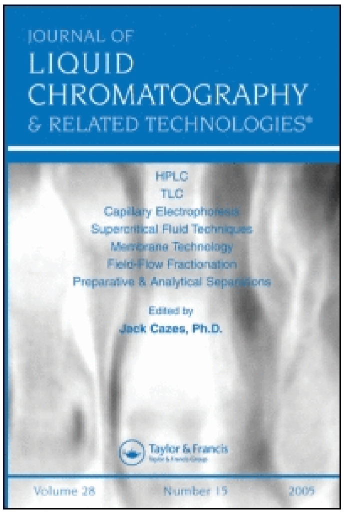

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Nitroaromatic Reagents for Determination of Amines and Amino Acids by Liquid Chromatography/Electrochemistry

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To cite this Article Jacobs, W. A. and Kissinger, P. T.(1982) 'Nitroaromatic Reagents for Determination of Amines and Amino Acids by Liquid Chromatography/Electrochemistry', Journal of Liquid Chromatography & Related Technologies, 5: 5, 881 — 895

To link to this Article: DOI: 10.1080/01483918208060620
URL: http://dx.doi.org/10.1080/01483918208060620

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(5), 881-895 (1982)

NITROAROMATIC REAGENTS FOR DETERMINATION OF AMINES AND AMINO ACIDS BY LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY

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ABSTRACT

Trinitrobenzene sulfonic acid, 2,4-dinitrofluorobenzene and 2-chloro-3,5-dinitropyridine were compared in terms of properties relevant to use as precolumn derivatization labels for amino compounds. This included consideration of chemical characteristics as well as purely detector oriented behavior. Trinitrobenzene sulfonic acid appears to be superior in all respects, yielding the most detectable derivatives with minimum production of interfering by-products.

INTRODUCTION

Electrochemical detectors for liquid chromatography utilize the inherent sensitivity of hydrodynamic electrochemistry to allow detection and quantitation of trace amounts of electroactive materials. They are the only commercially available LC detectors capable of competing with fluorescence detectors in terms of analyte detectability. We have been interested for some time in expanding the utility of EC detection through the application of effective precolumn derivatization schemes. Of the available "electrophoric" labels which one might consider, nitroaromatics appear to be the most generally useful. Nitroaromatic moieties are already among the most frequently used derivatizing groups due to the strong chromophoric properties of the resulting derivatives, and a variety of reagents are available which allow convenient derivatization of many classes of compounds.

The potential utility of nitroaromatics as labels for liquid chromatography/electrochemistry (LCEC) has been discussed previously in some detail (1,2) and is based on the particularly favorable electrochemistry of aromatic nitro groups. In general, they are reduced at moderate negative potentials in multi-electron steps (equations 1 and 2). The exact nature of the

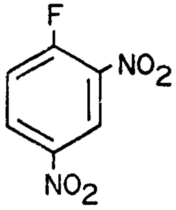
$$\text{R-NO}_2 \xrightarrow{+4e^-,\ 4H^+} \text{R-NHOH} \qquad (1)$$

$$\text{R-NHOH} \xrightarrow{+2e^-,\ 2H^+} \text{R-NH}_2 \qquad (2)$$

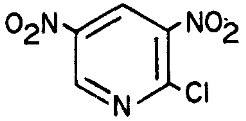
product(s) as well as the required potential(s) depends on a number of parameters including pH, electrode material, and the nature and orientation of other ring substituents (3). This behavior makes them ideally suited for reductive mode EC detection (4).

Nitroaromatics have been and continue to be widely used as reagents for derivatizing amino compounds, especially amino acids (5,6). These methods normally utilize TLC or LCUV for identification/quantitation of derivatives, but the lack of sensitivity limits the utility of these procedures for trace determinations. Recently, however, a method for determining γ-aminobutyric acid in brain tissue by LCEC, as its trinitrophenyl derivative was reported which demonstrates the significant improvement in sensitivity which can be achieved by the use of electrochemical detection (7).

