due to water vapor that passed the water trap.

It should be noted that the sample introduction efficiency could be estimated simply by comparing the emission intensities of volatile and nonvolatile species. All of the Os(III) introduced into the plasma was in liquid particles with method I, and approximately 100% of the Os(VIII) was in the gaseous phase with method II (2). Since the amount of liquid particles transported to the plasma was same with both methods, the physical properties of the plasmas were considered to be same. When the efficiencies of sample introduction were assumed to be a% and 100% with methods I and II, respectively, the following relationship was given:

$$100/a = 2.2 \times 10^6/2.8 \times 10^4$$

Then

$$a = 1.3$$

This value would be reasonable for sample introduction efficiency of ICP.

Interference, Recovery, and Precision. Interferences from coexisting ions and volatile organic compounds with determination of Os(III) are listed in Table III. Although Ru(III) apparently showed a positive interference, this is because the standard solution of Ru contained Os as impurity. The concentration of impurity was estimated to be 130 μg/g from the peak height. Spectral interference from Ru I at 225.552 nm was not conceivable because RuO_4 was not generated by the oxidation with $K_2Cr_2O_7$. Volatile organic compounds, which might be condensed with the low-temperature trap, gave no interference at the concentrations in Table III. Interferences for oxide generation were very small, as compared to those for hydride generation.

When 100 ng of Os(III) was added to 50 mL of lake and seawater samples, the recoveries were 104% and 98%, respectively. The concentrations of the original Os in these samples were below the detection limit.

The relative standard deviation of emission intensities at 5 ng/mL of Os(III) was estimated to be 4.0% (n = 27) by assuming that there were no interferences for the data in Table III.

This study demonstrates that the oxide generation/condensation method is very useful for raising sample introduction efficiency as well as the hydride generation method. This method seems to be applicable to atomic absorption spectrometry and inductively coupled plasma mass spectrometry.

Registry No. Os, 7440-04-2; OsO_4, 20816-12-0.

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Fluorescence Reagents for High-Sensitivity Chromatographic Measurements of Primary Amines

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Model fluorogenic reagents have been synthesized and evaluated toward the goal of ultrahigh-sensitivity chromatographic analysis of primary amines with laser-based detection. Chemical reactivity and spectral criteria were utilized to evaluate 12 potential reagents forming highly fluorescent isoindoles. 3-Benzoyl-2-quinolinecarboxaldehyde has been chosen at this time as the most promising reagent for the precolumn derivatization of model amino acids, followed by a chromatographic analysis of the formed isoindoles, and their detection with a helium–cadmium laser-based detector at the femtogram level.

Highly sensitive detection and quantitative measurements of biological compounds bearing the primary amino group are becoming increasingly important to modern biochemistry. For example, as our capabilities of isolating small amounts of proteins gradually improve, significantly greater demands will be placed on the sensitivity needed to measure their degradation products, the amino acids and peptides. Fluorescence spectroscopy, when applied as the final measurement technique after liquid chromatographic separation, offers perhaps the greatest potential for decreasing the detection limits below the femtomole levels.

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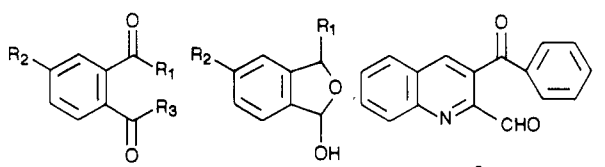
Table I. Compound Structures[a]



1 2 3

compd 1 or 2	R_1	R_2
a	phenyl	H
b	β-naphthyl	H
c	phenyl	Me_2N
d	β-naphthyl	Me_2N
e	β-naphthyl	phenyl
f	phenyl	β-styryl (PhCH=CH–)
g	p-$Me_2NC_6H_4$	Me_2N
h	p-$Me_2NC_6H_4$	β-styryl
i	4-stilbenyl (PhCH=CH–C_6H_4)	Me_2N
j	4-pyridyl	Me_2N
k	4-stilbenyl	H

[a] R_3 = H, unless otherwise noted in the text.

