

Quantitative Proteomic Analysis of the Secretory Proteins from Rat Adipose Cells Using a 2D Liquid Chromatography–MS/MS Approach

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We have developed two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC–MS/MS) and ¹⁸O proteolytic labeling strategies to identify and compare levels of secretory proteins with low abundance in the conditioned medium of rat adipose cells without or with insulin stimulation. Culture medium was concentrated and secreted proteins were separated on a RP–HPLC followed by LC–MS/MS analysis. For ¹⁸O proteolytic labeling, ¹⁶O- to ¹⁸O-exchange in the digested peptides from eight individual fractions was carried out in parallel in H₂¹⁶O and H₂¹⁸O with immobilized trypsin, and the ratios of isotopically distinct peptides were measured by mass spectrometry. A total of 84 proteins was identified as secreted adipokines. This large number of secretory proteins comprise multiple functional categories. Comparative proteomics of ¹⁸O proteolytic labeling allows the detection of different levels of many secreted proteins as exemplified here by the difference between basal and insulin treatment of adipose cells. Taken together, our proteomic approach is able to identify and quantify the comprehensive secretory proteome of adipose cells. Thus, our data support the endocrine role of adipose cells in pathophysiological states through the secretion of signaling molecules.

Keywords: comparative proteomics • proteolytic ¹⁸O labeling • secreted proteome • adipose cell • electrospray-ionization mass spectrometry

Introduction

In the past, adipose cells have generally been regarded as energy storage sites.^{1–5} Only very recently, the critical endocrine function of adipose cells to release signaling molecules has been recognized.^{1–5} It is now known that adipose cells play a central role in the pathogenesis of obesity and its related diseases such as type II diabetes, cardiovascular disease, dyslipidemia, and certain cancers by secreting endocrine and autocrine/paracrine factors.^{1–5} Dysregulation of adipose cell secretion may be the initial defect responsible for the development of disorders in other tissues or organs. Most of our current knowledge has been collected through mouse model studies.⁴ Recently, genomic approaches have been used to identify more adipose secretory factors (adipokines).^{6–7}

To our knowledge, only a few proteomic approaches have been described so far; some focus on the differentiation of 3T3-L1 preadipocytes to adipocytes.^{8–13} Not surprisingly, the early

studies document the changes on 2D gels upon differentiation, but they do not identify or even characterize the proteins.^{9–10} With the renewed interest in the study of adipokines, some of these proteins have been identified through cloning¹⁴ and proteomics studies.^{8,13} One proteomic study reported the discovery of twelve novel adipokines from 3T3-L1 adipocytes using one-dimensional gel electrophoresis and tandem mass spectrometry, and selected LC–MS/MS experiments.⁸ A more recent study relied on 2D gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS), and identified 41 candidate proteins, whereby 15 additional adipokines were identified that were not previously known to be secreted from 3T3-L1 adipocytes.¹³

However, no systematic approach has been applied to study the composition of the secretory proteome of primary adipose cells that presumably better reflect the physiology of adipose cells than do immortalized cell lines such as 3T3-L1 adipocytes. To accomplish this goal, we have developed a 2D-LC–MS/MS approach to catalog adipokines from primary adipose cells. In contrast to the popular MUDPIT (*Multidimensional Protein Identification Technology*) approach that was initially described by Yates et al.,^{15–22} intact proteins were separated in the first dimension and only then adipokines of the individual fractions were digested before analysis by LC–MS/MS. One of the reasons for the development of MUDPIT was the difficulty in detecting and analyzing hydrophobic membrane and membrane-associated proteins, which thus often remained uniden-

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tified. Since our goal was the detection of secreted soluble proteins, we chose to separate intact proteins before digestion.

Furthermore, to develop a quantitative method, which could ultimately be used to investigate differences in the secretory proteome of adipose cells in altered metabolic states, we determined the changes in the secretory proteome induced by insulin treatment by adapting the $H_2^{16}O/H_2^{18}O$ method.^{23–29} We needed a flexible stable isotope labeling strategy that is not limited to cell cultures, but could be extended to animal or human (clinical) studies. Thus, the very common and accurate metabolic labeling techniques, which metabolically introduce a label such as ^{13}C during growth in enriched medium, could not be applied.^{30–33} Instead, we chose enzymatic labeling using $H_2^{18}O$.^{23–29} Quantification was done by comparing the relative abundance of the isotopically labeled and unlabeled peptides.

A total of 183 candidates were identified with this technology. After scrutinizing these proteins for signal peptides, a signature of secreted proteins, using bioinformatic tools (SIGNAL P), 84 proteins were identified as adipokines. Fifty-three of these proteins have not previously been reported to be secreted by adipose cells and only thirteen were missed when compared to earlier proteomic studies. Upon chronic insulin treatment in primary culture, modulation of several secreted proteins was detected using the quantitative approach, including an increase in adiponectin, and decrease in PAI-1 and osteonectin. Since none of the currently identified adipokines provides more than a hint at the mechanisms relating adipose cell secretory activity to systemics glucose homeostasis, the current description of novel adipokines and report of a method for quantifying alterations in multiple secretory proteins simultaneously between cells in altered metabolic states represent a significant advance in defining the role of adipose cells in pathophysiology of obesity and type II diabetes.

Methods

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM) and collagenase type 1 were from Invitrogen (Carlsbad, CA) and Worthington Biochemical Co., respectively. Water and acetonitrile (HPLC gradient grade quality) were supplied by Fisher (Pittsburgh, PA); bovine serum albumin (BSA), adenosine, trifluoroacetic acid (99+%), glacial acetic acid (99%), dithiothreitol (DTT), iodoacetamide, and ^{18}O -water were from Sigma (St. Louis, MO). Endoproteinase Lys-C and trypsin (modified sequencing grade) were from Roche (Indianapolis, IN). Immobilized trypsin was from Applied Biosystems (Foster City, CA). All other chemicals and reagents were of the highest purity available.

