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Mass Spectrometric Analysis of Combinatorial Peptide Libraries Derived from the Tandem Repeat Unit of MUC2 Mucin

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Abstract: Four 19-member synthetic peptide libraries, based on the TX^1TX^2T epitope motif of the mucin-2 gastrointestinal glycoprotein (MUC2) and ranging in peptide length from dipeptides to 15-mers (XT, TXT, TQTXT and KVTPTPTPTGTQTXT), were synthesized by combinatorial solid phase peptide synthesis using the portioning-mixing combinatorial approach, and analysed by electrospray ionization mass spectrometry at different (1000–10 000) resolutions. Most of the components of the individual libraries could be easily identified in a single-stage molecular mass screening experiment. The resolving power of the instrument becomes an important factor above 800–1000 Da molecular mass, when predominantly multiply charged molecular ions are formed. Approaches to the identification of isobars (glutamine/lysine), isomers (leucine/isoleucine) and sequence variations by tandem mass spectrometry, and/or by high-performance liquid chromatography-mass spectrometry are outlined. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: combinatorial chemistry; HPLC-MS; peptide library; mass spectrometry; mucin-2 glycoprotein; portioning-mixing; tandem mass spectrometry

INTRODUCTION

Combinatorial chemistry [1,2] plays a major role in drug discovery and lead compound research. Automated synthetic and high-throughput screening methods make it possible to examine thousands of compounds and to select the biologically most effective one. The portioning-mixing combinatorial approach developed by Furka *et al.* [1] for peptide libraries was adopted for the synthesis of various types of organic compounds on solid phase and in solution as well [3–7]. Several analytical methods have been developed for the separation and the structural characterization of combinatorial mixtures and parallel compound libraries, including HPLC [8,9], CE [10], NMR [11–13] and mass spectrometry [3,4,14,15]. In the analysis of combinatorial mixtures, the use of mass spectrometry is the most widespread and its application is well described in recent reviews and research papers

Abbreviations: CID, collision induced dissociation; MUC2, mucin-2 glycoprotein; Q-TOF, quadrupole-time-of-flight; TES, triethylsilane.

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[3,4,16–19]. For peptide libraries, matrix-assisted laser desorption/ionization (MALDI) [20] and electrospray ionization (ESI) mass spectrometry [21,22] lead molecular ion production with high efficiency. Especially when ESI or MALDI is applied on high-resolution instruments, such as on a FT-ICR [3,23–26], the mass spectrometry based analysis can yield very useful information for the synthetic chemist. Single-stage analysis generally gives molecular mass information on the single components and thus provides molecular mass distribution also useful for semi-quantitative analysis. Tandem mass spectrometry (MS/MS) provides more structural information as demonstrated in the case of naturally occurring complex peptide mixtures [27,28].

In the case of peptide libraries, in which the amino acids vary only in one or a limited number of positions, several factors hinder the identification of the components. In an ideal case each peptide would yield a single, well-resolved molecular ion in the mass spectrum, peak intensity being proportional to concentration. However, this occurs very rarely, possibly in the case of libraries with a small number of components having the similar physicochemical properties. Peak overlapping is the most often experienced problem especially in a complex mixture. Isomers, e.g. leucine and isoleucine containing peptides, and positional isomers, having the same elemental composition cannot be distinguished by mass measurement only. Tandem mass spectrometry (usually high energy collision induced dissociation (CID) and/or multistage fragmentation) [29,30], or separation combined mass spectrometry, like HPLC-MS, may help in the identification of these species. In the case of isobar but not isomer peptides (e.g. glutamine vs lysine containing peptides), the small difference in the molecular mass may be used to distinguish them. This requires very high resolution (at 1000 Da molecular mass ca. 30 000 resolution is needed). Another problem arises for peptides having a molecular mass difference of only 1 Da (e.g. L/N, N/D or Q/E), later defined as ^{13}C-isobars. The molecular ion of one peptide and the ^{13}C isotope peak of the other one has the same nominal mass. If the peak corresponding to the lower mass peptide has a much higher abundance, it may not be possible to identify the peptide with higher mass. As before, these components may be resolved at high resolution — but the required resolution is even higher than in the case of the Q/K pair. A different type of problem is that the sensitivity of various components of a mixture is often different. Sensitivity is influenced by several factors, such as proton affinity, surface activity, etc. Relative sensitivity may depend on the concentration and the complexity of the mixture as well. Due to the different response factors and suppression effects, semi-quantitation of peptide libraries based on a simple mass measurement is not always straightforward.

To overcome the difficulties discussed above, special mass spectrometric techniques, such as high resolution FT-ICR [23–26,31–33] or ion-mobility/time-of-flight (TOF) mass spectrometry [34,35], have been utilized for the characterization of peptide libraries. FT-ICR and ion-mobility TOF mass spectrometry can differentiate successfully isobars and isomers, respectively. In the case of large libraries, (>100 compounds), detailed investigation of all components, however, is not always feasible. In such cases approximate comparison of the measured and computer-reconstructed theoretical mass spectra has led to useful information [36, 37]. These very powerful techniques, however, are relatively seldom used in practical applications, partly due to their high cost and complexity.

The objective of the present study was to assess the applicability of simple commonly used mass spectrometric techniques for the analysis of peptide libraries. To illustrate the analytical capabilities and limitations of these methods, four peptide libraries based on the TX^{1}TX^{2}T epitope motif of the mucin-2 gastrointestinal glycoprotein (MUC2) were produced by combinatorial solid phase peptide synthesis using the portioning-mixing combinatorial approach [1]. These libraries range in peptide length from XT-dipeptides to KVTPTPTPTGTQTXT (K12X) 15-mers, and contain 19 components with all proteinogenic amino acids (except cysteine), in position X. Mucins are high molecular weight glycoproteins with a molecular mass of more than 200 000 Da. The mucin-2 glycoprotein has been shown to be underglycosylated or overexpressed in the case of colon cancer, and therefore it is considered as a marker protein for cancer [38]. MUC2 is built up of tandem repeat units corresponding to the ^{1}PTTTPITTTTTVTPTPTPTGTQT^{23} sequence. From this repetitive sequence, the TX^{1}TX^{2}T motif was identified previously as the minimal epitope by the protein-specific monoclonal antibody mAb [39,40]. Applying a combinatorial approach, it was demonstrated that the sub-library containing Q in position X^1 has the highest affinity to mAb 994 [39,41,42].

