Flow Injection and Sequential Injection On-line Pre-column Derivatization for Liquid Chromatography

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Abstract

Approaches of on-line pre-column derivatization with flow injection (FI) and sequential injection (SI) for liquid chromatography (LC) are reviewed. Considerations in interfacing FI/SI to LC is discussed. Employing such on-line derivatization pre-column pretreatment approaches, applications to organic species (biomedical, food, pharmaceutical, environmental, and other types of samples) as well as metal ions are presented. Further developments in the approaches are recommended.

Introduction

Chromatography is the most widely accepted method for chemical separation and determination of the components in complex mixtures. High-performance liquid chromatography (HPLC) is one of the most versatile chromatographic techniques. Its popularity is due to the sensitivity, ready adaptability to accurate quantification, suitability for separation of a variety of substances including nonvolatile or semi-volatile species, which are not sufficient to be analyzed by gas chromatography (GC). HPLC is usually applied for amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, pesticides, antibiotics, steroids, metal-organic species, and a variety of inorganic substances. There are several modes of separation in HPLC, namely normal-phase, reversed-phase, ion-exchange, ion-pair, etc. However, HPLC signals are very susceptible to the compositions of the sample matrix or interfering constituents in the complex samples.

Derivatization in LC

Derivatization is an important sample pretreatment to minimize errors due to possible interferences from the sample matrices before chromatographic analysis and to improve the selectivity and detectability of the analysis. In many cases, the analytes can be preconcentrated at the same time. Attempts have been made in either off-line or on-line approaches. Off-line derivatization would be simple but time-consuming and labor-intensive, which is more troublesome when large numbers of samples are to be involved and may increase the chances that some of the analytes will be lost or contaminated. An alternative approach is that interface of the chromatographic system by the on-line derivatization unit using flow methods, whereby the drawbacks of the off-line procedures can be overcome.

On-line derivatization for LC can be performed either pre- or post-column. In the post-column procedure, a chromogenic reagent is mixed with column eluent in a post-column reactor and the product formed is detected. Therefore, the reaction(s) in this post-column derivatization should be rapid. A post-column reagent to generate low background signal is desired. To avoid the reaction, band broadening, dead volume associated with connecting tubing, mixing device, and detection flow cell must be minimized. It should be noted that with the post-column reactors, band broadening could be much reduced by using segmented-flow procedures. Nowadays, a variety of post-column reactors for different detection systems have been commercially available.

On the other hand, on-line derivatization can be performed prior to chromatographic separation. This would offer advantages for compounds with poor separation due to strong adsorption on the stationary phase, or labile compounds, which may easily decompose or react with other components during chromatographic separation. Changing the chemical and physical properties of the derivative or comparing with the original analytes would lead to changes in chromatographic characteristics (1). This would increase the selectivity of the analyte–stationary phase interaction when comparing to the underivatized analyte and the matrix components. Benefits in sensitivity and selectivity would be gained from on-line pre-column derivatization. In the pre-column derivatization procedure, the exceeded reagent is ensured to be completely separated from the derivatives and does not contribute to the increase in background signals at the peak position of the analyte of interest.

In the comparison between pre-column and post-column techniques, it should be stressed out for the former that it requires (i) a fast reaction, (ii) a quantitative reaction, and (iii) the formation of a single derivative (otherwise a single component would give several peaks during the HPLC step), which for compounds bearing several chemically reactive groups may be unsuitable. These drawbacks are not critical for post-column techniques, provided that a suitable calibration procedure is used.

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Flow Injection and Sequential Injection

Since its introduction in 1975 by Ruzicka and Hansen (2), flow injection (FI) has experienced wide acceptance in the chemical analysis community. As is well-known, FI is based on injection of a solution sample into a moving unsegmented continuous stream of a suitable liquid/solution. The injected sample forms a zone, which is then transported toward a detector that continuously records the signal, as it continuously changes as a result of the passage of sample zone through the flow cell. Sequential injection (SI) was first reported in 1990 by Ruzicka and Marshall (3). While most FI procedures employ continuous, uni-directional pumping of carrier and reagent streams, SI is based on using programmable, bi-directional discontinuous flow as precisely coordinated and controlled by a computer. Although the aspiration and propulsion systems in SI are different to those used in FI, the basic principles are quite similar. Via a selection valve, sample and reagent(s) are sequentially aspirated into a holding coil. The zones overlap by reversing the flow direction, and the reaction product is consequently formed. Apart from their uses as stand-alone analytical techniques, FI and SI can be utilized as effective on-line sample handling tools (4,5).

