Getting the best of both worlds: Ensuring the perfect partnership of chromatographic separation and MS detection for protein quantification

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The absolute quantification of proteins by stable isotopically labelled peptides as calibrators and selected reaction monitoring experiments has become a method commonly applied for the quantification of standard proteins [1,2], proteins in complex matrices [3] or post-translational modifications [4]. Assuring traceability, or the anchoring of the "absolute amount of substance" to the Système international d'unités (SI), and robustness in protein quantification is still a complex task however.
This article describes the digestion steps and the chromatographic and mass spectrometry conditions which need to be optimised when traceable quantification of proteins with minimised uncertainty has to be performed.
Examples will be given where optimising chromatographic and mass spectrometry conditions can help improve the overall confidence for the absolute quantification of proteins using stable isotopically labelled peptides as standards. The performance of a number of columns and critical mass spectrometry parameters such as number of points across the peak and scan time, and their influence on ratio measurements, and therefore measurement uncertainty will be discussed.

INTRODUCTION

Liquid chromatography mass spectrometry (LC-MS) based proteomics has evolved as an important tool to support molecular and cellular biology. While improvements in LC-MS technical platforms and bio-informatic tools have significantly contributed to the development of robust methods for bio-marker discovery and cell expression profiling, an increasing interest has emerged in protein quantification for clinical, environmental and nutritional applications.

Progress in this area has aided by the use of stable isotopes via tagging (ICAT [5], ICPL[6], iTRAQ[7]) or synthetic isotopically labelled peptides as internal standards. These strategies have been successfully applied for the quantitative comparison of protein samples and an example has been reported where iTRAQ has been used for absolute quantification of a protein by using standard peptides and monitoring the iTRAQ derivatisation reaction [8].

Currently the most common and successful method for absolute protein quantification is based on the use of stable isotopic labelled peptides as standards. This approach has been introduced by Dr. Steve Gygi and colleagues at Harvard Medical School [9] and is based on the selection of a tryptic digest peptide as a stoichiometric representative of the protein from which it is enzymatically cleaved. Quantification is performed by adding isotopically labelled internal standard peptides before digestion. C-reactive protein [10], apolipoprotein A [11] and prostate specific antigen [12] are examples of proteins quantified in plasma or serum using this approach.

LGC and other National Measurement Institutes (NMIs) have further developed the method introduced by Gygi et. al. by applying the principles of exact matching isotope dilution mass spectrometry [1,2,13], where quantification is performed by using traceable synthetic and isotopically labelled peptides as standards. Due to the lack of commercially available traceable quantified standard peptides, a method has been developed to quantify peptides employing traceable standard amino acids. Single peptides of known sequence are digested by oven or microwave and isotope dilution mass spectrometry combined with GC-MS or LC-MS/MS analysis is performed [1,14].

Selection of which peptides to use to represent the protein intended for quantification is a critical step in absolute protein assay. Ideally the selected peptides will be stable during digestion, will not be extensively modified and will be completely cleaved from their neighbouring peptides on digestion. Failure to realise any of these conditions will result in unwanted method bias and/or an increased measurement uncertainty. If working in complex mixtures of proteins, the necessity for the peptides to be unique to the protein of interest must also be considered. Time course experiments are generally performed to control the stability of the peptides during digestion and to help assess their complete release from the protein [8].

The equimolar release of the peptides (the number of peptides selected is often a judgment based on protein size and the position of the peptide in the protein

sequence. A minimum of two peptides should always be used) is generally a good indicator of the complete release of the peptides from the protein to be quantified. However in some cases ICAT experiments and standard addition experiments have been used to confirm the complete release of the peptides from the protein and to reduce the uncertainty associated to the digestion protocol [13].

Exact matching IDMS involves an iterative procedure culminating in the gravimetric preparation of a calibration blend having the same ion abundance, ratio and amount content of natural and labelled peptides as the sample blend. For protein quantification, the calibration blend contains quantified synthetic peptides and stable isotopic labelled peptides, whereas the sample blend contains the protein to be quantified and the stable isotopic labelled peptides. Both calibration blend and sample blend are digested and analysed by LC-MS/MS using selected reaction monitoring (SRM) or selected ion recording (SIR) experiments [1,8,13].

