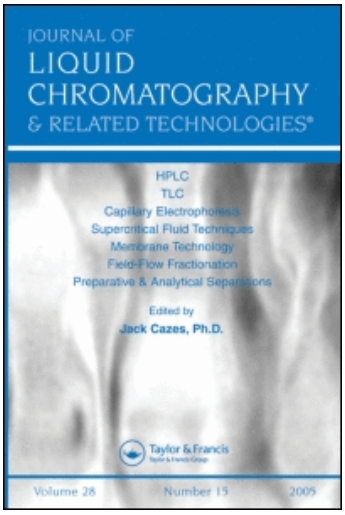

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RP-HPLRC Separation of Polyamines After Automatic FMOC-CI Derivatization and Precolumn Sample Clean-Up Using Column Switching

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RP-HPLC SEPARATION OF POLYAMINES AFTER AUTOMATIC FMOC-CI DERIVATIZATION AND PRECOLUMN SAMPLE CLEAN-UP USING COLUMN SWITCHING

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ABSTRACT

A fully automated RP-HPLC procedure including precolumn derivatization and sample clean-up was developed for the separation and determination of polyamines in plant tissue. The crude plant extract was derivatized with FMOC-Cl during 3 min without a prechromatographic purification step. The sample matrix, the reagent excess and most of the reaction by-products were effectively removed by a simple end-cut technique using column switching. The FMOC-polyamines were separated through an ODS microbore column with good resolution and detected with a fluorescence detector. The detection limit was less than 40 femtomoles for all analysed FMOC-polyamines.

INTRODUCTION

The growing interest in the polyamines and the clarification of their role in physiological and biochemical processes has led to a continuous development of simpler and more efficient methods of their determination, including TLC, GC and HPLC, in various types of biological fluids.

During the past ten years, the most wide-spread procedures were ion-exchange and ion-paired reversed-phase HPLC combined with pre- or postcolumn derivatization and UV or fluorescent monitoring, respectively. Recently, however, precolumn derivatization followed by RP-HPLC separation is the preferred method for the separation of polyamines, because ion-exchange HPLC and postcolumn derivatization is time-consuming and more expensive (1).

The reagents most widely used for the prechromatographic derivatization of polyamines are dansyl chloride (1,2), benzoyl chloride (1,3-5) and o-phthalaldehyde (OPA) (1,6,7). However, the application of these reagents involves a number of drawbacks. Dansyl chloride and benzoyl chloride are nonspecific reagents, since they react not only with primary and secondary amino groups, but also with phenols, aliphatic alcohols and some sugars. Various manual sample clean-up steps are therefore required to remove interfering compounds before sample injection. Furthermore, the relatively long reaction time makes automatic precolumn derivatization impossible. As the benzoylated polyamines do not fluoresce, these compounds are generally detected with the less sensitive UV detector. The application of OPA is more advantageous, since it reacts only with primary amines within a few minutes and forms strongly fluorescent derivatives. This reaction can easily be automated because of the short reaction time (8). However, OPA derivatives are not too stable; this is especially true for polyamines containing secondary amino groups, such as spermidine and spermine (6).

During the past five years, 9-fluorenylmethyl chloroformate (FMOC-Cl) has been used as a new precolumn derivatization reagent for polyamines (9-11). FMOC-Cl was earlier reported to be of use for the precolumn derivatization of amino acids (12). The application of FMOC-Cl combines the advantages of the above-mentioned derivatization reagents. It reacts rapidly only with primary and secondary amines forming stable and highly fluorescent derivatives.

The present paper describes a very sensitive and reproducible method for the determination of FMOC-polyamines in plant tissue. The

method is fully automated, from the precolumn derivatization through the sample clean-up to the fluorescent detection of the derivatives.

MATERIALS AND METHODS

Chemicals:

The polyamine standards putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPN), and also diaminohexane (DAH) as internal standard, were purchased from Aldrich (Steinheim, Germany).

The FMOC-Cl derivatizing reagent was obtained from Sigma (St.Louis, MO, U.S.A.).

Borate buffer (0.4 M, pH 10.4) was prepared from boric acid and potassium hydroxide (Reanal, Budapest, Hungary).

HPLC grade, membrane-filtered acetonitrile (ACN) and methanol (MeOH) were from Farmitalia Carlo Erba (Milano, Italy).

