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tert.-Butylcarbamoylquinine as chiral ion-pair agent in non-aqueous enantioselective capillary electrophoresis applying the partial filling technique☆

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Abstract

The potential of *tert.*-butylcarbamoylquinine as chiral selector (SO) added to a non-aqueous background electrolyte for the capillary electrophoretic separation of the enantiomers of *N*-derivatized amino acids (selectands, SAs) is evaluated. Separation is based on different ion-pair formation equilibrium constants of (*R*) and (*S*) enantiomers of the negatively charged chiral analytes with the positively charged quinine-derived chiral SO and on mobility differences of free and complexed SAs, so that differences in the overall migration behavior of the SA enantiomers result. To suppress problems associated with the high UV absorption of the chiral SO and thus the high detector background in the 'total filling technique', the 'partial filling technique' has been adopted. Several parameters including filling time and length of SO zone, respectively, SO concentration, type of background electrolyte, have been evaluated. Using such an optimized method, for example, (*R*) and (*S*) enantiomers of 2,4-dinitrophenyl (DNP)-protected proline could be separated with $\alpha=1.08$, $R_S=6.60$, and $N=130\,000$ theoretical plates within 15 min. Similar α values, resolution, and efficiencies were observed for other DNP-protected, as well as for diverse, *N*-derivatized amino acids like *N*-benzoyl, *N*-9-fluorenylmethoxycarbonyl, *N*-3,5-dinitrobenzyloxycarbonyl amino acids. A repeatability study clearly validated the robustness of the method and revealed its practical applicability.

Keywords: Chiral selectors; Enantiomer separation; Nonaqueous capillary electrophoresis; Partial-filling capillary electrophoresis; Butylcarbamoylquinine; Amino acids

1. Introduction

Capillary electrophoresis has become a popular and widely accepted liquid phase microseparation technique for the analysis of the enantiomers of chargeable chiral compounds, primarily due to its high separation efficiency. The current state-of-the-art in CE enantioseparation including a detailed discussion of the chiral selectors (SOs) and selectivity principles applied for this challenging task has been subject of a recent comprehensive book [1]. Also, various review articles are dealing with progresses made in this field [2–13].

Most of these enantiomer separations have been performed with aqueous solutions of background electrolytes (BGEs) to which the diverse chiral SOs have been added in various concentrations. However, in recent years non-aqueous CE (NACE) enantio-

☆Dedicated to Prof. Dr. J.F.K. Huber on the occasion of his 75th birthday.

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separation has become reasonably attractive, as non-aqueous BGE can clearly extend the versatility and scope of applicability of CE as a separation technique for the analysis of mixtures of enantiomers. One of the most pronounced advantages is the better solubility of many chiral SOs in organic solvents, so that higher SO concentrations can be used in the BGE to achieve useful separations. Such a technique has been employed for various types of SOs (native and derivatized cyclodextrins, chiral ion-pair agents, chiral crown ethers, etc.) and chiral selectivity principles [14–23].

Already in the mid-1980s the use of quinine and derivatives as chiral ion-pair agents in HPLC was described [24,25]. Problems associated with large SO consumptions could be overcome by conducting the separations in micro-LC separation techniques. Later, Stalcup and Gahm employed quinine as an ion-pair agent for CE enantioseparations under non-aqueous conditions (methanol as organic solvent and ammonium acetate as electrolyte) [18]. With this separation system they were able to separate *N*-3,5-dinitrobenzoylated (DNB) amino acid derivatives and a few other chiral acids. Recently, we have shown that *tert.*-butylcarbamoylated quinine (see Fig. 1) yields much higher enantioselectivity and efficiency in CE enantioseparation of DNB-derivatized amino acids than the native quinine analogue [23], which, in addition, exhibits reversed elution order of (*R*) and (*S*) enantiomers of DNB-amino acids providing experimental evidence for a changed stereochemical recognition mechanism. The same chiral *tert.*-butylcarbamoylated quinine SO has also been successfully employed for the capillary electrochromatographic (CEC) enantioseparation of *N*-derivatized acids using the ion-pair selectivity principle and a packed C_{18} reversed-phase column, thus combining chromatographic and electrophoretic separation principles [26].

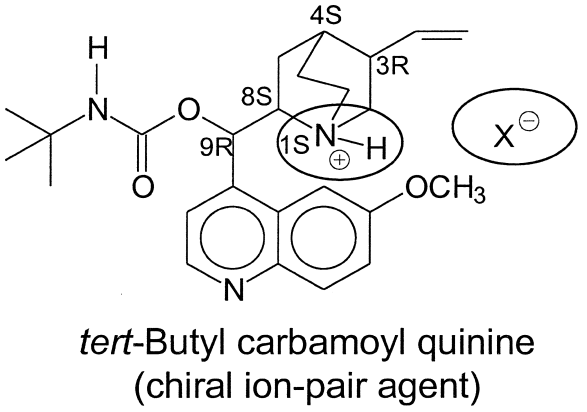

Fig. 1. Structure of the quinine-derived chiral SO and ion-pair agent used in this study.

While a common non-aqueous CE method utilizing *tert.*-butylcarbamoylated quinine as chiral ion-pair agent (counter-ion) (see Fig. 1) in a conventional 'total filling technique', with a continuous separation zone throughout the capillary and the chiral ion-pair SO in both inlet and outlet home vials during the runs in combination with UV detection, can be highly successful with regard to enantioselectivity and efficiency, the practical applicability of such a setup is limited by the high detector background of the strongly UV-absorbing chiral SO. To overcome such problems with detector noise the 'partial filling technique' is often employed in capillary electrophoretic and electrokinetic separation methods. The technique was first described by Valtcheva et al. [27]. A discontinuous separation zone is built up in the capillary by filling the SO-BGE solution from the injection end to a defined length before the detection cell, while the remaining section of the capillary is filled with plain BGE only. The runs are then carried out with pure BGE in inlet and outlet home vials. This prevents the SO entering the detection window during the runs, provided that the SO zone has no significant mobility in the separation direction. The 'partial filling technique' nowadays is frequently used for CE–UV [28–36], CE–MS [37–39], and micellar electrokinetic chromatographic (MEKC)–MS [40] separation techniques in enantioselective as well as in nonstereoselective analysis.

In this study, the 'partial filling technique' is adopted to the application of *tert.*-butylcarbamoylquinine (Fig. 1) as chiral ion-pair SO in non-aqueous CE for the enantioseparation of *N*-derivatized amino acids. The optimization of the separation conditions will be described here, and the practical applicability of this liquid phase separation method should be demonstrated by validation data.

