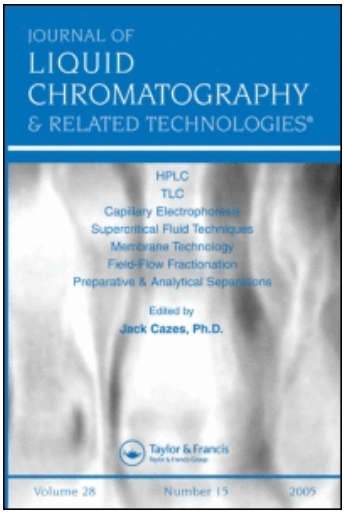


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# Fully Automated Analysis of Amino Acid Enantiomers by Derivatization and Chiral Separation on a Capillary Electrophoresis Instrument

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# FULLY AUTOMATED ANALYSIS OF AMINO ACID ENANTIOMERS BY DERIVATIZATION AND CHIRAL SEPARATION ON A CAPILLARY ELECTROPHORESIS INSTRUMENT

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## ABSTRACT

A completely automated method for determining the chiral purity of amino acids at large enantiomeric excess was developed based on automated pre-separation derivatization with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole, separation by capillary electrophoresis with cyclodextrin chiral selectors and detection by laser-induced fluorescence at 488 nm excitation.

The detection limit is 140 ppm L-phenylalanine in D-phenylalanine, linearity is more than three orders of magnitude and the coefficient of variation is 3 % at the 0.5 % enantiomer level.

## INTRODUCTION

High voltage capillary electrophoresis (CE) is a very efficient separation technique, but it suffers from a lack of detector sensitivity. Pre-separation derivatization can alleviate this problem without adversely affecting the efficiency. In HPLC

pre-column derivatization was not widely used until the introduction of standard protocols on fully automated instrumentation.

We therefore decided to explore the benefits of automated pre-separation derivatization on a commercially available CE-instrument. We shall focus here on enantiomeric analyses of some amino acids.

Two approaches can be used:

A. Derivatization with a chiral reagent and separation of the diastereomers.

B. Derivatization with a non-chiral reagent and separation of the enantiomers.



Approach A was used in the determination of D-valine in an excess of L-valine by derivatization with D-phthalaldehyde and N-acetylcysteine, separation by micellar electrokinetic capillary chromatography (MECC) at acidic pH and absorbance detection at 340 nm (1). This method suffices if the total analyte concentration is known and the enantiomer ratio is not extreme. The linear dynamic range (LDR) of the separation system is determined by the noise level of the detector at the lower limit and the buffer capacity of the MECC system at the upper analyte concentration.

At levels of less than 1 % enantiomeric impurity the separation system has to be overloaded with the major diastereomer to be able to determine the minor one.

The LDR can not be extended by increasing the buffer capacity of the MECC system, because of current limitations. A different derivative or wavelength for UV-detection could increase the LDR by a factor of three at the expense of derivatization speed, using Marfey's reagent, or selectivity, using 214 nm as the detection wavelength (1).

A variety of chiral reagents can be utilized not only with absorbance detection but also with fluorescence detection, which extends the LDR by several orders of magnitude. However, at extreme enantiomer ratios the enantiomeric impurity of the reagent can cause an error in the enantiomeric excess (ee) of the analyte. Approach B thus appears more appropriate for performing enantiomeric analyses at large ee.

Several off-line pre- and post-separation reactions with fluorogenic reagents were compared by Albin et al. (2). Fluorescein is accompanied by artifact and blank reagent problems, fluorescamine derivatives are generally too unstable to be used in an off-line method, so 9-fluorenylmethyl chloroformate (FMOC) was their preferred reagent, although the reagent itself is fluorescent and was extracted with pentane (2). This last drawback and the lack of a suitable laser for excitation (at 260 nm) of these derivatives of primary and secondary amino acids made us search for an alternative to be used in automated derivatization. Such a reagent - for primary amino acids - is naphtalene dicarboxaldehyde (3), which is included in our study. Liu et al. (4) demonstrated with a similar, new reagent that they synthesised a three order of

magnitude linearity in the determination of amino acid derivatives using laser induced fluorescence at 442 nm and MECC.
In 1989 thermooptical absorbance detection of 4-(dimethylamino) azobenzene-4'-sulfonylchloride derivatives was reported to be even superior over fluorescence detection (5). Unfortunately this detection mode is not available to us.

