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Development and application of radio-size-exclusion chromatography

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Abstract

The development of radio-size-exclusion chromatography and its application to two widely different areas, (a) the determination of the radiochemical purity of ^{3}H- and ^{14}C-labelled biopolymers and (b) the kinetics of epoxy resin formation, is reported. The versatility of the instrument is also reflected in the fact that it is able to function in one of four modes, i.e. HPLC, radio-HPLC, SEC and radio-SEC.

Keywords: Radiochromatography; Polystyrene; Methacryloyl phosphoryl choline; Dodecynyl methacrylate; Phenyl glycidyl ether; Aniline; Bisphenol-A diglycidyl ether; Dianilinoethane

1. Introduction

Gas (GC), thin-layer (TLC), high-performance liquid (HPLC) and size-exclusion chromatography (SEC) are widely used for the purification, isolation and identification of organic compounds and when coupled to infrared, mass or nuclear magnetic resonance spectrometers they provide analytical technology of extraordinary capability [1].

In view of the extensive use that is made in the life sciences of radioactive (particularly ^{3}H and ^{14}C) compounds a range of radio-chromatographic methods have also become available. [2,3]. These include radio-GC, radio-TLC and radio-HPLC but significantly no ratio-SEL. In view of the increasing use being made of tritiated macromolecules this is a serious deficiency; here we report on such a development and also its application in two separate areas.

The first of these areas concerns the preparation of phosphoryl choline containing polymers [4,5] and their application to surfaces which has led to the development of a new generation of coatings for medical devices which resist protein adsorption and are non-thrombogenic. Their adhesion and stability can best be determined by preparing a radioactive form of the polymer and measuring any leeching of the radioactivity away from the surface by liquid scintillation counting.

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A very small percentage of a radioactive impurity in the coated polymer could lead to anomalous findings during the leeching studies.

The second application is in the epoxy resin chemistry area. We have developed a radio-HPLC method which is ideal for studying the kinetics of the reaction between a model epoxide (phenyl glycidyl ether, PGE, **1**; see Fig. 1) and a tritiated primary amine (aniline, **2**) but when we employ a di-epoxide (bisphenol-A diglycidyl ether, BADGE, **3**) and a tritiated diamine (1,2-dianilinoethane, DAE, **4**) the limitations begin to appear. The reaction produces more than nine intermediates and as it proceeds the intermediates (oligomers) increase in molecular mass. This leads to longer analysis times and poorer resolution, features which would be overcome if radio-SEC were available.

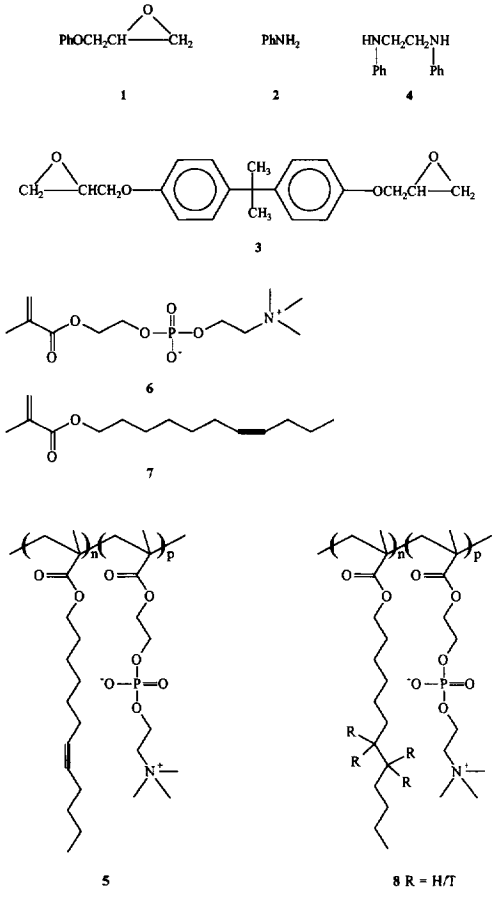

Fig. 1. Structures of the compounds used.

