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Polymeric dimethylaminopyridinium reagents for derivatization of weak nucleophiles in high-performance liquid chromatography–ultraviolet/fluorescence detection

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ABSTRACT

This paper introduces a novel polymeric dimethylaminopyridinium 9-fluorenylmethoxycarbonyl reagent for off-line derivatizations of *weak* nucleophiles in high-performance liquid chromatography. The method of synthesis and characterization of the polymeric reagent via loading determinations is presented and discussed. Derivatization conditions (solvent, time, and temperature) for primary and secondary alcohols were optimized. As one application, off-line derivatizations of 2-chloro-1-propanol, a potential carcinogen in foodstuffs, were carried out with this polymeric reagent with single-blind and standard addition techniques. A specific sample treatment procedure was also developed. The accuracy and precision of the method were determined and data were statistically evaluated.

INTRODUCTION

There are many important natural or man-made organic compounds that contain hydroxyl or thiol functionalities[1,2]. Virtually all such analytes must be derivatized prior to the application of any chromatographic method, mainly because of their often limited volatility [gas chromatography (GC)], poor chromatographic properties [thin-layer chromatography, high-performance liquid chromatography (HPLC)], and especially their limited detectability by all common detection techniques[3]. There are several classes of successful derivatizing reagents for soft nucleophiles, such as alkyl, acyl and silyl reagents, and a large number of reports exist on the use of such reagents for the successful derivatization of hydroxyl, sulfhydryl and/or carboxyl groups. Most of these reagents enhance detectability and sensitivity[4–6]. Almost all derivatizations have been performed in solution, requiring sample preparation, sample work-up, and lengthy reaction times at elevated temperatures.

We have described several polymeric reagents for strong nucleophiles in earlier publications[7–11]. It was shown that the use of solid-phase reagents in HPLC has many advantages over solution reactions. However, none of these polymeric reagents were useful for the derivatization of soft nucleophiles.

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There are very few examples in the literature of any successful solid-supported reagents for soft nucleophiles. Only Rosenfeld and co-workers[12–15] have described useful approaches for alcohols or carboxylic acids. However, these reagents were physically adsorbed on a solid support, for off-line reactions in GC, and none of them were covalently bonded solid-phase reagents that could be used off-line or on-line in HPLC. Thus, all derivatizations of these and other classes of weak nucleophiles are performed by solution reactions. They are generally inefficient and time-consuming. Thus, there is a great need for the development and application of faster and more efficient derivatizing reagents for alcohols, thiols, and related soft nucleophile classes, especially solid-phase-attached reagents for off-line and on-line HPLC applications.

In 1967, Litvinenko and Kirichenko[16] discovered that by replacing pyridine as the general base catalyst by 4-dimethylaminopyridine (DMAP) for the solution benzoylation of a *m*-chloroaniline reaction they could realize an increase in the overall rate of the reaction by *ca.* 10^4 times. In separate studies, Steglish and Hofle[17] described in 1969 the strong catalytic effect of DMAP in preparative-scale acylation. Since then, a large number of papers have been published in which advantage was taken of the high catalytic activity of DMAP. With acetic anhydrides of acyl halides, for example, for acylating sterically hindered secondary alcohols, such as 1-methylcyclohexanol, 1-ethylcyclohexanol, linalool, 5,5-dimethoxy-2-methyl-3-pentyn-2-ol[18], *cis*-4-(1-hydroxypropyl)-2-methylcyclohexanone[19], and 1-hydroxycholesterol[20]. DMAP has also been used for acylating sterically hindered hydroxyl groups in carbohydrates, such as methyl sibirosaminide[21] and related sugar derivatives[22]. All of these acylation have been performed under very mild conditions (room temperature, one-half to several hours) with high yields (87–93%).

The enormous increase in acylation rates and the quantitative conversions possible with DMAP as the catalyst have made its use attractive for heterogeneous reactions as well. Hierl *et al.*[23] reported that DMAP was covalently attached to poly(ethyleneimines), which then showed significant catalytic effects in the hydrolysis of nitrophenyl acylates. Shinkai *et al.*[24] described the use of polymer-bound DMAP as an effective catalyst for ester synthesis. Another research group in Japan has also reported a rapid amide synthesis, with high purity, using acid anhydrides with polymer-bound DMAP as the catalyst[25]. A French research group has published a paper showing that polystyrene-supported DMAP exhibited good catalytic activity in acylations of methylcyclohexanol[26].

Patchornik and co-workers[27,28] have described a method for the synthesis of peptides in a "two-polymer system" by using polymer-bound DMAP salts. This work demonstrated the applicability of polymer-bound DMAP as a storable acylium "bank", and its versatility for peptide synthesis was demonstrated. The acylation of amino acids by the acyl-DMAP support was faster than that of the corresponding polymeric *o*-nitrobenzophenone activated esters, reported earlier by the same group. All of these heterogeneous acylations were aided by the polymer-supported DMAP, and were carried out successfully in terms of batch syntheses in high purity, high yields and diminished reaction times. However, none of these homogeneous or heterogeneous acylation reactions aided by DMAP were related to any analytical or HPLC applications or interfacing.

We have synthesized a polymer-attached DMAP reagent, containing a 9-fluorenylmethoxycarbonyl (FMOC) detector sensitive tag, for the derivatization of

strong and *weak* nucleophiles. This has led to significant improvements in the detectability of primary and secondary alcohols, simplicity, and overall rates of reaction for off-line derivatizations in HPLC. In this paper we discuss the method of synthesis, characterization of the polymeric reagent via loading determinations, and optimization of derivatization conditions (solvent, time and temperature). A number of primary and secondary alcohols were derivatized, as a mixture, and separated under optimized conditions. These peaks could be well separated within 20 min isocratically, by reversed-phase HPLC with resolution (R_s) values of 1.1–1.5.

As one of the possible applications, a method of determination of 2-chloro-1-propanol (CP) in foodstuffs has been developed using "single-blind spiking" and standard addition techniques. CP is used as an important chemical intermediate in the polymer industry[29]. It may be found in wheat products after fumigation with propylene oxide, which reacts with moisture and chlorine from the natural inorganic chloride content of foodstuffs to form the corresponding CP. When CP was identified as a potential carcinogen, it became very significant to monitor it in foodstuffs and environmental samples. There have been a few publications, in the past two decades, describing analytical methods for CP: a chemical method involves the hydrolysis of the distillation product and titration of any chloride liberated from the expected CP[30], and several GC methods were used for the determination of CP in foodstuffs[31–33]. It was reported that the chromatographic performance and sensitivity of these methods were poor. There is no current HPLC method for CP in the literature.

Validated by "single-blind spiking" experiments, our method may become the first HPLC method for quantitation of CP in wheat flour. The accuracy and precision of the method were: standard deviation $< \pm 1.9$, relative standard deviation $< \pm 9.1\%$, and relative error $< 10\%$ for a CP concentration range of 10–50 ppm. The smallest detectable amount of CP recovered from wheat flour was *ca.* 150 ppb[a] after derivatization with this reagent.

