Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 231–235
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Rapid, automated screening method for enzymatic transformations using a robotic system and supercritical fluid chromatography

Li Di[a,*], Oliver J. McConnell[b], Edward H. Kerns[a], Alan G. Sutherland[c]

[a] *Wyeth Research, P.O. Box CN 8000, Princeton, NJ 08543-8000, USA*
[b] *500 Arcola Road, Collegeville, PA 19426, USA*
[c] *401 North Middletown Road, Pearl River, NY 10965, USA*

Received 27 January 2004; received in revised form 16 April 2004; accepted 14 June 2004

Available online 20 July 2004

Abstract

An automated screening method was developed for enzymatic transformations using a robotic system and rapid chiral supercritical fluid chromatography (SFC) analysis with a run time of 1.5 min. The method accelerates the enzyme selection process for screening biocatalysts, where a large number of enzymes are evaluated for activity and enantioselectivity. Kinetic resolution of secondary alcohols by enzymatic transesterification was used as a prototype for method development. The rapid automated method can be used effectively for screening enzymes and optimizing reaction conditions in biocatalysis.

Keywords: Enzyme screening; Biocatalysis; Hydrolases

1. Introduction

Chiral drugs play an important role in pharmaceutical research and development. Different enantiomers can have quite different pharmacokinetic and pharmacodynamic properties. More than 50% of the marketed drugs are chiral. Several approaches are being used to meet the increasing demands of producing enantiomerically pure synthetic intermediates and products, such as asymmetric synthesis, chiral separation or purification, and kinetic resolution. Enzymatic transformation is becoming one of the important techniques for kinetic resolution and asymmetric synthesis [1–3].

Recent advances in biochemistry and molecular biology have made many enzymes readily available. Although these versatile enzymes have drawn tremendous attention in organic synthesis and industrial applications [4–8], measuring their activity and selectivity remains tedious and time consuming. High throughput screening of diverse enzyme libraries is an effective way to rapidly identify enzymes for biocatalysis [9–15]. The bottlenecks of the screening process are (1) automated sample preparation and (2) rapid determination of chiral purity for enantioselectivity. Several high throughput enantiomeric excess (ee) screening systems have been developed, such as chiral chromatography with UV–vis, fluorescence, circular dichroism and mass spectrometry detection [16–24], capillary array eletctrophoresis [25], and GC [26]. Here, we discuss the development of an automated method for enzyme screening using a robotic liquid handler and rapid chiral SFC analysis to speed up the enzyme selection process for drug discovery and development. The method was developed with transesterification of alcohols using hydrolases. Enantiomerically pure alcohols are important synthetic intermediates and chiral auxiliaries [28]. Similar approaches can be applied to other biotransformations.

2. Experimental

2.1. Material

AMANO enzymes (Lombard, IL) provided samples for the following enzymes: Lipase D “Amano” Conc, Lipase

* Corresponding author. Tel.: +1 732 274 4489; fax: +1 732 274 4505.
E-mail address: dil@wyeth.com (L. Di).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.033

R "Amano" G, Lipase F-AP15, Lipase PS-C I, Lipase AK "Amano" 20, Lipase PS-D "Amano" I, Lipase PS-C "Amano" II, Lipase M "Amano" 10, Lipase G "Amano" 50, Newlase F, Lipase A "Amano" 12, Lipase AY "Amano" 30G, Technical Grade AS, and Lipase BC. A hydrolase kit was purchased from Biocatalytic (Pasadena, CA): Chirazyme E-1, Chirazyme E-2, Chirazyme L-2, Chirazyme L-3, Chirazyme L-3 (purified), Chirazyme L-4, Chirazyme L-5, Chirazyme L-6, Chirazyme L-7, Chirazyme L-8, Chirazyme L-9, and Chirazyme L-10. Several other enzymes were purchased from Sigma (St. Louis, MO): Lipase L-1754, Lipase L-9156, Acylase I A-8376, papain crude, protease type VIII bacterial, lipase type II crude from porcine, lipase type I from wheat germ, and lipase from *Candida cylindracea*. The alcohol substrate, (±)-1-phenylethanol, was purchased from Aldrich (Milwaukee, WI). Vinyl acetate and methyl *t*-butyl ether were obtained from EM Sciences (Gibbstown, NJ).

