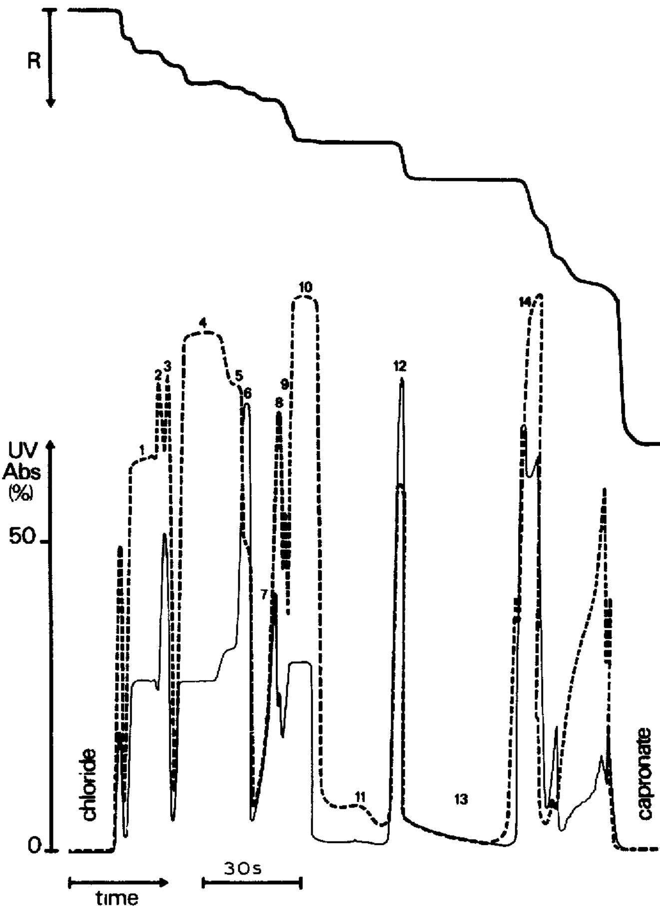

Fig. 2. Analysis of nucleotides after extraction with methanol. For further details refer to the legend of Fig. 1.

TABLE II

AVERAGE RECOVERIES OF NUCLEOTIDES (IN nmol/μl) WITH THE DIFFERENT EXTRACTION PROCEDURES

Values in parentheses indicate standard deviations; G6P = glucose 6-phosphate.

Nucleotide	Methanol—EDTA (n = 6)	Methanol (n = 4)	PCA (n = 6)
UTP	1 9 (0.2)	2.0 (0.5)	3.6 (0.2)
ITP	0.5*	N.D.**	2.7 (0.4)
GTP	0.6*	N.D.	3.2 (0.2)
ATP	4 7 (0.7)	4.2 (1.0)	9.3 (0.9)
UDP	1.8 (0.3)	2.4 (0.2)	3.7 (0.5)
CTP	1 0 (0.1)	1.2 (0.3)	2.5 (0.2)
GDP	1 1 (0.1)	0.7 (0.2)	1.7 (0.4)
ADP	2.7 (0.8)	3.2 (0.6)	5.2 (0.6)
CDP	0 5 (0.2)	0.8 (0.1)	1.3 (0.2)
G6P	14.1 (1.6)	17.9 (1.2)	16.7 (1.2)
GMP	0 9 (0.3)	N.D.	1.5 (0.3)
Total triphosphate/total diphosphate	1 43	1.04	1.79
Total tri- plus diphosphate	14.8	14.5	33.2

*Approximated (see text).
**N.D. = Not determined.

For most nucleotides, recovery is not significantly different (Table II). The lactate zone is shorter so that this procedure seems to be more specific for nucleotides as compared to methanol—EDTA. In contrast to total recovery, the total triphosphate/total diphosphate ratio is different, i.e. 1.04 and 1.43, respectively. This illustrates that, in methanolic extracts, enzymatic decomposition of triphosphates occurs. Fig. 3 shows the isotachopherogram of a PCA extract. The PCA zone migrates directly following the leading zone and does not disturb the nucleotide pattern. The ratio of nucleotide/non-nucleotide material recovered is also the highest of all procedures, so that the PCA method can be regarded as the most specific. The total nucleotide recovery was 33 nmol/μl, with a total triphosphate/total diphosphate ratio of 1.79. The mixed zone between ATP and UDP is caused by the higher sample load due to a higher extraction recovery. This can be coped with by having either a longer separation capillary or a smaller injection volume. As an alternative, we used an approximation procedure. As there is virtually no phosphate recovered, the GDP zone can be more accurately measured, except for the adjacent spike. Table II gives average recoveries for some of the nucleotides with the three different extraction procedures. The relative standard deviations (n = 6) for the PCA extraction were ca. 10%. Statistical analysis of these recovery data has shown that the PCA method is superior.

