Trypsin Catalyzed ^{16}O-to-^{18}O Exchange for Comparative Proteomics: Tandem Mass Spectrometry Comparison Using MALDI-TOF, ESI-QTOF, and ESI-Ion Trap Mass Spectrometers

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Quantitative or comparative proteome analysis was initially performed with 2-dimensional gel electrophoresis with the inherent disadvantages of being biased towards certain proteins and being labor intensive. Alternative mass spectrometry-based approaches in conjunction with gel-free protein/peptide separation have been developed in recent years using various stable isotope labeling techniques. Common to all these techniques is the incorporation, biosynthetically or chemically, of a labeling moiety having either a natural isotope distribution of hydrogen, carbon, oxygen, or nitrogen (light form) or being enriched with heavy isotopes like deuterium, ^{13}C, ^{18}O, or ^{15}N, respectively. By mixing equal amounts of a control sample possessing for instance the light form of the label with a heavy-labeled case sample, differentially labeled peptides are detected by mass spectrometric methods and their intensities serve as a means for direct relative protein quantification. While each of the different labeling methods has its advantages and disadvantages, the endoprotease ^{16}O-to-^{18}O catalyzed oxygen exchange at the C-terminal carboxylic acid is extremely promising because of the specificity assured by the enzymatic reaction and the labeling of essentially every protease-derived peptide. We show here that this methodology is applicable to complex biological samples such as a subfraction of human plasma. Furthermore, despite the relatively small mass difference of 4 Da between the two labeled forms, corresponding to the exchange of two oxygen atoms by two ^{18}O isotopes, it is possible to quantify differentially labeled proteins on an ion trap mass spectrometer with a mass resolution of about 2000 in automated data dependent LC-MS/MS acquisition mode. Post column sample deposition on a MALDI target parallel to on-line ESI-MS/MS enables the analysis of the same compounds by means of ESI- and MALDI-MS/MS. This has the potential to increase the confidence in the quantification results as well as to increase the sequence coverage of potentially interesting proteins by complementary peptide ionization techniques. Additionally the paired y-ion signals in tandem mass spectra of $^{16}O/^{18}O$-labeled peptide pairs provide a means to confirm automatic protein identification results or even to assist de novo sequencing of yet unknown proteins. (J Am Soc Mass Spectrom 2003, 14, 704–718) © 2003 American Society for Mass Spectrometry

Genome sequencing projects have led to the publication of an increasing number of genome sequence databases in recent years. These genome sequence databases are the basis for the automated identification of proteins by mass spectrometry and has led to an enormous boost in the area of protein discovery, protein-protein interaction and protein characterization sciences. The term "proteomics" was initially used in 1994 at the Siena meeting for protein separation based on 2-dimensional gel electrophoresis. Today the field of proteomics covers essentially all of the above described efforts. To understand complex cellular dynamics, powerful methods for quantification of the messenger ribonucleic acid (mRNA) synthesis have been developed [1–3]. Although these methods are very sensitive, mRNA expression levels do not necessarily reflect changes in the concentration of specific functional proteins in cells [4]. This is especially true if the final step for the synthesis of functional proteins encompasses post-translational modifications (PTMs).

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Hence, the analysis of the protein content (proteome) provides more accurate information about biological systems and pathways because the measurement is directly focused on proteins, the actual biological effector molecules.

In the case of blood plasma, proteome analysis is the only means to detect perturbation-related changes in its protein content because essentially every cell of an organism releases proteins into the plasma, hence there is no unique source of mRNA. Plasma proteins changing in their relative concentration between a control and a perturbed state can potentially serve as biomarkers, or may represent drug targets or protein therapeutics for a certain disease. Determination of protein concentration changes is therefore fundamental for the understanding of a biological system as well as for pharmaceutical research. There are currently various options for relative quantification of proteins in biological samples. Differential stable isotope labeling of proteins in comparative samples is a commonly used method for subsequent quantitative analysis by mass spectrometry [5].

There are several approaches available for incorporating the stable isotope label, and the most appropriate one depends on sample source and type. When proteome samples are of human origin, such as human plasma and serum, it is not practical to label proteins metabolically [6–8]. Post-biosynthesis/bioprocess labeling, in contrast to metabolic labeling, is more versatile. Chemical labeling by alkylation, esterification, or acylation [9–11] and endoprotease catalyzed labeling with ^{18}O at the C-terminus of peptides [12–14], present examples of post-biosynthesis labeling.

In analyzing systems as complicated as entire proteomes, by-products of labeling reactions need to be kept to a minimum in order to reduce the workload for data acquisition and analysis. Additionally, in order to obtain the maximal proteome coverage in a comparative protein quantification experiment, it is important to generate as many labeled peptides as possible.

Enzymatic labeling of proteins/peptides has been proposed to compare protein quantities in two counterpart proteomes mainly because, compared to chemical labeling methods, the enzymatic labeling is highly specific and is almost universally applicable [15]. Through its ability to universally label almost all the carboxyl termini, enzymatic labeling is an ideal choice to quantitatively study mixtures of low molecular weight proteins. These proteins may not contain many or any cysteine residues for example, a common target for chemical labeling reactions like ICAT [9]. Besides, when compared with large proteins, small proteins generate fewer peptides upon digestion, especially information-rich peptides (peptides that are useful to identify their precursor proteins unambiguously). In addition, by targeting all endoprotease-specific peptides, the enzymatic approach will label post-translationally modified peptides that go otherwise undetected with ICAT if they do not contain a cysteine residue. Furthermore not all the peptides generated can be ionized for mass spectrometric analysis via electrospray ionization or matrix-assisted laser desorption/ionization process, again limiting the usefulness for quantification of small proteins by labeling strategies which target only specific residues. Finally, isotopic ^{18}O labeling does not alter the physical properties of peptides, such that they ionize exactly as their unlabeled counterparts, and no shift in the chromatographic behavior has been reported.

We show here that trypsin-catalyzed ^{16}O-to-^{18}O exchange is a valid differential isotope-coding technology for comparative proteomics using mass spectrometry-based analysis of complex protein mixtures like a low molecular weight protein subfraction of human blood plasma. Ion trap and MALDI-TOF mass spectrometers are probably the most abundant instruments in protein mass spectrometry laboratories world-wide. We demonstrate that $^{16}O/^{18}O$-labeled peptide pairs, with a 4 Da mass differential, can successfully be quantified on relatively low resolving ion trap or higher resolving TOF mass spectrometers.

Experimental Methods

All protein/peptide standards were purchased either from Bachem (Bubendorf, Switzerland) or Sigma (Buchs, Switzerland). All chemicals were purchased from either Fluka/Riedel de Haen/Sigma/Aldrich (Buchs, Switzerland), or Merck (Dietikon, Switzerland), and were of the highest quality available and used without any further purification. Anhydrous dimethyl sulfoxide (DMSO) and HPLC grade acetonitrile (MeCN) were from Fluka/Riedel de Haen (Buchs, Switzerland), acetic acid (HAc) 100% from Amman-Technik AG (Koelliken, Switzerland), trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) from Pierce (Socochim SA, Lausanne, Switzerland), fused silica capillaries from Polymicro Technologies Inc. (MSP Friedli & Co, Köniz, Switzerland), PLRPS column from Ercatech AG (Bern, Switzerland), and nanobore reverse phase (RP) liquid chromatography (LC) columns from GROM GmbH (Rottenburg-Hailfingen, Germany). Water was in-house purified and desalted by reverse osmosis and a Milli-Q system (Millipore, Switzerland). α-Cyano-4-hydroxycinnaminic acid (HCCA) was purchased from Bruker Daltonics (Bremen, Germany).

Trypsin Catalyzed ^{16}O-to-^{18}O Oxygen Exchange

Proteins were digested with modified porcine trypsin from Promega (Catalys, Wallisellen, Switzerland) in 20 mM Tris/HCl pH 8.0 buffer at a substrate/protease ratio of 50:1 (wt/wt). Peptides were dried completely in a speedvac from Savant (Fisher Scientific, Wohlen, Switzerland). The dried peptides were resuspended in anhydrous DMSO at a concentration of 25 mg/ml. This solution was further diluted using normal water or 95%+ enriched ^{18}O-water (Isotec/Campro Scientific, Berlin, Germany) and stock solutions of 5 M NaCl, 5 M

$CaCl_2$, and 1 mg/ml trypsin made up in either normal water or 95%+ enriched ^{18}O-water. The final composition for the oxygen exchange reaction was 2.5 mg/ml peptide, 20 mM Tris/HCl pH 8.0, 50 mM NaCl, 50 mM $CaCl_2$, and trypsin at a substrate/protease ratio of 20:1 (wt/wt). This solution was incubated overnight at 30 °C. Samples were subsequently stored at −30 °C and mixed just prior to further chromatography and/or mass spectrometric analysis.

