Determination of organomercury compounds using horseradish peroxidase immobilised on a polyurethane foam

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Polyurethane foam as a novel support for immobilization of horseradish peroxidase has been used for the development of a test technique for the determination of trace organomercury cations ($MeHg^+$, $EtHg^+$, $PhHg^+$) based on their liberating effect on the enzyme inhibited by phenylthiourea in the *o*-dianisidine oxidation or on their effect on the duration of an induction period in the 3,3′,5,5′-tetramethylbenzidine oxidation in the presence of diethyldithiocarbamate.

The determination of trace inorganic and organic mercury has an increasing importance in environmental analysis. Organomercury compounds (OMCs) are the most toxic mercury species. Instrumental methods[1–4] for the determination of OMCs are highly sensitive and selective, but the majority of them are fairly complicated and require the use of specialised and expensive equipment. Test methods are characterised by simple experimental techniques and, as a rule, require little or no instrumentation. In enzymatic test methods, the advantages of enzyme assay (high sensitivity and selectivity) and immobilised enzymes (simplicity of application and storage) are combined.

Recently,[5] test methods for the determination of 0.02–1000 μmol dm^{-3} of OMCs were developed using horseradish peroxidase (HRP) immobilised in chitosan in the wells of a polystyrene plate and on chromatography paper. At the same time, it has been shown[6] that the most promising support for peroxidase immobilization is a polyurethane foam (PUF). The preparation of PUF-immobilised peroxidase retains a constant catalytic activity for longer than 1.5 years, whereas in the case of polystyrene plates and chromatography paper the enzyme preparations are stable for a year and 6 months, respectively.[7–8]

We have examined the effect of OMC cations (methyl-, ethyl- and phenylmercury) on HRP immobilised in chitosan on a polyurethane foam and developed a new test procedure for the determination of organic mercury.†

Determination of OMCs using (i) o-dianisidine oxidation in the presence of phenylthiourea, or (ii) TMB oxidation in the presence of DEDTC, catalysed by PUF-immobilised HRP. The required volumes of solutions of the reaction components were applied sequentially to the same point at the surface of a PUF tablet (diameter of 0.8 cm and thickness of 0.3 cm) with immobilised peroxidase using a micropipette.

(i) When *o*-dianisidine oxidation was used, 12 ml of a 0.1 mol dm^{-3} potassium hydrogen phthalate buffer (pH 5.0) and 2 μl of 0.1 mol dm^{-3} phenylthiourea were added to the PUF-immobilised peroxidase. Next, 6 μl of a methyl-, ethyl- or phenylmercury solution of a required concentration, 6 ml of 2.5 mmol dm^{-3} *o*-dianisidine and 6 μl of 5 mmol dm^{-3} hydrogen peroxide were added sequentially.

† *Experimental*. Horseradish peroxidase (1.11.1.7) from 'Reanal' (Hungary) was used (RZ = A_{403}/A_{430} = 3.28). The aqueous enzyme solutions were prepared by dissolving the commercial enzyme preparation in a sodium borate buffer (pH 7.0) containing 20 vol% of a 0.1 M sodium nitrate solution to maintain a constant ionic strength. Solutions of the enzyme substrates [*o*-dianisidine, *o*-phenylenediamine and 3,3′,5,5′-tetramethylbenzidine (TMB)], sulfur-containing organic compounds [phenylthiourea and diethyldithiocarbamate (DEDTC)], MeHgI, EtHgBr and PhHgCl were prepared daily by dissolving accurately weighed amounts (1.0–3.0±0.2 mg) in ethanol; chemicals from 'Soyuzreaktiv' (Russia) were used. The preparation of phenylmercury solutions included preliminary dissolution of an accurately weighed portion of PhHgCl in 3–4 drops of 0.1 M HCl in order to prevent decomposition. The solution obtained was diluted to the required volume with ethanol. Solutions of phenylthiourea, DEDTC and OMCs were stored in the dark. All the solutions were prepared using twice-distilled and demineralised water.

Peroxidase was modified and immobilised using water-soluble native chitosan[9] and PUF according to the procedure described in ref. 5.

Table 1 Colour changes of polyurethane foams in the oxidation of *o*-dianisidine, *o*-phenylenediamine and TMB, catalysed by immobilised peroxidase, in the presence of (i) phenylthiourea and (ii) DEDTC.

