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SPECTROPHOTOMETRIC DETERMINATION OF UREA IN NATURAL WATERS WITH HYPOCHLORITE AND PHENOL

By
Robert T. Emmet



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MACHINERY LABORATORY
RESEARCH AND DEVELOPMENT REPORT

May 1969

Report 2663

Spectrophotometric Determination of Urea in
Natural Waters with Hypochlorite and Phenol



NAVAL SHIP RESEARCH AND DEVELOPMENT CENTER
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Naval Ship Research and Development Center
Washington, D.C. 20007

**SPECTROPHOTOMETRIC DETERMINATION OF UREA
IN NATURAL WATERS WITH HYPOCHLORITE AND PHENOL**

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ABSTRACT

The yellow compound formed from urea, hypochlorite, and phenol with an absorbancy maximum at 454 millimicrons has been adapted to determine micromolar quantities of urea in natural waters. The urea is chlorinated in a solution of sodium hypochlorite at pH 7.7, and upon the addition of phenol, the intermediate condenses rapidly to form the colored product. In freshwater, the absorbancy index is 2.6×10^3 absorbancy units per centimeter cell times gram atom urea-N per liter, and in 35 parts per thousand seawater the color is twice as intense. The lower limit of detection of urea in seawater is 0.2 microgram atoms urea-N per liter, and the relative standard deviation is 10% at a urea-N concentration of 1 microgram atom per liter. An adaptation of the method to automatic analysis of clinical samples is suggested, and a sensitive analysis procedure for tyrosine is described.

ADMINISTRATIVE INFORMATION

This report is part of Sub-project SR104 03 01, Task 0590, Assignment 723 108, as described in the May 1968 program summary.

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SPECTROPHOTOMETRIC DETERMINATION OF UREA IN NATURAL
WATERS WITH HYPOCHLORITE AND PHENOL

By
Robert T. Emmet

INTRODUCTION

Urea is produced by ureatylic animals, including the crustacea, is hydrolyzed to ammonia,² and reabsorbed by the phytoplankton.¹² The urea forms a significant portion of the available nitrogen, particularly in waters polluted by domestic sewage. An analysis for urea would provide a more complete description of the partition and dynamics of nitrogen in natural systems.² Because of the myriad potential interferences and low urea concentrations, such an analysis for use in nutrient surveys should be specific and sensitive. The most specific sea-water method to date, a paper chromatographic method described by E. T. Degens and H. J. Reuter,³ requires a lengthy procedure for concentration. A more convenient sea-water method is the adaptation by B. S. Newell, B. Morgan, and J. Cundy⁸ of the Fearon carbamido-diacetyl reaction.^{1, 5, 13} However, the red color produced by combining urea with diacetyl monoxime in the presence of strong acid develops slowly, and the acidic terminal solution is difficult to handle.⁷ In addition, the calibrations and blank determination are carried out in sodium chloride solution rather than in the natural sample where unexpected chemical interferences could be detected.

In contrast, the hypochlorite-phenol method described in this article produces general, blank, and standard sample signals which rapidly develop in aliquots of the sample. The signal is a canary yellow dye with an absorbancy maximum at 454 millimicrons. This color reaction requires conditions similar to those of the hypochlorite-phenol-ammonia reactions,⁴ and was noticed by the author while making ammonia measurements of ocean surface-water samples.

EXPERIMENTAL INFORMATION

APPARATUS

The determination is made in small volumetric flasks by adding the reagents with Luer-type syringes. The Teflon syringe needles should reach beneath the meniscus of the sample to ensure replicate addition and rapid mixing of the reagents.

REAGENTS

Except where noted the reagents are stable for several months if kept in glass stoppered containers.

