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[Continued on next page]

(54) Title: THYROGLOBULIN QUANTITATION BY MASS SPECTROMETRY

Figure 1

P01266 Sequence

MALVLEIFTLLASICWVSANIFEYQVDAQPLRPCELQRETAFLKQADYVPQCAEDGSFQT
VQCQNDGRSCWCVGANGSEVLGSRQPGRPVACLSFCQLQKQQILLSGYINSTDTSYLPQC
QDSGDYAPVQCDVQQVQCWCVDAEGMEVYGTRQLGRPKRCPRSCEIRNRRLLHGVGDKSP
PQCSAEGEFMPVQCKFVNTTDMMIFDLVHSYNRFPDAFVTFSSFQRRFPEVSGYCHCADS
QGRELAETGLELLLDEIYDTIFAGLDLPSTFTETTLYRILQRRFLAVQSVISGRFRCPTK
CEVERFTATSFGHPYVPSCRRNGDYQAVQCQTEGPCWCVDAQGKEMHGTRQQGEPPSCAE
GQSCASERQQALSRLYFGTSGYFSQHDLFSSPEKRWASPRVARFATSCPPTIKELFVDSG
LLRPMVEGQSQQFSVSENLLKEAIRAIFPSRGLARLALQFTTNPKRLQQNLFGGKFLVNV
GQFNLSGALGTRGTFNFSQFFQQLGLASFLNGGRQEDLAKPLSVGLDSNSSTGTPEAAKK
DGTMNKPTVGSFGFEINLQENQNALKFLASLLELPEFLLFLQHAISVPEDVARDLGDVME
TVLSSQTCEQTPERLFVPSCTTEGSYEDVQCFSGECWCVNSWGKELPGSRVRGGQPRCPT
DCEKQRARMQSLMGSQPAGSTLFVPACTSEGHFLPVQCFNSECYCVDAEGQAIPGTRSAI
GKPKKCPTPCQLQSEQAFLRTVQALLSNSSMLPTLSDTYIPQCSTDGQWRQVQCNGPPEQ
VFELYQRWEAQNKGQDLTPAKLLVKIMSYREAASGNFSLFIQSLYEAGQQDVFPVLSQYP
SLQDVPLAALEGKRPQPRENILLEPYLFWQILNGQLSQYPGSYSDFSTPLAHFDLRNCWC
VDEAGQELEGMRSEPSKLPTCPGSCEEAKLRVLQFIRETEEIVSASNSSRFPLGESFLVA
KGIRLRNEDLGLPPLFPPREAFAEQFLRGSDYAIRLAAQSTLSFYQRRRFSPDDSAGASA
LLRSGPYMPQCDAFGSWEPVQCHAGTGHCWCVDEKGGFIPGSLTARSLQIPQCPTTCEKS
RTSGLLSSWKQARSQENPSPKDLFVPACLETGEYARLQASGAGTWCVDPASGEELRPGSS
SSAQCPSLCNVLKSGVLSRRVSPGYVPACRAEDGGFSPVQCDQAQGSCWCVMDSGEEVPG
TRVTGGQPACESPRCPLPFNASEVVGGTILCETISGPTGSAMQQCQLLCRQGSWSVFPPG
PLICSLESGRWESQLPQPRACQRPQLWQTIQTQGHFQLQLPPGKMCSADYADLLQTFQVF
ILDELTARGFCQIQVKTFGTLVSIPVCNNSSVQVGCLTRERLGVNVTWKSRLEDIPVASL
PDLHDIERALVGKDLLGRFTDLIQSGSFQLHLDSKTFPAETIRFLQGDHFGTSPRTWFGC
SEGFYQVLTSEASQDGLGCVKCPEGSYSQDEECIPCPVGFYQEQAGSLACVPCPVGRTTI
SAGAFSQTHCVTDCQRNEAGLQCDQNGQYRASQKDRGSGKAFCVDGEGRRLPWWETEAPL
EDSQCLMMQKFEKVPESKVIFDANAPVAVRSKVPDSEFPVMQCLTDCTEDEACSFFTVST
TEPEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDSPAVYLKK
GQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCCDGFVLT
QVQGGAIICGLLSSPSVLLCNVKDWMDPSEAWANATCPGVTYDQESHQVILRLGDQEFIK
SLTPLEGTQDTFTNFQQVYLWKDSDMGSRPESMGCRKDTVPRPASPTEAGLTTELFSPVD
LNQVIVNGNQSLSSQKHWLFKHLFSAQQANLWCLSRCVQEHSFCQLAEITESASLYFTCT
LYPEAQVCDDIMESNAQGCRLILPQMPKALFRKKVILEDKVKNFYTRLPFQKLMGISIRN
KVPMSEKSISNGFFECERRCDADPCCTGFGFLNVSQLKGGEVTCLTLNSLGIQMCSEENG
GAWRILDCGSPDIEVHTYPFGWYQKPIAQNNAPSFCPLVVLPSLTEKVSLDSWQSLALSS
VVVDPSIRHFDVAHVSTAATSNFSAVRDLCLSECSQHEACLITTLQTQPGAVRCMFYADT
QSCTHSLQGQNCRLLLREEATHIYRKPGISLLSYEASVPSVPISTHGRLLGRSQAIQVGT
SWKQVDQFLGVPYAAPPLAERRFQAPEPLNWTGSWDASKPRASCWQPGTRTSTSPGVSED
CLYLNVFIPQNVAPNASVLVFFHNTMDREESEGWPAIDGSFLAAVGNLIVVTASYRVGVF
GFLSSGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPRRVSLAADRGGADVASIHLLTAR
ATNSQLFRRAVLMGGSALSPAAVISHERAQQQAIALAKEVSCPMSSSQEVVSCLRQKPAN
VLNDAQTKLLAVSGPFHYWGPVIDGHFLREPPARALKRSLWVEVDLLIGSSQDDGLINRA
KAVKQFEESRGRTSSKTAFYQALQNSLGGEDSDARVEAAATWYYSLEHSTDDYASFSRAL
ENATRDYFIICPIIDMASAWAKRARGNVFMYHAPENYGHGSLELLADVQFALGLPFYPAY
EGQFSLEEKSLSLKIMQYFSHFIRSGNPNYPYEFSRKVPTFATPWPDFVPRAGGENYKEF
SELLPNRQGLKKADCSFWSKYISSLKTSADGAKGGQSAESEEEELTAGSGLREDLLSLQE
PGSKTYSK

(57) Abstract: Provided are methods for determining the amount of thyroglobulin in a sample using various purification steps followed by mass spectrometry. The methods generally involve purifying thyroglobulin in a test sample, digesting thyroglobulin to form peptide T129, purifying peptide T129, ionizing peptide T129, detecting the amount of peptide T129 ion generated, and relating the amount of peptide T129 ion to the amount of thyroglobulin originally present in the sample.

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THYROGLOBULIN QUANTITATION BY MASS SPECTROMETRY

FIELD OF THE INVENTION

[0001] The invention relates to the quantitation of thyroglobulin. In a particular aspect, the invention relates to methods for quantitation of thyroglobulin by mass spectrometry.

BACKGROUND OF THE INVENTION

[0002] The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

[0003] Thyroglobulin, or Tg, is a large dimeric secretary glycoprotein with a molecular weight of 660 kDa comprised of noncovalently bound homodimers.

[0004] Tg molecules exist in several forms. The three major Tg molecule sequences as found in the UniProt Knowledgebase (Swiss-Prot + TrEMBL) are P01266 (Human Thyroglobulin Precursor), P01266-2 (Isoform 2 of P01266), and Q59GF02 (Human Thyroglobulin Variant). (See Figures 1, 2, and 3, respectively.)

[0005] P01266 is the major variant of P01266 with a length of 2768 AA; P01266-2 is an isoform of P01266 with a length of 2711 AA. P01266-2 varies from P01266 at amino acid positions 1510 to 1567 of Tg; and Q59GF0 is a thyroglobulin fragment with a length of 1574 AA. Q59GF0 contains amino acids from positions 1212 to 2768 of Tg.

[0006] Tg can only be produced in the thyroid gland and may be produced by either normal well differentiated benign thyroid cells or thyroid cancer cells. It is the precursor protein for thyroid hormone syntheses and serves as the matrix for thyroid iodine storage. Tg is used by the thyroid gland to produce the thyroid hormones thyroxine (T4) and triiodothyroine (T3). Tg levels in the blood can be used as a tumor marker for differentiated thyroid carcinoma (DTC). A high level of Tg in the blood is not by itself an indicator of thyroid cancer, but persistence of Tg in the blood following surgical removal of the thyroid gland indicates persistence of thyroid tissue. A course of treatment following detection of Tg in the blood following surgical removal of the thyroid gland may include administration of radioiodine to ablate all remaining normal thyroid. Continued persistence of Tg in the blood following ablation of all normal thyroid could indicate that some amount of tumor is still present.

[0007] Several methods for quantaition of Tg have been developed. For example Spencer, *et al.*, Thyroid, 1999, 9(5):435-41 and Persoon, *et al.*, Clinical Chem 2006, 52(4):686-691 disclose immunometric, radioimmunometric, and immunochemiluminometric methods for quantitation of Tg. These methods are all subject to methodological problems such as differences in standardization, variability in interassay sensitivity and precision, hook effects, and interference attributable to Tg antibodies. The problem of interference attributable to Tg antibodies is particularly troubling for clinical application of monitoring Tg levels as a tumor marker because up to 20% of thyroid cancer patients have Tg autoantibodies.

SUMMARY OF THE INVENTION

[0008] The present invention provides methods for quantitation of Tg in a sample by mass spectrometry, including tandem mass spectrometry.

[0009] In one aspect, methods are provided for determining the amount of Tg in a test sample that include: (a) subjecting a Tg containing test sample to digestion resulting in creation of Tg peptides; (b) purifying one or more Tg peptides; (c) ionizing one or more Tg peptides; (d) detecting the amount of the Tg peptide ion(s) by mass spectrometry; and (e) relating the amount of detected Tg peptide ion(s) to the amount of Tg in the test sample. A preferred enzyme for preparing Tg peptides is trypsin. A suitable Tg peptide for the method is one that can be evaluated by mass spectrometry and can be sufficiently purified from related peptides that may be generated from proteins other than Tg. An example of one such peptide is peptide T129 (sequence VIFDANAPVAVR) (SEQ ID NO: 4) which contains amino acids from positions 1579 to 1590 of Tg, has a molecular weight of about 1,270 Da, and is present in all three isoforms of Tg. See Figure 4.

[0010] Formation of peptide T129 provides a unique trypsin generated peptide for thyroglobulin. Also, creation of peptide T129 from tryptic digestion of Tg should be unaffected by the presence or absence of the Tg antibodies. Thus, measurement of the increase in peptide T129 in a test sample offers a way of quantitating the amount of Tg originally in the test sample free from infercnce from Tg antibodies.

[0011] Any appropriate method may be used to determine the amount of Tg peptide resulting from digestion of Tg in a sample. In the event that a test sample may contain endogenous Tg peptide, steps may be taken to make certain that the endogenous peptide is not confused with peptide generated by digesting Tg in sample. One approach is to remove the endogenous Tg peptide from the sample before digesting Tg. This may done, for example, using a size separation technique. Another approach is to analyze a portion of a test sample according to the claimed methods but excluding the digestion step in order to establish a baseline level for the endogenous peptide in the test sample. In this approach, once a baseline is determined, it can be subtracted from the post-digestion level of the peptide, the later representing both the endogenous peptide and that generated by digestion.

[0012] Because the methods may be applied to complex test samples (particularly body fluids or test samples derived from tissue) which contain proteins other than Tg, steps may be taken to purify Tg in the test sample prior to digestion. This may done, for example, using a size separation technique.

[0013] In some embodiments, the methods include generating one or more Tg peptide ions in which at least one of the ions has a mass/charge ratio (m/z) corresponding to that of (singly or multiply charged) peptide T129 ions. In preferred related embodiments, the methods include generating one or more Tg peptide ions in which at least one has m/z of 1272.8 ± 0.5, 636.4 ± 0.5, or 424.3 ± 0.5 (corresponding to singly, doubly, or triply charged peptide T129 ions). In related preferred embodiments, the methods may include generating one or more fragment ions of a Tg peptide ion in which at least one has a m/z of 541.3 ± 0.5, 612.3 ± 0.5, 726.4 ± 0.5, 797.4 ± 0.5, 912.4 ± 0.5, or 1059.5 ± 0.5; preferably one or more of the fragment ions are selected from the group consisting of ions with a m/z of 797.4 ± 0.5, 912.4 ± 0.5, and 1059.5 ± 0.5.

