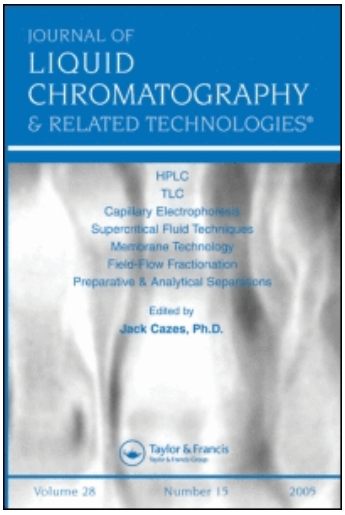

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REVIEW OF SUB-MICROLITER(NANOLITER) INJECTION TECHNIQUES IN LIQUID CHROMATOGRAPHY

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ABSTRACT

In liquid chromatography with "low-dispersion methods", there is an increasing need to reproducibly inject nanoliter sample volumes. Low-dispersion methods produce very narrow peaks because of short column length, narrow column bore, small particle packing, low particle surface area, open tubular configuration, or combinations of these parameters.

This paper reviews methods used for sub-microliter injections for use with these low-dispersion liquid chromatography methods. Some nanoliter injection methods for open-tubular electrophoresis are also described.

INTRODUCTION

For low-dispersion liquid chromatography (LC) methods, there is a need to inject samples at the 1-1,000 nanoliter range or less.

Conventional LC methods, using porous 5 to 10 micron diameter particles and relatively long columns (ca. 250 mm) of wide bores (4 to 5 mm), are being challenged by several new low-dispersion techniques: microbore LC, fast LC, non-porous particle LC, and open tubular LC. These may save on solvent costs (both purchase and disposal), greatly increase resolution, and/or dramatically reduce analysis costs by shortening analysis time and thus increase sample throughput.

A first low-dispersion LC method, microbore-LC, involves maintaining the conventional column length (250 mm) and particle diameter (5-10 um) but reducing the internal diameter to 2 mm, 1 mm or below. These reduce solvent costs in proportion to the reduction in column cross sectional area by factors of 5-fold to 20-fold or more. Microbore columns, especially those constructed with fused silica tubes, may take a leading role in the future because of new advantages, such as higher-than-predicted permeabilities (1), higher stability (2), higher plate count in internally-coated columns (3), better tolerance to voids (2), on-packing detection (through the transparent walls of the fused silica)

giving higher dynamic detector range and higher concentration peaks (higher by 1 + k' vs. when eluted) (2), and other yet-to-be-determined advantages.

A second low-dispersion method of high economic importance is fast LC, currently using 3 micron particles in conventional diameter columns (4-8 mm), but shorter than usual (10-100 millimeters). Fast LC simultaneously can (a) increase sample detectability, (b) reduce solvent costs, and (c) greatly increase the sample throughput (and reduce analysis costs) by up to 15-fold (4). Compared to conventional columns, the shorter columns can elute sample in a shorter time for the same eluent linear velocity, but, because mobile phase mass transport is greatly reduced in the smaller particles, even higher linear velocities can be used. In fact, with 2 micron particles in a van Deempter plot, Verzeles showed no detectable increase in peak spreading (H) to the limit of the usable velocity (limited by the 6,000 psi pump pressure) (5). Fast LC also can decrease solvent costs and increase sample detectability because the peaks elute more sharply with the 3 micron particles.

Gant and Dong showed that fast LC can cut the per analysis costs from $12.63 for a conventional column to $0.84, a 94% reduction, for fast LC primarily due to the 15-fold higher throughput, but solvent costs were also reduced (4). Costs were reduced to $12.50 for the

microbore separation; only 1% reduction, due to the reduced solvent costs. Thus. it seems that if a laboratory is to put effort into changing column types, currently, the benefits from fast LC outweigh those from microbore LC.

The two low-dispersion methods discussed above typically have similar retentions (capacity factors) for similar column lengths. However, the following two low dipersion methods, non-porous particle columns and open tubular columns, typically have very low internal surface areas compared to solvent volume, and capcity factors tend to be low. New ways of preparing stable columns of higher surface areas are certain to come along.

A third low dispersion method, very important to protein separations and bioengineering, is Unger's very small (ca. 1 micron) non-porous particles (4, 6) or Horvath's "pellicular" non-porous 2-5 micron particles (7). These columns are typically very short (5-30 mm), but of conventional diameters (5-10 mm). They show reduced peak broadening, especially for large, slowly diffusing proteins, since the mobile phase mass transfer by diffusion in and out of pores is reduced by eliminating the pores.

