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LC−MS/MS Analysis of Peptides with Methanol as Organic Modifier: Improved Limits of Detection

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With the advent of soft ionization methods such as MALDI and ESI, mass spectrometry has become the most important technique for the analysis of proteins and peptides. ESI-MS is often preceded by separation of the peptide sample by reversed-phase liquid chromatography (LC). Acetonitrile (ACN) is the most commonly employed organic solvent in LC−ESI-MS analysis of peptides. In this report, we demonstrate that the use of methanol (MeOH) as the organic modifier improves the detection limits for analysis of peptide mixtures such as those found in tryptic digests of proteins. A nanoLC−ESI-quadrupole ion trap instrument (LCQ Deca, ThermoFinnigan) was used to analyze peptide standards, protein digests of known concentrations, and tryptic digests of 2-DGE-separated proteins. MeOH displayed excellent chromatographic performance (separation and sensitivity), and shorter gradient times were possible for chromatographic separation with MeOH versus ACN. Sensitivity levels of a few hundred attomoles were achieved with MeOH; those levels could not be achieved with ACN. In addition, MeOH-based nanoLC-MS/MS yielded superior results for the analysis of digests of 2-DGE-separated proteins. For the 14 protein spots analyzed, the success rate of protein identification with MeOH-based nanoLC−ESI-MS/MS was 100%, with multiple proteins identified in several of the spots. In contrast, ACN-based procedure failed to identify any proteins in 21% of the spots and overall identified 33% fewer proteins than the MeOH-based procedure. In summary, higher sensitivity and shorter gradient times make MeOH an excellent organic modifier for the use in nanoLC−ESI-MS/MS analysis of peptides.

The completion of several genome projects, including the human genome, has generated an enormous amount of biological information at the molecular level. That information, most of which is contained in publicly available databases, enables a new phase in the study of biological systems, with a particular focus on proteins, including protein mapping, protein−protein interaction studies, and comparative protein expression studies, e.g., of physiological versus pathological cell state. The field of global protein analysis is generally referred to as proteomics.[1−6]

Mass spectrometry has been the most important technique for the analysis of proteins and peptides, and it is therefore a key component of most proteomics methods. The two ionization methods used in mass spectrometric analysis of biomolecules are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The identification of proteins with mass spectrometry (MS) is commonly based on the analysis of peptide mixtures produced by proteolytic digestion of the protein(s) under study. A widely used strategy involves the acquisition of tandem mass spectra (MS/MS) from the peptides in a digest and subsequent identification of the protein(s) through database searches.[7] Both MALDI and ESI, coupled with tandem mass analyzers, can be employed in this strategy. However, unless off-line prefractionation has been performed, MALDI-based analysis is not suitable for complex peptide samples. In contrast, ESI requires elution of a peptide sample in a solvent, and therefore, it can be easily coupled to a chromatographic device that will separate most of the components in the sample. The elution of peptides from a reversed-phase column directly into the ESI source is a very common configuration for liquid chromatography (LC)-ESI-MS-based analyses. Reduced flow rates and the selection of the appropriate mobile-phase composition are key factors for high sensitivity in LC−ESI-MS. The ion formation process in ESI involves the ionization of sample molecules in the liquid phase, charge separation by an electric field, formation of a charged aerosol, and the production of gas-phase ions from the charged droplets.[8] From a general perspective, a solvent with low surface tension, weak ion solvation properties, low viscosity, and low heat of vaporization would promote charge separation and the produc-

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tion of gas-phase ions. For those reasons, ESI measurements usually employ acidified solutions of acetonitrile (ACN)/water or methanol (MeOH)/water, in which the charge is carried by multiprotonated solvent clusters that are formed by reaction of the solvent with a weak acid [such as acetic acid (pK_a 4.75) or formic acid (pK_a 3.75)] added to the solvent. The acidic pH promotes protonation of the analyte molecules; therefore, acidic solvents are ideal for the analysis of analytes such as peptides that have basic functional groups. ESI performance can be modified significantly by the use of MeOH or ACN. Using representative drugs as test analytes, Temesi and Law[9] investigated the ESI response with various ESI-compatible LC solvents. Most of the compounds tested showed marked signal improvements with MeOH-based eluents. Nevertheless, ACN continues to be the solvent of choice for analysis of peptide mixtures by nanoLC−ESI-MS. Due to the low viscosity of H_2O/ACN mixtures, ACN-based solvent systems are suitable to reduce the high back pressure associated with low-permeability capillary columns. For that reason, in the past few years, the effect of eluent additives on peptide ESI response has been studied in ACN-based solvents.[10] Recently, solvent composition has also been shown to influence the maximum charge state and the charge-state distribution of ESI-produced protein ions.[11]

In the present study, ACN and MeOH were compared as organic modifiers for the analysis of peptide mixtures by nanoLC−ESI-MS/MS with the aim to maximize the limits of detection. Four commercially available angiotensin peptide standards and two tryptic digests from horse myoglobin and bovine catalase were used to evaluate the differences between the two solvent systems. The performance of the two solvent systems was also evaluated with digests of proteins extracted from a silver-stained 2D gel from a human pituitary tissue.

The results of this study show that MeOH as the organic modifier represents a better mobile-phase system for nanoLC−ESI-MS/MS analysis of peptide mixtures such as those found in tryptic digests of proteins.

EXPERIMENTAL SECTION

Peptide standards of human angiotensin I, [Val^4]-angiotensin III, human [Val^5]-angiotensin I, and Ala-Pro-Gly-[Ile^3, Val^5]-angiotensin II were purchased from Sigma (St. Louis, MO). Each peptide sample was weighed on a Cahn C-33 microbalance (Orion Research, Inc., Beverly, MA). Peptide stock solutions (500 pmol/μL) were made in H_2O/MeOH/formic acid (89.9:10:0.1, v/v/v). Dilutions of the peptide stock solutions were made immediately before analysis with H_2O/trifluoroacetic acid (TFA) (99.8:0.2, v/v).

Tryptic digests of two protein standards (bovine catalase and horse myoglobin) were purchased from Michrom Bioresources (Auburn, CA). The lyophilized tryptic digest of each protein standard was dissolved in 0.5 mL of H_2O/TFA (99.8:0.2, v/v). The peptide concentration of each protein digest was determined by amino acid analysis performed by AAA Service Laboratory, Inc. (Boring, OR).

Postmortem human pituitary tissue was obtained from the Shelby County Medical Examiner. The procedure for protein extraction was as follows: the sample removed during autopsy was immediately placed into liquid nitrogen and remained frozen (−80 °C) until it was processed. The entire pituitary gland (0.4−0.5 g), while partially frozen, was quickly minced into small pieces and was suspended in 1.0 mL of Trizol reagent (Invitrogen, Carlsbad, CA) that contained phosphatase (Sigma) and protease inhibitors (Roche, Indianapolis, IN). The suspension was homogenized with a tissue homogenizer. After homogenization, the solution was sonicated (10 s; 3×). Following sonication, the sample was vortexed (5 °C, ∼6 h), and the entire suspension (∼750 μL) was used for protein extraction, according to the manufacturer's recommendations. Briefly, after removal of RNA and DNA, the sample was divided into three equal fractions (200 μL each), and proteins were precipitated by the addition of 1 mL of acetone per fraction. After precipitation, the pellets were washed with a 0.3 M guanidinium hydrochloride solution (1 mL) of EtOH/H_2O (95:5, v/v) and stored (−80 °C).