EXPERIMENTAL

Chemicals

Macroporous chloromethylstyrene–divinylbenzene copolymer (4% cross-linked, 200–400 mesh, 4.2 mequiv. Cl/g) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). 4-Chloropyridine hydrochloride (99%) and CP were from Aldrich (Milwaukee, WI, U.S.A.), and methylamine (gas at room temperature, 97%) was from Fluka (Ronkonkoma, NY, U.S.A.). Other chemicals were obtained with the highest purity available from Aldrich and J. T. Baker (Phillipsburg, NJ, U.S.A.). HPLC solvents were obtained from EM Science (Cherry Hill, NJ, U.S.A.), (Omnisolv HPLC grade). All HPLC solvents were used after filtration through a 0.45-μm solvent filter (GVWP; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum with stirring.

Apparatus

The HPLC system consisted of a Waters Model 6000A solvent delivery system (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.), a Rheodyne

[a] Throughout this article, the American billion (10^9) is meant.

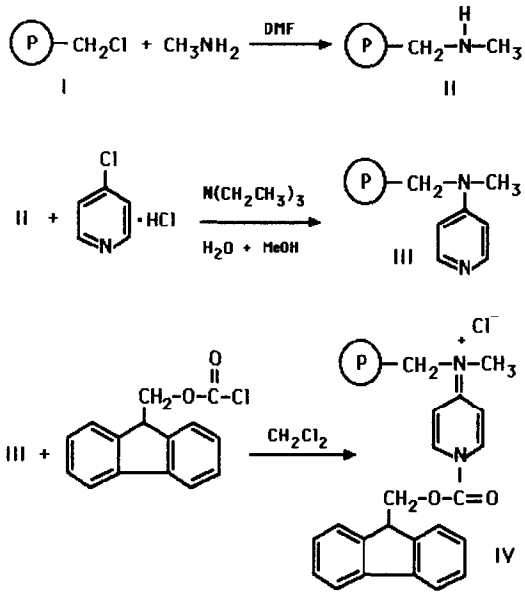

Fig. 1. Synthesis of polymeric DMAP/FMOC reagent. I = Chloromethyl styrene–divinylbenzene copolymer; II = polymeric N-methylene methylamine; III = polymeric DMAP; IV = polymeric DMAP/FMOC reagent. DMF = Dimethylformamide; MeOH = methanol.

Model 7010 injection valve with 10- and 20-μl sample loops (Rainin, Emeryville, CA, U.S.A.), a EM Science LiChrospher C_{18} reversed-phase column, 250 mm × 4.0 mm I.D., 5-μm particle size, a Waters Model 480 variable-wavelength UV–VIS detector, a Hitachi Model F1000 fluorescence spectrophotometer, and a Hitachi Model D-2000 ChromatoIntegrator (Hitachi, Naka Works, Mito City, Japan).

The instrumentation used to characterize the synthesized, purified standards consisted of a Varian 300 MHz nuclear magnetic resonance (NMR) spectrometer (Varian Assoc., Palo Alto, CA, U.S.A.), a Perkin-Elmer (PE) Model 599B infrared (IR) spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.), and a Thomas capillary melting point apparatus (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.).

Synthesis of polymeric DMAP/FMOC reagent

The dry polymer (I, Fig. 1) (10 g) was suspended in 15 ml N,N-dimethylformamide (DMF) and saturated with methylamine gas at 0°C. The vessel was sealed and agitated for 1 day. The polymer was washed successively in dioxane (3 × 30 ml), ethanol (3 × 30 ml), 2 *M* sodium hydroxide–2-propanol (1:1) (3 × 30 ml), water (until eluate neutral), ethanol (3 × 30 ml), and diethyl ether (2 × 30 ml). After drying in vacuum, a white product, polymeric N-methylene methylamine (II, Fig. 1) was obtained.

This product (3.5 g) was swelled in a mixture of water (1 ml), ethanol (2 ml) and triethylamine (5 ml). The excess solvent was evaporated until the polymer was slightly wet. This mixture was transferred to a pressure vessel (obtained from Ace Glass,

Vineland, NJ, U.S.A.), sealed, and heated for 4 days at 140°C. The polymer was washed as before, and unreacted amino groups were blocked by acetylation. This reaction was carried out by treating the polymer with acetic anhydride–dichloromethane (1:1) (5 ml) with stirring at room temperature for 1 h. The polymer was then washed with 2 × 10 ml, as above. The washed polymer was dried to constant weight at 140°C. The product, polymer-bound dimethylaminopyridine (DMAP) (III) was obtained (light brown, 3.4 g).

The anhydrous DMAP polymer (3 g) (III) was swelled in anhydrous dichloromethane and treated with excess FMOC-Cl (3 g) at room temperature with stirring for 1 h. The polymer product was filtered and washed with dichloromethane under anhydrous conditions until the washings contained negligible amounts of FMOC-Cl (blank tests by HPLC). The polymer was dried under vacuum at room temperature. The final, polymeric DMAP/FMOC reagent (IV) was obtained (light brown, 3.1 g).

Loading determination of the final polymeric reagent

Because the hydrolysis product of the fluorenyl tag was unstable under strong basic hydrolysis conditions[8], a pH-controlled (sodium carbonate buffer) hydrolysis procedure was then developed.

Hydrolysis procedure. The final polymeric reagent (IV), 0.2 g, was suspended in 20 ml of acetonitrile–sodium carbonate (4.1 m*M* in water) (50:50, v/v). A round-bottom flask (25 ml), containing the mixture, was placed into a water bath at constant temperature (70°C) for 1 h. After it had cooled to room temperature, the mixture was filtered, and washed with a 1:1 mixture of acetonitrile–0.2% HCl, and made to the

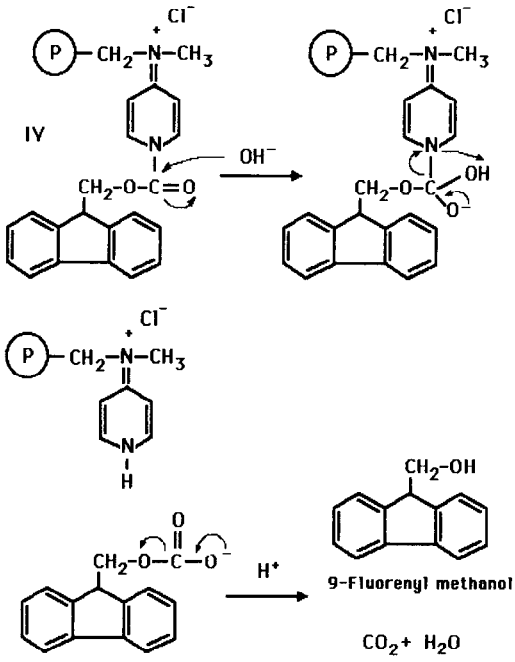

Fig. 2. Hydrolysis of the polymeric DMAP/FMOC reagent.

mark of a 10-ml volumetric flask. After dilution with the same acetonitrile–0.2% HCl mixture, 10 μl of the dilute solution was injected into the HPLC column.