²Superscripts refer to similarly numbered entries in Appendix A

1. Sodium hypochlorite, 2.5% NaOCl.
This analysis was developed using Fisher Scientific Company N. F. 5% NaOCl solution which was commercially prepared by passing Cl₂ through NaOH solution, and which contains equimolar concentrations of NaOCl and NaCl.
2. Sodium hypobromite; 0.050% v/v Br₂, pH 12.2.
Prepare 1 liter of pH 12.2 NaOH solution, add with a syringe 0.050 ml Br₂, and stir until homogenous.* This reagent contains equimolar concentrations of NaOBr and NaBr.
3. Hydrochloric acid reagent; 0.25N in HCl, 0.3% w/v MgCl₂.
4. Boric acid buffer stock solution; 0.16 M in H₃BO₃, 0.054 M in KCl and 0.090 M in NaOH.
Prepare the reagent daily by adding to 25 ml of the stock solution 0.15 ml 3% H₂O₂ solution.
5. Ethanolic phenol reagent; 10% v/v C₆H₅OH.
Prepare by diluting clear phenol 1:10 with 95% ethanol. To remove the yellow oxidation products, crystalline phenol may be distilled in the hood with a round bottomed flask and a short air condenser.

PROCEDURES

The sample and reagent volumes in the following procedures are chosen to maintain the meniscus in the flask neck while permitting adequate mixing. To closely approximate the stepwise pH measurements in Table 1, the reagent volumes should be within 5% of those specified. The flasks should be well washed and rinsed twice with distilled water.

Table 1

Interim pH's of a General and a Blank Sample should not vary appreciably from these values which were read with a Beckman Expandomatic pH Meter.

Reagent Addition	Distilled Water		Sea-Water	
	General Sample	Blank Sample	General Sample	Blank Sample
Original Sample	7.0	7.0	7.9	7.9
OBr ⁻	-	11.1	-	9.5
OCl ⁻	10.3	11.2	9.0	9.8
HCl	-	7.7	-	7.6
HCl + OBr ⁻	7.7	-	7.6	-
H ₃ BO ₃	8.3	8.3	8.1	8.1
C ₆ H ₅ OH	8.9	8.9	8.4	8.4

*Abbreviations used in this text are from the GPO Style Manual, 1967, unless otherwise noted.

The general procedure for the analysis of urea requires eight steps.

1. Transfer a 20.0 ml aliquot of sample, which should be colorless, free from solid matter, and at temperature (T) to a 25 ml volumetric flask containing a microstirring magnet. Do not cavitate the sample by rapid addition as the microbubbles contribute to the blank signal.
2. Add 3.0 ml of distilled water and begin to stir vigorously.
3. Add 0.30 ml of NaOC1 reagent.
4. After 3 to 5 seconds, add 0.50 ml NaOBr reagent with one hand.
5. After 1 to 2 more seconds, add (0.30+X) ml of HC1 reagent with the other hand. "X" is the volume in ml of HC1 reagent required to titrate 0.50 ml of NaOBr reagent to pH 7.0.
6. After 15 to 20 more seconds, add 0.50 ml of H₃BO₃ buffer reagent with one hand.
7. After 1 to 2 more seconds, add 0.30 ml ethanolic phenol reagent with the other hand and stir for 10 to 20 more seconds.
8. After 10 but not more than 20 more minutes, compare the absorbancy of the sample solution at 454 m μ against distilled water.

A separate blank sample should be obtained for every sample which differs from the previously accepted blank sample by more than 1 part per thousand in salinity or 2° C or if any of the reagents are renewed. The blank sample procedure is slightly different from that of the general sample.

1. Add a duplicate aliquot of the sample at $T \pm 2^{\circ}$ C to a 25 ml flask without cavitation.
2. Add 3.0 ml of distilled water and begin to stir vigorously.
3. Add 0.50 ml NaOBr reagent.
4. After 9 to 11 minutes add, 0.30 ml NaOC1 reagent with one hand.
5. After 1 to 2 more seconds add, (0.30+X) ml HC1 reagent with the other hand and continue with Step 6 in the general procedure.

Because the sensitivity of the color reaction varies with the composition of the sample, a standard sample should be reacted whenever it is found necessary to react a blank sample. Standardation is required more frequently at low salinities. If unusually high urea concentrations are found, the sample may be diluted with distilled water only if the blank and standard samples are similarly treated.

The standard sample is prepared by the general procedure with one change; the 3.0 ml addition of distilled water in Step 2 should be replaced by 3.00 ml of urea solution of known concentration 5 to 25 times that of the sample.

Urea is quite stable subject only to enzymatic hydrolysis by bacteria. A sample will keep for a day at room temperature, for several weeks under refrigeration and indefinitely at -20° C.