Results are then obtained by applying the double exact matching IDMS equation

$$C_x = C_z \cdot \frac{m_z}{m_{yc}} \cdot \frac{m_y}{m_x} \cdot \frac{R'_B}{R'_{BC}}$$

where C_x is the amount content (concentration, nmol/g) of the protein to be quantified, C_z is the amount content of the pure standard material in the standard solution, m_z and m_{yc} are respectively the mass of the natural standard and the labelled standard used to prepare the calibration solution, m_y and m_x are the mass of labelled standard and sample in the sample blend and R'_B and R'_{BC} are the measured ratios of the natural : labelled peptides signals in the sample and in the calibration blend.

By using this equation an uncertainty budget can be calculated which considers all the steps involved in the preparation and measurement of the peptides. A typical uncertainty budget for the quantification of small molecules by exact matching IDMS equation is reported in Figure 1. It is possible to observe that the ratio precision is the major component of the uncertainty budget. This is also true when protein quantification is performed provided the selection of peptides has been appropriately carried out, a low uncertainty is associated with the standard amino acids and peptides, and digestion has been properly optimised [13]. Reducing the uncertainty associated to the ratio measurements (natural/labelled) is a crucial task for the work carried out in our laboratories.

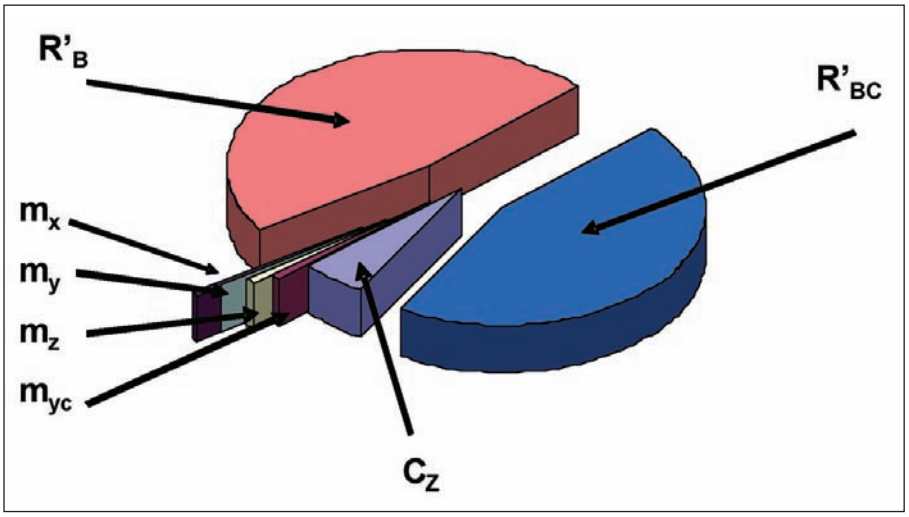

Figure 1. A typical uncertainly budget for exact matching IDMS equation

The main conditions that require optimisation in order to reduce the uncertainty associated with the natural/labelled peptide ratio measurements in the calibration and sample blends are here discussed in detail. The importance of optimising both mass spectrometer and chromatographic conditions is highlighted together with the need of combining innovative chromatographic strategies to improve sample throughput and reduce interferences.

An example is presented where a solution of human growth hormone has been tryptic digested and traceably quantified by using synthetic and isotopically labelled standard peptides [8,13]. Multiple reaction monitoring experiments have been optimised and a comparison of the ratios of natural/labelled peptides obtained by using diverse chromatographic strategies has been assessed. The performance of conventional C18 5µm particle columns has been compared with the performance of 2.5µm, 1.9µm and fused core particle columns.

