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# A General Precursor-Ion-Like Scanning Mode on Quadrupole Time-of-Flight Instruments Compatible with Chromatographic Separation

Ricarda Niggeweg*, Thomas Köcher*, Marc Gentzel, Alessia Buscaino, Mikko Taipale, Asifa Akhtar, Matthias Wilm

**Abstract**

Mass spectrometric protein identification and quantitation are key proteomic techniques in biological research. Besides identification of proteins, mass spectrometry is used increasingly to characterize secondary protein modifications. This often requires trimming the analytical strategy to a specific type of modification. Direct analysis of protein modifications in proteomic samples is often hampered by the limited dynamic range of current analytical tools. Here we present a fast, sensitive, multiplexed precursor ion scanning mode - implemented on a quadrupole time-of-flight instrument - that allows the specific detection of any modified peptide or molecule that reveals itself by a specific fragment ion or pattern of fragment ions within a complex proteomic sample. The high mass accuracy of the time-of-flight mass spectrometer is available for the marker ion specificity and the precursor ion mass determination. The method is compatible with chromatographic separation. Fragment ions and intact molecular ions are acquired quasi-simultaneously by continuously switching the collision energy between elevated and low levels. Using this technique many secondary modifications can be analyzed in parallel, however the number of peptides carrying a specific modification that can be analyzed successfully is limited by the chromatographic resolution or—more generally—by the depth of the resolved time domain.

* both authors contributed equally to the work
European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, 69117 Heidelberg, Germany

## Introduction

With the introduction of mass-spectrometry-based protein identification and sequencing, our ability to investigate biological systems on the protein level advanced considerably. Protein complexes, organelles or entire proteomes can now be mapped [1-9]; the functional relationship of proteins in their biological context is being revealed to an increasing extent [10-16]; quantitative protein expression patterns are now being analyzed for disease markers, allowing early diagnosis [17, 18]. First attempts to describe the temporal behaviour of entire biological systems have been made [19]. All of these experiments have been made possible by the analytical strength of mass spectrometers in identifying and quantifying proteins. At the same time, our ability to characterize secondary protein modifications has improved. Precursor ion scans on triple quadrupole or on quadrupole time-of-flight instruments allow the specific detection of peptides with a particular modification [20-30]. Modification-specific affinity enrichments in conjunction with capillary HPLC separation of the peptides and rapid sequencing by quadrupole time-of-flight or ion trap mass spectrometers extends protein modification mapping to a larger scale [29, 31-33]. Affinity enrichment appears to be necessary to overcome the dynamic range limitation of current analytical strategies [33, 34]. A complementary approach is the rapid, real-time analysis of fragmentation patterns to find and sequence modified peptides during chromatographic separation [35]. Bateman et al. showed that it is possible to record fragment spectra and intact molecular-mass ions quasi-simultaneously on a quadrupole time-of-flight instrument (Q-TOF 2) by continuously switching the collision energy between an elevated and a low level throughout a chromatographic run. They use this approach to detect and sequence phosphorylated peptides in real time. Neutral loss scans are implemented by searching for losses of 98 Da fragment spectra produced with a moderate collision energy. When such a loss is detected, the associated intact precursor is selected for fragmentation. The same mode is used for real precursor ion scanning for the immonium ion of phosphorylated tyrosine. However, when such an immonium ion is detected in the high collision energy spectra, all currently present ions have to be fragmented since no information about the origin of the immonium ion is available. This limits the application of this method to relatively simple samples because there is insufficient time to fragment many coeluting precursors during a chromatographic separation. Bateman et al. mentioned this limitation and proposed using an LC system with variable flow rate. With such an HPLC, the flow can be reduced when a precursor ion is detected to make sufficient time available to fragment all precursors present [35]. In our efforts to characterize protein modifications with our capillary HPLC–Q-TOF1 instrument, we were finally led to the same conclusion. The only way to record sensitive precursor ion spectra fast enough to be compatible with the HPLC separation is to switch the collision energy regularly between a high and a low level. We sought to implement a method that would be applicable to complex proteomic samples. The technical realization had to differ because we have no access to the acquisition code on our spectrometer, the electronics in our Q-TOF1 instrument are too slow for

real-time evaluation of the fragment spectra, and our capillary HPLC does not support stop-flow operation.

We used the dynamic switching of the collision energy to implement a global precursor ion scanning mode on our capillary HPLC–Q-TOF instrument. The instrument is operated permanently in MS mode with the collision cell filled with gas. The quadrupole is set to transmit all ions above m/z 400 Th with maximal efficiency. When the collision energy is at the lower level the m/z values of all the intact molecules eluting from the HPLC column are measured. In the next cycle, when the collision energy is at the higher level, fragments from all precursors are recorded. It is important for the sensitivity of the precursor ion scan that all detected ions with m/z below 400 Th are generated in the collision zone and are therefore fragments. The higher collision energy dataset can be searched for any fragment marker ion or pattern of marker ions. Once a marker ion has been found, its true precursor can be determined from the spectra generated in the lower energy mode by time course correlation of the elution profiles of marker and potential precursor ions. This parallel sequencing of many precursors followed by correlating fragments to their respective precursors using time course correlation was first demonstrated on quadrupole time of flight instruments equipped with ion mobility devices [36]. In this method, individual precursors are separated by different times of passage through a drift tube containing helium gas at a pressure of 2.5 Torr; ions are separated on a millisecond timescale before being fragmented in an octapole collision zone and analyzed in a time of flight instrument. The use of an ion drift device is certainly beneficial for a parallel sequencing approach. However, there is a loss in overall ion transmission. We demonstrate here that the moderate time resolution of a chromatographic system can be sufficient for targeted analysis of secondary protein modifications. This is significant because the overall sensitivity of the mass-spectrometric analysis is often the limiting factor for a biological experiment. Using a mass spectrometer with higher resolution and/or a ultra high pressure chromatographic system will be very beneficial for this type of investigation [37, 38].

Once a dataset containing elevated and low collision energy spectra is acquired, any marker or combination of markers that are charged in the chosen ionization mode can be scrutinized. In this sense, our method achieves a global precursor ion scan. As demonstrated in the articles of Hoaglund-Hyzer and Bateman, the entire precision of the time-of-flight mass spectrometer is available for the masses both of the marker ions and of the precursors [35, 36]. We separate detection and sequencing of the relevant precursors into two chromatographic runs. After m/z values and retention times of modified peptides have been identified, they can be sequenced in a targeted fashion, eliminating the bulk of unmodified peptides from the analysis. By switching the spectrometer to targeted mode, focusing exclusively on the previously identified, putatively modified peptides, the dynamic range of the tandem-MS-based analysis of complex mixtures can be extended.

This scanning regime exploits the geometry of the collision cell to produce fragment ions efficiently. Quadrupole time-of-flight machines optimized for the detection of small fragments will have an even higher sensitivity [22, 23, 39]. The ability to identify modified peptides improves with the resolution of the chromatographic separation. This analytical mode is therefore in harmony with efforts to improve mass resolution of tandem mass spectrometers and the separation of chromatographic systems. The high specificity and sensitivity of this approach reduces the requirement for affinity-based enrichments of modified peptides and thus simplifies the relative quantitation of secondary protein modifications. Being a general precursor ion scan, the analytical strategy proposed here is not limited to secondary modifications of proteins, but may be useful in the characterization of drugs or a combination of drugs and their metabolites in samples of biological origin.

It is useful that the method is not limited to newer quadrupole time-of-flight mass spectrometers that are fast enough to use real-time analysis of fragment spectra: it can be implemented on all Q-TOF instruments [35].

We assessed the global precursor ion scan by analyzing protein acetylations. To estimate the overall quality of the approach we compared the new strategy with a nano-electrospray precursor ion scan analysis on a triple quadrupole mass spectrometer (API III, Sciex). This method has a very high specificity for modified peptides, high sensitivity and robustness [20, 21]. But because of the lack of automation and chromatographic separation, the entire approach is limited to the investigation of samples with a small number of proteins, and cannot therefore be regarded as a proteomic technique. This is different for the global precursor ion scan on the quadrupole time-of-flight machine. Since this technique makes use of chromatographic separation it not only can be used on more complex samples but allows to differentiate between isobaric and isomeric molecules that fall onto the same m/z value in a nano-electrospray investigation. Comparing both methods on a synthetic peptide or on a single protein puts the global precursor ion scan at a disadvantage since this type of analysis is the specific strength of the nano-electrospray experiment. On the other hand, it is just this comparison that allows us to evaluate the quality of the new LC quadrupole time-of-flight-based approach.

