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C190-E113

HPLC Techniques to Improve LCMS Performance and Productivity

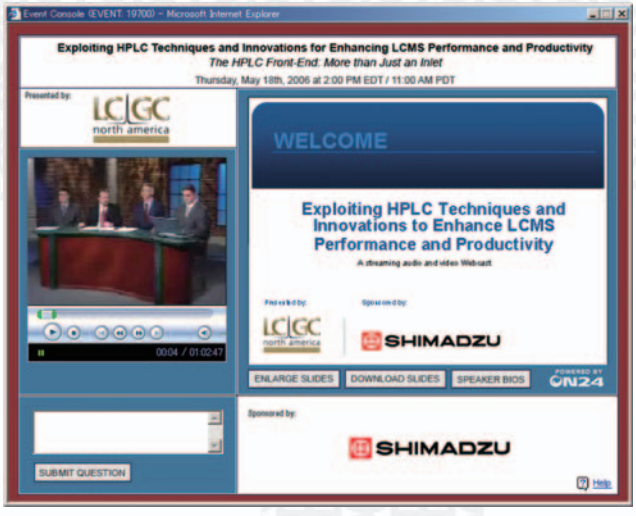
Technical Report vol.17

1. Introduction to Web Seminar "HPLC Techniques and Innovations to Improve LCMS Performance and Productivity"

A seminar featuring front-end LC (LC connected with mass spectrometer) was held over the Internet on May 18, 2006.
This Web seminar (webinar), sponsored by Shimadzu Scientific Instruments of the United States, was organized and presented by LCGC Magazine, known worldwide for its LC- and GC-related articles on technology trends, applications, and troubleshooting.
The seminar was entitled "Exploiting HPLC Techniques and Innovations to Enhance LCMS Performance and Productivity. The HPLC front-end: More than just an inlet." It consisted of a real-time, interactive lecture that could be attended via PC Web browser not just throughout the United States, but in countries around the world.
Mr. David Walsh, Editor in Chief of LCGC Magazine acted as the moderator, and Dr. John Dolan, Dr. Mauro Aiello and Mr. Mike Larson were panelists who made presentations and provided expertise according to themes, as described below.

- Theme 1
  Considerations in Autosampler Selection
- Theme 2
  Analysis of Ultra-low Concentrations using the Latest Technologies of LC-MS/MS
- Theme 3
  Implementation of Multiplexing in Bioanalytical LC-MS/MS

The seminar, including a question and answer session, lasted about one hour. All three themes were extremely interesting, but the discussion of Theme 1 only is presented here in abridged form. Content that was added to the original text, indicated by "(Note)", consists of supplementary comments provided by Shimadzu.



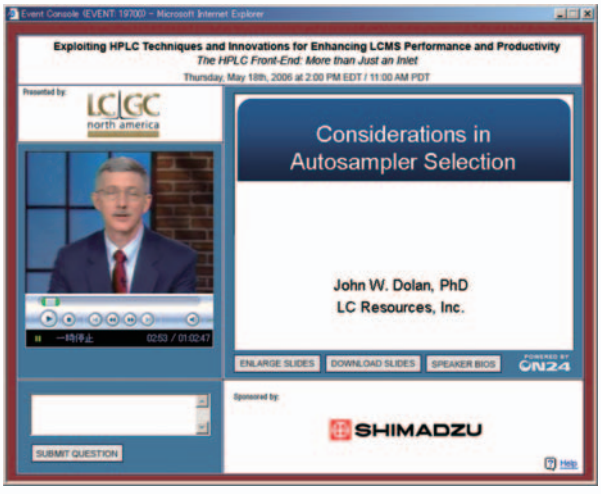
2. Webinar Overview

The adoption and utilization of MS detection for HPLC analysis has grown tremendously in the past several years. What was once an esoteric and problematic technique has almost become, in the case of a single quadrupole, a routine HPLC detection method.
With the advent of better interfaces and more advanced software control, the use of even the high-end instruments has become commonplace for many applications.
This discussion will focus on ways to improve the performance and productivity of the user's MS based on HPLC inlet considerations.

3. Theme 1: Considerations in Autosampler Selection

Dr. John W. Dolan (Note 1) (LC Resources, Inc.)