In this paper we explore the determination of the enantiomeric purity of some amino acids at large ee by fully automated pre-separation derivatization with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole (NBD-F), separation by CE with β-cyclodextrin (βCD) and dimethyl βCD as chiral selectors, and detection by laser-induced fluorescence (LIF) with an argon ion laser.



## THEORY

The purpose of any separation is to obtain adequate resolution. In CE the resolution of two components i and j, $R_{ji}$, can be expressed as:

$$R_{ji} = \frac{\Delta\mu_{ji} \cdot E}{\sqrt{\sigma^2_{L,inj} + 2Dt_j}} t_j \qquad (1)$$

in which:

$R_{ji}$ = resolution between components j and i, expressed in units of the average standard deviation of peaks j and i

$\Delta\mu_{ji}$ = selectivity, difference in electrophoretic mobility between component j and i ($m^2/V.s$)

E = electric field strength (V/m)

$\sigma^2_{L,inj}$ = injection contribution to the variance of the peaks ($m^2$)

D = average diffusion coefficient of components j and i ($m^2/s$)

$t_j$ = elution time of the last component (s)

The dependence of the selectivity on the selector concentration in an enantiomeric separation with chiral selectors was given by Wren and Rowe (6):

$$\Delta\mu = \frac{[CD]\ (\mu_o - \mu_\infty)\ (K_B - K_A)}{1 + [CD]\ (K_A + K_B) + K_A K_B [CD]^2} \qquad (2)$$

where:

[CD] = chiral selector concentration

$\mu_o$ = mobility of the enantiomers

$\mu_\infty$ = mobility of the enantiomer-selector complex

$K_A, K_B$ = equilibrium constants for enantiomers A and B, respectively

The apparent electrophoretic mobility of an enantiomer in this system is:

$$\mu = \frac{\mu_o + \mu_\infty K[CD]}{1 + K[CD]} \qquad (3)$$

The maximum selectivity is obtained at a selector concentration equal to the reciprocal average equilibrium constant (6), so with

equation 3 the optimum selector concentration can be evaluated from mobility data:

$$\mu_{opt} = \frac{\mu_o + \mu_\infty}{2} \qquad (4)$$

This means that for any analyte the optimum selector concentration can be obtained in a few experiments, but on the other hand each analyte will have a different optimum selector concentration depending on its affinity to the selector. This was demonstrated by Wren and Rowe (7) for compounds differing in hydrophobicity.



The analyte concentrations are much lower than the selector concentrations used so the selector concentration in the equations above can be considered constant.

Penn et al. (8) stressed that in order for the $\mu_\infty$ determination to be accurate the viscosity of the separation medium should be taken into account. At the relatively low selector concentrations used in our research viscosity corrections were not applied: they have only a minor effect on $\mu_{opt}$ (equation 4), and most optima are rugged (see table 2) and insensitive to small deviations. In addition $\mu_\infty$ is often not reached due to solubility limitations of the chiral selector in the run buffer.

## EXPERIMENTAL

Experiments were done on a PACE 2050 system (Beckman Instruments, Fullerton, CA, USA) equipped with a 3 mW argon-ion

laser-induced fluorescence detector. A 488 nm rejection filter and a 520 ± 20 nm bandpass filter were supplied with the detector and inserted in the filter holder on the emission side of the detector. The separations were carried out on a 970 x 0.05 mm (length x ID) fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The program for performing the analysis is given in table 1.

Except for the reaction microvials (30 µl, Beckman) standard 4 ml vials (Beckman) were used.
Triacetyl-β-cyclodextrin was obtained from Aldrich (Milwaukee, WI, USA), α-, β-, γ-, and 2,6-di-O-methyl-β-cyclodextrin from Fluka (Buchs, Switzerland).
4-Chloro-7-nitrobenzofurazan (NBD-Cl) was obtained from Janssen (Beerse, Belgium), its fluoro analog (NBD-F) from Sigma (St. Louis, MO, USA), naphthalene-2,3-dicarboxaldehyde (NDA) from Polysciences (Warrington, PA, USA) and the amino acids used were from a variety of sources.

