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Quantitative Analysis of Acrylamide Labeled Serum Proteins by LC−MS/MS

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Abstract: Isotopic labeling of cysteine residues with acrylamide was previously utilized for relative quantitation of proteins by MALDI-TOF. Here, we explored and compared the application of deuterated and ^{13}C isotopes of acrylamide for quantitative proteomic analysis using LC−MS/MS and high-resolution FTICR mass spectrometry. The method was applied to human serum samples that were immunodepleted of abundant proteins. Our results show reliable quantitation of proteins across an abundance range that spans 5 orders of magnitude based on ion intensities and known protein concentration in plasma. The use of ^{13}C isotope of acrylamide had a slightly greater advantage relative to deuterated acrylamide, because of shifts in elution of deuterated acrylamide relative to its corresponding nondeuterated compound by reversed-phase chromatography. Overall, the use of acrylamide for differentially labeling intact proteins in complex mixtures, in combination with LC−MS/MS provides a robust method for quantitative analysis of complex proteomes.

acrylamide isotope labeling • LTQ−FTICR • human serum

Introduction

Numerous methods have been introduced for quantitative analysis of proteins by mass spectrometry (MS). The choice of method for MS-based quantitation depends on the nature of the application and type of sample of interest. Intact cells can be labeled in vivo using cell culture media enriched with ^{15}N, or stable isotopes of amino acids. Alternatively, labeling can be performed with various reagents during or after enzymatic digestion, although samples to be compared have to be processed separately until the labeling step, which may introduce artifactual variations. Quantitative proteomics is reviewed in Ong and Mann, Sechi and Oda, and Julka and Regnier.[1−3]

Plasma proteome analysis represents a major challenge for quantitative proteomics because of the wide range of protein concentration and the occurrence of multiple isoforms that may need to be separately quantified. Our group has implemented an intact-protein based approach to serum profiling, with extensive fractionation of nondigested proteins to reduce sample complexity prior to mass spectrometry and to allow separation of isoforms and increased depth of analysis.[4] Among the amino acids that could be tagged in an intact-protein based approach, cysteine is a good target because it occurs in some 96% of all human proteins and in ∼27% of tryptic peptides.[5] Cysteine is efficiently alkylated by several classes of reagents,[6] and has been widely used in protein chemistry to facilitate enzymatic digestion and to prevent protein refolding.[9] Because of these advantages, the cysteine alkylation reaction has been extensively used to tag proteins with stable isotopes for mass spectrometry based quantitative analysis.[7−10]

While cysteine alkylation with acrylamide is an undesired reaction that frequently occurs during polyacrylamide gel electrophoresis,[11] there are several properties that make acrylamide a very useful tagging reagent for quantitative studies that rely on isotope labeling. First, it is a small reagent (mass = 71) that does not introduce significant mass shift or charge changes in the protein and does not negatively affect protein solubility, since it is an hydrophilic tag; second, the mass shift at the peptide level is also minimal, resulting in relative simple MS/MS spectra, compared to the effect of large tagging reagents such as isotope-coded affinity tags (ICAT);[7] third, the reaction is performed using standard protein solubilization solutions and with a virtually 100% yield; additionally the reagents are relatively inexpensive, making it practical to perform experiments starting with large amounts of protein as needed for extensive fractionation and in-depth analysis.

Acrylamide alkylation has been previously described as one way to obtain quantitation information by matrix assisted laser desorption/ionization- time-of-flight (MALDI-TOF) for proteins separated by gel electrophoresis.[8,9] In this study, we investigated differential labeling of cysteine residues of human serum samples depleted of abundant proteins with acrylamide and two different isotopes (2,3,3′-D_3-acrylamide, 1,2,3-$^{13}C_3$-acrylamide), for identification and quantitative analysis using reversed-phase liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS) and high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. We demonstrated that plasma proteins, most of which are globular and contain several cysteine residues, are quite suitable for alkylation with isotopes of acrylamide. Alkylation with acrylamide did not introduce detectable changes in protein solubility or difficulties in obtaining good collision induced dissociation (CID) spectra of labeled peptides. The use of a high sensitivity and high-resolution mass spectrometer such as the LTQ−FTICR provided excellent MS data, facilitating extraction of reliable relative quantitation for a wide range of serum proteins.

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Materials and Methods

Sample Preparation. A pool of sera from three healthy subjects was chromatographically immunodepleted of the top six most abundant proteins using HU-6 columns (4.6 × 100 mm; Agilent, Wilmington, DE) as previously described.[6] A total of 400 μg of immunodepleted sample, equivalent to 65μL of the sera pool was concentrated using an Amicon YM-3 device and rediluted in 8 M urea, 30 mM Tris pH 8.5, 0.5% OG (octyl-beta-D-glucopyranoside)(w/v). The reduction was performed by adding 0.66 mg dithiotreitol (DTT) per mg of protein and the reaction was carried out at room temperature for 2 h. Samples were split into three aliquots and alkylated with acrylamide, 2,3,3′-D_3-acrylamide, 1,2,3-$^{13}C_3$-acrylamide, designated here D0, D3, or ^{13}C-acrylamide. Acrylamide (>99.5% purity) was purchased from Fluka. Isotopes of acrylamide were acquired from Cambridge isotope laboratories (Andover, MA) with minimum chemical purity of 98%. Alkylation was performed by adding 7.1 mg of D0-acrylamide or 7.4 mg of D3 or ^{13}C-acrylamide per mg of total protein. This protocol was adapted from Sechi et al.[8,9] and the amount of acrylamide represents 25-fold molar excess over DTT. The reaction mixture was incubated in the dark for 1 h at room temperature. D3 or ^{13}C-acrylamide labeled samples were mixed at 1:1 ratio with D0-acrylamide and immediately cleaned-up on a reversed-phase trap column (2 × 10 mm packed with Poros R1, Applied Biosystems). The same conditions and reagent proportions were used to reduce and alkylate 50 μg of bovine serum albumin. Different ratios (1:1, 1:3 1:5 and 1:10) of D0 or D3-acrylamide alkylated albumin were used to evaluate the method.

Protein Digestion And Mass Spectrometric Analysis. Samples were resuspended in 0.25 M urea containing 50 mM ammonium bicarbonate and 4% of acetonitrile (v/v) and digested overnight with 2 μg of modified trypsin (Promega). The digestion was interrupted by addition of 5 μL of 10% formic acid solution (v/v). Samples were analyzed in a LTQ−FTICR mass spectrometer (Thermo-Finnigan) coupled with a nano-Acquity UPLC chromatography system (Waters). Liquid chromatography separation was performed in a 25 cm column (Picofrit 75 μm ID, New Objectives, in house-packed with MagicC18AQ resin) using a 140 min linear gradient from 5 to 40% of acetonitrile in 0.1% formic acid at 250 nL/min or 60 min gradient for bovine serum albumin (BSA). The spectra were acquired in a data-dependent mode in m/z range of 400 to 1800, with selection of the 5 most abundant +2 or +3 ions of each MS spectrum for MS/MS analysis. Mass spectrometer parameters were as follows: capillary voltage of 3.2 KV, capillary temperature of 200 °C, resolution of 100 000 and FT target value of 2 × 10^6. Acquired data was automatically processed by the Computational Proteomics Analysis System−CPAS,[12] using the Comet search algorithm. Minimum criteria for peptide matching was Peptide Prophet Score greater than 0.2. Peptides that met these criteria were further grouped to protein sequences using the Protein Prophet algorithm at an error rate of 5% or less.[13] For all databank searches, D0-acrylamide alkylation was considered as a fixed modification and heavy isotope labeled peptides were detected using a delta mass of 3.01884 and 3.01006 Da for D3 and ^{13}C-acrylamide, respectively.

