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DETERMINATION OF UREA AND AMMONIA USING ION-PAIR LIQUID CHROMATOGRAPHY WITH ON-LINE POST-COLUMN DERIVATIZATION IN AN ENZYMATIC SOLID-PHASE REACTOR

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SUMMARY

A quantitative method of analysis for urea and ammonia is described, which is based on ion-pair high-performance liquid chromatography with on-line post-column derivatization on immobilized urease.

In the urease solid-phase reactor, urea is quantitatively converted into ammonia, which reacts with *o*-phthalaldehyde and is detected by fluorescence monitoring. The method is sensitive, highly specific and easy to use, and has a linear range of *ca.* 1.5 orders of magnitude for urea and *ca.* 2.5 orders of magnitude for ammonia. Detection limits are 0.4 ng ($3 \cdot 10^{-7}$ *M*) and 0.3 ng ($9 \cdot 10^{-7}$ *M*) for urea and ammonia, respectively.

The method is used for the determination of the urea and ammonia content of samples from an urea plant and in wastewater samples.

INTRODUCTION

Post-column reaction detectors are widely used to enhance selectivity and sensitivity of detection in high-performance liquid chromatography (HPLC)[1]. Recently, several designs of post-column reactors and their applicability have been reviewed[1,2].

The use of solid-phase reactors (SPRs) has been reported by several workers[3–8]. An SPR can contain immobilized chemicals[6–8], in which case it has to be reloaded occasionally, or a solid catalyst[3–5]. SPRs are usually packed-bed reactors, but the concept can also be applied to open tubular reactors[9]. The use of immobilized enzymes as catalysts in SPRs has recently been reviewed[10]. Immobilized enzymes have a number of advantages over enzymes used in solution:(1) the elimination of enzyme solution pumps and mixing tees and, hence, the reduction of cost and the absence of mixing and dilution problems; (2) the possibility of working with enzymes that would otherwise interfere in the detection process; (3) immobilized enzymes can be re-

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used[10,11]; (4) immobilization often improves the storage properties and the thermal and pH stability of the enzymes[10,11].

A large number of techniques have been reported for immobilizing enzymes[10,12]. The immobilized enzyme used in an SPR should be mechanically stable under normal flow conditions, and the SPR should contribute as little as possible to band broadening. Good mechanical stability can be expected from enzymes that are immobilized on glass, silica or alumina particles[10]. Another approach, which can lead to mechanically stable SPRs, is to immobilize an enzyme on the inside wall of nylon tubing[9]; however, the unfavourable ratio of surface to volume, leading to low activity per unit volume, is a major disadvantage of this type of reactor. This ratio is more favourable for packed-bed SPRs. In addition, the use of small particles leads to minimal band broadening caused by the SPR.

The present paper describes an SPR containing urease covalently bonded to silica, which causes the hydrolysis of urea into carbon dioxide and ammonia. A similar type of SPR has been described earlier[13,14] for urea determinations. However, these methods do not permit determination of urea and ammonia in one assay. In the present study an HPLC separation coupled with an urease-SPR is described. In a second reactor, the ammonia produced is converted into a highly fluorescent *o*-phthalaldehyde (OPA) derivative. Application of this technique to urea analysis in production processes and wastewater is reported.

EXPERIMENTAL

Chemicals

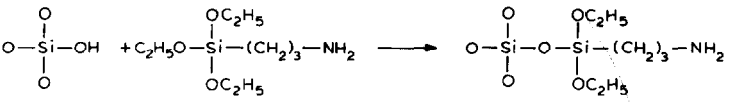
Demineralized water, which was purified by filtration in a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used throughout this work. The eluent was prepared by dissolving 0.005 *M* sodium octylsulphonate (Janssen, Beerse, Belgium) in a 0.03 *M* potassium phosphate buffer (pH 6.9), a medium well suited to urease action[15].

The OPA reagent was prepared by adding 0.8 g of OPA (Merck, Darmstadt, G.F.R.) dissolved in 10 ml of ethanol and 1 ml of mercaptoethanol (Fluka, Buchs, Switzerland) to a borate buffer, prepared by dissolving 24.7 g of boric acid in 1 l of water, and adjusting the pH to 10.2 with potassium hydroxide. The reagent, which was kept under nitrogen to avoid oxidation, is stable for at least two weeks. Test mixtures of urea and of ammonia were made by dissolving urea (purity >99.9%; Fluka) and carefully dried ammonium sulphate in water.