2. Experimental

2.1. Materials

The SO, *O*-(*tert*.-butylcarbamoyl)quinine (see Fig. 1), was synthesized by refluxing for 4 h anhydrous quinine base (Buchler, Darmstadt, Germany) and a 1.1 molar excess of *tert*.-butylisocyanate (Aldrich) in toluene in the presence of dibutyltin dilaurate (three drops) as catalyst. More details about the synthesis, purification and characterization are given elsewhere [41,42].

The chiral analytes, *N*-(9-fluorenylmethoxycarbonyl)leucine (Fmoc-Leu), *N*-benzoylleucine and -phenylalanine (Bz-Leu, Bz-Phe), *N*-(3,5-dinitrobenzoyl) derivatives of leucine, phenylglycine and phenylalanine (DNB-Leu, DNB-Phg and DNB-Phe) were supplied by Bachem (Bubendorf, Switzerland), Fluka, Aldrich or Sigma. *N*-(2,4-Dinitrophenyl) (DNP) derivatives of amino acids and amino-phosphonic acids, respectively, were prepared by derivatization with 2,4-dinitrofluorobenzene (Sanger's reagent) (Aldrich) following a standard derivatization protocol as described elsewhere [43]. *N*-(3,5-Dinitrobenzyloxycarbonyl) (DNZ)-protected amino acids were prepared as described previously [44]. DNB-β-aminobutyric acid (DNB-β-Abu) and DNB-β-phenylalanine (DNB-β-Phe) as well as Bz-β-Phe were synthesized from the free amino acids and the corresponding acid chlorides (benzoyl and 3,5-dinitrobenzoyl chloride, Aldrich) by a Schotten–Baumann reaction. The proteinogenic and non-proteinogenic amino acids, alanine (Ala), leucine (Leu), α- and β-aminobutyric acid (α-Abu and β-Abu), proline (Pro), phenylalanine (Phe), and β-phenylalanine (β-Phe), used for the above-mentioned derivatizations were from Bachem, Fluka, Sigma and Aldrich, respectively, and the phosphonic acid analogue of alanine, 1-aminoethylphosphonic acid, Ala(P), was provided by Aldrich. Samples have been prepared at a concentration of 0.1 mg/ml in the non-aqueous electrolyte solution.

HPLC-grade solvents, methanol (MeOH) and ethanol (EtOH), were from J.T. Baker. Triethylamine (Fluka), glacial acetic acid (Merck, Darmstadt, Germany), and octanoic acid (Aldrich) were of analytical grade and used for the preparation of the non-aqueous background electrolytes.

Separations have been carried out in 50-μm I.D. bare fused-silica (FS) capillaries (Composite Metal Services, Hallow, UK) with a total length of 45.5 cm and an effective length of 37 cm. Prior to use, capillaries were pre-conditioned by rinsing with 1 *M* sodium hydroxide (30 min), water (30 min) and non-aqueous electrolyte (without chiral SO) (10 min).

2.2. Instrumentation

An HP^{3D} capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector was used. Four different detection wavelengths (230, 250, 280 and 360 nm) were routinely set up for data acquisition. The capillary was thermostated (air ventilation) at 15°C.

2.3. Standard procedure for CE experiment with partial filling technique

Before each run, the capillary was pre-conditioned by flushing 10 min with the plain non-aqueous electrolyte solution containing no chiral SO, e.g. ethanol–methanol (60:40) mixture to which 100 m*M* octanoic acid or glacial acetic acid and 12.5 m*M* triethylamine were added. Then, the capillary was filled with non-aqueous electrolyte solution containing the chiral SO (10–80 m*M*) applying a pressure of 50 mbar to the injection end of the capillary over a period of 5 min. When the above-mentioned electrolyte solution with a 10 m*M* SO concentration is used, a plug of ca. 30 cm in length from the injection end of the 50-μm I.D. FS capillary (total length 45.5 cm, effective length 37 cm) is filled (calculated from the breakthrough curve). Then, the sample dissolved in BGE without SO was injected hydrodynamically by application of a pressure of 50 mbar for 5 s. Finally, the analytical run was performed applying a constant voltage of −25 kV using the plain non-aqueous electrolyte solution without chiral SO as running buffer at both inlet and outlet home vials.

3. Results and discussion

3.1. Separation mechanism

Recently, we have reported on the optimization of the capillary electrophoretic enantiomer separation of DNB-protected amino acids employing *tert.*-butylcarbamoylated quinine as SO under non-aqueous BGE conditions [23]. It has turned out that non-aqueous conditions using an ethanol–methanol (60:40) mixture containing 100 m*M* octanoic acid, 12.5 m*M* triethylamine, and reasonable amounts of chiral SO (e.g. 10 m*M*) are suitable to separate the enantiomers of DNB-protected amino acids with outstanding enantioselectivity, high efficiency, and good peak symmetry. Later, the scope of application of this NACE method has been extended also to a wide variety of other amino acid derivatives, e.g. DNP-protected aminocarboxylic and -phosphonic acids, but using a conventional 'total filling technique' (for selected separation examples see Fig. 2). The separation selectivity thereby is based on an ion-pairing mechanism.

Under the above-specified experimental conditions and apparent pH of the non-aqueous BGE, the quinine derivative used as chiral ion-pair agent (see Fig. 1) is positively charged, while the chiral analytes (selectands, SAs) to be separated into individual enantiomers are dissociated and negatively charged. In the course of the separation process, the negatively charged SAs (*N*-derivatized amino acids) intermolecularly interact with the positively charged quinine derivative to form a pair of electrically neutral diastereomeric SO–SA ion-pairs. Generally, in the non-aqueous medium the degree of ion-pair formation is supposed to be higher than in a purely