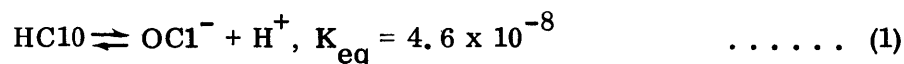
CALCULATION

To obtain the urea concentration of the sample, multiply the difference between the blank and general sample absorbancies by the difference between the general and standard sample absorbancies and divide by three twentieths the urea-N concentration of the added standard solution.

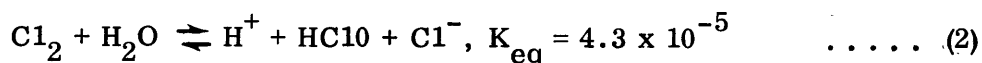
DISCUSSION

REACTIONS

When the sample is buffered by the NaOCl - HOCl equilibrium at pH 7.7, the urea is chlorinated to a mixture of mono-, di-, and tri-chlorourea.



The activity of the Cl₂ is influenced by the Cl⁻ activity.

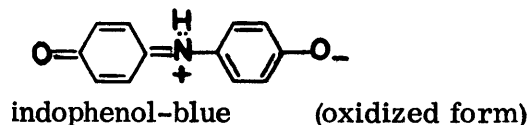


The pH and capacity of the buffer solution were adjusted to maximize the intensity of yellow color. Under optimal chlorination conditions, a variable decrease in color intensity was noticed when a large surface area of the reacting solution was exposed to the air. Escape of the Cl₂ or HOCl from the acidified hypochlorite solution was suspected. When a small reproducible area was exposed in the neck of a volumetric flask after the HOCl reagent addition, the yield and precision improved markedly.

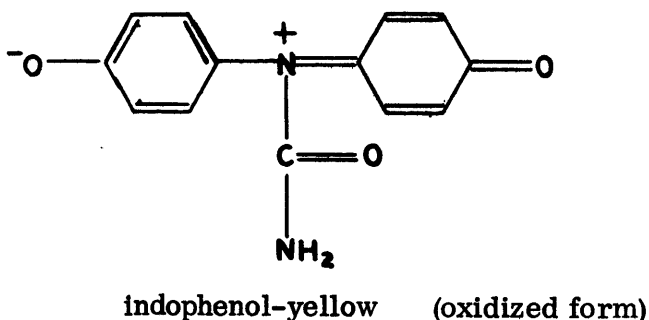
If phenol is added directly to the pH 7.7 solution, the blank signal is high because of side reactions between the halogens and phenol, and the yellow color is unstable. By first adding a H₃BO₃ buffer the blank is reduced and the pH is raised so that the rates of color development and fading become more manageable. In the presence of H₃BO₃ the conversion of urea-yellow to ammonia-blue is retarded and the sample does not turn green for several hours.

The rate of yellow color development is increased, particularly in the fresh-water samples if MgCl₂ is added with the HOCl reagent and KCl with the H₃BO₃ buffer reagent. The color development is also more rapid and the precision improved between replicate samples if H₂O₂ is added with the buffer. H₂O₂ reacts with OCl⁻ and OBr⁻ to produce O₂, and the oxidizing conditions evidently promotes color development. This last dependency lends support to the theory that the dyes in the ammonia and urea analyses are oxidized forms of indophenol compounds.¹⁰ E. H. Rodd⁹

describes an indophenol which absorbs strongly at 630 m μ and possesses a structure which could be directly obtained by para-condensations of the phenol molecules with the chlorinated ammonia intermediate followed by oxidation.

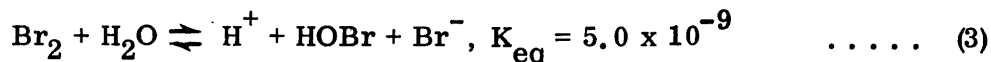


The leucophenol is reportedly stable at low pH whereas the oxidized form is stable in alkaline solution. The ammonia-blue compound similarly changes from clear to blue, above pH 8.1. The urea-yellow is probably an indophenol also because it is converted to ammonia-blue in alkaline solution and because, like the blue compound, it can be reversibly colorized and decolorized by varying the pH above and below 5.0, respectively.

