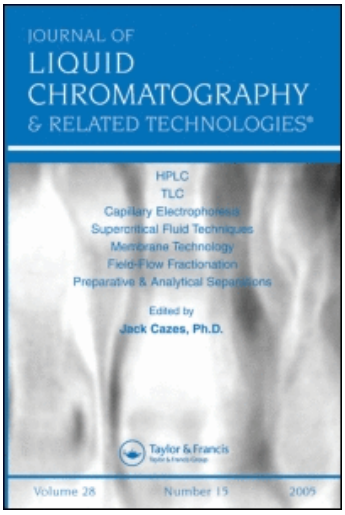

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Derivatization and Post-Column Reactions for Improved Detection in Liquid Chromatography/Electrochemistry

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To cite this Article Krull, Ira S. , Selavka, Carl M. , Duda, Chester and Jacobs, Wesley(1985) 'Derivatization and Post-Column Reactions for Improved Detection in Liquid Chromatography/Electrochemistry', Journal of Liquid Chromatography & Related Technologies, 8: 15, 2845 — 2870
To link to this Article: DOI: 10.1080/01483918508076607
URL: http://dx.doi.org/10.1080/01483918508076607

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(15), 2845-2870 (1985)

DERIVATIZATION AND POST-COLUMN REACTIONS FOR IMPROVED DETECTION IN LIQUID CHROMATOGRAPHY/ ELECTROCHEMISTRY

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ABSTRACT:

A summary is provided of most of the reported derivatizations that have been used for improved analyte detection in liquid chromatography with electrochemical detection (LCEC). These approaches include pre-column derivatizations and postcolumns chemical, photochemical or enzymatic reactions for oxidative EC detection. This review covers the literature up to early 1985, and includes information gathered from books, technical articles, previous reviews and scientific journal publications. Specific reagents, methods and instrumentation are described for those classes of compounds studied by derivatization-LCEC, and suggestions for future experiments are included, where applicable. It is concluded that the future will likely include the development of a great number of derivatizations which may be used in conjunction with LCEC for trace analysis.

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0148-3919/85/0815-2845$3.50/0

Introduction

Derivatizations have become very commonplace in LC, especially for ultraviolet-visible (UV-VIS) and fluorescence (FL) detection [1-19]. An extensive summary and review of post-column reactions in LC will shortly appear [20], but this text will only contain slight mention of EC detection. Therefore, other than for a very brief technical note in a commercial publication [21], this is the first review dedicated solely to derivatizations for LCEC.

Most of the derivatization approaches described for LCEC have involved off-line, pre-column chemical reactions as opposed to on-line, pre- or post-column reactions. Thus, derivatives are generally formed in a separate step, prior to injection onto the LC system. The off-line format allows great flexibility in selection and optimization of reaction chemistry since any necessary manipulations are readily carried out without special constraints. As is generally true, post-column reactions in LCEC are limited by the need for compatibility of reaction conditions with both chromatographic conditions and detector selectivity. Paramount among these requirements is detector transparency of reagents and side reaction products. Nonetheless, many useful post-column reactions for LCEC have been developed and their number will certainly expand.

Most of the chemical derivatizations thus far described have dealt with oxidative as opposed to reductive LCEC. Reductive LCEC has been hindered by operational difficulties based on the need to exclude oxygen from both mobile phase and sample. Until this difficulty is surmounted, it is unlikely that reductive LCEC will attain popularity comparable to oxidative approaches [22-24]. We will discuss here both, however, and also attempt to indicate the rationale to be used when selecting derivatizations for specific analytes in both the oxidative and reductive modes. The manuscript is divided to cover pre-column chemical derivatizations for reduction and oxidation, and post-column chemical, photolytic, and enzymatic reactions. Since a number of actual experimental parameters will be provided in the review of these derivatization approaches, all working electrode potentials are given with reference to the Ag/AgCl electrode, unless otherwise specified.

Pre-Column Derivatizations for Reductive LCEC

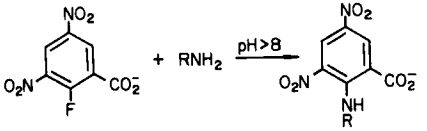
Rarely has a paper of speculation better presaged the actual development of the methods proposed than that by Kissinger *et al.* in 1979 [25]. A number of nitroaromatics were suggested as potential derivatization reagents for LCEC including alcohols, primary and secondary amines, amino acids, carboxylic acids, ketones, and aldehydes, all to be followed by reductive LCEC. Many of these proposed reagents have now been utilized and described in the literature for reductive LCEC. In 1982, Jacobs and Kissinger described, in two related papers, the use of various nitroaromatic reagents for carbonyl compounds, amines, and amino acids [26,27]. 2,4-dinitrophenylhydrazine was used for derivatization of aldehydes or ketones, leading to the reducible 2,4-dinitrophenylhydrazone products. Optimum performance for these derivatives was realized at an operating potential of -0.75 V vs Ag/AgCl. Detection limits were less than 100 pg for at least four separate aldehydes or ketone derivatives formed *via* this reaction. These detection limits were about 20 fold lower than was possible using LCUV at 254 nm.

A series of nitroaromatic reagents for the determination of amines and amino acids by reductive LCEC were described, including trinitrobenzene sulfonic acid, 2,4-dinitrofluorobenzene, and 2-chloro-3,5-dinitropyridine [27]. These three reagents were compared with respect to minimum detection limits, minimum production of interfering by-products, ease of derivatization, and stability of the derivatives. Trinitrobenzene sulfonic acid was found to be superior in all respects for these amino compounds. As expected, more nitro groups on the aromatic moiety leads to reduced detection limits, since the number of electrons transferred in the reduction increases.