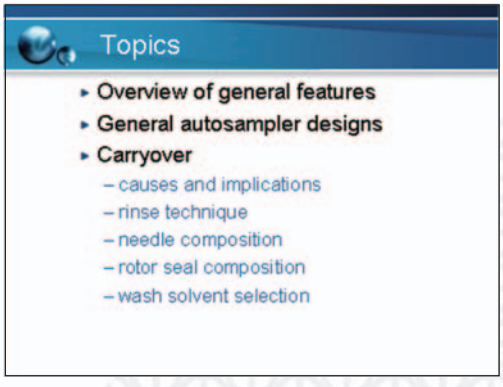
(Note 1) Panelist Introduction: Dr. Dolan has been in charge of writing the LC Troubleshooting section in LCGC Magazine for more than 20 years. In addition, as an instructor for LC Resources Inc. and the American Chemical Society, he has provided expert HPLC guidance to more than 10,000 people.



(1) Topics

Theme 1 provides explanations on 3 topics related to autosampler features, and in particular, microanalysis using LCMS, as follows:
- Overview of general features
- Features related to autosampler design
- Carryover causes and remedies

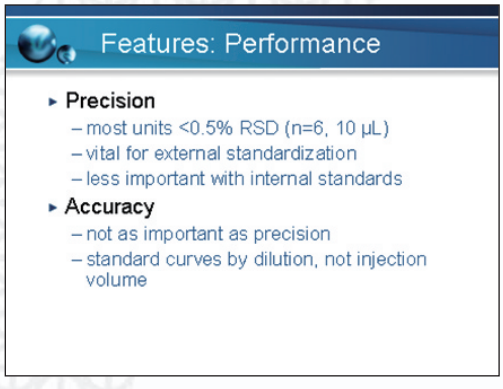
- These are items that should be considered when selecting an autosampler.



(2) Features: Autosampler Performance

When selecting an autosampler, concern is generally directed toward injection precision and accuracy. Precision refers the variation in results when conducting repeat analysis of the same sample. There are autosamplers currently on the market that can provide performance of 0.5% or less of injection repeatability (RSD) using 10 µL injections. Depending on the analysis, however, even higher injection repeatability is required, and in this situation, the selection of autosamplers is limited. In addition, precision is important when conducting quantitation by the absolute calibration curve method, but its importance is diminished when conducting quantitation by the internal standard method. Simply put, when an internal standard is used, accuracy is not as an important factor as precision because the amounts of internal standard and sample are equally affected by the performance of the autosampler.

- The analyst should always generate calibration curves by injecting different dilutions of standard samples at the same volume, instead of injecting different volumes of the same standard sample.

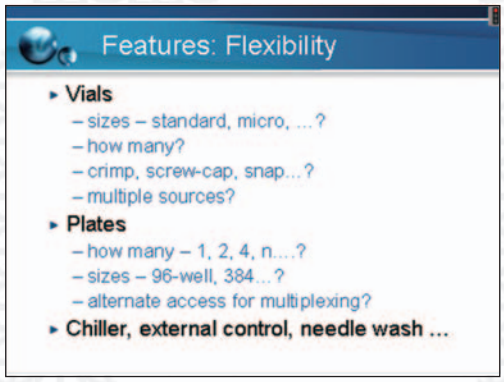


(3) Features: Autosampler Flexibility

When selecting an autosampler, one item that should be examined is the rack or tray types available, and those may vary depending on the substance to be analyzed. For example, the quantity of samples or their preparation may determine whether vials or micro titer plates will be used.
Moreover, there is also the possibility that analysis will be conducted with multiple LC systems connected to a single MS unit. In addition, it is also necessary to investigate whether the samples will require cooling, or whether an accessory like a fraction collector will be used in conjunction with the instrument.

