(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 June 2011 (09.06.2011)

PCT

(10) International Publication Number
WO 2011/068707 A1

(51) International Patent Classification:
G01N 33/00 (2006.01)

(21) International Application Number:
PCT/US2010/057627

(22) International Filing Date:
22 November 2010 (22.11.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12/630,790 3 December 2009 (03.12.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2011/068707 A1

(54) Title: VITAMIN D METABOLITE DETERMINATION UTILIZING MASS SPECTROMETRY FOLLOWING DERIVATIZATION

(57) Abstract: The invention relates to the detection of vitamin D metabolites. In a particular aspect, the invention relates to methods for detecting derivatized vitamin D metabolites by mass spectrometry.

VITAMIN D METABOLITE DETERMINATION UTILIZING MASS SPECTROMETRY FOLLOWING DERIVATIZATION

FIELD OF THE INVENTION

[0001] The invention relates to the quantitative measurement of vitamin D metabolites. In a particular aspect, the invention relates to methods for quantitative measurement of vitamin D metabolites by HPLC-tandem mass spectrometry.

BACKGROUND OF THE INVENTION

[0002] Vitamin D is an essential nutrient with important physiological roles in the positive regulation of calcium (Ca^{2+}) homeostasis. Vitamin D can be made *de novo* in the skin by exposure to sunlight or it can be absorbed from the diet. There are two forms of vitamin D; vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 is the form synthesized *de novo* by animals. It is also a common supplement added to milk products and certain food products produced in the United States. Both dietary and intrinsically synthesized vitamin D_3 must undergo metabolic activation to generate the bioactive metabolites. In humans, the initial step of vitamin D_3 activation occurs primarily in the liver and involves hydroxylation to form the intermediate metabolite 25-hydroxycholecalciferol (calcifediol; $25OHD_3$). Calcifediol is the major form of Vitamin D_3 in circulation. Circulating $25OHD_3$ is then converted by the kidney to form 1α,25-dihydroxyvitamin D_3 (calcitriol; $1\alpha,25(OH)_2D_3$), which is generally believed to be the metabolite of Vitamin D_3 with the highest biological activity.

[0003] Vitamin D_2 is derived from fungal and plant sources. Many over-the-counter dietary supplements contain ergocalciferol (vitamin D_2) rather than cholecalciferol (vitamin D_3). Drisdol, the only high-potency prescription form of vitamin D available in the United States, is formulated with ergocalciferol. Vitamin D_2 undergoes a similar pathway of metabolic activation in humans as Vitamin D_3, forming the metabolites $25OHD_2$ and $1\alpha,25(OH)_2D_2$. Vitamin D_2 and vitamin D_3 have long been assumed to be biologically equivalent in humans, however recent reports suggest that there may be differences in the bioactivity and bioavailability of these two forms of vitamin D (Armas *et. al.,* (2004) J. Clin. Endocrinol. Metab. 89:5387-5391).

[0004] Measurement of vitamin D, the inactive vitamin D precursor, is rare in clinical settings. Rather, serum levels of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, and total

25-hydroxyvitamin D ("25OHD") are useful indices of vitamin D nutritional status and the efficacy of certain vitamin D analogs. The measurement of 25OHD is commonly used in the diagnosis and management of disorders of calcium metabolism. In this respect, low levels of 25OHD are indicative of vitamin D deficiency associated with diseases such as hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, elevated alkaline phosphatase, osteomalacia in adults and rickets in children. In patients suspected of vitamin D intoxication, elevated levels of 25OHD distinguishes this disorder from other disorders that cause hypercalcemia.

[0005] Measurement of $1\alpha,25(OH)_2D$ is also used in clinical settings. Certain disease states can be reflected by circulating levels of $1\alpha,25(OH)_2D$, for example kidney disease and kidney failure often result in low levels of $1\alpha,25(OH)_2D$. Elevated levels of $1,25(OH)_2D$ may be indicative of excess parathyroid hormone or can be indicative of certain diseases such as sarcoidosis or certain types of lymphomas.

[0006] Detection of vitamin D metabolites has been accomplished by radioimmunoassay with antibodies co-specific for $25OHD_2$ and $25OHD_3$. Because the current immunologically-based assays do not separately resolve $25OHD_2$ and $25OHD_3$, the source of any nutritional deficiency of vitamin D cannot be determined without resorting to other tests. Reports have been published that disclose methods for detecting specific vitamin D metabolites using mass spectrometry. In some of the reports, the vitamin D metabolites are derivatized prior to mass spectrometry, but in others, they are not. For example Holmquist, et al., U.S. Patent Application Serial No. 11/946765, filed December 28, 2007; Yeung B, *et al.,* J Chromatogr. 1993, 645(1):115-23; Higashi T, et al., Steroids. 2000, 65(5):281-94; Higashi T, *et al.,* Biol Pharm Bull. 2001, 24(7):738-43; Higashi T, *et al.,* J Pharm Biomed Anal. 2002, 29(5):947-55; Higashi T, et al., Anal. Biochanal Chem, 2008, 391:229-38; and Aronov, et al., Anal Bioanal Chem, 2008, 391:1917-30 disclose methods for detecting various vitamin D metabolites by derivatizing the metabolites prior to mass spectrometry. Methods to detect underivatized vitamin D metabolites are reported in Clarke, *et al.*, in U.S. Patent Application Serial Nos. 11/101,166, filed April 6, 2005, and 11/386,215, filed March 21, 2006, and Singh, et al., in U.S. Patent Application Serial No. 10/977,121, filed October 24, 2004.

SUMMARY OF THE INVENTION

[0007] The present invention provides methods for detecting the amount of one or more vitamin D metabolites in a sample by mass spectrometry, including tandem mass

spectrometry. The methods include derivatizing vitamin D metabolites in the sample prior to analysis.

[0008] In one aspect, the invention provides methods for determining the amount of one or more vitamin D metabolites in a sample by mass spectrometry. In some embodiments, the methods include the steps of: (i) subjecting one or more 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)-derivatized vitamin D metabolites in the sample to an extraction column and an analytical column; (ii) subjecting one or more PTAD-derivatized vitamin D metabolites in the sample to an ionization source to generate one or more ions detectable by mass spectrometry; (iii) determining the amount of one or more of the PTAD-derivatized vitamin D metabolite ions by mass spectrometry; and (iv) relating the amount of PTAD-derivatized vitamin D metabolite ions determined in step (iii) to the amount of a vitamin D metabolite in the sample. In these methods, the sample is subjected to 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) under conditions sufficient to generate one or more PTAD-derivatized vitamin D metabolites prior to step (i). In some embodiments, the sample is subject to turbulent flow liquid chromatography (TFLC) as the extraction column, and no analytical column is used. In other embodiments, no chromatography, including extraction chromatography, is used. In these methods, the derivatized vitamin D metabolites may be ionized with laser diode thermal desorption (LDTD).

