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Tandem Mass Spectrometry for Simultaneous Qualitative and Quantitative Analysis of Protein

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1. Introduction

Tandem mass spectrometry has long been recognized as a technique for qualitative analysis of proteins via amino acid sequencing. The use of tandem mass spectrometry in quantitative analysis for proteins is much limited. Nevertheless, application of chromatographic separation prior to mass spectrometry analysis can be used as a device for quantitative analysis of proteins. Following chromatographic separation, specific compound in a complex mixture can be determined with minimal interference; this is possible by monitoring only the selected m/z ratio of the compound, which is the characteristic of the compound of interest, an approach known as selected ion monitoring (SIM). The application of SIM using a combined GC-MS instrument was first demonstrated by Sweeley *et al.* in 1966.

On the other hand, quantitative data can also be obtained by repetitive scanning of compounds during elution of a sample from the gas chromatograph (GC-MS) or liquid chromatograph (LC-MS). The detection limits for such techniques are generally much poorer than those of SIM; this is because the instrument spends very little time at each m/z ratio during scanning. Such techniques can either scan the entire mass range of analysis or only scan a limited mass range with a greater sensitivity; subsequently the quantitative data can be calculated from the peak area or peak height displayed by the extracted chromatograms of the selected masses (Shoemaker & Elliott, 1991). The selected masses can be a molecular ion or a fragment ion provided it is sufficiently intense. Repetitive scanning together with automated data processing (library search of the recorded spectra) for biological samples has been used to measure a large number of biological compounds in complex samples. Such technique was found to be reliable, accurate and considerably more cost-effective than operator-mediated methods (Slivon *et al.*, 1985).

Compared to a number of analytical techniques, tandem mass spectrometry is a technique which is able to provide more reliable data due to its high specificity and sensitivity. The major disadvantages of mass spectrometry are the high capital costs and the relatively low sample throughput. Therefore, the use of mass spectrometry in quantitative analysis is preferably dedicated to sensitivity and specificity rather than throughput. Since the original publication (Sweeley *et al.* 1966), technology and methodology on mass spectrometry have progressed and the applications of SIM for quantitative analysis, particularly in the analyses of small compounds have increased rapidly. Nevertheless, Bellar & Budde (1988) and Eichelberger, *et al.* (1983) cited that although improved sensitivity and precision is available

with SIM, the loss of qualitative information is significant and usually unacceptable. This is especially true for quantitative analysis of minute protein in biological samples. Although coupled with effective sample cleanups, it is common for such mass spectrometry analysis to generate large amount of data caused by interferences from unrelated compounds derived from the complex sample matrix. In this condition, false identification of protein in SIM quantitative analysis will concomitantly increase.

Other quantitative analyses available are selected reaction monitoring (SRM) and multiple reaction monitoring (MRM). Both SRM and MRM were based on the similar principle, where the target precursor ion will be isolated and subsequently one of the fragment ions (SRM) or multiple fragment ions (MRM) will be monitored and quantified. The specificity of SRM and MRM is better than that of SIM. However, in terms of quantification of peptides and proteins, the methods' specificity vary depending on the nature of the peptide or the protein. This is especially true when the protein or peptide is belonged to a family of proteins, where the proteins in the family shared a great extend of similarity in their amino acid sequence. Furthermore, it is relatively common that the target peptide or protein be found in other forms than its intact form in biological matrix, where these other forms of proteins or peptides are the result of partial digestion of the protein or peptide through the activity of proteases in biological matrix. In these instances, the qualitative data of the target protein or peptide becomes important feature to discriminate the targeted peptides from these other forms of proteins or peptides. An illustration of the complexity of protein or peptide analysis will be demonstrated in the quantification analysis of human chorionic gonadotropin (hCG), a glycoprotein belongs to gonadotropin family. Besides the closely resemblance of hCG and LH, there are also the presence of nicked hCG in the biological matrix. In these circumstances, a complete qualitative data to indicate the identity of the target peptide will surely lead to higher confident in protein or peptides quantification.

Other method for quantitative analysis of protein is by ELISA, where the amount of the protein present in a solution of biological matrix is measured by the degree of antibody-antigen interaction that is expressed in the intensity of color developed by the antibody-tag enzymatic reaction with its substrate. ELISA is a device for partial quantitative analysis. Furthermore, ELISA method may lead to false positive result caused by the cross reaction of the antibody with other unrelated proteins or compounds as the assay depends solely on the specificity of the antibody used.

An approach for simultaneous qualitative and quantitative analysis of proteins was developed. In this method, qualitative and quantitative analysis of proteins can be conducted in a single tandem mass spectrometry analysis. The method not only provides unambiguous identification of protein via amino acid sequencing, at the same time, quantitative data can be generated from the same tandem mass spectrometry data. This method is recommended as it can achieve a very low limit of quantification.

2. The concept of simultaneous qualitative and quantitative analysis of proteins

The main problem encounters in the quantification of minute protein in a biological sample matrix is the isolation of the target proteins from the sample matrix. One of the most direct approaches for isolation of protein of interest is by using immunoaffinity extraction of the targeted protein through specific antibody-antigen interaction (Gam et al, 2006), this approach is possible if the antibody for the protein is available. Although Such technique

can efficiently isolate target protein from the complex sample matrix, co-extraction of other unrelated proteins cannot be avoided. This may result from the non-specific interaction of the antibody with other proteins or may also result from the non-specific binding of other proteins with the antibody coating materials. Besides, it is also common that non-protein materials being extracted in such procedure. All these unrelated proteins or compounds will interfere with the subsequent analysis of the target protein using tandem mass spectrometry.

Mass spectrometer is an instrument with high sensitive but low selectivity, when analysis is carried out in the positive ion mode, the instrument will register all the positive charged ions that enter the detector. Therefore, quantification of minute protein extracted from complex biological matrix using mass spectrometer poses a great challenge. It is common that the target ion cannot be detected as it was suppressed or masked by other ions from the impurities in the sample. In this scenario, quantification of target ions cannot be achieved as it cannot fulfill the signal to noise ratio of greater than 3.

One way to solve such problem is to have a very clean sample, a demand that is hard to fulfill as the target protein is only present in trace quantity in the complex biological sample. The alternative way of solving this problem is to get rid of all the unrelated ions during mass spectrometry analysis making the spectrum to be very clean for quantification analysis. One way of getting rid of the unrelated ions is by filtering them out from the spectrum. Based on this understanding, we have developed a quantification method for minute protein in a complex mixture. The first issue to solve is how to filter the unrelated ions out from the target ion spectrum, surely we cannot place a mass filter at the inlet of sample nebulizer, where it will also filter off our target protein. The other filtering device that possible is by using the MS/MS scan, in this device, only the target ions that were exceeded the threshold programmed in MS scan will be isolated and excited to MS/MS scan. In a way, the target ion is being filtered from other unrelated ions.

In the analysis of protein using ion trap mass spectrometer, it is not advisable to analyze intact protein, where identification of intact protein is based solely on its deconvoluted molecular weight. Furthermore, analysis of intact glycoprotein by ion trap mass spectrometer possess additional problem where deconvolution of protein molecular weight may not be able to carry out successfully as the ionization of glycoprotein is inconsistent due to the presence of sugar components, which caused variation in the ionization of intact glycoprotein.