Isolation of Adipose Cells and Cell Culture. Animal handling followed National Institutes of Health guidelines, and experimental procedures were approved by the NIDDK animal care and use committee. Adipose cells from the epididymal, inguinal, and omental fat pads of male rats (CD strain, Charles River Breeding Laboratories, Inc.) were isolated by collagenase digestion as previously described.³⁴ After mincing, the fat pads were digested with collagenase (20 mg/mL solution) in digestion vials containing Krebs-Ringer bicarbonate Hepes buffer, pH 7.4, 200 nM adenosine (KRBH buffer), and 0.1% BSA. After a 2-hour digestion, adipose cells and stromal-vascular (SV) cells were separated by centrifugation at 1200 rpm for 10 min. The isolated adipose cells were washed twice with KRBH buffer containing 0.1% BSA, and twice with serum-free, BSA-free DMEM medium by centrifugation at 1000 rpm for 2 min. After the final wash, cells were cultured in serum-free, BSA-free

DMEM in the presence or absence of 67 nM insulin at 37 °C for 48 h in 5% CO_2 . After incubation, conditioned medium was collected and centrifuged at 1200 rpm for 10 min to separate cell debris. Supernatant medium was then filtered using a 0.45 μ m syringe-driven filter to remove the floating adipose cells. Conditioned medium was desalted and concentrated at a Macrosep centrifugal device with molecular weight cutoff of 1 kDa (Pall Life Sciences, MI). The protein concentration was approximately 650 ng/ μ L determined by the Pierce BCA Protein Assay.

Liquid chromatography (LC) of Proteins. For the LC of the proteins, a Hewlett-Packard HP1100 system (Hewlett-Packard, now Agilent Technologies, Palo Alto, CA) consisting of a quaternary pump, degasser, autosampler, and UV detector was used. The HP ChemStation data system was used for data acquisition. The culture medium was concentrated 5–6-fold and 100 μ L of concentrated extracts were used for each injection. Proteins were separated on a Zorbax 300SB–C3 reversed phase column (150 mm \times 4.6 mm ID, 5 μ m) that was equipped with a guard column (C3 reversed phase) at a flow rate of 700 μ L/min. The mobile phase was 0.1% (v/v) TFA (solvent A) and acetonitrile (solvent B). A gradient ramping from 5% to 40% acetonitrile within 55 min, followed by a gradient to 95% acetonitrile within 70 min, was used for the chromatographic separation. The eluent was monitored by UV at 254 nm. Fractions were collected in 2 min intervals and after evaporation of the solvent, the fractions were pooled to give 8 combined fractions.

Digestion of the Separated Proteins. Each combined fraction was dissolved and reduced in 20 μ L of 5M urea containing 5 mM DTT, and alkylated with 0.1 μ mol iodoacetamide. Reduced and alkylated proteins were digested with 0.5 μ g Lys-C, and diluted with water to give a final urea concentration of 2M, followed by tryptic digestion (1 μ g). To identify the digested proteins, their peptides were subjected to LC–MS/MS analysis.

Labeling of Digested Peptides with ^{18}O -Water. Aliquots of the tryptic peptides from basal and insulin-treated samples were evaporated to dryness and redissolved in $H_2^{16}O$ and $H_2^{18}O$, respectively. Immobilized trypsin was added to basal ($H_2^{16}O$) and insulin-treated ($H_2^{18}O$) samples, and incubated overnight in the presence of 0.1 M ammonium carbonate. The labeling procedure was stopped after 16 h. For quantitation, aliquots of untreated, unlabeled peptides (control) and treated, ^{18}O -labeled peptides were mixed at a 1:1 ratio, and subsequently analyzed by LC–MS.

LC–MS/MS System. For the separation of the peptides, a Micromass CapLC quadrupole time-of-flight QTOF2 system (Micromass, now Waters, Manchester, UK) consisting of a CapLC, an autosampler, and a QTOF2 mass spectrometer was used. Data were acquired and processed with MassLynx Software. Nitrogen was used as the nebulization and desolvation gas, and Argon as the collision gas. The instrument was calibrated with a Glu fibrinopeptide standard.

After digestion, peptides were concentrated on a Waters Symmetry300 C18 5 μ m OPTI-PAK trap column and after a 5 min delay, directed onto a ThermoHypersil C18 column (100 mm \times 150 μ m ID, 5 μ m) for separation. Solvent A was a mixture of 98.8% water, 1% acetonitrile, and 0.2% formic acid, and solvent B was a mixture of 98.8% acetonitrile, 1% water, and 0.2% of formic acid. A gradient was ramped from 5% B to 95% B within 175 min. The flow rate was 6 μ L/min and was split in a 10:1 ratio on the column. The column was kept at room

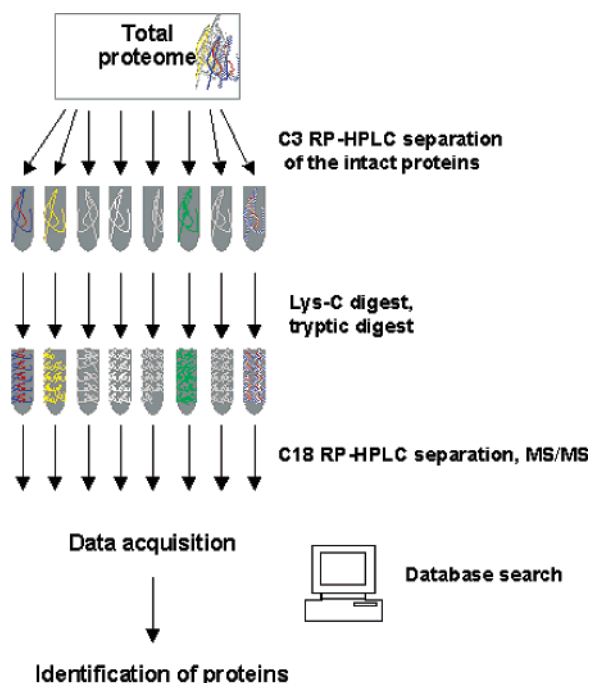


Figure 1. Adipose cell secretory proteome analysis using LC/LC-MS/MS. The secretory proteome was first separated by C3 reversed phase chromatography (70-min gradient). After reduction and alkylation, the resulting 8 fractions were double digested with Lys-C and trypsin. The resulting peptides of each fraction were separated on a C18 column (100 mm × 150 μm I.D.) coupled with a QTOF mass spectrometer. Data were acquired and processed with MassLynx software. Generated peaklist (pk1) files were searched against the NCBI protein database using MASCOT.

temperature. For protein identification, MS/MS data were acquired, whereas for quantification only MS data were acquired.

