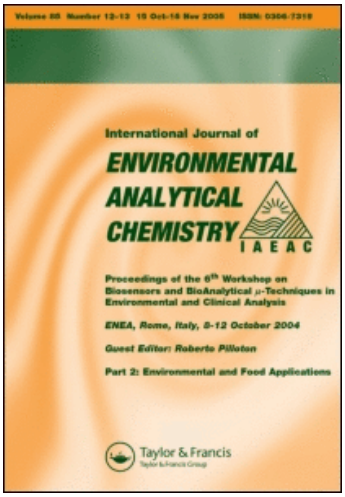

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Enzymatic Determination of Dimethylformamide in Waste Water Using Flow Injection Analysis

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The novel enzyme N,N-dimethylformamidase has been immobilized on controlled pore glass beads and introduced into a Flow Injection Analysis manifold. Using this system it was possible to determine the dimethylformamide content of industrial waste water and process effluents in 8 of 10 examined samples. Concentrations up to 15 mM could be determined with a detection limit of 0.05 mM. The immobilized enzyme is stable for months and has an operational lifetime of several days. The analytical system can be used for a permanent control of the dimethylformamide concentration in industrial waste water and process effluents.

KEY WORDS: Immobilized enzyme, flow injection analysis, waste water, N,N-dimethylformamide, N,N-dimethylformamidase.

INTRODUCTION

Organic solvents are generally used in large quantities in the production of specialities, such as drugs, agrochemicals, dyestuffs, etc. Part of the solvent often can be recycled, but a significant portion has to be disposed of. These organic solvents, present in the waste water, can cause a variety of environmental problems. Therefore, a

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permanent control of the solvent content of waste water and process effluents is very important. Since the composition of such waste water and process effluents is rather complex, a selective analytical method must be applied to determine one single compound.

Here we report a method, which offers the required selectivity by the application of an immobilized enzyme, introduced into an analytical system. This concept was realized by applying the following strategy:

A screening was set up for microorganisms which have the ability to use a given, critical compound as a single *C*- and/or *N*-source.[1] Such strains of microorganisms were isolated and their metabolic pathway used for the degradation of the critical compound was investigated.[1] Enzymes pivotal for the catabolism of the critical compound were isolated and characterized.[2] After immobilization of the enzyme(s) and their integration into an analytical system, selective and sensitive detection of the enzymatic reaction products was possible.

We report the immobilization of the novel enzyme N,N-dimethylformamidase (DMFase),* which catalyses the hydrolysis of DMF to dimethylamine (DMA) and formate. This enzyme has been purified from *Pseudomonas DMF 3/3*.[1,2] The immobilized enzyme is integrated into a Flow Injection Analysis (FIA) manifold used to detect DMA. The combination of immobilized enzyme and FIA can be used for a permanent control of the DMF content in industrial waste water and process effluents.

MATERIALS AND METHODS

DMFase was prepared as described by Schär *et al.*[2] and immobilized as follows: 200 mg aminopropyl controlled pore glass beads (pore size 51 nm) were added to a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. The reaction was performed under reduced pressure (to remove air bubbles from the glass beads) at room temperature for 1 h. The beads were then extensively washed with phosphate buffer and dried under vacuum. 0.5–1 ml of a DMFase solution (25–30 mg/ml; approx. 2 U/mg) were added. This

*Abbreviations: DMFase: N,N-dimethylformamidase; DMF: N,N-dimethylformamide; DMA: dimethylamine; FIA: Flow Injection Analysis.

solution was kept under reduced pressure for 1 h and afterwards extensively washed with phosphate buffer.[3] The beads were not allowed to dry during the washing. The immobilized DMFase was stored at 4 °C in phosphate buffer pH 7.0 and no loss of activity was observed during 9 months.

DMFase activity of soluble and immobilized enzyme was determined as described by Schär *et al.*[2] When DMF was determined using the FIA system described below, detection of DMA, the product of the enzymatic conversion, was done according to the method described by Benson and Spillane,[4] as modified by M. Garn for the use in a FIA system and with aqueous solutions. The color formed after polymerization of 1,4-benzoquinone was followed at a wavelength of 500 nm. It should be noted, that in the FIA system used, the determination of DMA is based on kinetic discrimination.[5] 1,4-benzoquinone polymerizes under the conditions used. This polymerization is accelerated by DMA. The advantage of the FIA system is, that no correction for the uncatalysed polymerization is necessary.