The reversible acetylation of the amino group of lysine residues is an important post-translational modification altering protein activity. A growing number of proteins

including histones [40], transcription factors [41] and nuclear import factors [42] are modified in this way.
To detect acetylated peptides specifically we used the reporter ion m/z 126.1 Da [43]. This ion is derived from the immonium ion of acetylated lysine by the loss of ammonia upon fragmentation. We evaluated the selectivity and the sensitivity of the approach with a synthetic acetylated peptide in a background of a tryptic peptide mixture. The technique was used to determine the acetylation sites of gel-isolated histone H4.
To test the ability of this scanning mode to detect several different secondary modifications simultaneously, we analyzed a mixture of 100 fmol of the acetylated peptide, 250 fmol of an RNAseB digest, a protein glycosylated on an individual site, and a digest of all proteins between 20 kDa and 60 kDa of a total E. coli lysate from a 1D gel.

## Material and methods

### Materials and reagents

β-galactosidase, RNAseB and bovine serum albumin were from Sigma (St. Louis, MO, USA); Coomassie brilliant blue was from Serva (Heidelberg, Germany); the synthetic acetylated peptide VLET[Acetyl-K]SLYVR was bought from Peptide Speciality Laboratories (Heidelberg, Germany); methanol, water, formic acid, acetic acid and acetonitrile in the highest purity grade came from Merck (Darmstadt, Germany); bovine trypsin (sequencing grade) was from Roche Diagnostics (Mannheim, Germany); POROS R2 and POROS oligoR3 material were from PerSeptive Biosystems (Framingham, MA, USA).

### Protein samples and in vitro acetylation assay

Drosophila MOF was expressed and purified as described recently [44]. Full-length human MOF was expressed as a GST fusion protein in E. coli using the vector pET41a (Novagen). The mutations K116R and K116G were introduced by site-directed mutagenesis. Recombinant Xenopus histones were expressed and purified as described [45]. In vitro acetylation was performed in a buffer containing 50 mM NaCl, 20 mM TrisHCl pH 8.8 and 1.5 mM $MgCl_2$. Acetylation reactions were allowed to proceed for 4 hours at 30℃ [45].

### In-gel digestion

Sodium dodecyl sulphate polyacrylamide gels were stained with silver or Coomassie brilliant blue [46]. The visualized protein bands of interest were excised, washed, reduced, alkylated and trypsinized overnight [47]. The peptides were extracted from the gel pieces and dried in a vacuum centrifuge.

### Nano-electrospray mass spectrometry

**T**ryptic peptide mixtures were desalted as previously described using a double column POROS R2, oligoR3, assembled in pulled glass capillaries [48]. The detection of acetylated peptides was performed using precursor ion scanning for the product ion m/z 126.1 Da. The experiments were carried out with a nano-electrospray source [48] mounted on a triple quadrupole instrument (API III, PE-Sciex, Ontario, Canada). The needle voltage was between 600 V and 650 V, the interface voltage was 100 V and the orifice potential was 55 V. The potential difference to the collision zone was 50 V. The precursor ion spectrum was acquired with a step width of 0.2 Th and a dwell time per m/z value of 3 ms. Resolution parameters were optimized for maximum sensitivity. Product ion spectra from the detected acetylated peptides were acquired on a quadrupole TOF hybrid instrument (Q-TOF1) with nano-electrospray in positive ion mode. The needle voltage was 650 V and the cone voltage was set to 35–40 V. Resolution for the precursor selection for tandem-MS was chosen to transmit a m/z window of 2 Th. The collision energies were adjusted individually for each peptide.

### Capillary LC tandem mass spectrometry

Peptide desalting and separation was done on a 150 $\mu$m x 2 cm pre-column followed by a 75 $\mu$m x 11 cm capillary column packed with YMC C-18 ODS AQ from YMC (Europe, Schermbeck, Germany). An Ultimate HPLC system (LC Packings, Dionex, Idstein, Germany) was used for loading, washing and generating the LC gradient. Loading and washing were done with a flow rate of 200 nl/min, peptide separation and analysis with 100 nl/min from 100% solvent A (0.2% formic acid, 2% acetonitrile) to 70% solvent B (80% acetonitrile, 0.5% formic acid). For the simultaneous identification of acetylated and glycosylated peptides, peptide separation and analysis were done with 100 nl/min from 100% solvent A (0.1% formic acid) to 70% solvent B (80% acetonitrile, 0.2% formic acid) using the Eksigent 1D NanoLC-System. The HPLC was interfaced to a quadrupole time-of-flight mass spectrometer (Q-TOF1, Micromass, Manchester, UK). The spray was generated from 50 $\mu$m x 150 $\mu$m fused silica tips pulled on a laser puller (Model P-2000, Sutter Instruments Co., Novato, CA, USA). Electrical contact between the electrospray source and the power supply was established by a 25 $\mu$m platinum wire (Goodfellow, Cambridge, UK) inserted into the liquid flow. Spraying voltage was between 1400 V and 2500 V depending on the current diameter of the electrospray emitter. The cone voltage of the mass spectrometer was set to 35 V. The mass spectrometer was operated by MassLynx version 3.4 (Micromass). For the precursor-ion—like scan the scan time per spectrum was set to 1.75 s, collision gas was permanently switched on but the mass spectrometer was operated in MS mode. The total mass range was set from 50 Th to 1800 Th. For transmitting ions coming from the ion source through the quadrupole to the time of flight mass spectrometer two transmission windows are set for

the quadrupole. During each scan of 1.75 seconds the transmission window of the quadrupole starts at 400 Th or at 700 Th each for 45% of the scanning time. In the remaining 10% the quadrupole scans from one window to the next. The potential in the collision zone was switched every 4 s between 4 V and 50 V throughout the entire LC run using Quickeys to manipulate the collision energy entry field in the user interface of MassLynx (CE Software, West Des Moines, Iowa, US). The total cycle time is 8 seconds. This is relatively long but since our average chromatographic peak has a width of 30 - 40 seconds we still can reconstruct the chromatographic elution curves. Switching times can be faster on our Q-TOFI instrument and will be certainly faster when this acquistion mode is natively implemented into the acquistion software. For the targeted tandem-MS acquisition of acetylated peptides we determined the m/z values of all peptide ions that generated the 126.1Th marker ion upon fragmentation. The 'include list' contained the m/z values within a window of ±0.2 Th around these precursor values. (MassLynx version 3.4 has a calibration-related bug when using include list masses: if an ion with a specific m/z value is to be selected, not this m/z value but a linearly dependent value has to be typed into the include list.) For the tandem-MS acquisition we allowed the selection of doubly and triply charged precursors, set the threshold for precursor selection to 5 ion counts per second, the scan time to 3 s, the mass tolerance window for the precursor selection from include lists to 0.25 Th, allowed the simultaneous selection of up to 4 cyclically fragmented precursors, and set the maximum tandem-MS acquisition time to 25 s per precursor. The tandem-MS acquisition was stopped when the maximum ion count in a scan dropped below 1. The collision energy applied depended on the precursor's m/z value and its charge state.

Data processing

Data processing routines were written in Igor Pro (WaveMetrics, Oregon, USA). A Macintosh application programmed in AppleScript Studio is used to supply the data to the Igor Pro routines and to read the processed data files. Spectra and chromatograms are displayed using Bio-Multiview (PE-Sciex, Ontario, Canada). The precursor ion scan data file contains two types of spectra – fragment data from elevated collision energy scans and intact molecular ion spectra from low collision energy scans. We process the chromatograms to separate these. In this way, continuous conventional chromatograms with half the number of spectra are generated, which we use for further evaluation. The original data are translated into ASCII (i.e. plain text) using the DataBridge program (Micromass). Then the file is read into Igor Pro, the zero intensity data points are removed and the mass spectra are separated into the two chromatographic sets. For both chromatograms, two indices are created to allow fast access to the mass spectra. The first index reports all indices of the data array where a new mass spectrum begins [49]. The second, deep index $i_{(r,s)}$ is two dimensional. The value of the index is an index number of a specific m/z - intensity data point in the complete data set of the chromatographic run. The first dimension r specifies the number of the spectrum of this data point, the second dimension s describes its m/z location. The two dimensional index is built such that $i_{(r,s)}$ is the index number of exactly that data point in the spectrum number (r-1) whose m/z value is the first being bigger than (10 times s) Th. Using this index any recorded m/z value in any spectrum can be rapidly found since the search in the complete data set starts already in the correct spectrum and in the correct decade of m/z values. In particular this deep index allows a very fast construction of mass chromatograms from large datasets.

