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Post-source Decay and Delayed Extraction in Matrix-assisted Laser Desorption/Ionization-Reflectron Time-of-Flight Mass Spectrometry. Are There Trade-offs?

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By the incorporation of delayed extraction (DE) into matrix-assisted laser desorption/ionization time-of-flight mass spectrometry a dramatic improvement of performance with respect to sensitivity, mass resolution and mass accuracy of precursor ions up to ~10 kDa has been achieved. Since DE reduces collisional in-source activation to a large extent, the rate of subsequent metastable decay is considerably reduced. Results are presented which demonstrate that under DE the loss of total post-source decay (PSD) fragment ion yield can be as large as one order of magnitude but that, in terms of sensitivity, part of this loss is balanced by a better S/N ratio which results from a significantly improved mass resolution of the PSD fragment ions ($M/\Delta M$ up to 1800 compared with $M/\Delta M = 200$–500 under prompt extraction). While this compensatory effect is true for the middle to high mass range of PSD fragment ions, it gradually vanishes towards the low mass end of the PSD mass scale where, in the case of linear peptides some important information (immonium ions) is lost. It appears, however, that in the majority of practical PSD work, DE improves the qualty of the PSD spectra and that high energy collisional post-source activation can compensate for the occasional loss of analytical information.

By utilizing the technique of delayed extraction (DE) in matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry, mass resolution and mass accuracy can be substantially improved over the 0.1%–0.01% reached in MALDI-TOF with linear or reflectron TOF (ReTOF) instruments.[1–6]

The rationale of the approach originates from the early work of Wiley and McLaren.[7] They devised a simple technique which, by means of pulsed extraction in a split acceleration field geometry, corrects ion flight times for distributions of either initial ion velocity or ion position (but not for both at the same time). Fortunately enough, in MALDI spatial and velocity distributions of freely expanding particles are correlated to each other which allows one to find conditions for a simultaneous velocity and spatial focusing. While DE parameters (delay time, pulsed field strength) which must be applied for ultimate improvements are mass dependent, broader m/z ranges of several kDa can be covered at slightly compromised performances.[5]

It has been recognized that in MALDI particles are ejected from the solid state sample in a jet-like manner[8–11] forming a forward peaking cone of neutral and charged particles which expand with a common velocity distribution.[10,11] In this almost uniform velocity plume, kinetic energies are roughly proportional to the particle's mass. If ions contained in such a plume are promptly accelerated by a strong continuous electrostatic field they will undergo multiple collisions with neutral particles and, hence, will acquire not only a further dispersion of kinetic energy but also a net energy deficit with respect to the nominal acceleration voltage. At the same time, the loss of correlation between ion position, ion velocity and ion energy destroys the option for time focusing by a classical reflectron. This scenario also explains why, in ReTOF instruments, peak widths strongly depend on the absolute intensity of the ion signal or, in other words, why, at laser irradiances even slightly above the threshold, mass resolution rapidly degrades.

Most of these undesirable conditions can be either prevented or corrected for by application of a split acceleration stage in which the acceleration voltage is turned on with a time delay (0.1–0.5 μs) after the laser pulse. It has been shown that, under DE conditions, reflectron instruments with very long flight paths can achieve mass resolution $M/\Delta M$ in excess of 10 000 (FWHM) for MALDI middle mass analytes (e.g. peptides up to 5 kDa).[5] This leads to a corresponding improvement of mass accuracy and precision to better than 10 ppm without the need to operate the laser near threshold for ion production.[5] In addition, it was felt that (1) the better S/N ratio, (2) the reduction of unimolecular decay rates and of chemical noise and (3) the suppression of unwanted matrix signals (by applying a weak retarding field prior to extraction) taken together not only result in a better quality of spectra but further expand the sensitivity limits of the method. Such beneficial effects of DE have been principally confirmed in other types of 'difficult' analytes such as oligonucleotides[6] or carotenoids[12] which otherwise undergo excessive metastable fragmentation.

While, for the purpose of molecular mass determination, metastable decay could generally be considered as an undesirable phenomenon, it has been found that in ReTOF mass spectrometry it can easily be exploited for obtaining structural information on various types of precursors. For time-of-flight mass spectrometry the so-called post-source decay (PSD) fragment ion mass analysis represents an equivalent to conventional (low energy collisionally-activated decomposition) tandem MS techniques.[13–18] The

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practical utility of this approach, e.g. for peptide sequencing in enzymatic digests, has been demonstrated[19] and the list of *successful* applications to other classes of analytes is rapidly expanding.[12, 20–22] The question if and to what extent DE will adversely (or favorably) affect the option for PSD analysis in MALDI ReTOF mass spectrometry in a given analytical situation has, however, not been systematically investigated so far. In the present study, we have tried to determine if trade-offs exist with respect to key instrumental performances under conditions of analytical routine work.

EXPERIMENTAL

Mass spectrometry

The two TOF instruments, employed a linear TOF(MS1) and a reflectron TOF(MS2) were both built in-house. Their principal characteristics have been extensively described elsewhere.[12, 16, 23] In both instruments, a two-stage ion source is used in conjunction with a high-voltage switch which provides for delayed extraction by supplying a HV step function to the first acceleration electrode. The second acceleration electrode is grounded. Three stabilized HV supplies are connected to the source in a way schematically shown in Fig. 1.

Delayed extraction (DE). Prior to turning on the extraction field, a small variable retarding field (50–250 Vcm^{-1}) between the sample and first acceleration electrode was set by means of a potentiometric voltage divider connecting the output of HV1 to ground. This allows one to selectively suppress matrix ions and other small prompt fragments. The HV-switch (Fa.Behlke, Frankfurt, FRG, Model HTS 15 PGSM) had nominal rise- and fall-times of 30 ns and was controlled by a digital delay gate generator (Stanford Research Systems, Sunnyvale, CA, USA, Model DG535). The switch could be operated in both polarities such as to allow DE experiments on both positive and negative precursor ions. Distances between sample and extraction electrodes (E1) were $d1 = 5$ mm, $d2 = 12$ mm in TOF(MS1) (gridded electrodes) and $d1 = 3$ mm, $d2 = 5$ mm (open aperture electrodes) in TOF(MS2). The length of the field-free drift path was 110 cm in TOF(MS1) and 205 cm in TOF(MS2). Nominal ion kinetic energy in TOF(MS1) was 20 keV and 10 keV in TOF(MS2). Standard delay time used throughout the present study was 250 ns (including 150 ns dead time of the delay gate generator) if not otherwise indicated. For the voltage settings during DE experiments see also Fig. 1.