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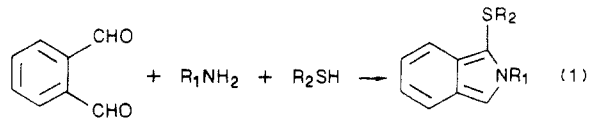
To convert amino acids and peptides into fluorescent derivatives, a number of reagents have been developed during recent years (*1–4*), notably fluorescamine (*2, 3*), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) (*4*), and *o*-phthalaldehyde (OPA) (*1, 5–7*).

Derivatization of primary amines with OPA (1; $R_1 = R_2 = R_3 = H$; refer to Table I for a description of all compounds discussed in this article) is particularly attractive in that the electronic spectra of the reagent and product are notably different. The dialdehyde undergoes condensation with an amine under the influence of a strong nucleophile (usually a thiol, RSH), leading to the formation of an isoindole (eq 1).



(1)

Hence, a common procedural complication—separation of fluorescent products from equally fluorescent excess reagent—is avoided; OPA is not fluorescent, while the isoindoles are among the most intensely fluorescent substances known (*1*). The OPA-derived isoindoles possess fluorescence excitation maxima of about 340 nm. Unfortunately, the utility of the isoindoles formed from OPA is severely limited by their instability with respect to light, attack by acids, and air oxidation (*8*).

Whereas it is highly desirable to decrease the fluorescence detection limits from the femtomole (10^{-15}) to the attomole (10^{-18}) levels, such dramatic improvements in sensitivity will ultimately need a combination of new chemical approaches and instrumentation. While miniaturized liquid chromatography combined with small-volume detectors featuring intense and highly focused beams considerably improves the prospects of ultrahigh-sensitivity measurements (*9, 10*), limitations are imposed by (a) background fluorescence of impurities originating from the mobile phases, detector cell materials, and sample matrices; and (b) various light-scattering processes. To reduce these limitations, it is generally desirable to excite the fluorescent molecules at higher wavelengths. As reviewed by Zare (*11*) and Sepaniak (*12*), ultrahigh-sensitivity fluorescence measurements greatly benefit from using lasers as the excitation sources. Ideally, then, the fluorescent derivatives of primary amines should have their absorption maxima near the output wavelengths of the readily available helium–cadmium (442 nm) or argon ion (488 nm) lasers.

This communication deals with the development of new fluorogenic reagents for primary amines, with two major objectives in mind: (1) forming fluorescent derivatives structurally similar to those based on OPA, but considerably more stable; and (2) increasing the excitation maxima of the isoindoles while maintaining or improving the fluorescence quantum yield. In addition, such derivatives should possess desirable chromatographic properties.

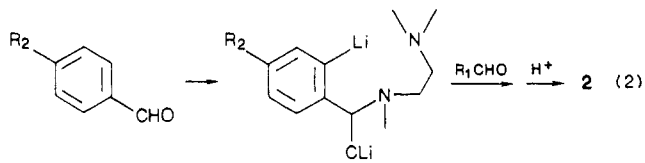
The stability of an isoindole can principally be enhanced by suitable substitution adjacent to the nitrogen atom. This initially led us to the synthesis of ortho diaroylarenes ($R_1 = R_3 = Ar$) designed to react with amines under reducing conditions to yield the more stable 2,7-diaroylisoindoles (*13*). Since even the simplest of these ortho diketones did not give rise to suitable derivatives with amino acids, we turned our attention to the less sterically demanding ortho aroylaraldehydes ($R_1 = Ar$; $R_3 = H$), the simplest of which is 2-benzoylbenzaldehyde (**1a**). Subsequently, several other 2-aroylaraldehydes were synthesized (**1b–1k**) and quantitatively evaluated as reagents in this work.