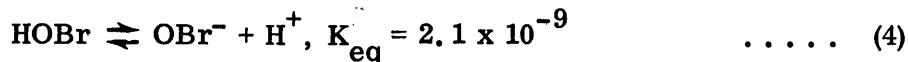


The indo-N:carbonyl-C bond, which is weakened by withdrawal of electrons to form the stable quinoid structure, is apparently hydrolyzed and the urea-yellow turns from yellow \rightarrow green \rightarrow blue more rapidly at higher pH's where the ammonia-blue is stable.

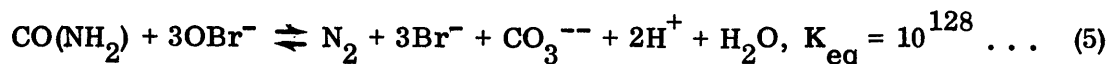
In the alkaline hypobromite reagent Br₂ is hydrolyzed.



OBr⁻ is in equilibrium with HOBr.



Urea is oxidized completely in 10 minutes in cold solutions more alkaline than pH 9.0. (6)



The OBr^- reagent never oxidizes the urea in the general procedure because the solution is always too acid. The Br_2 in the OBr^- reagent is destroyed by reaction with phenol in all samples, thus eliminating that source of absorbance interference. The slight absorbance which is added by side reactions between the OBr^- reagent and phenol is equivalent in all three procedures.

SIGNAL STABILITY

The rates of development and fading of the yellow color and the rate of development of the blank signal all increase with decreasing pH and increasing temperature and salinity. The pH's, after the successive additions as listed in Table 1, have been chosen so that the blank signal will be low and so that all samples between 3° and 30° C and between the salinities of fresh water and seawater will develop urea signals which are sufficiently stable for absorbance comparisons.

After maximal development at 25° C the yellow dye fades at rates of 1 to 2% per hour in distilled water and 3 to 4% per hour in seawater. The blank signal never reaches a maximum, but its growth can be retarded by keeping the temperature and pH of the terminal solution within the suggested limits.

TEMPERATURE AND SALT EFFECTS

The temperature of the sample does not effect the magnitude of the urea signal maximum. In a 3° C solution, however, the rates of the development of the urea signal and blank signal and the rate of fading of the urea signal are about 50% slower than in a 25° C solution. To ensure comparable blank signals in all samples and to ensure that urea signal maxima are being compared in general and standard samples, the three aliquots of a sample should be within 2° C of each other when reacted.

The sensitivity of the urea reaction increases about 90% with increasing salinity from freshwater to 35 parts per thousand seawater. The increase is evidently due to divalent cations.

SPECIFICITY OF THE METHOD

In distilled water standard samples of several other nitrogen-containing compounds were reacted by the general urea procedure, and the gram atom N absorbance indices in absorbance units per centimeter cell times gram atom N per liter at 454 μ are reported to determine the specificity of the urea-hypochlorite-phenol signal.

Negative reactions were obtained with acetamide, alanine, ammonia, arginine, asparagine, aspartic acid, barbital, caffeine, citrulline, creatine, creatinine, cysteine, cystine, diphenyl urea, glutamic acid, glutathione, glycine, hippuric acid, histidine, isoleucine, leucine, lysine, methionine, methyl urea, monoethanolamine, phenyl alanine, phenylurea, N-propylamine, semicarbazide, serine, and threonine. Compounds giving positive reactions are listed with their respective gram atom N absorbance indices: allantoin- 1.6×10^3 , buiret- 1.6×10^3 , tyrosine 1.0×10^3 and urea- 2.6×10^3 .

If the OBr^- reagent addition is omitted from the general procedure, the 454 $\text{m}\mu$ absorbancy index of ammonia is 0.35×10^3 . Ammonia is also oxidized by OBr^- in the blank procedure and would contribute to the urea signal except that the development of the ammonia signals at 630 and 400 $\text{m}\mu$ are inhibited by Br^- which is added in large quantities in the OBr^- reagent.⁴

Of the five compounds giving positive reactions, none are likely to be present in natural waters or in body fluids in concentrations approaching that of urea. No sources of positive interference are therefore expected.