Calibration plot for FMOC methanol. FMOC methanol was the final hydrolysis product (Fig. 2), and this was confirmed by quantitative HPLC–fluorescence detection (FL) *vs.* an authentic standard of FMOC methanol, which is commercially available. A calibration plot of 5–100 ppm of FMOC methanol was constructed to determine the load of final FMOC tag on the polymeric reagent (IV).

Recovery studies. FMOC methanol (100 μg) was added to the polymeric reagent intermediate III (0.3 g). The polymer-containing FMOC methanol was then treated, following the procedures described above. The final solution (20 μl) was injected into the HPLC column for quantitation of the recovered FMOC methanol. HPLC conditions: acetonitrile–0.1% aqueous acetic acid, pH 4 (70:30), 1.0 ml/min, LiChrospher C_{18}, 5 μm, 250 × 4.0 mm I.D., FL excitation wavelength 265 nm, emission wavelength 320 nm.

Elemental analysis for the polymeric reagent

Starting polymer I, polymer intermediate III, and final polymeric reagent IV were dried to constant weight. Three vials, containing 20 mg of each polymer product, were sealed and sent to Galbraith Labs. (Knoxville, TN, U.S.A.) for elemental analysis. Results of the elemental analysis were: starting material I (%C = 79.41, %H = 6.77, %N = <0.10), intermediate III (%C = 86.40, %H = 7.49, %N = 4.53), and final polymeric reagent IV (%C = 79.20, %H = 7.29, %N = 3.69).

Preparation and characterization of the external standards

FMOC-Cl (1.00 g, 3.87 mmol) was added into a 50-ml round-bottom flask containing excess ethanol or 2-propanol (5 ml) and 0.5 ml pyridine and kept at room temperature for 1 h with stirring. Afterwards, the excess solvent was evaporated, methanol (5 ml) was added to dissolve the product, and the mixture was heated to 60°C while distilled water was added dropwise until a turbidity appeared. The suspended mixture was kept at 5°C for crystallization. Pure ethanol FMOC derivative (white crystals) was obtained by recrystallization. Because the 2-propanol FMOC derivative was an oil at room temperature, preparative HPLC was used for purification (Waters μPorasil column, 300 × 7.8 mm I.D., *n*-hexane–2-propanol (90:10), 1.5 ml/min, FL excitation wavelength 265 nm, emission wavelength 320 nm). After purification, ethanol and 2-propanol FMOC derivative standards were characterized on the basis of NMR, IR, HPLC–UV/FL and melting point.

Off-line derivatization of alcohols

The substrate (primary or secondary alcohols, 100 ppm) in chloroform was added via a gas-tight syringe to the polymeric reagent (30 mg) in a closed vial. The capped vial, sealed with Parafilm, was placed in a constant-temperature Al_2O_3 bath at 60°C for 20 min (Fig. 3). The reaction mixture was rinsed with acetonitrile into a 1-ml volumetric flask under positive pressure, and 20 μl of this solution was injected into the HPLC column.

Determination of CP in wheat flour

Single blind spiking. CP solutions in chloroform were added to 1 g of wheat flour,

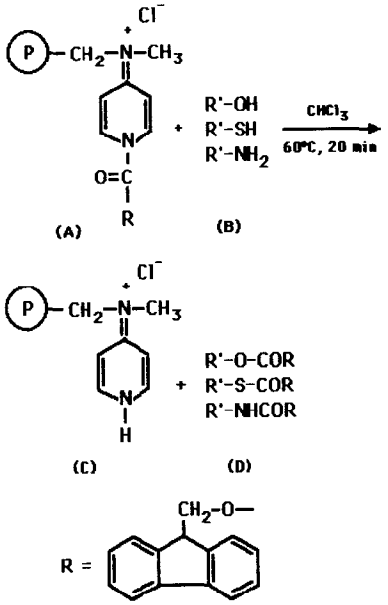

Fig. 3. Derivatization of nucleophiles with polymeric DMAP/FMOC reagent. A = Polymeric reagent; B = nucleophilic substrates; C = polymer backbone; D = derivatives of interest.

at three different concentrations, by another analyst. These spiked samples were then analyzed as samples containing CP at trace levels.

Sample extraction. Each spiked sample was extracted with 100 ml chloroform. After filtration through Waters Millex-SR filters (0.5 μm) to remove suspended wheat flour particles, the extract was ready for analysis.

Standard addition

Two different concentrations of CP (20 and 40 ppm in chloroform) were prepared as a set. To each spiked wheat flour sample two additional concentration levels of CP were added. One spiked flour sample without standard addition was used as a blank. These three samples represented one original wheat flour sample. Each derivatized sample was injected into the HPLC system in triplicate. Three-point calibration plots were constructed for the quantitation of CP in individual wheat flour samples.

RESULTS AND DISCUSSION

Nature of the polymeric DMAP/FMOC reagent

For derivatizing weak nucleophiles, the polymeric reagent should be extremely reactive. The polymeric reagent was intentionally designed to meet this need. Having a quaternary ammonium group in the para position, via a double bond (immonium ion), to the nitrogen containing the FMOC tag, the reagent IV (Fig. 1) is present as a loosely-bound ion pair. The reagent is very labile and highly reactive towards virtually all nucleophiles. However, the polymeric reagent is very sensitive to moisture

and polar solvents. Thus, on-line derivatizations met with failure in both normal- and reversed-phase modes, even when a switching valve was used for selecting derivatization solvents. Nevertheless, the polymeric reagent was very stable when stored at low temperature (5°C) under anhydrous conditions.

FMOC-Cl was again chosen as the tag because: (1) the detection properties of the tag are well known, as discussed in previous papers[8,10]; (2) in the acid chloride form, it can be well retained by the polymeric DMAP backbone[26,27]; and (3) it is commercially available from several manufacturers in high purity and at low cost.

The load, determined by the pH-controlled hydrolysis was 0.2 mequiv./g. Within experimental error, this number agreed with that calculated from the elemental analysis (0.3 mequiv./g). All these numbers were within the normal range of loads quoted in the literature[26,27], indicating that the correct synthetic route was followed, and that the desired polymeric reagent was obtained.

General mechanism of acylation reactions with DMAP

In all acyl-transfer reactions, it is assumed that there are three functions of DMAP. First, it serves as a Brønsted base, B, which can produce an anion, Y^-, from a substance H–Y.

$$H\text{–}Y + B \rightleftharpoons Y^- + H\text{–}B$$

This anion is a better nucleophile than H–Y, and therefore reacts more rapidly with the electrophilic acylating reagent. The extent of general base catalysis depends on the relative basicities of B and Y^-, *i.e.*, on the position of the acid–base equilibrium.

