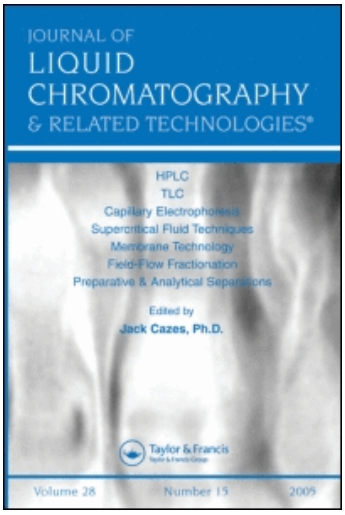

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SILICA BASED 3,5-DINITROBENZOYL (DNB) REAGENT FOR OFF-LINE DERIVATIZATION OF AMINE NUCLEOPHILES IN HPLC

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ABSTRACT

We describe here a new silica based derivatization reagent, containing the 3,5-dinitrobenzoyl tag, for solid phase derivatization of amines. It can be used for the off-line derivatization of primary and secondary amines. The amide derivatives can be easily detected under conventional UV detection modes. The entire synthetic method, structural characterization, and optimization of derivatization conditions of this solid phase derivatization reagent are described. Also, the reagent was tested in the on-line, pre-column derivatization mode for reversed phase HPLC, as well as for histamine analysis in fish samples.

INTRODUCTION

A solid phase reagent may be prepared on a variety of solid supports, which may be inorganic or polymeric in nature, such as

silica, alumina, crosslinked polystyrene, etc. The reactive tags can be physically adsorbed on, or covalently attached to, solid supports, in which the covalently attached moiety has better stability and other properties. Many kinds of silica based reagents have been reported and some of them dealt with reaction detection. Modified silica gels with a terminal functional 8-hydroxyquinoline group were used to extract some transition elements from solution (1-3). A silica based solid phase reagent was used to determine thiol compounds with a chromatographic system, in which a disulfide --S-SR' was the active functional group immobilized on the silica surface (4). UV/Vis detection was used to detect R'S- tagged products. A solid phase oxime reagent based on particulate bonded phase silica gel was claimed to be useful in quantification of analytes having primary or secondary amine or thiol functionalities (5). 3-Aminofluoranthene was immobilized on controlled pore glass beads for the determination of hydrogen peroxide in rain water samples (6). Immobilization of 1-bromonaphthalene on silica was used as a phosphor in quenched phosphorescence detection (7). Immobilized enzyme reactors (IMER) were also very useful for on-line sample modification in HPLC (8-10). Many papers have dealt with this research area (11, 12).

The monitoring of primary alkylamines in the atmosphere is important, as most of the alkylamines are toxic through exposure. Amine and amine-like compounds usually do not contain chromophores and fluorophores which are amenable to normal UV/FL detection, therefore, they cannot be detected directly without a pre- or post-column derivatization to introduce a detectable group or groups onto the original analyte. The most common derivatization method is converting analyte(s) to derivative(s) under homogeneous liquid phase conditions (13-15). In the past few years, successful developments have been made in the utilization of solid phase derivatization reagents for HPLC determination of bio-active amines, polyamines, and amine-like drugs (16-18). Alternatively, the solid phase derivatization reaction

occurs in a heterogeneous state, so that some unavoidable disadvantages associated with the homogeneous reaction are overcome. Some obvious advantages have been realized with the use of solid phase reagents, such as: 1) reduced toxicity of supported species compared with low molecular weight species; 2) improved chemical stability over time in comparison with the liquid reagent; 3) greater analyte selectivity with fewer side products; 4) derivatizations are often simpler than with the solution reaction analog, there can be much less contamination and/or background from excess derivatization reagent; 5) a high reaction capacity, it can be used many times without loss in reaction efficiency due to the high concentration of tag; and 6) the spent reagent can be regenerated easily by replacing the labeling moiety, often possible in situ.

Histamine analyses in food and clinical applications have been important for a long time (19, 20). Histamine is a potent mediator of physiologic processes; it also indicates the decomposition of fish products. Existing methods for determining histamine are based on bioassay, thin layer chromatography, gas chromatography, fluorometric batch method with *o*-phthalaldehyde (OPA) (21), and HPLC (22). As with all of the batch methods, time consuming sample preparation prior to the determination step were needed to avoid interference of the matrices in the fluorometric batch method. Interference of histidine and other biogenic amines was minimized by the fact that the retention time of these derivatives differed from that of the histamine derivative. A histamine/*o*-phthalaldehyde (OPA) condensation reaction was generally used for histamine detection with HPLC, even though some drawbacks exist. In this paper, a new silica based solid phase derivatization reagent was used for standard alkylamine analysis and histamine detection in canned tuna fish samples.

EXPERIMENTAL

Instrumentation and Chemicals

A. Chemical reagents and solvents

Bare silica (LiChrosorb Si-100, surface area 300 m^2/g, 10 μm) was obtained from E. Merck GmbH (Darmstadt, FRG). Bis-(2-hydroxyl)-3-amino-propyltriethoxysilane (purity 62%, solution in ethanol) was acquired from Petrarch Systems Co. (Bristol, PA) and used without further purification. Lithium chloride (99%), 3,5-dinitrobenzoyl chloride (DNB-Cl) (98%), thionyl chloride ($SOCl_2$) (99%), triethylamine (TEA, 98%), diethylamine (DEA) (98%), n-butylamine (BA) (99%), n-amylamine (99%), n-hexylamine (99%), n-heptylamine (99%), n-octylamine (99%), N,N-dimethylformamide (DMF) (99%), and dichloromethane (CH_2Cl_2) (99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC solvents were obtained form EM Science, Inc. (Gibbstown, NJ) as their Omnisolv® HPLC brand/grade. All HPLC solvents were used after filtration through a 0.45 μm HVHP® type solvent filter (Millipore Corp., Bedford, MA) and degassed under vacuum with stirring.

B. Instrumentation

The HPLC system consisted of a LDC Constametric III solvent delivery system (Riviera Beach, FL), a Rheodyne model 7125 injection valve with 20 μl injection loop (Rainin Instrument Co., Woburn, MA), a EM Science LiChrosphere C_{18}® reversed phase column (5μm, 250 mm x 4 mm I.D.), and a Waters model 480 variable wavelength UV-Vis detector.