CALIBRATION AND PRECISION

The data given in Table 2 reflect the reproducibility and the salt effect. In Table 2 at 1.0 microgram atoms urea-N per liter, the standard deviation of a set of seven sea-water absorbancies is equivalent to 0.1 microgram atoms urea-N per liter. The standard deviation of the nine sea-water blank samples in the sample groups of 0.5, 1.0, and 2.0 microgram atoms per liter of added urea-N is equivalent to 0.05 microgram atoms urea-N per liter, and the variances sum to 0.0125 microgram atom² urea-N per liter². The estimated standard deviation of the urea-N detected at 1.0 microgram atoms per liter is therefore 0.11 microgram atoms urea-N per liter. At the 95% confidence level, for a sample to be significantly different from the blank, it must contain urea equal to twice the standard deviation. The lower limit of detection of the analysis in seawater is thus 0.2 microgram atoms urea-N per liter, and the relative standard deviation is 10% at a urea-N concentration of 1.0 microgram atom per liter.

ADAPTATION TO CLINICAL SAMPLES

This urea method may be adapted for an automatic analysis of blood, serum, and urine samples. Deproteinization is best accomplished by dialysis with isotonic solutions because the reactions require that the sample be near neutral pH. This method is superior to the diacetyl monoxine method which is currently used for automatic analysis of clinical samples⁷ because (1) the color development is rapid; (2) its magnitude is independent of temperature; (3) the analyzed samples are not corrosive; and (4) the reaction is more specific for urea in biological fluids.^{1, 5, 13}

Table 2

Absorbancies Obtained by Adding Quantities of Urea Solution to Urea-Free Distilled Water and Urea-Free Seawater of Salinity 35 Parts Per Thousand

Microgram Atom per Liter of Added Urea-N	454 m μ Absorbancy in Distilled Water in a 5-CM Cell	454 m μ Absorbancy in Seawater in a 5-CM Cell	
		Blank Samples	Standard Samples
0.0	0.011		0.062
	0.011	0.062	0.063
	0.012	0.062	0.062
	0.012	0.063	0.062
	0.010		0.064
	0.011		0.065
	0.011		0.062
0.5	0.017		0.072
	0.017	0.064	0.074
	0.019	0.063	0.075
	0.018	0.062	0.076
	0.017		0.075
	0.017		0.076
	0.018		0.076
1.0	0.023		0.081
	0.023	0.064	0.083
	0.025	0.063	0.085
	0.025	0.065	0.087
	0.025		0.086
	0.024		0.087
	0.025		0.087
2.0	0.039		0.105
	0.040	0.066	0.108
	0.041	0.063	0.106
	0.040	0.064	0.109
	0.041		0.104
	0.039		0.110
	0.042		0.109
10	0.122		0.272
	0.120	0.064	0.268
	0.120	0.063	0.274
	0.122	0.064	0.270
	0.119		0.267
	0.122		0.268
	0.121		0.269
	0.123		

Table 2 (Cont)

Microgram Atom per Liter of Added Urea-N	454 m μ Absorbancy in Distilled Water in a 5-CM Cell	454 m μ Absorbancy in Seawater in a 5-CM Cell	
		Blank Samples	Standard Samples
20	0.231		0.461
	0.219	0.063	0.452
	0.224	0.061	0.455
	0.219	0.064	0.449
	0.218		0.465
	0.223		0.467
	0.219		0.462
	0.220		
40	0.425		0.830
	0.420	0.065	0.824
	0.427	0.064	0.838
	0.424	0.067	0.842
	0.425		0.846
	0.420		0.838
	0.428		0.846
	0.429		
80	0.834		1.54*
	0.841	0.065	1.54
	0.844	0.055	1.56
	0.839	0.050	1.54
	0.835		1.54
	0.846		1.53
	0.834		1.52
	0.839		
125	1.29*		2.30*
	1.26	0.051	2.31
	1.30	0.045	2.33
	1.28	0.046	
250	2.63*		
	2.57		
	2.63		
500	4.95*		
	4.91		
	4.97		
1000	9.25*		
	9.05		
	9.10		

*Absorbancies read in shorter cells and converted to a 5-cm path.

OTHER COLOR REACTIONS

As noted with regard to reaction specificity, several compounds other than urea react to give colored products when treated by the urea procedure. The biuret and allantoin products are thought to be identical with the urea inclophenol because of similarities in absorbency and reaction characteristics.

