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Conventional capillary electrophoresis in comparison with short-capillary capillary electrophoresis and microfabricated glass chip capillary electrophoresis for the analysis of fluorescein isothiocyanate anti-human immunoglobulin G

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Abstract

Fast, efficient analysis of fluorescein isothiocyanate anti-human IgG was achieved in a short-capillary and in a microfabricated glass chip. The capillary was 6 cm long from injection end to detector with electric field strength of 0.268 kV/cm. Analyses were performed within 1 min. A glass microchip device was fabricated using standard photolithographic procedures and chemical wet etching. The channels were sealed using a direct bonding technique. For an effective length of 2.8 cm with electric field strength of 0.526 kV/cm, electrophoretic analysis was achieved in less than 16 s. Conventional capillary electrophoresis gave highly reproducible results and good detection limits. Analysis time was 2.5 min for a capillary with 47 cm effective length and electric field strength of 0.383 kV/cm. © 1997 Elsevier Science B.V.

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1. Introduction

Miniaturized chemical analysis instruments are gaining increasing popularity. The combination of microlithography, etching techniques as well as controlled thin-film deposition, allows for the fabrication of three-dimensional structures on the micrometer scale [1].

Using micromachining techniques, Terry et al. [2] developed a gas chromatograph almost 20 years ago. Recently this technology has generated increasing interest and there are numerous studies where the use of these miniaturized analysis devices has been demonstrated [2–20]. Micromachined columns have since been fabricated for liquid chromatography (LC) [3,4], and capillary electrophoresis (CE) [5–25]. CE appears to be the most promising technique to be integrated in a single microfabricated device because of its experimental simplicity. In addition, CE requires few moving parts and electroosmotic flow (EOF) provides an excellent pumping system which eliminates the problem of high back pressures of conventional pumps. The flow-rate and flow direction can be easily controlled by applying voltages to the capillary channels in a specific manner. Due to the flat cross-section of the channel and the large thermal mass of the glass chip, temperature dissipation on chips is improved compared to conventional capillaries. This allows for the application of higher electric fields. As a result, the efficiencies

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of the separations improve greatly in two aspects. First, Joule heating which leads to the dispersion of the analyte band is minimized. Secondly, higher electric fields enable separations to be performed faster and therefore, the contribution to longitudinal diffusion to the dispersion of the analyte band is minimized. In addition, due to the small geometry of the channels which permits injection of rather small sample plugs, band dispersion is further minimized by the decrease in both injector and detector volumes.

Another advantage of CE performed in microstructures is that multiple channels of identical geometry can be easily generated on a single substrate, which would help to meet requirements of multiple biochemical sample analysis in parallel [5,6].

The use of microfabrication to produce electrophoretic separation capillaries was first introduced in 1992 by Manz et al. [7] and Harrison et al. [8]. Recently, several groups have performed electrically-driven separation in microfabricated CE devices on glass or silica chips. These include capillary zone electrophoresis (CZE) [9–11], synchronized cyclic CE [12,13], micellar electrokinetic CE [14,15], open channel electrochromatography [16] and capillary gel electrophoresis [5,6,17]. Devices that integrate chemical reactions with analysis include CZE with pre-column [18] or post-column [19,20] reactors.

In this work, CE on a microchip for the analysis of anti-human IgG was developed and evaluated. Results from conventional and short-capillary CE were obtained for comparison.

2. Experimental

2.1. Instrumental

For the experiments performed on a chip and a short-capillary, a lamp-based fluorescence detection system as described previously [21] was used. Briefly, a Nikon Labophot-2A episcopic fluorescence microscope (Nikon, Tokyo, Japan) and its accessories were used. The excitation lamp was a 100 W high-pressure mercury lamp. A filter block consisted of an excitation filter (EX 470–490 nm), a dichroic mirror (DM 510 nm) and a barrier filter (BA 520 nm). The fluorescence emission was collected with a Nikon CF Plan Achromat ELWD long working distance objective (5.08~6.84 mm; magnification, 40; numerical aperture, 0.55). A Hamamatsu silicon photodiode with a built-in amplifier (Model HC 220-21, Hamamatsu, Japan) was attached to the trinocular housing of the microscope. A Shimadzu integrator (C-R6A Chromatopac, Shimadzu, Kyoto, Japan) was used for signal recording. A laboratory-made light-proof box was used to cover the entire light sensitive area. A high-voltage CE power supply (Spellman High Voltage Electronics, Plainview, NY, USA) was used for injection and separation. For the conventional CE experiments a Shimadzu spectrofluorometer (Model RF-551) was used for peak detection. The excitation and emission wavelengths were 488 nm and 519 nm, respectively. Data were processed using a Shimadzu C-R6A Chromatopac integrator. A Spellman power supply (Model CZE 1000) was employed.