Database Searching. Data were processed using Masslynx to create so-called pk1 files (peaklist files). These were used for subsequent database searching using a MASCOT search engine.³⁵ The following parameters have been used: NCBIInr, MSDB, SPROT, and EST databases; 3 missed cuts; iodoacetamidation of cysteines; deamidation; and charge states +2, +3, and +4. A window of 1-Da mass accuracy for precursor ions and 0.4-Da mass accuracy for MS/MS data was chosen. Probability-based MASCOT scores were considered significant when greater than the cutoff score that indicates either identity or homology ($p < 0.05$) for individual ions. The sequence assignment of those occasional proteins that were only identified by one peptide was generally confirmed by de novo-sequencing using MassLynx. To increase confidence in the assignment as secretory proteins and reduce the risk of contamination from cellular material, all identified proteins were checked to meet the criteria set by SignalP predictions.

Results and Discussion

2D-LC-MS/MS Strategy to Separate and Identify the Secretory Proteome of Rat Adipose Cells. The 2D-LC-MS/MS approach used in this study is shown schematically in Figure 1 and described in detail in the Experimental Section. In contrast to the popular MudPIT approach, intact (and not digested) proteins of the secretory proteome of basal adipose cells were separated into eight fractions in the first dimension,

Table 1. Representative Secretory Proteins Identified by 2D-LC-MS/MS

| accession | secreted protein | total score | peptides matched |
|-------------|--|-------------|------------------|
| gi 14289336 | Adiponectin | 139 | 4 |
| gi 2507246 | Adipsin | 489 | 16 |
| gi 19705570 | Angiotensinogen | 422 | 9 |
| gi 4504165 | Gelsolin | 288 | 6 |
| gi 34858910 | Cystatin C | 665 | 4 |
| gi 123513 | Haptoglobin | 37 | 3 |
| gi 123036 | Hemopexin | 188 | 6 |
| gi 6981168 | Lipoprotein lipase | 94 | 4 |
| gi 1326433 | Neu-related lipocalin | 132 | 5 |
| gi 600381 | Osteonectin | 165 | 5 |
| gi 6996919 | Complement component factor B | 307 | 14 |
| gi 129575 | Plasminogen activator inhibitor-1 | 213 | 6 |
| gi 21426805 | Resistin | 134 | 4 |
| gi 13591991 | Matrix metalloproteinase 2 | 73 | 2 |
| gi 13591993 | Matrix metalloproteinase 9 | 64 | 1 |
| gi 202370 | Vimentin | 190 | 4 |
| gi 6978507 | Adrenomedullin | 54 | 1 |
| gi 12018262 | Cathepsin B | 450 | 9 |
| gi 2507388 | Contrapsin-like protease inhibitor 6 | 1157 | 32 |
| gi 20348290 | Complement component 2 | 401 | 8 |
| gi 1213490 | C4 complement protein | 231 | 5 |
| gi 20073175 | Complement component 1, s subcomponent | 95 | 2 |
| gi 6753218 | Complement component 1 inhibitor | 104 | 3 |
| gi 11120688 | CD14 antigen | 56 | 2 |
| gi 6978635 | CD59 antigen | 37 | 2 |
| gi 17985951 | Clusterin | 533 | 12 |
| gi 25006237 | GM2 activator protein | 72 | 2 |
| gi 20888903 | Retinol binding protein 4 | 112 | 3 |
| gi 6755911 | Thioredoxin | 123 | 3 |
| gi 203939 | Vitamin D-binding protein | 185 | 12 |
| gi 7657429 | Osteoblast specific factor 2 | 68 | 1 |
| gi 2500778 | Pigment epithelium-derived factor | 81 | 2 |
| gi 47117882 | Reticulocyte binding protein 1 | 36 | 4 |
| gi 7305463 | Small inducible cytokine A7 | 153 | 3 |
| gi 126846 | Small inducible cytokine A2 | 71 | 3 |
| gi 1174697 | Metalloproteinase inhibitor 1 | 130 | 2 |
| gi 6685639 | Macrophage migration inhibitory factor | 63 | 1 |

followed by Lys-C and tryptic digestion, and LC-MS/MS investigation.^{15–22} The analysis of all fractions led to the identification of a total of 183 candidate proteins that are listed in the Supporting Information. Representative adipokines that have been identified in previous studies and in this study are also given in Table 1. All of the proteins reported in the Supporting Information and Table 1 have been processed and identified using this approach. Adipose tissue consists of adipose and stromal-vascular (S-V) cells. The latter are a mixture of several cell types including preadipocytes, macrophages, endothelial, and other cells. By comparison to S-V cells (data not shown), mature adipose cells secrete at least twice as many identifiable proteins, only a few of which are the same, indicating the extensive secretory activity of adipose cells. Since it is possible that some proteins present in the conditioned medium are leaked from dead cells we set a control experiment, i.e., cells were treated with 20 μM KCN for 6 h and proteins identified from control medium were considered as leaked proteins. In this paper, we only report the methodology as proof of principle; detailed biological implications will be given in a separate paper (Chen, X.; Hess, S.; Xiang, C. C.; Ma, L.; Cam, M.; Smith, U.; Cushman, S. W., in preparation). To ensure that only adipokines secreted through endoplasmic reticulum(ER)/Golgi-dependent pathways are reported, each of the 183 candidates was validated for the presence of secretion signal peptides using SignalP. This bioinformatics tool

checks for signaling peptides that are indicative of secretory proteins. Only those 84 proteins that met these strict criteria were considered to be secreted through the ER/Golgi-dependent pathway. Microarrays were carried out to confirm the adipose origin of identified proteins. Among 84 proteins, mRNA expression of 37 proteins that are present on the microarray chip was also detected from adipose cells (data not shown). Five proteins that were not previously associated with adipose cells were further confirmed to be expressed in S–V as well as adipose cells by RT-PCR (data not shown). The majority of these 84 proteins, namely 53, have previously not been identified through proteomics and other studies.^{1,8,13} Thus, the discovery of additional novel secreted proteins through this study significantly increased our understanding of the secretory function of adipose cells. Among the adipokines listed in Table 1 are adiponectin, adipisin, β -2-microglobulin, cystatin C, gelsolin, haptoglobin, and osteonectin that have been previously identified by Mann and/or Wang using mainly gel electrophoresis approaches.^{8,13} Also listed are known adipokines such as angiotensinogen, C-reactive protein, hemopexin, and complement component factor B that have been missed by these gel-based studies, but have been identified in our study,^{1,8,13} and those that have not been described so far.