EXPERIMENTAL

Recombinant human growth hormone (hGH) was purchased from CytoShop and peptides T2 (sequence LFDNAMLR), T2* ([U-13C6, 15N]LFDNAMLR), T13 (TGQIFK), and T13* (TGQI[13C9, 15N]FK), all stated purities of >95%, were custom synthesized (Cambridge Research Biochemicals). Details are reported by Pritchard et. al. [13]

The evaluation of the performance of shell core (Halo 2.7 µm C18 2.1 x 100 mm, Hichrom), 1.9 µm (Hypersil Gold C18 2.1 x 100mm, ThermoFisher), 2.5 µm (Luna C18 2.1 x 100mm, Phenomenex), 5 µm (Atlantis C18 2.1 x 150mm, Waters) particle columns was carried out on a conventional HPLC system (HPLC 1100, Agilent Technologies) by injecting 5 µL of a mixture of synthetic peptides DGPLTGTYR, YVVDTSK, GTDVQAWIR, GFFYTPKAR (Cambridge Research Biochemicals) and by applying water 0.1% formic acid and acetonitrile 0.1% formic acid gradients. Detection of the peptides was performed by UV at a wavelength of 210nm.

Tryptic digestion of human growth hormone, selection of the peptides and optimisation of the digestion are detailed by Pritchard et al. [13]. The peptides selected for the quantification of hGH were TGQIFK and LFDNAMLR. Three sample blends containing 15 µg of hGH and labelled peptides, and the calibration blend containing labelled and synthetic peptides were prepared. Samples were dried (DNA 120SpeedVaC, Thermo Savant) and solubilised in 20µL of 0.5M triethylammoniumbicarbonate (TEAB) at pH 8.5 (Applied Biosystem). 1 µL of 2% sodium dodecyl sulfate (SDS) and 2 µL of 5mM tris-(2-carboxyethyl) phosphine (TCEP) (Applied Biosystem) were added and samples were incubated at 60°C for 1 hour. After addition of 1µL of cysteine blocking reagent (200mM methyl methanethiosulfonate, Applied Biosystem) and 10 µg of trypsin solubilised in water, samples were digested overnight at 37°C.

Absolute quantification of hGH has been performed by selected reaction monitoring experiments on a Waters Quattro Ultima triple-quadrupole instrument coupled with a Waters Alliance 2695 HPLC system. Mobile phases were: (A) water:0.1% formic acid (vol:vol) and (B) acetonitrile:0.1% formic acid (vol:vol). Linear gradient steps of 0–0.2 min

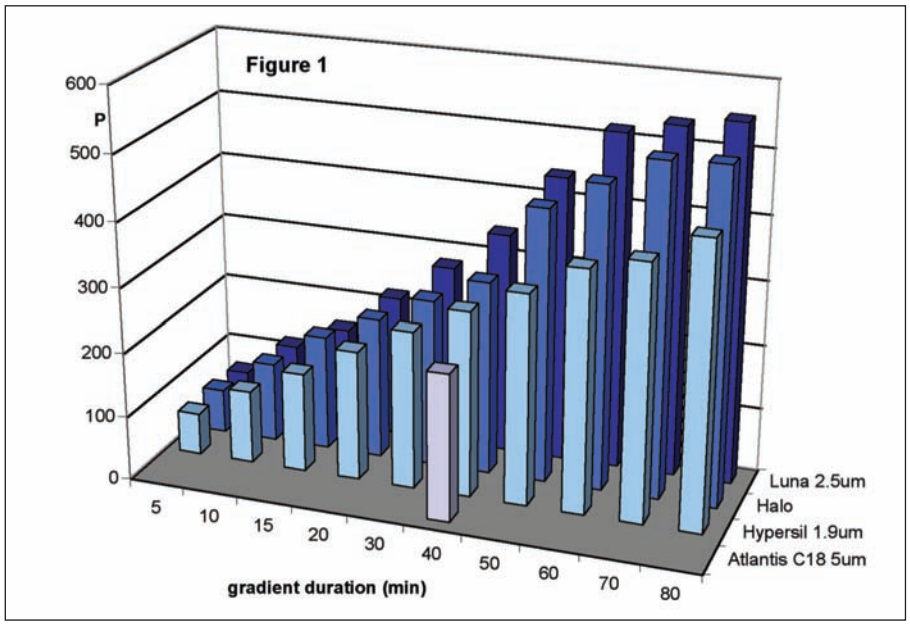

Figure 2. Comparison of the peak capacity (P) of Luna 2.5 µm, Hypersil 1.9 µm, Halo, Atlantis 5 µm columns obtained by injecting a mixture of four synthetic peptides and by applying gradients of 5, 10, 15, 20, 30, 40, 50, 60, 70 and 80min from 100% water 0.1% formic acid to 40% acetonitrile 0.1% formic acid. Flow rate: 0.3 mLmin^{-1}, temperature: 40 °C. Injection: 5 µL.