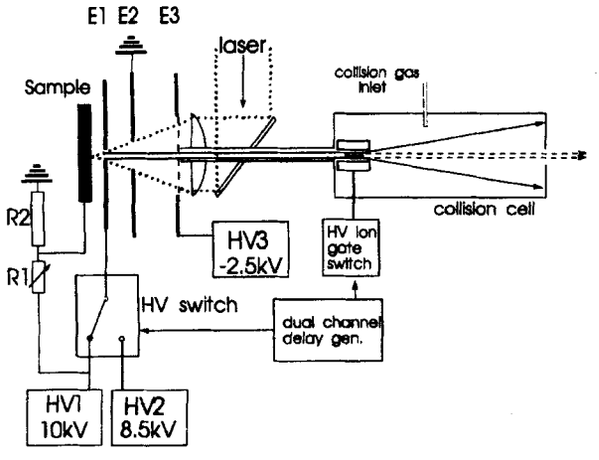

Figure 1. Block diagram of the electronics employed for delayed extraction and scheme of the MALDI ion source used in TOF(MS2). See text for further explanation.

Prompt extraction (PE). For PE experiments the HV switch was disconnected and the first extraction electrode (E1) directly biased by the output of HV2 (8.5 kV).

In both instruments large area (72 mm diameter of active area) dual microchannel plate (MCP) detectors were employed. Output signals were directly fed into a digital scope (LeCroy 9450A) where they were AD converted (8 bit) at sampling rates of either 100, 200 or 400 MS/s. Raw spectra were processed and stored in a 486PC by means of our Ulisses software (Chips at Work, Bonn, FRG) including a recent extension for processing PSD spectra (Ulisses, version 7.31, © Bernhard Spengler).

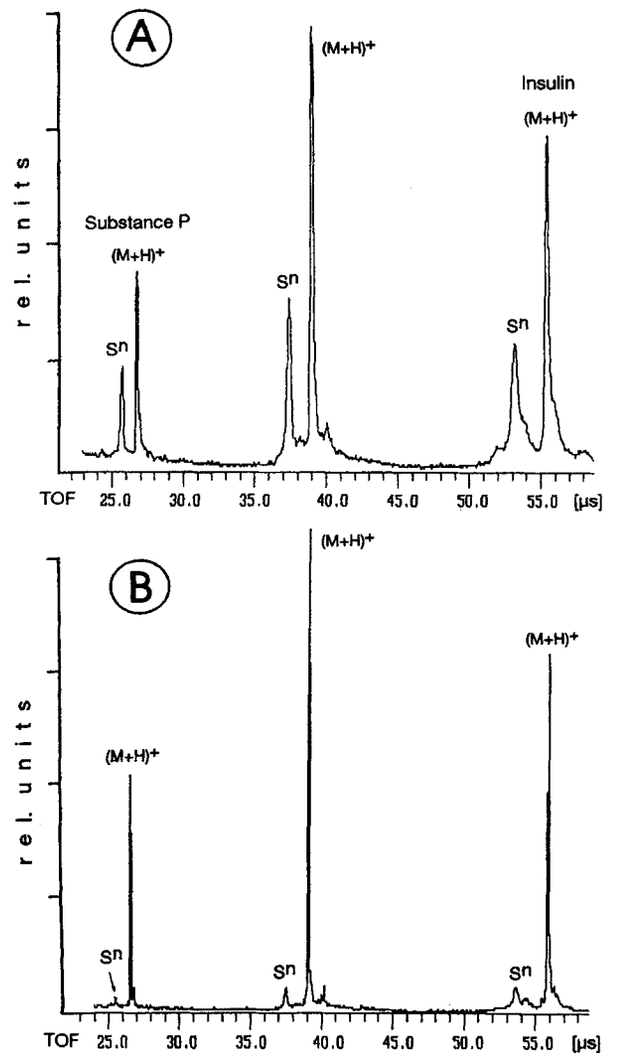

Figure 2. MALDI spectra recorded using the linear TOF(MS1) instrument with a retarding field active in front of the detector under the conditions of (A) prompt and (B) delayed extraction. The sample is a mixture of 3 peptides (substance P, melittin, insulin at 1 pmol/µL each in DHB). Signals labeled S^n represent all neutral fragments arising from metastable decay in the field-free drift path, those labeled $(M+H)^+$ are the unfragmented (charged) quasimolecular ions. Note that under DE (panel B) metastable decay is reduced by 1–2 orders of magnitude (see also Table 1). Spectra are sum of 50 laser shots each.

Sample preparation

Sample preparation followed a standard protocol. Analytes were dissolved in either distilled water or acetone (carotenoids) so as to contain final concentrations of ~1–10 pmol/L. Stock solutions of matrix were prepared by dissolving (1) 10 g/L 3,5-dihydroxybenzoic acid (DHB) in 1:1 acetonitrile/water, or (2) by dissolving to saturation 4-hydroxy-α-cyanocinnamic acid (HCCA) in either 1:1 H_2O/ethanol or acetone. Peptides were purchased from Sigma or synthesized (courtesy Dr. Koehrer, Biologisch-Medizinisches Forschungszentrum der Universität Düsseldorf). About 1–2 μL of analyte solution was mixed with the same volume of matrix solution on the sample holder and immediately air dried by a gentle air stream from a hair dryer.

RESULTS AND DISCUSSION

General aspects of PSD fragment ion formation and analysis after delayed extraction

In order to obtain a quantitative estimate as to what extent unimolecular decay rates are reduced by DE in MALDI we had to define some reference conditions. It has been shown in earlier work of this laboratory,[23] that, in MALDI-TOF-MS, metastable decay can vary over a rather wide range depending on instrumental conditions such as (1) laser irradiance, (2) acceleration field strength, (3) residual gas pressure, (4) ion flight time in the first field-free drift path, but also on sample parameters such as (5) the composition of the matrix[24] and (6) the chemical nature of the analyte. For the purpose of this study we have arbitrarily chosen the following standard conditions: (1) laser irradiance: 1.3–1.4× threshold irradiance for ion detection, (2) initial acceleration field strength: 5 kV cm^{-1}, (3) residual gas pressure: $<4\times10^{-7}$ mbar and, (4) DHB as matrix (if not otherwise stated).

In a first series of measurements the global rate of PSD fragmentation was determined in the linear TOF(MS1) equipped with a retarding field in front of the detector. With this field activated, all neutral fragments arising from metastable fragmentation show up in a peak preceeding the signal of the (retarded) intact molecular ions. The intensity ratio of the (unretarded) neutrals over the (retarded) charged analyte ions is a good global parameter for the extent of metastable decay which has occurred during post-source flight in the field-free drift path. Figure 2 shows the case of 3 test peptides (substance P, melittin and insulin) under conditions of either prompt or delayed extraction. It is immediately evident that, in all 3 test peptides, DE reduces the total amount of fragmentation by at least one order of magnitude but that some quantitative differences exist between the chosen precursors. Note in particular the case of substance P where the signal of neutrals barely exceeds noise level under DE conditions.