The FIA system used is shown in Figure 1. Teflon tubes (inner diameter 0.8 mm) were used and samples were injected with a Teflon low pressure injection valve (Rheodyne, Ismatec AG, Zürich, Switzerland). The pump was a Gilson minipuls 2 (Synmedic AG,

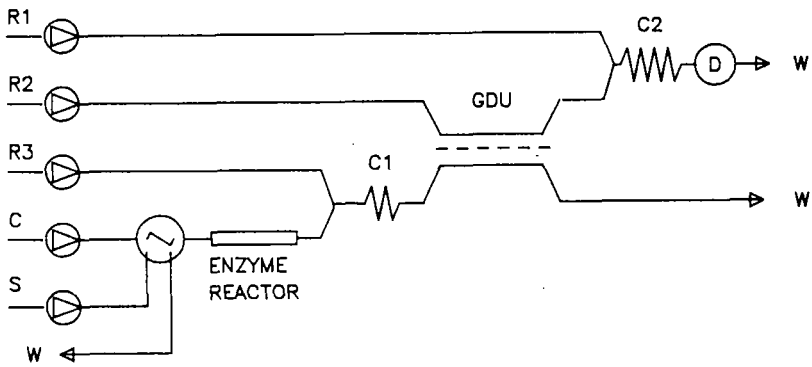

Figure 1 The reagents are: R1. 0.035 M 1,4-benzoquinone, 0.66 ml/min. R2. 0.05 M Na_2CO_3 + 0.025 M $Na_2B_4O_7$ pH 9.8, 0.66 ml/min. R3. 0.1 N NaOH, 0.28 ml/min. C. 0.067 M phosphate buffer pH 7.0, 0.32 ml/min. S. Sample diluted with 0.067 M phosphate buffer pH 7.0. Injection volume 0.1 ml. C1. Mixing coil 25 cm. C2. Mixing coil 150 cm. D. Detector at 500 nm. W. Waste. GDU. Gas diffusion unit. All reagents were filtered before use.

Zürich, Switzerland) and the detector was a HPLC detector from Kratos (Spectroflow 783, Kratos Analytical AG, Basel, Switzerland). The gas diffusion unit was built in house and contained a Teflon membrane (thickness 0.025 mm). It allows the passage of DMF, DMA and formate (M. Garn, unpublished results; see also van der Linden[6]). The enzyme reactor (volume 0.15 ml) was constructed as a normal glass column and contained the immobilized DMFase.

Aminopropyl controlled pore glass and 1,4-benzoquinone were from Fluka AG (Buchs, Switzerland) and glutaraldehyde (25% solution in water, stabilized with Amberlite A-21) was from Serva (Heidelberg, FRG). Samples of DMF containing waste water and process effluents were obtained from different plants of Ciba-Geigy AG in Switzerland.

RESULTS AND DISCUSSION

Immobilization of DMFase

DMFase could be immobilized by a standard method.[3] Determination of the protein content and DMFase activity in the supernatant obtained after immobilization of DMFase showed that it is possible to immobilize 50–75 mg of DMFase (100–150 units) per gram of aminopropyl controlled pore glass. No loss of enzymatic activity was observed after immobilization on the glass beads using glutaraldehyde as a crosslinking agent. Furthermore, if stored in phosphate buffer solution (pH 7.0) at 4 °C the immobilized DMFase did not show any loss of activity during 9 months.

The application of the DMFase for analytical purposes required not only the immobilization of the enzyme but also the set-up of a suitable detection method for one of the products of the enzymatic reaction (DMA or formate). Since a strength of FIA is the possible application in the continuous process control[7,8] it was straight forward to combine this method and an already established DMA determination with a reactor containing the immobilized DMFase. Such a system should allow the permanent control of DMF in e.g. waste water.

Determination of DMF in standard solutions using the immobilized DMFase

When the immobilized DMFase was introduced into the FIA system (Figure 1), it was possible to determine DMF in standard solutions

up to a concentration of 15 mM with a detection limit of 0.05 mM (Figure 2). Injecting DMA instead of DMF into the FIA system revealed, that in the linear range of the calibration curve a complete conversion of the DMF occurs within the enzyme reactor under the conditions used.