Synthetic Procedures

A: *Total synthesis of silica based reagent for derivatization*

1. Silanization of silica surface

Bare silica (intermediate **I**, Figure 1) was preconditioned for 2 weeks in a dessicator under a saturated aqueous LiCl solution. 5.0 g of preconditioned silica (**I**) and 8.4 g of a 62% solution of bis(2-hydroxyethyl)-3-aminopropyltriethoxysilane (in ethanol) were reacted in a 150 ml reaction vessel. 0.5% (v/v) pyridine and 40 ml ethanol (EtOH) were added into the flask as the reaction catalyst and solvent, respectively. The mixture was stirred at 78°C for 6 hours with EtOH refluxing under nitrogen. When the reaction system cooled to room temperature, the reaction solution was filtered, the silica was washed with 3 x 25 ml methanol and 4 x 25 ml CH_2Cl_2, and then dried (intermediate **III**) under a N_2 atmosphere at 40°C overnight.

2. Synthesis of acid chloride from 4-hydroxy-3-nitrobenzoic acid

4.7 g 4-hydroxy-3-nitrobenzoic acid (**IV**, Figure 1) and 7.5 ml of thionyl chloride were reacted in a three-neck reaction flask with 0.75 ml pyridine as catalyst and 60 ml benzene as solvent. The reaction temperature was kept at 55°C with a water bath for 4 hours. After cooling to room temperature, the reaction solution was filtered with a dry sintered glass funnel to remove the unreacted 4-hydroxy-3-nitrobenzoic acid. A rotary evaporator was used to remove excess thionyl chloride from the reaction product **V**. This compound was reported by Imai *et al.* (23). The IR and mass spectroscopy results of **V** indicated the monomeric acid chloride structure, instead of a dimer or oligomer (24).

3. Addition of nitrophenol ligand groups to silica intermediate **III**

Fresh intermediate compound **V**, 5.0 g, 0.3 ml pyridine, and silica intermediate **III**, 5.0 g, were added to a reaction flask, and 60 ml benzene (dried with anhydrous Na_2SO_4 before reaction) was used as the reaction solvent. Reaction was carried out at 70-75°C for 2 hours, the reaction solution was filtered and the surface modified silica **VI** was washed with 3 x 75 ml DMF and 3 x 75 ml CH_2Cl_2. This product, **VI**, was dried under vacuum at 40°C for 12 hours.

