Parts per Million Mass Accuracy on an Orbitrap Mass Spectrometer via Lock Mass Injection into a C-trap*[S]

Jesper V. Olsen‡§, Lyris M. F. de Godoy§, Guoqing Li§¶, Boris Macek§, Peter Mortensen‡, Reinhold Pesch‖, Alexander Makarov‖, Oliver Lange‖, Stevan Horning‖, and Matthias Mann‡§**

Mass accuracy is a key parameter of mass spectrometric performance. TOF instruments can reach low parts per million, and FT-ICR instruments are capable of even greater accuracy provided ion numbers are well controlled. Here we demonstrate sub-ppm mass accuracy on a linear ion trap coupled via a radio frequency-only storage trap (C-trap) to the orbitrap mass spectrometer (LTQ Orbitrap). Prior to acquisition of a spectrum, a background ion originating from ambient air is first transferred to the C-trap. Ions forming the MS or MS^n spectrum are then added to this species, and all ions are injected into the orbitrap for analysis. Real time recalibration on the "lock mass" by corrections of mass shift removes mass error associated with calibration of the mass scale. The remaining mass error is mainly due to imperfect peaks caused by weak signals and is addressed by averaging the mass measurement over the LC peak, weighted by signal intensity. For peptide database searches in proteomics, we introduce a variable mass tolerance and achieve average absolute mass deviations of 0.48 ppm (standard deviation 0.38 ppm) and maximal deviations of less than 2 ppm. For tandem mass spectra we demonstrate similarly high mass accuracy and discuss its impact on database searching. High and routine mass accuracy in a compact instrument will dramatically improve certainty of peptide and small molecule identification. ***Molecular & Cellular Proteomics 4:2010–2021, 2005.***

The data produced by a mass spectrometer are the mass and intensity of compounds and their fragments. The accuracy of mass measurement directly determines the usefulness of mass spectrometric experiments, and much effort in instrumentation development is directed at improving this key parameter. Mass accuracy and mass resolution are connected, and instruments introduced during the last decades radically improved in these two attributes. Traditionally accurate mass measurements, sufficient to determine the elemental composition of small molecules, were the province of magnetic sector instruments, but today TOF instruments equipped with energy correcting reflectrons can reach low ppm values. Triple quadrupole instruments or quadrupole ion traps, which are popular in proteomics research, however, have low resolution and mass uncertainties of typically half to several Da. At the other extreme, FT-ICR mass spectrometers reduce the mass measurement to a frequency measurement and are therefore potentially capable of exceedingly high mass accuracy. In practice, however, FT-ICR instruments have suffered from the requirement to precisely control the number of ions accumulated in the Penning trap. Over or under filling leads to mass shifts to high and low values, respectively. For example, Smith and co-workers (1) reported that in their measurements the mass determined over an LC peak varied by more than 10 ppm. The recent introduction of a linear ion trap-FT-ICR combination (2) largely solved this problem through a prescan in the ion trap to estimate ion current (called automatic gain control), which allows filling of the ICR cell with a predetermined number of ions. Using automatic gain control and narrow mass ranges (SIM[1] scans) we observed an average absolute mass error between 0.6 and 0.7 ppm in recent large scale proteomic analyses (3–5).

In a typical proteomics experiment, protein mixtures are digested to peptide mixtures that are separated by reversed phase HPLC and analyzed on-line by MS and MS/MS (6). The mass accuracy achieved in the instrument directly translates into the mass tolerances that can be specified in subsequent database searches of tandem mass spectra. Unambiguous protein identification in large data sets is by no means trivial (7), and any increase in achieved mass accuracy greatly aids the specificity of database searches in two ways (8, 9): High

From the ‡Center for Experimental BioInformatics (CEBI), Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark, §Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany, ¶Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 101300, China, and ‖Thermo Electron (Bremen) GmbH, Hanna-Kunath-Strasse 11, 28199 Bremen, Germany

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[1] The abbreviations used are: SIM, selected ion monitoring; MS/MS, tandem MS; LTQ, Thermo Electron linear quadrupole ion trap; RF, radio frequency; SILAC, stable isotope labeling by amino acids in cell culture; PCM, polycyclodimethylsiloxane.

precursor mass accuracy in the MS spectra directly translates into fewer "candidate sequences" that need to be considered as possible matches. High mass accuracy in the MS/MS spectra leads to fewer measured fragment masses that match the calculated fragments of a candidate sequence by chance and therefore decreases the scores of false positives in database search algorithms.

In 1923, Kingdon (10) devised a method to capture ions by causing them to orbit around a central electrode. Since then, the physics community has used "Kingdon traps" in a variety of experiments, but it was always used as a capturing device, not as a mass spectrometer. A few years ago Makarov invented a novel mass analyzer, which uses orbital trapping, termed the orbitrap (11–13). On the basis of this development, a new hybrid mass spectrometer was very recently introduced commercially. It consists of the linear ion trap (LTQ) mentioned above coupled to a radio frequency (RF)-only "C-trap" for intermediate storage of ions and of the orbitrap mass analyzer (LTQ Orbitrap) (14).

In contrast to the Penning traps used in FT-ICR, the orbitrap consists of two concentric electrodes around which injected ions circle. No magnetic fields are involved, and the size of the device is only a few centimeters. The detection principle is an image current of the axial motion of ion packages orbiting the inner electrode, and the mass spectrum is obtained as the Fourier transform of this current. As a result, the device has high resolution, and it should be capable of high mass accuracy. However, mass accuracy depends on the stability of an electric field, which is more difficult to achieve than stability of a magnetic field. No detailed studies of the mass accuracy of an orbitrap for complex proteomic samples have been reported so far.

Here we show that very high mass accuracy is possible on the LTQ Orbitrap. We use a known background ion produced by electrospray in ambient conditions and inject a defined number of this ion species into the C-trap, the RF-only trap connecting the LTQ to the orbitrap. The ions constituting the mass spectrum are then added to this "lock mass," and all are injected into the orbitrap together. We furthermore average the mass values over the chromatographic elution profile of every peptide peak. Together, these steps allow average absolute mass determination in the sub-ppm range for peptides yielding at least moderate signals. The masses of peptides close to the detection limit are still determined to within a few ppm.

EXPERIMENTAL PROCEDURES

In-solution Digestion of BSA—One mg of lyophilized BSA (Sigma-Aldrich) was resolubilized in a buffer containing 6 M urea (Invitrogen), 2 M thiourea (Fluka), and reduced, alkylated, and digested essentially as described (15). To reduce disulfide bonds 100 mM DTT was added to a final concentration of 10 mM in the protein solutions and incubated for 1 h at 56 °C in the dark. The resulting free thiol (-SH) groups were subsequently alkylated with iodoacetamide (55 mM final concentration) for 30 min at room temperature. The reduced and alkylated protein mixtures were digested and the resulting peptide mixtures were desalted on RP-C18 StageTips as described previously (16) and diluted in 0.1% TFA for nanoLC-MS/MS analysis.