Before analysis, a fraction was thawed and was immediately dissolved in 500 μL of 2-DGE rehydration buffer that contained urea (7 M), thiourea (2 M), CHAPS detergent (2% w/v), IPG buffer (2% v/v), DTT (0.3% w/v), and a trace of Bromophenol Blue dye. The protein concentration was determined (prior to addition of dye) with a PlusOne 2-D Quant kit from Amersham Biosciences (Piscataway, NJ). The pituitary proteins (200 μg) were separated by 2-DGE with a MultiPhor II apparatus (Amersham Biosciences), as described previously.[12] Briefly, the pituitary proteins were subjected to isoelectric focusing (IEF) in a precast pH 4−7 immobilized pH gradient gel strip (180 × 3 × 0.5 mm; Amersham Biosciences). IEF was performed at 20 °C under the following conditions: 100 (gradient over 1 min); 100 (fixed for 120 min); 500 (gradient over 1 min); 3500 (gradient over 90 min); and 3500 V (fixed for 8 h). For SDS−PAGE, a precast ExcelGel XL 12−14 gel (245 × 180 × 0.5 mm) was used; the proteins in the 2D gel were visualized with an MS-compatible silver-staining protocol.[13]

For protein identification, selected spots were manually cut from the gel, destained with a freshly made solution of sodium thiosulfate/potassium ferricyanide,[14] and washed with water and with ammonium bicarbonate (ABC) (50 μL, 200 mM). After the ABC wash, the gel fragments were dehydrated with ACN and dried in a speed vacuum centrifuge (30 min). Each dried gel fragment was rehydrated with 40 mL of 50 mM ABC containing 0.66 mg of trypsin (Promega, Madison, WI). Digestion was carried out overnight in a water bath (37 °C). The trypsin-digested samples were centrifuged (12000*g*, 1 min), and the supernatants were collected and placed in siliconized Eppendorf test tubes (Fisher Scientific, Pittsburgh, PA). To extract residual peptides, a solution of H_2O/TFA (10 μL; 96:4, v/v) was added to the remaining gel fragments, the samples were sonicated for 20 min, and they were centrifuged (12000*g* for 1 min). Following centrifugation, the supernatants were recovered and combined with the previously collected supernatants. The final volume was ∼40 μL/sample. Each peptide sample was split into two equal aliquots that were stored (−80 °C) until use. Shortly before the nanoLC−ESI-

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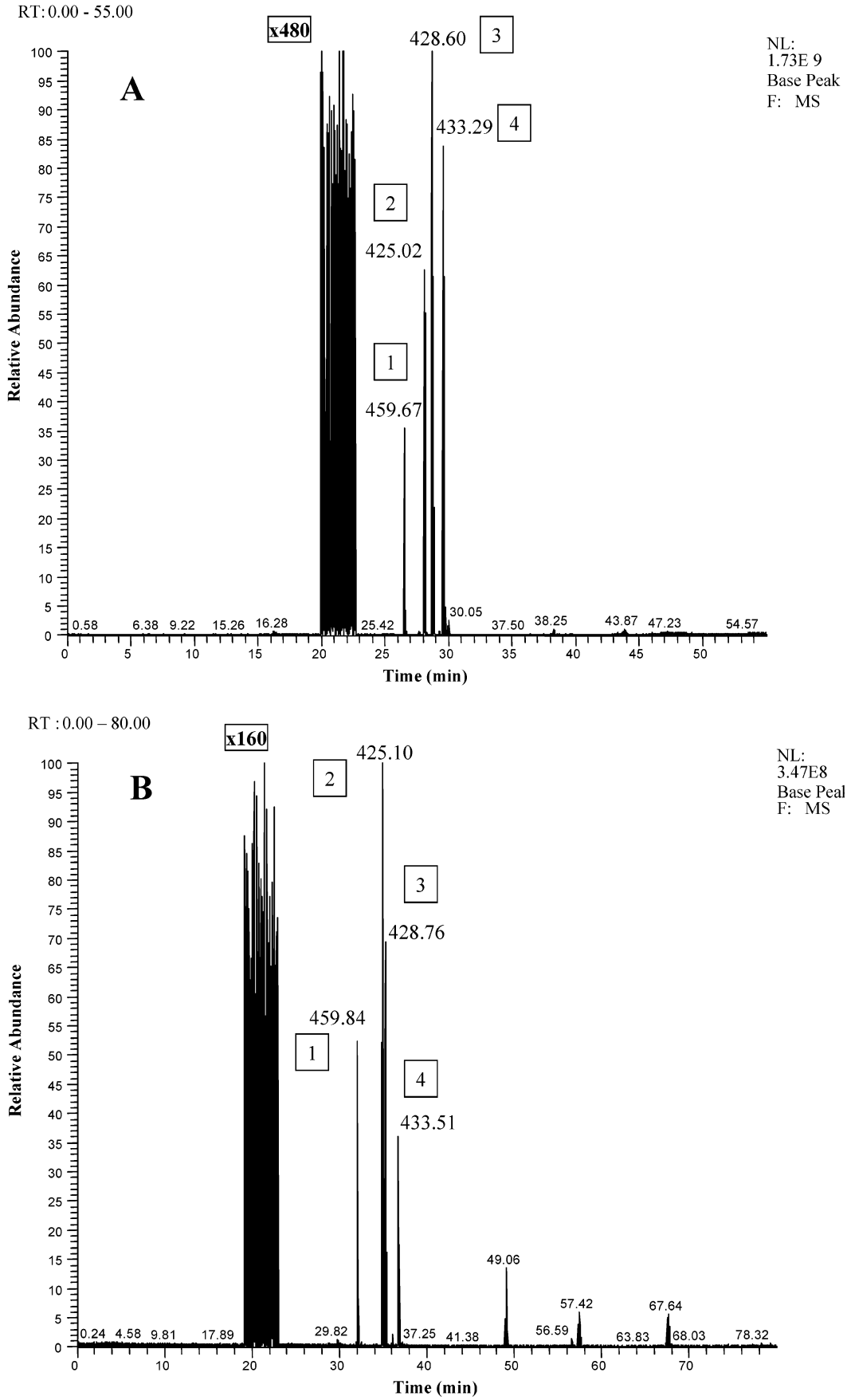

Figure 1. NanoLC−ESI-MS base peak chromatograms of a mixture of four peptide standards (50 fmol/peptide). Peak 1, RVYVHPF [$(M + 2H)^{2+}$ at *m*/*z* 459.25]; peak 2, APGDRIYVHPF [$(M + 3H)^{2+}$ at *m*/*z* 424.55]; peak 3, DRVYVHPFHL [$(M + 3H)^{2+}$ at *m*/*z* 428.50]; peak 4, DRVYIHPFHL [$(M + 3H)^{2+}$ at *m*/*z* 432.90]. (A) Base peak chromatogram obtained with a 55-min gradient of H_2O/MeOH. (B) Base peak chromatogram obtained with an 80-min gradient of H_2O/ACN.

