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Review

Solid-phase derivatization reactions for biomedical liquid chromatography

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Abstract

Polymeric reagents have been developed for performing off- and on-line derivatizations of numerous organic analytes in HPLC-detection modes. Such reagents utilize ionic or covalent attachment of labile tags that possess specific detector enhancement properties: ultraviolet, electrochemical, fluorescence, and so forth. Specific synthetic procedures have evolved to generate various linkages of the tag to the underlying, polymeric support, usually involving activated ester connections (leashes). The polymer itself may play a number of roles in the nature of the overall reactions, such as hydrophobic–hydrophillic exclusion, pore size restriction, stabilization of the attachment leashes, and protection of the tags from hydrolysis in aqueous media. The basic, underlying chemistry of polymeric reagents has evolved to the point where it is possible to engineer the polymer support itself, the attachment leash, and the various tags that are then transferred to the analyte molecules. These procedures have now reached the stage of commercialization and practical applicability for real-world drugs and bioorganics in complex biofluid type samples. Polymer supported reagents can now be used for direct injection of biofluids with solid-phase (hydrophobic) extraction of the analytes of interest, followed by sample cleanup, derivatization, elution onto the HPLC column, peak compression, gradient HPLC elution, multiple detection, and final data interpretation with quantitation. This review summarizes much or most of what has been described in the scientific literature over the past decade in the various areas where polymeric reagents are being used for derivatization in HPLC and in capillary electrophoresis as well.

Contents

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List of abbreviations

AFID	Alkali flame ionization detector
CD	Conductivity
CE	Capillary electrophoresis
DVB	Divinylbenzene
DMAP	Dimethylaminopyridine chloroformate
EC	Electrochemistry
ECD	Electron-capture detector
ELS	Evaporative light scattering
FID	Flame ionization detector
FL	Fluorescence
FMOC-L-pro	9-Fluorenylmethoxycarbonyl-L-proline
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NSD	Nitrogen selective detection
o-AC	*o*-Acetylsalicyl
PCD	Photoconductivity
PCR	Post-column reaction
ppb	Parts-per-billion
ppm	Parts-per-million
ppth	Parts-per-thousand
p-NP	*p*-Nitrophenyl
PS	Polystyrene
RI	Refractive index
SEC	Size-exclusion chromatography
SPR	Solid-phase reagent
TCD	Thermal conductivity detector
TEA	Thermal energy analyzer
UV	Ultraviolet
Vis	Visible
3,5-DNP	3,5-Dinitrophenyl or 3,5-dinitrobenzoyl
6-AQ	6-Aminoquinoline
9-FMOC	9-Fluorenylmethyl chloroformate
9-FA	9-Fluorenylacetic acid

1. Introduction

At first, solid supports containing immobilized reagents, also known as solid-phase or polymeric reagents (SPR), were developed by organic chemists for organic synthesis purposes. In such reactions a variety of solid supports could be used, *e.g.* silica gel, kieselguhr, clay, alumina, and organic polymers. The active reagents causing the actual reactions were either adsorbed, ionically bound, intercalated, and/or covalently linked to the underlying supports. Several reports and reviews have appeared over the years describing more and more of these reagents, but almost always for use in straight synthetic chemistry [1–12]. Until about 1977–78, there

were almost no publications describing the use of solid-phase reagents for derivatizations related to high-performance liquid chromatography (HPLC) [13]. There were, however, several papers describing the use of polymeric reagents in other forms of analytical chemistry, especially in gas chromatography (GC) [14,15]. To date this type of research is still being performed in these areas.

The use of polymers as reagents in organic synthesis has steadily increased since Merrifield [16] synthesized a tetrapeptide on chloromethylated polystyrene beads [17]. This was, perhaps, the most significant demonstration of the potential utility and applicability of solid-supported reagents in synthetic chemistry. An insoluble polystyrene support was used as an anchor to which an amino acid was added. The second amino acid was added using dicyclohexyl carbodiimide (DCC) mediated chemistry and so on. The final peptide was obtained by cleaving the anchored section from the support. This became known as the direct, polymer mediated peptide synthesis approach. It also became generally known as the solid-phase synthesis approach for peptide synthesis [16,17]. In the Merrifield approach, the growing peptide was covalently attached to an underlying polymer support, and reactions were performed on this substrate using traditional, liquid-phase chemistry. Various reactions could then be applied to the growing peptide, such as protection, deprotection, cleanup, release, and so forth.

In later work, Patchornik *et al.* used the inverse of this concept [8–12]. That is, the polymer now supported an activated ester which transferred a protected amino acid to the growing peptide in solution. Hodge and Sherrington have extended the use of these reagents to many different types of organic synthetic reactions and syntheses, quite different from the original work with amino acids [4]. The Merrifield concept is even being applied to the automation of oligonucleotide synthesis [2]. Much synthetic work with polymeric reagents today still follows the Merrifield concept, with reagents in solution; whereas analytical type work follows the style of Patchornik, with reagents attached to the polymer backbone.

2. Polymeric or solid-phase reagents; possible alternatives

The majority of the polymeric organic reagents used today are based on polystyrene crosslinked with small amounts of divinylbenzene (STY–DVB), because of its ease of functionalization and insolubility in most solvents [3–5]. Other supports have been used successfully, although they have not been as widely accepted. Oligonucleotide synthesis on polyamide resins is claimed to be superior to the STY–DVB approach since the resins, being more polar, are more compatible with the products [18]. Numerous other polymeric (organic and inorganic) supports have been described as supported or attached reagents, but again mainly for synthetic organic chemistry, rather than analytical chemistry or chromatography. Only over the past 15 years, since the late 1970s, the interest in the development and application of solid-phase reagents specific for analytical chemistry and/or chromatography has grown.

One of the largest commercial applications of solid-supported reagents, has been in the field of immobilized catalysts [3]. Immobilization is advantageous when the reagent is expensive or toxic or when a by-product is generated that could be separated only with difficulty from the final product. A clever application of this concept involves asymmetric syntheses using optically active catalysts [19]. Due to a chiral microenvironment, only one enantiomer is able to diffuse close enough to the active site for an effective collision to occur.

In addition to the variations possible in the nature of the underlying support, it is also possible to vary the particle size of the beads, their pore size, pore volume, surface area, percent crosslinking, and shape. As mentioned below, these variations may play different roles in the final, overall derivatization efficiency, selectivity, and longevity of the immobilized

reagents. Silica-based reagents have also been described, for both synthetic and analytical chemistry applications, but at present, it appears that silica-based reagents may suffer from poor lifetime, longevity, stability, and reproducibility, especially under continuous, on-line application conditions. Some of these reagents already described in analytical chemistry are discussed below. Though other supports have been described for synthetic chemistry, very few of these have been applied in analytical chemistry [13].

In addition to our ability to vary the nature of the support and its physical parameters, it is also possible to vary the nature of the reagent attachment. Thus, reagents have been physically adsorbed, ionically bound, or covalently attached to the underlying support for chromatographic applications [20–25]. In the case of covalent attachments, Fig. 1, it is possible to vary, as shown below, the nature of the leash being used to attach the final tag(s) to the polymer itself. Such leashes can be disulfides, activated esters, activated carbonates or carbamates, *i.e.* basically any leash that readily releases the final tag to the analyte of interest undergoing derivatization (*e.g.* nucleophiles). Finally, variations are possible in one single class of leashes, such as activated esters, where the chemistry is varied to provide differences in overall reactivity with a given tag for a given substrate. In addition to the above options, the tag itself can readily be altered to provide different products having different chromatographic, electrophoretic, and detector properties. Thus, there are really three basic components for any given polymeric reagent system: the underlying support, the leash

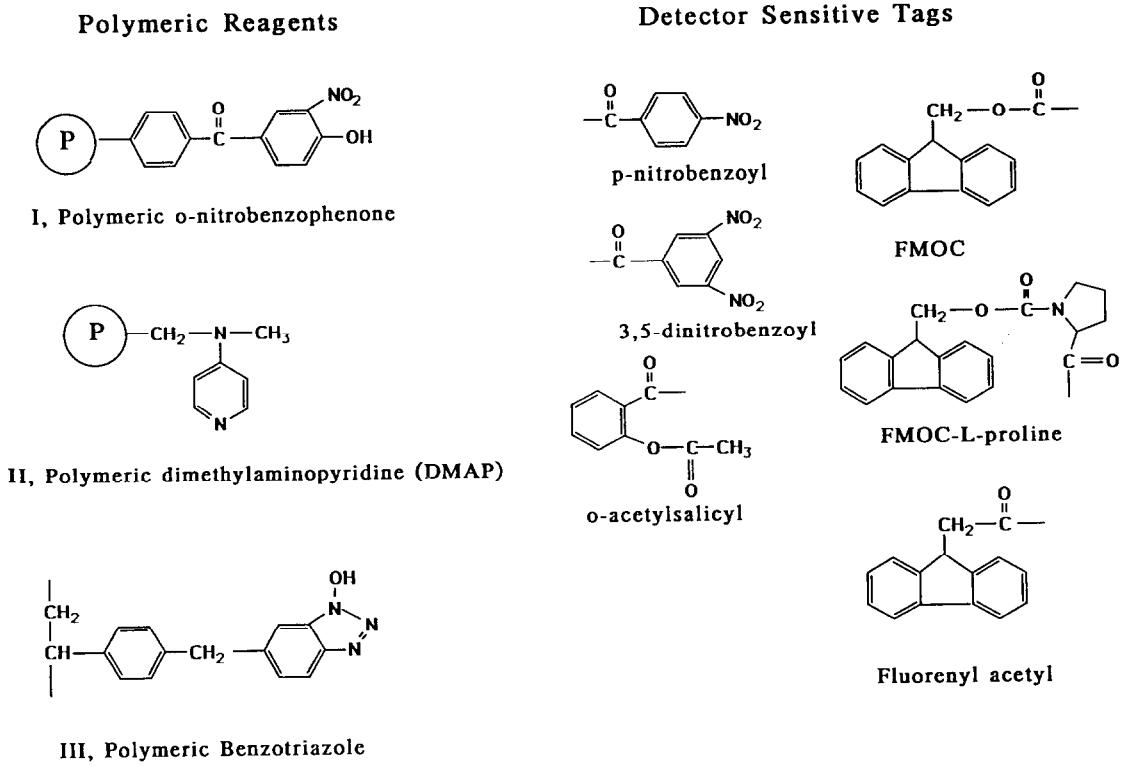

Fig. 1. Schematic illustration of the various parts of a covalently attached reagent–tag system, showing underlying support, leashes possible, tags possible, overall reagents possible.

that connects the support to the tag, and the final tag which will be transferred to the analyte via the derivatization or synthetic reaction.

3. Specific advantages of using immobilized reagents in analytical chemistry and chromatography

In part, several of the initially described synthetic approaches using immobilized reagents in synthetic chemistry were transferred to applications in analytical chemistry and chromatography. As this transformation ensued, specific modifications were made to the reagents, enhancing their detectability, chromatographic performance, analytical selectivity, and so forth. At present reagents are being developed with the initial intent to use them in chromatography, electrophoresis, or other analytical approaches. In synthetic chemistry, immobilized reagents were often used only once and they were not reused or continuously used on-line, with repeat sample introductions and reactions. However, on-line approaches are much more desirable in chromatography and electrophoresis, where the immobilized reactors can be placed in continuous, repetitive operation, often with buffers or solvents continuously flowing through the solid-phase reagent bed. Thus, the requirements for on-line operation in analytical chemistry, such as flow injection analysis (FIA), will often be substantially different from the requirements for conventional, batch-type, solid-phase synthetic organic chemistry.

The immobilization of analytical reagents on inert supports offers several advantages to the analytical chemist. Perhaps the most notable is the economic use of the derivatizing moiety since only the amount of reagent that reacts with the analyte is actually used. The remainder of the reagent, still bound to the insoluble support, can thus be easily separated from the derivatized analyte via filtration . After suitable washing and cleanup to remove prior sample components possibly adsorbed to the support, the polymeric reagent can be reused and/or regenerated, as needed. The extent to which the reagent can be reused depends on the relative concentrations of the analyte being derivatized and the loading of bound reagent. By loading, we mean the amount (meq or mmol) of reagent tag per gram of final polymeric reagent. If the loading is too low, for example <0.1 meq/g, then the overall rate of some reactions may be slower than ideal, and the lifetime of that particular reactor bed may be less than desirable. Even for polymeric reagents, there is a minimum amount of loading that still gives reasonable reaction rates and reproducibility of the reactions under observation.

For parts-per-million (ppm) concentrations of analyte, a reagent with a loading of 0.3 meq/g theoretically can be used several hundred times before a decrease in overall rate of reaction, percent derivatization, and therefore reactor response is observed. Most polymeric reagents can easily be regenerated with a few simple washing steps and reattachment of the solution-based analytical reagent. Although the initial costs for preparing the polymeric reagent are higher than for a soluble reagent, the final costs per assay may be lower. Some polymeric reagents allow easy handling of carcinogenic or foul smelling reagents. When bound to the polymer, these reagents are diluted from the pure form and rendered non-volatile, thus considerably lowering their toxicity [26–28].

Immobilized reagents can be designed to contain large amounts of derivatizing reagent (*e.g.* >1.5 meq/g). This high molar excess of the derivatization species forces reactions to completion, allowing for rapid derivatization of the analyte, even at trace levels. One major benefit of the immobilization step is that, ideally, only the reactive species (tag) remains covalently bound to the polymer. Most of the impurities are removed during the synthesis of the reagent. Thus, trace-level derivatizations using polymeric reagents are often less complicated by system peaks. However, this does not mean that system (*e.g.* hydrolysis) peaks cannot appear, they very often still do, usually due to solvent reactions competing with the immobilized reagent. In the ideal situation, there would be no leaching of the

reagent tag, no unwanted hydrolysis of the tag, and only the amount of tag being transferred to the analyte would be released from the polymeric support [29,30].

Finally, there is virtually no dilution of the sample when using immobilized reagents. Preconcentration and peak compression may occur. There is no mixing of the sample with the reagent solutions, as is required for a solution derivatization reagent. There is also no loss of sensitivity because of the derivatization reaction, and no additional, excess reagent is present (ideally) in the final derivatized solution ready for introduction onto the analytical/HPLC system.

4. Nature of the hydrophobic polymer support, effect of percent crosslinking, particle size, and pore size

Another possible advantage of using immobilized reagents in analytical chemistry, is that the microenvironment of the polymer may act to suppress or catalyze a reaction. This lends an extra mode of selectivity to the derivatization process. Derivatizations which proceed with charge formation in the rate limiting step, can be effectively catalyzed by manipulation of the polarity of the polymeric microenvironment. This is, in effect, extractive acylation conditions using an organic polymer as one of the immiscible phases. Reactions which require a very hydrophobic environment could be best performed on a hydrophobic polymer support, such as STY–DVB, rather than a more hydrophillic support, such as polyethylene glycol (PEG) or polymethylmethacrylate (PMMA). On the other hand, reactions that are catalyzed by a more hydrophillic or polar environment, might be best performed on a hydrophillic or polar polymeric support, as already described for many synthetic chemistry applications.