2.2. Automated reaction procedure

A Packard Multiprobe™ II EX HT (eight probes) robot with WinPrep™ software was used for the liquid handling (PerkinElmer, Downers Grove, IL). Reagents were added to the troughs and delivered to the plates by the robot. The layout of the robotic deck is shown in Fig. 1. Approximately, 1 mg of each enzyme was added to a separate reaction well, consisting of a one-dram vial held in a custom-made 24-well-plate (Fig. 1). Stock solutions containing vinyl acetate at 100 mM and the alcohol substrate at 50 mM were each prepared in methyl *t*-butyl ether and placed in liquid troughs labeled "reagent 1" and "reagent 2", respectively. The robot added 300 μL of vinyl acetate and 200 μL of alcohol substrate into the wells containing the enzymes. The total reaction volume was 0.5 mL, resulting in a concentration of the vinyl acetate and alcohol substrate of 60 mM and 20 mM, respectively. To maximize enzyme-substrate interaction, the samples were shaken vigorously on a plate shaker (Boekel Scientific, Feasterville, PA) for 24 h at room temperature. The enzyme material remains a suspension throughout the reaction. Subsequently, the solutions are filtered using a 1 mL syringe and 0.45 μm Nylon 66 disk filter and filtrate was placed in a 300 μL HPLC autosampler vial. Samples were either injected directly or diluted and then injected into the SFC.

2.3. Chiral SFC analysis

A chiral analytical method was developed using a SFC system (Berger Instruments, Newark, DE) that consists of a carbon dioxide pump, a modifier pump, an Alcott autosampler, an integrated and programmable Valco six-port valve for solvent selection, a temperature-controlled column oven that is capable not only of heating, but also cooling well below ambient temperature using the adiabatic expansion of carbon dioxide in the oven, a Chiralizer™ six-port programmable column-switching valve (Chiralizer™ Service, Newtown, PA), and an Agilent 1100 photodiode array UV–vis detector (Agilent Technology, Piscataway, NJ). Due to the non-polar nature of the substrate, a rapid yet shallow gradient method was used to select the chiral stationary phase that yielded the greatest resolution of enantiomers. The method consisted of a concave 1.2 min gradient of 2–30% MeOH/CO_2 with a 1.8 min hold at 30% MeOH/CO_2 and a 0.5 min gradient back to 2% MeOH/CO_2 at 25 °C, 2 mL/min, 150 bar outlet pressure, UV detection at 254 nm and injections of 5 μL of approximately 1–2 mg/mL solutions in methyl *t*-butyl ether with the following columns: Chiralcel OD (Diacel Chemical Industries, distributed by Chiral Technologies Inc., Exton, PA), Chiralpak AD (Diacel Chemical Industries), Kromasil TBB (O,O′-bis-(4-*t*-butylbenzoyl)-*N*,*N*′-diallyl-L-tartaric

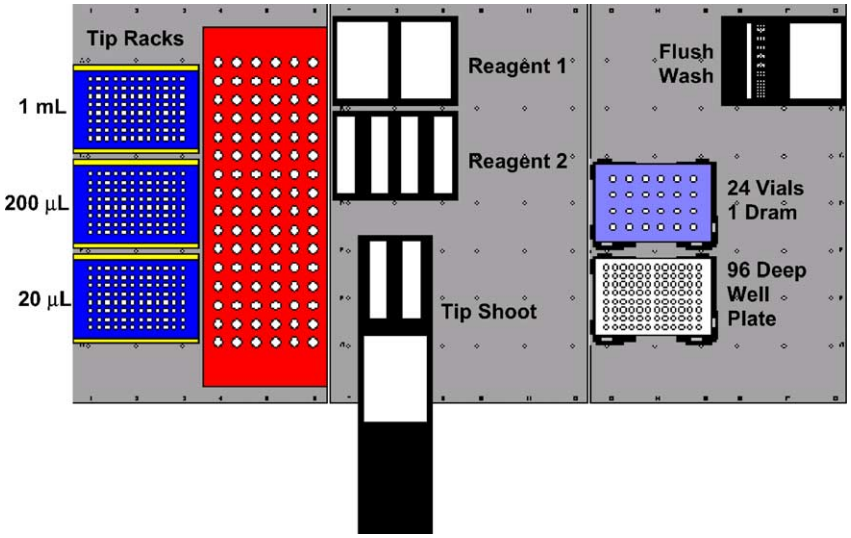


Fig. 1. Packard robot deck layout for transesterfication.