[0014] In some embodiments, the purification in step (b) is accomplished with at least one size separation technique. Preferably, size separation techniques may be filtration, LC, or any combination thereof. In certain preferred embodiments, the test sample is a body fluid or tissue. In some embodiments, an additional step is included where a second quantity of the test sample is subjected to steps (b) through (e) in order to establish a baseline level of one or more endogenous Tg peptides. In these embodiments, this baseline level can be subtracted from the amount of Tg peptide ion(s) detected in the test sample to determine the amount of Tg peptide ion(s) that result from Tg in the original test sample. In other embodiments, the methods include an additional initial step of purifying Tg in the test sample prior to digestion. In these embodiments, the pre-digestion purification and/or the purification in step (b) may each be accomplished with at least one size separation technique. Preferably, at least one size separation technique used in both pre-digestion purification and step (b) is filtration; more preferably, this filtration is done with a molecular weight cut-off filter with molecular weigh cut off that allows for retention of Tg above the filter and allows Tg peptides to pass through with the filtrate. In related embodiments, the molecular weigh cut-off is about 2 kD to 300 kD; more preferably about 100 kD to 300 kD. In these embodiments, the two filtrations (pre-digestion and step (b)) may be conducted with the same filter.

[0015] In a second aspect, methods are provided for determining the amount of Tg in a test sample that include: (a) subjecting a Tg containing test sample to digestion resulting in creation of peptide T129; (b) purifying peptide T129; (c) ionizing peptide T129 to generate a precursor ion with a m/z of 636.4 ± 0.5; (d) fragmenting the peptide T129 precursor ion to form one or more fragment ions in which at least one has a m/z of about 797.4 ± 0.5, 912.4 ± 0.5, or 1059.5 ± 0.5; detecting the amount of peptide T129 precursor ions, one or more fragment ions, or both, by mass spectrometry; and (e) relating the amount of detected ion(s) to the amount of Tg in the test sample. In certain preferred embodiments, the test sample is a body fluid or tissue or tissue. In some embodiments, an additional step is included where a second quantity of the test sample is subjected to steps (b) through (e) in order to establish a baseline level of one or more endogenous peptide T129. In these embodiments, this baseline level can be subtracted from the amount of peptide T129 ion(s) detected in the test sample to determine the amount of peptide T129 ion(s) that result from Tg in the original test sample. In other embodiments, the methods include an additional initial step of purifying Tg in the test sample prior to digestion. In these embodiments, the pre-digestion purification and/or the purification in step (b) may each be accomplished with at least one size separation technique. Preferably, at least one size separation technique used in both pre-digestion purification and step (b) is filtration; more preferably, this filtration is done with a molecular weight cut-off filter with molecular weigh cut off that allows for retention of Tg above the filter and allows Tg peptides to pass through with the filtrate. In related embodiments, the molecular weigh cut-off is about 2 kD to 300 kD; more preferably about 100 kD to 300 kD. In these embodiments, the two filtrations (pre-digestion and step (b)) may be conducted with the same filter.

[0016] As used herein, the term "purification" or "purifying" does not refer to removing all materials from the sample other than the analyte(s) of interest. Instead, purification refers to a procedure that enriches the amount of one or more analytes of interest relative to one or more other components of the sample. Purification, as used herein, does not require the isolation of an analyte from all others. In preferred embodiments, a purification step or procedure can be used to remove one or more interfering substances, *e.g.*, one or more substances that would interfere with the operation of the instruments used in the methods or substances that may interfere with the detection of an analyte ion by mass spectrometry.

[0017] As used herein, the term "about" in reference to quantitative measurements, not including the measurement of mass of an ion, refers to the indicated value plus or minus 10%.

[0018] As used herein, the term "substantially all" refers to any proportion greater than 50%, more preferably greater than 60%, more preferably greater than 70%, more preferably greater than 80%, and more preferably greater than 90%.

[0019] As used herein, the term "test sample" refers to any sample that may contain Tg. As used herein, the term "body fluid or tissue" means any fluid or tissue that can be isolated from the body of an individual. For example, "body fluid or tissue" may include blood, plasma, serum, bile, saliva, urine, tears, perspiration, and the like. If solid tissue is to be analyzed, it may be processed to release a liquid fraction that could contain any Tg present in the tissue. The liquid fraction can then be subject to the methods described herein.

[0020] As used herein, the term "digestion" means proteolytic cleavage of proteins into peptides. Digestion agents may include trypsin, Lyc-C, Arg-R, Asp-N and the like. Digestion is carried out by adding a digestion agent (i.e., an enzyme) to a sample and incubating for some period of time.

[0021] As used herein, "Tg" or "Tg molecule" means an intact Tg protein molecule.

[0022] As used herein, the term "Tg peptide" means any peptide of 100 amino acids or less that is a fragment of the native Tg. Tg peptides can be endogenous to a test sample or formed as a result of digestion of Tg. Peptide T129 is an example of a Tg peptide formed as a result of trypsin digestion of Tg.

[0023] As used herein, the term "size separation technique" means any technique (physical or chemical) that allows for the separation of at least one species from a test sample based on any one or more of molecular weight and shape. Examples of such techniques include, but are not limited to, filtration, chromatography, and certain aspects of mass spectrometry.

[0024] As used herein, the term "chromatography" refers to a process in which a chemical mixture carried by a liquid or gas is separated into components as a result of differential distribution of the chemical entities as they flow around, over, and/or through a stationary liquid or solid phase.

[0025] As used herein, the term "liquid chromatography" or "LC" means a process of selective retardation of one or more components of a fluid solution as the fluid uniformly percolates through a column of a finely divided substance, or through capillary passageways. The retardation results from the distribution of the components of the mixture between one or more stationary phases and the bulk fluid, (i.e., mobile phase), as this fluid moves relative to the stationary phase(s). "Liquid chromatography" includes reverse phase liquid chromatography (RPLC), high performance liquid chromatography (HPLC) and high turbulence liquid chromatography (HTLC).

[0026] As used herein, the term "high performance liquid chromatography" or "HPLC" refers to liquid chromatography in which the degree of separation is increased by forcing the mobile phase under pressure through a stationary phase, typically a densely packed column.

[0027] As used herein, the term "mass spectrometry" or "MS" refers to an analytical technique to identify compounds by their mass. MS refers to methods of filtering, detecting, and measuring ions based on their m/z. MS technology generally includes (1) ionizing the compounds to form charged species (e.g., ions); and (2) detecting the molecular weight of the ions and calculating their m/z. The compounds may be ionized and detected by any suitable means. A "mass spectrometer" generally includes an ionizer and an ion detector. In general, one or more molecules of interest are ionized, and the ions are subsequently introduced into a mass spectrographic instrument where, due to a combination of magnetic and electric fields, the ions follow a path in space that is dependent upon mass ("m") and charge ("z"). *See, e.g.*, U.S. Patent Nos. 6,204,500, entitled "Mass Spectrometry From Surfaces;" 6,107,623, entitled "Methods and Apparatus for Tandem Mass Spectrometry;" 6,268,144, entitled "DNA Diagnostics Based On Mass Spectrometry;" 6,124,137, entitled "Surface-Enhanced Photolabile Attachment And Release For Desorption And Detection Of Analytes;" Wright *et al.*, *Prostate Cancer and Prostatic Diseases* **2**:264-76 (1999); and Merchant and Weinberger, *Electrophoresis* **21**:1164-67 (2000).

[0028] As used herein, the term "operating in positive ion mode" refers to those mass spectrometry methods where positive ions are detected. Similarly, the term "operating in negative ion mode" refers to those mass spectrometry methods where negative ions are detected.

[0029] As used herein, the term "ionization" or "ionizing" refers to the process of generating an analyte ion having a net electrical charge equal to one or more electron units. Positive ions are those having a net positive charge of one or more electron units. Negative ions are those having a net negative charge of one or more electron units.

[0030] As used herein, the term "electron ionization" or "EI" refers to methods in which an analyte of interest in a gaseous or vapor phase interacts with a flow of electrons. Impact of the electrons with the analyte produces analyte ions, which may then be subjected to a mass spectrometry technique.

[0031] As used herein, the term "chemical ionization" or "CI" refers to methods in which a reagent gas (e.g. ammonia) is subjected to electron impact, and analyte ions are formed by the interaction of reagent gas ions and analyte molecules.

[0032] As used herein, the term "fast atom bombardment" or "FAB" refers to methods in which a beam of high energy atoms (often Xe or Ar) impacts a non-volatile sample, desorbing and ionizing molecules contained in the sample. Test samples are dissolved in a viscous liquid matrix such as glycerol, thioglycerol, m-nitrobenzyl alcohol, 18-crown-6 crown ether, 2-nitrophenyloctyl ether, sulfolane, diethanolamine, and triethanolamine. The choice of an appropriate matrix for a compound or sample is an empirical process.

[0033] As used herein, the term "matrix-assisted laser desorption ionization" or "MALDI" refers to methods in which a non-volatile sample is exposed to laser irradiation, which desorbs and ionizes analytes in the sample by various ionization pathways, including photo-ionization, protonation, deprotonation, and cluster decay. For MALDI, the sample is mixed with an energy-absorbing matrix, which facilitates desorption of analyte molecules.

[0034] As used herein, the term "surface enhanced laser desorption ionization" or "SELDI" refers to another method in which a non-volatile sample is exposed to laser irradiation, which desorbs and ionizes analytes in the sample by various ionization pathways, including photo-ionization, protonation, deprotonation, and cluster decay. For SELDI, the sample is typically bound to a surface that preferentially retains one or more analytes of interest. As in MALDI, this process may also employ an energy-absorbing material to facilitate ionization.

[0035] As used herein, the term "electrospray ionization" or "ESI," refers to methods in which a solution is passed along a short length of capillary tube, to the end of which is applied a high positive or negative electric potential. Solution reaching the end of the tube is vaporized (nebulized) into a jet or spray of very small droplets of solution in solvent vapor. This mist of droplets flows through an evaporation chamber, which is heated slightly to prevent condensation and to evaporate solvent. As the droplets get smaller the electrical surface charge density increases until such time that the natural repulsion between like charges causes ions as well as neutral molecules to be released.

[0036] As used herein, the term "atmospheric pressure chemical ionization" or "APCI," refers to mass spectrometry methods that are similar to ESI; however, APCI produces ions by ion-molecule reactions that occur within a plasma at atmospheric pressure. The plasma is maintained by an electric discharge between the spray capillary and a counter electrode. Then ions are typically extracted into the mass analyzer by use of a set of differentially pumped skimmer stages. A counterflow of dry and preheated N_2 gas may be used to improve removal of solvent. The gas-phase ionization in APCI can be more effective than ESI for analyzing less-polar species.

[0037] The term "Atmospheric Pressure Photoionization" or "APPI" as used herein refers to the form of mass spectrometry where the mechanism for the photoionization of molecule M is photon absorption and electron ejection to form the molecular M+. Because the photon energy typically is just above the ionization potential, the molecular ion is less susceptible to dissociation. In many cases it may be possible to analyze samples without the need for chromatography, thus saving significant time and expense. In the presence of water vapor or protic solvents, the molecular ion can extract H to form MH+. This tends to occur if M has a high proton affinity. This does not affect quantitation accuracy because the sum of M+ and MH+ is constant. Drug compounds in protic solvents are usually observed as MH+, whereas nonpolar compounds such as naphthalene or testosterone usually form M+. Robb, D.B., Covey, T.R. and Bruins, A.P. (2000): *See, e.g.*, Robb *et al.*, Atmospheric pressure photoionization: An ionization method for liquid chromatography-mass spectrometry. *Anal. Chem.* 72(15): 3653-3659.

[0038] As used herein, the term "inductively coupled plasma" or "ICP" refers to methods in which a sample is interacted with a partially ionized gas at a sufficiently high temperature to atomize and ionize most elements

[0039] As used, herein, the term "field desorption" refers to methods in which a non-volatile test sample is placed on an ionization surface, and an intense electric field is used to generate analyte ions.

[0040] As used herein, the term "desorption" refers to the removal of an analyte from a surface and/or the entry of an analyte into a gaseous phase.

[0041] As used herein, the term "limit of quantification" or "LOQ" refers to the point where measurements become quantitatively meaningful. The analyte response at this LOQ is identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80% to 120%.

[0042] In certain preferred embodiments of the methods disclosed herein, mass spectrometry is performed in positive ion mode. In certain particularly preferred embodiments of the methods disclosed herein, mass spectrometry is performed using ESI as the method of creating ions from Tg peptides.

[0043] In preferred embodiments, the ions from Tg peptide ionization detectable in a mass spectrometer are selected from the group consisting of ions with a m/z of 636.4 ± 0.5, 1059.5 ± 0.5, 921.4 ± 0.5, 797.4 ± 0.5, 726.4 ± 0.5, 612.3 ± 0.5, and 541.3 ± 0.5; the first ion listed (m/z of 636.4 ± 0.5) being a precursor ion with a net charge of positive 2 electron units and the latter six ions listed being fragment ions of the precursor ion. In particularly preferred embodiments, the precursor ion has a net charge of positive 2 electron units and a m/z of about 636.4 ± 0.5, and the fragment ions have a m/z of 1059.5 ± 0.5, 921.4 ± 0.5, or 797.4 ± 0.5.