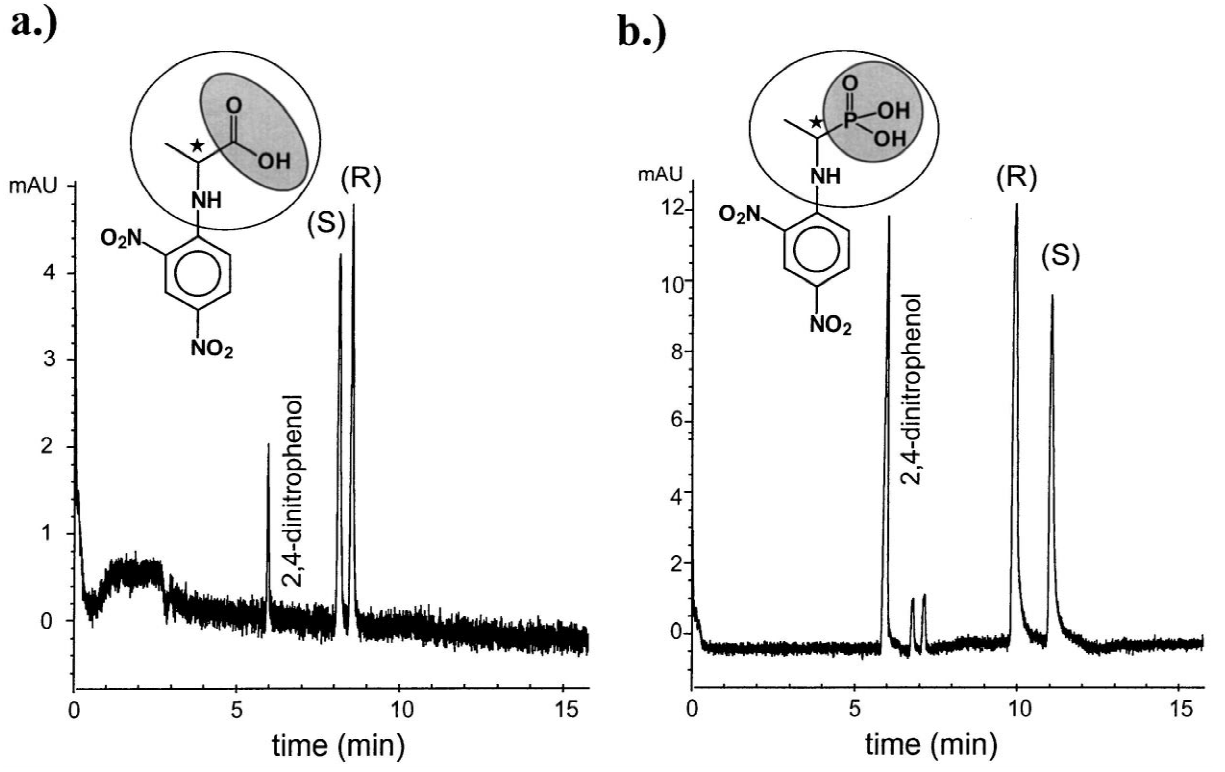

Fig. 2. Application of the conventional 'total filling method' for the CE enantioseparation of (a) (*R,S*)-Ala racemate and (b) (*R,S*)-Ala(P) enantiomers as *N*-DNP derivatives employing *tert.*-butylcarbamoylquinine as chiral ion-pair SO under non-aqueous BGE conditions. Experimental conditions: 100 m*M* octanoic acid, 12.5 m*M* triethylamine and 10 m*M* *tert.*-butylcarbamoylquinine in EtOH–MeOH (60:40); *T*, 15°C; voltage, −25 kV (−3.5 μA); detection, UV 280 nm (other conditions as specified in Section 2).

aqueous solution due to the lower dielectric constant of the EtOH–MeOH mixture compared to water and resulting stronger ionic interaction [45]. However, competing ion-pairing species in the BGE solution, namely negatively charged co-ions (octanoate) and positively charged buffer components (triethylammonium) balance the ionic interaction leading to dissociation of the ion-pairs. Zero electrophoretic mobility is assumed for the electrically neutral ion-pairs, which therefore migrate solely with the electroosmotic flow (EOF), while the negatively charged analytes being in the dissociated or uncomplexed state have significant effective electrophoretic mobility and migrate towards the anodic detection end of the capillary. Favorably, the free chiral SO migrates towards the cathodic injection end, in the same direction as the electroosmotically driven bulk liquid, resulting in a counter-current-like separation process. Separation of the corresponding *N*-derivatized amino acid enantiomers is based on (i) different ion-pair equilibrium constants (K_{ip}) of (*R*) and (*S*) enantiomers, and (ii) different electrophoretic mobility of free SAs and complexed SAs (ion-pairs). In a buffered liquid phase, the degree of ion-pair formation decreases with increasing concentrations of anionic co-ions due to competition for ion-pair formation. Recently, we have measured the ion-pair equilibrium constants of (*R*) and (*S*)-DNB-Leu and *tert.*-butylcarbamoylquinine in MeOH–0.1 *M* aqueous ammonium acetate (80:20) (pH_a 6.0) by a CE method, which yielded an ion-pair equilibrium constant in the range of $10^2\ M^{-1}$ for the stronger bound (*S*)-enantiomer and ca. $0.5\times10^1\ M^{-1}$ for the weaker bound (*R*) enantiomer [46]. The basis for the difference of $K_{ip(R)}$ and $K_{ip(S)}$ on the molecular level is discussed in detail in several previous papers [42,47–51] and is related to the stereoselective occurrence of supplementary intermolecular SO–SA interactions (hydrogen bonding, $\pi-\pi$ interaction, etc.) in addition to the primary ionic interaction and ion-pairing mechanism, respectively.

For such an enantioselective CE ion-pairing separation process, Bjornsdottir et al. [17] proposed a theoretical mobility model (analogues to that derived earlier for chiral separations with cyclodextrins by Wren and Rowe [52]) describing the mobility difference (separation selectivity) of (*R*) and (*S*) SA enantiomers in dependence of their ion-pair equilibrium constants ($K_{ip(R)}$ and $K_{ip(S)}$ values) and of the SO concentration. According to this model, the percentage difference in the equilibrium constants determines the magnitude of the difference in migration of (*R*) and (*S*) enantiomers of the analyte. Large differences of the K_{ip} values will lead to large selectivity. On the other hand, the absolute values of the equilibrium constants will determine whether optimum enantioselectivity is obtained at high SO concentrations (when K_{ip} have small absolute values) or at low SO concentrations (when K_{ip} adopt large absolute values) [17,52].

Applying this model to the currently discussed separation process, it can be argued that optimum separation selectivity will be obtained at SO concentrations above the earlier studied 1–10 m*M* *tert.*-butylcarbamoylquinine [23]. By using EtOH–MeOH mixtures as solvents, which exhibit reasonable solubility for electrolytes composed of organic acids and bases like triethylammonium octanoate, SO solubility above 10 m*M* is not a problematic issue. However, the strongly UV-absorbing quinoline moiety of the chiral SO prevents application of the conventional 'total filling technique' at such high concentrations due to rather poor detection sensitivity and baseline drifts during the runs. Further, it should be pointed out that in the 'total filling technique' diastereomers are detected, which may have significantly different detector response due to different absorption coefficients of (*R*) and (*S*) SO–SA ion-pairs, as can be seen from the electropherograms in Fig. 2. For example, the ratio of the peak areas of (*R*) and (*S*)-DNP-Ala (Fig. 2a) deviates significantly from 50:50, which is expected for a racemic mixture. In contrast, in the 'partial filling mode' no chiral SO reaches the detection window. Accordingly, much higher detection sensitivity can be obtained, and in addition, the same detector response is observed for (*R*) and (*S*) enantiomers, thus being less prone to errors in quantitation.

