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Preventing Carryover of Peptides and Proteins in Nano LC-MS Separations

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Sample carryover is a significant problem that occurs in high-performance liquid chromatography (HPLC) analysis. Carryover effects cannot be tolerated in any high-performance liquid chromatography—mass spectroscopy (HPLC-MS) separation system, and proteomics analysis must be performed in a separation system with virtually no carryover. Several procedures have been tested for effective and fast removal of interfering peptides and proteins originating from previous analyses in the HPLC system. We have developed and optimized a cleaning method for eliminating carryover caused by the autosampler and the trap column. The new washing method uses an injection of trifluoroethanol into the injection path and onto the trap column to remove strongly bound peptides and proteins, and it includes trifluoroethanol as an additional solvent in the chromatographic mobile phase for enhanced cleaning of the separation column. By application of this method, a significant reduction in carryover was achieved without any loss in the amount of proteins and peptides identified by MS.

When an analyte originating from a previously injected sample appears after the injection of buffer as a blank injection, this is called carryover. From a quantitative standpoint, carryover is a problem when the peak that results from it is large enough to be detected and interferes with the results generated with the current sample. Generally, carryover is unwanted and the chromatographic separation method must be developed in such a way to enable carryover-free analysis.

Carryover is one of the most commonly encountered problems for liquid chromatography/mass spectroscopy (LC/MS) analytics, which is gaining a completely new dimension in proteomics, because of the high sensitivity of modern mass spectrometers. For proteomic analytics, even the slightest carryover in the separation/detection system will result in false positive or false negative results in the database search of the acquired MS/MS spectra; therefore, carryover must be eliminated prior to sample analysis.

The susceptibility of miniaturized HPLC (nano HPLC) systems used for peptide and protein separations in proteomics to the carryover phenomenon has been discussed only sporadically in the literature,[1−3] and no satisfying solution was provided for carryover removal except for using very long wash runs[3] of the separation column, although the separation was performed on two separate columns.

Significant protein or peptide carryover and loss from solution can appear because of interaction with the surface of the sample vial or HPLC separation system or because of their aggregation.[4−7] This behavior can be significantly influenced, and carryover reduction achieved, by adjusting the pH of the sample solution and the injection solution, careful selection of the material of the sample vial or the material of the HPLC system (stainless steel, titanium, polyetheretherketone (PEEK), polyaryletherketone (PAEK), as well as the connection tubing, the frits of the separation column, and the housing of the trap column), and by the surfactants that are added to the solution.[8,9] Niederlaender et al.[10] injected 25 μL of digested protein in a 25% dimethylsulfoxide (DMSO) solution on a C18 separation column and, in addition to better peptide solubility, better electrospray stability and higher sequence coverage also was achieved, compared to conventional sample injection procedures and washing the needle and injection port with 20% isopropanol (*i*-PrOH).

A carryover-free system is of great importance for experiments when relative quantification, such as comparing the proteomes of cells grown under different conditions, is performed. When samples that originate from an identical source but are grown under different conditions are analyzed, the inability to eliminate remains of previously injected analytes will seriously hamper the

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analysis and make the identification and proper assignment of peptides and proteins impossible or erroneous.

The solution for preventing the carryover problem must be universal and applicable for all types samples injected onto the separation system.

The most frequent causes of carryover in an HPLC system are

(1) Improper tubing connections. With a properly plumbed nano HPLC system, no carryover should be observed; however, it is observed in a nano HPLC system with improper tubing connections.

(2) The use of connecting tubing and capillaries with a large inner diameter (ID > 20 μm) significantly increases the overall void volume of the separation system.

(3) Poorly cut capillaries and fused silica represent another possible source.

(4) Mechanical scratches of the valve's rotor or stator surface may cause a significant increase in carryover in a separation system by simply providing space for increasing the void volume and enabling sample remains to be "trapped" between the valve ports, to be eluted in later fractions.

(5) Analytes binding to the column and being eluted under regular elution conditions for reversed phase separations.

For better control of the described effects, we have studied the influence of different wash procedures on carryover within both the dual gradient separation system and the conventional single-column separation system.