SILAC Labeling of Yeast-K_out-strain—The *Saccharomyces cerevisiae* strain Y15969 (BY4742; MAT α; his3D1; leu2D0; lys2D0; ura3D0; YIR034c::kanMX4), which has a Lys1 gene deletion and which is therefore an auxotroph for lysine, was purchased from EuroScarf. Two populations of yeast cells were grown in yeast nitrogen base liquid medium containing either 20 mg/liter normal L-lysine or 20 mg/liter L-lysine-U-$^{13}C_6$,$^{15}N_2$ (Isotec-SIGMA) for 10 generations until they reached log-phase (A_{600} 0.7). Equal amounts of the normal and heavy SILAC-labeled yeast cells (as determined by A_{600} measurement) were then mixed 1:1, harvested by centrifugation for 5 min at 4,000 $\times$ *g* at 4 °C, washed two times with cold H_2O by centrifugation, and immediately lysed for protein extraction.

Cell membranes were disrupted by boiling in a SDS solution (50 mM Tris-HCl, pH 7.5; 5% SDS; 5% glycerol; 50 mM DTT; complete protease inhibitors mixture (Roche Applied Science)). The total yeast lysate was centrifuged to remove cellular debris, the supernatant was transferred to a fresh tube, and the protein concentration in the extract was determined by Bradford assay.

1D-SDS-PAGE and In-gel Digestion of Yeast Proteins—Yeast proteins (100 μg) were separated by one-dimensional SDS-PAGE using NuPage® Novex bis-Tris gels and NuPage® MES SDS running buffer (Invitrogen) according to the manufacturer's instructions. The gel was stained with Coomassie Blue using the colloidal blue staining kit (Invitrogen). Protein bands were excised and digested with endoproteinase Lys-C (Wako).

Gel bands were cut into 1 mm^3 cubes and washed four times with 50 mM ammonium bicarbonate, 50% ethanol. For protein reduction, gel pieces were incubated with 10 mM DTT in 50 mM ammonium bicarbonate for 1h at 56 °C. Alkylation of cysteines was performed by incubating the samples with 50 mM iodoacetamide in 50 mM ammonium bicarbonate for 1h at 25 °C in the dark. Gel pieces were washed two times with 50 mM ammonium bicarbonate, 50% acetonitrile, dehydrated with 100% ethanol, and dried in a vacuum concentrator.

The gel pieces were rehydrated with 12.5 ng/μl Lys-C in 50 mM ammonium bicarbonate and incubated for 16h at 37 °C for protein digestion. Supernatants were transferred to fresh tubes, and the remaining peptides were extracted by incubating gel pieces two times with 30% acetonitrile in 3% TFA followed by dehydration with 100% acetonitrile (MeCN). The extracts were combined and desalted using RP-C_{18} StageTip columns, and the eluted peptides used for mass spectrometric analysis.

NanoLC-MS/MS and Data Analysis—All digested peptide mixtures were separated by on-line nanoLC and analyzed by electrospray tandem mass spectrometry. The experiments were performed on an Agilent 1100 nanoflow system connected to an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). Binding and chromatographic separation of the peptides took place in a 15-cm fused silica emitter (75-μm inner diameter from Proxeon Biosystems, Odense, Denmark) in-house packed with reversed-phase ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany).

The peptide mixtures were injected onto the column with a flow of 500 nl/min and subsequently gradient eluted with a flow of 250 nl/min from 5–40% MeCN in 0.5% acetic acid. Gradients were 30 min for BSA analysis and 90 min for the yeast run. The mass spectrometer was operated in the data-dependent mode to automatically switch between orbitrap-MS and orbitrap-MS/MS (MS^2) acquisition. Survey full scan MS spectra (from *m/z* 300–1600) were acquired in the orbitrap with resolution r = 60,000 at *m/z* 400 (after accumulation to a target value of 1,000,000 charges in the linear ion trap). The most

intense ions (up to five, depending on signal intensity) were sequentially isolated for fragmentation in the linear ion trap using collisionally induced dissociation at a target value of 100,000 charges. The resulting fragment ions were recorded in the orbitrap with resolution r = 15,000 at m/z 400.

For accurate mass measurements the lock mass option was enabled in both MS and MS/MS mode and the polydimethylcyclosiloxane (PCM) ions generated in the electrospray process from ambient air (17) (protonated $(Si(CH_3)_2O))_6$; m/z = 445.120025) were used for internal recalibration in real time. For single SIM scan injections of the lock mass into the C-trap the lock mass "ion gain" was set at 10% of the target value of the full mass spectrum. The total lock mass cycle time including one orbitrap full scan and five subsequent orbitrap-MS/MS spectra was in general below 4 s. The time for the accumulation, isolation, and transfer into the C-trap of the lock mass was estimated to be a few msec at most because no time difference in cycles with and without lock mass was apparent. When calibrating in MS/MS mode the ion at m/z 429.088735 (PCM with neutral methane loss) was used instead for recalibration.

Target ions already selected for MS/MS were dynamically excluded for 30 s. General mass spectrometric conditions were: electrospray voltage, 2.4 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 125 °C; collision gas pressure, 1.3 mT; normalized collision energy, 32% for MS^2. Ion selection threshold was 500 counts for MS^2. An activation q = 0.25 and activation time of 30 ms was applied for MS^2 acquisitions.

Peptide Identification via MASCOT Database Search—Proteins were identified by automated database searching (Mascot Daemon, Matrix Science) against an in-house curated version of the yeast_orf (*S. cerevisiae*) protein sequence database or of the National Center for Bioinformatics, non-redundant database. This yeast database was complemented with frequently observed contaminants (porcine trypsin, achromobacter lyticus lysyl endopeptidase, and human keratins).

Search parameters specified an initial MS tolerance of 10 ppm and an MS/MS tolerance at 0.01 Da (minimum possible in MASCOT) and full Lys-C specificity allowing for up to 2 missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionines, N-protein acetylation, lysine-U-$^{13}C_6$,$^{15}N_2$, and *N*-pyroglutamate were allowed as variable modifications. Because of the high mass accuracy, the 99% significance threshold in the yeast database search was a MASCOT score of 25 even without taking into account the high fragment mass accuracy, and peptides with a greater score than this were accepted for analysis without further validation.

Averaging Precursor Masses Over the LC Profile—To correct for peptide mass errors due to imperfect peaks caused by weak signals we have implemented a script that averages all MS full scan mass measurements of a given peptide ion over the LC peak, weighted by signal intensity. For any LTQ Orbitrap raw file containing LC-MS/MS data our script calculates all peak centroids and charge state assignments independently, making use of the lock mass to eliminate systematic error. After the peptide is recognized in adjacent survey scans, the measured peptide masses are averaged over the elution profile, weighted by its signal intensity in each scan. The program was implemented in Microsoft DotNet (C#.NET) and works with Thermo Electron Xcalibur 2.0 data files. As output, the script generates a merged MASCOT generic search-file (extension "msm") in which all peptide ion masses have been substituted with the LC-profile corrected ones. Identified peptides are listed in supplemental material 1, and peak lists for the yeast peptides displayed in the figures are given in supplemental material 3.