Table 1. S/N and Retention Time (min) Values of Four Angiotensin Peptides (50 fmol/Peptide) ($p < 0.05$ Student's t Test)

analysis number	MeOH				ACN			
	peak 1	peak 2	peak 3	peak 4	peak 1	peak 2	peak 3	peak 4
1	105 (25.58)	200 (28.04)	245 (28.71)	192 (29.69)	75 (32.08)	144 (34.93)	100 (35.26)	52 (36.74)
2	178 (26.53)	314 (28.09)	501 (28.72)	419 (29.57)	38 (30.15)	71 (34.70)	97 (35.12)	42 (36.88)
3	209 (25.54)	356 (27.68)	491 (28.32)	410 (29.21)	58 (29.63)	177 (34.05)	162 (34.34)	52 (36.25)
S/N mean ± std	164 ± 53	290 ± 81	412 ± 145	340 ± 129	57 ± 19	131 ± 54	120 ± 37	49 ± 6

MS analysis, each sample was thawed and purified with a ZipTip C18 microcolumn (Millipore, Bedford, MA). The peptides were eluted from the ZipTip with a solution (1.5 mL) of ACN/H_2O/TFA (50:49.8:0.2, v/v/v). After elution, each sample was diluted with a solution (1.5 mL) of H_2O/TFA (99.8:0.2, v/v) to a final volume of 3.0 μL. Immediately following the ZipTip step, each sample was manually injected through a six-port NanoPeak valve (M-485) from Upchurch Scientific (Oak Harbor, WA), fitted with a 2-μL capillary PEEK sample loop or, alternatively, a 1-μL capillary PEEK sample loop (for peptide standards analysis). The samples were loaded onto a 50-μm-i.d. (360-μm-o.d., 8-μm tip, 10.5-cm length) Picotip capillary column/spray needle (New Objective, Woburn, MA) packed in our laboratory with Magic C18 silica-based reversed-phase material (5 μm, 200 Å) from Michrom Bioresources. The column was packed with a slurry packing technique.[15] The slurry was a suspension of the stationary phase in ACN mixed in a ratio of 1 mg of stationary phase to 4 mL of ACN. The suspension was shaken to make it homogeneous, and it was positioned into a stainless steel reservoir. Picotips were used as the column skeleton and were connected to the reservoir. The slurry was immediately transferred into the Picotip columns with a Varian, model 9012, HPLC pump (Varian, Walnut Creek, CA), to homogeneously pack the column (~10 min).

To improve the signal-to-noise ratio (S/N) of the LC−MS instrument, a precolumn splitter as described by Gatlin et al.[16] was used to reduce the flow rate. The lower elution flow rate was obtained by opening one of the outlets of a three-way valve (MicroTee P-775, Upchurch Scientific) that was positioned at the base of the Picotip column. The sample-loading flow rate was 110 nL/min. After the sample was completely loaded onto the column, the valve was opened at one end, to decrease the flow rate at the column tip to 40 nL/min. [With this precolumn splitter, a ~2-fold increase in the S/N was achieved for the angiotensin standards (data not shown).] All samples were eluted from the column with two different gradient elution programs: one for the MeOH-based system (I), and a second for the ACN-based system (II). Gradient I consisted of a 5-min initial isocratic elution with 0% B, a linear gradient 0−70% B in 35 min (2.0% MeOH min^{-1}), a 10-min isocratic elution with 70% B, and a linear gradient 70−0% B in 5 min (A, water/0.1% formic acid; B, 90% MeOH/10% water/0.1% formic acid). Gradient II consisted of a 5-min initial isocratic elution with 0% B, a linear gradient 0−70% B in 60 min (1.05% ACN min^{-1}), a 10-min isocratic elution with 70% B, and a linear gradient 70−0% B in 5 min (A, water/0.1% formic acid; B, 90% ACN/10% water/0.1% formic acid). MeOH (Burdick & Jackson, B&J Brand, 99.9% min), ACN (J.T. Baker, HPLC Grade), H_2O (Burdick & Jackson, B&J Brand, High Purity), and formic acid (EM, Guaranteed Reagent, 98% min) were all purchased from VWR Scientific Products (West Chester, PA).

The eluted peptides were introduced into a nanoESI-quadrupole ion trap MS (LCQ^{Deca}, ThermoFinnigan, San Jose, CA). The MS instrument was operated with the following settings: spray voltage 2.2 kV; heated capillary voltage and temperature 6.0 V and 150 °C, respectively; q_z 0.25; activation time 30 ms; collision energy value 35%; isolation width ±3.0 Da; three microscans for MS and MS/MS; and with the automatic gain control. All MS and MS/MS spectra were acquired in the data-dependent mode. The instrument executed one MS scan followed by an MS/MS scan of each one of the five most intense peaks from the MS scan.

For protein identification, the nanoLC−MS/MS data were used to search the SWISSPROT protein sequence database, using the SEQUEST Browser search engine (ThermoFinnigan). The SEQUEST search output data were evaluated in terms of the number of identified peptides and the magnitude of their X_{corr} values. (The X_{corr} is defined as the peptide identification cross-correlation score, which represents the degree of similarity between the experimental MS/MS spectrum and a theoretical MS/MS spectrum generated for a given peptide obtained from a database.)[17]

Statistical analysis of the data was performed with the SPSS statistical software package for Windows version 12.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The goal of this study was to compare MeOH- and ACN-based solvent systems for nanoLC−ESI-MS/MS analysis of peptides, to determine which solvent offered better performance in terms of the limit of detection without any loss of chromatographic resolution and analysis time. Formic acid was added as an additive to MeOH- and ACN-based eluent systems. Previous studies[9] clearly demonstrated that this weak acid is ideal to increase ESI response, when compared to other buffer additives used in LC−ESI-MS. To maximize the S/N for the peptide analytes, the concentration of formic acid in the mobile phase was evaluated, and the optimum response was obtained at a concentration of 0.1% (v/v) (data not shown).

Four commercially available angiotensin peptide standards, RVYVHPF (MW 917.1), APGDRIYVHPF (MW 1271), DRVYVHPFHL (MW 1282), and DRVYIHPFHL (MW 1296), were used to determine which solvent and gradient conditions would yield the

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Table 2. Analysis of Trypsin-Digested Bovine Catalase (25 fmol) and Horse Myoglobin (75 fmol) ($p < 0.05$ Student's t Test)