Preparation of the urease-SPR

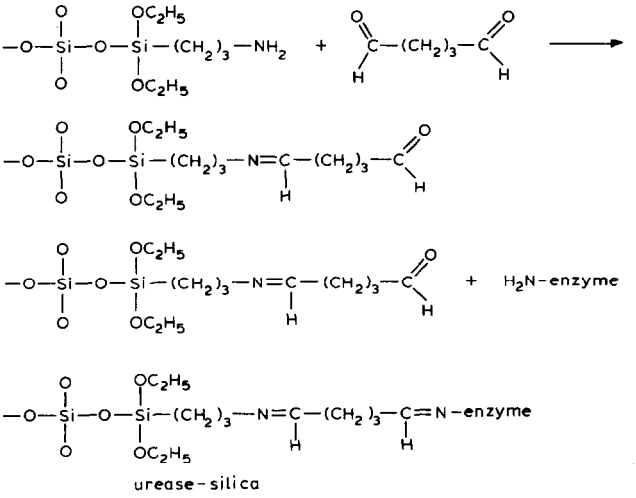
Urease (urea amidohydrolase EC 3.5.1.5, U-2000, Sigma, St. Louis, MO, U.S.A.) was covalently bonded to silica by modifying a technique for coupling to controlled-pore glass described in detail by Johansson and Ögren[13]. Modifications were the use of a wide-pore silica, LiChrospher SI 500 (particle diameter 10 μm, BET surface 45 m^2/g, pore diameter 50 nm; Merck) instead of glass, and the use of a potassium phosphate instead of a sodium phosphate buffer.

The first step is the preparation of the aminopropyl derivative by treating the silica with 3-aminopropyltriethoxysilane (Petrarch Systems, Levittown, PE, U.S.A.) which yields[16]:



(1)

In the next step, the enzyme is coupled to the aminopropyl derivative by using glutaraldehyde (25% solution; Janssen). A simplified reaction scheme is as follows[11]:



(2a)

(2b)

urease-silica

For packing the reactor, a slurry of the urease-silica in water was made which was packed with 0.01 *M* potassium phosphate buffer (pH 6.9) into a stainless-steel column (40 × 4.6 mm I.D.) containing a stainless-steel frit (2-μm pore width). The packing pressure was 110 bar and was maintained for 15 min. When not in use the urease-SPR was kept at 4°C.

Apparatus

A schematic diagram of the experimental set-up is given in Fig. 1. The apparatus consisted of two high-pressure pumps (Gilson 302 with 5S pump head, Villiers-le-Bel, France), a pressure transducer (Viatran Model 108, Buffalo, NY, U.S.A.), a sampling valve (Rheodyne 70-10, Berkeley, CA, U.S.A.) with a 20-μl loop, a filter fluorimeter (Waters 420C/420E, Milford, MA, U.S.A.), an integrator (Minigrator, Spectra-Physics, San José, CA, U.S.A.) and a stripchart recorder (Kipp & Zonen BD-8 Multirange, Delft, The Netherlands).

The OPA detection system consisted of a low-dead-volume mixing T-piece and a piece of stainless-steel tubing (13.3 m × 0.6 mm I.D.), resulting in a volume of 3.75 ml and tightly coiled to a diameter of *ca.* 8 mm. The fluorimeter was operated at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The eluent and OPA reagent were pumped at flow-rates of 1.15 and 1.00 ml/min, respectively. The urease-SPR was kept at 40°C in an oven (Becker type 110, GC-oven, Delft, The Netherlands). The OPA reactor was thermostatted at 20°C in a water-bath (P. M. Tamson, Zoetermeer, The Netherlands).

