Communication

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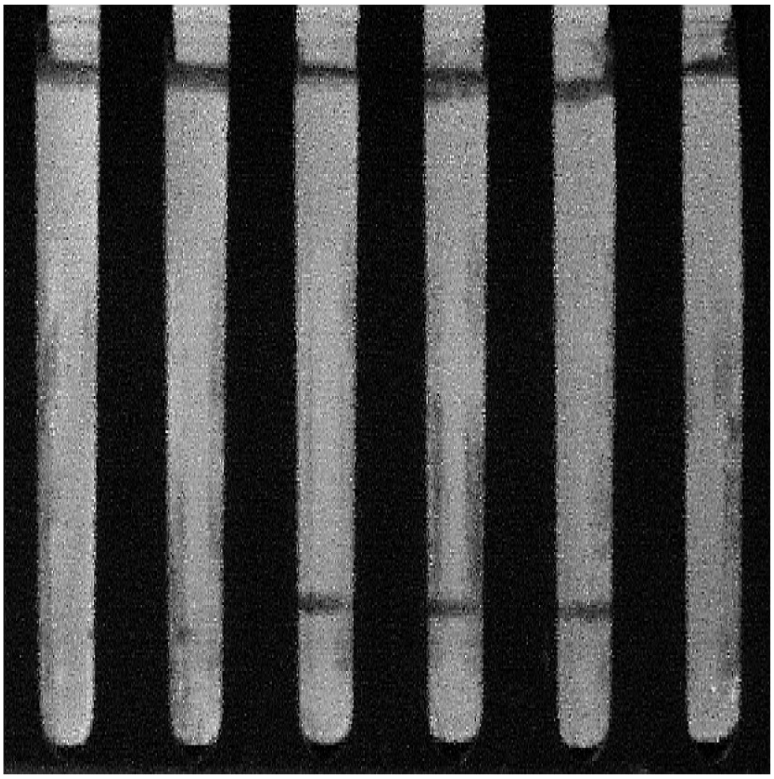
*J. Am. Chem. Soc.*, **2008**, 130 (8), 2426-2427 • DOI: 10.1021/ja067905i





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Published on Web 02/07/2008

# Separation of Racemate from Excess Enantiomer of Chiral Nonracemic Compounds via Density Gradient Ultracentrifugation

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The discovery of the importance of stereochemistry in biochemical environments dates back to 1860 when Pasteur reported[1] on the different destruction rates of *dextro* and *levo* ammonium tartrate by the mold, *Penicillium glaucum*. Since then, the resolution of racemic compounds has been of fundamental importance both for basic scientific and application purposes. In recent years, amplification of enantiomeric excess[2−6] (ee) has generated much interest for practical reasons and also in relation with the problem of molecular homochirality in living systems. For example, manipulation of amino acid phase behavior led to highly enantioenriched solutions,[3] and the faster sublimation of a racemic crystal could be used to amplify ee.[5] In many experiments, small enantiomeric excesses show high nonlinear effects that lead to chiral amplification, particularly, in asymmetric autocatalytic reactions. The asymmetric amplification of small amino acid ee had been proposed as a possible mechanism for the homochirality of biologically important molecules in prebiotic environment. While resolution of enantiomers can be achieved with various techniques,[7] methods for separation of racemate from excess enantiomers are very limited. As a conclusion, the development of an inexpensive and easy method to separate racemate from excess enantiomer (preferable on a larger scale) is very important.

In this study, we will show that density gradient ultracentrifugation can be applied to separate racemate crystals from excess enantiomer crystals. This technique is well-established and can be applied with high accuracy of up to 4 digits of the density in the density range of 0.8−2.0 g/mL, which covers almost the entire density range of organic substances. Although density gradient techniques have been well-established for decades to separate various biopolymers from mixtures or biopolymers with subtle density differences like single- and double-stranded DNA,[8] they have not been applied to the separation of chiral compounds. Solid racemic compounds differ significantly in density from the corresponding pure enantiomers. This difference can be as large as 5%.[9] On average, the racemic compounds in the solid state are denser than the corresponding enantiomers (Wallach's rule[10]). Therefore, the application of density gradient ultracentrifugation is well-suited to separate excess enantiomers from racemates with a high accuracy of the third digit in density and good recovery rates between 75 and 90%.