Second, it has been shown that the nucleophilic and sterically hindered base DMAP tends to attack the acylation reagent with formation of a salt-like intermediate. For example, carboxylic anhydrides and pyridine yield small amounts of N-acylpyridinium carboxylates, which, because of their charge, are more capable of transferring an acyl group to a nucleophile than the anhydride itself[33,34]. Even acyl halides react more slowly with nucleophiles than this acylium salt[33]. The extent of this nucleophilic catalysis depends on the reactivity and concentration of the acylium salt, which is, in turn, determined by the position of the equilibrium. However, as a referee has indicated, DMAP does not *always* lead to an increase in the rate of acylation reactions.

Third, once the DMAP salt is formed, it would be stable in solution and on a polymer support[27,28]. It has been estimated that the DMAP salt is about 25 kJ/mol lower in energy than the starting materials[35]. This large shift in equilibrium is caused by a lowering in energy due to the mesomeric stabilization of the N-acyl-4-dialkylamino pyridinium ion (Fig. 4). The mesomeric resonance structures do indeed contribute significantly to the stabilization of this anion, and this has been demonstrated by its NMR spectrum[35].

Mechanism of acylation reaction with polymeric DMAP/FMOC reagent

We believe there is a tetrahedral addition/elimination mechanism and a diffusion-controlled process in this heterogeneous reaction. In view of the above concepts and evidence, the polymeric DMAP/FMOC reagent is highly activated by the fact that: (1) the neighboring anion may act as a base catalyst in acetylation reactions; (2) the inductive effect caused by the delocalization of electrons through the pyridine ring

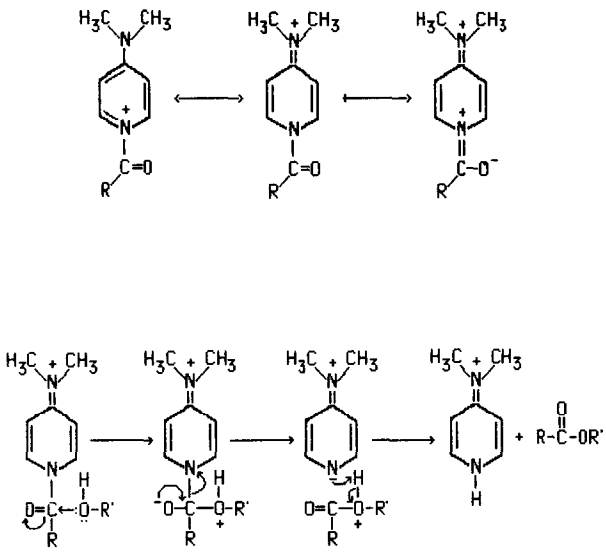

Fig. 4. Resonance structure of DMAP and the mechanism of its reaction with alcohols.

to the quaternary ammonium site makes the carbonyl carbon more electro-positive, susceptible to nucleophilic attack; and (3) attack by a nucleophile occurs at the carbonyl group, with transfer of the pair of electrons to nitrogen, resonance stabilized through the pyridine ring, eventually onto the quaternary nitrogen cation. With strong delocalization, the pyridinium ion may act as a good leaving group, perhaps one of the best imaginable, similar to that derived from the *o*-nitrobenzophenone phenoxide anion, as described in our earlier publications[8–11]. Thus, it would be expected, by analogy with all solution and polymeric reactions for DMAP already described, that IV should possess even higher reactivity than any of our previously described immobilized reagents.

Characterization of external standards

Simple primary and secondary alcohols (ethanol and 2-propanol) were chosen as the representative weak nucleophiles for evaluation of the polymeric reagent. Authentic tagged standards for each alcohol were synthesized and purified (see Experimental). This was followed by analytical and physical property measurements (HPLC–UV/FL, NMR, IR, melting point and elemental analysis), in order to prove structures. All data are shown in Table I.

All of the analytical and structural data were fully consistent with the expected structures, suggesting that correct and pure FMOC alcohol derivatives were obtained from the synthesis and purification. These compounds were then used as external standards to determine the extent of reactions with the polymeric reagent, under varying conditions.

Solvent optimization

As indicated in the literature[35], the degree of solvation has a strong effect on the

TABLE I

PHYSICAL AND SPECTRAL PROPERTIES OF FMOC ETHANOL AND 2-PROPANOL DERIVATIVES

HPLC conditions: 10-μl injection of 5 ppm of each standard in acetonitrile; acetonitrile–methanol–water (60:20:20), 1.5 ml/min, FL excitation wavelength 265 nm, emission wavelength 320 nm.

Property	*FMOC ethanol*	*FMOC 2-propanol*
Molecular weight	269	282
Melting point (°C)	56°C	liquid at 25°C
HPLC–FL	4.25 min	5.03 min
Elemental analysis[a]	C 76.1% (75.9%); H 6.0% (5.8%)	
NMR	$-CH_3$, 3H (triplet), 1.4 ppm	$-(CH_3)_2$, 6H (doublet), 1.3 ppm
	$-CH_2-$, 2H (multiplet), 4.2 ppm	$-CH_2-$, 2H (doublet), 4.4 ppm
	$-CH_2-$, 2H (doublet) 4.4 ppm	$-CH-$, 1H (multiplet), 4.9 ppm
	Aromatic 8H (multiplet), 7.5 ppm	Aromatic 8H (multiplet) 7.5 ppm
IR	Aromatic C–H stretch, 3040/cm	Aromatic C–H stretch, 3040/cm
	Aliphatic C–H stretch, 2960/cm	Aliphatic C–H stretch, 2960/cm
	Carbonyl C=O vibration, 1750/cm	Carbonyl C=O vibration, 1750/cm
	Ester C–O stretch, 1250/cm	Ester C–O stretch, 1250/cm
		Dimethyl C–H stretch, 1290/cm

[a] Numbers in parentheses are theoretical values.

reactivity of the DMAP reagent. Using an arbitrary, initial setting for temperature and time, four solvents were evaluated for optimum derivatization yield (Table II).

As expected, the derivatizations of ethanol with the polymeric DMAP/FMOC reagent proceeded much better in less polar solvents. This can be understood in view of the fact that the N-acylpyridinium ion pairs do not react themselves, but only dissociate and solvate in polar solvents, with charges further apart. More separated charges would decrease the mesomeric stabilization of the reagent, leading to decreased reactivity. In less polar solvents, charges on the reagent become less spread, and thus increasing mesomeric stabilization of the N-acylpyridinium ions results. This leads to an increased expansion of the ion pair, and this, in turn, activates the carbonyl carbon and facilitates attack of a nucleophile.