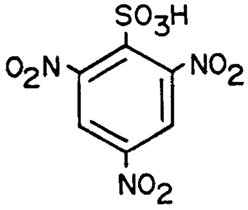
In selecting reagents for derivatization procedures it is important to consider not only the detectability of the resulting derivatives but also the convenience of application. Of particular concern is the propensity for formation of interfering by products and the ease with which these and excess reagent can be removed. Three of the most common reagents for introduction of a nitroaromatic moiety into amino compounds are shown below. All are chemically quite similar, being



DNFB



DNCP



TNBS

Abbreviations: DNFB = 2,4-Dinitrofluorobenzene
DNCP = 2-Chloro-3,5-dinitropyridine
TNBS = 2,4,6-Trinitrobenzene sulfonic acid

susceptible to nucleophilic substitution at a strongly activated ring position. We have evaluated these three reagents for use in conjunction with LCEC by comparing both voltammetric behavior of derivatives and relevant chemical properties of each reagent as discussed above. The approach we have taken is to provide an overview of important features, recognizing that specific needs vary from application to application.

EXPERIMENTAL

Chemicals: DNFB, DNCP, all amino acids, DNP-amino acids, and DNPy-amino acids were from Sigma (St. Louis, MO). TNBS and TNP-amino acids were from ICN Biochemicals (Cleveland, OH). Ammonium acetate, sodium acetate, and dibasic sodium phosphate (all reagent grade) were from Fisher (Pittsburg, PA). Citric acid was from Mallinckrodt (St. Louis, MO). Sodium tetraborate and 3,5-Dinitro-2-hydroxypyridine were from J. T. Baker (Phillipsburg, NJ).

Liquid Chromatography: All liquid chromatography as well as hydrodynamic voltammetry was carried out using a BAS LC-154 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN) equipped with a TL-5A glassy carbon thin layer amperometric transducer cell and modified to allow continuous deoxygenation of mobile phase and deoxygenation of samples prior to injection (8). The LC was equipped with a Biophase 6032 octyl or a Biophase 6017 ODS, both 0.46 x 25 cm with a 5 µm particles (BAS). Details of the mobile phase composition and detector potentials are provided in figure legends. For LCUV experiments a model LC-6 (BAS) fixed wavelength detector (254 nm) was used. Peak integration was done with a Minigrator (Spectra-Physics, Santa Clara, CA).

Derivatization of Synthetic Samples: TNBS: 1 mL of sample was mixed with 3 mL of borate buffer (0.2M, pH 9.0) and the reaction initiated by addition of 1 mL of 0.03M TNBS (prepared daily in distilled H_2O). The mixture was incubated at room temperature for 1 hour at which time the reaction was complete. An aliquot of the mixture was then either diluted and injected directly, or subjected to extraction.

DNFB, DNCP: The procedure was as for TNBS except that DNFB and DNCP were dissolved in acetone at a concentration of 0.15M. The mixture contained 0.8 mL H_2O and was initiated by addition of 0.2 mL of the appropriate acetone solution.

Notes: For each reagent the total incubation volume was 5 mL and the reagent concentration 0.006 mM. The reaction pH was between 8.8 and 8.9. For micro-scale derivatizations all volumes were decreased by a factor of 10.

Extraction of Derivatization Mixtures: In order to characterize the pH dependence of extraction, a series of standard mixtures were prepared in McIlvaine buffers ranging in pH from 2 to 9. All of the solutes of interest were present at a concentration of approximately 10^{-4}M. A 0.5 mL aliquot of each solution was extracted (vortexed for 15 seconds) 1 time with 2.5 mL of a 4:1 ethyl acetate:hexane mixture. Part of the organic layer (2.0 mL) was removed and evaporated and the residue redissolved in 0.5 mL of 0.1 M $HClO_4$. The reconstituted samples were diluted 1:200 prior to injection.

Derivatization mixtures were extracted in the same manner or, in the case of TNBS, acidified with 1 drop 6 M HCl (adjusted pH $\sim$ 2) prior to extraction. With DNFB and DNCP reaction mixtures, the organic phase was discarded and an aliquot of the aqueous phase injected onto the LC.

RESULTS AND DISCUSSION

Voltammetric Behavior: The hydrodynamic voltammetric behavior of DNP, DNPy, and TNP-alanine was taken as an indication of the suitability of each derivative type for reductive mode electrochemical detection. From a purely detector oriented viewpoint the most important parameters are the potential(s) at which reduction occurs and n, the number of electrons transferred. Since the sensitivity of EC detectors is directly proportional to n, it is desirable to maximize n while maintaining the operating potential in a region which minimizes steady-state background current and noise.

