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Accelerated ^{18}O-labeling in urinary proteomics

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ABSTRACT

Proteolytic ^{18}O-labeling of peptides has been studied and optimized in order to improve the labeling efficiency and to accelerate the process without increasing the degree of incomplete labeling. Using peptides generated from tryptic digested bovine serum albumin (BSA) and cytochrome *c* as model proteins, it was shown that complete labeling was achieved after 2 h at pH 6. To increase the sample throughput in a bottom-up proteomic setup, tryptic digestion of proteins *in-solution* was replaced with tryptic digestion using immobilized trypsin. As a result, an integrated approach was made possible, where both digestion (pH 8) and ^{18}O/^{16}O-labeling of the resulting peptides (pH 6) were done using immobilized trypsin beads. This simplified the sample handling and reduced the overall reaction time significantly: the setup enabled tryptic digestion and ^{18}O/^{16}O-labeling without sample transfer steps within 3.5 h with average ^{18}O/^{16}O-ratios of 0.96 ± 0.13 in aqueous buffer. The initial results were confirmed with a more complex matrix, by spiking urine with the model proteins, yielding results comparable with the ratios obtained in buffer. Satisfying ratios were also achieved regarding urinary proteins identified in a full scale bottom-up experiment. Average ^{18}O/^{16}O-peptide ratios of 0.83 ± 0.13 and 0.91 ± 0.27 indicated good performance in a highly relevant matrix for biomarker discovery.

1. Introduction

To improve the ability to accurately monitor changes in the protein expression both relative quantification and absolute quantification methodologies have been developed [1–5]. Regarding relative quantification, most techniques are based on incorporating a stable isotope tag which results in a mass shift and enables comparison with an unlabeled sample [6]. Several strategies for stable isotope labeling are available including isotope-coded affinity tags (ICAT) [2], isobaric tag for relative and absolute quantification (ITRAQ) [3], tandem mass tags (TMT) [4] and ^{18}O-labeling [5,7].

^{18}O-labeling is performed enzymatically mostly using trypsin, but enzymes like Lys-C and Glu-C are also used [8,9]. Labeling is performed at peptide level, and an incorporation of two ^{18}O atoms results in a mass shift of +4 Da for the labeled peptides. Advantages of this method are that all proteolytically generated peptides are labeled (except C-terminal peptides) and at low costs compared with e.g. ITRAQ. One major disadvantage is that the procedure is relatively time-consuming and labor-intensive [9]. This is partly due to the slow incorporation of the second ^{18}O atom into the peptides [6,10]. Several attempts have been made to accelerate this process, including the use of ultrasound during the labeling which seemed promising [11]. However, results from other groups suggest no improvement using this approach [9].

Another challenge with ^{18}O-labeling is back exchange to ^{16}O when labeled samples are mixed with unlabeled samples before LC–MS analysis [12], which is likely as long as trypsin is present. To reduce this effect, immobilized trypsin on solid supports can be used for labeling since the trypsin can be separated from the solution stopping the labeling reaction. Sevinsky et al. also applied immobilized trypsin for protein digestion prior to labeling, in order to reduce the risk of back exchange further [13]. Despite these efforts to improve labeling, many ^{18}O/^{16}O-labeling protocols still includes a time consuming reaction step, ranging up to 48 h, which is a bottleneck for the throughput of samples [13–15].

Tryptic digestion of proteins *in-solution* is another procedure which usually is time-consuming. Several groups have however described the use of immobilized trypsin for accelerated enzymatic digestion of proteins [16–18]. We also have recently demonstrated the application of protein digestion of urinary proteins using immobilized trypsin. The work described showed comparable digest quality compared to *in-solution* digestion, but with a significantly shorter time interval (1.5 h) [19].

The present study describes optimization of the ^{18}O-labeling procedure using immobilized trypsin with emphasis on accelerating the process without compromising the labeling quality. To achieve this, both reaction pH and reaction time was evaluated for the labeling procedure. Further, the *in-solution* digestion step preceding the labeling procedure has been replaced with an

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integrated approach using immobilized trypsin for both digestion and labeling. By optimizing the pH for each step separately, the overall digestion and labeling time was decreased without back exchange effects. Finally, the optimized procedure was tested and implemented in a full urinary proteomic workflow to evaluate the feasibility on clinically relevant samples.

2. Materials and methods

2.1. Chemicals

TPCK treated trypsin, bovine serum albumin (BSA), cytochrome *c* and ^{18}O-enriched water (97%) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). NHS activated Sepharose beads were purchased from GE Healthcare (Uppsala, Sweden). All other chemical used were of analytical grade.

2.2. Urine sampling

For the optimization of the procedure on BSA and cytochrome *c* in urine, mid-stream morning void urine from healthy individuals was used. The patient sample used was collected from an anonymous kidney transplant patient. After sampling, the urine was centrifuged at 1000 × *g* for 10 min and frozen at −70 °C until analyzed. Before the analysis, the samples were thawed and centrifuged at 9000 × *g*.

2.3. Preparation of immobilized trypsin beads

Preparation of the immobilized trypsin beads was carried out in-house as described earlier by Freije et al. [16].

2.4. HPLC-UV (HILIC), 1st dimension separation

In case of multidimensional separation, Hydrophilic Interaction Liquid Chromatography (HILIC) was used as the first dimension of separation. The chromatography was performed with an Ultimate 3000 system which consisted of a WPS-3000TSL autosampler, a HPGM-3200 pump, a VWD-3400 UV/VIS detector operated at 214 nm and 254 nm and Chromeleon software version 6.70 used for operation and data acquisition (all Dionex Corporation, Sunnyvale, CA, USA). Chromatographic separation was carried out on a 150 × 2.1 mm ID zwitterionic (ZIC®)-HILIC column, packed with 5 μm particles (pore size 200 Å, SeQuant AB, Umeå, Sweden). The flow was set to 0.2 mL/min and the injection volume was 50 μL. Mobile phases consisted of A: 20 mM ammonium acetate, pH 5.5 and acetonitrile (MeCN, 20/80, v/v) and B: 20 mM ammonium acetate, pH 5.5 and MeCN (60/40, v/v). A linear gradient was run to 82.5% A in 12.5 min using 100% mobile phase A/0% mobile phase B as starting point. Further, the gradient continued up to 35% mobile phase B in 17.5 min and was kept at 35% B for 1 min and the gradient returned to starting conditions during 1 min. The column was re-equilibrated for 18 min. Fractionation was done manually using short and narrow peek tubing at the outlet of the UV detector. Fractions were collected every minute, totally 30 fractions for each sample.