As expected, the excitation maxima (ca. 340 nm) of the isoindoles based on **1a** were too low to match the helium–cadmium laser wavelength (blue line, 442 nm); initial synthetic strategies, then, were primarily concerned with introducing additional aromatic rings so as to significantly increase the excitation maxima without decreasing the fluorescence intensities of the derivatives. Solubilization difficulties encountered in some of the early syntheses, however, made us aware of the desirability of incorporating solubilizing groups (amino, alkyl, methoxy, etc.) into the aromatic moieties of the target molecules.

EXPERIMENTAL SECTION

Synthesis of Reagents. 2-Benzoylbenzaldehyde (**1a**) has been synthesized according to Metlesics et al. (*14*) by selenium dioxide (SeO_2) oxidation of the corresponding dialcohol, itself obtained from commercially available 2-benzoylbenzoic acid by lithium aluminum hydride (LAH) reduction of its ethyl ester (*15*).

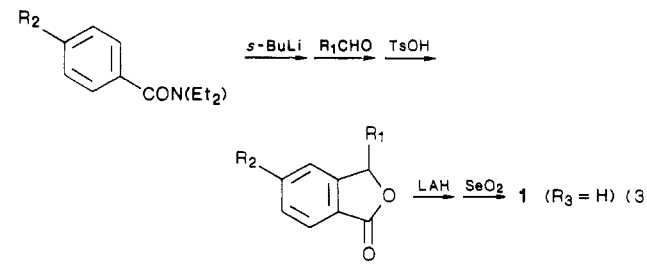
2-Aroylaraldehydes, as a class of compounds, have not been synthesized routinely. Our attention was directed toward the development of one or more synthetic strategies that might prove generally useful. Ortho metalation of a suitable araldehyde derivative followed by a reaction with an aroyl chloride appeared promising. Comins and Brown (*16*) have treated the adduct of benzaldehyde and *N,N,N'*-trimethylethylenediamine with excess *n*-butyllithium and affected subsequent ortho substituion by a number of electrophiles. Efforts on our part to aroylate this lithiated intermediate by using the Comins–Brown methodology were unsuccessful, but modest yields of lactols were obtained upon reaction of the lithiated intermediates derived from benzaldehyde and several substituted benzaldehydes with various aromatic aldehydes (eq 2). The lactols could be reduced by LAH to the corresponding diols, which were oxidized by SeO_2 to the desired 2-aroylaraldehydes (**1**).



(2)

Subsequent improvements in the methodology have included the following: (1) the direct SeO_2 oxidation of lactols (**2**) to 2-aroylaraldehydes (**1**); and (2) the inclusion of an equivalent of tetramethylethylenediamine (TMEDA) in the lithiated mixture to obviate the need for a large excess of *n*-butyllithium, hence also of possibly costly aldehyde R_1CHO. This modified Comins–Brown approach was used for the synthesis of reagents **1b–1j**.

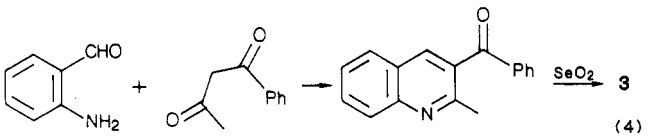
An alternative methodology is based upon the excellent ability of the tertiary amide group to direct ortho metalation (*17*). The metalated intermediate reacts with an aldehyde, R_1CHO, to yield (after short reflux in the presence of *p*-toluenesulfonic acid) a phthalide that can be reduced with LAH and oxidized with SeO_2 to yield a 2-aroylaraldehyde (eq 3). Reagent **1k** was prepared in this way.