diamide) (Regis Technologies Inc., Morton Grove, IL), Chirobiotic V (vancomycin) (Regis Technologies Inc., Morton Grove, IL) and Whelk-O 2 (Regis Technologies Inc., Morton Grove, IL). The Chiralcel OD column provided the greatest separation of enantiomers of 1-phenylethanol, and an isocratic method was developed employing the conditions above but using 2% MeOH/CO_2. This chiral SFC method is used for enzyme screening. The detection limit for 1-phenylethanol is 75 μg/mL. The reproducibility of percent ee is 1.7%. The runtime is 1.5 min.

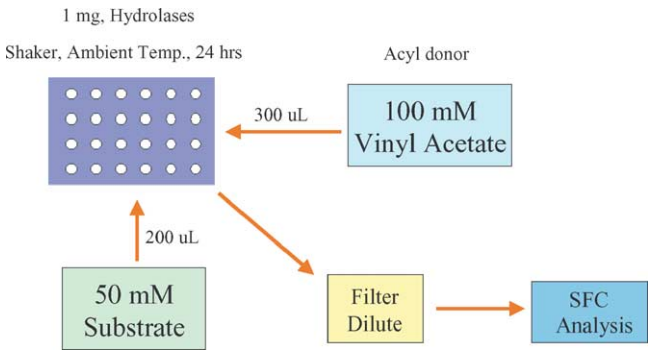


Fig. 3. Automated procedure for transesterfication.

3. Results and discussion

The prototype reaction of enzymatic transesterification for 1-phenylethanol is shown in Fig. 2 [27,28]. Selective acylation of one of the enantiomers under the catalysis of hydrolases resulted in kinetic resolution the alcohols. The screening of the selective enzymes for the transformation was setup with an automated procedure using a robotic liquid handler. The automation scheme is summarized in Fig. 3. Reagents were added automatically to the reaction wells to eliminate repetitive and tedious manual operations. This significantly increases the speed and throughput to screen a large number of diverse enzymes for specific transformations. The robotic programs can be modified easily and applied to different types of biotransformations.

In drug discovery, typically when a large quantity of a chiral intermediate or product is needed, enzymatic transformation is one of the approaches that is considered for scale-up and for supplying material for development and clinical trials. Rapid identification of specific enzymes for transformations and optimal reaction conditions leads to accelerated drug development. Due to the advancement in instrumentation, SFC has been widely used in the pharmaceutical industry for analytical, preparative, and chiral separations [29]. One of the advantages of chiral SFC as compared to chiral HPLC or chiral GC is its separation efficiency [30]. The low viscosity of carbon dioxide enables high flow rates without loss of chromatographic efficiency. This produces short run time, which is ideal for screening purposes where throughput is very important.

In this study, SFC conditions were developed using generic methods with a Chiralizer^{TM} and multiple chiral columns. An isocratic condition with a runtime of 1.5 min was selected to analyze the enantioselectivity of the enzymatic transformation. Under the optimal conditions, the throughput is 700 analyses/day. Combination of the robotic liquid handling system and rapid SFC analysis, allows screening of a large number of enzymes for reactivity and selectivity. If the enzymatic transformation is selected for scale-up, the fast method is transferred to development and used for optimization of reaction conditions in large scale. The short run time makes it possible to rapidly determine optimal reaction conditions for high yield, high ee, and minimum side-reactions.

A typical chiral SFC analysis of the alcohols is shown in Fig. 4. The first chromatogram is the racemate of 1-phenyl-ethanol. The two enantiomers were well separated in a 1.5 min run. The second chromatogram is after a 24-h reaction in the presence of lipase PS-C Amano II. The enzyme selectively acylated the (*S*, +)-alcohol to ester and left the (*R*, −)-enantiomer intact. The ester product was also detected under the same chromatographic conditions with a retention time of 0.27 min. The third chromatogram is after a 24-h reaction in the presence of lipase PS-D Amano I. In this case, the selectivity is reversed. The enzyme selectively acylated the (*R*, −)-alcohol and left the (*S*, +)-alcohol intact. The ester product appeared at 0.27 min. This example shows the effectiveness of the automated enzyme screening in identifying unique substrate selectivity of each enzyme, which