2.5. LC–MS/MS

Reversed phase (RP) separation was performed as second dimension in the case of multidimensional separation as well as the only dimension in the developing phase. An Ultimate 3000 system (Dionex) consisting of a LPG-3x00 micropump, WPS3000RS autosampler and FLM-3300 flow manager coupled to a LTQ-Orbitrap-MS (Thermo, San Jose, CA, USA). Xcalibur™ version 2.07 software (Thermo) was used to operate the system and to perform data acquisition.

Chromatographic separation was carried out on a 50 × 1 mm ID BioBasic-C8 column from Thermo (average pore size 300 Å and particle diameter 5 μm). The mobile phases consisted of A: 20 mM formic acid and methanol (95/5, v/v) and B: 20 mM formic acid and methanol (5/95, v/v). A linear gradient was run from 0% to 100% B in 55 min and kept constant at 100% B for 4 min before returning to starting conditions within 0.5 min. The column was regenerated for 10 column volumes. Flow rate was set to 50 μL/min and injection volume was typically 20 μL.

The electrospray ionization (ESI) source was operated in the positive ionization mode at a voltage of 5 kV. Experiments were performed in two scan events: Scan event 1: scan from *m*/*z* 250 to *m*/*z* 2000 in the FT-Orbitrap with resolution *R* = 30,000. Scan event 2: data dependent MS/MS with wide band activation carried out on the highest *m*/*z* value for a maximum of two spectra in the linear ion-trap. The *m*/*z* values fragmented were dynamically excluded for 30 s in order to fragment lower intensity *m*/*z* values. Helium gas was used to cause collision-induced fragmentation at 35% relative collision energy.

2.6. Procedures

2.6.1. Digestion of BSA and cytochrome c in-solution

Tryptic digestion of BSA and cytochrome *c in-solution* was done prior to labeling in the experiments carried out to optimize the labeling procedure. Reduction of the sample was done using DTT (1 μg per 50 μg protein) at 95 °C for 15 min, followed by alkylation with iodoacetic acid (5 μg per 50 μg protein) in the dark at room temperature for 15 min. Freshly prepared ammonium hydrogen carbonate buffer pH 8 was added to a final concentration of 50 mM before trypsin was added in the ratio of 1_{trypsin}:40_{protein}. The proteolysis was carried out overnight at 37 °C.

2.6.2. Digestion of proteins using immobilized trypsin beads

Digestion of proteins using immobilized trypsin beads was done by adding 50 μL of sample to 5 μL of sedimented immobilized trypsin beads (washed twice with freshly prepared ammonium hydrogen carbonate buffer). Acetonitrile was added to a final concentration of 20% to prevent unspecific binding of proteins to the beads. The sample was allowed to shake at 800 rpm at 37 °C for 90 min. In all experiments tryptic digestion was preceded by reduction and alkylation of the proteins as described above.

2.6.3. Optimization of ^{18}O/^{16}O-labeling using immobilized trypsin beads

BSA and cytochrome *c* were used as model proteins for optimization of post-digestion labeling using immobilized trypsin beads. Both labeling time and pH were varied separately while the other conditions were kept constant. Reaction times of 15, 30, 60, 120 and 240 min were tested. The buffer solutions used for pH optimization were 50 mM citric acid at pH 5 and 6 (adjusted by addition of NaOH), 50 mM phosphate at pH 7 and 8 (adjusted by addition of HCl) and 50 mM Tris at pH 9 (adjusted by addition of HCl). ^{18}O/^{16}O-labeling of the samples was done post-proteolytic using immobilized trypsin beads at the specified pH value. Prior to ^{18}O/^{16}O-labeling of the samples, 50 μL of digested sample (desalted, procedure see later), 40 μL buffer and 5 μL sedimented immobilized trypsin (washed twice with 50 mM ammonium hydrogen carbonate) were dried down completely by vacuum centrifugation using SpeedVac® (Thermo). For each sample to be labeled, the sample was reconstituted in either 40 μL $H_2{}^{18}O$ or $H_2{}^{16}O$ before transferred to the dried down buffer followed by transfer to the dried down trypsin beads where 10 μL MeCN was added to prevent peptide adsorption to the beads. Labeling was carried out under shaking (1200 rpm) at 37 °C