- *If micro titer plates will be used, consider how many titer plates the autosampler can handle, and whether MTP capacity can be increased.*

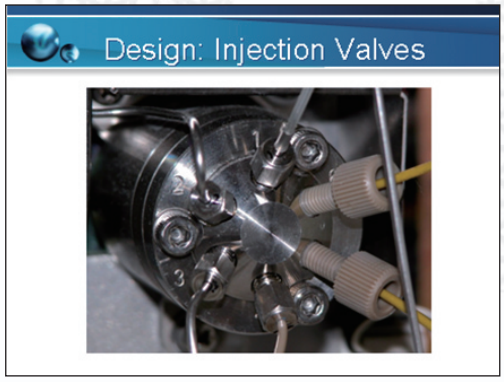
- *96 well plates are standard format, but 384 well plates are also becoming more and more popular. So investigate whether the autosampler you are considering can handle both 96 and 384 well plates.*



(4) Design: Injection Valves

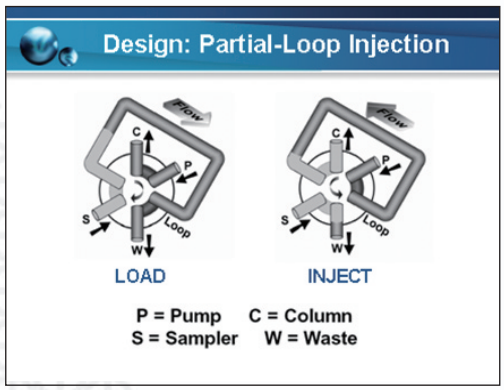
Six-port valves (Note 2) are used with autosamplers. Rheodyne LLC and Valco Instruments Co. Inc. are well-known manufacturers of 6-port valves, but other manufacturers are also currently producing them.

(Note 2) The endurance performance of a 6-port valve generally depends on the maximum pressure supported according to the specifications.
Therefore, for 6-port valves that adopt the same rotor seal material, the higher the maximum pressure, the greater will be the frequency of maintenance.
Moreover, the life (wear performance) of the rotor seal is determined by the tightening pressure applied when the 6-port valve is produced, and is not determined according to the pumping pressure used during analysis. This is because regardless of whether mobile phase is pumped or not, the tightening pressure applied when the valve is produced is continuously applied to the rotor seal. This holds true even in the case of manual injectors.



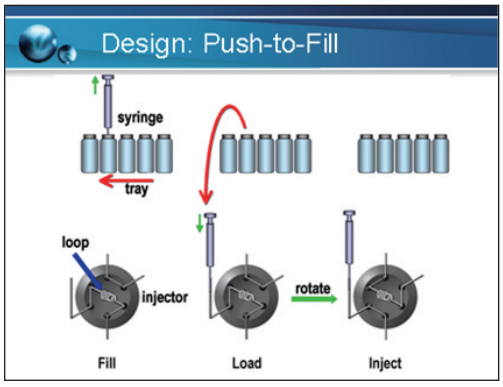
(5) Design: Partial-Loop Injection

The partial-loop injection method is a common sample measuring mechanism using a 6-port valve with a fixed volume loop, either with a manual injector or an autosampler.
As shown in the figure, in the LOAD position, the pump and column are directly connected, and in this state the sample is pushed directly into the sample loop. The valve then rotates to the INJECT position so the mobile phase flows into the loop and sends the sample onto the column.



(6) Design: Push-to-Fill System

Various injection methods can be used with a fixed-loop injector style.
The one described here is based on the push-to-fill system (Note 3), in which the injection operation normally conducted by the manual injector is automated.
As shown in the figure, when the needle enters the sample vial, the syringe aspirates the sample and moves to the needle seal (Note 4). The syringe then pushes the aspirated sample into the sample loop to completely fill the loop and send excess sample to the waste reservoir. The 6-port valve then rotates to put the loop in-line with the pump and column, and analysis starts.
In this push-to-fill system, some of the measured sample remains in the tubing connected between the needle seal and sample loop. Typically, about 10 µL in excess of the injection volume must be drawn into the syringe, and will be wasted.