Another approach to estimation of global rates of metastable decay can be conducted in a ReTOF-MS if a gridded two-stage reflectron is employed as in the present

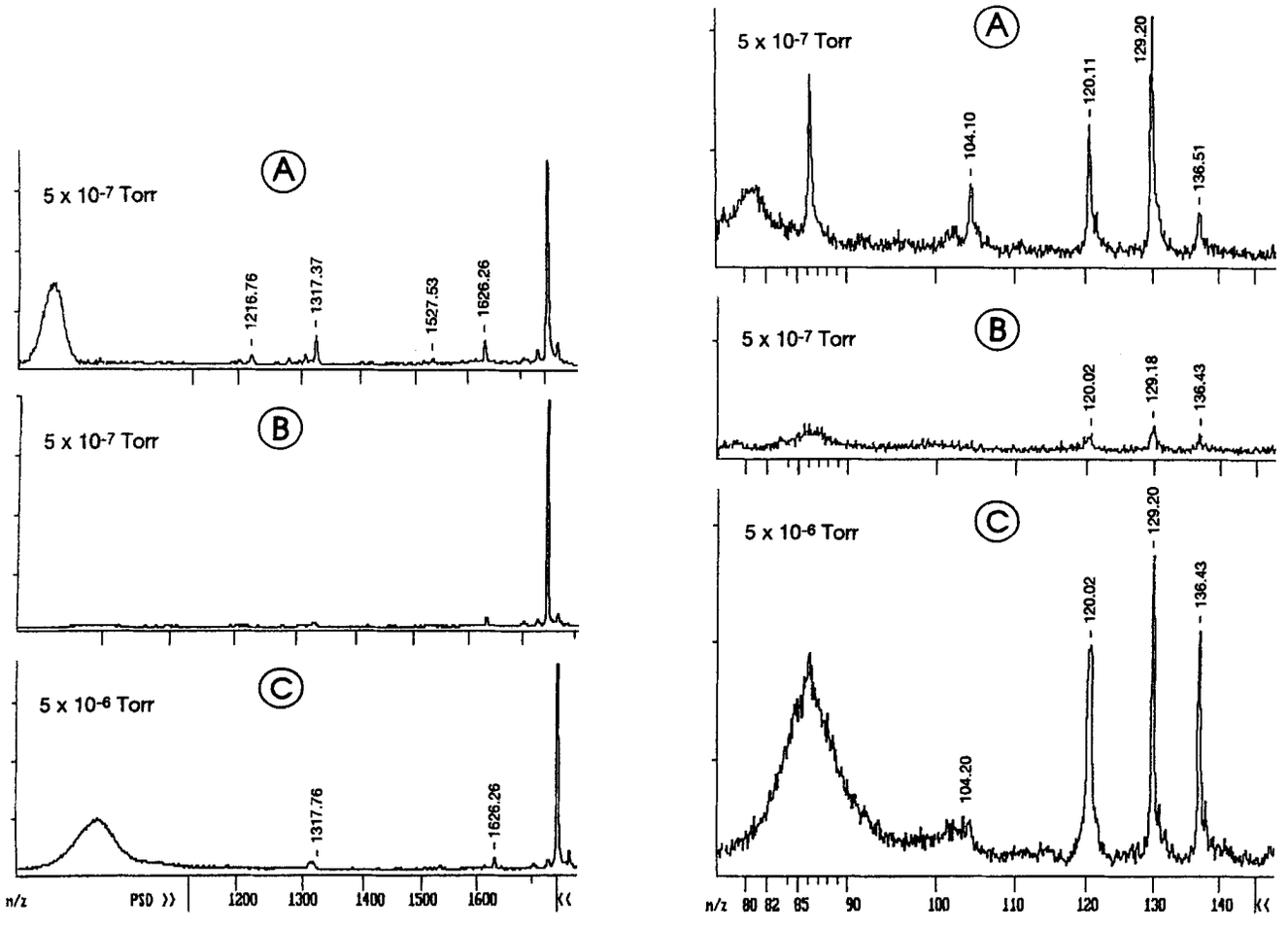

Figure 3. High mass (left hand row) and low mass (right hand row) PSD mass window of α-endorphin recorded under PE (a) and DE (b, c) conditions in ReTOF(MS2) instrument. Note that by an increase of the residual pressure from 5×10^{-7} to 5×10^{-6} Torr the loss of PSD ion yield induced by DE can be counteracted, especially in the low mass range. See text for further explanation.

TOF(MS2). If such a reflectron is operated at voltages set for precursor ion recording, all fragment ions below about $0.85 \times m_p$ (m_p=precursor ion mass) will fail to penetrate into the second stage of the reflectron and, thus, will be mirrored in the first stage with only a small mass-dependent flight-time dispersion. These fragment ions show up at the left edge of the PSD mass window as a broad common signal. Its area can be taken as a good quantitative estimate for the total amount of PSD fragments formed. Figure 3(a) shows such an experiment on the peptide α-endorphin. Again, it is obvious that the total PSD ion yield decreases under DE by more than one order of magnitude. Figure 3 also demonstrates that an increase of the residual gas pressure in the instrument from 5×10^{-7} to 5×10^{-6} Torr restores metastable decay to a large extent as a result of high energy collisions which occur in the field-free drift path. This induces a preferred formation of small fragment ions (see Fig. 3).

The dramatic reduction of metastable decay rates under DE has also been observed in other groups of compounds such as oligonucleotides, oligosaccharides, phospholipids, cerebrosides and various conjugates such as fatty acid esters of carotenoids.[12] In some of these rather labile compounds, in which an excessive metastable decay prevented recording of acceptable parent-ion signals under conditions of prompt extraction, DE usually produced a dramatic improvement in parent-ion signal intensity. It was interesting to note that the relative improvement in signal intensity was much more pronounced in protonated than in sodiated precursors. This is in line with the notion that sodiated precursors are less prone to metastable decay than their protonated counterparts. A similar gain of parent-ion S/N ratio was observed with negative analyte ions (e.g. phospholipids or oligonucleotides).