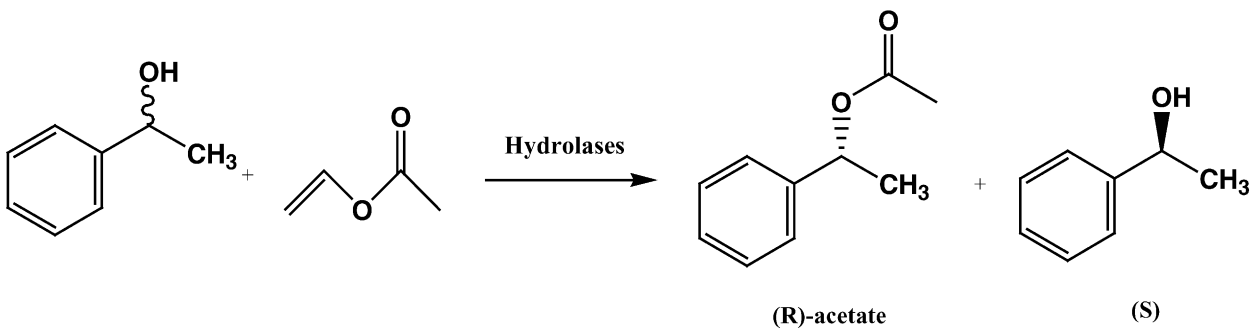


Fig. 2. Transesterification reaction of alcohols.

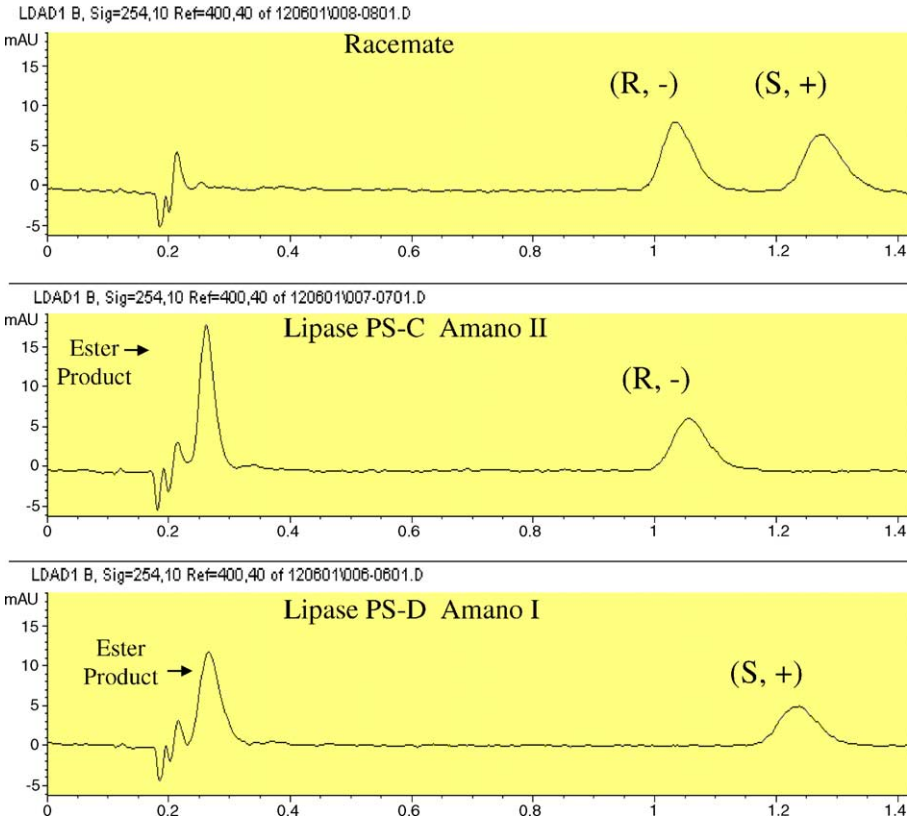
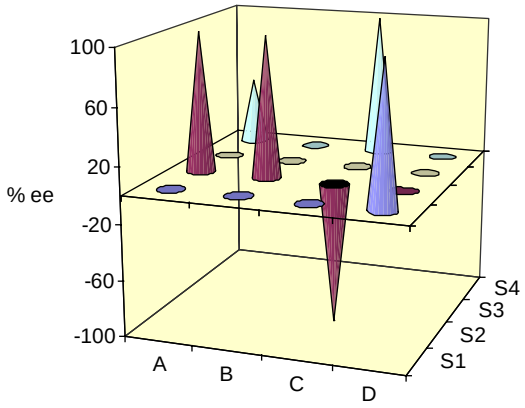