METHODS AND MATERIALS

Material

Boc-Thr(Bzl) resin (capacity: 0.68 mmol/g) was prepared using Boc-Thr(Bzl) and Bio-Beads S-X1 chloromethylated polystyrene resin containing 1% divinyl benzene (200–400 mesh, 1.26 mmol Cl/g; Bio-Rad Laboratories, Richmond, CA, USA) by anhydrous KF [43]. N^{α}-Boc-protected amino acid derivatives with appropriate side chain blocking were purchased from Sigma-Aldrich (Budapest, Hungary).

Fmoc-Thr(^{t}Bu) resin (capacity: 0.6 mmol/g) was prepared from *p*-benzyloxy-benzylalcohol resin containing 1 % divinyl benzene (Wang resin, 200–400 mesh, ~1 mmol/g, Fluka AG, Buchs, Switzerland). Fmoc-Thr(^{t}Bu) was attached to the resin by DMAP and DCC [44]. N^{α}-Fmoc-protected amino acid derivatives with appropriate side chain blocking were purchased from Fluka AG (Buchs, Switzerland) or from Sigma-Aldrich (Schnelldorf, Germany).

Reagents (acetic acid, DIC, DIEA, DMAP, HF, HOBt, *p*-cresol, piperidine, TES, TFMSA, TFA) were analytical grade products of Fluka AG (Buchs, Switzerland). Solvents (ACN, DCM, DMF, diethyl and *tert*-butyl ether) were products of Reanal (Budapest, Hungary). Synthesis was performed in 19 plastic tubes equipped with teflon frits using a vacuum manifold apparatus (Shimadzu Scientific Research Inc., Tokyo, Japan). The HF apparatus was a product of the Peptide Institute, Inc. (Minoh-Shi, Osaka, Japan).

Combinatorial Peptide Synthesis

The XT, TXT and TQTXT libraries were synthesized by the portioning-mixing method [1] using Boc/Bzl chemistry [41]. Briefly, after deprotection of 19 × 34 μmol (50 mg) Boc-Thr(Bzl)-resin with TFA/DCM (1 : 2, v/v), the resin samples were washed (DCM, MeOH, DCM, MeOH and DCM) and neutralized by DIEA/DCM (1 : 9, v/v). After appropriate washing (DCM) one of the 19 Boc-amino acid derivatives was coupled to the resin sample in 5-fold molar excess using the DIC/HOBt method in DCM/DMF (2 : 1, v/v). The coupling was monitored by ninhydrin [45] or isatin [46] assays. The protected dipeptidyl resin samples suspended in DCM were united and thoroughly mixed in a common tube. In the case of the library XT, the peptides were cleaved from the resin after the removal of N^{α}-Boc protecting group by TFA/DCM. In the other three cases the deprotected dipeptidyl resin was coupled with Boc-Thr(Bzl) in DCM/DMF (2 : 1, v/v) mixture using the same coupling method. For the TXT library the removal of Boc groups was followed by HF cleavage of the peptide mixture.

To obtain the TQTXT library Boc-Gln and Boc-Thr(Bzl) was coupled subsequently to the tripeptidyl TXT resin mixture. After the removal of N^{α}-Boc groups the peptides were cleaved from the resin using anhydrous HF containing 5% (m/V) *p*-cresol, at 0 °C for 1 h. HF was removed *in vacuo* and the crude peptides were precipitated with diethyl ether, filtered and extracted with 10% aqueous acetic acid. The solution was diluted with distilled water and freeze-dried.

The KVTPTPTPTGTQTXT (K12X) library was synthesized by Fmoc/^{t}Bu chemistry. Fmoc-Thr(^{t}Bu)-Wang resin samples (19 × 30 mg, 18 μmol) were swollen in 3 ml DMF, and the Fmoc-group was removed by 3 ml of 50% piperidine/DMF. After washing with 5 × 3 ml of DMF, one of the 19 Fmoc-amino acids (3-fold molar excess, 54 μmol dissolved in 100–200 μl DMF, preactivated with DIC (27 μl, 54 μmol) and HOBt (25 mg, 187 μmol)) was coupled to each sample for 1 h at RT. After washing (3 × 3 mL DMF and 3 × 3 ml MeOH), the success of the coupling was monitored by ninhydrin [45] or isatin [46] assays. The protected dipeptidyl resin samples were suspended in 2 ml DMF and poured into a common tube. The combined and thoroughly mixed resin (~570 mg, 342 μmol) was coupled with the next side chain protected Fmoc amino acids (1.03 mmol of each: Q, T, G, T, P, T, P, T, P, T, V, K) in DMF using the protocol described above.

After the removal of the *N*-terminal Fmoc-group the peptides were cleaved from the resin using 5% (v/v) aqueous TFA (15 ml) in the presence of 5% (v/v) TES in a tube equipped with teflon caps at RT for 3 h. The crude sample was precipitated with *tert*-butyl-ether in a 7-fold volume excess. After an overnight stand at −20 °C, the products were isolated by filtration, washed twice with ether and then dissolved in 10% acetic acid. The solution was diluted with distilled water and freeze-dried.

The Synthesis of Individual Peptides

Four individual pentapeptides (TQTQT, TQTKT, TQTLT and TQTIT), corresponding to the TQTXT library were synthesized by the Boc/Bzl protocol described above. The peptides were purified by RP-HPLC using a Phenomenex C_{18} semi-preparative column (250 × 10 mm, 5 μm, 300Å) and gradient elution (eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80 : 20, v/v)). Analytical

RP-HPLC was run on a Phenomenex C_{18} column (250 × 4.6 mm, 5 μm, 300Å). Linear gradient of eluent (0 min 0% B; 30 min 30% B) with 1 ml/min flow rate and UV detection at $\lambda = 214$ and $\lambda = 254$ nm was used.

