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RAPID AND SENSITIVE METHOD FOR MURAMIC ACID DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRECOLUMN FLUORESCENCE DERIVATIZATION

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SUMMARY

Muramic acid was analysed by high-performance liquid chromatography with precolumn derivatization using *o*-phthalaldehyde in a standard solution and in a culture of marine bacteria from a natural sample. The effects of buffer pH were studied in order to optimize the separation of muramic acid from interfering amino acids. A linear relationship was found between the fluorescence response and muramic acid concentration. The muramic acid–(*o*-phthalaldehyde) derivative gave a single sharp peak, and complete separation from interfering amino acids was achieved at the picomole level in a sort time (3 h for preparation and 10 min for chromatography with the bacterial sample).

INTRODUCTION

Many attempts have been made to estimate indirectly the microbiomass in biological and ecological samples. In order to estimate the bacterial biomass in environmental samples, especially in soils and in marine sediments, several authors have tried to measure the concentration of muramic acid (2-amino-3-O-(D-1'-carboxyethyl)-2-deoxy-D-glucose), which is a relatively specific compound of prokaryotic cell wall peptidoglycans, by chemical and biochemical methods[1–3].

In the past decade, analytical methods for muramic acid determination have been successively improved: colorimetric methods[1,3,4], enzymatic methods[2], gas chromatographic methods[5–7] and liquid chromatographic methods with an amino acid analyser[8] have all been used. The main purposes of these studies were to obtain better sensitivity, to eliminate interference by other compounds and to simplify the analytical procedure. Today, the gas chromatographic method is considered to be the most efficient tool for the muramic acid determination, allowing its evaluation in sediments[5,6], detritus and micro-fouling samples[9,10]. This method, however, still seems to be insufficiently sensitive for analysing this compound in samples of low bacterial biomass, such as seawater, and is, at least, time consuming for routine work.

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Recently, with the development of fluorescence labelling techniques in high-performance liquid chromatography (HPLC), many analyses have been performed including sugars, amino acids, amino sugars, etc.[11–16]. Lindroth and Mopper[17] have shown that amino compounds dissolved in seawater can be analysed by HPLC using precolumn fluorescence derivatization with *o*-phthalaldehyde (OPA) at extremely low concentrations, and some studies have been carried out on its application to amino sugars. Taking into account the chemical structure of muramic acid, we have applied this HPLC procedure to the quantitative analysis of muramic acid.

MATERIALS AND METHODS

Chemicals

Muramic acid, L-asparagine, L-histidine and L-glutamine were purchased from Sigma (St. Louis, MO, U.S.A.). L-Glutamic acid, L-aspartic acid, L-serine, OPA and 2-mercaptoethanol were from Fulka (Buchs, Switzerland). Methanol, sodium citrate, sodium acetate and other chemicals were chromatography or reagent grade.

Apparatus

The following chromatographic apparatus was used: two pumps (Altex, Model 110A), gradient programmer (Altex, Model 420), injector valve with 20-μl loop (Rheodyne, Model 7010), fluorescence detector (Schoeffel, FS 970) with an excitation wavelength of 340 nm and a 418 nm cut-off secondary filter, integrator (Shimadzu chromatopac C-R1B), column for reversed-phase chromatography (Beckman, Ultrasphere ODS 5 μm, 250 × 4.6 mm I.D.).