One of the advantages of using ion trap mass spectrometer is its ability to ionize peptide into multiple charged ions and to perform MS/MS scan, however it is not possible to perform MS/MS scan on most of the intact proteins, especially the high molecular weight proteins as the data generated cannot be interpreted. It is commonly understood that MS/MS scan is best performed on tryptic digested peptides, where the length of the peptides digested by trypsin is manageable by MS/MS scan of most types of mass spectrometer. Furthermore, the site of trypsin digestion is either Arginine or Lysine, the basic amino acids that will favor the formation of double charged ion to the peptides in positive ion mode scanning. The formation of double charged ion is an added advantage for collision induced dissociation (CID) in the MS/MS scan, according to the mobile proton hypothesis (McCormack et al, 1993), kinetic energy from collision induced dissociation will be converted to vibrational energy that releases through fragmentation reactions directed by the site of protonated amide bond. In this hypothesis, fragmentation of double charged peptide under the collision induced dissociation will result in detection of both y and b ions

from the fragmentation, this is because each of the product ions carry a proton to themselves. When comparing to single charged ions, only one of the product ions resulted from the fragmentation will carry a proton and thus carry a charge for detection. The generation of comprehensive product ions by the double charged peptide ion gives a higher confidence in determining its amino acid sequence, where the more y and b product ions identified, the more significance the identity of the peptide.

In the currently developed method, both the qualitative and quantitative analyses of protein are based on the data of MS/MS scan, which displays product ions of the selected peptide. In order to give significant qualitative value of a protein, the peptide marker of the target protein must be uniquely different from peptides of other proteins. In another word, the amino acid sequence of the peptide marker must be specific for the target protein. Furthermore, upon collision induced dissociation, such peptide must produce comprehensive spectrum of y and b product ions which give a definite identity to the target protein.

In terms of quantitative analysis of protein, the same peptide marker will be used. Similar with qualitative analysis, the peptide marker will be subjected to collision induced dissociation to generate y and b product ions, as mentioned earlier, these product ions are used to confirm the identity of target protein. Once the identity of the protein is confirmed, subsequence extracted product ions chromatogram will be performed. When performing extracted product ions chromatogram, two orders of mass filters were in place, the first mass filter is peptide marker mass filter that will single out the molecular ion for the peptide marker, while the second mass filter is quantitative ion (or selected product ions) mass filter that will single out the quantitative ion. In doing so, this method create a high selectivity to our currently developed quantitative method. This is because the first mass filter isolates the double charged m/z value of the peptide marker, subsequently the second mass filter isolates the selected quantitative ion/s that was generated from the peptide marker that has undergone the first mass filter. Quantitative ion/s selected must be the most stable and intense product ion/s (either y or b ions) from the fragmentation of the peptide marker. Quantitative ion can be a single product ion or can be a sum of a few product ions. In the event of the summation of a few product ions, limit of quantification (LOQ) of the method will concomitantly reduce, this is because the total peak areas of product ions will certainly enhance the signal to noise ratio. However, the usefulness of selected product ions to act as quantitative ions must be validated, where its peak area under extracted product ions chromatogram must be proportional to the concentration of the target protein. In the other word, the intensity of collision energy plays an important role in this currently developed method, where the collision energy used must be kept constant throughout the analysis.

The beauty of this method is it is able to simultaneously provide qualitative and quantitative data on trace amount of protein in a complex mixture. The two layers of filtering event will ensure only the correct ions being quantified. This method is very useful for quantification of trace amount of protein in a complex biological sample. It is not impossible that many compounds with similar masses can be filtered in by the first layer of mass filter, nevertheless, these similar masses from the impurities of the sample cannot generate the product ions spectrum similar to that of target peptide marker, as they do not contain the amino acid sequence of the peptide marker that is unique to the target protein. Therefore, these unrelated masses cannot pass the second layer of mass filter.

As a result of the selectivity of the two layers mass filter described above, the extracted product ion chromatogram produced is usually free from any back-ground noises, giving rise to a very low limit of quantification of the method and therefore trace protein in complex sample matrix can be quantified. Furthermore, the method gives a very high confident level in terms of qualitative information of the target protein, where false positive data are completely omitted.

In the course of my study, I was given a task to develop a quantitative method for human chorionic gonadotropin (hCG) in human urine. hCG is a glycoprotein misused by male athletes to induce endogenous secretion of testosterone. hCG is present as trace component in urine, a complex biological matrix. In order to concentrate and purify the glycoprotein from urine, I have implemented immunoaffinity purification technique to extract hCG from urine matrix. This purification technique did not produce pure hCG as expected, the numerous amount of contaminants can be visualized when subjecting the extracted hCG to mass spectrometry analysis.

3. Materials and methods

3.1 Immunoaffinity protein purification

Approximately 11 ml of urine was centrifuged at 1500 rpm for 5 minutes to precipitate any particulate matters. A volume of 10 ml of the centrifuged urine was transferred to a clean polypropylene tube for hCG extraction. The immunoaffinity column was first flushed with 6 ml distilled water to remove the storage buffer and then conditioned with 5 ml of 0.01 M PBS at pH 7.2. During this step, the column flow was adjusted to 7-9 drops per minute. A 2 ml volume of the centrifuged urine was loaded onto the immunoaffinity column (column volume was 2 ml). A 20 minutes incubation time was allowed for the antibody-antigen association to take place. The urine was then removed from the column by flushing with 2 ml of 0.01 M PBS pH 7.2. The column was then reloaded with another 2 ml aliquot of urine and incubated for 20 minutes. This process was repeated until all the 10 ml urine had passed through the column. Finally, the column was washed with 15 ml (7 bed volumes) of washing buffer (0.1% (v/v) Tween 20 in 0.1 M PBS, pH 7.2) followed by 2 ml of elution buffer (1 M citric acid adjusted to pH 2.2 with 10 M NaOH). After the first 1 ml of the elution buffer had entered the gel, the collection of the eluate began. When the 2 ml of the elution buffer had fully immersed in the gel, a 5 minutes equilibration time was allowed to enable complete antibody-antigen dissociation to take place. This was followed by 8 ml of elution buffer. A total of 9 ml eluate was collected. Eluate was concentrated and desalted using a protein concentrator column (Jones Chromatography).

3.2 Preparation of tryptic digestion product of hCG

3.2.1 Digestion of hCG using trypsin

Protein sample (hCG) was desalted using protein concentrator column (C_{18} , 2 cm x 4.6mm ID, Jones Chromatography). A syringe pump (Harward Apparatus) was used to pump the protein solution through the column at 1 ml/min. The column was then flushed with 25 ml of deionized distilled H_2O and the protein was recovered by eluting with 70% acetonitrile 0.1 % formic acid. The eluted protein was dried under N_2 at 37°C.

The dried protein was denatured using denaturing buffer (6M Guanidine HCl, 0.5M Tris, 2mM EDTA pH8.6). A volume of 10 μ l of 1 M dithiothrietol was added to the mixture and

incubated at 37°C for 30 minutes. After which 25 µl of 1M iodoacetic acid in 1M NaOH was added and the mixture was further incubated for 30 minute at room temperature.

The excess reagents were removed from the protein sample by using the protein concentrator column (as mentioned above). The dried hCG was then reconstituted in 50 µl of 50 mM NH_4HCO_3 . A volume of 2 µl (0.25 µg/µl) of trypsin solution was added and the mixture was incubated at room temperature for 20 hours. This was followed by another addition of the same amount of trypsin and the sample then further incubated for 4 hours at room temperature. The digested hCG was lyophilized and stored at -20°C.

3.3 HPLC separation

The tryptic digested hCG was first reconstituted in 25 µl of high purity distilled H_2O (Maxima, ELGA); 10 µl of the sample was then injected into the C-18 Vydac column (300 Å, 5 µm, 1 mm X 50 mm). Separation of the peptides was performed using a Hewlett Packard series 1100 HPLC. The flow rate was set at 1 ml/min and further split by a fused silica splitting device to 20 µl/min through-column flow rate. Mobile phase A was 0.05% TFA in H_2O and B was 0.05% TFA in ACN. The gradient used was 5-95% B for 20 minutes and held at 95% B constant for 5 minutes. The HPLC was interfaced to an ion trap mass spectrometer (LCQ, ThermoQuest).