Mass Spectrometry

Positive ion ESI-MS experiments were performed on three different instruments. Low resolution (approx. unit resolution) experiments were run on a PE SCIEX API 2000 triple quadrupole mass spectrometer (Toronto, Canada), in full scan mode. Spray voltage 5000 V and orifice voltage 30 V were applied. A Q-Tof Micro (Micromass, Manchester, UK) hybrid, quadrupole orthogonal acceleration time-of-flight instrument was used for the experiments at medium resolution (resolution ~5000 at half height), and for HPLC/ESI-MS analysis in TOF-MS mode. The capillary voltage was 3000 V, sample cone 30 V, desolvation temperature 180 °C and the ion source temperature was 80 °C. The high resolution (resolution ~10 000 at half height) experiments and MS/MS analysis of selected molecular ions were performed on a Q-Star Pulsar (Applied Biosystems) hybrid, quadrupole-time-of-flight instrument in TOF-MS and TOF-MS/MS mode, respectively. The capillary voltage was 5500 V, cone 35 V. For MS/MS analysis only the ^{12}C containing isotope of interest was transmitted to the collision quadrupole. Nitrogen was used as the collision gas for all experiments at a collision energy of 45 eV. Samples were dissolved in acetonitrile/water mixture (1 : 1, v/v), containing 0.1% acetic acid and analysed by flow injection at a flow rate of 10 μl/min.

High performance liquid chromatography was performed on a CapLC system (Micromass, Manchester, UK) equipped with low flow capillary HPLC pumps and with an auto sampler and directly coupled to the Q-Tof Micro instrument. A Vydac 150 × 0.3 mm C_{18} (5 μm, 300 Å) capillary column was used for separation. Eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile-water (95 : 5, v/v). Samples were dissolved in water in a concentration of 0.1 mg/ml in the case of the peptide library (TQTXT), and 5 μg/ml in the case of individual peptides. 0.5 μl sample was injected by the auto sampler in μl pick up mode. A linear gradient of eluents (0 min 0% B; 5 min 0% B; 50 min 45% B) with 5 μl/min flow rate was used as the mobile phase. Data were acquired on a Micromass Q-Tof Micro instrument (see above). Due to the high polarity of the sample, the built in stream select module and the precolumn were disconnected from the solvent line, allowing the sample to run directly to the capillary column.

RESULTS AND DISCUSSION

Identification of Peptide Library Components

Single-stage (MS1) ESI-MS at low, medium and high resolution, tandem MS and capillary HPLC-MS techniques were applied for the analysis of the XT, TXT, TQTXT, KVTPTPTPTGTQTXT (K12X) peptide libraries.

Low resolution spectra of the four peptide libraries acquired on the PE-SCIEX quadrupole mass spectrometer are shown in Figures 1–3. In the 2-, 3- and 5-mer libraries (XT, TXT, TQTXT) ESI produced abundant singly charged ions, and the unit resolution of the quadrupole instrument was sufficient to distinguish and identify most components (Figure 1, 3(A)). In the 15-mer K12X library with an average molecular mass of 1550 Da predominantly doubly charged ions were observed (Figure 2). In this case the nominal unit resolution of a quadrupole instrument was not sufficient fully to resolve the peaks, nevertheless tentative assignment of peaks with a 0.5 mass unit difference was still possible (insert in Figure 2(A)). Full peak resolution was achieved on hybrid quadrupole-time-of-flight (Figure 2(B)), and also on many ion-trap instruments.

Isobars (Q/K) and ^{13}C-isobars (e.g. L/N, N/D and Q/E) can not be distinguished at low resolution. The hybrid Q-Star instrument proved to have sufficient resolving power to differentiate them in the dimer XT and partially in the trimer TXT libraries, but not in the two higher 5- and 15-mer libraries; so this option has limited practical utility. If such isobars were to be distinguished in 5-mer or larger libraries, FT-ICR instruments should be used [41]. Identification of ^{13}C-isobars relies mainly on relative peak abundance and is particularly difficult at higher mass, when the isotope peaks have high abundance. A good example is the K12X library, when the first isotope peak is nearly as abundant, as the low mass isotope (Figure 2). Assuming equal sensitivity for all components in the mixture, the theoretical isotope pattern can be calculated, and it is also shown in the insert in Figure 2(B). Comparison of the theoretical and observed pattern clearly indicates that all ^{13}C isobars are present in the library (I(L)/N; N/D and Q(K)/E/M); and suggests that the relative sensitivity of these components is not very different. For the

