ELSEVIER

Journal of Chromatography A, 977 (2002) 239–246

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Aqueous two-phase extraction as an effective tool for isolation of geniposide from gardenia fruit

I.-Horng Pan[a,b,*], Hsi-Ho Chiu[b], Chu-Hsun Lu[b], Lain-Tze Lee[b], Yaw-Kuen Li[a]

[a]*Department of Applied Chemistry, National Chiao Tung University, Hsinchu, Taiwan, ROC*

[b]*Biomedical Engineering Center, Industrial Technology Research Institute, Hsinchu 30055, Taiwan, ROC*

Received 3 April 2002; received in revised form 7 August 2002; accepted 26 August 2002

Abstract

Natural products are normally obtained by organic solvent extraction and many subsequent chromatographic separations. Compounds of interest are often isolated with very low yield and limited purity. An aqueous two-phase extraction process combined with a simple ethanol treatment, for removing excess inorganic salt, has been developed for preparation of geniposide from gardenia. The system was comprised of PE62, a random copolymer composed of 20% ethylene oxide and 80% propylene oxide, KH_2PO_4 and ethanol. To find optimal conditions, the partition behavior of geniposide under an aqueous two-phase system was investigated. Various factors were considered, including the concentration of salt, the concentration of polymer, the sample loading, and the addition of ethanol. The experimental results demonstrated that increasing salt concentration or decreasing PE62 concentration results in enhancement of the geniposide partition in the salt-rich phase. The addition of ethanol and higher sample loading also promoted the partition efficiency of geniposide. Based on this study, an optimized system containing 5% PE62, 7.5% KH_2PO_4, and 10% ethanol was tested on a large-scale extraction. A 39.0-g aliquot of final product (in powder form) with 77% purity of geniposide can be effectively extracted from 500 g of gardenia fruit. This process is proved to be useful for industrial application of geniposide preparation.

Keywords: Aqueous two-phase systems; Extraction methods; Fruits; Gardenia fruit; Geniposide; Glycosides; Iridoids

1. Introduction

Geniposide is an iridoid glycoside purified from gardenia fruit, used as a traditional Chinese medicine to treat hepatic and inflammatory conditions [1,2].

Partial purification of bioactive small molecules is an interesting field in the pharmaceutical industry and in bioengineering. Generally, several solvents with different polarities (such as ether, chloroform, ethyl acetate, and so on) should be used to separate the desired molecules from other contaminants. The yield and/or purity of the product of interest is expected to be relatively low. Furthermore, such processes in industrial applications inevitably consume a massive amount of organic solvent and the danger of explosion necessitates special equipment leading to increased costs.

Thus, an aqueous two-phase system has been

*Corresponding author. Present address: Biomedical Engineering Center, Industrial Technology Research Institute, 321 Kuang Fu Road, Section 2, Hsinchu 30055, Taiwan, ROC. Tel.: +886-3-573-2615; fax: +886-3-573-2556.

E-mail address: 760404@itri.org.tw (I.H. Pan).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01385-7

considered as a useful method for purifying biological materials in biochemistry and biotechnology [3,4]. The system is composed of a polymer and salt or two incompatible polymers, e.g. dextran and poly(ethylene glycol). The partition behavior of molecules in these systems depends mainly on their physicochemical properties, such as hydrophobicity or charge [3,4]. The partition can be influenced by changing the composition of the phase system, including polymer type, polymer concentration, salt type and salt concentration [5]. Additionally, some organic solvents [6] or surfactants [7] also influence partition behavior in the system. Before such a scheme can be developed, it is necessary to determine the distribution of these molecules in an aqueous two-phase system and how to manipulate the system to achieve the desirable partition. Numerous reports dealing with macromolecule partition in an aqueous two-phase system have been published, mostly involving proteins or enzymes. Few cases of the partition of small molecules were studied [8–11]. The present work studies the partition behavior of geniposide in a small-scale aqueous two-phase extraction system containing PE62 (ethylene oxide–propylene oxide, 20:80) copolymer and phosphate. Factors such as polymer concentration, sample loading, and and salt concentration were investigated. In addition, the effects of alcoholic additives on phase composition were also studied. Based on the outcome of small-scale (4.8 g of gardenia powder) study, a scaled-up aqueous two-phase extraction process for purifying geniposide from 500 g of gardenia fruit powder has been developed and evaluated. This study turns out to be the first report presenting the development of a simple and successful process for geniposide preparation from its natural source.