An extremely useful signal, however, would be the tyrosine compound absorbency at 375 m μ , because tyrosine of all the amino acids tested gave the sole positive signal. As shown in Table 3 the tyrosine signal is linear through 100 microgram atoms tyrosine-N per liter. The distilled water samples were reacted by the general urea procedure excluding the OBr⁻ reagent. The signals develop within 2 minutes and are stable for several hours. If no ammonia is present the urea blank procedure will give a valid tyrosine blank signal.

Table 3
Absorbancies Obtained by Adding Quantities of Tyrosine
Solution to Tyrosine-Free Distilled Water

Microgram Atoms per Liter of Added Tyrosine-N	375 m μ Absorbancy in Distilled Water in a 5-CM Cell
0	0.034 0.031 0.033 0.032
25	0.287 0.292
50	0.585 0.580
100	1.142 1.130

INSIGHTS INTO THE AMMONIA METHOD

This work on urea suggested several refinements of the hypochlorite-phenol-ammonia analysis in Report 2570. (4)

The precision of a series of replicate ammonia samples can be increased by using teflon syringes which extend beneath the sample meniscus. The use of micro-stirring bars (Fisher Scientific Company, No. 9-312-102) reduces the vortex which also increases the precision.

In the search for a chemical reaction which would replace the tedious vacuum diffusion blank procedure, several compounds were studied that oxidized ammonia to nitrogen gas. Sodium nitrite was found unsatisfactory because, in addition to removing ammonia, it inhibited the blank signal development. Since ammonia is oxidized more readily than urea by hypobromite, this reagent would have worked as in the urea analysis except that it contains bromide which inhibits the development of the ammonia signal. Such a blank would not detect ammonia in the reagents, nor would it be possible to compensate by adding equivalent hypobromite to the general sample, for the 20% to 30% increase in the blank absorbency from the side reactions between hypobromite and phenol.

The recommended ammonia blank signal is composite and is obtained by reacting a series of three solutions. Solution 1 is an ammonia-free distilled water sample reacted by the general procedure. By comparing the distilled water blank with previous such values the degree of ammonia contamination in the reagents may be estimated. The ammonia contamination would not show up in Blank Solutions 2 and 3 because of Br^- interference. Solutions 2 and 3 are aliquots of the natural sample which should be stirred for 5 to 10 minutes with 0.20 and 0.40 ml, respectively of NaOBr reagent. They should then be developed by the general ammonia procedure, adding extra acid to compensate for the alkalinity as in the urea blank. The sample blank is determined by subtracting the difference between the absorbencies 3 and 2 from 2, the lowest absorbency [sample blank = $2 - (3 - 2)$]. The ammonia contribution to the blank is then corrected for the salt effect (see Table 2), and added to the sample blank to yield the true blank signal.

The urea interference in the ammonia analysis can become significant if the samples are left for more than 20 minutes before comparing the absorbancies, because of the transformation of urea - yellow to ammonia - blue. This reaction is 100% more rapid in seawater and is further encouraged by the alkaline terminal condition of the ammonia analysis. The urea interference in the ammonia analysis may be minimized by comparing the sample absorbancies promptly.

Appendix A

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SPECTROPHOTOMETRIC DETERMINATION OF UREA IN
NATURAL WATERS WITH HYPOCHLORITE AND PHENOL,
by Robert T. Emmet. May 1969 28 pp.
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The yellow compound formed from urea, hypochlorite, and phenol with an absorbancy maximum at 454 millimicrons has been adapted to determine micromolar quantities of urea in natural waters. The urea is chlorinated in a solution of sodium hypochlorite at pH 7.7 and upon the addition of phenol, the intermediate condenses rapidly to form the colored product. In freshwater, the absorbancy index is 2.6×10^3 absorbancy units per centimeter cell times gram atom urea-N per liter, and in 35 parts per thousand seawater, the color is (over)

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twice as intense. The lower limit of detection of urea in seawater is 0.2 microgram atoms urea-N per liter, and the relative standard deviation is 10% at a urea-N concentration of 1 microgram atom per liter. An adaptation of the method to automatic analysis of clinical samples is suggested, and a sensitive analysis procedure for tyrosine is described.

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