	MeOH X_{corr} (charge)			ACN X_{corr} (charge)		
peptide sequence	1	2	3	1	2	3
Bovine Catalase						
^{19}AAQKPDVLTTGGGNPVGDK^{37}			4.40 (2)			
^{47}GPLLVQDVVFTDEMAHFDR^{65}		3.55 (3)	4.45 (3)			
^{105}RTPIAVR^{111}			2.22			
^{106}TPIAVR^{111}				1.79 (2)		1.78 (2)
^{112}FSTVAGESGSADTVRDPR^{129}	3.27 (3)	3.25 (3)	1.94 (2); 3.01 (3)	3.17 (2); 3.04 (3)	2.81 (3)	2.05 (2); 3.07 (3)
^{135}FYTEDGNWDLVGNNTPIFFIR^{155}			3.67 (2)			5.33 (2)
^{221}LVNANGEAVYCK^{232}	3.60 (2)	3.73 (2)				
^{243}NLSVEDAAR^{251}	2.13 (2)	1.86 (2)	2.03 (2)	2.33 (2)		
^{252}LAHEDPDYGLR^{262}	3.47 (3)	2.97 (2); 3.10 (3)	2.76 (2); 3.08 (3)	2.96 (2); 3.09 (3)		2.80 (2); 3.19 (3)
^{354}LFAYPDTHR^{362}	2.51 (3)	2.19 (3)	2.05 (3)	2.36 (3)		
^{365}LGPNYLQIPVNCPYR^{379}	4.04 (2)	3.26 (2)	3.09 (2)			
^{422}THFSGDVQR^{430}	2.50 (2)	2.41 (2); 3.00 (3)	2.31 (2); 3.12 (3)		2.70 (3)	
^{431}FNSANDDNVTQVR^{443}	2.94 (2)	4.22 (2)	4.13 (2)	4.70 (2)	3.71 (2)	4.05 (2)
^{449}VLNEEQR^{455}	2.05 (2)					
^{457}RLCENIAGHLK^{467}	3.31 (2)		3.09 (2)			
^{458}LCENIAGHLK^{467}	3.00 (2); 2.83 (3)	3.11 (2)	2.87			
^{468}DAQLFIQK^{475}	3.13 (2)	3.05 (2)	3.13 (2)	2.74 (2)		2.96 (2)
^{468}DAQLFIQKK^{476}	2.58 (2)					
^{480}NFSDVHPEYGSR^{491}	3.45 (2); 2.09 (3)	3.21 (2); 2.05 (3)	3.61 (2); 2.03 (3)	3.39 (2); 1.93 (3)	2.71 (2)	3.61 (2)
^{492}IQALLDKYNEEKPK^{505}	4.81 (2)	4.98 (2)			3.21 (3)	
Horse Myoglobin						
^{1}GLSDGEWQQVLNVWGK^{16}		4.32 (2)	4.31 (2)		3.03 (2)	2.59 (2)
^{17}VEADIAGHGQEVLIR^{31}	4.19 (2)	3.71 (3)	3.92 (2); 3.23 (3)	4.54 (2); 3.18 (3)	4.05 (2); 3.84 (3)	3.94 (2); 3.25 (3)
^{32}LFTGHPETLEK^{42}	3.17 (2)	2.78 (2)	1.76 (3)			2.43 (2)
^{32}LFTGHPETLEKFDK^{45}	3.16 (3)	3.56 (3)		3.02 (2)		
^{48}HLKTEAEMK^{56}						3.06 (2)
^{57}ASEDLKK^{63}	1.98 (2)	2.07 (2)	2.00 (2)	2.08 (2)		
^{64}HGTVVLTALGGILK^{77}	5.00 (2); 3.23 (3)	5.08 (2); 3.16 (3)	3.23 (3)	5.03 (2); 3.59 (3)	4.88 (2); 3.22 (3)	4.92 (2); 3.29 (3)
^{64}HGTVVLTALGGILKK^{78}	5.22 (2); 4.35 (3)	4.20 (3)	2.98 (2); 4.00 (3)	4.07 (3)	4.99 (2); 4.09 (3)	4.30 (2); 4.08 (3)
^{79}KGHHEAEIQPLAQSHATK^{96}	4.24 (3)		4.26 (3)			
^{78}KKGHHEAEIQPLAQSHATK^{96}				2.92 (3)		
^{80}GHHEAEIQPLAQSHATK^{96}	3.53 (3)					3.52 (2)
^{97}HKIPIK^{102}	1.99 (2)					
^{103}YLEFISDAIIHVLHSK^{118}	3.53 (3)	2.12 (2); 4.00 (3)				2.94 (3)
^{119}HPGDFGADAQGAMTK^{133}	3.57 (2); 3.98 (3)	3.81 (2); 4.06 (3)	3.90 (2); 3.58 (3)	3.78 (2); 4.07 (3)	3.85 (3)	2.52 (2)
^{134}ALELFRNDIAAK^{145}	2.70 (3)	3.17 (2)	2.14 (3)			3.37 (2)
^{146}YKELGFQG^{153}	2.24 (2)	2.22 (2)	2.18 (2)	2.38 (2)	2.71 (2)	2.38 (2)
totals: no. of peptides; X_{corr}	28; 107.79	24; 98.02	25; 98.48	16; 69.81	11; 49.80	18; 75.43

best S/N and peptide separation. The analysis with each solvent system was performed in triplicate; the total amount injected onto the column was 50 fmol/peptide. The data in Figure 1 and Table 1 demonstrate that MeOH reproducibly yielded higher S/N for each of the peptides: from 2.2- (peak 2) to 6.9-fold (peak 4); the difference in S/N was statistically significant for all peptides (Student's t test $p < 0.05$). In addition, the MeOH-based system provided better peak separation with a significantly shorter gradient time as compared to the ACN-based system (a 55-min gradient for MeOH vs an 80-min gradient for ACN; Figure 1 and Table 1).

To further study the effects of the organic modifier on detection limit, a second set of experiments was performed with a mixture of two tryptic digests of standard proteins of known concentration. The digests used were of bovine catalase (SWISSPROT accession code P00432) and horse myoglobin (SWISSPROT accession code P02188). (The total peptide concentration of each protein digest sample, although known from the vendor, was confirmed by amino acid analysis). Bovine catalase has a molecular mass of 57 kDa and closely represents the average size of proteins found in mammalian cells (40−50 kDa). When digested with trypsin, bovine catalase will theoretically generate a total of 40 tryptic peptides (assuming no missed cleavages) with a MW range of 500−4401. In contrast, horse myoglobin is a small protein (MW 16 932) close to the low-molecular weight limit of proteins detected on standard 2D gels. Horse myoglobin will theoretically generate 12 peptides when treated with trypsin (no missed cleavages) with a MW range of 500−1885. For the study, bovine catalase and horse myoglobin tryptic digests were combined in 1:3 molar ratio of catalase/myoglobin. The four quantities (catalase/myoglobin, fmol/fmol) studied were 25/75, 5/15, 1/3, and 0.2/0.6. The protein digests were diluted to the desired final amount immediately before nanoLC−ESI-MS/MS analysis. Each digest mixture was analyzed with the two solvent systems, and the nanoLC−ESI-MS/MS data were used to search the SWISSPROT protein sequence database. Each analysis was performed in triplicate. The results of the analyses, summarized in Tables 2−5, show that the MeOH-based solvent system yielded overall superior data in terms of the number of peptides matched for each protein. At the 25 fmol/75 fmol catalase/myoglobin level, the comparative analysis showed a statistically significant difference between the MeOH- and ACN-based solvent systems (Student's t test performed either on the

Table 3. Analysis of Trypsin-Digested Bovine Catalase (5 fmol) and Horse Myoglobin (15 fmol) ($p < 0.05$ Student's t Test)