The polymeric support can also function as a preconcentration medium, extracting and preconcentrating the more hydrophobic analytes from the sample solution. Using hydrophobic, polystyrene-based supports, the analyte is extracted from an aqueous solution and derivatized in the gel-phase region or on the pore surface, if the resin is highly crosslinked. Polymeric reagents have been developed to selectively extract species based on micropolarity or hydrophobicity [31–36]. That is, the polymer can function as a solid-phase extraction (SPE) material, just as with conventional SPE cartridges used for sample cleanup in analytical chemistry, and at the same or a somewhat later time after extraction and cleanup on the support, the reagent part of the SPR can be made to react with the adsorbed analytes. Thus the desired derivatives are formed in a higher concentration and purity than can be obtained by any direct solution derivatization procedure feasible at present. This basic approach has recently been commercialized by two separate vendors, using two different types of reagents [37].

Both selectivity and sensitivity can be increased through the appropriate use of microenvironments. This is important, since these generally are mutually exclusive goals. Polymeric reagents can be used to increase the sensitivity of detection by preconcentrating the analyte prior to derivatization. However, only certain analytes will be extracted and even fewer are usually amenable to the derivatization scheme. This sensitivity and selectivity enhancement is generally difficult to attain in homogeneous, solution-phase type chemistry. Thus, polymeric reagents can be used to simultaneously extract and derivatize the analytes, eliminating steps which may introduce variation in the analysis [38]. Multifunctional analytes may reproducibly have only one site derivatized, depending upon the reaction conditions. Reaction conditions can be modified to label more than one site to introduce differential selectivity. That is, analytes that have several derivatizable functional groups on the same molecule, may show differences in their final, solid-phase reaction products when compared to solution chemistry. Numerous cases exist where solution chemistry provides different ratios of the derivatization products compared with the analogous, solid-phase reaction. This can often be used to advantage in analytical chemistry [27,28].

Microenvironments may lead to unexpected enhancements in reaction rates. This is partly due to extraction and concentration of analytes from solution, which effects the concentration term of the rate expression. However, other enhancements can be due to intermolecular catalysis. Although the reagents are technically immobilized and dilute due to the absence of Brownian diffusion, many site–site interactions on polymers have been recorded [39]. These may reduce the derivatization efficiency by hydrogen bonding or increase the efficiency by acting in an achimeric fashion to assist the reaction [40,41]. The interaction of neighboring groups can be of the acid–base type, where a proton is donated or accepted [42].

5. Large *versus* small analyte molecules and their derivatizations via polymeric reagents: size-selective polymeric reagents

In addition to chemical hydrophobicity, the physical character of the polymer can also lend special selectivity to solid-supported reactions. The choice of pore size and percentage of crosslinking of the polymer network will effect size exclusion, thus enabling derivatization of small molecules, but hindering or excluding derivatization of large molecules [35,36,43]. This is analogous to the use of restricted access media (RAM) in HPLC stationary phases which permit the separation of small molecular mass drugs from biopolymer type materials [44–47]. This can be used to the analyst's advantage when performing derivatizations in biological media, where proteins or other macromolecules might interfere with the reaction. The idea of developing controlled pore size polymeric reagents is quite recent, and evidence is accruing that suggests an entirely feasible approach for size-selective derivatization of analytes in a complex mixture.

Another mode of analyte selectivity is caused by the hydrophobic nature of the polymer support, which can prevent very hydrophillic analytes from reaching the region of the highest reagent concentration (within-pores) [48]. However, this hydrophobic–hydrophillic selectivity exhibited by the polymer support on the reactions possible, is quite different from the size-exclusion effect shown by the pore size of that same support. If the bulk of the reagent can be made to reside within the gel phase, rather than on the external surface and inside the pores, then very large analyte molecules will be excluded from the internal pores and not undergo derivatization. On the other hand, much smaller analytes, in principle and fact, will enter the small pores, encounter immobilized reagent sites, be derivatized, and then diffuse back out of the pores and into the solution. It is conceivable that for a given mixture of potential analytes with a widely varying size, only those within a certain size limit will enter the pores to react and diffuse back into the solution outside the polymer entirely. It is even conceivable, though not yet proven, that certain medium-sized analytes could enter the pores, become multiply derivatized on several reactive sites on a single molecule, and then are not able to escape from the pores to be detected, *e.g.* peptides or antibiotics.

6. Mixed-bed derivatization approaches

Quite aside from the area of sequential or consecutive reactions on different polymer beds in series [49,50], several different labels can be attached to the same polymer bed to obtain two or more different derivatives from one, reproducible injection of the sample, thus allowing quantitation and confirmation of the presence of the analyte in complex matrices [51,52]. The formation of multiple derivatives allows numerous opportunities for quantitation from the same injection, and also allows improved identification from the retention times of the multiple derivatives. A comparison between the derivatives formed from standards of the presumed analytes (knowns) and the actual sample, under identical derivatization and HPLC-detection conditions is, of course, necessary for optimum understanding of mixed-bed reactions with the unknown sample matrix. Thus, multiple reagents have been combined into a single, mixed-bed reactor, and used

for the simultaneously preparation of several, different derivatives from a single analyte. Each derivative possesses different chromatographic and detection properties, depending on the nature of the original polymeric supports containing the immobilized tagging species (9-FMOC, *p*-NP, *o*-Ac, *etc.*; *cf.* List of abbreviations).

Variations in the amounts/ratios of the polymeric reagents contained in a single mixed-bed reactor will lead to varying ratios of the final derivatives. These ratios can be predicted knowing the approximate reactivity of each polymeric reagent, percent derivatizations, and overall reaction rates for each reagent towards a given substrate. In addition, changing the reaction conditions changes the ratio of the products formed, so that changes in the ratio of peak heights and areas can also be used for analyte identification [52]. Changing the derivatization conditions, *e.g.* reaction time, temperature, solvent, presence of catalyst, and the components of the reactor, also changes the ratio of the derivatives formed. These changes in product formation with changing reaction conditions can be applied to the identification and quantitation of drugs in biofluids [52]. Again, the goal behind using mixed-bed derivatization schemes in analytical chemistry is to improve analyte identification, structural determination, and final quantitation. These approaches can be applied to both achiral and chiral analytes, in all forms of analytical chemistry, but are especially useful and applicable in various forms of chromatography and electrophoresis [53–56].

7. Use of polymeric reagents via off-line and on-line, pre-column and post-column arrangements in HPLC

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are separation techniques which often provide the selectivity necessary for determination of analytes in complex mixtures. However, analyses are often not sufficiently sensitive without a derivatization step, which enhances the detectability of the analyte. Derivatization can also often improve chromatographic performance, peak shape, efficiency, and resolution for many analyte species. Derivatization procedures for GC/HPLC separations can be grouped into two major techniques, pre- and post-column derivatizations. In addition, reactions can be performed separate from the instrumental set-up, termed off-line, or as an integral part of the instrumentation, then termed on-line. Hence, derivatizations can occur in HPLC in four distinct modes or approaches: (1) off-line, pre-column; (2) off-line, post-column; (3) on-line, pre-column; and (4) on-line, post-column. In general, there are distinct advantages and disadvantages inherent to each of these four modes, as will be described below.

7.1. Post-column derivatizations

Post-column, on-line modifications allow for enhanced detection using existing separation schemes [57,58]. The chromatographic conditions normally used to separate the analyte of interest usually suffice for post-column derivatizations, unless the mobile phase is incompatible with the reaction requirements and/or reagents. However, in general, there is no change in the chromatographic conditions, since the derivatization is occurring post-separation. The post-column, on-line reagent cannot interfere with the detection scheme. Though 100% derivatization is ideal, it is not required in this mode, but it should be reproducible. Rapid kinetics are desirable, since this avoids reaction band-broadening, reduces overall variance, and contributes to improved peak shape and chromatographic efficiency.

Solution, post-column, on-line derivatization techniques generally introduce such extra-column effects, that the separation efficiency for closely eluting components may be compromised. This fact has become more important with today's high-efficiency separations, since post-column techniques were originally designed for low-efficiency separations. In addition, more expertise, pumping hardware and low-volume mixers are needed. If the post-column reaction has exceptionally poor reaction kinetics, longer

reaction coils may be needed, at times with considerable loss of resolution [59]. This problem is often alleviated by the use of segmented reactors, or more recently by the use of knitted-open-tubular (KOT) reactors [60,61]. Segmented reactors compensate for the variance due to axial diffusion of the analyte and laminar flow characteristics, which induce band-broadening in open tubular reactors [62–67]. However, the immiscible fluid used to segment the stream may interfere with the detection, and membrane separation of phases is often required prior to detection; this again induces variance and discrimination bias to the analysis.

Post-column, off-line approaches have not been used often in any form in HPLC derivatizations, solution or solid-phase, and this is not expected to change very soon. In general, post-column, off-line approaches are impractical and difficult to automate or use in a routine manner. They tend to be impractical, unwieldy, and extremely labor/time intensive.

7.2. Pre-column derivatizations

Pre-column derivatizations are useful for compounds which chromatograph poorly due, for example, to strong hydrogen bonding with the chromatographic stationary phases, or for compounds containing labile functional groups which may decompose or react with components in the system during the separation process. By a judicious choice of the derivatizing reagent, the interaction of the analyte with the stationary phase can be altered, in order to make the separation of the analyte over the matrix components more selective. By changing the chemical and physical properties of the derivative compared with the original analyte, chromatographic properties and performances are usually altered. Pre-column derivatizations, as for post-column ones, can be grouped into two categories: off- and on-line.

Pre-column kinetics are not very important, since they have no effect on the chromatographic performance; they only effect the total time needed for derivatization and overall analysis. In order to reduce the total run-time and increase sample throughput, fast kinetics are obviously beneficial. Quantitative conversions are also not imperative, as long as they are reproducible and lead to a sufficient amount of derivative to allow for trace analysis at the levels present in real samples. Because of the presence of unreacted reagent and possible impurities or side products of the derivatization reaction, the chromatography must be able to resolve the derivative of interest from all other potentially interfering peaks.

7.3. Off-line, pre-column derivatizations

Off-line, pre-column derivatization has the major advantage that there is no extra-column loss of efficiency. That is, the reaction is occurring separate from the HPLC system, and an aliquot of the derivatized solution is then introduced onto the HPLC system. No additional instrumentation, mixing tees, reaction chambers, delay coils, and so forth are necessary. Derivatization conditions, such as time, reaction solvent and temperature, need not be compatible with the separation scheme. Consequently, longer reaction times and more demanding conditions can be employed for trace derivatization of unreactive analytes. Sample cleanup after derivatization can be performed to reduce the amount of extraneous sample matrix injected onto the analytical system. However, there is usually some dilution of the sample due to the introduction of reaction solvent and solution phase reagents, and an additional extraction step for isolation of the derivative from the reaction medium may be necessary. That is, usually an off-line, pre-column derivatization is followed by cleanup of the desired derivative prior to injection. Dilution of the analyte can be compensated by evaporation of the extraction and/or reaction solvents, and thus concentrate the desired derivative in a small volume. These methods require extra sample handling, and thus generally increase the variance of the methodology. However, since no extra hardware is required, off-line, pre-column derivatizations remain quite popular and most analyses which

require derivatization, utilize some form of off-line, pre-column modification of the analyte.

7.4. On-line, pre-column derivatization

Derivatizations can be performed on-line, pre-column by use of a switching valve or a high-volume reactor, if the kinetics allow for real-time derivatization. This type of approach has been commercialized for some time, *e.g.* in the Hewlett-Packard HPLC systems which contain automated, solution-phase derivatization systems, pre-column, on-line. Sensitivity of the analysis, *i.e.* the ability to detect small concentrations of analyte, can be increased if the reactions are performed on-line. The reaction medium and derivatization reagents must be compatible with the separation and detection scheme. As with off-line derivatizations, new separation schemes must be tailored to the chromatographic character of the final analytically labeled derivative. This usually increases the selectivity of the analyte–stationary phase interaction, when compared to the underivatized analyte and components of the matrix. Sensitivity and selectivity are two of the major benefits of on-line, pre-column derivatization. Band-broadening contributions from reactor hardware can be minimized, and these are less crucial to the final separation than is the same void volume for post-column procedures [68–70].

In the case of solid-phase derivatizations in HPLC, as will become evident below, most applications thus far have been on-line, pre-column or off-line, pre-column. In principle, if not in practice, it should be just as easy to apply such approaches in both post-column modes, but perhaps less practical and useful. In the on-line, post-column mode, solid-phase approaches should be utilizable, but they would only improve the detector response, as expected, but not the chromatographic performance properties. Also, because the post-column reactors would have to be on-line virtually all of the time, their lifetimes might be severely compromised, depending on the particular solvents used in the mobile phase and their pH. Also, this approach would require low flow-rates or extremely fast kinetics to avoid reaction rate band broadening. However, there are some examples where solid-phase reactions have been very practical on-line, post-column (*e.g.* Studebaker's, using disulfide interchange chemistry).

8. Immobilized reagents used in HPLC

Most of the work describing the use of polymeric reagents in HPLC has occurred since the initial paper by Studebaker *et al.* [13], who used cellulose-based supports, Sephadex or Sepharose, rather than silica or organic polymers. Polymeric reagents designed for HPLC have largely been STY–DVB based. This is due to their structural stability, well established chemistry and ease of modification. The literature contains many outstanding reviews of analytical uses of solid-phase reagents for HPLC analysis [49,50].

8.1. Silica-based reagents in HPLC

The high surface area and rigid structure of silica make it an ideal adsorbent for reactive species. Silica has been used for adsorption of sodium borohydride for the reduction of carbonyl compounds [22]. The reagent was utilized for on-line, pre- and post-column analyses of various organic carbonyl (aldehydes/ketones) containing compounds. Picric acid has also been adsorbed as a silver salt for the derivatization of alkyl halides [71]. This reagent activated the alkyl halide towards electrophilic addition by complexing the halide leaving group as an insoluble silver halide salt. This reagent was successfully used to determine trace levels of ethylene dibromide (EDB) in gasoline [28]. EDB is bifunctional and two derivatives resulted, the ratio of which was dependent upon the reaction conditions. This ratio was used to confirm the presence of EDB at low ppb concentrations. However, both of these reagents were adsorbed on the silica, and were only useful for anhydrous, aprotic solvent conditions. These reactions could be performed on-line, as pre-column reactions, but only with normal phase solvents of

low polarity. This was unfortunate, since polar aprotic solvents were found to yield the highest percent conversions. Polar aprotic solvents, *e.g.* acetonitrile (ACN), stabilize cations, leaving anions naked and highly reactive, and they are less viscous than protic solvents, thus enhancing the mass transfer to the reactive sites in the solid-phase reagent. Adsorbed reagents often suffer from mobile phase bleeding, *i.e.* they slowly diffuse into the flowing mobile phase and are depleted from the solid support. They thus create an elevated background, more rapid depletion of the reagent from the support, and can even modify the elution properties of the mobile phase.