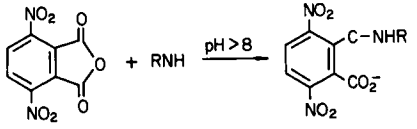
A similar derivatization approach was described in 1982 by Wightman and colleagues, wherein 2,4,6-trinitrobenzenesulfonic acid was used for the pre-column derivatization of alkylamines and amino acids, again based on reductive LCEC [22-24]. Gamma-aminobutyric acid was determined in rat brain homogenates using reverse phase LC and picomole detection limits for the final derivatives were possible. Wightman has also shown that dinitrobenzenesulfonyl chloride derivatives of amines can be detected at the picomole level using reductive

LCEC [28]. Meek recently described novel derivatizing reagents for determining peptides at the picomole level, again incorporating a nitroaromatic moiety into the final derivatives [29]. Reagents examined included 3,6-dinitrophthalic anhydride and 2-carboxy-4,6-dinitrophthalic anhydride (1a,b) for tagging peptides at the amino-terminus. The final derivatives could be detected electrochemically by reduction at -0.24 V, as well as by UV absorbance at 360 nm. Detection limits were improved in comparison with the underivatized peptides by 50- to 500-fold, depending on which reagent was used.

(1a)



(1b)



There have been no reports incorporating on-line, post-column reactions for reductive LCEC, though in principle this may be possible. Experimental difficulties are substantially increased due to the presence of unused reagents and hydrolytic byproducts in the eluent entering the detector. The use of solid phase derivatization reagents in a post-column, on-line approach might circumvent this problem, and work is being performed in this area [30-32]. Alkyl and allyl halides have been successfully derivatized, as shown in equation 2, both in solution and on silica gel, using a silver picrate tagging

(2) $$CH_3(CH_2)_3CH_2X + Ag^+C_6O(NO_2)_3^- \xrightarrow{MeCN} CH_3(CH_2)_3CH_2OC_6H_2(NO_2)_3$$

reagent which converts the starting halides into the corresponding alkyl/allyl picryl ether. Detection limits in the ppb range have been realized using both

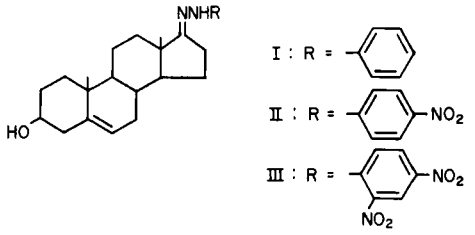


Figure 1. Phenylhydrazine Derivatives of Dehydroepiandrosterone (ref. 33).

reductive LCEC and oxidative LC-hv-EC (described below). These are 2-3 orders of magnitude lower than may be obtained using UV detection. Ultimate applicability in a post-column format will hinge on the ability to inhibit reagent bleed and improve substrate selectivity.

Pre-Column Derivatizations for Oxidative LCEC

Of the functional groups that possess no inherent electroactivity, carbonyl and amine containing compounds seem to be of the greatest interest, judging from the number of derivatization approaches reported for each. Phenylhydrazine (I), p-nitrophenylhydrazine (II) and 2,4-dinitrophenylhydrazine (III) have all been reported as useful off-line reagents for carbonyl compounds, allowing for detection at +0.8 V with a glassy carbon electrode after reverse phase LC separation [33]. These same authors earlier reported additional derivatization approaches for both primary and secondary amines in LCEC [34-37]. The phenylhydrazone derivatives of various steroids were prepared off-line, characterized, and evaluated as standards for detector conditions and detection limits. The p-nitrophenylhydrazone derivative was found to provide optimal sensitivity with a detection limit of about 200 pg/injection. Figure 1 indicates the specific derivatives studied for one particular steroid, dehydroepiandrosterone [33].

Another recent study involved oxidative LCEC for 2,4-dinitrophenylhydrazone derivatives of aldehydes and ketones [38]. For these authors, the use of a +1.10 V working electrode potential allowed for MDLs from 30-212 pg (S/N = 5) for 10 carbonyl compounds. Although the p-nitro- and 2,4-dinitrophenylhydra-

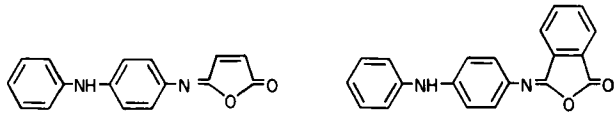


Figure 2. Structures of APIM and APIP (ref 40).

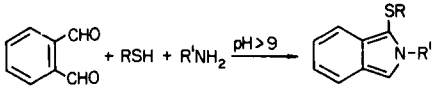
zones can be used both in the reductive and oxidative modes, it would appear that reductive detection limits are a bit lower [27,33,39].

Shimada et al. recently described two off-line derivatization approaches for LCEC determination of amines using N-(4-anilinophenyl) isomaleimide (APIM) and N-(4-anilinophenyl) isophthalimide (APIP) (Figure 2) [40].

Derivatization of typical amines, such as phenethylamine and piperidine with the indicated reagents was complete within 20 mins at room temperature in 1:1 acetonitrile:0.05 M borate buffer (pH 9.0). The detection limit for the phenethylamine-APIM adduct was about 0.1 pmol, and EC response was linear in the range of 0.1-10 ng of phenethylamine [40]. Similar reagents for thiols were also described based on N-substituted maleimides [41].