Hydrodynamic voltammograms, obtained under typical chromatographic conditions are shown in Figure 1. The results are plotted as n_{eff} vs E (see Appendix). As might be expected the TNP derivative yields the greatest number of electrons at the lowest (most positive) potentials. Although not fully resolved, each voltammogram certainly contains two or more waves. None of the derivative types are completely reduced within the potential range examined, however. At -1.1V the DNPy, DNP, and TNP derivatives yield 8, 10, and 12 electrons respectively. Complete reduction of all nitro groups to amino groups whould produce n_{eff} values of 12, 12, and 18. It is not clear why the DNPy derivative yields only 8 electrons rather than 10 as does the DNP compound. No effort was made to elucidate the actual reduction product(s)

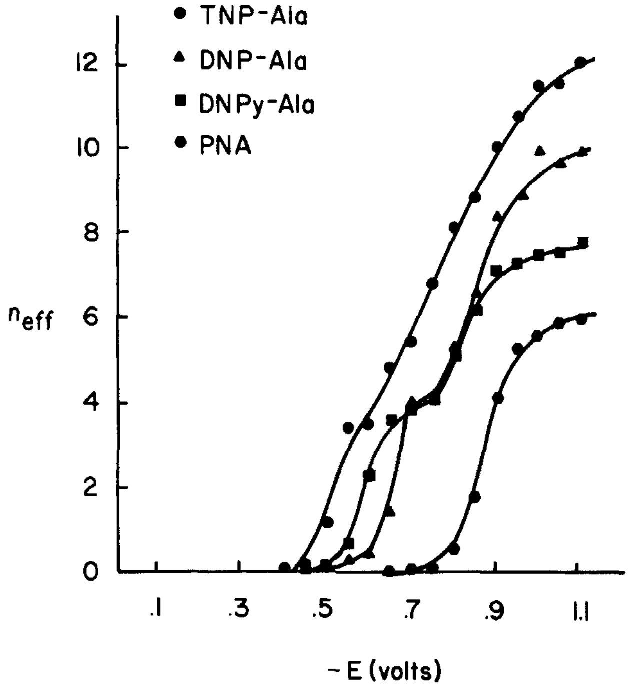


FIGURE 1. Hydrodynamic voltammograms of Ala derivatives. Conditions: 0.1 M NaOAc, pH 5.0, 10% 1-propanol, flow rate = 1.0 mL/min.

for any of the derivative types but several mechanisms could be formulated to explain the observed results. Nevertheless, the TNP derivatives should exhibit the most favorable detection characteristics with reductive LCEC.

Detectability: Figure 2 shows standard curves obtained for each derivative type using both EC detection at -0.85 vs Ag/AgCl and UV detection at 254 nm. These results were obtained by connecting the detectors in series (UV first) and recording data from both simultaneously. The enhanced sensitivity of EC detection is clearly illustrated (note the plots give S/N ratio vs injected amount to effectively normalize between detection modes). Detection limits at a signal to noise ratio of 3 are

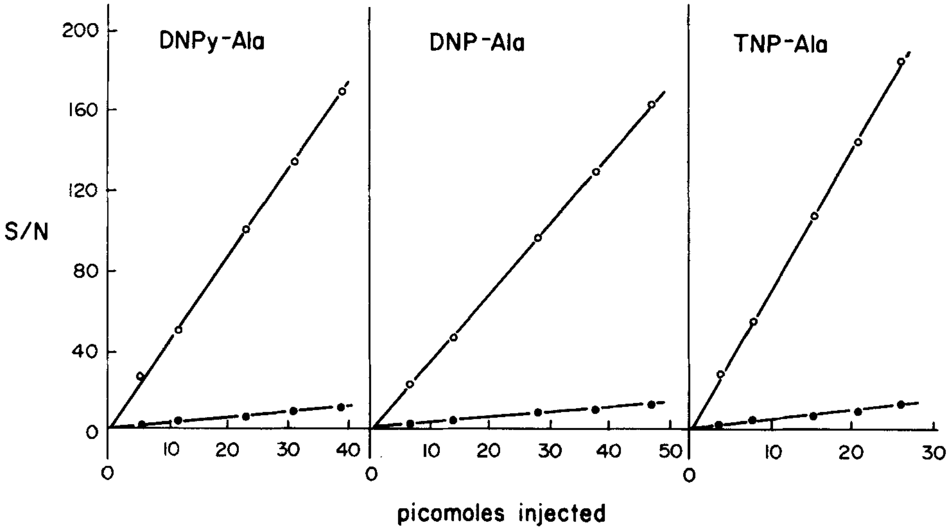


FIGURE 2. Calibration curves for Ala derivatives by LCUV and LCEC. Conditions as in Figure 4 •UV ○EC

listed in Table 1. A representative chromatogram is shown in Figure 3. As expected, the lowest detection limit is obtained for the TNP-derivative by both UV and EC but in all cases EC provides lower detection limits than UV by about one order of magnitude. Both detectors were linear over the range examined.