2.2. Reagents

Fluorescein isothiocyanate (FITC) anti-human IgG was purchased from Sigma (St. Louis, MO, USA), which was developed in goat using purified human IgG as the immunogen. The F(ab')2 fragment of the antibody was obtained from pepsin-digested antiserum by immunospecific methods of purification. Affinity isolation essentially removed all goat serum proteins, including immunoglobulins which did not specifically bind to the γ-chain of human IgG. Goat anti-human IgG was then conjugated to fluorescein isothiocyanate (FITC, Isomer I). Tris was purchased from Bio-Rad Labs. (Hercules, CA, USA). FITC was purchased from Fluka (Buchs, Switzerland). Acetic acid was of analytical-reagent grade and was purchased from Baker (Phillipsburg, USA)

2.3. Capillary and chip

The fused-silica capillary of 50 μm I.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the short capillary was 14 cm. The distance from injection end to the detection window was 6 cm. Two 50 μm plastic vials were used as buffer reservoirs. The two ends of

the capillary were inserted into the reservoirs through the holes made on the side by syringe needle, while electrodes were immersed into the buffer from the top of the plastic vials. The capillary and reservoirs were mounted onto the microscopes XY stage holder. In the conventional CE the total length of the capillary used was 47 cm and the effective length was 35 cm.

The glass structures were fabricated by using standard photolithographic and wet chemical etching techniques described elsewhere [5]. Fig. 1 shows the layout and dimensions of channels. Fifteen channels were etched in a 100 mm×80 mm×1.5 mm Hoya borosilicate glass (Hoya, Japan). Four holes were drilled through the top plate to contact four of the channels. After bonding, pipette tips were inserted into these holes to form small reservoirs. The micromachined substrate and the cover were joined by direct bonding. The top plate with holes and the bottom plate with the etched channels were submerged in hot H_2SO_4–H_2O_2 for 10 min prior to thermal bonding and rinsed thoroughly with Millipore water. Then the surfaces of both plates were hydrolyzed in dilute NH_4OH solution, rinsed again with Millipore water, and dried with N_2 gas. Subsequently the pair of glass plates were placed between two pieces of well polished steel plates and annealed in a Ney Model 2-525 programmable furnace (Barkmeyer Division, CA, USA). The temperature program was as follows: 20°C/min to 500°C for 1 h, 550°C for 0.5 h and 620°C for 2~3 h, 550°C for 1 h, followed by natural cooling of the furnace to room temperature. Some regions (especially in the channel area) were not totally bonded. Usually this cycle needed to be repeated about 3–4 times to eliminate bonding defects [8].

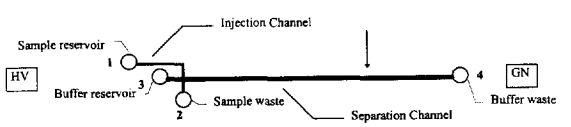

Fig. 1. Layout of the microfabricated channels. Channels referred to in Section 2.3 are identified by numbers. The channel function is indicated in the figure. The separation channel is 50 μm wide, 45 mm long; the injection channel is 30 μm wide, 7 mm long; all the channels are 8 μm deep. Access holes are 1 mm in diameter. The point of fluorescence detection is marked with an arrow.

2.4. Electrophoresis procedures

Capillaries were conditioned following the standard procedures i.e., 10 min NaOH (0.1 *M*), 5 min water and 10 min running buffer. Hydrodynamic injection was employed in short capillary for 10 s at a height difference of 14 cm. The power supply for the analysis was operated at 3.75 kV relative to ground. For conventional CE hydrodynamic injection at a height difference of 14 cm for 10 s was used. The applied potential was 18 kV.