[0044] In some preferred embodiments, a separately detectable internal standard peptide (e.g., T129) is introduced in the test sample after trypsin digestion. In these embodiments, all or a portion of the peptide present in the test sample both from digestion of endogenous Tg and the addition of the internal standard are ionized to produce a plurality of ions detectable in a mass spectrometer, and one or more ions produced from the peptide ionization are detected in a mass spectrometer.

[0045] In other preferred embodiments, a separately detectable internal Tg standard is provided in the test sample prior to trypsin digestion. In these embodiments, all or a portion of both the endogenous Tg and the internal standard present in the test sample are digested by trypsin resulting in formation of Tg peptides. Tg peptides are ionized to produce a plurality of ions detectable in a mass spectrometer, and one or more ions produced from Tg peptide ionization are detected by mass spectrometry.

[0046] In preferred embodiments, the ions detectable in a mass spectrometer produced from the ionization of Tg peptides resulting from Tg digestion are selected from the group consisting of ions with a m/z of 636.4 ± 0.5, 1059.5 ± 0.5, 921.4 ± 0.5, 797.4 ± 0.5, 726.4 ± 0.5, 612.3 ± 0.5, and 541.3 ± 0.5; the first ion listed (m/z of 636.4 ± 0.5) being a precursor ion with a net charge of positive 2 electron units and the latter six ions listed being fragment ions of the precursor ion. In particularly preferred embodiments, the precursor ion has a net charge of positive 2 electron units and a m/z of 636.4 ± 0.5, and the fragment ions have a m/z of 1059.5 ± 0.5, 921.4 ± 0.5, 797.4 ± 0.5.

[0047] In preferred embodiments, the presence or amount of Tg peptide ions is related to the presence or amount of Tg in the original test sample by comparison to a reference Tg sample.

[0048] In one embodiment, the methods involve the combination of LC with mass spectrometry. In another preferred embodiment, the mass spectrometry is tandem mass spectrometry (MS/MS).

[0049] The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] Figure 1 shows the amino acid sequence for P01266 (Human Thyroglobulin Precursor) (SEQ ID NO: 1).

[0051] Figure 2 shows the amino acid sequence for P01266-2 (Isoform 2 of P01266) (SEQ ID NO: 2).

[0052] Figure 3 shows the amino acid sequence for Q59GF0 (Thyroglobulin Variant-Fragment) (SEQ ID NO: 3).

[0053] Figure 4 shows a comparison of the three sequences contained in Figures 1-3 demonstrating that they all contain amino acids corresponding to positions 1579 to 1590 of Tg. Sequence P01266 is on top (SEQ ID NO: 1); sequence P01266-2 is in the middle (SEQ ID NO: 2); and sequence Q59GF0 is at the bottom (SEQ ID NO: 3).

[0054] Figure 5 shows the limit of quantitation verification for Tg peptide ion with m/z corresponding to peptide T129 by MS/MS. The equation describing the trend line for Figure 5 is as follows: $y = 1E\text{-}05x^4 - 0.0007x^3 + 0.0114x^2 - 0.0787x + 0.2606$. $R^2 = 0.9833$ for this fit. Details are described in Example 1.

[0055] Figure 6 shows the linearity of the quantitation of peptide T129 in serially diluted stock samples using an LC-MS/MS assay. The equation describing the trend line for Figure 6 is as follows: $y = 26.919x^2 + 2939.4x + 310.78$. $R^2 = 0.9988$ for this fit. Details are described in Example 1.

[0056] Figure 7 shows the limit of quantitation verification for peptide T129 in stripped serum by MS/MS. The equation describing the trend line for Figure 7 is as follows: $y = 1807.2x - 1975$. $R^2 = 0.9993$ for this fit. Details are described in Example 2.

[0057] Figure 8 shows the linearity of the quantitation of peptide T129 in peptide T129 spiked stripped serum using an LC-MS/MS assay. Details are described in Example 2.

[0058] Figure 9 shows the linearity of the quantitation of Tg peptide ions with m/z corresponding to peptide T129 using an LC-MS/MS assay in stripped serum spiked with Tg prior to processing and concentration according to the methods described herein. The equation describing the trend line for Figure 9 is as follows: $y = 218.15x + 8363.2$. $R^2 = 0.9681$ for this fit. Details are described in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

[0059] Methods are described for quantitatively measuring Tg in a test sample. This quantitative measurement is achieved through the use of LC-MS/MS techniques. Prior to the use of LC-MS/MS, samples may be prepared by the following technique, or any portion thereof. A first purification of Tg in a test sample may be conducted through the use of a size separation technique such that substantially all Tg in the test sample is retained and smaller proteins not of interest are removed. Following the first purification step, enzymatic digestion of Tg may be carried out creating Tg peptides of interest. After digestion, another utilization of a size separation technique may be employed such that a selected Tg peptide generated in the enzymatic digestion of Tg is purified. This second size separation technique can be used to remove substantially all undigested, higher-molecular weight species. Properly executed, the sample preparation techniques ensure that selected Tg peptides quantitated by LC-MS/MS directly result from enzymatic digestion of Tg originally in the test sample; thus, the level of selected Tg peptides in the test sample at the start of LC-MS/MS is directly proportional to the amount of Tg originally present in the test sample.

[0060] Any suitable size separation technique may be utilized, but in the examples that follow, both the first and second size separation techniques are filtration through a molecular weight cut-off filter. It is also possible, as discussed in the Examples that follow, to select a molecular weight cut-off filter with an appropriate molecular weight cut-off such that the same filter can be used for both the first size separation and the second size separation.

[0061] LC, most preferably HPLC, is utilized, may be utilized either alone or in combination with other purification methods, to purify selected Tg peptides. This purification is combined with MS/MS, thereby providing an assay system for quantifying selected Tg peptides in a test sample. The quantity of the selected Tg peptides in the test sample is then used to determine the quantity of Tg in the original test sample. The Tg quantitation methods provided herein have enhanced specificity and are less subject to methodological problems (such as Tg antibody interference).

[0062] Suitable test samples may include any test sample that may contain the analyte of interest. In some preferred embodiments, a sample is a biological sample; that is, a sample obtained from any biological source, such as an animal, a cell culture, an organ culture, and the like. In certain preferred embodiments, samples are obtained from a mammalian animal, such as a dog, cat, horse, etc. Particularly preferred mammalian animals are primates, most preferably humans. Particularly preferred samples include blood, plasma, serum, urine, saliva, tears, cerebrospinal fluid, or other body fluid or tissue samples. Such samples may be obtained, for example, from a patient; that is, a living person presenting oneself in a clinical setting for diagnosis, prognosis, or treatment of a disease or condition. The test sample is preferably obtained from a patient, for example, serum or plasma.

Sample Preparation for Mass Spectrometry

[0063] Samples may be processed or purified to obtain preparations that are suitable for analysis by mass spectrometry. Such purification will usually include chromatography, such as liquid chromatography, and may also often involve an additional purification procedure that is performed prior to chromatography. Various procedures may be used for this purpose depending on the type of sample or the type of chromatography. Examples include filtration, centrifugation, combinations thereof and the like. In certain preferred embodiments, Tg present in a test sample prior to enzymatic digestion.

[0064] Filtration is one preferred method of preparing a test sample, especially a biological test sample, such as serum or plasma, for chromatography. Such filtration is carried out by filtering a test sample through a molecular weight cut-off filter to separate species with molecular weights higher than the filter's cut-off (including Tg) from those with molecular weights lower than the filter's cut-off. The test sample remaining above the filter following complete (or near complete) filtration is substantially free of potentially interfering species with molecular weights lower than the filter's cut-off.

[0065] The pH of the test sample may then be adjusted to any point required by a digestion agent. In certain preferred embodiments, the digestion agent is trypsin and pH can be adjusted with a solution of ammonium acetate to have a pH suitable for this enzyme. In these preferred embodiments, the sample is then digested with trypsin to form Tg peptides (including peptide T129).

[0066] After trypsin digestion, the sample may be purified with a second filtration. This post-digestion filtration can be carried out similarly to the pre-digestion filtration described above (with the exception that the filtrate is retained), in order to separate Tg fragments from potentially interfering species with molecular weights higher than the filter's cut-off that may also be present in the sample. The filtrate from this post-digestion filtration can then be purified by liquid chromatography and subsequently subjected to mass spectrometry analysis.

[0067] Various methods have been described involving the use of HPLC for sample clean-up prior to mass spectrometry analysis. See, *e.g.*, Taylor *et al.*, *Therapeutic Drug Monitoring* **22**:608-12 (2000) (manual precipitation of blood samples, followed by manual C18 solid phase extraction, injection into an HPLC for chromatography on a C18 analytical column, and MS/MS analysis); and Salm *et al.*, *Clin. Therapeutics* **22** Supl. B:B71-B85 (2000) (manual precipitation of blood samples, followed by manual C18 solid phase extraction, injection into an HPLC for chromatography on a C18 analytical column, and MS/MS analysis). One of skill in the art may select HPLC instruments and columns that are suitable for use in the methods. The chromatographic column typically includes a medium (i.e., a packing material) to facilitate separation of chemical moieties (i.e., fractionation). The medium may include minute particles. The particles include a bonded surface that interacts with the various chemical moieties to facilitate separation of the chemical moieties. One suitable bonded surface is a hydrophobic bonded surface such as an alkyl bonded surface. Alkyl bonded surfaces may include C-4, C-8, or C-18 bonded alkyl groups, preferably C-8 bonded groups. The chromatographic column includes an inlet port for receiving a sample and an outlet port for discharging an effluent that includes the fractionated sample.

[0068] In certain embodiments, an analyte may be purified by applying a sample to a column under conditions where the analyte of interest is reversibly retained by the column packing material, while one or more other materials are not retained. In these embodiments, a first mobile phase condition can be employed where the analyte of interest is retained by the column and a second mobile phase condition can subsequently be employed to remove retained material from the column, once the non-retained materials are washed through. Alternatively, an analyte may be purified by applying a sample to a column under mobile phase conditions where the analyte of interest elutes at a differential rate in comparison to one or more other materials. Such procedures may enrich the amount of one or more analytes of interest relative to one or more other components of the sample.

[0069] In one embodiment, the sample to be analyzed is applied to the column at the inlet port, eluted with a solvent or solvent mixture, and discharged at the outlet port. Different solvent modes may be selected for eluting the analytes of interest. For example, liquid chromatography may be performed using a gradient mode, an isocratic mode, or a polytyptic (i.e. mixed) mode. In preferred embodiments, HPLC is performed on an analytical HPLC system with a C8 solid phase using 0.2% formic acid in HPLC Grade Ultra Pure Water and 0.2% formic acid in 100% methanol as the mobile phases.

[0070] Numerous column packings are available for chromatographic separation of samples and selection of an appropriate separation protocol is an empirical process that depends on the sample characteristics, analyte of interest, presence of interfering substances and their characteristics, etc. Commercially available HPLC columns include, but are not limited to, polar, ion exchange (both cation and anion), hydrophobic interaction, phenyl, C-2, C-8, C-18, and polar coating on porous polymer columns.

[0071] In one embodiment, the HPLC column has a C8 solid phase with a median particle size of 5μm (nominal) and a median particle pore size of 100 Å. In a preferred embodiment the column dimensions are 1.0 mm ID x 50 mm length (Phenomenex Corp. Luna 5μ C8(2) 100 Å New Column 50 x 1.0 mm, Phenomenex Cat. No. 00B-4249-A0 or equivalent).

[0072] During chromatography, the separation of materials is effected by variables such as choice of eluent (also known as a "mobile phase"), choice of gradient elution and the gradient conditions, temperature, etc.

Detection and Quantitation by Mass Spectrometry

[0073] In various embodiments, Tg peptides may be ionized by any method known to the skilled artisan. Mass spectrometry is performed using a mass spectrometer, which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. Ionization sources used in various MS techniques include, but are not limited to, electron ionization, chemical ionization, electrospray ionization (ESI), photon ionization, atmospheric pressure chemical ionization (APCI), photoionization, atmospheric pressure photoionization (APPI), fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray ionization, surface enhanced laser desorption ionization (SELDI), inductively coupled plasma (ICP) and particle beam ionization. The skilled artisan will understand that the choice of ionization method may be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc.

[0074] In preferred embodiments, Tg peptides are ionized by electrospray ionization (ESI) creating Tg peptide precursor ions. In related preferred embodiments, Tg peptide precursor ions are in a gaseous state and the inert collision gas is argon.

[0075] After the sample has been ionized, the positively charged ions thereby created may be analyzed to determine m/z. Suitable analyzers for determining m/z include quadrupole analyzers, ion trap analyzers, and time-of-flight analyzers. The ions may be detected using one of several detection modes. For example, only selected ions may be detected using a selective ion monitoring mode (SIM), or alternatively, multiple ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). In preferred embodiments, ions are detected using SRM.