(R3 = H) (3)

The compound 3-benzoyl-2-quinolinecarboxaldehyde (Table I; structure 3) (*18*) was synthesized in 70% overall yield from *o*-aminobenzaldehyde by a Friedländer condensation (*19*) and SeO_2 oxidation of the resulting 3-benzoyl-2-methylquinoline (eq 4). The oxidation was effected in 12 h in glacial acetic acid at 82 °C to yield 85% of the product. Typical experimental methods were as follows (structures of the compounds described below were verified by routine MS, IR, and NMR data):

Lactols (2) from Araldehydes. **2h** (R_1 = 4-(dimethylamino)-phenyl, R_2 = 2-styryl). To 0.385 mL (306 mg, 3.0 mmol) of *N,N,N'*-trimethylethylenediamine in 10 mL of tetrahydrofuran



(4)

(THF) (freshly distilled from sodium benzophenone ketyl) under argon at −20 °C was added 1.2 mL (3.0 mmol) of 2.5 M *n*-butyllithium in hexane. The solution was stirred for 15 min and then subjected to dropwise addition of a solution of 625 mg (3.0 mmol) of 4-stilbenecarboxaldehyde in 8 mL of dry THF. After another 15 min, 0.910 mL (696 mg, 6.0 mmol) of *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) was added, followed by a second injection of 1.2 mL of *n*-butyllithium. The solution was stored 20 h at −20 °C (in an argon-filled Ziploc bag containing Drierite), cooled to −40 °C, and treated dropwise with a solution of 470 mg (3.15 mmol) of 4-dimethylaminobenzaldehyde in 5 mL of dry THF. A nearly instantaneous color change from dark blue-green to light yellow-orange was noted. After 2.5 h at −40 °C, the mixture was quenched with 80 mL of cooled 10% HCl, stirred 10 min, adjusted to pH 6, and subjected to ether extraction (three 40-mL portions). The ether solution was dried over Na_2SO_4 and concentrated to yield 1.10 g of orange semisolid. Chromatography through a 2-cm column of silica gel (grade 60, 230–400 mesh) using methanol (0–5%) in dichloromethane as eluent yielded 525 mg (1.47 mmol, 49%) of lactol.

Phthalides via Ortho Metalation of Tertiary Amides (17). 3-(4-stilbenyl)phthalide. To 845 mL (651 mg, 5.61 mmol) of *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) in 41 mL of tetrahydrofuran (THF) (freshly distilled from sodium benzophenone ketyl) under nitrogen at −78 °C was added 8.0 mL (11.2 mmol) of 1.4 M *s*-butyllithium in cyclohexane. The solution was stirred 15 min and then subjected to the addition (over 10 min) of a solution of 900 mg (5.09 mmol) of *N,N*-diethylbenzamide in 10 mL of dry THF. After another 15 min, a solution of 1.06 g (5.09 mmol) of 4-stilbenecarboxaldehyde in 10 mL of dry THF was added over 10 min. The mixture was allowed to warm to 25 °C and was quenched with water. An ether extract was washed with 3 M HCl, water, $NaHCO_3$, and water and dried over $MgSO_4$. Most of the solvent was evaporated, yielding 2.07 g; by NMR spectroscopy of this unpurified material, the reaction was estimated to have proceeded nearly quantitatively.

Of this residue, 1.06 g (2.60 mmol maximum) was heated 4 h in 25 mL of refluxing toluene with 173 mg (0.91 mmol) of *p*-toluenesulfonic acid. Cooling and partial removal of the solvent left 289 mg (1.57 mmol, 61% based on starting amide) of crystals.

Reduction of Lactols (e.g., Lactol 2) or Phthalides with Lithium Aluminum Hydroxide (LAH). To 30 mg (79 mmol) of LAH in 20 mL of anhydrous ether at 5 °C was added gradually a solution of 200 mg (0.76 mmol) of lactol **2b** (R_2 = 2-naphthyl; R_2 = H) in 15 mL of anhydrous ether. The mixture was heated under reflux 2 h, cooled, and quenched with 2 mL of 1 M HCl. The ether layer was washed with water, dried (Na_2SO_4), and concentrated, leaving 172 mg (0.65 mmol, 86%) of residue deemed sufficiently pure for the next step.