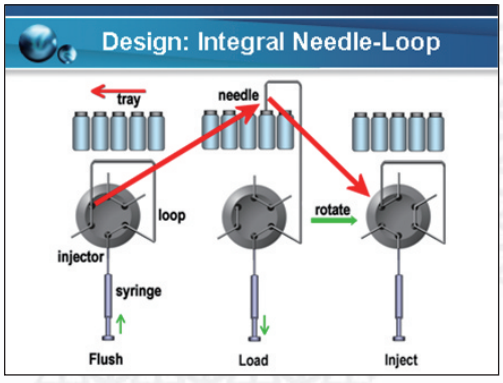


(Note 3) This system is used in the SIL-10AF/SIL-10AP in the Shimadzu LC. The figure in the above paragraph (5) shows the 6-port valve flow diagram used in the push-to-fill system.
(Note 4) The needle seal is the plastic part at the bottom of the injection port which is pierced by the needle point, and is the hole through which the liquid passes. The needle is sealed by this part, and the needle flow line and 6-port valve flow line are connected via this part.

- If enough sample is available, the excess volume and waste associated with the push-to-fill system is not a problem. But, if the available sample is scarce, a wasted 10 µL is a volume that is significant enough that an alternate injection method should be considered.

(7) Design: Direct Injection System

Another syringe measuring method is the direct injection system (integral needle-loop system), and this is a mainstream system used in many analytical laboratories. As can be seen from the figure, this is a design in which the needle and sample loop are constructed as a combined unit (Note 5).
In the injection operation using this system, the needle separates from the needle seal, enters the sample vial, and then the syringe aspirates the sample through the needle into sample loop. After the needle then returns to the needle seal, the 6-port valve rotates, and analysis starts (Note 6). This injection method is also referred to as "no sample loss", so there is no waste of limited quantities of sample.

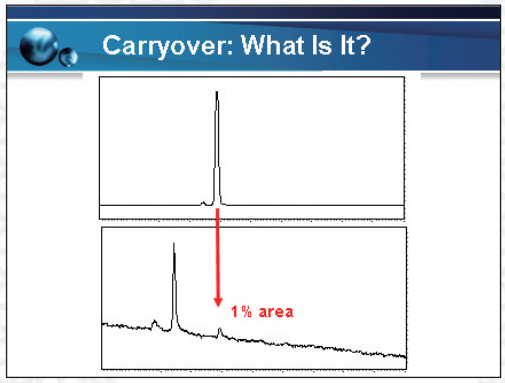


(Note 5) The direct injection system is used in Shimadzu's SIL-20A/AC (HT), SIL-HTa/c, LC-2010a/c (HT), and SIL-10ADvp autosamplers. This injection style uses a metering pump rather than a syringe, so the term "syringe measuring system" is used here to represent both plunger measuring and syringe measuring.
(Note 6) In the direct injection system, the mobile phase coming from the solvent delivery pump passes through the sample loop and needle, and then flows onto the column. The sample loop and needle operate as an in-line component during analysis and analysis standby. Conversely, in injection systems other than the direct injection system, the needle and sample loop are not continuously part of the flow path.

- Because the needle is part of the flow path, no extra sample is wasted. So 100 % of the sample that is drawn up is introduced into the loop.

(8) Carryover: What Is It?

From here we will pursue the subject of Carryover.
Carryover, using a simple example, refers to the appearance of a peak in the chromatogram of a blank analysis due to sample remaining from the previous analysis. In the figure at right, a 1% peak appears in the second analysis, and this is carryover (Note 7).

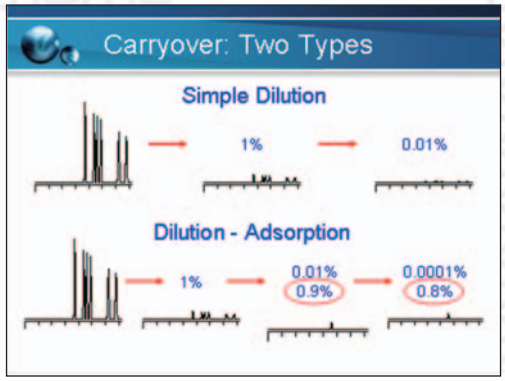


(Note 7) The term "carryover" is used nearly interchangeably with "cross-contamination". Carryover indicates the carrying over of a substance from a previous analysis to the current one. On the other hand, cross-contamination indicates the existence of contamination in the current analysis, but this contamination need not necessarily be carried over from a previous analysis.