2. Experimental

2.1. Instrument design

The design of the instrument can best be understood by reference to a block diagram (Fig. 2) showing both the long established radio-HPLC and the new radio-SEC set-up. A 12.5-cm reversed-phase HPLC column and a Polymer Laboratories PL-Gel Mixed-D 60-cm SEC column were used together with a Nuclear Enterprises ISOFLO-1 radio-detector and an ERMA 3570 refractive index (RI) detector, the latter maintained at 35°C. Data were collected via a Waters SAT/IN satellite interface to a NEC computer operating Millipore's Millennium 2010 Version 2 software. The SEC carrier solvent was tetrahydrofuran (THF) delivered by a Waters 510 pump whilst for HPLC a Spectraphysics SP8700 ternary solvent pump was used. Unisolve E scintillant (NBS Biologicals) was delivered at a flow-rate of 2 ml/min by a Milton Roy LDC pump.

2.2. Tritiation of polystyrene standards

In order to calibrate the radio-SEC instrument it was necessary to prepare a number of tritiated standards. Tritiated polystyrene samples were prepared by a catalysed ^{3}H–^{1}H exchange reaction according to previously reported procedures [6], a summary of which follows. Platinum oxide catalyst was reduced with sodium borohydride in distilled water (0.4 g of BH_4^- for 0.1 g of PtO_2). Excess hydride was destroyed by heating and the catalyst washed with water and acetone.

A sample of narrow-molecular-mass range polystyrene (100 mg) and an amount of freshly reduced platinum oxide catalyst (75 mg) were placed in a thick walled glass tube. Tritiated water (6 μl at approximately 20 mCi/ml) were