[0009] In some embodiments, the extraction column of step (i) is a solid-phase extraction (SPE) column; preferably, the extraction column of step (i) is a turbulent flow liquid chromatography (TFLC) column. In some embodiments, the analytical column of step (i) is a high performance liquid chromatography (HPLC) column.

[0010] In some embodiments, the methods include the steps of : (i) subjecting the sample to turbulent flow liquid chromatography (TFLC); (ii) ionizing one or more Cookson-type vitamin D metabolite derivatives in the sample to generate one or more ions detectable by mass spectrometry; (iii) determining the amount of one or more of the Cookson-type vitamin D metabolite derivative ions by mass spectrometry; and (iv) relating the amount of Cookson-type vitamin D metabolite derivative ions determined in step (iv) to the amount of a vitamin D metabolite in the sample. In these embodiments, the sample is subjected to a Cookson-type derivatizing reagent under conditions sufficient to generate one or more Cookson-type vitamin D metabolite derivatives prior to step (i). In some embodiments, the sample is subjected to high performance liquid chromatography (HPLC) after step (i) but prior to step

(ii). In some related embodiments, the TFLC of step (i), the HPLC, and the ionization of step (ii) are conducted in an on-line fashion.

[0011] Cookson-type derivatizing reagents useful for these methods may be selected from the group consisting of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD), 4-(4-nitrophenyl)-1,2,4-triazoline-3,5- dione (NPTAD), and 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD), and isotopically labeled variants thereof. In preferred embodiments, the Cookson-type derivatizing reagent is 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) or an isotopically labeled variant thereof.

[0012] In some embodiments, the methods include the steps of: (i) subjecting the sample to 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) under conditions sufficient to generate one or more PTAD-vitamin D metabolite derivatives; (ii) ionizing one or more PTAD-vitamin D metabolite derivatives in the sample with laser diode thermal desorption to generate one or more ions detectable by mass spectrometry; (iii) determining the amount of one or more of the PTAD-vitamin D metabolite derivative ions by mass spectrometry; and (v) relating the amount of PTAD-vitamin D metabolite derivative ions determined in step (iv) to the amount of a vitamin D metabolite in the sample; wherein the one or more vitamin D metabolite derivatives are selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$). In some embodiments, the one or more vitamin D metabolite derivatives comprise two or more selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$). In related embodiments, the two or more vitamin D metabolite derivatives are ionized simultaneously. In some embodiments, the sample is not subjected to chromatography. In some embodiments, the sample is not subjected to liquid chromatography.

[0013] In the methods described herein, mass spectrometry may be tandem mass spectrometry. In embodiments utilizing tandem mass spectrometry, tandem mass spectrometry may be conducted by any method known in the art, including for example, multiple reaction monitoring, precursor ion scanning, or product ion scanning.

[0014] In the methods described herein, the one or more vitamin D metabolites may comprise one or more vitamin D metabolites selected from the group consisting of 25-

hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$); such as comprising 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$), or comprising 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

[0015] Thus, in some embodiments, the one or more Cookson-type derivatized vitamin D metabolites, or the one or more PTAD-derivatized vitamin D metabolites, comprise one or more of the group consisting of PTAD-derivatized 25-hydroxyvitamin D_2 (PTAD-$25OHD_2$), PTAD-derivatized 25-hydroxyvitamin D_3 (PTAD-$25OHD_3$), PTAD-derivatized 1α,25-dihydroxyvitamin D_2 (PTAD-$1\alpha,25(OH)_2D_2$), and PTAD-derivatized 1α,25-dihydroxyvitamin D_3 (PTAD-$1\alpha,25(OH)_2D_3$).

[0016] In embodiments where the one or more Cookson-type derivatized vitamin D metabolites, or the one or more PTAD-derivatized vitamin D metabolites, comprise PTAD-derivatized 25-hydroxyvitamin D_2 (PTAD-$25OHD_2$), the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 570.3 ± 0.5 and 298.1 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 570.3 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 298.1 ± 0.5.

[0017] In embodiments where the one or more Cookson-type derivatized vitamin D metabolites, or the one or more PTAD-derivatized vitamin D metabolites, comprise PTAD-derivatized 25-hydroxyvitamin D_3 (PTAD-$25OHD_3$), the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 558.3 ± 0.5 and 298.1 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 558.3 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 298.1 ± 0.5.

[0018] In embodiments where the one or more Cookson-type derivatized vitamin D metabolites, or the one or more PTAD-derivatized vitamin D metabolites, comprise PTAD-derivatized 1α,25-dihydroxyvitamin D_2 (PTAD-$1\alpha,25(OH)_2D_2$), the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized

vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 550.4 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 277.9 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 568.4 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 298.0 ± 0.5.

[0019] In embodiments where the one or more Cookson-type derivatized vitamin D metabolites, or the one or more PTAD-derivatized vitamin D metabolites, comprise PTAD-derivatized 1α,25-dihydroxyvitamin D_3 (PTAD-1α,25$(OH)_2D_3$), the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 538.4 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 278.1 ± 0.5. In some embodiments, the one or more Cookson-type or PTAD-derivatized vitamin D metabolite ions comprise a parent ion with a mass/charge ratio (m/z) of 556.4 ± 0.5 and a fragment ion with a mass/charge ratio (m/z) of 298.0 ± 0.5.

[0020] In embodiments which utilize two or more of an extraction column, an analytical column, and an ionization source, two or more of these components may be connected in an on-line fashion to allow for automated sample processing and analysis.

[0021] As used herein, the term "vitamin D metabolite" refers to any vitamin D analog or any chemical species related to vitamin D generated by a biotransformation of vitamin D, such as intermediates and products of vitamin D metabolism. Vitamin D metabolites may include chemical species generated by biotransformation of analogs of, or a chemical species related to, vitamin D_2 or vitamin D_3. Vitamin D metabolites may be found in the circulation of an animal and/or may be generated by a biological organism, such as an animal. Vitamin D metabolites may be metabolites of naturally occurring forms of vitamin D or may be metabolites of synthetic vitamin D analogs. In certain embodiments a vitamin D metabolite is one or more compounds selected from the group consisting of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 1α,25-dihydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_2.

[0022] As used herein, "derivatizing" means reacting two molecules to form a new molecule. Thus, a derivatizing agent is an agent that may be reacted with another substance to derivatize the substance. For example, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is a

derivatizing reagent that may be reacted with a vitamin D metabolite to form a PTAD-derivatized vitamin D metabolite.

[0023] As used here, the names of derivatized forms vitamin D metabolites include an indication as to the nature of derivatization. For example, the PTAD derivative of 25-hydroxyvitamin D_2 is indicated as PTAD-25-hydroxyvitamin D_2 (or PTAD-25OHD_2).