Other ion-exchange supported polymeric reagents have been described in recent years, usually on anion-exchange supports [72,73]. In one example, Colgan *et al.* derivatized a series of alkyl halides, acid chlorides, and other electrophiles with a 2-naphthoxide based ion-exchange reagent, leading to UV/FL/EC sensitive derivatives for HPLC [72]. The reaction could be performed both off- and on-line under reversed-phase conditions, but bleeding of the UV/FL tag could not be prevented. The reagent could be used in both the pre- and post column modes, with detection limits after derivatization in the ppb or sub-ppb ranges. In a more recent report, Idowu and Adewuyi described the use of sodium benzoxazole-2-sulfonate as a derivatization reagent for the analysis of amines and amino acids in HPLC-UV/FL [73]. This approach used a non-fluorescing reagent for both solution and solid-phase tagging of amines and amino acids; the final derivatives were fluorescent at wavelengths where the excess, unreacted tagging reagent was not. Excess reagent present in the sample injected did not produce peaks that interfered with the FL tagged derivatives. The sulfonate reagent was ionically attached to an anion-exchange support, commercially available, which could be used to retain unreacted, excess reagent from the HPLC injected sample. In the immobilized form, the reagent could be used for heterogeneous reactions on-line, pre- or post-column for both amines and amino acids [73]. This basic approach of using an immobilized FL reagent which does not show fluorescence at wavelengths where the derivatives do, is very similar to the work described below using a 6-aminoquinoline, covalently tagged, polymeric reagent for amines, amino acids, and peptides in HPLC-UV/FL [74].

Thus, the best route for the immobilization of reactive species is covalent bonding. This obviates the problems associated with leaching of the reactive species from the reagent, and gives better control over the reaction conditions, *e.g.* solvent and temperature. The use of both adsorbed and immobilized silica-based reagents for HPLC analyses has been recently reviewed [75]. A post column, silica-based, solid-phase reactor has been used for the detection of thiols in reversed-phase HPLC (RP-HPLC) [76]. The reagent was not effected by the mobile phase conditions and exhibited excellent kinetics. A similar reagent was prepared which exhibited better linearity, better detection limits and longer lifetimes; this reagent was attached to a polymer coating that had been applied to the silica [77]. The immobilization of an active ester bound to an alkyl spacer on silica, allowed for derivatization of amines at low ppm concentrations [78,79]. This reagent was not stable in on-line, pre-column RP-HPLC conditions, but could be used off-line for batch derivatizations. The optimal pH range for the reaction of the reagent with the analyte was 9–10. However, at this pH, the silica support, the chemical grafting on the silica, and the active ester, were all simultaneously attacked by hydroxide. In addition, the temperature which was used to attain fast reaction kinetics was 50–60°C. At this temperature and pH, the analytical reagent could only be used for 10–15 analyses. The possibility to stabilizing these active esters on silica supports using hydrophobic shielding from adjacent alkyl ($C_{8/18}$) groups remains to be explored.

8.2. Organic, polymer-based reagents in HPLC

Based on the work of Shambhu and Digenis [80] and Martin *et al.* [81,82], analytical reagents for the derivatization of nucleophiles have been prepared. These reagent were then transferred

to HPLC applications. An immobilized anhydride was prepared on a microporous STY–DVB support for the analysis of primary and secondary amines, Fig. 2 [83]. The reagent was based on 1% crosslinked (DVB) chloromethylated polystyrene. It was the first reported use of a covalently bound, polymeric reagent for improvements in HPLC detection. Two previous publications from the same group dealt with ionically bound reagents for the oxidation of aldehydes [84] and the derivatization of alkyl halides and other electrophiles [85]. However, these approaches were not amenable to on-line conversions in aqueous environments, due to leaching of the analytical label from the reactor. The anhydride was capable of rapid conversion of amines in aqueous/organic environments to amides, which were chromatographically stable and possessed excellent UV absorbance and EC detection properties. The derivatization procedure lowered the limit of detection 3 to 4 orders of magnitude compared with UV detection of the underivatized amines. However, the polymeric anhydride, although covalently bound, was also not stable in on-line, pre-column RP-HPLC conditions because of rapid hydrolysis. It is possible that on a lightly crosslinked polymeric material with a large surface area, as below, this reagent might also work quite well in the on-line mode. In any case, it was then necessary to prepare a reagent that was not as moisture sensitive, yet still provided high conversions under mild conditions.

Following the work of Kalir *et al.* [86], an

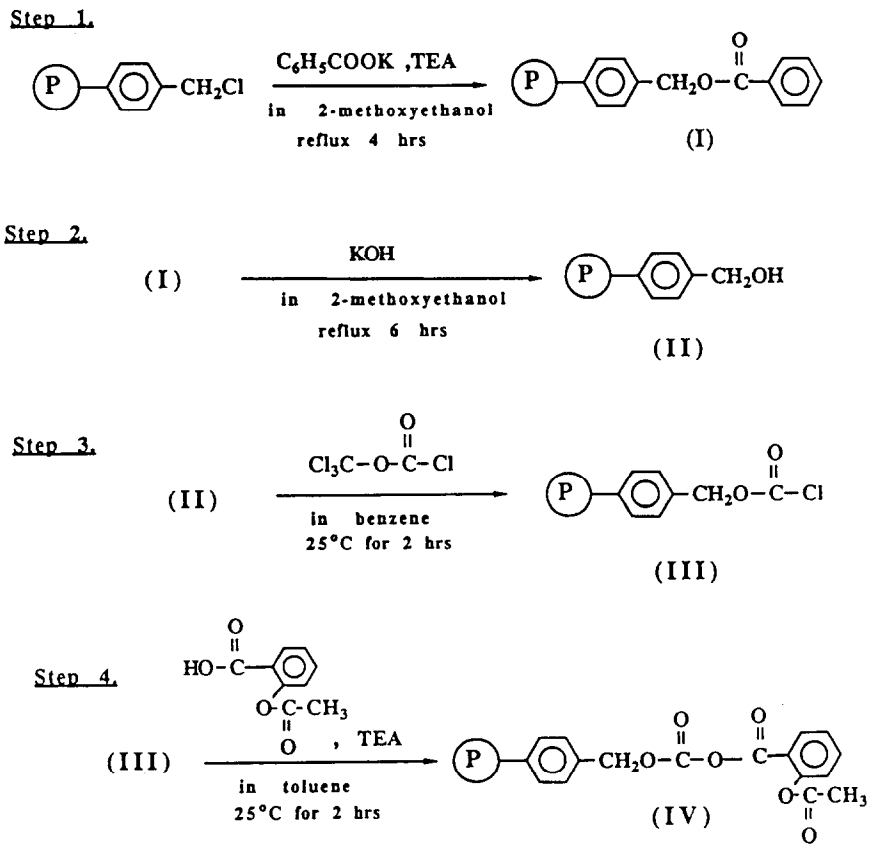

Fig. 2. A polymeric anhydride containing *o*-acetylsalicyl reagent for off-line derivatizations of amines and polyamines in HPLC. Reproduced from ref. 83 with permission.

active ester was prepared based on a 1-hydroxybenzotriazole leash [87]. This polymer was prepared on a polymeric support very different from the previous activated anhydride. The anhydride was prepared on a 1% DVB, microporous support, while the benzotriazole was prepared on a highly (*ca.* 80% DVB) crosslinked macroporous support. The benzotriazole activated ester on the macroporous support proved to be a powerful acylating reagent when labeled with 9-fluorenylmethyl chloroformate (9-FMOC) [88]. Urinary diamines were analyzed successfully using this reagent, but on-line derivatizations in RP-HPLC were still not possible. The chemical structure of the benzotriazole was even more sensitive to moisture than the anhydride. The high surface area and lack of hydrophobic shielding in the macroporous support only made the situation worse.

Cohen *et al.* had described a reagent that was not as sensitive to moisture, yet still allowed excellent conversion of amino acids to peptides [89]. The chemical leash, *o*-nitro-*p*-carbonyl-benzophenol (also known as *o*-nitrobenzophenone) (Fig. 1, I) was prepared on a 4% crosslinked microporous STY–DVB polymer, Fig. 3. This choice of polymer was based on the commercial availability. The polymer, labelled with a 9-FMOC tag, proved to be stable for on-line pre-column conversions of amines in RP-HPLC [90]. This particular reagent again contained an activated ester linkage, which imparted both UV and FL detector properties to the final derivatives, mainly for amines. Kinetic studies of these solid-phase reactions were conducted, and specific rate constants were compared with those of the analogous solution reactions for the same substrates. Percent derivatizations reached 90% and 70% for primary and secondary amines, respectively, under fully optimized conditions. High reaction reproducibility was obtained by using the on-line approach for more than 50 separate injections of the same amine substrate with a single solid-phase reactor. These solid-phase derivatizations, perhaps the very first ever done on-line, pre-column in HPLC with good reproducibility, led to detection limits for typical amines in the low ppb range. The final, overall

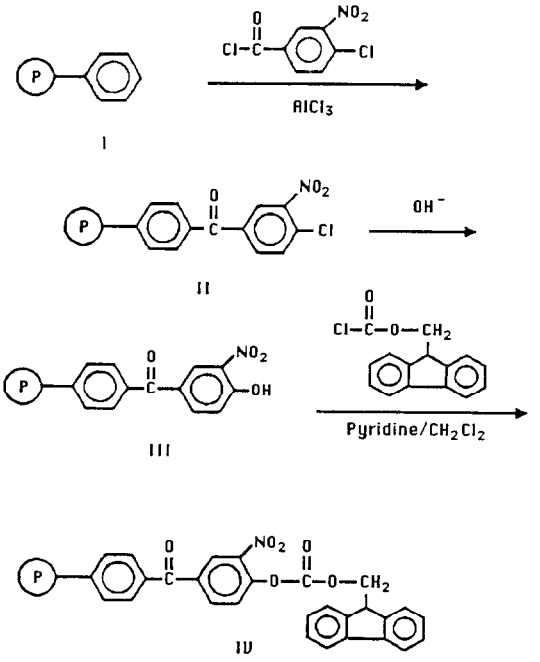

Fig. 3. A polymeric *o*-nitrobenzophenone reagent containing 9-FMOC for off-line and on-line derivatizations of amines and polyamines in HPLC. Reproduced from ref. 88 with permission.

methods provided rapid, automatable, accurate, and precise detection and quantitation of primary/secondary amines and amine-like compounds in real-world sample matrices. Thus, amphetamine spiked in urine has been derivatized off- and on-line, with minimum sample preparation, and detected via HPLC-UV/FL with acceptable accuracy and precision [90].

This same reagent as used above, was again used on-line, pre-column, in a reaction cartridge, Fig. 4, placed just before the separation column [91]. Trace levels of aliphatic amines and a polyamine in environmental air samples were trapped with commercially available silica gel tubes. The amines were then desorbed with an acidic aqueous–organic solution and neutralized with NaOH prior to HPLC injection. Recovered amine solutions were then directly injected into the on-line, pre-column derivatization, HPLC-UV/FL detection system for identification and quantitation of individual amine components. No further sample workup was required. The per-

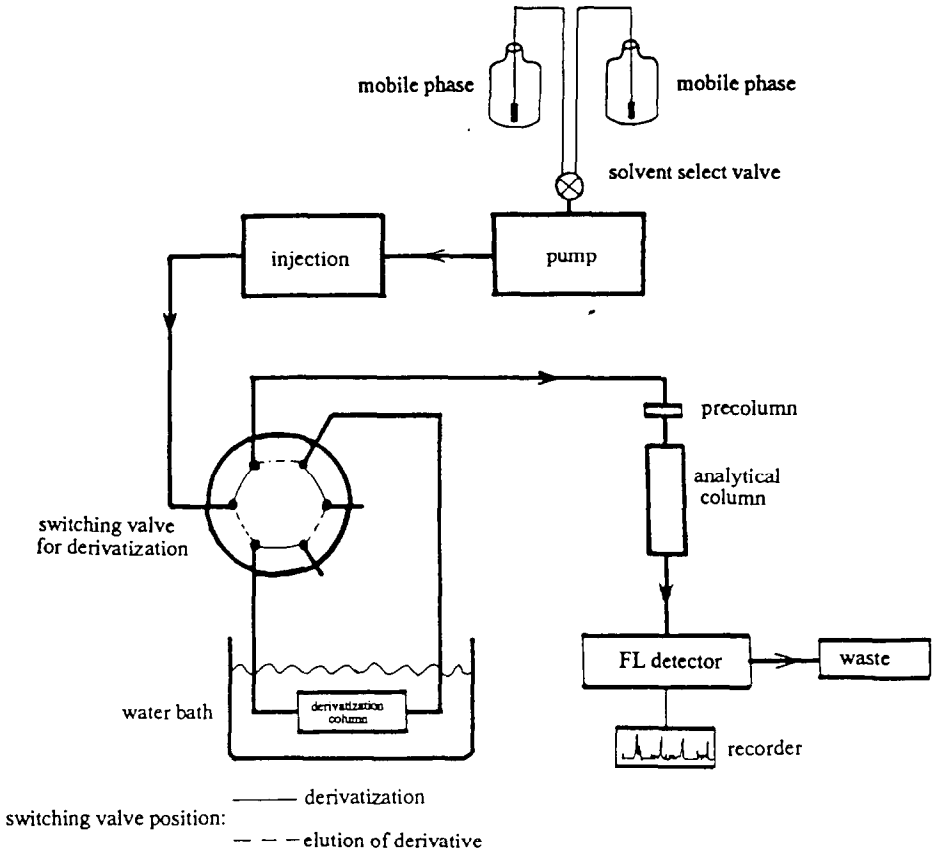

Fig. 4. Schematic diagram of an on-line, pre-column reaction cartridge, placed just before the HPLC separation column. Reproduced from ref. 34 with permission.

cent derivatizations were as high as 88 ± 5% for primary amines and 75 ± 4% for diethylamine under optimized conditions of 60°C for 10 min. The recoveries for all amines were higher than 90% from the air sampler cartridge. The overall method was validated by a single-blind, spiked experiment with 1.1–4.4% R.S.D. in the range of 15–47 ppm of amines. Amines were quantitated via calibration plots, with final concentrations from 0.02 to 0.38 mg/m^3 air. This was a totally new and general approach for the determination of amines and polyamines, using polymeric solid-phase reagents on-line, pre-column in HPLC [92].

Approaches completely analogous to those described above for HPLC applications to air sampling have also been described in recent years for GC analysis [14,15]. Reagents very analogous to those first used in HPLC were modified to contain GC-detector sensitive tags, with air sampling and derivatization occurring on the same air sampling adsorbent/support. Waters /Millipore has recently introduced a commercial 2,4-dinitrophenylhydrazine (2,4-DNPH) reagent adsorbed onto a silica support for performing HPLC analyses of volatile aldehydes and/or ketones [37].

Solid-phase derivatization of weak nucleophiles was not possible using any of the above polymeric reagents, because the nature of the leash and tags were such that insufficient activation of the tag ensued for release by alcohols, thiols, phenols, and so forth. However, Gao and Krull [93] demonstrated, following the lead of Shai *et al.* [12], that polymeric dimethylaminopyridinium (DMAP) 9-FMOC re-

agent could be used for off-line derivatizations of weak nucleophiles in HPLC under normal-phase conditions (organic solvents). Derivatization conditions in terms of solvent, time, and temperature, for both primary and secondary amines, were fully optimized in a univariate manner. As a typical example of the possible applications, off-line derivatizations of 2-chloro-1-propanol, a potential carcinogen in foodstuffs, were carried out with this polymeric DMAP reagent using single-blind and standard addition quantitation techniques. A specific sample treatment procedure was also developed. These particular DMAP reagents, with varying tags, could not be used under aqueous conditions, but required purely organic solvents for derivatizations. Separations of the once-formed derivatives could then be performed using any form of HPLC. Polymeric, chiral DMAP reagents can also be used, *e.g.* for chiral recognition (enantiomeric excess) of weak nucleophiles via the off-line formation of diastereomers prior to HPLC [94].