Using this 2D-LC–MS/MS approach, most adipokines have been identified by two or more peptide matches (Table 1). Adiponectin (also known as adipocyte complement-related protein), for example, has been identified by four different peptide matches. Figure 2a shows an m/z 752.90 with two charge states yielding a molecular weight of 1503.78. After collision-induced fragmentation, the amino acid sequence of AVLFTYDQYQEK was determined as shown in Figure 2b. This sequence represents the tryptic peptide fragment 184–195 from adiponectin. Other tryptic peptide fragments of adiponectin were found with m/z 457.63 (3+), m/z 497.83 (2+), and m/z 591.29 (3+), corresponding to the amino acid sequences VTVPNVPIRFTK (aa 126–137), VTVPNVPIR (aa 126–134), and IFNQNHYDGGSTGK (aa 138–152).

A protein that has previously not been known to be associated with adipose tissue, retinol binding protein 4, was identified by three peptide matches. As shown in Figure 2c an m/z 613.84 was observed with two charge states yielding a molecular weight of 1225.67. The amino acid sequence of YWGVASFLQR (aa 131–140) was determined after collision-induced fragmentation (Figure 2d). The other two tryptic peptide fragments of retinol binding protein 4 were found with m/z 539.29 (2+), and m/z 578.34 (2+), corresponding to the amino acid sequences QEELCLER (aa 197–206) with a deamidated Q and FSGLW-YAIAK (aa 61–70).

In those few cases where only one peptide was identified (matrix metalloproteinase 9, adrenomedullin, osteoblast specific factor 2, and macrophage migration inhibitory factor) the probability score was still greater than 54. To avoid false positives, the sequence of these few single hit identifications was additionally confirmed by de novo-sequencing using MassLynx. A substantial number of proteins were identified by more than five different peptides. Contrapsin-like protease inhibitor 6, for example, was identified by 32 peptide matches yielding a total probability score of 1157. Thus, the confidence in our assignments was generally high.

Out of the 183 candidates, 99 proteins did not meet our validation test for signaling peptides. Therefore, these are listed as nonsecreted proteins. They might, however, have unidentified signal peptides, or might be secreted through endoplasmic

reticulum/Golgi-independent pathways, and/or might be contaminations from cellular material. For instance, Wang et al.¹³ proposed that α -enolase, actin, heat-shock protein 70, and the coatamer protein, together with 11 other proteins, are secreted through endoplasmic reticulum/Golgi-independent pathways as determined through experiments that blocked secretion. Some of these proteins e.g., γ -actin, enolase and peroxiredoxin have also been identified as adipose tissue proteins.³⁶ It is conceivable that proteins that are expressed in the cytoplasm could also be secreted proteins, although pathways for such secreted activity are not known. Nevertheless, these proteins also could be contamination from cellular material. Since we do not currently have evidence of a signaling peptide and to avoid reporting false positives, we list them as nonsecreted proteins.

Only 26 proteins of the 41 candidates that Wang and colleagues discovered through two-dimensional gel electrophoresis and MALDI–MS are secreted through endoplasmic reticulum/Golgi-dependent pathways.¹³ A considerable overlap (16 out of 26) exists between the adipokines identified by Wang et al. and those identified in this study. Adipokines that were missed in our study were mostly the different types (I, II, VI) of procollagens, procollagen C-proteinase enhancer protein, protein-lysine 6-oxidase, complement C3 β chain, galectin, and cyclophilin C. Nevertheless, we have identified an additional 68 adipokines through our approach. To the best of our knowledge, 53 of these 84 adipokines have never been described before to be secreted by adipose cells, neither by proteomics nor by other method.^{1,8,13} However, this likely reflects the 3T3-L1 adipocytes investigated in previous studies, which have different biological characteristics than the primary adipose cells that we used in our study. Although it is presently unknown whether these two different cell types have differences in secretory activity, there are several facts suggesting that. First, 3T3-L1 cell lines and adipose cells undergo different physiological processes. For instance, 3T3-L1 adipocytes have already adapted to the culturing environment during the development, while primary adipose cells are adapting to the environment when cultured in vitro. Second, 3T3-L1 cells are adherent to culture dishes when compared to floating primary adipose cells. Last, among 13 proteins missed by our studies, many of them are collagens that are involved in interaction with substrates, indicating that secretion of collagen is stimulated in the culture of 3T3-L1 cells. Thus, undetection of 13 proteins in our study is most likely due to the difference in cell models rather than proteomics approaches.

The power of our 2D-LC–MS/MS approach is apparent when the number of proteins that have been identified and the number of peptides that were identified for each protein hit are compared between our approach and gel-electrophoresis based proteomics studies.^{8,13} Our approach has several advantages over traditional 2D gel electrophoresis: As a complicated multistep process, 2D gel electrophoresis is considered time-consuming and poorly reproducible at bench-scale. In addition, it has also been recognized that one visible spot does not necessarily equal one protein, but might actually represent any number of proteins that have similar pI 's and molecular weights. It is therefore conceivable that one protein is upregulated and another protein is downregulated in the same spot. The common practice only to analyze those spots that have seemingly changed might thus lead to false conclusions. In addition, it has been reported that membrane proteins, proteins with extrem pI 's and low abundant proteins