We have chosen alanine as a model system because its enantiomer and racemate crystals have a different density. We first performed density measurements of the alanine solutions in a density oscillation tube to obtain information about the needed density range for the Nycodenz density gradient (not shown).

The density gradient experiment was performed with DL-alanine-saturated Nycodenz solution (Nycodenz AG Axis-Shield PoCAS, OSLO, Norway) if not otherwise stated to prevent dissolution of the crystals. A solution of DL-alanine, saturated water, and 50% w/w Nycodenz with a density of 1.391 mg/mL was prepared, and 10 mg of the enantiomer and the racemate crystals were added. The gradient was formed in 67 h at 16 000 rpm in a SW55 swing out rotor in a Beckman L70 preparative ultracentrifuge. Quantitative information about the density gradient was obtained by measuring the densities of fractions (0.5 mL) along the density gradient.

Powder mixtures with 20% enantiomeric excess (ee) of the L enantiomer and D enantiomer were prepared by mixing 20 wt % of D-alanine crystals with 80 wt % DL crystals and 20 wt % L-alanine with 80 wt % DL-alanine. The powder mixtures were grinded manually to obtain fine powders (see Figure S1 for SEM image of powder mixtures).

From Figure 1a, it can be seen that DL-alanine can be separated from D- and L-alanine with the applied Nycodenz gradient. The buoyant densities of the alanine crystals determined from this density gradient experiment include the following: DL-alanine = 1.400 g/mL, L-alanine = 1.395 g/mL, and D-alanine = 1.393 g/mL (Figure 1a). These values differ from those determined for dissolved alanine because, in the crystal, the packing of the alanine molecules in the unit cell is critical for the density of the crystal. From this gradient, the densities of the enantiomers and the racemates could be determined with an accuracy of 0.002 g/mL as deduced from the determined densities of D- and L-alanine, which should be equal. Nevertheless, the bands are broad in the low-density region of the gradient due to the very small density gradient differences limiting the separation of racemate crystals from excess enantiomers and causing an experimental inaccuracy. Because a clear separation of the enantiomeric and racemic crystals could not be achieved, as can be seen in Figure 1a, the gradient needed to be modified for separation purposes. In an additional experiment with 50.8% Nycodenz (density of 1.394 g/mL) a clear boundary of the DL-alanine can be obtained after 21 h of centrifugation at 16 000 rpm, whereas D- and L-alanine crystals are floating (Figure 1b). The apparent contradiction that the crystals float in the density gradient material with an equal initial density to the crystals can be resolved if it is considered that, after 21 h, a flat density gradient will already be formed with a density in the bottom region, which is higher than that of the crystals, which makes them float toward the meniscus, where the gradient density is slightly lower than that of the crystals.

In this experiment, a crystallization of DL-alanine solution could be observed because the concentration is higher at the bottom and the mixture is oversaturated. Therefore, an L-alanine-saturated solution with L-alanine crystals was added as a control sample. Crystallization of the alanine used to saturate the density gradient solution to prevent crystal dissolution can occur occasionally. As DL-alanine can be separated from the pure enantiomers, this is no

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10.1021/ja067905i CCC: $40.75 

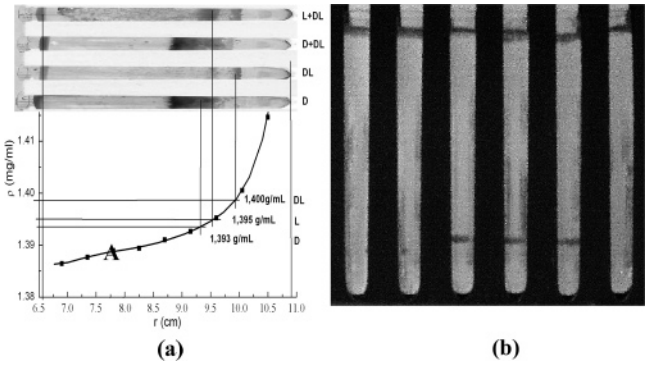


***Figure 1.*** (a) Upper centrifuge tubes with 50 wt % Nycodenz gradient and different alanine crystals after 67 h and (lower) the radial density gradient (line) based on density measurements at different positions in the centrifuge tube (squares). The radial position of the sample band can be converted into a sample density. (b) Centrifuge tubes with 50.8 wt % Nycodenz gradient after 21 h from left to right: D-alanine, L-alanine, DL-alanine, D+DL-alanine, and L+DL-alanine in DL-saturated solution; L-alanine in L-saturated solution.