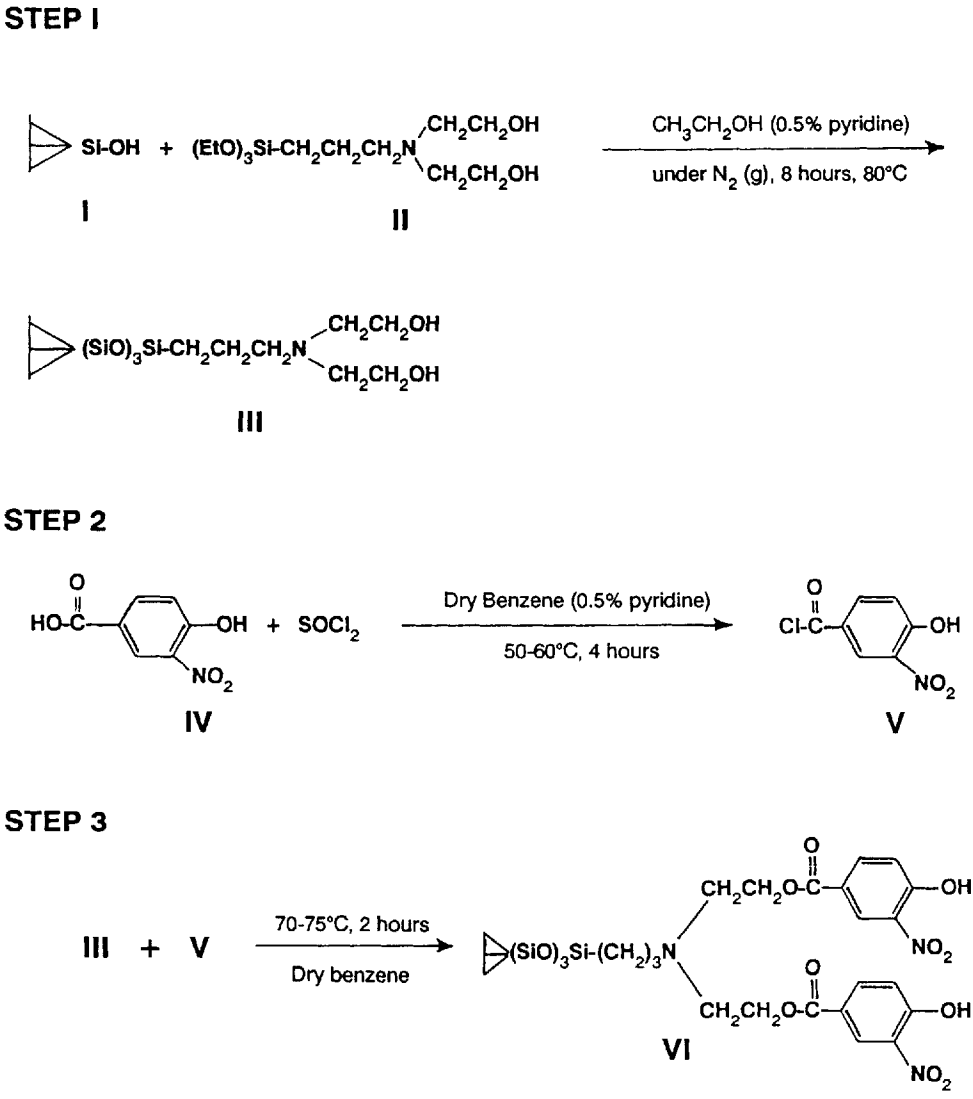

Figure 1a: Synthesis of Silica Based Derivatization Reagent Intermediates

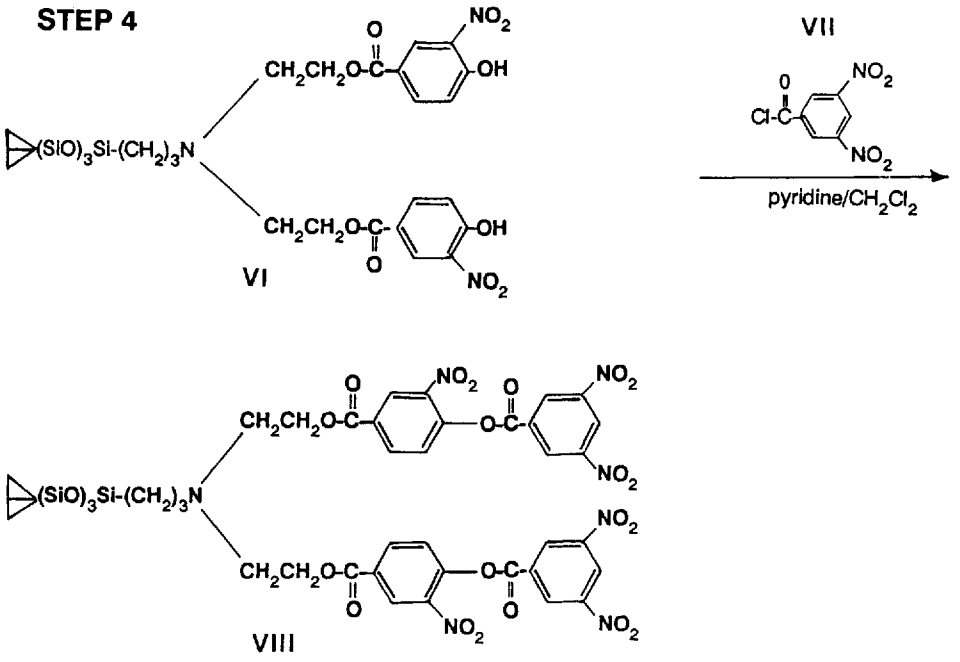

Figure 1b: Synthesis of Silica Based 3, 5-Dinitrobenzoyl (DNB) Tagged Derivatization Reagent

4. Addition of 3,5-dinitrobenzoyl chloride reagent to modified silica **VI**

0.4 g silica **VI**, and 0.6 g 3,5-dinitrobenzoyl chloride reagent **VII** were added to a flask. 25 ml benzene and 0.3 ml pyridine were added as solvent and catalyst, respectively. The reaction mixture was stirred at room temperature for 24 hours, and then the mixture was filtered and washed with 3 x 25 ml dichloromethane (CH_2Cl_2). This product was the final silica based derivatization reagent, **VIII**.

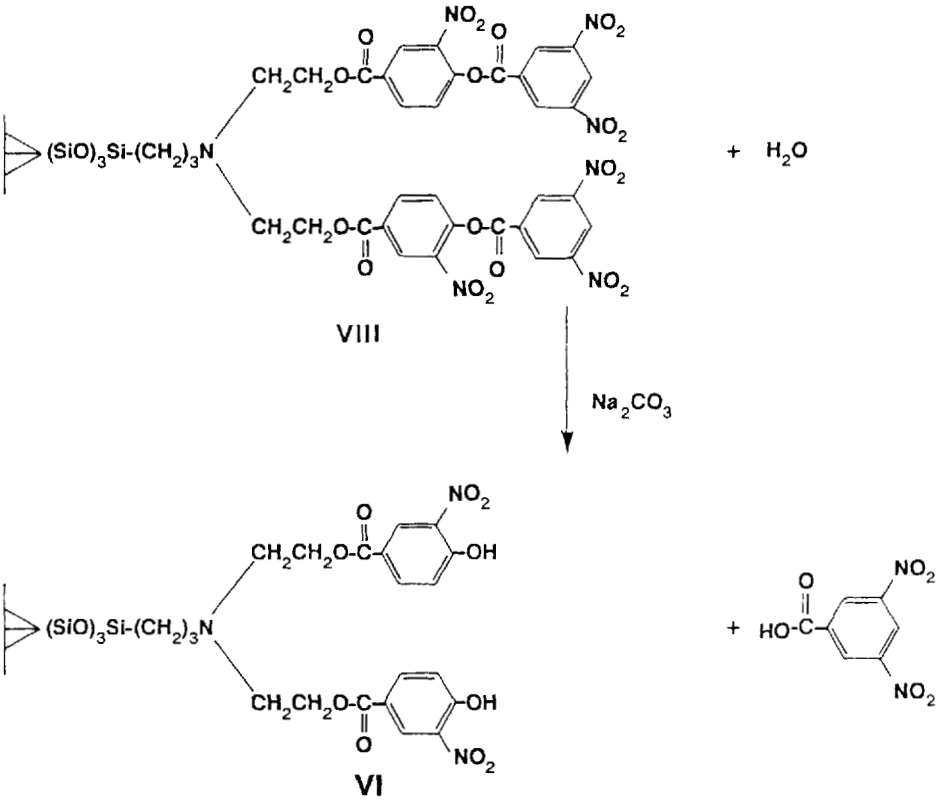

Figure 2a: Hydrolysis of Silica Based DNB Reagent

B: *Characterization of the silica based reagents with loading capacity determination*

1. Hydrolysis of 3,5-DNB tagged silica reagent

a. Hydrolysis

A weak base solution was used to hydrolyze the silica based reagent because silica could be dissolved in a strong base solution. Sodium carbonate was used as the hydrolysis medium (Figure 2a).