Determination of DMF in waste water and process effluents

The results obtained with diluted waste water and process effluent samples are shown in Figure 3. Some of the waste water samples had a strong absorbance at 500 nm. This did not interfere with the detection of DMA using 1,4-benzoquinone, since none of the colored compounds passed through the Teflon membrane of the gas diffusion unit.

Changing from standard solutions to "real" samples one has to be aware of the following facts:

—Some of the components present in the sample may inhibit the enzyme activity and therefore the measured concentration of

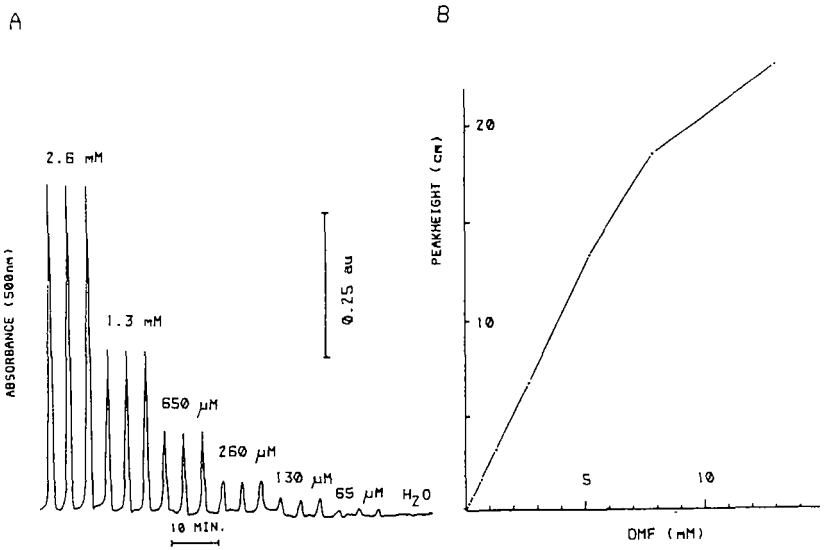

Figure 2 Standard DMF solutions in 0.067 M phosphate buffer pH 7.0 were injected into the FIA system shown in Figure 1. A: Original trace of the calibration. B: The peak height is plotted against DMF concentrations. au: Absorbance units.

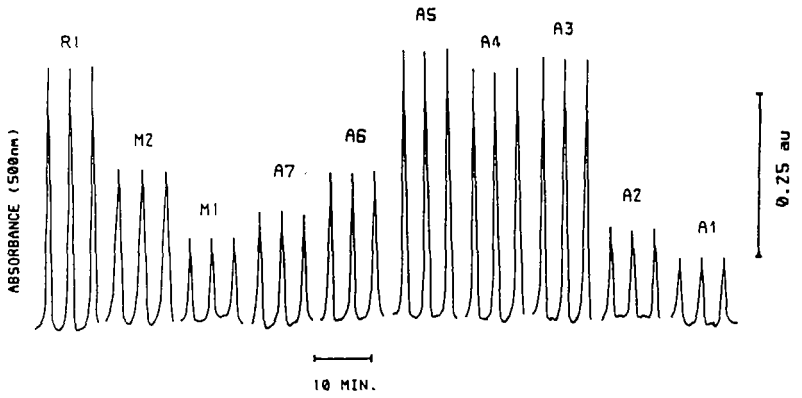

Figure 3 Waste water samples (A1–A6), process effluents (M1, M2 and R1) were injected into the FIA system shown in Figure 1. The samples were diluted in 0.067 M phosphate buffer solution pH 7.0 to give a signal which falls in the linear range of the calibration curve shown in Figure 2. au: Absorbance units.

DMF is too low. This was not observed in our samples, probably due to high dilution necessary to adjust the DMF concentration to a level within the linear range of the calibration curve.

—In the FIA system used every compound which passes through the Teflon membrane of the gas diffusion unit and which catalyses the polymerization of 1,4-benzoquinone is detected. This is especially important for DMA present in the sample. Therefore, if the product of the enzymatic reaction is already present in the sample, it is necessary to carry out control experiments using a FIA system without an enzyme reactor. Alternatively, a reactor without immobilized DMFase can be included. The DMF concentration in the sample is then determined by the difference of the measurements with and without enzyme reactor.