When the 'partial filling technique' is used, the SO plug which represents the active separation zone should ideally not move, either towards the detection end or to the injection end. If the SO zone moves toward the detection end, usually a shift of the signal is observed and the signal noise reduces the detection sensitivity. If the SO zone moves too fast towards the injection end, enantioselectivity will be reduced,

since the SA analytes will migrate a long time in a zone containing no chiral SO. Therefore, as an ideal prerequisite for application of the 'partial filling technique' the chiral SO should have (i) no significant self-electrophoretic mobility or at least the SO mobility should be significantly lower than SA mobility, and (ii) also no significant EOF should exist. The above-discussed separation process fits both requirements relatively well, although uncoated capillaries have been used in this study. Under the conditions specified in Fig. 2, both the SO mobility as well as the EOF (due to low ε/η ratios of EtOH–MeOH mixtures and high buffer concentrations) were negligible, so that they could not be determined accurately. However, it should be pointed out that the strongly basic quinine derivative may adsorb to the negatively charged capillary wall modifying the EOF characteristics and leading to relatively worse run-to-run repeatability.

3.2. 'Partial filling' method

The separation of the SA enantiomers occurs while they migrate through the SO zone and interact with the SO in this separation zone. On the other hand, both (*R*) and (*S*) SA enantiomers migrate with same velocity in the plain BGE zone. Accordingly, the length of the separation zone containing chiral SO will affect the observed migration behavior and apparent mobility of the analytes, as well as enantioselectivity, resolution and also separation efficiency. Therefore, we studied the influence of different filling times and SO zone lengths, respectively, on these separation parameters. First, breakthrough curves have been monitored with the SO-BGE solution, 100 m*M* octanoic acid, 12.5 m*M* triethylamine and 10 m*M* chiral SO added to a mixture of EtOH–MeOH (60:40). The SO zone reached the detection window after 6 min, when the 45.5 cm (effective length 37 cm)×50 μm I.D. capillary was filled hydrodynamically with a constant pressure of 50 mbar. From this breakthrough time the approximate length of the SO zone can be calculated for different filling times, yielding a SO plug of approximately 6, 15 and 30 cm for filling times of 1, 2.5 and 5 min, respectively, for the above-specified SO-BGE solution.

Fig. 3 illustrates the dependence of the separation parameters of selected analytes (DNB-Leu and DNZ-Phe) on the filling times. Generally, the longer the SO-BGE plug or SO zone the longer the observed migration time and the lower the apparent mobility (μ_a) (see Fig. 3a). The time average of the SAs being in complexed form (ion-pair) increases with increasing filling time. As the complex or ion-pair has nearly zero mobility this results in longer migration times. Simultaneously, enantioselectivity (α) values increase as can be seen from the plot in Fig. 3b. Also separation efficiency is effected by the length of the zone filled with SO. With increasing filling times theoretical plate numbers (*N*) increase (see Fig. 3c). As a result, resolution R_S between corresponding (*R*) and (*S*) enantiomers improves with the effective length of the separation zone (see Fig. 3d).

This behavior applies to most of the studied analytes. For a few SAs, however, a slightly different behavior was observed. For example, migration time of the weak bound (*R*)-enantiomer of DNB-β-Abu (ca. 49 min) was more or less unaffected by increasing the filling time (see Fig. 4), while migration times for the enantiomer with higher affinity to the chiral SO increased in the expected way from 56.12 min (at a filling time of 1 min) to 64.14 min (filling time of 2.5 min) to 82.74 min (when the capillary is filled 5 min with the SO-BGE solution). In agreement with the data in Fig. 3, this leads to highly improved enantioselectivity (increase from 1.13 to 1.29 to 1.71) as well as resolution (increase from 4.08 to 7.44 to 17.47). The results obtained for other analytes at a filling time of 5 min and employing the same method are summarized in Table 1. It can be seen that R_S values for different *N*-derivatized amino acids are large enough for practical application.

With a filling time of 5 min (ca. 30 cm) there is still a safety zone of about 7 cm between SO zone and detection window, so that no baseline drifts have been observed throughout the study. Generally, if SO-BGE solutions with significantly different viscosities are used, the filling time has to be optimized again. However, the same method presumably may work well, if only the SO concentration is varied in a limited range. Overall, it has to be pointed out that enantioselectivity and resolution values have been slightly higher in the corresponding 'total filling method' than in the optimized 'partial filling