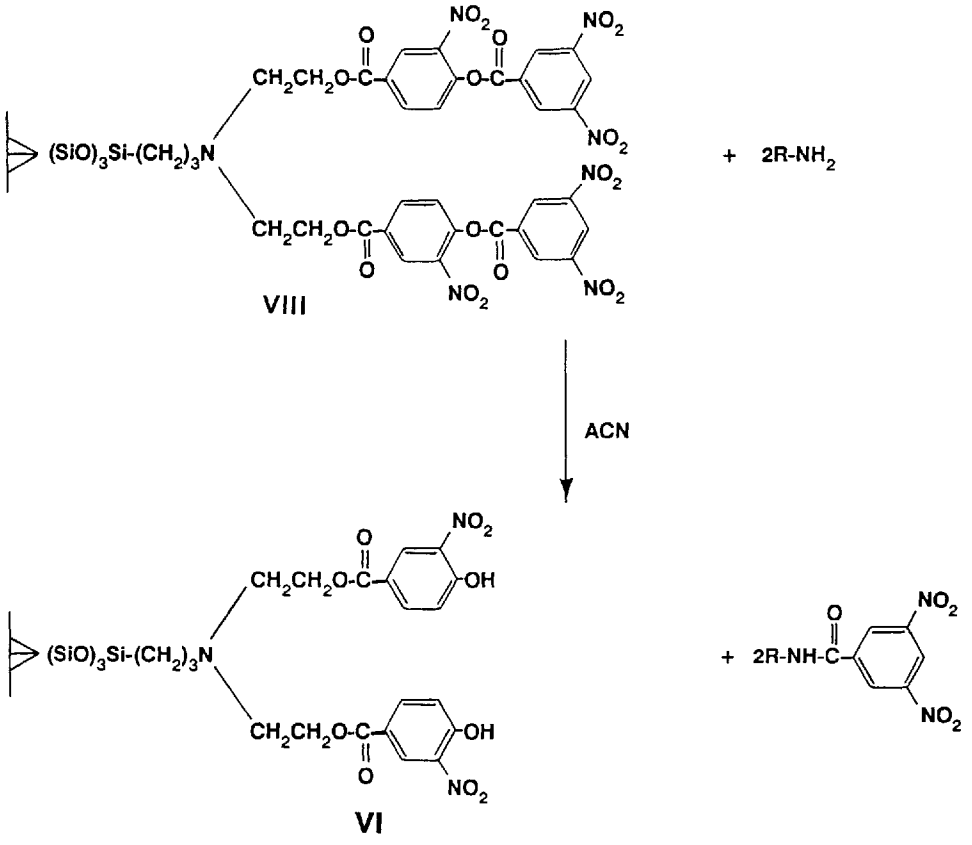

Figure 2b: Aminolysis of Silica Based DNB Reagent and Derivatization of Amine Analytes

200-300 mg of the 3,5-DNB tagged silica was suspended and vigorously agitated in 5 ml of 4.1 mM Na_2CO_3/ACN (1/1, v/v) solution. This slurry was heated for 10 minutes in a 70°C water bath. The released 3,5-dinitrobenzoic acid was diluted and determined by HPLC-UV (25). A calibration plot of 3,5-dinitrobenzoic acid was used for concentration determinations.

b. Recovery tests of 3,5-dinitrobenzoic acid

The hydrolytic product of the 3,5-dinitrobenzoyl (DNB) solid phase reagent **VIII** contains 3,5-dinitrobenzoic acid (Figure 2a). 2.25 ppm of 3,5-dinitrobenzoic acid in 50% ACN/ 4.1 mM Na_2CO_3 (v/v) was sealed in a bottle and heated under 70°C for 10 min. After cooling, 20 µl of the heated solution was analyzed by HPLC. HPLC conditions were: 50% ACN/H_2O (0.1M phosphoric acid, pH=3.5) as mobile phase, 1.5 ml/min, LiChrosphere® C_{18} HPLC column, 5 µm, 250 mm x 4.0 mm i.d., UV 254 nm detection.

2. Aminolysis of 3,5-DNB tagged silica reagent

This procedure was similar to the off-line derivatization of primary amines, except that n-butylamine in a large excess was used to take off all the 3,5-dinitrobenzoyl tag from the silica based reagent **VIII**. The two products from the aminolysis of the silica based DNB reagent would be 3,5-dinitrobenzoyl butylamide and 4-hydroxy-3-nitrobenzoyl butylamide (Figure 2b). Butylamine was a strong nucleophile but weak base, so that decomposition of the silica backbone was minimal. Based on the quantification of 3,5-dinitrobenzoyl butylamide from aminolysis, the loading capacity was obtained.

3. Elemental analysis of 3,5-DNB tagged silica reagent

Silica intermediate **I**, **III**, **VI** and the final DNB tagged reagent **VIII** were dried to constant weight, and approximately 20 mg of each product was sealed and sent to be analyzed at Galbraith Laboratories, Inc. (Knoxville, TN). The results were: intermediate **I**: C%=0.31, H%=0.64, N%<0.10; intermediate **III**: C%=6.27, H%=1.55, N%=0.75; intermediate **VI**: C%=16.50, H%=1.63, N%=2.51; intermediate **VIII**: C%=21.00, H%=1.76, N%=4.05. Loading capacity was calculated based on increase in nitrogen from intermediate **VI** to intermediate **VIII**.

C: *Synthesis and characterization of standard DNB amides of n-C_4-C_8 primary amines and histamine*

1. Synthesis of DNB amides from n-C_4-C_8 primary amines

DNB-standards of amines were prepared via solution derivatization of the amine with triethylamine (TEA) catalyzed 3,5-dinitrobenzoyl chloride according to literature (26). An amine (8 mmol) solution in 25 ml ethyl acetate (EtAc) was prepared in a 100 ml reaction vial. In another reaction vessel, 1.7 ml (8 mmol) of triethylamine, 1.8 g (8 mmol) DNB-Cl and 25 ml of EtAc were mixed with ice-water cooling. The acid chloride slurry was added to the amine solution drop-by-drop with stirring (room temperature) and reacted for 1 hour. After reaction, the amides were washed with 2 x 50 ml 0.5 N HCl, 2 x 70 ml 0.05 N NaOH as well as 2 x 30 ml saturated NaCl aqueous solution. Rotary evaporation of these amides led to a thick solution, and they were purified by recrystallization (2 times) from MeOH solution (with a small amount of water).

2. Synthesis of DNB amide from histamine

8 mmol histamine was prepared in water and 8 mmol of 3,5-dinitrobenzoyl chloride was prepared in EtAc solution with TEA (1/1 molar ratio to the acid chloride). Then an acid chloride solution was added drop-by-drop to the histamine solution at room temperature, and the reaction was continued for 1 hour. The derivative was washed with NaOH and HCl consecutively and purified by extraction. Elemental analysis results were: C%=46.81 (47.19), H%=3.51 (3.60), N%=22.89 (22.94). Numbers in parentheses represent the theoretical values. Melting point and spectral identification were used to confirm structures of these DNB tagged amides.

D. *Off-line derivatization and optimization*

Off-line solid phase derivatizations of amine samples were performed in either disposable pipets or small capped reaction vials.

25 µl of a sample solution was added into a reaction cartridge containing 10 mg of silica based reagent. Derivatization was performed in a 60°C water bath. For histamine analysis, fish extract was prepared in methanol with 1200 ppm triethylamine (TEA) before derivatization. During the optimization, derivatization temperature and time were varied in order to get the maximum percent derivatization.