DMSO is as efficient in resuspending peptides as formic acid. In contrast to formic acid and its salts, the presence of 10% (vol/vol) DMSO increases the apparent trypsin activity compared to water-based buffer alone as measured with a commonly used kinetic trypsin activity assay using Nα-benzoyl-L-arginine ethyl ester as substrate (results not shown). In addition, we have not detected any problems on chromatographic separations of samples containing residual traces of DMSO. It has recently been reported that the addition of 10% DMSO to reverse phase chromatography solvents can even improve the detection of hydrophobic peptides [16]. DMSO is known for its tendency to oxidize peptides, but this can be taken into account during peptide identification searches by allowing for oxidation as a variable modification.

Preparation of a $^{16}O/^{18}O$-Labeled Complex Standard Protein Mixture Digest

Ten commercially available human proteins (total of 260 nmole) were dissolved in 3 ml 80:20 (vol/vol) of 20 mM Tris/HCl pH 8.0, 8 M urea/DMSO. The protein composition was as follows, with the SWISS-PROT accession number given in parenthesis: Atrial natriuretic factor (P01160)/β-endorphin (P01189)/galanin (P22466)/calcitonin (P01258)/growth hormone-releasing factor (P01286)/pancreatic polypeptide (P01298)/peptide YY (P10082)/gastrin-releasing peptide (P07492)/α-lactalbumin (P00709)/apolipoprotein A-II (P02652), 4:4:4:4:2:2:2:2:1:1 (molar ratio). Two equal portions of 1.4 ml of this protein stock solution were reduced, alkylated, digested and $^{16}O/^{18}O$-labeled in parallel. Briefly, proteins were reduced for 3 h at 37 °C by the addition of 33 μl of 200 mM dithioerythritol (DTE) in 20 mM Tris/HCl pH 8.0, followed immediately by addition of 200 μl of 100 mM iodoacetamide in 20 mM Tris/HCl pH 8.0 for alkylation at 37 °C in the dark. After 0.5 h, 75μl of 2-mercaptoethanol was added to the solution. The proteins were then digested overnight at 30 °C with porcine trypsin at a substrate/enzyme ratio of 50:1, after dilution to a final volume of 10 ml in 20 mM Tris/HCl pH 8.0, 50 mM NaCl, 50 mM $CaCl_2$. The pH was lowered to 3 using a minimal volume of 10% (vol/vol) TFA. Both samples were desalted using a SepPak C-18 light cartridge (Waters, Rupperswil, Switzerland) according to the manufacturers recommendations. Peptides were eluted from the SepPak resin with 70% (vol/vol) methanol. Both peptide solutions were dried completely by vacuum centrifugation and were subsequently labeled with either ^{16}O or ^{18}O as described above. For LC-MS/MS analysis of $^{16}O/^{18}O$-labeled mixtures, approximately 5 pmol/μl total peptides at the desired $^{16}O/^{18}O$-ratio were prepared in 1% (vol/vol) TFA, loading 0.5 μl on the nanobore column of the ESI-QTOF system as described below.

Preparation of $^{16}O/^{18}O$-Labeled Horse Apomyoglobin Digest

To 50 nmole of lyophilized apomyoglobin powder in a vial, as delivered by Sigma, 90 μl of 20 mM Tris/HCl pH 8.0 and 10 μl of 1 mg/ml porcine trypsin were added without shaking and incubated for 2 h at 37 °C. A volume of 785 μl of 20 mM Tris/HCl pH 8.0 together with 100 μl MeCN and 5 μl 2-mercaptoethanol were added and mixed gently. Digestion of the apomyoglobin was completed by further addition of 10μl of 1 mg/ml porcine trypsin and incubation for 16 h at 37 °C. The final digest was aliquoted and stored until further use at −30 °C. The isotopic labeling of tryptic apomyoglobin was performed as described above.

Preparation of a Human Plasma Low Molecular Weight Protein Subfraction

Blood was obtained using standard veinous puncture procedures in a medical centre setting after informed written consent of donors. Plasma was prepared by centrifugation and removal of white cells on filters by standard techniques. Protease inhibitor (Complete, Roche) was added according to the manufacturer's instructions and mixed gently to ensure dissolution. Plasma samples were then frozen and stored at −80 °C. Plasma was thawed, 10 ml were filtered through a 0.45 μm filter and applied to a proprietary column combination (36 ml bed volume) that adsorbs serum albumin and immunoglobulins removing close to 100% of these highly abundant plasma proteins as assessed by 2-D gel electrophoresis. The flow-through fraction was applied to gel filtration chromatography on a 1.6 litre bed volume column that was equilibrated and percolated with a buffer containing 8 M urea, monitoring at 280 nm. Low pressure chromatography equipment (Akta Purifier) was from Amersham Bioscience (Uppsala, Sweden). After elution of the larger proteins (as monitored in prior experiments by gel electrophoresis) the equivalent of one column volume of the effluent was diverted to a column of reversed phase medium that adsorbed the low molecular weight proteins and peptides (ca. Mr 20–25 kDa cutoff). After washing, the capture column was developed with two column volumes of a 0–80% MeCN gradient containing 0.2% (vol/vol) TFA. Protein content in the collected eluate

was determined by analytical size exclusion HPLC using BSA as a standard, and this fraction was stored at −30 °C until further use.

Digestion, $^{16}O/^{18}O$ labeling, and SCX Fractionation of a Low Molecular Weight Protein Human Plasma Subfraction

A human plasma subfraction containing low molecular weight proteins in a volume of 3 ml at a protein concentration of 0.76 mg/ml, prepared as described above, was diluted with 3 ml of 20 mM Tris/HCl pH 8.0, 8 M urea. Proteins were reduced by the addition of 10 mM DTE at 37 °C for 1 h, followed by alkylation with 40 mM iodoacetamide at 37 °C for 30 min in the dark. Immediately after alkylation the sample was diluted 1:9 in 4 M urea and acidified to pH 3 by addition of 10% (vol/vol) TFA. Proteins were loaded on a reverse phase PLRPS column with a bed volume of 0.3 ml. After extensive washing of the column with 0.2% (vol/vol) TFA in water, proteins were eluted by a two column volume gradient of 0–80% MeCN containing 0.2% TFA. The protein solution was frozen in liquid nitrogen and lyophilized. The dry protein powder was digested with trypsin at an enzyme/substrate ratio of 1:50 as described for apomyoglobin. The sample was then split into two equal portions followed by C-terminal ^{16}O-to-^{16}O oxygen exchange with one portion and ^{16}O-to-^{18}O oxygen exchange with the other as described above. The final reaction volume of each sample was 460 μl.

After the labeling procedure, 4.3 μl of ^{16}O-labeled apomyoglobin digest (200 pmol) were added to the ^{16}O-labeled plasma peptide sample and 2.15 μl (100 pmol) of ^{18}O-labeled apomyoglobin digest was added to the ^{18}O-labeled plasma peptide sample. Aliquots of 175μl from both samples were mixed together and diluted in 700 μl 0.1% (vol/vol) formic acid containing 20% (vol/vol) MeCN resulting in a theoretical ^{16}O-to-^{18}O-ratio of 2:1 and 1:1 for apomyoglobin and plasma protein peptides respectively. 1 ml of this mixture was loaded on a strong cation exchange (SCX) column (PL-SCX 8um 1000A, 50 × 2.1 mm; Polymer labs) equilibrated with 0.1% (vol/vol) formic acid in 20% (vol/vol) MeCN. The column was developed with a step gradient beginning with 2.5% and followed by 0.5% steps (3 column volumes each) of a 1 M ammonium acetate buffer in 0.3% (vol/vol) formic acid/20% (vol/vol) MeCN at a flow rate of 200 μl/min. Chromatography was performed on an Alliance 2795 separation module equipped with a photodiode array detector and a fraction collector (Waters, Millford, MA). The chromatography was monitored at 280 nm. Peptides in the eluate were collected according to the salt steps in 1.5 ml polypropylene tubes. Fractions were dried in a speedvac and peptides redissolved in 20% formic acid prior to LC-MS/MS analysis.

LC-ESI/MALDI-MS/MS Analysis of $^{16}O/^{18}O$-Labeled Peptides

LC-ESI/MALDI. The ion trap system consisted of an Esquire3000plus (Bruker Daltonics, Bremen, Germany) equipped with a standard electrospray (ESI) source and coupled to an Alliance 2795 separation module delivering a flow rate of 3 μl/min with an in-house built flow splitter. Reverse phase liquid chromatography was performed on a nanobore GROM-SIL C8 column, 5 μm, 0.1 × 100 mm, using a bi-phasic gradient of 0–10% solvent B in 2 min, followed by 10–40% solvent B in 30 min. Solvent A consisted of 5% (vol/vol) MeCN in 0.4% (vol/vol) HAc, 0.005% (vol/vol) HFBA; solvent B was 95% (vol/vol) MeCN containing 0.4% HAc. The post column flow was split with a micro-T (Valco-Vici, Schenkon, Switzerland) diverting the column eluate by a ratio of 2:1 to the ESI source and a LC-MALDI interface, respectively.