Quantitation Algorithm. Acrylamide ratios were determined using a script designated "Q3" developed in-house to obtain the relative quantities for each pair of peptides identified by MS/MS that contained cysteine residues. Essentially, the algorithm reconstructs the ion chromatogram for both light (D0) and heavy (D3 or ^{13}C) forms for each identified peptide containing cysteine residues and computes the intensities for each form. More specifically, from the MS/MS peptide identification list, peptides containing cysteine with Peptide Prophet scores greater than 0.75 were selected. We then obtained the theoretical mass-charge of the monoisotopic light and monoisotopic heavy labeled peptides as well as the theoretical mass-charge of each respective ^{13}C isotope peak. Since the mass difference between light and heavy is approximately three times (3.01884 or 3.01006 for D3-acrylamide or ^{13}C-acrylamide, respectively) the number of cysteines present in the peptide, the analysis was restricted to either the monoisotopic and the first 2 ^{13}C isotopic peaks of the light form (if there is one cysteine) or the first 5 ^{13}C isotopic peaks (if there were two or more cysteines) and their corresponding peaks from the heavy form. The intensity of peaks in each MS scan was computed by centering the MS scan to the location of its maximum closest to the theoretical m/z for each peak within a 25 ppm window. Each light peak was paired with the corresponding heavy peak and if both peaks were present, we considered this a match. In general, since peaks occasionally occur stochastically, only matched peaks contributed to the quantitation. For the final quantitation, we used the primary scan (i.e., the one that immediately preceded the MS/MS identification) as well as a certain number of MS scans immediately before and after the primary scan, up to a limit of 10 scans. For a scan without any matched peaks, this scan and any subsequent scan in both directions was excluded. As a special case applied to low intensity peaks, if no more than one matched pair of ions was found in the primary scan, then all of the isotopic peaks found were used, whether matched or not (i.e., if the monoisotopic peak of both heavy and light form matched, but the heavy form also presented the first ^{13}C isotope, then we considered the sum of both peaks from the heavy and only the monoisotopic peak from the light form in the final quantiation). The resulting ratio was adjusted slightly for all the peptides with mass greater than 1800 Da by subtracting from the derived quantity of the heavy form, the fraction that can be expected to result from overlapping isotopes of the light form. Proteins containing more than one pair of peptides that yielded quantitation had all the ratios

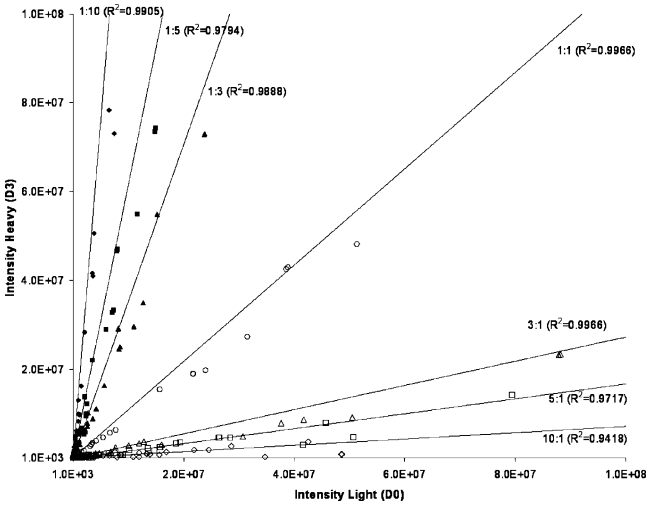

Figure 1. Quantitation of different ratios of BSA. The graph shows the linear distribution of an average of 28 data points for each of the 7 different ratios of BSA analyzed. Although the data indicates greater variability for extreme ratios (1:5, 1:10, and 10:1) all the data points present good correlation coefficients (R2).

Table 1. Serum Proteins Identified and Quantified using Isotopic Alkylation with Acrylamide[g]

