# Method Development for the High-Performance Liquid Chromatographic Enantioseparation of 2-Cyanocycloalkanols

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## Abstract

**An indirect high-performance liquid chromatographic method is developed for the enantioseparation of *cis*- and *trans*-2-cyanocyclopentanol and -cyclohexanol. The racemic *cis*-(1*S*,2*S* and 1*R*,2*R*)- or *trans*-(1*S*,2*R* and 1*R*,2*S*)-2-cyanocycloalkanols are converted to their diastereomers formed with (*S*)-(+)- or (*R*)-(–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. The diastereomers are separated on a reversed-phase column, and the conditions of derivatization and HPLC analysis are optimized.**

## Introduction

2-Cyanocycloalkanols with two stereogenic centers are important synthetic targets for the preparation of 1,2-disubstituted 1,3-difunctional cycloalkanes, which can be subjected to further transformation to produce 1,3-heterocycles (1). Thus, hydrogenation of the cyano group provides a valuable amino group in the formation of alicyclic 1,3-amino alcohols. A chemical or enzymatic hydrolysis of the cyano group leads to the formation of amides and carboxylic acids (2,3). Methods have been developed for the preparation of racemic *cis*- and *trans*-2-cyanocycloalkanols and their enantiomers (4–6). As targets for biologically active or pharmaceutically important compounds, the enantiopurity of 2-cyanocycloalkanols (IA–ID and IIA–IID in Figure 1) is crucial.

The gas chromatographic (GC) enantioseparation of *cis*- and *trans*-2-cyanocyclopentanols and *cis*-2-cyanocyclohexanol was carried out on a Chrompack (Middelburg, The Netherlands) CP-Cyclodextrin-β-2,3,6-M-9 column, and the enantiomers of *trans*-2-cyanocyclohexanol were separated on a Chirasil-L-Val column (Chrompack) (6). Before GC analysis, the alcohols were derivatized with propionic or pentanoic anhydride in the presence of 4-dimethylaminopyridine and pyridine.

Successful high-performance liquid chromatographic (HPLC) methods for the resolution of stereoisomers include direct and indirect methods. Direct methods are performed by ligand-exchange chromatography (7–10) or by the application of chiral stationary phases (CSPs) (11–13). Indirect methods involve a precolumn derivatization reaction with chiral derivatizing agents (CDAs) with a subsequent separation of the diastereomers on an achiral column (14,15). Many attempts have been made in connection with the derivatization and subsequent separation of compounds containing hydroxy groups. Most of the CDAs applied were of acyl halide type. Kim et al. (16) used *S*-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxyl chloride (III); Ishida et al. (17) used camphanoic chloride (IV); Toyo'oka et al. (18,19) used 4-(2-chloroformylpyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (V) and 4-(2-chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (VI); Liebmann et al. (20) used *S*-(+) flunoxaprofen chloride (7); Vogt and Kiessig (21) used (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) (8); Harvey and Cho (22) used menthoxy chloride (IX); Aycard et al. (23) used menthyl chloroformate (MCF) (X); Nusser et al. (24) used (*R*)-(–)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPA-Cl) (XI); Shimizu et al. (25,26) used

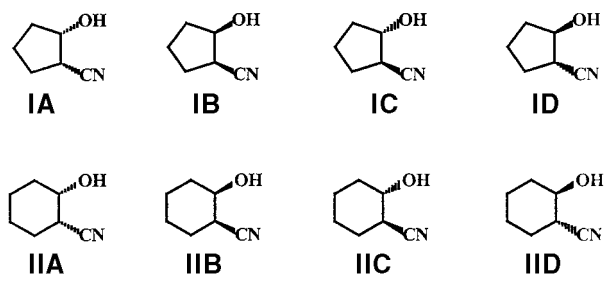


**Figure 1.** Structures of 2-cyanocycloalkanols and their enantiomers: 2-cyanocyclopentanol, I; 2-cyanocyclohexanol, II; *cis*-(1*S*,2*S*), A; *cis*-(1*R*,2*R*), B; *trans*-(1*S*,2*R*), C; and *trans*-(1*R*,2*S*), D.