The tests performed with different wash solvents and procedures yielded important information about the wash efficiencies of some solvents that are considered to be effective, if applied with reversed-phase columns.

We present a wash protocol for the autosampler, the trap column, and the separation column that can reliably remove carryover from HPLC systems and can be widely applied in the field of mass-spectrometry-based proteomics.

EXPERIMENTAL PROCEDURES

The nano HPLC system used in all experiments was an UltiMate 3000 Dual Gradient HPLC system (Dionex, Amsterdam, The Netherlands), coupled to an LTQ-FT mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

The HPLC system consisted of (i) a WPS-3000 autosampler; a separation column (PepMap C18; pore size = 100 Å; particle size = 3 μm; 0.075 mm ID × 150 mm length); and (iii) a trapping column (trap column) (PepMap C18; pore size = 100 Å; particle size = 5 μm; 0.3 mm ID × 5 mm length). Both the separation column and the trap column were operated at 30 °C in a column oven.

Acetic acid (AA) 100% Suprapur, formic acid (FA), 98%−100% Suprapur, and acetonitrile (ACN), HPLC grade, were purchased from VWR International (Vienna, Austria). 2,2,2-Trifluoroethanol (TFE), isopropanol (*i*-PrOH), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany).

In-house synthesized peptides and tryptic digests of BSA and Cytochrome c were used for all experiments except for the flushing procedure with DMSO solution. Five hundred femtomoles of tryptically digested apo-transferrin, phosphorylase b, catalase, myoglobin, glutamic hydrogenase, and lactoperoxidase were added to the standard mix of 10 synthetic peptides and tryptic digests of BSA and Cytochrome c, to mimic a complex peptide sample. The procedure for the synthesis of standard synthetic peptides and for tryptic digests was as described previously.[13]

A tryptically digested HeLa cell extract, prepared as described previously,[15] was used to test the wash procedure after injection of a complex sample of biological origin.

The following mobile phases were used for additional rinsing of the injection needle, injection valve, and sample loop:

- W1: 60% ACN, 30% *i*-PrOH, 10% water, 0.1% TFA
- W2: 1% DMSO in 60% ACN, 30% *i*-PrOH, 9% water, 0.1% TFA
- W3: 100% TFE

The data for the system wash with mobile phases W1 and W2 are available in the Supporting Information.

The sample was loaded on a C18 trap column with 0.1% TFA, and the separation on a nano C18 column was performed by applying an acetonitrile gradient composed by mixing the following mobile phases for test substances:

- MP1: 95% water, 5% ACN, 0.1% FA
- MP2: 20% water, 80% ACN, 0.08% FA

The tryptically digested HeLa cell extract was separated by applying an acetonitrile gradient formed with following mobile phases:

- MP3: 95% water, 5% ACN, 0.1% FA
- MP4: 70% water, 30% ACN, 0.1% FA
- MP5: 10% water, 10% TFE, 80% ACN, 0.08% FA

Before injection of the biological sample, separation system performance tests were conducted with tryptic digests of Cytochrome c and BSA mixed with synthetic peptides. The separation gradients for the system test and the separation of the biological sample are available in the Supporting Information.

A user-defined injection program (UDP) was programmed with *Chromeleon* (6.8 SR5), which enabled definition of the repetitive aspiration of the wash solvent by the autosampler into the injection needle and sample loop of the injection valve, without the need to disrupt a separation run. The UDP, the full program file (as text), and detailed description of programmed steps are available in the Supporting Information.

Generally, the following procedure was applied:

(i) After loading the sample onto the trap column, the injection valve on the autosampler was switched to the "load" position, and wash solvent was aspirated into the sample loop.

(ii) After bypassing the injection valve into the "injection" position, the wash solvent was flushed through the tubing that

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connected the injection valve and the 10-port switching valve, with a mounted trap and separation column, and was directed to the waste.

Loading solvent (0.1% TFA) was injected to remove any remaining wash solvent, followed by an additional flush with the loading mobile phase (0.1% TFA) from the loading pump.