Among the solvents tested, chloroform provided the highest derivatization yield. It was therefore used as derivatization solvent throughout the study. After derivatiza-

TABLE II

SOLVENT OPTIMIZATION FOR OFF-LINE DERIVATIZATIONS OF ETHANOL WITH POLYMERIC DMAP/FMOC REAGENT

Conditions: 60°C, 20 min, 100 ppm ethanol; acetonitrile–water (60:40), 1.5 ml/min, LiChrospher C_{18}, 5 μm, 250 × 4.0 mm I.D., FL excitation wavelength 265 nm, emission wavelength 320 nm.

Solvent	*Derivatization (%) (average ± S.D.)*
Acetonitrile	7.8 ± 0.6 (n = 6)
Dioxane	10.1 ± 1.1 (n = 6)
Dichloromethane	27.8 ± 4.7 (n = 9)
Chloroform	30.4 ± 3.2 (n = 9)

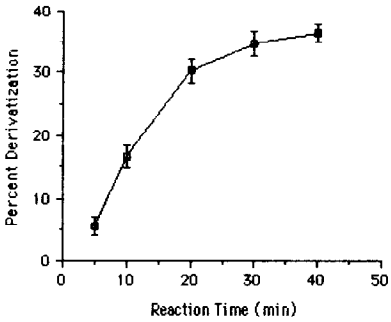

Fig. 5. Optimization of reaction time for off-line solid-phase derivatizations of ethanol. Reaction temperature 60°C; times: 5–40 min; acetonitrile–water (60:40), 1.4 ml/min, LiChrospher C_{18}, 5 μm, 250 × 4.0 mm I.D., FL excitation wavelength 265 nm, emission wavelength 320 nm.

tion in chloroform, acetonitrile was used as washing solvent in view of its good dissolving ability for the derivative and its compatibility with reversed-phase mobile phases.

Time optimization

Using chloroform as the best derivatizing solvent, off-line derivatizations of ethanol were attempted at room temperature. Low yields of derivatizations were obtained. Thus, a higher temperature was needed for more efficient reaction. This is true, especially for a heterogeneous reaction, where the rate of molecular collision/diffusion is highly increased with temperature, leading to a faster reaction. Since chloroform has a low boiling point (62°C), the temperature of 60°C was fixed for the optimization of reaction time and for all experiments throughout the study.

Holding the temperature constant at 60°C, the reaction time was then varied from 5 to 40 min. The results (Fig. 5) show a plot of percent derivatizations *vs.* reaction times. More than 30% derivatization for ethanol was obtained after 20 min at 60°C. Low-percent conversions for these alcohols may be due to high energy barriers in the reaction pathway to the product, leading to a decrease in reaction rates. The frequency factor, A, for alcohols, especially for secondary alcohols, may be inherently small, resulting in unfavorable orientation and slow collision rates in the reaction.

This may explain why it is always difficult to derivatize weak nucleophiles, even in solution reactions with highly reactive reagents. Higher-percent derivatization yields can be obtained by using longer reaction times, owing to an increase in collision probability. However, these low-percent derivatizations were still acceptable, because the method was highly sensitive and the results were reproducible. In addition, short reaction times could provide a great saving in analysis time and could reduce the chance of decomposition of the derivatives. Thus, the final, practical derivatization conditions used throughout were: chloroform as derivatizing solvent, acetonitrile as washing solvent, 60°C, and 20 min reaction times. Fig. 6 shows typical chromatograms of ethanol, derivatized with the polymeric DMAP/FMOC reagent, off-line, under optimized conditions, with confirmation by the external standard. Baseline separation and symmetric peaks were obtained.

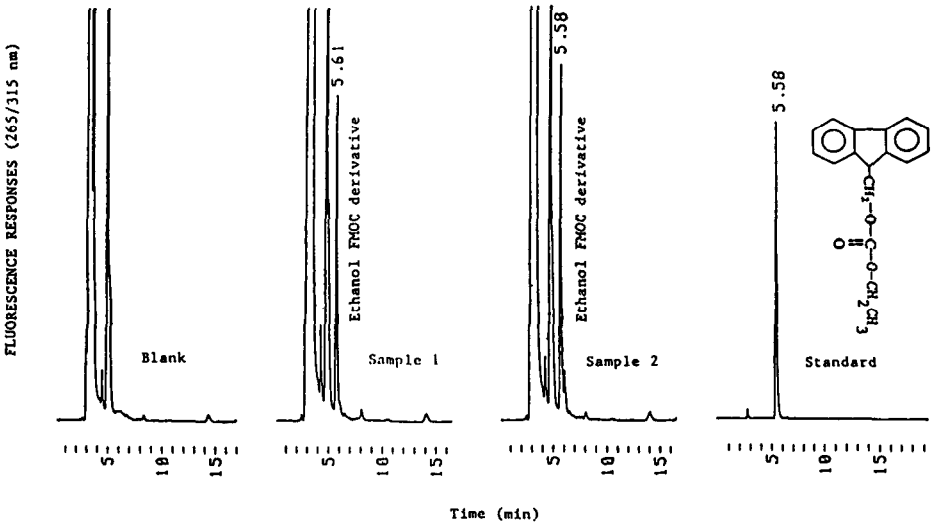

Fig. 6. Chromatograms of ethanol derivatized with polymeric reagent and of the external standard. Conditions: ethanol in dichloromethane (100 ppm), 60°C for 20 min, 10-μl injections for samples and the standard (20 ppm); other conditions as in Fig. 5.

Derivatizations of nucleophiles with the polymeric reagent under optimized conditions

To evaluate the reactivity of the polymeric reagent towards different nucleophiles, further off-line solid-phase derivatizations of strong and weak nucleophiles were investigated under optimized conditions. Ethanol, 2-propanol, and propylamine were prepared in chloroform separately (each at 100 ppm). Off-line derivatizations for individual nucleophiles were then followed at 60°C for 20 min. The results are given in Table III.

The polymeric DMAP/FMOC reagent was very reactive, in view of high-percent yields for a strong nucleophile, propylamine, and lower-percent yields for weak nucleophiles, both primary and secondary alcohols, under optimized conditions. Compared to the percent derivatizations obtained under the same conditions, difficulties were realized in derivatizing weak alcohol nucleophiles (less reactive). It is very clear that alcohols, especially secondary ones, do not show very high reactivity when compared with primary amines. Therefore, it was the reactivity of the analyte,

TABLE III

DERIVATIZATIONS OF DIFFERENT NUCLEOPHILES WITH THE POLYMERIC REAGENT UNDER OPTIMIZED CONDITIONS

60°C for 20 min, off-line derivatizations of separate nucleophiles (each at 100 ppm).

Substrate	*Derivatization (%) (average ± S.D., n = 9)*
Propylamine	84.2 ± 2.1
Ethanol	31.5 ± 1.5
2-Propanol	7.8 ± 0.7

but not of the polymeric reagent, that dominated the yields in these particular reactions.