E. *Extraction of histamine from canned tuna fish sample*

1. Extraction procedure

10.0 g of canned tuna fish and 50 ml of HPLC grade methanol were added to a 150 ml beaker. The mixture was sonicated for 10 minutes and the solution was filtered. To prevent any clogging of the HPLC column, a 0.45 µm filtering kit was used to filter the extract solution again, and this final solution was used for derivatization. The derivatization procedure was as previously described.

2. Extraction efficiency test

Histamine exists as a free base, its salt, and in protein bound forms in food products. There are many types of extraction methods to recover histamine from a food sample (19-22). Some of these decompose the protein-histamine bond with acid hydrolysis to get a higher recovery. Typical methods include perchloric acid ($HClO_4$), trichloroaceatic acid (CCl_3COOH), or methanol (MeOH) extraction, and others. A good recovery of histamine is always desired in order to get high sensitivity of analysis, although recovery will not be taken into consideration when the standard addition technique is used. This experiment was designed to show a valid sampling method and sampling efficiency with methanol as extraction solvent. The concentration of histamine (free base) standard in different concentrations in MeOH, before and after spiking into tuna fish followed by extraction, were determined using the above off-line derivatization conditions.

RESULTS AND DISCUSSION

The purpose of this work was to develop a silica based, covalently attached reagent with a detectable functional group to recognize nucleophilic analytes for improved chromatographic and detection performances. A silica based reagent, compared with similar polymeric reagents that have been described (16,17), may possess some desirable, optional properties, e.g.: 1) good pressure stability in HPLC; 2) usable pH range of 2-8 or greater, 3) narrow particle size distribution for narrow band variance in on-line chromatography; 4) high surface area for maximum attachment of reagents; 5) ease of incorporation into TLC plates for in situ derivatizations; 6) low cost of small particle diameter silica gel, with narrow size distribution as a substrate; and 7) well established chemistry for the attachment of various ligands and final tagging groups. These advantages are in addition to those already described for polymeric, solid phase reagents in HPLC, such as: 1) the absence of excess reagent; and 2) improved stability of the final tagged reagents at known concentration levels.

3,5-Dinitrobenzoyl chloride (DNB-Cl) and 9-fluorenylmethyl chloroformate (FMOC-Cl) are known to be very effective reagents for the derivatization of amines and amino acids with high sensitivity in UV, fluorescence and/or electrochemical detection in HPLC. We have been synthesizing and evaluating a silica based reagent with an *o*-nitrobenzoyl linkage for sensitive tags (Figure 1). Methods for the synthesis of silica bonded phases have been described in the literature for many years (27, 28), and a standard silanization method for attaching a hydroxyl group containing chain onto the silica surface was used (step 1, Figure 1). An analogous procedure in the synthesis of organic polymer derived *o*-nitrobenzophenone reagents for the attachment of the nitrophenol group to the PS-DVB (polystyrene-divinylbenzene) surface was used (17).

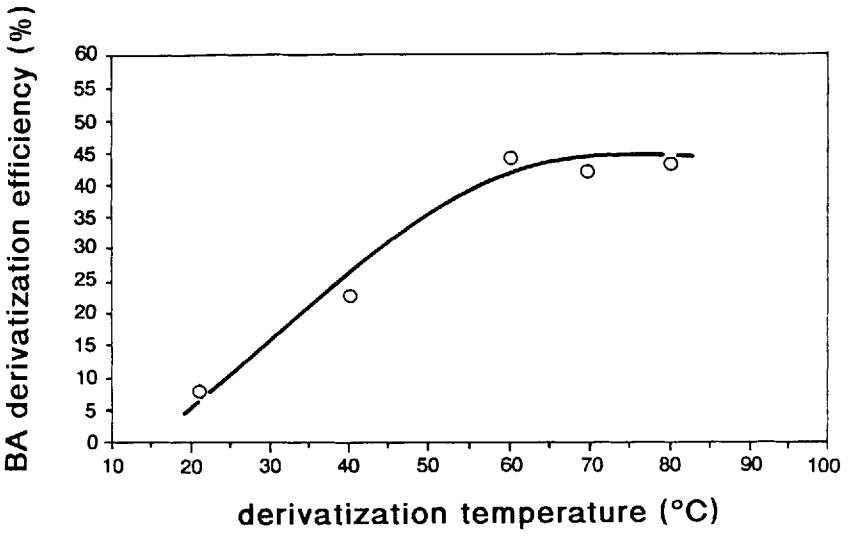

Figure 3: Derivatization Temperature Optimization for Silica Based DNB Reagent with Butylamine

Temperature and Time Optimization

To obtain an efficient derivatization of amine samples, the solvent, temperature and other derivatization conditions need to be optimized. Acetonitrile was determined to be a good solvent for amine derivatizations with the silica based reagent after solvent optimization studies (24). Temperature optimization was performed for the silica based DNB reagent by using ACN as a derivatization solvent. Figure 3 is the result of temperature optimization. The results showed that at about 60°C, an optimal derivatization efficiency was realized within 10 minutes. It was unnecessary to use a higher temperature, because derivatization efficiency did not increase, and lower derivatization temperatures would increase stability of this silica based reagent. The off-line, time optimization curve is shown in Figure 4. A short derivatization time was used to

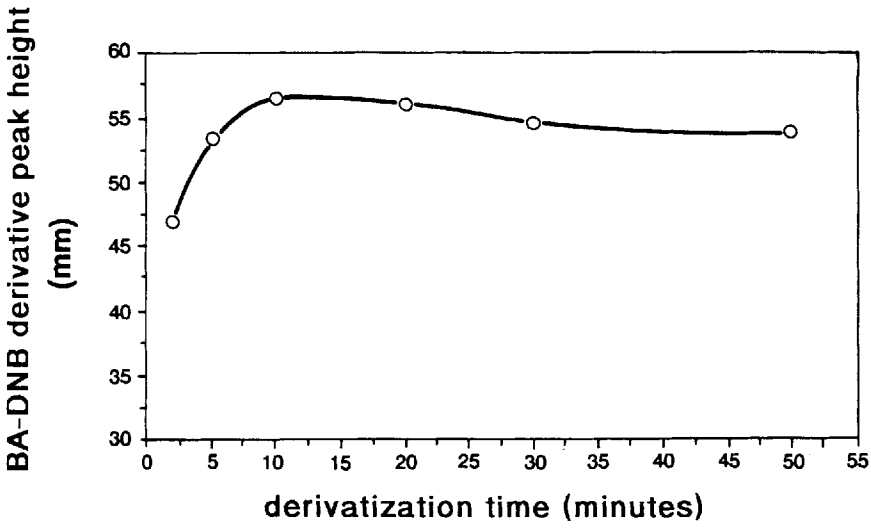

Figure 4: Derivatization Time Optimization for Silica Based DNB Reagent with Butylamine

reduce overall analysis time and the chance of derivative decomposition. A short derivatization time resulted in less decomposition of the solid phase reagent, longer reagent life, higher selectivity, and less interferences in the chromatographic separation. Based on this experiment, 60°C for 10 min were chosen as the optimized conditions.