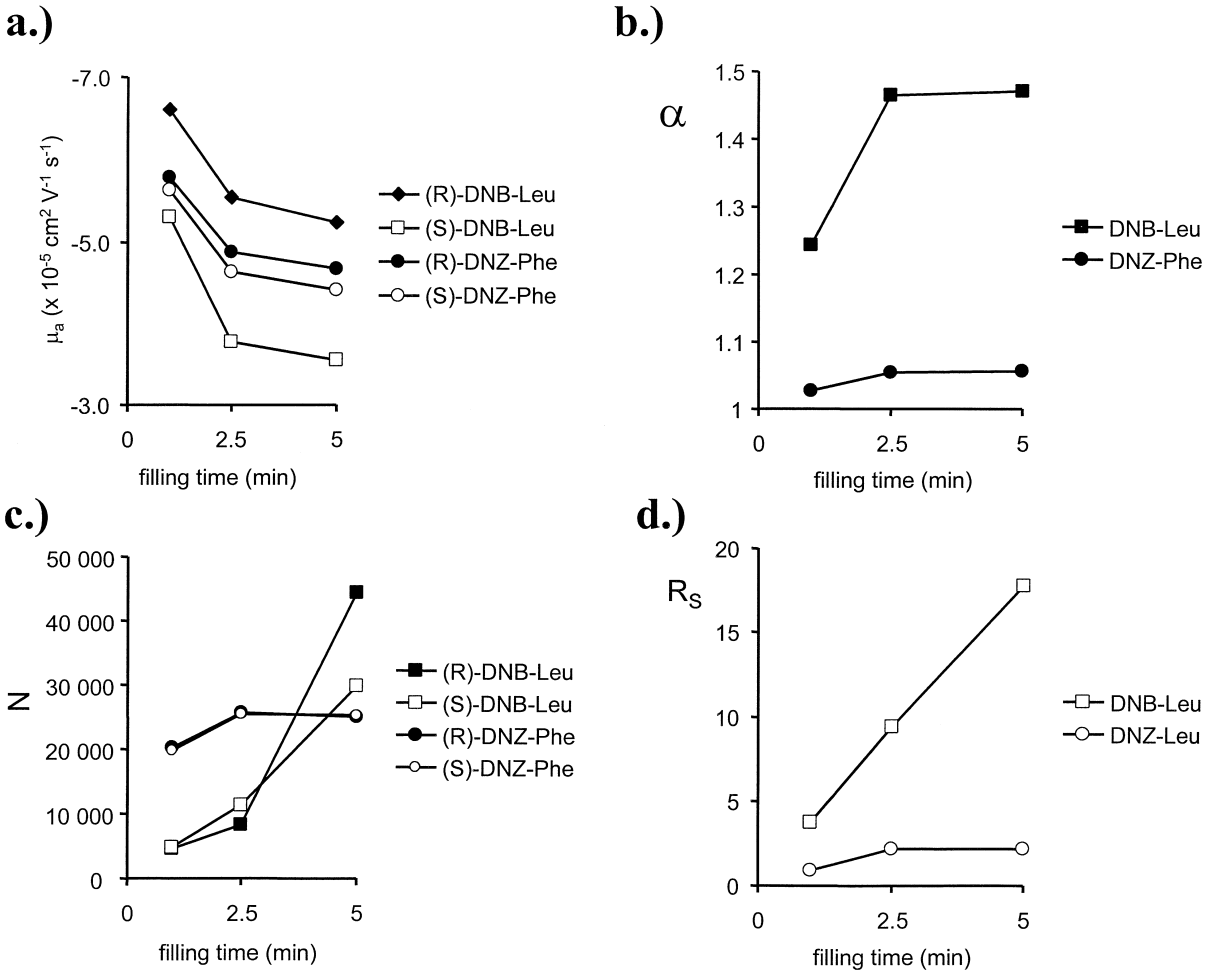

Fig. 3. Separation of (*R*,*S*)-DNB-Leu and (*R*,*S*)-DNZ-Phe by non-aqueous CE employing *tert*.-butylcarbamoylquinine as chiral ion-pair SO and applying the 'partial filling technique': influence of the filling time (min) at constant pressure of 50 mbar on (a) apparent mobility (μ_a), (b) enantioselectivity (α), (c) theoretical plate numbers (N), and (d) resolution (R_s). Experimental conditions: 100 m*M* octanoic acid, 12.5 m*M* triethylamine, and 10 m*M* *tert*.-butylcarbamoylquinine in EtOH–MeOH (60:40); *T*, 15°C; voltage, −25 kV; detection, UV 250 nm (other conditions as specified in Section 2).

method', but this might be compensated by the use of higher SO concentrations in the BGE.

3.3. Influence of SO concentration

As pointed out earlier, separation selectivity strongly depends on the SO concentration in the non-aqueous BGE. As in NACE applying the 'partial filling technique' solubility and detection problems no longer prohibit the use of enhanced SO concentrations, we could study the influence of the SO concentration on the separation parameters of (*R*,*S*)-DNP-Pro above 10 m*M* *tert*.-butylcarbamoylquinine (filling time of 5 min at 50 mbar). Due to enhanced ion-pair formation, migration times increased and apparent mobilities decreased with increasing amounts of SO in the BGE (see Fig. 5a), leading to a steady increase of α values in the investigated range (between 10 and 80 m*M* *tert*.-butylcarbamoylquinine) (see Fig. 5b). Since efficiency values decline at the same time, when the amount of SO in the BGE is increased (see Fig. 5c), optimum res-

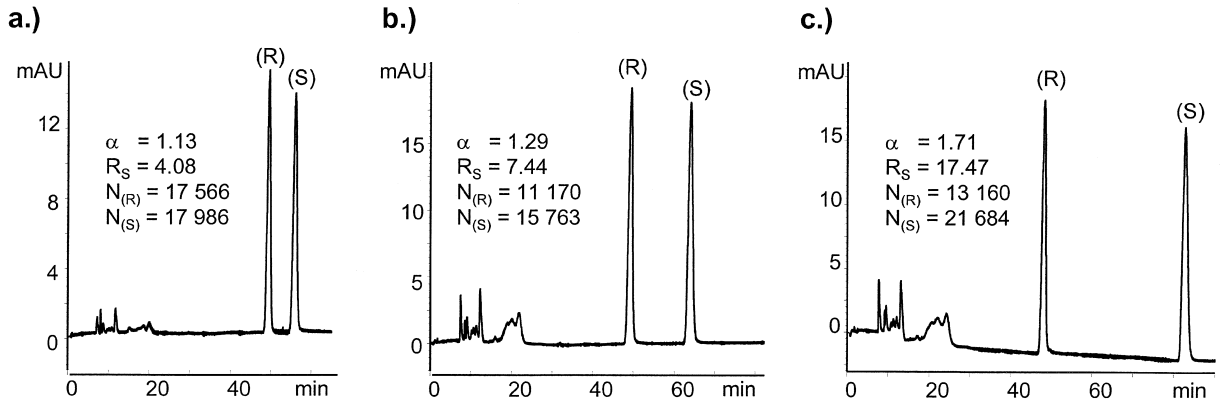

Fig. 4. Influence of the filling time and SO plug length on the enantioseparation of (*R,S*)-DNB-*β*-Abu by non-aqueous CE employing *tert*.-butylcarbamoylquinine as chiral ion-pair SO and the 'partial filling technique'. (a) Filling time of 1 min (corresponding to a SO zone of ca. 6 cm), (b) filling time of 2.5 min (corresponding to a SO zone of ca. 15 cm), and (c) filling time of 5 min (corresponding to a SO zone of ca. 30 cm) (experimental conditions as in Fig. 3).

olution was observed at a *tert*.-butylcarbamoylquinine concentration around 20 m*M* (see Fig. 5d), reaching, e.g. R_S values for (*R,S*)-DNP-Pro higher than 6. Further, it should be mentioned that the length of the SO plug has not been re-investigated again for the changed SO-BGE compositions and the filling time has not been re-optimized or adjusted to the new conditions, as the effect has been thought to be negligible. In any case, no baseline drifts have been observed throughout the studies.