The desire to develop an activated polymeric reagent that would react with both weak and strong nucleophiles eventually led Bourque and Krull to prepare the polymeric *o*-nitrobenzophenone and benzotriazole, 3,5-dinitrophenyl (3,5-DNP) containing reagents, Fig. 5 [95,96]. These reagents were easily prepared and could be regenerated after use to attain their original activity. The final chromatograms were again

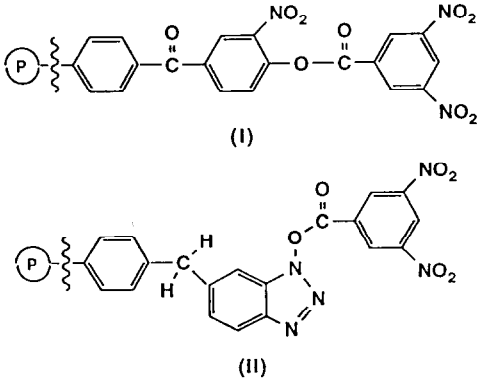

Fig. 5. Polymeric *o*-nitrobenzophenone and benzotriazole, 3,5-dinitrophenyl, activated ester reagents. Reproduced from refs. 95 and 96 with permission.

free of system peaks from excess derivatizing reagent, and sample handling was kept to a minimum. The reagents could be used in conjunction with both reversed- and normal-phase HPLC, as well as with off-line GC. These reagents could also be used on-line for derivatizations in HPLC, as for the 9-FMOC analogs. Since the labeling reagent was a strong Pi-acid, chiral substrates could also be derivatized and separated on a Pirkle-type, Pi-donor column. The confirmation and quantitation of amphetamine in urine was accomplished using a polymer containing two labeling moieties, *p*-nitrobenzoyl and 3,5-dinitrobenzoyl. The derivatization and separation of both chiral and achiral amines, amino alcohols and amino acids was described. These types of 3,5-DNP reagents thus proved useful for tagging of achiral substrates with conventional, normal or reversed-phase HPLC separation conditions. At the same time, they proved very useful for performing tagging of chiral analytes, especially amine derived, prior to Pirkle-type chiral recognition using a Pi-base column. The alternative arrangement, using a Pi-base tag with a Pi-acid chiral stationary phase (CSP) is also feasible, though not yet demonstrated in practice [97]. As discussed elsewhere in this Special Volume, such derivatizations are important in performing successful CSP resolutions of enantiomeric substrates.

The polymeric benzotriazole derived 3,5-DNP reagent proved of interest because it is the fastest, most reactive reagent thus far described for derivatizations of amines and virtually all weak nucleophiles [95]. These reactions, with both achiral and chiral substrates, for nucleophiles such as alcohols, thiols, phenols, and even anilines, occurred at room temperature in less than 30 s, often within 5–10 s. Again, such derivatives could be used for both achiral and chiral separations. Bourque and Krull [96] described a series of microporous and macroporous PS–DVB beads modified to contain various activated centers to which the 3,5-DNP label was attached. The effects of surface area, porosity, pore size, and percent crosslinkage of the polymeric support containing the *o*-nitroben-

zophenone leash were reported. Such polymers were again characterized by a loading experiment to calculate the amount of active acylating reagent/tag incorporated per gram of dry polymer. The kinetics of reaction with various substrates of varying steric hindrance *versus* solvent strength were determined with the optimized polymeric support, and subsequent polymeric reagents were synthesized based on that very support. The second polymeric reagent studied here contained the hydroxybenzotriazole leash to the same 3,5-DNP tag. This polymeric leash had a largely increased electrophilicity and was used to prepare a much stronger acylating reagent than the corresponding polymeric *o*-nitrobenzophenol when labelled with the same 3,5-DNP tag.

9. Chiral *versus* achiral polymeric reagents. Chiral recognition using polymeric reagents: direct *versus* indirect modes

In current HPLC approaches to chiral recognition of enantiomeric analytes, there are basically two general approaches that have become popular. The first uses direct injection of the enantiomers, at times without prior derivatization, often with achiral tagging to form Pi-acid or Pi-base derivatives for Pirkle-type CSP recognition [97–103]. In this case, the enantiomers are recognized by the chiral portion of the stationary phase, and some type of diastereomeric interaction ensues, of a temporary nature, so that differences in ΔG eventually lead to chiral resolutions. However, by and large, there is no improvement of detection limits or detectability of the resolved enantiomers. This direct chiral separation approach has become very popular, since it provides unambiguous demonstration of both chemical and chiral purities with single, repetitive injections. However, most of these CSPs are quite expensive and do not survive repeated injections of crude biofluids [53]. Thus, CSP approaches are valid only for relatively clean samples, such as formulations, aqueous solutions, and neat drugs. A variation of the direct chiral recognition approach uses an achiral stationary phase and a chiral mobile phase, but it usually becomes prohibitively expensive to run such analyses for large numbers of samples.

The direct recognition approach, now using polymeric 3,5-DNP reagents to tag enantiomers prior to Pirkle CSP recognition, has been discussed above. It presents a perfectly general approach for chiral recognition via the formation of amide derivatives, separable on many Pirkle-type CSPs (Fig. 5). An alternative approach is to use an immobilized carbamate reagent to form ureas, rather than amides, of typical amine analytes (Fig. 6). Such ureas are more readily separated on CSPs than the amides, and several examples of such separations using polymeric carbamate reagents with the 3,5-DNP tag have been reported [104]. Fig. 7 illustrates the use of a mixed-bed approach to chiral recognition, again using a CSP based on the Pirkle recognition approach. In this particular example, the mixed polymeric reagent bed contained predominantly the immobilized 3,5-DNP as the carbamate leash rather than the activated ester leash. It therefore formed predominantly the expected urea derivatives (enantiomers), separable as indicated. The 3,5-DNP activated ester leash again formed the urea derivatives (enantiomers), which were less readily separable on this same Pirkle-type CSP. It is clear that the ratios of the peak areas for both pairs of enantiomers are identical, confirming the exact enantiomeric excess present in the original sample of 2-aminooctane analyte. These reagents are thus useful for Pirkle-type recognition of stereochemical compositions of amines and all related nucleophiles, often in a fully automated manner [30]. The ability to stabilize the 3,5-dinitrophenylisocyanate by covalent immobilization through a carbamate leash on several polymeric supports varied greatly with the nature of the support chosen. This was the first demonstration of an immobilized isocyanate reagent leading to the formation of ureas from typical amines. The kinetics of the activated dinitrophenylcarbamate reagents with various nucleophiles were derived and then compared to similar, polymeric activated ester reagents.

An alternative approach, termed indirect chiral recognition, uses the formation of diastereo-

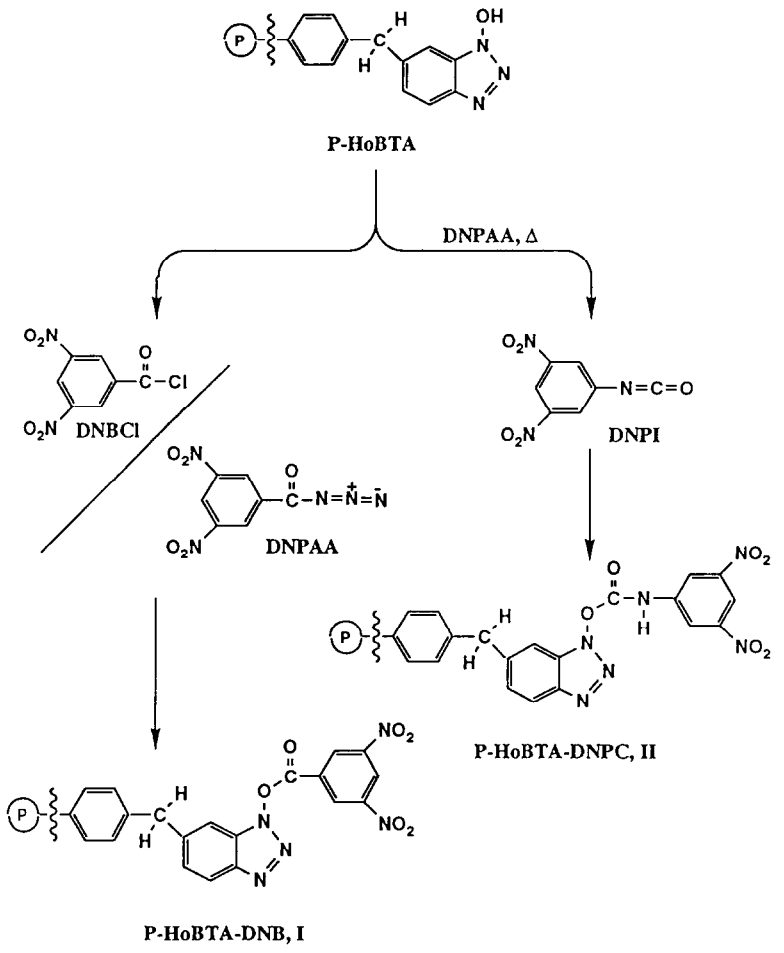

Fig. 6. Polymeric benzotriazole-3,5-dinitrophenyl, activated ester and carbamate reagents. Reproduced from ref. 104 with permission.

mers from the enantiomeric analytes, then separated on achiral stationary phases. In this approach, actually one of the oldest methods used for chiral recognition, a chemically and optically pure enantiomeric reagent must be available to form diastereomers of the substrate analytes. There have always been potential pitfalls with such an approach: *e.g.* the derivatizing chiral reagent must be 100% optically and chemically pure in order to avoid incorrect enantiomeric excess determinations. Also, in the solution approach, there may always be kinetic resolution, *i.e.* prior to full conversion of each enantiomer into its corresponding diastereomer, the specific rate constants and overall rates of conversion may be different from one enantiomer to another. If the reaction was not allowed to proceed to completion, then the final ratio of the diastereomers might not reflect the original ratio of the enantiomers and the analysis would be invalid. Finally, it is also possible that two diastereomers do not have exactly the same detector response, resulting in a different output for each enantiomer. Peak heights/areas would then not reflect the original enantiomeric ratio present in the original sample. This too could lead to an invalid assay for the enantiomeric excess. Finally, for this indirect method of chiral recognition to be useful, it is necessary to baseline resolve the diastereomers under achiral

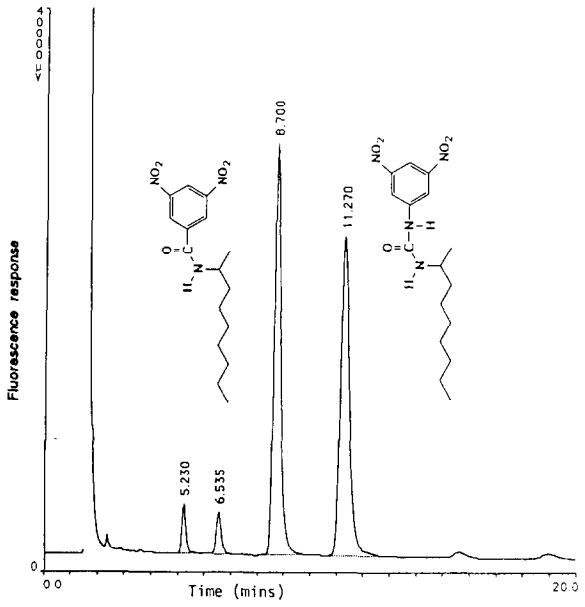

Fig. 7. Use of a mixed-bed, polymeric reagent approach to chiral recognition, using a CSP based on the Pirkle recognition approach. The enantiomers of (+)-2-aminooctane were derivatized, off-line, with the polymeric benzotriazole-3,5-dinitrophenylcarbamate reagent. A small amount (10%) of the mixed-bed reactor contained the same 3,5-DNP label on the same polymeric support through the activated ester leash (Fig. 5), leading to two pairs of tagged enantiomers being formed. The chromatogram shows the difference in chiral recognition for this Pirkle-type CSP between the urea and amide derivatives of the same d/l pair of enantiomers. Analytical conditions: derivatization: 50 μl × 200 ppm amine in ACN × 25 mg polymeric reagents, 60 s at room temperature, eluted to 1 ml with THF–HEX (1:3, v/v). Separation conditions: hexane–ethanol–dichloromethane (90:5:5, v/v) at 2.0 ml/min flow-rate on a 250 × 4.6 mm I.D. LC-(*R*)-naphthylurea column, 5-μl injection, UV detector at 0.004 AUFS at 254 nm. Reproduced from ref. 104 with permission.

HPLC conditions. Chiral HPLC conditions might be used, but there is no obvious advantage for most cases of diastereomer separation.

In 1989, Chou *et al.* described perhaps the first polymeric chiral reagent for both off- and on-line derivatizations of enantiomers in HPLC with both UV and FL detection [105]. This was then termed an enantiomer recognition approach in HPLC. In this approach (Fig. 5), optically active and detector sensitive, polymeric reagents were synthesized, loadings determined, derivatizations/separations/detection optimized, and applications were described for simple amines and amino alcohols. These polymeric chiral reagents contained different chiral centers, usually amino acids, leashed via an activated ester attachment to the insoluble, organic polymer backbone. Again, 9-FMOC tags were attached to the amino acids (*e.g.* L-proline) as UV and FL sensitive detector probes to the final diastereomers of enantiomeric substrates. These diastereomers were readily separated by isocratic or gradient elution modes. The overall rates of formation of various diastereomers from several pairs of enantiomeric substrates were determined, in terms of specific rate constants, overall rates of reaction. In every single case, the overall rates of formation for a given pair of enantiomers were identical, at any time during the reactions. That is, no kinetic resolution could be discerned for these enantiomeric reactions, despite the fact that solution chemistry using analogous chiral reagents would be expected to show such discriminations, especially at the initial phases of the reactions. Though an exhaustive demonstration of the absence of kinetic resolution has not been performed as yet with these polymeric chiral reagents, it is hypothesized that the slow, rate determining step in these enantiomeric reactions is diffusion through the achiral polymer matrix, rather than the actual reaction between enantiomers. If this is true, and diffusion is now rate-controlling the final, overall rate expression, then chiral discrimination (kinetic resolution) would be impossible to observe. If the hypothesis is indeed correct, then these polymeric chiral reagents may be the only chiral reagents thus far described that may never show kinetic resolution for any enantiomeric substrate.

In the initial studies with polymeric chiral reagents, the final UV/FL detector responses for known mixtures of enantiomers have been compared in order to demonstrate the overall validity of this indirect chiral recognition method [105]. In one case, that of d,l-napththylethylamine, derivatized off-line with the polymeric 9-FMOC-L-proline chiral reagent (Fig. 1), the resultant diastereomers showed different FL responses but identical UV responses (Fig. 8).

