# A comparative study of large volume injection techniques for microbore columns in HPLC

# A Comparative Study of Large Volume Injection Techniques for Microbore Columns in HPLC

H. A. Claessens*

Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands.

M. A. J. Kuyken

N. V. Philips, Central Laboratory Elcoma, P.O. Box 218, 5600 MD Eindhoven, The Netherlands.

**Key Words**

Column liquid chromatography
Microbore columns
Injection techniques

**Summary**

This paper focuses attention on the potentially larger signal-to-noise ratios produced by microbore columns in comparison with conventional columns. The increased chromatographic signals by the application of microbore columns are due to the lower chromatographic dilution of elution profiles which are proportional to the square of the column inner radius. Generally less than 1 $\mu$l sample should be injected into microbore systems to obtain the full benefit of the column performance. However, since more sample can be loaded on conventional columns compared to microbore columns the advantage of improved signal-to-noise ratio can only be realised in situations where very little sample is available. To inject more than 1 $\mu$l sample, at the same time avoiding extra band-broadening effects, suitable injection techniques must be available.

In this study three injection methods for microbore systems that meet this condition, are studied and compared.

## Introduction

Miniaturization is an important, rather recent development, in high performance liquid chromatography (HPLC). The main advantages of miniaturized HPLC are increased efficiencies in shorter times, significantly decreased eluent consumption and increased signal-to-noise ratios of chromatographic peaks, using concentration sensitive detectors [1–3].

Moreover, these miniaturized techniques offer attractive possibilities for coupling with advanced detection systems like mass spectrometry [4–7].

Micro HPLC columns can be divided into three main types:

1. microbore columns;
2. packed capillary columns;
3. open tubular columns.

All column types have their specific demands with respect to the chromatographic equipment like injectors, detection devices, pump systems and electronics. Packed capillary and open tubular columns for HPLC purposes are in the research stage and not commercially available at present.

The application of microbore columns ($\leqslant$ 1 mm i.d.) is a first logical step towards miniaturization. The equipment and column technology are derived from conventional techniques.

The maximum concentration ($C_m$) of a solute in the mobile phase at the end of the column is given by:

$$C_m = \frac{\varphi}{\sqrt{2\pi \cdot \epsilon_c \cdot A \cdot (1+k')\,(HL)^{1/2}}} \qquad (1)$$

where:

$\varphi$ = injected amount of solute,
$\epsilon_c$ = column porosity,
A = cross-sectional area of column,
L = column length,
H = theoretical plate height of solute.

Compared to conventional columns miniaturized HPLC systems in principle offer improved signal-to-noise ratios, depending strongly on the column inner diameter and assuming the same sample sizes in both micro- and conventional systems.

In modern clinical chemistry and biochemistry many pretreatment processes result in sample sizes in the range 10–100 $\mu$l and containing low concentrations of the components under investigation. For these situations the application of microbore HPLC can be profitable. However, the



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sample capacity of columns is proportional to the square of the column inner diameter, for microbore columns sample volumes less than 1μl should be injected [8]. As a result, the advantage of an improved signal-to-noise ratio is only obtained when appropriate injection techniques are employed. In this study a number of injection techniques for microbore systems, based on peak compression, are investigated. These are:

i) on-column concentration;
ii) partial bracketing of the sample plug;
iii) complete bracketing of the sample plug.

These three methods are based on strong retardation and compression of the solute(s) as a narrow band after the injection stage on top of the column. In the widely applied reversed-phase chromatography this can be achieved by adjusting the relative polarities of the mobile phase and the injection liquid.

Polarities of liquids can be expressed in terms of the solubility parameter [9] ($\delta$):

$$\delta = \left(-\frac{E}{V}\right)^{1/2} \quad (2)$$

where:

E = cohesive energy required to transfer one mole of component from the ideal gas phase to the liquid state.
V = molar volume of the liquid.

For mobile phases in reversed-phase chromatography normally consisting of aqueous-organic mixtures the solubility parameter can be calculated by the following formula:

$$\delta_m = \Sigma_p \phi_p \cdot \delta_p \quad (3)$$

where:

$\delta_m$ = solubility parameter of the mobile phase
$\phi_P$ = volume fraction of component p in the mobile phase
$\delta_P$ = solubility parameter of component p.