	IPI[a]	description	gene name	concentration in plasma (ng/mL)[b]	D3/D0 unique peptide[c]	D3/D0 ratio[d]	D3/D0 ratio stdev[d]	13C/D0 unique peptide[c]	13C/D0 ratio[d]	13C/D0 ratio stdev[d]
1	IPI00164623	complement C3 precursor	C3	9.5E+05	175	1.17	0.12	90	1.02	0.01
2	IPI00478003	alpha-2-macroglobulin precursor	A2M	1.4E+06	126	1.14	0.08	89		
3	IPI00556148	complement factor H	CFH		102	1.13	0.06	110	0.96	0.16
4	IPI00418163	complement C4 precursor	C4	1.7E+05	114			80		
5	IPI00555812	vitamin D-binding protein,	GC		88			87		
6	IPI00298828	beta-2-glycoprotein I precursor	APOH		53	1.17	0.01	79	1.06	0.04
7	IPI00019580	plasminogen precursor	PLG	1.4E+05	76	1.19	0.08	56	1.02	0.04
8	IPI00017601	ceruloplasmin precursor	CP	2.1E+05	81	1.14	0.00	43	1.08	0.05
9	IPI00019568	prothrombin precursor	F2		63	1.13	0.03	54	1.00	0.04
10	IPI00021841	apolipoprotein A−I precursor	APOA1	1.4E+06	47			70		
11	IPI00022488	hemopexin precursor	HPX	7.5E+05	55	1.08	0.02	47	1.02	0.03
12	IPI00032328	kininogen precursor	KNG		51	1.12	0.05	48	1.05	0.11
13	IPI00019591	complement factor B precursor	BF		48	1.18	0.10	49	1.04	0.03
14	IPI00021727	C4b-binding protein alpha chain precursor	C4BPA		44	1.38	0.18	39	0.93	
15	IPI00294193	inter-alpha-trypsin inhibitor heavy chain H4 precursor	ITIH4		51			31		
16	IPI00022418	fibronectin; cold-insoluble globulin; migration-stimulating factor	FN1		45	0.89	0.13	35	1.12	0.18
17	IPI00022463	serotransferrin precursor*	TF	2.3E+06	49	1.25	0.11	30	1.03	0.04
18	IPI00022895	alpha-1B-glycoprotein precursor	A1BG		29	1.15	0.13	45	1.01	0.03
19	IPI00022229	apolipoprotein B-100 precursor	APOB	7.2E+05	63	1.04	0.03	10	0.94	0.02
20	IPI00019943	afamin precursor	AFM		35	1.11	0.16	27	1.12	0.24
21	IPI00026314	gelsolin precursor	GSN		29	1.25	0.12	31	0.89	0.06
22	IPI00022431	alpha-2-HS-glycoprotein precursor	AHSG		29	1.07	0.02	30	1.04	0.03
23	IPI00032179	antithrombin-III precursor	SERPINC1	3.2E+05	37	0.92	0.09	19	0.95	0.08
24	IPI00022434	serum albumin precursor	ALB	4.0E+07	43	1.05	0.03	12	0.96	0.19
25	IPI00305461	inter-alpha-trypsin inhibitor heavy chain H2 precursor	ITIH2		36	0.76		15	0.77	
26	IPI00292530	inter-alpha-trypsin inhibitor heavy chain H1 precursor	ITIH1		38	0.97	0.03	12		
27	IPI00304273	apolipoprotein A-IV precursor	APOA4		24			25		
28	IPI00022426	AMBP protein precursor	AMBP		28	1.18	0.11	21	0.95	0.01
29	IPI00478493	haptoglobin precursor e	HP	8.8E+05	22	1.40	0.46	24	1.08	
30	IPI00550991	alpha-1-antichymotrypsin precursor	-		37			8		
31	IPI00021854	apolipoprotein A-II precursor	APOA2	3.0E+05	19	1.17	0.01	27	1.20	0.08
32	IPI00032291	complement C5 precursor	C5		37	0.95	0.08	7	1.00	0.03
33	IPI00022371	histidine-rich glycoprotein precursor	HRG		27	1.00	0.08	17	0.98	0.14
34	IPI00291867	complement factor I precursor	IF		26	0.97	0.02	17	0.88	
35	IPI00292950	heparin cofactor II precursor	SERPIND1		21	1.22	0.43			
36	IPI00479867	similar to Complement C1r component precursor	C1R		29	1.07	0.04	13	1.01	0.16
37	IPI00298971	vitronectin precursor	VTN		21	1.10	0.07	20	0.93	0.03
38	IPI00009920	complement component C6 precursor	C6		23	1.29	0.24	18	1.12	0.19
39	IPI00008558	plasma kallikrein precursor	KLKB1		22	1.11	0.02	16	1.01	0.04
40	IPI00022432	transthyretin precursor	TTR	2.6E+05	16			22		
41	IPI00007240	coagulation factor XIII B chain precursor	F13B		25	1.07	0.06	12	1.00	0.07
42	IPI00022429	alpha-1-acid glycoprotein 1 precursor	ORM1	6.1E+05	17	1.00	0.10	19	0.98	0.02
43	IPI00020091	alpha-1-acid glycoprotein 2 precursor	ORM2	6.1E+05	18	1.07	0.02	17	0.94	0.04
44	IPI00011252	complement component C8 alpha chain precursor	C8A		21	0.90	0.03	14	1.15	0.43
45	IPI00291262	clusterin precursor	CLU		15	1.41	0.30	17	1.03	0.01
46	IPI00291866	plasma protease C1 inhibitor precursor	SERPING1		21	1.21	0.20	12		
47	IPI00166729	alpha-2-glycoprotein 1, *z*inc	AZGP1		14	1.02	0.06	17	0.95	0.00
48	IPI00021885	fibrinogen alpha/alpha-E chain precursor	FGA		17	1.20	0.24	12	0.94	
49	IPI00017696	complement C 1s subcomponent precursor	C1S		15	1.26	0.03	14	1.06	0.03
50	IPI00025426	pregnancy zone protein precursor	PZP		13	1.07	0.17			
51	IPI00296608	complement component C7 precursor	C7		16	1.08	0.03	9	1.05	0.27
52	IPI00022395	complement component C9 precursor	C9		15	1.05	0.19	10	1.06	0.20
53	IPI00163207	*N*-acetylmuramoyl-L-alanine amidase precursor	PGLYRP2		14	1.03	0.01	11	1.01	0.12
54	IPI00021842	apolipoprotein E precursor	APOE	3.4E+04	16			8	0.79	0.08
55	IPI00022445	platelet basic protein precursor	PPBP		10	1.05	0.06	13	1.07	0.05
56	IPI00021364	properdin precursor	PFC		6	1.05	0.02	17	0.96	0.07
57	IPI00006662	apolipoprotein D precursor	APOD		8	0.95	0.05	15	0.88	0.10
58	IPI00294395	complement component C8 beta chain precursor	C8B		13	1.08	0.16	9	0.92	0.04

Table 1. (Continued)