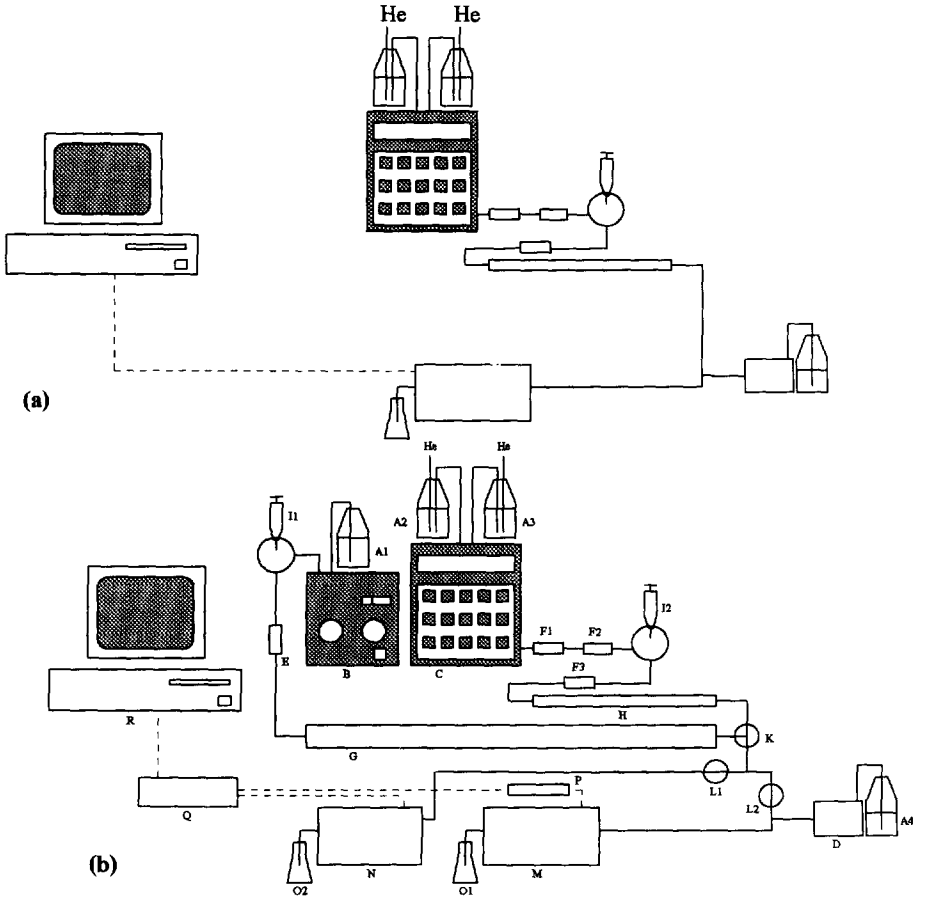

Fig. 2. Schematic diagram of (a) radio-HPLC, (b) radio-/non-radio-HPLC/GSEC. (A1) SEC solvent reservoir, (A2) HPLC solvent reservoir, (A3) HPLC solvent reservoir, (A4) scintillant reservoir, (B) SEC pump, (C) HPLC pump, (D) scintillant pump, (E) SEC guard column, (F1) HPLC solvent filter column, (F2) HPLC solvent mixer column, (F3) HPLC guard column, (G) SEC column, (H) HPLC column, (I1) SEC injector, (I2) HPLC injector, (K) 3-way tap, (L1) 2-way tap, (L2) 2-way tap, (M) radio-detector, (N) RI detector, (O1) waste solvent flask (radioactive), (O2) waste solvent flask (non-radioactive), (P) radio interface, (Q) Waters SAT/IN interface, (R) computer/database.

washed into the tube with 200 μl of THF (HPLC grade, 99.9 + %). The tube was evacuated, flame sealed and placed in an oil bath maintained at 110°C for several days. After cooling the seal was broken and anhydrous magnesium sulphate was added followed by a small volume of THF. The contents were then transferred and filtered through a bed of activated charcoal prior to removing the solvent by evaporation. A small quantity of the resulting white solid was then dissolved in THF prior to being counted in a liquid scintillator and the specific activity of the polystyrene determined.

2.3. Tritiation of a biocompatible polymer

A copolymer (**5**) of methacryloyl phosphoryl choline (MPC, **6**) and dodec-7-yn-1-yl methacrylate (**7**), where *n:p* was 2:1, was prepared [7] and then reduced with tritium gas ($^3H_2$) in the presence of Wilkinson's catalyst, to give the tritiated biocompatible polymer (**8**) [4].

2.4. Determination of radiochemical purity

The pattern of labelling within the tritiated polystyrenes was determined using ^{3}H NMR spectroscopy; the results showed that typically more than 80% of the tritium was located in the aromatic part of the polymer (Fig. 3). The same spectrum showed that the product was also radiochemically pure.

2.5. Radio-HPLC analysis of the early and later stages of the BADGE–1,2-dianilinoethane reaction

DAE (993:3 mg) and tritiated DAE (9.7 mg) were combined with BADGE (1.6052 g) to give a reaction mixture of 1.00:1.00 DAE–BADGE (mol/mol). The mixture was dissolved in a small amount of chloroform (300 μl, HPLC grade) so that the solution may be separated into 30 equal portions before commencing the reaction rather than take aliquots during the reaction. This is simply because after approximately 6 h of reaction using 1:1 molar ratios the brown material is highly viscous and very adhesive making accurate withdrawal of samples difficult. The 30 samples were placed in 1-ml Eppendorf sample tubes and the chloroform left to evaporate without heating. When the solvent had been removed, the tubes were placed upright in a wire cradle and the latter lowered into an oil bath maintained at 90°C. At the allotted time the appropriate sample tube was removed from the oil bath and the reaction quenched by dissolving the sample in excess cold THF.

The radio-HPLC chromatograms of the initial stages (up to 5 h) are shown in Fig. 4. The components were eluted using a gradient program of a mixture of THF and water (containing 0.1% trifluoroacetic acid) at a flow-rate of 1 ml/min according to the gradient program shown in Table 1.