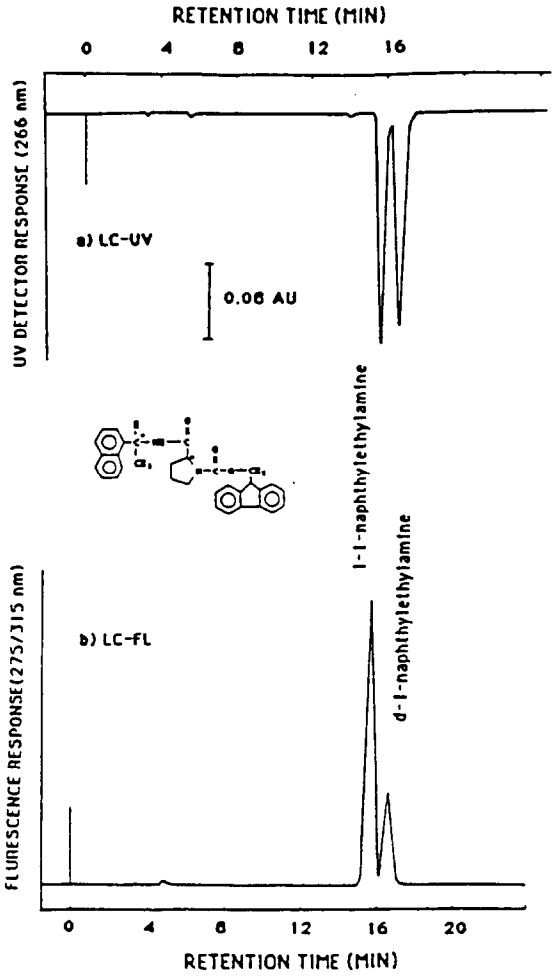

Fig. 8. HPLC-UV/FL chromatograms for a 50:50 mixture of the diastereomers of d,l-naphthylethylamine, derivatized off-line with the polymeric chiral reagent 9-FMOC-L-proline. Conditions of analysis: 11% IPA–HEX, 0.7 ml/min flow-rate, 1 ppth (thousand) of each enantiomer present; derivatization volume, 50 μl; dilution volume, 2 ml; injection, 20 μl; LiChrospher Si-60 column, 5 μm, 250 × 4 mm I.D.; FL 275/315 nm; UV 266 nm. Reproduced from ref. 105 with permission.

This may have been due to internal fluorescence quenching for one of the two diastereomers, thus leading to a lowered FL response. As discussed elsewhere, this is one potential pitfall in using the indirect method of chiral recognition, *i.e.* a pair of diastereomers may show different detector responses, as here. Using multiple detection, such as UV/FL in combination, should highlight this potential deficiency and allow for its correction.

In simple biofluid matrices, such as urine, Gao *et al.* [90] and Gao and Krull [106] described in 1989 the use of this indirect approach for a typical drug, amphetamine. This was perhaps the first time that a polymeric chiral reagent, *e.g.* 9-FMOC-L-proline (Fig. 1), was used to perform indirect chiral recognition from a biofluid. Several other enantiomeric drugs were also investigated using the same method, in order to demonstrate the generality of the approach and the lack of kinetic resolution. The overall method, using the instrumental set-up of Fig. 4, was validated by several experiments, including: (1) kinetic studies for the reaction of each enantiomeric drug with the solid-phase chiral reagent; (2) single blind spiking experiments; and (3) polarimetry for confirmation of the enantiomeric composition determined by the solid-phase diastereomer formation, HPLC-UV/FL method. All resulting diastereomers from urine were well resolved (Fig. 9), $R_s = 1.05–1.40$, under typical reversed-phase conditions. Enantiomeric contamination at the 1.1% level and below could be detected. The lowest amount of d,l-amphetamine that could be simultaneously derivatized and detected was *ca.* 50 ng/ml in the biofluid. The linearity of the overall measurement was 3–4 orders of magnitude. d,l-Amphetamine spiked into urine at different concentrations and different d/l ratios, followed only by filtration, was directly injected onto the on-line, solid-phase derivatization HPLC-UV/FL system for quantitation with relative standard deviations of 1.8–6.4% and relative errors of 0.6–9.8% [106].

10. Direct injection of biofluids with solid-phase derivatizations in HPLC

In most derivatizations for analytes in biofluids, prior to HPLC or any other separation–detection scheme, it has been necessary to first remove the analyte from its matrix. Thus, a great deal of sample preparation was necessary, filtration, protein precipitation, release of drug from

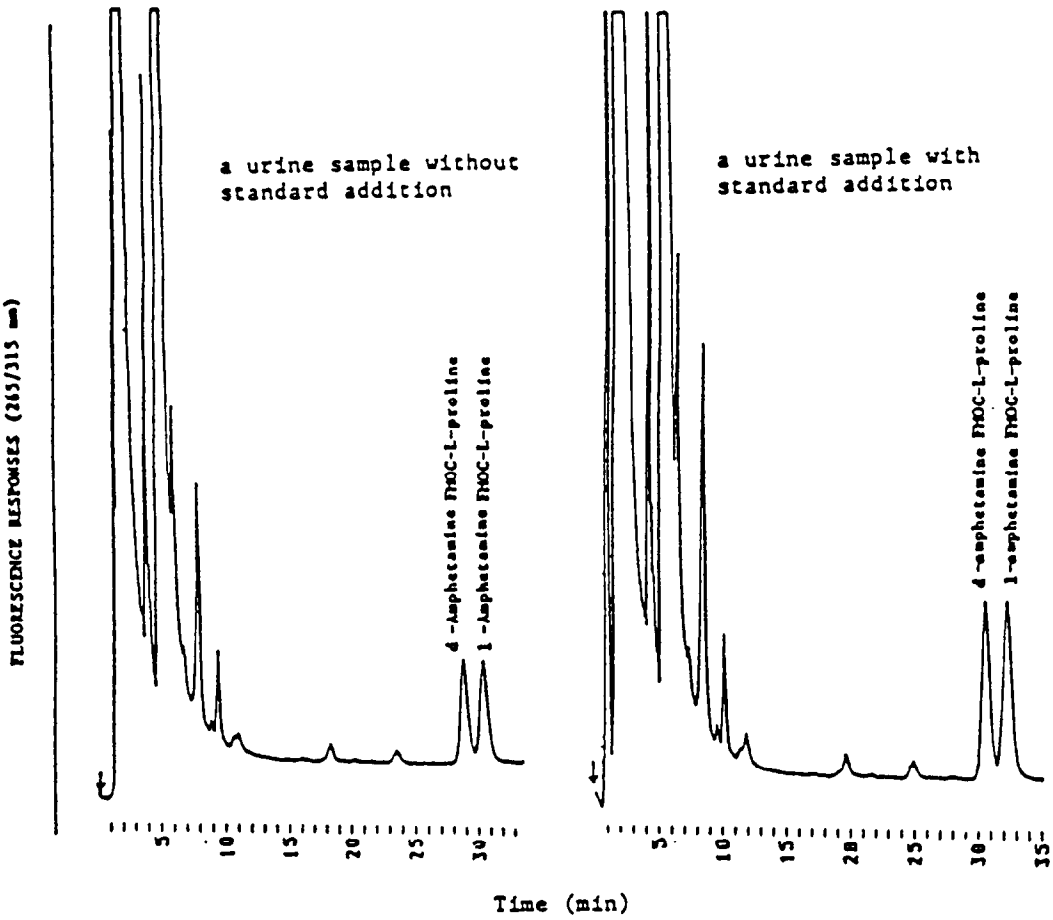

Fig. 9. HPLC-FL chromatogram for low levels of d,l-amphetamine in urine derivatized on-line, pre-column, with the 9-FMOC-L-proline chiral reagent from direct urine injections. Analytical conditions: reaction cartridge 27 × 2.0 mm I.D. at 60°C for 5 min, 10-μl injections of amphetamine in urine, mobile phase 40–48% ACN–H_2O isocratic, flow-rate 1.5 ml/min, separation column LiChrospher C_{18}, 5 μm, 250 × 4.0 mm I.D., UV 265 nm, FL 265/315 nm. Reproduced from ref. 106 with permission.

proteins, solid-phase extraction of the drug from the biofluid, centrifugation of precipitated proteins, cleanup of the isolated analyte/drug, derivatization in solution, removal of excess unreacted reagent, concentration of the final derivatized analyte, and only then injection–detection. However, it appeared that a much simpler approach might be to combine solid-phase extraction on a polymeric, hydrophobic support along with immobilized reagent derivatizations.

Urine is a somewhat cleaner biofluid matrix than plasma or whole blood, and thus it can be directly injected onto a RP-HPLC column without concern about protein precipitation, clogging of the frits/column, and the system will tolerate repeated injections with good overall reproducibility. However, much more complex biofluids such as plasma or serum eluded this direct injection approach until 1992. Direct injections allow for several sample pretreatment steps to be performed on-line, often with full automation. Zhou *et al.* have now described the use of a 9-fluorenylacetyl (9-FA) solid-phase reagent for derivatization in direct plasma injection [34]. Using a controlled pore support of 60 Å, made by silica template polymerization of the PS–DVB resin, it was possible to analyze for drugs such as amphetamine in plasma with virtually no

sample pretreatment prior to injection onto the polymeric reagent/support. On-line, pre-column derivatization was performed by direct injection of diluted plasma sample into a sodium dodecyl sulfate (SDS) containing mobile phase. The SDS was used to stabilize the plasma samples to gel formation, thus allowing them to remain on an autosampler for very long periods of time.

Second, the SDS provided long-term solubilization of the proteins in the plasma, so that no precipitation occurred when the plasma matrix reached the hydrophobic polymeric support. Thus, a combination of surfactant enhanced HPLC conditions for solubilization of the biopolymers, together with polymeric reagent derivatization of the drugs present in the plasma, was developed. The SDS solubilized proteins eluted early in the final chromatogram, with longer retention for the derivatized drug analytes, depending on which tag was actually being used. Because SDS was present in the mobile phase as well as in the sample itself, column efficiencies were compromised by adsorption of the surfactant onto the C_{18} surface. As described below, this could be improved by voiding the matrix components after adsorption of the hydrophobic drug analytes, again using SDS in the sample but now *not* in the mobile phase. This provided increased long-term stability of the stationary phase and improved peak shapes and resolutions, overall. The drug, such as amphetamine, after sample introduction, was trapped in the hydrophobic derivatization column and derivatized at elevated temperature by the activated solid-phase reagent. The derivatized 9-FA amphetamide was then separated by reversed-phase HPLC with a step gradient and determined by either UV/FL detection. This solid-phase reagent combined with a surfactant containing mobile phase, provided a sensitive and simple system for on-line derivatization in direct injection analysis of biofluids.

Fig. 10 illustrates a series of repeated, on-line derivatizations of 10 μg/ml (10 ppm) amphetamine spiked plasma, with chromatographic conditions as indicated. The injections were performed manually with valve switching, using the instrumental arrangement indicated in Fig. 4. The use of SDS in the samples and mobile phase leads to early elution of the more hydrophillic plasma components, such as amino acids, peptides, lipids, and proteins.

However, the same SDS also causes a loss of peak efficiency and peak shape, as illustrated by the rather broad peaks obtained for the FA-amphetamine derivatives. This can be improved, as described below, by using an automated valve switching arrangement that passes the plasma/biofluid components to waste after extraction of the desired analytes/drugs onto the hydrophobic solid-phase reagent. Thus, SDS is only needed in the plasma/biofluid sample, but not in the mobile phase, since no plasma components ever reach the HPLC column in this sample venting mode of operation [36]. Peak shapes, plate counts, resolutions, and overall chromatographic performance is greatly enhanced, as shown below, in comparison to this manual valve switching operation that passes the entire biofluid sample through the HPLC column [34].

An analogous study was recently reported by Zhou and Krull, in which they performed direct enantiomeric analysis of amphetamine in plasma by simultaneous solid-phase extraction and chiral derivatization [33]. This approach, now combining a polymeric chiral reagent, as used above for amphetamine in urine, with on-line, solid-phase extraction and diastereomer formation from plasma, was performed both manually and with full automation, using a Gilson sample preparation station and gradient elution HPLC conditions. Thus, this represented a completely automated approach for performing indirect chiral recognition of drugs in plasma, combining all of the procedures described above for manual operations, but now with full automation via the Gilson sample preparation station system. Spiked plasma samples were directly injected onto the solid-phase reactor column automatically, and a series of washing steps were then performed to elute the larger molecular mass components prior to derivatization of the smaller analytes on the same support. High-molecular-mass protein components were excluded from the bulk of the reagent by the small pore structure of the polymeric support and the pres-

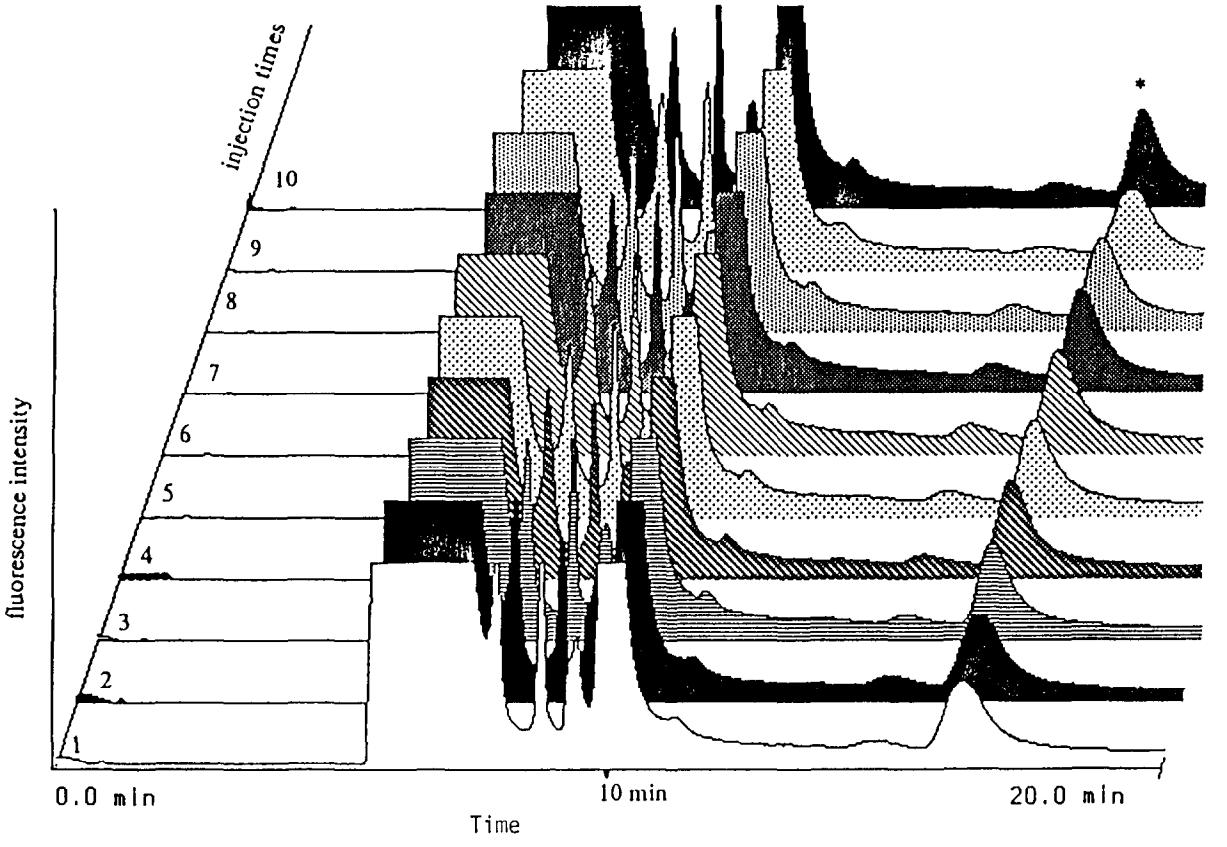

Fig. 10. Chromatograms for repeated, on-line derivatizations of 10 μg/ml amphetamine-spiked plasma. Chromatographic conditions: mobile phase: step gradient from 1 m*M* SDS in ACN–H_2O (10:90, v/v) to 1 m*M* SDS in ACN–H_2O (55:45, v/v) after derivatization; flow-rate 1.5 ml/min; injection volume: 20 μl; separation column, Supelcosil LC-ABZ, 150 × 4.6mm I.D.; derivatization column: 35 × 2 mm I.D.; derivatization temperature: 75°C; derivatization time: 8.0 min. Reproduced from ref. 34 with permission.

ence of SDS. These were thus washed out of the reactor, as described elsewhere in this paper, before derivatization occurred. Spiked amphetamine in plasma was thus extracted and derivatized by the polymer-based 9-FMOC-L-proline solid-phase reagent. The derivatized diastereomers, incorporating a strong chromophore, were then separated on a conventional, achiral C_{18} column with an ACN–H_2O mobile phase under gradient or isocratic conditions. Calibration plots and reproducibility experiments were performed to demonstrate the overall validity of this newer approach. Automation of the entire chiral recognition procedure provided a simple and reproducible method for the direct injection of plasma samples with indirect chiral recognition and absolute quantitation [33].