RESULTS

LC-MS/MS on the LTQ Orbitrap—Whereas the principles of the orbitrap mass spectrometer have been explained in the literature (11–13), we briefly describe the hybrid LTQ Orbitrap for better understanding of the mass accuracy issues addressed here. As can be seen in Fig. 1*a*, it consists of three parts. The front part is a LTQ capable of detecting MS and MS^n spectra at very high sensitivity but relatively low resolution and mass accuracy. Ions accumulated in the LTQ can be transferred into a C-trap, an RF-only quadrupole that accumulates and stores the ions. The name refers to the shape of the RF section in the form of the letter "C." In the C-trap, ions are collisionally damped by a low pressure of nitrogen, and they come to rest in the middle of the trap. From here they are injected into the orbitrap using high voltage electric pulses with short rise times (of few hundreds nanoseconds). Ions are then captured in the orbitrap by lowering the electric potential of the inner, spindle-like electrode simultaneously with entry of the ion packet. Ions begin to circle around the inner electrode and spread into rings that oscillate along the electrode (axial motion in z-direction). The amplitude of oscillations is determined by the initial offset of ion injection from the orbitrap center, whereas coherency of axial oscillations is ensured by the short temporal duration of injected ion packets. The frequency of this oscillating motion is inversely proportional to the square root of the mass to charge ratio of the ions and is detected by a differential amplifier connected to both halves of the outer electrode comprising the orbitrap. For a single compound a sine wave is produced and the overlapping signals of an ion mixture can be converted into a mass spectrum by Fourier transformation.

Importantly, ions are brought to rest in the C-trap, and therefore the C-trap decouples the LTQ and orbitrap. In our experience, the C-trap can be filled with about a million charges (data not shown). This contrasts with the total capacity of the LTQ (without mass selection) of about 10 million charges and the capacity of the orbitrap, which has not been determined yet, but which is larger than the capacity of the C-trap. Nitrogen is used as a bath gas because it is already used in the source region of the instrument and because it is efficient at capturing and cooling the ions.

In the LTQ Orbitrap combination, the linear ion trap accumulates ions, isolates them, and fragments them. Although it may be possible to fragment ions in the orbitrap, it is more practical and faster to manipulate them in the linear ion trap and in the C-trap. By employing the linear ion trap as a "front end" the well known low mass cutoff for fragments applies to the LTQ Orbitrap as well. The mass range of the LTQ employed here extends to m/z 2000. Generally, fragmentation behavior in the LTQ Orbitrap will be the same as in the LTQ alone (which in turn is quite similar to three-dimensional ion traps (18, 19)) because the orbitrap solely functions as a high performance mass analyzer.

To characterize LC-MS/MS in the LTQ Orbitrap combination, we injected 50 femtomole of a tryptic digest of BSA onto a 75-μm column, performed a 30-min gradient, and measured MS and MS/MS spectra with the orbitrap as the detector.

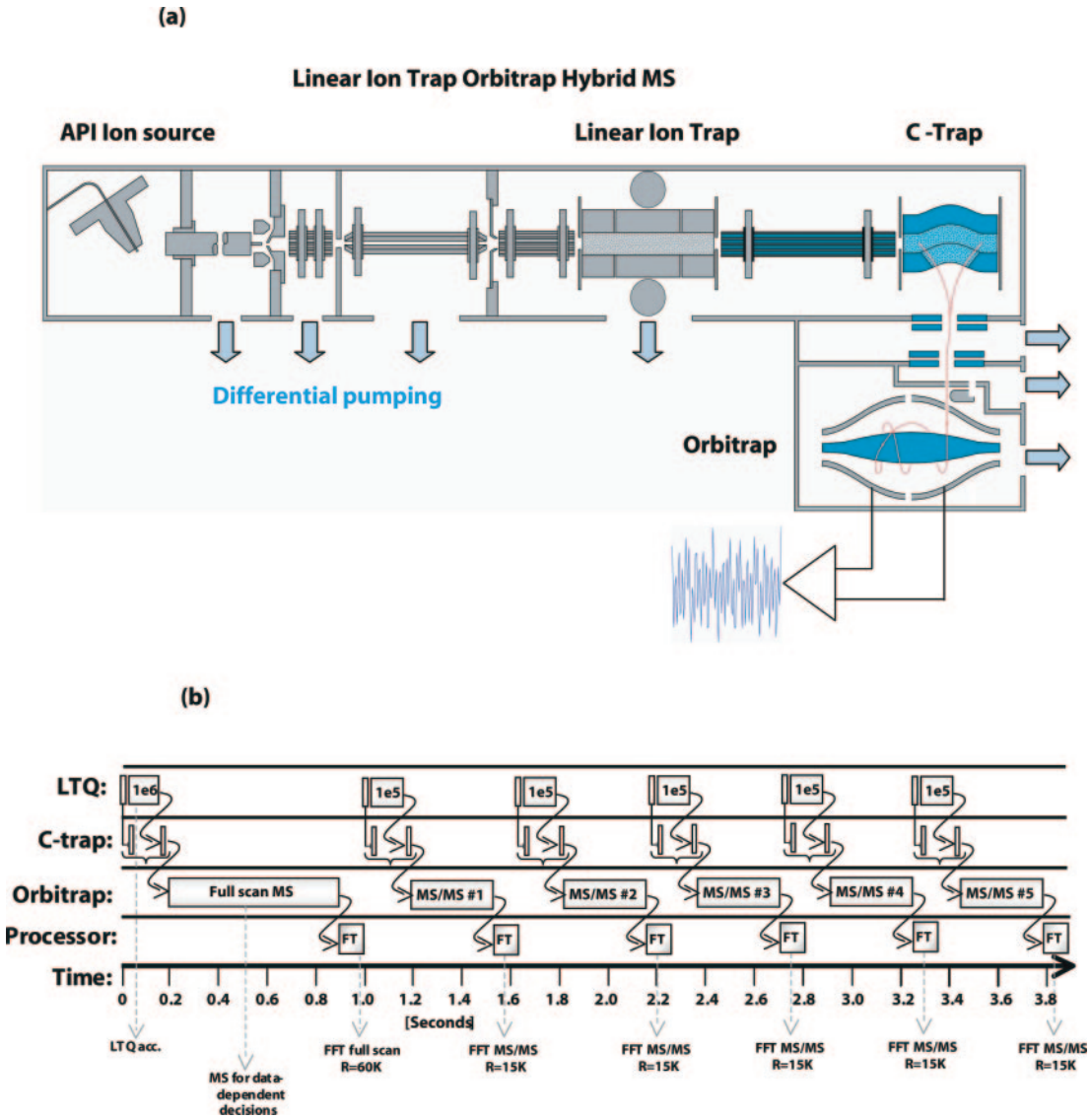

FIG. 1. a, schematic of the LTQ Orbitrap. The front part of the instrument is a standard LTQ linear ion trap mass spectrometer capable of detecting MS or MS^n ions. Accumulated ion populations are moved into the C-trap via an octapole ion guide. In the C-trap, the motion of the ion population is damped by a residual pressure of nitrogen. Ions are then injected into the orbitrap in a short pulse and begin to circle the central electrode. The signal is detected via a differential amplifier between the two halves of the outer orbitrap electrodes. *b*, analysis scheme on the LTQ Orbitrap. *Rectangles* in the *first row* represent filling times for the lock mass ion (*small rectangle*) and precursor ions in the LTQ. The *second row* represents fill times in the C-trap, and the *third row* represents transient accumulation in the orbitrap. One million ions are accumulated for a full scan spectrum and 50,000–100,000 ions for an MS/MS experiment with detection in the orbitrap. The *bottom row* represents processing time for Fourier transformation. *FFT*, fast Fourier transform.