The resolution of the peaks is good enough for quantitative determination since the radioactivity of any given species, represented by the area under the peak, is directly proportional to the concentration of the species present at that time. Fig. 5 depicts the radio-HPLC chromatograms of later stages of the reaction (6–10 h). It is clear that the large number of species present makes accurate determination of component concentrations difficult. It is also clear that the high-

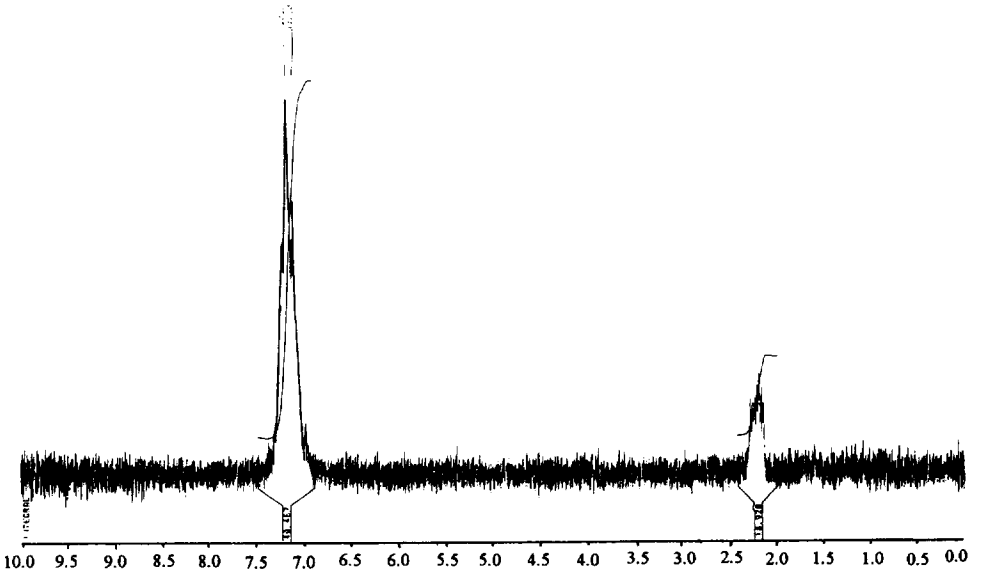

Fig. 3. ^{3}H NMR spectrum (^{1}H decoupled) of [G-^{3}H]-polystyrene.

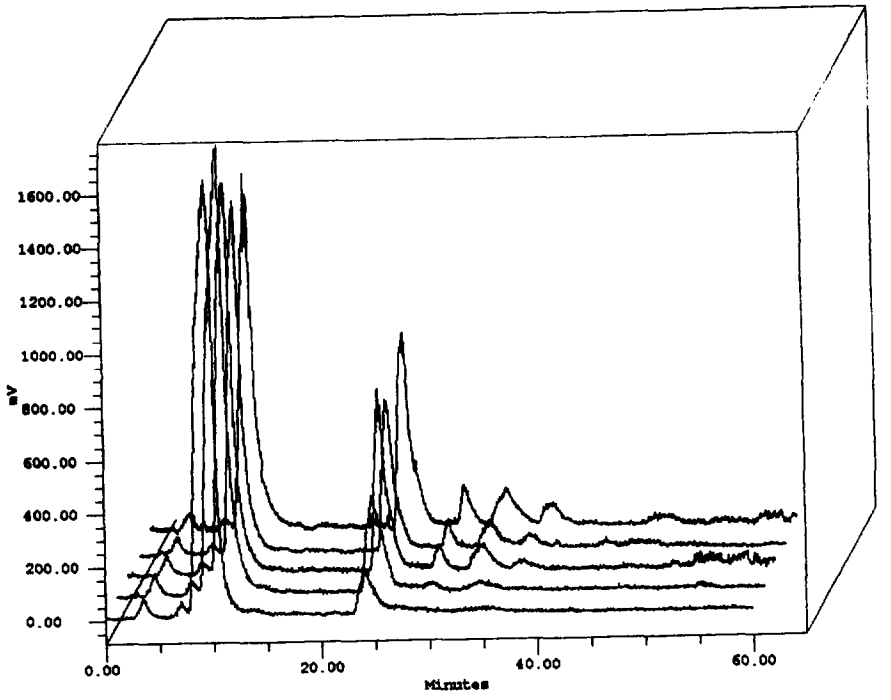

Fig. 4. Radio-HPLC of the early stages of the DAE–BADGE reaction.

molecular-mass species are not easily resolved and that no longer can their concentrations be determined with confidence.