[0024] As used herein, a "Cookson-type derivatizing agent" is a 4-substituted 1,2,4-triazoline-3,5-dione compound. Exemplary Cookson-type derivatizing agents include 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD), 4-(4-nitrophenyl)-1,2,4-triazoline-3,5- dione (NPTAD), and 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD). Additionally, isotopically labeled variants of Cookson-type derivatizing agents may be used in some embodiments. For example, the $^{13}C_6$-PTAD isotopic variant is 6 mass units heavier than normal PTAD and may be used in some embodiments. Derivatization of vitamin D metabolites by Cookson-type reagents can be conducted by any appropriate method. See, e.g., Holmquist, et al., U.S. Patent Application Serial No. 11/946765, filed December 28, 2007; Yeung B, *et al.,* J Chromatogr. 1993, 645(1):115-23; Higashi T, et al., Steroids. 2000, 65(5):281-94; Higashi T, *et al.,* Biol Pharm Bull. 2001, 24(7):738-43; Higashi T, *et al.,* J Pharm Biomed Anal. 2002, 29(5):947-55; Higashi T, et al., Anal. Biochanal Chem, 2008, 391:229-38; and Aronov, et al., Anal Bioanal Chem, 2008, 391:1917-30.

[0025] In certain preferred embodiments of the methods disclosed herein, mass spectrometry is performed in positive ion mode. Alternatively, mass spectrometry is performed in negative ion mode. Various ionization sources, including for example atmospheric pressure chemical ionization (APCI), laser diode thermal desorption (LDTD), or electrospray ionization (ESI), may be used in embodiments of the present invention. In certain preferred embodiments, vitamin D metabolites are measured using APCI or LDTD in positive ion mode.

[0026] In preferred embodiments, one or more separately detectable internal standards are provided in the sample, the amount of which are also determined in the sample. In these embodiments, all or a portion of both the analyte(s) of interest and the internal standard(s) present in the sample are ionized to produce a plurality of ions detectable in a mass spectrometer, and one or more ions produced from each are detected by mass spectrometry. Preferably, the internal standard(s) are one or more of 25OHD_2-[6, 19, 19]-$^2H_3$, 25OHD_2-[26,

26, 26, 27, 27, 27]-$^2H_6$, 25OHD$_3$-[6, 19, 19]-$^2H_3$, 25OHD$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_2$-[6, 19, 19]-$^2H_3$, 1α,25(OH)$_2$D$_2$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_3$-[6, 19, 19]-$^2H_3$, and 1α,25(OH)$_2$D$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$.

[0027] One or more separately detectable internal standards may be provided in the sample prior to treatment of the sample with a Cookson-type derivatizing reagent. In these embodiments, the one or more internal standards may undergo derivatization along with the endogenous vitamin D metabolites, in which case ions of the derivatized internal standards are detected by mass spectrometry. In these embodiments, the presence or amount of ions generated from the analyte of interest may be related to the presence or amount of analyte of interest in the sample. In some embodiments, the internal standards may be isotopically labeled versions of vitamin D metabolites, such as 25OHD$_2$-[6, 19, 19]-$^2H_3$, 25OHD$_2$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 25OHD$_3$-[6, 19, 19]-$^2H_3$, 25OHD$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_2$-[6, 19, 19]-$^2H_3$, 1α,25(OH)$_2$D$_2$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_3$-[6, 19, 19]-$^2H_3$, and 1α,25(OH)$_2$D$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$.

[0028] Ions detectable in a mass spectrometer may be generated for each of the above identified exemplary internal standards, as demonstrated in Examples 14 and 15, and Figures 7-8, 10-11, 13, and 15-16.

[0029] As used herein, an "isotopic label" produces a mass shift in the labeled molecule relative to the unlabeled molecule when analyzed by mass spectrometric techniques. Examples of suitable labels include deuterium (^2H), ^{13}C, and ^{15}N. For example, 25OHD$_2$-[6, 19, 19]-$^2H_3$ and 25OHD$_3$-[6, 19, 19]-$^2H_3$ have masses about 3 mass units higher than native 25OHD$_2$ and 25OHD$_3$. The isotopic label can be incorporated at one or more positions in the molecule and one or more kinds of isotopic labels can be used on the same isotopically labeled molecule.

[0030] In other embodiments, the amount of the vitamin D metabolite ion or ions may be determined by comparison to one or more external reference standards. Exemplary external reference standards include blank plasma or serum spiked with one or more of 25OHD$_2$-[6, 19, 19]-$^2H_3$, 25OHD$_2$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 25OHD$_3$-[6, 19, 19]-$^2H_3$, 25OHD$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_2$-[6, 19, 19]-$^2H_3$, 1α,25(OH)$_2$D$_2$-[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25(OH)$_2$D$_3$-[6, 19, 19]-$^2H_3$, and 1α,25(OH)$_2$D$_3$-[26, 26, 26, 27, 27, 27]-$^2H_6$. External standards typically will undergo the same treatment and analysis as any other sample to be

analyzed, including treatment with one or more Cookson-type reagents prior to mass spectrometry.

[0031] In certain preferred embodiments, the limit of quantitation (LOQ) of $25OHD_2$ is within the range of 1.9 ng/mL to 10 ng/mL, inclusive; preferably within the range of 1.9 ng/mL to 5 ng/mL, inclusive; preferably about 1.9 ng/mL. In certain preferred embodiments, the limit of quantitation (LOQ) of $25OHD_3$ is within the range of 3.3 ng/mL to 10 ng/mL, inclusive; preferably within the range of 3.3 ng/mL to 5 ng/mL, inclusive; preferably about 3.3 ng/mL.

[0032] As used herein, unless otherwise stated, the singular forms "a," "an," and "the" include plural reference. Thus, for example, a reference to "a protein" includes a plurality of protein molecules.

[0033] 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 other components in the sample that may interfere with detection of the analyte of interest. Purification of the sample by various means may allow relative reduction of one or more interfering substances, e.g., one or more substances that may or may not interfere with the detection of selected parent or daughter ions by mass spectrometry. Relative reduction as this term is used does not require that any substance, present with the analyte of interest in the material to be purified, is entirely removed by purification.

[0034] As used herein, the term "solid phase extraction" or "SPE" refers to a process in which a chemical mixture is separated into components as a result of the affinity of components dissolved or suspended in a solution (i.e., mobile phase) for a solid through or around which the solution is passed (i.e., solid phase). In some instances, as the mobile phase passes through or around the solid phase, undesired components of the mobile phase may be retained by the solid phase resulting in a purification of the analyte in the mobile phase. In other instances, the analyte may be retained by the solid phase, allowing undesired components of the mobile phase to pass through or around the solid phase. In these instances, a second mobile phase is then used to elute the retained analyte off of the solid phase for further processing or analysis. SPE, including TFLC, may operate via a unitary or mixed mode mechanism. Mixed mode mechanisms utilize ion exchange and hydrophobic retention in the same column; for example, the solid phase of a mixed-mode SPE column

may exhibit strong anion exchange and hydrophobic retention; or may exhibit column exhibit strong cation exchange and hydrophobic retention.

[0035] 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 or over a stationary liquid or solid phase.

[0036] 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). Examples of "liquid chromatography" include reverse phase liquid chromatography (RPLC), high performance liquid chromatography (HPLC), and turbulent flow liquid chromatography (TFLC) (sometimes known as high turbulence liquid chromatography (HTLC) or high throughput liquid chromatography).