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D-2-(2-naphthyl)propionyl chloride (XII) and *N*-1-(2-naphthylsulfonyl)-2-pyrrolidinecarbonyl chloride (XIII); and Wang (27) used naproxen chloride (XIV). An isocyanate-based CDA, *S*-(+)-α-(1-naphtylethyl)isocyanate (XV), was used by Ruettimann et al. (28), and carboxylic acid type CDAs were used by Nishida et al. (29), Chen et al. (30), Banfield and Rowland (31), and Carter et al. (32). Carboxylic acid type CDAs have some disadvantages in regards to the derivatization procedure. Applications of acyl halide and isocyanate-based CDAs for the derivatization of analytes containing hydroxy groups are summarized in Table I. Despite a great assortment of CDAs applied for the derivatization of a hydroxy group, a selection of the most adequate one requires some circumspection. Certain CDAs are not available commercially, and the difference in the price in some cases is significant. After derivatization, the diastereomers formed were separated by normal-phase (NP) or reversed-phase (RP) methods, and in some cases both separation methods were applied. Taking into account the availability of the CDA, the duration, and the temperature of the reaction, the elaboration of a procedure involving the application of FLEC and MCF seemed to be favorable. However, the selection of an appropriate CDA is dictated not only by the properties of the CDA, but also by the reactivity of the analyte. In this study, the hydroxy group on the cycloalkane ring displayed very low reactivity, which made the selection of the CDA and derivatization difficult.

This study describes the enantioseparation of racemic *cis*-(1*S*,2*S* and 1*R*,2*R*)- or *trans*-(1*S*,2*R* and 1*R*,2*S*)-2-cyanocycloalkanols by an indirect HPLC method. The 2-cyanocycloalkanols (Figure 1) were synthesized in the racemic form, and lipase-catalyzed enzymatic resolution was applied for the preparation of the pure enantiomers. For enantiomeric purity control, racemic 2-cyanocycloalkanols were converted to their diastereomers formed with (*S*)-(+)- or (*R*)-(–)-MTPA-Cl. The diastereomers were separated under RP conditions. The conditions for derivatization and HPLC analysis were optimized.

## Experimental

### Chemicals and reagents

Racemic *trans*-2-cyanocyclopentanol (IC,D) and -cyclohexanol (IIC,D) were obtained from 1,2-epoxycyclopentane and -cyclohexane, respectively, with hydrogen cyanide (4). The corresponding racemates of *cis*-2-cyanocyclopentanol (IA,B) and -cyclohexanol (IIA,B) were prepared by the decarboxylative ring opening of 3-carboxyisoxazolines obtained by the hydrolysis of 3-carbethoxyisoxazolines formed in the (3+2) cycloaddition of carbethoxyformonitrile oxide and the corresponding cycloalkene (5). These racemates were resolved through lipase PS-catalyzed (from *Pseudomonas cepacia*) asymmetric acetylation, allowing for the simultaneous preparation of (1*S*,2*S*)-IA and (1*S*,2*S*)-IIA alcohols and (1*R*,2*R*) ester enantiomers (IB and IIB) for the *cis* isomers and (1*S*,2*R*)-IC and (1*S*,2*R*)-IIC alcohols and (1*R*,2*S*) ester enantiomers (ID and IID) for the *trans* isomers (6).

The preparation of the analytes depicted in Figure 1 has been reported elsewhere (4,5). The reagents used for derivatization—benzoic anhydride, FLEC, and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)—were purchased from Sigma-Aldrich (Steinheim, Germany). The reagents (–)-MCF and (*S*)-(+)- or (*R*)-(–)-MTPA-Cl (Mosher's reagent) were obtained from Fluka (Buchs, Switzerland).

HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), triethylamine (TEA), pyridine, 4-dimethylaminopyridine, and other reagents of analytical grade were also from Merck.