In reversed-phase chromatography water, for instance, is a liquid of low eluting power, $\delta_H = 25.5$, while methanol has strong eluting power $\delta_m = 15.9$.

The three injection methods can be briefly characterized as follows.

i) On-column concentration.
Relatively large volumes of sample, dissolved in a liquid of lower eluting power compared to the eluent, can be injected by this technique. The components are focused at the top of the column during the injection stage [10].

ii) Partial bracketing of the sample.
In this technique the sample introduced is immediately followed by an amount of water as a liquid of low eluting power, so that a high degree of focusing can be achieved (Fig. 1).

iii) Two-sided bracketing of the sample.
In this injection technique the sample plug introduced is completely bracketed by water, yielding optimum focussing of the injected sample (Fig. 2).

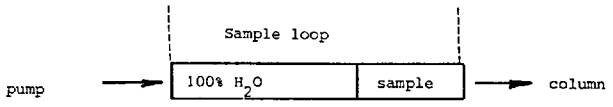


**Fig. 1**
Partial bracketing of sample in injection loop schematic.

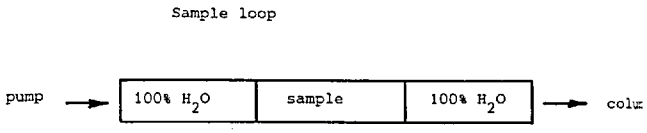


**Fig. 2**
Complete bracketing of sample in injection loop schematic.

The injection techniques investigated in this study are compared in terms of both maximum volume loading of the column and improvement of signal-to-noise ratios.

## Experimental

*Liquid chromatograph*: Shimadzu LC-5A microbore chromatographic system, equipped with a UV-detector (SPD-2AM), 0,5μl cell variable wavelength type, operating at 254nm. Kipp and Zonen recorder BP 40, Delft, The Netherlands. Columns: microcolumns 250 × 1.35mm, packed with Chromospher C-18 of Chrompack, Middelburg, the Netherlands. Flow: 200μl min$^{-1}$; eluent: $H_2O$: methanol 50:50v/v. Test mixture: resorcinol, phenol, benzaldehyde, nitrobenzene,nitrotoluene, dissolved in three injection liquids 100% $H_2O$(a), water: methanol 80:20v/v (b) and water:methanol 50:50v/v (c). The solubility parameters of a, b and c are 25.5, 23.6 and 20.7 respectively, the latter value being the same for the eluent.

Injection devices:
- 0.5μl internal loop Rheodyne 7520 injector
- Valco injector equipped with the following exchangeable external loops:
  i) 509 × 0.25mm 25μl
  ii) 1018 × 0.25mm 50μl
  iii) 2037 × 0.25mm 100μl
  iv) 2546 × 0.5mm 500μl
  v) 5093 × 0.5mm 1000μl

Applying the on-column concentration technique Slais *et al.* [11] calculated the maximum internal diameter of the sample loop from:

$$d_l < \frac{V_0}{V_{inj}} \cdot d_p^2 \cdot g(k') \quad (4)$$

where:

$d_1$ = internal diameter of loop
$v_0$ = column void volume
$V_{inj}$ = injection volume
$d_p$ = particle diameter of packing
$g(k')$ = function of capacity factor

Table I Injected mass of test components and capacity factors.

| Component | Injected mass (moles) | Capacity factor |
|---|---|---|
| Resorcinol | $4.38 \times 10^{-9}$ | 0.36 |
| Phenol | $5.53 \times 10^{-9}$ | 1.57 |
| Benzaldehyde | $7.08 \times 10^{-10}$ | 3.26 |
| Nitrobenzene | $7.60 \times 10^{-10}$ | 5.56 |
| Nitrotoluene | $6.35 \times 10^{-10}$ | 10.26 |

For practical reasons 0.5mm tubing has been applied for the larger injection volumes.