For example, instead of the second analysis being a blank analysis, let us assume that it is analysis of sample that is a 10% concentration of the first analysis. In this situation, since the amount of sample carried over from the first analysis is the same absolute quantity, the result is that a 10% error will occur in the second analysis. Therefore, the reduction of carryover is extremely important.

(9) Carryover: Two Types

When classifying carryover, two types are commonly defined.
One type is due to dilution, and the other is due to a combination of dilution and adsorption (Note 8).

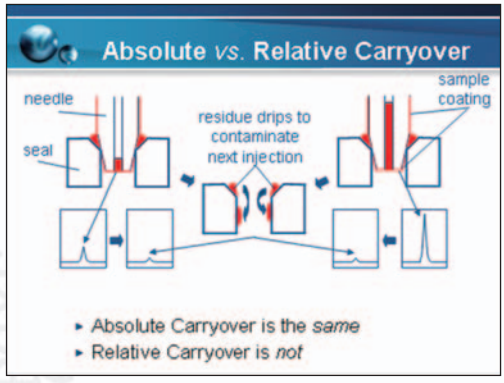


(Note 8) In the case of dilution, the sample starts off adhering to the surface of some part of the autosampler (needle, needle seal, rotor) and through each subsequent run, more is washed away from the material surface. This dilution rapidly decreases the magnitude of the carryover.
On the other hand, adsorption indicates a situation where the sample chemically adheres to the surface of a material and may be difficult to completely remove even after repeated blank measurements.

- When adsorption of a sample constituent occurs, it is not easily washed out of the system. In the case of carryover according to the figure above, it decreases from 1% to 0.9% in the second blank analysis, to 0.8% in the third blank analysis, and so on, demonstrating the rather gradual reduction. This cannot be ignored in low-level analysis.

(10) Absolute vs. Relative Carryover

During the injection sequence, the needle enters the sample vial and aspirates the sample. Since the needle is immersed in the sample, a thin film of the sample remains on the surface of the needle even after it is withdrawn from the sample vial. Then, after the needle returns to the needle seal, analysis starts. At this time, since the needle comes into contact with the needle seal, the sample remaining on the needle surface is transferred to the surface of the needle seal, as shown in the figure. This appears as carryover in the next analysis.
Here, as is clear from the figure, the absolute quantity of sample that adheres to the needle seal surface is constant if samples of the same concentration are analyzed. Therefore, the carryover peak that occurs in blank analysis has the same area value regardless of the injection volume. However, since carryover is calculated as an area ratio (%), the smaller the injection volume, the larger the carryover value will become relative to the sample value (Note 9). Therefore, carryover becomes a serious problem in low-level analysis (Note 10).



(Note 9) Carryover is obtained as a % by [area value in blank analysis] ÷ [area value in sample analysis] x 100. Therefore, if the area value in blank analysis is constant, the smaller the area value in sample analysis, that is, from smaller injection volumes, the greater the calculated carryover value.
(Note 10) That is why, in LCMS and other high sensitivity analysis, carryover is a real problem.

- The first place to combat carryover is at the sample vial where the needle withdraws after aspiration of the sample. Vials with rubber septa or a silicone microtiter plate mat are recommended instead of a sealing mat or cap with aluminum foil. The septa or mat will wipe the needle surface and remove much of the adsorbed sample before it reaches the injection port.

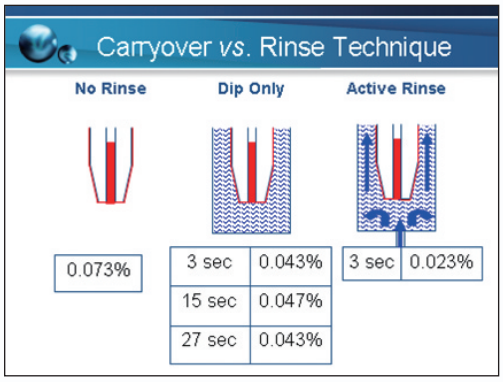
(11) Carryover vs. Needle Rinse Technique

A thin film of the sample may remain on the surface of the needle, but there are 3 approaches that can be taken to address this problem, namely, (1) No Rinse at all, (2) Dip Only in wash solution, and (3) Active Rinse (pumping of wash solution to wash off). In this example, chlorhexidine (Note 11) is used for the investigation.