TABLE 1

Detection Limits for Ala Derivatives by LCUV and LCEC (pmole, S/N = 3)

	EC	UV
DNP-Ala	1.0	10
DNPy-Ala	0.60	12
TNP-Ala	0.31	5.7

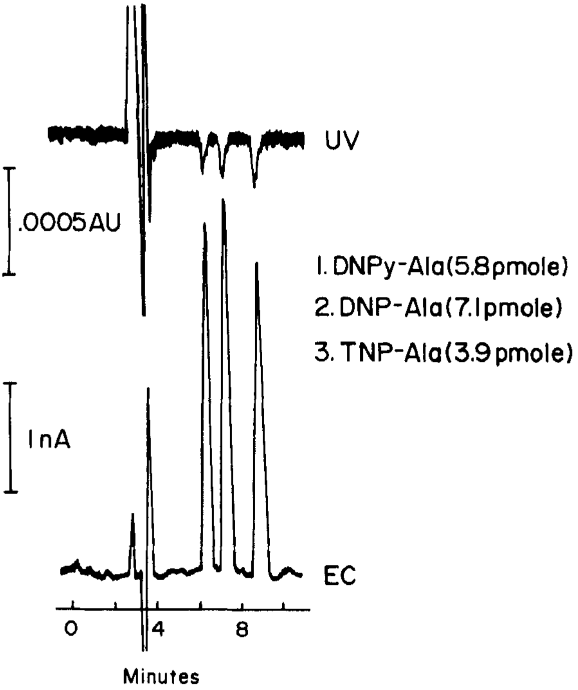


FIGURE 3. Chromatograms of standard Ala derivatives by LCUV and LCEC. Conditions: Mobile phase: 0.1 M NaOAc, pH 5.0, 12.5% 1-propanol. Column: Biophase C_{18}, 5 µm, 25 x 0.46 cm. Flow rate: 1.0 mL/min. UV at 254 nm. EC at -0.85V vs Ag/AgCl.

Occurrence of and Removal of Interferences: A factor of primary concern with precolumn derivatization schemes is the ease with which excess reagent and/or by-product can be removed (assuming they are detector responsive). In the case of the reagents used in this study, interference from the parent reagent must in fact be considered. In addition, each reagent hydrolyzes to some extent under the derivatization conditions employed, resulting in phenolic by-products which also constitute potential interferences. The problem of these interferences may be dealt with by utilizing a prechromatographic clean up step

TABLE 2

Summarized Acid/Base Properties of Reagent, Derivatives, and Hydrolytic Products

Reagent	Derivatives	Hydrolysis
TNBS - acid ($pK_a<1$)	neutral or acid ($pK_a \sim 4$)	acid ($pK_a<1$)
DNFB - neutral	neutral or acid ($pK_a \sim 4$)	acid ($pK_a \sim 4$)
DNCP - neutral	neutral or acid ($pK_a \sim 4$)	acid ($pK_a \sim 4$)

and/or by proper selection of chromatographic conditions for the final separation.

The acid-base properties of each reagent and their major hydrolytic products and derivatives are summarized in Table 2. All of the derivatives will be neutral or weakly acidic depending on whether the substrate is a simple amine or an amino acid. The data in Table 2 suggest that extraction schemes as outlined in Figure 4 would be useful as pre-chromatographic clean up procedures. With DNFB and DNCP, interferences from hydrolytic products would not be removed, however. The extraction profiles in Figure 5 verify the predicted extraction behaviors except for picric acid, the hydrolysis product of TNBS. Despite its low pK_a (<1), PA is extracted to a significant extent over the entire pH range examined. The reason for this unusual behavior is not clear but it may reflect a tendency to form extractable ion-pairs or dimers in the buffer system used. The extraction profiles of DNFB and DNCP were not determined as a function of pH since they possess no ionizable groups. It should also be noted that while the shape of the extraction profile is primarily due to the acid base properties of the solute, the relative extraction efficiencies at the extremes of the curve depend on the hydrophobicity of the non-ionic portion of the molecule. This is to