Before use, the fabricated channels were washed with H_2SO_4/H_2O_2 for 5 min, water for 10 min, NaOH for 2 min, and water for 2 min by applying a moderate pressure with a pipette tip filled with the appropriate solution and connected to a nitrogen gas cylinder. The solution was flushed away using pressurized nitrogen gas. The pressure used was less than 20 p.s.i. (1 p.s.i.=6894.76 Pa). After the channels were filled with buffer solution, pipette tips were inserted into the four holes to form reservoirs into which buffer and Pt electrodes were placed. In this work, reservoir 1 was filled with the sample and the rest of the reservoirs were filled with separation buffer. For the injection of a sample plug, 2.5 kV potential was applied between reservoirs 1 and 4 during 2 s. Analysis was carried out between reservoirs 3 and 4 by applying 2.5 kV. The distance from the injection point to the detection window was 2.8 cm.

3. Results and discussion

Fig. 2 shows the electropherogram of FITC anti-human IgG obtained using the 47 cm capillary. Analysis time for the separation of FITC-Ig G from free FITC reagent was 335 s. Fig. 3 shows an electropherogram obtained using the 14 cm capillary. Analysis time was 84 s. A typical electropherogram obtained in the glass chip is illustrated in Fig. 4; the migration time was 16 s. The analysis time was reduced by decreasing the separation length and increasing the field strength and consequently, the linear velocity of the sample. The efficiencies were measured in all three systems. Results are presented in Table 1. The microchip provided the greatest efficiency with values about 49 000 theoretical plates

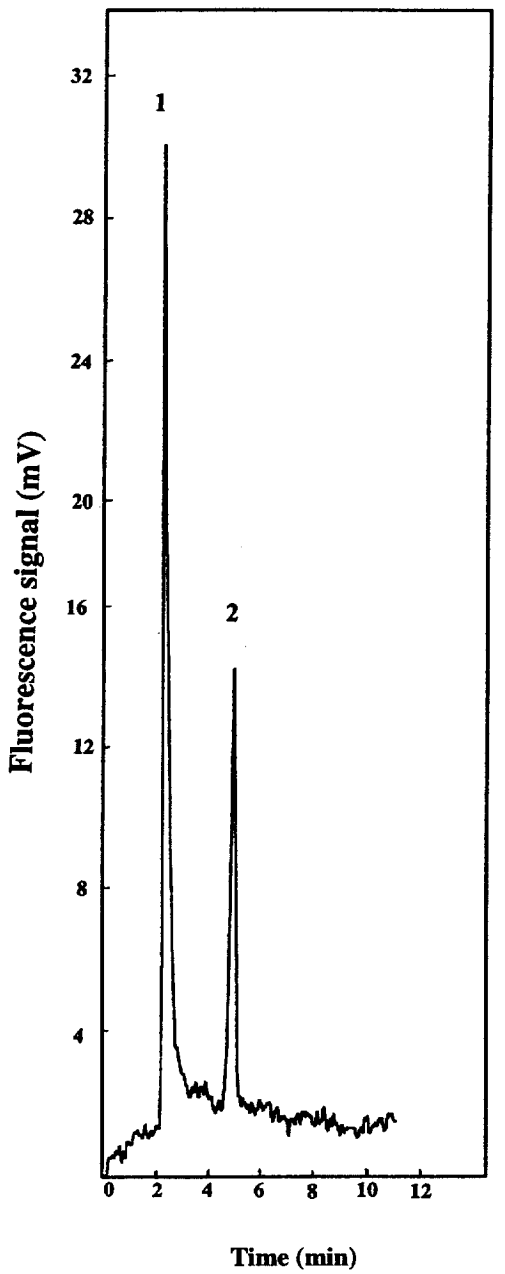

Fig. 2. Electropherogram obtained for FITC-IgG using fused-silica capillary. Buffer: 30 mmol/l Tris–AcOH, pH 8.6; capillary: 47 cm (effective length 35 cm)×50 μm I.D.; voltage: 18 kV; detection: excitation wavelength 488 nm, emission wavelength 519 nm; injection: hydrodynamic for 10 s; peak identities: 1= FITC-IgG, 2=FITC. Concentration of peak is ca. 1000 ppm.