The mass resolution (FWHM) of parent-ion signals which could routinely be attained under standard conditions in our ReTOF instrument increased from $M/\Delta M$ 1500–3000 (PE) to $M/\Delta M$ 4500–6000 (DE) in the m/z 1000–5000 mass range. This is about half the ultimate resolution which, in a smaller mass window, could be obtained by fine tuning the critical parameters. While, in the PE mode, mass resolution rapidly degrades with increasing laser irradiance, it becomes remarkably tolerant of higher irradiances under conditions of DE. Since, in practical PSD fragment-ion mass analysis, an optimal yield and energy uptake of the precursor ion can rarely be achieved near the threshold for ion formation, rather high irradiances (1.5–3 times threshold intensity) must usually be employed. The concomitant degradation of the precursor-ion mass resolution also limits the attainable PSD fragment ion resolution which, in practical work under PE conditions, usually does not exceed the 200–500 $M/\Delta M$ range (see Figs 4 and 5).

It appears that, with the improved mass resolution of MALDI precursor ions and its enhanced operational stability under DE, the PSD fragment-ion mass resolution is also substantially improved. Since PSD fragment-ion mass

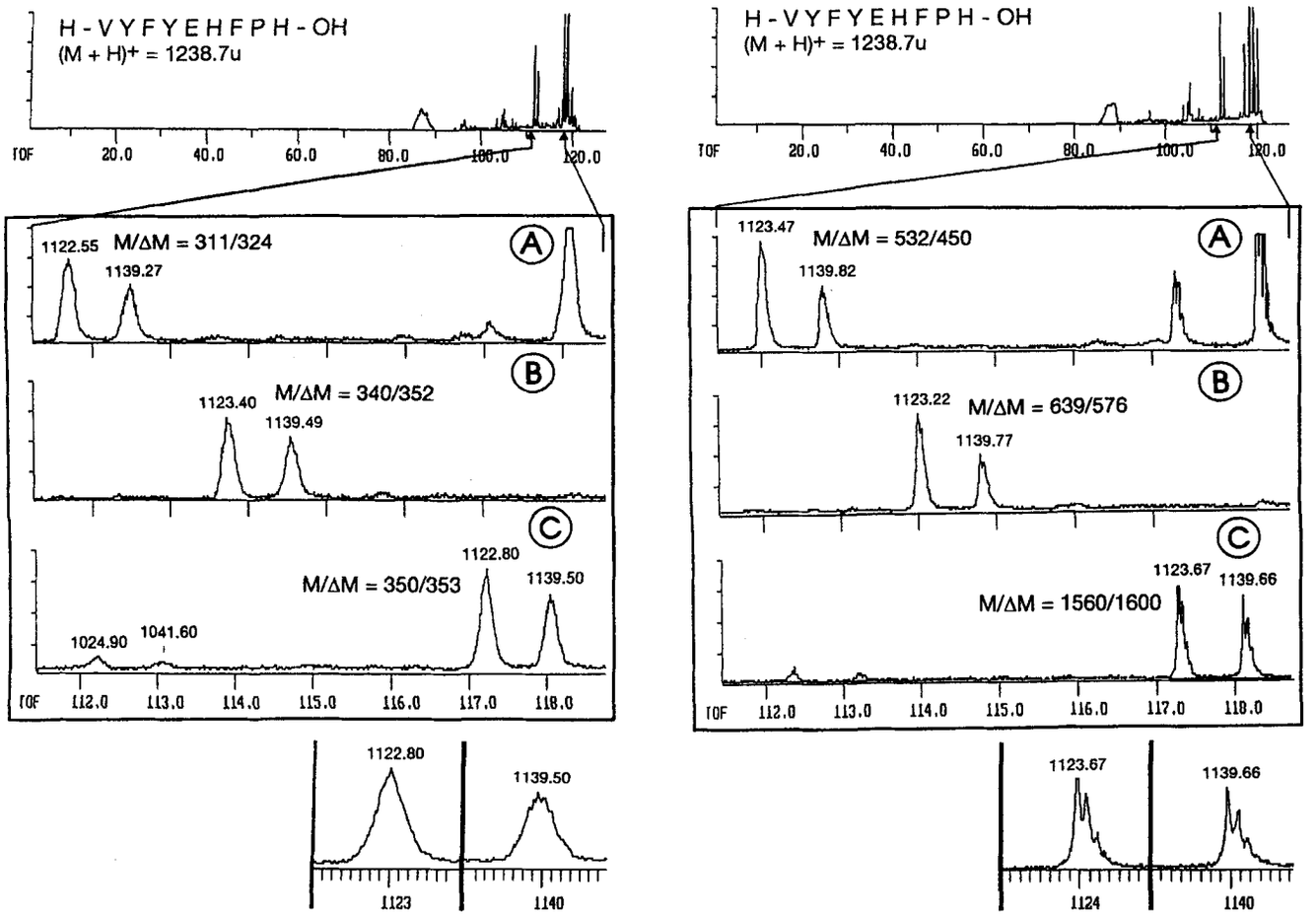

Figure 4. Expanded high mass PSD windows recorded from a synthetic peptide under PE (left panel) and DE (right panel) conditions. The PSD ion signals shown represent the $[b_8+Na/K+OH]^+$ fragments (m_{calc}=1123.65 and 1139.67 Da respectively). Windows labelled A, B and C show this PSD ion pair recorded at reflectron voltages of 10 800 V, 10 400 V and 9800 V respectively. Note that under DE (1) mass resolution of PSD ions is generally improved but that (2) a more dramatic improvement occurs within a rather narrow section of the PSD-window centered around the nominal flight time of the precursor ion (see also Fig. 5).