Tanaka et al. recently reported the use of a novel reagent, N-succinimidyl-3-ferrocenylpropionate, for derivatization of arylalkylamines, such as phenethylamine and tryptamine [42]. Hydrodynamic voltammetry of the final derivatives showed that maximum sensitivity was possible at +0.40 V, and detection limits of 0.2 pmol (S/N = 2) were realized. Final detection of these derivatives was also accomplished using an oxidative and reductive (upstream = +0.6 V; downstream = 0.0 V) series dual electrode detection system.

(3)



O-phthalaldehyde (OPA) has been one of the most popular derivatizing reagents for amines and amino acids (equation 3), especially for LCUV and LCFL [1-7]. In recent years it has also been incorporated by many investigators in

LCEC, and a number of papers have appeared which discuss the use of this particular reagent [46-51]. Joseph and Davies first suggested the use of LCEC for determination of OPA derived isoindoles and described the use of this reagent for a number of amino acids, followed by combined fluorescence and EC detection [49,50]. The optimized derivatization methods were applied, with dual detection for the determination of these amino acids in plasma and other biological materials. Using a series detection method, confirmation of the identities of the amino acids, based on EC/FL response ratios, could be obtained in a single run. Improved selectivity for certain amino acids was possible at lower working potentials, in that the OPA derivatization of amines was shown to be an example of a reaction in which the product is electroactive at a lower potential than the reactant. It was suggested that peptides and proteins whose OPA derivatives have little or no fluorescence activity should be electroactive, thus permitting their detection by the use of OPA derivatization. A number of dual detector chromatograms were included in these papers, indicating the nature of the final separations and relative responses (FL/EC).

Leroy et al. have analyzed a series of sympathomimetic drugs, such as heptaminol and related compounds, by derivatization with OPA followed by both UV spectrophotometry at 340 nm and amperometry at +0.9 V [46]. It was assumed that the OPA-amino acid drug adducts were derivatives of isoindoles, both by analogy with the known structure of amino acid-OPA adducts, and using new structural data. HPLC was carried out on a reverse phase column with a phosphate buffer (pH 7.2):MeOH mobile phase. Detection limits were lower by oxidative amperometry, usually in the picomole range, for original analytes. Other drugs studied included amphetamine, norephedrine, phenethylamine, and 2-heptylamine.

Shoup et al. recently described the use of this reagent to form OPA-amino acid and OPA-amine adducts for high-speed LCEC [48]. Derivative stability was vastly improved over that obtained using 2-mercaptoethanol by using t-butyl thiol as coreagent wherein the increased bulk of the thiol decreased the rate of product degradation. With this reagent, half-lives for the derivatives in

excess of several hours were realized, and the thiol used for formation of the isoindole products had little effect on the final electrochemistry of the derivatives. Gradient separations of these derivatives on short 3-μm reverse phase columns allowed LCEC detection limits of less than 500 fmol for each amino acid, and separations of 22 amino acids could be obtained in less than 10 minutes. However, detection limits were lowered to 30-150 fmol in the isocratic mode. A comparison was also made with LCFL detection of these same adducts.

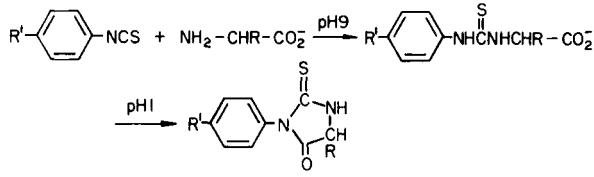
In a related area of derivatization, Granberg described the use of phenylisothiocyante (PITC) conversion of amino acids to phenylthiocarbamyl derivatives (equation 4) prior to reverse phase LC and combined (series) UV and oxidative EC detection [47]. Separation and detection of all amino acids from a calibration standard and an insulin hydrolysate was achieved in 25 min using a convex gradient of acetonitrile and methanol in sodium acetate at pH 6.5. Picomole detection limits were possible for all amino acids by both UV (254 nm) and EC (+0.85 V).

Amino acids have also been derivatized with substituted phenylisothiocyanates, as reported by Mahachi et al. [52]. In this case the initially formed PTC-amino acids were further converted to the cyclic phenylthiohydantoins (equation 5). The amines or amino acids reacted with an isothiocyanate such as

(4) $$H_2N-CHR-CO_2^- + C_6H_5-NCS \longrightarrow C_6H_5-NH-\overset{\overset{S}{\|}}{C}-NH-CHR-CO_2^-$$

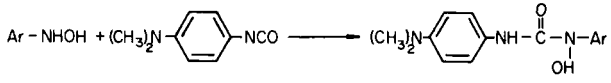
p-N,N-dimethylaminophenylisothiocyante (DMAPI) to form the corresponding substituted phenylthiohydantoins, which were then isolated and characterized. These workers demonstrated that these adducts could be reversibly oxidized at a glassy carbon electrode at pH 2 with a $E_{\frac{1}{2}}$ = 0.68 V, while final LCEC conditions employed a C_8 column, a mobile phase of 75:25 0.1 M phosphate buffer (pH 2 or 6):acetonitrile, and detection at +0.85 V. A mixture of 21 amino acids could be separated with 85-90% recovery, and the response was linear from 1 ng to over 150 ng/injection. Detection limits ranged from 0.5-1.0 ng.