[0037] As used herein, the term "high performance liquid chromatography" or "HPLC" (sometimes known as "high pressure liquid chromatography") 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.

[0038] As used herein, the term "turbulent flow liquid chromatography" or "TFLC" (sometimes known as high turbulence liquid chromatography or high throughput liquid chromatography) refers to a form of chromatography that utilizes turbulent flow of the material being assayed through the column packing as the basis for performing the separation. TFLC has been applied in the preparation of samples containing two unnamed drugs prior to analysis by mass spectrometry. See, e.g., Zimmer *et al.*, *J Chromatogr A* 854: 23-35 (1999); see also, U.S. Patents No. 5,968,367, 5,919,368, 5,795,469, and 5,772,874, which further explain TFLC. Persons of ordinary skill in the art understand "turbulent flow". When fluid flows slowly and smoothly, the flow is called "laminar flow". For example, fluid moving through an HPLC column at low flow rates is laminar. In laminar flow the motion of the particles of fluid is orderly with particles moving generally in straight lines. At faster velocities, the inertia of the water overcomes fluid frictional forces and turbulent flow results. Fluid not in contact with the irregular boundary "outruns" that which is slowed by friction or

deflected by an uneven surface. When a fluid is flowing turbulently, it flows in eddies and whirls (or vortices), with more "drag" than when the flow is laminar. Many references are available for assisting in determining when fluid flow is laminar or turbulent (e.g., Turbulent Flow Analysis: Measurement and Prediction, P.S. Bernard & J.M. Wallace, John Wiley & Sons, Inc., (2000); An Introduction to Turbulent Flow, Jean Mathieu & Julian Scott, Cambridge University Press (2001)).

[0039] As used herein, the term "gas chromatography" or "GC" refers to chromatography in which the sample mixture is vaporized and injected into a stream of carrier gas (as nitrogen or helium) moving through a column containing a stationary phase composed of a liquid or a particulate solid and is separated into its component compounds according to the affinity of the compounds for the stationary phase.

[0040] As used herein, the term "large particle column" or "extraction column" refers to a chromatography column containing an average particle diameter greater than about 50 μm.

[0041] As used herein, the term "analytical column" refers to a chromatography column having sufficient chromatographic plates to effect a separation of materials in a sample that elute from the column sufficient to allow a determination of the presence or amount of an analyte. In a preferred embodiment the analytical column contains particles of about 5 μm in diameter. Such columns are often distinguished from "extraction columns", which have the general purpose of separating or extracting retained material from non-retained materials in order to obtain a purified sample for further analysis.

[0042] As used herein, the terms "on-line" and "inline", for example as used in "on-line automated fashion" or "on-line extraction" refers to a procedure performed without the need for operator intervention. In contrast, the term "off-line" as used herein refers to a procedure requiring manual intervention of an operator. Thus, if samples are subjected to precipitation, and the supernatants are then manually loaded into an autosampler, the precipitation and loading steps are off-line from the subsequent steps. In various embodiments of the methods, one or more steps may be performed in an on-line automated fashion.

[0043] 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 mass-to-charge ratio, or "m/z". MS technology generally includes (1) ionizing the compounds to form charged compounds; and (2) detecting the molecular weight of the charged compounds and calculating a mass-to-charge ratio. 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 spectrometric 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* 1999, 2: 264-76; and Merchant and Weinberger, *Electrophoresis* 2000, 21: 1164-67.

[0044] As used herein, the term "operating in negative ion mode" refers to those mass spectrometry methods where negative ions are generated and detected. The term "operating in positive ion mode" as used herein, refers to those mass spectrometry methods where positive ions are generated and detected.

[0045] 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. Negative ions are those having a net negative charge of one or more electron units, while positive ions are those having a net positive charge of one or more electron units.

[0046] 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.

[0047] 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.

[0048] 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.

[0049] 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.

[0050] 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.

[0051] 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.

[0052] 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.

[0053] 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 ion 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+. See, e.g., Robb *et al.*, *Anal. Chem.* 2000, 72(15): 3653-3659.

[0054] As used herein, the term "inductively coupled plasma" or "ICP" refers to methods in which a sample interacts with a partially ionized gas at a sufficiently high temperature such that most elements are atomized and ionized.

[0055] 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.

[0056] 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. Laser diode thermal desorption (LDTD) is a technique wherein a sample containing the analyte is thermally desorbed into the gas phase by a laser pulse. The laser hits the back of a specially made 96-well plate with a metal base. The laser pulse heats the base and the heat causes the sample to transfer into the gas phase. The gas phase sample may then be drawn into an ionization source, where the gas phase sample is ionized in preparation for analysis in the mass spectrometer. When using LDTD, ionization of the gas phase sample may be accomplished by any suitable technique known in the art, such as by ionization with a corona discharge (for example by APCI).

[0057] As used herein, the term "selective ion monitoring" is a detection mode for a mass spectrometric instrument in which only ions within a relatively narrow mass range, typically about one mass unit, are detected.

[0058] As used herein, "multiple reaction mode," sometimes known as "selected reaction monitoring," is a detection mode for a mass spectrometric instrument in which a precursor ion and one or more fragment ions are selectively detected.

[0059] As used herein, the term "lower limit of quantification", "lower limit of quantitation" or "LLOQ" refers to the point where measurements become quantitatively meaningful. The analyte response at this LOQ is identifiable, discrete and reproducible with a relative standard deviation (RSD %) of less than 20% and an accuracy of 80% to 120%.

[0060] As used herein, the term "limit of detection" or "LOD" is the point at which the measured value is larger than the uncertainty associated with it. The LOD is the point at which a value is beyond the uncertainty associated with its measurement and is defined as three times the RSD of the mean at the zero concentration.

[0061] As used herein, an "amount" of an analyte in a body fluid sample refers generally to an absolute value reflecting the mass of the analyte detectable in volume of sample. However, an amount also contemplates a relative amount in comparison to another analyte amount. For example, an amount of an analyte in a sample can be an amount which is greater than a control or normal level of the analyte normally present in the sample.

[0062] The term "about" as used herein in reference to quantitative measurements not including the measurement of the mass of an ion, refers to the indicated value plus or minus 10%. Mass spectrometry instruments can vary slightly in determining the mass of a given analyte. The term "about" in the context of the mass of an ion or the mass/charge ratio of an ion refers to +/- 0.50 atomic mass unit.

[0063] 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

[0064] Figures 1A-D show exemplary chromatograms for 25OH-D_3-PTAD, [6, 19, 19]-$^2H_3$-25OHD_3-PTAD (internal standard), 25OHD_2-PTAD, and [6, 19, 19]-$^2H_3$-25OHD_2-PTAD (internal standard), respectively. Details are discussed in Example 3.

[0065] Figures 2A and 2B show exemplary calibration curves for 25OHD_2 and 25OHD_3 in serum samples determined by methods described in Example 3.