In order to study the effect of the injected volumes for the three injection methods in all cases, the masses of the test components introduced were kept constant. In Table I the injected mass of the components and capacity factors are summarized.

## Results and Discussion

The criteria for judging the various injection methods and volumes were peak height (h) and peak width at half peak height ($W_{0,5}$). For a positive estimation of a certain injection technique, the peak height must not be smaller and the peak with not larger than the standard injections of 0.5µl of the same test mixture.

Table II contains a summary of the results of the on-column concentration injection experiments.

As regards the on-column injection technique: for the several test solutions used in solvents a, b and c, the following maximum volumes (µl) could be injected taking into account the above criteria for peak height and peak width.

| *Component* | *Test solution* | | |
|---|---|---|---|
| | *a* | *b* | *c* |
| Resorcinol | 500 | 100 | – |
| Phenol | 100 | 50 | – |
| Benzaldehyde | 100 | 25 | – |
| Nitrobenzene | < 25 | – | – |
| Nitrotoluene | < 25 | – | – |

The results for partial (E) and complete (T) bracketing of sample injections of 25, 100 and 500µl are given in Tables III, IV and V. In the case of injection method T, the two plugs of water bracketing the sample are equal in volume.

When the test solution contains a higher proportion of water, in some cases a greater peak height and decreased peak width can be observed when compared to the 0.5µl standard injections. This is due to the smaller contribution of the injection methods to band broadening.

The influence of the injection liquid composition on the injected volumes decreases as the k' value of the components increases. The results of the partial and complete bracketing injection techniques can be summarized as follows.

- When the sample is dissolved in pure $H_2O$(a) or in a mixture of $H_2O$:MeOH, 80:20 v/v(b), up to 100µl test solution can be injected for each component, having regard to the above criteria. Moreover, an improvement in the signal-to-noise ratio of the peaks to 30% is observed, compared to 0.5µl injections.
- When the sample is dissolved in $H_2O$:MeOH, 50:50 v/v (c), up to 25µl test solution of each component can be injected and an increase in the signal-to-noise ratio to 20% is observed.

Typical chromatograms resulting from these injection techniques are shown in Fig. 3.

**Table II** Results of peak height and peak width measurements of on column concentration injection. a = test mixture in 100% $H_2O$; b = test mixture in water: methanol 80:20 v/v; c = test mixture in water: methanol 50:50 v/v.

| Injection | Resorcinol | | Phenol | | Benzaldehyde | | Nitrobenzene | | Nitrotoluene | |
|---|---|---|---|---|---|---|---|---|---|---|
| | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ |
| 0.5µl injection | 8.7 | 1.08 | 14.8 | 1.58 | 10.3 | 3.63 | 13.1 | 3.4 | 9.1 | 5.8 |
| 25µl (a) | 10.7 | 0.85 | 15.3 | 1.55 | 11.0 | 3.45 | 12.7 | 3.48 | 8.6 | 5.85 |
| 25µl (b) | 9.7 | 0.9 | 14.4 | 1.55 | 10.7 | 3.5 | 12.4 | 3.55 | 8.4 | 6.0 |
| 25µl (c) | 5.4 | 1.8 | 10.7 | 2.2 | 9.7 | 3.95 | 11.2 | 3.9 | 8.0 | 6.25 |
| 50µl (a) | 10.8 | 0.9 | 14.8 | 1.6 | 10.8 | 3.55 | 12.1 | 3.6 | 8.4 | 6.0 |
| 50µl (b) | 9.45 | 0.95 | 14.2 | 1.65 | 10.0 | 3.75 | 12.1 | 3.6 | 8.2 | 6.0 |
| 50µl (c) | 3.65 | 3.2 | 8.0 | 3.3 | 8.0 | 4.65 | 10.1 | 4.65 | 8.1 | 6.8 |
| 100µl (a) | 11.6 | 0.9 | 15.4 | 1.6 | 11.2 | 3.5 | 12.4 | 3.45 | 8.9 | 5.85 |
| 100µl (b) | 8.7 | 1.1 | 13.8 | 1.8 | 10.3 | 3.7 | 12.3 | 3.6 | 8.8 | 5.95 |
| 100µl (c) | 1.8 | 6.3 | 4.6 | 6.3 | 6.3 | 7.3 | 7.3 | 6.8 | 6.9 | 8.1 |
| 500µl (a) | 9.8 | 0.95 | 13.9 | 1.65 | 10.0 | 3.65 | 12.5 | 3.55 | 8.6 | 6.0 |
| 500µl (b) | 2.8 | 3.7 | 6.8 | 3.6 | 8.3 | 4.2 | 10.1 | 4.2 | 8.4 | 6.0 |
| 500µl (c) | x | | x | | x | | x | | x | |
| 1000µl (a) | 5.2 | 1.73 | 11.5 | 2.08 | 9.4 | 3.43 | 12.8 | 3.5 | 8.1 | 6.2 |
| 1000µl (b) | x | | x | | 6.0 | 5.8 | 6.4 | 6.8 | 7.1 | 7.0 |
| 1000µl (c) | x | | x | | x | | x | | x | |