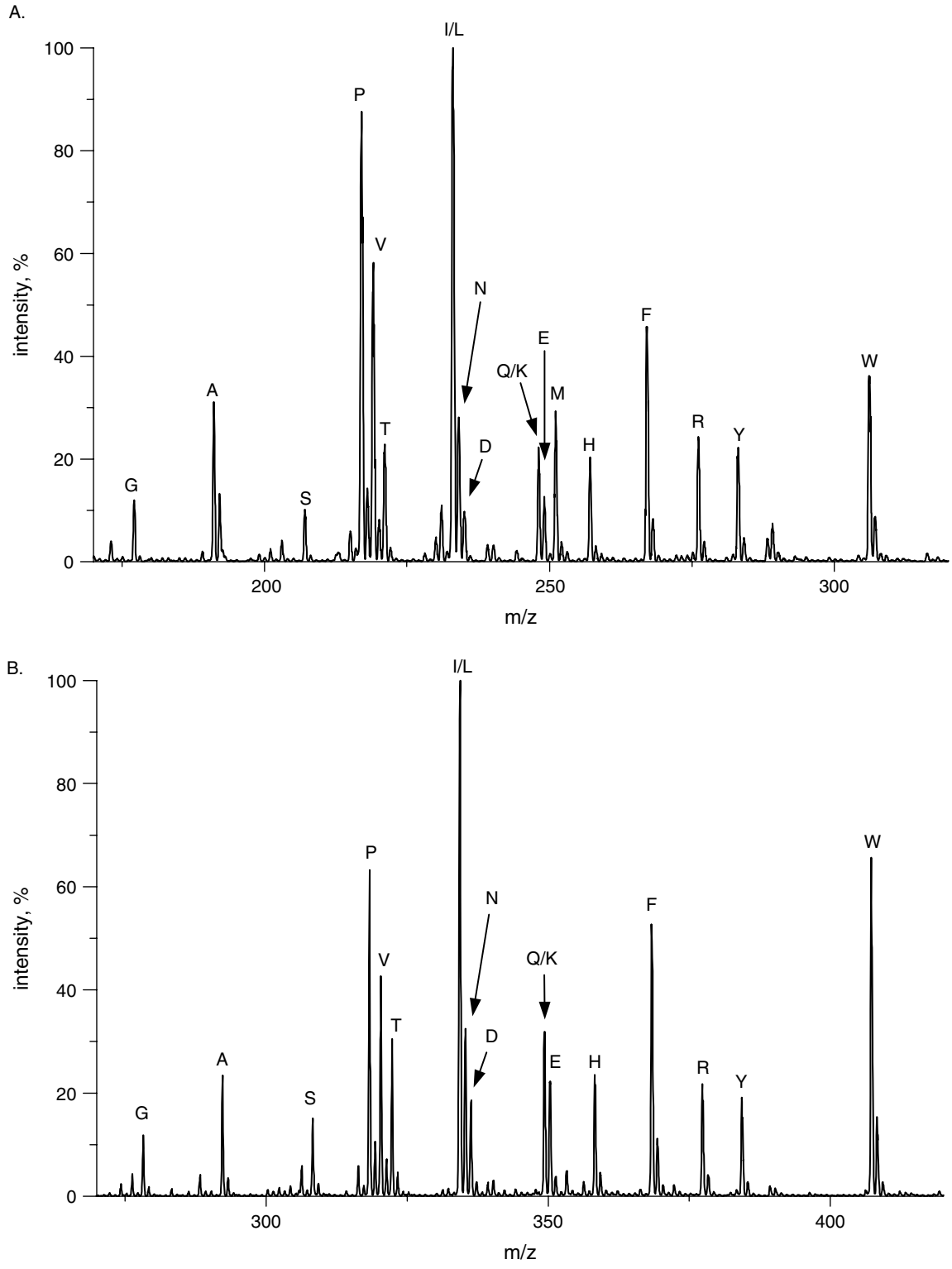

Figure 1 The molecular ion region of the ESI-MS spectrum of the (A) XT and (B) TXT libraries acquired on a low resolution PE-SCIEX API 2000 triple quadrupole mass spectrometer. Letters in the figures represent the amino acids in position X.