[0076] Preferably, m/z is determined using a quadrupole instrument. In a "quadrupole" or "quadrupole ion trap" instrument, ions in an oscillating radio frequency field experience a force proportional to the DC potential applied between electrodes, the amplitude of the RF signal, and m/z. The voltage and amplitude may be selected so that only ions having a particular m/z travel the length of the quadrupole, while all other ions are deflected. Thus, quadrupole instruments may act as both a "mass filter" and as a "mass detector" for the ions injected into the instrument.

[0077] One may enhance the resolution of the MS technique by employing "tandem mass spectrometry," or "MS/MS." In this technique, a precursor ion (also called a parent ion) generated from a molecule of interest can be filtered in an MS instrument, and the precursor ion subsequently fragmented to yield one or more fragment ions (also called daughter ions or product ions) that are then analyzed in a second MS procedure. By careful selection of precursor ions, only ions produced by certain analytes are passed to the fragmentation chamber, where collision with atoms of an inert gas produce the fragment ions. Because both the precursor and fragment ions are produced in a reproducible fashion under a given set of ionization/fragmentation conditions, the MS/MS technique may provide an extremely powerful analytical tool. For example, the combination of filtration/fragmentation may be used to eliminate interfering substances, and may be particularly useful in complex samples, such as biological samples.

[0078] Additionally, recent advances in technology, such as matrix-assisted laser desorption ionization coupled with time-of-flight analyzers ("MALDI-TOF") permit the analysis of analytes at femtomole levels in very short ion pulses. Mass spectrometers that combine time-of-flight analyzers with tandem MS are also well known to the artisan. Additionally, multiple mass spectrometry steps may be combined in methods known as "MS/MS". Various other combinations may be employed, such as MS/MS/TOF, MALDI/MS/MS/TOF, or SELDI/MS/MS/TOF mass spectrometry.

[0079] The mass spectrometer typically provides the user with an ion scan; that is, the relative abundance of each ion with a particular m/z over a given range (*e.g.*, 400 to 1600 amu). The results of an analyte assay, that is, a mass spectrum, may be related to the amount of the analyte in the original sample by numerous methods known in the art. For example, given that sampling and analysis parameters are carefully controlled, the relative abundance of a given ion may be compared to a table that converts that relative abundance to an absolute amount of the original molecule. Alternatively, molecular standards may be run with the samples and a standard curve constructed based on ions generated from those standards. Using such a standard curve, the relative abundance of a given ion may be converted into an absolute amount of the original molecule. In certain preferred embodiments, an internal standard is used to generate a standard curve for calculating the quantity of Tg. Methods of generating and using such standard curves are well known in the art and one of ordinary skill is capable of selecting an appropriate internal standard. Numerous other methods for relating the amount of an ion to the amount of the original molecule will be well known to those of ordinary skill in the art.

[0080] One or more steps of the methods may be performed using automated machines. In certain embodiments, one or more purification steps are performed on-line, and more preferably all of the LC purification and mass spectrometry steps may be performed in an on-line fashion.

[0081] In certain embodiments, techniques such as MS/MS are used to isolate precursor ions for further fragmentation. In these embodiments, collision activation dissociation (CAD) may be used to generate the fragment ions for further detection. In CAD, precursor ions gain energy through collisions with an inert gas, and subsequently fragment by a process referred to as "unimolecular decomposition". Sufficient energy must be deposited in the precursor ion so that certain bonds within the ion can be broken due to increased vibrational energy. In alternative embodiments, electron transfer dissociation (ETD) may be used to generate the fragment ions. In ETD, radical anions are used to transfer electrons to multiply charged peptide or protein cations resulting in random cleavage along the peptide backbone.

[0082] In particularly preferred embodiments, Tg is detected and/or quantified using LC-MS/MS as follows. A Tg peptide enriched test sample prepared as described above is subjected to LC. The flow of liquid solvent from the chromatographic column enters the heated nebulizer interface of a LC-MS/MS analyzer and the solvent/analyte mixture is converted to vapor in the heated tubing of the interface. The analyte (e.g., Tg peptides), contained in the nebulized solvent, is ionized by the corona discharge needle of the interface, which applies a large voltage to the nebulized solvent/analyte mixture. The ions (i.e. Tg peptide precursor ions) pass through the orifice of the instrument and enter the first quadrupole. Quadrupoles 1 and 3 (Q1 and Q3) are mass filters, allowing selection of ions (i.e., "precursor" and "fragment" ions) based on their m/z. Quadrupole 2 (Q2) is the collision cell, where ions are fragmented. Q1 selects for ions with m/z of peptide T129 precursor ions (m/z of 636.4 ± 0.5). Selected precursor ions are allowed to pass into the collision chamber (Q2), while ions with any other m/z collide with the sides of Q1 and are eliminated. Precursor ions entering Q2 may be fragmented with collision activated dissociation (CAD) through collisions with neutral argon gas molecules. Alternatively, if the precursor ions entering Q2 are multiply charged cations, they may be fragmented with electron transfer dissociation (ETD). The fragment ions generated are passed into Q3, where selected fragment ions are collected while other ions are eliminated.

[0083] Using standard methods well known in the art, one of ordinary skill is capable of identifying one or more fragment ions of a particular Tg peptide precursor ion that may be used for selection in Q3. A specific fragment ion is one that will not be formed in significant amounts by other molecules with similar molecular structures. In contrast, a non-specific fragment ion is one that is formed by molecules other than the desired analyte. Suitable specific fragment ions can be identified by testing various molecular standards to determine whether fragment ions formed by a selected Tg peptide are also formed by other molecules with similar structures or features. Preferably, at least one fragment ion specific for Tg peptide ions with m/z corresponding to that of peptide T129 ions are identified. More preferably, one or more of these fragment ions have m/z of 797.4 ± 0.5, 912.4 ± 0.5 or 1059.5 ± 0.5.

[0084] As ions collide with the detector they produce a pulse of electrons that are converted to a digital signal. The acquired data is relayed to a computer, which plots ion counts per unit time. The areas under the peaks corresponding to particular ions, or the amplitude of such peaks, are measured and the area or amplitude is correlated to the amount of the analyte of interest. In certain embodiments, the area under the curves, or amplitude of the peaks, for fragment ion(s) and/or precursor ions are measured to determine the amount of Tg peptides with m/z corresponding to peptide T129. As described above, the relative abundance of a given ion may be converted into an absolute amount of the original analyte using calibration standard curves based on peaks of one or more ions of an internal molecular standard. The absolute amount of an analyte detected by LC-MS/MS can then be converted into an absolute amount of Tg that was present in the original test sample.

[0085] The following examples serve to illustrate the invention. These examples are in no way intended to limit the scope of the methods.

EXAMPLES

Example 1: Demonstration of MS quantitation of peptide T129

[0086] Several samples with various known concentrations of peptide T129 were prepared by series dilution starting with a sample of known peptide T129 concentration. Peptide T129 LOQ and calibration curves were developed from LC-MS/MS analysis of these samples.

[0087] LC was performed with a Phenomenex analytical column (Phenomenex Corp. Luna 5μ C8(2) 100 Å New Column 50 x 1.0 mm). A binary HPLC eluent composed of 0.2% formic acid in ultra pure water (HPLC grade) (mobile phase A) and 0.2% formic acid in 100% methanol (mobile phase B) was applied to the analytical column to separate selected Tg peptides from other species contained in the sample. The binary eluent was applied according to the following gradient profile: as a first step, an 80/20 mixture of mobile phase A/mobile phase B was applied for 120 seconds; as a second step, a 30/70 mixture of mobile phase A/mobile phase B was applied for 60 seconds; as a third step, the relative amount of mobile phase B in the mixture was ramped to a 5/95 mixture of mobile phase A/mobile phase B over a period of 120 seconds; as a fourth step, a 5/95 mixture of mobile phase A/mobile phase B was applied for 60 seconds; as a fifth and final step, an 80/20 mixture of mobile phase A/mobile phase B was applied for 240 seconds.

[0088] The separated sample was then subjected to MS/MS for quantitation of one or more Tg peptides with m/z corresponding to peptide T129.

[0089] MS/MS was performed using a Finnigan TSQ Quantum Ultra MS/MS system (Thermo Electron Corporation). The following software programs all from ThermoElectron were used in the Examples described herein: Tune Master V 1.2 or newer, Xcalibur V 2.0 SR1 or newer, TSQ Quantum 1.4 or newer, LCQuan V 2.0 or newer, and XReport 1.0 or newer. Liquid solvent/analyte exiting the analytical HPLC column flowed to the heated nebulizer interface of a Thermo Finnigan MS/MS analyzer. The solvent/analyte mixture was converted to vapor in the heated tubing of the interface. Analytes in the nebulized solvent were ionized by the corona discharge needle of the interface, which applied voltage to the nebulized solvent/analyte mixture.

[0090] Ions passed to the first quadrupole (Q1), which selected ions with a m/z of 636.4 ± 0.5. Ions entering Quadrupole 2 (Q2) collided with argon gas to generate ion fragments, which were passed to quadrupole 3 (Q3) for further selection. Mass transitions used for quantitation of precursor ions with m/z corresponding to peptide T129 during validation on positive polarity are shown in Table 1.

Table 1. Mass transitions for precursor ions with m/z corresponding to peptide T129 (Positive Polarity)

Precursor Ion (m/z)	*Fragment Ion (m/z)*
636.4 ± 0.5	797.4 ± 0.5, 912.4 ± 0.5 & 1059.5 ± 0.5

[0091] To determine the limit of quantitation (LOQ) with a precision of 20% and an accuracy of 80% to 120%, seven different samples at varying concentrations were assayed and the reproducibility (CV) determined for each. The LOQ for one or more Tg peptides with m/z corresponding to peptide T129 was defined at about 67 amol/μl.

[0092] Data collected and used to develop the LOQ and Calibration curves in Figs. 5 and 6 is shown in Table 2.

Table 2. Data collected and used to develop LOQ and Calibration curves for peptide T129 in spiked stripped serum samples

Peptide T129 Concentration (Attomoles/μl)	*Femtomoles of peptide T129 in 30 μl sample*	*Average Ion Counts per Second*	*CV (%)*
2.5	0.075	1471.6	0.264429
25	0.75	2435.6	0.188653
75	2.25	6455.4	0.147946
150	4.5	13322.4	0.075327
300	9	28805	0.073374
450	13.5	46199.6	0.067088
600	18	61302.2	0.030893

Example 2: Demonstration of quantitation of peptide T129 in peptide T129 spiked processed, concentrated and digested stripped serum

[0093] A 500 μl sample of stripped serum (e.g., the test sample in this Example) was added atop the filter element of a commercially available 300 kDa molecular weight cut-off filter cartridge (Pall Corp. Nanosep 300kDa, Pall Corp. Cat. No. OD300C33).

[0094] The test sample was completely filtered upon centrifugation of the cartridge at 13 kg for 6 minutes. The filtrate was removed and discarded. 500 μl of HPLC grade water was then added to the top of the filter and the cartridge was again centrifuged at 13 kg for 6 minutes. The filtrate was again removed and discarded. Next, 200 μl of 20 mM ammonium acetate was added to the top of the filter. The cartridge was again centrifuged at 13 kg for 3 minutes. The filtrate was again removed and discarded and 100 μl of 20 mM ammonium acetate was added to the top of the filter.

[0095] Then, 15 μg of trypsin (Promega Trypsin Gold, Mass Spec Grade, Promega Corp. Cat. No. V5280 or equivalent) was added to the test sample remaining on top of the filter. The resulting mixture was incubated without removal from the filter cartridge at 37 C for up to 17 hours.

[0096] After incubation, the filter cartridge was centrifuged at 13 kg for 6 minutes, and the filtrate retained. The filter cartridge was then washed by adding 50 μl of 20 mM ammonium acetate to the top of the filter and centrifuged at 13 kg for 6 minutes. Test samples for analysis by LC-MS/MS were created by pooling the two retained post-digestion filtrates.

[0097] The starting volume of stripped serum samples subjected to the above processing and concentration was about 500 µl. The final volume of each pooled post-digestion filtrate was about 130 µl. Thus the above process concentrates samples by a factor of 3.83.

[0098] Peptide T129 was then added to the pooled post-digestion filtrates in varying concentrations. 30 µl samples were then analyzed for quantitation of peptide T129 by LC-MS/MS according to the procedure described in Example 1 with the exception that the mass transitions shown in Table 3 were used. The fragment ion with a m/z of 797.4 ± 0.5 was not used due to increased background generated by the processed, concentrated stripped serum.

Table 3. Mass transitions for precursor ions with m/z corresponding to peptide T129 from peptide T129 spiked stripped serum samples (Positive Polarity)

Precursor Ion (m/z)	*Fragment Ion (m/z)*
636.4 ± 0.5	912.4 ± 0.5 & 1059.5 ± 0.5

[0099] Data collected and used to develop the LOQ and Calibration curves found in Figs. 7 and 8 is shown in Table 4.