Sodium hydroxide, tris(hydroxymethyl)aminomethane (TRIS), boric acid, potassium cyanide and ethanol were purchased from Merck (Darmstadt, Germany) and all water was produced by a Milli-Q apparatus (Millipore, Bedford, MA, USA). The 0.1 M TRIS-borate buffer used throughout this paper was prepared by adjusting a 0.1 M boric acid solution to pH 8.3 by addition of a 0.1 M TRIS solution. The reagent for derivatization (autosampler vial # 4) was prepared daily by dissolving 0.5 mg NBD-F in 310 mg of ethanol.

TABLE 1

Program for Automated Derivatization Followed by Separation of the Derivatives.

| inlet vial[a] | contents | outlet vial | contents |
|---|---|---|---|
| 11 | separation buffer | 1 | separation buffer |
| 12-x | analyte | 3 | water |
| (x+1)-30 | empty | 4 | reagent |
| | microvial | 5 | ethanol |
| 31 | 1M sodium hydroxide | 8 | empty |
| | | 10 | empty |
| 32 | water | | |
| 33 | empty | | |
| 34 | ethanol | | |

[a] x = 20 for single analysis of nine samples; x = 14 for fivefold analysis of three samples.

Separation buffer = 0.1 M TRIS-borate pH 8.3

| step | process | duration | inlet | outlet | control summary |
|---|---|---|---|---|---|
| 1 | set detector | | | | LIF: 488:520 nm<br>Rate: 5 Hz<br>Zero 2.0 min |
| 2 | set temp. | | | | Temp.: 25 °C |
| 3 | rinse | 1.0 min | 31 | 8 | Forward: High Pressure |
| 4 | rinse | 1.0 min | 32 | 8 | Forward: High Pressure |
| 5 | rinse | 1.0 min | 33 | 8 | Forward: High Pressure |
| 6 | rinse | 2.5 min | 12 | 8 | Forward: High Pressure |
| 7 | wait | 0.0 min | 32 | 3 | |
| 8 | rinse | 0.1 min | 22 | 5 | Reverse |
| 9 | rinse | 2.0 min | 22 | 4 | Reverse |
| 10 | rinse | 0.1 min | 22 | 5 | Reverse |
| 11 | rinse | 2.0 min | 22 | 10 | Reverse |
| 12 | wait | 5.0 min | 34 | 3 | |
| 13 | rinse | 3.0 min | 11 | 8 | Forward: High Pressure |
| 14 | inject | 3.0 sec | 22 | 8 | Pressure |
| 15 | wait | 0.0 min | 34 | 3 | |
| 16 | inject | 3.0 sec | 32 | 10 | Pressure |
| 17 | separate | 18.0 min | 11 | 1 | Constant Voltage: 30.00kV<br>Current Limit: 100.0 µA<br>Integrator On |
| 18 | wait | 0.0 min | 34 | 3 | |

## RESULTS AND DISCUSSION

### Selection of Derivatization Reagent and Chiral Selector

Our selection of reagent was determined by the following limitations:

- fast reaction with amines,
- derivatives to be detected by LIF using 442 nm (He-Cd laser) or 488 nm (Ar-ion laser) excitation,
- enantiomeric derivatives separable by CE or MECC.

Two reagents fulfilled these requirements: naphthalene-2,3-dicarboxaldehyde (NDA) (3,9) and NBD-F (10). In experiments using 0.1 M TRIS-borate buffer pH 8.3 containing α-, β-, γ-, dimethylβ- or triacetylβ-cyclodextrin we found that the optimum selector concentration and the selectivity were much lower with NDA-derivatized amino acids than with NBD-derivatives. Selectivities at the optimum cyclodextrin concentrations (see equation 4) for some NBD-amino acid derivatives using β-CD and dimethylβ-CD are given in table 2.

Applying a buffer of lower pH (11) and/or MECC (10) offers opportunities for resolution enhancement, but were considered to be outside the scope of this paper.