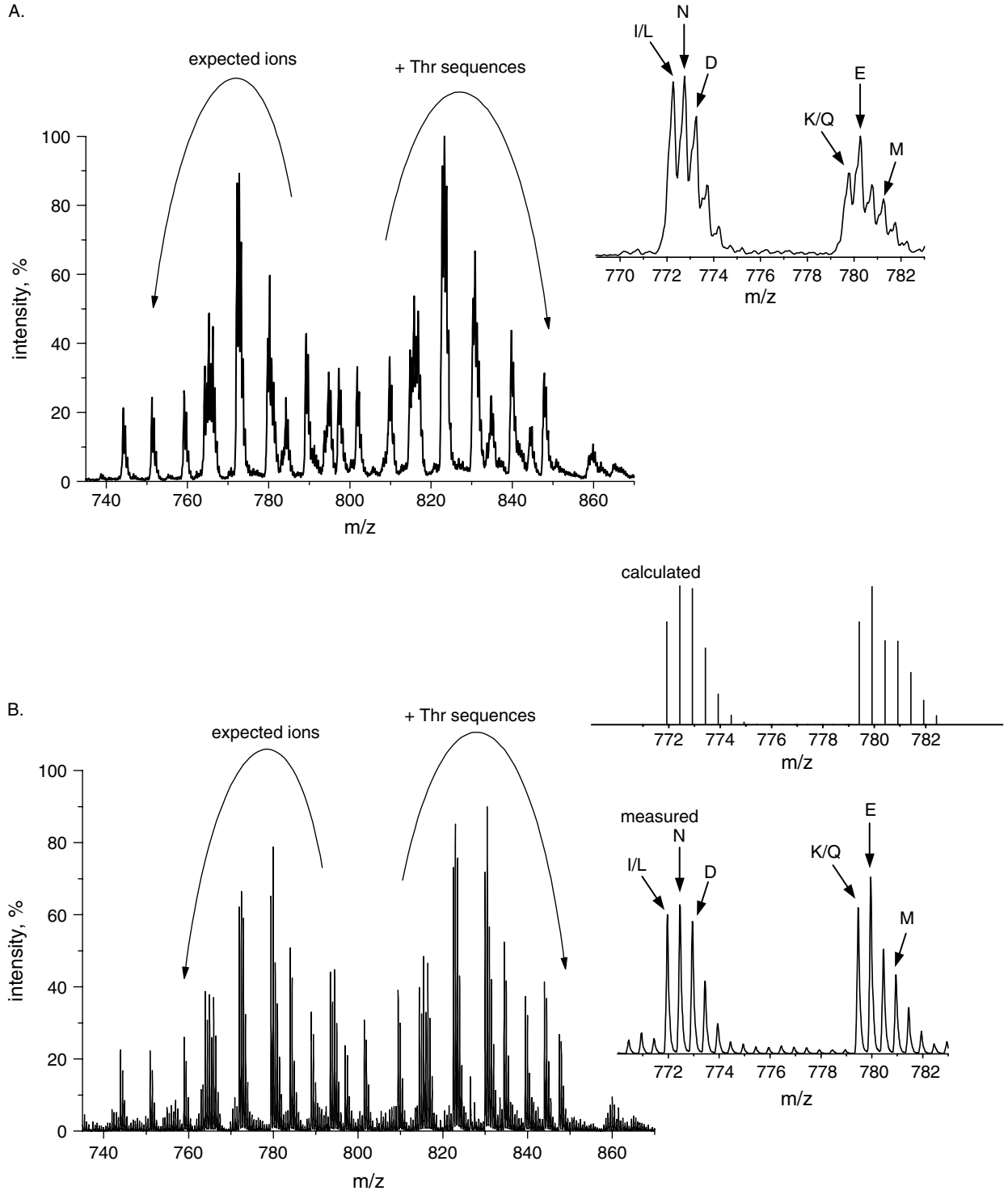

Figure 2 The doubly protonated molecular ion region of the KVTPTPTPTGTQTXT library acquired (A) on a low resolution PE-SCIEX API 2000 triple quadrupole mass spectrometer; (B) at 10.000 resolution on a hybrid PE-SCIEX Q-Star Pulsar instrument. The inserts show the critical ^{13}C-isobar region in an expanded scale. Letters in the figures represent the amino acids in position X.

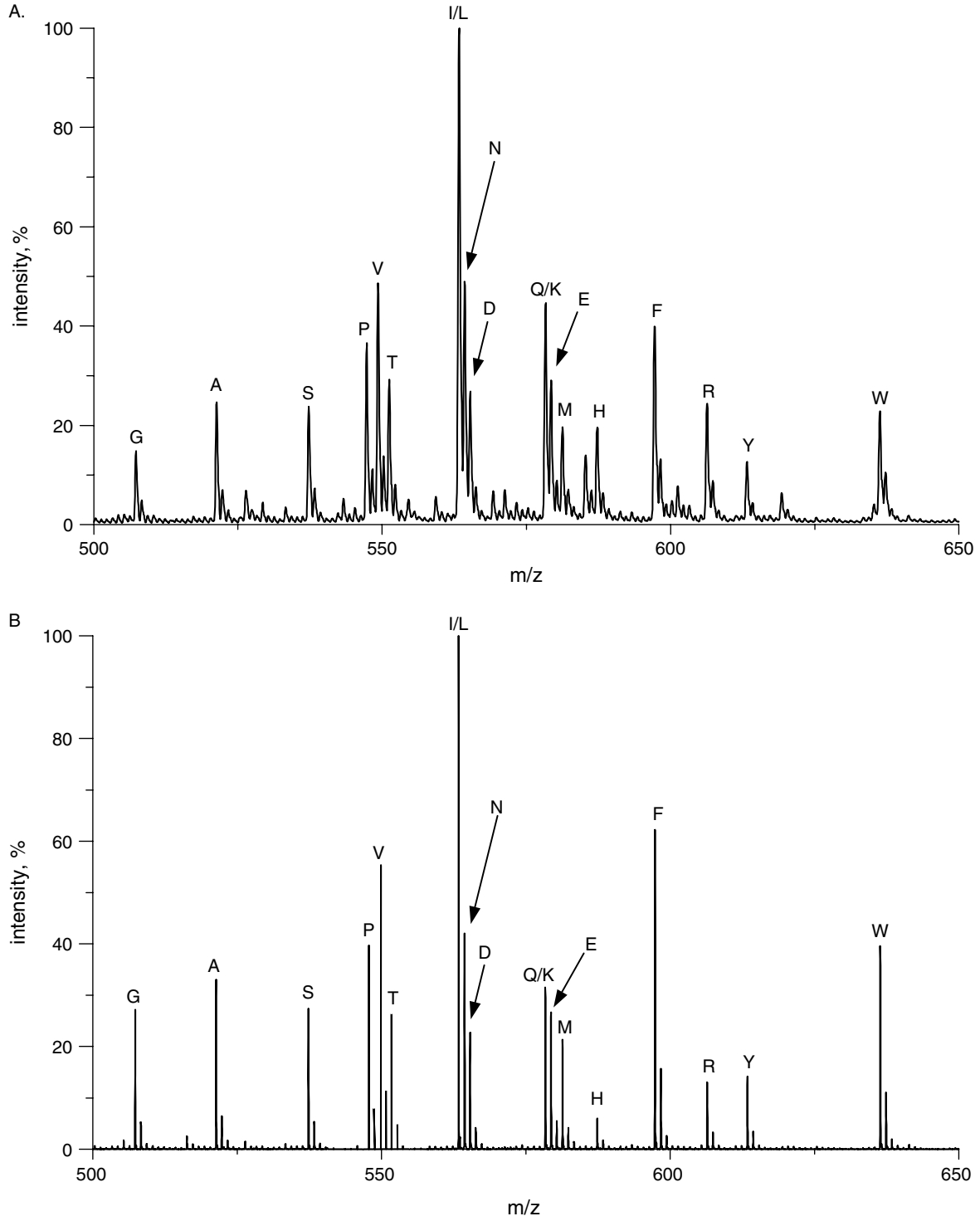

Figure 3 ESI-MS spectrum of the library TQTXT, acquired on a (A) low resolution PE-SCIEX API 2000 triple quadrupole mass spectrometer (B) high resolution PE-SCIEX Q-STAR Pulsar instrument. Letters represent the amino acids in position X.

peptide libraries studied, the relative sensitivities of individual components (taking into account the influence of isotope peaks) were determined from the spectra shown in Figure 1, 2(A) and Figure 3(A), and the results are shown in Table 1. These data indicate that relative sensitivities in these peptide libraries vary approximately within a factor of 2. It is interesting to note that the sensitivity variations are less for larger peptides. This is due to the physico-chemical differences introduced by one variable amino acid residue, and this has a larger effect on the properties of a small, than a large peptide library.