	IPI[a]	description	gene name	concentration in plasma (ng/mL)[b]	D3/D0 unique peptide[c]	D3/D0 ratio[d]	D3/D0 ratio stdev[d]	13C/D0 unique peptide[c]	13C/D0 ratio[d]	13C/D0 ratio stdev[d]
59	IPI00009028	tetranectin precursor	TNA		16	1.10	0.04	6	1.08	0.07
60	IPI00022420	plasma retinol-binding protein precursor	RBP4		14	1.13	0.04	6	0.90	0.02
61	IPI00385058	hypothetical protein	-		11	1.05	0.04	9	1.07	0.03
62	IPI00218732	serum paraoxonase/arylesterase 1	PON1		14	1.33	0.11	6	0.95	0.03
63	IPI00294004	vitamin K-dependent protein S precursor	PROS1		14	1.01	0.06	6	1.23	0.28
64	IPI00032220	angiotensinogen precursor	AGT		15			4		
65	IPI00019581	coagulation factor XII precursor	F12		12	1.06	0.01	7	1.18	0.15
66	IPI00027235	attractin precursor	ATRN		13	0.98	0.16	5	0.85	0.03
67	IPI00218816	HBB protein	HBB		11	1.11	0.11	8		
68	IPI00022446	Row70Platelet factor 4 precursor	PF4		7	1.06	0.04	11	1.03	0.03
69	IPI00029863	alpha-2-antiplasmin precursor	SERPINF2		12	0.95		5	0.84	0.01
70	IPI00011261	complement component C8 gamma chain precursor	C8G		13	1.00	0.08	4	1.00	
71	IPI00218746	complement component 1, *q* subcomponent,	-		12	1.06	0.06	4		
72	IPI00020996	insulin-like growth factor binding protein complex acid labile chain	IGFALS		13	1.43	0.34	3	1.02	0.13
73	IPI00061977	MGC27165 protein	IGHA1		9	1.09	0.08	7	1.05	0.07
74	IPI00019399	serum amyloid A-4 protein precursor	SAA4		6			10		
75	IPI00030739	apolipoprotein M	APOM		9	1.22		6	1.15	
76	IPI00303963	complement C2 precursor	C2		12	1.25	0.00	3	1.03	
77	IPI00022392	complement C1q subcomponent, A chain precursor	C1QA		7	1.10				
78	IPI00006114	pigment epithelium-derived factor precursor	SERPINF1		10	1.13		4		
79	IPI00292946	thyroxine-binding globulin precursor	SERPINA7		12			2		
80	IPI00328609	kallistatin precursor	SERPINA4		12	1.01	0.01	2		
81	IPI00021856	apolipoprotein C−II precursor	APOC2		7			6		
82	IPI00166930	carboxypeptidase N 83 kDa chain (regulatory subunit)	-		11	0.93	0.20	2		
83	IPI00020986	lumican precursor	LUM		11			2		
84	IPI00021857	apolipoprotein C−III precursor	APOC3		6			7		
85	IPI00410714	hemoglobin alpha-1 globin chain	HBA1		6			7		
86	IPI00022417	leucine-rich alpha-2-glycoprotein precursor	LRG1		7	0.98	0.11	5	1.51	
87	IPI00029168	apolipoprotein(a) precursor	LPA		5	0.99	0.02	6	0.89	0.04
88	IPI00218803	fibulin-1 precursor	FBLN1		7	1.07		4	0.75	
89	IPI00021855	apolipoprotein C−I precursor	APOC1		6			5		
90	IPI00025864	cholinesterase precursor	BCHE	8		1.00	0.03	3		
91	IPI00027507	complement factor H-related protein 3 precursor	CFHL3		3	1.10	0.03	8	1.01	0.03
92	IPI00041065	HGF activator like protein	HABP2		6	1.01	0.09	5	0.92	0.03
93	IPI00399007	Ig gamma-2 chain C region	IGHG2		6	1.02	0.05	5	0.96	0.06
94	IPI00382937	IGHM protein	-		6	0.95	0.02	5	1.23	0.34
95	IPI00025862	C4b-binding protein beta chain precursor	C4BPB		6	1.03	0.10	4	0.87	0.13
96	IPI00168728	FLJ00385 protein	FLJ00385		7	1.08	0.02	4	0.97	0.01
97	IPI00386158	hypothetical protein	-					5		
98	IPI00220327	keratin, type II cytoskeletal 1	KRT1		5					
99	IPI00005439	fetuin-B precursor	FETUB		7	1.37	0.09	3	1.26	0.03
100	IPI00296170	haptoglobin-related protein	hpr		9	1.21	0.04	1	1.01	0.09
101	IPI00029193	hepatocyte growth factor activator precursor	HGFAC		7	1.04	0.04	3	0.91	0.10
102	IPI00305457	alpha-1-antitrypsin precursor	SERPINA1		8			2		
103	IPI00218413	biotinidase precursor [*Homo sapiens*]	BTD		5	0.97				
104	IPI00003351	extracellular matrix protein 1 precursor	ECM1		5	1.15	0.11	4	1.45	0.16
105	IPI00024825	megakaryocyte stimulating factor	PRG4		3			6		
106	IPI00029061	selenoprotein P precursor	SEPP1		4			5	0.83	
107	IPI00027482	corticosteroid-binding globulin precursor	SERPINA6		4					
108	IPI00154742	IGLC1 protein	IGLC1		5			4	1.08	0.05
109	IPI00009865	keratin, type I cytoskeletal 10	KRT10		4					
110	IPI00299503	phosphatidylinositol-glycan-specific phospholipase D 1 precursor	GPLD1		6			3		
111	IPI00177869	apolipoprotein-L1 precursor	APOL1		5			3		
112	IPI00006543	complement factor H-related protein 5 precursor	CFHL5		5	1.39	0.26	3	1.01	0.08
113	IPI00292218	hepatocyte growth factor-like protein precursor	MST1		5	1.23		3	0.89	
114	IPI00018305	insulin-like growth factor binding protein 3 precursor	IGFBP3	5.9E+01	3			5		

Table 1. (Continued)

	IPI[a]	description	gene name	concentration in plasma (ng/mL)[b]	D3/D0 unique peptide[c]	D3/D0 ratio[d]	D3/D0 ratio stdev[d]	13C/D0 unique peptide[c]	13C/D0 ratio[d]	13C/D0 ratio stdev[d]
115	IPI00296176	coagulation factor IX precursor	F9		5	1.13	0.07	2	0.92	
116	IPI00006154	complement factor H-related protein2 precursor	CFHL2		4	1.06	0.03	4	1.04	0.01
117	IPI00061246	hypothetical protein	-		5	1.01	0.02	2	1.04	0.06
118	IPI00026199	plasma glutathione peroxidase precursor	GPX3		4	0.97	0.15			
119	IPI00299435	apolipoprotein F [*Homo sapiens*]	APOF					3	1.13	0.15
120	IPI00022937	coagulation factor V	F5		3	0.81				
121	IPI00008556	coagulation factor XI precursor	F11		4	0.96	0.02	3	1.09	0.02
122	IPI00297550	coagulation factor XIII A chain precursor	F13A1					3		
123	IPI00293925	ficolin 3 precursor	FCN3		3	1.00				
124	IPI00028413	inter-alpha-trypsin inhibitor heavy chain H3 precursor	ITIH3		5			2		
125	IPI00032311	lipopolysaccharide-binding protein precursor	LBP		3					
126	IPI00027350	peroxiredoxin 2	PRDX2		3					
127	IPI00007221	plasma serine protease inhibitor precursor	SERPINA5		3					
128	IPI00419630	tax_Id = 9606 DPKL1915	-		3					
129	IPI00296099	thrombospondin-1 precursor	THBS1		4			2		
130	IPI00021817	vitamin K-dependent protein C precursor	PROC		3	1.07	0.13			
131	IPI00022394	complement C1q subcomponent, C chain precursor	C1QG		4			2		
132	IPI00022391	serum amyloid P-component precursor	APCS		4			2		
133	IPI00028030	cartilage oligomeric matrix protein precursor	COMP		3	1.09	0.17			
134	IPI00011264	complement factor H-related protein 1 precursor	CFHL1		2	0.90	0.01	3	0.96	0.13
135	IPI00004798	cysteine-rich secretory protein-3 precursor	CRISP3		3	1.00	0.04			
136	IPI00019359	keratin, type I cytoskeletal 9	KRT9		3					
137	IPI00218795	L-selectin precursor	L-selectin	1.7E+01	3	1.01	0.04	1	0.73	
138	IPI00022368	Row70Serum amyloid A protein precursor	SAA2					3		
139	IPI00023019	sex hormone-binding globulin precursor	SHBG		4			2	0.91	
140	IPI00550315	Ig kappa chain C region	IGKC		2	0.99		3	1.09	0.09
141	IPI00001611	insulin-like growth factor II precursor	IGF2		3	0.97	0.69	2	0.94	0.09
142	IPI00022731	apolipoprotein C−IV precursor	APOC4		3			2		
143	IPI00004656	beta-2-microglobulin precursor	B2M	1.1E+03	2	1.04	0.03	2	1.05	0.07
144	IPI00027462	calgranulin B	S100A9		2					
145	IPI00329775	carboxypeptidase B2 precursor	CPB2		2					
146	IPI00025204	CD5 antigen-like precursor	CD5L		2	1.15		1	1.06	0.07
147	IPI00019576	Coagulation factor X precursor	F10		2			1	5.51(0.93)[f]	
148	IPI00242956	IgG Fc binding protein [*Homo sapiens*]	-		2			2	0.76	
149	IPI00247295	nesprin 1	SYNE1		2	0.03				
150	IPI00022331	phosphatidylcholine-sterol acyltransferase precursor	LCAT		2					
151	IPI00007199	protein Z-dependent protease inhibitor precursor	SERPINA10		2					
152	IPI00029699	Ribonuclease 4 precursor	RNASE4		2	4.92 (0.78)[f]				
153	IPI00178926	similar to immunoglobulin J chain	-		3	0.95	0.06	2	1.16	
154	IPI00009793	complement C1r-like proteinase	C1RL		2			2		
155	IPI00023673	galectin-3 binding protein precursor	LGALS3BP		2			1		
156	IPI00550640	IGHG4 protein	-		2					
157	IPI00019038	lysozyme C precursor	LYZ		2	1.01		1	0.91	
158	IPI00029260	monocyte differentiation antigen CD14 precursor	CD14		2			1		
159	IPI00003590	quiescin Q6	QSCN6		2			2		
160	IPI00395488	vasorin	MRX85		2			1		
161	IPI00374068	thrombospondin repeat containing 1	TSRC1					1		
162	IPI00020019	adiponectin precursor	ADIPOQ		1					
163	IPI00452748	amyloid protein A	SAA1		1					
164	IPI00008554	angiogenin precursor	ANG					1		
165	IPI00215983	carbonic anhydrase I	CA1		1					