In a related study, Fisher and Bourque have recently described the determination of amphetamine in urine using solid-phase extraction, polymeric reagent derivatization, and reversed-phase HPLC with UV detection [107]. The limit of detection here was 14 ppb (ng/ml), and the limit of quantification was 47 ppb. The calibration curve for this approach was linear from 0.01 to 4.0 μg/ml (ppm).

11. Hydrophobic *versus* hydrophillic analytes in polymeric reagent derivatizations

In general, the ability of an analyte to be derivatized on a polymeric support is dependent on the degree of transport or diffusion through the polymer into the hydrophobic pores. The bulk of the active ester reagent resides within such hydrophobic pockets, while much less is present on the surface, also available for reactions. The more hydrophobic the analyte, the easier it will enter these hydrophobic domains and be reacted. The less hydrophobic or more hydrophillic the analyte, such as amino acids and peptides, the more difficult they will enter the pores and the less reaction will occur. Thus, if one simply takes an amino acid and tries to derivatize it in water with these polymeric reagents based on PS–DVB supports of controlled pore sizes, *no* reactions can be detected [48]. If the amino acids have any degree of overall charge, *i.e.* when they are not at their isoelectric points, it becomes almost impossible to efficiently derivatize them within the pores of the polymeric reagent. Zhou *et al.* have recently demonstrated improved derivatizations of amino acids and peptides using ion-pairing and phase-transfer catalysis approaches with achiral polymeric reagents [48]. Using cationic surfactants, such as cetyltrimethylammonium bromide (CTAB) and others, the at basic pHs negatively charged amino acids and peptides, were neutralized by ion-pairing complexation and derivatized by a 9-FA tagged *o*-nitrobenzophenol reagent. Using an off-line derivatization, the effects of ion-pairing reagent, concentration of reagent, buffer pH, reaction temperature, time, and other derivatization conditions were evaluated and optimized, again in a univariate approach. The derivatization of amino acids from protein hydrolysates was demonstrated for several proteins, performing off-line tagging of the thus formed amino acid mixtures separated under gradient elution HPLC-UV/FL conditions. The derivatization of peptides from enzymatically digested proteins, such as cytochrome C, was also demonstrated, in order to show the overall feasibility of peptide mapping with solid-phase derivatization. It should also prove possible to use these same approaches, perhaps in an automated, on-line manner, in order to derivatize peptides in aqueous solutions and biofluids. A combination of surfactant (micellar) enhanced mobile phase conditions, again using SDS or SOS as additives in the mobile phase or only in the biofluid, together with on-line solid-phase tagging, sample cleanup and removal of biopolymers, along with preconcentration of the peptide from the aqueous sample, *should* allow for direct injection of aqueous and biofluid type samples under fully automated analysis conditions. However, the use of basic conditions and a surfactant in the sample may cause unacceptable degradation of the polymeric reagent in an on-line set-up. If ways can be found around this last-mentioned problem, then these approaches should enable trace detection and quantitation of peptides in biofluids, by using a combination of on-line peptide preconcentration, cleanup of the sample matrix, backflushing, peak compression under gradient elution RP conditions, and finally, rapid elution of the tagged peptide with multidetection.

An illustration of the use of this approach for peptide mapping is given in Fig. 11a–c, for a solid-phase derivatized mixture of trypsin digested cytochrome C [48]. In this case, the protein was first digested off-line, detached from the derivatization and HPLC systems, and then the entire peptide mixture was derivatized on the above described polymeric reagent. The resultant mixture of tagged peptides was then manually injected onto the HPLC system with dual detection. Fig. 11a shows a blank test of the solid-phase reagent with digestion solution alone, Fig. 11b shows a chromatogram of the digested cytochrome C without solid-phase tagging, and finally, Fig. 11c presents the solid-phase derivatization chromatogram of the trypsin digested cytochrome C. In comparison with the HPLC-UV analysis for the same sample of peptides formed by digestion with trypsin, the 9-FA tagged peptides offer a complementary chromatogram under analogous RP conditions, but

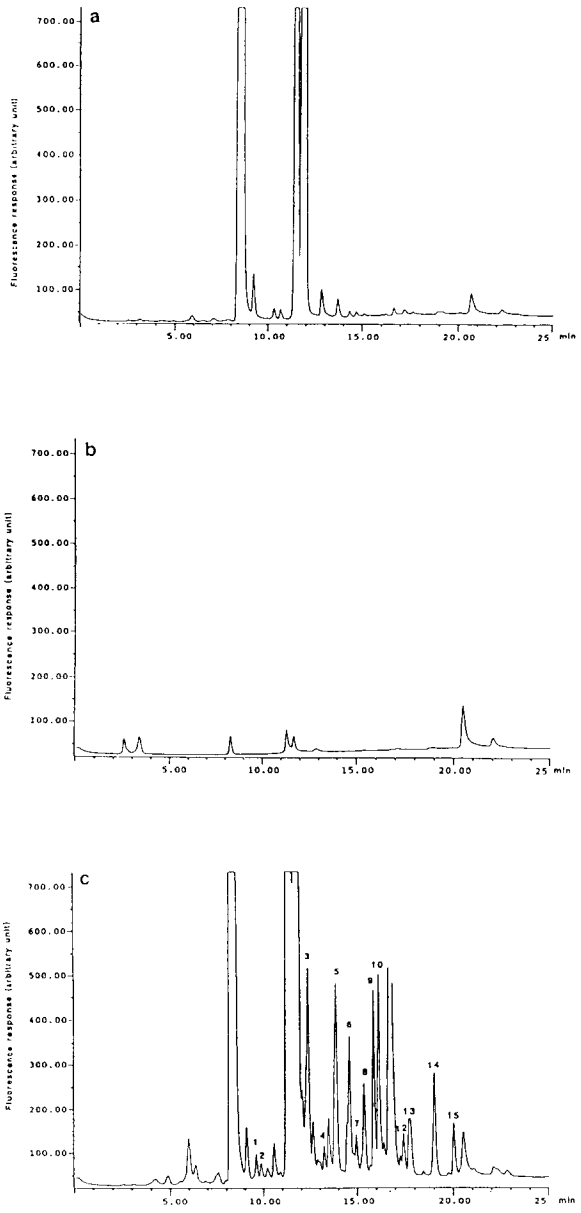

Fig. 11. Solid-phase (9-FA) derivatization of trypsin digested cytochrome C. Separation with a YMC AP-303 300A ODS column, 250 × 4.6 mm I.D.; detection: UV at 214 nm, 0.05 RFU; sample: 20 μl of trypsin digested cytochrome C; gradient separation in 25 min at a flow-rate of 1.5 ml/min; mobile phase A: 0.05% TFA–H_2O, B: 0.05% TFA–ACN in a stepwise gradient. Chromatograms: (a) blank test of solid-phase reagent with digestion solution alone (trypsin with buffer); (b) digested cytochrome C without derivatization; and (c) solid-phase derivatization of trypsin digested cytochrome C. Reproduced from ref. 48 with permission.

now with enhanced detectability due to the FL tags. Thus, chromatographic and detector properties are often improved or at least altered compared with the direct HPLC-UV/FL analysis of the untagged peptide mixture. The advantages of these approaches for the analysis of peptides or amino acids in protein hydrolysates, aqueous stabilized solutions of peptides/proteins, and eventually peptides in biofluids, should be apparent [108].

A serious draw-back in the use of ion-pairing techniques to draw the hydrophillic analytes into the reactive pores is that base (*e.g.* NaOH) may also wet the reagent walls and hydrolyze the tag. This results in a large peak due to the hydrolysis product of the 9-FA or 9-FMOC tagged reagent, which may, at times, cause interference with the desired amino acid or peptide derived product [48]. This is less of a problem when reactions are performed off-line, but becomes a major problem on-line, when larger volumes of buffer continuously pass through the reactor leading to much larger hydrolysis. At times, the hydrolysis peak, which is often 9-fluorenylacetic acid (9-FA) originating from the polymeric 9-FA tagged reagent, overshadows the derivatized amino acids with similar capacity factors. Even using a 12% crosslinked support with a narrow pore size does not protect the reagent within the pores from hydrolysis due to the wetting action of the surfactants present. With cationic surfactants the problem is only magnified. It is possible that with other tags, hydrolysis products may elute away from the derivatized amino acids or peptides, *e.g.* 9-fluorenylmethanol from the 9-FMOC tag. In other cases, the use of a 6-aminoquinoline (6-AQ) tag may lead to a hydrolysis product, namely 6-AQ itself, which fluoresces at wavelengths different than the 6-AQ tagged amino acids or peptides [109–111]. This is an approach that is now being pursued, using immobilization of the 6-AQ onto a polymeric support via the carbamate linkage [74]. In this procedure, the 6-AQ is linked/leashed to the polymer via an activated carbamate, similar to the previously described 3,5-DNP immobilized carbamate reagent for chiral recognition. Thus, this is another way to overcome the problems that increased

hydrolysis of the tag results in peaks in the chromatogram interfering with those of the desired analyte derivative peaks.

It is possible that other approaches may allow for improved derivatizations of very hydrophillic analytes, such as larger peptides. Though for smaller peptides and most amino acids the conversion is $>50\%$ at basic pH, this may become $<50\%$ when the peptide grows and becomes more hydrophillic due to side-chain carboxylic groups present. This will then lead to higher detection limits, poorer sensitivity, and so on. Other methods to neutralize the ionic charges could involve metal–ligand complexation, as in ligand-exchange chromatography [112–114]. However, this could result in binding of the free amino groups needed for tagging, as well as in a increased hydrolysis of the tag.

One other report exists on the solid-phase derivatization of amino acids, that by Zhang *et al.* [115]. These authors used a polymeric benzotriazole leash to the same polymer support reported earlier by Gao *et al.* [88]. To this leash they then attached the same 9-FMOC-L-proline chiral tag. They derivatized a number of amino acids in ACN or ACN–H_2O. This led to the expected diastereomers, which could then be separated as their individual peaks under conventional RP conditions. Chiral recognition and enantiomeric excess were readily quantifiable, although the approach was neither on-line or automated. The nature of the underlying polymer was mainly micro- or non-porous, and thus the reactions with the amino acids were actually occurring on the surface and not within the pores. Entry of the hydrophillic amino acids into the pores was not necessary with this type of support. However, hydrolysis of the tag must have been considerable.

12. Automated derivatization approaches, on-line in HPLC via polymeric reagents: simultaneous solid-phase extraction and derivatization

The first attempts to automate solid-phase derivatizations involved the use of a Gilson Model 231 or 232 automated sample preparation station, interfaced with a commercial Gilson gradient elution, reversed-phase HPLC system with dual detection modes (UV/FL) [36]. In this overall approach (Fig. 12), using the 9-FA tagged reagent, numerous samples could be automatically processed and introduced into the polymeric reagent cartridge. This was placed on a 6-port switching valve, microprocessor controlled via a PC station, with an automated syringe pump to withdraw samples from sealed vials and to delivered them to the polymeric reagent cartridge. The cartridge could be equilibrated and washed by a series of preparation solutions, again microprocessor controlled, prior to introduction of the samples. The samples could also be bracketed with ACN–H_2O mixtures to improve reagent access, push the sample further and further into the reactor bed, and then elute matrix (biofluid) components to waste without them ever reaching the RP column. It was possible to inject very large volumes of crude samples onto the reactor in this manner, thereby effecting improved concentration detection limits, overall. After washing and cleanup of the analytes, now hydrophobically adsorbed onto the polymer support, the temperature could be raised or maintained at 60–70°C throughout the process, reactions of the adsorbed and preconcentrated analytes would ensue together with sample cleanup, and the final, more hydrophobic derivatives would remain physically adsorbed onto the polymer support. The analytes were derivatized as they were extracted and preconcentrated, thus allowing almost unlimited sample volumes to be injected. The derivative was then backflushed from the reaction cartridge onto the head of the RP column, and as a function of the gradient composition, peak compressed before elution. Thus, both preconcentration of the analytes and peak compression could be done in an automated manner. Gradual gradient elution would then elute the desired derivative separated from any remaining sample matrix components and/or hydrolysis products of the reagent itself. This led to relatively simple and clean chromatograms for typical drugs in urine and plasma, with very few potentially interfering peaks other than,

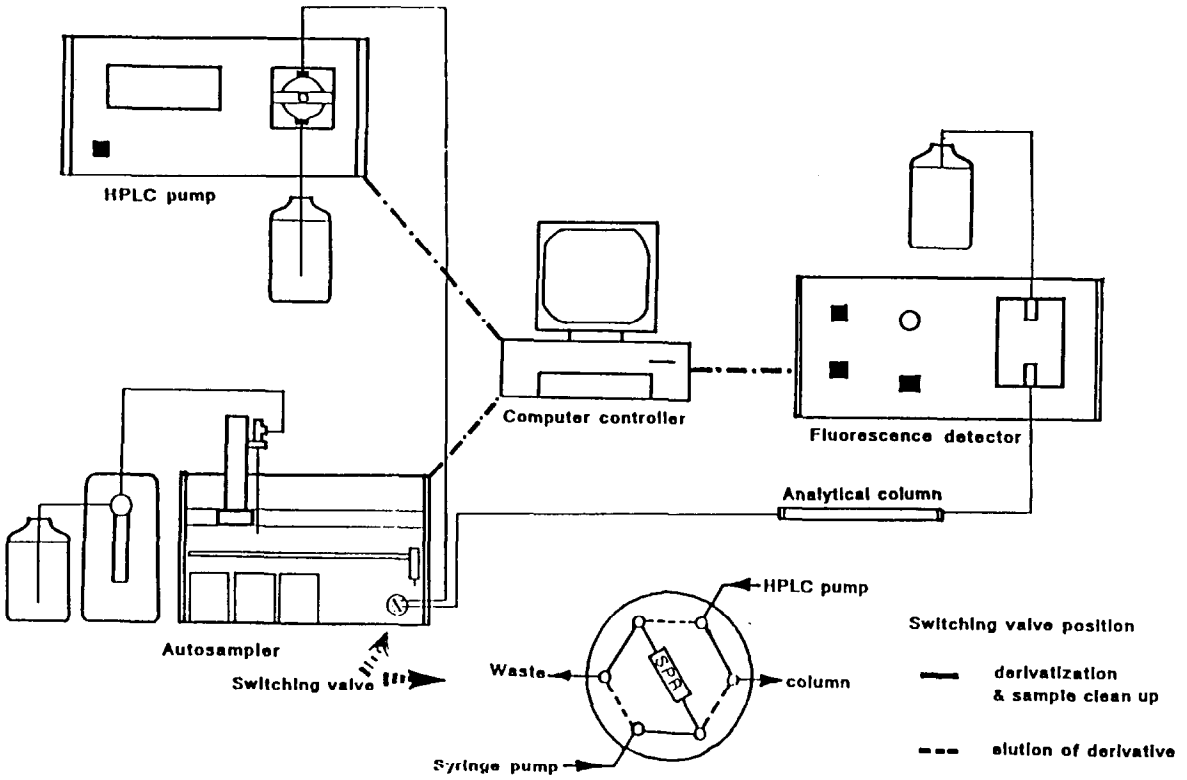

Fig. 12. A schematic illustration of the overall Gilson automated apparatus for sample preparation, cleanup, and solid-phase derivatization with gradient elution HPLC-UV/FL conditions. Reproduced from refs. 35,36 with permission.

most often, the 9-FA or 9-FMOC hydrolysis peaks [36]. The polymeric cartridge could be used for at least 100 injections without accruing an unacceptable reduction in sensitivity. A detection limit of at least 500 ppt (0.5 ppb) of amphetamine in urine was realized with this system, using less than 0.5 ml of sample injected. Fig. 13 shows a typical chromatogram using this completely automated, on-line solid-phase derivatization scheme, with conditions indicated. Both amphetamine and methamphetamine were analyzed in these particular samples, with good quantitation in terms of accuracy, precision, and overall reproducibility. With plasma samples, quantitation was also possible, and chromatograms were just as simple. Again, the only interfering peak of any size was the 9-FA hydrolysis product.