3.4. Influence of anionic co-ion

In our previous study, we have found that octanoic acid yields better peak efficiencies and improved peak symmetry in the separation of DNB-amino

Table 1
Enantiomer separation data of various acidic SAs using the 'partial filling technique' and *tert*.-butylcarbamoylquinine as ion-pair SO by non-aqueous CE[a]

SA	PG[b]	t_{R1} (min)	Configuration	t_{R2} (min)	Configuration	μ_{a1} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	μ_{a2} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	α[c]	R_S	N_1	N_2
(*R,S*)-Leu	DNB	21.48	(*R*)	31.62	(*S*)	−5.22	−3.55	1.47	17.73	44 379	29 721
(*R,S*)-Phg	DNB	15.17	(*R*)	19.67	(*S*)	−7.40	−5.71	1.30	7.72	10 400	19 006
(*R,S*)-*α*-Phe	DNB	16.38	(*R*)	22.76	(*S*)	−6.85	−4.93	1.39	12.83	19 183	30 673
(*R,S*)-*β*-Phe	DNB	38.00	(*S*)	67.96	(*R*)	−2.95	−1.65	1.79	12.28	10 839	23 616
(*R,S*)-*β*-Abu	DNB	48.33	(*R*)	82.74	(*S*)	−2.32	−1.36	1.71	17.47	13 160	21 684
(*R,S*)-Leu	DNZ	36.23	(*R*)	39.17	(*S*)	−3.10	−2.87	1.08	2.70	17 391	21 361
(*R,S*)-*α*-Phe	DNZ	24.08	(*R*)	25.43	(*S*)	−4.66	−4.41	1.06	2.16	25 009	25 132
(*R,S*)-*β*-Phe	DNZ	57.46	(*S*)	65.67	(*R*)	−1.95	−1.71	1.14	2.29	3997	5539
(*R,S*)-*α*-Abu	DNZ	25.10	(*R*)	30.95	(*S*)	−4.47	−3.63	1.23	5.90	7812	21 506
(*R,S*)-Ala	DNP	12.58	(*S*)	13.22	(*R*)	−8.93	−8.49	1.05	1.52	12 475	17 906
(*R,S*)-Leu	Bz	30.63	(*R*)	31.98	(*S*)	−3.66	−3.51	1.04	1.35	14 441	17 642
(*R,S*)-*α*-Phe	Bz	21.13	(*R*)	21.82	(*S*)	−5.31	−5.14	1.03	0.99	12 820	17 649
(*R,S*)-*β*-Phe	Bz	43.47	(*S*)	46.62	(*R*)	−2.58	−2.41	1.07	2.12	12 851	16 895

[a] Experimental conditions: 100 m*M* octanoic acid, 12.5 m*M* triethylamine, 10 m*M* *tert*.-butylcarbamoylquinine in EtOH–MeOH (60:40); −25 kV (−4.2 mA); filling time of 5 min at 50 mbar (corresponding to a SO zone length of 30 cm) (for other conditions, see Section 2).
[b] PG, protection group.
[c] $\alpha = \mu_{a1}/\mu_{a2}$.

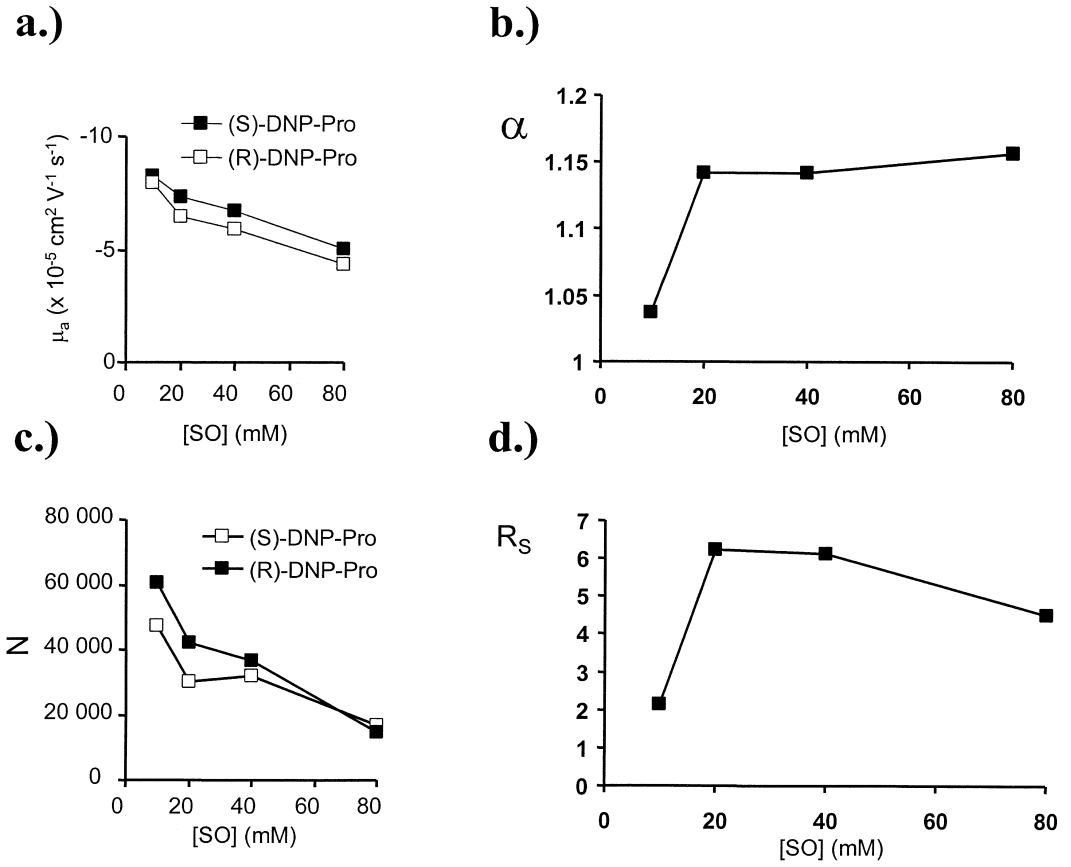

Fig. 5. Enantiomer separation of (*R,S*)-DNP-Pro by non-aqueous CE employing *tert.*-butylcarbamoylquinine as chiral ion-pair SO and the 'partial filling technique'. Effect of SO-concentration in the BGE on (a) apparent mobility (μ_a), (b) enantioselectivity (α), (c) theoretical plate numbers (N), and (d) resolution (R_S). Experimental conditions: 100 m*M* octanoic acid, 12.5 m*M* triethylamine and 10 m*M* *tert.*-butylcarbamoylquinine in EtOH–MeOH (60:40); filling time, 5 min at 50 mbar; *T*, 15°C; voltage, −25 kV; detection, UV 250 nm (other experimental conditions as specified in Section 2).