When a marker ion is detected during the LC run we determine possible precursor ion candidates by a time course correlation. A chromatogram is calculated from the low energy data for each ion above a certain intensity threshold at the time of maximal marker ion generation and compared to the chromatogram of the marker ion. We currently use two different curve characteristics to assess a score for the correct precursor ion: a quadratic norm representing the intensity displacement of the normalized elution profiles of precursor and marker ions ( $\sum_i [\, I_1(t_i) - I_2(t_i)\, ]^2$ ), and the time difference between the maxima of these two profiles. The chromatographic displacement is calculated for the peak width of the marker ion by interpolating both chromatograms in steps of 0.5 s, smoothing the curves, scaling both to 100 in a window three times the peak width of the marker ion to cover the maximum intensity of precursor ions with different elution times, calculating the displacement using the formula above for the width of the marker ion peak. We take the score value for curve displacement as the reciprocal of the displacement scaled to 100. The time difference between the maxima of the curves is measured in seconds. We take its score value as the reciprocal of this time difference plus the average time difference between two scans to avoid divisions by zero. All score values for the time difference are scaled to 100. The combined score value is the sum of these two individual scores rescaled to 100. We currently consider two criteria for matching elution profiles to find out in the light of increasing experience whether it is sufficient to consider only the time difference between the maxima of the elution profiles.

We export fragment spectra from targeted tandem-MS investigations as pkl files, involving as little processing as possible. The spectra in the pkl files are centroided, fragment spectra originating from the same precursor are joined, multiple charge states deconvoluted and fragments are deisotoped using our own algorithms [50]. After processing, the pkl files are submitted to a database search

using Mascot on the internet (Matrixscience, London UK, http://www.matrixscience.com).

Database searches

The fragment spectra were searched against the NCBI non-redundant database without any restrictions in the choice of the organism using Mascot on-line. Carbamidomethylated cysteines, oxidized methionines, deamidations and lysine acetylations were set as variable modifications. Only tryptic peptides were considered, but we allowed up to five missed cleavage sites since Mascot counts an acetylated lysine as a missed cleavage site. The fragment mass tolerance was set to ±0.3 Da, the precursor mass tolerance to ±0.6 Da. these high values are because we do not recalibrate the fragment spectra – in general, we use trypsin autolysis fragment spectra to recalibrate our fragment spectra automatically [50]. However, in the targeted analysis mode the (m/z, retention time) pairs of these peptides were not part of the include lists and therefore not acquired.

## Results and discussion

### Global precursor ion scanning mode on the quadrupole time-of-flight machine

The introduction of temporary ion storage in the collision zone of a quadrupole time-of-flight mass spectrometer improved the native precursor ion scan ability of this type of mass spectrometer [23, 24, 39]. On our quadrupole time-of-flight mass spectrometer a native precursor ion scan is too slow and not sensitive enough to be used on-line with chromatographic separation. We chose a simpler approach to record fragments and intact molecular masses quasi-simultaneously [35]. We switched the potential in the collision zone every four seconds between elevated and low values. The potential in the collision zone alternates regularly between 4 V and 50 V. The mass spectrometer does not adjust the potentials during a single scan, so every scan is recorded entirely either under low collision energy or elevated collision energy settings. We separate the mixed datastream into two chromatograms, one representing all spectra with elevated collision energy and a second with all spectra of low collision energy. Data separation occurs in two steps: the base peak chromatogram between 50 Th and 200 Th is used to decide whether a particular spectrum was recorded with high or low collision energy settings (see figure 1).

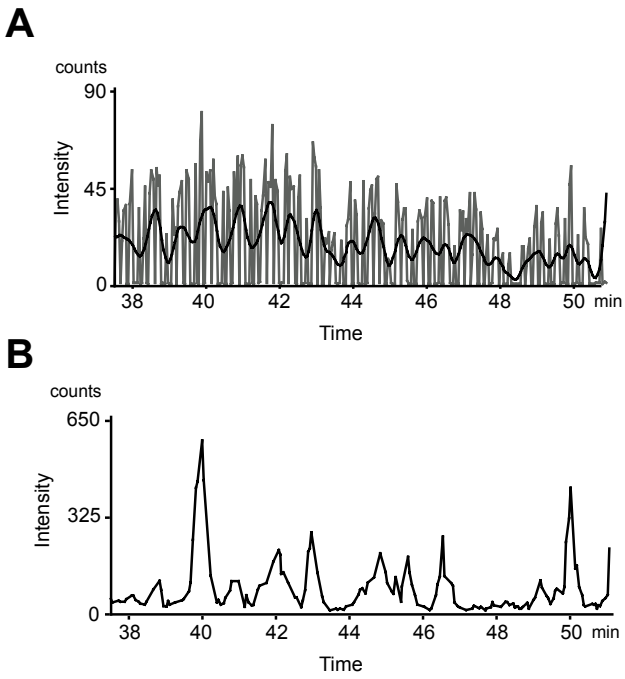


**Figure 1**

Data separation of a chromatogram containing low and elevated collision energy spectra. Panel A shows the base peak chromatogram of the m/z region between 50 Th and 200 Th. The quadrupole is set to transmit ions only from 400 Th upwards so there are hardly any ions detected in this mass range when the collision energy is low. Spectra with intensities above the average line are considered to be part of the elevated collision energy chromatogram, those below it belong to the low collision energy spectra. Panel B shows the base peak chromatogram of the low collision energy spectra after separation. The achievable chromatographic separation of a little less than 1 min per peak is maintained even though there are only half the number of spectra as in an ordinary LC run.

If the maximal ion intensity in this region of one particular spectrum at a specific time point in the elution profile is above the averaged maximal ion intensity of this m/z region then it belongs to the elevated collision energy chromatogram, and otherwise to the low collision energy chromatogram. In a second pass the intensity in this m/z region of every scan is compared to the time-averaged intensity of the now classified high and low collision energy spectra to find incorrectly classified individual spectra. The averaging is more precise within each group of spectra since the vast majority of them are classified correctly.

The purpose of this scanning mode is to record the intact masses of all ions that produce a specific marker ion upon fragmentation. However, the data have more aspects than just this one. Since the fragment spectra are complete, they allow for simultaneous investigation of different marker ions specific to different modifications or even to the fragmentation pattern, i.e. logical combinations of fragment masses. The multiplexing of marker ions and the higher resolution for precursors and markers are the major advantages of precursor ion scans on quadrupole

time-of-flight machines in comparison to triple quadrupole mass spectrometers [22, 35, 39, 51].

There are three features that are responsible for the high sensitivity for this scanning mode in comparison to the traditional precursor ion scanning mode or orifice fragmentation scans on quadrupole time of flight instruments: the high transmission of the quadrupole for intact molecular ions with m/z > 400 Th (since it is not used to select a specific one); the efficient generation of fragment ions in the collision zone; the absence of background ions coming directly from the ion source with m/z < 400 Th. The mass spectrometer is set to operate continuously in MS mode while generating fragments in the collision zone. In this mode the transmission window of the quadrupole starts at m/z 400 Th ensuring that all ions detected in this window are generated in the collision zone.

In comparison with precursor ion scans on our triple quadrupole instrument, the resolution for the marker and the precursor ion is much higher. The higher marker ion resolution is useful for increasing the specificity of the scan [28, 35].

It is certainly a disadvantage that the fragments are generated from all simultaneously eluting precursors. We handle the uncertainty about the real precursor molecule by two means: time-course correlation of the elution profiles of precursor and marker ions, and confirmation of the primary sequence of the detected molecule by targeted tandem-MS investigations. To prove the concept we mixed 1 pmol of the acetylated peptide VLET[Acetyl-K]SLYVR into 1 pmol of an in-gel digest of BSA. Panel A of figure 2 shows the base peak chromatogram of the low collision energy spectra, panel B the chromatogram of the 126.1 Da marker ion from the high collision energy spectra. Panel C shows an overlay of the marker ion chromatogram and the chromatogram of the doubly charged precursor ion of the acetylated peptide from the low collision energy spectra.