2.6. Radio-SEC analysis of the BADGE–DAE reaction

The samples dissolved in THF were used directly for analysis by SEC using THF as carrier solvent at a flow-rate of 0.8 ml/min. A calibration curve was constructed using the tritiated polystyrene samples as narrow molecular mass standards. Both SEC (RI detection) and radio-SEC chromatograms were monitored for comparison and a typical example is given in Fig. 6.

Table 1

Time (min)	THF (%)	Water (%)
0	40	60
5	40	60
10	50	50
15	50	50
25	60	40
35	60	40
60	80	20

3. Results and discussion

In several studies (see Ref. [6] and references cited therein) the versatility of the radio-HPLC method has been recognized. As long as the intermediates are not too numerous and are of low molecular mass the method works very well and as the radioactivity is proportional to concentration the latter can be determined directly, which is not the case with UV or IR detection (some form of calibration is necessary). With more complex reactions, e.g., when more intermediates are formed in a similar time interval, improvements in solvent gradient programs can be of assistance, but a limit arrives when the

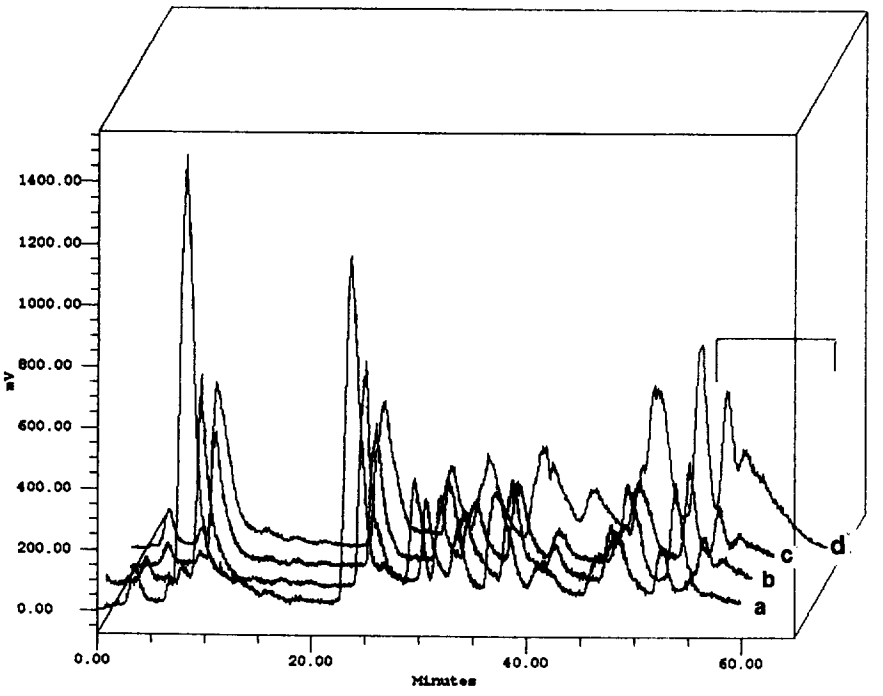

Fig. 5. Radio-HPLC of the later stages of the DAE–BADGE reaction.

oligomers (small and medium sized polymers) all elute together or not at all (Fig. 5).

Through appropriate manipulation of the taps K, L1 and L2 (Fig. 2) the designed instrument can function in one of four modes: HPLC, radio-HPLC, SEC and radio-SEC; it was the latter two that were employed for the higher-molecular-mass intermediates. The instrument performance was tested using a number of tritiated polystyrenes (six, covering a molecular mass range of 1200 to 35 000). A comparison of the size-exclusion chromatograms before and after tritiation showed no difference so that the polymers had withstood the harsh tritiation conditions without any decomposition. This was confirmed by the corresponding radio-size-exclusion chromatograms (Fig. 7). In some other studies small amounts of monomers, too low to be detected by SEC, have been preferentially tritiated, giving rise to additional peaks in the radio-size-exclusion chromatograms, thereby illustrating the power and potential of the method.

Samples taken after 10 h (trace d, Fig. 5) or more provided unsatisfactory radio-high-performance liquid chromatograms in the sense that the latter half of the trace (50 min onwards, as indicated) were unresolved. If this sample was now subjected to radio-SEC analysis the resulting chromatogram (Fig. 6) was very satisfactory, thereby making it possible to extend our investigations to reactions of direct industrial interest, rather than have to rely on model compounds.