[0066] Figure 3A shows a plots of coefficient of variation versus concentration for 25OHD_2 and 25OHD_3. Figure 3B shows the same plot expanded near the LLOQ. Details are described in Example 4.

[0067] Figures 4A-B show linear regression and Deming regression analyses for the comparison of mass spectrometric determination of 25OHD_2 with and without PTAD derivatization. Details are described in Example 10.

[0068] Figures 5A-B show linear regression and Deming regression analyses for the comparison of mass spectrometric determination of $25OHD_3$ with and without PTAD derivatization. Details are described in Example 10.

[0069] Figure 6A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_2 ions. Figure 6B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for fragmentation of the PTAD-25-hydroxyvitamin D_2 precursor ion with m/z of about 570.3. Details are described in Example 14.

[0070] Figure 7A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$ ions. Figure 7B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for fragmentation of the PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$ precursor ion with m/z of about 573.3. Details are described in Example 14.

[0071] Figure 8A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ ions. Figure 8B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for fragmentation of the PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 576.3. Details are described in Example 14.

[0072] Figure 9A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_3 ions. Figure 9B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for fragmentation of the PTAD-25-hydroxyvitamin D_3 precursor ion with m/z of about 558.3. Details are described in Example 14.

[0073] Figure 10A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ ions. Figure 10B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for fragmentation of the PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ precursor ion with m/z of about 561.3. Details are described in Example 14.

[0074] Figure 11A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ ions. Figure 11B shows an exemplary product ion spectra (covering the m/z range of about 200 to 400) for

fragmentation of the PTAD-25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 564.3. Details are described in Example 14.

[0075] Figure 12A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-1α,25-dihydroxyvitamin D_2 ions. Figure 12B shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2 precursor ion with m/z of about 550.4. Figure 12C shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2 precursor ion with m/z of about 568.4. Figure 12D shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2 precursor ion with m/z of about 586.4. Details are described in Example 15.

[0076] Figure 13A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ ions. Figure 13B shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 556.4. Figure 13C shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 574.4 Figure 13D shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 592.4. Details are described in Example 15.

[0077] Figure 14A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-1α,25-hydroxyvitamin D_3 ions. Figure 14B shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3 precursor ion with m/z of about 538.4. Figure 14C shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3 precursor ion with m/z of about 556.4. Figure 14D shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3 precursor ion with m/z of about 574.4. Details are described in Example 15.

[0078] Figure 15A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ ions. Figure 15B shows

an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ precursor ion with m/z of about 541.4. Figure 15C shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ precursor ion with m/z of about 559.4. Figure 15D shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the 1,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$-PTAD precursor ion with m/z of about 577.4. Details are described in Example 15.

[0079] Figure 16A shows an exemplary Q1 scan spectrum (covering the m/z range of about 520 to 620) for PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ ions. Figure 16B shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 544.4. Figure 16C shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 562.4. Figure 16D shows an exemplary product ion spectra (covering the m/z range of about 250 to 350) for fragmentation of the PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ precursor ion with m/z of about 580.4. Details are described in Example 15.

DETAILED DESCRIPTION OF THE INVENTION

[0080] Methods are described for measuring vitamin D metabolites in a sample. More specifically, mass spectrometric methods are described for detecting and quantifying vitamin D metabolites in a sample. The methods utilize Cookson-type reagents, such as PTAD, to generate derivatized vitamin D metabolites, and may use an extraction chromatography technique, such as turbulent flow liquid chromatography (TFLC), to perform a purification of the derivatized analytes, combined with methods of mass spectrometry (MS), thereby providing a high-throughput assay system for detecting and quantifying vitamin D metabolites in a sample. In some methods, no chromatography, including extraction chromatography, is necessary for sample analysis. In these methods, the derivatized vitamin D metabolites are ionized with LDTD. Preferred embodiments are particularly well suited for application in large clinical laboratories for automated vitamin D metabolite quantification.

[0081] Suitable test samples for use in methods of the present invention 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, etc. 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 male or female humans. Preferred samples comprise bodily fluids such as blood, plasma, serum, saliva, cerebrospinal fluid, or tissue samples; preferably plasma (including EDTA and heparin plasma) and serum; most preferably serum. Such samples may be obtained, for example, from a patient; that is, a living person, male or female, presenting oneself in a clinical setting for diagnosis, prognosis, or treatment of a disease or condition.

[0082] The present invention also contemplates kits for a vitamin D metabolite quantitation assay. A kit for a vitamin D metabolite quantitation assay may include a kit comprising the compositions provided herein. For example, a kit may include packaging material and measured amounts of a Cookson-type reagent and an isotopically labeled internal standard, in amounts sufficient for at least one assay. Typically, the kits will also include instructions recorded in a tangible form (e.g., contained on paper or an electronic medium) for using the packaged reagents for use in a vitamin D metabolite quantitation assay.

[0083] Calibration and QC pools for use in embodiments of the present invention are preferably prepared using a matrix similar to the intended sample matrix. If no appropriate biological matrix is commercially available with an endogenous concentration of $25OHD_3$ sufficiently low to act as a blank, a solution of 5% bovine serum albumin in phosphate buffered saline (with an estimated 1.5 ng/mL $25OHD_3$) may be used for calibration and QC pools.

Sample Preparation for Mass Spectrometric Analysis

[0084] In preparation for mass spectrometric analysis, vitamin D metabolites may be enriched relative to one or more other components in the sample (e.g. protein) by various methods known in the art, including for example, liquid chromatography, filtration, centrifugation, thin layer chromatography (TLC), electrophoresis including capillary electrophoresis, affinity separations including immunoaffinity separations, extraction

methods including ethyl acetate or methanol extraction, and the use of chaotropic agents or any combination of the above or the like.

[0085] Protein precipitation is one method of preparing a test sample, especially a biological test sample, such as serum or plasma. Protein purification methods are well known in the art, for example, Polson *et al., Journal of Chromatography B* 2003, 785:263-275, describes protein precipitation techniques suitable for use in methods of the present invention. Protein precipitation may be used to remove most of the protein from the sample leaving vitamin D metabolites in the supernatant. The samples may be centrifuged to separate the liquid supernatant from the precipitated proteins; alternatively the samples may be filtered to remove precipitated proteins. The resultant supernatant or filtrate may then be applied directly to mass spectrometry analysis; or alternatively to liquid chromatography and subsequent mass spectrometry analysis. In certain embodiments, samples, such as plasma or serum, may be purified by a hybrid protein precipitation / liquid-liquid extraction method. In these embodiments, a sample is mixed with methanol, ethyl acetate, and water, and the resulting mixture is vortexed and centrifuged. The resulting supernatant is removed, dried to completion and reconstituted in acetonitrile. The purified vitamin D metabolites may then be derivatized with any Cookson-type reagent, preferably PTAD or an isotopically labeled variant thereof.