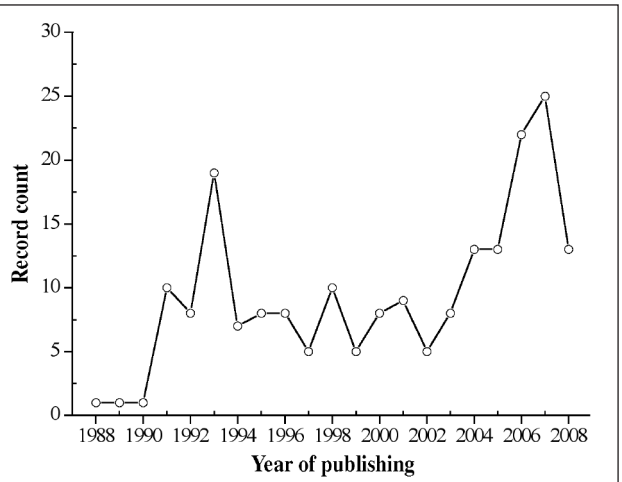

Figure 1. Contributions published in the period 1988–Nov 2008 containing the terms "online derivatization" and "liquid chromatography" (source: ISI Web of Knowledge[SM])

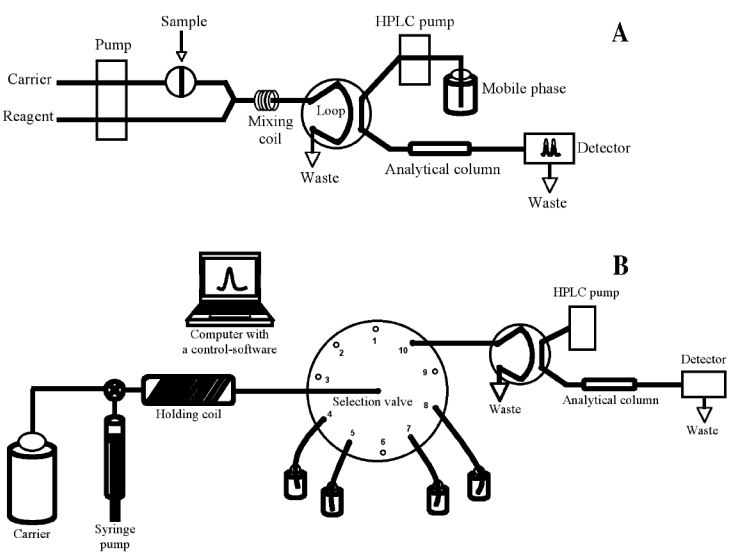

Figure 2. FI-LC (A) and SI-LC (B) instrumentation set-up.

FI or SI can be hyphenated with LC to perform on-line sample pretreatments, mainly preconcentration, matrix removal, and derivatization. In the period of 20 years (1988–2008), more than 190 articles, demonstrating the on-line derivatization for liquid chromatography, have been published, as presented statistically in Figure 1. Within these literatures, those coupling FI or SI for on-line derivatization prior to LC will be discussed in this review.

Interfacing FI or SI to LC

There are several possible arrangements for coupling FI and SI to HPLC for on-line pre-column derivatization, and the most commonly used are depicted in Figure 2. The FI/SI manifold can be directly interfaced with the injection valve of the LC by a PTFE tube. In the FI-HPLC system, the sample is injected into the carrier stream via a common low pressure 6-port injection valve and merged with the reagent stream. The derivative would form in-line along the mixing coil as well as during transfer to the HPLC system. The reaction time for on-line derivatization is precisely controlled by the flow rate and the dimensions of the mixing reactor. The experimental parameters of the FI system should be optimized in order to improve the sensitivity. The concentrated zone of the derivative is to be selected to fill the HPLC loop. This is determined by the travel time of the product zone between the injection port of FI and that of HPLC system and the flow rate of the FI system. In some cases, reverse FI (rFI) may be used instead of the common "normal" FI as describe earlier. In that case, a small volume of reagent is injected via the injector, while the sample flows continuously. To improve the analytical sensitivity for some reactions, a heating system is required. Therefore, the mixing coil is immerged in the temperature-controlled water bath or heating oven.