Target values were one million ions for MS ("survey") scans and 50,000 ions for MS/MS in the LTQ. Analysis of LTQ generated fragments was performed in the orbitrap. When recording the transients of the image currents, different times can be chosen, with a balance between fast acquisition cycles and high resolution. We chose "1-s survey scans," corresponding to 60,000 resolution at *m/z* 400 and .25-second MS/MS scans, corresponding to a resolution of 15,000. Total cycle time for one survey scan followed by MS/MS scans for up to five of the most intense ions was about 4 s on average, resulting from orbitrap transient accumulation (750 msec for MS and 190 msec each for MS/MS) and the ion fill times (Fig. 1*b*).

Fig. 2*a* shows a zoom of a typical mass spectrum. High resolving power and good signal to noise are evident. Part *b* of the figure contains a typical MS/MS spectrum acquired with 15,000 resolution and a target value of 50,000. The *inset* demonstrates that charge assignment of fragment ions is trivial given the high resolution and excellent peak shapes. Although fragmentation solely occurs in the LTQ, the user can choose to detect fragments either in the LTQ or in the orbitrap (Fig. 2*c*). As can be seen in the figure, the intensities of all

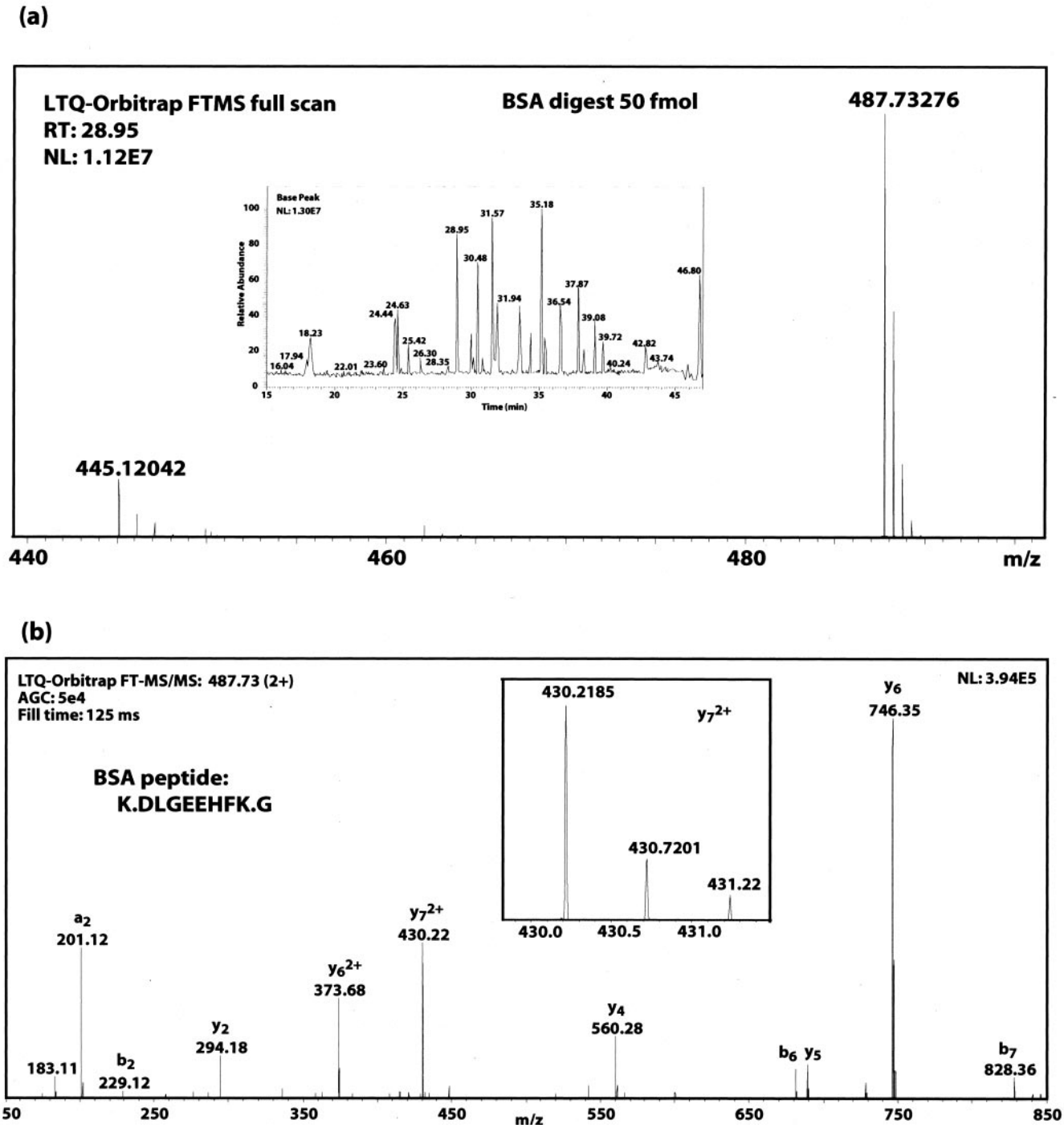

FIG. 2. *a*, part of a full scan spectrum of a BSA peptide separation (*inset*, total ion current of the separation). *b*, MS/MS spectrum of a BSA peptide fragmented in the LTQ and analyzed in the orbitrap. *Inset*, magnification of the ion at 430.22 Da. Note that the isotope spacing makes it easy to assign the charge state. *c*, comparison of MS/MS analyzed in the orbitrap (*black*) *versus* in the LTQ (offset by 10 *m/z* units). The *inset* shows the complete MS/MS spectrum in the ion trap.

major ions are comparable, as expected. However, the b_2 ion is more abundant in the LTQ spectrum, perhaps indicating that this species fragmented further upon injection into the C-trap at the voltages chosen in our experiment. The agreement between LTQ and LTQ Orbitrap MS/MS spectra contrasts with our previous observation that on an LTQ FT certain mass ranges were better represented than others and that it was necessary to stitch three mass ranges together to obtain a representative MS/MS spectrum (20). This difference is explained by the absence of ion loss caused by time-of-flight