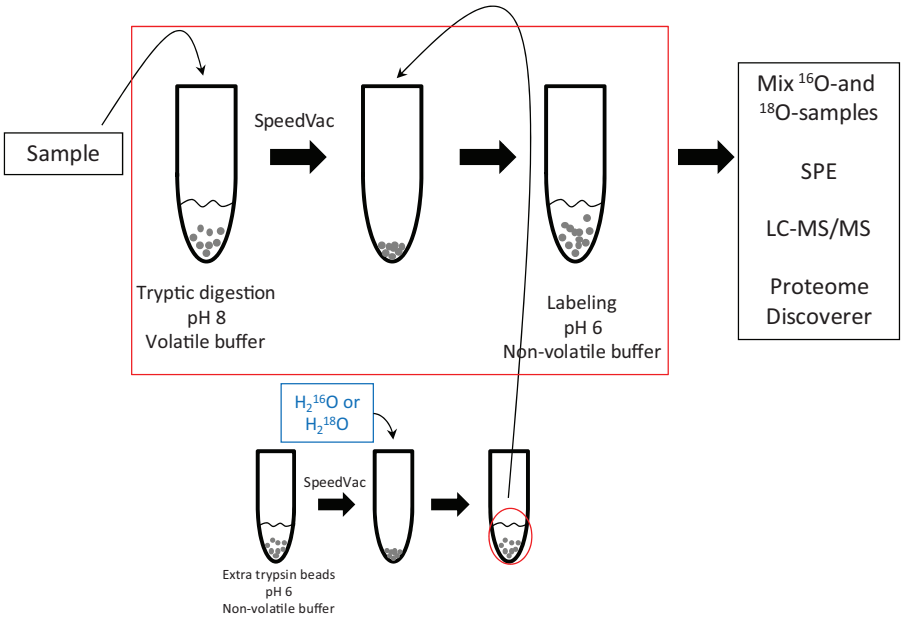

Fig. 1. Overview of the integrated digestion and labeling procedure on immobilized trypsin beads. The sample is first digested in ammonium hydrogen carbonate buffer (in $H_2{}^{16}O$) followed by evaporation. The sample is then reconstituted in $H_2{}^{18}O$ containing buffer and extra trypsin beads. The labeling is stopped by removing trypsin beads and addition of 8 M urea. The corresponding ^{16}O- and ^{18}O-labeled samples are then mixed in a 1:1 ratio before LC–MS/MS analysis.

for the given period of time. The supernatants were subsequently removed after centrifugation in order to separate the trypsin beads from the peptides. As a precaution, in case of any residual trypsin beads in the sample, urea was added to a final concentration of 8 M to prevent potential back exchange of ^{18}O to ^{16}O. Mixing of the ^{18}O-labeled sample and the ^{16}O-sample was done in a 1:1 ratio. Finally, the samples were dried down using vacuum centrifugation and stored at −32 °C. The optimized conditions are described in the following section.

2.6.4. Integrated digestion and $^{18}O/^{16}O$-labeling of proteins using immobilized trypsin beads

Tryptic digestion and $^{18}O/^{16}O$-labeling carried out using immobilized trypsin beads was integrated as illustrated in Fig. 1. Tryptic digestion on immobilized trypsin beads was done as described above. To exchange the buffer between the digestion and labeling step, the digested sample was dried down by vacuum centrifugation followed by addition of extra trypsin beads suspended in 40 μL of buffer (50 mM citric acid–NaOH pH 6, $H_2{}^{16}O$ or $H_2{}^{18}O$). Besides this, labeling was done as described above using 2 h reaction time.

2.6.5. Sample desalting by solid phase extraction

Before analysis the labeled samples were reconstituted in 20 mM formic acid and desalted using in-house prepared C18-tips. A pasteur pipette was used to punch 6 small cushions (diameter approx. 1 mm) from a EMPORE® C18 disk (3 M, St. Paul, MN, USA). The C18 material was transferred from the pasteur pipette to the bottom of a 10 μL pipette tip (Finntip® 10, Thermo) using a thin metal wire. After transfer, the 6 cushions were carefully pressed together in the narrow lower part of the tip. The tips were activated using 100 μL 50% MeCN in water and washed with 100 μL 20 mM formic acid prior to use. The whole sample was applied (approximately 120 μL) to each tip and the application was followed by a washing step (100 μL 20 mM formic acid). The peptides were eluted using 50 μL of HILIC mobile phase A.

2.6.6. Preparation of urine samples

Preparation of urine from both healthy individuals and anonymous kidney transplanted patients was done as earlier described [20]. In brief: Vivaspin centrifugal filters with a cut-off membrane off 5 kDa (Vivascience Sartorius Group, Stonehouse, UK) were used for desalting and concentration. For each experiment, 5 mL urine was applied to the filter followed by washing with equal volume of 10 mM Tris–HCl/150 mM NaCl (pH 7.4) after centrifugation. Subsequent to spin down, 1200 μL of the same buffer was used to resolubilize the sample on top of the filter. The resulting volume was transferred to Vivapure Anti-HSA kit (Vivascience Sartorius Group) for albumin depletion. Tryptic digestion and labeling was performed as described above. Since samples from anonymous human subjects were used, the Regional Committee for Medical Research Ethics allows analyses without preapproval.

2.7. Data analysis

The acquired mass spectrometric data were analyzed and processed using Proteome Discoverer 1.0 (Thermo). For the optimization experiments the software was used to identify ^{16}O- and $^{18}O_2$-peptides of BSA and cytochrome *c* in order to evaluate the ratios. The .raw files were analyzed using the SEQUEST™ search algorithm [21] and searched against a FASTA file from the NCBInr database containing the genome of both horse and bovine. Carboxymethyl (C) was set as constant modification while oxidation (M) and ^{18}O (2) on the C-terminal were chosen as variable modifications. The peptide tolerance was set to 10 ppm while MS/MS tolerance was ±0.8 Da and 2 "missed cleavages" were allowed using trypsin as enzyme.

For identification of proteins in urine, the FASTA file ipi.HUMAN.v3.66 containing 86,845 protein entries was used. Peptide and MS/MS tolerance in addition to variable and constant modification was set as described above. A decoy database search was performed by searching against a database containing the reversed protein sequences to determine the false discovery rate. Only "rank 1" peptides passing the significance threshold ($p < 0.05$) were accepted to ensure confident identification of urinary proteins. Although more complex algorithms to calculate accurate ratios exists [5,22], only signal for the fully labeled peptide was used to calculate the $^{18}O/^{16}O$-ratio using the following

Table 1
Tryptic peptides originating from BSA and cytochrome *c* used for evaluation of $^{18}O/^{16}O$-labeling efficiency.