Table 1. (Continued)

	IPI[a]	description	gene name	concentration in plasma (ng/mL)[b]	D3/D0 unique peptide[c]	D3/D0 ratio[d]	D3/D0 ratio stdev[d]	13C/D0 unique peptide[c]	13C/D0 ratio[d]	13C/D0 ratio stdev[d]
166	IPI00010295	carboxypeptidase N catalytic chain precursor	CPN1		1					
167	IPI00029658	EGF-containing fibulin-like extracellular matrix protein 1 precursor	EFEMP1					1	0.90	
168	IPI00218834	Fc of IgG, low affinity IIIa, receptor for	FCGR3A		1			1		
169	IPI00473011	hemoglobin delta chain	HBD					1		
170	IPI00301143	hypothetical protein PSEC0164	PI16		1					
171	IPI00385985	Ig lambda chain V−III region LOI	-		1			1		
172	IPI00009477	intercellular adhesion molecule-2 precursor	ICAM2		1					
173	IPI00216651	interleukin-28 receptor alpha chain precursor	IL28RA		1					
174	IPI00384401	myosin-reactive immunoglobulin kappa chain variable region	-					1		
175	IPI00299059	neural cell adhesion molecule	CHL1		1					
176	IPI00022733	phospholipid transfer protein precursor	PLTP		1					
177	IPI00013179	prostaglandin-H2 D-isomerase precursor	PTGDS		1					
178	IPI00216882	similar to mannan-binding lectin serine protease 1	-		1					
179	IPI00018136	vascular cell adhesion protein 1 precursor	VCAM1					1		

[a] The databank IPI version 3.09 was used for protein identification. [b] Values reproduced from Haab et al.[14] [c] Different charge states and different states of oxidation as well as heavy or light form of the peptide were considered different peptides in deriving the final number of unique peptides. [d] Standard deviation of ratios was calculated for the protein ratios across duplicates. [e] Residual protein from immunodepletion. [f] Value generated automatically (value manually calculated). [g] A total of 160 proteins were identified with more than 2 peptides in all 4 replicates of unfractionated serum; 113 proteins (70%) presented at least one peptide containing cysteine which provided quantitation information.

averaged. This quantitation algorithm will be incorporated in the near future to the open source distributions of the Mass Spectrometry in silico Peptide Characterization Tool (msInspect)[15] (http://proteomics.fhcrc.org) and integrated into the Computational Proteomics Analysis System (CPAS).[12]

Results and Discussion

Alkylation Of Cysteines With Acrylamide. The feasibility of acrylamide labeling for protein identification and quantitation has been demonstrated previously using MALDI-TOF for proteins separated by two-dimensional electrophoresis,[10,11] but not with high-throughput LC−MS/MS. In the present work, alkylation with acrylamide, 2,3,3′-D_3-acrylamide, 1,2,3-$^{13}C_3$-acrylamide, designated here D0, D3, or ^{13}C-acrylamide, respectively, was investigated with bovine serum albumin and applied to human immunodepleted serum. For both types of samples, no protein precipitation was observed, indicating that acrylamide based alkylation is compatible with intact-protein based approaches. A very high yield of cysteine alkylation was achieved, since databank searches for nonmodified cysteines did not return matches and we could not observe nonalkylated peptides by manual inspection of spectra. However, for several ion pairs of D0/D3 or D0/^{13}C-acrylamide labeled peptides, we observed a small set of adducts also in pairs separated by 3 Da, corresponding to less than 5% of the main pair. These adducts have mass increments of +16, +32, and +48. Analysis of the MS/MS spectra of these adducts (data not shown) indicated that addition of +16 and +32 occurred in the Cys-propionamide residue. Our explanation for these adducts would be oxidation of the labeled cysteine residues as observed for methionine during the electrospray ionization, forming Cys-propionamide sulfoxide (+16) or Cys-propionamide sulfone (+32). However, the third state of oxidation occurs at another site, since the sulfur atom of the Cys-propionamide could not accommodate one more atom of oxygen.

Bovine serum albumin is a protein rich in cysteine residues and represents a good standard to evaluate the approach presented here. Seven different D0/D3 labeled albumin ratios were evaluated. An average of 28 cysteine residues, out of 35 present in BSA, provided quantitation information for the ratios tested. Exemples of spectra obtained for 7 different ratios of D0/D3 acrylamide are shown in supplementary data. The result was obtained in a LTQ−FTICR mass spectrometer at 100 000 resolution, as utilized for analysis of complex samples in the data dependent acquisition mode. The spectra were well-defined and showed clearly the presence of both envelopes of ions for D0 and D3 labeled peptides even for ratios 10:1 (supplementary figure). Such resolution is important to resolve individual isotopic peaks from the D0 or D3-labeled peptide, directly impacting on peptide identification and accuracy of quantitation and on separation of different coeluting peptides especially in high complex mixtures such as human biological samples. Also the high-resolution ensures that background noise has minimal interference in quantitation.

Figure 1 shows the plot of all quantitation data points obtained, illustrating the linearity of this approach. The coefficient of variation calculated for all the albumin peptide ratios for the D0/D3 pairs 1:3, 1:1, and 3:1 were respectively 17.3%, 13.2%, and 19.2%, indicating accurate quantitation. These values are similar to those obtained in a recent ICAT evaluation that resulted in a coefficient of variation of 18.6%.[16] For ratios above 10-fold, these variations increased significantly to about

35−50%, especially for ratios where the peptide labeled with the light form of acrylamide is more intense, due to interference of isotopic peaks representing 3 or 4 ^{13}C. Similar results have been reported for other quantitation methods in which the separation of light and heavy envelopes is only 3 Da.[17,18] Although this decrease in quantitative accuracy for extreme ratios (≫10-fold) is significant, the method is still valuable for assessing large biological quantitative variations. For these particular cases, occasionally only the light or heavy form of the peptide is detected (unpublished data—not shown), which have to be individually evaluated to avoid distortions in the final calculation of the protein ratio.