x = denotes no peaks.

**Table III** Partial and complete bracketing injections of 25µl of test mixture in a, b, and c; sample loops 100, 500 and 1000µl. E = partial bracketing; T = complete bracketing of sample.

| Injection | Resorcinol | | Phenol | | Benzaldehyde | | Nitrobenzene | | Nitrotoluene | |
|---|---|---|---|---|---|---|---|---|---|---|
| | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ |
| 0.5µl injection | 8.7 | 1.08 | 14.8 | 1.58 | 10.3 | 3.63 | 13.1 | 3.4 | 9.1 | 5.8 |
| *25µl sample solved in a.* | | | | | | | | | | |
| 100µl, E | 13.2 | 0.85 | 16.7 | 1.45 | 11.7 | 3.4 | 13.1 | 3.2 | 9.3 | 5.5 |
| 100µl, T | 13.2 | 0.8 | 17.1 | 1.4 | 12.2 | 3.3 | 13.5 | 3.1 | 9.4 | 5.35 |
| 500µl, E | 12.9 | 0.7 | 17.2 | 1.35 | 11.7 | 3.3 | 12.9 | 3.3 | 9.1 | 5.7 |
| 500µl, T | 13.2 | 0.7 | 18.0 | 1.4 | 12.0 | 3.45 | 14.2 | 3.2 | 9.3 | 5.65 |
| 1000µl, E | 12.6 | 0.75 | 17.6 | 1.35 | 11.8 | 3.45 | 13.5 | 3.2 | 9.1 | 5.7 |
| 1000µl, T | 11.8 | 0.85 | 17.6 | 1.35 | 11.8 | 3.2 | 14.4 | 3.0 | 9.6 | 5.3 |
| *25µl sample solved in b.* | | | | | | | | | | |
| 100µl, E | 11.8 | 0.83 | 15.2 | 1.55 | 10.7 | 3.53 | 13.2 | 3.33 | 9.2 | 5.7 |
| 100µl, T | 12.5 | 0.75 | 16.3 | 1.43 | 11.5 | 3.35 | 13.3 | 3.2 | 9.3 | 5.63 |
| 500µl, E | 12.7 | 0.7 | 17.1 | 1.35 | 11.5 | 3.3 | 13.3 | 3.2 | 9.2 | 5.7 |
| 500µl, T | 12.4 | 0.7 | 17.3 | 1.45 | 11.6 | 3.4 | 14.1 | 3.2 | 9.3 | 5.65 |
| 1000µl, E | 12.1 | 0.75 | 17.1 | 1.3 | 10.9 | 3.45 | 13.9 | 3.25 | 9.3 | 5.6 |
| 1000µl, T | 9.4 | 0.9 | 17.3 | 1.4 | 11.8 | 3.3 | 14.2 | 3.1 | 9.7 | 5.25 |
| *25µl sample solved in c.* | | | | | | | | | | |
| 100µl, E | 8.0 | 1.18 | 13.8 | 1.7 | 10.1 | 3.65 | 12.3 | 3.43 | 8.4 | 5.9 |
| 100µl, T | 10.7 | 0.95 | 15.7 | 1.45 | 11.1 | 3.48 | 13.6 | 3.3 | 9.3 | 5.6 |
| 500µl, E | 9.5 | 1.0 | 15.7 | 1.5 | 10.7 | 3.5 | 13.7 | 3.2 | 9.1 | 5.8 |
| 500µl, T | 10.3 | 1.0 | 16.0 | 1.5 | 12.0 | 3.3 | 14.0 | 3.2 | 9.7 | 5.6 |
| 1000µl, E | 6.8 | 1.3 | 15.0 | 1.65 | 10.0 | 3.7 | 13.4 | 3.3 | 9.2 | 5.75 |
| 1000µl, T | 9.4 | 1.05 | 16.2 | 1.4 | 12.4 | 3.25 | 14.0 | 3.2 | 9.8 | 5.25 |