### Apparatus and chromatography

The HPLC system consisted of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector, and a Millenium$^{32}$ Chromatography Manager data system (Waters Chromatography, Milford, MA) and was equipped with an injector Model

**Table I. Data on the Derivatization of Compounds Containing Hydroxy Groups with Different CDAs**

| | Conditions of derivatization | | | | | | |
|---|---|---|---|---|---|---|---|
| Type of CDA | CDA (mg) | Analyte (mg) | Reaction time (h) | Temperature (°C) | Price of CDA* (DM) | Type of separation | References |
| 3† | 24 | 17 | 2 | 25 | – | NP | 16 |
| 4 | 10 | urine‡ | 24 | 25 | 106/g | NP | 17 |
| 5 | 0.4 | 1.0 | 3 | 25 | –§ | NP | 18 |
| 6 | 0.15 | 0.01 | 4 | 80 | –§ | NP | 19 |
| 7† | 2 | blood‡ | 0.25 | 110 | – | RP | 20 |
| 8 | 0.4 | blood‡ | 1 | 45 | 235/mL | RP | 21 |
| 9 | 16 | 8 | 24 | 25 | 20/g | NP,RP | 22 |
| 10 | 22 | blood‡ | 0.3 | 30 | 93/5 mL | NP,RP | 23 |
| 11 | 250 | urine‡ | 12 | 25 | 560/g | RP | 24 |
| 12 | 75 | 30 | 0.25 | 25 | – | RP | 25 |
| 13 | 20 | 2.0 | 0.25 | 25 | – | NP | 26 |
| 14† | 0.03 | blood‡ | 20 | 25 | 57/5 g | RP | 27 |
| 15† | 0.2 | blood‡ | 12 | 85 | 182/mL | RP | 28 |

* Price list from Sigma-Aldrich and Fluka catalogues.
† Before chromatographic analysis, the derivatives were prepared in further time-consuming procedures.
‡ Analyses were performed on either urine or blood matrices.
§ Available from Tokyo Kasei (TCI America, Portland, OR).

7125 (Rheodyne, Cotati, CA) with a 20-µL loop.

The RP column used was a Vydac 218TP54 $C_{18}$ (250- × 4.6-mm i.d., 5-µm particle size) (The Separations Group, Hesperia, CA). The CSPs applied were the Chiralcel OD-R (150- × 4.0-mm i.d., 5-µm particle size) (Daicel, Tokyo, Japan) and the Cyclobond I and III 2000 (250- × 4.6-mm i.d., 5-µm particle size) (Astec, Whippany, NJ).

The inorganic part of the mobile phase was an aqueous buffer or water, which was mixed with the organic modifier (MeOH or MeCN). Ultrapure water from the Millipore (Milford, MA) Milli-Q system was used for the preparation of all the solutions and eluents. The pH was measured with an OP 208/1 precision pH-meter (Radelkis, Budapest, Hungary). The flow rate was 1.0 mL/min and detection was carried out at 205 nm.

In the case of the separation of MTPA derivatives, the following gradient systems were applied. For gradient 1, component A was water–MeCN (80:20, v/v) and component B was water–MeCN (20:80, v/v). The gradient was run from 0 to 100% for B within 30 min. For gradients 2, 3, 4, and 5, component C was water–MeOH (95:5, v/v) and component D was water–MeOH (5:95, v/v). The gradient was run from 30 to 100% for D within 20, 45, 60, or 90 min respectively for gradients 2, 3, 4, and 5.

**Derivatization of 2-cyanocycloalkanols**

Different methods were applied for the derivatization of the 2-cyanocycloalkanols. For the derivatization of the analytes with MCF, aqueous- or organic-phase reactions were applied. In the former case, the 50 µL of 0.1M aqueous NaOH and 50 µL of 0.15M MCF that were dissolved in MeCN or MeOH were added to 50 µL of 0.025M analyte dissolved in MeCN or MeOH (32). In the case of organic-phase reactions, the analytes and MCF were dissolved in dichloromethane in the same concentrations as mentioned previously and dry TEA (water-free) was used as the catalyst (23,34). In both cases, the reactions were carried out at an elevated temperature (40°C) and the reaction time was increased to several hours. After 12–24 h, the reaction mixture was dried under argon pressure and the residue was then redissolved in the eluent.