Fig. 4. Enantioselective transesterification of 1-phenyl-ethanol. Absolute stereochemistry of the enantiomers is identified using authentic samples.

otherwise would be difficult to predict. The reaction was also studied in the absence of any enzymes. No nonspecific hydrolysis was detected, presumably due to the non-aqueous reaction environment. Non-aqueous conditions for enzymatic transformation have been very successful in producing chiral materials with high chemical and optical purity [31]. The absolute stereochemistry of the enantiomers was determined using authentic pure enantiomers.

The results of the screen can be plotted graphically for easier visualization of the effective enzymes and chiral purity. Representative results for selective enzymes are shown in Fig. 5. Positions of the enzymes were defined by “A, B,



	A	B	C	D
1	Lipase D "Amano" Conc	Lipase R "Amano" G	Lipase F-AP15	Lipase PS-C I
2	Lipase AK "Amano" 20	Lipase PS-D "Amano" I	Lipase PS-C "Amano" II	Lipase M "Amano" 10
3	Lipase G "Amano" 50	Newlase F	Lipase A "Amano" 12	Lipase AY "Amano" 30G
4	Lipase AH	Lipase L-1754	Lipase L-9156	Acylase I A-8376

Fig. 5. Representative results of chiral resolution of 1-phenyl-ethanol through enzymatic transesterification.

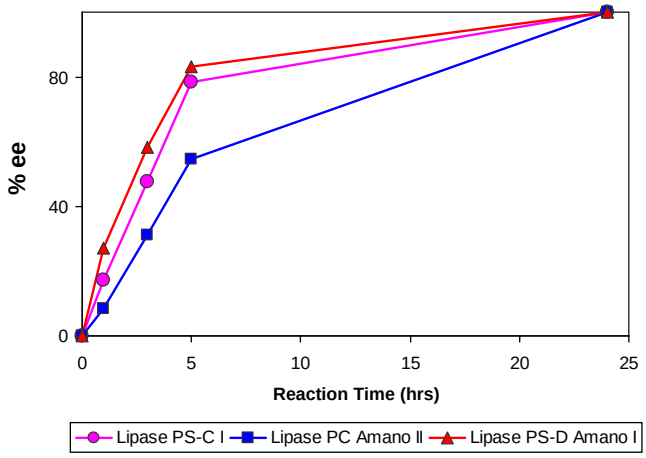


Fig. 6. Kinetics of transesterification for 1-phenyl-ethanol.

C, and D" and "1, 2, 3, and 4". The identities of the enzymes in each reaction vial are shown in the table under the figure. The height of each cone in Fig. 5 represents the percent ee. Positive represents excess in (*S*,+)-enantiomer. Negative represents excess in (*R*,−)-enantiomer. For 1-phenyl-ethanol, several enzymes were identified to give stereospecific resolution of the alcohol (Fig. 5).

For screening purposes, a single reaction time is usually tested initially to find out the reactivity and selectivity of the enzymes. A long reaction time (e.g., 18–24 h) is usually selected to ensure sufficient progress of the reactions. If an enzyme does not catalyze reactions in this time frame, it is considered a slow reaction and it would not be very practical to use the enzyme for production. The reaction conditions can be optimized later for the active enzymes, especially if there are competitive reactions. Different time points can be analyzed to monitor the rate of conversion and selectivity to maximize yield and ee. To study the rate of acylation for 1-phenol-ethanol, three active enzymes were selected and the kinetics of the asymmetric transformation is shown in Fig. 6. The rate of the reaction for each enzyme is slightly different, however, they all reached completion in 24 h with 100% ee. For this reaction, 24 hr is an appropriate time for screening purposes, since the enzymes are highly selective. Longer incubation time did not cause the enzymes to catalyze the reaction of the second enantiomer and reduce percent ee. If time is of the essence, the data in Fig. 6 indicate that 5 h is sufficient to determine whether or not an enzyme will affect a transformation. In scale-up settings, the reactions are monitored at different time points.

4. Conclusions

An automated screening method for enzymatic transformations was developed using a robotic system and rapid chiral SFC analysis. The method is simple, rapid, and high throughput. Similar approaches can be applied to other biotransformations.