The fluidic path of the dual nano HPLC system is shown in Figure 1. Peptides eluted from the nano separation column were detected with both UV detection at 214 nm and MS/MS detection by nanoscopic electrospray ionization (nano ESI) in positive mode.

RESULTS AND DISCUSSION

In our laboratory, the HPLC separation systems are generally tested for their separation efficiency, their separation selectivity, and the presence of residual peptides from previous sample injections (carryover) before running a set of biological samples. This test is performed by injecting a test mixture that contains 500 fmol of tryptically digested BSA and Cytochrome c, plus 11 synthetic peptides (see the Supporting Information for UV chromatograms and mass spectrometric BPC).

A certain level of carryover is always present in every HPLC system; therefore, a wash step with high organic percentage is programmed to remove sample remains from the trap column, the separation column, and the injection path. The use of the dual or parallel separation system for the nanoscopic HPLC (nano HPLC) separation, which shares the autosampler and sample tubing lines, opens a new dimension of potential carryover with the possibility of cross contamination from one separation system to the other. The selection of solvents used for flushing the injection path and the columns is not always a simple task, and factors such as the type of the stationary phase or the material used for sample loop and tubing must be considered before finally applying them to the HPLC system.

Major sources of carryover in a nano chromatographic separation system are the stationary phase (trap column and the separation column) and autosampler components.

For the injection system, different types of carryover can be distinguished:

- Sample residues can be trapped in parts of the injection system or between the transfer tubing system and the trap column.
- The rotor and/or stator surface in the injection or switching valves is damaged by scratches (e.g., from precipitating salt or precipitated peptides and proteins from samples). This increases the void volume and acts as a reservoir for the remaining sample and slowly releases it into the separation system.

In all cases, the residues of the sample trapped in the HPLC system will show up in the following runs and will interfere with the separation of the following samples. This effect could even multiply via the injection of other samples, generating complex carryover effects originating and accumulating from several preceding sample injections.

Void volume in the nano HPLC system can be significantly more problematic than in a conventional HPLC system, because of a lower flow rate (100−300 nL/min). Here, even the smallest gap of 1 μL can cause a flow delay of up to 10 min. We have previously published an effective method for monitoring and eliminating the void volume in nano HPLC systems for proteomic separations that would be also applicable for this purpose.[11]