For the SI-HPLC system, derivative would form in a holding coil by sequential aspiration of sample and reagent zones using the bi-directional syringe pump and a multi-position selection valve. Apart from putting the reaction coil in the heating oven or water bath, in the SI system a higher degree of mixing can be promoted by several flow reversals of the syringe pump. The appropriated product zone can be selected by determining the dispensed volume of the syringe pump after forming a derivative.

In both FI and SI systems, coupling to HPLC, the connection between the FI/SI to HPLC loop should be as short as possible in order to prevent further dispersion of the product zone. Another consideration involves compromising between volume of derivative product and volume of the HPLC loop. When injecting a large volume in an HPLC system, peak broadening will be observed; although, theoretically, the sensitivity of the FI/SI system should increase by increasing a sample volume. Therefore, the optimum volume of the HPLC loop should be considered, and the derivative should be formed such that it is just enough for filling in the HPLC loop.

Application to Organic Analytes

FI or SI coupled to LC for on-line pre-column derivatization of organic analytes has been applied to various types of samples with diverse matrices. In the past 20 years, 1988–2008, in literature, there were 23 methods reported for the determination of organic compounds by HPLC with on-line pre-column derivatization. Of the methods, 57% were used for the determination of the organic compounds in biological samples, 26% for the determination in environmental samples, 17% for food (and beverages) and pharmaceutical samples.

Biomedical samples

The application of FI or SI for on-line pre-column derivatization of organic analytes in biomedical samples has received more attention than in other areas. Selective reactors/reagents can be easily incorporated in FI/SI manifolds to allow the on-line derivatization of compounds of biological and clinical importance. Nozaki and Ohba (6) reported the on-line extraction and fluorescence derivatization of urinary free noradrenaline by column switching and reversed-phase HPLC. Urinary noradrenaline was simultaneously extracted and derivatized with alkaline mobile phase containing *o*-phthalaldehyde (OPA) and 2-mercaptoethanol in a boric acid gel column. After switching the column, the fluorescent derivatized catecholamines were separated with an ODS-4PW column, and the fluorescence was monitored. The instability of OPA derivatives of catecholamines after derivatization was eliminated because the derivatives were produced on-line and analyzed within 20 min after sample injection.

In 1990, the on-line micellar phase transfer catalysis (MPTC) was developed for the automated determination of free fatty acids in plasma by reversed-phase HPLC (7). The mechanism of MPTC is similar to that of phase transfer catalytic (PTC); a charged analyte is extracted with a lipophilic ion-pair agent into a suitable organic solvent, in which the analyte can be directly derivatized. In MPTC, an organic pseudo-phase using non-ionic micelle is used instead of a separate organic phase. The plasma samples were analyzed after derivatization with the fluorophore 9-bromomethylacridine (Br-MAC) in an on-line MPTC unit. The complications of protein precipitation during derivatization and chromatography could be avoided using an in-line filter.

Another application of on-line derivatization HPLC for biomedical samples was reported as a short communication paper in 1994 by Funakoshi et al. (8). It was the determination of busulfan in human serum by on-line derivatization and column switching. Busulfan was derivatized with sodium diethyldithiocarbamate on the first short column. The back-flushed derivative was then separated on the second column. Finally, the heart-cut fraction containing the derivative was further analyzed on the analytical column of HPLC and monitored with UV detection.

On-line derivatization HPLC was developed for in vivo monitoring of brain extracellular glutamate, as well as other amino acids in anesthetized rat (9). The method involved microdialysis perfusion technique and an HPLC system. The microdialysate was on-line derivatized with OPA through a mixing tee prior to automatic injection into the HPLC column. Glutamate concentrations determined by this system were similar to those obtained from an off-line derivatization procedure. This on-line system would be useful for various research projects, including the neuroscience investigations when analyses of amino acids are required.

A new application using photocatalytic oxidation for the derivatization of 5-hydroxyindoles (5-His) was proposed by Todoroki et al. (10). A photocatalytic column comprising of tefzel tubing packed with TiO_2-coated soda-lime glass beads was used as a pre-column reactor. The fluorescence given derivatization with benzylamine proceeded during the passage of the sample through the reaction column while under near-UV irradiation. The derivatives were separated continuously on the reversed phase HPLC system. This method was applied to the determination of 5-hydroxyindole-3-acetic acid in urine.