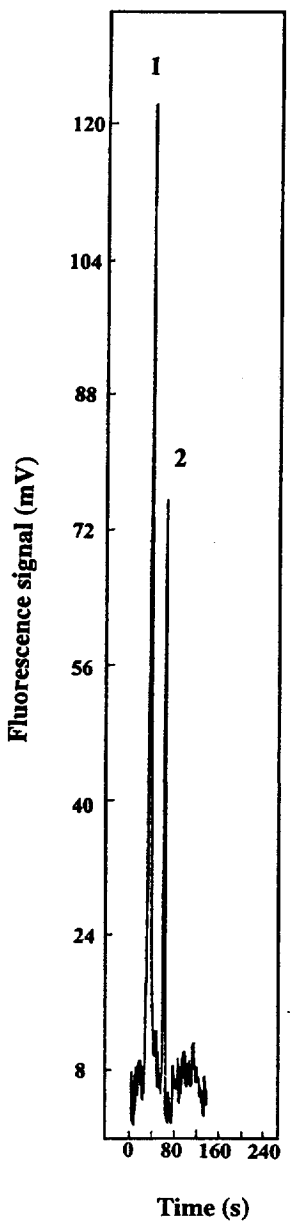

Fig. 3. Electropherogram obtained for FITC-IgG using fused-silica capillary. Buffer: 30 mmol/l Tris–AcOH, pH 8.6; capillary: 14 cm (effective length 6 cm)×50 μm I.D.; voltage: 3.75 kV; detection: excitation wavelength 470–490 nm, emission wavelength 520 nm, injection: hydrodynamic for 10 s; peak identities: 1=FITC-IgG, 2=FITC. Concentration of peak is ca. 100 ppm.

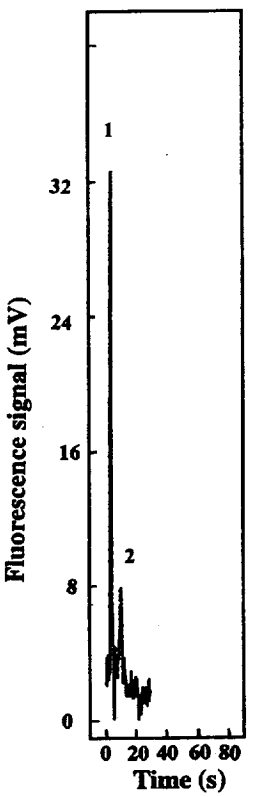

Fig. 4. Electropherogram obtained for FITC-IgG using glass chip. Buffer: 30 mmol/l Tris–AcOH, pH 8.6; separation channel: 4.5 cm (effective length 2.8 cm)×50 μm wide×8 μm deep; electrokinetic injection at 2.5 kV, 2 s; running voltage: 2.5 kV; detection: excitation wavelength 470–490 nm, emission wavelength 520 nm; peak identities: 1=FITC-IgG, 2=FITC. Concentration of peak is ca. 1000 ppm.

(19.89 plates/V), corresponding to a plate height of 0.56 μm. The 47 cm capillary was less efficient, it gave only 27 750 plates, or 1.5 plates/V. Plate height was 1.54 μm. However, the 14 cm capillary gave good efficiency of 41 816 plates (11.15 plates/V), and a plate height of 11.15 μm. The lower efficiency obtained for the 47 cm capillary was most probably due to diffusion in this longer capillary, which resulted in additional peak broadening.