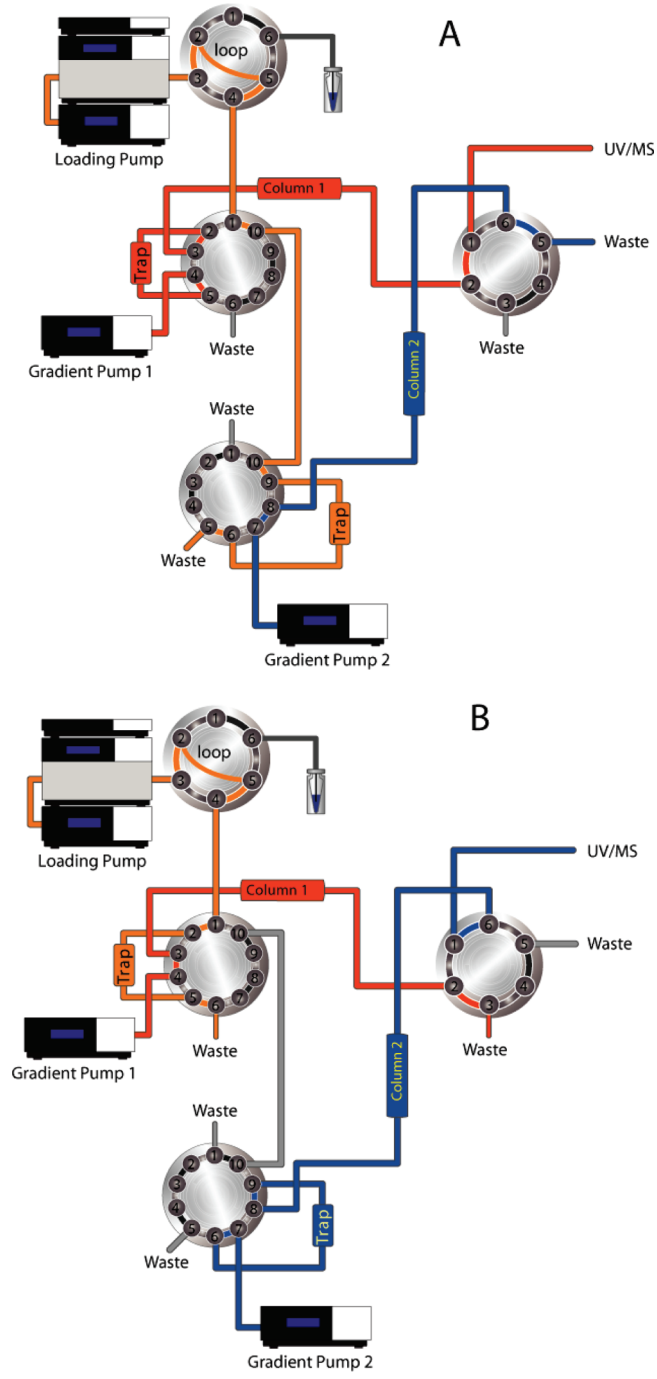

Figure 1. Plumbing scheme of the dual gradient HPLC system used for experiments described in this manuscript. Samples were injected on the trap columns and the separation columns in an alternating manner, to increase the sample throughput and enable proper cleaning of the stationary phases. (A) The sample is loaded on trap column 1 and separated on separation column 1. Simultaneously, trap column 2 and separation column 2 are washed and equilibrated for the following sample injection. (B) The sample is loaded on trap column 2 and separated on the separation column 2. Trap column 1 and separation column 1 are simultaneously washed and equilibrated for the following sample injection. During the wash procedure for the trap column and the separation column, the injection needle, the sample loop, and the sample transfer tubing were flushed with TFE.

The amount of carryover originating from the autosampler is strongly dependent on the material used for injection valves and the injection needles. A certain affinity of proteins and peptides