Indicator reaction	Colour change	
	(i)	(ii)
o-Dianisidine–H_2O_2	blue→green→red[a]	brown→red-brown
o-Phenylenediamine–H_2O_2	pale red→pale blue	pale red→pale blue
TMB–H_2O_2	pale blue→grey→colourless	blue→brown

[a]The colour appearance was monitored visually.

(ii) When TMB oxidation was used, 12 μl of a 0.1 mol dm^{-3} potassium hydrogen phthalate buffer (pH 5.0), 6 μl of 5 mmol dm^{-3} TMB, 4 μl of 7.5 mmol dm^{-3} DEDTC, 6 μl of an OMC solution of a required concentration and 6 μl of 5 mmol dm^{-3} hydrogen peroxide were added sequentially to the PUF-immobilised peroxidase.

In both procedures, at the instant when hydrogen peroxide was added, a stopwatch was started, and the time taken for the spot to develop a red (i) or brown (ii) colour was measured. The calibration graph was plotted as the time of appearance of the corresponding colour *vs*. OMC concentration.

Choice of the indicator system. For the development of the test method for determinating OMCs, peroxidase-catalysed reactions of *o*-dianisidine, *o*-phenylenediamine and TMB oxidation in the presence of phenylthiourea and DEDTC were used as indicators. Phenylthiourea and DEDTC were introduced in order to enhance the effect of OMCs on the enzymatic processes.[7–8,10] The indicator reaction rate was monitored visually by colour changes of the intermediate and final products, presented in Table 1.

The most contrasting colour changes on PUF were observed in the cases of *o*-dianisidine oxidation in the presence of phenylthiourea (blue–green–red) and TMB oxidation in the presence of DEDTC (blue–brown). The colour changes in the *o*-phenylenediamine oxidation gave poor contrasts in all cases.

Thus, the *o*-dianisidine–H_2O_2 in the presence of phenylthiourea and TMB–H_2O_2 in the presence of DEDTC indicator systems seem to be most promising for the development of the test method for determining OMCs.

We found that the introduction of an OMC to the indicator reactions with PUF-immobilised peroxidase led to the same effect as in the presence of native peroxidase. Thus, methyl-

Table 2 Optimum concentrations of the components of the reactions of (i) *o*-dianisidine and (ii) TMB oxidation in the presence of PUF-immobilised peroxidase (0.1 mol dm^{-3} potassium hydrogen phthalate buffer, pH 5.0).

Component	Optimum concentration/mmol dm^{-3}	
	(i)	(ii)
o-Dianisidine	0.4	—
TMB	—	0.8
H_2O_2	0.8	0.8
Phenylthiourea	0.2	—
DEDTC	—	1.25

Table 3 Analytical characteristics of the test procedures for determining the OMCs using *o*-dianisidine oxidation in the presence of phenylthiourea, catalysed by (i) PUF and (ii) paper-immobilized peroxidase[5] (higher limit of analytical concentrations is 1 mmol dm^{-3}).

OMC	Calibration equation	*r*	(i)		(ii)	
			c_l[b]/μmol dm^{-3}	RSD[c] (%)	c_l[b]/μmol dm^{-3}	RSD[c] (%)
Methylmercury	$y = -13.5x + 4.5$	0.9998	0.008	11	1	23
Ethylmercury	$y = -13.3x + 3.9$	0.9998	0.01	12	11	18
Phenylmercury	$y = -11.1x + 3.7$	0.9998	25	12	12	18

[a]*y* is the time of the appearance of a red colour, *x* is the OMC concentration (μmol dm^{-3}). [b]Lower limit of analytical concentrations. [c]Calculated at c_l (n = 3).

Table 4 Analytical characteristics of the test procedures for determining the OMCs using TMB oxidation in the presence of DEDTC, catalysed by (i) PUF and (ii) paper-immobilized peroxidase[5] (higher limit of analytical concentrations is 1 mmol dm^{-3}).