The sensitivity provided by the epifluorescence microscope proved to be higher than that of the conventional fluorescence detector. Detection limits in Table 2 show a 10-fold improvement in sensitivity. In addition, the size of the injection plug might have influenced the sensitivity for the microscope compared to a conventional detector. The higher detection limits obtained for the glass chip were attributed to the short path length of the channel of only 8 μm depth. However, there were other factors that contributed to the increase of background noise and as a result reduced the sensitivity. Scattered light and leakage of sample at the T-junction were among these factors. The contributions to scattered light include stray room light, scattered excitation light and inelastic scattering from the microscope objective and microchip substrate. It is expected to have less scattering from the fused-silica capillary than from the glass plate. The floating model with "injection stack" [5,8] was used to introduce sample into the separation channel. Voltage was applied between reservoirs 1 and 4, while the reservoirs 3 and 2 were left floating. The FITC-IgG was then analyzed by applying a potential between reservoirs 3 and 4 with the reservoirs 1 and 2 floating. Many authors [8,9,22–24] have found that during analysis, if the injection channel were left floating, the sample would be free to leak into the separation channel due to convective flow and diffusion of the sample at the

Table 1
Comparison of the performance of FITC anti-human IgG in a glass microstructure and fused-silica capillary

	Glass chip	14 cm fused-silica capillary	47 cm fused-silica capillary
Channel length (cm)	4.5	14	47
Separation length (cm)	2.8	6	35
Field strength (V/cm)	526	268	383
Migration time (s)	11.22	44.7	152
Theoretical plates, (N)	49 583	41 816	27 750
Plates per volt, (N/V)	19.83	11.15	1.54
Plate Height (μm)	0.56	1.43	12.61
Linear velocity (cm/s)	0.25	0.13	0.23
Apparent mobility (10^{-4} cm^2/V s)	4.74	5.00	6.01

Table 2
Detection limits, reproducibility of migration times (t_m) and areas (A), (n =6)

	14 cm capillary	47 cm capillary	4.5 cm chip channel
Detection limits (ppm)	10	100	250
t_m, R.S.D. (%)	0.81	0.25	0.90
A, R.S.D. (%)	4.17	2.51	5.78

intersection point. This leakage would contaminate the buffer solution which would increase the background signal in the detector. The background noise was measured experimentally using a 0.6 m*M* FITC solution. Plug lengths were estimated by driving sample electrokinetically into the separation channel at the injection voltage until the detector window was reached. In the case of the capillary for an injected plug of ~10 nl we obtained a *S/N* ratio of 210. In the case of the chip for a ~0.299 nl plug the obtained *S/N* ratio was 4–5. Proportionally, the *S/N* for the microchip was smaller, thus explaining in part the decrease in sensitivity.

We studied the reproducibility of the different systems. Relative standard deviations (R.S.D.s) of the migration times for six successive injections can be seen in Table 2. Since the three systems were manually operated, the standard deviations would be mainly due to operational errors. We attributed the high values of R.S.D. for the migration times in the cases of short-capillary and chip to the variations in the positions of the detection window after injection, since the migration times were of the order of seconds, slight changes from the exact point of detection became very critical. Since the injection amount in the chip was considerably reduced, parameters affecting injection amount were critical as well. The volume of the injection plug is a function of the injection time, electric field strength and electrophoretic mobility. Assuming a constant electric field delivered by the power supply, the injection plug would be a function of the injection time [8], and therefore, the reproducibility of the peak areas was dependent on how well the injection time was controlled. The R.S.D. for the area can be seen in Table 2. The short-capillary and chip showed high values. In the case of the chip the phenomena of leakage might have also contributed to the increase in the R.S.D. values since occasionally erratic changes in the baseline signal were observed. In addition, this effect was variable for different experiments.

Several approaches have been developed to alleviate the problem of sample leakage. The principle behind it is to maintain, during the separation, a potential in the sample reservoirs 1 and 2 that is lower than that in the buffer reservoirs 3 and 4 in order to drive the mobile phase back away from the injection cross. This is the so called pinched sample loading method [10,11,24]. This method also offers further advantages, such as better control of the plug shape, injection volume not being related to time or electric field applied for injection, and absence of electrophoretic bias. On the other hand, the "stack injection" method offers the possibility of improving the signal in the detector by modifications in the injection amount or sample plug concentration. Since the sample is electrokinetically loaded into the separation channel, field amplified injection or sample staking can be performed. The disadvantage of this method is that it is electrophoretic biased. Jacobson and coworkers [18,19] have described the gated injection method which combines both advantages of suppressing the leakage of sample into the separation channel and the possibility of concentrating sample by staking [25].