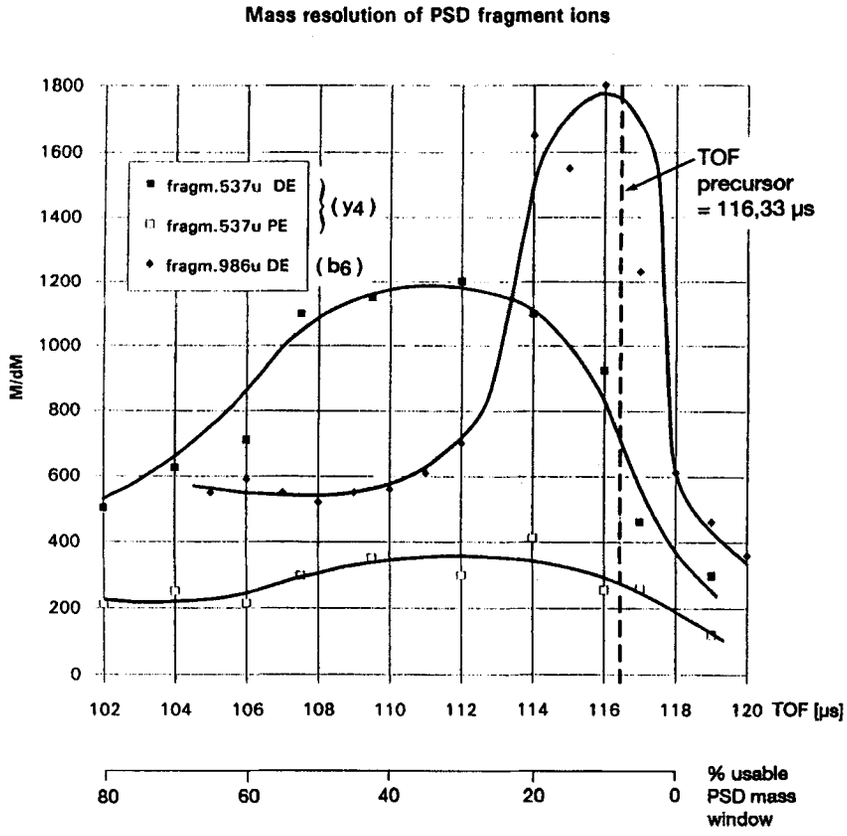

Figure 5. Mass resolution of selected PSD fragment ions (precursor: H–VYFYEHFPH–OH) as a function of the flight time (function of the reflectron voltages) in the ReTOF(MS1) instrument. Note the sharp increase of mass resolution around the nominal flight time of the precursor ion.

resolution under DE becomes roughly proportional to mass (for a more specific discussion of this phenomenon see below) it is mainly the middle to high mass range which profits from these conditions. Here, PSD mass resolution reached $M/\Delta M = 1000–2000$ in many instances, such that the mass patterns of isotope distributions or of previously skewed adjacent masses were clearly discernable (see Fig. 4). Needless to say, this also translates into an improved PSD mass accuracy provided that (1) operational parameters (e.g. reflector voltages) are reasonably stable (short and long term drifts <100 ppm) and (2) that appropriate calibration protocols (internal or external) are applied. We will address the problem of PSD mass accuracy in a separate paper. For the sake of this work we may state that the average PSD fragment mass error presently achievable under our DE conditions amounts to ±0.12–0.25 u. This means that a PSD fragment ion at m/z 1000 can be mass assigned with 120–250 ppm accuracy (which is about by a factor of 2–3 better than previously achieved under PE conditions) whereas for an m/z 100 fragment accuracy would remain in the 0.12–0.25% range (which is basically good enough for most practical purposes).

It is worth noting that in the DE mode the peaks of PSD fragment ions tend to adopt a shape which can be predicted if the effect of kinetic energy release on the spread of ion arrival times is taken into account.[25] In contrast to the quasi-Lorentzian profile of the precursor ion signal, PSD signals under DE look in fact more like the 'Eiffel tower', i.e. out of a rather wide base they form needle-like tops with symmetrical flanks of steadily increasing slope. This shape allows the secure discrimination of adjacent peaks even on a 5–10% valley basis and, thus, possibly, the achievement of better mass accuracies than by relying on conventional peak centroiding.

While an improved mass resolution of PSD fragment ions is certainly a highly welcome achievement, it must be noted, however, that the improved performance is restricted to a rather small section (20–50%) of a given usable PSD mass window (see Figs 4 and 5). Thus, about twice the number of mass segments (~20–24 instead of 12–14) must be recorded in sequence to obtain a full PSD mass spectrum at optimal mass resolution. In addition, since the improvement of mass resolution decreases towards the low mass end ($<m/z$ 200) of the PSD mass scale a drop of S/N ratio and, hence, a loss of sensitivity in this mass region is another part of the price to be paid.

Practical PSD fragment ion analysis under conditions of delayed extraction

Peptides. On the basis of the above results one may predict that under conditions of DE the yield of metastable fragments available for MALDI-PSD fragment ion mass analysis in a ReTOF instrument could possibly become too low in critical cases for recording PSD mass spectra over the full mass range. Moreover, since collisional in-source activation of analyte ions is drastically reduced by DE one might speculate not only about quantitative but also about qualitative differences with respect to those fragmentation processes which are energetically demanding.

Figure 6 exemplifies that, under delayed extraction, the PSD signal intensities of a chosen peptide (in this case H–CDPGYIGSR–OH) are substantially reduced and that some of the smaller fragment ions have fully disappeared while others are barely above noise level. In this particular case, obviously a good deal of analytical information (e.g.

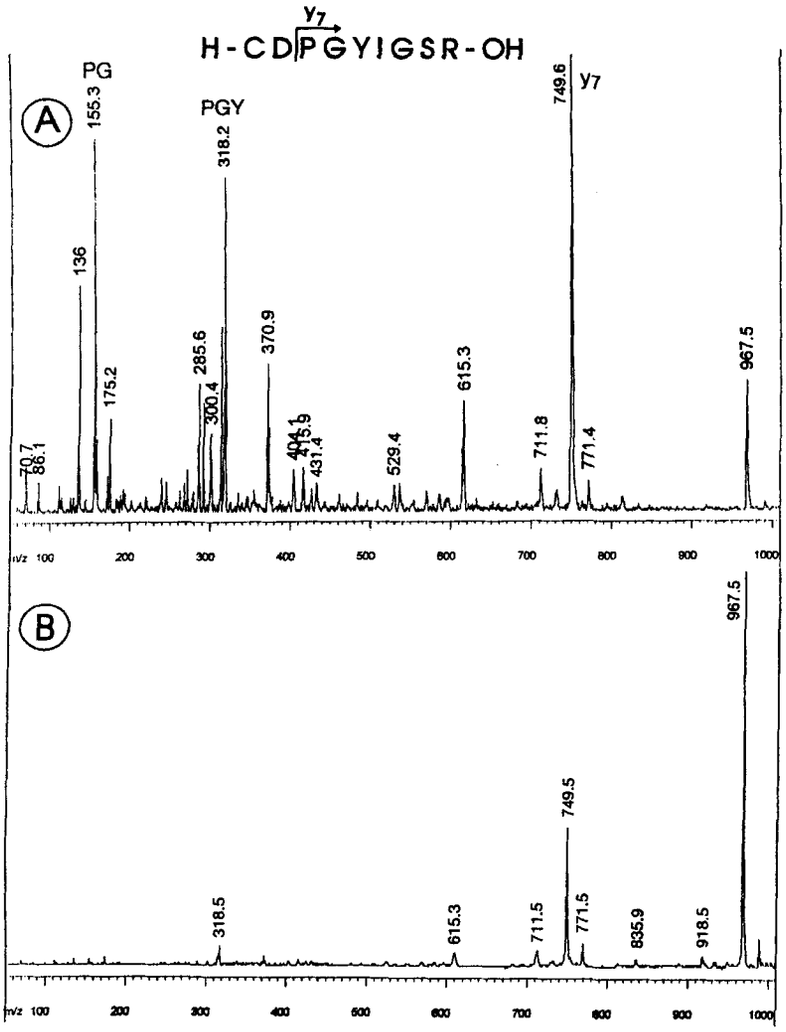

Figure 6. Full range PSD mass spectra (compilation of 14 segments with the sum of 50 laser shots each) of a synthetic peptide under PE (a) and DE (b) conditions. Note the complete loss of information at the low mass end of the PSD spectrum recorded under DE conditions.

immonium ions) contained in the PSD-spectrum of promptly extracted precursors is lost under the conditions of DE. This case is particularly remarkable because this peptide exhibits an extraordinary high fragmentation rate under conditions of PE. It further demonstrates that cleavages which are energetically favorable, such as that of the Asp–Pro amide-bond, become more dominant under DE while energetically demanding cleavages are suppressed.