Buffer and eluent

Buffer A was 0.05 *M* sodium citrate (pH 5.6–6.6) and buffer B was 0.05 *M* sodium citrate–0.05 *M* sodium acetate (50:50, pH 5.3). Eluent A was buffer A–methanol–tetrahydrofuran (80:18.5:1.5) and eluent B was methanol–buffer B (80:20). The flow-rate was 1 ml/min and chromatography was performed in the pressure range 150–250 bar. The elution gradient was programmed as follows:

Time (min)	0	1.5	10.2	10.5	14.5	21.5
Eluent B (%)	0	24	28	35	37	100

Derivatization procedure

The OPA–mercaptoethanol reagent was prepared according to Lindroth and Mopper[17]. A 100-μl volume of the final sample solution was placed in a Rhesus tube and 500 μl of OPA reagent were added. After 2 min of incubation at room temperature, a full loop (20 μl) was always injected and chromatographed. Under these conditions, the ratio of the amount of muramic acid injected to the amount of muramic acid per microlitre of the final solution was always 3.3; *e.g.* with 100 pmole/μl as the final concentration of the standard solution, 330 pmole were injected. Glassware was acid-washed and dried at 550°C.

Experimental

Effect of the eluent pH on the separation of muramic acid from amino acids. A standard mixture containing 100 pmole/μl each of muramic acid and six amino acids, Asp, Glu, Asn, His, Ser and Gln, was prepared and analysed as described above. The pH of Buffer A tested varied from 5.6 to 6.6.

Relative fluorescence versus muramic acid concentration. The standard solution of muramic acid was analysed over the concentration range 1–100 pmole/μl. The pH of buffer A was adjusted to 6.6 for this assay.

Bacterial sample assay. Seawater was enriched by adding Bacto-peptone (Difco), and after 5 days of incubation a sufficient bulk of packed cells was obtained by centrifugation and then freeze-dried. Then 10 mg of the freeze-dried sample were placed in a screwcap test-tube. At this point, some samples were spiked with standard muramic acid to help determine the recovery of muramic acid. After hydrolysis at 105°C in 3 ml of 6 *N* hydrochloric acid for 2.5 h under nitrogen, the samples were cooled and 300 μl of hydrolysate were evaporated under reduced pressure at 40°C (Büchi, Rotavapor) to dryness. The residue was dissolved in 3 ml of doubly distilled water and chromatographed as described above. For this assay, the pH of buffer A was adjusted to 6.6.

RESULTS AND DISCUSSION

Muramic acid assay by amino acid analysis

The chemical structure of muramic acid, which is close to those of amino acids (especially the presence of a free NH_2 radical) suggests the possibility of an application of the HPLC analysis by OPA derivatization[12,13,17] with slight modification to the measurement of this compound. In the first assay, standard muramic acid was added to a mixture of amino acids and assayed according to the method for amino acid analysis[12]. At pH 6.2 for buffer A, which corresponds to a classical amino acid analysis, a single sharp peak of muramic acid appears in the beginning of the analysis between Glu and Asn around 26–28% of eluent A in A + B in the elution gradient. But owing to the net predominance in the amount of amino acids in natural samples compared with the muramic acid fraction, we have tried to optimize its separation from a possible amino acid interference.

pH effects on the separation of muramic acid

Because the muramic acid peak appears at the beginning of the gradient, as previously observed, it can be deduced that the pH value of Eluent A has made influence on the separation than that of eluent B. We have thus tested seven pH values of buffer A (5.8–6.6) to study the behaviour of muramic acid against interfering amino acids. Some chromatograms obtained are presented in Fig. 1. Three points are to be noted. First, between pH 5.8 and 6.6, the muramic acid is gradually delayed as the pH value of buffer A is increased. Second, the relative fluorescence response of this compound is nearly constant, which allows the optimization of buffer pH without significant loss of sensitivity of the analysis. At pH 5.8, the muramic acid peak overlapped that of Glu. At lower pH, this peak is between Asp and Glu. Because of the possible presence of other unknown peaks in this region, there is a risk of interference with the analysis of natural samples. As the pH is increased from 6.0,