### Derivatization Protocol

The autosampler program is similar to that used before (1, c.f. table 1) except for ethanol enclosed transport of reagent to

TABLE 2

Selectivities at Optimum Cyclodextrin Concentrations

| amino acid | $\Delta\mu$ $10^{-10}$ $m^2/V.s$ | [βCD] mM | $\Delta\mu$ $10^{-10}$ $m^2/V.s$ | [dimethylβCD] mM |
|---|---|---|---|---|
| alanine | 4.2 | ≥ 14 | | |
| leucine | 5.5 | ≥ 8 | 2 | ≥ 4 |
| valine | 3.7 | > 14 | | |
| proline | 5.5 | ≥ 14 | 3.3 | > 8 |
| phenylglycine | 3.1 | 2 | 7 | 2 |
| phenylalanine | 5.7 | 2 | 10 | 1.5 |
| methionine | 3.5 | > 14 | 2 | > 8 |
| Phe-amide | 3.5 | > 14 | 7 | 2 |



the reaction vial. If this is omitted the reagent tends to adhere to the wall and precipitates during the mixing-step.

To obtain sufficient resolution the temperature during separation should not exceed 35 °C. With our instrument the derivatization is performed at the same temperature as the separation. Derivatization with NBD-CL takes a few minutes at 60 °C.
Therefore, the reaction was done with NBD-F, which reacts much faster (10). Unfortunately, the NBD-F solution is not very stable and has to be prepared daily.

In a more recent version of the CE-instrument the vials of the autosampler can be heated separately so that the reaction might be performed with the more stable NBD-Cl at elevated temperature while separation takes place at lower temperature.

TABLE 3

Precision of the determination of enantiomeric purity of phenylglycine at 0.5 % level.

A.

| duration of injection (s) | L area (counts) | D area (counts) | D/L (%) |
|---|---|---|---|
| 2 | 20399.5 | 99.4 | 0.58 |
| 2 | 21398.0 | 107.1 | 0.60 |
| 1 | 9287.5 | 46.8 | 0.60 |
| 1 | 12034.2 | 62.0 | 0.62 |
| 5 | 41046.1 | 205.4 | 0.60 |
| 5 | 32074.3 | 168.8 | 0.63 |

cv (%) 2.7

B.

| D area (counts) | L area (counts) | L/D (%) |
|---|---|---|
| 12177 | 78.7 | .54 |
| 24904 | 164.7 | .55 |
| 27232 | 172.9 | .53 |
| 24900 | 167.0 | .56 |
| 21236 | 136.8 | .54 |
| 19032 | 117.0 | .51 |