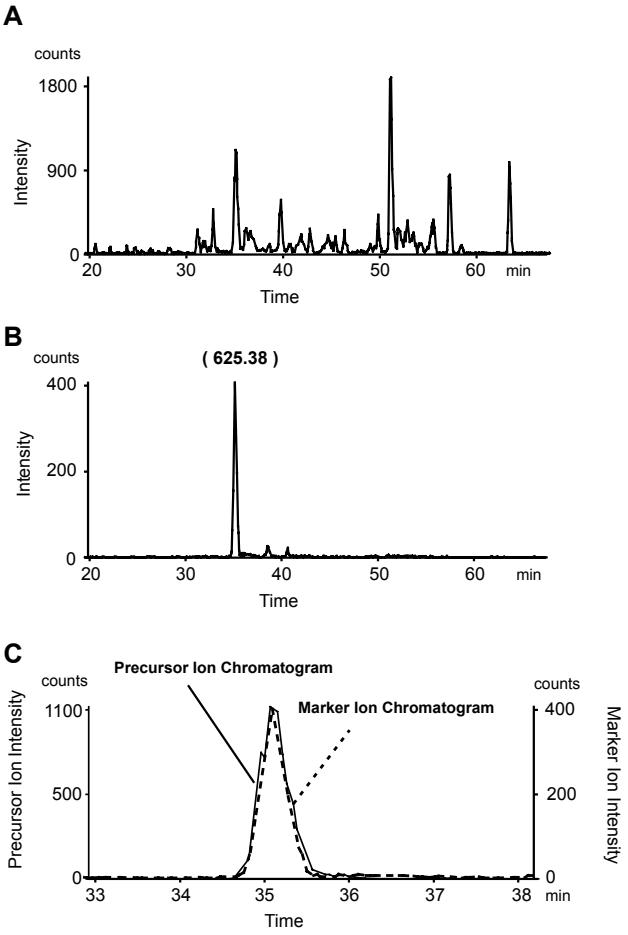


**Figure 2**

Demonstration of the precursor-ion-like scan on a quadrupole time of flight instrument. 1 pmol of the acetylated peptide VLET[Acetyl-K]SLYVR was mixed into 1 pmol of a BSA digest. Panel A shows the base peak chromatogram of the low collision energy spectra, panel B the precursor ion chromatogram for the 126.1 Da marker ion for acetylated lysines from the elevated collision energy spectra, and panel C shows both the marker precursor ion chromatograms. Their time profiles match very well.

The intensities of these two ions are different, but their elution profiles correspond very well. The ability to correlate a fragment ion to its real precursor depends directly on the time resolution of the intensity profiles.

This time resolution increases when using an ultra high pressure chromatography or an ion drift device [37, 52].

We generate a list of candidate molecules and program an experiment so that the mass spectrometer will exclusively fragment molecules with predetermined m/z values and retention times. This targeted mode investigation is in general complete, in the sense that if the suspected molecules reoccur, they will be fragmented and the existence of the characteristic fragment confirmed.. The intensity of the precursor is a secondary criterion for its selection for

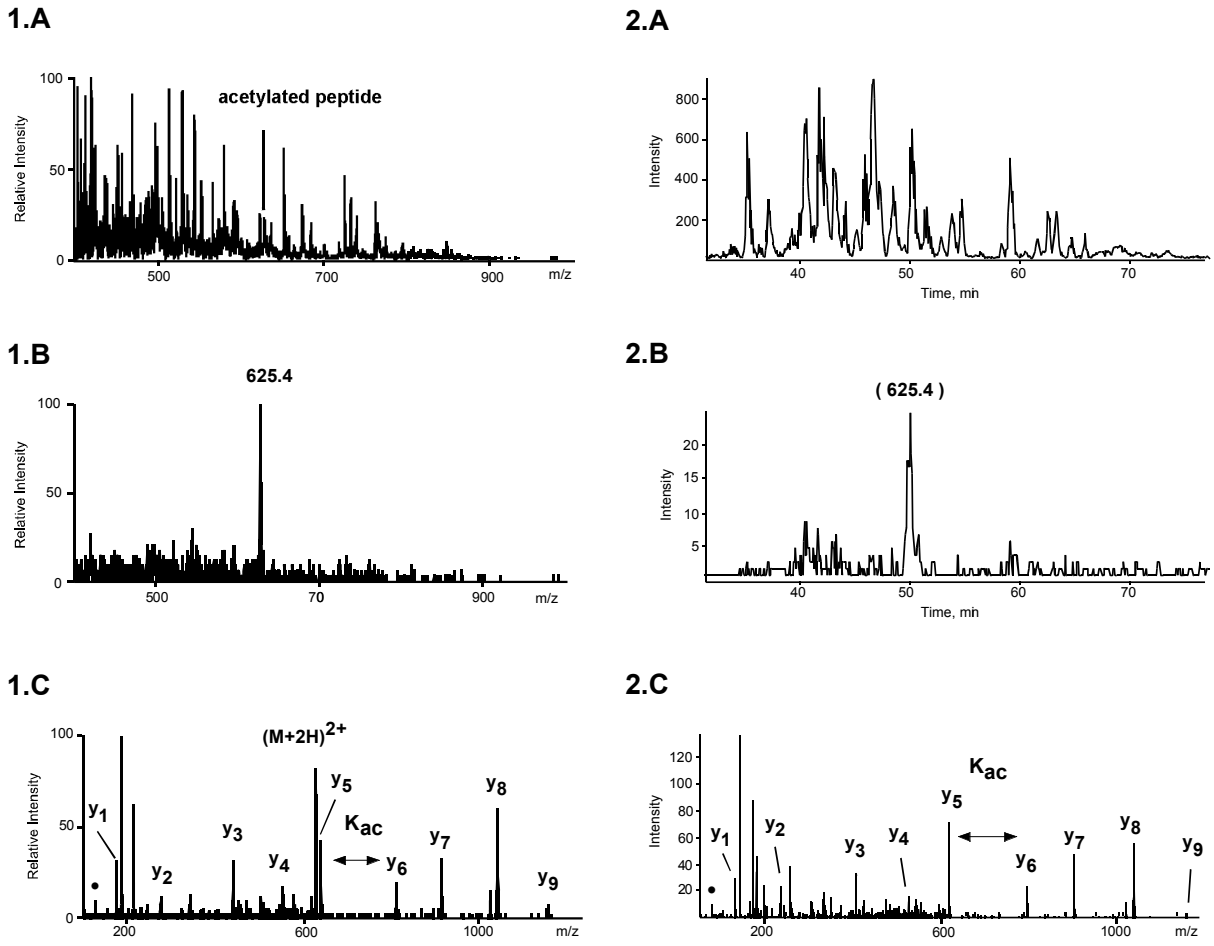


**Figure 3**

Comparison of the sensitivity of the precursor-ion-like scan on our Q-TOF1 instrument with a nano-electrospray investigation on the API III instrument. The first column shows the analysis done on the API III instrument, the second on the Q-TOF1 machine. 200 fmol of the acetylated peptide VLET[Acetyl-K]SLYVR was mixed into 1 pmol of a digest of the 116 kDa protein β-galactosidase. For the Q-TOF investigation this sample was split into two samples for the precursor-ion-like scan and the targeted tandem MS investigation. Panel A shows the initial mass spectrum (column 1) and the low collision energy base peak chromatogram (column 2). Panel B shows the precursor ion spectrum for the acetylated lysine on 126.1 Da (column 1) and the marker ion mass chromatogram from the elevated collision energy spectra (column 2). Panel C shows the tandem MS spectra of the detected acetylated peptide from the triple quadrupole (column 1) and the quadrupole time of flight instrument (column 2). The overall sensitivity and signal to noise ratios of the different spectra are similar, even though the nature of the datasets and the way they had been generated are very different.

fragmentation, so we do not expect a major limitation of the analysis in terms of dynamic range at this stage. With increasing complexity of the sample the number of possible precursor ions per detected marker ion will eventually grow. The total capacity of the analysis will depend on the specificity of the marker ion for the interrogated molecular structure, the resolution of the mass spectrometer and the discrimination of the chromatogram. The latter in particular can be adapted to experimental conditions, for instance by using two-dimensional chromatography, ultra-high-pressure chromatography or very long gradients [53, 54].

**Selectivity and sensitivity of the method**

We evaluated the suitability of the 126.1 Da marker ion for the detection of acetylated peptides using the precursor scanning techniques. 200 fmol of the synthetic acetylated peptide VLET[Acetyl-K]SLYVR was mixed into an in-solution digest of 1 pmol of the 116 kDa protein β-galactosidase. Figure 3 panel 1.A shows the nano-electrospray spectrum of the peptide mixture on our triple quadrupole mass spectrometer. Panel 1.B shows the precursor ion scan. The acetylated peptide is detected with high specificity. Panel 1.C shows the sequenced peptide from the same sample. The acetylation site can be located by interpreting the y-ion series. For the analysis on the HPLC-quadrupole time-of-flight instrument we divided the sample into two parts. The first was used to detect the acetylated peptide, the second to sequence it. Figure 3 panel 2.A shows the base peak chromatogram of the intact ions eluting from the chromatographic system, panel 2.B the marker ion chromatogram from the elevated colli-

sion energy spectra and panel 2.C the sequenced precursor ion detected in the first chromatographic run. It is remarkable that the signal to noise ratios of theprecursor ion scans and the fragment spectra are almost exactly match those for the nano-electrospray triple quadrupole results even though the spectra are very different. The triple quadrupole loses ions because of the scanning first quadrupole. However, nearly all marker ion fragments produced in the collision cell are transmitted to the detector. The quadrupole time of flight instrument loses marker ions because of the skimmer between the collision cell and the time of flight part of the instrument and the TOF part has only a limited duty cycle. The Q-TOF instrument is comparable in sensitivity only because the quadrupole is not used for ion selection in this acquisition mode, but transmits all ions with m/z > 400 Th. A possible explanation of the similarity of the two spectra is that the analysis is limited by the chemical noise still present in the precursor ion scans.