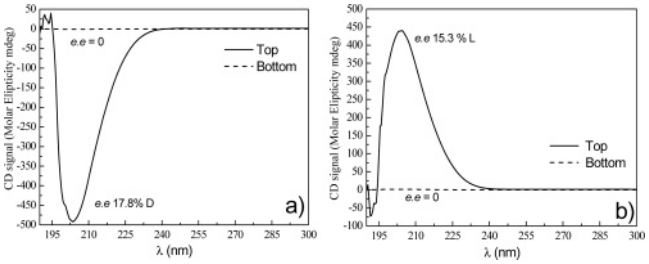


***Figure 2.*** Determination of the enantiomeric excess (e) in chiral powder mixtures after separation in a density gradient: (a) 20 wt % D-alanine from 80% DL-alanine; (b) 20 wt % L-alanine from 80 wt % DL-alanine. The curves denoted as "Bottom" represent the recovered DL-alanine.

problem for the separation purpose and the solution can also be saturated with one of the enantiomers if the DL-alanine shall be recovered.

To test the recovery of the excess enantiomer crystals from the mixture with the racemate crystals, we prepared a solid mixture of pure enantiomer and racemic crystals. In the density gradient (Figure 1a), the DL-alanine band is clearly visible, narrow, and separated from the L- and D-alanine. Therefore, the enantiomeric powder mixtures (20% enantiomer excess of D- and L-alanine) were subjected to the above-described separation in a 50% Nycodenz density gradient. After 21 h, the alanine enantiomer crystals were separated from the denser racemic sample by traditional techniques for sample recovery from density gradients. Afterward, the crystals from the different AUC fractions were collected and the enantiomeric excess was determined by calibrated circular dichroism (CD). The enantiomeric excess was measured from the CD signal based on calibration curves (tested concentrations were in range of 0.01−0.5 M). The CD signal versus the enantiomeric excess was linear with a correlation coefficient ($r^2$) of 0.9985.

Figure 2 shows that the recovery rate of the pure enantiomers is between 75 (for L-alanine) and 90% (for D-alanine). The recovered DL-alanine does not show any enantiomeric excess as expected for a pure racemate. The recovery rate is good if it is considered that recovery of a sample is always associated with a sample loss, especially if the two sample bands are not clearly separated. Therefore, the recovery rate of <100% is not a problem of sample separation but of fraction recovery, which should even be easier for the gradient shown in Figure 1b. Upscaling experiments have demonstrated a separation of 25 mg pure L-alanine in a 5 mL tube from a 100 mg 1:1 mixture within 2 h (Supporting Information).

In conclusion, we have shown that it is possible to separate alanine excess enantiomer crystals from racemate crystals on a preparative scale by simple density gradient ultracentrifugation applying a 50 wt % Nycodenz gradient. The samples can be recovered from the gradient material fractions by simple filtration, and the gradient material can be reused. As many chiral substances crystallize and show differences between enantiomer crystal density and racemate crystal density, our method should be widely applicable with the potential possibility of up-scaling (see Supporting Information). Another advantage of our method is that preparative (ultra)centrifuges are standard separation equipment in many laboratories. Our future work will explore the general applicability of our approach also to samples of pharmaceutical relevance and other crystal systems, such as polymorphic crystals. Our work is in line with the current motivations in the area of chiral resolution to develop new methods that can overcome several limitations of existing technologies, such as large-scale resolution and pure enantiomer availability. Therefore, the separation by the well-established density gradient ultracentrifugation has a great feasibility. Finally, separation of racemate crystals from excess enantiomer crystals by ultracentrifugation can also be used as an analytical method for the determination of enantiomeric excess in powder samples, without the necessity to dissolve the powder. This is particularly important in the case of chiral nanocrystals.

**Acknowledgment.** We thank the Max-Planck Society for financial support of this work.

**Supporting Information Available:** Experimental details on the preparation of alanine crystals, density gradient ultracentrifugation, upscaling and experiment optimization are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA067905I