Regeneration of the polymeric reagent

Since this solid-phase reagent was a stoichiometric type, a prolonged, continuous usage for a given amount of material resulted in a gradual decrease in percent derivatizations and overall reactivity. Since only the FMOC tag was consumed in reactions with nucleophiles, this left the activated pyridine site on the polymeric support (Fig. 1, III). It was possible to generate the spent polymeric reagent, using the same, last synthetic step initially employed to prepare reagent IV (Fig. 1). Since the pyridinium ion was left after the reaction, the spent polymeric reagent (100 mg) was then washed with a basic mixture, 50% aqueous acetonitrile, 4.2 m*M* Na_2CO_3, to remove all unreacted tags. The recovered polymeric intermediate, III, was then obtained. The regeneration (tagging) of the intermediate was performed using the conditions described in the Experimental section. The percent derivatizations with this batch before intentional consumption of tag and after regeneration were measured under optimized off-line reaction conditions (Table IV).

The final reactivity, as evidenced by percent derivatization, was almost equal to that of the fresh reagent. This result suggested that the reactivity of the polymeric reagent could be regenerated by just a simple, one-step tagging reaction. This experiment showed a very significant feature. If the spent reagent is intended to be used again with the original reactivity, it is not necessary to repeat the synthetic procedures from the beginning, but rather to repeat the very last, tagging step of the overall synthetic scheme (Fig. 1). The reagent reactivity could be easily regenerated, leading to simplification of the reagent recycling process and lower cost per analysis.

Shelf-life determination

One of the advantages of using solid-phase reagents is that they obviate the preparation of reagent solutions. The polymeric reagent can be stored at 5°C for prolonged periods of time between use without decrease in reactivity. The shelf-life stability of this polymeric reagent was determined. Percent derivatizations were determined immediately after synthesis of a fresh batch of the polymeric reagent and after it had been stored at room temperature, on the laboratory shelf. The results in Table V suggest that there was very little change in reactivity or percent derivatizations after at least four months of such storage.

TABLE IV

REGENERATION OF POLYMERIC DMAP/FMOC REAGENT

Conditions as in Table II.

Reagent status	*Substrate*	*Derivatization (%) (average ± S.D., n = 9)*
Fresh	Ethanol	28.9 ± 3.0
	2-Propanol	7.1 ± 0.6
Base washed	Ethanol	Not detectable
	2-Propanol	Not detectable
Regenerated	Ethanol	27.8 ± 2.8
	2-Propanol	6.7 ± 0.5

TABLE V

DETERMINATION OF SHELF-LIFE STABILITY FOR THE POLYMERIC DMAP/FMOC REAGENT

Mobile phase: acetonitrile–water (60:40); other conditions as in Table II.

Storage status	*Substrate*	*Derivatization (%) (average ± S.D., n = 9)*
Fresh	Ethanol	30.4 ± 3.1
	2-Propanol	7.8 ± 0.3
Four months	Ethanol	28.9 ± 3.0
	2-Propanol	7.1 ± 0.5

Calibration plots for alcohol derivatives and detection limits

This study was performed to understand the working concentration range over which the FL responses would be linear. Calibration plots of FMOC alcohol standard derivatives were constructed, using concentrations *vs.* FL response.

Ethanol and 2-propanol FMOC derivatives were prepared separately at various concentrations (10–15 000 ppb in acetonitrile). Three injections were made for each concentration, and linear calibration plots were then obtained with line equations ($y = -0.1748 + 0.9496x$, $r = 1.00$ and $y = -0.2324 + 0.9066x$, $r = 1.00$). This concentration range was within the normal concentration range for all analytes investigated. These calibration plots were linear, providing validated measurements via external standards.

The fluorescence detector was set at the maximum excitation and emission wavelengths (265/320 nm). Using a signal-to-noise ratio of 2:1, the detection limits of the FMOC alcohol derivatives were 10 ppb and 15 ppb, for the ethanol and 2-propanol FMOC derivative, respectively. The sensitivity of the method is comparable with those reported in the literature[12–16].

Separation of a mixture of alcohols after off-line derivatization

We demonstrated that a variety of typical primary and secondary alcohols could be simultaneously derivatized by a single cartridge of polymeric reagent. In this study, five different alcohols (methoxyethanol, ethanol, 2-propanol, 1-pentanol and 1,8-octanediol) were reacted as a mixture, off-line, under optimized conditions, and derivatives were detected by HPLC–FL. Typical chromatograms for the final derivative mixture are shown in Fig. 7. All five alcohols could be simultaneously derivatized and separated within 16 min. Baseline resolutions were obtained with R_s 1.3–1.5.

A mixture of another class of compounds, nitro alcohols, was also derivatized and separated successfully, using the polymeric reagent under optimized conditions. These compounds are well known as important chemical intermediates in the chemical industry and as bactericides in environmental applications. Baseline separation was also obtained for 2-ethyl-2-nitro-1-propanol (ENP) and 2-nitro-2-methyl-1-propanol (NMP), with R_s value 1.4. The result is shown in Fig. 8.

This method was versatile, and was further validated by the results obtained from the derivatization and separation of additional alcohol substrates. These

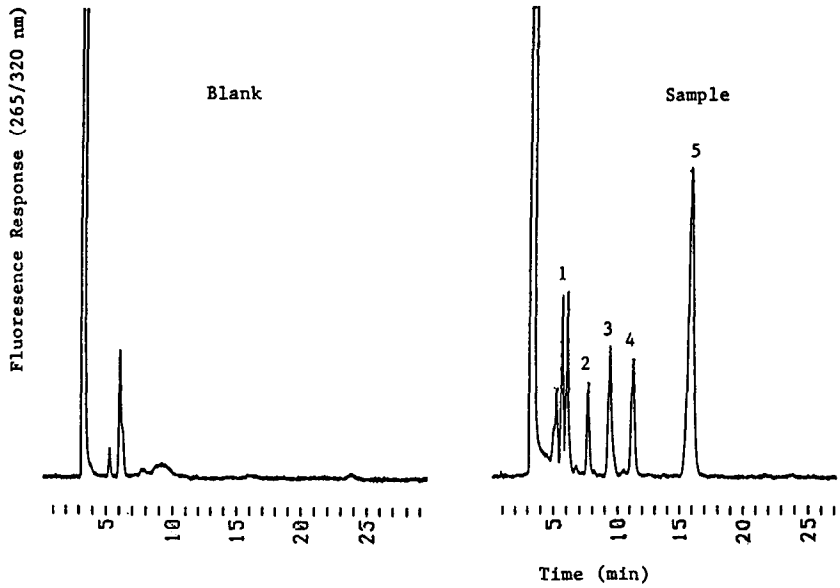

Fig. 7. Chromatograms for off-line solid-phase derivatization of a typical alcohol mixture. Peaks: 1 = methoxyethanol, 100 ppm; 2 = ethanol, 100 ppm; 3 = 2-propanol, 400 ppm; 4 = 1-pentanol, 100 ppm; 5 = 1,8-octanediol, 200 ppm. Mobile phase, acetonitrile–methanol–water (50:10:40); other conditions as in Fig. 5.