Water was prepared and membrane-filtered by using a Barnstead (Dubuque, IA, U.S.A.) NanoPure II water purification system.

For calibration, three standard mixtures of polyamines (0.5, 1.5 and 2.5 ng/µl of each, except DAH) were prepared with 0.1 N hydrochloric acid and frozen at -20 °C until use. The concentration of internal standard (DAH) in each standard solution was 1.0 ng/µl. Under these conditions, the standard mixtures were stable for at least three months.

Equipment:

The derivatization, sample clean-up and RP-HPLC separation were performed on a Hewlett-Packard HP 1090M liquid chromatograph equipped with a PV5 ternary solvent-delivery system, a variable volume auto-injector, an autosampler, a thermostatically controlled column compartment with an air-actuated Rheodyne 7010 (Rheodyne, Cotati, CA, U.S.A.) six-port column switching valve (CSV).

Two columns (guard and analytical) were connected to the CSV: a 200x2.1 mm analytical column with Hypersil ODS 5 µm packing

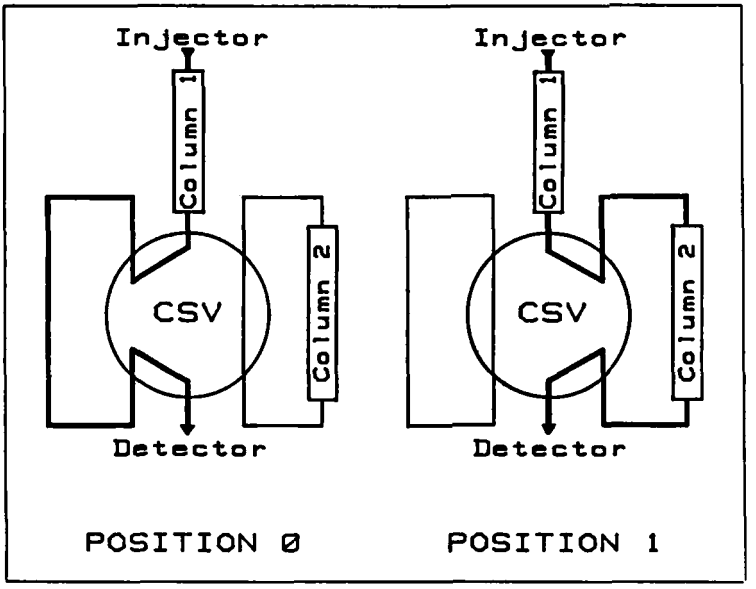

FIGURE 1. Switching valve positions during sample clean-up and separation. CSV = Rheodyne 7010 six-port, air-actuated column switching valve; Column 1 = Supelcosil C18 18 µm, 20x2.1 mm guard column; Column 2 = Hypersil ODS 5 µm, 200x2.1 mm microbore analytical column.

(Shandon, Cheshire, England) and a 20x2.1 mm guard column, which was dry-packed with Supelcosil C18 18 µm (Supelco S.A., Gland, Switzerland) packing material (Figure 1). The columns and CSV were thermostated in the column compartment at 40°C.

Extraction of polyamines from plant tissue:

Free polyamines were extracted from carrot leaves (1 g fresh weight) on ice using a silanized, ground-glass potter with 5 ml of 0.2 N perchloric acid containing 10 µg DAH as internal standard. After homogenization, the mixture was immediately placed in a refrigerator at 4 °C

TABLE 1

Injector Program for Derivatization of Polyamines with FMOC-Cl

Line#	Function	Amount (µl)	Details	Substance
1	Draw	5	From vial 2	Borate buffer (pH 10.4)
2	Draw	1	From sample	Crude extract (filtered)
3	Draw	0	From vial 3	Water for needle wash
4	Draw	0	From vial 4	Anhydr. ACN for needle wash
5	Draw	1	From vial 1	FMOC-Cl reagent
6	Draw	0	From vial 100	Water for needle wash
7	Mix	7	Eight cycles	Reaction
8	Inject			

for one hour and then centrifuged at 5000 g for 15 min. The supernatant was filtered through a 0.45 µm Millex FH membrane (Millipore, Bedford, MA, U.S.A.) into a HPLC autosampler vial and was used directly for the determination of polyamines.