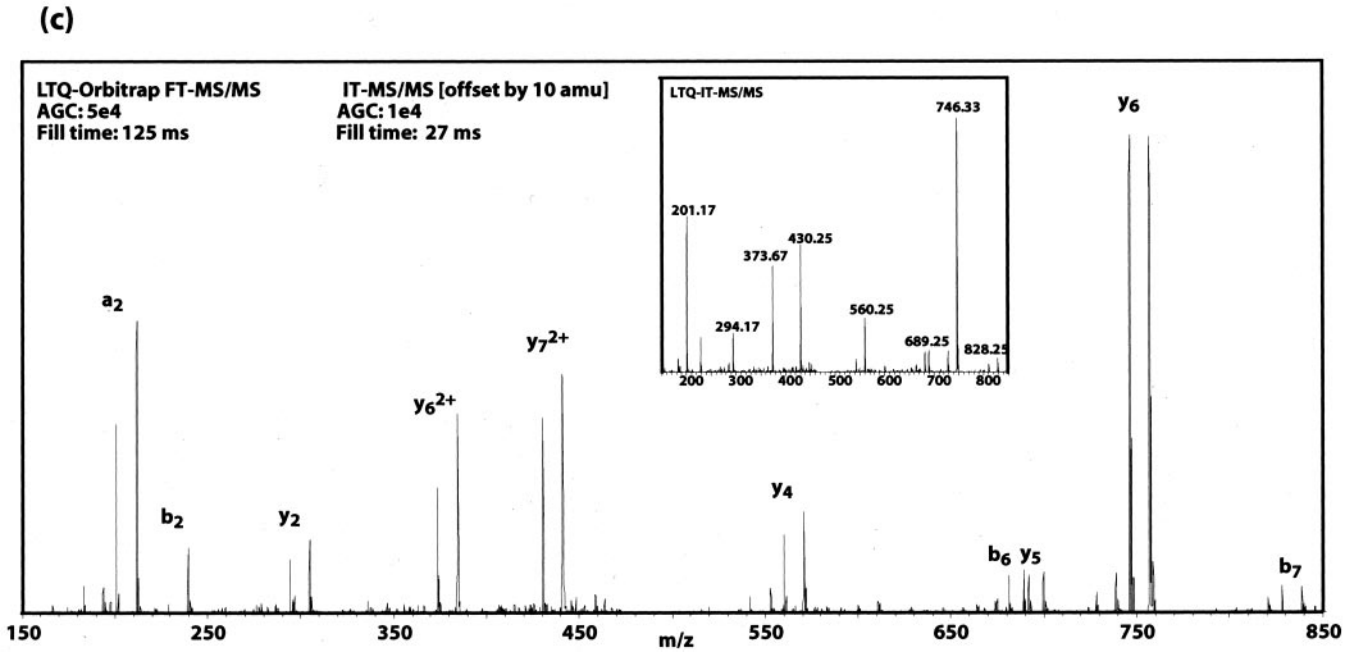

FIG. 2— *continued*

effects over the short distance from the LTQ to the C-trap and from the C-trap to the orbitrap.

Detection sensitivity in the orbitrap was very good as seen in the figure. We observed that target values of 50,000 were generally sufficient to obtain good signal to noise MS/MS spectra. In this experiment, fill times were of the order of 100 ms. They would have been even shorter if peptides were not "picked" for sequencing as soon as the signal is detected, at the beginning of the chromatographic peak. When using the LTQ as a detector, we usually employ target values of 5,000 or 10,000, leading to filling times that are 5–10 times shorter. However, in the analysis of complex mixtures the overall cycle is typically dominated by the time spent in acquisition of the transients rather than the ion fill times, as was the case in this example.

Finally, we observed that MS/MS spectra recorded in the orbitrap are "cleaner" (contain less noise ions) than spectra recorded in the LTQ. This is most likely caused by the high resolution of the orbitrap and its image current detection. At least 30 ions of the same species are needed for detection (14); therefore chemical background ions derived from electrospray generated solvent clusters, which are usually heterogeneous, tend not to register in the spectra. The vastly higher quality of MS/MS spectra achievable by analysis in the orbitrap will usually outweigh the longer acquisition cycle; therefore orbitrap MS/MS detection will often be preferable to LTQ MS/MS detection.

Low ppm Calibration Drift of the Orbitrap and Correction via Lock Mass—Next we wanted to study the mass accuracy achievable on the LTQ Orbitrap. Although mass accuracy was generally excellent, we observed some drift with time, most likely caused by ambient temperature changes. It can clearly be seen in Fig. 3*a* that short term mass precision is much better than long term mass accuracy, which changes by a few parts per million. In mass spectrometry a lock mass is sometimes employed to compensate for such drifts and to provide an internal standard in the same spectrum containing the analyte of interest. For example, insulin was measured in the presence of a known polymer leading to a sub-ppm mass accuracy (21), and MALDI-FTICR with a dual source for analyte and internal standards resulted in similar mass accuracies (22). We used a protonated electrospray ion of polycyclodimethylsiloxane (PCM-6), which has a composition of $(Si(CH_3)_2O))_6$ H^+ and an exact mass of 445.120025 and is present in electrospray spectra (17). As displayed in Fig. 3*b*, applying a potential of a few kilovolts to an electrospray emitter generates a number of singly charged background PCM ions, which can all be used as lock masses for internal calibration during online HPLC-MS analysis. As shown in Fig. 3*c*, mass accuracy of the BSA peptide YLYEIAR improved from 3.7 ppm to 0.87 ppm by employing PCM as lock mass. In every mass spectrum, we utilized the lock mass information to calculate new calibration coefficients and corrected all measured *m/z* values essentially by applying the same ppm deviation for all masses as for the lock mass.

Although the concept of a lock mass is well known it is not frequently employed. This is due, in part, to the effort of introducing a lock mass into the analyte. Here we use PCM, which is always being generated during the electrospray process. However, it is not always detectable in LTQ or LTQ-ICR-FT spectra because intense peptide signals lead to short fill times, making the background ion signals difficult to de-