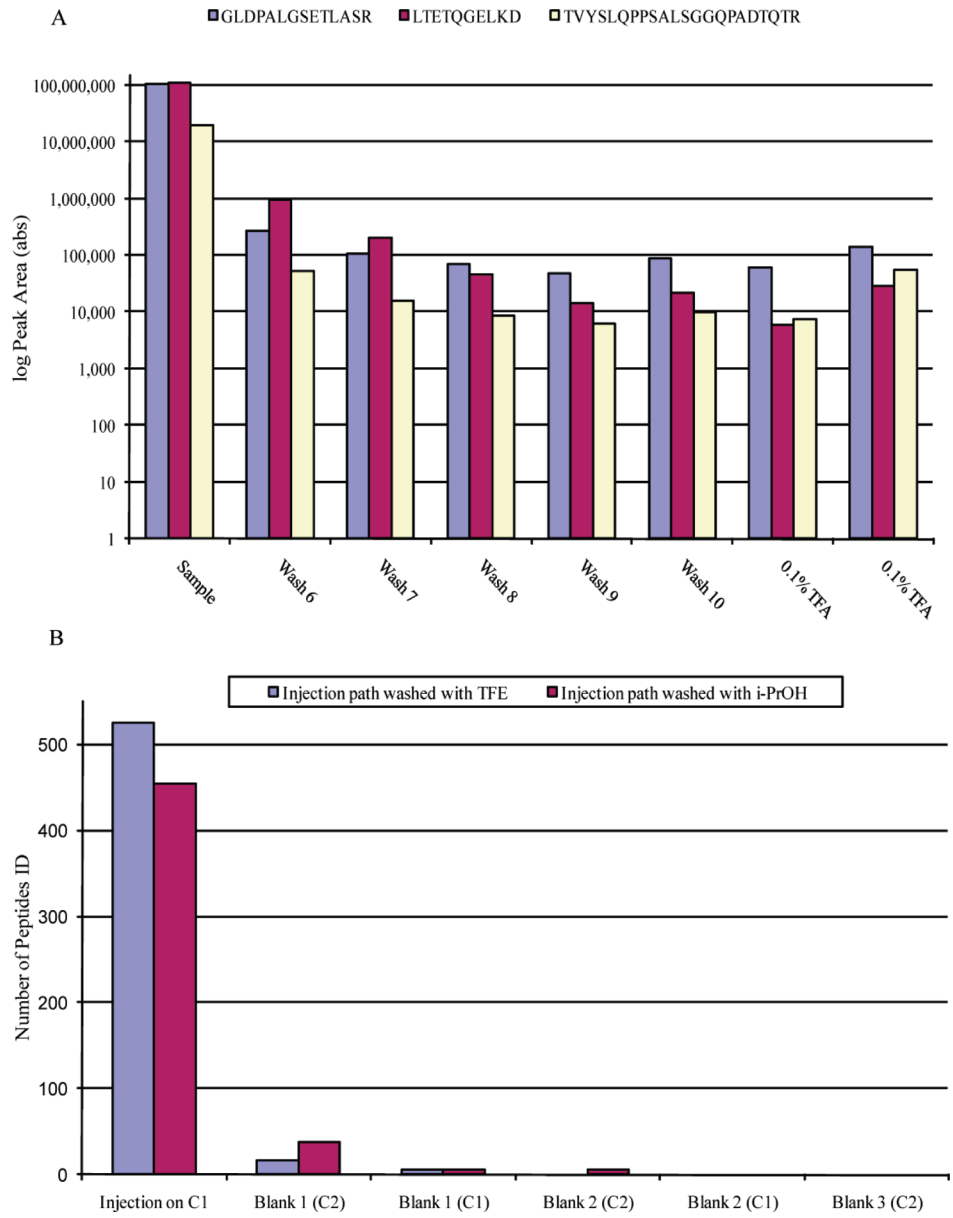

Figure 2. Flushing injection path with TFE reduces and eliminates carryover. It was not possible to remove sample remains from the injection path and the trap column when applying *i*-PrOH solution. (A) The peak areas of the most abundant peptides that were detected decreased very slowly, even after 10 wash cycles applied. (B) For TFE washes, its use in the mobile phase, and as a wash solvent for the injection path and transfer tubing, resulted in significantly fast and efficient removal of residual sample from the HPLC system, in comparison to *i*-PrOH washes. The sample was loaded on trap column 1 and separated on column 1. Subsequent injections were blanks (0.1% TFA). Blank injections were performed on both trap columns.

toward the hydrophobic surface of PAEK and PEEK materials in valve rotors, valve stators, and the tubing in nano HPLC system can be observed. Today, PEEK and PAEK injection and switching valves and sample loops prevail in the biocompatible HPLC systems, and a careful selection of the wash solvents must be made for their cleaning.

The flushing and washing procedure should not require much of the valuable measurement time; therefore, the method applied for the system wash should ideally be implemented during the sample separation run. The tests performed with different wash solvents and procedures yielded important information about the wash efficiency of some solvents that are considered to be effective if applied with reversed phase columns.

One solvent that is known for dissolving proteins prior to enzymatic digestion and enhancing the peptide solubility is TFE.[12,16−18] The use of polyfluorinated alcohols was also reported to prolong the life of the reversed phase column.[14] A new approach for washing the injection path and the stationary phase of the trap and the separation column was designed, using TFE in both wash solvent and in the mobile phase for the loading pump and the microgradient pump, and for the flushing of injection needle and the sample loop by aspirating TFE from a separate solvent vial in the autosampler and performing a single injection of 100 μL TFE into the injection path.

TFE was applied as both a flushing solvent for the injection path, trap column, and separation column, and as an additional