Automatic precolumn derivatization:

The calibration standards and the plant extract were derivatized automatically with FMOC-Cl using the computer-controlled auto-injector and autosampler.

Five vials - needed for derivatization - containing borate buffer, FMOC-Cl reagent (2.5 mg/ml anhydrous ACN) and injection needle washing solvents, were placed into the autosampler. The other positions in the autosampler were maintained for the sample vials. The buffer, the reagent and the sample were drawn up by the injection needle into a reaction capillary. This reaction mixture was mixed by moving the syringe plunger back and forth with a stepping motor to achieve the derivatization. During the injector program, the outer surface of the injection needle was rinsed three times to prevent cross-contamination between vials.

The derivatization reagent dissolved in anhydrous ACN was stable for at least one week when stored at 4 °C under argon in a tightly closed vial. The complete injector program is shown in Table 1.

Precolumn sample clean-up:

In order to ensure automatic precolumn sample clean-up, the crude reaction mixture (7 µl) was injected onto the guard column equilibrated with 44% ACN in water, while the analytical column was bypassed (Figure 1). The sample clean-up took 4.5 min, with a flow rate of 0.45 ml/min.

HPLC conditions:

After column switching at 4.5 min, the FMOC-polyamines were analysed at a slightly increased flow rate (0.5 ml/min) with a gradient of solvent A (44% ACN in water) and solvent B (70% ACN/30% MeOH). Both solvents were filtered through a 0.45 µm Nylon 66 membrane filter (Millipore, Bedford, MA, U.S.A.) and intensively degassed with helium before use. During the analysis, a low helium flow rate was maintained through the solvents. The gradient used for the separation of polyamine derivatives started with 100% solvent A and was increased to 100% solvent B in 14 min. After elution of the polyamines, the flow rate was increased to 0.85 ml/min and the columns were reequilibrated by washing with 100% solvent A for 6 min. At the end of the analysis (26 min), the CSV was turned back to the original position, making measurement of the next sample possible, and the flow rate was decreased to 0.45 ml/min (Table 2).

Detection:

The separated polyamine derivatives were detected by using a Hewlett-Packard HP 1046A programmable fluorescence detector supplied with a pulsed xenon flash lamp and a 5 µl flow cell. The excita-

TABLE 2

Time-Table for Separation of FMOC-Polyamines by HP 1090M LC

Time (min)	Gradient Composition (%) Solvent A	Solvent B	Flow (ml/min)	CSV Position
0	100	0	0.45	0
4.5				1
4.6			0.45	
4.7			0.50	
8.0	55	45		
14.0	0	100		
18.0	0	100		
19.0			0.50	
19.4			0.85	
20.0	100	0		
23.0			0.85	
23.4			0.50	
26.0	100	0	0.45	1

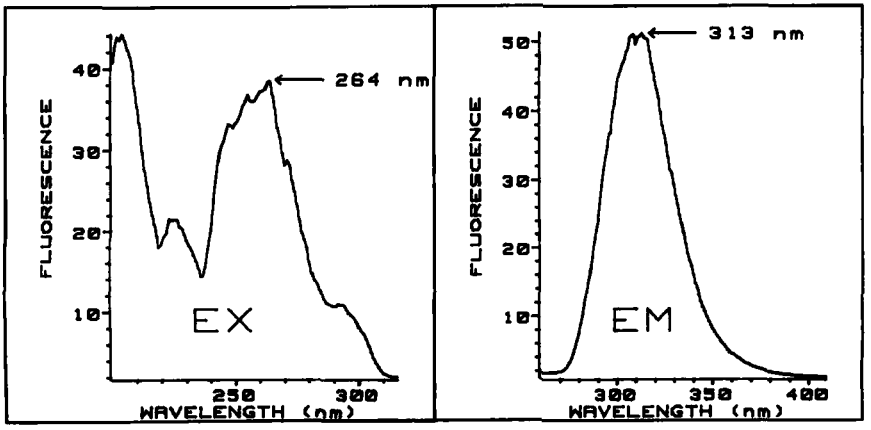

FIGURE 2. Excitation (EX) and emission (EM) spectra of FMOC-PUT. Arrows indicate maxima.

tion and emission wavelengths were 264 and 313 nm, respectively (Figure 2). To reduce the background noise, a 300 nm cut-off filter was placed between the flow cell and the emission grating.