[0086] Another method of sample purification that may be used prior to mass spectrometry is liquid chromatography (LC). Certain methods of liquid chromatography, including HPLC, rely on relatively slow, laminar flow technology. Traditional HPLC analysis relies on column packing in which laminar flow of the sample through the column is the basis for separation of the analyte of interest from the sample. The skilled artisan will understand that separation in such columns is a diffusional process and may select LC, including HPLC, instruments and columns that are suitable for use with derivatized vitamin D metabolites. 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, or may include a monolithic material with porous channels. A surface of the medium typically includes 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, cyano bonded surface, or highly pure silica surface. Alkyl bonded surfaces may include C-4, C-8, C-12, or C-18 bonded alkyl groups. In preferred embodiments, the column is a highly pure silica column (such as a

Thermo Hypersil Gold Aq column). The chromatographic column includes an inlet port for receiving a sample and an outlet port for discharging an effluent that includes the fractionated sample. The sample may be supplied to the inlet port directly, or from an extraction column, such as an on-line SPE cartridge or a TFLC extraction column.

[0087] In one embodiment, the sample may be applied to the LC 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 analyte(s) of interest. For example, liquid chromatography may be performed using a gradient mode, an isocratic mode, or a polytyptic (i.e. mixed) mode. During chromatography, the separation of materials is effected by variables such as choice of eluent (also known as a "mobile phase"), elution mode, gradient conditions, temperature, etc.

[0088] 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.

[0089] In one preferred embodiment, HPLC is conducted with an alkyl bonded analytical column chromatographic system. In certain preferred embodiments, a highly pure silica column (such as a Thermo Hypersil Gold Aq column) is used. In certain preferred embodiments, HPLC and/or TFLC are performed using HPLC Grade water as mobile phase A and HPLC Grade ethanol as mobile phase B.

[0090] By careful selection of valves and connector plumbing, two or more chromatography columns may be connected as needed such that material is passed from one to the next without the need for any manual steps. In preferred embodiments, the selection of valves and plumbing is controlled by a computer pre-programmed to perform the necessary steps. Most preferably, the chromatography system is also connected in such an on-line fashion to the detector system, e.g., an MS system. Thus, an operator may place a tray of

samples in an autosampler, and the remaining operations are performed under computer control, resulting in purification and analysis of all samples selected.

[0091] In some embodiments, an extraction column may be used for purification of vitamin D metabolites prior to mass spectrometry. In such embodiments, samples may be extracted using a extraction column which captures the analyte, then eluted and chromatographed on a second extraction column or on an analytical HPLC column prior to ionization. For example, sample extraction with a TFLC extraction column may be accomplished with a large particle size (50 μm) packed column. Sample eluted off of this column may then be transferred to an HPLC analytical column for further purification prior to mass spectrometry. Because the steps involved in these chromatography procedures may be linked in an automated fashion, the requirement for operator involvement during the purification of the analyte can be minimized. This feature may result in savings of time and costs, and eliminate the opportunity for operator error.

[0092] In some embodiments, protein precipitation is accomplished with a hybrid protein precipitation / liquid-liquid extraction method which includes methanol protein precipitation and ethyl acetate/water extraction from serum. The resulting vitamin D metabolites may be derivatized prior to being subjected to an extraction column. Preferably, the hybrid protein precipitation / liquid-liquid extraction method and the extraction column are connected in an on-line fashion. In preferred embodiments, the extraction column is a C-8 extraction column, such as a Cohesive Technologies C8XL online extraction column (50 μm particle size, 0.5 x 50 mm) or equivalent. The eluent from the extraction column may then be applied to an analytical LC column, such as a HPLC column in an on-line fashion, prior to mass spectrometric analysis. Because the steps involved in these chromatography procedures may be linked in an automated fashion, the requirement for operator involvement during the purification of the analyte can be minimized. This feature may result in savings of time and costs, and eliminate the opportunity for operator error.

Detection and Quantitation by Mass Spectrometry

[0093] In various embodiments, derivatized vitamin D metabolites 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. For example ionization of the sample may be performed by electron ionization, chemical ionization, electrospray ionization (ESI), photon

ionization, atmospheric pressure chemical ionization (APCI), photoionization, atmospheric pressure photoionization (APPI), Laser diode thermal desorption (LDTD), fast atom bombardment (FAB), liquid secondary ionization (LSI), 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.

[0094] Derivatized vitamin D metabolites may be ionized in positive or negative mode. In preferred embodiments, derivatized vitamin D metabolites are ionized by APCI or LDTD in positive ion mode.

[0095] In mass spectrometry techniques generally, after the sample has been ionized, the positively or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio. Suitable analyzers for determining mass-to-charge ratios include quadrupole analyzers, ion traps analyzers, and time-of-flight analyzers. Exemplary ion trap methods are described in Bartolucci, *et al.*, *Rapid Commun. Mass Spectrom.* 2000, 14:967-73.

[0096] The ions may be detected using several detection modes. For example, selected ions may be detected, i.e. using a selective ion monitoring mode (SIM), or alternatively, mass transitions resulting from collision induced dissociation or neutral loss may be monitored, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). Preferably, the mass-to-charge ratio is determined using a quadrupole analyzer. For example, 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 the mass/charge ratio. The voltage and amplitude may be selected so that only ions having a particular mass/charge ratio 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.

[0097] 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 collisions 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.

[0098] Alternate modes of operating a tandem mass spectrometric instrument include product ion scanning and precursor ion scanning. For a description of these modes of operation, see, e.g., E. Michael Thurman, et al., Chromatographic-Mass Spectrometric Food Analysis for Trace Determination of Pesticide Residues, Chapter 8 (Amadeo R. Fernandez-Alba, ed., Elsevier 2005) (387).

[0099] The results of an analyte assay 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, external 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 vitamin D metabolites. 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. For example, in preferred embodiments one or more isotopically labeled vitamin D metabolites (e.g., $25OHD_2$-[6, 19, 19]-$^2H_3$ and $25OHD_3$-[6, 19, 19]-$^2H_3$) may be used as internal standards. 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.

[00100] As used herein, an "isotopic label" produces a mass shift in the labeled molecule relative to the unlabeled molecule when analyzed by mass spectrometric techniques. Examples of suitable labels include deuterium (^2H), ^{13}C, and ^{15}N. For example, $25OHD_2$-[6, 19, 19]-$^2H_3$ has a mass about three units higher than $25OHD_2$. The isotopic label can be

incorporated at one or more positions in the molecule and one or more kinds of isotopic labels can be used on the same isotopically labeled molecule.

[00101] 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 purification and mass spectrometry steps may be performed in an on-line fashion.

[00102] In certain embodiments, such as MS/MS, where precursor ions are isolated for further fragmentation, collision activated dissociation (CAD) is often used to generate 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.