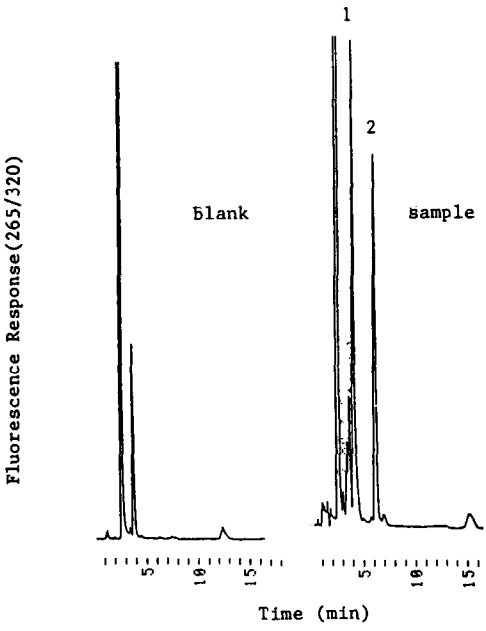

Fig. 8. Chromatograms for off-line solid-phase derivatization of a nitro alcohol mixture. Peaks: 1 = ENP, 200 ppm; 2 = NMP, 200 ppm. Conditions as in Fig. 5.

examples are but a few of the imaginable applications to many important compounds containing hydroxyl groups. We believe that most alcohol-like compounds, especially with primary hydroxyl groups, can be derivatized with this polymeric DMAP/FMOC reagent, and the resulting derivatives can be separated by either normal- or reversed-phase HPLC.

Derivatization of CP with the polymeric reagent

As an application of this polymeric reagent to actual samples, the determination of CP in wheat flour was performed. As stated in the Introduction, this is a potential carcinogen occurring in foodstuffs after fumigation. Because the current GC methods suffer from poor column performance and poor specificity, and there is no HPLC method in the literature for the determination of this compound, it was important for us to develop a new HPLC approach using the polymeric reagent, for the derivatization and quantitation of CP in a complex matrix.

First, we accomplished off-line derivatizations of standard CP after dissolving it in chloroform, using the polymeric reagent under optimized conditions. A single peak was observed, as compared with blanks analyzed under identical conditions (Fig. 9). The peak height for the suspected CP derivative increased with increasing concentration levels of CP derivatized, off-line, with good reproducibility. This confirmed that CP could be derivatized and separated under these conditions.

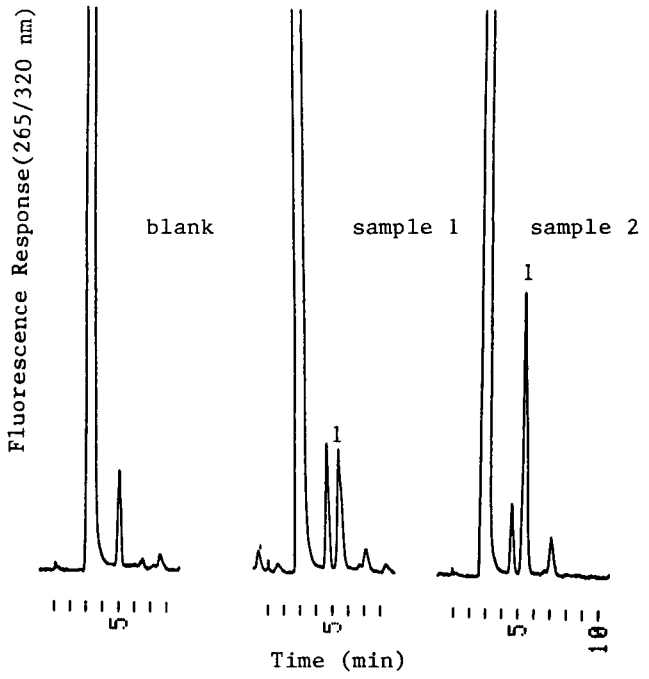

Fig. 9. Chromatogram of CP FMOC derivative after solid-phase derivatization. Peak: 1 = CP FMOC derivative. Conditions as in Fig. 5.

Recovery experiment

Wheat flour sample work-up involved only extraction. Good recovery for CP from the matrix is always desirable, although the recovery is taken into account when the standard addition technique is used. This experiment was designed to show a valid sampling method and sampling efficiency. The concentrations of CP standard (50 ppm in chloroform) before and after addition to wheat flour, followed by the extraction, were determined using the same off-line derivatization conditions. The recovery was 92 ± 3% (n = 3), indicating a high sampling efficiency.

Determining minimum detectable amount of CP in wheat flour

To demonstrate the sensitivity of the method for CP recovered from wheat flour, CP standards at different concentrations (0.15–1 ppm in series) were prepared and derivatized off-line, with the polymeric reagent under optimized conditions (60°C for 20 min). Each resulting solution was injected in triplicate into the HPLC system for quantitation. Peak heights and noise levels were measured for each CP concentration tested. The minimum amount of CP that could be derivatized, using the polymeric reagent and FL detection, was 150 ± 15 ppb (n = 6) with signal-to-noise ratio 3:1 (Fig. 10). The results showed high sensitivity or low detection limits of the method for the determination of CP in a complex matrix. These detection limits are lower than or comparable to those reported in the literature[29–35].

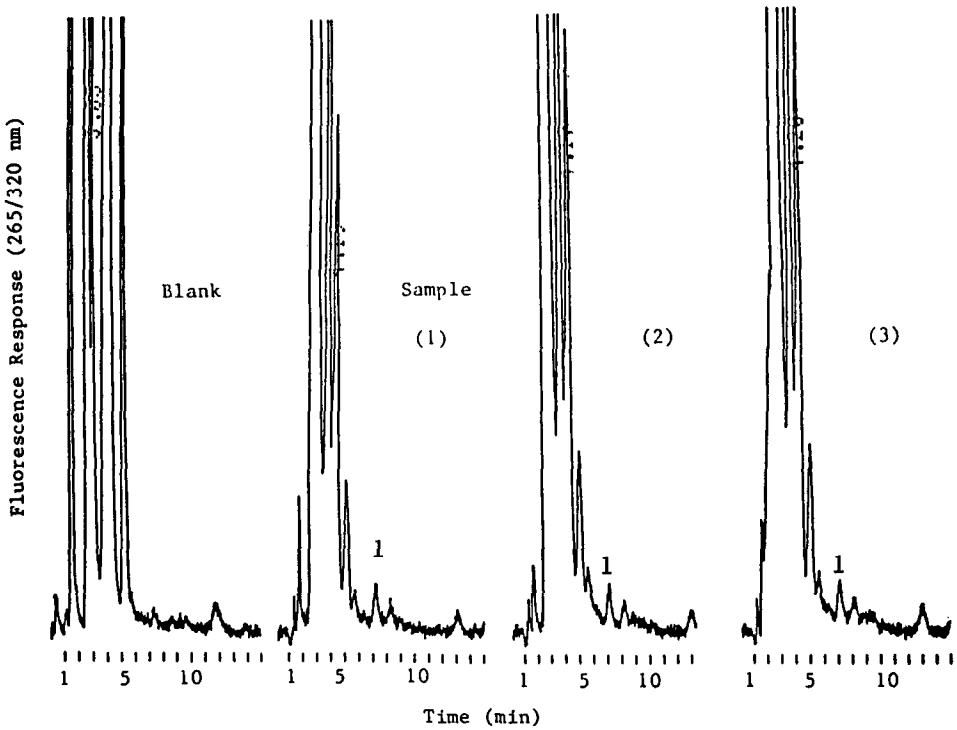

Fig. 10. Chromatogram of minimum detectable amount of CP in wheat flour after derivatization with the polymeric reagent. Peak: 1 = CP, at 150 ppb. Conditions as in Fig. 5.