In the case of the biocompatible polymer which had been prepared by the hydrogenation of an acetylene precursor (**5**) using $^{3}H_2$ gas and Wilkinson's catalyst the radio-SEC (Fig. 8a) gave a single relatively sharp peak, confirming its radiochemical purity (100%). However, a ^{14}C-labelled version of the same polymer, which had been stored for some time, gave additional peaks (Fig. 8b), showing that it was no longer radiochemically pure and could not be used without further purification.

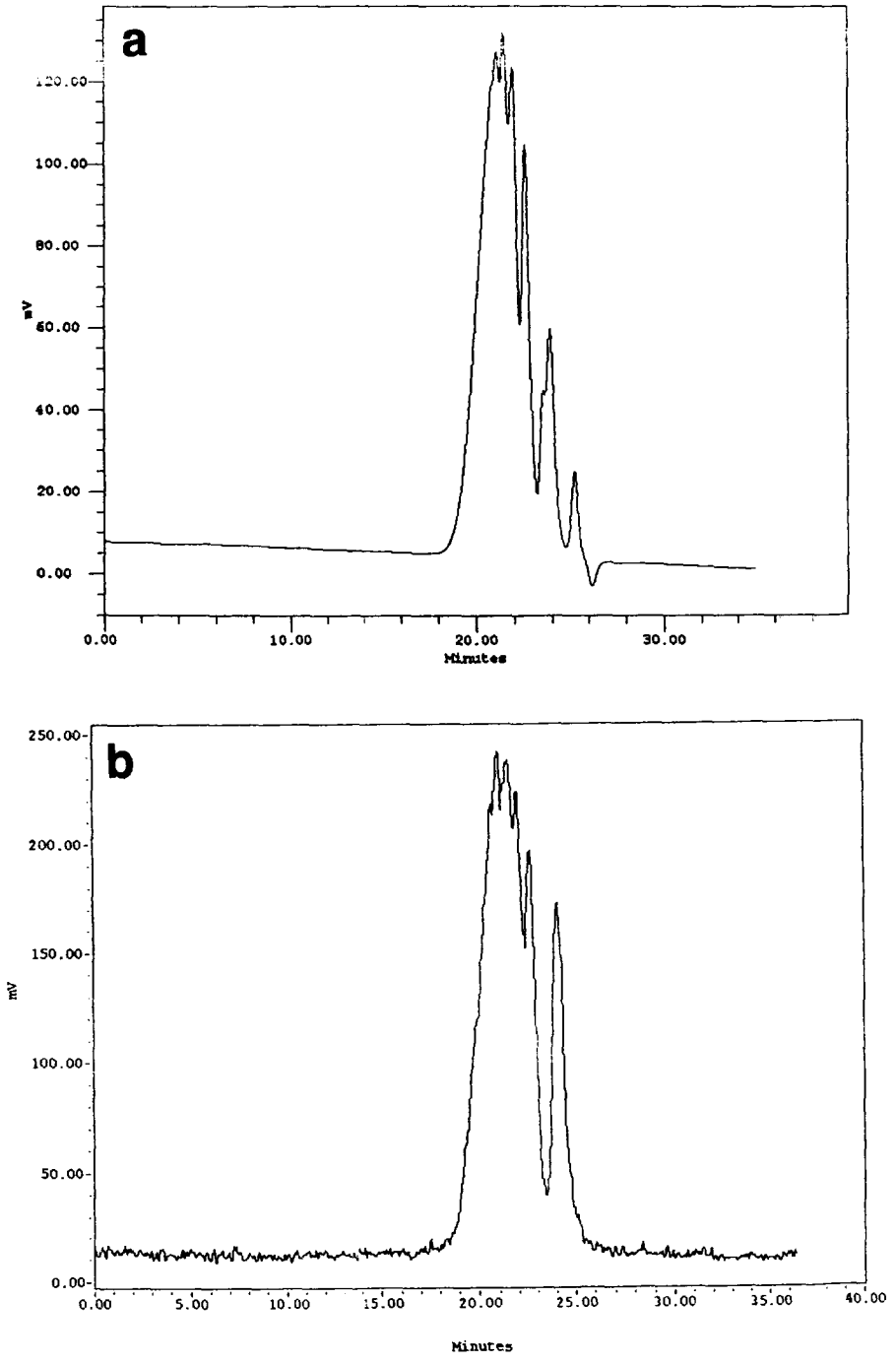

Fig. 6. (a) SEC and (b) radio-SEC of a sample from the DAE–BADGE reaction.