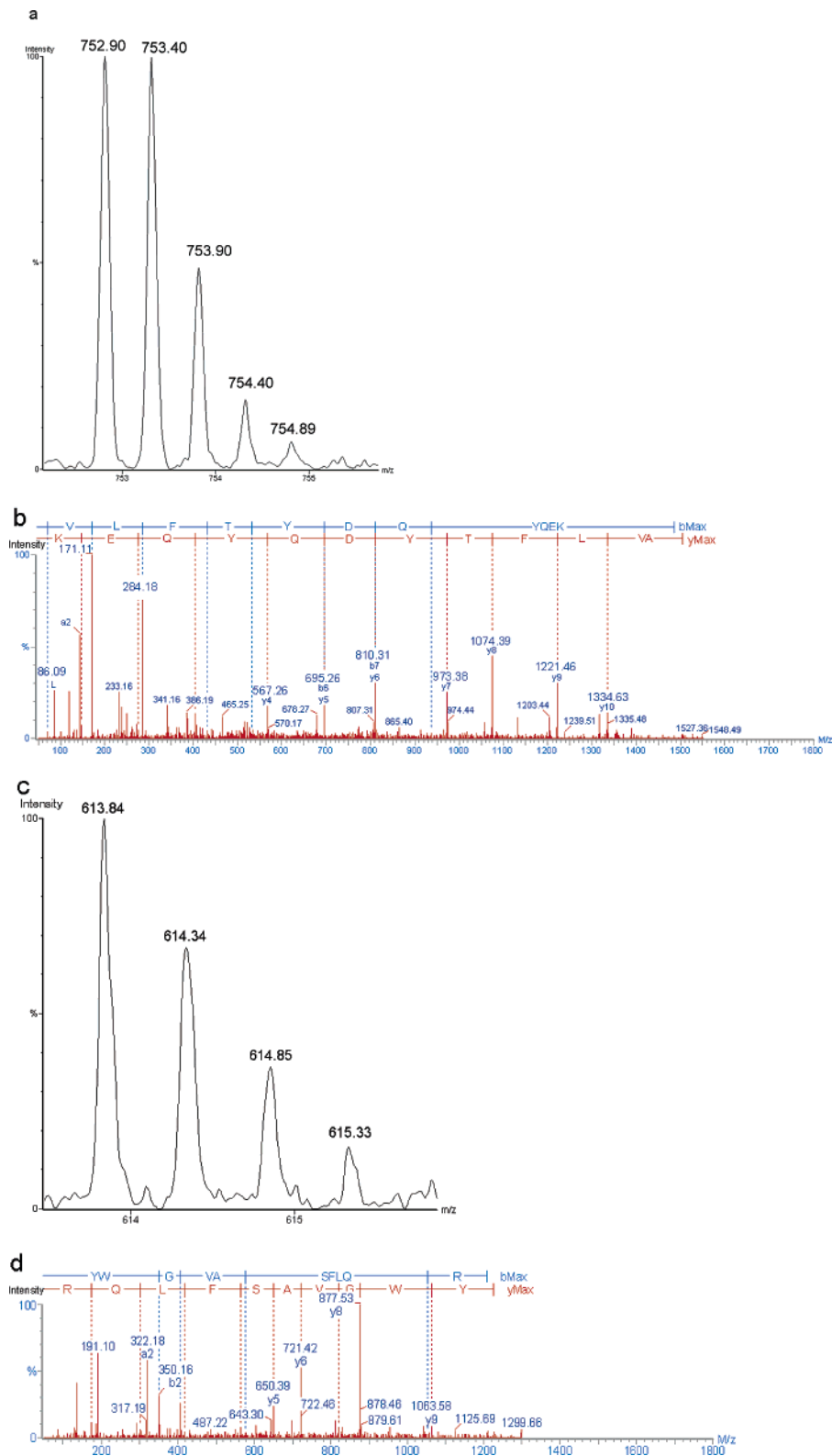


Figure 2. (a) MS spectrum of m/z 752.90 and its MS/MS spectrum (b) showing the fragmentation pattern for the amino acid sequence AVLFTYDQYQEK of adiponectin with the corresponding b and y ion series. (c) MS spectrum of m/z 613.84 and its MS/MS spectrum (d) showing the fragmentation pattern for the amino acid sequence YWGVASFLQR of retinol binding protein 4 with the corresponding b and y ion series.

are underrepresented in 2D gels.³⁷ Advantages of a 2D gel are certainly the high resolving power and the ability to detect

modified proteins, even though the nature and location of the modifications often remain unidentified.

The disadvantages have led to new developments that allow for simpler automation and higher throughput, most prominently the direct analysis of large protein complexes or MudPIT approach.^{15–22} MudPIT uses a total proteome digest that is separated in the first dimension using a strong cation exchange column, followed by a reversed-phase separation of the elute. This allows for automation and thus high throughput, but has the disadvantage of adding more complexity to the analysis since the peptides of one protein might be distributed throughout all fractions. To reduce the complexity of the proteome, we have followed a different path that is so far not widely used in high-throughput proteomics applications.³⁸ We have also used a 2D LC separation, but unlike MudPIT, the first dimensional separation is done using the undigested proteome resulting in 8 combined fractions of *intact* proteins. The secreted proteins are considered to be soluble and thus, precipitation of membrane bound proteins was not expected to occur. Each of these fractions is then double-digested with Lys-C and trypsin, followed by a second dimensional separation using a C18 column online-coupled to a QTOF instrument. The double digestion strategy with Lys-C and trypsin also proved to be advantageous. The endoproteinase Lys-C, which tolerates higher urea concentrations and thus limits the potential of precipitation prior to digestion, cuts C-terminal to Lys. Therefore, it does not add complexity to the following digestion with trypsin. After dilution of the urea, a tryptic digestion increases the sequence coverage. The second dimensional separation of the peptides is coupled online to the QTOF2, and thus fully automated.

The reduction of the complexity of the proteome was identified as the major advantage of this approach. It required less computational power, and greatly increased the confidence in the assignments due to the number of peptides that could be unambiguously assigned to one protein and thus, the obtained probability scores. In preliminary studies, we also found that a number of proteins that contain homologous sequences such as enolase 1, 2, and 3 could appear as three hits when a consensus sequence such as AAVPSGASTGIYEALRLR was determined. The identity of enolase 2 and 3 would only be certain when a unique peptide for these sequences was identified. Unless a unique peptide was identified for a protein, a methodological reductionism was applied according to Occam's Razor theorem and protein identifications were not multiplied unnecessarily. In other words, only those proteins that were uniquely identified were reported (<http://math.ucr.edu/home/baez/physics/General/occam.html>).