In the case of derivatization with FLEC, 50 µL of 18mM FLEC that was dissolved in acetone was added to 50 µL of 2.0mM analyte dissolved in 0.1M sodium hydrogencarbonate or 1.0M borate buffer (pH 8.4). The reaction mixture was kept at 40°C for 24 h and was diluted with eluent before injection (35,36).

For the derivatization with Mosher's reagent, the original proto-

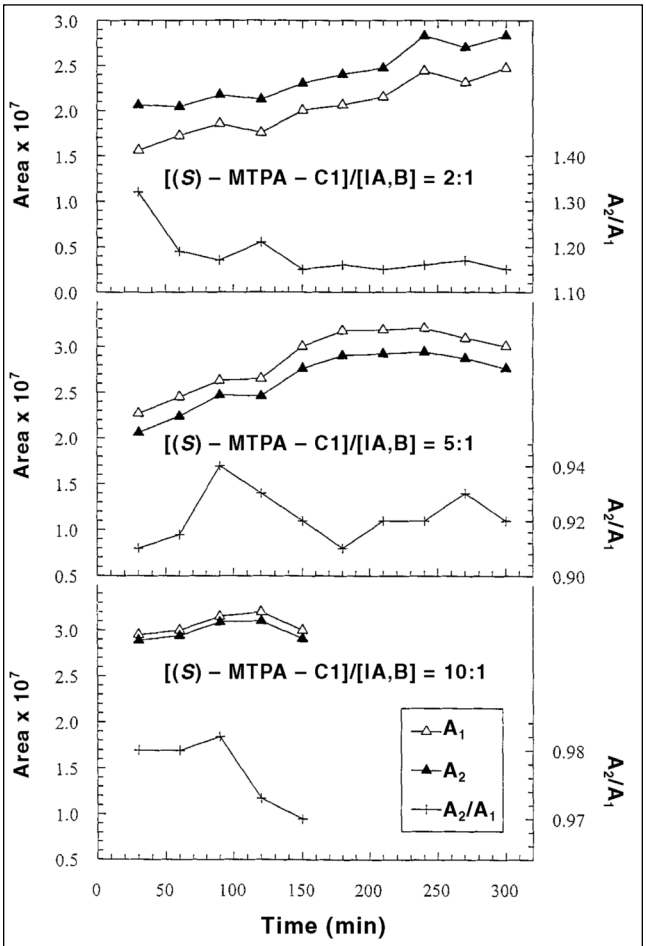


**Figure 2.** Dependence of peak areas and ratio of peak areas ($A_2/A_1$) of *cis*-(1*S*,2*S*)- and (1*R*,2*R*)-1-(*S*)-MTPA derivatives on the molar ratio [(*S*)-MTPA]/[IA,B] and on the time of reaction. The sequence of elution was (1*S*,2*S*)-1-(*S*)-MTPA diastereomer < (1*R*,2*R*)-1-(*S*)-MTPA diastereomer. Area of peak eluting first, $A_1$; area of peak eluting second, $A_2$; area of peak of (1*S*,2*S*)-1-(*S*)-MTPA diastereomer, ∅; area of peak of (1*R*,2*R*)-1-(*S*)-MTPA diastereomer, ▲.