Protein	Peptide sequence	Sequence position	*m/z*
BSA	LVTDLTK	257–263	395.24^{+2}
	LVTDLTK	257–263	789.47^{+1}
	AEFVEVTK	249–256	461.75^{+2}
	YLYEIAR	161–167	464.25^{+2}
	HLVDEPQNLIK	402–412	653.36^{+2}
	KVPQVSTPTLVEVSR	437–451	547.32^{+3}
	LVNELTEFAK	66–75	582.32^{+2}
	LGEYGFQNALIVR	421–433	740.40^{+2}
	QTALVELLK	549–557	507.81^{+2}
Cytochrome *c*	MIFAGIK	80–86	390.23^{+2}
	TGPNLHGLFGR	28–38	390.21^{+3}
	EDLIAYLK	92–99	482.77^{+2}
	EETLMEYLENPK	61–72	748.35^{+2}

equation.

$$\text{ratio} = \frac{\text{peak area}_{^{18}O_2}}{\text{peak area}_{^{16}O}}$$

Peak areas were calculated for each isotope using a mass width of 20 ppm to prevent inclusion of other isotopes in the peak area.

3. Results and discussion

The labeling efficiency was evaluated using BSA and cytochrome *c* as model proteins calculating $^{18}O/^{16}O$-ratios of the labeled peptides shown in Table 1. The criteria for selection, was to include both arginine and lysine terminated peptides with different hydrophobic properties. In addition, no interfering masses should be observed in the mass spectra for the peptide to ensure correct quantification. In every experiment, equal amounts of digested protein after ^{18}O- and ^{16}O-labeling were mixed. The $^{18}O/^{16}O$-ratio should then, under ideal conditions, be 1:1. In the current work, main focus has been put on optimizing the quantification. Other parts of the proteomic workflow used have not been modified, but is comparable to earlier published studies [23].

3.1. Evaluation of reaction time

To investigate the reaction time needed for complete labeling, experiments were carried out using a labeling buffer of pH 6 (see next section) where the reaction was stopped at different time points between 15 min and 4 h and compared with labeling overnight. Fig. 2 shows the average ratios of the $^{18}O/^{16}O$-labeled peptides at the different time points. This figure indicates that extensive but not complete labeling was achieved already after 15 min with an average ratio >0.5. A high standard deviation indicates a large variation in the labeling rate depending on peptide properties. The same trend was observed also after 30 min and 1 h. After 2 h however, the average ratio reached approximately 1.0 with a substantially lower standard deviation. The ratio in combination with the consistency between the peptides clearly suggests that complete incorporation of two ^{18}O atoms was achieved at this time point. Increasing the reaction time to 4 h, or even over night, did not improve the labeling any further. Looking at the ratio of the individual peptides, there seems to be a tendency of faster labeling of peptides with arginine at the C-terminal compared to peptides with C-terminal lysine, and 3 out of 4 of the selected arginine peptides were completely labeled already after 15 min and the ratios remained stable for the later time points on the curve. An earlier report have described problems with incorporating two oxygen efficiently into lysine terminated peptides [24]. Those results were, however, obtained using ammonium bicarbonate as labeling buffer. In this work, the ratio of the lysine peptides increased more gradually at a much slower rate than the arginine peptides and was also very peptide dependent. Additionally, relative large variations between replicates were observed at the 30 min and 1 h time points particularly for the lysine peptides. This might explain why there seems to be no increase in the average ratios between 15 min and 1 h displayed in Fig. 2. Our experiments showed that after 2 h reaction time at pH 6, complete labeling of lysine terminated peptides was achieved as well. Thus, the reaction time was set to 2 h for the further experiments.

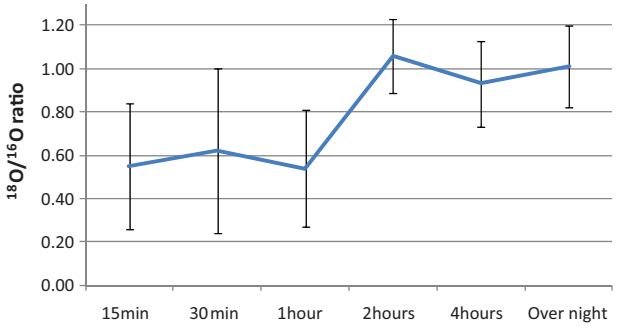

Fig. 2. Average $^{18}O/^{16}O$-ratios (±SD) of 12 BSA/cytochrome *c* peptides (Table 1) at different time points ($n = 3$). Labeling was done at pH 6.

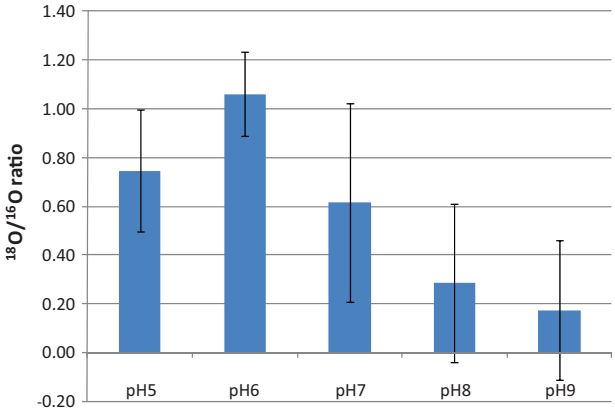

Fig. 3. Average $^{18}O/^{16}O$-ratios (±SD) of 12 BSA/cytochrome *c* peptides (Table 1) using labeling buffer of pH 5, 6, 7, 8 or 9 ($n = 3$). Reaction time was 2 h for all samples.