Acknowledgements

The authors thank Dimas Pridady and Nelson Kung for their help on the method development; Guy Carter and Magid Abou-Gharbia for their support; Mike Kagan for useful discussions.

References

[1] K.M. Koeller, C.H. Wong, Nature 409 (2001) 232.
[2] A.M. Klibanov, Nature 409 (2001) 241.
[3] S.C. Stinson, C & EN, 1 October 2001.
[4] U.T. Bornscheuer, C. Bessler, R. Srinivas, S. Hari Krishna, Trends Biotechnol. 20 (2002) 433.
[5] U.T. Bornscheuer, R.J. Kazlauskas, Hydrolases in Organic Synthesis—Regio and Stereoselective Biotransformation, Wiley–VCH, Weinheim, 1999.
[6] A. Liese, M. Villela Filho, Curr. Opin. Biotechnol. 10 (1999) 595.
[7] A. Liese, K. Seelbach, C. Wandrey, Ind. Biotransform., Wiley–VCH, Weinheim, 2000.
[8] S. Hari Krishna, N.G. Karanth, Catal. Rev. 44 (2002) 499.
[9] D. Wahler, J.L. Reymond, Curr. Opin. Chem. Biol. 5 (2001) 152.
[10] B. van Beilen, Z. Li, Curr. Opin. Biotechnol. 13 (2002) 338.
[11] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Nature 409 (2001) 258.
[12] M. Chartrain, R. Greasham, J. Moore, P. Reider, D. Robinson, B. Buckland, J. Mol. Catal. B: Enzym. 11 (2001) 503.
[13] S. Stahl, R. Greasham, M. Chartrain, J. Biosci. Bioeng. 89 (2000) 374.
[14] C.J. Welch, B. Grau, J. Moore, D.J. Mathre, J. Org. Chem. (2001) 66.
[15] H.J.W. Henderickx, A.L.L. Duchateau, P.C. Raemakers-Franken, J. Chromatogr. A 1020 (2003) 69.
[16] G.T. Copeland, S.J. Miller, J. Am. Chem. Soc. 121 (1999) 4306.
[17] M.T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, K.E. Jaeger, Angew. Chem. 109 (1997) 2961.
[18] L.E. Janes, A.C. Löwendahl, R.J. Kazlauskas, Chem. Eur. J. 4 (1998) 2324.
[19] M.T. Reetz, K.E. Jaeger, Chem. Eur. J. 6 (2000) 407.
[20] G. Klein, J.L. Reymond, Helv. Chim. Acta 82 (1999) 400.
[21] M.T. Reetz, M.H. Becker, H.W. Klein, D. Stöckigt, Angew. Chem. 111 (1999) 1872.
[22] K. Ding, A. Ishii, K. Mikami, Angew. Chem. Int. Ed. 38 (1999) 497.
[23] U.T. Bornscheuer, J. Altenbuchner, H.H. Meyer, Bioorg. Med. Chem. 7 (1999) 2169.
[24] J. Guo, J. Wu, G. Siuzdak, M.G. Finn, Angew. Chem. 111 (1999) 1868.
[25] M.T. Reetz, K.M. Kühling, S. Wilensek, H. Husmann, U.W. Häusig, M. Hermes, Catal. Today 15 (2001) 389.
[26] M.T. Reetz, K.M. Kühling, A. Deege, H. Hinrichs, D. Belder, Angew. Chem. Int. Ed. 39 (2000) 3891.
[27] K. Laumen, D. Breitgoff, M.P. Schneider, J. Chem. Soc. Chem. Commun. (1988) 1459.
[28] M. Bakker, A.S. Spruijt, F. van Rantwijk, R.A. Sheldon, Tetrahedron: Asymmetry 11 (2000) 1801.
[29] T.A. Berger, K. Fogleman, T. Staats, P. Bente, I. Crocket, W. Farrell, M. Osonubi, J. Biochem. Biophys. Methods 43 (2000) 87.
[30] S.H. Hoke II, J.D. Pinkston, R.E. Bailey, S.L. Tanguay, T.H. Eichhold, Anal. Chem. 72 (2000) 4235.
[31] H. Kitaguchi, P.A. Fitzpatrick, J.E. Huber, A.M. Klibanov, J. Am. Chem. Soc. 111 (1989) 3094.