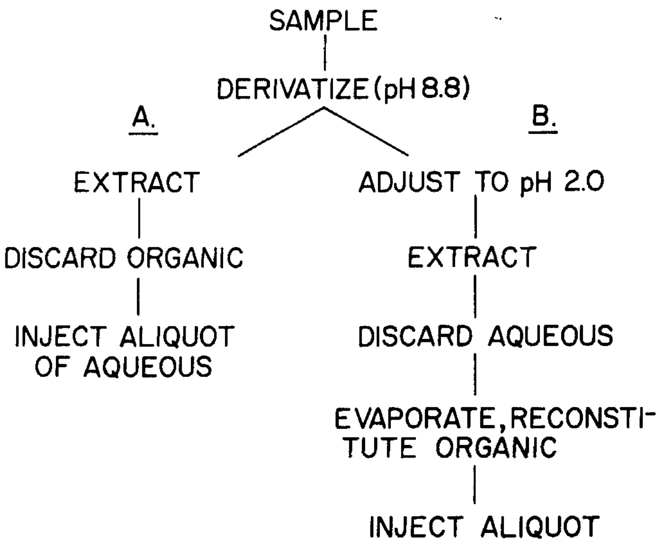


FIGURE 4. Proposed extraction schemes based on acid/base properties of reagents and their derivatives.

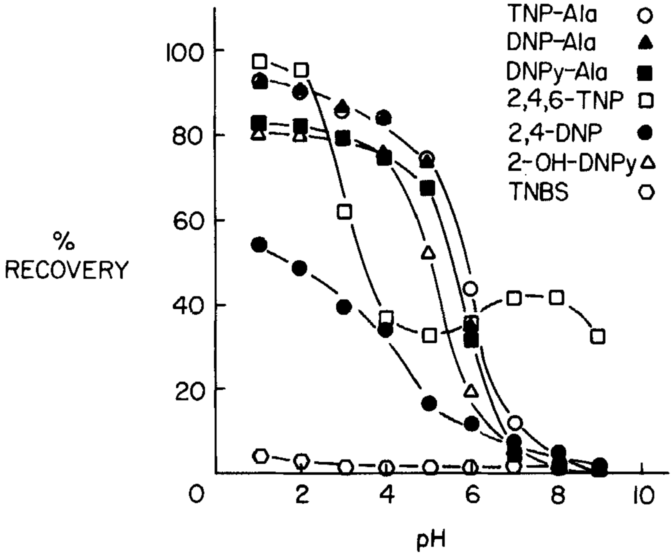


FIGURE 5. pH dependence of extraction efficiency for reagents, their Ala derivatives, and expected phenolic by-products (see experimental section for details)

say that, for example, a phenylalanine derivative may show significant extractability even at pH 9 while the corresponding alanine derivative is only very slightly extracted. Therefore, for relatively hydrophobic amino acids a high pH extraction for reagent removal (as in the case of DNFB and DNCP) could result in a significant loss of the desired derivatives. In the case of simple amine derivatives removal of excess DNFB and DNCP would be very difficult if not impossible.

Besides the extraction behavior considered above, it is desirable to compare the extent to which interfering reaction by-products are actually formed with each reagent. Again, the greatest concern is focused on the simple hydrolysis products. Figure 6 shows chromatograms obtained from reagent blank and synthetic mixture incubations with each reagent. It is striking that the blank for DNCB shows a significant number of unidentified peaks as well as the major peaks for the reagent and its hydrolytic product. The TNBS blank, on the other hand is quite clean and the PA Peak is small compared to the interfering by-product peaks from the other reagents. The chromatograms in Figure 7 are from synthetic samples identical to those in Figure 6 except that they were subjected to an extraction prior to injection (see experimental section). The extraction step does very little to remove interferences from the DNCP incubation. Neither the reagent itself product are removed to a significant extent. In the case of DNFB, extraction greatly decreases interference from the parent reagent but has essentially no effect on the hydrolytic product (as expected). With TNBS the effect of extraction is similar to that for DNFB. Even after inclusion of an extraction step, the useful elution "window" is larger with TNBS than with DNCP or DNFB. In fact, the limited region of interference with TNBS may make clean up unnecessary for many applications, a feature which would be particularly desirable for micro-scale derivatizations where manipulation of small volumes is difficult and tedious.