2-Aroylaraldehydes by Selenium Dioxide Oxidation of Diols or Lactols. A variety of 2-aroylaraldehydes was made in fair to good yield as described by Metlesics et al. (*14*) from the corresponding diols made by the previously described procedure. For the SeO_2 oxidation of various lactols (example: lactol **2g**, R_1 = *p*-dimethylaminophenyl; R_2 = Me_2N) a modification was developed that permitted a considerable shortening of reaction time: a mixture of 75 mg (0.25 mmol) of lactol **2g** and 36 mg (0.33 mmol) of SeO_2 in 2 mL of pyridine was heated under reflux until the reaction was complete, as judged by thin-layer chromatography (roughly 2–3 hours), and quenched with 2 M KOH. An ether extract (three 40-mL portions) was subjected to column chromatography (40 × 15 mm, hexanes–dichloromethane) to give 57 mg (77%) of 2-aroylaraldehyde **1g**.

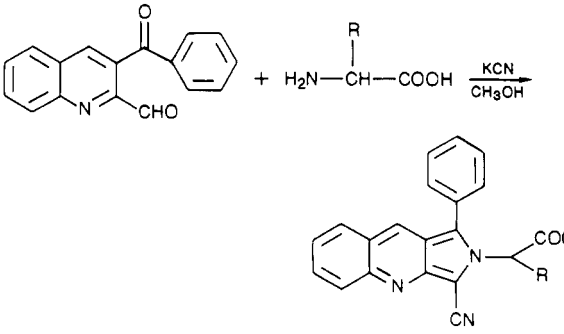
3-Benzoyl-2-Quinolinecarboxaldehyde (3). A mixture of 80 mg (0.32 mmol) of 3-benzoyl-2-methylquinoline (*20*) and 40 mg (3.6 mmol) of SeO_2 in 2 mL of acetic acid was heated 12 h at 82 °C and then cooled to 5 °C, and 0.5 mL of 1 M KOH was added. An ether extract was washed with water, dried over Na_2SO_4, and concentrated to yield 72 mg (0.28 mmol, 86%) of yellow crystals.

Derivatization and Fluorescence Measurements. It has been shown that stability and fluorescence intensity of the isoindoles could be enhanced by substituting cyanide for the thiol commonly used in OPA derivatizations (*21*). The general reaction scheme for isoindole formation is given by eq 5, and fluorescence quantum yields for many of these reactions were calculated and are tabulated in Table II.

Table II. Spectral Characteristics and Fluorescence Intensities of Isoindole Products[a]

reagent	max excitation wavelength, nm	max emission wavelength, nm	quantum yield
o-phthalaldehyde	340	455	0.3
1a	348	460	0.35
1b	355	480	0.31
1c[b]			
1d	335	450	0.34
1e	370	515	0.51
1f[c]			
1g	385	535	0.27
1h	365	530	0.35
1i	375	540	0.25
1j	410	560	0.27
1k	375	500	1.0
3	460	560	0.68

[a] All reagents were derivatized with glycine by utilizing the cyanide nucleophile (except OPA, for which β-mercaptoethanol was used) for this study. [b] We were unable to purify **1c** sufficiently for spectral analysis. [c] The absorption spectrum of glycine-derived isoindole with **1f** was not significantly shifted from that of **1f** to be considered for our purposes.



(5)

Methanol was chosen over the more commonly used borate buffer as the solvent in order to eliminate precipitation problems often encountered with buffers. This posed no problems with the reaction, since the optimum reaction pH is between 7 and 9 (see Figure 1).

Absorbance spectra of the isoindoles were obtained by using a Hewlett-Packard (Palo Alto, CA) 8450A diode-array spectrophotometer. Fluorescence spectra were obtained with a Perkin-Elmer (Norwalk, CT) 650 spectrofluorimeter by scanning the emission at 240 nm/min with the excitation fixed. All fluorescence emission spectra were corrected according to the method described by Parker (*22*).