	MeOH X_{corr} (charge)			ACN X_{corr} (charge)		
peptide sequence	1	2	3	1	2	3
Bovine Catalase						
[19]AAQKPDVLTTGGGNPVGDK[37]					3.90 (3)	2.39 (2); 4.16 (3)
[47]GPLLVQDVVFTDEMAHFDR[65]						
[105]RTPIAVR[111]	1.71 (2)					
[106]TPIAVR[111]	1.62 (2)	1.93 (2)	1.47 (2)	1.43 (2)		
[112]FSTVAGESGSADTVR[126]		2.73 (2)				
[112]FSTVAGESGSADTVRDPR[129]	1.73 (2)		1.60 (2)	3.01 (3)	1.65 (2)	3.40 (3)
[135]FYTEDGNWDLVGNNTPIFFIR[155]						
[221]LVNANGEAVYCK[232]						
[243]NLSVEDAAR[251]	2.33 (2)	2.27 (2)	2.26 (2)	2.36 (2)	2.01 (2)	
[252]LAHEDPDYGLR[262]	3.34 (3)	3.19 (3)	2.08 (2); 3.39 (3)	2.35 (2)	2.10 (2)	
[354]LFAYPDTHR[362]	2.64 (2); 2.24 (3)	2.26 (2); 2.19 (3)	2.10 (2)	3.16 (2); 2.29 (3)	3.14 (3); 2.31 (3)	2.19 (2)
[365]LGPNYLQIPVNCPYR[379]					2.68 (2)	
[422]THFSGDVQR[430]	2.54 (2); 2.08 (3)	2.75 (2)	1.87 (2)	2.19 (2)	2.43 (2)	2.40 (2)
[431]FNSANDDNVTQVR[443]	4.42 (2)	4.36 (2)	4.14 (2)	4.46 (2)		4.02 (2)
[449]VLNEEQR[455]						
[457]RLCENIAGHLK[467]						
[458]LCENIAGHLK[467]						
[468]DAQLFIQK[475]		2.36 (2)				
[468]DAQLFIQKK[476]			2.42 (2)			2.24 (2)
[480]NFSDVHPEYGSR[491]	3.62 (2)	3.42 (2); 2.08 (3)	3.62 (2); 2.33 (3)	1.91 (2)	3.45 (2); 2.50 (3)	2.87 (2); 2.23 (3)
[492]IQALLDKYNEEKPK[505]				2.46 (3)		
Horse Myoglobin						
[1]GLSDGEWQQVLNVWGK[16]						
[17]VEADIAGHGQEVLIR[31]		3.77 (3)	3.27 (3)	4.13 (3)		3.29 (3)
[32]LFTGHPETLEK[42]	2.76 (2)	1.67 (3)	2.69 (2)		2.45 (2)	1.75 (3)
[32]LFTGHPETLEKFDK[45]						
[48]HLKTEAEMK[56]		2.62 (2)	3.10 (2)			
[57]ASEDLKK[63]				3.21 (3)		
[64]HGTVVLTALGGILK[77]	4.96 (2); 2.18 (3)	4.64 (2)	4.74 (2); 2.76 (3)	5.02 (2)	2.55 (2); 2.10 (3)	
[64]HGTVVLTALGGILKK[78]	4.36 (3); 4.06 (3)	4.09 (3); 3.72 (3)	3.23 (3)	3.98 (3)	2.80 (3)	4.06 (3)
[79]KGHHEAEIQPLAQSHATK[96]						
[78]KKGHHEAEIQPLAQSHATK[96]						
[80]GHHEAEIQPLAQSHATK[96]						
[97]HKIPIK[102]		1.84 (2)				
[103]YLEFISDAIIHVLHSK[118]						
[119]HPGDFGADAQGAMTK[133]	3.52 (3)	3.80 (3); 2.82 (2)	3.17 (2)	2.40 (2); 3.73 (3)	1.43 (2); 3.58 (3)	
[134]ALELFR[139]	1.96 (2)		1.97 (2)	1.86 (2)	1.89 (2)	1.85 (2)
[134]ALELFRNDIAAK[145]	3.86 (2)	3.45 (2); 2.49 (3)	3.52 (2)	3.28 (2); 2.28 (3)	3.28 (2)	3.10 (2)
[146]YKELGFQG[153]	2.69 (2)	2.21 (2)	2.37 (2)	2.53 (2)	2.27 (2)	2.61 (2)
totals: no. of peptides; X_{corr}	16; 58.62	18; 66.66	18; 58.10	17; 58.04	15; 48.52	13; 42.56

total number of identified peptides or the total X_{corr} gave a p value of <0.05; Table 2). This difference is due to the higher number of detected peptides; it is not the result of higher X_{corr} scores for the individual peptides—the average X_{corr} value was 3.20 ± 0.11 and 3.33 ± 0.20 for MeOH and ACN, respectively. These results indicate that the observed difference between the two solvents lies in the number of detected peptides rather than in other factors such as solvent-dependent changes in peptide fragmentation behavior, which could influence the X_{corr} values.

The 5 fmol/15 fmol measurements showed no statistically significant difference in the number of peptides (or X_{corr} scores) detected; that result suggested that no quantitative or qualitative advantage exists with one solvent versus the other at this amount level (Table 3). A possible explanation of this result is that at 25 fmol/75 fmol, the better separation achieved by the MeOH-based system resulted in a more efficient selection of the peptide ions for MS/MS data-dependent analysis. At 5 fmol/15 fmol, there were fewer peptides detected than at 25 fmol/75 fmol and therefore improved separation with MeOH did not offer an advantage; at the same time, the peptide amount was still above the detection limit for both solvent systems. However, when lower amounts of peptide digests were used (1 fmol/3 fmol and 0.2 fmol/0.6 fmol), the MeOH-based solvent system produced strikingly better results. As shown in Tables 4 and 5, the ACN-based analysis did not yield any peptide identifications at levels of ≤1 fmol, and only one peptide was identified in one analysis for horse myoglobin at the 3-fmol level. In contrast, the MeOH-based analysis identified both proteins at all amounts studied, including the 0.2 fmol/0.6 fmol level, where several peptides with good X_{corr} values were matched (Table 5). The MS/MS spectra for two representative peptides from analyses at the 0.2 fmol/0.6 fmol level are depicted in Figure 2; these spectra display a number of intense product ions of the b- and y-series that define the amino acid sequence of the peptides. The data obtained for analyses of standard protein digests demonstrate that with MeOH it is possible to identify more peptides than with ACN at amounts higher than the detection limit and also that the detection limit is improved with MeOH by a factor of 5 or higher. Two-way ANOVA statistical analysis performed on the total number of peptides (Tables 2−5) for all four data sets confirmed that the solvent system used for the

Table 4. Analysis of Trypsin-Digested Bovine Catalase (1 fmol) and Horse Myoglobin (3 fmol) ($p < 0.05$ Student's t Test)