Analysis Of Immunodepleted Human Serum. To assess the merits of isotopic acrylamide labeling of intact proteins, a pool of serum from three healthy subjects was immunodepleted to remove the top six most abundant proteins, after which the remainder of the proteins were reduced with DTT, split into three aliquots and labeled with D0, D3-acrylamide, or ^{13}C-acrylamide. Mixtures of equal amounts of D0 and D3 or D0 and ^{13}C-labeled aliquots were analyzed in duplicate using a 2 h reversed-phase gradient in a LTQ−FTICR mass spectrometer.

Analysis of immunodepleted but otherwise unfractionated serum labeled with D0/D3 or D0/^{13}C yielded 160 proteins identified with two or more peptides in replicate runs (Table 1). A total of 121 of these proteins (76%) were present in four replicates, indicating good reproducibility of the overall method. Among the proteins identified in this study, complement C3 is found in plasma at 1 mg/mL, β-2-microglobulin at 1 μg/mL and insulin-like growth factor binding protein 3 precursor at 60 ng/mL,[14] which corresponds to more than 5 orders of magnitude in protein concentration in a single run. This is particularly noteworthy given that no fractionation step was applied.[4]

Quantitation ratios were calculated for those peptides containing cysteine residues identified by MS/MS, with a Peptide Prophet score >0.75. The presence of a coeluting pair of ions separated by multiples of 3 Da also indicates correct assignment of the peptide sequence containing cysteine.[8] Cysteine containing peptides provided quantitation information for a total of 113 proteins (69 present on all four replicates) (Table 1). Several proteins presented multiple peptides containing cysteine as observed in Figure 2 for plasminogen, for which out of 35 peptides containing cysteine in the sequence, 24 were detected in this study. Among the 2493 unique peptides identified (unique sequences) in our entire data set (duplicates of D0/D3 and D0/^{13}C), 857 peptides (34.4%) contained at least one cysteine residue. This value is slightly higher than the estimate of tryptic peptides containing cysteine predicted based on the human genome (26.6%).[5] It is likely that serum proteins are particularly rich in cysteine residues compared for example to membrane proteins.

Quantitation of individual proteins, represented by a particular IPI number, was calculated by averaging the ratios obtained for cysteine labeled peptides. In particular cases for which one peptide ratio represented an outlier among several others from the same protein, this ratio was excluded from the final protein ratio computation. As can be observed in Table 1, few replicates presented standard deviations for duplicates greater than 0.25. Only two proteins, coagulation factor X precursor and Ribonuclease 4, each containing a single peptide ratio measurement, presented unexpected high values. Manual inspection of these outliers indicated that for coagulation factor X precursor and ribonuclease 4, incorrect ratios were computed

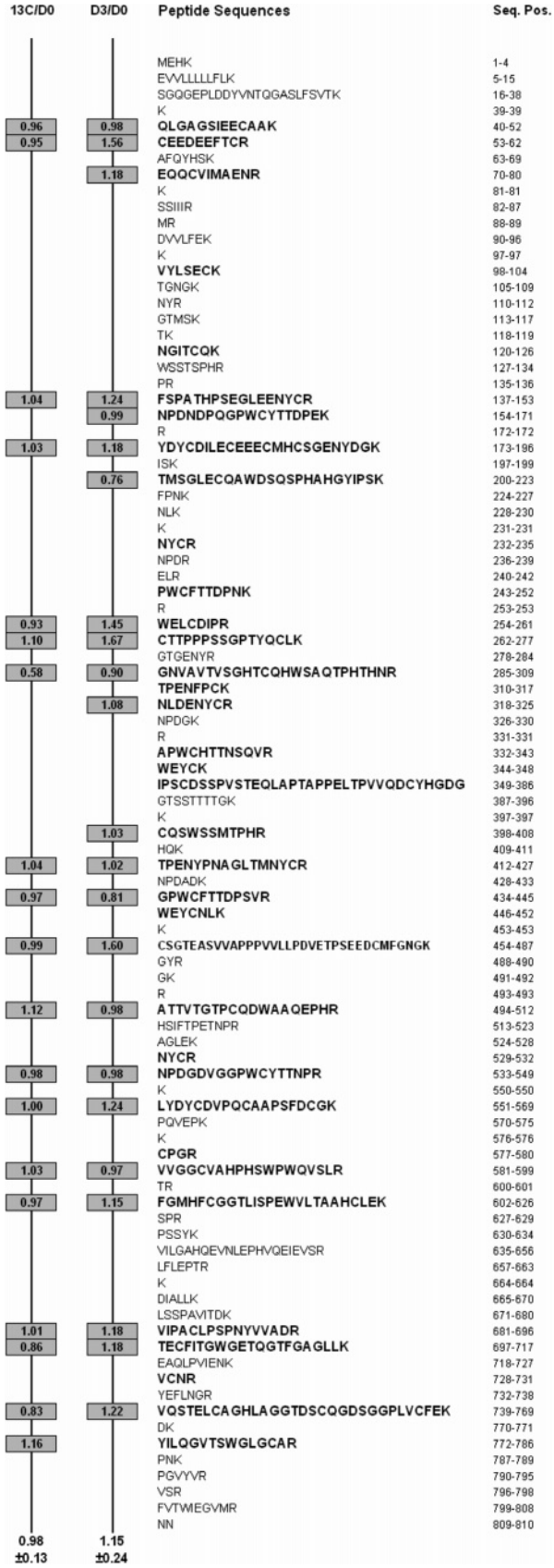

Figure 2. Quantitation of plasminogen. Of the 35 peptides containing cysteine in plasminogen, 24 were detected and provided quantitation data. The standard deviations calculated for the data (at the bottom) indicates that the pair of acrylamide isotopes D0/^{13}C provides highly accurate data.