3.2. pH dependency of the labeling procedure

One reason for long reaction times in the protocols described earlier, is the slow incorporation of the second ^{18}O atom which increases the risk of getting a peptide mixture with both one and two ^{18}O atoms incorporated [6]. It has later been demonstrated that the carboxyl oxygen exchange rate can be greatly accelerated by optimizing pH for the labeling reaction separately and independent of the conditions used for protein digestion. The pH optimum found here was at pH 6 – when trypsin was used as protease, but a confirmation of this was called for by the authors [10]. In light of this, a simple pH study was performed to see if labeling at other pH levels could produce complete labeling within 2 h which we showed in the previous section was sufficient for complete labeling at pH 6. To study this, $^{18}O/^{16}O$-labeling of the tryptic peptides of cytochrome *c* and BSA were carried out at pH 5, 6, 7, 8 and 9 and the reaction time was set to 2 h. The results are shown in Fig. 3 where the average ratios of the $^{18}O/^{16}O$-labeled peptides are presented for each pH value. There was a well defined optimum at pH 6 where the ratio was close to 1.0 and the standard deviation was low compared to the other pH values thus confirming the uniformity of the data.

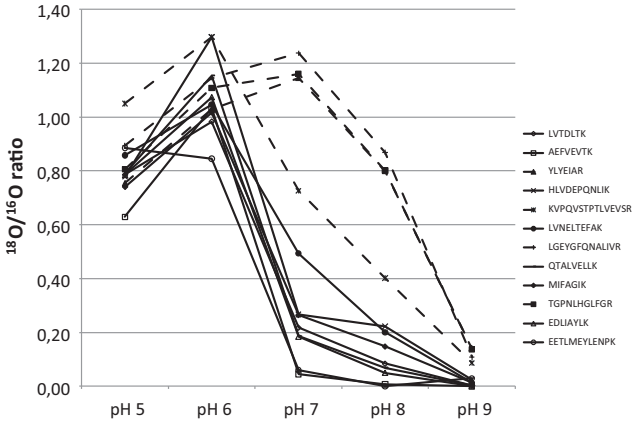

Fig. 4. $^{18}O/^{16}O$-ratios of the arginine (- - -) and lysine (—) terminated peptides from the model proteins (BSA and cytochrome *c*) monitored at various pH levels during labeling.

Moving one unit down or up on the pH scale resulted in a distinct decrease of the $^{18}O/^{16}O$-ratio and also increased the standard deviation. Compared with the results reported by Hajkova et al. [10], the shape of the curves are very similar and shows the same trend of increasing labeling rate towards more acidic conditions than that used for protein digestion. It must however be noted that these experiments were not carried out by measuring the initial rate of the reaction which is required to obtain accurate quantitative data on the pH dependency of the reaction rate [10]. Fig. 4 shows ratio of each peptide monitored. The results show that the labeling efficiency is highly peptide dependent at the different pH levels. The data also indicates differences between the peptides with arginine or lysine C-terminally in this experiment as well. While lysine peptides were poorly labeled at pH 7, the arginine peptides showed almost complete labeling at this pH. Although the overall efficiency dropped further at pH 8, the same trend was observed. At pH 5 no differences were observed between arginine and lysine terminated peptides, but EETLMEYLENPK was low as compared with pH 6. The reason for why this specific peptide behaves different is not clear, however, its labeling efficiency did not differ significantly between pH 5 and 6. In general, lysine has a lower pKa than arginine and the different degree of protonation probably plays a role in the labeling process. Even though complete labeling of several arginine peptides was achieved both at pH 6 and 7 after 2 h, shorter reaction times are required to decide the pH optimum for these peptides. In view of the fact that some of the arginine peptides were incomplete labeled at pH 7, a pH optimum closer to pH 6 seems most likely.

3.3. Integrated digestion and labeling on trypsin beads

As mentioned earlier there is a large potential in speeding up the workflow in traditional bottom-up proteomics. In addition to the labeling procedure already pointed out, other steps like tryptic digestion of proteins still remain time consuming. The idea of tryptic digestion using immobilized trypsin prior to the labeling step has been described earlier [13]. The reported work was however not done using the most favorable pH conditions for the labeling step resulting in a time consuming protocol (over night for digestion and 5 h for labeling). In our setup, the digestion buffer consisted of the volatile ammonium hydrogen carbonate (pH 8) which enables buffer exchange before labeling by simply evaporating the digestion buffer and reconstituting the sample in the labeling buffer. As displayed in Fig. 1, all these steps were done without any sample transfer, greatly reducing the risk of sample loss during the procedure. The initial experiments yielded acceptable average ratios (0.94 ± 0.35), but the variation was noteworthy larger compared to overnight *in-solution* digestion combined with labeling on trypsin beads where a standard deviation of 0.17 was observed. This was solved by adding an aliquot (equal amount as starting conditions) of fresh trypsin beads to the reaction vial between the digestion and labeling step which resulted in average ratios of 0.96 ± 0.13. Hence, the standard deviation was reduced significantly to a level below what was observed using the original setup with *in-solution* digestion. There is no evident explanation to this, but it seems like even though the trypsin beads can be reused, some loss of functionality occurs. Reduced degree of sample transfer during the procedure could also be a factor which contributed to the low variation observed in the integrated approach. A typical chromatogram of the integrated approach using immobilized trypsin beads for both digestion and labeling is presented in Fig. 5. From a qualitative point of view the chromatogram contains a wide range of peptide peaks supporting proof of concept. Nonspecific binding of peptides to the trypsin beads is a potential challenge. Earlier published data, using the same trypsin beads, showed varying peptide intensities where some of the peptides were more intense using *in-solution* digestion while other products had a similar intensity for both approaches [19]. To evaluate the effect on final peptide concentration in this work after replacing *in-solution* digestion to immobilized trypsin digestion, peak intensities of the peptides in Table 1 were compared for the different setups. The results are displayed in Table 2, showing the relative intensity change of the peptides going from *in-solution* to immobilized trypsin. All of the peptides evaluated, except LVTDLTK and LVNELTEFAK, actually increased in signal intensity when using immobilized trypsin beads. The peak intensity of some of the peptides increased dramatically, especially EDLIAYLK and EETLMEYLENPK, which increased by 1704% and 3794% respectively. Some of the increase could possibly be due to different kinetics resulting in different tryptic peptides from the two methods. It seems like introducing the use of immobilized trypsin beads in the digestion step does not increase peptide loss due to nonspecific binding. On the contrary, almost every peptide increases in peak intensity, most likely because of the reduced degree of sample transfer. Since immobilized beads were used for labeling in both cases, the results are inconclusive regarding nonspecific binding in this step.