Labeling of the Peptides of the Secretory Proteome of Adipose Cells. Because of the proposed role of the adipose cell secretory proteins in altered systemic metabolic states, we were interested in extending this newly established 2D-LC-MS/MS method to quantifying differences in adipose cell secretion under different conditions. As a model system, we chose to examine differences in the conditioned medium of rat adipose cells treated without and with insulin. Principally, two major methods are available for relative quantitation measurements: metabolic isotopic labeling and chemical labeling.³⁹ Even though metabolic labeling is generally considered to be the more accurate of the two procedures, its use is limited to cell cultures and cannot be used in human subjects. Among the chemical labeling methods, the so-called ICAT (Isotope Codes Affinity Tag) technique is a powerful approach.^{39–41} Some limitations include tag fragmentation leading to difficult to

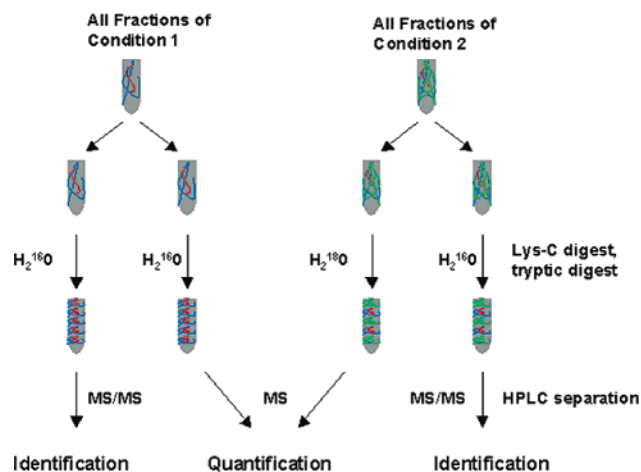


Figure 3. Labeling strategy for the differential quantification of the secretory proteome of rat adipose cells. Proteins from Condition 1 and 2 were split into two aliquots, and digested with trypsin and H_2^{16}O or H_2^{18}O , respectively. The H_2^{16}O digests of Condition 1 and 2 were used for the identification of the proteome of the two conditions. The H_2^{18}O -labeled tryptic peptides of the fractions of Condition 2 were mixed 1:1 with the H_2^{16}O -labeled tryptic peptides of the fractions of Condition 1 and used for differential quantification. This procedure was repeated with all eight fractions.

interpret MS/MS spectra and identification of significantly fewer proteins due to the specific selection of cysteine-containing peptides.^{39,42}

We therefore explored the possibility of using isotopic labeling through proteolytic cleavage with trypsin, which has been described by several groups,^{23–26} but not been applied to quantitation of adipokines in adipose cells. Fenselau et al.³³ recently reported how to successfully dissect the enzymatic cleavage from the labeling procedure by using immobilized trypsin during labeling with H_2^{18}O . We tested this technique with BSA and were able to achieve enrichment higher than 90% (data not shown).

Quantification of the Tryptic Peptides of the Secretory Proteome of Adipose Cells. The labeling strategy that we used is outlined in Figure 3. Secreted proteins from the 8 individual separated fractions were split into two aliquots, and Lys-C- and trypsin-digested, and labeled with H_2^{16}O and H_2^{18}O , respectively. The resulting peptides of the treated and untreated samples were then mixed at a ratio of 1:1 and analyzed in the MS mode, whereas the H_2^{16}O -only labeled peptides were used for identification of the peptides by MS/MS. The data were analyzed by the following steps: first, the elution time and isotopic pattern of particular peptides of identified proteins were used as signatures to find the corresponding peptides in the mixture of ^{16}O - and ^{18}O -labeled samples. Second, the ratios of ^{16}O - and ^{18}O -labeled peptides were calculated. Two methods for calculating the ratios of isotopically distinct peptides were reported and the calculations from two different methods generate very similar values.²⁴ Here, we used one of the methods, calculating the ^{18}O (I_4) to ^{16}O (I_0) ratio for multiple peptides of the same protein, where I_4 and I_0 are the observed base peak intensities for the monoisotopic peak for the peptides with and without ^{18}O label, respectively. Conveniently, the efficiency of the labeling can be checked with autocatalytically cleaved tryptic fragments, which was done with each fraction to ensure sufficient labeling of at least 95%.

Table 2. Quantitation of Representative Secretory Proteins in the Conditioned Medium of Rat Adipose Cells Cultured in the Absence and Presence of Insulin for 48 h

| protein | peptide | <i>m/z</i> _{obs} | ratio (<i>I</i> ₄ / <i>I</i> ₀) ^a |
|------------------------------|-----------------------|---------------------------|--|
| Clusterin | SLLSLEEK | 552.29 | 1.08 ± 0.11 |
| | LFSDPITVVLPEEVSK | 944.46 | |
| Complement factor B | YGLVTYATVPK | 606.34 | 0.39 ± 0.12 |
| | LKDEDLGFL (C-term.) | 525.29 | |
| Complement component 4 | YVLPNFEVK | 554.24 | 0.65 ± 0.09 |
| | LTVQAPPSR | 484.23 | |
| | ATETQGVNLLFSSR | 761.3 | |
| | YIAPCLDSELTEFPLR | 962.49 | |
| Osteonectin | FFETCDLDNDK | 702.31 | 0.44 ± 0.09 |
| | NVLVTLYER | 553.77 | |
| | LHLDYIGPCK | 608.3 | |
| | FIINDWVER | 596.31 | |
| PAI-1 | DVPLSAITNILDAELIR | 926.95 | 0.38 ± 0.10 |
| | AVLDVAETGTEAAAATGKV | 887.47 | |
| Serine protease inhibitor 2c | EVFSTQADLSGITGDK | 834.41 | 0.64 ± 0.04 |
| | IQGLITNLAK | 535.83 | |
| | LINDYVSK | 476.24 | |
| | VIVPNVPIR | 497.76 | |
| Adiponectin | AVLFTYDQYQEK | 752.78 | 1.30 ± 0.10 |
| | SLTLQPDPIVPGDVIVSAEGK | 1117.52 | |
| GM2 | TSIPLTSPQK | 536.27 | 1.13 ± 0.03 |

^a ¹⁸O (*I*₄) to ¹⁶O (*I*₀) ratio, where *I*₄ and *I*₀ are the observed base peak intensities for the monoisotopic peak for the peptides with and without ¹⁸O label, respectively. Results are the means ± sem of the means obtained from all peptides from the same protein in multiple fractions from two independent experiments.