[00103] In particularly preferred embodiments, vitamin D metabolites in a sample are detected and/or quantified using MS/MS as follows. The samples are first purified by protein precipitation or a hybrid protein precipitation / liquid-liquid extraction. Then, one or more vitamin D metabolites in the purified sample are derivatized with a Cookson-type reagent, such as PTAD. The purified samples are then subjected to liquid chromatography, preferably on an extraction column (such as a TFLC column) followed by an analytical column (such as a HPLC column); the flow of liquid solvent from a chromatographic column enters the nebulizer interface of an MS/MS analyzer; and the solvent/analyte mixture is converted to vapor in the heated charged tubing of the interface. The analyte(s) (e.g., derivatized vitamin D metabolites), contained in the solvent, are ionized by applying a large voltage to the solvent/analyte mixture. As the analytes exit the charged tubing of the interface, the solvent/analyte mixture nebulizes and the solvent evaporates, leaving analyte ions. The ions, e.g. 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., selection of "precursor" and "fragment" ions in Q1 and Q3, respectively) based on their mass to charge ratio (m/z). Quadrupole 2 (Q2) is the collision cell, where ions are fragmented. The first quadrupole of the mass spectrometer (Q1) selects for molecules with the mass to charge ratios of derivatized vitamin D metabolites of interest. Precursor ions with the correct mass/charge ratios are allowed to pass into the collision chamber (Q2), while unwanted ions with any other mass/charge ratio collide with the sides of the quadrupole and are eliminated.

Precursor ions entering Q2 collide with neutral argon gas molecules and fragment. The fragment ions generated are passed into quadrupole 3 (Q3), where the fragment ions of derivatized vitamin D metabolites of interest are selected while other ions are eliminated.

[00104] The methods may involve MS/MS performed in either positive or negative ion mode; preferably positive ion mode. Using standard methods well known in the art, one of ordinary skill is capable of identifying one or more fragment ions of a particular precursor ion of derivatized vitamin D metabolites that may be used for selection in quadrupole 3 (Q3).

[00105] 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 counts of the ions collected versus time. The resulting mass chromatograms are similar to chromatograms generated in traditional HPLC-MS methods. The areas under the peaks corresponding to particular ions, or the amplitude of such peaks, may be measured and 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 a particular vitamin D metabolite. 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.

EXAMPLES

Example 1: Hybrid Protein Precipitation / Liquid-Liquid Extraction and Cookson-type Derivatization

[00106] The following automated hybrid protein precipitation / liquid-liquid extraction technique was conducted on patient serum samples. Gel Barrier Serum (i.e., serum collected in Serum Separator Tubes) as well as EDTA plasma and Heparin Plasma have also been established as acceptable for this assay.

[00107] A Perkin-Elmer Janus robot and a TomTec Quadra Tower robot was used to automate the following procedure. For each sample, 50 μL of serum was added to a well of a 96 well plate. Then 25 μL of internal standard cocktail (containing isotopically labeled $25OHD_2$-[6, 19, 19]-$^2H_3$ and $25OHD_3$-[6, 19, 19]-$^2H_3$) was added to each well, and the plate vortexed. Then 75 μL of methanol was added, followed by additional vortexing. 300 μL of ethyl acetate and 75 μL of water was then added, followed by additional vortexing, centrifugation, and transfer of the resulting supernatant to a new 96-well plate.

[00108] The transferred liquid in the second 96-well plate from Example 1 was dried to completion under a flowing nitrogen gas maniforld. Derivatization was accomplished by adding 100 μL of a 0.1 mg/mL solution of the Cookson-type derivatization agent PTAD in acetonitrile to each well. The derivatization reaction was allowed to proceed for approximately one hour, and was quenched by adding 100 μL of water to the reaction mixture.

Example 2: Extraction of Vitamin D Metabolites with Liquid Chromatography

[00109] Sample injection was performed with a Cohesive Technologies Aria TX-4 TFLC system using Aria OS V 1.5.1 or newer software.

[00110] The TFLC system automatically injected an aliquot of the above prepared samples into a Cohesive Technologies C8XL online extraction column (50 μm particle size, 005 x 50 mm, from Cohesive Technologies, Inc.) packed with large particles. The samples were loaded at a high flow rate to create turbulence inside the extraction column. This turbulence ensured optimized binding of derivatized vitamin D metabolites to the large particles in the column and the passage of excess derivatizing reagent and debris to waste.

[00111] Following loading, the sample was eluted off to the analytical column, a Thermo Hypersil Gold Aq analytical column (5 μm particle size, 50 x 2.1 mm), with a water/ethanol elution gradient. The HPLC gradient was applied to the analytical column, to separate vitamin D metabolites from other analytes contained in the sample. Mobile phase A was water and mobile phase B was ethanol. The HPLC gradient started with a 35 % organic gradient which was ramped to 99 % in approximately 65 seconds.

Example 3: Detection and Quantitation of derivatized vitamin D metabolites by MS/MS

[00112] MS/MS was performed using a Finnigan TSQ Quantum Ultra MS/MS system (Thermo Electron Corporation). The following software programs, all from Thermo Electron, were used in the Examples described herein: Quantum Tune Master V 1.5 or newer, Xcalibur V 2.07 or newer, LCQuan V 2.56 (Thermo Finnigan) or newer, and ARIA OS v1.5.1 (Cohesive Technologies) or newer. Liquid solvent/analyte exiting the analytical column flowed to the nebulizer interface of the MS/MS analyzer. The solvent/analyte mixture was converted to vapor in the tubing of the interface. Analytes in the nebulized solvent were ionized by ESI.

[00113] Ions passed to the first quadrupole (Q1), which selected ions for a derivatized

vitamin D metabolite. Ions with a m/z of 570.32 ± 0.50 were selected for PTAD-25OHD$_2$; ions with a m/z of 558.32 ± 0.50 were selected for PTAD-25OHD$_3$. Ions entering quadrupole 2 (Q2) collided with argon gas to generate ion fragments, which were passed to quadrupole 3 (Q3) for further selection. Mass spectrometer settings are shown in Table 1. Simultaneously, the same process using isotope dilution mass spectrometry was carried out with internal standards, PTAD-25OHD$_2$-[6, 19, 19]-^{2}H$_3$ and PTAD-25OHD$_3$-[6, 19, 19]-^{2}H$_3$. The following mass transitions were used for detection and quantitation during validation on positive polarity.

Table 1. Mass Spectrometer Settings for Detection of PTAD-25OHD$_2$ and PTAD-25OHD$_3$

Mass Spectrometric Instrument Settings	
Discharge Current	4.0 μA
Vaporizer Temperature	300 C
Sheath Gas Pressure	15
Ion Sweep Gas Pressure	0.0
Aux Gas Pressure	5
Capillary Temperature	300 C
Skimmer Offset	-10 V
Collision Pressure	1.5 mTorr
Collision Cell Energy	15 V

Table 2. Mass Transitions for PTAD-25OHD$_2$, PTAD-25OHD$_2$-[6, 19, 19]-^{2}H$_3$ (IS), PTAD-25OHD$_3$, and PTAD-25OHD$_3$-[6, 19, 19]-^{2}H$_3$ (IS) (Positive Polarity)

Analyte	***Precursor Ion (m/z)***	***Product Ion (m/z)***
PTAD-25OHD$_2$	570.32	298.09
PTAD-25OHD$_2$-[6, 19, 19]-^{2}H$_3$ (IS)	573.32	301.09
PTAD-25OHD$_3$	558.32	298.09
PTAD-25OHD$_3$-[6, 19, 19]-^{2}H$_3$ (IS)	561.32	301.09

[00114] Exemplary chromatograms for PTAD-25OHD$_3$, PTAD-25OHD$_3$-[6, 19, 19]-^{2}H$_3$

(internal standard), PTAD-25OHD$_2$, and PTAD-25OHD$_2$-[6, 19, 19]-$^2H_3$ (internal standard), are shown in Figures 1A, 1B, 1C, and 1D, respectively.