Verification of the presence of the isobaric (or ^{13}C-isobaric) components in the pentamer library is possible not only by high resolution on FT-ICR instrument [23], but also by tandem mass spectrometry. An example is shown in Figure 4 for Q and K containing pentapeptides. The MS/MS spectrum of the respective molecular ion (m/z 578) obtained from the TQTXT combinatorial library was compared with the spectra of individual TQTQT and TQTKT peptides. The most important difference between the two pentapeptides is the formation of a fragment at m/z 129. This is the second most abundant peak in the spectrum of peptide TQTKT (Figure 4(A)), while it is absent in the case of peptide TQTQT (Figure 4(B)). The tandem mass spectrum obtained from the TQTXT library (Figure 4(C)) clearly shows the m/z 129 peak. This verifies the presence of peptide TQTKT in the library. However, its relative abundance is much lower in the mixture, than in the single peptide preparation (Figure 4(A)). This suggests that the other isobar, i.e. peptide TQTQT is also present in the library.

The presence of the two isomeric peptides, TQTIT and TQTLT, were also investigated by tandem mass spectrometry at low collision energy and by on-line HPLC-MS. The tandem mass spectrometric experiments performed on the protonated molecular ions of the two peptides result in identical spectra (data not shown) under the experimental conditions applied. These peptides were, however, successfully distinguished by chromatography (Figure 5), and identified by on-line HPLC-MS with the respective molecular ion (m/z 563) and appropriate control peptides. Thus TQTIT and TQTLT components were successfully resolved in the TQTXT library. As could be expected, the isoleucine containing peptide has the lower retention time on a reversed phase column.

Table 1 Relative Intensity and Standard deviation values for the Peptide Libraries

Residue	Relative intensity XT	Relative intensity TXT	Relative intensity TQTXT	Relative intensity K12X	Average	Standard deviation
G	0.41	0.41	0.57	0.77	0.54	0.17
A	1.07	0.82	0.95	0.76	0.90	0.14
S	0.35	0.53	0.91	0.88	0.67	0.27
P	3.01	2.22	1.41	1.31	1.99	0.79
V	2.00	1.50	1.87	0.77	1.53	0.55
T	0.79	1.07	1.12	0.95	0.98	0.15
I/L[a]	1.72	1.76	1.92	1.05	1.61	0.38
N	0.54	0.54	1.03	0.50	0.65	0.25
D	0.27	0.56	0.73	0.76	0.58	0.22
Q/K[a]	0.38	0.56	0.85	1.10	0.73	0.32
E	0.34	0.59	0.35	0.83	0.53	0.23
M	1.01	n.d.[b]	0.75	0.87	0.88	0.13
H	0.70	0.83	0.71	1.72	0.99	0.49
F	1.57	1.86	1.53	1.12	1.52	0.30
R	0.84	0.76	0.93	1.49	1.01	0.33
Y	0.76	0.67	0.48	0.81	0.68	0.14
W	1.24	2.31	0.88	1.32	1.44	0.61
Standard deviation	0.73	0.65	0.45	0.31	0.44	

[a] Due to the full peak overlapping, the intensity of these peptides was divided with a factor of two.
[b] Not determined. The peptide TMT was not present in this library. This value was eliminated from the calculations.

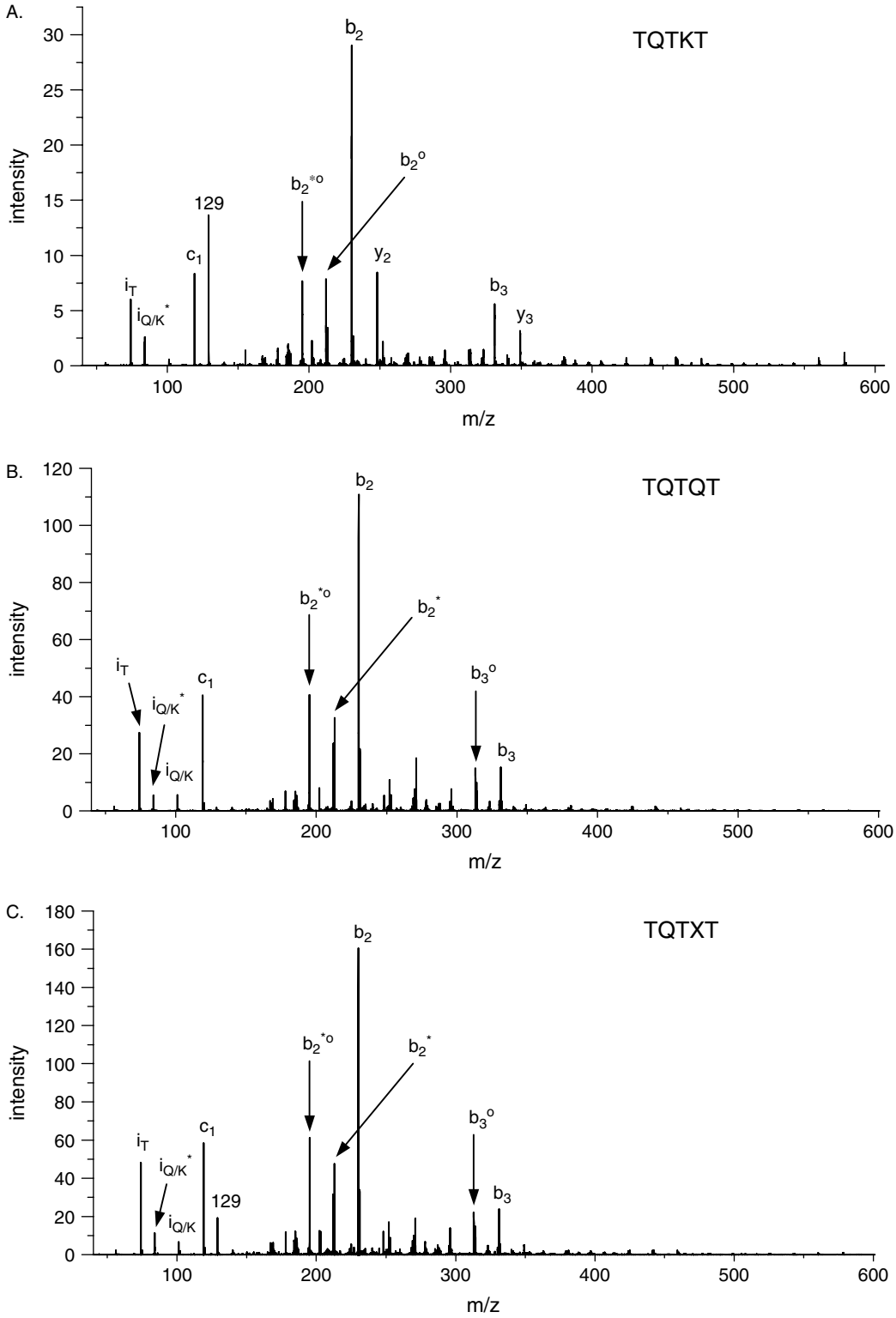

Figure 4 MS/MS spectrum of (A) the peptide TQTKT, (B) TQTQT and (C) the corresponding molecular ion (578 *m/z*) from the TQTXT library. Symbols represent sequence ions, water losses are indicated by a circle, ammonia losses by asterisk.