	MeOH X_{corr} (charge)			ACN X_{corr} (charge)		
peptide sequence	1	2	3	1	2	3
Bovine Catalase						
[19]AAQKPDVLTTGGGNPVGDK[37]	3.23 (3)					
[47]GPLLVQDVVFTDEMAHFDR[65]						
[105]RTPIAVR[111]						
[106]TPIAVR[111]						
[112]FSTVAGESGSADTVRDPR[129]			2.93 (2)			
[135]FYTEDGNWDLVGNNTPIFFIR[155]						
[221]LVNANGEAVYCK[232]						
[243]NLSVEDAAR[251]	2.18 (2)		2.14 (2)			
[252]LAHEDPDYGLR[262]						
[354]LFAYPDTHR[362]	2.02 (3)	1.79 (3)				
[365]LGPNYLQIPVNCPYR[379]						
[422]THFSGDVQR[430]						
[431]FNSANDDNVTQVR[443]	4.35 (2)	3.74 (2)				
[449]VLNEEQR[455]						
[457]RLCENIAGHLK[467]						
[458]LCENIAGHLK[467]						
[468]DAQLFIQK[475]						
[468]DAQLFIQKK[476]						
[480]NFSDVHPEYGSR[491]	3.37 (2)	3.07 (2)	2.96 (2); 2.15 (3)			
[492]IQALLDKYNEEKPK[505]						
Horse Myoglobin						
[1]GLSDGEWQQVLNVWGK[16]						
[17]VEADIAGHGQEVLIR[31]						
[32]LFTGHPETLEK[42]						
[32]LFTGHPETLEKFDK[45]		1.80 (3)				
[48]HLKTEAEMK[56]						
[57]ASEDLKK[63]						
[64]HGTVVLTALGGILK[77]						
[64]HGTVVLTALGGILKK[78]	4.18 (3)	4.17 (3)				
[79]KGHHEAEIQPLAQSHATK[96]						
[78]KKGHHEAEIQPLAQSHATK[96]						
[80]GHHEAEIQPLAQSHATK[96]						
[97]HKIPIK[102]						
[103]YLEFISDAIIHVLHSK[118]						
[119]HPGDFGADAQGAMTK[133]	4.00 (3)		3.41 (2)			
[134]ALELFRNDIAAK[145]	2.83 (2)					
[146]YKELGFQG[153]	2.35 (2)	2.05 (2)	2.14 (2)	2.33 (2)		
totals: no. of peptides; X_{corr}	9; 28.51	6; 16.62	5; 15.73	1; 2.33	0; 0	0; 0

analysis had a statistically significant effect on the number of identified peptides ($p < 0.01$).

To compare the performance of MeOH- and ACN-based LC solvent systems for "real-world" samples, a set of digests of 2-DGE-separated proteins were studied. Fourteen protein spots of various intensities and pI/MW values were analyzed from a 2D gel of a human pituitary proteome (Figure 3). The spots were excised from a single silver-stained 2D gel, and the proteins in these spots were digested with trypsin. The tryptic peptides were extracted from the gel plugs, the extracts were split in two equal-volume aliquots, and the peptides in each aliquot were purified and analyzed by nanoLC−ESI-MS/MS with either MeOH- or ACN-based solvent gradient. The proteins were identified through SWISSPROT database searches. The results of the analyses of the 14 spots are summarized in Table 6. With the MeOH-based LC solvent system, all 14 spots yielded a positive identification of at least one protein per spot. In 6 of the 14 spots (43%), multiple proteins were detected, which attests to the excellent sensitivity of the MeOH-based method. In contrast, the ACN-based analysis failed to detect any proteins in 3 of the 14 spots and overall detected 33% fewer proteins that the MeOH-based method. Statistical analysis (Mann−Witney nonparametric test performed on the total number of peptides identified per protein) showed a significant difference between the two groups ($p < 0.05$). Similar to the standard peptide digests, the differences between the two solvent systems were more evident for lower intensity protein spots, e.g., spots 4 and 11. (Although protein abundance cannot be determined from silver-stained spot intensity, smaller or weakly stained spots generally have less protein amount.) That result is particularly significant because it demonstrates that often the main reason for failing to identify a protein from a gel is simply due to the low detection sensitivity in the nanoLC−MS/MS step.

The findings of this study show that MeOH is a better alternative to ACN as a solvent for nanoLC−MS/MS of peptides. In general, MeOH is a common organic modifier used in reversed-phase chromatography; however, for analysis of peptides, the solvent used in an overwhelming majority of applications is ACN. Analysis of peptides by LC−MS/MS has inherited much of its LC methods from the classical HPLC/UV−visible methodology where peptides are usually monitored in the low-UV range (commonly 210 nm). Because ACN has a lower UV cutoff than MeOH (190 nm for ACN vs 205 nm for MeOH),[18] it is preferred

Table 5. Analysis of Trypsin-Digested Bovine Catalase (200 amol) and Horse Myoglobin (600 amol) ($p < 0.05$ Student's t Test)

	MeOH X_{corr} (charge)			ACN X_{corr} (charge)		
peptide sequence	1	2	3	1	2	3
Bovine Catalase						
^{19}AAQKPDVLTTGGGNPVGDK^{37}						
^{47}GPLLVQDVVFTDEMAHFDR^{65}						
^{105}RTPIAVR^{111}						
^{106}TPIAVR^{111}						
^{112}FSTVAGESGSADTVRDPR^{129}		2.86 (3)				
^{135}FYTEDGNWDLVGNNTPIFFIR^{155}						
^{221}LVNANGEAVYCK^{232}						
^{243}NLSVEDAAR^{251}						
^{252}LAHEDPDYGLR^{262}			2.70 (3)			
^{354}LFAYPDTHR^{362}						
^{365}LGPNYLQIPVNCPYR^{379}						
^{422}THFSGDVQR^{430}						
^{431}FNSANDDNVTQVR^{443}						
^{449}VLNEEQR^{455}						
^{457}RLCENIAGHLK^{467}						
^{458}LCENIAGHLK^{467}						
^{468}DAQLFIQK^{475}						
^{468}DAQLFIQKK^{476}						
^{480}NFSDVHPEYGSR^{491}		1.50 (2)	1.76 (3)			
^{492}IQALLDKYNEEKPK^{505}			1.81 (3)			
Horse Myoglobin						
^{1}GLSDGEWQQVLNVWGK^{16}						
^{17}VEADIAGHGQEVLIR^{31}	3.04 (3)	2.29 (3)				
^{32}LFTGHPETLEK^{42}						
^{32}LFTGHPETLEKFDK^{45}						
^{48}HLKTEAEMK^{56}						
^{57}ASEDLKK^{63}						
^{64}HGTVVLTALGGILK^{77}	5.21 (2)					
^{64}HGTVVLTALGGILKK^{78}	4.40 (3)					
^{79}KGHHEAEIQPLAQSHATK^{96}						
^{78}KKGHHEAEIQPLAQSHATK^{96}						
^{80}GHHEAEIQPLAQSHATK^{96}						
^{97}HKIPIK^{102}						
^{103}YLEFISDAIIHVLHSK^{118}						
^{119}HPGDFGADAQGAMTK^{133}		3.01 (2); 2.23 (3)				
^{134}ALELFRNDIAAK^{145}						
^{146}YKELGFQG^{153}	2.24 (2)		2.22 (2)			
totals: no. of peptides; X_{corr}	4; 14.89	4; 11.89	4; 8.49	0; 0	0; 0	0; 0

for UV-based detection of peptides in HPLC. In LC−MS/MS-based analysis, where UV monitoring is usually unnecessary, the higher UV absorbance of the MeOH-based solvent system is not an issue. In terms of reversed-phase elution strength, MeOH is a weaker solvent than ACN (eluotropic value for C18 of 1.0 for MeOH vs 3.1 for ACN).[18] Consequently, a higher percentage of MeOH is needed to elute a particular peptide in gradient elution,[18,19] which is advantageous for subsequent electrospray ionization. In our study, faster gradients and hence shorter analysis times were possible with MeOH versus ACN without any decrease in chromatographic performance. It should also be noted that high-purity MeOH is significantly less expensive that ACN. Because in nanoLC low flow rates are usually generated by flow splitting, solvent consumption in high-throughput settings can be significant, and the use of MeOH would bring substantial cost savings. A slight disadvantage of MeOH use is the fact that, unlike ACN, MeOH forms relatively viscous mixtures with water,[20] giving rise to increasing pressures in the LC system during the gradient run. However, in our study, the maximum pressures were still well below the acceptable limits and no problems with the LC components were observed.