(5)



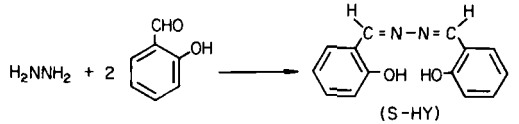
Musson and Sternson have described an off-line derivatization approach for arylhydroxylamines, using the reagent p-dimethylaminophenylisocyanate, which leads to stable hydroxyurea products [43]. These could be separated by reverse phase LC and detected both spectrophotometrically (254 nm) and amperometrically (+0.5 V, glassy carbon electrode) with detection limits of 9 x 10^{-7} M and 1 x 10^{-8} M respectively.

(6)



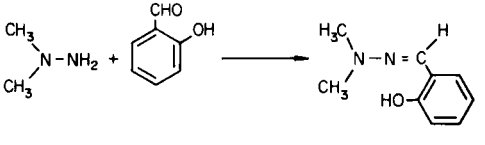
Kester and Danielson recently reported on the determination of hydrazine and 1,1-dimethylhydrazine as their salicylaldehyde derivatives in LCEC [44,45]. The oxidation of the phenolic group of salicylaldazine (S-HY) and salicylaldehyde-1, 1-dimethylhydrazone (S-UDMH) could be optimized with regard to ionic strength and pH of the mobile phase, as well as the applied oxidative potential. Detection limits were less than 5 ng/injection for hydrazine and 1,1-dimethylhydrazine as their derivatives. Detection limits for hydrazine and 1,1-dimethylhydrazine solutions, in terms of underivatized analytes, were approximately 25 ppb and 200 ppb, respectively. Equation 7 indicates the substrates, derivatizing reagent, and final derivatives determined by LCEC.

(7a)



(S-HY)

(7b)

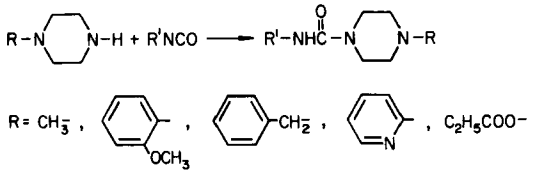


(S-UDMH)

Derivatization of isocyanates for LCEC was the subject of two recent reports [53,54]. In the first of these, Meyer and Tallman utilized p-aminophenol as the reagent for toluene diisocyanate. The separated products were detected amperometrically in the oxidative mode at a Kel-F-graphite composite electrode following reversed phase LC. The final LCEC detection limit for toluene diisocyanate was about 94 pg/injection. Complications arose since the isocyanate function can react with both the free amino and phenol functions of p-aminophenol, resulting in complex product mixtures.

A second derivatization approach for aromatic and aliphatic isocyanates in LCEC was described by Warwick et al. [54]. Their approach used a series of piperazine analogs as the derivatization reagent, the best being 1-(2-methoxyphenyl)piperazine, which formed EC and UV active derivatives. Equation 8 illustrates the reagents utilized in this study, all of which react to form a single stable derivative. A comparison was made between the EC and UV detection methods for final isocyanate analyses. As expected, the EC method was more sensitive than UV for determination of compounds such as phenyl isocyanate, toluene diisocyanate, hexamethylene diisocyanate, and (4,4-diisocyanatodiphenyl)methane in air samples.

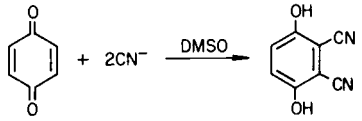
(8)



In a rather novel approach, spin adduct nitroxides produced from the derivatization of short-lived alkyl radicals using alpha-phenyl-tert-butyl nitrone (PBN) were determined using LCEC (+0.70 V) [55]. The sensitivity of the EC detector exceeded that offered by UV by about 2 orders of magnitude for 5 PBN spin adduct aminoxyls, and confirmation by ESR analysis of fractions validated the method.

A great deal more work has been reported for organic pre-column derivatization in LCEC than inorganic, and other than the work with metal chelations/complexations to follow, only the recent report by Mayer describes an inorganic derivatization for oxidative LCEC [59]. In this report, cyanide was derivatized with p-benzoquinone in the presence of dimethyl sulfoxide to form 2,3-dicyanohydroquinone (equation 9). The derivative was quantitated using reverse phase LCEC and oxidation at a glassy carbon electrode at +0.7 V. Attempted reaction of p-benzoquinone with Cl^-, SCN^- and $S_2O_3^=$ did not produce fluorescent or EC active products. The detection limit was calculated at 0.25 ug/ml KCN (74 pmoles CN^- injected), and it was noted that preconcentration of the final derivatization solution could lead to lower detection limits, if desired. The method has appeal as an alternative LC approach for cyanide determination because the derivatization is simple, reproducible, and leads to a single product in high yield.

(9)



Inorganic metal species have been determined using pre-column, in situ, and post-column derivatizations for LCEC, and the earliest report, by Takata and Muto, involved post-column chelation and coulometric detection of metals as the diethylenetriaminepentaacetate complexes [60]. Though a large amount of work has been described with these and similar chelation reagents for metal ions in LCUV/FL, until the work by Bond and Wallace, little research was performed in which these derivatizations were utilized in LCEC.