2. Experimental

2.1. Materials and chemicals

PE62 (a random copolymer comprising of 20% ethylene oxide and 80% propylene oxide, with molecular mass of 2475, and cloud point of 32±2 °C) was obtained from Sino–Japan, Taiwan. Gardenia fruit (*Gardenia jasminoides* Ellis) were obtained from local markets in Taiwan. All other chemicals were GR grade. The geniposide standard was obtained from Yoneyama, Japan. The fine powder of gardenia fruit for extraction study was prepared by milling dry gardenia fruit with a mechanical grinder (Tai Cheer Machinery Enterprise, Taiwan) and sieving through a 20-mesh metal sieve (Kuang Yang, Taiwan).

2.2. Small-scale aqueous two-phase extraction

A 4.8-g sample of the fine powder was mixed with 100 ml water. After boiling for 30–40 min, the mixture was subject to filtration with a 100-mesh metal sieve. The filtrate (crude water extract) was collected and used directly for further experiment.

An aqueous two-phase system was prepared by adding various amounts of PE62 (5, 10 and 20%), salt and ethanol (indicated in the text) to 5 ml of crude water extract. The system was vortexed to form a homogeneous phase. For visualizing the phase formation and to allow the system to equilibrate, the resulting solution was allowed to stand for 1–2 h at 40 °C room temperature (above the cloud point of PE62) and further centrifuged at 1500 *g* for 30 min to facilitate the phase separation. The geniposide-containing salt-rich layer (~4.8 ml) was separated and subject to back-extraction three times (5 ml each time) with 95% ethanol. The ethanol extracts were collected and kept at 4 °C overnight to allow salt to precipitate. After removing the precipitant by centrifugation, the amounts of geniposide were analyzed by HPLC as described in Section 2.4.

2.3. Scale-up of the aqueous two-phase extraction

For larger scale extraction, the manipulation of each step was similar to that of the small-scale study with only a few minor modifications, as described below. A 500-g sample of the gardenia fine powder was mixed with 10 l water and then boiled for 40 min. After filtration with 100-mesh metal sieve, the filtrate was concentrated to ~1100 ml (~1200 g) by rotary evaporator with temperature controlled at ~60 °C. The concentrated crude extract was then employed for the aqueous two-phase extraction study. The aqueous two-phase system with 10% solid content was prepared by mixing PE62 (60 g),

KH_2PO_4 (90 g), and ethanol (120 g), and pouring them into the above crude water extract (1200 g). The resulting mixture was then thoroughly agitated (500 rpm, 30 min) with a mechanical stirrer (Hsiang Tai, Taiwan) to form a homogeneous phase. This mixture was kept in a 40 °C water bath for 2 h to allow the preliminary phase formation. Centrifugation was then performed at 1500 *g* for 60 min to facilitate phase separation. The salt-rich phase, containing most geniposide (lower layer), was collected and vacuum dried by rotary evaporator at 60 °C. The resulting product was then subject to back-extraction three times (600 ml each time) with 95% ethanol. The ethanol extracts were collected and kept at 4 °C overnight to precipitate the salt. The precipitant was further removed by gravity filtration with 400-mesh metal sieve (Kuang Yang, Taiwan). The filtrate was subject to product analysis and subsequently vacuum dried by rotary evaporator to form a powder.

2.4. Detection and analysis

High-performance liquid chromatography was used to determine the concentration of geniposide in all samples. The HPLC system consisted of two pumps (series 4 type, Lab Alliance) linked with an autosampler (Basic$^+$ Marathon, Spark) and connected to a UV–Vis detector (wavelength: 260 nm, UV-620 type, GL Science). An Inertsil 5 ODS-2 C_{18} (250 mm×4.6 mm column) reversed-phase column was employed for chromatographic separation. Samples were filtered through a 0.45 μm syringe filter (poly(vinylidene difluoride) (PVDF) membrane, Millipore, USA) before injection. The mobile phase comprised a solution composed of 10% methanol in acetonitrile and 0.1% phosphoric acid with a ratio of 10:90 and a flow-rate of 1.0 ml/min. After 20 min, the ratio was linearly changed to 17:83, then changed again according to the following time dependent ratios: 20–30 min, linear to 50:50; 30–45 min, linear to 80:20. All quantitative data were calculated using peak area ratio and the internal standard method with butyl benzoate (2.49 mg/ml) as the internal standard. Fig. 1 presents the chromatogram of geniposide in the crude water extract and various manipulation steps. The standard curve for geniposide is $y = 1.410x + 0.0017$, $R^2 = 0.9997$, where y is the peak area of geniposide/area of butyl benzoate, and x is the concentration of geniposide (mg/ml). This equation is employed for calculating the amount of geniposide in this study.