**Table IV** Partial and complete bracketing injections of 100µl of test mixture in a, b, and c; sample loops 500 and 1000µl.
E = partial bracketing; T = complete bracketing of sample.

| Injection | Resorcinol | | Phenol | | Benzaldehyde | | Nitrobenzene | | Nitrotoluene | |
|---|---|---|---|---|---|---|---|---|---|---|
| | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ |
| 0,5µl injection | 8.7 | 1.08 | 14.8 | 1.58 | 10.3 | 3.63 | 13.1 | 3.4 | 9.1 | 5.8 |
| *100µl sample solved in a.* | | | | | | | | | | |
| 500µl, E | 11.4 | 0.8 | 18.5 | 1.3 | 12.1 | 3.3 | 14.8 | 3.1 | 9.8 | 5.25 |
| 500µl, T | 11.2 | 0.8 | 16.3 | 1.45 | 11.3 | 3.5 | 13.7 | 3.35 | 9.3 | 5.6 |
| 1000µl, E | 10.8 | 0.85 | 17.5 | 1.4 | 11.5 | 3.4 | 14.1 | 3.15 | 9.3 | 5.6 |
| 1000µl, T | 10.2 | 0.9 | 17.4 | 1.4 | 11.4 | 3.4 | 13.3 | 3.3 | 9.2 | 5.65 |
| *100µl sample solved in b.* | | | | | | | | | | |
| 500µl, E | 9.0 | 1.05 | 16.4 | 1.5 | 12.1 | 3.35 | 14.2 | 3.2 | 9.4 | 5.45 |
| 500µl, T | 8.4 | 1.1 | 14.5 | 1.65 | 11.2 | 3.5 | 13.5 | 3.35 | 9.3 | 5.6 |
| 1000µl, E | 7.2 | 1.35 | 15.6 | 1.5 | 11.3 | 3.5 | 13.4 | 3.35 | 9.3 | 5.6 |
| 1000µl, T | 9.8 | 0.95 | 16.4 | 1.5 | 11.1 | 3.5 | 13.3 | 3.25 | 9.1 | 5.65 |
| *100µl sample solved in c.* | | | | | | | | | | |
| 500µl, E | x | | x | | 7.4 | 5.7 | 9.3 | 5.1 | 7.9 | 6.55 |
| 500µl, T | x | | 8.7 | 3.1 | 9.2 | 4.0 | 11.6 | 3.9 | 8.7 | 6.0 |
| 1000µl, E | x | | 7.7 | 3.5 | 8.0 | 4.6 | 11.6 | 4.0 | 9.1 | 5.7 |
| 1000µl, T | x | | 9.8 | 2.6 | 11.5 | 3.5 | 13.4 | 3.35 | 9.5 | 5.45 |

X = denotes no peaks.