cv (%) 3.1

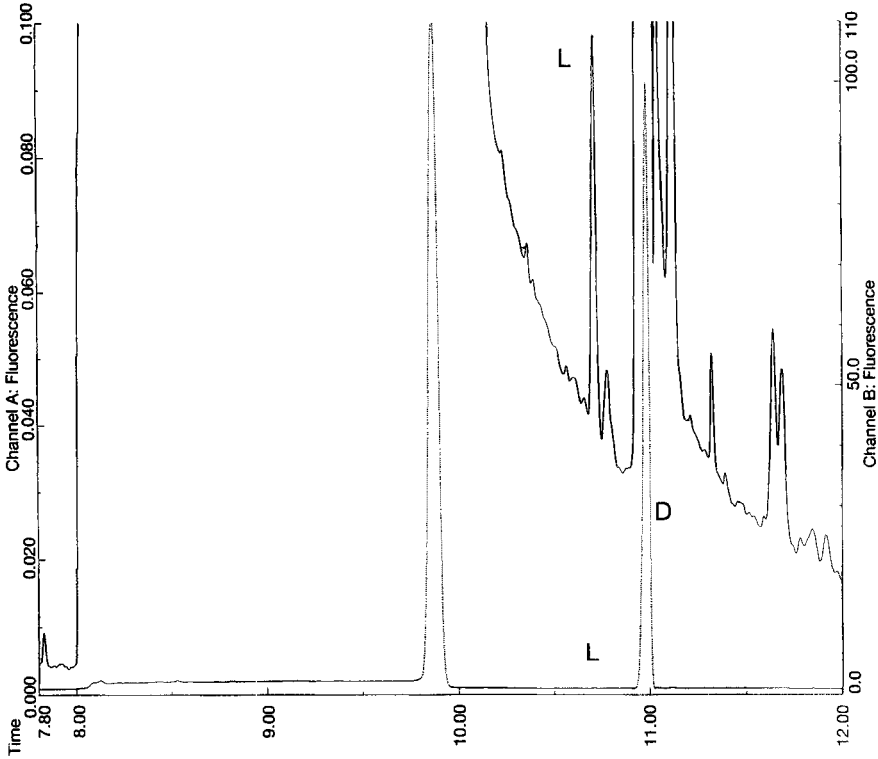


FIGURE 1. Electropherogram for determination of enantiomeric purity of D-phenylalanine.
Derivatization and separation program as in table 1. The scale on the right belongs to the lower tracing, the expanded scale on the left to the upper trace of the same electropherogram.
The wide band eluting at 8-10' is caused by the reagent.
The run buffer contains 2 mM dimethyl βCD, the sample 0.5 mg/ml D-Phe.

## Determination of Enantiomeric Purity of Phenylglycine and Phenylalanine

In chiral analysis the content of one of the enantiomers in the sample can be used as an internal standard if the LDR of the

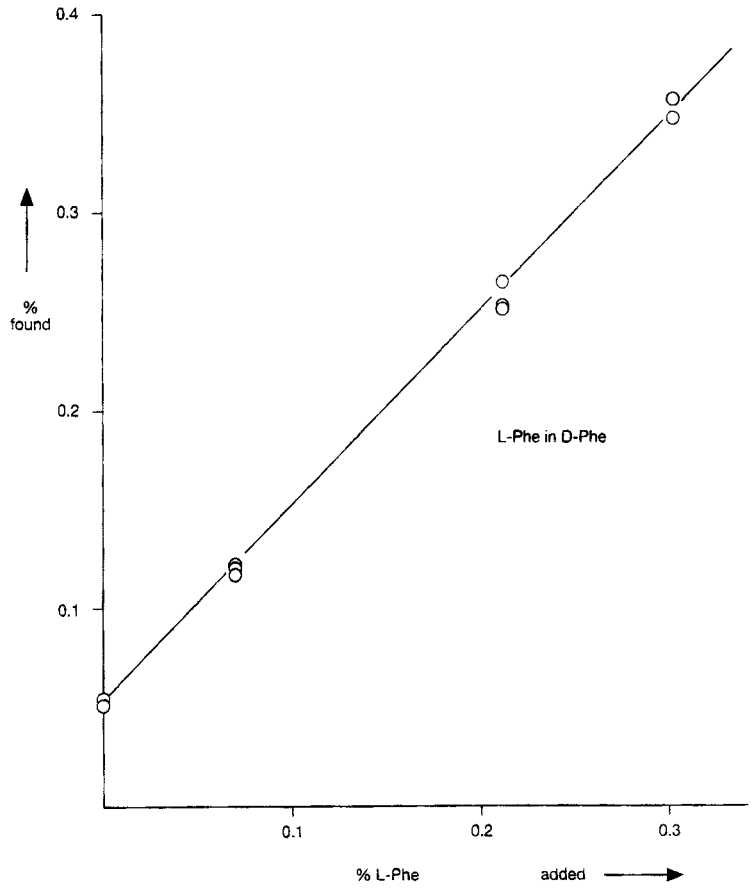


FIGURE 2. Calibration curve for determination of L-Phe in D-Phe by standard addition.
Derivatization and separation program as in table 1; sample as in fig. 1.

system is sufficient. The determination of L-phenylglycine in D-phenylglycine and D-phenylglycine in L-phenylglycine under these circumstances (data in tables 3A and 3B) shows that the precision at the 0.5 % level is about 3 % CV.

Analysis of a racemic mixture indicates that the intrinsic fluorescence sensitivities of the cyclodextrin complexes of the derivatives of D- and L-phenylglycine are different. This difference is taken into account in table 3.

In table 3A an amount of derivative varying over a factor of four was introduced into the system. The result indicates that any non-linearity of the system is limited to less than the three percent variation coefficient of this table. A calibration curve for L-Phe in D-Phe was made by standard-addition to determine the detection limit and the purity of D-phenylalanine (see fig. 1). The D-phenylalanine sample used contained 0.05 % L-phenyl-alanine (fig. 2), which is well above the detection limit calculated from the calibration curve: 0.014 % L- in D-phenylalanine (95 % confidence interval).



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