4. Conclusions

Fast, efficient separations of FITC antihuman IgG by CE with a short-capillary and microfabricated glass chip have been demonstrated. The advantages of the miniaturized systems are high efficiencies, fast analysis rates and very low requirement of samples and solvents. The main disadvantage of the chip method is the lower sensitivity obtained. Nevertheless, it is expected that detection sensitivity could be enhanced by concentrating the sample through stacking, controlling the injection channel potentials dur-

ing separation to eliminate leakage, or by using better optics, a brighter source and a more sensitive photon detector. In addition, these systems require more skilful operations and, especially for routine monitoring purposes, automatization.

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References

[1] A. Manz, E. Verpoorte, C.S. Effenhauser, N. Burggraf, D.E. Raymond, D.J. Harrison, H.M. Widmer, J. High Resolut. Chromatogr. 16 (1993) 433.
[2] S.C. Terry, J.H. Jerman, J.B. Angell, IEEE Trans. Electron Devices 26 (1979) 1880.
[3] A. Manz, Y. Miyahara, J. Miura, Y. Watanabe, H. Miyagi, K. Sato, Sens. Actuators B 1 (1990) 249.
[4] G. Ocvirk, E. Verpoorte, A. Manz, M. Grasserbauer, H.M. Widmer, Anal. Methods Instr. 2 (1995) 74.
[5] A.T. Woolley, R.A. Mathies, Proc. Natl. Acad. Sci., USA 91 (1994) 11348.
[6] A.T. Woolley, R.A. Mathies, Anal. Chem. 67 (1995) 3676.
[7] A. Manz, D. J Harrison, E.M.J. Verpoorte, J.C. Fettinger, A. Paulus, H. Ludi, H.M. Widmer, J. Chromatogr. 593 (1992) 253.
[8] D.J. Harrison, A. Manz, Z.H. Fan, H. Lüdi, H.M. Widmer, Anal. Chem. 64 (1992) 1926.
[9] D.J. Harrison, K. Fluri, K. Seiler, Z.H. Fan, C.S. Effenhauser, A. Manz, Science 261 (1993) 895.
[10] S.C. Jacobson, R. Hergenröder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 1114.
[11] L.B. Koutny, D. Schmalzing, T.A. Taylor, M. Fuchs, Anal. Chem. 68 (1996) 18.
[12] N. Burggraf, A. Manz, C.S. Effenhauser, E. Verpoorte, N.F. de Rooij, H.M. Widmer, J. High Resolut. Chromatogr. 16 (1993) 594.
[13] N. Burggraf, A. Manz, E. Verpoorte, C.S. Effenhauser, H.M. Widmer, Sensors Actuators B 20 (1994) 103.
[14] A.W. Moore Jr., S.C. Jacobson, J.M. Ramsey, Anal. Chem. 67 (1995) 4184.
[15] F. von Heeren, E. Verpoorte, A. Manz, W. Thormann, Anal. Chem. 68 (1996) 2044.
[16] S.C. Jacobson, R. Hergenröder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 2369.
[17] C.S. Effenhauser, A. Paulus, A. Manz, H.M. Widmer, Anal. Chem. 66 (1994) 2949.
[18] S.C. Jacobson, R. Hergenröder, A.W. Moore Jr., J.M. Ramsey, Anal. Chem. 66 (1994) 4127.
[19] S.C. Jacobson, L.B. Koutny, R. Hergenröder, A.W. Moore Jr., J.M. Ramsey, Anal. Chem. 66 (1994) 3472.
[20] K. Fluri, G. Fitzpatrick, N. Chiem, J.D. Harrison, Anal. Chem. 68 (1996) 4285.
[21] K.T. Chan, Y.J. Yao, C.L. Ng, S.F.Y. Li, Fresenius J. Anal. Chem. 349 (1994) 487.
[22] S.C. Jacobson, A.W. Moore, J.M. Ramsey, Anal. Chem. 67 (1995) 2059.
[23] Z.H. Fan, D.J. Harrison, Anal. Chem. 66 (1994) 177.
[24] S.C. Jacobson, R. Hergenröder, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Anal. Chem. 66 (1994) 1107.
[25] S.C. Jacobson, J.M. Ramsey, Electrophoresis 16 (1995) 481.