Table 4. Data collected and used to develop LOQ and Calibration curves for peptide T129

Femtomoles of Tg in spiked serum sample	*Average Ion Counts per Second*	*CV (%)*
0.75	203	0.348839
1.5	957.25	0.263782
3	2984.75	0.269659
4.5	6504.75	0.063318
11.25	18210.5	0.097296
22.5	37620	0.085823
30	51451	0.035083

Example 3: Demonstration of quantitation of peptide T129 in stripped serum containing various concentrations of added Tg.

Several 500 µl samples of stripped serum containing various concentrations of added Tg were prepared according to the procedure detailed in Example 2. LC-MS/MS of the resulting test samples was carried out following the steps detailed in Example 1.

[00100] Data collected and used to develop the calibration curve found in Fig. 9 are found in Table 6.

Table 6. Data collected and used to develop the calibration curve for peptide T129 MS/MS in Tg spiked stripped serum (processed and condensed as described in Example 3).

Femtomoles of Tg in spiked serum sample	*Average Ion Counts per Second*	*CV (%)*
0	8784.667	0.176987
1.5	8259.5	0.246833
4.5	9953.25	0.186588
11.25	9696.25	0.23816
22.5	13848.25	0.225496
45	18125.5	0.110826

[00101] The contents of the articles, patents, patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

[00102] The methods illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the invention embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00103] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the methods. This includes the generic description of the methods with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00104] Other embodiments are within the following claims. In addition, where features or aspects of the methods are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

THAT WHICH IS CLAIMED IS:

1. A method for determining the amount of thyroglobulin in a test sample, comprising:

(a) digesting thyroglobulin in said test sample to form Tg peptides;

(b) purifying said Tg peptides from step (a);

(c) ionizing said Tg peptides from step (b) to produce one or more Tg peptide ions detectable by mass spectrometry; and

(d) detecting the amount of the ion(s) from step (c) by mass spectrometry; wherein the amount of the ion(s) detected in step (d) is related to the amount of thyroglobulin in said test sample.

2. The method of claim 1, wherein step (b) is accomplished by utilizing at least one size separation technique.

3. The method of claim 2, wherein said at least one size separation technique is filtration.

4. The method of any one of claims 2-3, wherein said at least one size separation technique is liquid chromatography.

5. The method of any one of claims 1-4, said method further comprising analyzing a second quantity of said test sample by steps (b) through (d) to determine the baseline amount of endogenous Tg peptides in the test sample; wherein said baseline can be subtracted from the amount of Tg peptides detected in the digested test sample before relation to the amount of Tg in said test sample.

6. The method of any one of claims 1-5, said method further comprising purifying thyroglobulin in said test sample prior to step (a).

7. The method of claim 6, wherein purifying thyroglobulin prior to step (a) is accomplished by utilizing at least one size separation technique.

8. The method of claim 7, wherein said at least one size separation technique is filtration.

9. The method of claim 8, wherein a molecular weight cut-off filter is used and said filter is able to retain Tg above the filter.

10. The method of claim 9, wherein said molecular weight cut-off filter has a molecular weight cut-off of about 2 kDa to 300 kDa.

11. The method of claim 9, wherein said molecular weigh cut-off filter has a molecular weight cut-off of about 100 kDa to 300 kDa.

12. The method of claim 7, wherein step (b) is accomplished by utilizing at least one size separation technique.

13. The method of claim 12, wherein said at least one size separation technique utilized in both pre-digestion purification and step (b) is filtration.

14. The method of claim 13, wherein filtration is achieved with molecular weight cut-off filters that keep Tg a top the filter and pass Tg peptides to the filtrate.

15. The method of claim 14, wherein said filters have a molecular weight cut-off of about 100 kDa to 300 kDa.

16. The method of claim 14, wherein the same molecular weight cut-off filter is used for both pre-digestion filtration and step (b).

17. The method of claim 16, wherein said filter has a molecular weight cut-off of about 100 kDa to 300 kDa.

18. The method of any one of claims 1-17, wherein Tg peptide ions produced in step (c) comprise one or more ions selected from the group of ions with a mass/charge ratio of 541.3 ± 0.5, 612.3 ± 0.5, 636.4 ± 0.5, 726.4 ± 0.5, 797.4 ± 0.5, 912.4 ± 0.5, or 1059.5 ± 0.5.

19. The method of any one of claims 1-17, wherein said ionizing comprises generating a Tg peptide precursor ion with a mass/charge ratio of 636.4 ± 0.5, and generating one or more fragment ions selected from the group consisting of ions with a mass/charge ratio of 797.4 ± 0.5, 912.4 ± 0.5, and 1059.5 ± 0.5.

20. The method of any one of claims 1-19, wherein said test sample is body fluid or tissue.

21. A method for determining the amount of thyroglobulin in a test sample, comprising:

(a) digesting thyroglobulin in said test sample to form peptide T129;

(b) purifying said peptide T129 from step (a);

(c) ionizing said peptide T129 from step (b) to generate a precursor ion detectable by tandem mass spectrometry; wherein said precursor ion has a mass/charge ratio of 636.4 ± 0.5;

(d) fragmenting said precursor ion in said mass spectrometric instrument to generate one or more fragment ions detectable by mass spectrometry, wherein one or more of said fragment ions are selected from the list of ions having a mass/charge ratio of 797.4 ± 0.5, 912.4 ± 0.5, or 1059.5 ± 0.5; and

(e) detecting the amount of said precursor ions of step (c), one or more of said fragment ions of step (d), or both, by mass spectrometry;

wherein the amount of ion(s) detected in step (e) is related to the amount of said thyroglobulin in said test sample.

22. The method of claim 21, said method further comprising analyzing a second quantity of said test sample by steps (b) through (e) to determine the baseline amount of endogenous peptide T129 in the test sample; wherein said baseline can be subtracted from the amount of peptide T129 detected in the digested test sample before relation to the amount of Tg in said test sample.

23. The method of any one of claims 21-22, further comprising purifying thyroglobulin in said test sample prior to step (a).

24. The method of claim 23, wherein purifying thyroglobulin prior to step (a) is accomplished by utilizing at least one size separation technique.

25. The method of any one of claims 21-24, wherein step (b) is accomplished by utilizing at least one size separation technique.

26. The method of claim 25, wherein said at least one size separation technique utilized in both pre-digestion purification and step (b) is filtration.

27. The method of claim 26, wherein filtration is achieved with molecular weight cut-off filters that keep Tg a top the filter and pass Tg peptides to the filtrate.

28. The method of claim 27, wherein the same molecular weight cut-off filter is used for both pre-digestion filtration and step (b).

29. The method of claim 28, wherein said filter has a molecular weight cut-off of about 100 kDa to 300 kDa.

30. The method of any one of claims 21-29, wherein said test sample is body fluid or tissue.

Figure 1

P01266 Sequence

MALVLEIFTLLASICWVSANIFEYQVDAQPLRPCELQRETAFLKQADYVPQCAEDGSFQT
VQCQNDGRSCWCVGANGSEVLGSRQPGRPVACLSFCQLQKQQILLSGYINSTDTSYLPQC
QDSGDYAPVQCDVQQVQCWCVDAEGMEVYGTRQLGRPKRCPRSCEIRNRRLLHGVGDKSP
PQCSAEGEFMPVQCKFVNTTDMMIFDLVHSYNRFPDAFVTFSSFQRRFPEVSGYCHCADS
QGRELAETGLELLLDEIYDTIFAGLDLPSTFTETTLYRILQRRFLAVQSVISGRFRCPTK
CEVERFTATSFGHPYVPSCRRNGDYQAVQCQTEGPCWCVDAQGKEMHGTRQQGEPPSCAE
GQSCASERQQALSRLYFGTSGYFSQHDLFSSPEKRWASPRVARFATSCPPTIKELFVDSG
LLRPMVEGQSQQFSVSENLLKEAIRAIFPSRGLARLALQFTTNPKRLQQNLFGGKFLVNV
GQFNLSGALGTRGTFNFSQFFQQLGLASFLNGGRQEDLAKPLSVGLDSNSSTGTPEAAKK
DGTMNKPTVGSFGFEINLQENQNALKFLASLLELPEFLLFLQHAISVPEDVARDLGDVME
TVLSSQTCEQTPERLFVPSCTTEGSYEDVQCFSGECWCVNSWGKELPGSRVRGGQPRCPT
DCEKQRARMQSLMGSQPAGSTLFVPACTSEGHFLPVQCFNSECYCVDAEGQAIPGTRSAI
GKPKKCPTPCQLQSEQAFLRTVQALLSNSSMLPTLSDTYIPQCSTDGQWRQVQCNGPPEQ
VFELYQRWEAQNKGQDLTPAKLLVKIMSYREAASGNFSLFIQSLYEAGQQDVFPVLSQYP
SLQDVPLAALEGKRPQPRENILLEPYLFWQILNGQLSQYPGSYSDFSTPLAHFDLRNCWC
VDEAGQELEGMRSEPSKLPTCPGSCEEAKLRVLQFIRETEEIVSASNSSRFPLGESFLVA
KGIRLRNEDLGLPPLFPPREAFAEQFLRGSDYAIRLAAQSTLSFYQRRRFSPDDSAGASA
LLRSGPYMPQCDAFGSWEPVQCHAGTGHCWCVDEKGGFIPGSLTARSLQIPQCPTTCEKS
RTSGLLSSWKQARSQENPSPKDLFVPACLETGEYARLQASGAGTWCVDPASGEELRPGSS
SSAQCPSLCNVLKSGVLSRRVSPGYVPACRAEDGGFSPVQCDQAQGSCWCVMDSGEEVPG
TRVTGGQPACESPRCPLPFNASEVVGGTILCETISGPTGSAMQQCQLLCRQGSWSVFPPG
PLICSLESGRWESQLPQPRACQRPQLWQTIQTQGHFQLQLPPGKMCSADYADLLQTFQVF
ILDELTARGFCQIQVKTFGTLVSIPVCNNSSVQVGCLTRERLGVNVTWKSRLEDIPVASL
PDLHDIERALVGKDLLGRFTDLIQSGSFQLHLDSKTFPAETIRFLQGDHFGTSPRTWFGC
SEGFYQVLTSEASQDGLGCVKCPEGSYSQDEECIPCPVGFYQEQAGSLACVPCPVGRTTI
SAGAFSQTHCVTDCQRNEAGLQCDQNGQYRASQKDRGSGKAFCVDGEGRRLPWWETEAPL
EDSQCLMMQKFEKVPESKVIFDANAPVAVRSKVPDSEFPVMQCLTDCTEDEACSFFTVST
TEPEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDSPAVYLKK
GQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCCDGFVLT
QVQGGAIICGLLSSPSVLLCNVKDWMDPSEAWANATCPGVTYDQESHQVILRLGDQEFIK
SLTPLEGTQDTFTNFQQVYLWKDSDMGSRPESMGCRKDTVPRPASPTEAGLTTELFSPVD
LNQVIVNGNQSLSSQKHWLFKHLFSAQQANLWCLSRCVQEHSFCQLAEITESASLYFTCT
LYPEAQVCDDIMESNAQGCRLILPQMPKALFRKKVILEDKVKNFYTRLPFQKLMGISIRN
KVPMSEKSISNGFFECERRCDADPCCTGFGFLNVSQLKGGEVTCLTLNSLGIQMCSEENG
GAWRILDCGSPDIEVHTYPFGWYQKPIAQNNAPSFCPLVVLPSLTEKVSLDSWQSLALSS
VVVDPSIRHFDVAHVSTAATSNFSAVRDLCLSECSQHEACLITTLQTQPGAVRCMFYADT
QSCTHSLQGQNCRLLLREEATHIYRKPGISLLSYEASVPSVPISTHGRLLGRSQAIQVGT
SWKQVDQFLGVPYAAPPLAERRFQAPEPLNWTGSWDASKPRASCWQPGTRTSTSPGVSED
CLYLNVFIPQNVAPNASVLVFFHNTMDREESEGWPAIDGSFLAAVGNLIVVTASYRVGVF
GFLSSGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPRRVSLAADRGGADVASIHLLTAR
ATNSQLFRRAVLMGGSALSPAAVISHERAQQQAIALAKEVSCPMSSSQEVVSCLRQKPAN
VLNDAQTKLLAVSGPFHYWGPVIDGHFLREPPARALKRSLWVEVDLLIGSSQDDGLINRA
KAVKQFEESRGRTSSKTAFYQALQNSLGGEDSDARVEAAATWYYSLEHSTDDYASFSRAL
ENATRDYFIICPIIDMASAWAKRARGNVFMYHAPENYGHGSLELLADVQFALGLPFYPAY
EGQFSLEEKSLSLKIMQYFSHFIRSGNPNYPYEFSRKVPTFATPWPDFVPRAGGENYKEF
SELLPNRQGLKKADCSFWSKYISSLKTSADGAKGGQSAESEEEELTAGSGLREDLLSLQE
PGSKTYSK