Bond and Wallace established a series of chelation reactions for various metal cations, such as copper, nickel, cobalt, chromium (VI), chromium (III), and lead [61-64]. In a typical approach, a dithiocarbamate salt of copper can be prepared by using a mobile phase containing the chelating reagent. Injections of aqueous solutions of copper onto the column then forms the copper dithiocarbamate complex, $Cu(dtc)_2$ on-line. This amounts to continuous pre-

column derivatization, since it occurs before the column itself [64]. These workers have also shown that the $Cu(dtc)_2$ complex can be formed off-line, in a more conventional manner, and then injected onto the LC system if interference by a large concentration of another metal poses a problem in the analysis [65]. The complex undergoes a reversible, one-electron oxidation at platinum, gold and glassy carbon working electrodes in acetonitrile/water mobile phases. Detection limits were reported as about 1 ng/injection of Cu. A subsequent paper in this series demonstrated that metals such as nickel, cobalt, chromium (III), and chromium (VI) form dtc complexes, and these too could be detected using reversed phase LCEC [61]. Detection limits substantially below 1 ng/injection of metal were achieved for all metals. In order to determine all five metal species, it was necessary to form the dtc complexes off-line, prior to injection onto the column. For some metals, it was still possible to use *in situ* formation followed by LCEC, depending on the rates of formation of the various metal complexes and the final equilibrium constants involved. Other dialkyldithiocarbamates were studied as well. In a 1983 publication Bond and Wallace described an automated determination of nickel and copper by LCEC and LCUV, again making use of *in situ* generation of dithiocarbamate complexes [62]. In this report, both UV and single electrode EC detection were used as components of the total monitoring system. All of the EC detection involved oxidation at a glassy carbon electrode. These authors also described an automated, microprocessor based system developed for multielement determination, again with either spectrophotometric or electrochemical detection [63]. Limits of detection of 1 ng/10 μl injected were realized with spectrophotometric detection, and depending on the particular metal, higher or lower limits were possible by EC. Pre-column and *in situ* formation of the metal complexes with diethyl- or dipyridyldithiocarbamate were performed, depending on the application, and it was found that EC detection was considerably more sensitive for Ni and Cu than for the other elements determined.

In a separate study, Roston described the applicability of 4-(2-pyridylazo)resorcinol (PAR) as a pre-column chelating agent for LCEC multielement determinations [66]. Fixed-wavelength UV absorption and oxidative thin-layer

amperometry were used together for detection of these PAR chelates. Preliminary studies showed that determination of metal ions such as Cu^{+2}, Co^{+2} and Fe^{+2} as PAR chelates could be realized at the ppb levels.

Post-Column Photochemical Reactions

A number of different approaches have been described for the use of light as a post-column "reagent", together with LCEC, but most notable in these areas have been Johnson, Weber and Krull. Johnson's approach [68] used light to generate nitrite from N-nitrosamine analytes. This nitrite was pre-concentrated and collected on an anion-exchange column, after which it was quantitatively transferred onto a rotating ring-disk electrode detector operated oxidatively. Krull et al. have developed this approach further, in that the photolysis unit is now placed directly on-line, between the column and a conventional amperometric detector that may be operated either oxidatively or reductively. Figure 3 illustrates this LC-hv-EC system. Using this method, compounds which exhibit no inherent electroactivity at oxidative potentials may be directly photolyzed to form long-lived, EC-active species, which are detected using a thin-layer, amperometric detector [69,70].

This technique, which appears to offer several advantages over classic chemical derivatization methods, has now been used to study a wide variety of analyte classes which are not directly amenable to oxidative detection in LC. These classes include: organic nitro compounds (nitrate esters, aromatic nitro compounds, nitramines, etc.) [71,72], organothiophosphate pesticides (malathion, parathion, etc.) [73], beta-lactams (penicillins and cefoperazone) [74], barbiturates [75], cocaine [76], benzodiazepines, aromatic esters and amides.

LC-hv-EC offers three modes of selectivity, in that the retention time, dual (parallel) electrode response ratio and the analyte's lamp on/off behavior may all be used to lend greater confidence in assigning peak identities in the chromatogram. This is portrayed in Figure 4, which displays the two chromatograms obtained by the injection of a serum extract containing an unknown barbiturate and the internal standard, hexobarbital, onto the system in both the lamp on and lamp off modes. In the lamp-on chromatogram, the appearance of a peak having a retention time and dual electrode reponse ratio identical to that

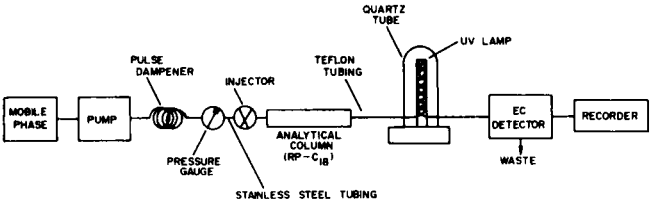


Figure 3. Schematic Diagram of the LC-hv-EC System.

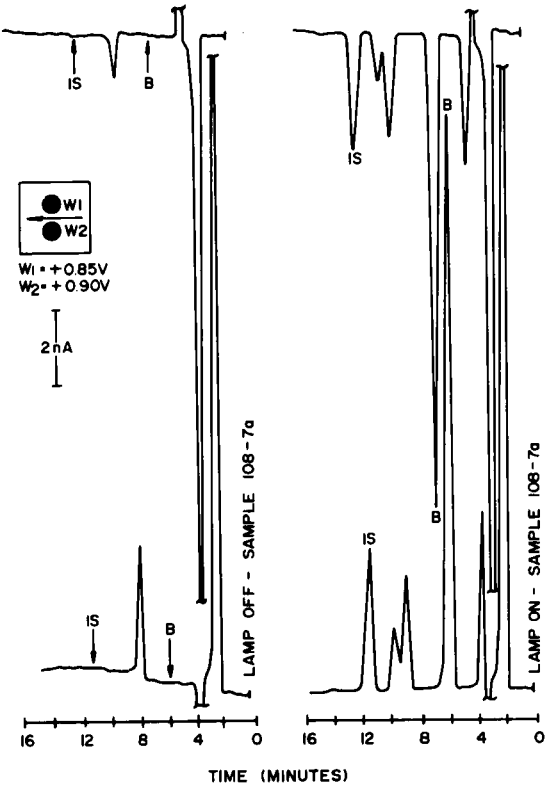


Figure 4. LC-hv-EC Determination of Butabarbital in Serum. See text for discussion.