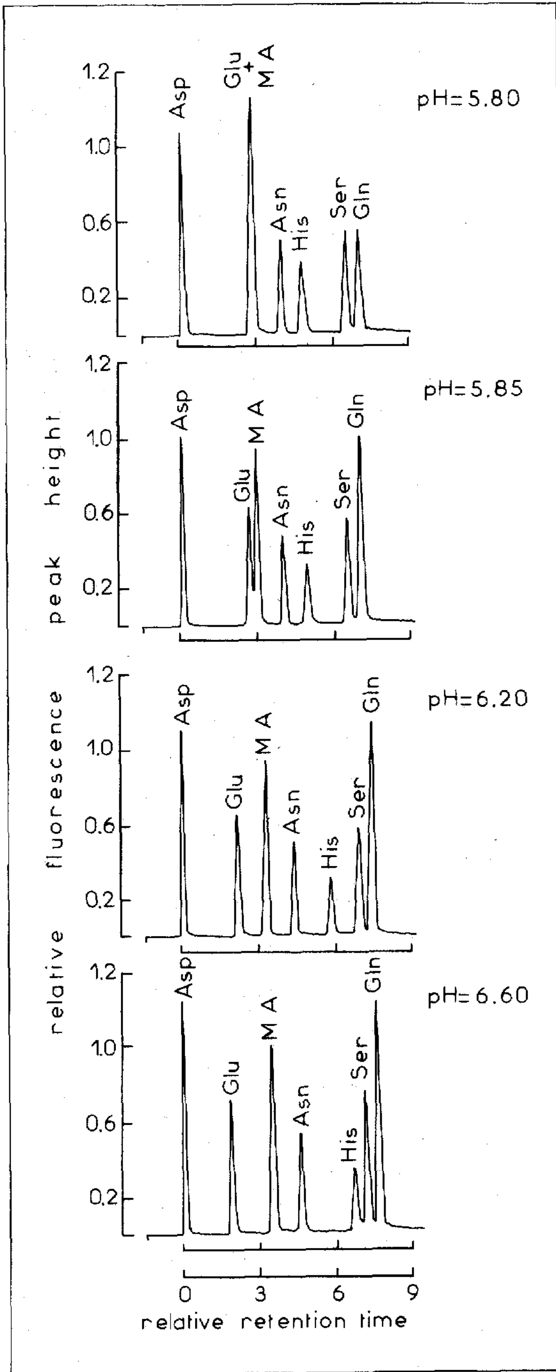

Fig. 1. Influence of Buffer A pH on the separation of muramic acid (MA) from amino acids (experimental conditions as described under Materials and Methods). Retention times relative to Asp = 0.

TABLE I

INFLUENCE OF THE pH OF BUFFER A ON RELATIVE RETENTION TIMES AND CAPACITY FACTORS OF GLUTAMIC ACID, MURAMIC ACID AND ASPARAGINE

pH of Buffer A	*Relative retention time (min)**			*Capacity factor, k'***		
	Glu	*MA*	*Asn*	*Glu*	*MA*	*Asn*
5.85	2.69	3.01	4.13	3.53	3.69	4.25
6.0	2.39	3.03	4.13	3.22	3.54	4.09
6.2	2.27	3.41	4.56	3.27	3.84	4.42
6.4	2.01	3.42	4.59	3.04	3.75	4.35
6.5	1.98	3.55	4.74	3.00	3.78	4.38
6.6	1.93	3.60	4.79	3.01	3.85	4.49

* Retention time relative to Asp.

** $k' = \frac{t_R - T_m}{T_m}$ (T_m = retention time of unretained compound).

the separation of muramic acid from Glu progressively improved (Fig. 1). However, at pH values higher than 6.8, erosion of the stationary phase may occur[17], shortening the column life without great improvement of separation.

To optimize and visualise the muramic acid (MA) separation, two parameters, capacity factor (k'), and separation factor $\alpha = k'_2/k'_2$ (associated numbers were in order of elution) were employed (Table I, Fig. 2). The values of $\alpha_{MA/Glu}$ and $\alpha_{Asn/MA}$ clearly show that muramic acid separation from Glu improves as the pH of buffer A increases while that from Asn remains constant. Therefore, the best separation of muramic acid from interfering amino acids without erosion of the column resin is obtained at pH 6.6 of Buffer A. This allows, in the bacterial materials, the omission of preliminary purification steps reported by some authors[7,16]. In samples where ratio of amino acids (mainly with Asp and Glu) to muramic acid exceed the capacity of analysis, such as in sediments, this purification procedure may be necessary[18].