A stock solution of 0.1 mg/mL of each the following amino acids (Sigma Chemical) in distilled water was prepared: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine. Derivatization for chromatographic separation was carried out by the addition of 20 μL of amino acid stock solution, 20 μL (0.2 mM) of reagent, and 20 μL (10 mM) of potassium cyanide in 500 μL of methanol. The solution was agitated by ultrasonic vibration and allowed to react for 1 h at room temperature.

For micro-scale liquid chromatographic separations, a Varian 8500 syringe pump (Palo Alto, CA) and a Valco Model CI4W (Houston, TX) injection valve with an injection volume of 100 nL were used. A laser-based fluorescence detector, designed and constructed in our laboratory (*23*), was the detector used in these studies. The laser employed was an Omnichrome (Chino, CA) He–Cd laser, operating at the blue line (442 nm). The column

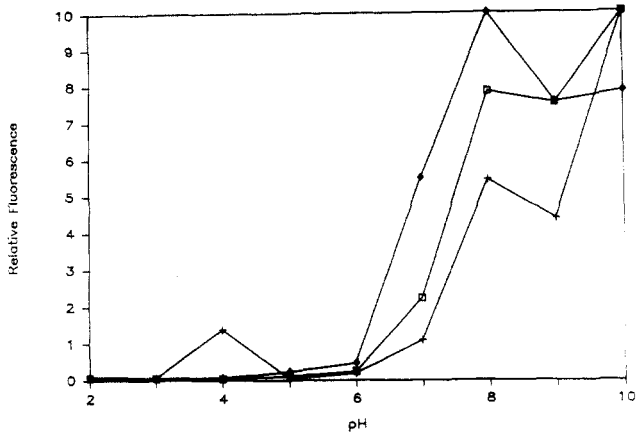


Figure 1. Effect of reaction pH on the BQCA derivatization of methionine (+), lysine (◊), and glycine (□).

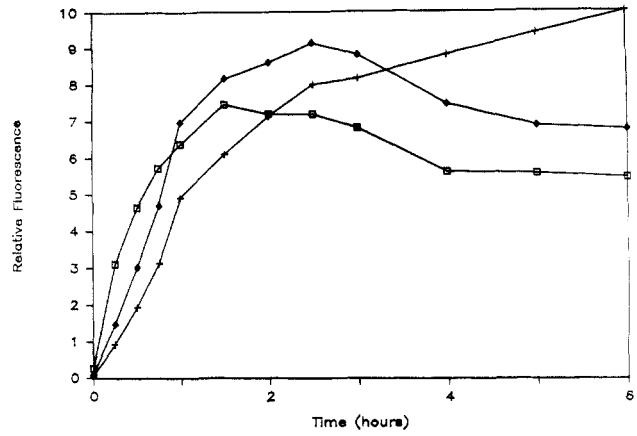


Figure 2. Stability, over time, of three amino acid derivatives of BQCA: (□) glycine, (+) methionine, (◊) lysine.

was made of fused silica tubing (Polymicro Technologies, Phoenix, AZ) (1000 mm × 0.25 mm) and slurry-packed with 5-μm Spherisorb ODS-2 particles.

RESULTS AND DISCUSSION

To compare the potential of various fluorogenic reagents for the determination of primary amines, four criteria were established. A high fluorescence quantum yield is the first obvious requirement. Quinine bisulfate was used as the standard for quantum efficiency measurements. Actual quantum yields were calculated by the method elucidated by de Montigny et al. (*21*). It should be noted that there is a 15–20% error in the quantum yield calculations by this indirect method. The quantum yields (as glycine derivatives) are listed in Table II. With only two exceptions, reagent **c** (which we were unable to purify) and reagent **f**, which has an absorption maximum for the derivatized species very close to that of the parent ketoaldehyde, all isoindoles were found to have good, workable fluorescence levels. In fact, derivatives of **1k**, which contain the stilbene moiety, exhibited an extremely high quantum yield.