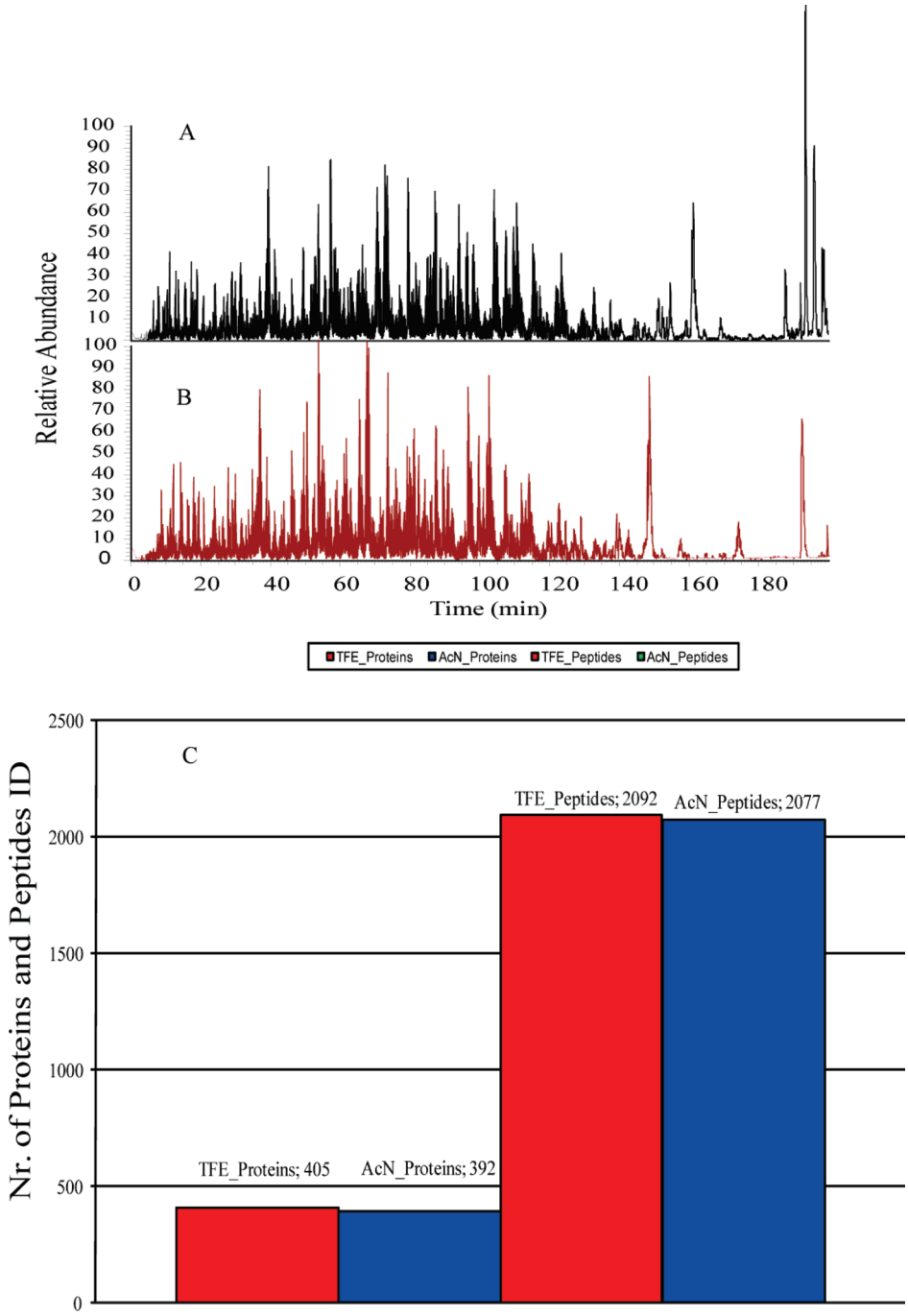

Figure 3. Using TFE in the mobile phase and for system wash increases the amount of identified peptides and proteins. Mass spectroscopy (MS) base peak chromatograms for separations of HeLa tryptic peptides acquired with mobile phases (A) containing TFE and (B) with conventional mobile phases. (C) The amount of peptides identified when a TFE-containing mobile phase is used for peptide elution is higher in comparison to the number of peptides identified with the conventional mobile phase. The results shown are the mean values of triplicate experiments.

solvent in the organic mobile phase. Injections of 100 μL of TFE into the chromatographic system yielded good cleaning results and the removal of sample residues trapped either in the injection system or on the trap column.