Application on biological fluids of FI-HPLC has been extended to the determination of amines in urine samples (11–13) using solid-phase derivatization. Solid-phase derivatizations are selective reactions between the reagent immobilized on a solid support and the analyte in solution (13). The polymeric reagents were synthesized and packed in a short column attached to the HPLC system. Recently, two or more reagents can be used in a single reactor (called a mixed-bed reactor) to form multiple derivatives simultaneously. Solid-phase derivatizations avoid dilution of the analyte with reagent as only the amount that reacts with the analyte is used, excess reagent remains bound to the solid matrix. Thus, several derivatizations can be performed using the same on-line reactor column.

The first automated derivatization protocol based on the on-line coupling of SI to HPLC was reported in 2004 by Zacharis et al. (14). The feasibility of SI-HPLC was demonstrated for the determination of γ-aminobutyric acid (GABA) in human biological fluids based on the reaction with OPA and fluorimetric detection. The set-up was fully automated enabling precise computer-control of all major reaction parameters, such as sample and reagent volumes and reaction time, using cost-effective and simple low-pressure instrumentation.

Food and pharmaceutical samples

The trace-level analysis of foods, beverages, and drugs by HPLC often requires derivatization of the analytes in order to attain the desired sensitivity and selectivity. Recently, hyphenation of on-line clean-up and/or derivatization for food and pharmaceutical samples have been proposed as alternatives to overcome sample handling problems. Farjam et al. (15) investigated an on-line automated immunoaffinity sample pretreatment of aflatoxins in milk prior to column LC. The system consisted of an immuno pre-column packed with immobilized monoclonal or polyclonal antibodies, a second pre-column packed with C_{18} bonded silica, and a C_{18} analytical column. Defatted milk was loaded onto the immuno pre-column and desorbed by eluting the immuno pre-column with methanol–water. The analytes were subsequently on-line concentrated on the C_{18} bonded silica pre-column before separation on the analytical column.

A concept to combine multi-dimensional LC and on-line derivatization for determination of biogenic amines in wine was proposed by Hyötyläinen et al. (16). Sample clean-up, derivatization, and separation of amines could be operated in a closed on-line system, consisting of a cation-exchange pre-column, a derivatization coil, and the analytical column. The sample was

injected into the pre-column. After washing, the amines were eluted to a derivatization coil and mixed with the derivatization reagent in a T-piece, and the reaction took place in the reaction coil. The derivatives formed were then separated on the analytical column. In this work, OPA was used as the derivatization reagent, which was well suited for on-line derivatization, as the reaction was fast. The problem in instability of the OPA derivatives could be overcome because the analytes were immediately transferred to the analytical column.

In 2005, García-Villar et al. (17) proposed an HPLC method for determination of histamine in red wine samples, based on continuous flow derivatization with 1,2-naphthoquinone-4-sulfonate (NQS). This approach resulted in an attractive alternative to conventional off-line labeling techniques and allowed full automation of the derivatization process and a minimization of by-products from the hydrolysis or oxidation processes. In contrast to other pre-column methods proposed in the literature for histamine analysis, the removal of excess reagent was avoided as it does not cause any interference. The coupling of on-line pre-column derivatization to the HPLC system reduces the analysis time as well as minimizes the possible systematic errors arising from sample handling procedures. Furthermore, degradation of reagent derivatives is minimized. The simplicity of sample treatment is another advantage deserving attention.

Application of on-line derivatization with LC for pharmaceutical samples was proposed by Zacharis et al. in 2006 (18). A fully automated pre-column derivatization protocol for the determination of 14 amino acids was developed using OPA as the reagent. The SI was used for sampling, reagent mixing, and introducing into LC loop. The SI and the LC may run in parallel modes: the LC may analyze the sample, while at the same time the SI can process the next sample. The instrumentation simplicity of SI offers significant flexibility in method development due to the possibility to perform multivariate studies in the same set-up.

Environmental samples

The determination of organic contaminants in environmental samples requires appropriate sample pretreatments aimed at removing interfering constituents from the sample, as well as preconcentration and derivatization for improving analyte detectability. Chun and Krull (19) developed an on-line solid-phase derivatization-HPLC system for determination of volatile amines. A reaction column containing the polymeric fluorenyl reagent was connected to the loop position on the valve and placed into a constant-temperature water bath. The sample solution was injected and the analyte was held within the reaction column for 5 min before flushing the derivative formed into the separation/analytical column. One single analysis, from injection, derivatization, separation, to detection, of the aliphatic amines and polyamines was achieved within 30 min. The possible advantages in performing on-line solid-phase derivatizations in HPLC with this polymeric reagent were fast and efficient analysis, sensitive for most amines in air samples, accurate and precise analyses, less sample work-up, inexpensive, and great potential for automation.