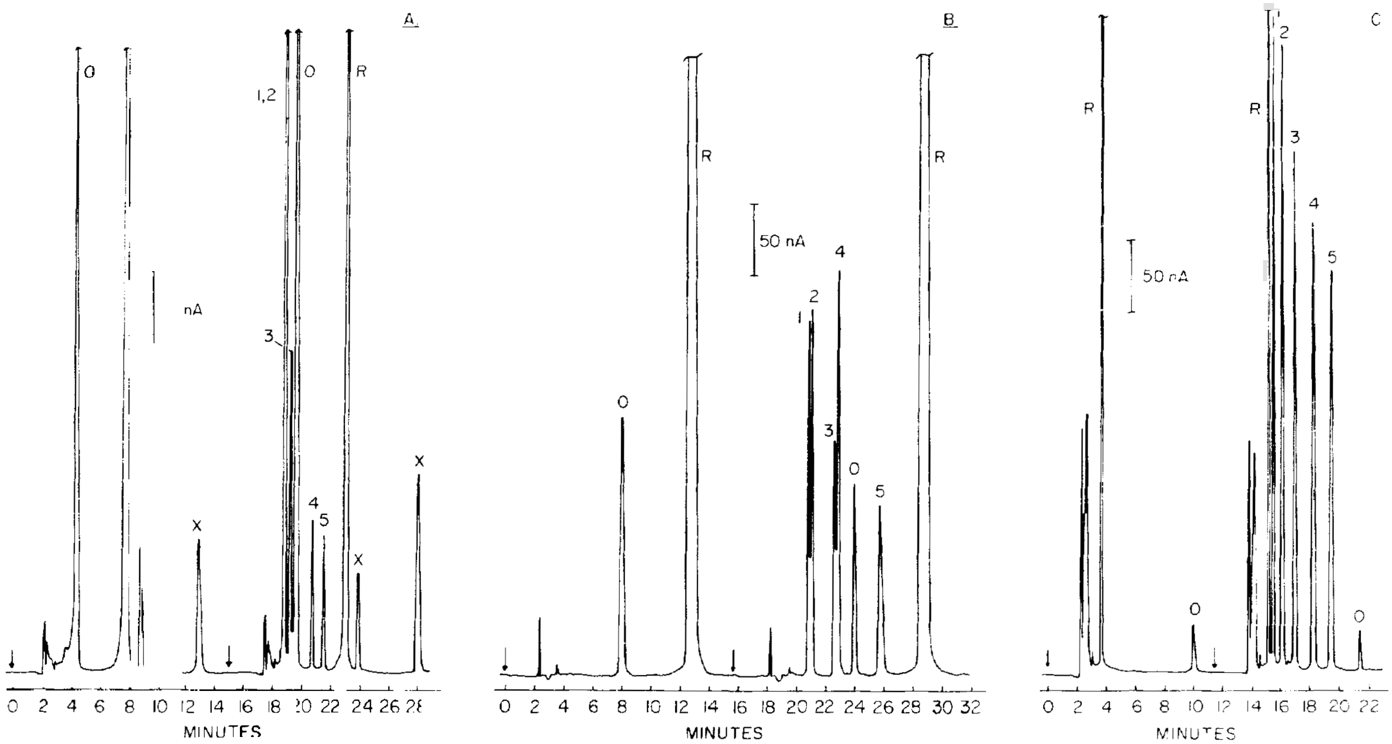


FIGURE 6. Chromatograms of blank (left) and synthetic incubations for A. DNFB B. DNCP C. TNBS peaks: 1 Glutamic acid, 2. Serine, 3. Threonine, 4. Glycine, 5. Alanine, R reagent, O predicted major hydrolysis product. All amino acids present in incubation mixture at 10^{-4}M. Conditions same as in Figure 4 except column was a Biophase C_8, 5 µm, 25 x 0.46 cm.