2.5. Quantification

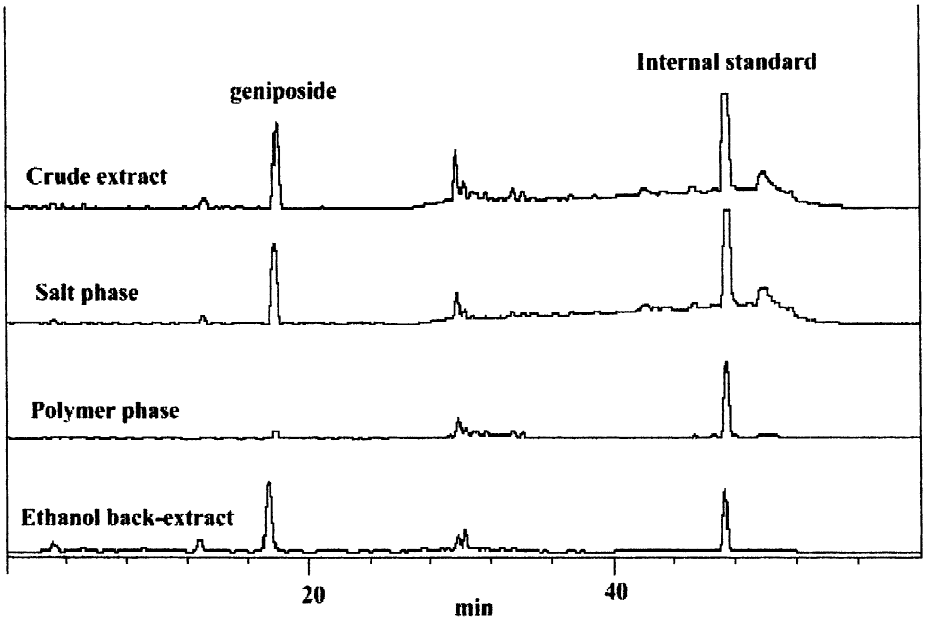
The partitions of geniposide between the various phases were characterized by various parameters including the partition coefficient (K), volume ratio (V), and distribution ratio (G). The parameters of K and V were defined as follows: $K = C_p/C_s$, where C_p and C_s denote the concentration of the partitioned substance in the polymer and salt phases, respectively; $V = V_p/V_s$, where V_p and V_s represent the volumes of the PE62 and salt phases, respectively. Under conditions of equilibrium, the distribution ratio (G) is equal to KV, giving the ratio of the total amount of geniposide in the polymer to that in the salt phase. The recovery yield (Y), equal to $1/(1 + G)$, is simply calculated by the amount of geniposide in each step of extraction versus the total amount of geniposide in water extract.

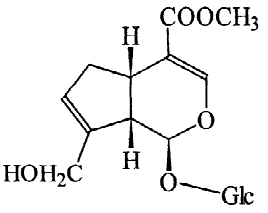
The geniposide content or purity was defined as the mass percentage of geniposide in total solid.

3. Results and discussion

3.1. Partition in a system with PE62 and a different salt

For classical natural product purification, one or several organic solvents are used to extract the desired molecules, and the molecules are recovered after the evaporation of the solvent in the final extraction step. To date, separation using the aqueous two-phase system is a rapid means of purifying biomolecules [3,4]. The partition behavior of some small organic molecules has been studied previously [8–11]. A suitable process for partially purifying geniposide from gardenia by using the aqueous two-phase extraction system is investigated here. Various salts, including $MgSO_4$, $(NH_4)_2SO_4$, NaH_2PO_4, KH_2PO_4, $CaCl_2$, NaCl, sodium acetate, sodium propionate, and glycine, were tested for their phase-forming characteristics with PE62, with the two-phase system being composed of 10% PE62 and 10% salt, and an attempt was made to determine the





geniposide

Fig. 1. HPLC analysis of the samples obtained from various extraction steps. The final product was obtained from the ethanol back-extraction of the dry substance derived from the salt-rich phase. Geniposide and the internal standard were eluted at 16.63 and 46.73 min, respectively.