The LC-MALDI interface was achieved by pulling a 75 μm inner diameter fused silica capillary through one of the needles of a MAP II/8 preparation robot (Bruker Daltonik, Bremen, Germany). Sample deposition on a prestructured sample support (384 anchor target, Bruker Daltonik, Bremen, Germany) was performed by moving the steel needle with the fixed capillary in z-direction towards the sample support. Upon contact with the target, the eluate droplet of 250 nl forming at the tip of the capillary within the 15 s sampling time was dispensed to the 600 μm diameter matrix precoated hydrophilic anchor. All movements of the robot and the synchronization with the HPLC were controlled by an Excel VBA script within the MAP-control software supplied by the manufacturer. The matrix precoating of the target was made by placing a small volume of HCCA matrix solution [1 g/l in Acetone/TFA (0.1%) 97:3 vol/vol] on each of the individual anchors by the help of a small pipet tip (GELoader, Eppendorf) [17]. A consecutive addition of 1 μl of a diluted matrix solution (0.1 g/l HCCA in ethanol/acetone/TFA (0.1% vol/vol) at a volume ratio of 60:30:10) was applied by another MAPII/8 robot.

Ion trap MS/MS. The ion trap (ESI-IT) was tuned to allow the isolation of the entire $^{16}O/^{18}O$-isotopic envelope of doubly and higher charged peptides, irrespective of which of the labeled forms was chosen as the precursor for MS/MS as shown in Figure 1 (isolation width of 5 *m/z*, isolation coarse high of 150, isolation fine high of 10, and isolation fine low of 70). Otherwise the ion trap was operated in data-dependent MS to MS/MS switching mode using two precursors detected in the 350–1600 *m/z* unit window and excluding singly charged ions. The duty cycle for such a data dependent MS/MS cycle was in the order of 8 to 9 s depending on the precursor ion intensity. This dependency is due to the fact that this instrument regulates ion accumulation times in the trap based on detected ions. The charge state of precursor ions is detected instantaneously dur-

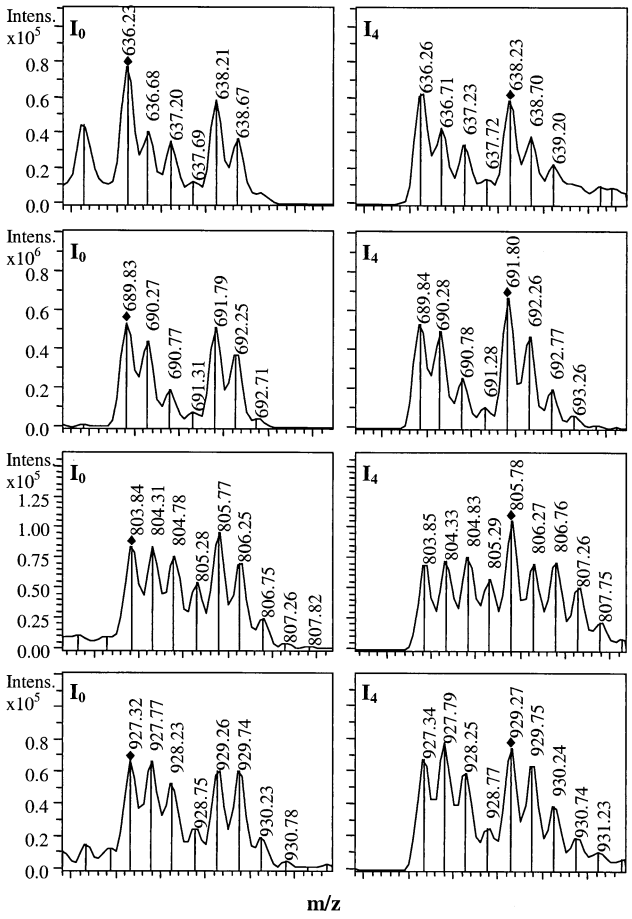

Figure 1. Tuning of ESI-IT for optimal isolation efficiency of the entire isotope cluster of $^{16}O/^{18}O$-labeled peptide ions. A horse apomyoglobin digest labeled with ^{16}O- or ^{18}O-water, respectively, was mixed at a theoretical ratio of 1:1 in 1% acetic acid/MeCN 1:1 at 1 pmol/μl concentration. This solution was delivered to the ESI-IT using a syringe pump at a flow rate of 2 μl/min. The doubly charged tryptic peptide ions LFTGHPETLEK (m/z = 636.34), HGTVVLTALGGILK (m/z = 689.93), VEADIAGHGQEVLIR (m/z = 803.93), and GHHEAELKPLAQSHATK (m/z = 927.49), respectively were isolated in the ion trap with the isolation parameters set as described in the Experimental Methods section. Isolation was done either on the I_0 (left panel) or the I_4 isotope (right panel) resulting in mean $^{18}O/^{16}O$-ratios of 0.94 $\pm$ 0.17 and 1.13 $\pm$ 0.31. The small black diamonds denote the isolated target mass.

ing acquisition by a Bruker proprietary algorithm. Precursors were excluded for one min after one MS/MS acquisition and the scan range was kept between 100 to 1600 m/z.

MALDI-TOF MS. MALDI-TOF spectra of positively charged ions were recorded in reflector mode on either a Bruker ReflexIII or a Bruker Ultraflex MALDI-TOF mass spectrometer (Bremen, Germany), both equipped with a Scout 384 ion source. Both instruments were operated under delayed ion extraction conditions and optimized to achieve a mass resolution of 3000–15000 (up to 20000 for the Ultraflex) over the whole mass range of interest (600–4000 Da), using a total acceleration voltage of 25 kV. A deflection of matrix ions up to 600 Da was furthermore applied to prevent detector saturation. Calibration was performed by external calibration over a wide mass range using bradykinin, angiotensin II, substance P, bombesin and ACTH clip 18-39.

MALDI-TOF/TOF MS/MS. MALDI-TOF/TOF MS/MS was carried out using the MALDI post-source-decay (PSD) in combination with the LIFT-cell TOF/TOF set-up of the Ultraflex mass spectrometer [18, 19]. Briefly, after the first TOF stage with an acceleration voltage of 8 kV, parent and metastable fragment ions induced by the PSD process were selected simultaneously by a timed ion gate. The ion package was then lifted in the timed LIFT-cell by a 19 KV potential. Data dependent single-scan MS/MS experiments were carried out in an automated fashion with the FlexControl 1.2 software that applies several filters for parent selection, e.g., signal intensity, signal-to-noise ratio or resolution. The calibration of the MS/MS spectra is performed automatically by the XMASS data processing software, using mass dependent higher order machine calibration curves in combination with the parent ion lock mass.

ESI-QTOF MS/MS. The Q-Tof micro was equipped with a Micromass CapLC with autosampler (Micromass, UK) using a ten port zero dead volume valve (Valco-Vici) enabling fast sample loading on a pre-column (Opti-Guard 1mm, Symmetrie C18, Optimize Technologies Inc., OR) at a flow rate of 15 μl/min isocratically with solvent A delivered by auxiliary pump C. The composition of solvents A and B was 0.1% (vol/vol) formic acid in 3% (vol/vol) MeCN or 95% (vol/vol) MeCN, respectively. After washing of the pre-column, the ten-port valve was switched allowing delivery of a tri-phasic MeCN gradient at 300–400 nl/min onto the analytical column (GROM Ruby C8, 2 μm, 0.1 $\times$ 100 mm) by back-flushing the pre-column. The three phases of the gradient were from 0 to 25% B in 2 min, then 5 min isocratically at 25% B, followed by 25 to 40% B during 30 min. The Q-TOF was operated in DDA mode using a 1-s MS survey scan followed by 2-s scans, each on three different precursor ions. CID spectrum acquisition was allowed for up to a total of 12 s on each precursor ion or stopped when the signal intensity fell below three counts per s respectively, before a new MS to MS/MS cycle was started. This set of parameters allows for acquisition of good quality MS/MS spectra of low intensity ions but can increase the duty cycle of the instrument to approximately 40 s in case of three intense precursors present. Tapered fused silica capillaries with a 10 μm aperture (PicoTip, New Objectives, Woburn, MA) served as spraying emittors. Precursors were excluded from any further MS/MS experiment for one min and singly charged ions were excluded as precursors for MS/MS.