Figure 2

P01266-2 Isoform 2 Sequence

>sp_vs|P01266-2|THYG_HUMAN Isoform 2 of P01266 - Homo sapiens (Human)
MALVLEIFTLLASICWVSANIFEYQVDAQPLRPCELQRETAFLKQADYVPQCAEDGSFQT
VQCQNDGRSCWCVGANGSEVLGSRQPGRPVACLSFCQLQKQQILLSGYINSTDTSYLPQC
QDSGDYAPVQCDVQQVQCWCVDAEGMEVYGTRQLGRPKRCPRSCEIRNRRLLHGVGDKSP
PQCSAEGEFMPVQCKFVNTTDMMIFDLVHSYNRFPDAFVTFSSFQRRFPEVSGYCHCADS
QGRELAETGLELLLDEIYDTIFAGLDLPSTFTETTLYRILQRRFLAVQSVISGRFRCPTK
CEVERFTATSFGHPYVPSCRRNGDYQAVQCQTEGPCWCVDAQGKEMHGTRQQGEPPSCAE
GQSCASERQQALSRLYFGTSGYFSQHDLFSSPEKRWASPRVARFATSCPPTIKELFVDSG
LLRPMVEGQSQQFSVSENLLKEAIRAIFPSRGLARLALQFTTNPKRLQQNLFGGKFLVNV
GQFNLSGALGTRGTFNFSQFFQQLGLASFLNGGRQEDLAKPLSVGLDSNSSTGTPEAAKK
DGTMNKPTVGSFGFEINLQENQNALKFLASLLELPEFLLFLQHAISVPEDVARDLGDVME
TVLSSQTCEQTPERLFVPSCTTEGSYEDVQCFSGECWCVNSWGKELPGSRVRGGQPRCPT
DCEKQRARMQSLMGSQPAGSTLFVPACTSEGHFLPVQCFNSECYCVDAEGQAIPGTRSAI
GKPKKCPTPCQLQSEQAFLRTVQALLSNSSMLPTLSDTYIPQCSTDGQWRQVQCNGPPEQ
VFELYQRWEAQNKGQDLTPAKLLVKIMSYREAASGNFSLFIQSLYEAGQQDVFPVLSQYP
SLQDVPLAALEGKRPQPRENILLEPYLFWQILNGQLSQYPGSYSDFSTPLAHFDLRNCWC
VDEAGQELEGMRSEPSKLPTCPGSCEEAKLRVLQFIRETEEIVSASNSSRFPLGESFLVA
KGIRLRNEDLGLPPLFPPREAFAEQFLRGSDYAIRLAAQSTLSFYQRRRFSPDDSAGASA
LLRSGPYMPQCDAFGSWEPVQCHAGTGHCWCVDEKGGFIPGSLTARSLQIPQCPTTCEKS
RTSGLLSSWKQARSQENPSPKDLFVPACLETGEYARLQASGAGTWCVDPASGEELRPGSS
SSAQCPSLCNVLKSGVLSRRVSPGYVPACRAEDGGFSPVQCDQAQGSCWCVMDSGEEVPG
TRVTGGQPACESPRCPLPFNASEVVGGTILCETISGPTGSAMQQCQLLCRQGSWSVFPPG
PLICSLESGRWESQLPQPRACQRPQLWQTIQTQGHFQLQLPPGKMCSADYADLLQTFQVF
ILDELTARGFCQIQVKTFGTLVSIPVCNNSSVQVGCLTRERLGVNVTWKSRLEDIPVASL
PDLHDIERALVGKDLLGRFTDLIQSGSFQLHLDSKTFPAETIRFLQGDHFGTSPRTWFGC
SEGFYQVLTSEASQDGLGCVKCPEGSYSQDEECIPCPVGFYQEQAGSLACVPCPVGRTTI
SAGAFSQTHLMQKFEKVPESKVIFDANAPVAVRSKVPDSEFPVMQCLTDCTEDEACSFFT
VSTTEPEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDSPAVY
LKKGQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCCDGF
VLTQVQGGAIICGLLSSPSVLLCNVKDWMDPSEAWANATCPGVTYDQESHQVILRLGDQE
FIKSLTPLEGTQDTFTNFQQVYLWKDSDMGSRPESMGCRKDTVPRPASPTEAGLTTELFS
PVDLNQVIVNGNQSLSSQKHWLFKHLFSAQQANLWCLSRCVQEHSFCQLAEITESASLYF
TCTLYPEAQVCDDIMESNAQGCRLILPQMPKALFRKKVILEDKVKNFYTRLPFQKLMGIS
IRNKVPMSEKSISNGFFECERRCDADPCCTGFGFLNVSQLKGGEVTCLTLNSLGIQMCSE
ENGGAWRILDCGSPDIEVHTYPFGWYQKPIAQNNAPSFCPLVVLPSLTEKVSLDSWQSLA
LSSVVVDPSIRHFDVAHVSTAATSNFSAVRDLCLSECSQHEACLITTLQTQPGAVRCMFY
ADTQSCTHSLQGQNCRLLLREEATHIYRKPGISLLSYEASVPSVPISTHGRLLGRSQAIQ
VGTSWKQVDQFLGVPYAAPPLAERRFQAPEPLNWTGSWDASKPRASCWQPGTRTSTSPGV
SEDCLYLNVFIPQNVAPNASVLVFFHNTMDREESEGWPAIDGSFLAAVGNLIVVTASYRV
GVFGFLSSGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPRRVSLAADRGGADVASIHLL
TARATNSQLFRRAVLMGGSALSPAAVISHERAQQQAIALAKEVSCPMSSSQEVVSCLRQK
PANVLNDAQTKLLAVSGPFHYWGPVIDGHFLREPPARALKRSLWVEVDLLIGSSQDDGLI
NRAKAVKQFEESRGRTSSKTAFYQALQNSLGGEDSDARVEAAATWYYSLEHSTDDYASFS
RALENATRDYFIICPIIDMASAWAKRARGNVFMYHAPENYGHGSLELLADVQFALGLPFY
PAYEGQFSLEEKSLSLKIMQYFSHFIRSGNPNYPYEFSRKVPTFATPWPDFVPRAGGENY
KEFSELLPNRQGLKKADCSFWSKYISSLKTSADGAKGGQSAESEEEELTAGSGLREDLLS
LQEPGSKTYSK

Figure 3

Q59GF0 (Tg variant-Fragment) Sequence

>Q59GF0|Q59GF0_HUMAN Thyroglobulin variant (Fragment) - Homo sapiens (Human).

IPRKPISKRPVRPSLPRSPRCPLPFNASEVVGGTILCETISGPTGSAMQQCQLLCRQGSW
SVFPPGPLICSLESGRWESQLPQPRACQRPQLWQTIQTQGHFQLQLPPGKMCSADYAGLL
QTFQVFILDELTARGFCQIQVKTFGTLVSIPVCNNSSVQVGCLTRERLGVNVTWKSRLED
IPVASLPDLHDIERALVGKDLLGRFTDLIQSGSFQLHLDSKTFPAETIRFLQGDHFGTSP
RTWFGCSEGFYQVLTSEASQDGLGCVKCPEGSYSQDEECIPCPVGFYQEQAGSLACVPCP
VGRTTISAGAFSQTHCVTDCQRNEAGLQCDQNGQYRASQKDRGSGKAFCVDGEGRRLPWW
ETEAPLEDSQCLMMQKFEKVPESKVIFDANAPVAVRSKVPDSEFPVMQCLTDCTEDEACS
FFTVSTTEPEISCDFYAWTSDNVACMTSDQKRDALGNSKATSFGSLRCQVKVRSHGQDSP
AVYLKKGQGSTTTLQKRFEPTGFQNMLSGLYNPIVFSASGANLTDAHLFCLLACDRDLCC
DGFVLTQVQGGAIICGLLSSPSVLLCNVKDWMDPSEAWANATCPGVTYDQESHQVILRLG
DQEFIKSLTPLEGTQDTFTNFQQVYLWKDSDMGSRPESMGCRKNTVPRPASPTEAGLTTE
LFSPVDLNQVIVNGNQSLSSQKHWLFKHLFSAQQANLWCLSRCVQEHSFCQLAEITESAS
LYFTCTLYPEAQVCDDIMESNAQGCRLILPQMPKALFRKKVILEDKVKNFYTRLPFQKLT
GISIRNKVPMSEKSISNGFFECERRCDADPCCTGFGFLNVSQLKGGEVTCLTLNSLGIQM
CSEENGGAWRILDCGSPDIEVHTYPFGWYQKPIAQNNAPSFCPLVVLPSLTEKVSLDSWQ
SLALSSVVVDPSIRHFDVAHVSTAATSNFSAVRDLCLSECSQHEACLITTLQTQPGAVRC
MFYADTQSCTHSLQGQNCRLLLREEATHIYRKPGISLLSYEASVPSVPISTHGRLLGRSQ
AIQVGTSWKQVDQFLGVPYAAPPLAERRFQAPEPLNWTGSWDASKPRASCWQPGTRTSTS
PGVSEDCLYLNVFIPQNVAPNASVLVFFHNTMDREESEGWPAIDGSFLAAVGNLIVVTAS
YRVGVFGFLSSGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPRRVSLAADRGGADVASI
HLLTARATNSQLFRRAVLMGGSALSPAAVISHERAQQQAIALAKEVSCPMSSSQEVVSCL
RQKPANVLNDAQTKLLAVSGPFHYWGPVIDGHFLREPPARALKRSLWVEVDLLIGSSQDD
GLINRAKAVKQFEESQGRTSSKTAFYQALQNSLGGEDSDARVEAAATWYYSLEHSTDDYA
SFSRALENATRDYFIICPIIDMASAWAKRARGNVFMYHAPENYGHGSLELLADVQFALGL
PFYPAYEGQFSLEEKSLSLKIMQYFSHFIRSGNPNYPYEFSRKVPTFATPWPDFVPRAGG
ENYKEFSELLPNRQGLKKADCSFWSKYISSLKTSADGAKGGQSAESEEEELTAGSGLRED
LLSLQEPGSKTYSK

DLMR_560807.1

Figure 4

```
        10         20         30         40         50         60
MALVLEIFTL LASICWVSAN IFEYQVDAQP LRPCELQRET AFLKQADYVP QCAEDGSFQT
MALVLEIFTL LASICWVSAN IFEYQVDAQP LRPCELQRET AFLKQADYVP QCAEDGSFQT

        70         80         90        100        110        120
VQCQNDGRSC WCVGANGSEV LGSRQPGRPV ACLSFCQLQK QQILLSGYIN STDTSYLPQC
VQCQNDGRSC WCVGANGSEV LGSRQPGRPV ACLSFCQLQK QQILLSGYIN STDTSYLPQC

       130        140        150        160        170        180
QDSGDYAPVQ CDVQQVQCWC VDAEGMEVYG TRQLGRPKRC PRSCEIRNRR LLHGVGDKSP
QDSGDYAPVQ CDVQQVQCWC VDAEGMEVYG TRQLGRPKRC PRSCEIRNRR LLHGVGDKSP

       190        200        210        220        230        240
PQCSAEGEFM PVQCKFVNTT DMMIFDLVHS YNRFPDAFVT FSSFQRRFPE VSGYCHCADS
PQCSAEGEFM PVQCKFVNTT DMMIFDLVHS YNRFPDAFVT FSSFQRRFPE VSGYCHCADS

       250        260        270        280        290        300
QGRELAETGL ELLLDEIYDT IFAGLDLPST FTETTLYRIL QRRFLAVQSV ISGRFRCPTK
QGRELAETGL ELLLDEIYDT IFAGLDLPST FTETTLYRIL QRRFLAVQSV ISGRFRCPTK

       310        320        330        340        350        360
CEVERFTATS FGHPYVPSCR RNGDYQAVQC QTEGPCWCVD AQGKEMHGTR QQGEPPSCAE
CEVERFTATS FGHPYVPSCR RNGDYQAVQC QTEGPCWCVD AQGKEMHGTR QQGEPPSCAE

       370        380        390        400        410        420
GQSCASERQQ ALSRLYFGTS GYFSQHDLFS SPEKRWASPR VARFATSCPP TIKELFVDSG
GQSCASERQQ ALSRLYFGTS GYFSQHDLFS SPEKRWASPR VARFATSCPP TIKELFVDSG

       430        440        450        460        470        480
LLRPMVEGQS QQFSVSENLL KEAIRAIFPS RGLARLALQF TTNPKRLQQN LFGGKFLVNV
LLRPMVEGQS QQFSVSENLL KEAIRAIFPS RGLARLALQF TTNPKRLQQN LFGGKFLVNV

       490        500        510        520        530        540
GQFNLSGALG TRGTFNFSQF FQQLGLASFL NGGRQEDLAK PLSVGLDSNS STGTPEAAKK
GQFNLSGALG TRGTFNFSQF FQQLGLASFL NGGRQEDLAK PLSVGLDSNS STGTPEAAKK

       550        560        570        580        590        600
DGTMNKPTVG SFGFEINLQE NQNALKFLAS LLELPEFLLF LQHAISVPED VARDLGDVME
```