A fourth low-dispersion method, possibly representing the future direction of LC (and electrophoresis), is open tubular microbore chromatography. These use un-packed fused silica or

glass tubes from 2 to 50 microns i.d. and have the potential for producing very high resolution. e.g. sufficient to separate molecules of ca. 500 MW differing in 3 mass units due to a deuterated vs. a hydrogenated methyl group (8). Runs currently take several hours and instrumental complexities of injection, detection, and forming and maintaining columns are formidable.

Some efforts have even combined several factors that lead to low dispersion LC. Some recent work by Verzeles et al. involves a combination of reduced column length (to 10 mm) with very small particles (1, 2 and 3 micron). (9). Future work for proteins could combine several of these parmeters, e.g. a short 10 millimeter column of narrow 100 micron i.d., packed with small 1 micron diameter particles of non-porous morphology.

These low-dispersion methods put special demands on reducing the extra-column contributions to peak spreading. Often injectors, transfer lines, column frits, detectors, and even pumps have to be re-designed.

This review considers techniques used for nanoliter size injections useful with many of these low-dispersion liquid chromatography methods.

"NON-ELUTING" SOLVENT INJECTION

An injection approach using a "non-eluting solvent" to dissolve the sample permits very sharp peaks even with large microliter sized injections, if the sample can be

dissolved in a solvent weaker than used for elution. For example, such a system involves dissolving sample in 5% acetonitrile in water, when components of interest are eluted using 10% acetonitrile in water. Many gradient methods offer no problems with even large injection volumes because samples typically are loaded in non-eluting solvents. Takeuchi and Ishii (10) used this method in what they called injection by the "micro pre-column". With this approach, trace levels in water of aromatic hydrocarbons or phthalates were determined. Samples as large as one million nanoliters (1 milliliter) could be concentrated on a packed micro precolumn (5 X 0.2 mm i.d.) and later eluted with a stronger eluent (e.g. 65% acetonitrile) on an ultra-micro glass LC column (100 X 0.12 mm i.d.).

DIRECT MANUAL INJECTION

An early 1980 stopped-flow direct manual injection technique from Ishii and Takeuchi (11) involved disconnecting a short length of 0.13 mm i.d stainless steel tubing from the column inlet. This was allowed to draw in about 20 nanoliters of sample and then manually was re-connected with their special Teflon tubing fittings onto their open tubular columns (5 meters X 50 micron i.d.). (Alkaline etched glass capillaries were dynamically coated with non-polar phases such as SE-30, as done in gas chromatography).

Instead of the moderate-pressure Teflon press fittings of Ishii, more recent (1986) work using open tubes by Capacci and Sedaniak (12) used more conventional, higher pressure Swagelok fittings for direct manual injection. This permitted injection of nanoliter biological plasma samples directly on open tubular fused silica columns (6 m long X 25 microns i.d.) for on-column laser fluorescence detection. The open tubular surface was electro-etched to improve sorption of an ion pairing agent for a "solvent generated" stationary phase. (These micellelar systems hold plasma proteins in solution to prevent column clogging, but resultant slow kinetics can lead to broad peaks.) For this "sample tube" injection, samples were drawn by capillarity into a short piece of column tubing (24 micron i.d.) and these tubes very carefully connected by hand to the end of the column. Individual tubes when re-used gave sampling reproducibility of 5-8% RSD, however, variations from tube-to-tube was "rather poor" (and not given). An advantage of this technique is that small samples can be field-collected remotely from the LC instrument and stored directly in the tube that will become the injector.

A very clever "in-column" injection method was described in 1981 by Tseuda et al. (13) using long (5 m) and narrow (20 micron i.d.) soda-lime capillary columns and normal phase system with hexane as eluent. The

column was pointed down, and an electric hair dryer was used to evaporate about a 50 millimeter length of hexane from the inlet (ca. 15 uL; the volume to eventually be injected). The column head was then dipped into the sample, and a point about 100 millimeters from the column head was heated with a microburner. When the hexane boiled at the 100 millimeter point, it expanded, forcing out all but a tiny bubble of air. When the flame was removed, the condensed liquid produced a suction which drew in sample. The precise volume of sample drawn in could be measured under a microscope as the length of sample in front of the air bubble (ca. 50 millimeters or 15 nL). Reproducibility and accuracy of this clever technique were not discussed, nor was it applied to other than normal phase eluents.