A 150 mm × 4.6 mm I.D. stainless-steel column, packed with 5-μm Polygosil

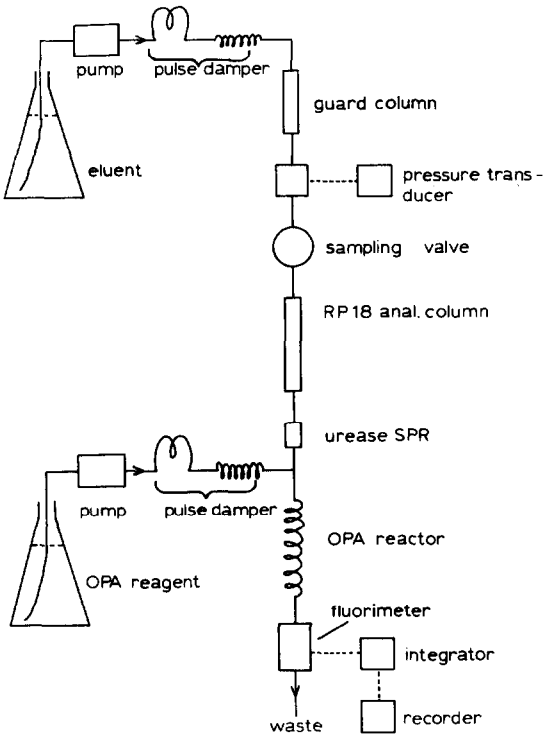


Fig. 1. Experimental set-up of the HPLC/urease-SPR/OPA reactor/fluorescence detector system.

60-5 C_{18} (Macherey-Nagel, Düren, G.F.R.) was used for separations.

A 100 mm × 4.6 mm I.D. stainless-steel guard column packed with a C_{18}-bonded phase was used to protect the HPLC system.

RESULTS AND DISCUSSION

Chromatography and reactor performance

Analysis of a test mixture of urea and ammonia in the system C_{18}-bonded silica/octylsulphonate containing aqueous phosphate buffer, and subsequent conversion of urea into ammonia in an urease-SPR and fluorigenic labelling of ammonia, yields the chromatogram in Fig. 2a. Obviously, complete resolution is obtained after a total analysis time of less than 6 min. When the urease-SPR is removed from the system, while all other conditions are kept the same, a chromatogram with the ammonia peak only is obtained (Fig. 2b). Urea, which does not react with OPA to form fluorescent products, is not detected in this case. Data for retention times, peak broadening and peak asymmetry for the above system are presented in Table I. From these measurements it can be concluded that band broadening is caused mainly by the OPA reactor, whereas there is hardly any contribution to total band broadening from the urease-SPR. The asymmetry of the ammonia peak (*cf.* Fig. 2) is caused by the separation system. Without the analytical column, peak shapes obtained after injection of an urea solution and an ammonia solution are excellent ($A_{0.1}$ = 1.1) and

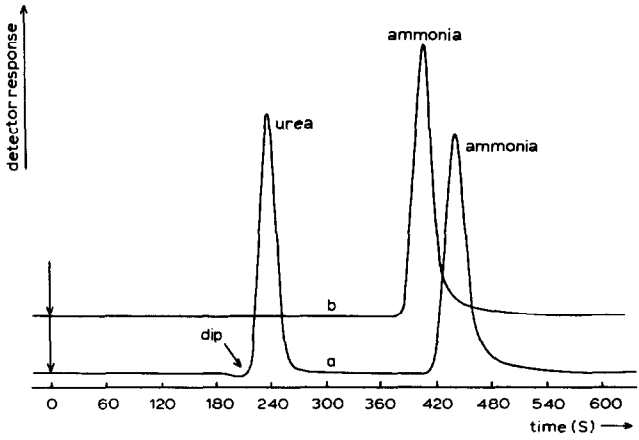


Fig. 2. (a) Chromatogram of a test mixture of urea and ammonia. Column, 150 × 4.6 mm I.D. Polygosil 60-5 C_{18}; eluent, 0.03 *M* potassium phosphate buffer (pH 6.9) with 0.005 *M* sodium octyl sulphonate; urease-SPR, 40 × 4.6 mm I.D. stainless-steel column packed with urease-silica; flow-rates of eluent and OPA reagent, 1.15 and 1.00 ml/min, respectively; fluorescence detection, 344 (ex) and 455 (em) nm. (b) As (a), but without urease-SPR.

almost identical. It is seen also that there is no significant difference in total retention time between urea and ammonia in the absence of the analytical column; *i.e.*, the hold-up time of both analytes in the urease-SPR is essentially the same (*ca.* 34 sec). This is slightly surprising: since urea must couple to the enzyme for its conversion into ammonia to occur, one would expect a larger hold-up time for this analyte. Or, in other words, conversion must be very rapid.