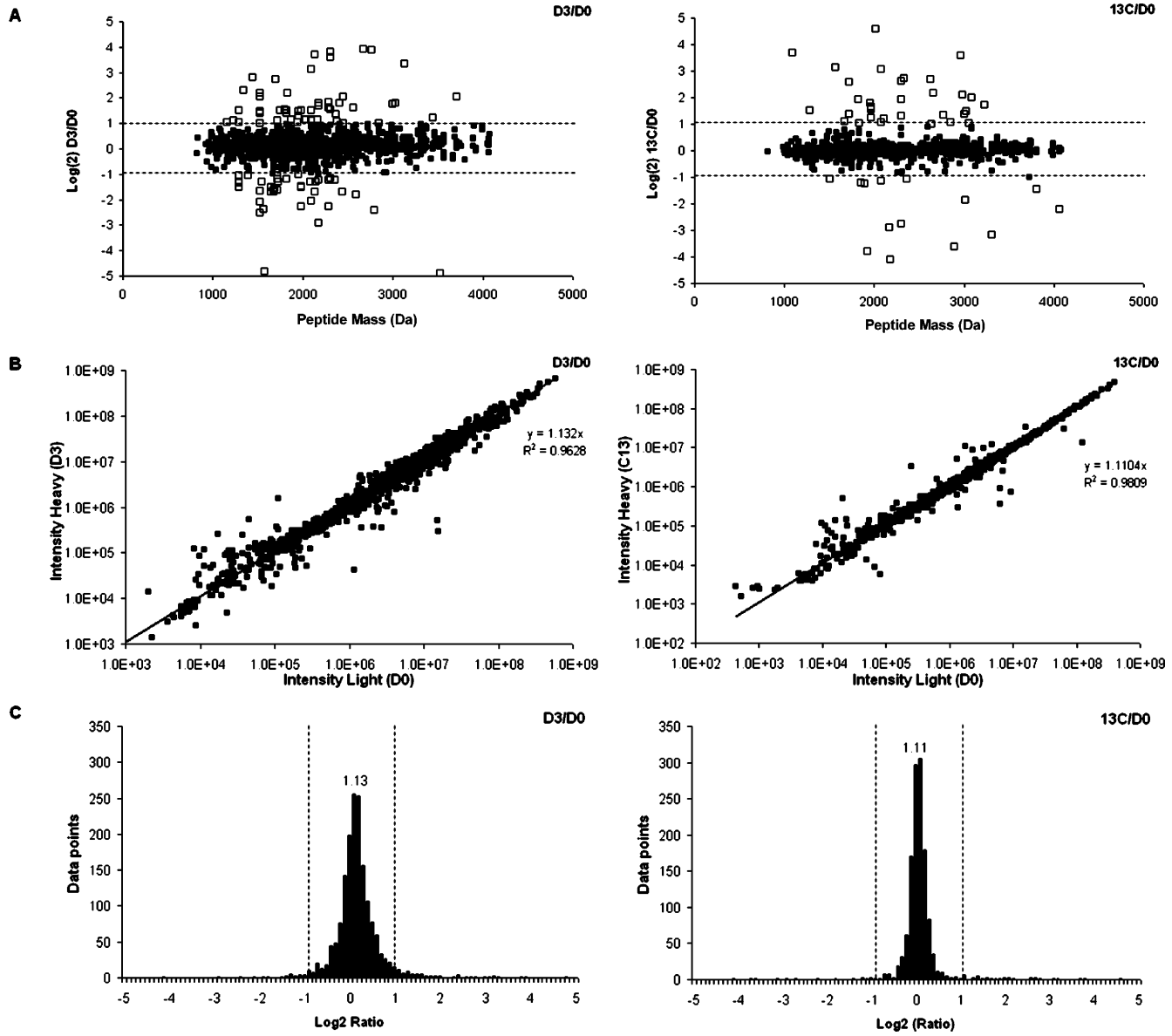

Figure 3. Quantitation of serum proteins. A mixture of 1:1 immunodepleted human serum sample was labeled with D0/D3-acrylamide (right panels) or D0/^{13}C-acrylamide (left panels). These data points represent an average of two measurements per peptide and this redundancy in quantification results from redundancy in peptide identification, which increases confidence in the data. Panel A illustrates the distribution of quantitation data correlated to peptide mass. Most of the data points (89%) were acquired for peptides with mass lower than 3000 Da. The plots in panel B show the distribution of ratios obtained for intensities of the heavy and light forms of acrylamide. The points are linearly distributed over 5 orders of magnitude based on ion intensities. Panel C shows the distribution of quantitation ratios obtained for the pairs D0/D3 and D0/^{13}C. 94 and 96% of the data points are within a 2-fold ratio threshold (indicated by dashed lines) and 86 and 94% within a 1.5-fold for D0/D3 and D0/^{13}C, respectively.

due to low intensities of the peptide ions ($s/n < 4$) and ratios manually calculated from peak intensities were respectively 0.93 and 0.78. Also, it can be observed in Table 1 that often the number of peptides obtained for the pair D0/D3 was greater than for D0/^{13}C. We attribute this difference solely to a lower recovery of the D0/^{13}C proteins during samples cleanup, since no chemical reactivity difference can be expected from acrylamide isotopes and the purity grade of the reagents was similar (>98%).

A complicating factor with the acrylamide labeling method is overlapping of light and heavy labeled isotopes, due to the low mass difference (3 Da). However, this effect would be more noticeable for peptides with mass above 3000 Da, for which the relative intensity of the third ^{13}C isotope of the light form could contribute about 46% of the intensity of the monoisotopic peak of the heavy form. In the present work, the script that calculates intensities of the quantitation pairs took into consideration the predicted contribution of the third, fourth and fifth ^{13}C isotope of the light form which was subtracted from the corresponding ions of the heavy form envelope for all peptides with single cysteine residues and mass greater than 1800. As can be noted in Figure 3a, peptides above 3000 Da provided reliable data after this correction for both D0/D3 and D0/^{13}C-acrylamide pairs. Also it would be expected that the larger the peptide, the greater the chance for occurrence of more than one cysteine residue. In this study, we detected peptides containing up to 4 cysteine residues, although the number of these high mass peptides was small, due to