3.4 Mass spectrometry

Mass spectrometric analysis was carried out using the ion trap mass spectrometer (LCQ, ThermoQuest). Data dependent experimental method was created for the analysis of tryptic peptides of hCG.

3.4.1 Creating a data dependent experimental method for qualitative analysis

The MS data was acquired at heating capillary temperature 200°C, sheath gas flow rate is 60arb, spray voltage at 4kV, tube lens offset is -60V and the capillary voltage is at 38V.

Data dependent experimental method was created for the analysis. The experimental method was consisted of 2 scan events. The first scan event was full scan MS, the second was MS/MS scan, which were dependent on the results of the full scan MS. This linkage is known as data dependent scan. The parameters of data dependent scan were default collision energy of 25, charge state of 2, minimum signal acquired was 1×10^5 counts, isolation width was 2 m/z.

3.4.2 Creating a data dependent experimental method for simultaneous qualitative and quantitative analysis of hCG

Doubly charged parent ion for peptide VLQGVLPALPQVVCNYR, [964.7]²⁺ was programmed into the parent ion list in data dependent scan. The parameters set for data dependent scan (MS/MS scan) were default collision energy = 25, default charge state = 2, minimum signal acquired = 1×10^4 counts, and the isolation width = 2 m/z.

4. A demonstration of the method by using human chorionic gonadotropin hormone

Human chorionic gonadotropin or hCG is a hormone misuses by male athlete to induce endogenous production of testosterone (Boer et al, 1991). It will be interesting to use hCG for

the demonstration of this analysis method, this is because the hormone belongs to a family of gonadotropin. Other hormones in the same family are follicle stimulating hormone (FSH), lutropin hormone (LH) and thyroid stimulating hormone (TSH) (Canfield et al, 1976). All these hormones share a similar characteristic in molecular structure, they are glycoprotein hormones that made up of one alpha-subunit and one beta-subunit. The alpha-subunit of the hormones is identical (Vaitukaitis et al, 1976), hence, it cannot serve as marker for hCG. On the other hand, the beta-subunits are basically similar with only minor differences in certain amino acid residues. This is especially true between the beta-subunits of hCG and LH (Figure 1). Due to the high resemblance of the hormones, they share relatively similar electrophoretic mobility in gel electrophoresis separation. Therefore, qualitative and quantitative analysis of hCG possess extra challenges.

Human Chorionic gonadotropin (hCG) is synthesized by the trophoblast cells of the placenta (Canfield, *et al.*, 1971). The hormone is release in the first few weeks of pregnancy. Between the 7th to 12th week of pregnancy, the plasma level of hCG rises to extremely high levels where approximately 11,000 – 289,000 mIU/ml of hCG were released. This is followed by a decline during the last two trimester (Braunstein, *et al.*, 1978).The molecular weight of intact hCG, α hCG (α -subunit) and β hCG (β -subunit) are approximately 36.7, 14.5 and 22.2 kDa, respectively. It has been estimated that 30% of the total weight of hCG is contributed by the carbohydrate content (Canfield, *et al.*, 1976) and these carbohydrates account for the heterogeneity properties of hCG. Besides the intact hCG, α hCG and β hCG, the other commonly found hCG fragments are β -core fragment and nicked hCG fragment. These fragments are formed by proteolytic degradation of hCG in kidney. The β -core fragment composes of two polypeptides linked by a disulfide bond. The molecular weight of the purified β -core fragment is between 12-16kDa. Reduction of β -core fragment resulted in its dissociation into two fragments between 8-12kDa and 5-6kDa molecular weights (Endo, *et al.*, 1992).



Fig. 1. Amino acid sequence for hCG β -subunit, (-) indicates the site of trypsin digestion. The amino acid residues which are different from those in LH are in italic and red. The site of glycosylation is indicated by g.

In our method, hCG was subjected to reduction, alkylation and digestion using trypsin enzyme prior to tandem mass spectrometry analysis. The expected peptide fragments derived from the digestion were listed in Table 1. A total of 16 peptides and glycopeptides

fragment were expected from the tryptic digestion of hCG, these peptides were (S **K** E P L R P **R**), (C R P I N **g** A **T** L A V E K), (E G C P V C I T V N **g** T T I C A G Y C P T M **T** R), (V L Q **G** V L P **A** L P Q V V C **N** Y R), (D V R), (F E S I R), (L P G C P R), (G V **N** P V V S **Y** A V A L S C **Q** C A L C R), (R), (S **T** **T** D C G G P K), (D H P L T C D **D** P R), (**F** **Q** **D** **S** **S** **S** **S** **g** K), (**A** **P** **P** **P** **S** **g** L P S P **S** **g** R), (**L** **P** **G** **P** **S** **g** D T P I L P Q). (note: amino acid residue in red is different from that of LH while **g** indicated the site of glycosylation). Amongst these peptides, only 10 peptides show at least one amino acid residue different from that of LH, the difference in amino acid residues can be used to distinguish hCG from LH in tandem mass spectrometry analysis.

Position no.	[M + H] ⁺	Selected data dependent parent ions	Sequence
1-2	234.1	-	SK
3-8	767.4	[384.5] ²⁺	EPLRPR
1-8	983.2	[491.8] ²⁺	SKEPLRPR
9-20	Glycopeptide	-	^a CPINATLAVEK
21-43	Glycopeptide	-	^a EGCPVCITVNTTICAGYTCPT MTR
44-60	1928.4	[964.7] ²⁺	VLQGVLPALPQVVCNYR
61-63	389.2	[389.2] ⁺	DVR
64-68	651.3	[326.1] ²⁺	FESIR
69-74	700.8	[350.5] ²⁺	LPGCPR
75-94	2228.6	[743.5] ³⁺	GVNPVVSYAVALSCQCALCR
95	175.2	-	R
96-104	924.0	[462.5] ²⁺	STTDCGGPK
105-114	1227.3	[614.1] ²⁺	DHPLTCDDPR
115-122	Glycopeptide	-	^b FQDSSSSK
123-133	Glycopeptide	-	^b APPPSLPSLSR
134-145	Glycopeptide		^b LPGPSDTPILPQ

a shows N-link glycopeptide at the bold amino acid
b shows O-link glycopeptide at the bold amino acid/s

Table 1. Predicted tryptic digested βhCG fragments.

4.1 Selection of peptide marker

In the selection of suitable peptide marker for simultaneous qualitative and quantitative analysis of protein, three criteria are implemented:

- a. The amino acid sequence of the peptide marker must be unique to the protein. This is important as the marker will be used in the qualitative analysis to differentiate the protein of interest from other unrelated proteins.
- b. The length of the peptide marker must be suitably long. The long marker will lead to generation of a more comprehensive spectrum of product ions which will give higher confident level in the qualitative analysis. Moreover, the generation of greater number of product ions will also lead to greater choice of quantifying ions to be used in subsequent quantitative analysis.

- c. The degree of ionization of the peptide marker. Only the peptide marker that can be easily ionized by the ionization mode of mass spectrometer to produce high abundant molecular ions will be selected as peptide marker. This is important in the analysis of protein from complex biological sample, where interferences from the matrix and chemical noise may mask the low abundance ions.

The β -subunit of hCG has an extension of 30 amino acid residues at its C-terminus compared to LH. This distinctive region of hCG is used as the antigenic epitope for raising of hCG-specific antibodies. This region is highly glycosylated and following trypsin digestion, a total of 3 glycopeptides, **FQDSSSSgK**, **APPPSgLPSPSgR**, **LPGPSgDTPILPQ** are expected to be formed from this particular region. However, we did not detect any of these glycosylated peptides in MS scan, the possible reason is the presence of carbohydrate in the peptides suppressed the ions signals of glycopeptides and therefore reduced the detection of these glycopeptides.