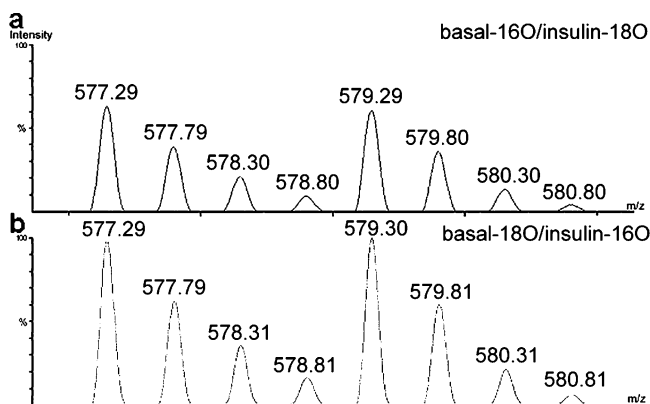


Figure 4. Isotopic pattern of H₂¹⁶O and H₂¹⁸O cross-labeled trypsin peptide SSGTSPDVLK in basal and insulin-treated samples. (a) H₂¹⁶O-labeled basal and H₂¹⁸O-labeled insulin-treated samples were mixed and gave a 1:1 ratio. (b) Similarly, H₂¹⁶O-labeled insulin-treated and H₂¹⁸O-labeled basal samples were mixed and gave the same 1:1 ratio.

Figure 4a shows that a representative ratio of ¹⁶O- to ¹⁸O-labeled peptide SSGTSPDVLK of trypsin (aa 132–142), which is expected to be present at the same concentration in two samples, was about 1:1. This indicated that the labeling efficiencies for ¹⁶O and ¹⁸O were equal between the two samples. To further examine the efficiency for ¹⁶O- and ¹⁸O-labeling, peptides from one fraction of the insulin-treated and untreated samples were cross-labeled with H₂¹⁶O and H₂¹⁸O yielding the same results as shown Figure 4b for the same tryptic fragment.

Figure 5 demonstrates the similar isotopic patterns of two different peptides, namely EVFSTQADLSGITGDK (aa 323–338) and AVLDVAETGTEAAAATGKV (aa 349–367) from serine protease inhibitor 2c (gi 13928716). In general, a total of 14 secreted proteins with valid *I*₄ to *I*₀ ratio were quantified. Two of them were up-regulated, while 11 were down-regulated; one was not changed by insulin stimulation. Table 2 shows the mean ± SEM of insulin (*I*₄) to basal (*I*₀) ratios for the peptides from selected secretory proteins in the conditioned media of rat adipose cells

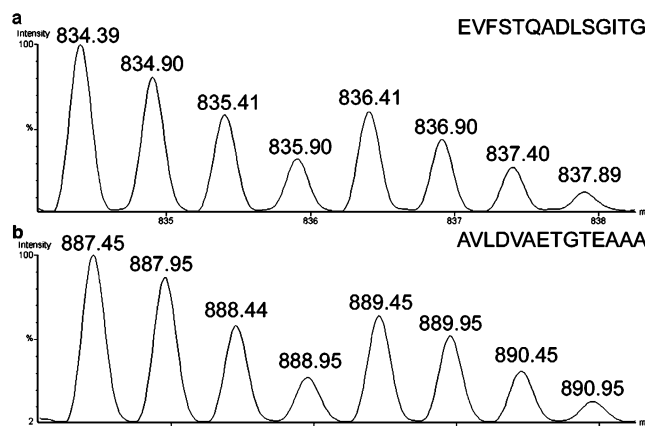


Figure 5. Mass spectra of the ¹⁶O- and ¹⁸O-labeled peptides EVFSTQADLSGITG (A) and AVLDVAETGTEAAA (B) of Serine Protease Inhibitor 2c showing a consistent *I*₄/*I*₀ ratio of 0.64, indicating a reduction of the secretion of Serine Protease Inhibitor 2c after insulin treatment.

from two independent experiments. As shown in Table 2, the ratio values for each individual peptide from one protein in multiple fractions from two independent experiments were averaged. The data clearly demonstrate that the variation of ¹⁸O labeling among peptides from the same protein and between experiments was, with the exception of Complement factor B, generally between 2% and 20% (mean 10%). This clearly shows that the proposed methodology is capable of quantifying the up- or down-regulation trend upon insulin treatment. In no case was a contradictory trend found for the individual peptides of one protein. Next to the expected biological variations, the variations that were found reflect the minimum criteria set for complete exchange of ¹⁶O with ¹⁸O. To minimize the contributions of the ¹³C isotopes, only doubly charged ions and no higher charged ions were considered for the quantification.

The levels of clusterin were not significantly different between basal and insulin-treated samples. Adiponectin and GM2 levels were slightly increased with insulin treatment, whereas

insulin reduced the secretion of complement factor B, complement component 4, PAI-1, osteonectin, and serine protease inhibitor 2c. Importantly, insulin-stimulated adiponectin secretion quantified by our ^{18}O -labeling method is consistent with the results measured by radioimmunoassay (RIA) from a study with human cultured adipose cells treated with insulin for 24 h.⁴³ In another study, insulin treatment reportedly suppresses the increased plasma levels of PAI-1 seen in ST-segment-elevation myocardial infarction (STEMI) patients, consistent with our results, that PAI-1 secreted into the culture medium is decreased in the presence of insulin.⁴⁴ All this together indicates that the ^{18}O -labeling methodology used to measure the secretion of adipokines is sound and solid.

Taken together, we have been able to identify the secretory proteome of rat adipose cells. It is obvious that our 2D-LC-MS/MS approach is more powerful than the gel electrophoresis-based proteomics studies in terms of the following aspects: first, the number of proteins identified by our approach is larger than that by any reported gel electrophoresis-based method. Second, the proteins identified using our approach have a larger number of peptides matched with higher statistical confidence. Third, our approach is truly quantitative. Both the profiling and the monitored changes upon insulin treatment lead to an improved understanding of the endocrine function of adipose cells, the role of adipose cells in several pathophysiological states, and to the identification of potential sites of therapeutic intervention.

Acknowledgment. The authors declare that they have no competing financial interests.