Althogh a few other cases like this have been observed amongst the 36 model peptides comparatively investigated so far, the majority of peptide analytes, however, behaved less critically at least as long as enough precursor ions were produced from a given sample. In Fig. 7, complete PSD mass spectra of a synthetic peptide H–HPFHEYFYV–OH $[(M+H)^+_m = 1238.6$ Da] are presented. Here, the overall yield of PSD fragment ions, after changing from PE to DE conditions, was reduced to roughly 50%. Again, small fragment ions were more affected than the more massive fragments but are far from being fully lost as in the previous case. The expanded views of PSD mass windows shown in Fig. 8 nicely demonstrate how, in the middle to high mass range, the loss of absolute PSD ion yield under DE is compensated for by an improved S/N ratio and how previously skewed mass signals are resolved (e.g. the *m/z* 648 signal in Fig. 8).

Although, from the analytical point of view, the concomitant gain in the accuracy of PSD mass measurement in the middle to high mass range is an important advantage for the task of sequencing it must be balanced against a certain loss of information at the low mass end. This can become particularly critical when analyzing peptide precursors with a molecular weight above 2000 Da in the subpicomole range (enzymatic digests).

In real-world peptide samples, such as tryptic digests, the general trade-offs between DE and PE have been more difficult to establish. On the one hand, parent ion mass measurements and subsequent PSD confirmation of highly unstable precursors (such as phosphorylated or lipidated peptides) were largely facilitated by the DE mode. On the other hand, many stable low-intensity precursors *did not* produce enough PSD ions even to allow the definition of a sequence tag. In particular, the loss of information on small fragments (residue-specific immonium ions or proline directed internal fragments) made peptide characterization sometimes difficult or even impossible. On the one hand, the cleavage of energetically favorable amide bonds (Asp–Pro, Asp–X, X–Pro, Glu–X, see also Fig. 3 and previous section) could be recorded even in precursor peptides as large as 3500 Da and could be mass assigned to such an accuracy that, within the known sequence of the target

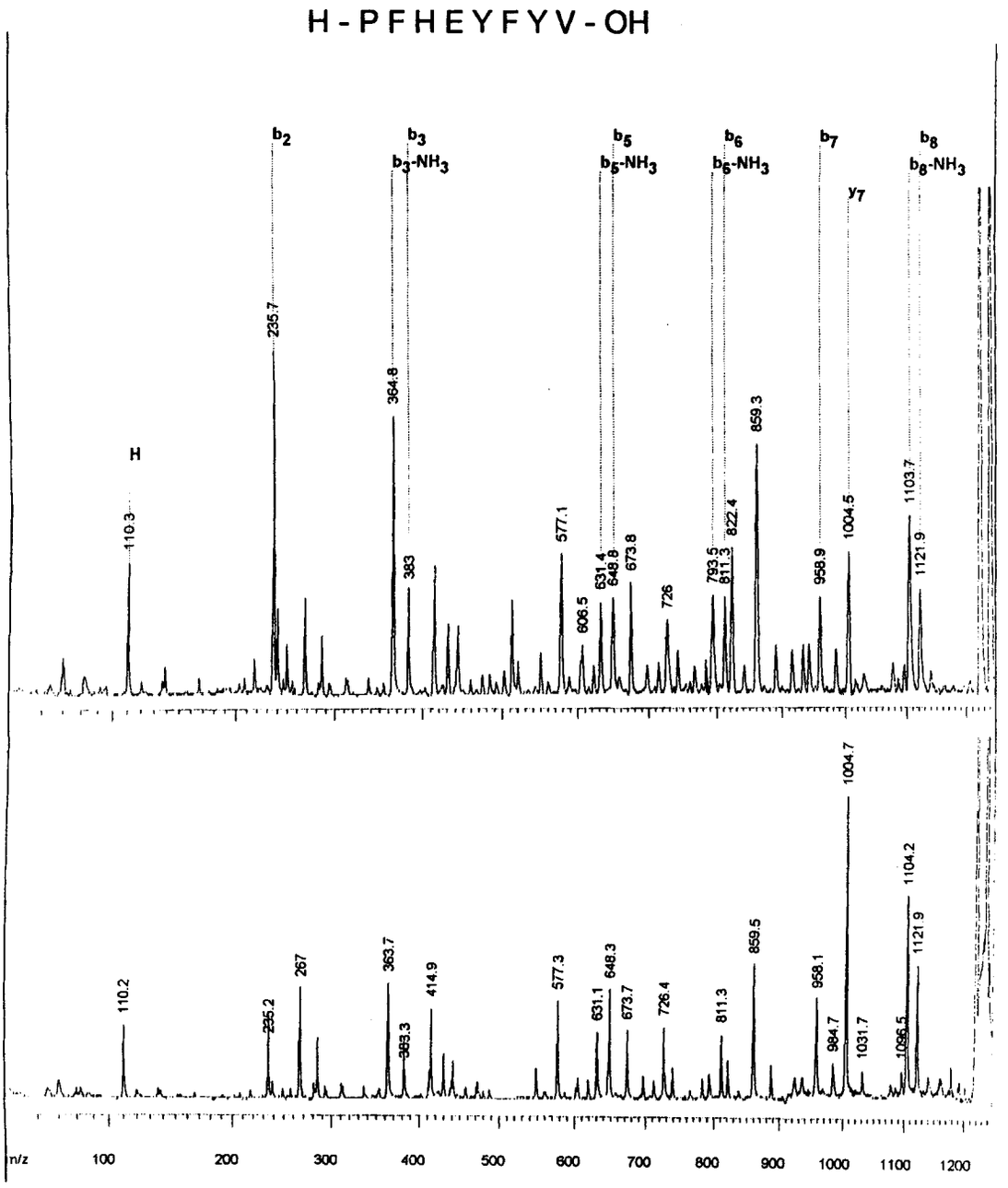

Figure 7. Full range PSD mass spectra (compilation of 14 segments with the sum of 50 laser shots each) recorded from a synthetic peptide under PE (top) and DE (bottom) conditions. Only moderate reduction of PSD fragment ion intensities under DE is seen (cf. Fig. 6).

protein, the digest peptide could unambiguously be identified. On the other hand, whenever the task was a full or near complete sequencing (unknown target protein, substituted or tagged AA) the higher PSD ion yields obtainable under PE became mandatory.