On the other hand, the molecular ions of two other glycopeptides **CRPINgATLAVEK** and **EGCPVCITVNgTTICAGYCPMTTR** derived from the β -subunit of hCG were detected in different glycoforms, which indicates more than one type of sugar moieties were present in each individual glycopeptide. However, when subjecting these molecular ions to MS/MS scan, there is no useful MS/MS data to indicate the identity of these glycopeptides. Once the sugar moieties were removed (deglycosylation), four out of the five deglycosylated glycopeptides derived from the β -subunit of hCG were detected (Gam *et al*, 2006). Nevertheless, the deglycosylation process was time consuming and also resulted in sample lost. Hence, the glycopeptides do not serve as suitable marker for hCG.

In terms of peptides, only the peptides that are differ in at least one amino acid residue were targeted in the analysis. This is possible as a different in one amino acid residue will be resulted in a different product ions masses upon tandem mass spectrometry analysis. An example is for a peptide sequence of **VLQGVLPA LPQVV CN YR** of hCG. The amino acid residues in red are the different amino acid residues than those of LH, the expected product ions series of these closely resemblance peptides of hCG and LH is listed in Table 2.

It is clear that out of 32 product ions expected from the peptides, there were only three identical product ions between hCG and LH, namely b_1^+ , b_2^+ , b_3^+ product ions. The extensive difference in the product ions series between the seemingly similar peptides of hCG and LH was great, which allow distinctive identification of these two closely resemblance hormones. Due to the high similarity between the two hormones, false identification of LH as hCG in ELISA was reported as a result of cross reaction of hCG antibodies with LH (Bottger *et al*, 1993).

Peptide **SEPLRP R** was formed from an incomplete digestion of β -subunit at the 2nd to 3rd amino acid residue. It is reasonable to suggest that the formation of this peptide was not due to insufficient enzyme used but rather it was due to the presence of a carboxyl side chain group (glutamic acid, E) on the digestion site, which remarkably reduced the rate of hydrolysis. The use of this peptide as hCG marker may not be suitable as its formation may vary due to incomplete trypsin digestion. In general, taking into the consideration of all the peptides and glycopeptides derived from hCG, only 4 peptides fulfilled the first two criteria (A and B) of peptide marker, these peptides were (**VLQGVLPA LPQVV CN YR**), (**GVNPVVS YAVALSC QCALCR**) (**ST TDCGGPK**), (**DHPLTCD DPR**).

y-fragment ions	M/z LH	M/z hCG	b-fragment ions	M/z LH	M/z hCG
y ₁ ⁺	175.1	182.1	b ₁ ⁺	100.1	100.1
y ₂ ⁺	338.1	337.4	b ₂ ⁺	213.2	213.3
y ₃ ⁺	439.2	451.5	b ₃ ⁺	341.2	341.4
y ₄ ⁺	560.2	613.7	b ₄ ⁺	412.3	398.5
y ₅ ⁺	699.3	712.9	b ₅ ⁺	511.3	497.6
y ₆ ⁺	780.3	812.0	b ₆ ⁺	624.4	610.8
y ₇ ⁺	926.4	940.1	b ₇ ⁺	721.4	707.9
y ₈ ⁺	1023.5	1037.2	b ₈ ⁺	818.5	779.0
y ₉ ⁺	1136.5	1150.4	b ₉ ⁺	931.6	892.1
y ₁₀ ⁺	1233.6	1221.5	b ₁₀ ⁺	1028.6	989.3
y ₁₁ ⁺	1330.6	1318.6	b ₁₁ ⁺	1156.7	1117.4
y ₁₂ ⁺	1443.7	1431.7	b ₁₂ ⁺	1255.8	1216.5
y ₁₃ ⁺	1542.8	1530.9	b ₁₃ ⁺	1300.8	1315.6
y ₁₄ ⁺	1613.8	1587.9	b ₁₄ ⁺	1515.8	1476.8
y ₁₅ ⁺	1683.9	1716.1	b ₁₅ ⁺	1616.9	1590.9
y ₁₆ ⁺	1855.0	1829.1	b ₁₆ ⁺	1779.9	1754.1

Table 2. The predicted product ions of V L Q **G** V L P **A** L P Q V V C **N** Y R and V L Q **A** V L P **P** L P Q V V C **T** Y R of hCG and LH, respectively.

Figure 4 shows the total ion chromatogram (TIC) of the tryptic digested peptides of hCG, a total of 3 out of 4 potential peptide markers were detected, where V L Q **G** V L P **A** L P Q V V C **N** Y R were presented as the most abundant ion followed by G V **N** P V V S **Y** A V A L S C **Q** C **A** L C R as the second most abundant ion in the analysis. Nevertheless, the detection of G V **N** P V V S **Y** A V A L S C **Q** C **A** L C R peptide by mass spectrometry is anomalous. In some analyses, the peptide cannot be detected. The rationale for this irregularity in the detection of this peptide is not understood, therefore the use of this peptide as hCG marker is not indicated. Hence, in terms of the ease of peptide ionization (criteria C), V L Q **G** V L P **A** L P Q V V C **N** Y R was found to be the most suitable peptide marker for hCG.

The molecular ion of V L Q **G** V L P **A** L P Q V V C **N** Y R peptide was [964.7]²⁺ while the corresponding peptide of LH has an amino acid sequence of V L Q **A** V L P **P** L P Q V V C **T** Y R and the doubly charged ion was [977.6]²⁺. This LH peptide differs from the hCG peptide by 3 amino acid residues (red). With these minor differences, a completely different set of parent ions and product ion spectra were expected (Table 2). These differences can be used to distinguish hCG from LH. Therefore, this peptide is unique to hCG for tandem mass spectrometric qualitative and quantitative analysis. Furthermore, the long amino acid sequence (17 amino acid residues) of the peptide increases the method specificity by preventing false identification of the peptide. In addition, the high m/z ratio of its parent ion avoids interference from chemical noise, which normally found at low m/z ratios. All these features of the peptide fulfilled the requirement of peptide marker. Hence, this peptide was selected as the peptide marker for identification and quantification of hCG.

Nicking of hCG occurs at position between 47-48 amino acid residue of the β-subunit. After tryptic digestion, the nicked hCG peptide which comprises amino acid residue 48 to 60 was formed. This amino acid sequence is within the amino acid sequence of V L Q **G** V L P **A** L P

Q V V C **N** Y R peptide (from amino acid residue 44 to 60). For this reason, the nicked hCG peptide produces the y^+ product ions (y_1^+ to y_{12}^+) which are identical to some of the y^+ product ions of V L Q **G** V L P **A** L P Q V V C **N** Y R peptide. The similarity in the y^+ ions of the two peptides did not interfere with the quantitative result. This is because the specificity of the data dependent mass spectrometric method is able to eliminate the interference of nicked hCG peptide as the two peptides have distinctly different parent ion masses ($[964.7]^{2+}$ and $[765.9]^{2+}$).