In conclusion, it must be observed that of all the procedures investigated, PCA extraction gives the best recovery of nucleotides from our specific samples. In addition, it is evidently more specific for nucleotides. Experiments have shown that for PCA extraction, enzymatic activity is sufficiently suppressed so that EDTA is not necessary. With methanol, being a milder denatur-

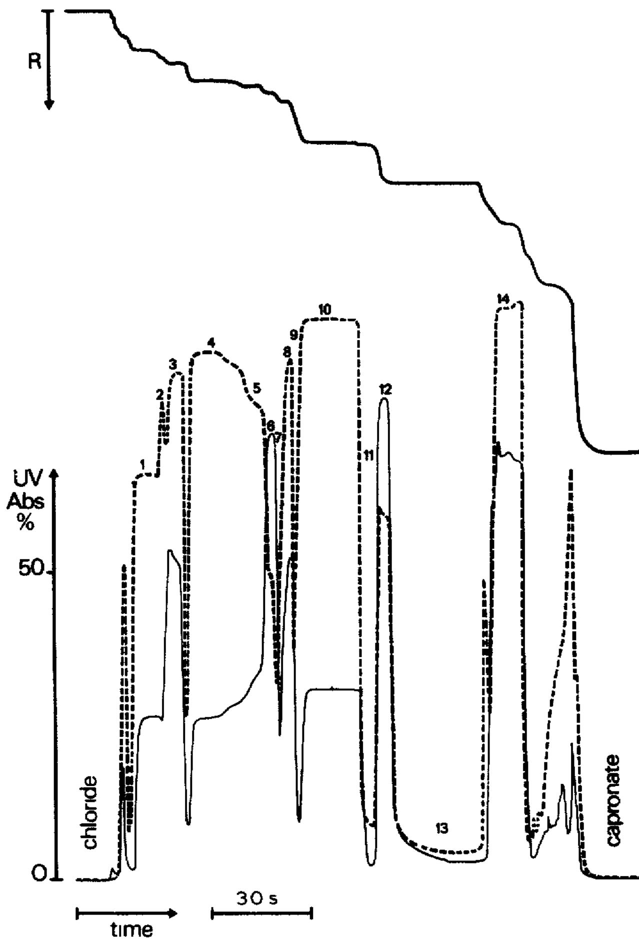

Fig. 3. Analysis of nucleotides after extraction with perchloric acid. For further details refer to the legend of Fig. 1.

ing agent, the use of EDTA is imperative. Although the methanol method is less laborious, and can be carried out at lower temperature, it is less selective and consequently yields a more complicated isotachopherogram, in addition to a lower recovery.

Some authors reported specific preference for the methanol—EDTA procedure, e.g. Oerlemans [16] in a comparative study of muscle extracts. From this, it can be concluded that for any material, a comparison of extraction procedures should be carried out and that the results for our specific sample material should not be generalized.

As column-coupling for isotachophoresis is, as yet, not widely available, the higher ionic strength of the PCA extracts might necessitate the use of methanol—EDTA instead, even though it has a lower recovery. In this respect it should be noted that, for our sample material, the recovery variances are within reasonable limits for both methods.

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REFERENCES

1 C.J. Holloway and J. Luestorff, Electrophoresis (Weinheim), 1 (1980) 129.
2 D.C. Gower and R.C. Woledge, Proc. Anal. Div. Chem. Soc., 14 (1977) 83.
3 R.C. Woledge and P. Reilly, Anal. Chem. Symp. Ser., 5 (1980) 103.
4 F. Oerlemans, C. van Bennekom, C. De Bruijn and S. Kulakowski, J. Inherited Metab. Dis., 4 (1981) 109.
5 M. Aomine, M. Arita, S. Imanishi and T. Kiyosue, Jpn. J. Physiol., 32 (1982) 741.
6 M. Aomine, M. Arita, S. Imanishi and T. Kiyosue, Jpn. J. Physiol., 32 (1982) 895.
7 J.A. Perez, F. Mateo and E. Melendez-Hevia, Electrophoresis (Weinheim), 3 (1982) 102.
8 G. Eriksson, Anal. Biochem., 109 (1980) 239.
9 G. Eriksson and D. Straath, FEBS Lett., 124 (1981) 39.
10 A. Talbot, Acta Med. Okayama, 36 (1982) 407.
11 A. Talbot, Acta Med. Okayama, 36 (1982) 431.
12 C.A.M. van Dongen, F.E.P. Mikkers, Th.P.E.M. Verheggen and C. De Bruijn, Anal. Chem. Symp. Ser., 6 (1981) 207.
13 C.A.M. van Dongen, J.H. Wes, J.H. Goedemans and J.C. Reijenga, Exp. Cell Res., in press.
14 Th.P.E.M. Verheggen, F.E.P. Mikkers and F.M. Everaerts, Protides Biol. Fluids, Proc. Colloq., 27 (1980) 723.
15 J.C. Reijenga, G.V.A. Aben, Th.P.E.M. Verheggen and F.M. Everaerts, J. Chromatogr., 260 (1983) 241.
16 F. Oerlemans, personal communication.