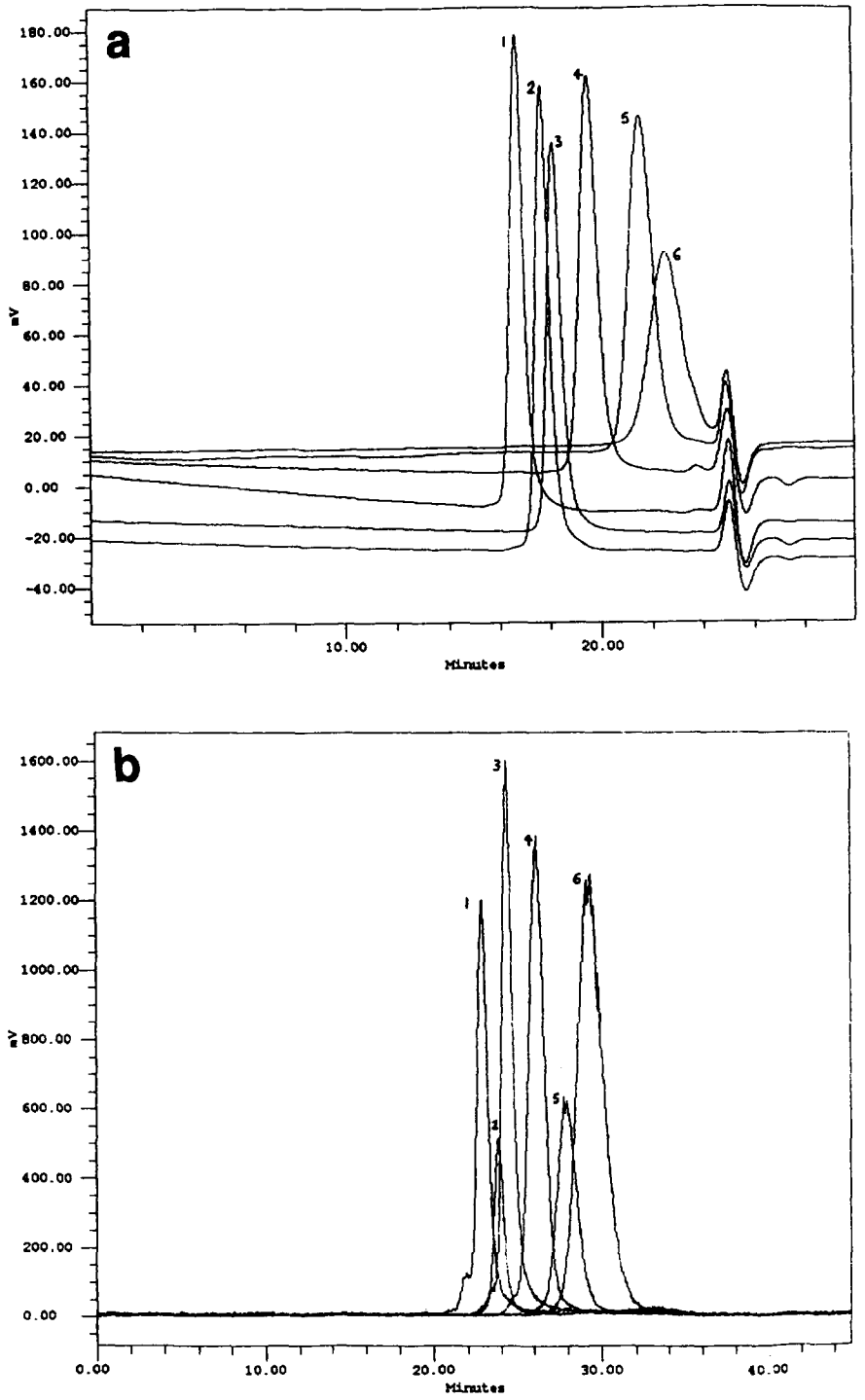

Fig. 7. (a) SEC and (b) radio-SEC chromatograms of tritiated polystyrenes of average molecular masses (1) 35 000, (2) 19 000, (3) 13 000, (4) 5200, (5) 2100 and (6) 1200.