In practice, the following strategies have been found useful and applicable with not too much additional effort. (1) Consecutive application of DE for high-precision precursor ion mass measurement and of PE for subsequent PSD fragment ion mass analysis, (2) exchanging the matrix for one which favors PSD fragmentation rates (e.g. HCCA for DHB), (3) introducing post-source collisional activation. Such post-source collisional activation can be most easily achieved by admitting into the mass spectrometer either ambient air or a chosen collision gas via a leak valve. As already shown in Fig. 3, increasing the residual gas pressure by roughly one order of magnitude from 5×10^{-7} to 5×10^{-6} mbar induces a dramatic increase of metastable decay with a preferential incidence of smaller fragments. This is in line with earlier observations that post-source collisional activation in MALDI-PSD analysis of peptides leads to the occurrence of energetically demanding cleavages such as the formation of d_n or w_n fragments.[16, 26]

Other compounds (oligonucleotides, oligosaccharides, carotenoid fatty acid esters). The observations reported above for peptides basically also apply to other compounds. In Fig. 9 the example of an oligosaccharide (6-*N*-diaminobutylcyclodextrin) demonstrates once more that under the conditions of DE the loss of total fragment-ion yield (by about one order of magnitude) is largely balanced by an improved S/N ratio and that the loss of qualitative information at the low mass end of the PSD mass scale can

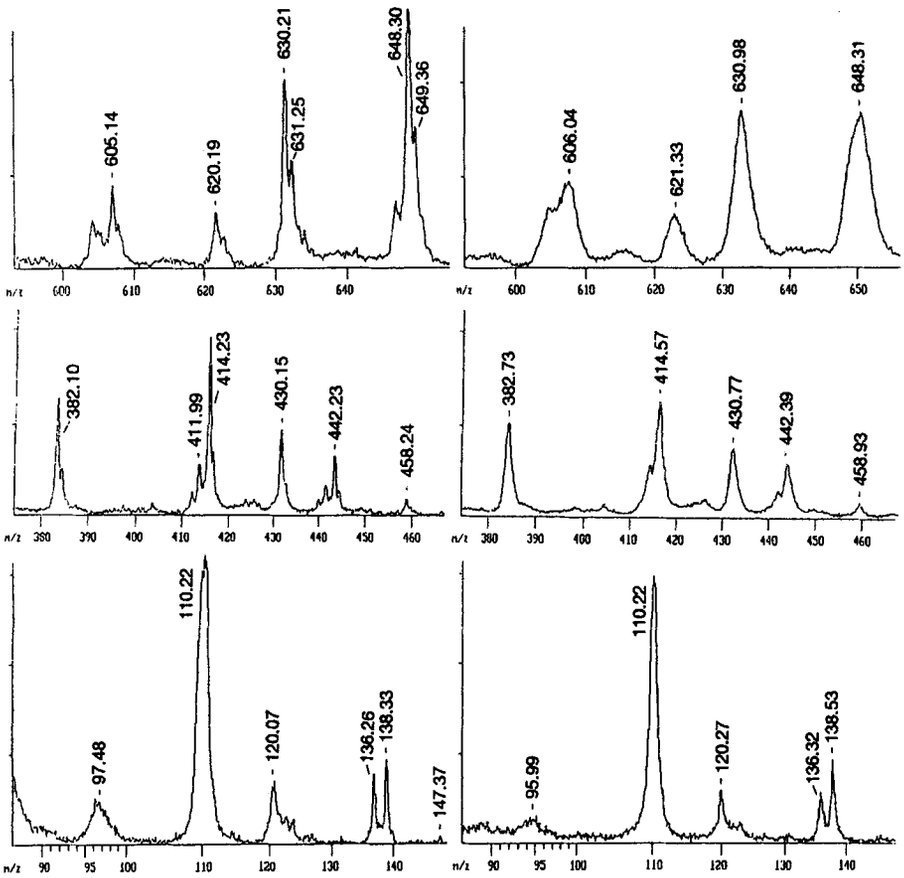

Figure 8. Expanded views of various PSD mass windows recorded from the same synthetic peptide as in Fig. 7. The left panel displays spectra (sum of 50 laser shots each) recorded under conditions of DE while the right panel shows the corresponding spectra (on the same *y*-scale) obtained under PE. See text for further information.

be compensated for by post-source collisional activation.

In an extended MALDI-PSD study on carotenoids and their fatty acid esters[23] it was found that, under the conditions of DE, the PSD ion yield and incidence of diagnostically unspecific low-mass fragments was reduced in favor of the diagnostically more relevant middle to high mass fragment ions. Some of these compounds (e.g. gluthathione-S-S-CoA) were so labile that parent ions could hardly be recorded under conditions of prompt extraction.

CONCLUSIONS AND OUTLOOK

Post-source decay fragment-ion mass analysis has largely extended the analytical capabilities of MALDI-TOF-MS so as to make this technique a valuable approach for MS based structural elucidation of large analytes, especially peptides. Under the usual mode of MALDI-TOF-MS operation, PSD is a unimolecular process related to the internal energy which MALDI ions take up via collisional events during the early phase of ion acceleration. With the recent advent of DE, many of the undesirable effects of in-source collisions on mass resolution and mass accuracy can be overcome to a large extent. With the reduction of internal ion energy, however, unimolecular decay occurring during post-source flight also reduces significantly. In the present work a decrease of the global PSD ion yield by 1–2 orders of magnitude has been observed after a transition from PE to DE under otherwise unchanged standard conditions.

Under these circumstances the options for PSD fragmentation mass analysis must necessarily involve key instrumental parameters such as sensitivity and, most probably, selectivity. It has become clear, however, that in the majority of analytes investigated so far a considerably improved mass resolution of the PSD fragment ions balances the loss of absolute ion yield such that the S/N ratio remains nearly unchanged except for the low mass PSD region where occasionally a loss of information occurs.