These aspects are outlined in Table 1. In column B the results obtained after the injection of the samples in a FIA system which does *not* contain the enzyme reactor are shown. The system is calibrated with DMA, though other unknown compound of the sample might contribute to the signal obtained (see above). A

Table 1 Enzymatic versus gaschromatographic determination of dimethylformamide in industrial samples

Substances determined	*A* *DMF+DMA* *(M/l)*[a]	*B* *DMA* *(M/l)*[b]	*C* *DMF* *(M/l)*	*D* *DMF* *(M/l)*
Method	*FIA with enzyme reactor*	*FIA without enzyme reactor*	*B–A*[c]	*GC*[d]
Number of sample				
A1[e]	0.49	0.018	0.47	0.45
A2	$7.2 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$7.1 \cdot 10^{-3}$	0.0
A3	0.20	0.23	0.0	0.0
A4	0.21	0.22	0.0	0.0
A5	0.20	0.22	0.0	0.0
A6	1.20	0.0	1.20	1.28
A7	8.35	0.0	8.35	8.78
M1[e]	6.30	0.0	6.30	6.73
M2	2.00	0.0	2.00	2.24
R1[e]	0.20	0.23	0.0	0.007

[a]According to the calibration curve using dimethylformamide standard solutions.
[b]According to the calibration curve using dimethylamine standard solutions.
[c]The difference between the concentrations given in column B and A gives the dimethylformamide content determined with FIA.
[d]The detection limit of the GC-method is $5 \cdot 10^{-4}$ M/L.
[e]Origin of sample: A: Waste water. M&R: Process effluents.

significant amount of presumably DMA was observed in samples A3, A4, A5 and R1. When these experiments were repeated using the FIA system which includes the enzyme reactor, calibration was done using DMF standard solutions and the results obtained are listed in column A of Table 1. The concentration of DMF in the sample is calculated from the difference between the concentrations listed in column B and A and is given in column C. It is obvious, that samples A3, A4, A5 and R1 contained only DMA and no more DMF, due to spontaneous chemical hydrolysis during prolonged storage.

The results obtained for the DMF concentration in waste water and process effluents using the enzymatic assay (Table 1, column C) were then compared to the concentrations of DMF determined by routine gaschromatography. In general there is a good agreement between the values obtained with the two completely different

methods. Samples A3, A4 and A5 did not contain any DMF, as already observed with the enzymatic assay. In sample R1, a very small amount of DMF was detected using the chromatographic method. Since the amount of DMF was about 30 times smaller than the amount of DMA, DMF could not be detected using the enzymatic assay.

The only significant difference between the enzymatic and chromatographic determination was found in sample A2. Since presumably some DMF was detected in the enzymatic assay and no DMF was detected with the gaschromatographic method the discrepancy is not due to enzyme inhibition. Furthermore, it could be shown, that in the enzymatic assay, the addition of known amounts of DMF to sample A2 did not result in the expected (calculated) increase in signal height. Therefore the measured DMF concentration was too low. The above mentioned phenomena could be explained by the presence of an unknown substrate (e.g. diethylformamide)[2] of the DMFase in the sample. This assumption was proved by the following experiment: A solution of 0.9 M diethylformamide gave rise to a signal which correspond to a concentration of 0.55 mM DMF. Addition of 1.3 mM DMF to the diethylformamide solution did not increase the signal height to the expected level, corresponding to 1.85 mM DMF, but only to the level of 0.8 mM DMF. This can be explained by the competition of the two substrates for the active site of the DMFase. It should be noted that these phenomena were not observed with any of the other samples, where the addition of a known amount of DMF increased the signal height to the expected level and the concentration of added DMF could be determined within 5% of the calculated value (not shown).

Stability: Samples of waste water A1 were injected every 5 minutes during 50 hours into the FIA system containing the immobilized DMFase. The signal height continuously decreased to 80% of the original size during this time. Since the system was periodically calibrated, this effect could be corrected and had no influence on the measured concentration of DMF in the sample. It should be noted, that incubation of the immobilized DMFase with a sample of waste water A1, even when diluted to the same degree as obtained in the FIA system, completely inhibited the enzymatic activity within 10–20 minutes.

SUMMARY

In 8 of 10 cases a continuous control of DMF using immobilized DMFase and a FIA system is possible. Introducing a hydrodynamic dilution unit into the FIA manifold allows the dilution of the sample within the analyzing system.[9] Measurements are then carried out in the linear range of the calibration curve. If necessary in parallel to the enzyme reactor a reference reactor is introduced to perform the difference measurement. To correct for changes in temperature, flow rate and enzyme activity periodic intercalibration is necessary.

Acknowledgements

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