The second criterion was the rapidity of isoindole formation upon derivatization. When OPA reacts with primary amines, fluorescent product forms within 2 min. Although none of the fluorogenic reagents examined in this study reacted as quickly, all isoindoles were produced in good fluorescent yields within 30–60 min. Although these reaction times might still be shortened through optimization with respect to a catalyst, they are acceptable for the purpose of precolumn derivatization.

Stability of the isoindole over a long period of time at its maximum fluorescence was the third criterion examined. This stability is essential in the use of precolumn derivatization, necessary for capillary liquid chromatography (LC). Figure 2 depicts a typical stability study for one of the more promising reagents, 3-benzoyl-2-quinolinecarboxaldehyde (BQCA). As was the case with all reagents studied, it is shown here that, once the fluorescence maximum has been reached, the intensity remains stable for several hours, as compared to OPA derivatives, which tend to be unstable. After 28 h in solution, the derivatives exhibited only ca. 10% of their peak fluorescence.

The final and most important criterion of this comparative study was the proximity of the excitation maximum to 442 nm, the output wavelength of the helium–cadmium laser detector. Table II lists the excitation maximum for the isoindoles produced with each reagent studied in this work. Although some of the synthesized reagents could be used with alternative light sources, only one of the candidate reagents exhibited appreciable fluorescence at 442 nm. 3-Benzoyl-2-quinolinecarboxaldehyde (BQCA) formed sufficiently fluorescent derivatives with the primary amines (amino acids) to be used in further characterization studies.

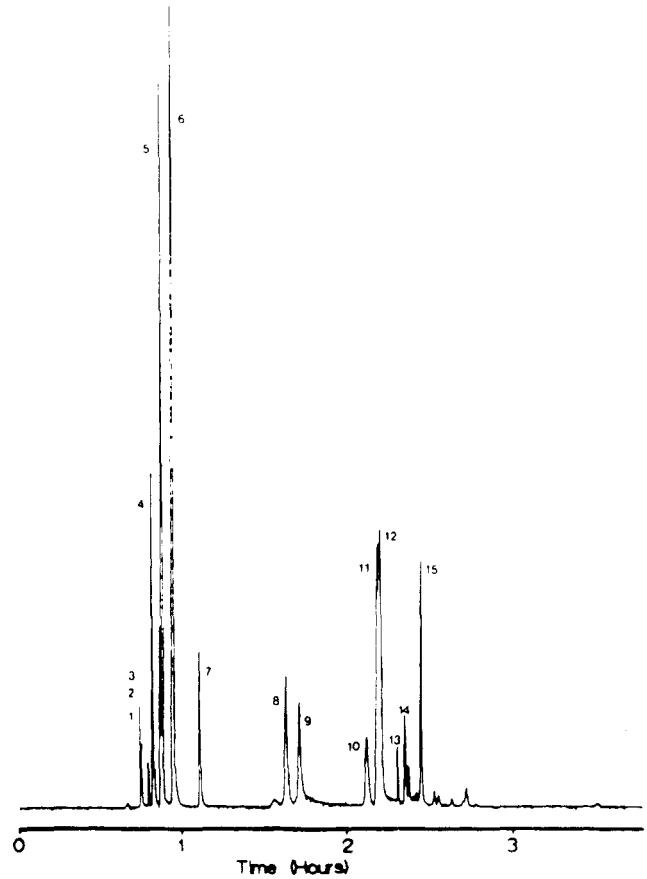


Figure 3. Separation of 15 amino acids via microcolumn liquid chromatography: peak 1, aspartic acid; 2, glutamic acid; 3, threonine; 4, histidine; 5, serine; 6, glycine; 7, alanine; 8, tyrosine; 9, arginine; 10, valine; 11, isoleucine; 12, leucine; 13, methionine; 14, phenylalanine; 15, lysine.