obtained for a standard of butabarbital injected onto the same system, as well as the disappearance of this peak in the second, lamp-off injection of the same extract, allows the analyst to easily identify this peak as butabarbital.

In addition to the improved selectivity realized with LC-hv-EC, minimum detection limits for almost all of the analytes studied have been between 1 ppb and 100 ppb, and the method exhibits linearity over roughly 3 orders of magnitude. A number of validation studies have been performed, and quantitative results have been obtained using LC-hv-EC for the determination of: malathion in wheat samples, bacampicillin HCl in formulations for oral suspension, cefoperazone in simulated saline infusion solutions, chlordiazepoxide and norchlordiazepoxide in human urine, barbiturates in human serum, and cocaine in simulated illicit preparations. All have been compared to results obtained from the analysis of these samples using accepted methods. The levels of the analytes of interest determined using the newer method have always been within ±6% of those levels determined using well-established procedures.

Although this hybrid technique has shown applicability to organics, there is evidence to suggest that LC-hv-EC may have a great deal of utility for inorganics as well. Such work is now in progress, especially with regard to the detection of anions. However, as is the case for both fluorescence and UV absorbance detection, not all classes of compounds which have no inherent electroactivity may be photolyzed to form electroactive products. Therefore, to demonstrate some possible off-line, pre-column derivatizations possible for such analytes, a study was recently completed in which a number of nitroaromatic reagents were used to derivatize amines, amino acids and aminoalcohols to a form which was then amenable to LC-hv-EC determination [77]. Amine-containing compounds were derivatized with 2,4-dinitroflurobenzene (Sanger's reagent) in solution, and the final derivatives were isolated, purified, and characterized, and the percent formation determined. Analyses for amino acids were performed in spiked water, beer and spiked beer samples. This study demonstrated that all of the nitroaromatic derivatization schemes and final derivatives already described in the literature for reductive LCEC should be fully amenable to oxidative LC-hv-EC.

The other major approach that has been used with post-column irradiation in LCEC involves irradiation within the thin-layer cell, and the work of both Weber and Krull is relevant. Weber has irradiated across the working electrode surface of a wall-jet electrode, using an intermediate $Ru(byp)_2^{+3}$ species to carry out the derivatizations of analytes injected [78-80]. This is a redox system wherein one of the intermediate species necessary for detection is generated photochemically. The recent approach of Krull and LaCourse has incorporated direct irradiation of the working electrode surface in a thin-layer, flow through, amperometric cell. Their approach involves irradiation of the analytes as they pass across the working electrode surface, as well as the surface itself, and has been limited to carbonyl containing compounds [81,82]. It is possible that some type of (as yet undefined) intermediate species, derived from the carbonyl analytes injected, has been formed photochemically, and this species is then detected oxidatively. However, sufficient evidence is not yet available to confirm a specific mechanism involved in this type of photoelectrochemical detection for LC (LC-PED), and additional studies are now in progress. Specific applications to actual samples, using LC-PED, are nearing completion, including trace analysis for benzaldehyde in almonds, liquors, and other foods [83,84]. The nature of the species generated and detected in the PED appears to be quite different, at this time, from the species generated in LC-hv-EC. Those compounds amenable to one technique are not usually suitable for the other. One appears to involve photochemical excitation or promotion, followed by EC detection (PED), while the other involves photolysis and cleavage of an analyte to form stable inorganic or organic anions and stable hydrolysis products (hv-EC). Obviously, more work is needed to establish the mechanisms operative in each post-column derivatization approach.

Post-Column Chemical Reactions

Post-column reactions in LC have become more and more commonplace, and there is a considerable interest in the use of novel reagents and reactions for this approach [85]. The use of post-column reactions increases instrument complexity while providing convenience and sometimes improved precision. It is

of particular advantage when chromatographic resolution is impaired by derivative formation or derivative stability is especially poor. The use of post-column reactions is also very useful for continuous monitoring of preparative separations via stream splitting. Post-column implementation is mandatory when the detectable species generated is not unique to each individual analyte (e.g. many enzyme systems). Little et al. have described a low dead volume mixer which contributes very little to the overall dispersion of the chromatographic peak [86]. It was especially suited for use with fast post-column reactions, such as the formation of OPA derivatives of amines or amino acids followed by UV-VIS detection. Additionally, it was well suited for use in connection with EC detection (reductive), where addition of reagents to control pH and electrolyte composition was very important for sensitive detection. These authors were able to convert a series of aldehydes and ketones post-column, into semicarbazone derivatives by addition of semicarbazide reagent, and detect the final products downstream using electroreduction. In the examples presented, EC detection limits were at least one order of magnitude lower than those obtained using UV.