The derivatization efficiency of this silica based reagent was not high (about 40% for BA and other primary amines) when compared with other polymeric reagents (15, 21, 23). These low percent derivatizations were still acceptable because: 1) the method was sensitive and provided stable derivatives for UV detection; and 2) results were reproducible.

Recovery Test of 3,5-Dinitrobenzoic Acid

A 3,5-dinitrobenzoic acid solution in ACN was heated under the above hydrolysis conditions (Experimental). The recovery was 99.2 ± 1.1% (n=3), meaning 3,5-DNB acid was stable under the 50/50 ACN/4.1 mM Na_2CO_3 hydrolysis conditions and the loading determination by quantification of 3,5-dinitrobenzoic acid would be accurate.

Loading Capacity

Three methods for the determination of loading capacity are available with HPLC quantification. When using the above hydrolysis method, 3,5-DNB acid was used as the quantitative product. The loading capacity of DNB tag for this silica based reagent was 0.53 ± 0.05 meq/g (n=6). By using the aminolysis method with n-butylamine, the loading capacity was 0.54 ± 0.07 meq/g (n=3). These results were in good agreement with the elemental analysis result, which was 0.55 ± 0.02 meq/g (n=2).

Off-line Derivatization of Primary and Secondary Amines

The DNB tagged, silica based reagent was effective for the derivatization of both primary and secondary amines. Figures 5a and 5b are the chromatograms from pure ACN and standard butylamine solution without derivatization. Figures 5c and 5d are the blank test of reagent (with ACN) and a derivatization of a C_4-C_8 amine mixture in ACN with 100 ppm of each component. Figure 6 is the derivatization of diethylamine with a silica based DNB reagent. Both primary and secondary amine compounds could be effectively analyzed by HPLC-UV with the silica based DNB reagent.

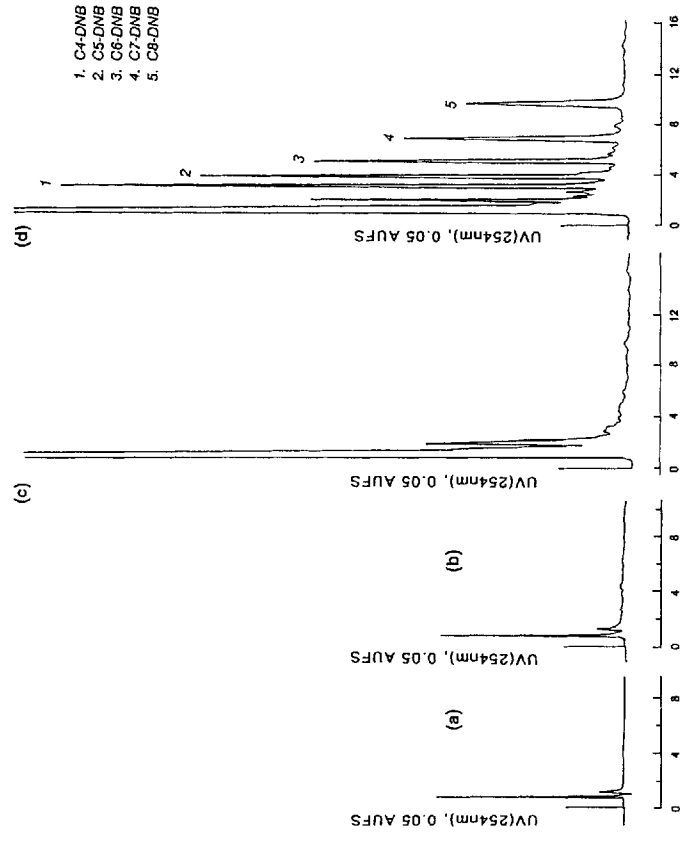

Figure 5: Chromatogram of Off-line Amine Derivatizations with Silica Based Reagent
Chromatographic conditions: 50% ACN/H_2O (v/v) as mobile phase; flow rate: 1.5 ml/min; injection volume: 20 µl; separation column: LiChrosphere 4x250 mm ODS column; UV detection at 254 nm, 0.05 AUFS;
Derivatization conditions: 25 µl of amines in ACN reacted with 10 mg solid phase reagent under 60°C for 10 min, washed into 1 ml ACN
a: pure ACN
b: n-C_4-C_8 primary amines in ACN (100 ppm each)
c: blank test of silica based DNB reagent with ACN
d: derivatization of n-C_4-C_8 primary amines in ACN (100 ppm)