Supporting Information Available: Analysis of all of the fractions leading to the identification of the 183 candidate proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- Ahima, R. S.; Flier, J. S. *Trends Endocrinol. Metab.* **2000**, *11*, 327–332.
- Goldfine, A. B.; Kahn, C. R. *Lancet* **2003**, *362*, 1431–1432.
- Steppan, C. M.; Lazar, M. A. *Trends Endocrinol. Metab.* **2002**, *13*, 18–23.
- Rajala, M. W.; Scherer, P. E. *Endocrinology* **2003**, *144*, 3765–3773.
- Havel, P. J. *Diabetes* **2004**, *53 Suppl. 1*, S143–151.
- Lan, H.; Rabaglia, M. E.; Stoehr, J. P.; Nadler, S. T.; Schueler, K. L.; Zou, F.; Yandell, B. S.; Attie, A. D. *Diabetes* **2003**, *52*, 688–700.
- Nadler, S. T.; Stoehr, J. P.; Schueler, K. L.; Tanimoto, G.; Yandell, B. S.; Attie, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11371–11376.
- Kratchmarova, I.; Kalume, D. E.; Blagoev, B.; Scherer, P. E.; Podtelejnikov, A. V.; Molina, H.; Bickel, P. E.; Andersen, J. S.; Fernandez, M. M.; Bunkenborg, J.; Roepstorff, P.; Kristiansen, K.; Lodish, H. F.; Mann, M.; Pandey, A. *Mol. Cell. Proteomics* **2002**, *1*, 213–222.
- Sidhu, R. S. *J. Biol. Chem.* **1979**, *254*, 11111–11118.
- Spiegelman, B. M.; Green, H. *J. Biol. Chem.* **1980**, *255*, 8811–8818.
- Wilson-Fritch, L.; Burkart, A.; Bell, G.; Mendelson, K.; Leszyk, J.; Nicoloro, S.; Czech, M.; Corvera, S. *Mol. Cell. Biology* **2003**, *23*, 1985–1094.
- Tsuruga, H.; Kumagai, H.; Kojima, T.; Kitamura, T. *Biochem. Biophys. Res. Commun.* **2000**, *272*, 293–297.
- Wang P.; Mariman, E.; Keijer, J.; Bouwman, F.; Noben, J.-P.; Robben, J.; Renes, J. *Cell. Mol. Life Sci.* **2004**, *61*, 2405–2417.
- Scherer, P. E.; Bickel, P. E.; Kotler, M.; Lodish, H. F. *Nat. Biotechnol.* **1998**, *16*, 5481–5486.
- Washburn, M. P.; Wolters, D.; Yates, J. R. 3rd. *Nat. Biotechnol.* **2001**, *19*, 242–247.
- Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. 3rd. *Nat. Biotechnol.* **1999**, *17*, 676–682.
- Wolters, D. A.; Washburn, M. P.; Yates, J. R. 3rd. *Anal. Chem.* **2001**, *73*, 5683–5690.
- Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R. 3rd. *Anal. Chem.* **2002**, *74*, 1650–1657.
- MacCoss, M. J.; McDonald, W. H.; Saraf, A.; Sadygov, R.; Clark, J. M.; Tasto, J. J.; Gould, K. L.; Wolters, D.; Washburn, M.; Weiss, A.; Clark, J. I.; Yates, J. R. 3rd. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7900–7905.
- Liu, H.; Lin, D.; Yates, J. R. 3rd. *Biotechniques* **2002**, *32*, 898–902.
- Sanders, S. L.; Jennings, J.; Canutescu, A.; Link, A. J.; Weil, P. A. *Mol. Cell Biol.* **2002**, *22*, 4723–4738.
- Peng, J.; Elias, J. E.; Thoreen, C. C.; Licklider, L. J.; Gygi, S. P. *J. Proteome Res.* **2003**, *2*, 43–50.
- Stewart, I. I.; Thomson, T.; Figeys, D. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2456–2465.
- Mirgorodskaya, O. A.; Kozmin, Y. P.; Titov, M. I.; Korner, R.; Sonksen, C. P.; Roepstorff, P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1226–1232.
- Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. *Anal. Chem.* **2001**, *73*, 2836–2842.
- Reynolds, K. J.; Yao, X.; Fenselau, C. *J. Proteome Res.* **2002**, *1*, 27–33.
- Flory, M. R.; Griffin, T. J.; Martin, D.; Aebersold, R. *Trends Biotech.* **2002**, *20*, S23–S29.
- Aebersold, R. *J. Infect. Dis.* **2003**, *187*, S315–320.
- Krijgsveld, J.; Ketting, R. F.; Mahmoudi, T.; Johansen, J.; Artal-Sanz, M.; Verrijzer, C. P.; Plasterk, R. H.; Heck, A. J. *Nat. Biotechnol.* **2003**, *21*, 927–931.
- Hess, S.; van Beek, J.; Pannell, L. K. *Anal. Biochem.* **2002**, *311*, 19–26.
- Schnolzer, M.; Jedrzejewski, P.; & Lehmann, W. D. *Electrophoresis* **1996**, *17*, 945–953.
- Shevchenko, A.; Chernushevich, I.; Ens, W.; Standing, K. G.; Thomson, B.; Wilm, M.; Mann, M. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1015–1024.
- Yao, X.; Afonso, C.; Fenselau, C. *J. Proteome Res.* **2003**, *2*, 147–152.
- Quon, M. J.; Guerre-Millo, M.; Zarnowski, M. J.; Butte, A. J.; Em, M.; Cushman, S. W.; Taylor, S. I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5587–5591.
- Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20*, 3551–3567.
- Corton M.; Villuendas, G.; Botella, J. I.; San Milan J. L.; Escobar-Morreale H. F.; Peral, B. *Proteomics* **2004**, *4*, 438–441.
- Frey S. J.; Larsen, P. M. *Curr. Opin. Chem. Biol.* **2001**, *5*, 26–33.
- Chong, B. E.; Hamler, R. L.; Lubman, D. M.; Ethier, S. P.; Rosenspire, A. J.; Miller, F. R. *Anal. Chem.* **2001**, *73*, 1219–1227.
- Lill, J. *Mass Spectrom. Rev.* **2003**, *22*, 182–194.
- Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994–999.
- Smolka, M. B.; Zhou, H.; Purkayastha, S.; Aebersold, R. *Anal. Biochem.* **2001**, *297*, 25–31.
- MacCoss, M. J.; Yates, J. R., III. Proteomics: Analytical tools and techniques. *Curr. Opin. Clin. Nutr. Metab. Care* **2001**, *4*, 369–375.
- Motoshima, H.; Wu, X.; Sinha, M. K.; Hardy, V. E.; Rosato, E. L.; Barbot, D. J.; Rosato, F. E.; Goldstein, B. J. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 5662–5667.
- Chaudhuri, A.; Janicke, D.; Wilson, M. F.; Tripathy, D.; Garg, R.; Bandyopadhyay, A.; Calieri, J.; Hoffmeyer, D.; Syed, T.; Ghanim, H.; Aljada, A.; Dandona, P. *Circulation* **2004**, *109*, 849–854.

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