An analogous study was recently reported by Zhou *et al.* for the determination of adamantanamine in plasma and urine by direct injection onto the HPLC system [35]. The method now involved a fully automated on-line simultaneous extraction and derivatization of the drug in the biofluids (urine/plasma) with the 9-FA solid-phase reagent. This approach again eliminated tedious sample preparation steps and provided automated derivatization with selective and efficient sample cleanup for direct injection of virtually all biofluids. Derivatized adamantanamine was separated under reversed-phase conditions and detected by FL. The optimization and validation of the derivatization–detection method was described, again using an on-line solid-phase reactor (cartridge) placed on a microprocessor controlled switching valve placed after the automated injector and before the HPLC column (Fig. 14) [35]. Full automation of these

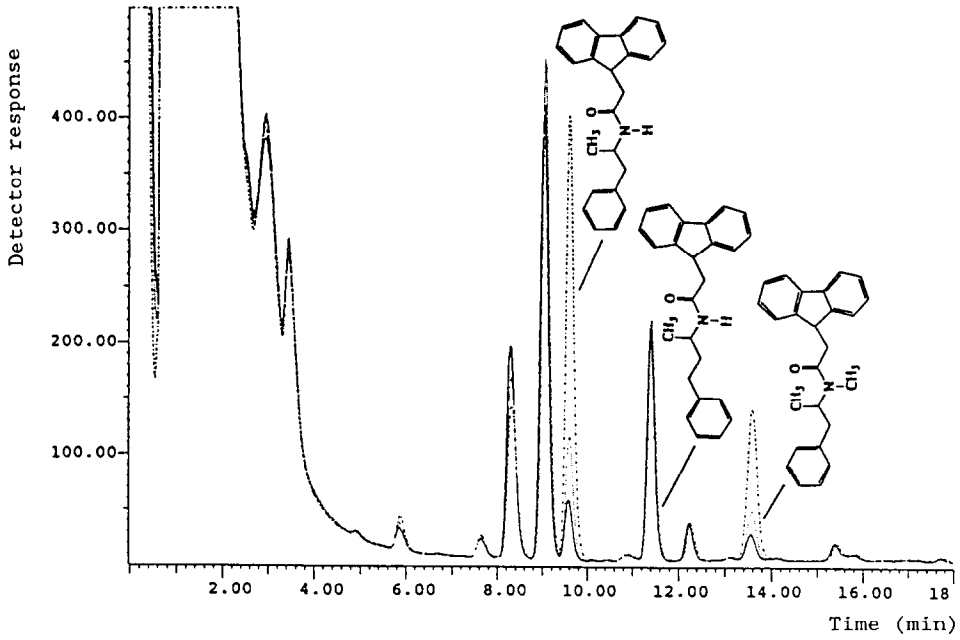

Fig. 13. Automated analysis for amphetamine and methamphetamine in urine using on-line, pre-column solid-phase derivatizations. The concentrations were 25, 50, and 200 ppb for each drug with constant levels of the internal standard. HPLC conditions: step-gradient elution: 0–3.5 min, 50% ACN; 3.5–15.5 min, 50–70% ACN; 15.5–18 min, 70% ACN; 18–19 min, 70–50% ACN; column: 150 × 4.6 mm I.D. LC-18-DB column with a guard column in-place; derivatization conditions: 100 μl sample at 60°C for 30 s. Reproduced from ref. 36 with permission.

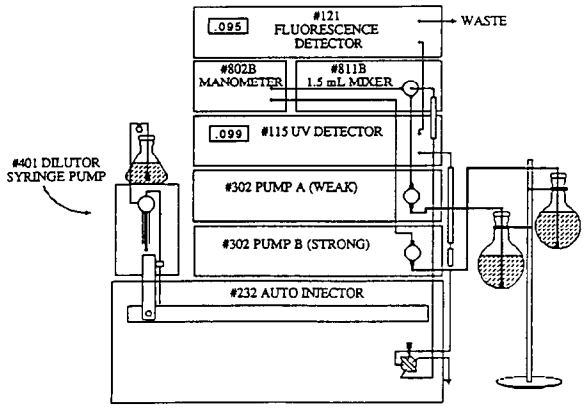

Fig. 14. Diagram of the fully automated Gilson sample preparation station and HPLC system for performing on-line, pre-column, solid-phase derivatizations of drugs in biofluids. Reproduced from ref. 36 with permission.

techniques was described in this study, using a Gilson automated sample preparation station with full microprocessor control. The sample preparation station was fully interfaced with a Gilson microprocessor controlled gradient elution HPLC system, with multi-detection modes (UV/FL). Reproducibility of the overall analytical results was easy to demonstrate, again with direct injection of both urine and plasma spiked samples. Accuracy and precision of the quantitative determinations was also demonstrated. Fig. 15 illustrates a typical automated, pre-column, on-line derivatization of adamantanamine in plasma, with conditions as indicated. More specific conditions showing the use of SDS in the rinse (cleanup) buffers and sample injections is found elsewhere [35]. A series of 59 automated, repeat injections of the same sample of adamantanamine in plasma was performed using identical injection, on-line sample preparation (cleanup), derivatization, and separation–

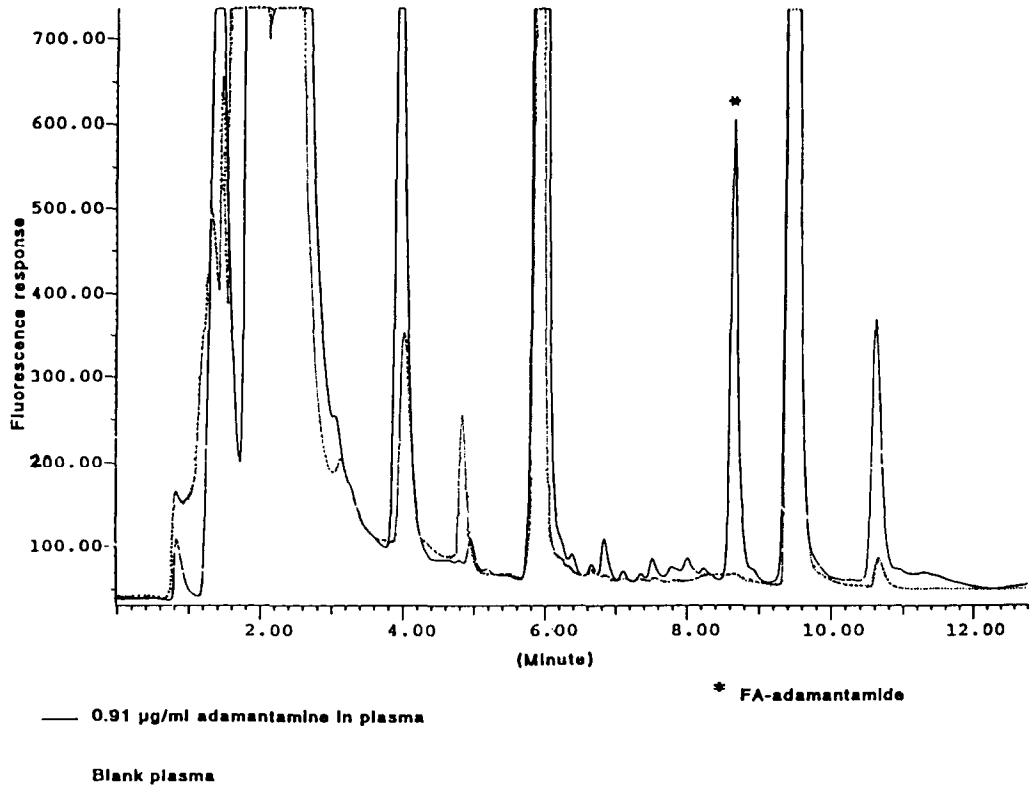

Fig. 15. A typical HPLC-FL chromatogram illustrating the automated, pre-column, on-line derivatization of adamantanamine in plasma. Derivatization was performed using the 9-FA tagged reagent on 12% DVB–PS substrate in a 27 mm × 2.1 mm I.D. reactor; derivatization at 75°C for 3 min; 50 μl of 1.36 μg/ml adamantanamine in 50 m*M* NaOH. Chromatographic conditions: mobile phase A, 100% H_2O; mobile phase B, 100% ACN; mobile phase flow-rate, 1.5 ml/min; total separation gradient time, 13.47 min (0.00 min = 55% B; 1.50 min = 55% B; 5.50 min = 85% B; 10.50 min = 85% B; 11.50 min = 55%B). Both blank (· · · · ·) and sample (——) chromatograms are presented. Reproduced from ref. 35 with permission.

detection conditions, with the peak areas indicated *versus* injection number in Fig. 16 [35].

13. Conclusions and future prospects

The potential applications of manual or automated solid-phase derivatizations of suitable nucleophilic species in organic or aqueous solutions has become a firm reality within recent years. Any number of polymeric reagents can be synthesized, purified, characterized, retagged, reproducibly used for numerous samples on each batch, and modified in terms of leash, support, tag, hydrophobicity and so forth. Though most previously used polymeric reagents made use of a hydrophobic support, *i.e.* PS–DVB, this too can be modified to make it less hydrophobic and more hydrophillic. However, this may increase the rate of hydrolysis of the tag. The addition of ion-pairing reagents to the sample allows for ion-pair formation and phase-transfer catalysis of the derivatization, when the hydrophillic analyte can enter the hydrophobic pores through ion-pairing mechanisms. The use of chiral polymeric reagents permits indirect enantiomeric recognition processes to occur in neat samples, formulations, stabilized solutions, and biofluids, always with acceptable accuracy, precision, and reproducibility. Mixed-bed approaches permit the formation of several derivatives at the same time, and by varying reaction and reagent con-

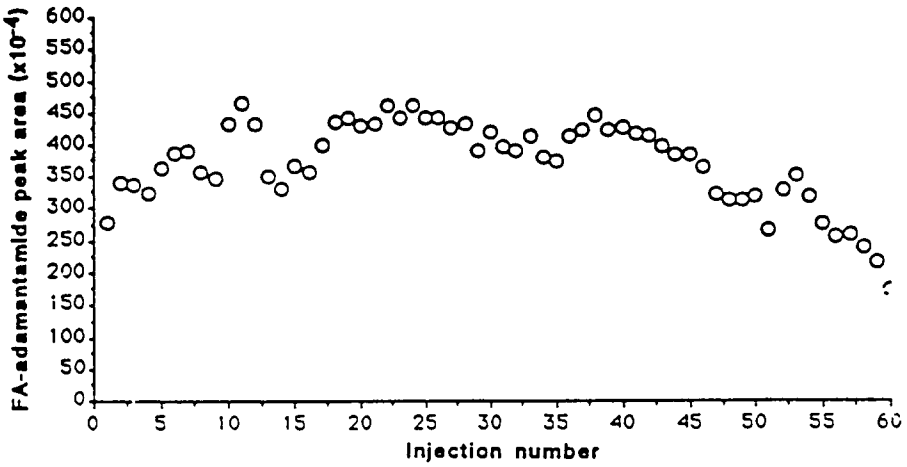

Fig. 16. Demonstration of reproducibility for a series ($n = 60$) of automated, repeated injections of the same sample of 0.91 μg/ml adamantanamine in plasma, using identical injection, on-line sample preparation (cleanup), derivatization, and separation–detection conditions, as in Fig. 15, with the peak areas indicated *vs.* injection number. Reproduced from ref. 35 with permission.

ditions, improved qualitative and quantitative determinations become feasible and practical. The use of 3,5-DNP or Pi-base tagging of enantiomeric, nucleophilic species permits direct recognition of these chiral species via the use of CSPs. This technique too is fully automatable, so that any number of chiral drug samples could be analyzed for both chemical and chiral purity using the polymeric reagent approaches in HPLC or HPCE [116]. Enantiomeric purity determinations of virtually all primary/secondary amines, alcohols, thiols, anilines, phenols, and other weak nucleophiles should also be entirely feasible. Automated tagging of achiral substrates, again strong or weak nucleophiles, is now also possible, so that 100 samples can be analyzed overnight for chemical purity, using polymeric achiral reagents.

However, perhaps the most exciting areas for further development involve direct injection of crude samples, especially biofluids. It should be apparent by now that the *direct* injection approach for drugs and bioorganics in samples of virtually all types of biofluid is a truly general approach that should be immediately applicable to the study of *in vivo* metabolism, pharmacokinetics, and for drug screening. Even fermentation broths of recombinant peptides (size dependent) should be suitable samples. Also amenable would be drugs of abuse that can be easily derivatized by these reagents. The ability to eliminate almost all sample preparation and pretreatment steps, other than simple filtration and perhaps pH adjustment, which are also automatable, represents a significant step forward in the ability to determine trace levels of various nucleophilic type drugs in biofluids. Applications of these automated approaches to the analysis of amino acids in foods/beverages/drugs, amino acid hydrolysates of proteins, peptide maps, peptides in biofluids, and so forth, should all be suitable areas for future/current studies.

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will appear, it is hoped, on future publications, several currently being in press or submitted for publication (1994).

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References

[1] Abstracts of the 3rd International Conference on Polymer Supported Reactions in Organic Chemistry, Jerusalem, July, 1986.

[2] N.K. Mathur, C.K. Narang and R.E. Williams, *Polymers as Aids in Organic Chemistry*, Academic Press, NY, 1980.