Figure 3 shows a typical chromatogram obtained with the microcolumn liquid chromatographic system utilizing a stepwise gradient system and laser-induced fluorescence detection. The mobile phase consists of a stepwise gradient, beginning with 25% acetonitrile in water doped with 0.1% triethylamine and 0.2% acetic acid and concluding with 50% acetonitrile in water doped with 0.1% triethylamine and 0.2% acetic. This chromatogram represents a 100-fold increase in sensitivity over the conventional chromatographic detection systems.

In order to determine detection limits, glycine solutions of decreasing concentration were prepared. Each solution was

individually derivatized such that, upon injection, known quantities of glycine would be introduced into the system. With the current laser detection system, the minimum detectable amount for the glycine derivative has been determined to be 17 fg at a signal-to-noise ratio of 3. Further improvements of this detection limit are currently being sought.

In summary, we have developed some general syntheses of fluorogenic reagents for the derivatization of primary amines for subsequent separation with high-sensitivity laser-induced fluorescence detection. The reagent of choice at this point, BQCA, has been evaluated on the basis of a number of criteria, including quantum yield and product stability. Our reagent is similar to OPA in that the absorption maximum of the resulting isoindole is well removed from that of the parent molecule. However, BQCA features some advantages over OPA; for example, the isoindole products are substantially more stable than OPA derivatives, facilitating precolumn derivatization. Moreover, the BQCA derivatives display an absorption maximum closely matching a convenient laser line, leading to increased sensitivity with a relatively inexpensive detection system. The fact that the rate of the BQCA reaction is significantly less than that of OPA is of little consequence in our studies. The strict volumetric requirements of capillary LC almost necessitate precolumn derivatization methods. Therefore, an improved stability, not reaction rate, was our primary concern.

It is noteworthy that the one quinoline-based reagent we have synthesized (BQCA) has emerged from this study as more acceptable for our purposes than the best of the benzene-based reagents, which suffer primarily from insufficiently high absorption maxima. Design of additional reagents of this type is under way, facilitated by the knowledge we have gained in these early efforts.

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Atomization Characteristics and Direct Determination of Manganese and Magnesium in Biological Samples Using a Magnetically Altered Thin-Film Plasma

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A magnetic field with peak value of 3.7 kG is used to improve the atomization characteristics of an electrically vaporized thin-film plasma for the direct determination of Mg and Mn in solid biological materials. Plasmas are generated by high-current capacitive discharges through 350-μg Ag or Au thin films formed on polypropylene substrates. Radiation intensity vs time plots are compared with and without the magnetic field for the NBS materials bovine liver, oyster tissue, orchard leaves, citrus leaves, tomato leaves, and pine needles. Analytical standards for Mg are prepared from suspensions of MgO powder, and standards for Mn are prepared from aqueous solutions of $Mn(NO_3)_2$ or $MnSO_4$. Analytical accuracy usually is improved with the presence of the magnetic field.

In a recent feature article in this journal, Scheeline and Coleman (*1*) pointed out the desirability of direct analysis of solids without prior dissolution, which is often a time-consuming process that can introduce contaminants. Numerous attempts have been made to perform direct and near-direct elemental analysis of solids by sensitive atomic spectrometric techniques. Mohamed et al. (*2*) reported analysis of coal slurries by flame atomic absorption, with 51% losses of the slurries in transport. Hoenig and Van Hoeyweghen (*3*) determined Pb and Cd in biological samples by using slurries to which a matrix modifier was added for platform electrothermal atomic absorption spectrometry. Rettberg and Holcombe (*4*) analyzed a variety of solid NBS reference materials with platform techniques without sample pretreatment or matrix modifier addition. McCurdy et al. (*5*) successfully analyzed NBS coal by introduction of slurries into a direct current plasma (DCP) after reduction of the coal to roughly 5.7-μm particles.

Considerable work has been done on the introduction of solids into inductively coupled plasma (ICP) torches. Sugimae

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