A refinement of the above technique, applied to reversed phase LC using aqueous eluents, was described by Capacci and Sepaniak (12). By replacing the manual torch heating step with an electrically heated element, they also could avoid the need to use a hair dryer for the initial step of evaporating out a plug of solvent to contain the sample. In this "heating injection" technique, the column is manually disconnected from the pump and a section about 100 millimeters from the inlet end of the column is heated reproducibly for 1-2 min by a short, electrically heated "C-clamp" that fits around the column. With a microscope, they observed that boiling of

solvent emptied ca. 80 millimeters of tubing (ca. 40 nanoliters). When the heater was turned off, this then drew in sample, as in the method of Taseuda et al. described above. Reproducibity was about 12% RSD, and the method is influenced by properties of the eluent, heater position, air currents, heating time, heating voltage, etc.

DIRECT VALVE INJECTION

Direct injection of nanoliter volumes with a valve is possible using 4-port valves offered by several comercial sources. In these, sample is contained across the two ports that connect the pump to the column, either (a) in a groove in the valve core or (b) in tiny metal external loops that connect two ports. The two remaining ports permit filling the groove or loop with sample. Most manufacturers offer both electric and pneumatic actuation of their valves, proprietary polymer seals that are resistant to most LC solvents, and valve-bodies of stainless steel or more chemically resistant Hastelloy-C.

The 3XL valve injector from SSI (State College, PA) uses a single loop disc with 3 different injection volumes (200, 500, and 1,000 nanoliter or 200, 1,000, and 10,000 nanoliter). The advantages of this injector are: direct coupling with their "soft-seal" columns, straight-through flow for sample solution; loop volumes changeable in less than 15 seconds without disassembly; valve-

contained (0.5 micron) filter; and conversion of the internal loop valve (200-10,000 nanoliter) to an external loop valve (10,000 nanoliter and higher) (14). For low k' samples, where column efficiency is least, and using a 75 mm X 3 mm i.d. column, up to 50% decrease in column efficiency was found with the direct coupling compared to using a 50 X 0.007 inch connector tube with this valve.

The 7410 valve injector from Rheodyne (Cotati, CA) uses a loop disc with a fixed loop (either 500, 1,000 or 5,000 nanoliter) but the 7413 valve can be ordered with a triple loop disc of various combinations of the volumes (500, 1,000, 2,000 and 5,000 nanoliter) (14). Rheodyne also offers a fixed volume injector (Model 7520) "designed for microbore LC" with volumes of 200, 500, or 1,000 nanoliters. This valve is similar to the earlier 100 nanoliter JASCO valve (ML-422) that was reduced to 20 nanoliter by Takeuchi and Ishii (15). Rheodyne also offers three 6-port valves that can use loops from 5,000 nL up, some with smaller internal channels, for sharper peaks, (but requiring more pressure to load large samples). They note that in addition to the factors sample volume, or pressure to load, other factors may be more important in a particular application, such as volume to properly load (flush) the valve, ease of setting tension to prevent leaks, port spacing for inserting fittings, peak spreading (especially at k' below ca. 5, with non-gradient elution), and wastage

during partial loop filling. Concerning this wastage, their 8125 valve permits the needle of a new type of Hamilton syringe (2.5 ul full scale) to directly abut on the ceramic stator face so all sample exiting the needle is injected, down to 100 nL; no sample is lost in internal connecting passages (important if sample is precious).

Rather than flat sealing surfaces, as used by all other manufacturers, valves from Valco (Houston TX) use a tapered core that potentially can seal more easily, turn with less pressure, and turn at higher speed (16). In early designs using tapered cores, the possiblity existed for the holes to increasing mis-allign as the valve seated more tightly or the seal "wore", a problem less likely in the flat design. However, this seems not to be a problem with current (proprietary) seal materials. The extreme reduction in force required for actuation led to replacing the usual "T" handle with a small knob. Changing seals or sample size is simple, with no effect on the factory-set tension (5,000 psi, but settable to 7,000 psi). Grooves cut in the surface of the core contain the sample. The 4-port sub-microliter model (CI4W) can be cut with two different types of grooves (four in all, three being used at any one time). By inserting the core into the valve 180 degrees apart, the two different sample volumes can be obtained. The lowest nominal volume of a these grooves is 60 nL with

other volumes available (100, 200, 500, and 1,000 nL). Valco also offers a number of external loop injectors (6, 10, 12 and, recently, 14-ports) which can be used with external loops down to 1,600 or 2,000 nL. Both internal-loop and external-loop valves were used with Valco valves in the "moving-injection" technique described later.