**Table V** Partial and complete bracketing injections of 500µl of test mixture in a, b, and c; sample loop 1000µl.

| Injection | Resorcinol | | Phenol | | Benzaldehyde | | Nitrobenzene | | Nitrotoluene | |
|---|---|---|---|---|---|---|---|---|---|---|
| | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ | h | $w_{0.5}$ |
| 0.5µl injection | 8.7 | 1.08 | 14.8 | 1.58 | 10.3 | 3.63 | 13.1 | 3.4 | 9.1 | 5.8 |
| *500µl sample solved in a.* | | | | | | | | | | |
| 1000µl, E | 7.6 | 1.15 | 14.5 | 1.6 | 11.4 | 3.25 | 13.5 | 3.2 | 9.4 | 5.45 |
| 1000µl, T | 7.3 | 1.25 | 14.6 | 1.6 | 11.1 | 3.3 | 13.5 | 3.2 | 9.3 | 5.5 |
| *500µl sample solved in b.* | | | | | | | | | | |
| 1000µl, E | x | | 5.2 | 4.4 | 9.4 | 4.0 | 10.4 | 4.0 | 9.3 | 5.7 |
| 1000µl, T | x | | 6.6 | 3.6 | 9.8 | 3.75 | 12.1 | 3.85 | 9.6 | 5.45 |
| *500µl sample solved in c.* | | | | | | | | | | |
| 1000µl, E | x | | x | | x | | x | | x | |
| 1000µl, T | x | | x | | x | | x | | x | |

x = denotes no peaks.

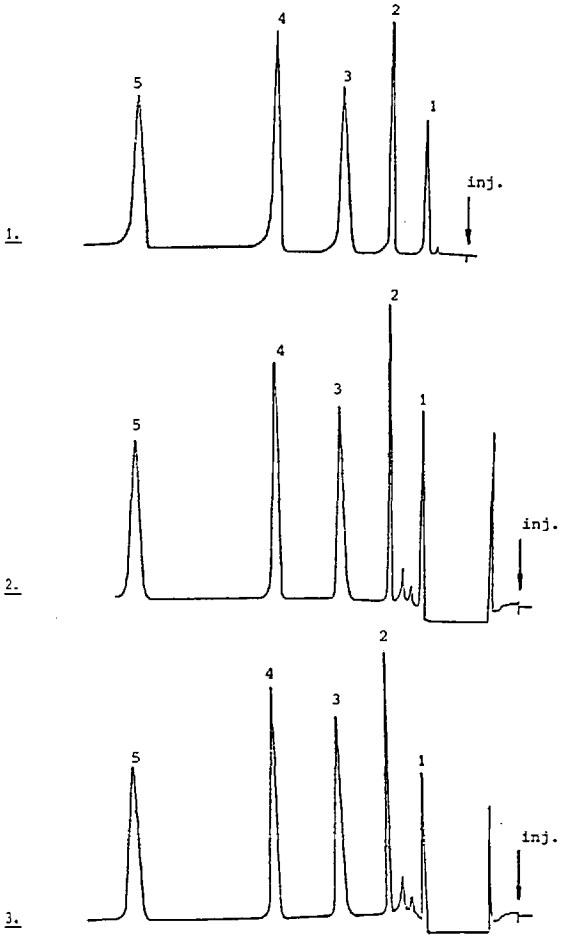


**Fig. 3**

Typical chromatograms of three different injections of test mixture under same chromatographic conditions.

(1) injection of 0.5µl; (2) partial bracketing injection of 100µl (a) in 500µl sample loop; (3) partial bracketing injection of 100µl (b) in 500µl sample loop.

The results of the different injection methods are graphically presented for nitrobenzene in Fig. 4, where the peak neight as a function of the injected volume is plotted.

Application of on-column concentration-injection offers a limited solution to the problem of injection of the test components and solvents under study. Partial and complete bracketing injection techniques in general permit larger volumes of the test solutions of all components to be injected.

The last two techniques mentioned require no special equipment and can easily be performed. Moreover, sample solutions up to 100µl, containing components with widely differing k′ values, can be introduced in HPLC microsystems, while retaining the full benefit of the lower chromatographic dilution of microbore systems.