(Note 11) Selected as a test compound because its functional groups and hydrophobicity are prone to carryover.

In No Rinse, there is absolutely no rinsing of the needle surface, and about 0.07% carryover occurs.
In Dip Only, where the needle is only immersed in the wash solution, carryover is reduced to about half as compared with the No Rinse measure. As the wash solution is static in the Dip Only approach, the contaminating substance only separates from needle surface due to diffusion, and therefore, the rinse effectiveness is not improved much regardless of how long the needle remains immersed.
In the Active Rinse method, when the wash solution is replaced while the needle remains immersed in the rinse port, carryover is reduced to about 1/3 as compared to the No Rinse approach.

- The hole at the needle tip through which the sample is aspirated is small, and the needle is plugged at the other end. Therefore, there is no loss of sample from the sample tip during rinsing, even if the needle is immersed in the wash solution.

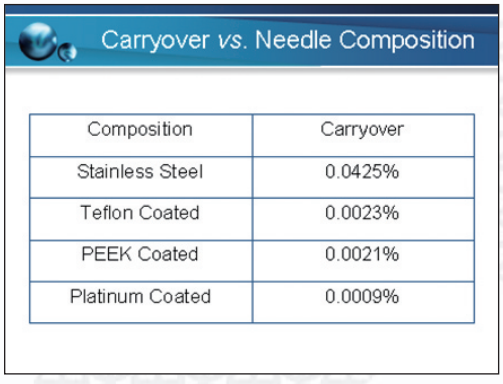


(12) Carryover vs. Needle Composition

Chlorhexidine is also used for the investigation in this example, however, here we look at the factor of chemical adsorption of the sample on the needle surface. Generally, the needle material consists of stainless steel, and in this case, about 0.04% carryover occurs. Since PTFE resin and PEEK resin have the property of hydrophobicity, applying them to the needle surface can reduce carryover, although the short longevity of the coating effect is a drawback.
On the other hand, the use of platinum-coated needles (Note 12) reduces carryover to 0.0009%.

(Note 12) A Pt-coated needle is standard in the SIL-20A/AC (HT), SIL-HTa/c, and LC-2010a/c (HT) autosamplers. Moreover, this needle is available as an option for the LC-2010a/c and SIL-10ADvp autosamplers.

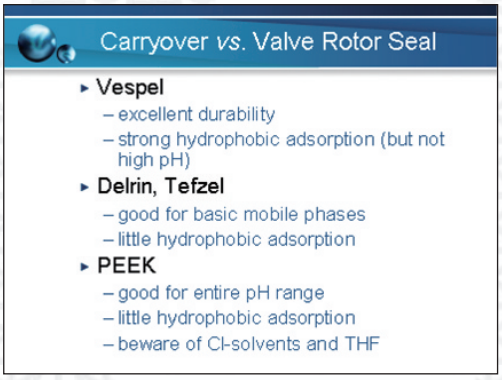
-And this is very durable lasting 20,000 injections and more.



Composition	Carryover
Stainless Steel	0.0425%
Teflon Coated	0.0023%
PEEK Coated	0.0021%
Platinum Coated	0.0009%

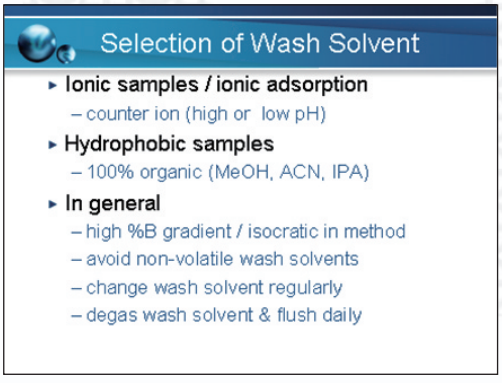
(13) Carryover vs. Valve Rotor Seal Composition

A possible source of carryover other than the needle seal and needle is the 6-port valve rotor seal.
Vespel® is a highly anticorrosive resin, and for that reason is adopted in many 6-port valves as the material used for the rotor seal. However, in addition to the ease with which hydrophobic samples can be adsorbed on it, its chemical resistance in the high pH region is inferior. Delrin® and Tefzel® show no problem with chemical resistance at high pH. PEEK resin, on the other hand, displays its usefulness not only because hydrophobic samples are not easily adsorbed, but it can be used over the total pH range as well.



