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Note

Column lifetime of a new agarose medium for hydrophobic interaction chromatography

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The column lifetime of fast protein liquid chromatography (FPLC) columns has recently been investigated[1–3]. These studies cover cation-exchange, anion-exchange and gel filtration columns. It was observed that all columns withstand 1000 repetitive injections. This report provides an extention of these investigations by evaluating the column lifetime of a newly launched gel for hydrophobic interaction chromatography (HIC), Phenyl-Superose™. This HIC gel was developed by reaction of phenyl glycidyl ether with Superose 12, an agarose-based gel for high-performance gel filtration[4]. The lifetime of a Phenyl-Superose HR column was studied by making 1000 injections of a protein mixture and by continously monitoring the retention times, the peak heights, the peak areas and the column back-pressure. Furthermore, the ligand content was analysed before and after the test.

EXPERIMENTAL

Myoglobin and lysozyme were purchased from Sigma (St. Louis, MO, U.S.A.) and ribonuclease-A was obtained from Pharmacia (Uppsala, Sweden). All inorganic compounds were of p.a. quality. Mobile phase B contained 0.1 *M* phosphate buffer (pH 7.0) and 0.02% sodium azide, whereas mobile phase A contained B with the addition of 1.7 *M* ammonium sulphate. The eluents were filtered through a 0.45-μm filter.

A 50-μl injection of myoglobin (0.67 mg/ml), ribonuclease A (0.63 mg/ml) and lysozyme (0.028 mg/ml) served as test sample. From a stock solution of the proteins in solvent B the sample was prepared by five-fold dilution with solvent A. The sample was prepared daily from frozen aliquots of this stock solution and was filtered through a 0.22-μm bacteriological filter before use.

Chromatographic measurements were carried out on a Pharmacia-prepacked Phenyl-Superose column HR 5/5 with a Pharmacia FPLC system consisting of a LCC-500 control unit, two P-500 high-precision pumps, a UV-1 UV monitor (280 nm, HR 10 cell), a MV-7 sample injector with a 50-μl loop, and a REC-482 recorder. The column temperature was controlled by the ambient temperature (*ca.* 20°C).

The protein mixture was repetitively injected every 15 min and eluted according to the gradient programme in Table I. After 550 injections the column was washed

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TABLE I

GRADIENT PROGRAMME FOR ONE INJECTION SEQUENCE

Time (min)	*Instruction*	*Value*	*Note*
0	Conc. % B	10	Equilibration
0	ml/min	0.5	Flow-rate
1.0	Valve pos.	1.2	Injection
3.0	Conc. % B	10	Isocratic elution
8.0	Conc. % B	54	Linear gradient elution (8.8%/min)
10.0	Conc. % B	100	Linear gradient elution (23%/min)
12.0	Conc. % B	10	Regeneration (−43%/min)
15.0	Conc. % B	10	Equilibration

with 6 ml of 43% acetic acid and 3 ml of 100% acetic acid. The column was subsequently reequilibrated before the lifetime test was completed (1000 injections).

The chemical stability was evaluated by measuring the ligand content before and after the lifetime test. The ligand content was determined by cleavage of the ether bond between the gel and the phenyl substituent, and the subsequent determination of the amount of phenol produced[5].

RESULTS AND DISCUSSION

The quality of prepacked liquid chromatographic columns embodies criteria such as column efficiency, selectivity, peak symmetry, column permeability and the concentration of bonded organic phases[6]. Another aspect of the column quality is its lifetime in use. A long lifetime means that the contribution of the column to the cost per analysis is negligible. In this study the lifetime of a Phenyl-Superose HR 5/5 column was investigated by making 1000 repetetive injections of a protein mixture.