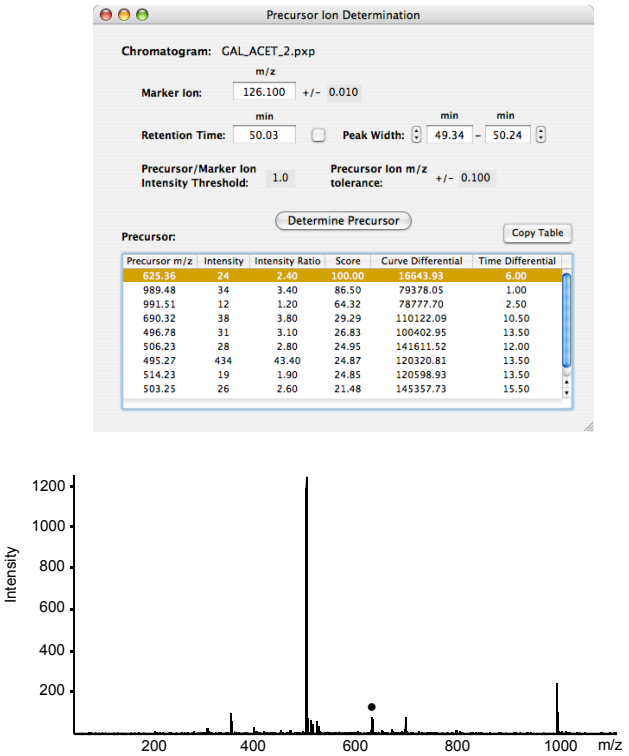


**Figure 4**

Precursor identification of the investigation shown in figure 3. The precursor ion of a given marker ion is determined by time course correlation of the chromatograms. The panel shows the parameter typically used for a precursor ion determination. The doubly charged 625.36 Th ion of the acetylated peptide scored highest even though it belongs to the smaller ions in the low collision energy spectrum at the time point of maximal marker ion intensity.

This would mean that sample and ionization characteristics are limiting the analysis in both cases and not the lower ion transmission as in earlier implementations of precursor ion scans on quadrupole time of flight machines. A higher mass resolution might help to reduce the remaining chemical background noise.

Part of the analysis on the LC quadrupole time-of-flight mass spectrometer is the determination of the true precursor ion. Figure 4 shows the spectrum of intact molecular ions at the time of maximal marker ion intensity. The doubly charged acetylated peptide is marked. During automatic precursor ion determination, chromatograms of all intact molecular ions whose intensity per scan is higher than a parameterized multiple of the marker ion intensity are calculated. As can be seen from figure 4 the true precursor scored highest.

This result is quite acceptable in view of the fact that the sample concentration was at the lower limit of allowing a successful analysis including the sequencing of the peptide using the nano-electrospray ion source on our triple quadrupole machine. It is possible to detect an acetylated peptide from a clean sample using a precursor ion scan on the API III triple quadrupole instrument down to the level of 1 fmol/$\mu$l = 1 nM (data not shown). However, the more common situation is to detect and sequence a peptide on a background of abundant, unmodified peptides.

### Identification of acetylation sites on individual gel-separated proteins

Once the procedure was established, we tested it on an in-gel digest of histone H4 acetylated in vitro with human MOF (males-absent on the first) protein. We performed histone acetyltransferase (HAT) assays [55] using recombinant Xenopus histone octamers [45] and recombinant human MOF protein (as described in methods). After in-gel digestion the proteins were analyzed on the triple quadrupole and the quadrupole time of flight mass spectrometers. Figure 5 shows the triple quadrupole precursor scan in panel A and the corresponding chromatogram of the 126.1 Th marker ion from the LC–Q-TOF instrument in panel B.

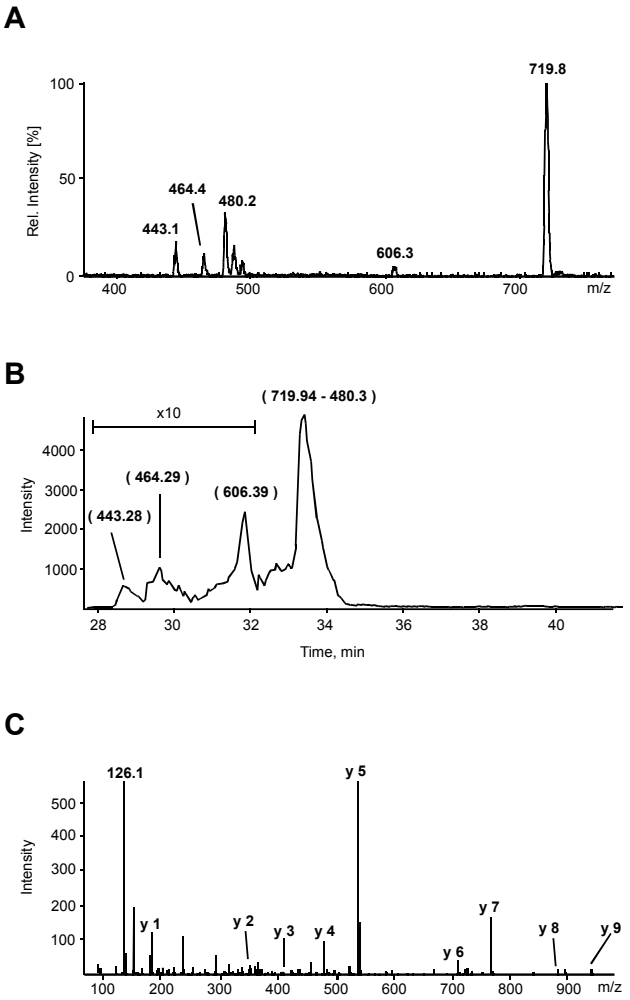


**Figure 5**

Application of the new precursor ion scan mode to the determination of the acetylation sites in Histone H4 acetylated in vitro with human MOF (males-absent on the first) protein. Panel A shows the triple quadrupole investigation of the in-gel digested protein, panel B the marker ion chromatogram of the Q-TOF investigation. Panel C shows the targeted tandem MS investigation of the 464.29 Th ion.

The observed peptides in (m/z, sequence) pairs were (443.28, GLGKGGAKR), (464.29, GLGKGGAKR), (606.35, GGKGLGKGGAKR), (719.94 - doubly charged, 480.3 - tripply charged, GKGGKGLGKGGAKR). The underlined lysines are acetylated. Both types of investigations gave the same acetylated peptides.

The peaks in the chromatogram are labelled with the m/z values of the automatically determined putative precursors. A comparison between panels A and B shows that there is a complete correspondence between the detected acetylated peptides. The observed peptides in (m/z, sequence) pairs were (443.28, GLGKGGAKR), (464.29, GLGKGGAKR), (606.35, GGKGLGKGGAKR), and (719.94, 480.3, GKGGKGLGKGGAKR). The measured intensity ratio between the marker and precursor ions corresponded to the number of acetylated lysines in the peptide. It was highest for the highly acetylated 719.94 Th peptide (1/0.91) and lowest for the 443.28 Th precursor (1/7.9).