In a 1980 paper, King and Kissinger described the use of LCEC with electrogenerated reagents, wherein an amperometric or coulometric generator electrode could be used, post-column, to generate new reagents from the mobile phase [87]. These reagents then react with an analyte between the upstream, generator electrode and the downstream, detector electrode. Alternatively, a reagent could be placed in the mobile phase post-column, mixed with the analytes of interest after introduction, and differences in the relative levels of the reagent concentration could then be detected by a downstream EC monitor. An example of this was the reaction of unsaturated organic compounds with molecular bromine, which could be generated in situ or added post-column. The technique was used to determine ng-levels of underivatized fatty acids, prostaglandins, and phenols after initial separation by reversed phase LC.

Similarly, Kok et al. have used electrochemically generated bromine for the determination of a number of phenolic ethers [88]. These authors also characterized the generator/detector cell with respect to band broadening characteris-

tics, conversion efficiency and generating current. With an optimized system, detection limits for a number of opiates were found to be between 0.4 ng and 300 ng. In the previously cited work by Takata and Muto [60], the workers used electrochemical generation of ferricyanide for the determination of sugars, using a coulometric cell at +0.08 V *vs.* a ferro-ferricyanide reference electrode.

In related work, Watanabe and Inoue described the amperometric LCEC detection of reducing sugars by the use of a copper phenanthroline $[Cu(phen)_2]^{+2}$ reagent added post-column [89]. The reagent was added in an alkaline solution, and reacted with eluting sugars in a reaction coil, placed after the column and reagent introduction tee. In this reaction, the $[Cu(phen)_2]^{+2}$ was reduced to $[Cu(phen)_2]^{+}$ by the reducing sugar present in the reaction coil, and this reduced form was detected amperometrically at oxidative potentials. The technique allowed for highly sensitive detection, in that glucose could be determined at levels down to 1 pmol (0.2 ng/injection). In addition to high sensitivity, there was selectivity only for those compounds capable of reducing the initially added chemical reagent. The overall reaction sequence is indicated in equation 10.

(10) $$[Cu(phen)_2]^{+2} + \underset{\text{sugar}}{\text{reducing}} \xrightarrow[\Delta]{} [Cu(phen)_2]^{+} \xrightarrow{EC} [Cu(phen)_2]^{+2}$$

In a more recent paper, Honda *et al.* described another LCEC method for detection of reducing carbohydrates by post-column derivatization with 2-cyanoacetamide [90]. This reagent reacted by addition to the sugar, rather than by a redox reaction, and the final derivatives possessed UV, FL and oxidative EC properties. Glucose could be monitored in this manner with a detection limit of 20 pmol and a linear calibration range of 50 pmol to 2 nmol. Other reducing carbohydrates were amenable to the same post-column technique, and LC separations could involve reverse phase, gel-permeation, ion-exchange, and other partition modes. The specific structure or nature of the intermediate derivative(s) has not been described as yet.

Elchisak described another type of post-column derivatization in LCEC, which does not involve the addition or generation of any reagents [91]. In

this study, dopamine conjugates, such as dopamine-3-O-sulfate and dopamine-4-O-sulfate were first separated by reversed phase, ion-pair LC. Each of these was separately hydrolyzed to free dopamine immediately after elution *via* an acid catalyzed incubation in a reaction coil at an elevated temperature. Each isomer could then be detected as free dopamine by oxidative EC using a glassy carbon working electrode. Use of the technique resulted in a 15-fold improvement in S/N ratio for each of the dopamine-sulfate isomers when compared with a previous detection method using UV detection.

Post-Column Enzymatic Reactions

A number of reports have appeared in which immobilized enzymes have been used for post-column derivatization and EC detection. Kamada *et al.* described one such approach using immobilized 3-alpha-hydroxysteroid dehydrogenase together with oxidative EC, for the determination of individual bile acids in serum and bile [92]. Bile acids eluting from the HPLC column reacted with NAD, which had been pumped to the enzyme reactor, to generate NADH. The NADH was then reacted downstream with a phenazine methosulphate solution, and the final product of this reaction was detected electrochemically. Using this approach, each bile acid could be detected at the 20 pmol level. Figure 5 illustrates the overall LC-detection system, which involves mixing of NAD with the column effluent, reaction in the mixing coil at 30°C, mixing with phenazine methosulphate, and final EC detection.

Post-column enzymatic reactions have also been described by Dalgaard *et al.* for the LCEC detection of phenolic glycosides [93]. This work utilized β-glucuronidase immobilized on porous glass beads packed into a short column and placed post-column. Enzyme catalyzed cleavage of the glycosides occurred during passage of the analytes through the reaction column at room temperature, and the newly formed phenols could then be readily detected downstream at oxidative working potentials. Detection limits of the various phenolic glycosides were in the range of 3-23 pmol.

Immobilized glycosidases have also been used in the LCEC detection of cyanogenic glycosides (94). The first post-column reaction consists of the

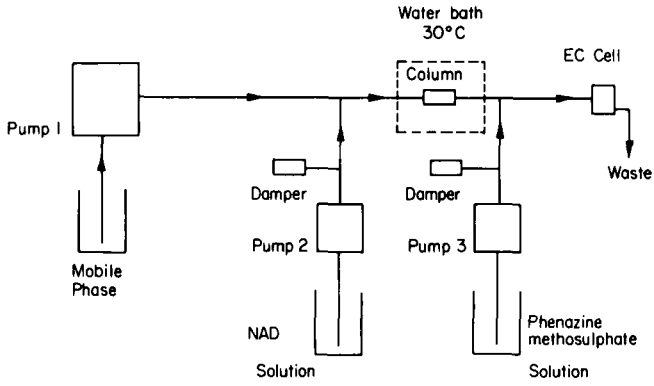


Figure 5. Schematic Diagram of Enzyme Reaction System for Determination of Bile Acids (ref. 92).

enzymatic hydrolysis of the separated cyanogenic glycosides. Hydrolysis leads to the formation of a cyanohydrin. Sodium hydroxide added to the flowstream after the enzyme-reactor hydrolyses cyanohydrin yielding cyanide, which can be detected at a silver electrode. The detection limit was about 20 pmol for all the glycosides examined. This method was utilized for the determination of cyanogenic glycosides in crude plant extracts (95).