Figure 2 (lower panel) shows the MS/MS spectrum of V L Q **G** V L P **A** L P Q V V C **N** Y R peptide, where the fragmentation of the peptide produced a comprehensive product ions spectrum, which provides a convincing identification of the peptide, and subsequently hCG. Once the targeted peptide markers were identified, the subsequently analysis of protein using tandem mass spectrometry can be carried out. Since this method only emphasizes tandem mass spectrometry analysis and its data interpretation, any peptides chromatography separation device can be applied. In our laboratory, we used either HPLC or 2D-LC dependent on the quantity of the protein being analyzed, in 2D-LC, we included an enrichment column prior to chromatographic separation by reversed-phase column suitable for separation of peptides. This device helps to concentrate the peptides and therefore increase the sensitivity of the analysis method. The parameter for HPLC separation method for hCG analysis was 20 $\mu\text{l}/\text{min}$ through-column flow rate at a gradient of 5-95% B for 20 minutes and held at 95% B constant for 5 minutes. Mobile phase A was 0.05% TFA in H_2O and B was 0.05% TFA in ACN. The column used was C-18 Vydac column (300 \AA , 5 μm , 1 mm X 50 mm).

As for the parameters for tandem mass spectrometry analysis, it is advice to apply the optimum parameters for respective mass spectrometers. In our laboratory, an ESI - ion trap mass spectrometer (LCQ, ThermoQuest) is used, where data dependent experiment method consisting of MS and MS/MS scan was created. The peptide markers ions were programmed and MS data were acquired at heating capillary temperature 200°C, sheath gas flow rate was 60 arb, spray voltage at 4 kV, tube lens offset was -60 V and capillary voltage was at 38 V. MS/MS scan was conducted at default collision energy of 25, charge state of 2, minimum signal acquired was 1×10^5 counts, isolation width was 2 m/z.

4.2 Quantitative and qualitative analysis of hCG using selected peptide marker

The qualitative and quantitative experimental method involved the introduction of the marker to the tandem mass spectrometric analysis. This is to obtain product ions spectrum that reveals the amino acid sequence of the peptide marker, which will then confirm the presence of hCG. The experiment was carried out in the data dependent acquisition mode, where the parent ion mass of the marker was programmed in the parent ion list. The programmed software will run in such a way that as the parent ion is detected in the full scan MS, they will be selectively excited for the MS/MS scan. The resulting product ions produced by CID fragmentation of the parent ion will be shown as the MS/MS spectrum that provides characteristic of the amino acid sequence of the peptide.

The determination of amino acid sequence of the peptide marker was included in hCG confirmatory analysis to avoid the possibility of producing false positive results, which is more likely to happen in selected ion monitoring (SIM) data acquisition method. SIM depends solely on the parent ion mass and the retention time of the compound of interest, which results in the loss of qualitative information of the compound (Gam et al 2003).

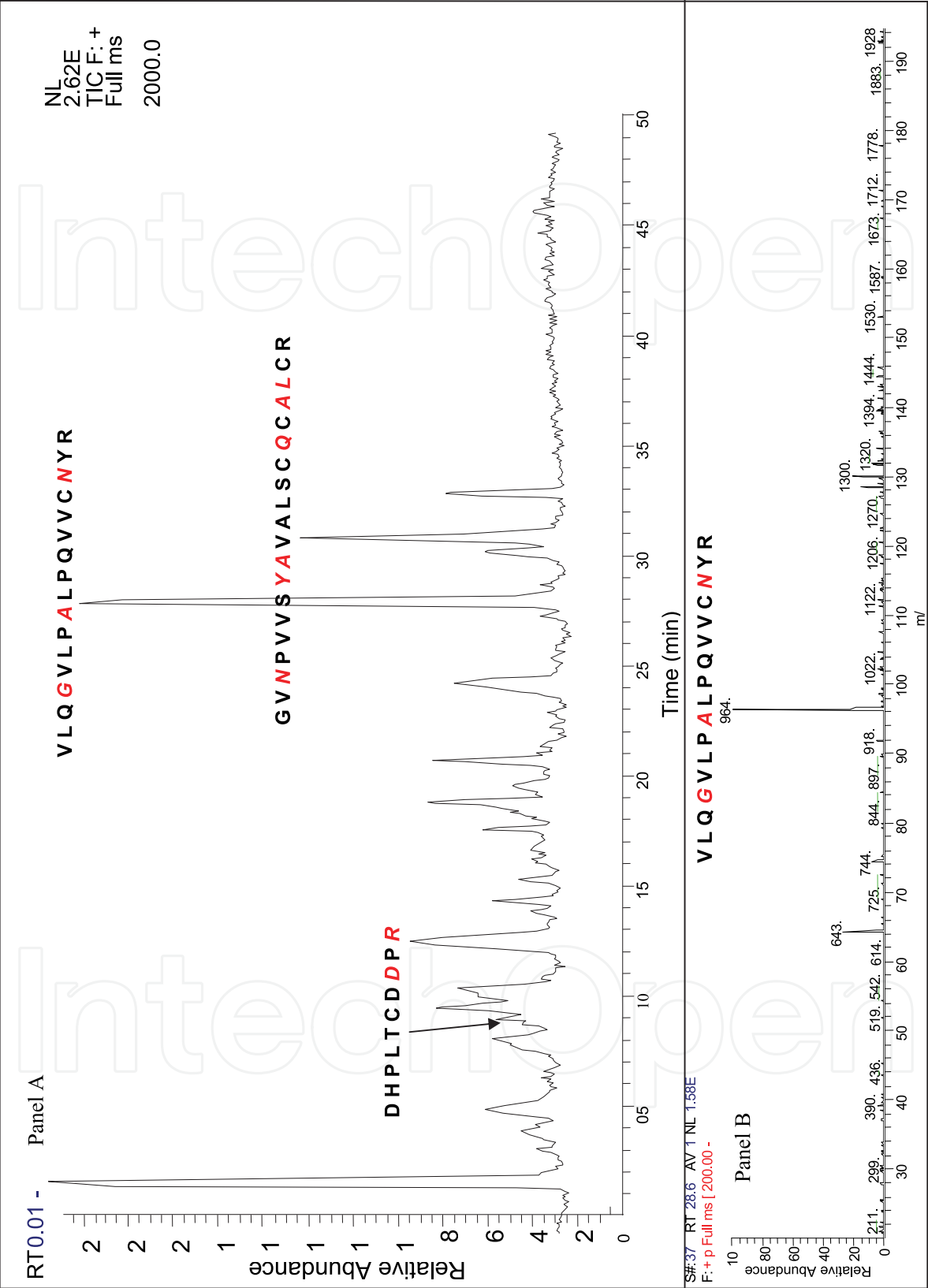


Fig. 2. Panel A; Based Peak Chromatogram for tryptic digested peptides. The y-axis is relative abundance and x-axis is retention time. Panel B; spectrum for peptide VLQGVLPALPQVVCNRYR. The y-axis is relative abundance and x-axis is mass to charge ratio.

Full scan MS/MS not only gives enough information for the qualitative data of hCG but at the same time, the intensity of the product ions of the peptide marker were used to quantify concentrations of hCG. The quantitative method using product ions spectrum allows relatively low quantification limit as compared to SIM method. This is because the MS/MS experiment is a technique that will minimize or eliminate all chemical and background noises.

For quantification purpose, three most abundant product ions of $[964.7]^{2+}$, namely b_6^+ , b_9^+ and y_{11}^+ with the m/z ratios of $[610.3]^+$, $[891.5]^+$ and $[1317.8]^+$ respectively were selected as the quantitative markers. These ions can be evaluated individually by peak area display in the extracted product ion chromatogram or by the summation areas of the three product ions (Figure 3). The summation of three product ions increases the total peak area and therefore greatly reduced the quantification limit as the signal to noise ratio is tremendously increased.

At 5 mIU/mL which approximately equal to 1 pg/mL of hCG in urine matrix, the signal to noise ratio for each product ions chromatogram of the peptide marker were exceeded one hundred (Figure 3, upper panel). The high signal to noise ratio reveals that this method would be able to detect a much lower concentrations of hCG. This detection limit of hCG (5 mIU/mL) using our current method is superior than SIM method where a detection limit of 25 mIU/mL was reported (Liu & Bowers 1997).