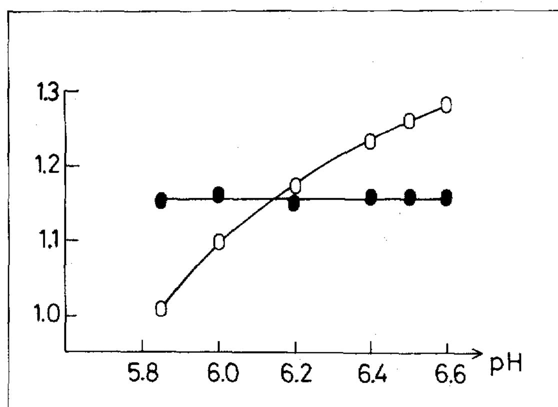

Fig. 2. Relationships between separation factors and Buffer A pH. ○, k'_{MA}/k'_{Glu}; ●, k'_{Asn}/k'_{MA}.

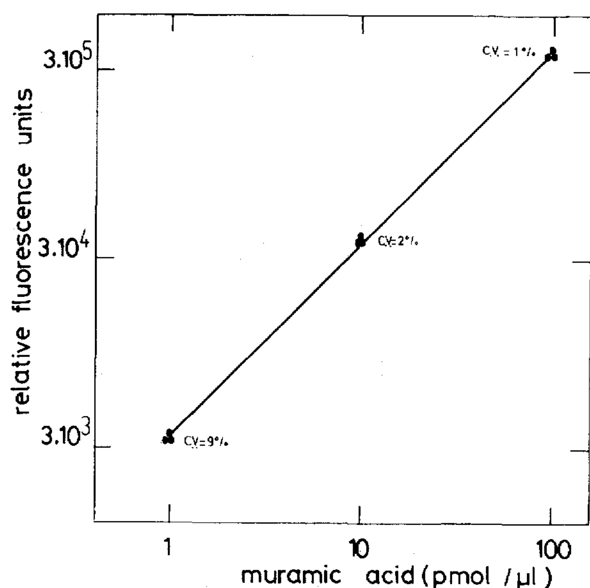

Fig. 3. Log–log plot of muramic acid concentration *versus* relative fluorescence.

Relative fluorescence response to muramic acid concentration

Under the experimental procedure described above, the fluorescence response were determined for increasing amounts of muramic acid in a wide concentration range (3.3–330 pmole per injection, which corresponds to a real concentration range of 1–100 pmole/μl of final standard solution). In this range, the relationship between muramic acid concentration and fluorescence response was linear (Fig. 3). Moreover,

TABLE II

COMPARISON OF METHODS FOR MURAMIC ACID ANALYSIS

Author (method)	*Detection sensitivity*	*Recovery (%)*	*Reproducibility (%)*	*Analysis time (h)*
Millar and Cassida, 1970 (col)*	–	79.0	5.1	13**
King and White, 1977 (col)*	5–20 μg/ml	–	17.0**	12**
Moriarty, 1977 (enz)*	0.28 μg/ml**	–	10.0	8.5**
Casagrande and Park, 1978 (GLC)*	–	83.0**	–	5**
Fazio *et al.*, 1979 (GLC)*	0.11 μg/inj**	99.8	9.5	17
Hicks and Newell, 1983 (split GLC)*	1.15 μg/inj**	93.0	7.8	10
(splitless GLC)*	0.01 μg/inj	–	–	10
Mimura and Delmas, this report (HPLC)	0.1 ng/inj	92.8	4.7	3.5

* col = Colimetric; enz = enzymatic; GLC = gas–liquid chromatographic.