Enzymes have also been used in homogeneous post-column reactions in LCEC, such as in the determination of acetylcholine (ACh) and choline (Ch) in neuronal tissue [96]. ACh and Ch were first separated by reversed phase LC, and as each eluted from the column they were mixed with acetylcholinesterase and choline oxidase. Endogenous Ch, and Ch formed by the enzymatic hydrolysis of ACh, were both hydrolyzed by choline oxidase to betaine (non-EC active) and hydrogen peroxide (H_2O_2). The peroxide generated was then detected downstream using oxidative EC. Equation 11 indicates the overall reaction utilized in this approach. The detection limits were 1 pmol for Ch and 2 pmol for ACh. Specificity of the method was based on LC, two specific enzyme catalyzed reactions, and EC detection of hydrogen peroxide on a Pt electrode at a fixed working potential.

(11a) $$CH_3-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_2CH_2O-\overset{\overset{O}{\|}}{C}-CH_3 + H_2O \xrightarrow[\text{Cholinesterase}]{\text{Acetyl-}} \text{Choline + acetate}$$

(11b) $$CH_3-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_2CH_2OH + 2O_2 + H_2O \xrightarrow[\text{Oxidase}]{\text{Choline}} \text{Betaine} + 2H_2O_2$$

LCEC detection of ACh has also been used to develop a sensitive assay for choline acetyltransferase (97). This enzyme catalyzes the formation of ACh from acetyl-CoA and Ch. Enzyme concentration is proportional to the amount of ACh formed under standard incubation conditions. The sensitivity of the assay is high enough to determine transferase activity in submilligram samples of brain tissue.

The homogeneous enzymatic procedure for the detection of ACh, as outlined above, does not allow for the utilization of the full catalytic potential of the enzymes. Enzyme is used for a single determination and then directed to waste. An immobilized enzyme post-column reactor for the detection of ACh and Ch by LCEC has been described by Meek and Eva (98), allowing for the catalytic activity of the enzymes to be recycled. Acetylcholinesterase and choline oxidase are adsorbed to a commerical anion-exchange cartridge. Conversion of ACh to peroxide is quantitative during the residence time in the cartridge. The reactor can be replenished by addition of fresh enzyme (required every 5 to 10 days). Such an enzyme-loaded cartridge has been used for the determination of ACh and Ch levels in brain extracts (99). The detection limit is less than 5 pmol for both compounds. Although performance is not improved over the homogeneous enzymatic technique, the reactor does decrease the amount of enzyme consumed resulting in lower operational costs.

Oxalate oxidase also has been immobilized on an ion-exchange resin and used in a post-column reactor (100). Oxalate oxidase catalyzes the oxidation of oxalate to H_2O_2 and CO_2. Peroxide is detected amperometrically on a Pt electrode at +0.7 V. The enzyme was immobilized on a commercial cation-exchange cartridge, by a simple injection procedure. Oxalate is anionic and the elution

profile would be affected by its interaction with an anion exchanger. The reactor was stable during 2 weeks of use, and after an additiona 2-week storage period.

Summary

In this manuscript, we have discussed chemical approaches that have been used, to date, to expand the range of analytes accessible to LCEC. These involve chemical, photochemical or enzymatic processes, and they may occur pre-column, post-column, or in situ. Judging from the trends in the literature observed during the preparation of this manuscript, it is likely that a number of improved derivatization techniques will be described in the near future. The methods utilized will become more refined, perhaps involving more complex chemistry, but at the same time will likely become more straightforward in the actual system design and application. Post-column, on-line methods may be used more frequently, since these offer the possibility for automation and are generally less time consuming. In any case, regardless of the specific mode of derivatization, all of these future efforts will increase the usefulness of LCEC and enable the analyst to incorporate this sensitive and selective method in the determination of an ever increasing number of compounds.

Acknowledgements

Some of the results described in the area of post-column photolytic derivatizations in LCEC were provided by a number of graduate students and visiting scientists at Northeastern University. These include: William LaCourse, Robert Nelson, John Burton, Xiang-Dong Ding and Li-Ren Chen. The last two mentioned contributors were visting Chinese Scholars from the People's Republic of China. We are very grateful to their country, and their Institutes, for allowing them to come to the U.S. and collaborate with us in this research program. Funding for some of this work was provided, in part, by an unrestricted grant from the Analytical Research Department, Pfizer Central Research, Pfizer, Inc., Groton, CT 06340. We are very grateful to our colleagues within Pfizer, especially Karl Bratin and George Forcier, for making these grants

possible, as well as for their technical assistance and collaboration. Additional funding was provided, in part, by a Biomedical Science Research Support Grant, No. RR07143, to Northeastern University, from the Department of Health and Human Services (DHHS). This is contribution number 216 from the Barnett Institute of Chemical Analysis and Materials Science at Northeastern University.

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