Upon elution from the nanoLC, the peptides are ionized by ESI. The detailed mechanistic aspects of ESI are complex and still a matter of some debate.[21,2221−22] The basic concept of the ion formation process in ESI includes several steps. First, the liquid sample is dispersed from a tip of a capillary in an electric field into an aerosol of charged droplets. Second, evaporation of the solvent from these initial droplets results in their shrinkage into smaller and more highly charged droplets. Third, when the Coulomb repulsion within the droplet approaches the surface tension (a so-called Rayleigh stability limit), solvated ions are released from the surface of the droplet into the gas phase or the droplet undergoes fission into smaller "offspring" droplets and repeated fissions/solvent evaporation ultimately produces gas-

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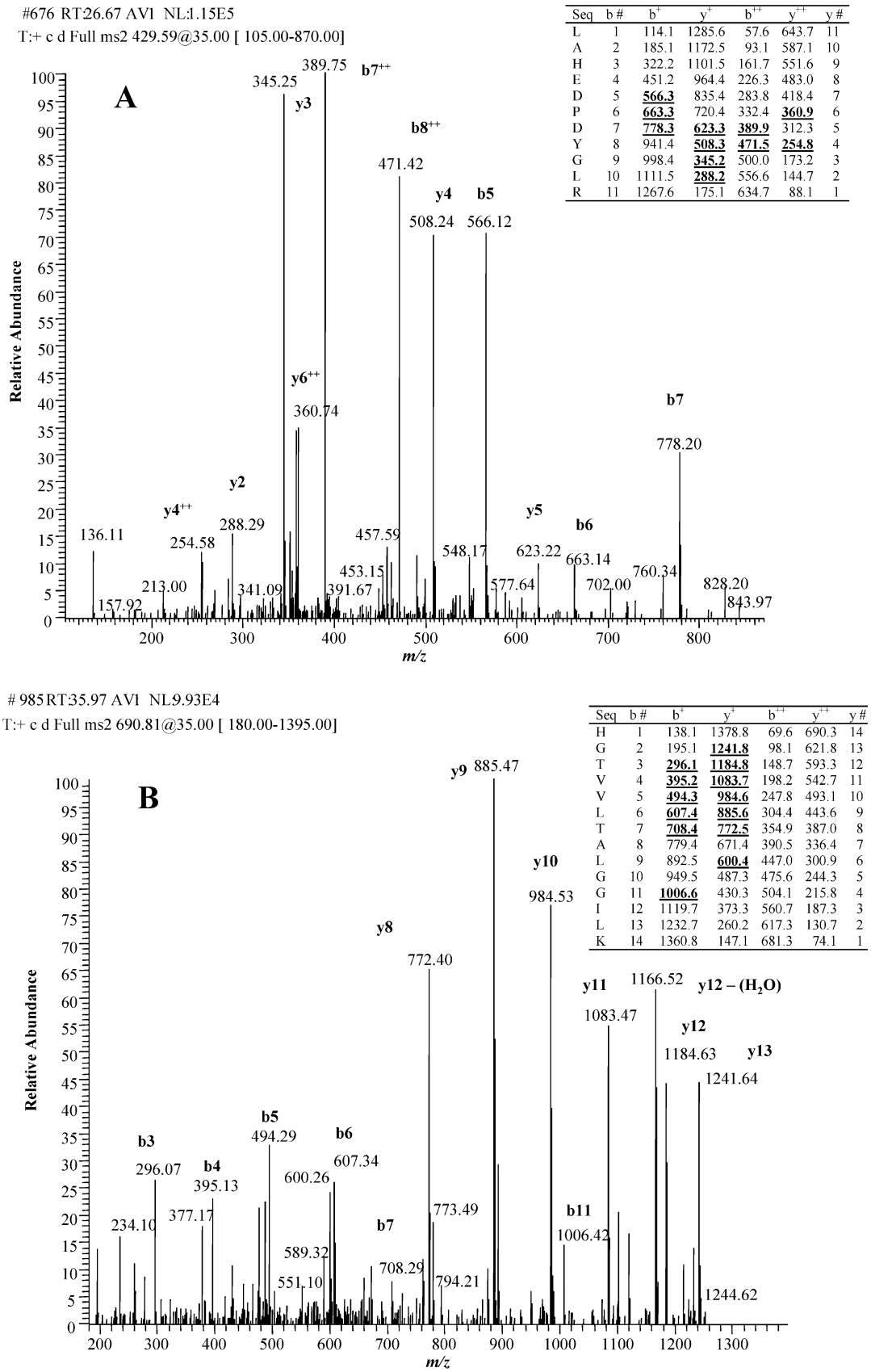

Seq	b #	b+	y+	b++	y++	y #
L	1	114.1	1285.6	57.6	643.7	11
A	2	185.1	1172.5	93.1	587.1	10
H	3	322.2	1101.5	161.7	551.6	9
E	4	451.2	964.4	226.3	483.0	8
D	5	**566.3**	835.4	283.8	418.4	7
P	6	**663.3**	720.4	332.4	**360.9**	6
D	7	**778.3**	**623.3**	**389.9**	312.3	5
Y	8	941.4	**508.3**	**471.5**	**254.8**	4
G	9	998.4	**345.2**	500.0	173.2	3
L	10	1111.5	**288.2**	556.6	144.7	2
R	11	1267.6	175.1	634.7	88.1	1

Seq	b #	b+	y+	b++	y++	y #
H	1	138.1	1378.8	69.6	690.3	14
G	2	195.1	**1241.8**	98.1	621.8	13
T	3	**296.1**	**1184.8**	148.7	593.3	12
V	4	**395.2**	**1083.7**	198.2	542.7	11
V	5	**494.3**	**984.6**	247.8	493.1	10
L	6	**607.4**	**885.6**	304.4	443.6	9
T	7	**708.4**	**772.5**	354.9	387.0	8
A	8	779.4	671.4	390.5	336.4	7
L	9	892.5	**600.4**	447.0	300.9	6
G	10	949.5	487.3	475.6	244.3	5
G	11	**1006.6**	430.3	504.1	215.8	4
I	12	1119.7	373.3	560.7	187.3	3
L	13	1232.7	260.2	617.3	130.7	2
K	14	1360.8	147.1	681.3	74.1	1

Figure 2. MS/MS spectra of selected peptides from a trypsin-digested mixture of bovine catalase and horse myoglobin. Bovine catalase (0.2 fmol) and horse myoglobin (0.6 fmol) tryptic digests were combined and manually injected. The peptides were eluted with a 55-min gradient of H_2O/MeOH, and MS and MS/MS spectra were acquired in the data-dependent mode. The peptides and proteins were identified through SWISSPROT database searches. (Note that no peptides were detected at the 0.2 fmol/0.6 fmol level with H_2O/ACN solvent system—see Table 5.) (A) MS/MS spectrum of a precursor ion of *m*/*z* 429.59; these MS/MS data were matched to the peptide LAHEDPDYGLR from bovine catalase. (B) MS/MS spectrum of a precursor ion of *m*/*z* 690.81; these MS/MS data were matched to the peptide HGTVVLTALGGILK from horse myoglobin.