Results and Discussion

Evaluation of Relative Protein Quantification by ^{18}O Labeling

It has been shown that the catalysis of the C-terminal ^{18}O labeling of peptides can be dissected from the actual proteolysis of proteins by endoproteases like trypsin [15, 20, 21]. During this process, two oxygens are exchanged by trypsin into the α-carboxyl group of peptides C-terminally ending with a lysine or arginine residue. This is because tryptic peptides continue to interact with trypsin by forming covalent intermediates with repeated binding/hydrolyses cycles. In order to assess whether this technique allows for a proteome-wide protein quantification, first tests were performed with a mixture of ten standard proteins labeled differentially with either ^{16}O or ^{18}O, ranging in Mr from 2184 to 16225 Da, mixed together at various concentrations and analysed by nanoLC-MS on an ESI-QTOF mass spectrometer (see Experimental Methods). Initially a two-fold change in protein concentration was chosen to evaluate the lower quantification limit of the ^{18}O labeling method.

For this, either the ^{16}O pool or the ^{18}O pool was set at the higher concentration. For quantification, the ratios of the first mono-isotopic peaks I_0/I_4 (I_0 and I_4 stand for the first isotopic peak of the ^{16}O- and ^{18}O-labeled peptides, respectively, recalling that two oxygens are incorporated) and the second isotopic peaks, I_1/I_5, were calculated. While the statistical averages were basically the same for a given sample analyzed, the ratio I_1/I_5 better represents the relative quantity for larger peptides. The switch from I_0/I_4 to I_1/I_5 should be applied in the mass range of 1500 to 1800 Da where the second isotopic peak becomes more intense than the first one and the intensity contribution of the fifth isotope of the ^{16}O-labeled peptide to the intensity of I_4 becomes significant. A total of 17 precursor peptides, present as singly, doubly, or triply charged ions, were considered from the ion chromatography of TOF MS on the ESI-QTOF instrument. The ratios of a theoretical 1:2 $^{16}O/^{18}O$ mixture ranged from 0.41 to 0.56. The average was 0.50 ± 0.05 (panel a in Figure 2). Duplicating the experiment by mixing a freshly prepared sample gave ratios in the range of 0.49 to 0.71 and an average ratio of 0.61 ± 0.06. The difference between the two averages is likely due to errors during mixing and/or differential losses during desalting on the SepPak cartridges rather than the labeling procedure itself. A sample set with the $^{16}O/^{18}O$ reversed to 2:1 resulted in a mean ratio of 2.2 ± 0.2 for the same 17 peptides (Figure 2, panel b). In these three sample sets the standard deviations were always ±10% and the extreme ratios in each sample set differed less than 50% from the theoretical ratio values of 0.5 or 2.0 respectively. It can therefore be concluded that the ^{18}O labeling method enables quantitative proteomics with a confidence in changes of protein concentrations as low as 2-fold.

The high-end limit of relative quantification is determined by the mass spectrometer, acquisition parameter settings, and also by the complexity of the sample. The higher the signal-to-noise ratio (S/N), the higher the dynamic range of the method will be. With standard parameter settings on the QTOF, 10- to 20-fold differences were detected by averaging all MS spectra acquired during the chromatographic elution of each individual peptide pair. A sample set consisting of a 1:10 $^{16}O/^{18}O$ mixture of the same protein digest mixture as described above resulted in a mean ratio of 0.13 ± 0.02, with a range of values between 0.11 and 0.20. With this and other results (not shown), we concluded that it is possible to measure semi-quantitatively 10- to 20-fold differences with ^{18}O-labeled samples on a time-of-flight instrument.

In order to improve the data quality on samples with higher differences, longer acquisition times were required to increase the S/N ratios. However, the need to perform LC separation of complex samples on-line to ESI-MS imposes a limit on increased acquisition times due to chromatographic peak width and the need to obtain high sequence coverage of the peptides present in the sample. Today, ion trap mass spectrometers are cheaper and have faster cycle times in automatic, data-dependent MS to MS/MS acquisitions than a quadrupole/time-of-flight instrument (8 to 9 s for ESI-IT compared to up to 24+ s for ESI-QTOF with 2 precursors, as described in Experimental Methods section). An alternative is MALDI-TOF-MS as the samples are static. With MALDI, it can be envisaged to acquire MS spectra until the entire sample is ablated from the sample target plate. Compared to direct MALDI-MS analysis of a digest, coupling an LC separation of peptide mixtures with fraction collection directly on MALDI plates (LC-MALDI) drastically reduces sample complexity resulting in an increase in peptide concentration from a chromatographic peak, and decreased ion suppression by contaminants. All these effects add up to an increase in the S/N. For these reasons, we evaluated the possibility to use an ion trap instrument for quantification of ^{18}O-labeled samples with combined on-line LC-ESI and LC-MALDI.

Protein Quantification by LC-ESI-MS/MS on Ion Trap

Peptide pairs C-terminally labeled by ^{16}O or ^{18}O differ only by 4 Da in mass. Under electrospray ionization multiply charged peptide ions are formed which are detected as doubly, triply, or quadruply charged species. The resulting differences in *m/z* units are therefore of 2, 1.33, and 1 units, respectively, between the ^{16}O- and the ^{18}O-labeled version. Due to the close spacing of the isotopes it was always considered to be necessary to use a mass spectrometer with high resolution, e.g., MALDI-TOF or ESI-QTOF. Use of ion trap MS was therefore suggested to be difficult [15]. Recently, man-