The flow diagram of the method described above is shown in Figure 3.

RESULTS

We have developed a fully automated procedure, including precolumn derivatization and sample clean-up, for the RP-HPLC analysis of polyamines.

The polyamines could be derivatized automatically with FMOC-Cl under basic conditions (pH 10.4), using a computer-controlled auto-injector and autosampler in eight steps. During the injector program, a stepping motor-driven syringe drew up an exact volume of buffer, reagent and sample, and mixed them in a reaction capillary to complete the derivatization. The injector program took only 3 min (Table 1).

The crude reaction mixture containing FMOC-derivatized polyamines, amino acids, reagent excess and other interfering compounds could be injected directly onto the guard column, which was equilibrated with 44% ACN similarly to the analytical column. Under these conditions, FMOC-polyamines were retained, and all components which were more polar than FMOC-PUT passed through the guard column, while the analytical column was bypassed. Removal of the sample matrix during the precolumn sample clean-up not only improved the resolution of the FMOC-polyamines, but also prolonged the lifetime of the analytical column. In order to maintain a good separation, it was necessary to replace the guard column by a new one after the injection of every 50 samples. At 4.5 min, the guard and analytical columns were switched in series with each other through the CSV, to carry out the chromatographic separation of the FMOC-polyamines.

The polyamines were analysed with a gradient of 44% ACN (solvent A) and 70% ACN/30% MeOH (solvent B) during 19 min (Table

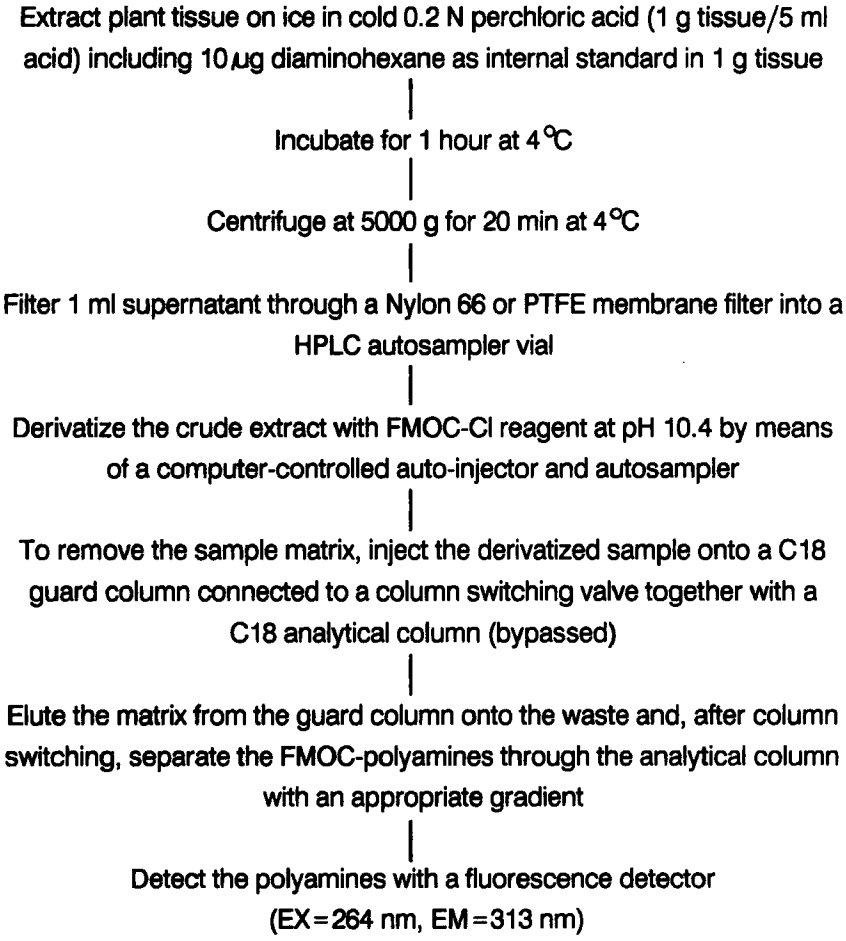

FIGURE 3. Flow diagram for the extraction, automatic FMOC-Cl derivatization, sample clean-up and RP-HPLC analysis of polyamines.