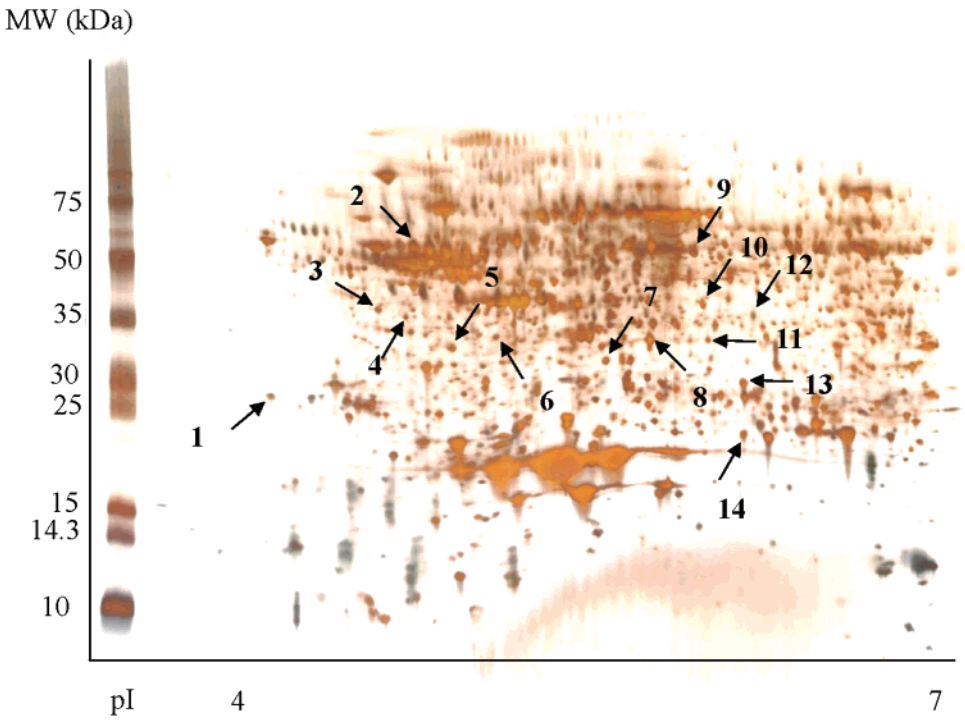

Figure 3. 2D gel image of the proteome from a human pituitary. To produce this gel, 200 μg of protein extract was separated by 2-DGE, and the proteins were visualized by silver staining. Selected protein spots (labeled 1−14) were excised from the gel and digested with trypsin. Half of each digested sample was analyzed by nanoLC−MS/MS with a MeOH-based LC solvent system and the other half with ACN-based solvent system. The proteins identified in these spots are listed in Table 6.

Table 6. Identification of 2-DGE-Separated Proteins (p < 0.05 Mann−Witney Nonparametric Test)

	MeOH		ACN	
spot no.	identified protein (SWISSPROT accession no.)	no. of identified peptides	identified protein (SWISSPROT accession no.)	no. of identified peptides
1	complement component 1 (Q07021)	1	complement component 1 (Q07021)	1
2	α-1-antitrypsin precursor (P01009)	11	α-1-antitrypsin precursor (P01009)	11
	secretogranin I precursor (P05060)	8	secretogranin I precursor (P05060)	10
	carboxypeptidase H precursor (P16870)	7	carboxypeptidase H precursor (P16870)	5
	vimentin (P08670)	6	vimentin (P08670)	4
	tubulin β-2 chain (P05217)	5	tubulin β-2 chain (P05217)	3
	tubulin α-1 chain (P18258)	3	tubulin α-1chain (P18258)	2
	antithrombin-III precursor (P01008)	2		
	microsomal stress 70 protein ATPase core precursor (P48723)	2		
3	40S ribosomal protein SA (P40) (P08865)	5	40S ribosomal protein SA (P40) (P08865)	3
	vimentin (P08670)	4		
4	arsenical pump-driving ATPase (O43681)	3	none	
5	μ-crystallin homolog (Q14894)	7	μ-crystallin homolog (Q14894)	4
	tubulin β-1 chain (P07437)	4		
	somatotropin precursor (P01241)	2		
6	SEC13-related protein (P55735)	4	none	
7	actin 1 (P02578)	3	none	
8	L-lactate dehydrogenase B chain (P07195)	13	L-lactate dehydrogenase B chain (P07195)	10
	annexin A4 (P09525)	10	annexin A4 (P09525)	8
9	aldehyde dehydrogenase class 2 (P05091)	10	aldehyde dehydrogenase class 2 (P05091)	5
10	leukocyte elastase inhibitor (P30740)	11	leukocyte elastase inhibitor (P30740)	9
	macrophage capping protein (P40121)	2	macrophage capping protein (P40121)	4
11	60S acidic ribosomal protein P0 (P05388)	10	60S acidic ribosomal protein P0 (P05388)	3
	serine protease HTRA2, mitochondrial (O43464)	2		
12	Aclid ceramidase (Q13510)	4	Acid ceramidase (Q13510)	3
13	Glutathlione transferase ω 1 (P78417)	8	glutathione transferase ω 1 (P78417)	7
14	thioredoxin-dependent peroxide reductase, mitochondrial (P30048)	4	thioredoxin-dependent peroxide reductase, mitochondrial (P30048)	4

phase ions. Detection sensitivity and linearity for a given analyte are determined by the mutual interaction among the experimental parameters involved in the entire ionization process (electric potential, mobile-phase flow rate and composition, interface geometry). The composition of the eluting solvent has a marked effect on the efficiency of the ESI process. MeOH is a weaker solvent than ACN in reversed-phase chromatography, and therefore, a higher concentration of MeOH is required for peptide

elution. This results in a higher proportion of the organic solvent in the eluent during peptide ionization and, hence, in lower surface tension. The lower surface tension induces an easier elongation of the Taylor cone and a better aerosol formation during the spray process. Higher volatility of MeOH will facilitate solvent evaporation and ion desolvation. Furthermore, a decrease in the surface tension in the droplets will facilitate the desorption of ions from the droplet surface, droplet fission, or both.

In summary, the results presented in this report demonstrate that MeOH is a superior organic modifier for nanoLC−MS/MS analysis of peptides. MeOH-based solvent system allows the use of faster gradient times and yields better detection limits. High success rates of protein identification from silver-stained 2D gel spots can be achieved without gel pooling.

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