most effective salt. No phase separation can be observed when $CaCl_2$, NaCl, sodium acetate, sodium propionate, or glycine were applied. In general, the results revealed that the effectiveness of phase formation varies in a similar pattern to lyotropic series of anion, as discussed by Ananthapadmanabhan and Goddard [12]. The higher the charge density anion the better the phase-formation tendency, i.e. higher charge density anion needs lower concentration for phase formation. This phenomena can be explained by the salting-out ability of anion, in which the relative effectiveness of various salts in promoting phase separation reflects the lyotropic series [13]. For geniposide molecules, the higher the salting-out potency of the salt, the greater the K value observed in the system. For instance, $MgSO_4$ (1.55)$>KH_2PO_4$ (0.91). Though the K values of the systems containing different salts reflect the lyotropic series, the G values did not exhibit a systematic trend but exhibited a small range of variation (0.36–0.65).

Table 1
Partitioning behavior of geniposide from gardenia in system composed of PE62 and different salts

Salt type	*V*	*K*	*G*
$MgSO_4$	0.27	1.55	0.42
$(NH_4)_2SO_4$	0.26	1.40	0.36
NaH_2PO_4	0.51	1.25	0.64
KH_2PO_4	0.71	0.91	0.65

Phase systems were composed of 10% (w/v) PE62, 10% (w/v) salt, and 5-ml extract (0.12 g of gardenia/ml). *V*, *K*, and *G* were obtained from phase separation at 40 °C room temperature. Note that no phase was formed when 10% (w/v) $CaCl_2$, NaCl, sodium acetate, sodium propionate, or glycine were used separately as salt.

We, therefore, arbitrarily selected KH_2PO_4 as the salt for the following experiments. Some calculated parameters are listed in Table 1 for reference.

3.2. Sample loading effect

The influence of the loaded mass on the partition and purification of macromolecules is important. The loaded biomass can alter not only the phase volume ratio of the aqueous two-phase system [4] but also the partitioning behavior of target compounds. In this study, different concentrations of extracts derived from 0.048–0.12 g of gardenia per 1 ml of water served as original samples. A phase system composed of 10% (w/v) PE62, 10% (w/v) KH_2PO_4 and 5 ml of the original samples was employed. With increasing sample loading in the system, the volume ratio (*V*) was slightly increased (0.61–0.71), but the partition coefficient (*K*) and distribution ratio (*G*) were decreased with values of 1.25–0.91 and 0.76–0.65, respectively. The *Y* values, however, showed only a 4% difference (57–61%), indicating that the loaded mass in a reasonable range is not a crucial factor in this case.

3.3. Salt and PE62 concentration effect

Changing the properties of the aqueous two-phase system influences the partitioning solutes. The properties of a phase system largely depend on the type and concentration of polymer and salts [3,4]. The influence of the concentrations of both KH_2PO_4 and PE62 in the aqueous two-phase system were also investigated. A 5-ml extract was applied (0.048 g of gardenia/ml) for each of the following experiments. The results showed that when the concentration of PE62 (5–20%) was increased at a fixed level of KH_2PO_4 (10%), the *K*, *V*, and *G* values constantly increased from 1.21 to 1.53, 0.15 to 0.86, and 0.18 to 1.32, respectively, indicating that geniposide partitioned to the salt-enriched phase more efficiently when less concentrated PE62 was used. Consequently, 85% of geniposide was recovered with the system containing 10% salt and 5% PE62. However, a lower concentration of PE62 ($<5\%$) resulted in an obscure phase formation. In the case of increasing salt concentration (5–15%) at a constant PE62 (5%), the *K* value was increased (1.0–1.45), while *V* (0.28–0.11) and *G* (0.28–0.16) values were slightly decreased. Based on the values of *Y* (78–86%), the result clearly demonstrated that the partition efficiency of geniposide was enhanced by the increasing salt concentration. With application of an extraction system containing 10% salt and 5% PE62, the recovery yield can reach 86%.