Table 2
Intensity change of tryptic peptides from BSA and cytochrome *c* after replacing tryptic digestion *in-solution* with digestion using immobilized trypsin beads.

Protein	Peptide sequence	*m*/*z*	Intensity change
BSA	LVTDLTK	395.24^{+2}	−27.7%
	LVTDLTK	789.47^{+1}	−51.0%
	AEFVEVTK	461.75^{+2}	23.5%
	YLYEIAR	464.25^{+2}	11.3%
	HLVDEPQNLIK	653.36^{+2}	12.7%
	KVPQVSTPTLVEVSR	547.32^{+3}	240.2%
	LVNELTEFAK	582.32^{+2}	−3.8%
	LGEYGFQNALIVR	740.40^{+2}	71.2%
	QTALVELLK	507.81^{+2}	366.9%
Cytochrome *c*	MIFAGIK	390.23^{+2}	269.9%
	TGPNLHGLFGR	390.21^{+3}	231.8%
	EDLIAYLK	482.77^{+2}	1704.5%
	EETLMEYLENPK	748.35^{+2}	3794.3%

All these results indicate that digestion and labeling can be performed in a very satisfying manner using immobilized trypsin in both steps. An important aspect is the time-efficiency of this procedure, enabling reduction of total reaction time of tryptic digestion and labeling from approximately 32 h to a total of 3.5 h. This will facilitate a higher throughput of samples which is important in the biomarker search field where large set of samples are necessary to obtain results that are statistically significant. With the reduced reaction times needed for digestion and labeling, the bottleneck is now more in terms of how many samples that are possible to

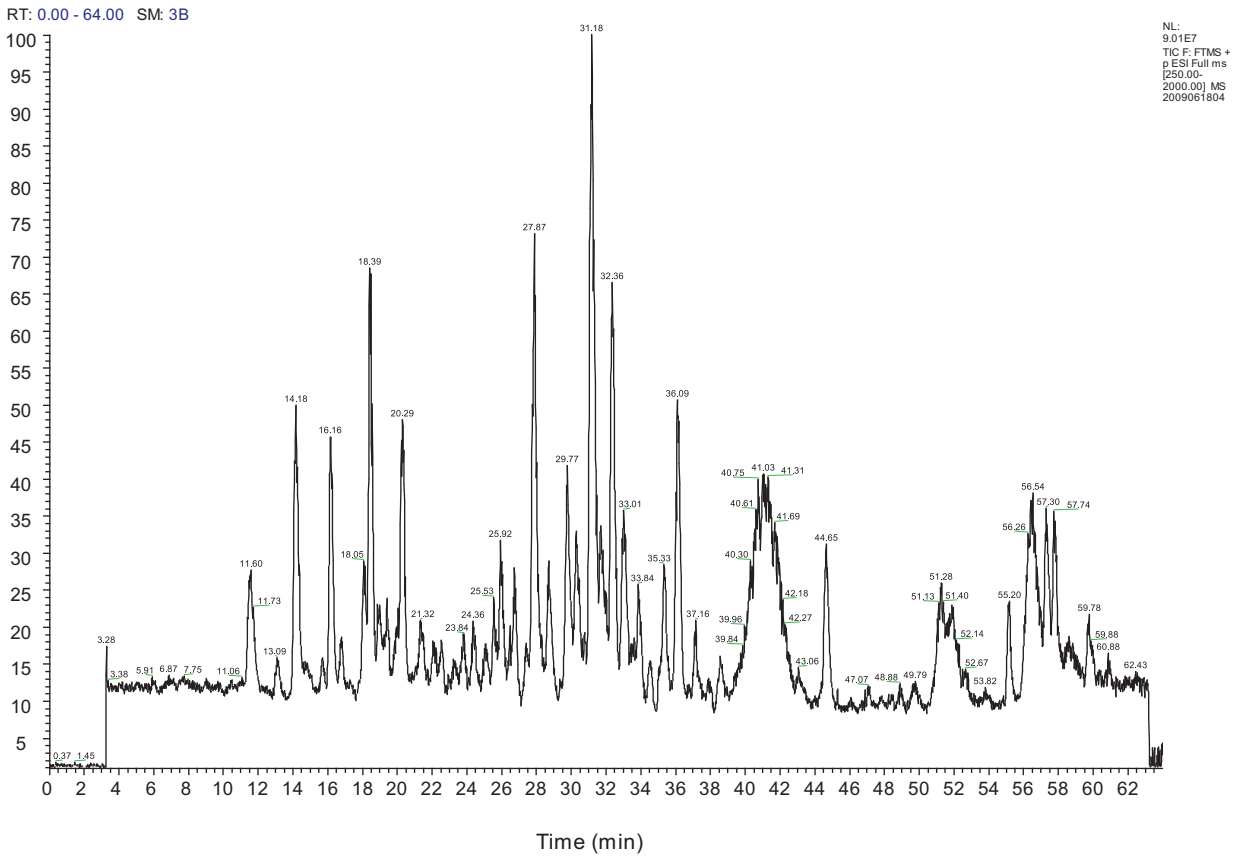

Fig. 5. TIC chromatogram of tryptic digested and $^{18}O/^{16}O$-labeled BSA/cytochrome *c*. Both tryptic digestion and labeling were done using immobilized trypsin beads.

handle in practice. This is due to the labor intensive nature of the procedure combined with concurrent manual handling of samples setting a limit of approximately 10 samples a day. Automation of this integrated method might allow for an even higher throughput. This would, however, require some changes to the procedure where especially modifications of the buffer exchange step are needed to achieve this. Another key point in this method is the use of only volatile buffers in the steps prior to labeling. This complicates the use of for example urea in the denaturation of the proteins initially, and only high temperature (95 °C) was used in the current protocol which could in worst case lead to precipitation of proteins with low solubility. High organic content in combination with high temperature could be a possible solution to this, especially in matrices where proteins of low solubility are dominant.