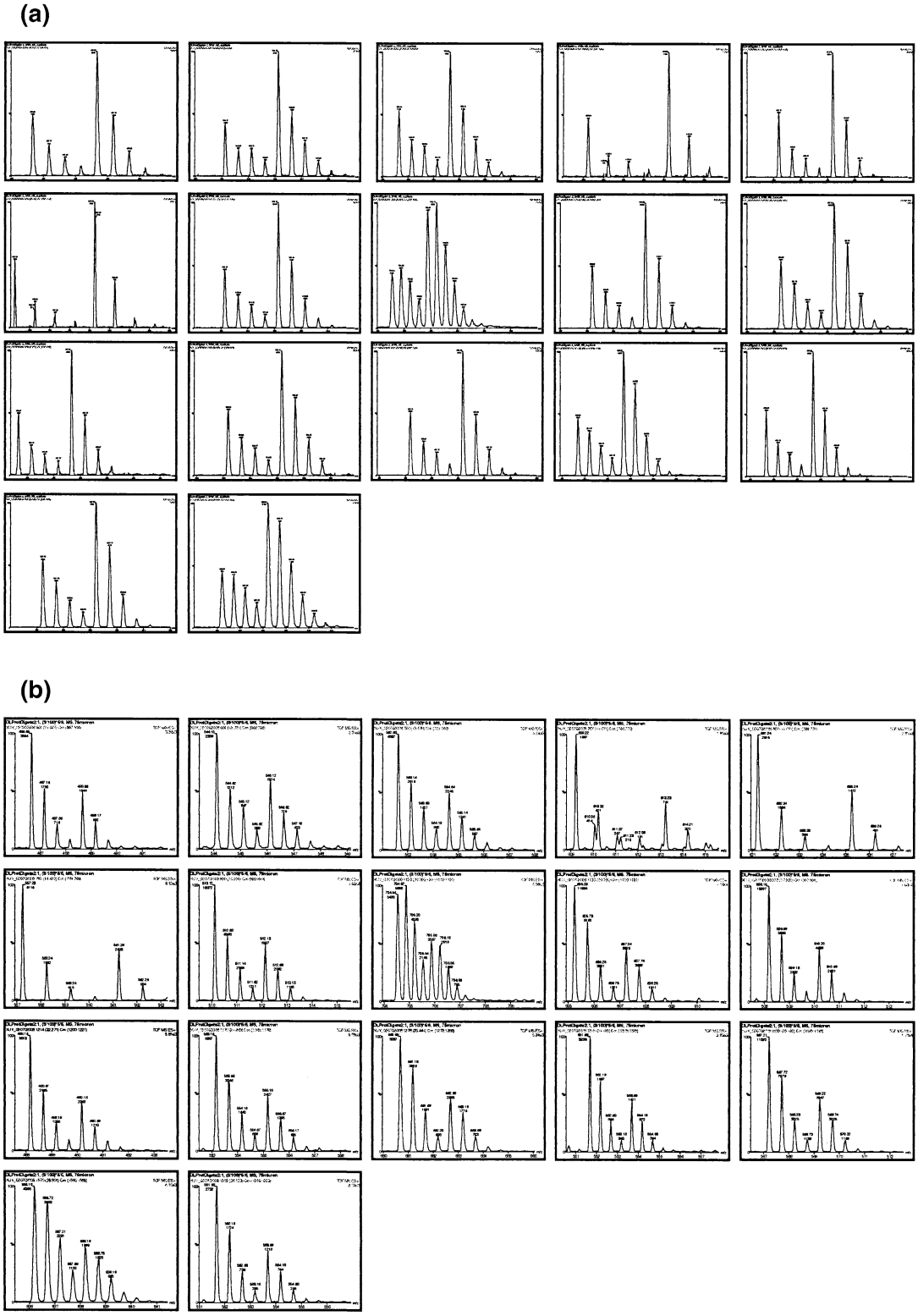

Figure 2. Proof of concept for the quantification of differential protein expression with C-terminally ^{18}O-isotope labeled peptides. Ten standard proteins were digested, the mixture split in half and subsequently labeled by enzyme catalyzed oxygen exchange at the C-terminal Lys and Arg residues of the tryptic peptides either with ^{16}O- or ^{18}O-water. The two samples were mixed at ratios 1:2 or 2:1 and analyzed by LC-nanoESI-MS on a QTOF instrument. A total of 17 representative peptides, as 1+, 2+, and 3+ ions, were chosen for the calculation of the peak intensity ratios of the ^{16}O- and ^{18}O-labeled peptides. ESI-QTOF MS spectra of these ions are shown in panel A for the 1:2 mixture and in panel B for the 2:1 mixture, respectively. The average $^{16}O/^{18}O$-ratio in case of the 1:2 mixture was calculated as 0.50 ± 0.05 and 2.2 ± 0.2 for the 2:1 mixture, respectively.

Table 1. Relative $^{18}O/^{16}O$-ratio quantification of different apomyoglobin mixtures using precursor ion intensities of MS scans, CID y-ion fragment intensities, and extracted ion chromatogram (EIC) peak areas from ion trap LC-ESI-MS/MS acquisition data

$^{18}O/^{16}O$ expected	MS signal	Number of ions[a]	$^{18}O/^{16}O$ observed (mean ± SD)	R.D. (%)	Relative error[b] (%)
1	Precursor ion	6	1.06 ± 0.26	24.5	+6
	y-Ions	4	1.06 ± 0.60	56.6	+6
	EIC	6	1.35 ± 0.65	48.1	+35
5	Precursor ion	7	4.21 ± 1.18	28.0	−16
	y-Ions	17	4.39 ± 0.50	11.4	−12
	EIC	6	8.81 ± 6.76	76.7	+76
0.2	Precursor ion	7	0.28 ± 0.15	53.6	+29
	y-Ions	20	0.27 ± 0.08	29.6	+26
	EIC	6	0.65 ± 0.59	90.8	+69
10	Precursor ion	7	9.13 ± 3.76	41.2	−9
	y-Ions	17	7.98 ± 2.82	35.3	−20
	EIC	6	8.81 ± 7.18	81.5	−12
0.1	Precursor ion	6	0.16 ± 0.07	43.8	+38
	y-Ions	17	0.25 ± 0.09	36.0	+60
	EIC	5	0.15 ± 0.11	44.0	+33
20	Precursor ion	7	15.10 ± 7.46	49.4	−25
	y-Ions	19	14.32 ± 16.74	116.9	−28
	EIC	6	18.82 ± 8.21	43.6	−6
0.05	Precursor ion	7	0.14 ± 0.15	107.1	+64
	y-Ions	15	0.13 ± 0.08	61.5	+62
	EIC	5	0.16 ± 0.11	68.8	+69

[a]Number of ions where the pair of ^{18}O- and ^{16}O-labeled peptides was clearly detected. For precursor ion calculations only the more intense signal between a 2+ or 3+ ion signal was included.
[b]For relative error calculation, the intensity of the higher abundant isotopic peptide form was divided by the intensity of the less concentrated form.

ufacturers of ion trap instruments made some major improvements to the performance of their instruments. For instance, the Bruker Esquire3000plus has improved ion optics coupled with a higher sensitivity compared to its predecessor. In addition, the MS instrument can be run in the so-called "enhanced mode" with 20 acquired data points per *m/z* unit allowing for isotopic resolution of triply, and under ideal conditions, even quadruply charged ions. We were therefore interested to test this instrument for quantification of ^{18}O-labeled peptides. A horse apomyoglobin digest was labeled with ^{18}O and mixed with ^{16}O-labeled peptides in the ratios of 1:1, 1:5, 1:10, 1:20, 5:1, 10:1, and 20:1. An aliquot corresponding to 100 fmol of the lower abundant isotope of each mixture was analyzed by LC-ESI-MS/MS using the tuning parameters described under Experimental Methods.

The LC-MS/MS data provides three possibilities for ratio calculation. First, there is the MS scan on the intact peptide precursor or alternatively a summed MS spectrum over the entire chromatographic peak of a precursor. The latter gave increased S/N ratios, hence more accurate $^{18}O/^{16}O$-ratios and should definitely be applied when dealing with higher concentration differences, e.g. exceeding 10-fold limits.

Second, $^{18}O/^{16}O$-ratios could also be calculated from MS/MS spectra [22] because y-ions retain the C-terminally labeled lysine or arginine residues. However, special care needs to be taken when using these ions. A peptide can fragment in many ways resulting in different types of ions. Therefore chances are high that the signal of a y-ion isotope can be disturbed by the presence of an underlying fragment ion of the b, a, or c-series. An example is the y_4-ion of monoisotopic mass 500.356 from the apomyoglobin peptide VEADIAGH-GQEVLIR that is almost isobaric with the a_5-ion at 500.272. Although the $^{18}O/^{16}O$-labeled pair of the y_4-ion was detected in the ESI-MS/MS spectra on the ion trap as well as in the MALDI PSD-MS/MS spectra, the $^{18}O/^{16}O$-ratios were far from ratios calculated on other y-ions (not shown). Another observation was that the calculated $^{18}O/^{16}O$-ratios of low mass y-ion pairs differed in most cases considerably from the ones calculated in the higher mass range of the MS/MS spectra (700–1500 *m/z* units). This effect could be explained by decreased peak intensities, thus reduced S/N, at the lower end of the mass range in ion trap generated MS/MS spectra.

Third, extracted ion chromatograms (EIC) of the pair of ^{18}O- and ^{16}O-labeled peptides could be used to compare the area under the chromatographic peaks. This approach was initially demonstrated for quantification of ICAT-labeled samples [9]. The mass units of the extracted ion signals need to be chosen with a very narrow tolerance window (±0.2 *m/z*) in order to prevent contribution of the I_2 to the ^{16}O or ^{18}O signal.

From the summarized results on apomyoglobin peptides presented in Table 1, several trends can be extracted. The observed mean ratios seemed to be biased in favor of the less abundant isotope regardless of whether this was the ^{16}O- or ^{18}O-labeled peptide. This observation corresponded with other data acquired on

Table 2. Quantification results on MS and MS/MS data acquired on ESI-IT in comparison with MALDI-TOF (Bruker ReflexIII and Ultraflex) and MALDI-TOF/TOF (Ultraflex)

$^{18}O/^{16}O$ expected	MS Instrument and signal		Number of ions[b]	$^{18}O/^{16}O$ observed (mean ± SD)	R.D. (%)	Relative error[c] (%)
	ESI-IT	MALDI-TOF[a]				
5	Precursors		7	4.21 ± 1.18	28.0	−16
5	y-Ions		17	4.39 ± 0.50	11.4	−12
5		ReflexIII, MS	17	4.76 ± 0.87	18.3	−5
5		Ultraflex, MS	13	4.24 ± 0.44	10.4	−15
5		Ultraflex, y-ions	42	3.67 ± 1.40	38.1	−27
0.2	Precursors		7	0.28 ± 0.15	53.6	+29
0.2	y-Ions		20	0.27 ± 0.08	29.6	+26
0.2		ReflexIII, MS	13	0.24 ± 0.05	20.8	+17
0.2		Ultraflex, MS	15	0.20 ± 0.03	15.0	0
0.2		Ultraflex, y-ions	64	0.29 ± 0.09	31.0	+31

[a]One chromatographic peak of a RP nanobore LC separation was collected in several MALDI fractions, resulting in an increased number of MS analyses on the same $^{18}O/^{16}O$-labeled peptide pair.
[b]Number of ions where the pair of ^{18}O- and ^{16}O-labeled peptides was clearly detected. For ESI precursor ion calculations only the more intense signal between a 2+ or 3+ ion signal was included.
[c]For relative error calculation, the intensity of the higher abundant isotopic peptide form was divided by the intensity of the less concentrated form.