At 5 mIU/mL hCG, the parent ions of the peptide marker was indistinguishable from the background noise in full scan MS. Nevertheless, as long as the parent ion intensity surpasses the threshold set, the ion will be isolated and excited to data dependent scan to generate MS/MS data. The MS/MS data obtained not only gives identification to the peptide; furthermore, the product ions were used to quantify the protein concentration. At 5 mIU/mL hCG concentration, our currently developed method yields minimal, if there is any background interference. Quantitative analysis of hCG using SIM method at this concentration is not possible due to the same reason discussed above, where the parent ion was indistinguishable from the background noise, in this situation, the signal to noise ratio valid for quantification analysis could not be established.

Using this approach, we are able to conduct simultaneous qualitative and quantitative analysis on protein. The qualitative data (MS/MS scan data) confirmed the identity of the protein via its unique peptide marker while the product ions (quantifying ions) of the peptide marker were subjected to product ion extracted chromatogram to generate quantitative data of the protein. This approach avoids false quantification of ions, which is possible in SIM.

4.3 Method validation

A standard curve was constructed using hCG at 5 mIU/mL (1pg/mL), 8 mIU/mL (1.6 pg/mL), 10 mIU/mL (2 pg/mL), 15 mIU/mL (3 pg/mL), 20 mIU/mL (4 pg/mL) and 30 mIU/mL (6 pg/mL) concentrations. The protein was subjected to tryptic digestion and analyzed by HPLC/MS/MS according to the method described. In which, the peptide marker was eluted from the column at 12 minutes retention time.

Each of the standard points was performed in triplicate. Tables 3 to 6 show the peak areas (triplicate) of the extracted product ions chromatograms for the chosen quantifying ions. The reliability of each product ions as a quantitative marker was measured by their coefficient of variance (r^2) values. The coefficient of variance for $[610.0]^+$, $[891.8]^+$, $[1317.8]^+$ and the summation of $[610.0]^+ + [891.8]^+ + [1317.8]^+$ were 0.998 (Figure 4), 0.993 (Figure 5),

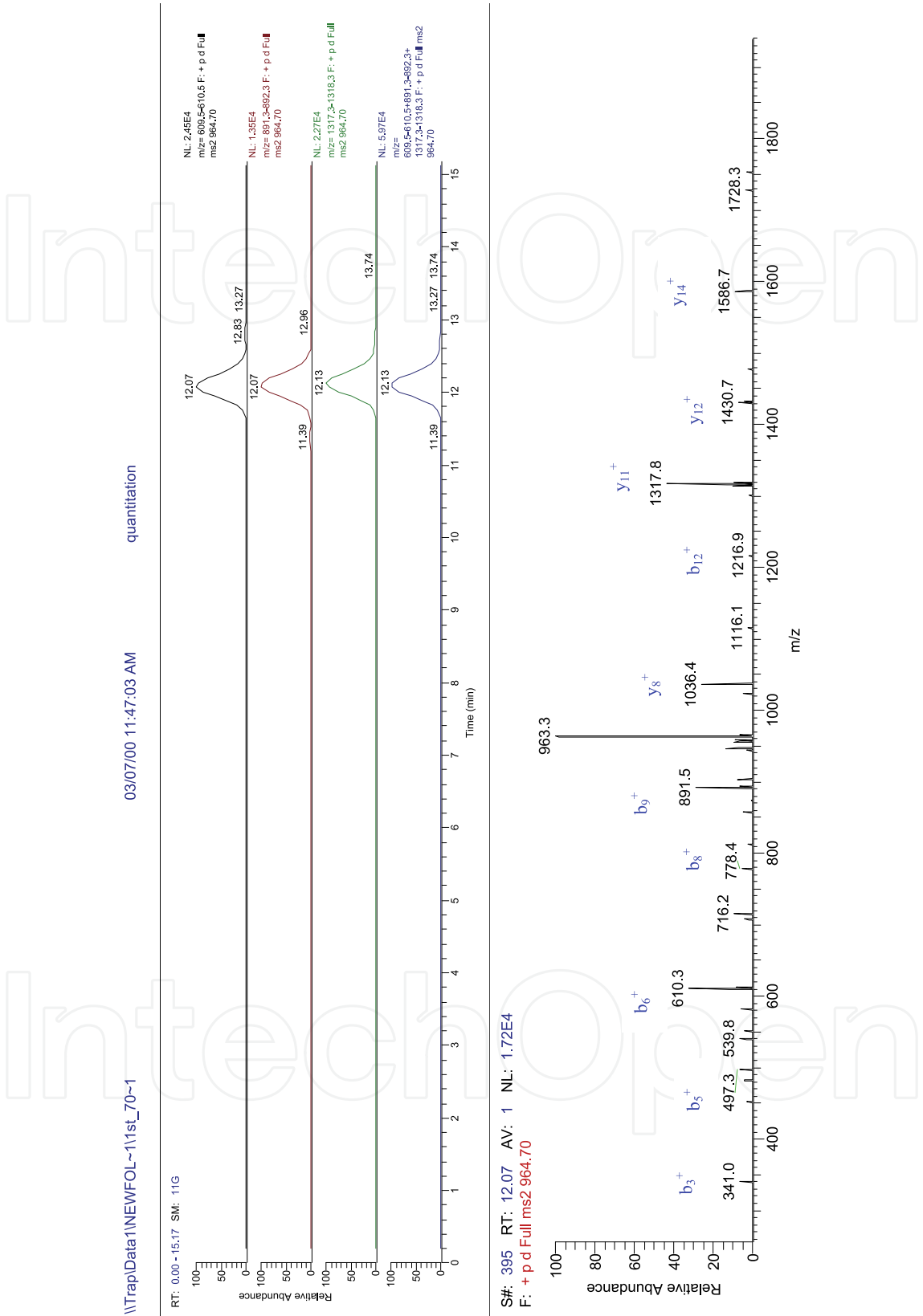


Fig. 3. Data for quantitative and qualitative analysis of hCG at 5 mIU/mL. Upper panel: extracted product ions chromatogram; Lower panel: MS/MS spectrum of the selected peptide marker (Gam *et al*, 2003).

0.997 (Figure 6) and 0.995 (Figure 7), respectively. Thus, it is obvious that the intensity of the product ions formed correlates well with the concentrations of hCG. The reliability of using the selected product ions as the quantitative markers is remarkable as the precision (C.V) of each standard point in triplicate is always < 10% (Tables 3 to 6). The linearity of the standard curve was only obtained in a narrow range of hCG concentration between 5 mIU/ml to 30 mIU/ml. Above this range, the curve deviates from linearity.