3.4. Application on urine samples using model proteins

To test the applicability of the optimized digestion/labeling method in a more complex matrix, the procedure was carried out using urine. This biological matrix was chosen since the labeling method described will be used in further research in finding diagnostic proteins that might predict kidney rejection in transplanted patients. Urine samples from healthy volunteers (protein concentration, 60 μg/mL) were spiked with a mixture of BSA and cytochrome *c* prior to tryptic digestion, this to allow comparison with the results described above. Peptide ratios ($^{18}O/^{16}O$) were calculated to evaluate the labeling efficiency in urine, using the same peptides which were monitored in the experiments in aqueous buffer. The $^{18}O/^{16}O$-ratios of the peptides in the 5 replicates ranged from 0.73 to 1.05 with an average of 0.88. Overall, the results show successful labeling in urine as well. Even though the variation is slightly larger than in aqueous buffer, RSD values below 16% were observed in 4 out of 5 samples. This is comparable to work published using ITRAQ, where average standard deviations less than 23% were reported [3]. Another group investigating the variation of ITRAQ labeling has previously reported a coefficient of variation (CV) = 0.24 [25], while Gan and coworkers classified the variation into different sources: technical (±11%), experimental (±23%) and biological (±25%) variation [26]. Further, a Student's *t*-test comparing the lowest and the highest ratio also indicates, that at the chosen alpha level (0.05), no significant difference between these ratios was found. These findings are also supported by Fig. 6, where mass spectra of six ^{18}O-labeled BSA peptides in urine before mixing with samples of unlabeled peptide are displayed. Ideally there should be no ^{16}O-labeled peptides present. The spectra strongly indicate complete labeling and a very low degree of back exchange or incomplete labeling. Traces of unlabeled and singly ^{18}O-labeled peptides are visible, but in low amounts relative to doubly ^{18}O-labeled (<3%). This is explained by the use of 97% pure $H_2{}^{18}O$ (thus having 3% $H_2{}^{16}O$ present).

3.5. Application on patient sample

In order to confirm the results obtained using model proteins, urinary proteins were labeled to test the applicability on a real biological sample. The digestion/labeling procedure on immobilized trypsin beads was tested on a urine sample from a kidney transplant patient (protein concentration, 669 μg/mL), collected in a stable phase during the third post transplant month. Labeling efficiency was evaluated using proteins identified from urine. The urine sample was treated as described in the experimental section including, desalting, depletion and a comprehensive 2D-LC–MS/MS analysis. This was performed to test if the procedure would produce complete labeling of peptides from other proteins than the