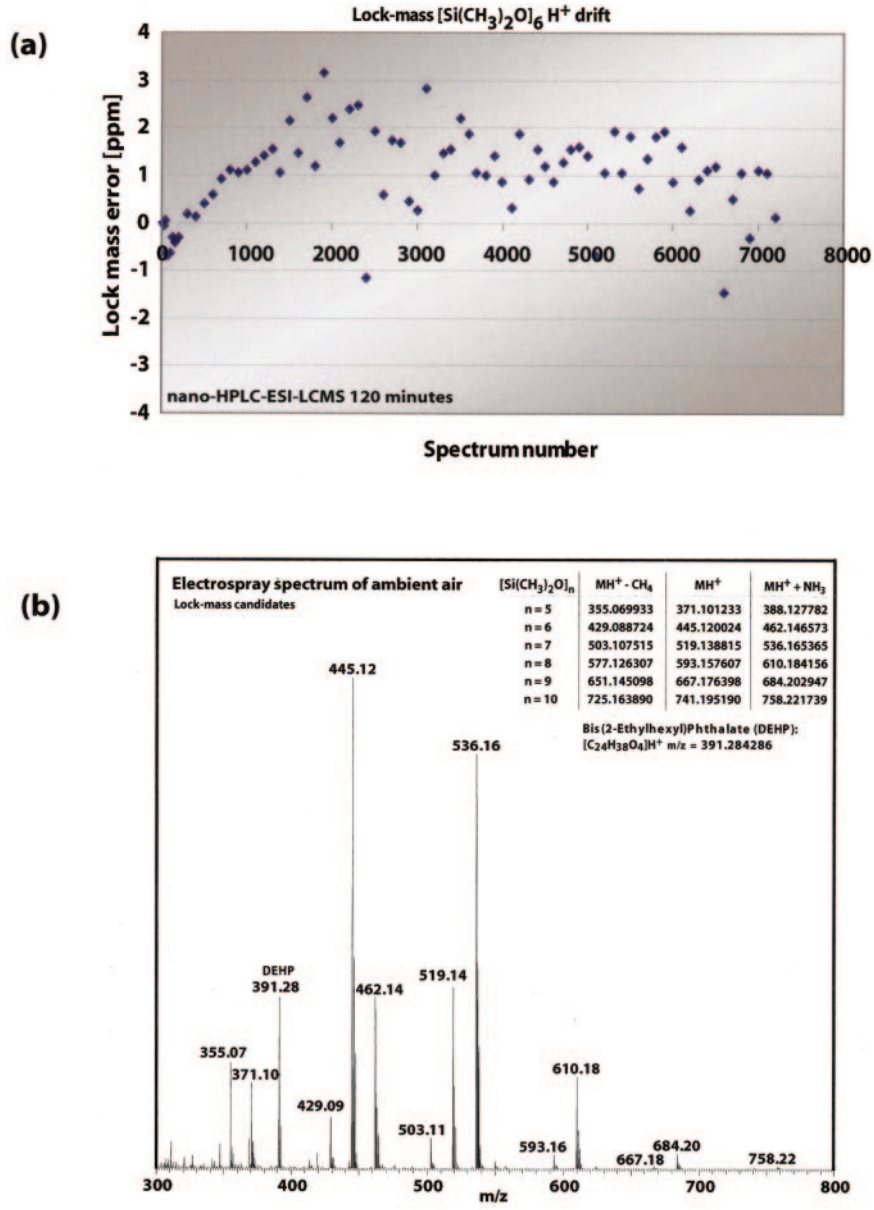

FIG. 3. *a*, the measured mass of the background ion PCM-6 as a function of time. The observed mass is stable within less than 1 ppm during short time intervals but fluctuates in the 30-min time frame, possibly because of temperature changes. *b*, electrospray spectrum of ambient air. Applying a voltage of 2 kV to an electrospray emitter ionizes PCMs present in the ambient air. Any of these ions can be used as lock masses during online LC-MS analyses. *Inset*, table of theoretical PCM masses. *c*, correction of the measured mass of a BSA peptide using the known mass of the background ion PCM.

tect. Therefore we made use of a unique feature of the LTQ Orbitrap combination, namely its C-trap. A predefined number of PCM ions (we used a target value of 5000) is accumulated in the LTQ and transferred to the C-trap. In this way an adequate signal of PCM is always present, regardless of the accumulation time used for the mass spectrum. This number of ions does not appreciably limit the usable capacity of the C-trap. Accumulation of PCM ions and the whole isolation and transfer protocol adds no more than a few msecs to the scan. Furthermore, the lock mass can be added to any spectrum. For example, 5000 ions of PCM can be accumulated in the C-trap, and subsequently a precursor ion can be accumulated and fragmented in the LTQ, which is followed by transfer of all MS/MS ions into the C-trap. Detection of an arbitrary lock mass or set of lock masses has been integrated into the data system and is transparent; the lock mass signal is removed from the spectra.

Evaluation of Lock Mass for Complex Peptide Mixtures—Next we tested the performance of lock mass injection for the analysis of a complex peptide mixture. SILAC (23) labeled yeast was lysed, in-gel digested with Lys-C, and analyzed by LC-MS/MS on the LTQ Orbitrap. The acquisition scheme was as depicted in Fig. 1*b*, using lock mass injection for all spectra. After identification of yeast peptides by database searching, mass differences between calculated and measured peptide masses were binned in 0.5-ppm windows. Fig. 4*a* shows that the distribution of mass errors is roughly Gaussian with almost all errors within plus or minus two ppm. To investigate the cause of the few outliers at 3–5-ppm mass deviation, we plotted the mass deviation as a function of peptide intensity.

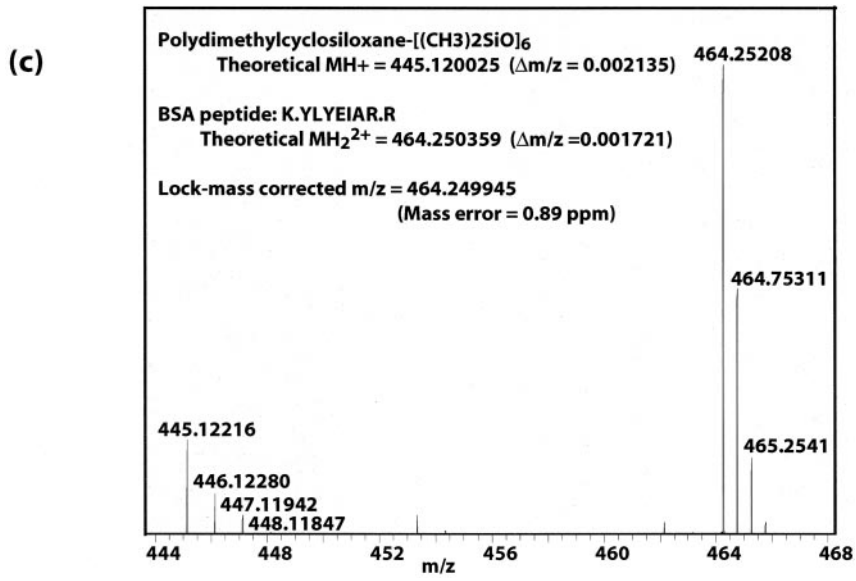

FIG. 3—*continued*

Fig. 4*b* suggests that the outliers are mainly caused by low signal. Makarov *et al.* (14) have reported previously that signal to noise is a determinant of achieved mass accuracy in orbitrap mass spectrometry, which is also in agreement with previous observations on other instruments (24).

The above data show that the lock mass strategy can reduce the mass error to a few ppm on the basis of the single mass measurement that is the basis for peptide picking for MS/MS. To further improve accuracy, next we made use of the fact that several mass measurements are made of the same precursor as it elutes from the capillary column and that these measurements typically are of higher intensity than the one used for precursor picking. Fig. 4*c* shows the intensity of a yeast SILAC peptide pair as it elutes from the column. As can be seen in the figure, the precursor was picked for sequencing and its mass determined when it was less than 10% of its maximal intensity. Altogether, five mass measurements of the precursor pair were acquired, and it is clear from Fig. 4*d* that mass accuracy is much better at the center of the LC peak than it is at its beginning.

Therefore we implemented a script to follow precursors over the LC peaks, which averages the mass measurements weighted by signal intensity. Fig. 5 shows the results of these calculations. As expected, mass accuracy was further improved and was generally within a 1 ppm absolute deviation from the calculated values. Fig. 5*b* demonstrates that all peptides after the LC-profile correction have a maximal mass error of less than 2 ppm (absolute average mass accuracy of 0.48 ppm and a standard deviation of 0.38 ppm).

As mentioned above, the lock mass strategy can equally be applied for MS/MS spectra. We noticed, however, that the PCM ion underwent substantial neutral loss of methane in the C-trap during the time it took to isolate and fragment the precursor ion. Therefore, we used this ion (m/z = 429.088735) for mass scale correction. Fig. 6, *a* and *b*, show analysis of two peptide fragmentation spectra recalibrated in real time with the lock mass ion. Both spectra were acquired with a target value of 50,000 and a resolution of 15,000 at m/z 400. We expected the mass accuracy to be somewhat lower in the MS/MS scan compared with the MS scan because of the shorter transient and consequently lower resolution as well as the fact that a single tandem mass spectrum was acquired for each precursor. Nevertheless, all fragments matched within 2 ppm of their calculated value provided that the intensity was higher than 5000 counts. Fragments with less than 5000 counts were still within 4 ppm of the calculated value in all cases. Note that the elemental composition of low mass ions can be determined uniquely given a mass accuracy of about 1 ppm, a fact that can be useful in interpretation of tandem mass spectra, for example, to define peptide sequence tags (25), end sequence tags (26), S-scores (27, 28), or in *de novo* composition based sequencing (29). When searching orbitrap MS/MS spectra with the MASCOT database searching program the achieved mass accuracy is much higher than what can be specified as a search parameter. Furthermore, the program does not decrease the statistical significance score for mass accuracies better than 0.25 Da or assign better scores to fragment matches with extremely high mass accuracy (30). However, the "delta score," the difference in score to the next best matching peptide sequence, was very large with these high mass accuracy tandem mass spectra; in fact, for most spectra no second peptide sequence was found at all. Data on the peptides can be found in supplemental material 1 and 2 ("msm" search files with and without LC mass correction) and supplemental material 3 (Excel sheet with peptide identifications with and without mass corrections).

