**Introduction**
As the sequenced human genome and levels of expression of many proteins do not fully describe the current state of a living cell, in order to understand complex cellular processes and pathways relevant to environmental cancer research conducted at EDC NHEERL EPA, it is important to be able to analyze the complete protein composition. Proteomics has recently emerged as a scientific discipline with the main purpose to identify, in a comprehensive manner, proteins in complex biological systems. Such comprehensive methods combining separation and detection techniques have been developed for protein identification. These techniques include 2D gel electrophoresis and mass spectrometry. Prior to the digestion with trypsin, the samples are reduced and alkylated with different cysteine modifying reagents: DISS, IAM, VP. The alkylated and digested samples (HSA - IAM, VP, APTA) were combined together and injected onto the 2D LC System.

**Specificity-Related peptides eluting in SCX chromatography:**
Retention increases with increasing number of basic residues, and decreases with increasing number of acidic residues. Adaptor residues of the same length have a linear effect probability due to the same – charge repulsion.

**HSA Digest:** reverse-phase base-peak LC/MS chromatograms for the SCX runs 5 – 40% "B", pH 3.00 – 4.62, show no carry-over between consecutive pH - gradient - eluted SCX fractions.

**Conclusions:** This new automated 2D-LC/MSMS method, designed for protein digests analyses, is based on the principle of simultaneous, pH - gradient and salt - gradient - induced SCX elution, followed by peptide trapping and RP nanospray-MSMS. It is characterized by low carry-over, using a reversed-phase salt gradient, based on sequential elution, for a comprehensive sequencing of peptides. This new method combines a simultaneous pH - gradient and salt gradient - induced SCX elution, followed by RP nanospray-MSMS. The sample volume was adjusted to approx. 50 ul with water and the pH was adjusted with 2M acetic acid to pH 3.7. An aliquot of 10 ul volume was removed from each of the three sample solutions and analyzed individually by 2D/LC/MSMS.

**Experimental**
Protein Extraction
Cellular protein extract was prepared from human lung epithelial cells collected from eight 150 mm dishes. Cells were washed in PBS twice and resuspended in 50 mM Tris-HCl, pH 7.5. Three cycles of freezing and thawing were applied followed by 15 min centrifugation at 4°C. Only the soluble protein fraction was used.

Human Serum Albumin (HSA) digest was purchased from Sigma-Aldrich, Inc. Reduction, Alkylation and Purification
The test protein sample was split into 2 aliquots, each 80 ul; ca. 2 ug/l. In the first aliquot, the samples were reduced with dithiothreitol (DTT) in 6M urea and 0.1M Tris buffer at pH 7.8, and alkylated with iodoacetic acid amide (IAM). The sample was then purified using semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) of IAM (eluted with 50% acetonitrile/49.9% water/0.1% trifluoroacetic acid). In the second aliquot, the samples were reduced and alkylated with different cysteine modifying reagents: DISS, IAM, VP. The alkylated and digested samples (HSA - IAM, VP, APTA) were combined together and injected onto the 2D LC System.

**2D (SCX/RP) – LC/MSMS experimental layout: SCX – gradient pH elution followed by SCX elution at constant pH 8.0**

**Top 70 proteins out of the total of 900 protein hits passing BioworksTM multiscorer filter.** The data set was displayed as a multi-consensus report from the IAM1, IAM2, and APTA1 cell lysate protein 2D-LC/MSMS runs

**Analysis of Protein Digests by Nano-SCX/RP/MSMS with pH/salt Gradient SCX Elution**
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