acids than shorter and longer-chain aliphatic organic acids. This effect has been interpreted by reduced electrokinetic dispersion effects. However, it turns out that for other analytes efficiencies, also enantioselectivities can greatly be enhanced by using acetic acid instead of octanoic acid as anionic co-ion, thereby improving also resolution values significantly (see Table 2). For example, α values of Bz-β-Phe improved from 1.03 to 1.14 with 100 m*M* acetic acid instead of 100 m*M* octanoic acid in the BGE, while also efficiencies increased from ca. 7800 to 55 500 theoretical plates. This leads to an improvement of the separations from $R_S = 0.62–7.54$. Similarly significant was the improvement of the separations of many other SAs as shown by some selected examples in Table 2. This method can be utilized for enantioseparation of a wide variety of *N*-derivatized amino acids including the various proteinogenic and non-proteinogenic amino carboxylic, sulfonic and phosphonic acids and employing diverse *N*-protection groups. For example, Fig. 6 shows the electropherograms of DNP-, Bz- and Fmoc-protected (*R,S*)-Leu achieved with these optimized conditions. Efficiencies of DNP-labeled amino acids reached remarkable numbers (e.g., up to 130 000 theoretical plates for DNP-Leu and for DNP-Pro) (see Fig. 7).

3.5. Repeatability studies

In order to show the practical applicability of this

Table 2
Influence of type of electrolyte and anionic co-ion, respectively, on separation parameters: acetic acid vs. octanoic acid[a]

SA	100 m*M* octanoic acid						100 m*M* acetic acid					
	μ_{a1} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	μ_{a2} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	α	R_S	N_1	N_2	μ_{a1} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	μ_{a2} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})	α	R_S	N_1	N_2
(*R*,*S*)-DNZ-Leu	−2.82	−2.45	1.15	4.98	19 468	22 481	−4.35	−3.45	1.26	12.3	50 540	42 544
(*R*,*S*)-Bz-*β*-Phe	−1.41	−1.37	1.03	0.62	7782	7629	−4.95	−4.35	1.14	7.54	56 147	53 749
(*R*,*S*)-Bz-Leu	−3.35	−3.16	1.06	2.53	29 023	28 464	−3.48	−3.29	1.06	3.32	58 768	56 856
(*R*,*S*)-Fmoc-Leu	−1.65	−1.58	1.04	1.33	15 585	17 588	−3.61	−3.29	1.10	5.61	58 037	54 516
(*R*,*S*)-DNP-Leu	−9.11	−8.78	1.04	1.74	34 659	40 212	−9.31	−8.70	1.07	5.52	109 252	105 756
(*R*,*S*)-DNP-Pro	−8.24	−7.95	1.04	2.12	47 198	60 634	−8.35	−7.76	1.08	6.6	133 341	132 913

[a] Experimental conditions: 100 m*M* octanoic acid, 12.5 m*M* triethylamine, 10 m*M* *tert*.-butylcarbamoylquinine in EtOH–MeOH (60:40); −25 kV (−4.2 mA); filling time of 5 min at 50 mbar (corresponding to a SO-zone length of 30 cm); *T*, 15°C (for other conditions see Section 2).

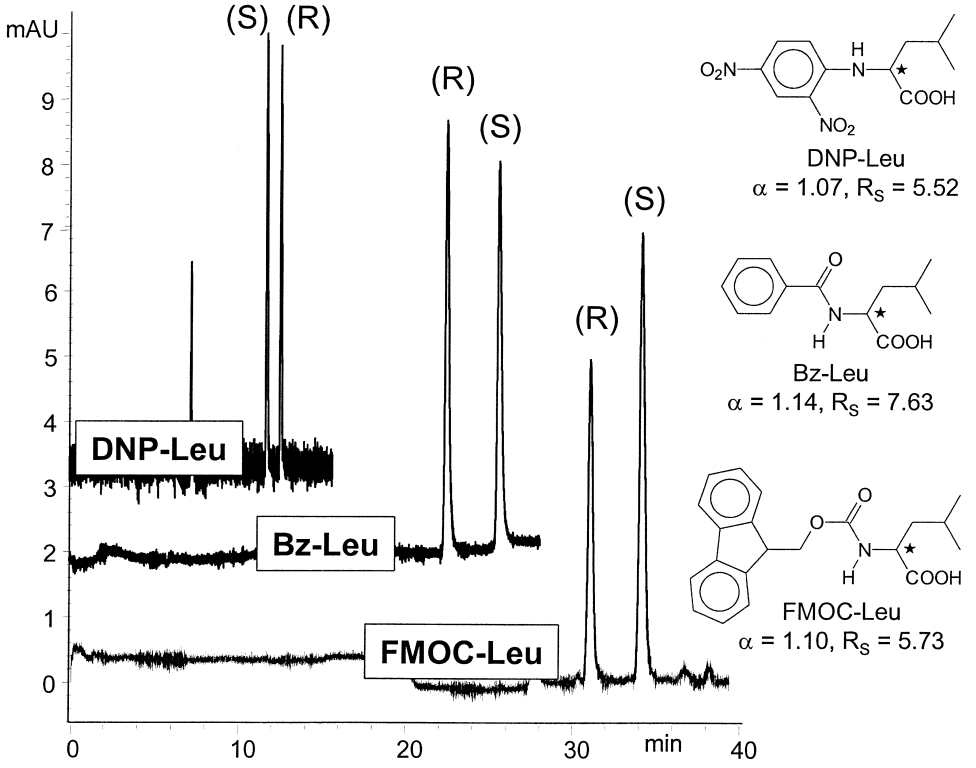

Fig. 6. Enantioseparations of different leucine derivatives by non-aqueous CE employing *tert*.-butylcarbamoylquinine as chiral ion-pair SO and applying the 'partial filling technique'. Experimental conditions: 100 m*M* acetic acid, 12.5 m*M* triethylamine and 10 m*M* *tert*.-butylcarbamoylquinine in EtOH–MeOH (60:40); filling time, 5 min at 50 mbar; *T*, 15°C; voltage, −25 kV; detection, UV 250 nm (other conditions as specified in Section 2).