ESI-QTOF or MALDI-TOF instruments using the apomyoglobin test sample (see also Tables 2 and 3). There are several reasons behind the observed bias, based on calculations using I_4/I_0 and I_5/I_1 ratios. Here we extracted intensity values directly from the raw data, consequently the first reason is the artificial contribution of the background chemical and electronic noise in mass spectra that increases intensities of weak peaks. Indeed subtraction of the background signals by an in-house developed peak detection algorithm for MALDI-TOF mass spectra could diminish this kind of bias (not shown). Second, the residual 5% of ^{16}O in the ^{18}O-water theoretically increases I_0 intensities by 0.25% relative to the monoisotopic peak of the ^{18}O-labeled peptide. In the same manner I_2 intensities are increased by 9.5%. This in turn decreases the intensities of I_4 and I_5 and as such can have negative effects in detecting relative differences at higher ratios [23]. The use of purer ^{18}O-water will decrease these contributions. Other factors contributing to the observed bias include incomplete ^{18}O incorporation, slow ^{18}O-to-^{16}O back exchange and ^{13}C contributions to the different isotopic peak intensities. More sophisticated ratio calculations can be applied to correct for the bias [13]. However this bias does not prevent the ^{18}O labeling method from detecting protein concentration changes as low as 2-fold using the simple I_4/I_0 and I_5/I_1 ratio calculations.

It is also apparent with $^{18}O/^{16}O$ mixtures of up to 1:5 ratios that the comparison with EIC traces resulted in relative errors that were greater compared to precursor ion MS and fragment ion MS/MS scans. As explained above, due to residual 5% ^{16}O in the ^{18}O-water the I_2 is relatively increased, hence extraction of signal intensity coming from the I_2 peak can contribute to the EIC of both monoisotopic peptide forms. Interestingly, the EIC trace calculations became at least as reliable, if not more, than the two other modes with ratios exceeding the 1:5 level. Otherwise it was observed that the MS and the MS/MS signals gave a similar level of accuracy on the ratio, independent of the $^{18}O/^{16}O$ mixture subjected to ESI ion trap MS analysis.

As can be seen from Table 1 and Figure 1, the ion trap MS and MS/MS analysis resulted in rather high standard deviations for $^{18}O/^{16}O$-ratios. It was therefore interesting to compare directly with results obtained by LC-MALDI-TOF analysis. The LC-MALDI approach

Table 3. Comparison of $^{18}O/^{16}O$-ratio quantification based on $^{18}O/^{16}O$ peak intensities determined on different mass spectrometers in MS or MS/MS mode

Peptide	$^{18}O/^{16}O$ expected	MS instrument MS signal	Number of ions	$^{18}O/^{16}O$ observed (mean ± S.D.)
ALELFR	0.5	MALDI-TOF-MS	1	0.41
	0.5	ESI-IT, MS	1	0.44
	0.5	ESI-QTOF, MS	2	0.49
NWGLSVYADKPETTK	1.0	MALDI-TOF-MS	1	0.91
	1.0	ESI-IT, MS	2	0.91
	1.0	ESI-QTOF, MS	2	1.01
	1.0	MALDI-TOF/TOF-MS/MS	9	0.87 ± 0.12
	1.0	ESI-IT, MS/MS	5	0.90 ± 0.21
	1.0	ESI-QTOF, MS/MS	11	0.91 ± 0.19

allowed for the collection of several fractions from the same chromatographic peptide peak, hence increasing the statistics for mean ratio and standard deviation calculations. However, despite improved statistics and improved MS resolution on the MALDI-TOF data compared to the ESI-IT data, there was no significant improvement on the relative error of the calculated ratios nor on the standard deviations apparent (Table 2). It could be concluded from all these data that relative quantification with ion trap mass spectrometry of $^{18}O/^{16}O$-labeled peptide ions is feasible and the results are comparable with MALDI-MS data. Even though the variability within one data set can be rather high, with relative standard deviation values in the range of 20–50%, it should be possible to measure reliably protein concentration differences as low as 2- to 3-fold with ESI-QTOF (Figure 2) by combining precursor MS, y-ion fragment MS/MS, EIC trace and LC-MALDI-MS data.

Application of ^{18}O Exchange to Relative Protein Quantification in a Human Plasma Subfraction

The availability of quantitative information on protein expression levels greatly increases the value of protein identification for a given proteome. We therefore applied the trypsin-catalyzed ^{16}O-to-^{18}O exchange to a total trypsin digest of an albumin- and immunoglobulin-depleted human plasma fraction consisting of low molecular weight proteins (MW cutoff ca. 20 kDa) and combined it with an equivalent sample prepared in parallel for ^{16}O labeling. To this mixed plasma sample, 200 pmol ^{16}O-labeled and 100 pmol ^{18}O-labeled peptides of a tryptic digest of horse apomyoglobin were added as an internal standard. A portion corresponding to 30% of the total peptide mixture was separated into 17 fractions by strong cation exchange (SCX) chromatography. The peptides collected in fraction 6, eluting with 50 mM ammonium acetate, were subsequently analysed by LC-MS/MS using the ESI-IT system with on-line LC-MALDI fraction collection, as well as with the ESI-QTOF system as described in the Experimental Methods section. A database search of the MS/MS data thus obtained against the SWISS-PROT protein database identified human plasma proteins of low molecular weight together with the horse apomyoglobin peptide ALELFR. As an example, a peptide with the sequence NWGLSVYADKPETTK from α-1-acid glycoprotein 1 (A1AG_HUMAN) eluted together with the horse apomyoglobin peptide and they were subsequently sampled on the same MALDI target anchor. The MALDI-MS is shown in the top panel of Figure 3. The same peptides were also detected with the ESI-IT as well as with the ESI-QTOF systems. As can be seen in Figure 3 the two peptides were present as an $^{16}O/^{18}O$-labeled pair and the $^{18}O/^{16}O$-ratios of 0.44 and 0.91 (expected 0.5 and 1.0) calculated from the ion trap MS and MS/MS peak intensities were confirmed by both MALDI-TOF and ESI-QTOF MS and MS/MS (Table 3).

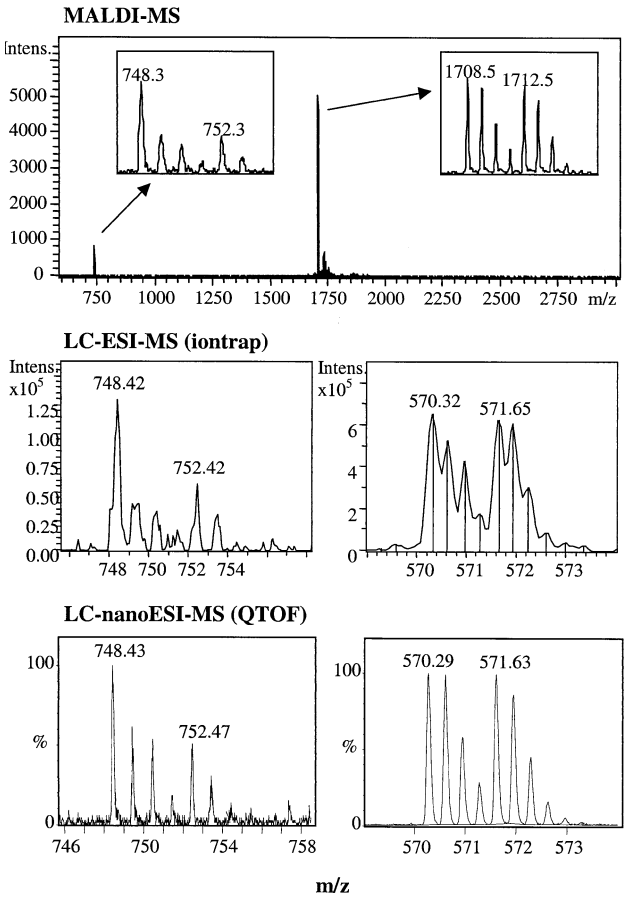

Figure 3. MS analysis with MALDI-TOF and LC-ESI on ion trap and QTOF of one peptide isolated from a complex, $^{16}O/^{18}O$-labeled protein sample. The low molecular weight protein fraction (MW cutoff of approximately 20 kDa, 2.28 mg total) of an albumin and immunoglobulin depleted human plasma sample was digested. The digest was split in two equal portions. Peptides in the first portion were labeled by trypsin catalyzed ^{16}O-to-^{16}O oxygen exchange at the C-terminus in normal water, the second portion by ^{16}O-to-^{18}O oxygen exchange in ^{18}O-water, respectively. The combined 1:1 mixture was subsequently spiked with a $^{16}O/^{18}O$ 2:1 mixture of a horse apomyoglobin tryptic digest. One fraction was analyzed by LC-MS/MS on QTOF and IT coupled with LC-MALDI. The horse apomyoglobin peptide ALELFR ($[M + H]^+$ = 748.44), ($[M + H]^{2+}$ = 374.72) and the peptide NWGLSVYADK-PETTK ($[M + H]^+$ = 1708.7), ($[M + H]^{3+}$ = 570.26) from A1AG_ human were identified among others in this particular sample and eluted at the same time from the column on the ion trap system. The relatively short apomyoglobin peptide was detected only in the singly protonated form on the ion trap, but was detected as a singly and doubly charged ion on the QTOF, whereas the larger A1AG_HUMAN peptide was detected as a doubly and triply charged ion on both systems. The top panel is the MALDI-MS spectrum showing both ions with the insets as a zoom into the isotopic peak cluster of the two peptide pairs. The middle and the lower panel show the apomyoglobin and the A1AG_HUMAN ions as detected on the ion trap and QTOF, respectively. For the $^{18}O/^{16}O$-based quantification please refer to Table 3.