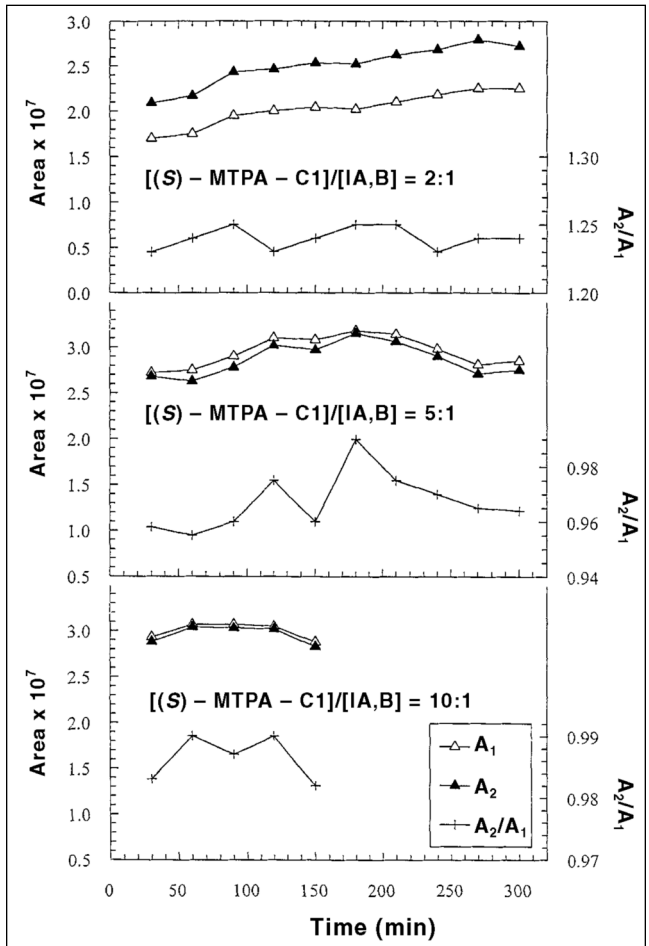


**Figure 3.** Dependence of peak areas and ratio of peak areas ($A_2/A_1$) of *cis*-(1*S*,2*S*)- and (1*R*,2*R*)-1-(*R*)-MTPA derivatives on the molar ratio [(*R*)-MTPA]/[IA,B] and on the time of reaction. The sequence of elution was (1*R*,2*R*)-1-(*R*)-MTPA diastereomer < (1*S*,2*S*)-1-(*R*)-MTPA diastereomer. Area of peak eluting first, $A_1$; area of peak eluting second, $A_2$; area of peak of (1*R*,2*R*)-1-(*RR*)-MTPA diastereomer, ∅; area of peak of (1*S*,2*S*)-1-(*R*)-MTPA diastereomer, ▲.

**Table II. Chromatographic Data, Retention Factor*, Separation Factor, and Resolution of the Separation of (*R*)- or (*S*)-MTPA Derivatives of 2-Cyanocycloalkanols**

| Compound | CDA | $k_1$ | $k_2$ | Separation factor | Resolution | Elution sequence | Gradient |
|---|---|---|---|---|---|---|---|
| IA,B | (*R*)-MTPA | 7.19 | 7.31 | 1.02 | 1.11 | (1*R*,2*R*) < (1*S*,2*S*) | I |
| | (*R*)-MTPA | 5.11 | 5.27 | 1.03 | 2.19 | (1*R*,2*R*) < (1*S*,2*S*) | II |
| | (*S*)-MTPA | 5.16 | 5.30 | 1.03 | 2.22 | (1*S*,2*S*) < (1*R*,2*R*) | II |
| IC,D | (*R*)-MTPA | 7.57 | 7.57 | 1.00 | 0.00 | (1*R*,2*S*) < (1*S*,2*S*) | I |
| | (*R*)-MTPA | 5.26 | 5.30 | 1.01 | < 0.40 | (1*R*,2*R*) < (1*S*,2*R*) | II |
| | (*R*)-MTPA | 8.68 | 8.81 | 1.01 | 0.95 | (1*R*,2*R*) < (1*S*,2*R*) | III |
| | (*S*)-MTPA | 10.47 | 10.73 | 1.02 | 1.34 | (1*S*,2*R*) < (1*R*,2*S*) | IV |
| | (*S*)-MTPA | 13.75 | 14.00 | 1.02 | 1.40 | (1*S*,2*R*) < (1*R*,2*S*) | V |
| 2A,B | (*R*)-MTPA | 7.74 | 7.87 | 1.02 | 1.31 | (1*R*,2*R*) < (1*S*,2*S*) | I |
| | (*R*)-MTPA | 5.47 | 5.62 | 1.03 | 2.11 | (1*R*,2*R*) < (1*S*,2*S*) | II |
| | (*S*)-MTPA | 5.43 | 5.51 | 1.02 | 2.15 | (1*S*,2*S*) < (1*R*,2*R*) | II |
| 2C,D | (*R*)-MTPA | 7.86 | 7.98 | 1.02 | 1.25 | (1*R*,2*S*) < (1*S*,2*R*) | I |
| | (*R*)-MTPA | 5.41 | 5.55 | 1.03 | 1.78 | (1*R*,2*S*) < (1*S*,2*R*) | II |

* Retention factor, k.