DGTMNKPTVG SFGFEINLQE NQNALKFLAS LLELPEFLLF LQHAISVPED VARDLGDVME

610 620 630 640 650 660
TVLSSQTCEQ TPERLFVPSC TTEGSYEDVQ CFSGECWCVN SWGKELPGSR VRGGQPRCPT
TVLSSQTCEQ TPERLFVPSC TTEGSYEDVQ CFSGECWCVN SWGKELPGSR VRGGQPRCPT

670 680 690 700 710 720
DCEKQRARMQ SLMGSQPAGS TLFVPACTSE GHFLPVQCFN SECYCVDAEG QAIPGTRSAI
DCEKQRARMQ SLMGSQPAGS TLFVPACTSE GHFLPVQCFN SECYCVDAEG QAIPGTRSAI

730 740 750 760 770 780
GKPKKCPTPC QLQSEQAFLR TVQALLSNSS MLPTLSDTYI PQCSTDGQWR QVQCNGPPEQ
GKPKKCPTPC QLQSEQAFLR TVQALLSNSS MLPTLSDTYI PQCSTDGQWR QVQCNGPPEQ

790 800 810 820 830 840
VFELYQRWEA QNKGQDLTPA KLLVKIMSYR EAASGNFSLF IQSLYEAGQQ DVFPVLSQYP
VFELYQRWEA QNKGQDLTPA KLLVKIMSYR EAASGNFSLF IQSLYEAGQQ DVFPVLSQYP

850 860 870 880 890 900
SLQDVPLAAL EGKRPQPREN ILLEPYLFWQ ILNGQLSQYP GSYSDFSTPL AHFDLRNCWC
SLQDVPLAAL EGKRPQPREN ILLEPYLFWQ ILNGQLSQYP GSYSDFSTPL AHFDLRNCWC

910 920 930 940 950 960
VDEAGQELEG MRSEPSKLPT CPGSCEEAKL RVLQFIRETE EIVSASNSSR FPLGESFLVA
VDEAGQELEG MRSEPSKLPT CPGSCEEAKL RVLQFIRETE EIVSASNSSR FPLGESFLVA

970 980 990 1000 1010 1020
KGIRLRNEDL GLPPLFPPRE AFAEQFLRGS DYAIRLAAQS TLSFYQRRRF SPDDSAGASA
KGIRLRNEDL GLPPLFPPRE AFAEQFLRGS DYAIRLAAQS TLSFYQRRRF SPDDSAGASA

1030 1040 1050 1060 1070 1080
LLRSGPYMPQ CDAFGSWEPV QCHAGTGHCW CVDEKGGFIP GSLTARSLQI PQCPTTCEKS
LLRSGPYMPQ CDAFGSWEPV QCHAGTGHCW CVDEKGGFIP GSLTARSLQI PQCPTTCEKS

1090 1100 1110 1120 1130 1140
RTSGLLSSWK QARSQENPSP KDLFVPACLE TGEYARLQAS GAGTWCVDPA SGEELRPGSS
RTSGLLSSWK QARSQENPSP KDLFVPACLE TGEYARLQAS GAGTWCVDPA SGEELRPGSS

1150 1160 1170 1180 1190 1200
SSAQCPSLCN VLKSGVLSRR VSPGYVPACR AEDGGFSPVQ CDQAQGSCWC VMDSGEEVPG

SSAQCPSLCN VLKSGVLSRR VSPGYVPACR AEDGGFSPVQ CDQAQGSCWC VMDSGEEVPG
IPRKPI

1210 1220 1230 1240 1250 1260
TRVTGGQPAC ESPRCPLPFN ASEVVGGTIL CETISGPTGS AMQQCQLLCR QGSWSVFPPG
TRVTGGQPAC ESPRCPLPFN ASEVVGGTIL CETISGPTGS AMQQCQLLCR QGSWSVFPPG
SKRPVRPSLP RSPRCPLPFN ASEVVGGTIL CETISGPTGS AMQQCQLLCR QGSWSVFPPG

1270 1280 1290 1300 1310 1320
PLICSLESGR WESQLPQPRA CQRPQLWQTI QTQGHFQLQL PPGKMCSADY ADLLQTFQVF
PLICSLESGR WESQLPQPRA CQRPQLWQTI QTQGHFQLQL PPGKMCSADY ADLLQTFQVF
PLICSLESGR WESQLPQPRA CQRPQLWQTI QTQGHFQLQL PPGKMCSADY AGLLQTFQVF

1330 1340 1350 1360 1370 1380
ILDELTARGF CQIQVKTFGT LVSIPVCNNS SVQVGCLTRE RLGVNVTWKS RLEDIPVASL
ILDELTARGF CQIQVKTFGT LVSIPVCNNS SVQVGCLTRE RLGVNVTWKS RLEDIPVASL
ILDELTARGF CQIQVKTFGT LVSIPVCNNS SVQVGCLTRE RLGVNVTWKS RLEDIPVASL

1390 1400 1410 1420 1430 1440
PDLHDIERAL VGKDLLGRFT DLIQSGSFQL HLDSKTFPAE TIRFLQGDHF GTSPRTWFGC
PDLHDIERAL VGKDLLGRFT DLIQSGSFQL HLDSKTFPAE TIRFLQGDHF GTSPRTWFGC
PDLHDIERAL VGKDLLGRFT DLIQSGSFQL HLDSKTFPAE TIRFLQGDHF GTSPRTWFGC

1450 1460 1470 1480 1490 1500
SEGFYQVLTS EASQDGLGCV KCPEGSYSQD EECIPCPVGF YQEQAGSLAC VPCPVGRTTI
SEGFYQVLTS EASQDGLGCV KCPEGSYSQD EECIPCPVGF YQEQAGSLAC VPCPVGRTTI
SEGFYQVLTS EASQDGLGCV KCPEGSYSQD EECIPCPVGF YQEQAGSLAC VPCPVGRTTI

1510 1520 1530 1540 1550 1560
SAGAFSQTHC VTDCQRNEAG LQCDQNGQYR ASQKDRGSGK AFCVDGEGRR LPWWETEAPL
SAGAFSQTHL
SAGAFSQTHC VTDCQRNEAG LQCDQNGQYR ASQKDRGSGK AFCVDGEGRR LPWWETEAPL

1570 1580 1590 1600 1610 1620
EDSQCLMMQK FEKVPESKV**I** **FDANAPVAVR** SKVPDSEFPV MQCLTDCTED EACSFFTVST
MQK FEKVPESKV**I** **FDANAPVAVR** SKVPDSEFPV MQCLTDCTED EACSFFTVST
EDSQCLMMQK FEKVPESKV**I** **FDANAPVAVR** SKVPDSEFPV MQCLTDCTED EACSFFTVST

1630 1640 1650 1660 1670 1680
TEPEISCDFY AWTSDNVACM TSDQKRDALG NSKATSFGSL RCQVKVRSHG QDSPAVYLKK
TEPEISCDFY AWTSDNVACM TSDQKRDALG NSKATSFGSL RCQVKVRSHG QDSPAVYLKK
TEPEISCDFY AWTSDNVACM TSDQKRDALG NSKATSFGSL RCQVKVRSHG QDSPAVYLKK

1690 1700 1710 1720 1730 1740

GQGSTTTLQK RFEPTGFQNM LSGLYNPIVF SASGANLTDA HLFCLLACDR DLCCDGFVLT
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GQGSTTTLQK RFEPTGFQNM LSGLYNPIVF SASGANLTDA HLFCLLACDR DLCCDGFVLT

1750 1760 1770 1780 1790 1800
QVQGGAIICG LLSSPSVLLC NVKDWMDPSE AWANATCPGV TYDQESHQVI LRLGDQEFIK
QVQGGAIICG LLSSPSVLLC NVKDWMDPSE AWANATCPGV TYDQESHQVI LRLGDQEFIK
QVQGGAIICG LLSSPSVLLC NVKDWMDPSE AWANATCPGV TYDQESHQVI LRLGDQEFIK

1810 1820 1830 1840 1850 1860
SLTPLEGTQD TFTNFQQVYL WKDSDMGSRP ESMGCRKDTV PRPASPTEAG LTTELFSPVD
SLTPLEGTQD TFTNFQQVYL WKDSDMGSRP ESMGCRKDTV PRPASPTEAG LTTELFSPVD
SLTPLEGTQD TFTNFQQVYL WKDSDMGSRP ESMGCRKNTV PRPASPTEAG LTTELFSPVD

1870 1880 1890 1900 1910 1920
LNQVIVNGNQ SLSSQKHWLF KHLFSAQQAN LWCLSRCVQE HSFCQLAEIT ESASLYFTCT
LNQVIVNGNQ SLSSQKHWLF KHLFSAQQAN LWCLSRCVQE HSFCQLAEIT ESASLYFTCT
LNQVIVNGNQ SLSSQKHWLF KHLFSAQQAN LWCLSRCVQE HSFCQLAEIT ESASLYFTCT

1930 1940 1950 1960 1970 1980
LYPEAQVCDD IMESNAQGCR LILPQMPKAL FRKKVILEDK VKNFYTRLPF QKLMGISIRN
LYPEAQVCDD IMESNAQGCR LILPQMPKAL FRKKVILEDK VKNFYTRLPF QKLMGISIRN
LYPEAQVCDD IMESNAQGCR LILPQMPKAL FRKKVILEDK VKNFYTRLPF QKLTGISIRN

1990 2000 2010 2020 2030 2040
KVPMSEKSIS NGFFECERRC DADPCCTGFG FLNVSQLKGG EVTCLTLNSL GIQMCSEENG
KVPMSEKSIS NGFFECERRC DADPCCTGFG FLNVSQLKGG EVTCLTLNSL GIQMCSEENG
KVPMSEKSIS NGFFECERRC DADPCCTGFG FLNVSQLKGG EVTCLTLNSL GIQMCSEENG

2050 2060 2070 2080 2090 2100
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GAWRILDCGS PDIEVHTYPF GWYQKPIAQN NAPSFCPLVV LPSLTEKVSL DSWQSLALSS
GAWRILDCGS PDIEVHTYPF GWYQKPIAQN NAPSFCPLVV LPSLTEKVSL DSWQSLALSS

2110 2120 2130 2140 2150 2160
VVVDPSIRHF DVAHVSTAAT SNFSAVRDLC LSECSQHEAC LITTLQTQPG AVRCMFYADT
VVVDPSIRHF DVAHVSTAAT SNFSAVRDLC LSECSQHEAC LITTLQTQPG AVRCMFYADT
VVVDPSIRHF DVAHVSTAAT SNFSAVRDLC LSECSQHEAC LITTLQTQPG AVRCMFYADT

2170 2180 2190 2200 2210 2220

QSCTHSLQGQ NCRLLLREEA THIYRKPGIS LLSYEASVPS VPISTHGRLL GRSQAIQVGT
QSCTHSLQGQ NCRLLLREEA THIYRKPGIS LLSYEASVPS VPISTHGRLL GRSQAIQVGT
QSCTHSLQGQ NCRLLLREEA THIYRKPGIS LLSYEASVPS VPISTHGRLL GRSQAIQVGT

2230 2240 2250 2260 2270 2280
SWKQVDQFLG VPYAAPPLAE RRFQAPEPLN WTGSWDASKP RASCWQPGTR TSTSPGVSED
SWKQVDQFLG VPYAAPPLAE RRFQAPEPLN WTGSWDASKP RASCWQPGTR TSTSPGVSED
SWKQVDQFLG VPYAAPPLAE RRFQAPEPLN WTGSWDASKP RASCWQPGTR TSTSPGVSED

2290 2300 2310 2320 2330 2340
CLYLNVFIPQ NVAPNASVLV FFHNTMDREE SEGWPAIDGS FLAAVGNLIV VTASYRVGVF
CLYLNVFIPQ NVAPNASVLV FFHNTMDREE SEGWPAIDGS FLAAVGNLIV VTASYRVGVF
CLYLNVFIPQ NVAPNASVLV FFHNTMDREE SEGWPAIDGS FLAAVGNLIV VTASYRVGVF

2350 2360 2370 2380 2390 2400
GFLSSGSGEV SGNWGLLDQV AALTWVQTHI RGFGGDPRRV SLAADRGGAD VASIHLLTAR
GFLSSGSGEV SGNWGLLDQV AALTWVQTHI RGFGGDPRRV SLAADRGGAD VASIHLLTAR
GFLSSGSGEV SGNWGLLDQV AALTWVQTHI RGFGGDPRRV SLAADRGGAD VASIHLLTAR