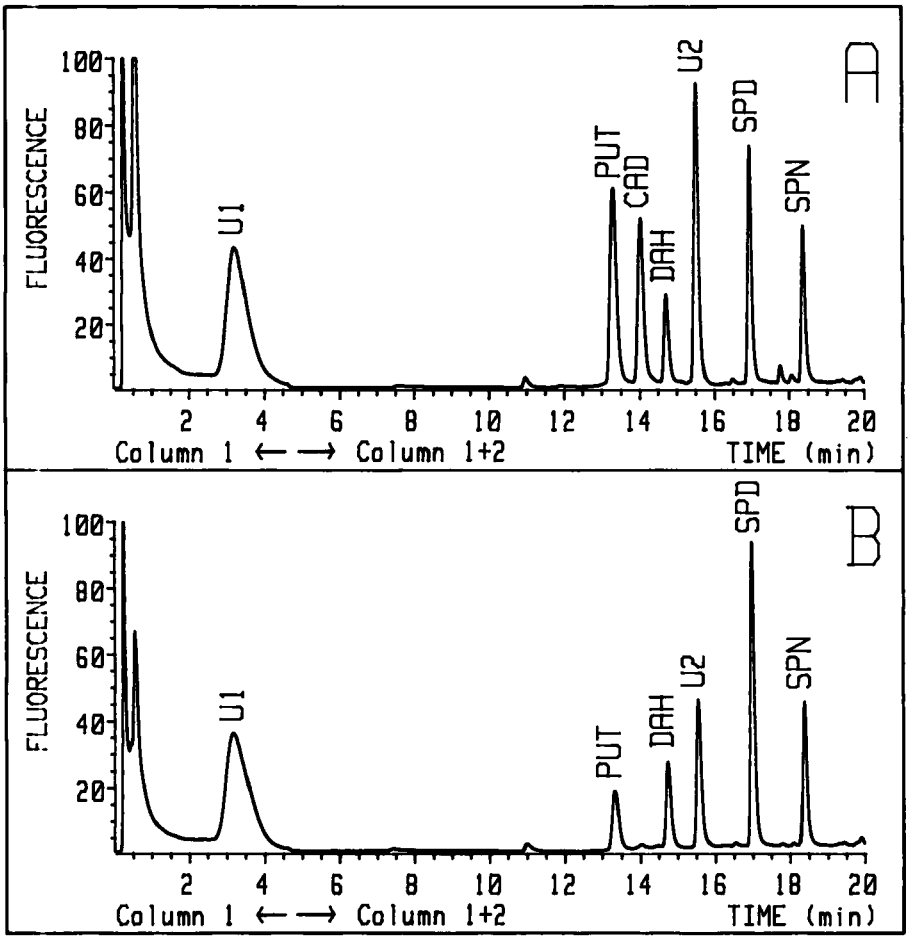

FIGURE 4. Separation of FMOC-polyamines using column switching. A) Standard mixture of polyamines (each peak represents 2.5 ng of polyamine injected, with the exception of DAH, whose content was 1.0 ng. B) Polyamines from carrot leaves, including DAH as internal standard. Column 1 = Supelcosil C18 18 µm, 20x2.1 mm guard column; Column 2 = Hypersil ODS 5 µm, 200x2.1 mm microbore analytical column; Column compartment temperature: 40°C;
Detection: fluorescence (EX = 264 nm, EM = 313 nm). For other conditions, see TABLE 2. Peaks: putrescine (PUT), cadaverine (CAD), diaminohexane (DAH), spermidine (SPD), spermine (SPN), unknown 1 (U1) and unknown 2 (U2).

TABLE 3

Precision Calculation for Retention Times of FMOC-Polyamines (n = 5)

Polyamine	Mean (min)	S.D. (min)	R.S.D. (%)
Putrescine	13.404	0.152	1.14
Cadaverine	14.105	0.136	0.97
Diaminohexane	14.788	0.111	0.75
Spermidine	16.989	0.063	0.37
Spermine	18.389	0.042	0.23

2). Higher selectivity was obtained by using solvent B containing MeOH instead of ACN alone. After the elution of the last FMOC-polyamine (SPN), both columns could be effectively cleaned with an increased flow of solvent B. Reequilibration of the columns for 6 min was required to restore the initial conditions. The total analysis time, including derivatization, sample clean-up, separation, column washing and equilibration cycle, was 26 min.