Discussion and Perspectives—Here we describe a strategy for obtaining very high mass accuracy with an LTQ Orbitrap hybrid mass spectrometer. By separately injecting a background ion of known composition into the C-trap, we com-

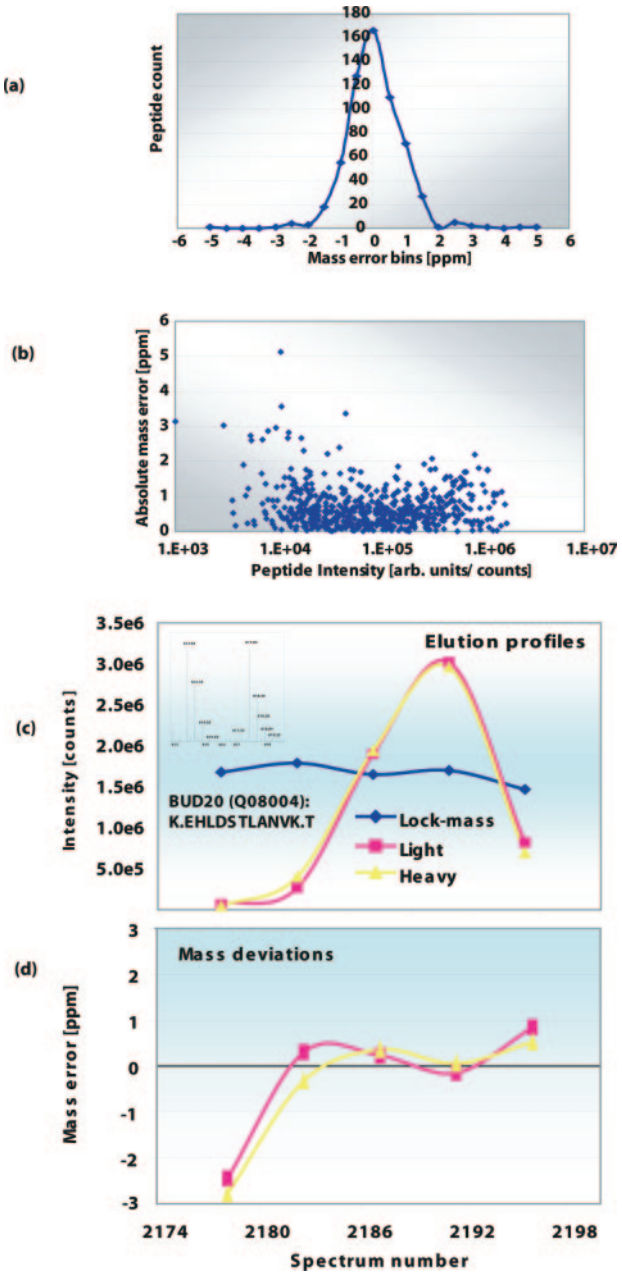

FIG. 4. *a*, mass errors binned into 0.5-ppm windows for peptides identified during LC-MS/MS of a gel band of a yeast lysate. Peptide masses were recalibrated in real time using the lock mass. *b*, mass error as a function of ion intensity. Outliers are concentrated at low signal intensities. *c*, signals of a 1:1 SILAC peptide pair as it elutes from the column. The intensity of accumulated PCM is also shown. *d*, measured mass deviations of the peptide pair shown in *c*. Mass error is lowest at the top of the LC peak. *arb.*, arbitrary.

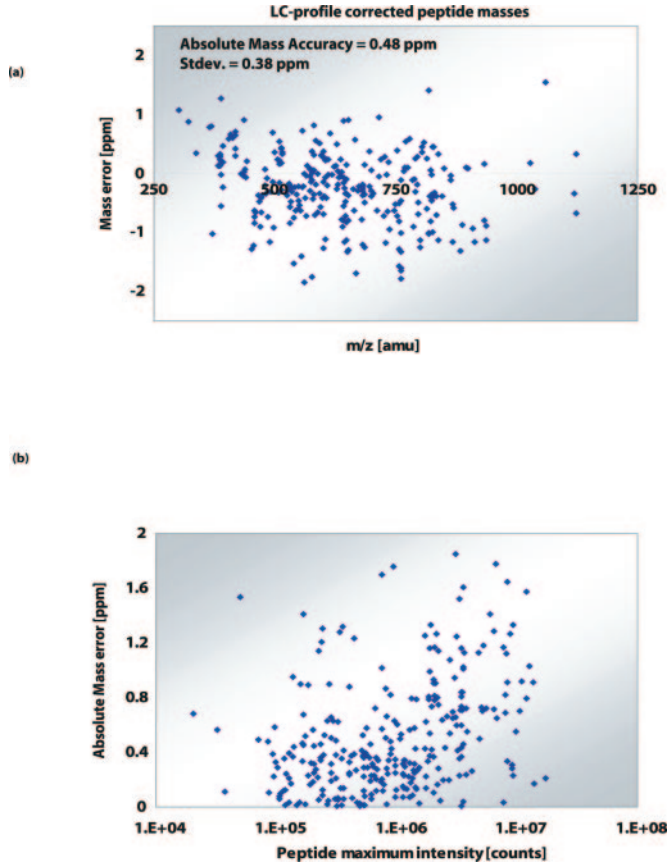

FIG. 5. *a*, masses determined using the lock mass and averaging over the LC peak. *Stdev.*, standard deviation. *b*, same data as in *a*; the absolute deviation is displayed as a function of total signal intensity. All peaks are within 2 ppm of their true value, and the absolute average mass deviation is 0.48 ppm.

pensate for drift in the electric field over time. The success of this strategy shows that the mass scale of the orbitrap, that is the proportionality of the frequency and square root of the *m/z*, is extremely linear, at least to better than 1 part per million. We find that the remaining mass error depends mainly on the signal intensity of the peptide peaks. For any peaks that are not close to the detection limits, we obtained a mass accuracy close to 1 ppm. Even for weak peaks the mass accuracy is still within a few ppm. To further increase the mass accuracy, we averaged mass measurements over the LC peak weighted by signal intensity. In this way, several mass measurements contribute to the final determination, and the mass determination is not based on a signal close to the detection limit as is normally the case when the precursor mass is the same as the one which is the basis for peak selection for MS/MS.