2410 2420 2430 2440 2450 2460
ATNSQLFRRA VLMGGSALSP AAVISHERAQ QQAIALAKEV SCPMSSSQEV VSCLRQKPAN
ATNSQLFRRA VLMGGSALSP AAVISHERAQ QQAIALAKEV SCPMSSSQEV VSCLRQKPAN
ATNSQLFRRA VLMGGSALSP AAVISHERAQ QQAIALAKEV SCPMSSSQEV VSCLRQKPAN

2470 2480 2490 2500 2510 2520
VLNDAQTKLL AVSGPFHYWG PVIDGHFLRE PPARALKRSL WVEVDLLIGS SQDDGLINRA
VLNDAQTKLL AVSGPFHYWG PVIDGHFLRE PPARALKRSL WVEVDLLIGS SQDDGLINRA
VLNDAQTKLL AVSGPFHYWG PVIDGHFLRE PPARALKRSL WVEVDLLIGS SQDDGLINRA

2530 2540 2550 2560 2570 2580
KAVKQFEESR GRTSSKTAFY QALQNSLGGE DSDARVEAAA TWYYSLEHST DDYASFSRAL
KAVKQFEESR GRTSSKTAFY QALQNSLGGE DSDARVEAAA TWYYSLEHST DDYASFSRAL
KAVKQFEESQ GRTSSKTAFY QALQNSLGGE DSDARVEAAA TWYYSLEHST DDYASFSRAL

2590 2600 2610 2620 2630 2640
ENATRDYFII CPIIDMASAW AKRARGNVFM YHAPENYGHG SLELLADVQF ALGLPFYPAY
ENATRDYFII CPIIDMASAW AKRARGNVFM YHAPENYGHG SLELLADVQF ALGLPFYPAY
ENATRDYFII CPIIDMASAW AKRARGNVFM YHAPENYGHG SLELLADVQF ALGLPFYPAY

2650 2660 2670 2680 2690 2700
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EGQFSLEEKS LSLKIMQYFS HFIRSGNPNY PYEFSRKVPT FATPWPDFVP RAGGENYKEF
EGQFSLEEKS LSLKIMQYFS HFIRSGNPNY PYEFSRKVPT FATPWPDFVP RAGGENYKEF

2710 2720 2730 2740 2750 2760
SELLPNRQGL KKADCSFWSK YISSLKTSAD GAKGGQSAES EEEELTAGSG LREDLLSLQE
SELLPNRQGL KKADCSFWSK YISSLKTSAD GAKGGQSAES EEEELTAGSG LREDLLSLQE
SELLPNRQGL KKADCSFWSK YISSLKTSAD GAKGGQSAES EEEELTAGSG LREDLLSLQE

PGSKTYSK
PGSKTYSK
PGSKTYSK

Figure 5

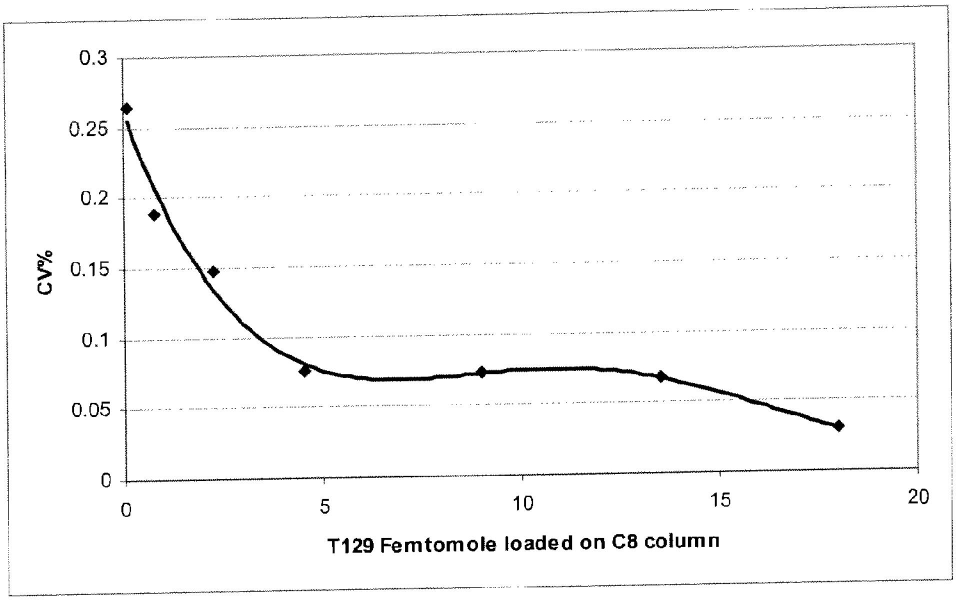


Figure 6

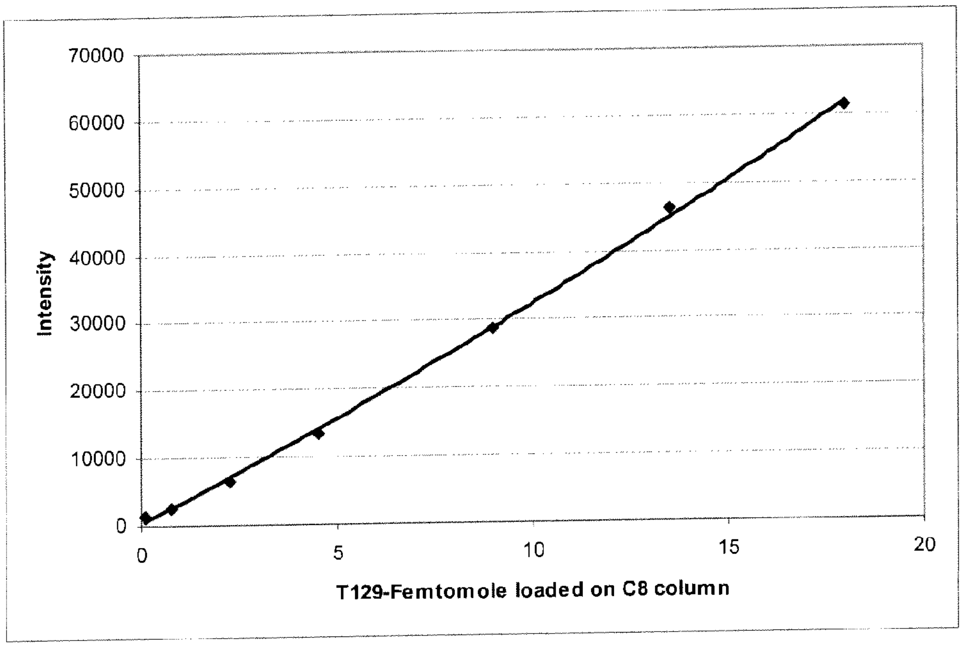


Figure 7

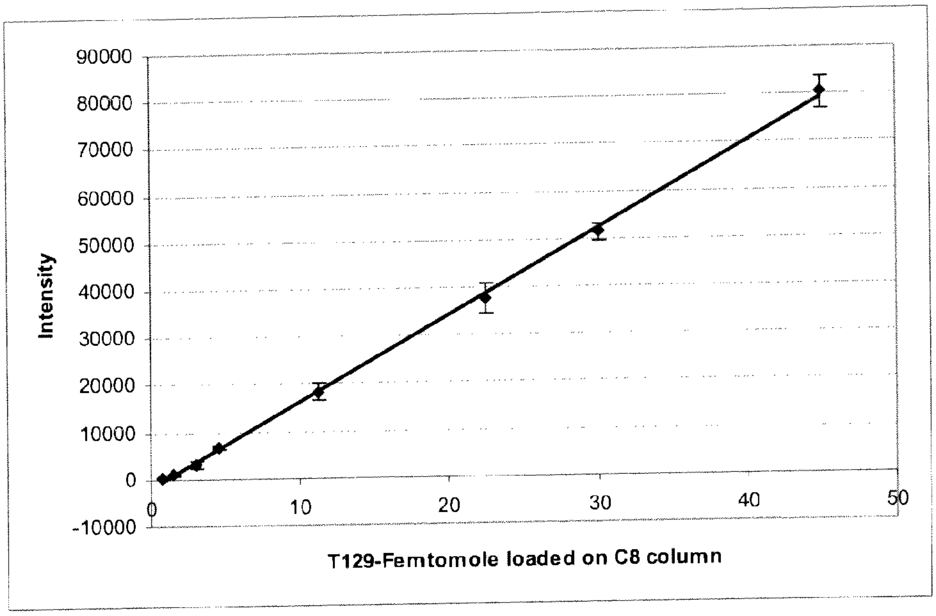


Figure 8

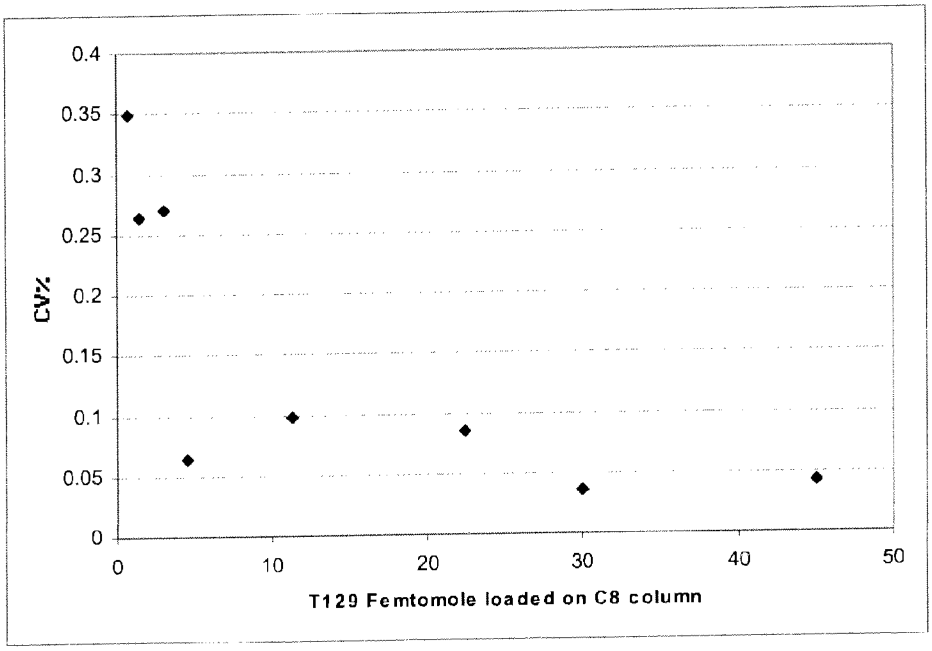
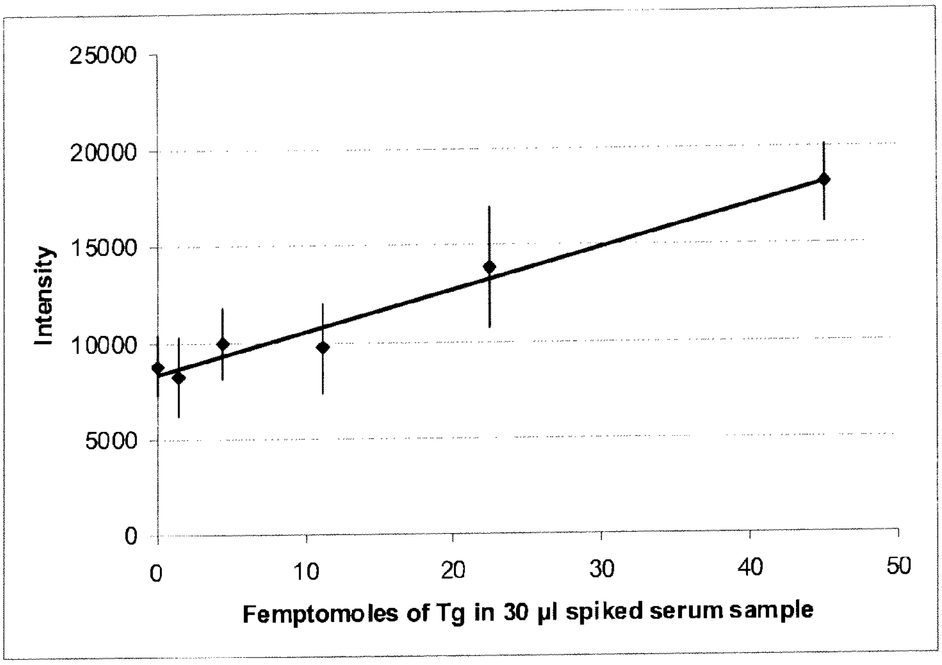


Figure 9


25000
20000
15000
10000
5000
0
Intensity
0
10
20
30
40
50
Femptomoles of Tg in 30 µl spiked serum sample