(14) Selection of Needle Wash Solution

When analyzing ionic samples, selection of the needle wash solution is important. For example, a wash solution to which counterions have been added provides effective adsorption suppression, thereby reducing carryover because the counter-ions are competing with the sample for active sites on the needle surface.
Moreover, selection of the needle wash solution is important also when analyzing hydrophobic samples. In this case, using an organic solvent as the needle wash solution can reduce carryover because it acts to solubilize and wash away the adsorbed sample components.
On the other hand, for hydrophobic samples that are adsorbed to the interior of the needle (Note 13) or column, when conducting gradient analysis, flushing can be performed by pumping a high concentration organic solvent as the mobile phase in the final stage of analysis. This washing using high concentration organic solvent is also useful in isocratic analysis to rinse the interior of the needle and sample loop.
Regarding wash solutions, in order to prevent liquid leaks in the autosampler, non-volatile wash solutions such as phosphate buffer solution should be avoided as a needle wash solution. As an alternative, formic acid is recommended for low pH analyses, and ammonium hydroxide is recommended for high pH analyses.
In addition, stable performance can be maintained by degassing and daily purging of the needle wash solution (Note 14).



(Note 13) Since the Shimadzu SIL-20A/AC (HT), SIL-HTa/c, LC-2010a/c (HT), and SIL-10ADvp autosamplers adopt the direct injection system (mobile phase passes through the needle), washing of the interior of the needle discussed here is performed. However, since the mobile phase doesn't pass through the needle in the SIL-10AF/SIL-6A etc., the needle interior is not washed with mobile phase during the separation. The needle and sample loop are rinsed before each injection through the standard pretreatment routine. The SIL-6A adopts the sample aspiration system (pull-to-fill system), which is a generation behind the push-to-fill injection system of the SIL-10AF, and two generations behind the integral needle-loop injection system used in the SIL-20A(HT), etc.

(Note 14) In Shimadzu's autosamplers with the needle in the flow path (SIL-20A/AC (HT), SIL-HTa/c, LC-2010a/c (HT), and SIL-10ADvp), a part of the needle washing flow line is used as a flow line for measuring sample quantity. By degassing and purging, bubble formation in the needle washing flow line can be suppressed to achieve stable performance.

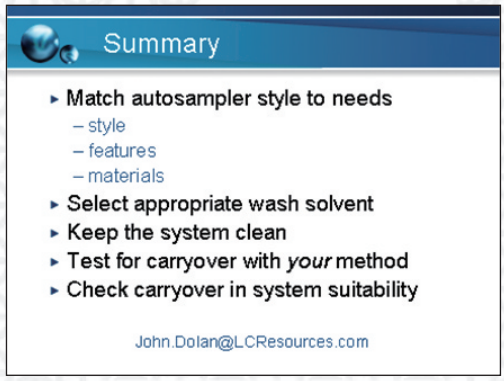
- The needle wash solution should be treated like the mobile phase. It should be changed periodically, and moreover, the wash solution reservoir bottle should periodically be replaced or thoroughly washed to prevent the growth of microorganisms.

(15) Summary

Following is a summary of all the points discussed above.

- When selecting an autosampler, consider the types of analyses for which the autosampler will be used (LCMS, low-level concentration, sample vials vs. MTP, etc.)
- Select a needle wash solution appropriate for reducing carryover of the analyte (Note 15).
- Maintain the system (for example, the needle wash solution, etc.) in a clean condition.
- The source of carryover varies according to the sample. Therefore, it is necessary to test for carryover for each set of analytical conditions used.
- It is recommended to include a carryover test among the other items in the system suitability test. Although the carryover test is extremely easy, its purpose is to confirm that there is no problem of carryover.

(Note 15) With the SIL-20A/AC (HT), not only can needle washing be conducted using two types of wash solutions independently, a 4-wash solution mode and 5-wash solution method are also available. This effectively provides additional means of suppressing adsorption according to the properties of the analytical sample (a second type of needle wash function as an option).



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