TABLE I

RETENTION TIMES, PEAK BROADENING AND PEAK ASYMMETRY

Experimental conditions: column, 150 × 4.6 mm I.D. packed with Polygosil 60-5 C_{18}; eluent, 0.03 *M* potassium phosphate buffer (pH 6.9) with 0.005 *M* sodium octylsulphonate; urease-SPR, 40 × 4.6 mm I.D. stainless-steel column packed with urease-silica; flow-rates of eluent and OPA reagent, 1.15 and 1.00 ml/min, respectively, fluorescence detection, 344 (ex) and 455 (em) nm.

*Equipment**	*Sample*	*Retention time (sec)*	*Peak width at half height (sec)*	*Asymmetry***
A	Urea	240	18.5	1.2
	Ammonia	440	22.1	1.8
B	Ammonia	407	21.3	1.8
C	Urea	145	17.7	1.1
	Ammonia	144	17.6	1.1
D	Ammonia	111	18.0	1.2

* A, equipment as described under *Apparatus*; B, as A, without urease-SPR; C, as A, without analytical column; D, as A, without urease-SPR and without analytical column.

** Asymmetry calculated as the quotient of peak width after and before the peak maximum at 10% of the peak height.

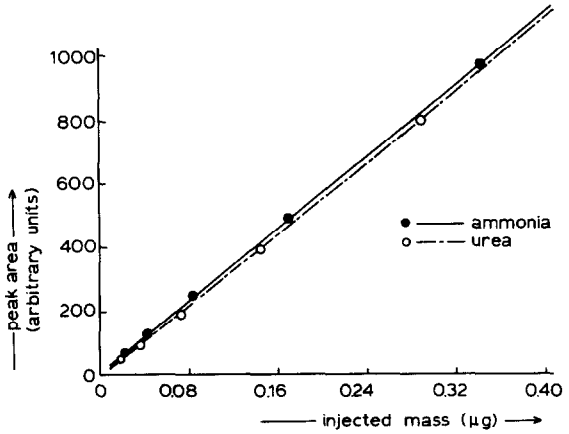


Fig. 3. Calibration plots used for the quantitative determination of urea (--------, O) and ammonia (——, ●). The injected mass of urea is expressed as the equivalent amount of ammonia present (mass of urea times 34/60).

Analytical data

The relationships between peak area and injected mass were measured for urea and ammonia. Detection limits (signal-to-noise ratio = 3:1) were found to be 0.4 ng for urea and 0.3 ng for ammonia. With the 20-μl injection volume, this corresponds to 20 ppb and 15 ppb for urea and ammonia, respectively. At low concentrations of urea, the peak area determination is slightly influenced by the baseline dip that occurs just before the urea peak (Fig. 2a). As a result, the linear range is *ca.* 1.5 orders of magnitude for urea, whereas for ammonia it is *ca.* 2.5 orders of magnitude. Extension of the linear ranges to higher concentrations was limited by the maximum fluorescence that could be measured with the fluorimeter used.

Calibration plots are shown in Fig. 3. The amount of urea injected is expressed as the equivalent amount of ammonia present (mass of urea multiplied by 34/60). The two calibrations plots nearly coincide, which indicates a quantitative conversion of urea into ammonia at 34 sec retention time. Repeatability was found to be 0.35% and 0.65% R.S.D. for injections of 300 ng of urea and 200 ng of ammonia, respectively (n = 12).

Application to urea plant samples

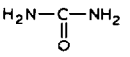
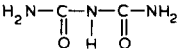
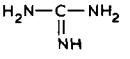
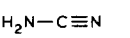
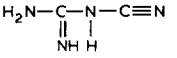
The present method has been used for the determination of urea and ammonia in samples from an urea plant. In these samples, low concentrations of other compounds such as biuret, guanidine, cyanamide and dicyandiamide may well be present. As can be seen from the data in Table II, they all show very small responses compared with urea and will, therefore, hardly interfere in the analysis of real samples. This is all the more true since, with each of these analytes, a single peak was detected with the same retention time as urea; when the urease-SPR was removed, no peak was detected. In other words, the low responses observed are probably (mainly) caused by the presence of small amounts of urea in the samples of the several analytes.

Sample pretreatment simply consists of diluting the sample with water in order to work within the range of the calibration plot of Fig. 3. Table III summarizes

TABLE II

RESPONSE OF BY-PRODUCTS RELATIVE TO RESPONSE OF UREA

Experimental conditions as in Table I.