3.4. Effect of ethanol in extraction system

Effects of organic solvents on the partitioning of molecules in aqueous-two phase systems have been reported [6,9,11], revealing that the phase-forming properties can be influenced by the addition of an organic solvent, such as alcohol. This study investigates the effects of the partition behavior of geniposide by adding ethanol to a PE62–KH_2PO_4 system. As can be seen in Table 2, the presence of ethanol unequivocally enhanced the partition efficiency of geniposide to the salt-rich phase. For instance, in the system containing PE62 (20%), KH_2PO_4 (10%) and various amounts of ethanol, the recovery yields are increased from 43 to 54% as ethanol concentration is increased from 0 to 10%. For cases of lower concentrations of PE62 and KH_2PO_4, the addition of 10% ethanol could lead to 90% recovery yield of geniposide.

Table 2
The influence of ethanol in a system with various salt and PE62 concentrations[a]

PE62 (%)	KH_2PO_4 (%)	Ethanol (%)	*V*	*K*	*G*	*Y* (salt) (%)
20	10	0	0.84	1.56	1.31	43.3
20	10	2	0.60	1.71	1.03	49.4
20	10	5	0.53	1.78	0.94	51.5
20	10	10	0.56	1.51	0.85	54.0
20	7.5	10	0.86	1.11	0.95	51.3
20	7.5	0	1.5	1.30	1.95	34.0
10	10	0	0.61	1.25	0.76	56.8
10	10	10	0.59	1.10	0.65	60.6
10	7.5	0	0.44	1.24	0.54	64.9
10	7.5	10	0.39	1.13	0.44	69.4
5	10	0	0.15	1.21	0.18	84.7
5	10	10	0.09	1.23	0.11	89.9
5	7.5	0	0.22	1.20	0.26	79.4
5	7.5	10	0.1	1.08	0.11	90.1

[a] A 5-ml extract (0.048 g of gardenia/ml) was applied for each experiment. *K*, *V*, *G*, and *Y* values were obtained from phase separation at 40 °C room temperature.

3.5. Ethanol back-extraction

In this study, ethanol back-extraction is designed for removing inorganic salt from the product. Since geniposide is mainly present in the salt-rich layer of the extraction system, a large amount of salt is retained in the product. Triplicate back-extraction with 95% ethanol (5 ml each time for small-scale and 600 ml each time for scaled-up) can recover nearly 100% of geniposide from the crude product (the dry powder of the salt-rich extract). A fair amount of salt, re-dissolved in ethanol back-extract, can be precipitated by keeping the solution in 4 °C overnight and further eliminated by centrifugation or filtration. With this simple salt-removing step, the purity of geniposide can be greatly enhanced from 41 to 81% (Table 3).

3.6. Evaluation of the extraction system

In order to test the efficiency of the optimized condition, 90 mg of pure geniposide was added to the extraction system containing 10 ml water, 0.48 g PE62, 0.72 g KH_2PO_4 and 0.96 g ethanol. A total of 90 mg solid powder containing 81 mg of geniposide, can be recovered. The recovery yield and purity are 90%. By spiking a similar amount of geniposide in raw material, nearly 80% of the total geniposide (the spiked and the estimated amounts in gardenia) was recovered, indicating that this aqueous-two phase system can effectively extract geniposide from gardenia.

3.7. Scale-up of aqueous two-phase extraction

Based on all studies described above, the optimal condition for geniposide partition was found to be the system containing 5% PE62, 7.5% KH_2PO_4, and

Table 3
The purity and recovery yield of each step in scaled-up process

Purification step	Purity[a] (%)	Recovery yield (%)
Crude water extraction	28	100
Salt-rich phase	12	89
Final ethanol back-extraction[b]	41	81
Filtration[c]		
Diatomite (SABH)	81	66
Cellulose membrane	72	78
Filtration paper	74	77
400-mesh sieve	77	80

[a] Purity (%) is the mass percentage of geniposide in the total solid content.

[b] The yield and purity were calculated without removing the salt contamination.

[c] Note that four different filtration methods were employed and compared. The yield and purity were measured directly after the filtration of ethanol back-extraction by each method, separately.