95% A and 0.2–7min 95%–55% A were applied on the Luna 2.5 µm column. Linear gradient steps of 0-2min 90% A, 2-30min 90-40% A were applied on the Atlantis 5 µm column.

The following transitions were used to monitor for the two peptides: T2 *m/z* 490.3 _233.1, T2* *m/z* 493.8 _ 240.2, T13 *m/z* 347.2 _ 294.1, T13* *m/z* 352.2_304.1 for the Luna 2.5 µm and T2 *m/z* 490.3 _419.2, T2* *m/z* 493.8 _ 419.2, T13 *m/z* 347.2 _ 294.1, T13* *m/z* 352.2_304.1 for the Atlantis 5 µm. A collision energy of 18 V and dwell time of 0.1 s were used for all transitions

RESULTS AND DISCUSSION

The tryptic digestion of the proteins to be quantified and the appropriate selection of the standard peptides to be used are undoubtedly critical steps to be optimised in order to obtain reliable results. However chromatographic and mass spectrometry parameters play an important role in the development of robust LC-MS based methods where low uncertainty/good precision is desirable.

Although triple quadrupole mass spectrometers are generally used to quantify small molecules and peptides due to their high selectivity, the selection of the product ions to be monitored, MS and chromatographic conditions have to be carefully chosen and optimised. This is extremely important when quantification of protein via their constituent peptides has to be performed due to the high number of different peptides contained in the samples after tryptic digestion.

Good separation of the peptides to be quantified and use of columns characterised by high peak capacity are generally recommended to reduce the number of SRMs to be monitored at one time and to minimise potential interferences. However, it has to be underlined that transitions also need to be optimised to reduce interferences between the natural and labelled co-eluting peptides.

Quantification of multiply charged peptides can be challenging when triple quadrupole mass spectrometers with nominal mass resolution are employed. To improve ratio reproducibility of multiply charged ions in these cases, it is often desirable to isolate the monoisotopic peak and the associated isotopes of the most abundant charge state.

The resolution of the first quadrupole should be therefore reduced to assure selective transmission of all the isotopes of the multiply charged precursor. This reduces somewhat the selectivity of the SRM and therefore further increases the importance of chromatographic separation, since MS selectivity is often reduced when precursors are isolated in this manner.

Absolute protein quantification by isotopically labelled standard peptides and selective reaction monitoring experiments are generally performed by conventional chromatographic columns (3-5 µm particle size, 2.1 mm inner diameter). This is mainly due to the robustness of these columns, their excellent resolving properties and their optimum operative flow rates, which correspond to the optimum flow rates to be used with the standard electrospray ionisation sources provided with the majority of triple quadrupole mass spectrometers. Despite the advantages associated with these columns, long run times and reduced mass spectrometry utilisation are often a major drawback when several samples have to be analysed. The use of submicron particles and high back pressure liquid chromatography systems to improve efficiency without compromising resolution represents an important alternative.