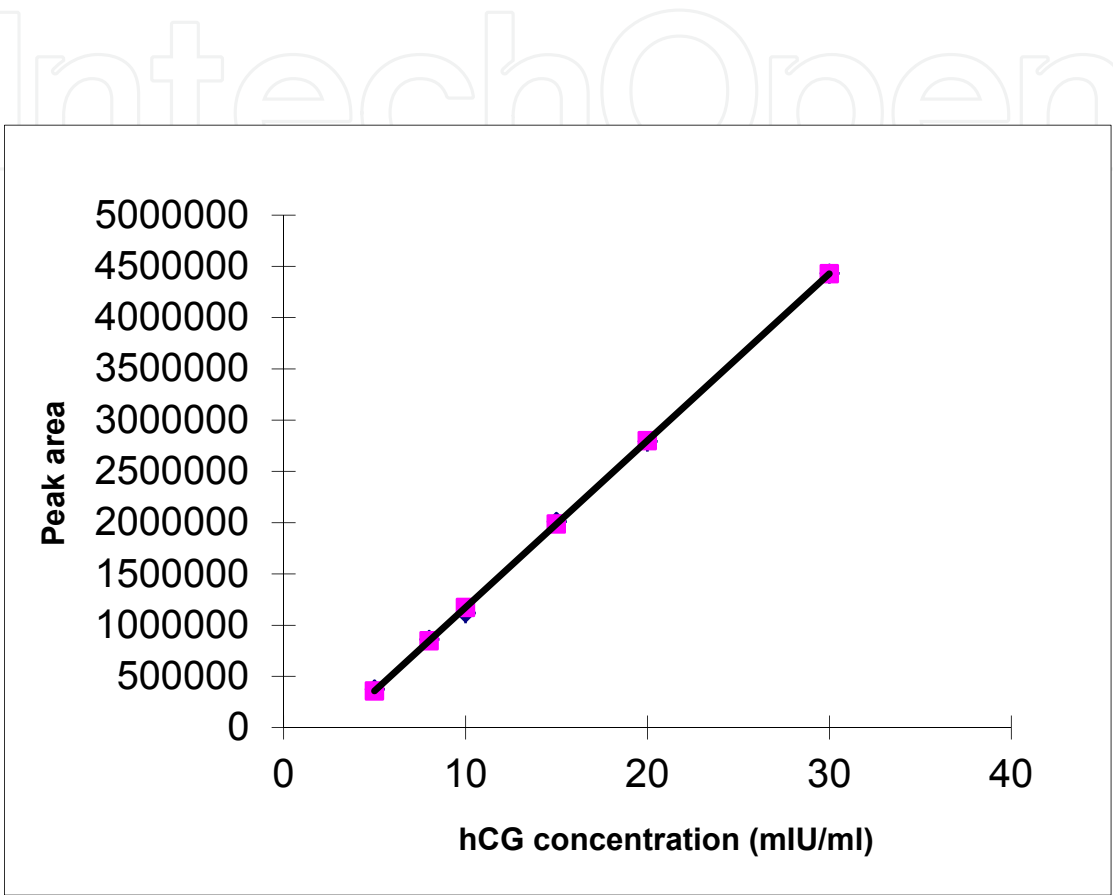


Fig. 4. The standard curve constructed using the b_6^+ product ion. Intercept = -457221, X variable = 1628623, $r^2 = 0.999647$.

hCG mIU/ml	Peak area			means	SD	CV
5	364576	395481	367517	375858	17057.52	5 %
8	856239	894572	826756	859189	34004.11	4%
10	1173659	1127543	1055078	1118760	59776.41	5%
15	1995623	1973176	2060943	2009914	45595.35	2%
20	2748931	2865973	2762191	2792365	64090.25	2%
30	4468624	4487696	4341096	4432472	79706.43	2%

Table 3. Standard curve data quantify using the b_6^+ , $[610.0]^+$ product ion.

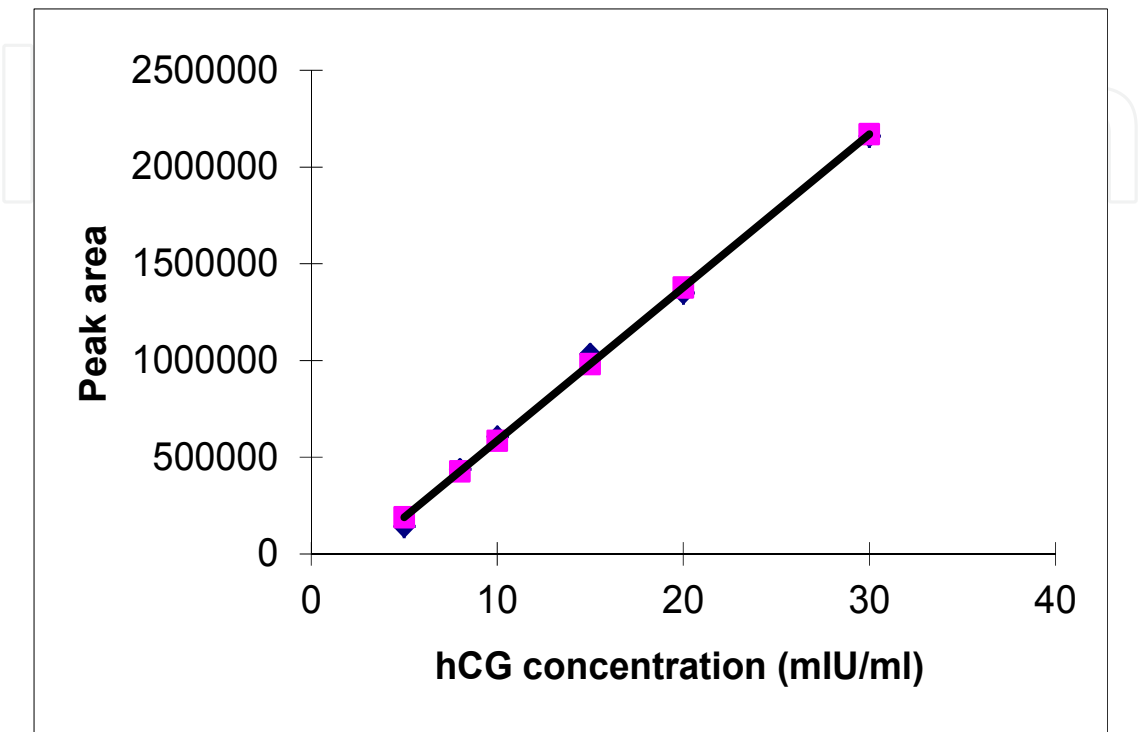


Fig. 5. The standard curve constructed using the b_9^+ product ion. Intercept = -207664, X variable = 79251.53, $r^2 = 0.99765$.

hCG mIU/ml	Peak area			means	SD	CV
	140221	152539	134155			
5	419835	449395	442145	437125	15406.13	4%
8	623484	601678	594782	606648	4876.21	1%
10	1017695	1013689	1065890	1032425	36911.68	4%
15	1280853	1400054	1365535	1348814	24408.62	2%
20	2198756	2231539	2052210	2160835	12680.80	6%
30						

Table 4. Standard curve data quantify using the b_9^+ , $[891.8]^+$ product ion.