In addition, the gain in PSD mass accuracy (by a factor of 2–3) and the capacity to separate previously unresolved peaks makes structural elucidation (especially peptide sequencing) much more unambiguous. In the rather exceptional cases where low PSD ion yields prevented structural elucidation PSD 'boosting' options (e.g. in-flight collisional activation or replacing DHB by HCCA) could be invoked without greatly compromising the beneficial effects of DE. In essence, DE improves the quality of PSD spectra and, in practical work, the positive aspects of this feature clearly outweigh the negative.

It appears that PSD fragment-ion flight-time dispersion (ΔTOF_{KER}) due to kinetic energy release is becoming the limiting factor for PSD mass resolution under DE. Since the mean ΔTOF_{KER} value scales with $(KER/m_{fr})^{1/2}$ one can understand why, under conditions of DE, PSD fragments in the middle and high-mass region profit much more from the improved mass resolution of their precursors than do low-mass fragments.

Although the actual state of performance achieved in MALDI-ReTOF-PSD fragment ion mass analysis already match most of the requirements of analytical MS/MS work

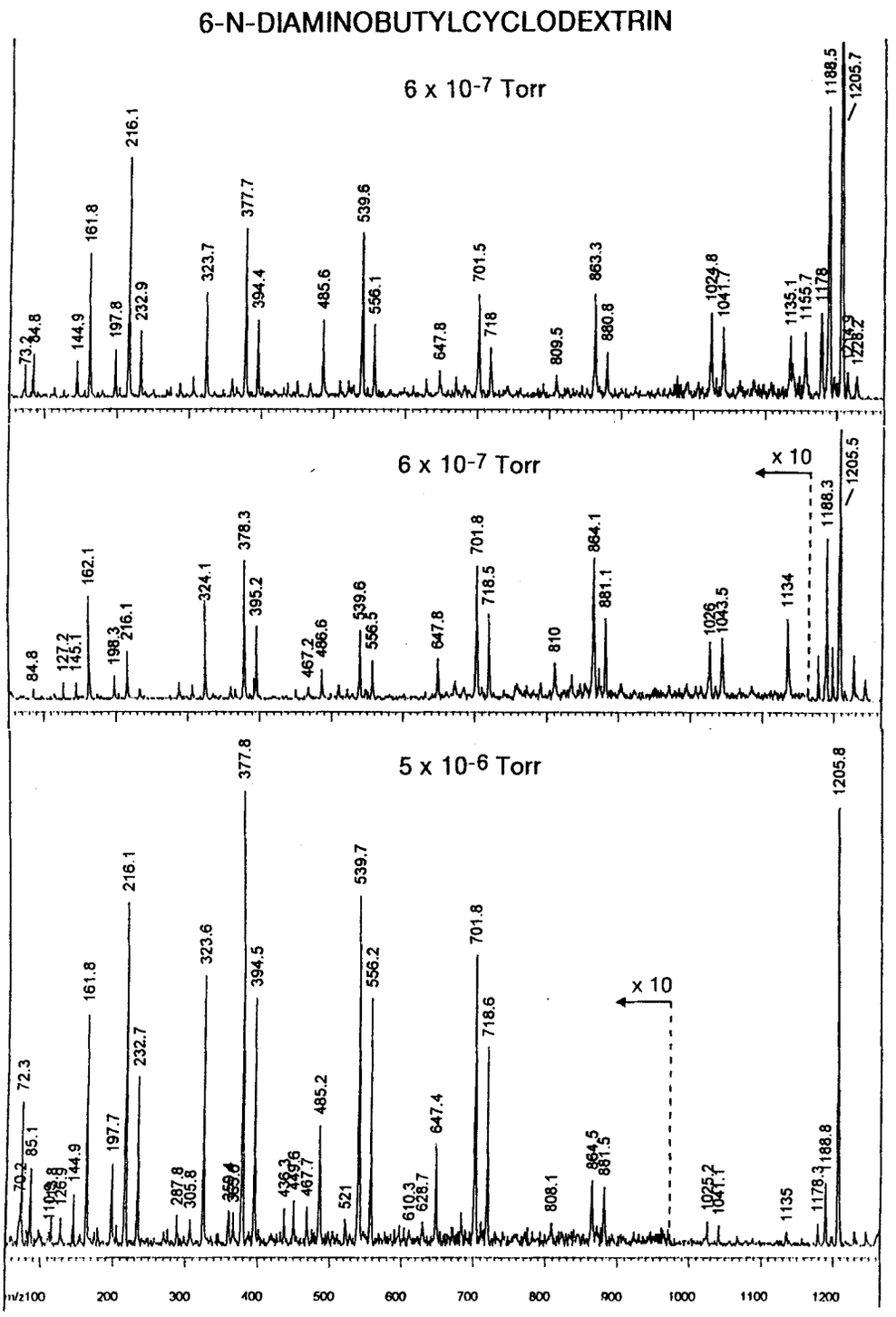

Figure 9. Full range PSD mass spectra (compilation of 14 mass segments with the sum of 30 laser shots each) of a cyclic oligosaccharide (3 pmol sample loaded in DHB) obtained under PE (top), DE (middle) and DE in combination with reduced residual pressure (bottom).

one might, nonetheless, look for possible further improvements. It appears that any attempt to bring PSD mass resolution beyond the actual limitations imposed by KER must rely on the application of some additional time lag focusing principles.

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Note added in proof

After the submission of this manuscript a paper by Stahl-Zeng, Hillenkamp and Karas appeared in *Eur. Mass. Spectrom.* **2**, 23–32 (1996) entitled 'Metastable fragment-ion analysis in a reflectron instrument with a gridless ion mirror'. This work deals mainly with an attempt to optimize a gridless reflectron for the conditions of PSD fragment ion mass analysis. In a short paragraph the authors also address the aspect of PSD ion yield and detectability under conditions of delayed extraction with the following statement: 'It was somewhat surprising that the delayed extraction did not change the fragment spectra substantially, neither qualitatively nor quantitatively for a variety of tested peptides and glucans. Even though the overall yield of the fragment ions is expected to decrease somewhat under delayed extraction conditions this appears to be more than compensated for by an improved signal-to-noise ratio and possibly a more efficient detection efficiency and/or generation of sequence specific ions'. We think that there is no principle discrepancy with the present results especially if one takes into account that instrumental factors such as ion optical effects (which are notably critical in the case of the 'vision-2000' instrument) may fold with the basic phenomena to a rather large extent. This point is also considered by the authors themselves. In the present study we have taken some precautions to avoid or at least minimize such uncontrollable effects either by eliminating ion optical effects at all (strictly gridded field borders in conjunction with large detector areas as in MS1) or by keeping the ratios of open aperture diameters to field lengths as small as possible (MS2).