Compound	*Structural formula*	*Relative response*
Urea		100
Biuret		0.29
Guanidine		0
Cyanamide		1.5
Dicyandiamide		0.64

results for the urea and ammonia concentrations in these samples. The values are compared with values obtained by using the reference methods B′ and B″. In reference method B′, urea is determined by a colour reaction with dimethylaminobenzaldehyde[2]. This method has an overall R.S.D. of 0.6%. Reference method B″, for ammonia, involves titration with an alkaline solution after the addition of an excess of acid and removal of carbon dioxide. The overall R.S.D. of this method is 0.4%. There is good agreement between the present method and the reference methods.

Applications to wastewater samples

The present method was also used for the analysis of wastewater samples. Separations were carried out on a 150 × 4.6 mm I.D. Hypersil-ODS column using

TABLE III

COMPARISON OF THE PRESENT METHOD, A, AND TWO REFERENCE METHODS, B′ AND B″

Experimental conditions as in Table I.

Sample	*Urea content*			*Ammonia content*		
	*Method A** *(mass %)*	*Method B″*** *(mass %)*	*Difference (%)*	*Method A** *(mass %)*	*Method B″*** *(mass %)*	*Difference (%)*
1	0.598	0.598	0.00	2.458	2.415	1.78
2	1.273	1.302	−2.23	2.296	2.302	−0.28
3	0.742	0.753	−1.46	4.844	4.887	−0.88

* Mean from three determinations.

** Value from one determination.

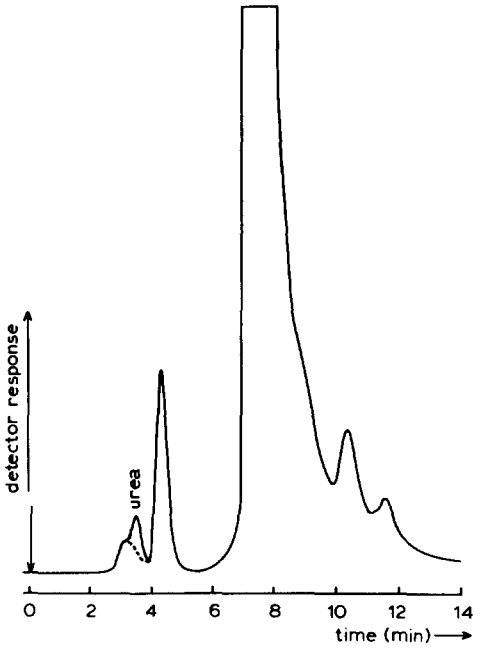


Fig. 4. Chromatogram of a wastewater sample (------, blank; ——, spiked with 1.7 ppm urea). Column, 150 × 4.6 mm I.D. Hypersil-ODS; other conditions as in Fig. 2.

the same mobile phase as before. Samples of 20 μl were injected without any pretreatment. Fig. 4 shows the chromatogram of a blank sample and the same sample spiked with 1.7 ppm urea. After 10-fold injection of these heavily contaminated samples, the urease-SPR still showed its original activity.

CONCLUSIONS

The urease-SPR is a suitable tool for efficient conversion of urea into ammonia, and its contribution to total band broadening is minimal. The elution profiles of urea and of ammonia obtained after direct injection into the reactor, *i.e.* without the analytical column, are identical, which indicates instantaneous conversion of urea or identical retention behaviour of urea and ammonia in the urease-SPR[17]. Since the eluent is a suitable medium for the urease action, no changes in the composition of the column effluent are required, *i.e.* mixing and dilution problems are completely avoided. The urease-SPR has been regularly used during a ten-week period and no change in reactor performance has been observed. This does not necessarily mean that the enzyme as such keeps its full activity. It is possible that the reactor contains such a large excess of enzyme that a decrease in activity does not result in a noticeable change in behaviour.

The present method allows the fast, simple and highly specific determination of urea and ammonia in urea plant samples with only minimal sample handling. The application to the trace-level determination of urea in polluted water samples also seems feasible. It is promising that a relatively large number of such contaminated

samples can be analysed without an apparent change in urease-SPR performance. Current research is directed at the characterization of the immobilized enzyme surface, and the miniaturization and adaptation of the present principle to bioanalytical work.

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