Joshua et al. show some typical reproducilbity results possible with direct valve injection (17). With a 40 degrees Centigrade thermostated column, they showed that the Valco valve with interchangable 200 and 500 nL shafts and 1 mm i.d. columns (250 mm long) gave good area reproducibility of 0.5% and 2.1% RSD, respectively. However, when area was corrected for slight changes in flow from the Varian single piston displacment pump by adjusting the area to an average retention time, for 200 and 500 nL injections, their error dropped from 0.5% and 2.1 % RSD, respectively, to 0.2 % and 0.3% % RSD. With a Rheodyne 7410 valve, and a different pump (Milton Roy Constametric II dual piston small-piston pump) reproducibility varied from 0.3% to 0.7% and because flow effect on data was random and not from the pump; the correction used above gave no improvement in reproducibility.

NANOLITER AUTOSAMPLER INJECTION

Joshua et al. modified the Waters WISP autosampler for small injection volumes. The usual 250 microliter

syringe was replaced with a 25 microliter (18) or 40 microliter syringe (Ace Scientific Supply Co., E. Brunswick, NJ). The nominal minimum injection volume (1,000 nL) was thus reduced to ca. 400 nL with the possibility of 100 nL increments, with good reproducibillty (ca. 3% RSD) and the advantage that different samples could be injected in an automated manner.

The Hewlett-Packard 1090 liquid chromatograph also has an autosampler with the excellent capability of injecting down to 500 nL with good precision (0.3 % volume RSD) (19).

THE "MOVING-INJECTION" TECHNIQUE

The "moving-injection" technique, described by Harvey, Stearns, and Averette (20, 21, 22), moves the valve from the "load" to the "inject" and back to the "load" position very rapidly so that only part of the sample in the groove or loop is injected. From 100 nanoliters down to 3 nanoliters, with reproducibility from 2% up to 5% RSD, respectively, can be injected depending on the flow rate, the time the valve stays in the "inject" position, and whether a 4-port or 6-port valve is used (20). Samples as small as 0.003 nanoliters (3 picoliters) could be injected with tolerable reproducibility (24% RSD) and samples to 3.3 nL with good reproducibility (5% RSD) using microbore flows (20

microliter/min) and very fast valve actuation time (20). A "pilot valve" at 100 psi pressure is used to put gas (low viscosity helium) at a much higher volumetric flow on the actuation mechanism for faster (20 - 300 millisecond) valve movement than would be possible by the usual gas actuation using more narrow gas connection tubes.

The moving-injection technique is affected by the eluent flow, valve actuation time, the "tightness" to which the valve body and seat are adjusted, the long-term wear of the seals, and the gas flow (flow channel size, viscosity and hence type of gas, temperature, pressure, etc.). The smallest injection volumes require pilot valves, a 100 psi helium supply, and a milli-second timer. Recently, the gas activated moving-injection approach has been made available in several commercial supercritical fluid chromatographs. Injection times can be programmed from the computer based controller. This injector system is also commercially available (23).

Instead of gas actuation, electrical actuation with the moving injection technique was also shown useful. This technique was originally used with milliliter per minute flows for microliter size injections. Potentially, electric actuation valves could give nanoliter size injections if flows were low enough (microliter range). For electrically actuated valves of 1/3, 1/2, or 1 second and a high eluent flow of 0.95

ml/min, sample volumes from 2,200 nL to ca. 100,000 nL could be injected with good reproduciblity (1-2% RSD) when the time the valve remained activated varied from 0.2 to 7.9 seconds. (Regular full-loop injection with the same valve gives reproducibility of ca. 0.5%).

SPLIT-INJECTION

Split-injection can be used for nanoliter size injections. For split-injection, the major part of both the sample and solvent are vented to waste, as demsonstrated by Yang (24). Often split-ratios of 50:1 through 500:1 are used to provide injection volumes in the 20 to 200 nL range. Splitting may be used continuously (a method suitable for use with conventional pumps with microbore columns) or only during injection. McGuffin and Novotny (25) showed a "heart-cut" injection technique in which most of a 1,000 to 10,000 nL sample from a conventional 6-port valve is split away only during the time of injection via a "purge valve". As the sample passes the capillary column, samples from 1 to 1,000 nL could be forced to enter the column due to the back pressure of a splitter resistor. The range of injection volumes could be roughly controlled by choosing the flow, heart-cut time, and splitter resistor, although injection volume vs. splitter time was non-linear.