In order to evaluate the advantages of novel particle column technologies, the performance of a shell core particle column, 1.9 µm particle column and 2.5 µm particle columns were compared with the performance of a standard 5 µm C18 column commonly used in our laboratory for absolute quantification of peptides. Four synthetic peptides DGPLTGTYR, YVVDTSK,

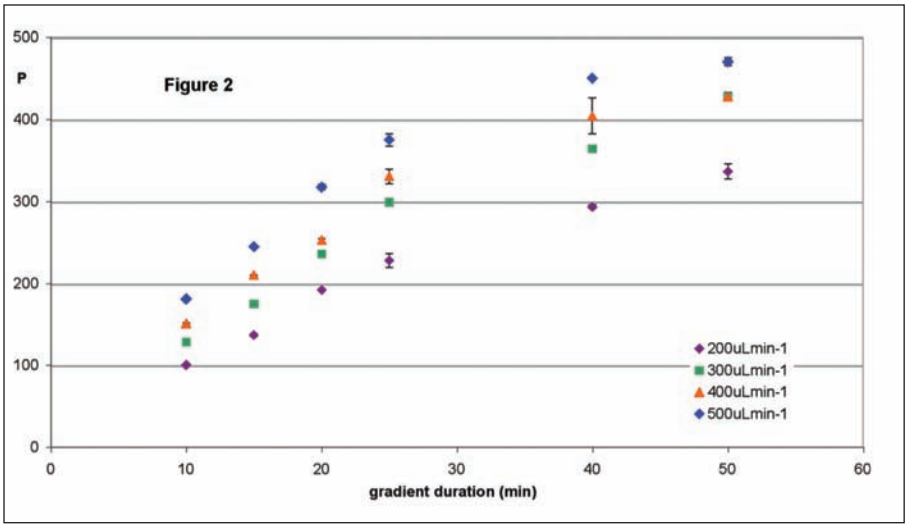

Figure 3. Peak capacity calculated by injecting 5 µL of a mixture of four synthetic peptides (DGPLTGTYR, YVVDTSK, GTDVQAWIR, GFFYTPKA) on a Halo column. A gradient from 100% water 0.1% formic acid to 40% acetonitrile 0.1% formic acid was applied at increasing flow rates (range 0.2 mLmin^{-1}-0.5 mLmin^{-1}).

GTDVQAWIR, GFFYTPKAR were injected and the peak capacity of the columns was calculated as P= 1 + tg/w, where tg is the gradient duration and w is the average peak width [15]. A flow rate of 0.3 mLmin^{-1} was chosen for these experiments, as this was the maximum flow rate compatible with the electrospray sourse of the Ultima triple quadrupole under the applied conditions.

Results are reported in Figure 2. It is possible to observe that the best performing column under these conditions was the Luna 2.5 µm. This is probably due to the low flow rate employed. An increased peak capacity was in fact observed for the Halo and for the 1.9 µm particle columns when increased flow rates were applied (Figure 3).

The performance of the Luna 2.5 µm was compared with the performance of a standard Atlantis column 5 µm for the quantification of a solution of human growth hormone by using two standard synthetic and labelled peptides (TGQIFK T13, LFDNAMLR T2 and TGQI[13C9,15N]FK, [13C6,15N]LFDNAMLR). Digestion optimisation and selection of the standard peptides have been reported by Pritchard et. al. [13]. The separation of the two peptides by using the two columns is reported in Figure 4. In order to compare the performance of the two columns, calibration blends containing natural:labelled peptides at ratio 1:1 and sample blends containing labelled peptides and the digested proteins were injected on both columns and the %RSDs of the area ratios of natural/labelled peptides (n=5) were calculated. %RSD ≤ 1 are generally obtained when standard peptides or small molecules are injected. However, since tryptic digestion of calibration and sample blends is performed, %RSD of ~2 were considered appropriate for this evaluation.

Ratio %RSDs for the calibration blends injected on the Atlantis 5 µm column and on the Luna 2.5 µm column were respectively 2.2 and 1.3 for peptide T2 and 2.3 and 2.0 for peptide T13. Ratio %RSD for the sample blends injected on the Atlantis 5 µm column and on the Luna 2.5 µm column were respectively 1.4 and 1.9 for peptide T2 and 2.7 and 2.0 for peptide T13.