10% ethanol. This extraction system was further tested on a 500-g gardenia. Manipulation steps were similar to those of the small-scale extraction except for the filtration after ethanol back-extraction. A total of 134 g of crude product (in solid form) with 28% (w/w) geniposide purity, which was analyzed by HPLC with calculated internal standard, was obtained from the water extraction step. Since no significant amount of geniposide was detected in the third and later water extraction steps, the geniposide content in gardenia fruit was estimated to be 7.5%. This larger scale extraction was performed with a recovery yield of 89% (in salt-rich phase), which is nicely consistent with the small-scale manipulation. However, owing to the large amount of salt contamination, the purity of larger scale extraction was only 12%. In order to remove salt which remained in the sample, ethanol back-extraction and further filtration using diatomite (SABH, Grefco, USA), cellulose membrane (0.22 μm, MSI Micron Separations), filtration paper (Adventec, Toyo, Japan), and 400-mesh metal sieve, were performed, with final recovery yields of 66, 77, 78, and 80%, and correspondent purity of 81, 72, 74, and 77%, respectively. Results are shown in Table 3. Note that the yields were calculated based on the equation derived from the standard curve of the peak area ratio of geniposide versus butyl benzoate (see Detection and analysis section). Considering the recovery yield, purity, and cost, the filtration process with 400-mesh metal sieve is a better choice for practical application. This simple extraction system has been shown to be an effective method for isolation of geniposide from gardenia fruit. For example, 39.0 g of final solid product containing 30 g of geniposide, corresponding to 77% of purity, can be easily extracted from 500 g of gardenia fruit powder.

4. Conclusions

The quantitative partition of geniposide derived from the crude extract of gardenia in an aqueous two-phase system containing PE62 copolymer, salts and a limit amount of ethanol has been reported. Large scale isolation of geniposide by the developed system was also investigated. The major conclusions of this study can be summarized as follows.

(1) We demonstrated that increasing salt concentration or decreasing PE62 concentration resulted in an enhancement of the geniposide partition in salt-rich phase.

(2) The addition of ethanol unequivocally improved the partition efficiency of geniposide to the salt-rich phase.

(3) An optimized system containing 5% PE62, 7.5% KH_2PO_4, and 10% ethanol was developed and shown to be effective for the extraction of geniposide from gardenia fruit.

(4) Ethanol back-extraction following by filtration process provides a simple way to remove the precipitants of salt and others. The purity of geniposide can be largely increased without losing its recovery yield.

(5) This simple system has been evaluated with a 500-g scaled extraction and turns out to be a very useful process for industrial application of geniposide preparation.

Acknowledgements

The authors would like to thank the Ministry of Economic Affairs for financially supporting this research under Contract No. MOEA 89-EC-2-A-17-0170 and a research grant of the National Science Council of Taiwan, ROC.

References

[1] S.W. Wang, C.J. Lai, C.J. Wang, Cancer Lett. 65 (1992) 133.
[2] C.M. Harada, N. Tenmyo, M. Aburada, T. Endo, Yakugaku Zasshi 94 (1974) 157.
[3] P.Å. Albertsson, Partition of Cell Particles and Macromolecules, 3rd ed, Wiley, New York, 1986.
[4] H. Walter, D.E. Brooks, D. Fisher, Partition in Aqueous Two-phase Systems, Academic Press, Orlando, FL, 1985.
[5] K. Berggren, H.-O. Johansson, F. Tjerneld, J. Chromatogr. A 718 (1995) 67.
[6] G. Johansson, G. Kopperschläger, J. Chromatogr. 388 (1987) 295.
[7] M.T. Cunha, F. Tjerneld, J.M.S. Cabral, M.R. Aires-Barros, J. Chromatogr. B 711 (1998) 53.
[8] R.D. Rogers, H.D. Willauer, S.T. Griffin, J.G. Huddleston, J. Chromatogr. B 711 (1998) 255.
[9] P.A. Alred, F. Tjerneld, J. Chromatogr. 628 (1993) 205.

[10] J. Planas, V. Varelas, F. Tjerneld, B.H. Hagerdal, J. Chromatogr. B 711 (1998) 265.
[11] R.F. Modlin, P.A. Alred, F. Tjerneld, J. Chromatogr. A 668 (1994) 229.
[12] K.P. Ananthapadmanabhan, E.D. Goddard, Langmuir 3 (1987) 25.
[13] J. Huddleston, A.V. Veide, K. Kohler, J. Flanagan, S.-O. Enfors, A. Lyddiatt, Trends Biotechnol. 9 (1991) 381.