Many different types of splitting resistors have been used in LC, including a "controlled leak" through a

Swagelok union, (26) a "microflow valve" (27), fused silica capillaries, and packed columns. Splitting can be complicated, and may spread a peak (27). In addition, the split ratio may change, and hence injection size may change with: (a) variations in solvent viscosity in the splitter (and thus "local" composition and temperature) or, (b) drift in permeability of the analytical column or splitter resistor (for example, either as a result of the packing material settling with time or clogging with sample impurities).

CONVENTIONAL MULTI-PORT VALVE FOR "GROOVE-INJECTION"

Recently, Berry and Lawson described the use of a (slow) 1/3 second electrically actuated multi-port valve to make reproducible injections ranging from 30 to 2,000 nanoliters (nearly two orders of magnitude) (28). One of the ports of a conventional multiport valve (8 or more ports) is equipped with two outlets so during the short (1.2 second) injection, sample contained in the groove in the rotor is split between the column and resistor (a short packed column). With a low resistor, the groove-injector acts like a split injector, injecting only part of one groove-volume (down to 30 nL). This volume depends only slightly on flow, resistor size, etc. With a high resistor, the groove-injector acts like a splitter-injector with various fractions of the zone above one port being swept onto the column (to 2,000 nL), depending

on the flow rate, resistor size, etc. Compared to the usual 4-port valves for nanoliter injections, multi-port valves are more versatile and, thus, a more desirable investment. Multiport valves can be used for a great variety of applications, including, column switching, sample clean-up, sample re-cycle, automated injection of two different volumes of sample, and partial loop filling for variable volume injections.

INJECTION IN MICROBORE ELECTROPHORESIS

The problems of injecting nanoliter size samples in microbore open tubular electrophoresis are similar to those for low dipsersion LC methods. A number of clever methods have been used in this parallel field.

In a "hydrostatic" method, usable for larger bore (100-300 micron i.d.) columns developed by Everaerts, Verheggen, and Mikkers (29) and used by Jorgenson (30), the outlet from an open tubular electrophoresis column is temporarily closed. A conventional sample valve then puts a plug of sample into a tee to which the inlet of electrophoresis column is attached. On repressurizing the system, some sample (nanoliter size) is then forced into the column (outlet still closed) due to the slight compressibility of the liquid and expansion of the column, fittings, etc. The side arm of the tee is then opened to flush out most of the sample, and finally the

actual injection is made when the outlet of the electrophoresis column is unplugged.

In other work using micro-manipulator pipets, Jorgenson et al. (31), showed the processing, injection and detection of as few as 200,000 molecules. Detection limits to ten attomoles of amines in a single (snail) nerve cell involved sucking out the contents of the cell using a micro-manipulator pipet,centrifuging the disrupted cell contents in 500 nanoliters of saline, and injecting one nanoliter into the end of the electrophoresis column using a micro-manipulator pipet. With amperiometric detection they generated very high resolution "chromato-voltamograms" (current vs. voltage vs. time) showing about 100 peaks with sufficient resolution to quantify ten biogenic amines in the single cell (e.g. serotonin, tryptophane, and dopamine).

An earlier method from Jorgenson and Lukacs, using columns in the tens-of-microns diameter range, made "injection" by electrophoretic migration or "electromigration" (32, 33). Sample is electromigrated by replacing the anode buffer reservoir with a buffer containing sample solution and a high voltage is applied for a few seconds. With electromigration, sample enters the column by electrophoresis and electro-osmosis. In later applications, to automate this electromigration sampling (34), Jorgenson showed that an autosampler could be used to automatically dip the inlet of the

electrophoresis capillary into a sample, and then electromigrate sample into the capillary.

It is probable that some of the electrophoresis sampling techniques can be adapted for nanoliter and, more importantly, picoliter and lower injection volumes in liquid chromatography. The potential exists with such methods for injecting not only small charged molecules, but most large molecules like proteins, and even neutral molecules by sorption onto charged species, such as micelles, such as has been shown for electrophoretic separations by Karger et al. (35).

CONCLUSIONS

This paper has reviewed techniques for submicroliter (nanoliter) samples injections. As packed column LC (using microliter injections) moves toward open tubular LC columns of 2-5 micron i.d. (using nanoliter and smaller injections) new ways for injecting sample will have to be developed. New injection techniques are a challenge to this future technology, along with new ways of detecton, pumping, and gradient generation.

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