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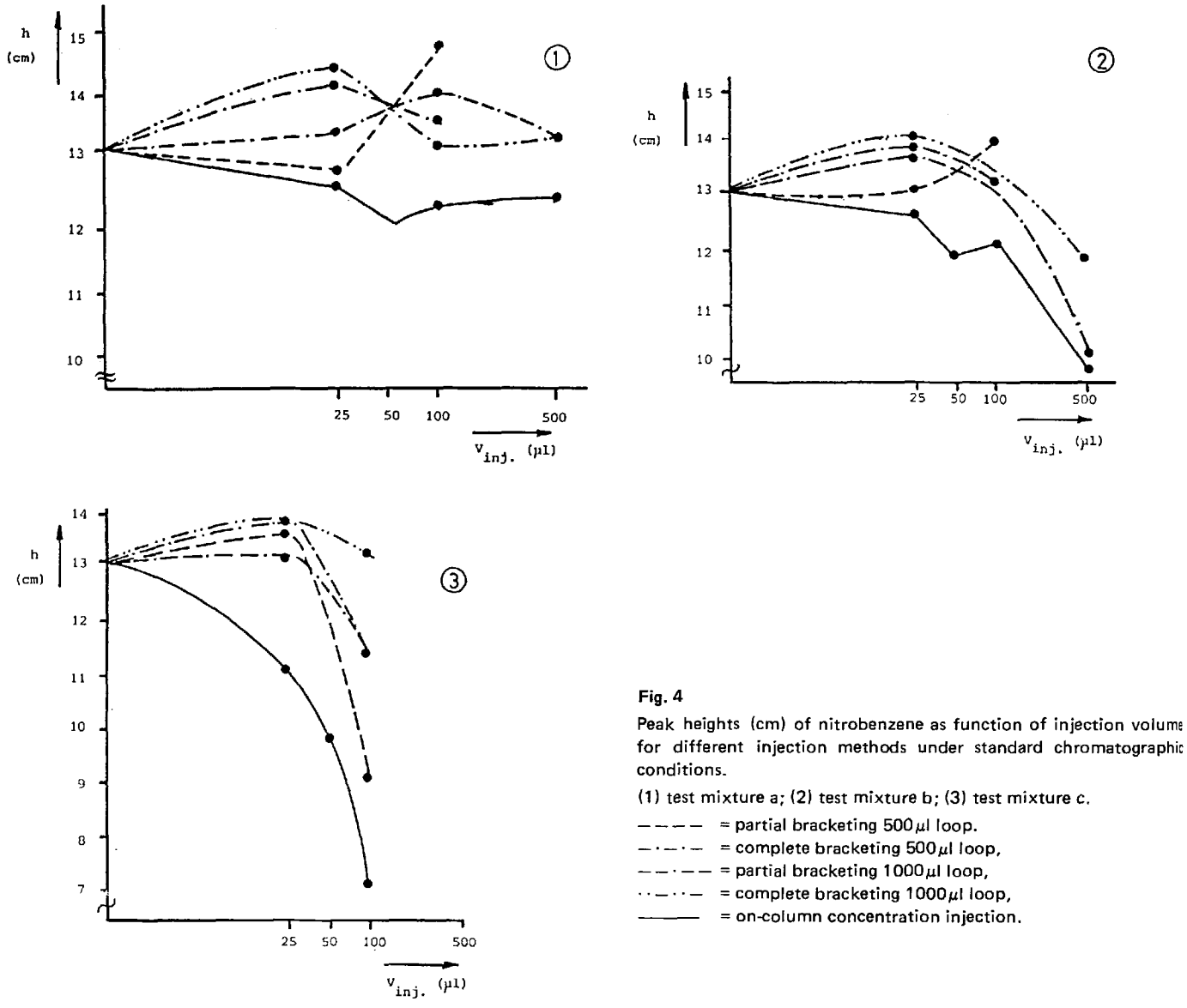


**Fig. 4**

Peak heights (cm) of nitrobenzene as function of injection volume for different injection methods under standard chromatographic conditions.

(1) test mixture a; (2) test mixture b; (3) test mixture c.

– – – – = partial bracketing 500 µl loop.
– · – · – = complete bracketing 500 µl loop,
– – · – – = partial bracketing 1000 µl loop,
·· – ·· – = complete bracketing 1000 µl loop,
——— = on-column concentration injection.

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