It is interesting to note that the illustrated A1AG_HUMAN peptide is the 3+ ion. Although the isotopic peaks were not completely resolved in the ion trap mass spectrum, the ratio calculated on the first isotopic peaks of the ^{16}O- and ^{18}O-labeled isotopic peak

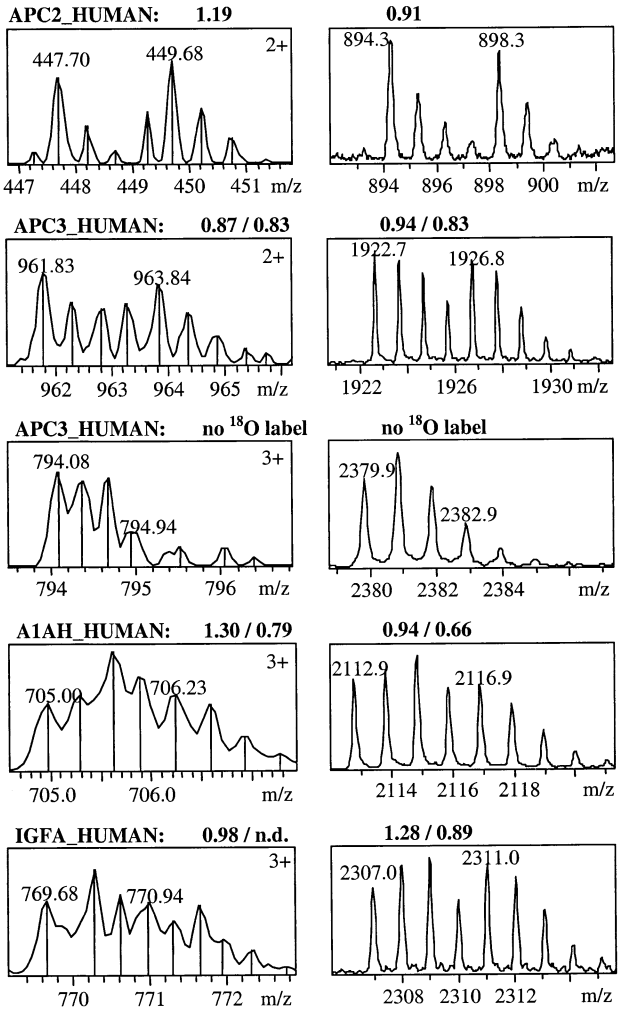

Figure 4. Comparison of ion trap LC-ESI-MS and MALDI-TOF-MS peak resolution for $^{18}O/^{16}O$-ratio calculations. A human plasma subfraction containing low molecular weight proteins was digested with trypsin and one half each was enzymatically labeled with either ^{16}O or ^{18}O, respectively, as described under Experimental Methods and in Figure 3. The peptides of the combined aliquots were fractionated by cation exchange chromatography and one particular fraction was subjected to RP chromatography with on-line ESI-MS/MS on an ion trap and fraction collection for MALDI-TOF. On the left, the combined MS survey scans of the precursor ion pairs submitted to MS/MS in the ion trap are shown together with the corresponding MALDI-MS of the same peptide precursor ion on the right. At the top of each pair of MS spectra, the protein identified by means of the ESI-IT MS/MS spectra is given together with the corresponding $^{18}O/^{16}O$-ratio as calculated by I_4/I_0 and I_5/I_1. The charge state of the detected ion on the ESI-IT MS as well as the *m/z* values for each of the first monoisotopic peak in the $^{16}O/^{18}O$ peptide pair are marked as well.

cluster was well within the calculated value from the time-of-flight measurements. The measured ratios for the apomyoglobin and the plasma protein peptides corresponded very well with the values that could be expected from the experimental setup.

Figure 4 is a representation of another five different peptides out of four different proteins identified in the same sample. The first two examples represent the ideal case. The ESI-generated ions are doubly charged and the resolution achieved on the ESI-IT is largely sufficient to allow accurate $^{18}O/^{16}O$-ratio calculation. Both examples validate the method, as the experimental ratio of around 0.90 corresponds well with the theoretically expected value of 1.0. The APC2 peptide had the sequence LRDLYSK, the APC3 peptide SEAEDASLLSFM(ox)QGYMK. The third example represents a C-terminal peptide derived from apolipoprotein C3 with the sequence DKFSEFWDLDPEVRPTSAVAA. As there is neither a lysine nor an arginine C-terminal residue, no ^{18}O exchange occured, hence the peptide appears correctly as a single isotopic cluster. Although such singular peptide peaks can be used to identify C-terminal peptides of proteins quickly [21, 24], caution is required because concentration differences in $^{16}O/^{18}O$-labeled peptides exceeding 20:1 might also be detected as singlets only.

The last two examples [A1AH peptide of sequence EQLGEFYEALDC(cam)LC(cam)IPR and IGFA peptide GPETLC(cam)GAELVDALQFVC(cam)GDR] are cases where the incorporation of two ^{18}O atoms was less than complete. The intensity of the third isotopic peak (I_2) of both peptides should theoretically show an intensity that is between 70–75% of the one of I_1. However I_2 in the ^{16}O-labeled peptide isotope cluster is the most intense peak as detected in both ESI-IT and MALDI-TOF MS spectra. On the contrary, I_6 (the third isotopic peak in the ^{18}O-labeled peptide) shows only about 50% of the intensity of I_2. As mentioned above, there is normally little difference between the ratios calculated by dividing I_5 by I_1 or I_4 by I_0 in the case where the ^{16}O-to-^{18}O exchange proceeded quantitatively. This fact is well demonstrated with example two in Figure 4 (second panel) where the two values differ only by 0.04 for the ESI-IT and 0.09 for the MALDI-TOF spectrum respectively. Nevertheless there were major differences between those two ratios in the last two examples of Figure 4, which were calculated to be 0.61 and 0.86, respectively. This suggests that the differences between those ratios could serve as an automatic quality control over successful oxygen exchange and as a trigger to initialize accurate calculations using I_0, I_2, and I_4 intensities, as well as natural isotope distributions of peptides as described by Yao and colleagues [13]. It is not obvious why the oxygen exchange reactions for these peptides did not reach completion but it had been shown earlier by Yao et al. [15] and Schnölzer et al. [21] that the kinetics for the oxygen exchange reaction can differ considerably between different peptides. Coincidently both peptides are rather large and contain two alkylated cysteine residues. The hydrophobicity and p*I* characteristics of both peptides compare well with the APC3 peptide (second example from top) that showed complete ^{16}O-to-^{18}O exchange. Immobilized trypsin, providing high enzyme concentration, should always be an option when the exchange reaction needs to be pushed to completion.

Finally, the last example given in Figure 4 (panel 5)

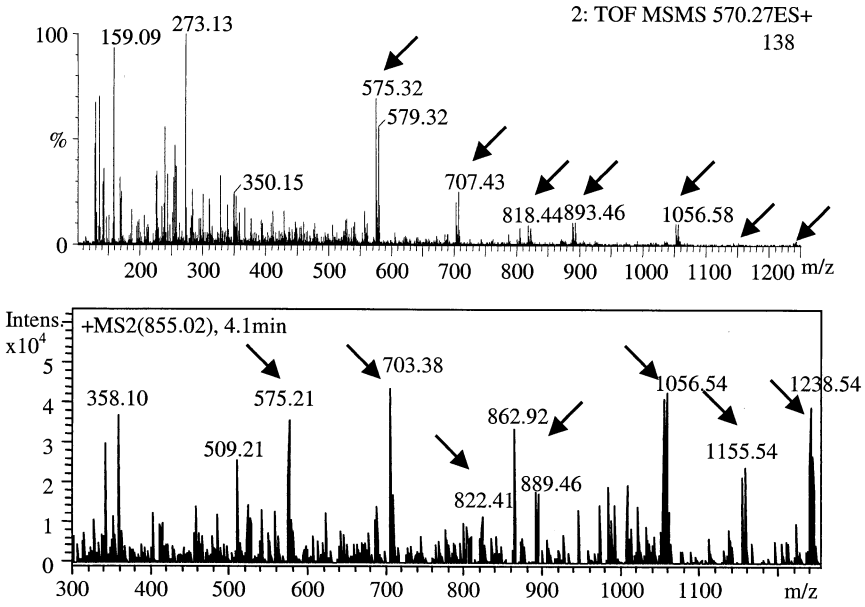

Figure 5. Paired $^{16}O/^{18}O$ peaks of y-ions assist validation of automated sequence identification. ESI-QTOF and ESI-IT allow the concurrent isolation of both ^{18}O- and ^{16}O-labeled parent ions in the CID cell. The y-ions bearing the C-terminal labeled Lys or Arg residue appear as paired peaks with a 4 Da mass difference. The paired ion signals marked with arrows in the QTOF (top) and ion trap (bottom) MS/MS spectra were readily identified as y-ions and the sequence SVYADQ/K could easily be assigned. A BLAST search with this sequence tag retrieved sequence similarity to a peptide sequence of A1AG_HUMAN. Identification of A1AG_HUMAN was confirmed by attributing the N-terminal mass difference of 470.1 to the NWGL and the C-terminal mass difference of 428.2 to the PETTK sequences, respectively.