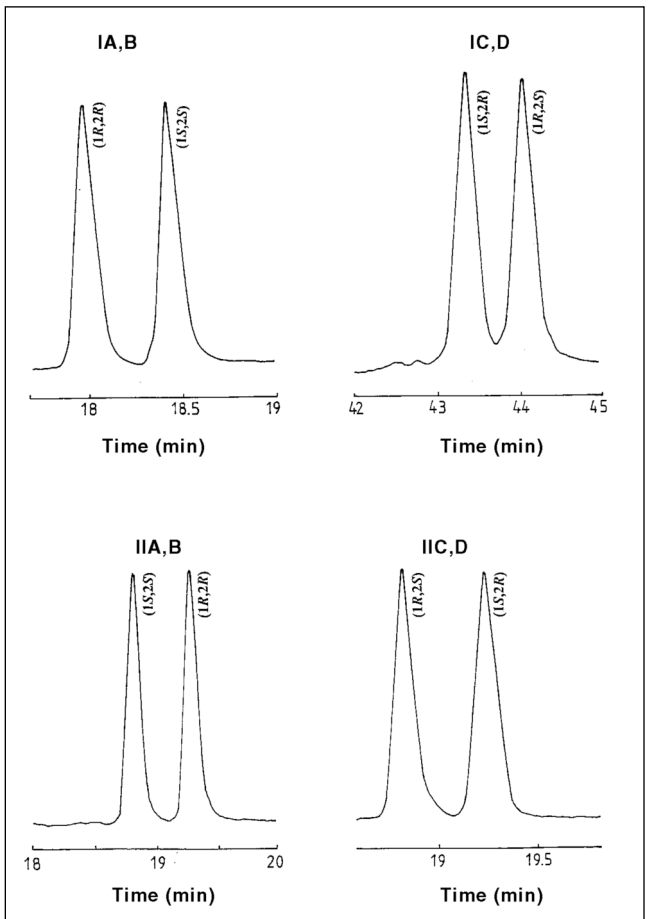


**Figure 4.** Selected chromatograms for the enantiomeric separation of 2-cyanocycloalkanols. Gradient II was used for analytes IA,B; IIA,B; and IIC,D and gradient V for analyte IC,D. IA,B and IIC,D were derivatized with (*R*)-MTPA and IC,D and IIA,B with (*S*)-MTPA. Sequence of elution: IA,B (1*R*,2*R*) < (1*S*,2*S*); IC,D (1*S*,2*R*) < (1*R*,2*S*); IIA,B (1*S*,2*S*) < (1*R*,2*R*); and IIC,D (1*R*,2*S*) < (1*S*,2*R*).