The chromatograms in Fig. 1 show that the gradient separation was retained throughout the test. Furthermore the retention times (t_R) of the three main protein peaks exhibit no trends with the number of injections (Table II). The relative standard deviation (R.S.D.) of the t_R values was less than 1% for all peaks. These results indicate that the Phenyl-Superose gel is unaffected by 1000 injections. The unaltered

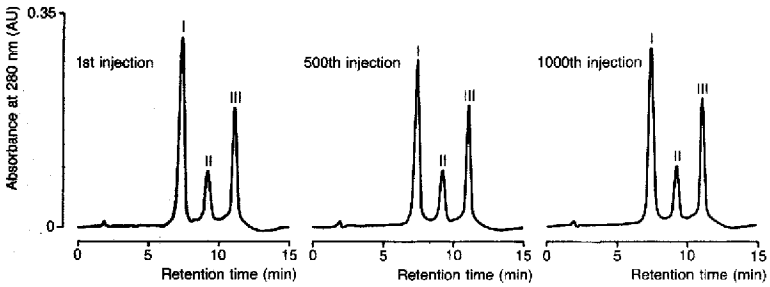

Fig. 1. Chromatograms from the 1st, the 500th and the 1000th injections of a protein mixture on a Phenyl-Superose HR 5/5 column. Peaks: I = myoglobin; II = ribonuclease; III = lysozyme. For chromatographic conditions, see Table I and the Experimental section.

TABLE II

VARIATION OF RETENTION TIMES (t_R) AND PEAK HEIGHTS (h') OF THE THREE PROTEIN PEAKS AND THE TOTAL ACCUMULATED AREA DURING 1000 INJECTIONS ON A PHENYL-SUPEROSE HR 5/5 COLUMN AT pH 7.0

Injection number	*Myoglobin*		*Ribonuclease*		*Lysozyme*		*Total accumulated peak area above 0.006 a.u. (min a.u.)*
	t_R *(min)*	h' *(a.u.)*	t_R *(min)*	h' *(a.u.)*	t_R *(min)*	h' *(a.u.)*	
1	7.32	0.305	9.23	0.095	11.12	0.203	0.292
50	7.45	0.288	9.19	0.100	11.11	0.210	0.307
100	7.46	0.288	9.27	0.098	11.09	0.205	0.292
150	7.50	0.290	9.27	0.103	11.10	0.210	0.286
200	7.34	0.300	9.18	0.098	11.06	0.208	0.293
250	7.40	0.280	9.20	0.098	11.09	0.208	0.282
300	7.38	0.290	9.20	0.100	11.09	0.208	0.287
350	7.35	0.285	9.21	0.100	11.07	0.213	0.297
400	7.34	0.285	9.20	0.098	11.07	0.210	0.294
450	7.34	0.280	9.20	0.098	11.08	0.205	0.297
500	7.36	0.270	9.24	0.093	11.07	0.200	0.284
550	7.40	0.290	9.21	0.100	11.08	0.208	0.300
600	7.34	0.290	9.18	0.095	11.06	0.208	0.293
650	7.47	0.305	9.24	0.098	11.09	0.213	0.273
700	7.38	0.288	9.22	0.098	11.09	0.213	0.298
750	7.53	0.293	9.27	0.100	11.08	0.210	0.294
800	7.42	0.295	9.24	0.103	11.06	0.215	0.316
850	7.42	0.295	9.21	0.098	11.09	0.210	0.283
900	7.30	0.278	9.16	0.095	11.08	0.205	0.296
950	7.29	0.300	9.12	0.098	11.12	0.205	0.290
1000	7.35	0.288	9.22	0.098	11.09	0.208	0.278
Mean	7.39	0.290	9.21	0.098	11.09	0.208	0.292
R.S.D. (%)	0.89	3.00	0.40	2.50	0.16	1.75	3.29

ligand content, which was 0.58 μmol/g dry gel before and after the test, also verifies the resistance of the gel to the washing procedure and the injection sequences.

The peak heights (h') of the three peaks, evaluated as the distance between the peak maximum and the baseline at injection time, were at the same level throughout the test (Table II). Therefore it can be concluded that the efficiency of the column did not change during the test.

The continously registered back-pressure was 1.5 MPa (the value at the equilibrium conditions; see Table I) at all 1000 injections. In addition, no significant drift was observed of the total accumulated peak area (Table II). These results indicate that the sample was not adsorbed on the column filter or in the gel matrix during the test.

To summarize, the results clearly show that Phenyl-Superose HR 5/5 columns can withstand 1000 repetetive injections without any significant decrease in performance.

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