The ability of autosampler to implement a UDP for sample injection enabled programming the specific preinjection and postinjection steps on the autosampler. During the flushing procedure, the injection valve was switched three times between the injection position and the load position, to ensure that all valve ports in contact with the sample were flushed. The complete procedure was executed while one trap column was on-line with the separation column and the second one was being conditioned. The separation of the previously injected sample was not influenced or hindered, and there was a significant saving of operational time. Multiple (three) aspirations and dispensing of TFE into the sample loop and injection needle removed sample remains from both the injection needle and sample loop simultaneously. Multiple switching of the injection valve from "load" to "inject" and back ensured proper flushing of all valve ports with TFE and removal of any possible sample remains.

In comparison to the wash solvent containing 20% *i*-PrOH, a system wash with TFE resulted in significantly less peptides being detected in the subsequent blank injection (0.1% TFA) on either

column 1 or column 2 (see Figure 2). Although TFE provided effective removal of residual sample from the injection system and the trap column, considerably longer time was needed for conditioning the stationary phase, compared to the 15−20 min that was needed after simple acetonitrile runs and a system flush with *i*-PrOH (see the Supporting Information). The removal of TFE from the injection path was achieved by pumping 0.1% TFA through the sample loop at 400 μL/min. The loading pump flow rate was reduced to 20 μL/min prior to switching the trap column in-line, to prevent overpressure.

Comparing the data obtained with a simple acetonitrile gradient (MP1, MP2) and enhanced gradient with TFE (MP3, MP4, MP5), a slight increase in the number of identified peptides was observed, which can be explained with improved elution of analytes from the C18 stationary phase (see Figure 3). In addition, TFE is able to stabilize the hydrophobic C18 chains of the stationary phase and prevent their partial collapse, thus enabling better recovery of peptides from the column.

Without proper removal of residual TFE from the injection path and from the stationary phase, a loss of peptides was observed with both ultraviolet (UV) and MS detection (see the Supporting Information). It was observed that the best procedure for TFE removal from the C18 trap column and the C18 separation column was flushing the columns with a gradient that contains 5%−80% ACN, followed by subsequent re-equilibration with the starting aqueous mobile phase. Without the complete removal of TFE from the trap column with an acetonitrile gradient, sample loss due to lower binding capacity of the trap column can occur.

CONCLUSION

2,2,2-Trifluoroethanol (TFE) was introduced as an alternative solvent for a system cleanup, because of its properties to decoy the proteins and peptides, and the ability to simply clean all parts of the high-performance liquid chromatography (HPLC) system. The injection of TFE resulted in effective column and injector cleaning, and the addition of TFE to the organic mobile phase additionally improved the wash process of the stationary phase without causing sample loss or damaging the HPLC system. Before applying TFE for system wash, cross contamination from the first separation system to the second one was commonly observed and a significant number of wash runs was needed to obtain a clean HPLC system. For TFE as a wash solvent, however, no cross contamination was observed on any of the columns in the chromatographic system.

Advanced programming options and the flexibility of the WPS3000 autosampler enables easy programming of different flushing and wash combinations. The wash procedure described can be used with any available HPLC system. However, if the autosampler does not support user-defined programming steps, a separate wash run can be performed following the sample injection.

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LIST OF ABBREVIATIONS USED

HPLC = high-performance liquid chromatography
MS = mass spectrometry
BPC = base peak chromatogram
PEEK = polyetheretherketone
PAEK = polyaryletherketone
DMSO = dimethyl sulfoxide
ACN = acetonitrile
i-PrOH = 2-propanol
FA = formic acid
TFA = trifluoroacetic acid
TFE = trifluoroethane
UDP = user-defined program
CC = Cytochrome c
PP = peptides

SUPPORTING INFORMATION AVAILABLE

Tables giving data for synthetic phophopeptides and nonphosphorylated peptides used to test the separation system (Table 1 in the Supporting Information) and describing the features of the user-defined injection program (Table 2 in the Supporting Information). Figures describing the HPLC separation process, carryover, the effects of flushing the HPLC system with DMSO, and the importance of peptide detection on column re-equilibration after flushing with TFE. This material is available free of charge via the Internet at http://pubs.acs.org.

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