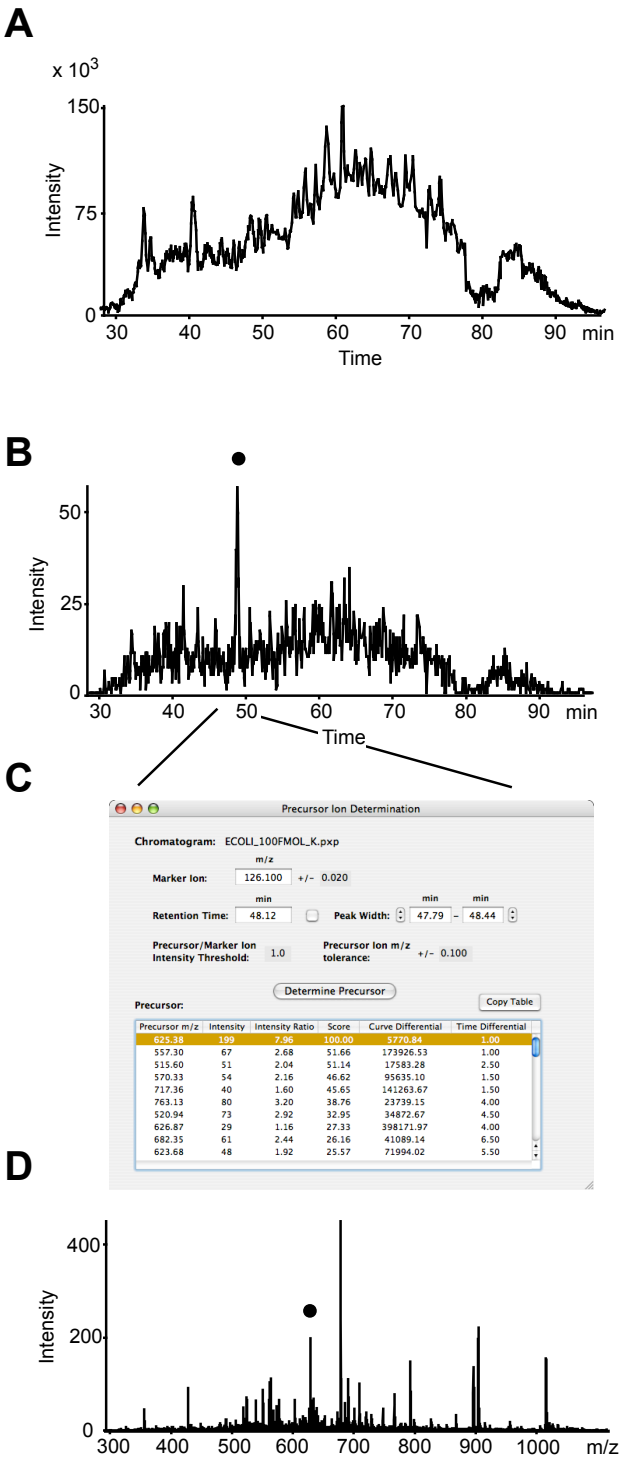


**Figure 6**

Test of the specificity of the precursor-ion-like investigation on the quadrupole time of flight instrument. 100 fmol of the acetylated peptide VLET[Acetyl-K]SLYVR was 'spiked' into a digest of all proteins between 20 kDa and 60 kDa of a total lysate of E.coli separated on a 1D gel. Panel A shows the total ion chromatogram of the low collision energy spectra, panel B the marker ion chromatogram of the high collision energy spectra, panel C the determination of the precursor ion and panel D the low collision energy spectrum at the time point of highest marker ion intensity. Even though there are many different peptide ions present, the correct precursor ion was determined as having the highest score.

In a second experiment we were able to sequence all but one of the peptides. The missing one with m/z = 443.28 Th had the lowest abundance, and was not found in the chromatographic run, possibly because of a short transient instability of the spray. All fragment spectra of the acetylated peptides were identified automatically using Mascot.

**Analysis of an acetylated peptide in a proteomic sample**

To test the ability of our scanning regime to detect and sequence an acetylated peptide on a background of peptides from many proteins, we mixed 100 fmol of the synthetic peptide VLET[Acetyl-K]SLYVR into a crude digest of a mixture of proteins. A total cell lysate from E. coli was separated on a short one-dimensional gel. All proteins between 20 kDa and 60 kDa were cut out and digested, and 100 fmol of the acetylated peptide was added to the digest. Figure 6 shows the result of the precursor-ion-like scanning experiment on our capillary HPLC–Q-TOF instrument. Panel A displays the total ion chromatogram of the intact molecular ions, panel B the chromatogram of the 126.1 Th marker ion in the elevated collision energy spectra. Panel C shows the result of the automatic determination of the acetylated peptide detected in the chromatographic peak of panel B. The correct ion (625.38 Th) scored highest when its elution profile was compared to the elution profile of the marker ion. Panel D shows the spectrum of the intact molecular masses at the time of maximum marker ion intensity. This spectrum demonstrates that it is possible to determine the correct precursor ion from a relatively complex spectrum. The acetylated peptide is marked. In a second targeted tandem MS investigation the acetylated peptide was sequenced successfully.

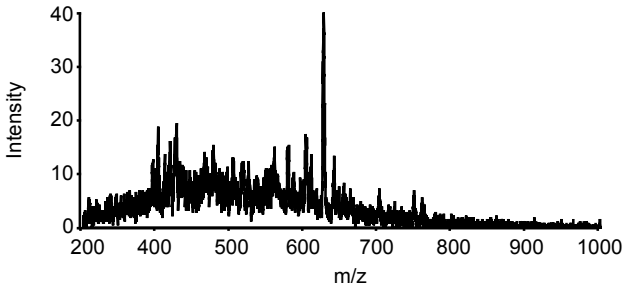


**Figure 7**
Precursor ion scan for acetylated lysines of the same sample shown in Figure 6 done on the API III triple quadrupole instrument. The acetylated peptide was detected with the same signal to noise ratio as with the precursor-ion-like scan on the quadrupole time of flight instrument.

Again it is interesting to note that a nano-electrospray-based precursor ion scan on the triple quadrupole instrument from the same sample generates a spectrum with a signal to noise ratio very similar to that of the precursor-ion-like scan on the quadrupole time of flight instrument (see figure 7). Technically it is much faster and easier to do the precursor ion scan on the triple quadrupole. However, the available information is then limited to the chosen precursor ion(s).

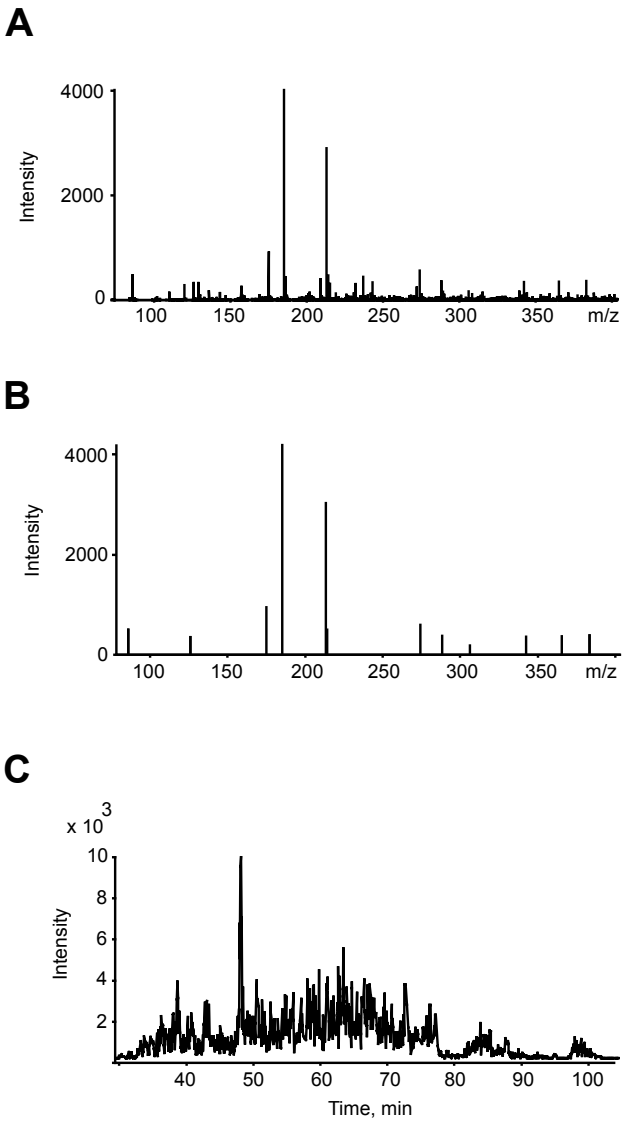


**Figure 8**
Detection of the acetylated peptide within all the elevated collision energy spectra using a pattern-based search. A pattern (panel B) can be generated with a known fragment spectrum of the acetylated peptide (panel A) that reflects the most abundant ions in each 100 Th-wide window. This pattern can be matched against the elevated collision energy spectra to find if the peptide was present in the sample. Panel C was generated from the same dataset used for figure 6. The acetylated peptide was detected with a very similar signal to noise ratio. The ion intensity of the chromatogram reflects at every time point the multiplication of all the intensities in the elevated collision energy spectra at the m/z values of fragment ions in the pattern spectrum. The peak at 48 minutes represents an elevated collision energy spectrum with overall maximal intensity at all these locations. This is the elution time of the acetylated peptide.