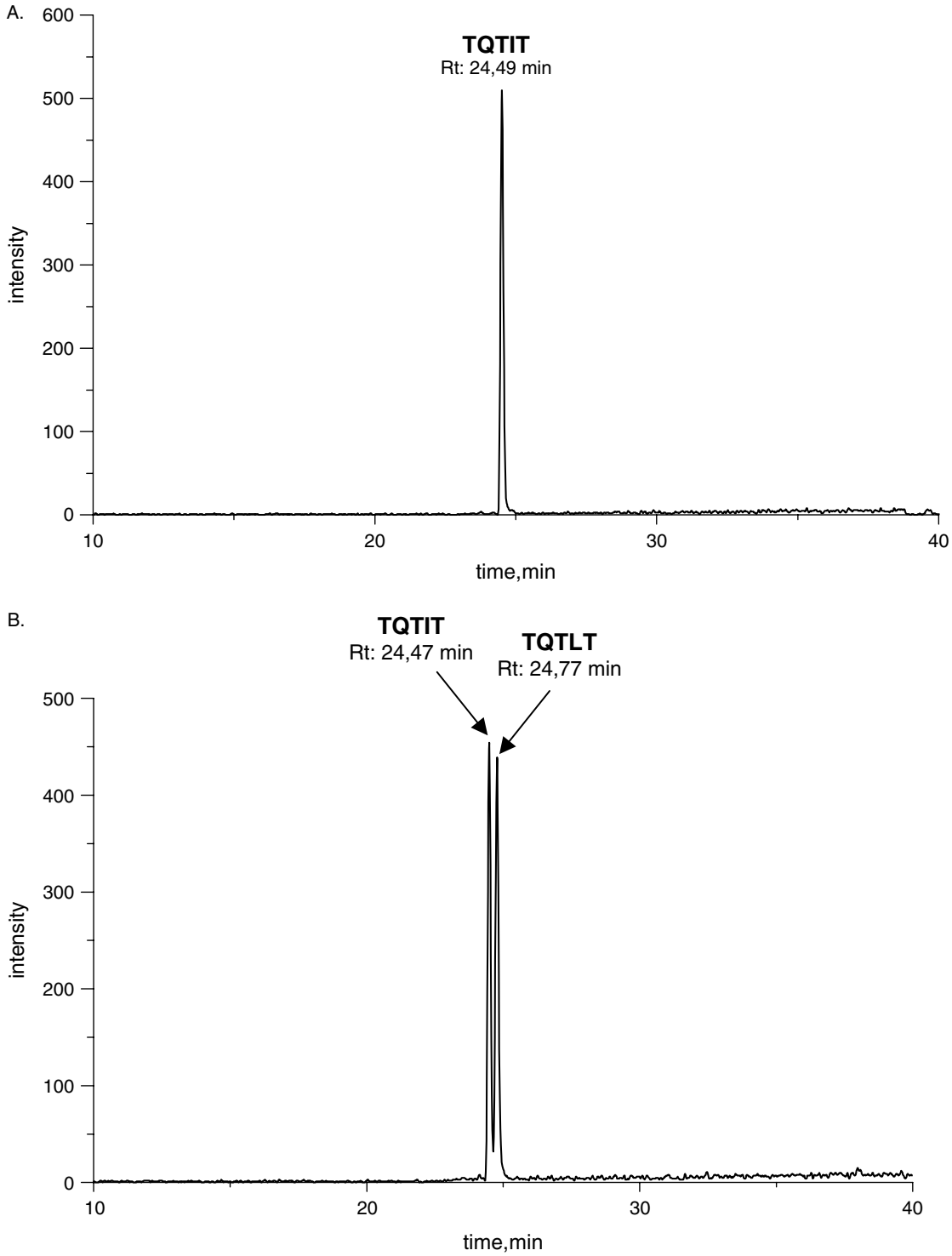

Figure 5 Selected ion chromatogram of the 563 m/z ion corresponding to the protonated molecular ion of the isomer TQTLT and TQTIT peptides from the HPLC/ESI-MS chromatogram of the (A) peptide TQTIT, (B) TQTXT library. The two peaks in the spectrum indicate that both components are present in the library in approximately the same quantity.

Identification of Synthesis-related Pitfalls

The low (and in the case of the 15-mer library the medium) resolution mass spectra can be used to identify the side reactions that occurred during the synthesis of peptide libraries. Our data suggest that there are no apparent failures in the XT and in the TQTXT libraries (Figure 1(A), Figure 3). In the TXT library, however, the TMT component is absent (Figure 1(B)). As the analogous di- penta- and 15-mer peptides (MT, TQTMT and KVTPTPTPTGTQTMT) were identified without difficulties in the respective libraries, this might indicate that peptide TMT is a missing component.