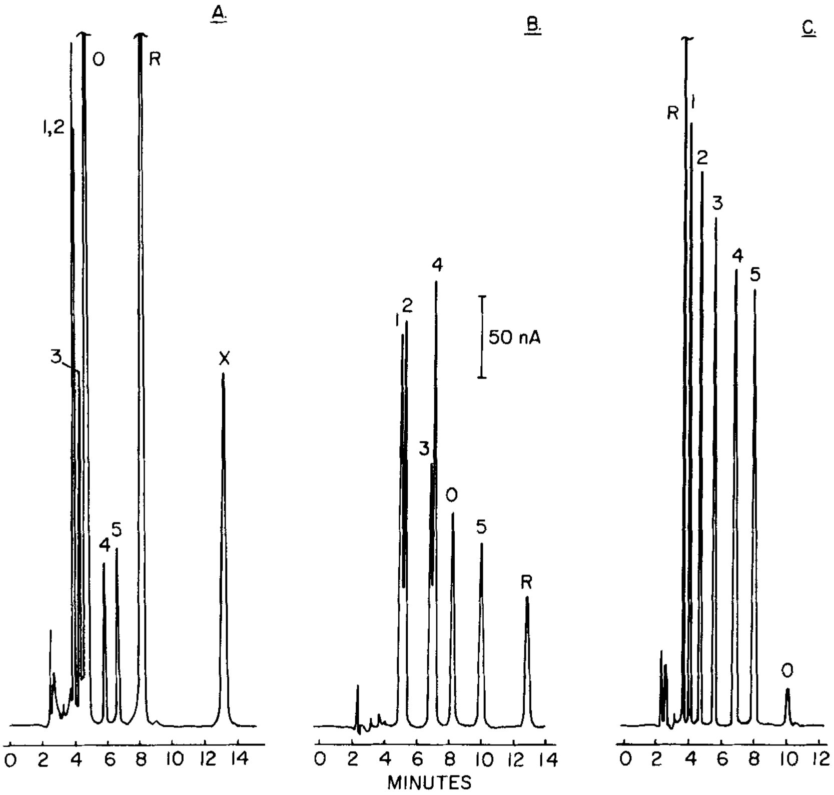


FIGURE 7. Chromatograms from the same synthetic incubations as Figure 6 except subjected to extraction as outlined in Experimental. Conditions as in Figure 6.

Of the three reagents considered in this work, TNBS appears best suited to application as a pre-column derivatization reagent for LCEC. This conclusion is based not only on the favorable electrochemical properties of the trinitrophenyl derivatives but also the minimal occurrence of extraneous reaction by-products and/or reagent impurities. TNBS offers the further advantage of water solubility thus making its use with aqueous samples convenient. It may well be that DNFB and DNCP would be better suited to application in non-aqueous systems although reagent removal would still pose a problem.

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APPENDIX

The fundamental process occurring in the flow cell of the electrochemical detector is governed by Faraday's Law (equation 1) modified to reflect the fact that the detector

$$Q = fnFN \qquad (1)$$

behaves amperometrically (i.e. less than 100% of electroactive material entering the flow cell is actually reacted). Q is the number of Coulombs produced by the n electron reduction (oxidation) of N moles of analyte. F is the Faraday Constant (96,500 coulomb/equivalent) and f is the conversion efficiency for the analyte, where $0 \leq f \leq 1$. The conversion efficiency for a selected analyte is dictated by a combination of thermodynamic

(The Nerstian behavior of the redox couple) and mass transport (cell geometry, flow rate, analyte diffusion coefficient) and in some cases electron transfer kinetics (if slow) parameters. On the plateau region of the HDV the conversion efficiency is determined soley by mass transport, the so-called diffusion current region, thus for a pair of analytes we can write equation 2. If the respective diffusion coefficients are very

$$n_2 = \frac{Q_2 f_1 FN_1}{Q_1 f_2 FN_1} \cdot n_1 \qquad (2)$$

nearly the same, then (2) simplifies to (3) and true n values

$$n_2 = \frac{Q_2 N_1}{Q_1 N_2} \cdot n_1 \qquad (3)$$

for an unknown may be approximated by comparison to a reference compound whose n value is known.

For the potential region where the unknown HDV is rising this simplification is not valid and equation 4 is appropriate

$$n_{2eff} = \frac{Q_2 N_1}{Q_1 N_2} \cdot n_1 \qquad (4)$$

where $n_{2eff} = n_2 \cdot \frac{f_2}{f_1}$. The value n_{2eff} is now an apparent or <u>effective</u> n value which reflects the differing conversion efficiencies between analyte and reference compound.

The use of n_{eff} values is valid under any conditions as long as the true n value of the reference material is known. Under conditions of diffusion limited conversion $n_{eff} \rightarrow n$ only <u>if</u> the reference and analyte diffusion coefficients are very nearly equal.

In this study p-nitroaniline (n = 6) was used as a reference compound and n_{eff} values were calculated for the derivatives of interest over the entire potential range examined by comparing integrated peak areas to that obtained for p-nitroaniline under solely mass transport limited conditions.