** Estimated by Hicks and Newell[7].

under the reported experimental conditions, the absolute retention time (t_R) of muramic acid appears to be stable (mean t_R = 9.8 min; coefficient of variation, C.V. = 1.4%, n = 9). The ordinate value at the origin of the regression line (b = 0.09 in relative fluorescence units) corresponds to the background noise of the protocol, but its low value allows a detection threshold below 1 pmole/μl. Taking into account all possible interferences linked to the method and apparatus, the practical detection limit with our experimental procedures is *ca.* 0.3 pmole of muramic acid per injection (*i.e.* 0.1 pmole/μl of final standard solution). This detection threshold is close to that of the direct fluorescent assay with OPA derivatization on standard amino sugars which had been previously reported[15].

But, as shown in Table II, the detection limit of this method is much better than that of muramic acid assay by gas–liquid chromatography[5–7], by a factor of 10^2–10^4.

Marine bacterial population assay

This analytical method was also tested on a marine bacterial population which had been cultured on nutrient-enriched seawater (see Methods). The dry particulate matter obtained was hydrolysed and chromatographed (see Methods) (Fig. 4).

The large difference between the concentration of muramic acid and its nearer amino acids in this natural sample provides a justification of the pH value which was chosen for a better separation. Without any purification steps, we ought to obtain 1.52 μg of muramic acid per milligram of dry matter using 10 mg of dry sample. Recovery yields in the course of the whole procedure were estimated by the addition of a standard of muramic acid to this sample prior to the hydrolysis step. The approximative ratio of spiked standard to sample muramic acid concentration was *ca.* 1. As shown in Table II, the recovery of added internal standard was 92.8% (n = 3, C.V. = 4.8%). This loss may be put down to the condensation of muramic acid during the evaporation step. In order to obtain a better recovery, glycerine may be

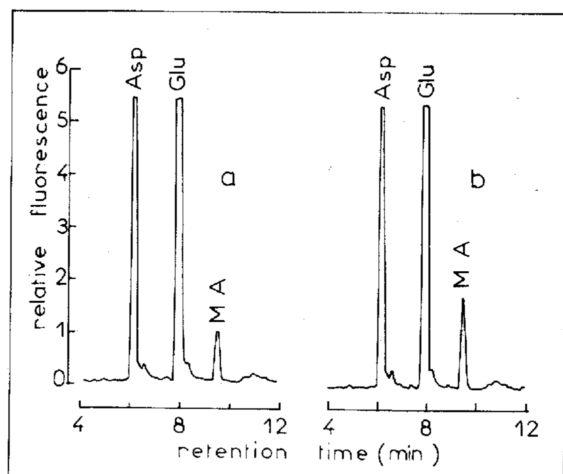

Fig. 4. Bacterial extract chromatograms: (a) direct analysis; (b) analysis of the same sample spiked with standard muramic acid prior to hydrolysis (the ratio of spiked standard to sample muramic acid concentration was *ca.* 1).

added prior to evaporation[19]. The effect of the sample extraction (pH, salts, etc.) on the fluorescence yield of muramic acid was studied by adding a known amount of standard muramic acid solution to the extract at the derivatization step. Recovery of this standard of muramic acid was 100.4% (n = 4, C.V. = 4.8%). So we must conclude that there is no effect of sample extract on OPA derivatization and on fluorescence response under our experimental conditions.

CONCLUSION

Table II lists some literature data concerning the measurement of muramic acid by different methods. The results show that HPLC analysis with precolumn fluorescence derivatization provides a better sensitivity. The complete separation and quantitative analysis of muramic acid were achieved without sample purification in both standard solution and cultured marine bacterial samples. The enhancement of the sensitivity, good reproducibility and reduced analysis time may permit smaller sample sizes and automation of the analysis of muramic acid in large variety of natural samples in routine work.

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