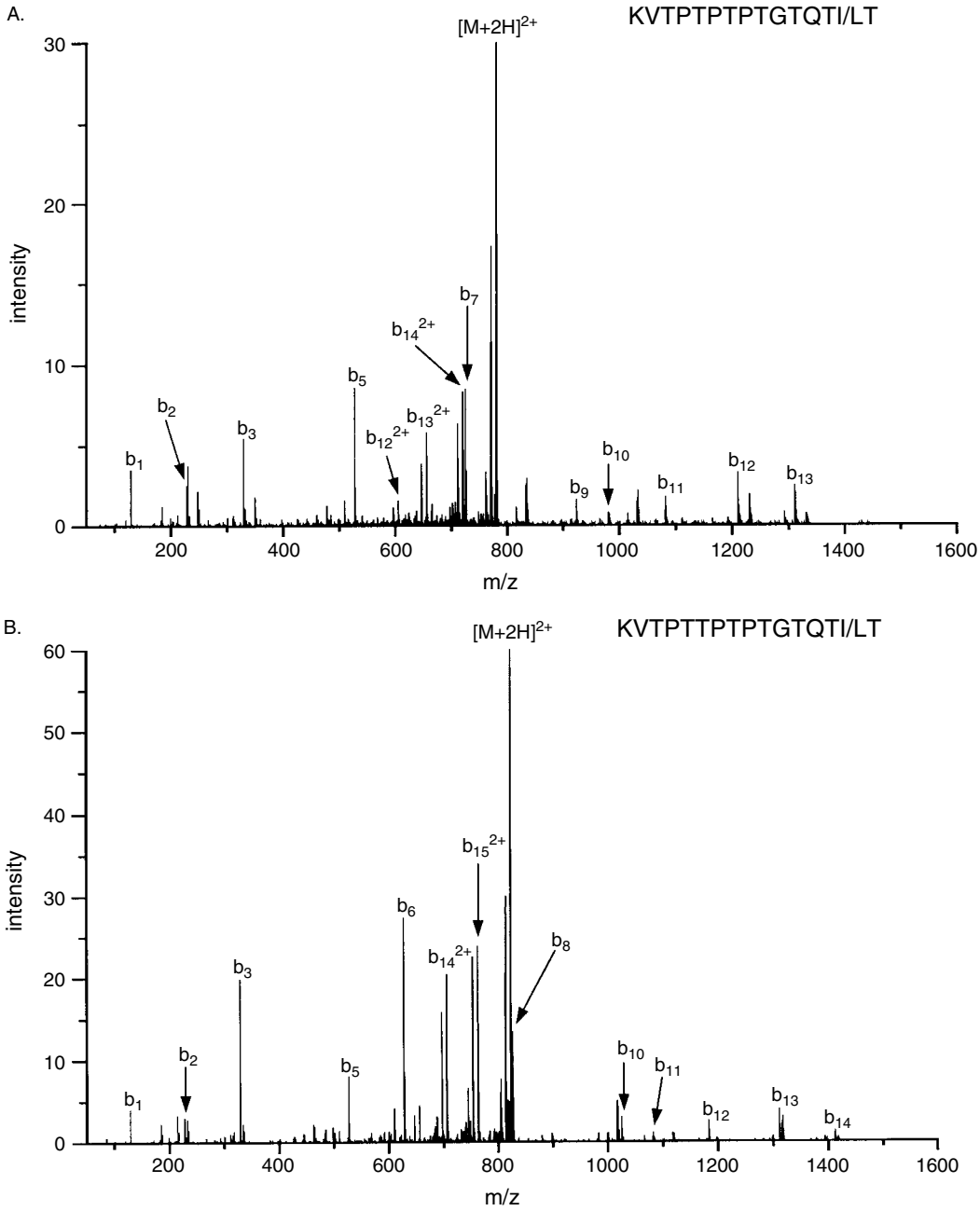

Figure 6 MS/MS spectrum of the (A) KVTPTPTPTGTQTI(/L)T (B) KVTPTTPTPTGTQTI(/L)T peptides from the K12X library, acquired on a high resolution hybrid PE-SCIEX Q-Star Pulsar instrument. The most characteristic sequence ions are indicated.

For the interpretation of this experimental observation the most likely explanation is that it is possible that an oxidative side reaction occurred and the oxidized products are isobars of peptide TFT and TYT.

The analysis of the K12X library led to the identification of a different problem. Beside the expected molecular mass envelope (Figure 2) a second envelope corresponding to a 101 Da shift to higher masses was also observed. This suggests that the 15-mer library also contains peptides with one additional amino acid (threonine) residue, indicating a mistake in the synthesis performed. From ion intensity data the relative amount of the elongated peptides can be estimated. Since the ion intensities of the second series are almost identical to those of the 15-mer K12X peptides, one can conclude that the quantity of the longer peptides is approximately 50%.

In order to locate the site of the extra threonine residue in the K12X library, MS/MS experiments were performed. MS/MS spectra of the 15-mer components containing I/L in position X (KVTPTPTPTGTQTI/LT) are shown in Figure 6(A). The spectrum shows a nearly complete set of b ions, which confirms the expected sequence. It should be noted that b_4, b_6 and b_8 fragments are practically missing from the spectrum — these correspond to cleavages at the *C*-terminal site of proline residues. On the other hand, cleavages at the *N*-terminal side of proline residues (b_3, b_5 and b_7) have a very high abundance. These features can be explained by the known pattern of peptide fragmentations. The tandem mass spectrum of the elongated component containing one additional threonine residue (Figure 6(B)) was analysed in an analogous manner. Not only the masses of the b_3, b_5, b_6, b_8, b_{10-14} ions, but also the missing b_4, b_7, b_9 fragments identify unequivocally that the sequence of this peptide is KVTPTTPTPTGTQTI/LT. This suggests that threonine residue at position 5 was also introduced during the synthesis.

CONCLUSION

Based on these results it can be concluded that even simple mass spectrometric techniques are valuable for the analysis of synthetic peptide libraries. For small peptide libraries below mass 800–1000 and containing 20–40 components, low resolution, single-stage electrospray mass spectrometry may be sufficient for a quick control of the quality of synthetic products. For larger peptides or more complex mixtures medium or high resolution techniques are preferable. Tandem mass spectrometry (which is fast becoming a routine technique) and chromatographic separation with MS detection may yield more information on peptide sequences and the presence of isobar or isomer peptides in combinatorial libraries.

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