Despite the shorter gradient (7 min vs 40 min), smaller peak width (0.2 min instead of 0.6 min) and the lower number of points across the peaks, no significant changes in the %RSD of the ratios natural/labelled peptides was observed. This is probably due to the better peak shape and therefore integration

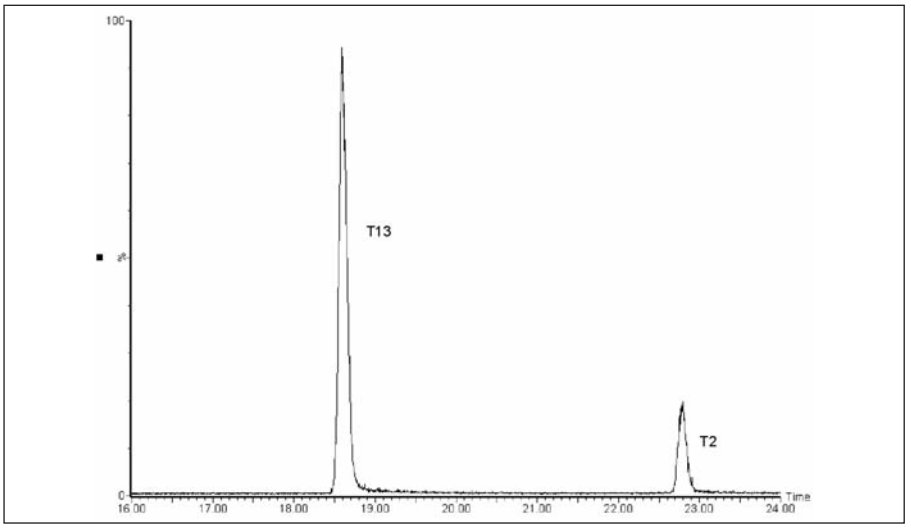

Figure 4. A: SRM experiments of T2 and T13 peptides injected on an Atlantis column 5 µm. B: MRM experiments of T2 and T13 peptides injected on a Luna column 2.5 µm

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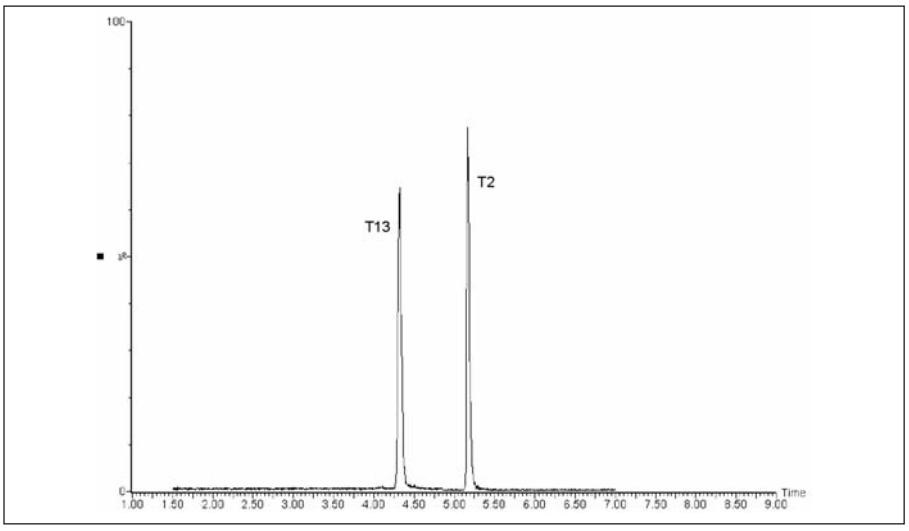

B:

obtained by using the Luna 2.5 µm. The number of points across the peak is a very important factor in the optimisation of the ratio measurements. Desirable ratio precisions (%RSD ≤ 1) are generally obtained by using a minimum of 30 points. Too few points may result in loss of information e.g. peak apex and therefore irreproducible integration. In order to increase the number of points across the peak fast scans can be performed by optimising dwell and electronic settle time in the mass spectrometer. An example of the influence of dwell time (time which the mass spectrometer spends on the selected masses) and span factor (mass window selected to compensate mass shift calibration) on the %RSD of a mixture natural:labelled peptide is shown in Figure 5. It is possible to observe that in this case lower dwell times and higher span factors are preferable. However it has to be underlined that dwell time and span factor need to be optimised for each analyte, chromatographic method and instrumentation.