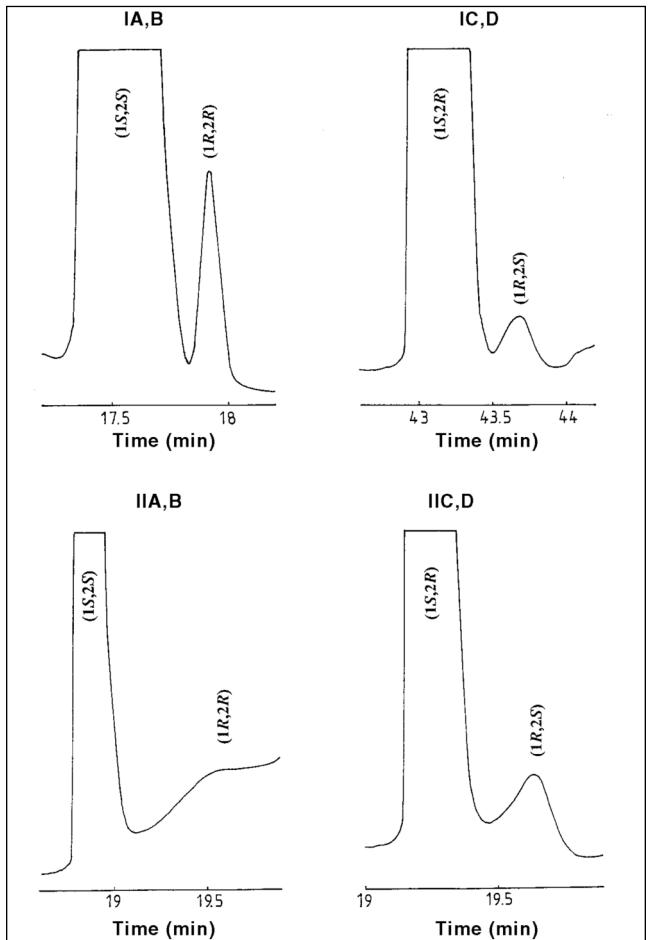


**Figure 5.** Separation of enantiomers after enzymatic resolution (enantiomeric purity control). Gradient II was used for analytes IA,B; IIA,B; and IIC,D and gradient V for analyte IC,D. All of the analytes were derivatized with (*S*)-MTPA. Sequence of elution: IA,B (1*S*,2*S*) < (1*R*,2*R*); IC,D (1*S*,2*R*) < (1*R*,2*S*); IIA,B (1*S*,2*S*) < (1*R*,2*R*); IIC,D (1*S*,2*R*) < (1*R*,2*S*).

cols were modified (24,37). In a test tube, 10 µL (*R*)- or (*S*)-MTPA-Cl was added to 0.5 mg analyte dissolved in 100 µL dry pyridine and the reaction mixture was kept at 40°C for 120 min. The molar ratio of MTPA-Cl and the analyte was approximately 10 to 1. The excess of reagent could readily be removed by the addition of 50 times the glycine to the reaction mixture, which would then be kept for another 30 min at 40°C. Before the HPLC analyses, the reaction mixture was diluted 5 to 10 times with the eluent.

Derivatization with Marfey's reagent (38) and nonchiral derivatization with benzoic anhydride (39) were carried out by the literature methods.

## Results and Discussion

The direct separation of the enantiomers of IA,B; IC,D; IIA,B; and IIC,D was attempted on two types of CSPs. The cellulose-based Chiralcel OD-R has been recommended for compounds containing such groups as the free carboxyl, hydroxy, and cyano groups (40). The α- and β-cyclodextrin-based CSPs Cyclobond III and I 2000, respectively, have been recommended for the separation of enantiomers containing aromatic rings (41). For this reason, the *O*-benzoyl derivatives of the analytes were prepared. No conditions were found for direct enantioseparation either on Chiralcel OD-R or the Cyclobond columns.

For indirect separation, different acyl halide-based CDAs were applied (listed in the Experimental section). Of these CDAs, only the most electrophilic CDA (MTPA-Cl) displayed a suitable reactivity towards the hydroxy group of 2-cyanocycloalkanols. The yield of derivatization with all other CDAs remained below 5–10%.

### Optimization of MTPA-Cl derivatization and chromatography

Because the hydroxy group in 2-cyanocycloalkanols has low reactivity, the conditions for the derivatization described in the literature needed to be optimized (24,36). The results of the optimization are depicted in Figures 2 and 3. The racemic analyte IA,B was chosen as the model compound, and derivatization was carried out with both enantiomers of the reagent—(*S*)-MTPA-Cl and (*R*)-MTPA-Cl—at three different ratios of MTPA-Cl and analyte (2:1, 5:1, and 10:1). The reaction time was varied between 30 and 300 min, and the temperature was raised to 40°C (at ambient temperature the rate of reaction was low). Figures 2 and 3 clearly demonstrate that with an increasing MTPA-Cl/analyte ratio, the yield of the reaction increased. It reached its maximum at an approximate MTPA-Cl/analyte ratio of 5:1 or 10:1. The duration of the reaction also influenced the yield. At a molar ratio of 2:1 for MTPA-Cl and analyte the yield constantly increased with an increasing duration of the reaction, and at the higher molar ratios of MTPA-Cl and analyte the yield reached a maximum at approximately 120–180 min. A slow decomposition of the derivatized analyte was subsequently observed at 40°C.