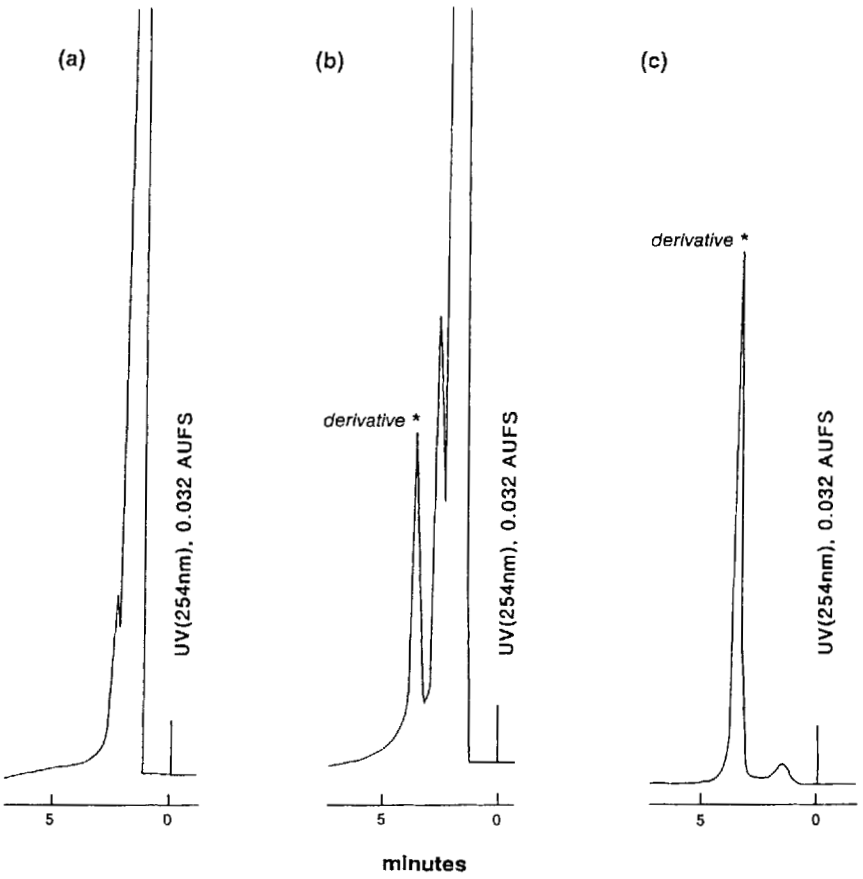

Figure 6: Off-line Derivatization of A Secondary Amine with Silica Based DNB Reagent
Chromatographic conditions: 50% ACN/H_2O (v/v) as mobile phase; flow rate: 1.5 ml/min; injection volume: 20 µl; separation column: LDC 4x250 mm ODS column; UV detection with LDC UVIII monitor at 254 nm, 0.032 AUFS
Derivatization conditions are same as in Figure 5.
(a) blank test of silica based DNB reagent with ACN
(b) derivatization of diethylamine(100 ppm) in ACN
(c) DNB-diethylamine standard derivative 5 ppm

On-line Derivatization with Silica Based, DNB Tagged Reagent

The silica reagent was packed into a 30 mm x 3 mm i.d. stainless steel reaction column. This packed reaction column was installed on-line, before the separation column and after the injection valve in the HPLC system (18). By using two valves, a defined amount of sample was injected into the reaction column and held with the reagent for a certain time to complete derivatization in a 60°C water bath. However, decomposition of reagent was very fast and derivatization was impossible. This may have been due to the high reactivity of the DNB tagged reagent, and further research will be needed in the modification of silica based intermediate **VI** (Figure **1**) and the final tagged structures.

Calibration Plot for Histamine Derivatization

The calibration equation for off-line histamine derivatizations (without triethylamine catalyst) by silica based, DNB tagged reagent was:

$$y = 0.51\ x + 138\ ,\ r^2 = 0.842$$

where x was the concentration of standard free histamine in ppm, y was peak area counts of histamine-DNB derivative from solid phase derivatization. With the 1200 ppm triethylamine as catalyst, the calibration equation was:

$$y = 2.15\ x + 138\ ,\ r^2 = 0.988$$

With the catalysis of triethylamine, much better linear calibration was obtained. This result may arise from protonation of the strongly basic histamine in the derivatization reactions, which decreases nucleophilicity and reactivity of histamine.

Regeneration of Reagent and Shelf Life Determination

The silica based, DNB tagged reagent was stored for 3 months in a capped glass bottle without any special protection. Its

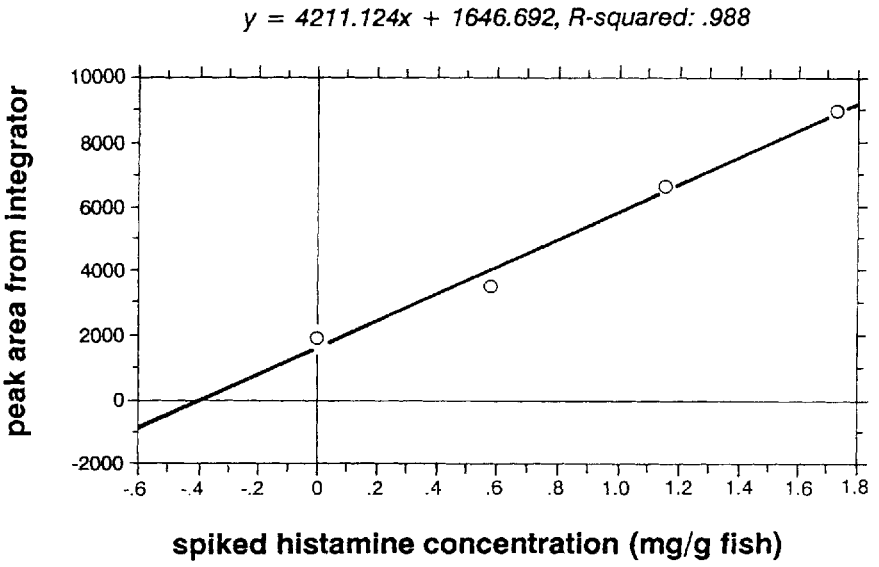

Figure 7: Histamine Analysis in a Fish Sample by Standard Addition

derivatization efficiency for a standard butylamine solution (100 ppm in ACN) changed from 43.2 ± 1.0% (n=6) to 4.7 ± 0.6% (n=6). Also, several decomposition peaks were observed in the chromatogram when using the stored reagent for off-line derivatizations. After washing this reagent with ACN and DMF, it could be regenerated by the same procedure as shown in Figure 1, step 4. Good derivatization efficiency (47.4 ± 1.2%, n=6 for butylamine) was again obtained from the regenerated DNB tagged reagent.