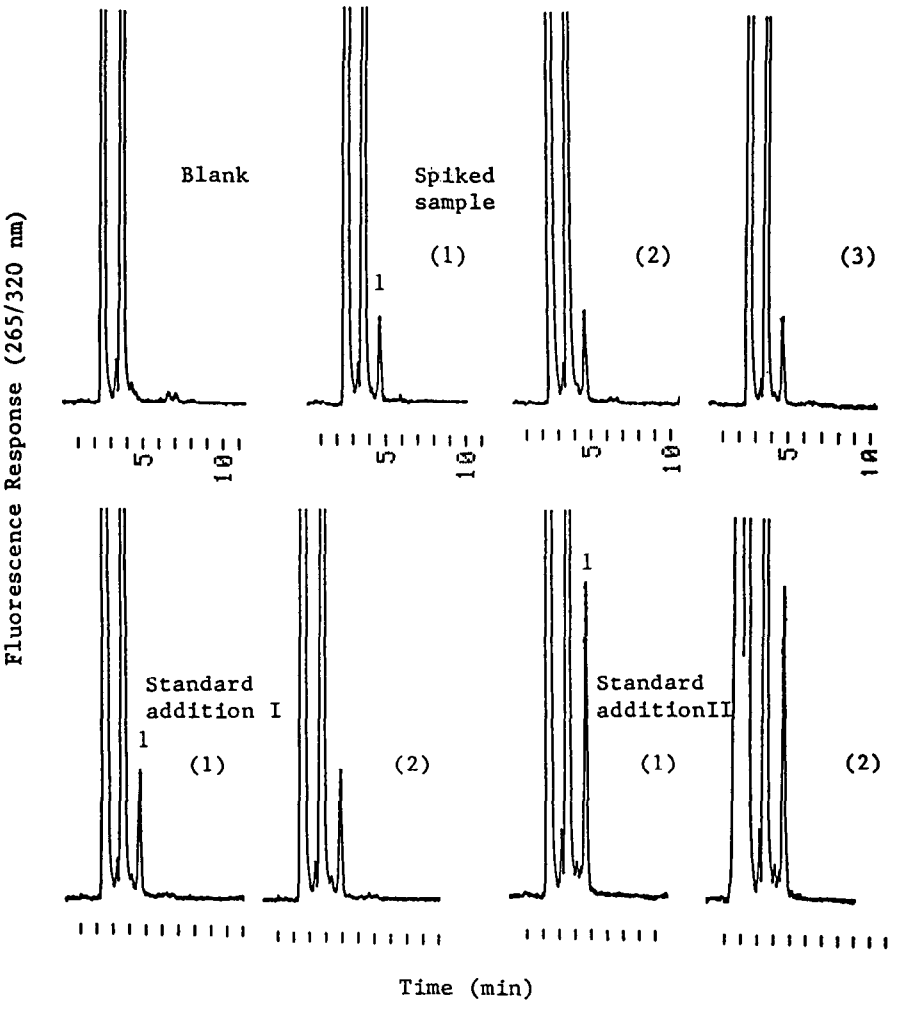

Fig. 11. Chromatograms of CP in wheat flour, derivatized with polymeric reagent and quantitated via standard addition. Peak: 1 = CP FMOC derivative. Reaction detection conditions as in Fig. 5.

Quantitation of CP in wheat flour via standard addition

CP in wheat flour was determined using single-blind spiking procedures. With a minimum of sample work-up (Experimental), off-line solid-phase derivatization, reversed-phase separation, and final FL detection could be accomplished in 45 min per analysis. A standard addition method was used for final quantitation. This could take into account the matrix effect and percent derivatization yields for the analyte, leading to reliable results with good precision and accuracy.

Fig. 11 illustrates typical HPLC–FL chromatograms for one of the spiked wheat flour samples and standard-addition results (increased peak heights), with off-line solid phase derivatization under optimized conditions. Peak symmetry was excellent. Baseline separation with good reproducibility and stability was obtained for each

TABLE VI

DETERMINATION OF 2-CHLORO-1-PROPANOL SPIKED IN WHEAT FLOUR BY STANDARD-ADDITION TECHNIQUE

Reaction detection conditions: 60°C for 10 min. R.S.D. = Relative standard deviation = (S.D./$\bar{X}$) × 100, n = 9; R.E. = Relative error = (value found − true value)/true value × 100.

Spike level (ppm)	*Found (ppm)*	*R.S.D. (%)*	*R.E. (%)*
10	11.0	9.1	+10
15	13.9	8.6	−7.3
30	28.1	7.1	−6.3

wheat flour sample. Most interferences in the wheat flour sample were removed after extraction and filtration. Finally, by carefully choosing the mobile phase composition, any interfering derivatized and/or underivatized impurity could be eluted with the solvent front, or as earlier-eluted peaks, totally separated from the CP FMOC derivative of interest.

Each wheat flour sample (with standard added samples as a set of three) was injected in triplicate. Three-point calibration plots were then constructed for each spiked wheat flour sample. The quantitation results are given in Table VI. The relative standard deviations (R.S.D.) were <9.1%, indicating good reproducibilities for these spiked wheat flour samples. These errors could be further reduced with more careful sample handling, preparation, and spiking. R.S.D. values were comparable to those for other CP assays in the literature[29–35]. Accuracy of the method is evident from the relative errors, which were less than 10%.

A polymeric DMAP chiral reagent for enantiomer recognition

We have prepared the first polymeric DMAP chiral reagent containing the FMOC-L-proline tagging species. This polymeric chiral reagent has been characterized via hydrolysis and elemental analysis. The polymeric chiral reagent was used successfully, off-line, for the derivatizations of chiral primary alcohols, such as (±)-cyclohexenyl-4-methanol. The expected diastereomers were formed and confirmed by external standards prepared via solution reactions. The resulting diastereomers were separated by normal-phase TLC. We are now optimizing reaction conditions, such as solvent, temperature, time, and so forth, to increase percent derivatizations towards chiral or achiral alcohols. The resulting diastereomers should be separable using reversed- or normal-phase HPLC.

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