The ability to scan the data for different precursors can be used to do a pattern-based search. Figure 8 gives an example. The lower part of the fragment spectrum of the acetylated peptide was used to generate a peak pattern characteristic of the peptide. This pattern was matched against the higher collision energy spectra. We produce two spectra, one representing at each time-point the sum of all intensities at the locations of fragments in the patterned fragment spectrum, and a second representing their product. Figure 8 C shows the chromatogram from the multiplied intensities. It was indeed possible to detect the eluting peptide in a specific way. The peak in the chromatogram represents the time-point when all fragments of the pattern are present simultaneously. Once a chromatogram has been constructed we can use time course correlation to determine the underlying precursor. A pattern search does not give this kind of result every time: there still needs to be at least one peak in the pattern that are specific for the peptide against the background from other fragmented peptides. The difference from a precursor ion scan is that it is not necessary to know precisely which peak is the specific one,. Its absence from the elevated collision energy spectra when the sought peptide is not eluting from the column will reduce the calculated intensity in other parts of the chromatogram when a multiplicative correlation is used.

**Parallel detection of glycosylated and acetylated peptides**

One advantage of using the precursor ion scan mode on a quadrupole time of flight instrument is that the fragment spectra cover a wide range on the m/z scale. This allows us to interrogate the elevated energy spectra for several precursor ions simultaneously.

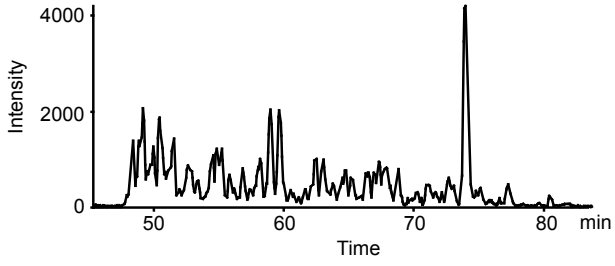


**Figure 9**

Base peak chromatogram of the low collision energy spectra of a digest of all proteins between 20 kDa and 60 kDa of an E.coli lysate 'spiked' with 100 fmol of an acetylated peptide and 250 fmol of an RNAseB digest.

To demonstrate this we 'spiked' the digest of all E.coli proteins between 20 kDa and 60 kDa with 100 fmol acetylated peptide and 250 fmol of RNAseB digest. The digest was analyzed on the capillary HPLC (Eksigent) using dynamic collision energy switching. Figure 9 shows the low energy base peak chromatogram. Figure 10 shows the precursor ion chromatograms of m/z = 126.1 Th for the acetylated peptide and m/z = 204.1 Th for glycosylated peptides. The acetylated peptide was easily detected and the true precursor determined. The second peak originates from a very abundant molecule (see figure 9) whose ion signal leaks into the precursor ion channel. For acetylated peptides the precursor ion to marker ion signal ratio was always between 4 and 8. For the second peak this ratio was 19 indicating a very inefficient generation of the fragment ion with the marker's ion mass. Upon targeted fragmentation this precursor did not yield any interpretable peptide sequence.

The chromatogram for the marker ion of glycosylated peptides is completely different from that of the acetylated peptide. This time, it was not possible to determine easily the m/z values of the precursor ions. There are four doubly charged glycosylated peptides eluting roughly at the same time, two pairs 162 Da apart (one hexose), m/z 846.4 Th, 927.4 Th and 874.9 Th, 955.9 Th. The mass difference between the two members of each pair could be explained by a reaction with our blocking reagent for cystein carbamindomethyl. There is no noticeable chromatographic separation between the two members of each group. There is a small, unresolved, difference in elution time between the two groups. Because of this lack of chromatographic separation it was not possible to determine the precursors' m/z unambiguously using their time course correlation with the ion signal of the 204.1 Da fragment. Detailed inspection of the intact molecular mass spectrum at the time of detection of the 204.1 Da fragment revealed the two groups of precursors ions by their characteristic separation of 162 Da. Targeted fragmentation during a second chromatographic run confirmed that these peptides were indeed glycosylated [20].

**Concluding remarks**

The precursor-ion-like scanning regime combines fragment and intact molecular mass information within one datastream by dynamically switching the collision energy between elevated and low values throughout an entire LC run, as proposed by Hoaglund-Hyzer and Bateman et al. [35, 36]. The data can be separated into two distinct LC-mass traces by computational means, one representing the intact molecular masses of molecules eluting from the LC column and a second containing all the fragment spectra generated with elevated collision energy settings.

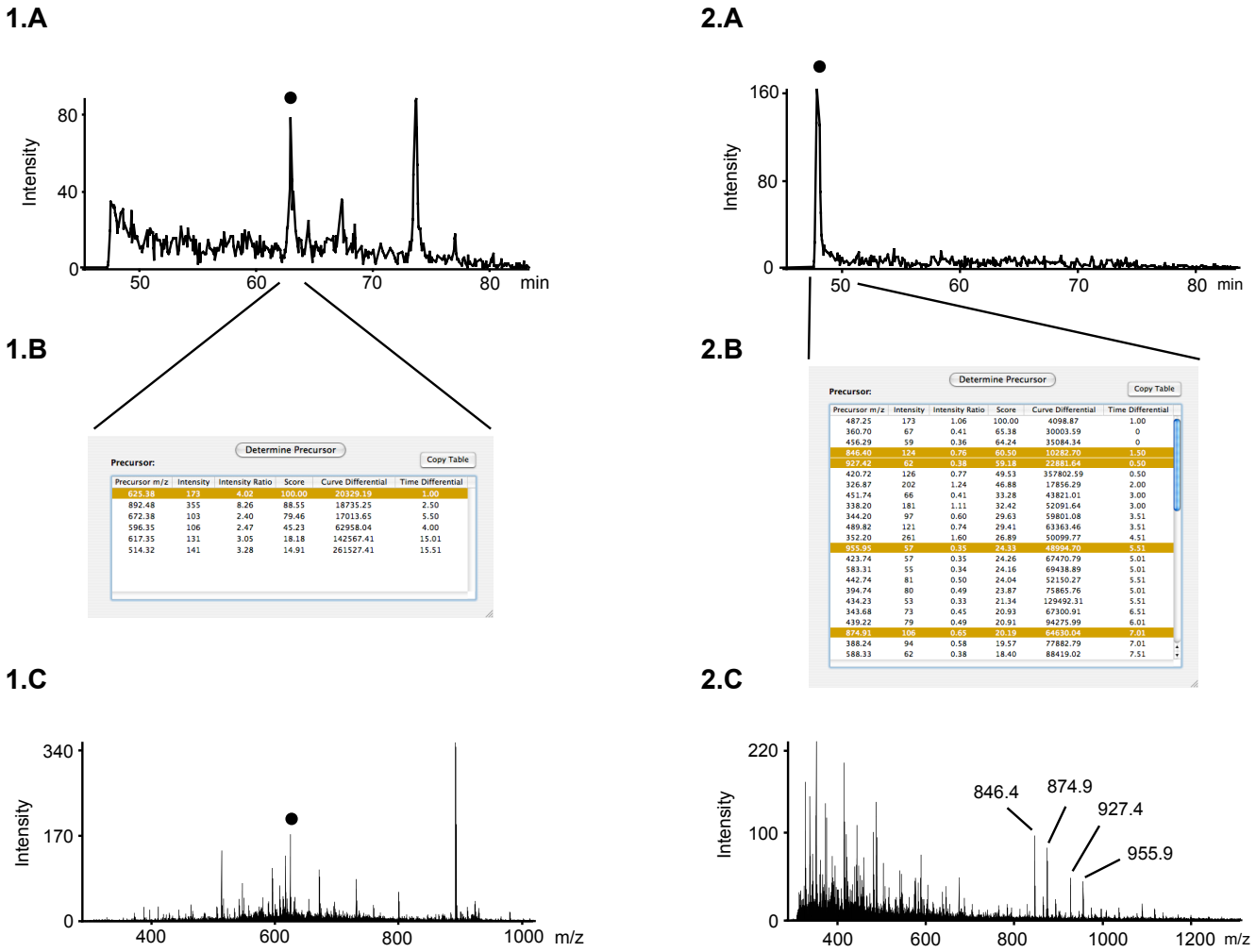


**Figure 10**

Precursor ion scans for acetylated lysines (126.1 Th, column 1) and glycosylated residues (204.1 Th, column 2) of the same dataset as shown in figure 9. The acetylated peptide was detected clearly (column 1, panel A) and the precursor ion determined with highest score (panel B, C). The second peak in the marker ion chromatogram is a minor signal of a major peak in the chromatogram in the 126.1 Da mass channel (see figure 9). The second column shows that the chromatogram for the marker ion of glycosylated peptides is completely different. The glycosylated peptides all elute at the start of the separation and are not chromatographically resolved. This is why the determination of the precursor ions is not straightforward using time course correlation. The true precursors are indicated in column 2, panel B. Only a careful evaluation of the low collision energy spectrum at this time-point revealed two pairs of doubly charged ions with the mass separation of a hexose group (162 Da) (m/z 846.4, 927.4 and 874.9, 955.9). Targeted sequencing confirmed the presence of the glucosylation.