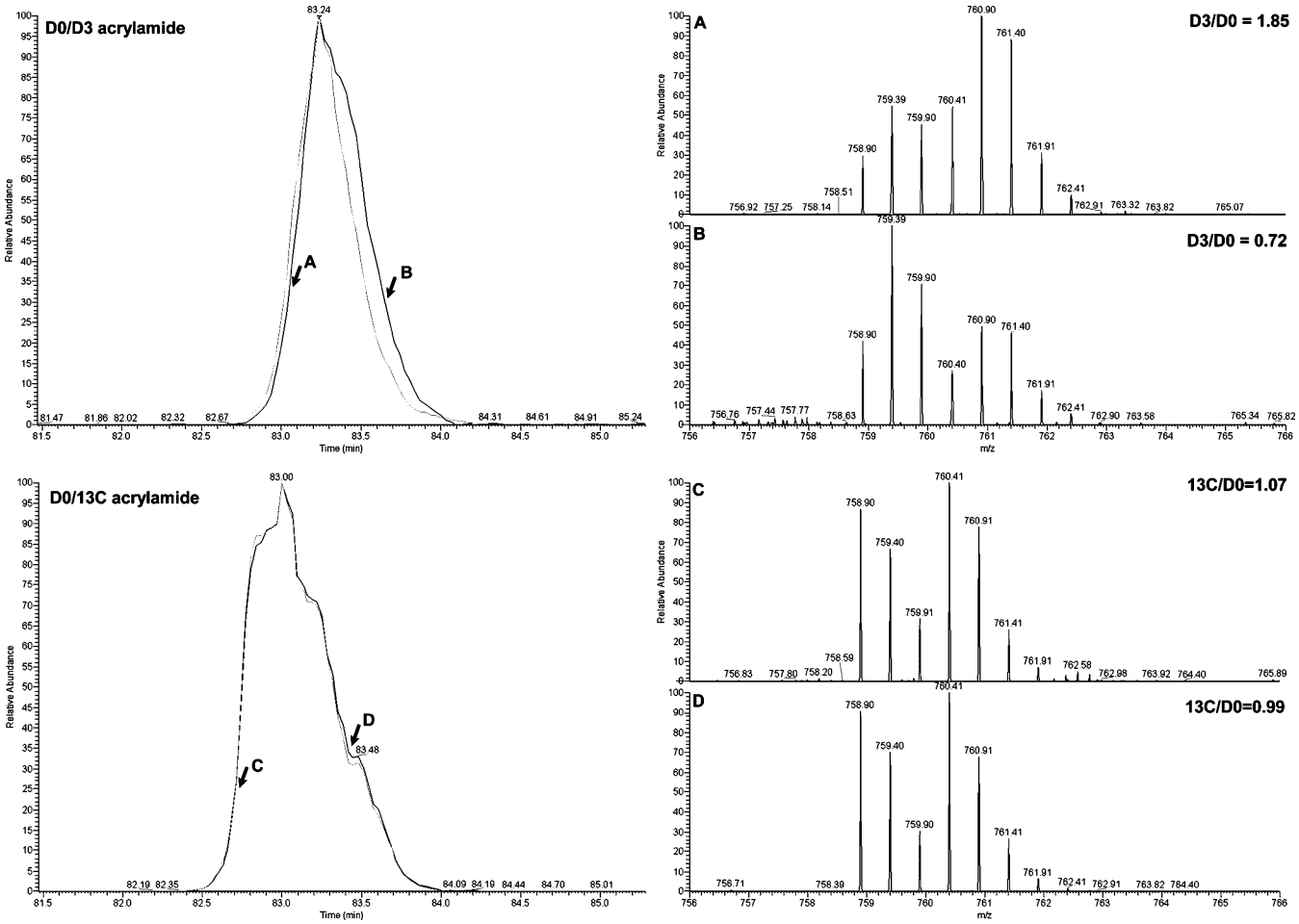

Figure 4. Influence of deuterated acrylamide peptide elution. The reconstructed ion chromatograms compare the elution profiles of the peptide VCPFAGILENGAVR from β-2-glycoprotein I precursor labeled with the pairs D0/D3 (upper panel) and D0/^{13}C (lower panel). The elution profile of the heavy form (D3 or ^{13}C-acrylamide) is represented in gray and in black for the light form (D0 acrylamide). Early elution of the D3-acrylamide labeled peptide is noticeable interfering with the quantitation ratios obtained at different retention times. Spectra on the right correspond to the D0/D3 or D0/^{13}C pairs obtained at the critical retention times indicated with arrows.

limitations in ionization and fragmentation as well as MS/MS data complexity.

As previously indicated, proteins identified in this study occur in human plasma in concentrations ranging from mg/mL to ng/mL (Table 1). The levels of ion intensities for peptides that provided quantitation information were also distributed across some 5 orders of magnitude as illustrated in Figure 3b. This correspondence indicates that the combination of a high accuracy and sensitivity mass spectrometer and simple and efficient isotopic tagging with acrylamide provides a robust method for quantitative analysis of complex proteomes.

Comparison Of D3-Acrylamide And ^{13}C-Acrylamide. A concern with the use of deuterated compounds for quantitation in LC−MS is a small difference in hydrophobicity between deuterated and nondeuterated pairs. This difference may result in small shifts in chromatographic retention time of peptides as observed with the first generation of ICAT reagents in which peptides labeled with the deuterated tag eluted some seconds earlier than the nondeuterated tag that contains only hydrogen.[19] This effect is not expected for isotopes containing ^{13}C. To evaluate the consequence of these effects on quantitation results, we compared the performance of D0/D3 and D0/^{13}C pairs using data obtained for serum proteins.

Hydrophobicity of the domain into which heavy isotopes are incorporated is the most important indicator of whether a coding agent will exhibit a deuterium isotope effect during reversed-phase chromatography.[19] Deuterium atoms in a polar and small functional group such as acrylamide would be expected to present a small resolution effect. We observed consistent differences in retention time of about 1−2 s between the D3 and the D0-acrylamide across the entire chromatogram. Figure 4 presents the elution profile of one peptide containing a single cysteine residue with about a 6 s-shift between D0/D3 labeled peaks. The quantitation script takes the MS/MS sequencing event as the starting point to extract the ion intensities and a 10-MS scan (~14 s) window is considered for the calculations. Consequently, significant shifts can lead to errors. For this particular example, a total of 6 ratios (6 MS/MS events for the peptide) were obtained for the peptide as a result of wide elution of the corresponding peak. The values have an average of 1.06 (D3/D0), but when considered individually, we observed influence of the shift in elution into the quantitation (1.85, 1.02, 1.33, 0.87, 0.60, 0.72). Points taken at the beginning of the elution had greater contribution of the D3-labeled isotope, increasing the ratio, while points at the end had greater contribution of the D0-labeled ion (see Figure 3 left panels). This same peptide, labeled with the pair D0/^{13}C, is illustrated

in Figure 4. As can be observed, no difference in elution of isotopes of acrylamide can be detected for the pair D0/^{13}C as predicted. Several quantitation data points were also obtained for the D0/^{13}C-labeled peptide, due to its broad elution profile. In contrast to D0/D3 findings, basically the same ratio was obtained across all elution points (1.07, 1.01, 0.98, 1.05, 1.05, 0.90, 1.00, 0.92, 0.99) for the D0/^{13}C-labeled peptide. The standard deviation for the ratios was ±0.06, compared to ±0.46 for the D0/D3 pair.

Other undesirable effects resulting from distinct elution of deuterated and nondeuterated peptides may complicate analysis since they can be ionized at different times or may overlap with other peptides with similar mass, increasing the possibility of producing both systematic and random quantitation errors.[19] These effects could be reflected in quantitation data obtained across the entire experiment. Figure 2 illustrates peptide quantitation findings for plasminogen. Greater quantitative variability was observed for D0/D3, with a standard deviation that was 2-fold higher than that obtained for D0/^{13}C-acrylamide. We attribute this variability to the deuterium effect, since the samples compared were the same. In the same way, the influence of resolution of deuterated compounds is evident in all the panels presented in Figure 3. Though both D0/D3 and D0/^{13}C pairs presented almost the same modes of 1.13 and 1.11 (Figure 3C) and percentage of data points within a 2-fold threshold (94% and 96%), when this threshold was lowered to 1.5-fold, the differences became significant (86% and 94%). In both the correlation ratio/peptide mass (Figure 3a) and the distribution plot of the quantitation data (Figure 3b), there was evident spreading of values for the pair D0/D3. On the basis of all the comparisons, we can conclude that for more accurate analysis, the D0/^{13}C-acrylamide pair should be used, though the D0/D3 pair can also provide useful data.

Conclusions

The quantitative LC−MS/MS approach presented is simple, low cost and takes advantage of the high-sensitivity/high resolution provided by an instrument like the LTQ−FTICR mass spectrometer and provides both high confidence identification and quantitative data for complex protein mixture. Differential alkylation of intact serum proteins with acrylamide isotopes provided relative quantitation for about 70% of proteins identified (113 proteins quantified from 160 proteins identified with two or more peptides). These proteins were distributed over 5 orders of magnitude of abundance levels based on the range of intensity of peptide ions and known protein concentration in plasma. The variations caused by chromatographic resolution of deuterated and nondeuterated acrylamide labeled peptides lead to the conclusion that use of ^{13}C isotope of acrylamide is preferred because of greater accuracy. Implementation of this methodology in combination with intact protein separation methods would be a valuable tool for the analysis of complex biological fluids proteomes.

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Supporting Information Available: High resolution mass spectra of D0 and D3 labeled peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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