Other types of samples

The feasibility of on-line pre-column derivatization LC has also been investigated in a variety of samples. Eikenes et al. (20) developed a selective and sensitive method for the determination of chitosan using acidic hydrolysis and on-line derivatization with OPA in the LC system. The developed method was applied for wooden and aqueous samples. The method offered a high degree of repeatability. The problem with degradation of the fluorescent product between the analytes and OPA was avoided using on-line derivatization. Chen et al. (21) proposed a simple and sensitive method for the determination of formaldehyde in textile samples. The derivatization occurred on a cation-exchange column attached in the injection valve of HPLC system. The resin used as solid support for the derivatization was previously charged with 2,4-dinitrophenylhydrazine (DNPH). Formaldehyde reacted with DNPH to form the corresponding hydrazone before LC analysis.

Application to Metal Ion Analysis

Apart from the element-selective techniques, such as, atomic spectrometry, inductively coupled plasma spectrometry, LC has been recognized as one of the methods for multi-element analysis. The RP-HPLC technique with pre-column derivatization has been indicated to be a favorable and reliable technique for the separation and determination of trace amounts of metal ions. Most of the reports on metal analysis by chromatographic method are based on the separation of their chelates (22–27).

In 1998, Ali et al. (28) interfaced the FI on-line derivatization and preconcentration system to the LC equipment using a home-designed micro-preconcentration column containing RP-C_{18} material as solid sorbent for preconcentration of Ni(II), Cu(II) and Hg(II) as their diethyldithiocarbamate (DDC) chelates. The micro-column was accommodated in a pressure-tight housing of stainless steel combined with the LC six-port valve. The performance of the developed system was demonstrated by determination of these metals in waste water samples from an electric power station and a machine tool factory.

Another application of FI-HPLC for on-line metal complexation was reported in 2006 by Srijaranai et al. (23). The reverse FI was chosen instead of normal FI because of its low background noise for HPLC baseline as well as low reagent [4-(2-pyridylazo) resorcinol, PAR] consumption. The system was fit for the analyses of Cr(VI), Co(II), Ni(II), and Cu(II) in chrome plating waste water samples.

In 2007, we reported the coupling of SI with HPLC for on-line derivatization of some heavy metals [Co(II), Ni(II), Cu(II) and Fe(II)] using 2-(5-nitro-2-pyridylazo)-5-[*N*-propyl-*N*-(3-sulfopropyl)amino]phenol (nitro-PAPS) as the chelating reagent (29). To the best of our knowledge, it was the first time that SI has been exploited as the on-line pre-column derivatization system for metal analysis by HPLC. The SI system offered automated handling of sample and reagent, on-line pre-column derivatization and propulsion of the derivatives formed into the HPLC system. By parallel operation of derivatization and separation, advantages of less consumption of sample/reagent, as well as better analysis time could be gained. Such a system has been investigated for the determination of metals in various samples, including food, pharmaceutical, and water samples.

Conclusion and Outlook

In this review article, FI and SI are presented as a front-end to liquid chromatography for on-line derivatization. The feasibility of the hyphenated technique for multi-component analysis has been demonstrated by particular application in a wide variety of analytes in diverse matrices.

It would be of interest for further explorations directed towards miniaturization of an on-line pre-column derivatization system. The third generation of flow injection, the so-called micro-sequential injection lab-on-valve (μSI-LOV) or a simple and economic sequential injection lab-at-valve (SI-LAV) (30,31), may be excellent alternatives to conventional flow injection and sequential injection. In combination with bead injection-lab-on-valve (BI-LOV) approach (32), on-line solid phase derivatization and/or preconcentration prior to liquid chromatography should be explored. This could be extended to the discovery of the novel solid phase for handling within the BI-LOV format. The renewable bead injection in the LOV conduit has proven to be a very powerful methodology for on-line sample pretreatment for trace metal prior to electrothermal atomic absorption spectrometry (ETAAS) (33,34), but it should be noted that there is only one report exploiting the coupling of BI-LOV and LC system (35). It would be also interesting to explore the μSI-BI-LOV-LC for various applications as well as on-line renewable solid-phase derivatization using μSI-BI-LOV prior to capillary electrophoresis.

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