The fragment spectra can be interrogated for any marker ion or combination of marker ions specific for a particular chemical structure. Using time correlation analysis it is possible to reduce the number of possible precursor ions for a specific marker ion to very few candidates, often only a single one, depending on the complexity of the sample [36]. These candidates can be fragmented in a second LC run by a targeted tandem-MS experiment. In this way, it is possible to implement a multiplexed, sensitive, and fast precursor ion scan experiment on a quadrupole time-of-flight machine, which is fully compatible with on-line chromatographic separation. The high precision of the time-of-flight mass spectrometer is available for the marker ion selection and the precursor mass determination. This scanning regime allows us to investigate complex proteomic peptide samples for multiple peptide modifications and analyze them by tandem-MS. When comparing this analysis regime with a nano-electrospray investigation on an API III triple quadrupole machine about the same sensitivity is obtained. Though the nano-electrospray investigation is more robust and much simpler to implement, the new scanning mode benefits from the higher mass accuracy of the time of flight measurement and covers many precursor ions simultaneously. We see two disadvantages of the LC–Q-TOF approach using dynamically changing collision energies: a relative uncertainty about the true precursor ion and the requirement for a second LC experiment to

fragment any peptides thought to have been modified. The sample complexity that can be covered is limited by the time axis resolution. The chromatographic resolution is too low to characterize many peptides with the same type of modification. This changes considerably if higher resolving chromatographic systems or ion drift devices are combined with mass spectrometers [37, 52]. But considering the high sensitivity and analytical value of the initial combined dataset, we accept these limitations.

This scanning regime will be useful for the analysis of secondary protein modifications in proteomic experiments and the analysis of complex secondary modification of individual proteins. It may also become significant for the targeted analysis of small molecules within complex samples of chemical and biological origin.

**References**


[1] Neubauer, G., Gottschalk, A., Fabrizio, P., Seraphin, B., et al., Proc. Natl. Acad. Sci. USA 1996, 94, 385 - 390.

[2] Grant, S. G. and Blackstock, W. P., J Neurosci 2001, 21, 8315-8318.

[3] Inada, T., Winstall, E., Tarun, S. Z., Jr., Yates, J. R., 3rd, et al., Rna 2002, 8, 948-958.

[4] Andersen, J. S., Lyon, C. E., Fox, A. H., Leung, A. K., et al., Curr Biol 2002, 12, 1-11.

[5] Carucci, D. J., Yates, J. R., 3rd and Florens, L., Int J Parasitol 2002, 32, 1539-1542.

[6] Lasonder, E., Ishihama, Y., Andersen, J. S., Vermunt, A. M., et al., Nature 2002, 419, 537-542.

[7] Bauer, A. and Kuster, B., Eur J Biochem 2003, 270, 570-578.

[8] Foster, L. J., De Hoog, C. L. and Mann, M., Proc Natl Acad Sci U S A 2003, 100, 5813-5818.

[9] Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., et al., Nature 2003, 426, 570-574.

[10] Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., et al., Nat Biotechnol 1999, 17, 1030-1032.

[11] Puig, O., Caspary, F., Rigaut, G., Rutz, B., et al., Methods 2001, 24, 218-229.

[12] Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., et al., Nature 2002, 415, 180-183.

[13] Gavin, A. C., Bosche, M., Krause, R., Grandi, P., et al., Nature 2002, 415, 141-147.

[14] Hazbun, T. R., Malmstrom, L., Anderson, S., Graczyk, B. J., et al., Mol Cell 2003, 12, 1353-1365.

[15] Graumann, J., Dunipace, L. A., Seol, J. H., McDonald, W. H., et al., Mol Cell Proteomics 2003.

[16] Forler, D., Kocher, T., Rode, M., Gentzel, M., et al., Nat Biotechnol 2003, 21, 89-92.

[17] Guillaume, E., Zimmermann, C., Burkhard, P. R., Hochstrasser, D. F. and Sanchez, J. C., Proteomics 2003, 3, 1495-1499.

[18] Hochstrasser, D., Sanchez, J. C., Binz, P. A., Bienvenut, W. and Appel, R. D., Novartis Found Symp 2000, 229, 33-38.

[19] Lin, D., Tabb, D. L. and Yates, J. R., 3rd, Biochim Biophys Acta 2003, 1646, 1-10.

[20] Wilm, M., Neubauer, G. and Mann, M., Anal Chem 1996, 68, 527-533.

[21] Neubauer, G. and Mann, M., Analytical Chemistry 1999, 71, 235 - 242.

[22] Chernushevich, I., Eur. J. Mass Spectrom. 2000, 4, 471-479.

[23] Steen, H., Kuster, B. and Mann, M., J Mass Spectrom 2001, 36, 782-790.

[24] Steen, H., Kuster, B., Fernandez, M., Pandey, A. and Mann, M., Anal Chem 2001, 73, 1440-1448.

[25] Steen, H., Kuster, B., Fernandez, M., Pandey, A. and Mann, M., J Biol Chem 2002, 277, 1031-1039.

[26] Steen, H. and Mann, M., J Am Soc Mass Spectrom 2002, 13, 996-1003.

[27] Steen, H., Pandey, A., Andersen, J. S. and Mann, M., Sci STKE (http://stke.sciencemag.org/) 2002, 2002, PL16.

[28] Steen, H. and Mann, M., Anal Chem 2002, 74, 6230-6236.

[29] Mann, M. and Jensen, O. N., Nat Biotechnol 2003, 21, 255-261.

[30] Ferguson, P. L. and Smith, R. D., Annu Rev Biophys Biomol Struct 2003, 32, 399-424.

[31] Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., et al., Nat Biotechnol 1999, 17, 994-999.

[32] Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., et al., Nat Biotechnol 2002, 20, 301-305.

[33] Nuhse, T. S., Stensballe, A., Jensen, O. N. and Peck, S. C., Mol Cell Proteomics 2003, 2, 1234-1243.

[34] Kocher, T., Allmaier, G. and Wilm, M., J Mass Spectrom 2003, 38, 131-137.

[35] Bateman, R. H., Carruthers, R., Hoyes, J. B., Jones, C., et al., J Am Soc Mass Spectrom 2002, 13, 792-803.

[36] Hoaglund-Hyzer, C. S., Li, J. and Clemmer, D. E., Anal Chem 2000, 72, 2737-2740.

[37] Plumb, R., Castro-Perez, J., Granger, J., Beattie, I., et al., Rapid Commun Mass Spectrom 2004, 18, 2331-2337.

[38] Castro-Perez, J., Plumb, R., Granger, J. H., Beattie, I., et al., Rapid Commun Mass Spectrom 2005, 19, 843-848.

[39] Ekroos, K., Chernushevich, I. V., Simons, K. and Shevchenko, A., Anal Chem 2002, 74, 941-949.

[40] Turner, B. M., Bioessays 2000, 22, 836-845.

[41] Chen, H., Tini, M. and Evans, R. M., Curr Opin Cell Biol 2001, 13, 218-224.

[42] Bannister, A. J., Miska, E. A., Gorlich, D. and Kouzarides, T., Curr Biol 2000, 10, 467-470.

[43] Kim, J. Y., Kim, K. W., Kwon, H. J., Lee, D. W. and Yoo, J. S., Anal Chem 2002, 74, 5443-5449.

[44] Akhtar, A., Zink, D. and Becker, P. B., Nature 2000, 407, 405-409.

[45] Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. M. and Richmond, T. J., J Mol Biol 1997, 272, 301-311.

[46] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M., Anal. Chem. 1996, 68, 850-858.

[47] Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., et al., Nature 1996, 379, 466-469.

[48] Wilm, M. and Mann, M., Anal Chem 1996, 68, 1-8.

[49] Pedrioli, P. G., Eng, J. K., Hubley, R., Vogelzang, M., et al., Nat Biotechnol 2004, 22, 1459-1466.

[50] Gentzel, M., Kocher, T., Ponnusamy, S. and Wilm, M., Proteomics 2003, 3, 1597-1610.

[51] Rappsilber, J., Friesen, W. J., Paushkin, S., Dreyfuss, G. and Mann, M., Anal Chem 2003, 75, 3107-3114.

[52] Liu, X., Plasencia, M., Ragg, S., Valentine, S. J. and Clemmer, D. E., Brief Funct Genomic Proteomic 2004, 3, 177-186.

[53] Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., et al., Nat Biotechnol 1999, 17, 676-682.

[54] Wolters, D. A., Washburn, M. P. and Yates, J. R., 3rd, Anal Chem 2001, 73, 5683-5690.

[55] Akhtar, A. and Becker, P. B., Mol Cell 2000, 5, 367-375.

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