Detection limits of 3 ng (152 ppb, 20 µl) and 0.78 ng (39 ppb, 20 µl) were obtained for his-DNB and BA-DNB standard derivatives, respectively (with signal/noise normalized to 2/1). A linear detection range for this his-DNB standard derivative was obtained from 0.025 ppm to 400 ppm with the calibration equation:

$$y = 4.00\ x - 2.3\ ,\ r^2 = 0.999.$$

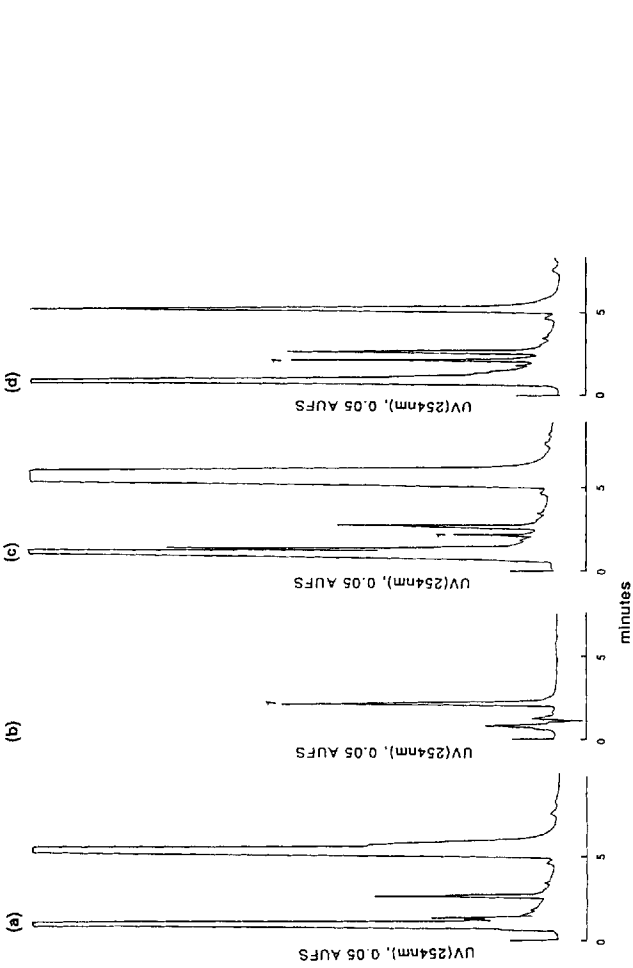

Figure 8: Chromatogram of Histamine Analysis in Fish Sample
Chromatographic conditions: 50% ACN/H_2O with 0.05% NH_4OH (v/v) as mobile phase; flow rate: 1.5 ml/min; injection volume: 20 µl; separation column: LiChrosphere 4x250 mm ODS column; UV detection at 254 nm, 0.05 AUFS; Derivatization conditions: 50 µl of extract with 1200 ppm triethylamine are reacted with 10 mg solid phase reagent under 60°C for 10 min, washed into 1 ml ACN, injection volume: 20 µl
a: blank test of silica based DNB reagent with MeOH
b: 10 ppm histamine-DNB standard derivative in ACN
c: derivatization of fish sample extract
d: derivatization of fish sample extract spiked with histamine

1---histamine-DNB derivative

where x was the concentration of authentic standard histamine-DNB derivative in ppm, y was peak area counts of it. To demonstrate the sensitivity of the derivatization with a silica based 3,5-dinitrobenzoyl (DNB) reagent, a butylamine standard at different concentrations (0.15-1 ppm) was prepared in ACN and derivatized off-line, with the DNB reagent under optimized conditions (100 µl sample in ACN, 60°C for 10 min). Each resulting solution was injected in triplicate onto the HPLC for quantification. The minimum amount of butylamine that could be derivatized using the silica based DNB reagent and detected using UV detection, was 99 ± 11 ppb (n=4) with S/N ratio 2/1. Histamine was not tested because its concentration in real samples was not very low and thus the minimum detection amount was not important.

Fish Sample Analysis

Histamine analyses in fish samples were performed using the standard addition method. This could eliminate the matrix effect, leading to results with good accuracy and precision. The samples were extracted with MeOH and derivatized under the optimal conditions described (Experimental). Each fish sample (with standard added samples as a set) was injected three times. The typical HPLC-UV chromatogram for a fish sample is given in Figure 8c. Four point calibration plot was then constructed for fish sample (Figure 7). The calibration equation was:

$$y = 4211.1\ x + 1646.7,\ r^2 = 0.988$$

in which x was the concentration of added standard histamine (mg/g fish) and y was the peak area counts of histamine-DNB derivative from the HPLC integrator. From the equation, if y=0, -0.391 mg histamine/g fish was obtained for x. Thus, 39.1 mg histamine/100 g fish was the histamine concentration in the fish sample. This was below toxic concentration levels (29).

Table 1. Determination of Histamine Spiked in Fish Using Standard Addition Technique [a]

spiked level (mg/100g)	found (SD) [b] (mg/100g)	% RE [c]
34.2	35.3 (0.2)	3.2
49.0	49.4 (2.4)	0.8
115.8	114.4 (1.4)	-1.2

a. Derivatization conditions: 60°C for 10 min, other conditions as in Figure 8.
b. Average number (standard deviation), n=3.
c. % Relative error = (found-true)/true x 100.

Table 2. Extraction Efficiency of Histamine Spiked in Tuna Fish [a]

histamine spiked level (mg of free base/100g)	extraction efficiency (SD)[b] (%)
30.0	83.1 (4.8)
34.2	81.2 (3.6)
49.0	83.2 (0.3)
60.0	84.4 (2.2)
90.0	83.1 (0.4)

a. Data obtained by using operating conditions given in experimental.
b. Average number (standard deviation) (n=3).

After storage of this opened, canned fish meat in a freezer for 30 days at -20°C and one day at 4°C, the histamine level rose to 90 mg/100 g (n=3), using the same extraction and standard addition methods. This result quantitatively showed the degradation extent of opened, canned fish during storage was much more serious than that of canned fish. A large amount of histidine contained in fish and exposure of a sample to a bacterial environment, are two reasons for the occurrence of high histamine concentrations.

Single Blind Spiked Histamine Detection

Three single blind, spiked fish samples were analyzed to validate this derivatization method. The extraction and derivatization procedures were the same as those used in the above standard addition method. The determined and spiked histamine concentrations are shown in Table 1. The detected concentrations were in good agreement with the spiked levels.

A chromatogram of a fish sample analysis is shown in Figure 8. Histamine extraction efficiencies were reproducible (Table 2). With the standard addition technique, quantitative results will not be affected by the extent of extraction efficiency.

CONCLUSIONS

We have demonstrated the synthesis and evaluation of a silica based derivatization reagent containing the 3,5-dinitrobenzoyl tag. This reagent was used for the off-line derivatization of amine nucleophiles. Under optimized conditions, low detection limits could be obtained for typical amines. This approach has been shown to be practical and valid by application to fish samples. The overall approach was a simple and accurate method for sample pre-treatment in HPLC analysis.

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