The reaction was accompanied by kinetic resolution. At the molar ratio of 2:1 for MTPA-Cl and analyte a significant difference was observed in the areas of the peaks of the diastereomers formed. The second peak was higher than the first, and the ratio of the areas was $A_2/A_1 > 1.0$. At molar ratios of 5:1 and 10:1 the ratio of the peak areas $A_2/A_1$ approached 0.98–0.99. When (*S*)-MTPA-Cl was applied in a small excess, it had a higher reactivity towards the (1*R*,2*R*) stereoisomer than the (1*S*,2*S*) stereoisomer. (*R*)-MTPA-Cl displayed a higher reaction rate with the (1*S*,2*S*) stereoisomer than with the (1*R*,2*R*) stereoisomer when the (*R*)-MTPA-Cl/analyte ratio was near 2:1. This meant that (*S*)-CDA preferred to react with hydroxy groups attached to carbon atoms with the *R* configuration and (*R*)-CDA preferred to react with hydroxy groups attached to carbon atoms with the *S* configuration. When an increase of the excess of CDA in the reaction mixture occurred, this difference in the reaction rate was diminished in order for the two enantiomers to react with CDA with almost equal reaction rates.

HPLC analyses of the derivatives were performed on a Vydac 218TP54 $C_{18}$ column. Two mobile phase systems were applied with MeCN or MeOH as the organic modifier. The enantiomers were separated by means of a gradient elution (results presented in Table II). MeOH seems to be a better organic modifier than MeCN in the separation of MTPA derivatives. With gradient program 1, despite the higher retention factors the resolutions were lower than with gradient program 2. MeCN was unsuitable for the separation of IC,D (these stereoisomers practically coeluted). With MeOH as the organic modifier, baseline separations were obtained for all of the derivatives of the enantiomers, but when the total separation of IC,D is desirable, the slope of the gradient should be decreased. Selected chromatograms for the separation of the stereoisomers of the 2-cyanocycloalkanols are depicted in Figure 4.

The method was applied to control the enantiomeric purity of the stereoisomers after enzymatic resolution. For the determination of enantiomeric impurity, it may be important to ensure that the impurity elutes before the main peak, but the efficiency of the method can be characterized if the peak eluting second is the minor component. Figure 5 provides examples of the enantiomeric purity control when the peak eluting second is the minor component. Analytes (1*S*,2*S*)-IA and -IIA contain small amounts of (1*R*,2*R*)-IB and -IIB, respectively, and analytes (1*S*,2*R*)-IC and -IIC contain small amounts of (1*R*,2*S*)-ID and -IID, respectively. On the $C_{18}$ column, the (*S*)-MTPA derivatives of these stereoisomers furnished chromatograms in which the minor component was the peak eluting second. The ratio of the minor and major peak areas gave the following results concerning the enantiomeric impurity: the IB content in IA was 1.23%, the ID content in IC was 0.16%, the IIB content in IIA was approximately 0.1%, and the IID content in IIC was 0.14%. For the derivatives of (*R*)-MTPA, the component eluting first was the minor one (not shown). Calculations relating to the enantiomeric impurities for the (*R*)-MTPA derivatives gave results that were similar within experimental error to those observed for (*S*)-MTPA derivatives. The very low level of enantiomeric impurities meant that the derivatization reaction took place without racemization and was instead a result of the incomplete enzymatic resolution.

## Conclusion

Despite the low reactivity of the hydroxy groups of 2-cyanocycloalkanols, a successful procedure was developed for their

derivatization with MTPA-Cl. By the variation of different parameters (such as the molar ratio of CDA and analyte, the reaction time, and the temperature of reaction), the conditions of derivatization were optimized. The kinetic resolution observed in the derivatization reaction was explained. The derivatives of the enantiomers were separated by means of the gradient HPLC elution on an RP column with the application of a water–MeOH-containing mobile phase.

## Acknowledgments

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