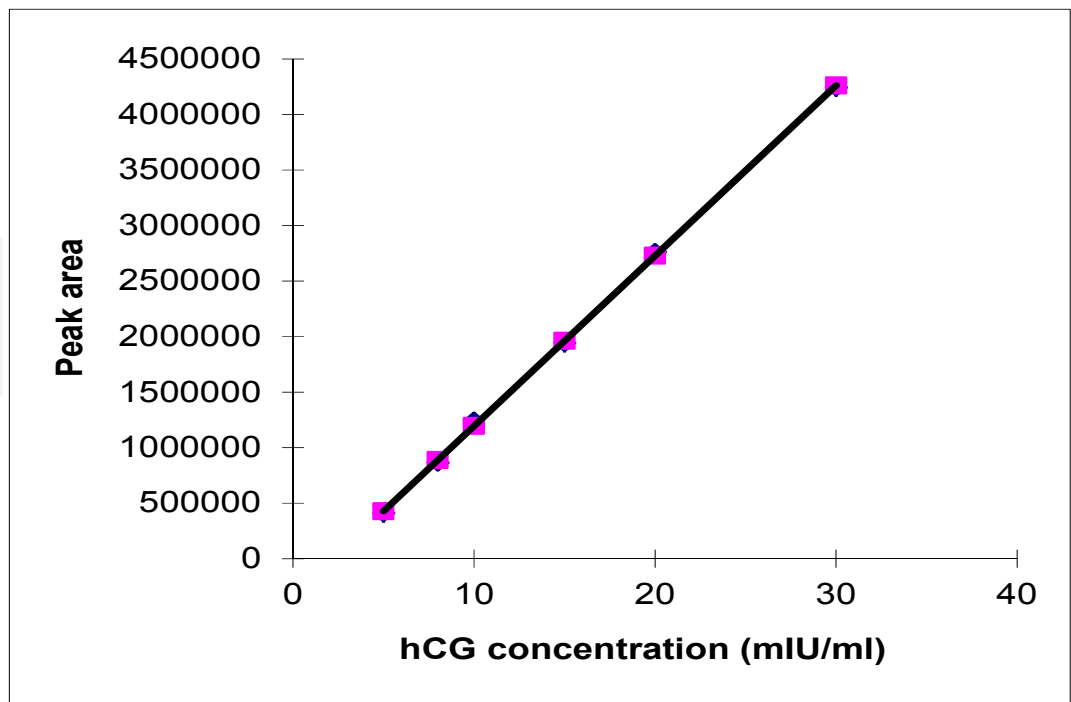


Fig. 6. The standard curve constructed using the y_{11}^{+} product ion. Intercept = -338677, X variable = 153377.7, $r^2 = 0.999483$.

hCG mIU/ml	Peak area			means	SD	CV
5	396514	419764	411420	409233	5900.10	1%
8	859176	883765	850385	864442	23603.22	3%
10	1327647	1262137	1141016	1243600	85645.48	7%
15	2032478	1863586	1929342	1941805	46496.51	2%
20	2759796	2789313	2737455	2762188	36669.14	1%
30	4245795	4353626	4132309	4243910	156494.8	4%

Table 5. Standard curve data quantify using the y_{11}^{+} , $[1317.8]^{+}$ product ion.

5. Future work

I believed the method demonstrated here will be of help to protein chemists whom struggle in protein quantitative analysis, especially for analysis of trace amount of protein in complex biological sample. This method may be useful not only in doping analysis for hCG, it can be applied to other doped proteins such as erythropoietin, growth hormone and ext. This is because an accurate quantitative data (definite amount of doped substances) is needed in doping analysis to differentiate between endogenous and exogenous protein, a fine line between doped and non-dope level. At this time, my works are mainly focused on the identification of biomarkers in diseases, where the biomarkers can be used as diagnostic markers or therapeutic markers for the diseases. The quantitative data of the expression of the biomarkers are important in determining the usefulness of individual biomarker, therefore, this developed method has created a good platform for conducting quantitative analysis on the identified biomarkers.

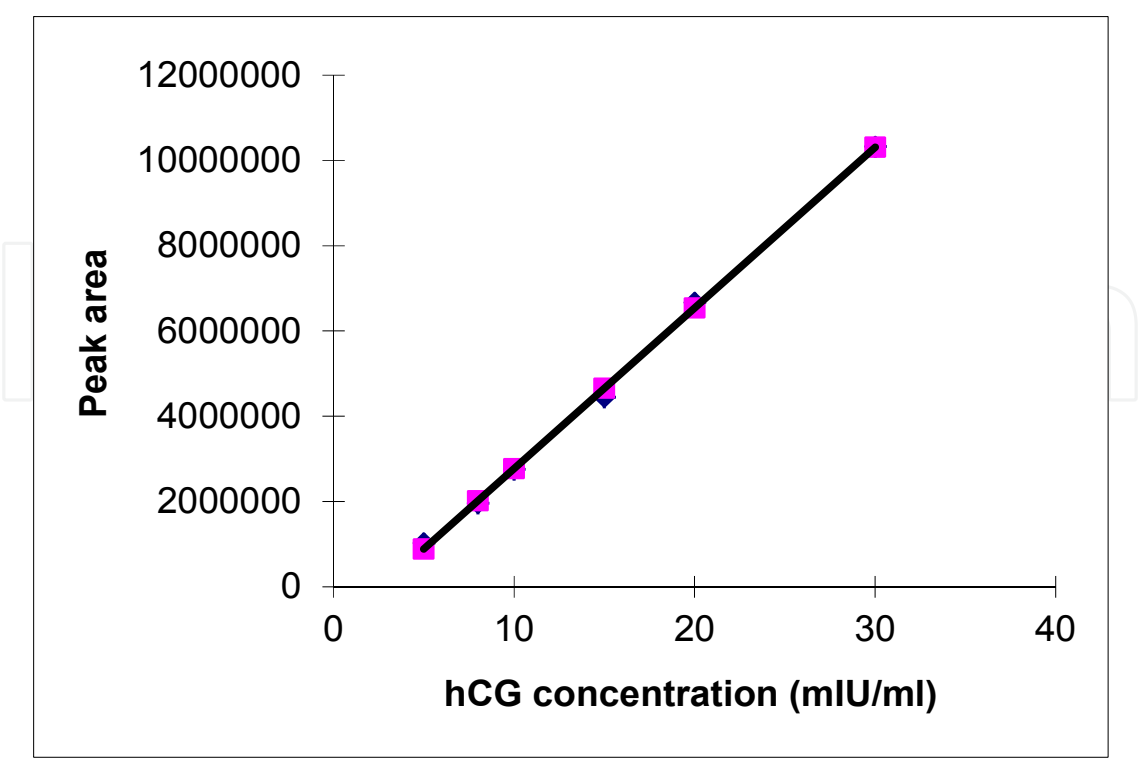


Fig. 7. The standard curve constructed using the summation of three product ions. Intercept = -1000044, X variable = 376994.7, $r^2 = 0.998635$.

hCG mIU/ml	Peak area			means	SD	CV
5	985703	1083863	998570	1022712	60311.26	6%
8	1903745	2014766	1954676	1957729	42490.05	2%
10	2869837	2729474	2664510	2754607	45963.48	2%
15	4375897	4400165	4563987	4446683	115839.60	3%
20	6542834	6742038	6713948	6666273	19862.62	0%
30	10428756	10514285	10038730	10327257	336238.20	3%

Table 6. Standard curve data quantify using the summation of three product ions: b_6^+ , b_9^+ and y_{11}^+ .

6. Conclusion

Using hCG as an example, the approach for simultaneous qualitative and quantitative analysis of protein by using tandem mass spectrometry has been demonstrated. Since the qualitative data for identification of protein was carried out through product ions profiling which revealed the amino acid sequence of the protein, this analysis method give a high confident level of protein identity. Subsequently, the high abundant product ions are selected as quantifying ion for quantitative analysis of the protein. This quantification approach eliminates all background noises. This is because the quantifying ions were chosen from the fragmentation of the selected peptide marker, which was isolated from the remaining ions in MS scan and excited to collision induced dissociation. Using this

approach, it is not possible to quantify a false ion, where only the correct parent ion will produce the expected product ions profile and subsequently the product ions were chosen as quantifying ions. Thus, our current method satisfies both the qualitative and quantitative requirements for protein analysis, which normally can be achieved for one aspect but not the other.

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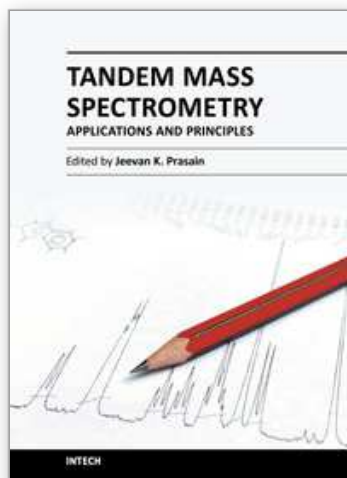
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Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

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