shows a case where the second isotopic peak of the ^{16}O-labeled peptide was disturbed to such an extent that the peak was not detectable. This is due to the space-charge effect in the ion trap, an effect that could prevent the correct ratio calculation for triply and higher charged peptides on this machine. The space-charge effect is caused by a heterogenous distribution of energy in cases where only a few ion species are trapped, resulting in a perturbation of close ion trajectories, like in the case of isotopes. The Esquire tries to regulate the trap filling by a predetermined value (ICC for ion charge control). By setting this ICC value relatively low, it is rather unlikely to risk ion trajectory perturbation, hence detecting a mass shift of the first isotope, due to overloading of the trap with only one ion species. Therefore analysis of fairly complex mixtures with a homogenous energy distribution is an advantage with regards to avoiding space charge effects. Correspondingly, the samples analyzed so far in our laboratory by using optimized ESI-IT tuning parameters resulted in a rather low occurrence of the space-charge effect. Furthermore, accurate isotope intensities can be measured more reliably on doubly charged ions, if present, where the space charge effect has less impact.

Database Searches

When running an LC-MS/MS experiment where the mass spectrometer automatically switches from MS to MS/MS mode by choosing the most intense ions present in the MS survey scan for fragmentation, it is obvious that ^{18}O-labeled peptide precursors are subjected to MS/MS. As the precursor mass of such an ion is 4 Da larger than the naturally occurring peptide mass, this could prevent a correct identification during the database search. However, database search algorithms can account for this by allowing a variable 4 Da modification at the C-termini.

Ions containing the peptide C-terminus appear as doublets spaced by 4 mass units in MS/MS spectra. Others have already exploited this feature of peak doublets to do peptide de novo sequencing by applying a 50:50 $^{16}O/^{18}O$ labeling of the C-termini that results in a mass difference of 2 Da [25, 26]. By recognizing y-ions this way, we were for instance able to deduce easily the sequence SVYADQ/K from the MS/MS spectrum acquired on the ESI-QTOF and the ESI-IT of the plasma peptide shown in Figure 3 with a monoisotopic mass of 1708.5 (Figure 5). A Blast search revealed sequence alignment with the human α-1-glycoprotein 1 protein. As expected, the N- and C-terminal mass differences of 470.1 and 428.2 fitted exactly to the missing N-terminal and C-terminal sequences NWGL and PETTK of α-1-glycoprotein 1, respectively. The endoprotease catalyzed ^{16}O-to-^{18}O exchange provides therefore additional value to a proteome analysis by enabling the facile confirmation of a database search identification result in addition to relative quantification data.

Conclusions

The trypsin catalyzed ^{16}O-to-^{18}O oxygen exchange reaction presents a highly specific and versatile method for the labeling of tryptic peptides with two stable ^{18}O isotopes, thus increasing the mass of the labeled peptide by four mass units. The labeling reaction is generally independent of the amino acid sequence of the peptide (an exception to this rule is given in Figure 4) and it is therefore possible to detect peptides being post-translationally modified. We show here that the 4 Da mass difference between labeled and unlabeled peptide is sufficient for the relative quantification of peptides/proteins using either one of the three mass spectrometers tested, namely ESI-QTOF, ESI-IT, and MALDI-TOF. Despite the fact that the resolution attainable on an ion trap mass spectrometer is about four times lower compared to the two TOF instruments, the results presented here show no obvious difference in measured $^{16}O/^{18}O$-ratios. Nevertheless, because of the lower mass resolution and the space-charge effect with the ion trap mass detector, problems of accurate isotopic peak detection can occur, especially with ions charged 3+ and 4+.

Although the relative standard deviations of the calculated mean $^{16}O/^{18}O$-ratios were in the order of 20–50%, it was still possible to distinguish between a theoretical 1:1 and a 2:1 or 1:2 ratio. A difference of at least 2- to 3-fold in expressed protein concentrations between two biological samples is generally accepted to be of biological significance. A larger cutoff value such as 5-fold changes can also be applied to focus on the most important proteins only [27]. The present approach of conducting comparative proteomics by means of separating differentially labeled peptide samples prior to tandem mass spectrometry offers at least two, and in the case of on-line ESI-MS even three, possibilities to verify $^{16}O/^{18}O$-ratios. The first and most accurate is by measuring intensities of the signals generated by the intact peptides in the MS-survey scans. By averaging all MS scans acquired over the chromatographic peak of a peptide the S/N ratio can be increased, hence the accuracy of the ratio value between the two isotopic forms is improved. Second, fragmentation of tryptic peptides at low collision energies or in MALDI-PSD mode produces usually C-terminal y-ion fragments detected as doublets with a 4 Da mass difference. We show here that, by tuning the mass spectrometer, both light and heavy precursor ions are isolated quantitatively for CID or PSD. Hence, y-ion series can also be used for relative protein quantification. Yet caution must be taken in order to avoid using y-ion signals that can be influenced by underlying signals of a fragment from the unlabeled N-terminus or an internal fragment. Programs automatically extracting intensity ratios of y-ion series directly from MS/MS peak lists, could use the sequence identified by a database search to apply a filter removing masses belonging potentially to N-terminal fragment ions of the b and a series in order to avoid false ratio calculations. Third, the chromatographic peaks of EIC traces from the light and heavy form of a peptide can be integrated to provide another means of $^{16}O/^{18}O$-ratio calculation based on peak area. It is, however, the least accurate of the three methods, and it requires substantial raw data reprocessing based on knowledge of the sample acquired beforehand with the other two, above mentioned methods, and a protein identification. Taken together, these results suggest that it is possible to relatively quantify with confidence proteins whose concentration differ by a factor of two and more.

The direct combination of LC-ESI-MS/MS on an ion trap or QTOF with parallel fraction deposition of the RP column eluate on a MALDI target for subsequent MALDI-TOF/TOF MS/MS offers many interesting features. First, the two ionization modes provide complementary data for a potential increase in protein sequence coverage. Second, the acquisition in ESI mode on-line to LC separation is prone to miss out on peptides due to the cycle time of the mass spectrometer switching from MS to MS/MS mode and due to the presence of many co-eluting peptides at a given time. In contrast, once peptides are deposited on a MALDI target they remain amenable to analysis for a long period of time. Hence, it is imaginable to do a first survey scan in MS mode providing a first dataset on $^{16}O/^{18}O$-ratios of differentially labeled peptides. In a second MS experiment, using the tandem MS capability of new generation MALDI-TOF/TOF instruments, one can perform MS/MS only on those peptides that showed significantly different expression levels in the MS survey scan, thus saving a lot of machine time. We have developed, in-house, a peak detection software that can calculate isotopic ratios of predetermined mass differentials. The use of such software tools enables the creation of preferred mass lists for the subsequent MS/MS experiment spontaneously.

The combined ESI/MALDI approach would ensure that interesting proteins showing differences in expression level would not go undetected. This approach is fast, parallel and only one aliquot of a particular sample is needed, in contrast to Griffins approach, where a first aliquot was used to do an MS survey scan on a ESI-TOF mass spectrometer followed by a second analysis on a different mass spectrometer doing MS/MS with a preferred mass list on fractions containing peptides of interest [28].

The protease-catalyzed ^{16}O-to-^{18}O oxygen exchange methodology for comparative proteomics relies heavily on a successful separation of highly complex peptide mixtures. This is because the total protein content of a biological sample needs to be digested without preseparation of the intact proteins in order to avoid differential sample loss due to chromatographic irreproducibility during the separation of two different samples. We

have followed the 2-D LC approach to separate very complex peptide mixtures that was introduced by Yates and colleagues [29, 30]. Mass spectrometry, especially in the form of LC-MALDI, adds yet another separation dimension based on mass.

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