[3] W.T. Ford (Editor), *Polymeric Reagents and Catalysts*, ACS Symposium Series 308, American Chemical Society, Washington, DC, 1986.

[4] P. Hodge and D.C. Sherrington (Editors), *Polymer-Supported Reactions in Organic Synthesis*, J. Wiley and Sons, New York, 1980.

[5] P. Hodge and D.C. Sherrington (Editors), *Syntheses and Separations Using Functional Polymers*, J. Wiley and Sons, New York, 1988.

[6] P. Laszlo (Editor), *Preparative Chemistry Using Supported Reagents*, Academic Press, San Diego, CA, 1987.

[7] A. Akelah and A. Moet, *Functionalized Polymers and Their Applications*, Chapman and Hall, London, 1990.

[8] A. Patchornik and M.A. Kraus, *in Encyclopedia of Polymer Science and Technology*, Suppl. 1, J. Wiley and Sons, New York, 1976, p. 468.

[9] A. Patchornik, *Nouv. J. Chimie*, 6 (1982) 639.

[10] R. Kalir, A. Warshawsky, M. Fridkin and A. Patchornik, *Eur. J. Biochem.*, 59 (1975) 55.

[11] M. Kraus and A. Patchornik, *Chemtech*, Feb. (1979) 118.

[12] Y. Shai, K.A. Jacobson and A. Patchornik, *J. Am. Chem. Soc.*, 107 (1985) 4249.

[13] J.F. Studebaker, S.A. Slocum and E.L. Lewis, *Anal. Chem.*, 50 (1978) 1500.

[14] K. Jedrzejczak and V.S. Gaind, *Fresenius' J. Anal. Chem.*, 344 (1992) 133.

[15] K. Jedrzejczak and V.S. Gaind, *Analyst*, 117 (1992) 1417.

[16] R.B. Merrifield, *J. Am. Chem. Soc.*, 85 (1963) 2149.

[17] J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis*, W.H. Freeman, San Francisco, CA, 1969.

[18] C.K. Narang, K. Brunfeldt and K.E. Norris, *Tetrahedron Lett.*, 19 (1977) 1819.

[19] C.U. Pittman, in P. Hodge and D.C. Sherrington (Editors), *Polymer Supported Reactions in Organic Synthesis*, John Wiley and Sons, New York, 1980, Ch. 5.

[20] I.S. Krull, K.-H. Xie, S. Colgan, T. Izod, U. Neue, R. King and B. Bidlingmeyer, *J. Liq. Chromatogr.*, 6 (1983) 605.

[21] K.-H. Xie, S. Colgan and I.S. Krull, *J. Liq. Chromatogr.*, 6 (1983) 125.

[22] I.S. Krull, S. Colgan, K.-H. Xie, U. Neue, R. King and B. Bidlingmeyer, *J. Liq. Chromatogr.*, 6 (1983) 1015.

[23] K.-H. Xie, C. Santasania, I.S. Krull, U. Neue, B. Bidlingmeyer and A. Newhart, *J. Liq. Chromatogr.*, 6 (1983) 2109.

[24] I.S. Krull, S. Colgan, K.-H. Xie, C. Santasania, U. Neue, R. King, A. Newhart and B. Bidlingmeyer, in E. Reid and I.D. Wilson (Editors), *Methodological Surveys in Biochemistry and Analysis*, Plenum Press, London, 1984, p. 173.

[25] S.T. Colgan, I.S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr. Sci.*, 26 (1988) 501.
[26] J.M. Rosenfeld, O. Hammerberg and M.C. Orvidas, *J. Chromatogr.*, 378 (1989) 9.
[27] S.T. Colgan, I.S. Krull, U. Neue, A. Newhart, C. Dorschel, C. Stacey and B. Bidlingmeyer, *J. Chromatogr.*, 333 (1985) 349.
[28] S.T. Colgan, I.S. Krull, C. Dorschel and B. Bidlingmeyer, *Anal. Chem.*, 58 (1986) 2366.
[29] J.F. Stobaugh, *Paper T-L5 presented at the Fourth International Symposium on Pharmaceutical and Biomedical Analysis*, Baltimore, MD, April 18–21, 1993.
[30] A.J. Bourque, I.S. Krull and B. Feibush, *Anal. Chem.*, 65 (1993) 2983.
[31] F. Montanari and P. Tundo, *J. Org. Chem.*, 46 (1981) 2125.
[32] T. Balakrishnan, S.H. Babu and A. Perumal, *J. Polymer Sci., Part A: Polymer: Polymer Chem.*, 28 (1990) 1421.
[33] F.-X. Zhou and I.S. Krull, *Chromatographia*, 35 (1993) 153.
[34] F.-X. Zhou, I.S. Krull and B. Feibush, *J. Chromatogr.*, 609 (1992) 103.
[35] F.-X. Zhou, I.S. Krull and B. Feibush, *J. Chromatogr.*, 619 (1993) 93.
[36] A.J. Bourque, I.S. Krull, and B. Feibush, *Biomed. Chromatogr.*, in press (1994).
[37] (a) Technical Bulletins on DerivaChrom-OH and Derivatizing Reagents, Supelco Corporation, Supelco Park, Bellefonte, PA; (b) Technical Bulletins on 2,4-DNPH on Silica Reagent, Waters Chromatography Division, Millipore Corporation, Milford, MA.
[38] J.M. Rosenfeld, M. Mureika-Russell and A. Phatak, *J. Chromatogr.*, 283 (1984) 127.
[39] G.E. Martin, M.B. Shambhu, S.R. Shakhshir and G.A. Digenis, *J. Org. Chem.*, 43 (1978) 4571.
[40] J.J. Rebek and J.E. Trend, *J. Amer. Chem. Soc.*, 101 (1979) 737.
[41] K. Lloyd and G.T. Young, *J. Chem. Soc.*, 93 (1971) 2890.
[42] A. Patchornik and M.A. Kraus, *J. Am. Chem. Soc.*, 92 (1970) 7587.
[43] F.-X. Zhou, A.J. Bourque, M.E. Szulc and I.S. Krull, Unpublished results (1992–93).
[44] T.C. Pinkerton, T.D. Miller, S.E. Cook, J.A. Perry, J.D. Rateike and T.J. Szczerba, *BioChromatography*, (1986) 96.
[45] J.A. Perry, *J. Liq. Chromatogr.*, 13 (1990) 1047.
[46] J.A. Perry, L.J. Glunz, T.J. Szczerba and J.D. Rateike, *LC/GC*, 8 (1990) 832.
[47] J. Haginaka, *TrAC*, 10 (1991) 17.
[48] F.-X. Zhou, I.S. Krull and B. Feibush, *J. Chromatogr.*, 648 (1993) 357–365.
[49] C.-X. Gao and I.S. Krull, *BioChromatography*, 4 (1989) 222.
[50] S.T. Colgan and I.S. Krull, in I.S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 5.
[51] C.-X. Gao, D. Schmalzing and I.S. Krull, *Biomed. Chromatogr.*, 5 (1991) 23.
[52] M.E. Szulc and I.S. Krull, *Biomed. Chromatogr.*, 6 (1992) 269.
[53] I.S. Krull, F.-X. Zhou and J.-H. Yu, *TrAC*, 12 (1993) 159.
[54] I.S. Krull, J. Mazzeo, M. Szulc, J. Stults and R. Mhatre, in E. Katz (Editor), *Liquid Chromatography Analytical Techniques in Biotechnology*, J. Wiley and Sons, New York, in press (1994).
[55] M.E. Szulc and I.S. Krull, *J. Chromatogr. A*, 659 (1994) 231–245.
[56] I.S. Krull and M.E. Szulc, *LC/GC* , 11 (1993) 350.
[57] R.W. Frei, H. Jansen and U.A.Th. Brinkman, *Anal. Chem.*, 57 (1985) 1529a.
[58] B. Lillig and H. Engelhardt, in I.S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 1.
[59] U.A.Th. Brinkman, *Chromatographia*, 24 (1987) 190.
[60] J.R. Poulsen, K.S. Birks, M.S. Gandelman and J.W. Birks, *Chromatographia*, 22 (1986) 231.
[61] C.M. Selavka, K.-S. Jiao and I.S. Krull, *Anal. Chem.*, 59 (1987) 2221.
[62] U.A.Th. Brinkman, *Chromatographia*, 24 (1987) 190.
[63] L.D. Bowers, in I.S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 4.
[64] C.M. Selavka and I.S. Krull, *J. Energetic Mat.*, 4 (1986) 273.
[65] C. M. Selavka, I.S. Krull and K. Bratin, *J. Pharm. Biomed. Anal.*, 4 (1986) 83.
[66] C.M. Selavka, I.S. Krull and I.S. Lurie, *Forensic Sci. Int.*, 31 (1986) 103.
[67] L. Dou, J.R. Mazzeo and I.S. Krull, *BioChromatography*, 5 (1990) 74.
[68] H.P.M. Van Vliet, T.C. Bootsman, R.W. Frei and U.A. Th Brinkman, *J. Chromatogr.*, 185 (1979) 483.
[69] H. Jansen, U.A. Th. Brinkman and R.W. Frei, *Chromatographia*, 20 (1985) 453.
[70] C.-Y. Jeng and S.H. Langer, *J. Chromatogr.*, 589 (1992) 1.
[71] S.T. Colgan, I.S. Krull, U. Neue, A. Newhart, C. Dorschel, C. Stacey and B. Bidlingmeyer, *J. Chromatogr.*, 333 (1985) 349.
[72] S.T. Colgan, I.S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr. Sci.*, 26 (1988) 501.
[73] O.R. Idowu and G.O. Adewuyi, *J. Liq. Chromatogr.*, 16 (1993) 2501.
[74] J. Yu and I.S. Krull, *Paper presented at the 23rd Annual Northeast Regional Meeting (NERM) of the American Chemical Society*, Boston, MA, June, 1993.
[75] F.-X. Zhou, J.M. Thorne and I.S. Krull, *TrAC*, 11 (1992) 80.
[76] M.-C. Milliot, B. Sebille and J.-P. Mahieu, *J. Chromatogr.*, 354 (1986) 155.
[77] M.-C. Millot and B. Sebille, *Reactive Polymers*, 13 (1990) 177.
[78] F.-X. Zhou, J. Wahlberg and I.S. Krull, *J. Liq. Chromatogr.*, 14 (1991) 1325.

[79] H.-M. Zhang, F.-X. Zhou and I.S. Krull, *J. Pharm. Biomed. Anal.*, 10 (1992) 577.
[80] M.B. Shambhu and G.A. Digenis, *J. Chem. Soc., Chem. Comm.*, (1974) 619.
[81] G.E. Martin, S.B. Manvendra and G.A. Digenis, *J. Pharm. Sci.*, 67 (1978) 110.
[82] G.E. Martin, M.V. Shambhu, S.R. Shakhshir and G.A. Digenis, *J. Org. Chem.*, 43 (1978) 4571.
[83] T.-Y. Chou, S.T. Colgan, D.M. Kao, I.S. Krull, C. Dorschel and B.A. Bidlingmeyer, *J. Chromatogr.*, 367 (1986) 335.
[84] K.-H. Xie, C.T. Santasania, I.S. Krull, U. Neue, B. Bidlingmeyer and A. Newhart, *J. Liq. Chromatogr.*, 6 (1983) 2109.
[85] S.T. Colgan, I.S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr. Sci.*, 26 (1988) 501.
[86] R. Kalir, A. Warshawsky, M. Fridkin and A. Patchornik, *Eur. J. Biochem.*, 59 (1975) 55.
[87] T.-Y. Chou, C.-X. Gao, S.T. Colgan, I.S. Krull, C. Dorschel and B.A. Bidlingmeyer, *J. Chromatogr.*, 454 (1988) 169.
[88] C.-X. Gao, T.-Y. Chou, S.T. Colgan, I.S. Krull, C. Dorschel and B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 26 (1988) 449.
[89] B.J. Cohen, H. Karoly-Hafeli and A. Patchornik, *J. Org. Chem.*, 49 (1984) 922.
[90] C.-X. Gao, T.-Y. Chou and I.S. Krull, *Anal. Chem.*, 61 (1989) 1538.
[91] C.-X. Gao, I.S. Krull and T.M. Trainor, *J. Chromatogr.*, 463 (1989) 192.
[92] C.-X. Gao, I.S. Krull and T.M. Trainor, *J. Chromatogr. Sci.*, 28 (1990) 102.
[93] C.-X. Gao and I.S. Krull, *J. Chromatogr.*, 515 (1990) 337.
[94] M.E. Szulc and I.S. Krull, unpublished results (1992).
[95] A.J. Bourque and I.S. Krull, *J. Chromatogr.*, 537 (1991) 123.
[96] A.J. Bourque and I.S. Krull, *J. Chromatogr. Sci.*, 29 (1991) 489.
[97] A.J. Bourque, R. Strong and I.S. Krull, unpublished results (1992–93).
[98] M. Zief and L.J. Crane (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1988.
[99] I.W. Wainer and D.E. Draye (Editors), *Drug Stereochemistry, Analytical Methods and Pharmacology*, Marcel Dekker, New York, 1988.
[100] W.H. Pirkle and J. Finn, in J.D. Morrison (Editor), *Asymmetric Synthesis, Analytical Methods, Vol. 1*, Academic Press, NY, 1983, Ch. 6.
[101] W.H. Pirkle and T.C. Pochapsky, in K.K. Unger (Editor), *Packings and Stationary Phases in Chromatographic Techniques*, Marcel Dekker, NY, 1990, p. 783.
[102] S.G. Allenmark, *Chromatographic Enantioseparation: Methods and Applications*, Halsted Press, J. Wiley and Sons, Chichester, 1988.
[103] R.W. Souter, *Chromatographic Separations of Stereoisomers*, CRC Press, Boca Raton, FL, 1985.
[104] A.J. Bourque and I.S. Krull, *J. Pharm. Biomed. Anal.*, 11 (1993) 495.
[105] T.-Y. Chou, C.-X. Gao, N. Grinberg and I.S. Krull, *Anal. Chem.*, 61 (1989) 1548.
[106] C.-X. Gao and I.S. Krull, *J. Pharm. Biomed. Anal.*, 7 (1989) 1183.
[107] D.H. Fisher and A.J. Bourque, *J. Chromatogr.*, 614 (1993) 142.
[108] S. Agayna, J. Yu, D. Chang and I.S. Krull, unpublished results (1993).
[109] P.J. Brynes, P. Bevilacqua and A. Green, *Anal. Biochem.*, 116 (1981) 408.
[110] S.A. Cohen and D.P. Michaud, *Anal. Biochem.*, 211 (1993) 279.
[111] S.A. Cohen and D.J. Strydom, *Anal. Biochem.*, 174 (1988) 1.
[112] J.F. Lawrence, U.A.Th. Brinkman and R.W. Frei, in I.S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 6.
[113] M.T.W. Hearn (Editor), *Ion-Pair Chromatography, Theory and Biological and Pharmaceutical Applications*, Marcel Dekker, New York, 1985.
[114] P.E. Hare, in M. Zief and L.J. Crane (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1988, Ch. 6.
[115] Z. Zhang, G. Malikin and S. Lam, *J. Chromatogr.*, 603 (1992) 279.
[116] M.E. Szulc and I.S. Krull, *Paper presented at the 23rd Annual Northeast Regional Meeting (NERM) of the American Chemical Society*, Boston, MA, June, 1993.