OMC	Calibration equation	*r*	(i)		(ii)	
			c_l[b]/μmol dm^{-3}	RSD[c] (%)	c_l[b]/μmol dm^{-3}	RSD[c] (%)
Methylmercury	$y = -16.9x + 31.3$	0.9998	0.01	9	5	30
Ethylmercury	$y = -7.8x + 27.6$	0.9996	1	11	120	26
Phenylmercury	$y = -6.9x + 30.4$	0.9994	75	12	110	23

[a]*y* is the time of the appearance of a brown colour, *x* is the OMC concentration (μmol dm^{-3}). [b]Lower limit of analytical concentrations. [c]Calculated at c_l (n = 3).

mercury (as well as other OMCs) decreases the inhibiting effect of phenylthiourea on the enzyme catalysing *o*-dianisidine oxidation. This results in a decrease in the time of the appearance of a red colour of the final product of *o*-dianisidine oxidation. Thus, methylmercury acts as a liberator of the inhibited immobilised enzyme[11] as in the case of native peroxidase. Such a liberating effect can be explained by the interaction between methylmercury and phenylthiourea (as we earlier showed by spectrophotometry).

The combined effect of DEDTC and an OMC (methyl-, ethyl- or phenylmercury) results in a decrease in the induction period in comparison with that in the absence of OMCs. The time of the appearance of a brown colour of the final product of TMB oxidation in the presence of DEDTC decreased proportionally to the OMC concentration. Note that the nature of the anion has no influence on the rate of the indicator process.

Optimisation of conditions for the determination of OMCs in the presence of phenylthiourea. The OMC effect on the rate of *o*-dianisidine oxidation in the presence of PUF-immobilised peroxidase was examined under optimum conditions that were chosen by a detailed investigation (Table 2). The optimum concentrations of the indicator reaction components were selected to give a contrasting colour change on PUF after 30–40 s. This period of time is sufficient to measure reliably the inhibiting effect of phenylthiourea and to make the procedure reasonably rapid.

The analytical characteristics of the developed procedures are presented in Table 3. Note that, in contrast to the earlier developed procedures for determining OMCs with another solid supports for immobilised peroxidase, it is not necessary to preincubate the enzyme with components of the reaction. Thus, this procedure is more rapid.

Optimisation of conditions for the determination of OMCs in the presence of DEDTC. The rates of TMB oxidation catalysed by PUF-immobilised HRP in the presence of DEDTC as functions of the concentrations of TMB, H_2O_2 and DEDTC were investigated. The rate was characterised by the time of appearance of a brown colour on a PUF tablet. After varying the concentration conditions for the indicator reaction in the presence of DEDTC, optimum concentrations that gave a contrasting colour change on PUF after 60 s were chosen (Table 2).

We found that at DEDTC concentrations higher than 100 mmol dm^{-3} a brown colour did not appear at all. For DEDTC concentrations from 0.75 to 10 mmol dm^{-3}, there is no induction period, and the reaction proceeds with immediate appearance of a blue colour further changed to a brown colour. A DEDTC concentration range of 75–100 mmol dm^{-3} was chosen as optimum because it led to a maximum induction period.

Under the optimum conditions, OMCs decreased the induction period in proportion to their concentration. This fact allowed us to develop a test procedure for the determination of OMCs with the analytical characteristics presented in Table 4. Inorganic mercury(II) does not influence the rate of the indicator reactions in the presence of phenylthiourea and DEDTC. Mercury(II) inhibits the peroxidase activity only in the presence of thiourea.[12] Thus, the proposed test procedures for the determination of OMCs are selective for mercury(II). The investigation of the selectivity of the determination of OMCs in the presence of other known effectors of native peroxidase (heavy metals, sulfur-containing organic compounds) showed that a 100-fold excess of Pb^{II} or Bi^{III} interfered with the determination of OMCs at a level of C_L using procedure (ii) because of the capability of these cations to interact with DEDTC, changing the duration of the induction period. Sulfur-containing organic compounds (such as acetylthiourea, 1,4-dithiotrietole, 1,2,4-triazolethiole), which are also peroxidase inhibitors (weaker than phenylthiourea), can interfere with the determination of OMCs according to procedure (i) at their 10^6-fold excesses.

The procedure developed was successfully applied to the determination of methylmercury in water of the Kara Sea.[13]

The use of PUF-immobilised peroxidase allowed us to develop test procedures for the determination of methyl-, ethyl- and phenylmercury cations, which are more sensitive, reproducible and rapid than those with the use of peroxidase immobilised in polystyrene plate wells and on chromatography paper. It should be emphasised that the catalytic activity of the PUF-immobilised enzyme is stable for a much longer time. The procedures are simple, rather selective (without complicated sample preparation), inexpensive and usable for the determination of organomercury compounds in natural water under field conditions.

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