Absolute quantification of a standard human growth hormone solution was finally achieved by using a Luna 2.5 µm column [13]. The calculated concentration of the sample was 0.69±0.1mg g^{-1}. The major contribution in the uncertainty budget was the uncertainty associated to the purity of the standard amino acids utilised to absolutely quantify the standard peptides. Studies are currently carried out in collaboration with other National Measurement Institutes to improve the uncertainty of the standards used in protein quantification.

CONCLUSIONS

Absolute protein quantification by isotopic labelled standards and exact matching IDMS is still a difficult task when robust methods have to be optimised with minimum uncertainty. Tryptic digestion,

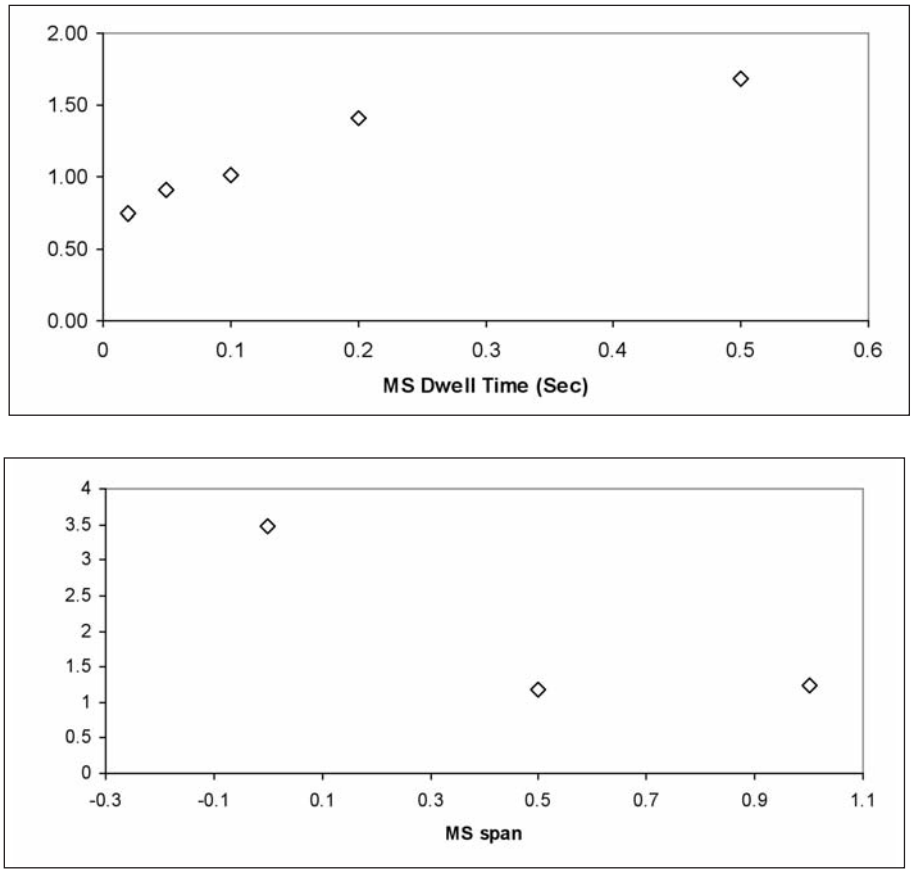

Figure 5: Influence of dwell time and span factor on %RSD of the area ratios obtained by injecting 5 µL of a mixture 1:1 natural:labelled peptide (YVVDTSK).Column C18 Aqua 2.1 x 150mm. Mobile phase: A: water 0.1% formic acid, B: methanol 0.1% formic acid. Flow rate 0.2 mLmin^{-1}

chromatographic and mass spectrometry conditions need to be carefully developed in order to obtain reproducible results. Innovative particle size technology such as fused core particles or submicron particles can improve chromatographic resolution and sample throughput, reduce potential interferences from other peptides and maximise instrument time. However careful method optimisation and choice of appropriate instrumentation need to be performed when high speed chromatographic separation is carried out in order to obtain appropriate ratio precisions.

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