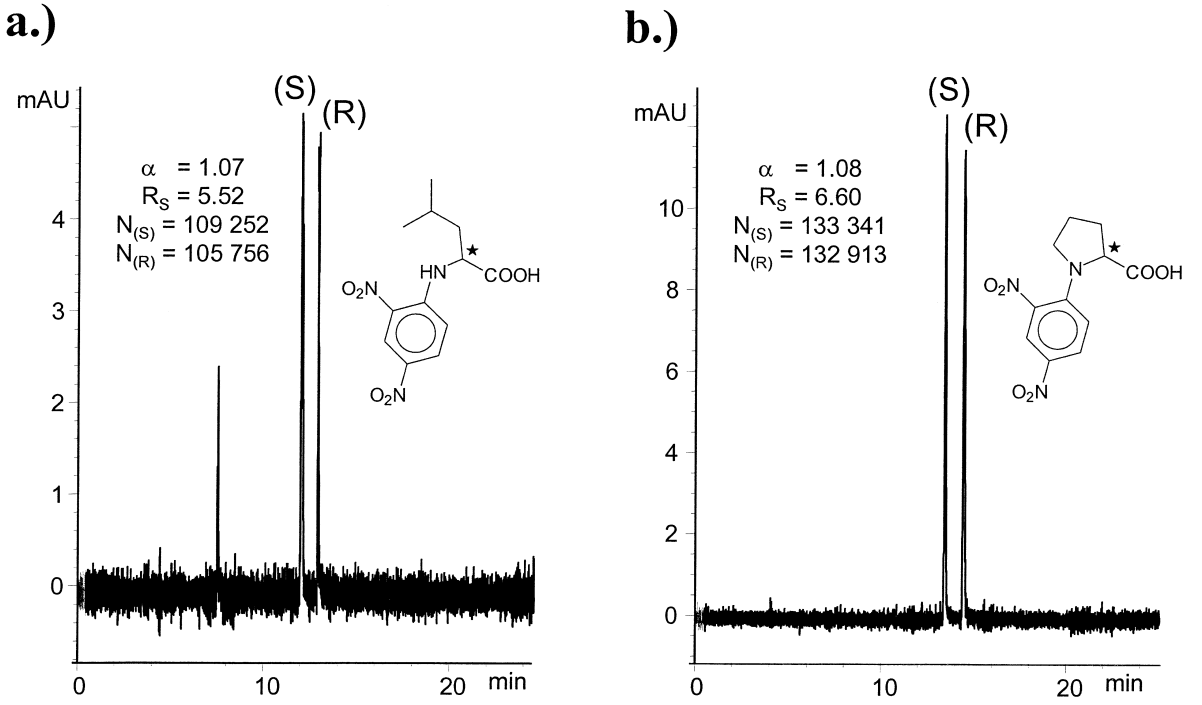

Fig. 7. Enantioseparations of *N*-DNP-labeled leucine and proline by non-aqueous CE employing *tert*.-butylcarbamoylquinine as chiral ion-pair SO and applying the 'partial filling technique' (experimental conditions as in Fig. 6).

optimized NACE method applying the 'partial filling technique', run-to-run and day-to-day repeatabilities have been evaluated for DNP-Ala(P) and DNP-Pro as analytes. Run-to-run repeatability ($n=4$) (see Table 3) has proven to be fairly good and comparable to conventional CE methods, yielding relative standard deviations (RSDs) in the range of 0.0–1.0% for migration times and α values, while RSD values of resolution and plate numbers were slightly higher ranging between 2 and 6% for both investigated analytes.

The same two series have been repeated on two subsequent days yielding statistical data for run-to-run repeatability which are in good agreement with the above results. The resulting day-to-day repeatabilities for these two SAs are also sufficiently

Table 3
Run-to-run repeatability evaluated for different *N*-DNP protected amino carboxylic and phosphonic acids[a]

	t_{R1} (min)	t_{R2} (min)	α	R_S	N_1	N_2
(*R*,*S*)-DNP-Ala(P) ($n=4$)						
Mean	16.65	18.34	1.10	2.10	7493	7715
SD	0.05	0.06	0.00	0.07	468	421
RSD (%)	0.28	0.30	0.04	3.21	6.25	5.46
(*R*,*S*)-DNP-Pro ($n=4$)						
Mean	13.93	15.05	1.08	5.72	87 991	88 776
SD	0.09	0.09	0.00	0.12	4259	3999
RSD (%)	0.62	0.57	0.08	2.05	4.84	4.51

[a] For experimental conditions see Table 2.
SD, standard deviation; RSD, relative standard deviation.

Table 4
Day-to-day repeatability evaluated for different *N*-DNP-protected amino carboxylic and phosphonic acids[a]

	t_{R1} (min)	t_{R2} (min)	α	R_S	N_1	N_2
(*R*,*S*)-DNP-Ala(P)						
Day 1: mean ($n=4$)	16.65	18.34	1.10	2.10	7493	7715
Day 2: mean ($n=3$)	16.43	18.13	1.10	1.94	5976	6583
Day 3: mean ($n=3$)	17.55	19.34	1.10	1.89	6153	6214
Mean of 1,2,3	16.88	18.60	1.10	1.98	6540	6837
SD	0.49	0.53	0.00	0.09	677	639
RSD (%)	2.88	2.85	0.07	4.63	10.35	9.34
(*R*,*S*)-DNP-Pro						
Day 1: mean ($n=4$)	13.93	15.05	1.08	5.72	87 991	88 776
Day 2: mean ($n=3$)	14.58	15.75	1.08	5.12	67 673	73 661
day 2: mean ($n=3$)	15.58	16.86	1.08	6.29	102 200	100 065
Mean of 1,2,3	14.70	15.89	1.08	5.71	85 955	87 506
SD	0.68	0.75	0.00	0.48	14 169	10 817
RSD (%)	4.61	4.70	0.13	8.34	16.48	12.36

[a] For experimental conditions see Table 2
SD, standard deviation; RSD, relative standard deviation.

good (Table 4). Both RSD values for migration times and resolutions clearly ranged below 8%. In this context it has to be pointed out that no special rinsing procedures (with sodium hydroxide, etc.) have been carried out between these sequences. A part of the variance therefore may be attributed to changes in the EOF, caused by partial adsorption of the basic *tert.*-butylcarbamoylquinine SO to the negatively charged FS capillary wall. Therefore, it is supposed that repeatabilities can be improved by carrying out rinsing steps between sequences. However, preferentially the use of coated capillaries may be recommended for the application of the 'partial filling technique', as demonstrated in several studies with macrocyclic antibiotic selectors [29,53,54]. Coated capillaries should certainly improve run-to-run, but in particular day-to-day repeatabilities.

4. Conclusion

The presented data clearly demonstrate that *tert.*-butylcarbamoylated quinine is a very useful chiral SO for the separation of the enantiomers of *N*-derivatized amino acids and aminophosphonic acids as well as many other chiral acidic racemates. Practical applicability is achieved by (i) adaption of the non-aqueous CE mode overcoming the problem with limited SO solubility and (ii) application of the 'partial filling technique' avoiding high detector background resulting from strong UV absorbance of the quinoline moiety of the SO in the conventional 'total filling' mode.

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