Dependence of achieved mass accuracy on signal or signal to noise is not unique to the LTQ Orbitrap. Unfortunately, in current proteomic practice, a "least common denominator" strategy is pursued when searching databases. The precursor and fragment mass tolerances are set to encompass even the worst mass measurements. After systematic mass errors have been removed by iterative recalibration (31) or by lock mass as shown here, a better strategy would be to specify very tight mass tolerances for well defined peaks and larger mass tolerances for weak peaks. Such a strategy could be implemented in a second step, after all peptides have been searched with a

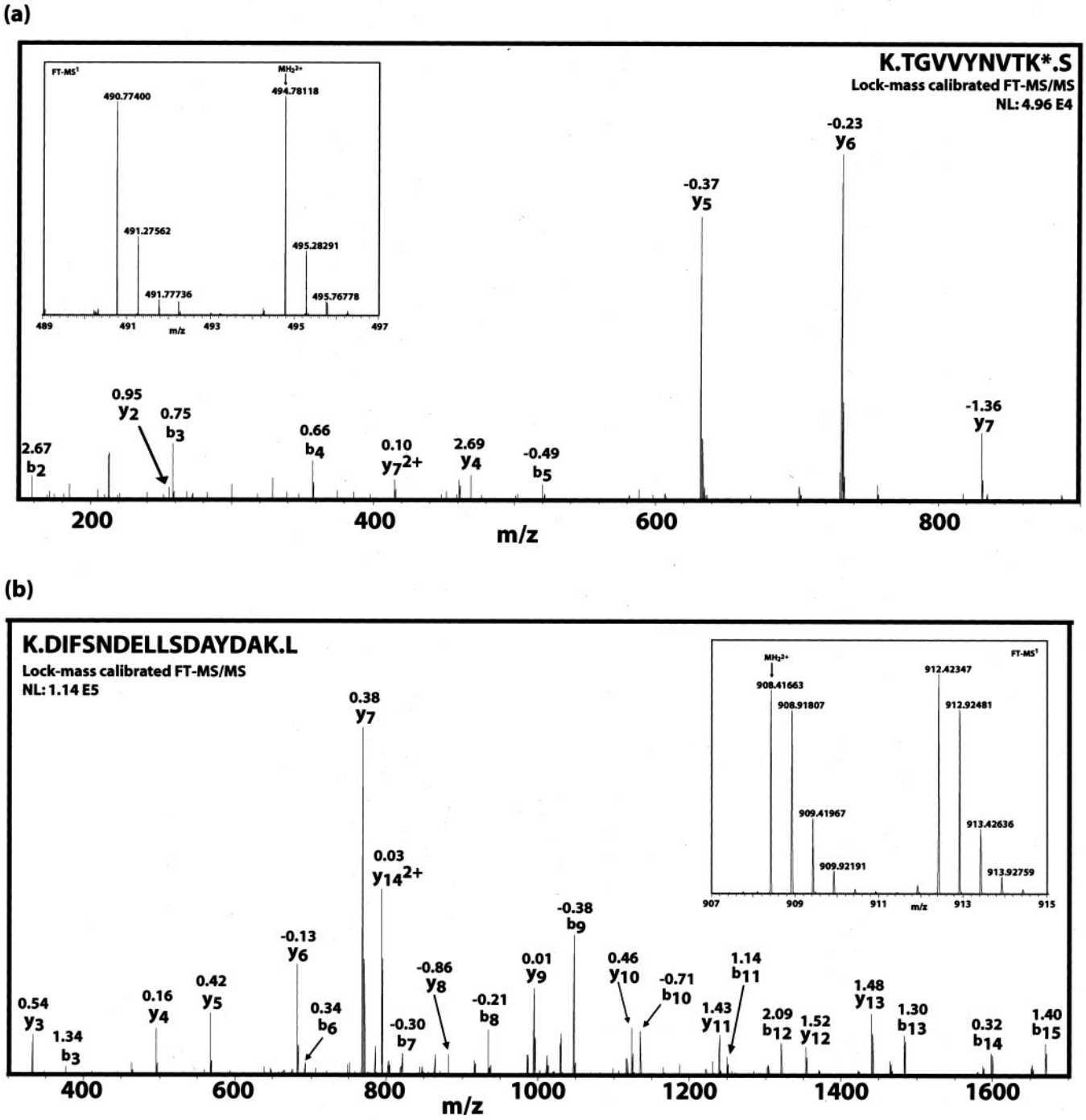

FIG. 6. ***a*, lock mass calibrated MS/MS spectrum of a small and (*b*) of a larger tryptic peptide.** Masses are within 2 ppm of their true values for all peptides with intensities more than 5000, and average absolute mass accuracy is 0.89 ppm for the fragments assigned.

larger mass tolerance. In this second step peptides with good signal but large deviation from the calculated masses would be removed from consideration, or at least obtain a reduced score. Ultimately, each precursor mass measurement should be accompanied by its own standard deviation. Statistical evaluation tools such as PeptideProphet (32) can already accommodate such additional evidence in a quantitative way.

The lock mass employed here, PCM, is ubiquitously present in laboratory air. However, other background ions could be used as well, and it is also possible to use more than one lock mass in the same spectrum.

Lock mass injection made use of the intermediate storage compartment provided by the C-trap. Although we have used the C-trap for storing a constant number of lock mass ions, it

clearly can be used for many other interesting purposes as well. For example, the C-trap could be filled rapidly with several narrow mass ranges of interest or several MS/MS fragment ions generated from different precursor ions before high resolution analysis of the accumulated ions in the orbitrap.

In comparison with the LTQ-FTICR, the LTQ Orbitrap is capable of similar mass accuracy, as shown here. However, here we achieve the high mass accuracy with the help of a lock mass whereas in the LTQ-FTICR we used "SIM scans," the isolation and injection of a narrow mass range into the ICR cell (3). An advantage of the lock mass strategy is that no extra time needs to be spent on SIM scans. Moreover, when the LTQ-FTICR is operated without SIM scans and with a high ion load at the limit of its space charge capacity to maximize dynamic range, then mass accuracy is about an order of magnitude worse than what we demonstrate here for the LTQ Orbitrap.

What is the utility of the robust, high mass accuracy demonstrated here? Zubarev *et al.* (33) have pointed out that a mass accuracy of 1 ppm could constrain peptide candidates to just a few sequences and thereby greatly facilitate peptide identification. Smith and co-workers added a retention time criterion and suggested that such "accurate mass and time tags" could be sufficient for peptide identification (34). This concept has been difficult to evaluate because mass accuracies actually achieved so far seem to have been relatively modest (see for example, Ref. 35). Even with the accuracy reported here, we do not believe that the mass alone is sufficient to identify peptides in typical proteomic experiments given the complexities introduced by incomplete cleavage and peptide modifications. However, for specialized situations, such as re-analysis of subproteomes, the number of candidate peptide sequences will be very small, and even low accuracy tandem mass spectra can then unequivocally decide if the candidate peptide is indeed present. Likewise, this mass accuracy will be extremely helpful in the elucidation of post-translational modifications, which presents a special problem because of the "combinatorial explosion" caused when considering many different types of modifications (36).

In conclusion, we have shown that a new compact hybrid mass spectrometer, the LTQ Orbitrap, is capable of very high mass accuracy using a lock mass strategy. High mass accuracy is routinely achievable in both the MS and MS/MS mode, and alone or in combination with MS^3 strategies (37) should help to virtually eliminate the problem of false positive peptide identification in proteomics and to identify post-translational modifications much more easily than currently possible.

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** To whom correspondence should be addressed: Dept. of Proteomics and Signal Transduction, Max-Planck Institute for Biochemistry, Am Klopferspitz 18, D-82512 Martinsried, Germany. E-mail: mmann@biochem.mpg.de.

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