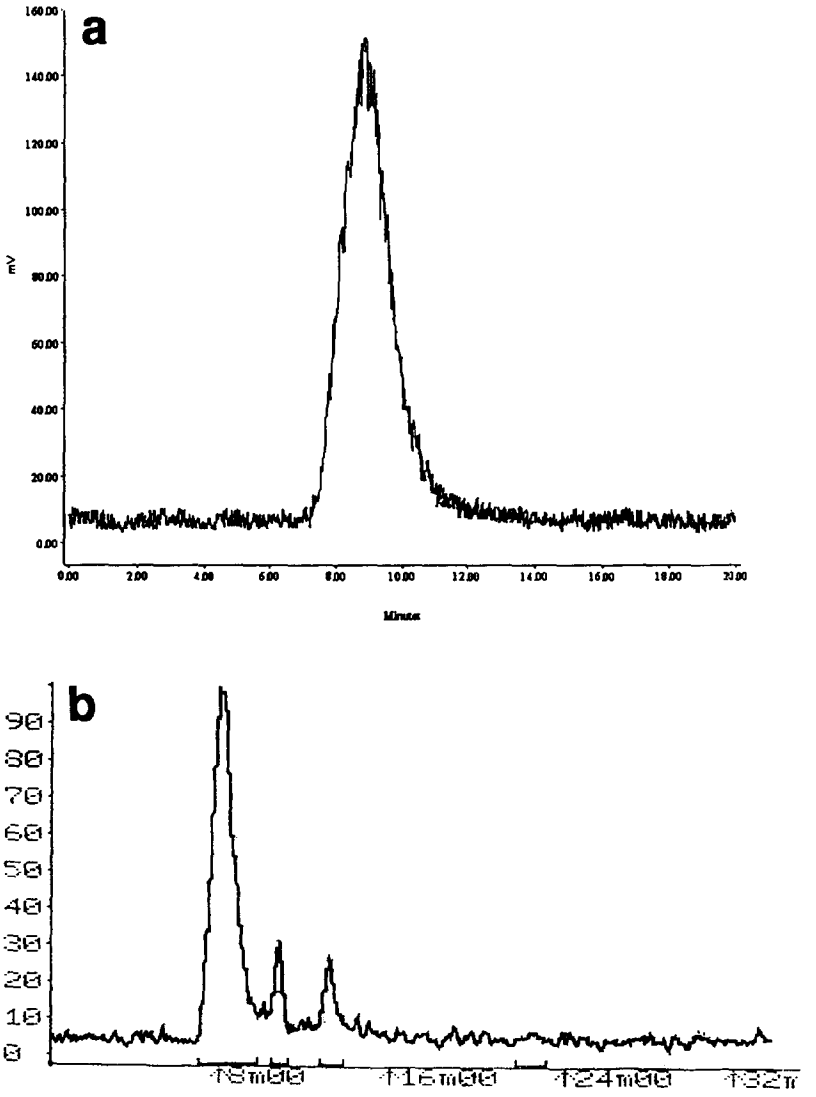

Fig. 8. Radio-SEC chromatograms of (a) the tritiated biocompatible polymer and (b) the ^{14}C-labelled version.

4. Conclusions

A radio-SEC instrument has been designed in such a way that it can operate in a non-radioactive mode as well as a radio- and non-radio-HPLC instrument. The potential of the technique is wide-ranging; in the present study its use in ascertaining the radiochemical purity of biomaterials, specifically polymers, has been illustrated as well as in the area of epoxy resin chemistry. Further developments, based on the use of higher resolution columns, will widen still further the range of potential applications.

Acknowledgements

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