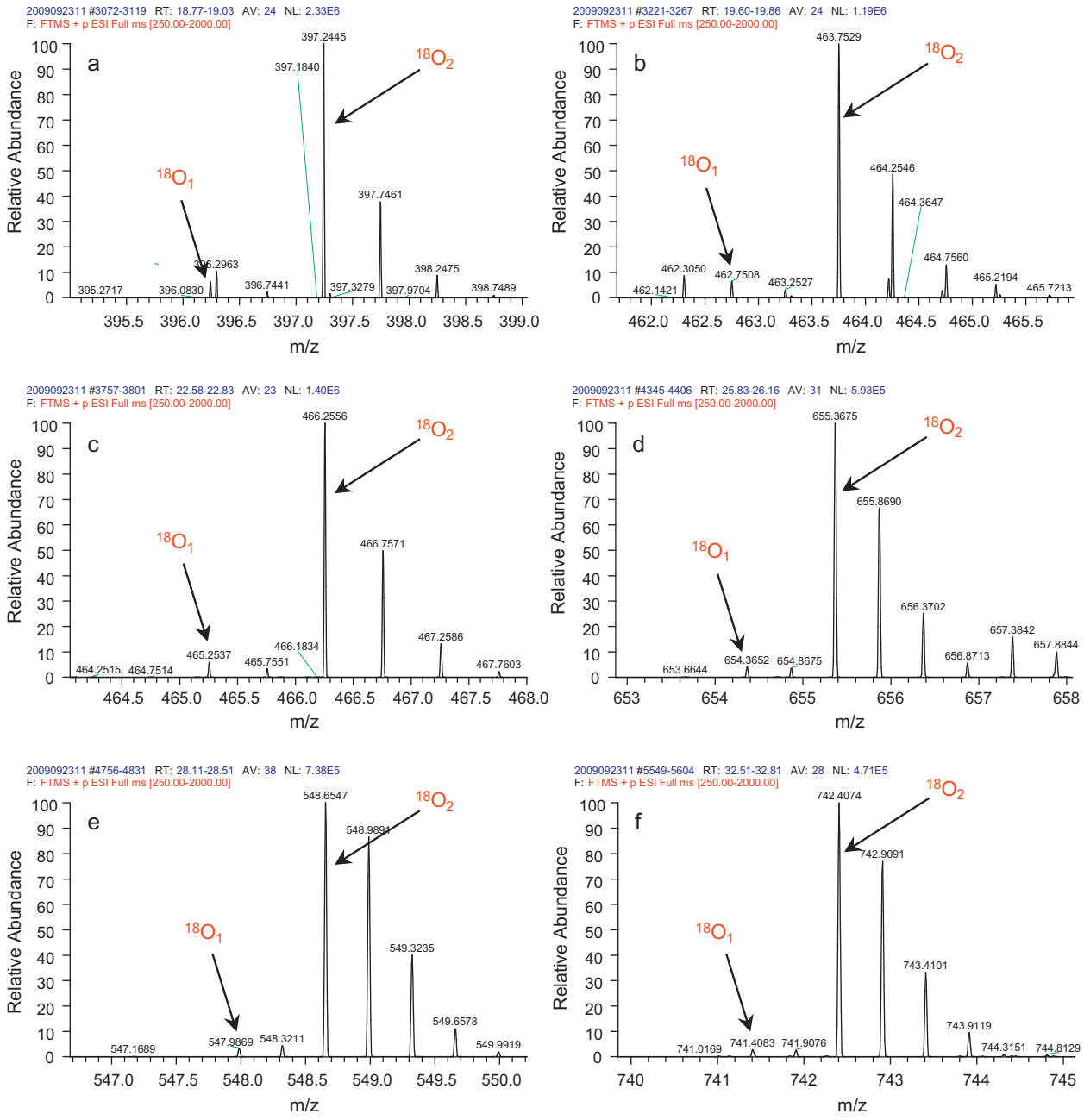

Fig. 6. Orbitrap mass spectra of six ^{18}O-labeled BSA peptides before mixing with unlabeled peptides. (a) LVTDLTK, (b) AEFVEVTK, (c) YLYEIAR, (d) HLVDEPQNLIK, (e) KVPQVSTPTLVEVSR, and (f) LGEYGFQNALIVR. Spectra were obtained from urine spiked with BSA, digested and labeled by immobilized trypsin.

model proteins used earlier, and also how introduction of the procedure in a comprehensive proteomic experiment would affect the result. Only peptides from proteins identified with a high degree of confidence were used to measure ^{18}O/^{16}O-ratios, where 50 and 62 proteins passed the significance threshold in the two replicates. Fig. 7 shows the ratio distribution of the identified peptides in both replicates. Average peptide ratios of 0.83 ± 0.13 and 0.91 ± 0.27 (no statistical significant difference) suggest that a high degree of labeling is achieved and the standard deviation also shows a relatively low degree of variation between the peptides (the respective medians were 0.84 and 0.94, indicating symmetric distribution). In practice, protein ratios are more relevant to calculate in the search for biomarkers. However, since the labeling is done at the peptide level, calculation of the average ratios of the peptides labeled is considered to be more suitable to evaluate the labeling method itself. The number of peptides quantified in the replicate samples was 78 and 97 respectively, and the figure shows a large majority of the peptides centered around the average ratios calculated with only a few peptides outside of the standard deviation. This trend is also confirmed by the isotope pattern of each peptide, where only small amounts (<3%) of singly ^{18}O-labeled peptides are observed in the mass spectra (data not shown). These typical isotope patterns are clear indicators of complete labeling since deviations from the normal pattern are usually easily revealed in the mass spectra. However, the average ratios shows a slight deviation from the theoretical achievable target ratio of 0.97 (97% pure $H_2{}^{18}O$). One explanation to this could be incomplete labeling not discovered, in particular for peptides where no manual inspection of the mass spectra was performed. In addition, small amounts of incompletely labeled peptide could be difficult to detect since the singly ^{18}O-labeled peptide has the same mass as the $^{13}C_2$ isotope of the peptide. Even though 2 missed cleavages were allowed for identi-

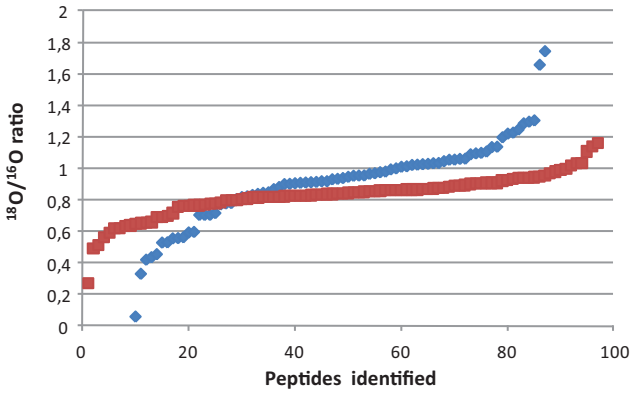

Fig. 7. Ratio distribution of all $^{18}O/^{16}O$-labeled peptides identified (*x*-axis) in urine from a kidney transplanted patient ($n=2$). Both tryptic digestion and $^{18}O/^{16}O$-labeling were done using immobilized trypsin.

fication, 90% of the identified peptides were completely digested, indicating good digestion efficiency of the method. The $^{18}O/^{16}O$ ratios for the incompletely digested peptides are also comparable with the completely digested. However, it is probably advisable to avoid use of incompletely digested peptides for quantification purposes.

The number of identified proteins in this study turned out be relatively small, suggesting a more simple separation approach using only a long reversed-phase gradient could be more appropriate in this case. The Patterson group successfully identified parts of the urinary proteome using such a setup [27,28].

4. Conclusions

Integration of tryptic digestion and ^{18}O-labeling using immobilized trypsin beads has resulted in a reduction of reaction time from typically 24–48 h to 3.5 h. One of the key factors is the optimization of the two steps separately allowing for different conditions, e.g. reaction pH. The optimum of the labeling reaction has been confirmed to be at pH 6, and adjustment of the pH level either up or down, significantly reduces the labeling efficiency. At pH 6 complete labeling was achieved in 2 h for peptides from model proteins and urinary proteins. In addition to increased time efficiency, no sample transfer (a potential source of variation) is needed in the integrated approach on trypsin beads. The optimized procedure also showed promising performance in a full scale proteomic experiment resulting in complete labeling of peptides from several urinary proteins. This confirms the feasibility of the method in a highly relevant matrix for biomarker discovery, where the advantage of increased throughput of samples can be utilized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.119.

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