This method was developed by using a microbore analytical column, which made it possible to reduce the analysis costs because of the lower solvent consumption, but with enhanced detectability.

Chromatograms of the standard polyamine mixture and of a crude plant extract from carrot leaves are shown in Figure 4. The two unknown reaction by-products (U1 and U2) which can be seen on both chromatograms are probably 9-fluorenylmethanol and bis(9-fluorenylmethyl) carbonate. These unknown compounds can be identified by means of mass spectrometry.

When the above-mentioned gradient was applied, all five FMOC-polyamines, including the internal standard (DAH), were resolved and well separated from each other and from the interfering compounds, with a resolution of at least 1.63.

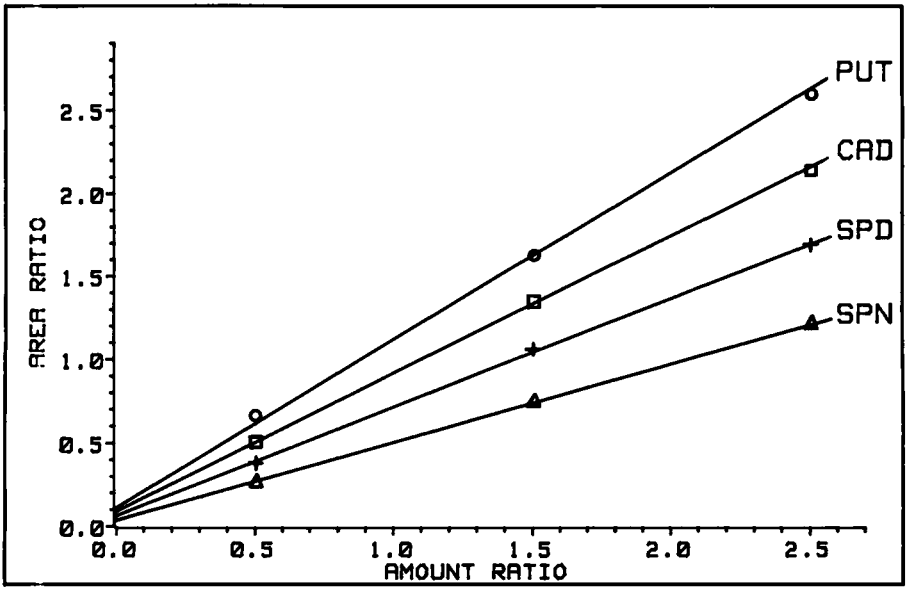

FIGURE 5. Calibration curves for FMOC-polyamines (0.5, 1.5 and 2.5 ng of each polyamine injected, with the exception of DAH, whose content was 1.0 ng).

As shown in Table 3, the retention times were reproducible, due to the automatic precolumn derivatization and sample clean-up.

Figure 5 shows calibration curves for FMOC-polyamines with DAH as internal standard. The linear relationships between the area ratios and amount ratios were obtained with correlation coefficients of r = 0.997-1.000.

The detection limit at a signal to noise ratio of 4/1 was less than 40 femtomoles.

DISCUSSION

We have presented an on-line RP-HPLC method for the determination of polyamines in plant tissues. This method can easily be auto-

mated, from precolumn derivatization through sample clean-up to fluorescence detection.

The simple end-cut technique using column switching allows the analysis of the derivatized polyamines from a crude plant extract without any prechromatographic purification step except membrane filtration.

The FMOC-Cl derivatization offers advantages over other methods (9-12). FMOC-Cl reacts only with compounds containing primary and secondary amino groups, in contrast with the less specific reagents most frequently used for the derivatization of polyamines (1-5).

Through use of the unattended precolumn derivatization and sample clean-up, possible errors due to manual sample handling and derivative instability could be avoided.

Application of the automatic precolumn derivatization in conjunction with the sample clean-up can significantly increases both the lifetime of the separation system and the reproducibility of the analysis.

The FMOC-Cl-derivatized polyamines are stable, highly fluorescent and can be detected in an extremely low concentration.

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