[00115] Exemplary calibration curves for the determination of 25OHD$_2$ and 25OHD$_3$ in serum specimens are shown in Figures 2A and 2B, respectively.

Example 4: Analytical Sensitivity: Lower Limit of Quantitation (LLOQ) and Limit of Detection (LOD)

[00116] The LLOQ is the point where measurements become quantitatively meaningful. The analyte response at this LLOQ is identifiable, discrete and reproducible with a precision (i.e., coefficient of variation (CV)) of greater than 20% and an accuracy of 80% to 120%. The LLOQ was determined by assaying five different human serum samples spiked with PTAD-25OHD$_2$ and PTAD-25OHD$_3$ at levels near the expected LLOQ and evaluating the reproducibility. Analysis of the collected data indicates that samples with concentrations of about 4 ng/mL yielded CVs of about 20%. Thus, the LLOQ of this assay for both PTAD-25OHD$_2$ and PTAD-25OHD$_3$ was determined to be 4 ng/mL. The graphical representations of CV versus concentration for both analytes are shown in Figures 3A-B (Figure 3A shows the plots over an expanded concentration range, while Figure 3B shows the same plot expanded near the LOQ).

[00117] The LOD is the point at which a value is beyond the uncertainty associated with its measurement and is defined as three standard deviations from the zero concentration. To determine the LOD, generally, blank samples of the appropriate matrix are obtained and tested for interferences. However, no appropriate biological matrix could be obtained where the endogenous concentration of 25OHD$_3$ is zero, so a solution of 5% bovine serum albumin in phosphate buffered saline (with an estimated 1.5 ng/mL 25OHD$_3$) was used for LOD studies. The standard was run in 20 replicates each and the resulting area rations were statistically analyzed to determine that the LOD for 25OHD$_2$ and 25OHD$_3$ are 1.9 and 3.3 ng/mL, respectively. Raw data from these studies is presented in Table 3, below

Table 3. Limit of Detection Raw Data and Analysis

Replicate	*25OHD$_2$ (ng/mL)*	*25OHD$_3$ (ng/mL)*
1	0.0	0.0
2	1.1	2.0
3	0.1	2.4
4	0.3	1.1
5	0.5	1.9
6	0.4	1.8
7	0.2	1.9
8	0.5	2.3
9	1.1	2.3
10	0.5	2.1
11	0.4	1.5
12	1.2	1.9
13	0.4	1.8
14	0.3	1.6
15	0.0	1.3
16	0.9	1.3
17	0.8	1.5
18	0.1	1.9
19	0.5	1.7
20	0.4	1.8
Mean	0.4	1.7
SD	0.5	0.5
LOD (Mean + 3SD)	1.9	3.3

Example 5: Reportable Range and Linearity

[00118] Linearity of derivatized vitamin D metabolite detection in the assay was determined by diluting four pools serum with high endogenous concentration of either 25OHD$_2$ or 25OHD$_3$ and analyzing undiluted specimens and diluted specimens at 1:2, 1:4, and 1:8, in quadruplicate. Quadratic regression of the data was performed yielding correlation coefficients across the concentration range tested of R^2=0.97. These studies demonstrated that specimens may be diluted at 1:4 with average recovery of 101%, permitting a reportable range of about 4 to about 512 ng/mL. Average measured values for each of the specimen dilution levels and correlation values from linear regression analysis are presented in Table

4A, below. Percent recoveries for each of the specimen dilution levels are presented in Table 4B, below.

Table 4A. Linearity Data and Linear Regression Analysis over Reportable Range

Dilution Level	*25OHD$_2$ (ng/mL)*		*25OHD$_3$ (ng/mL)*	
	Pool 1	Pool 2	Pool 1	Pool 2
Undiluted	110.0	75.6	73.3	60.6
1:2	55.5	39.3	35.7	28.7
1:4	26.2	19.4	18.1	16.3
1:8	14.3	10.9	9.7	8.3
R^2	0.9744	0.9721	0.9705	0.9601

Table 4B. Percent Recovery at Various Specimen Dilution Levels

Dilution Level	*25OHD$_2$ (ng/mL)*		*25OHD$_3$ (ng/mL)*	
	Pool 1	Pool 2	Pool 1	Pool 2
Undiluted	(100%)	(100%)	(100%)	(100%)
1:2	100.9	104	97.4	94.8
1:4	95.5	102.7	98.6	107.3
1:8	104.2	115.0	106.0	109.0

Example 6: Analyte Specificity

[00119] The specificity of the assay against similar analytes was determined to have no cross reactivity for any vitamin D metabolite tested with the exception of 3-epi-25OHD$_3$, which behaves similarly to 25OHD$_3$ in the assay. The side-chain labeled stable isotopes of 25OHD2 and 25OHD$_3$ also showed cross-reactivity owning to hydrogen exchange that occurs in the ion source. Thus, side-chain labeled stable isotopes of 25OHD$_2$ and 25OHD$_3$ should not be used as internal standards. Table 5, below, shows the compounds tested and the results of the cross-reactivity studies.

Table 5. Cross-Reactivity Studies (Compounds tested and results)

Analyte	*25OHD$_2$*	*25OHD$_3$*	*Cross-Reactivity*
1,25(OH)$_2$D$_3$	-	-	No
1,25(OH)$_2$D$_2$	-	-	No

$1,25(OH)_2D_3$-[6,19,19']-^2H	-	-	No
$1,25(OH)_2D_3$-[26,26,26,27,27,27]-^2H	-	-	No
$1,25(OH)_2D_2$-[26,26,26,27,27,27]-^2H	-	-	No
$25OHD_3$	-	**(100%)**	-
$25OHD_2$	**(100%)**	-	-
$25OHD_3$-IS-[6,19,19']-^2H	-	-	No
$25OHD_2$-IS-[6,19,19']-^2H	-	-	No
$25OHD_3$-IS-[26,26,26,27,27,27]-^2H	-	**13.8%**	Yes
$25OHD_2$-IS-[26,26,26,27,27,27]-^2H	**2.7%**	-	Yes
vitamin D_3	-	-	No
vitamin D_2	-	-	No
vitamin D_3-[6,19,19']-^2H	-	-	No
vitamin D_2-[6,19,19']-^2H	-	-	No
vitamin D_3-[26,26,26,27,27,27]-^2H	-	-	No
vitamin D_2-[26,26,26,27,27,27]-^2H	-	-	No
1-OH-D_3 (Alfacalcidiol)	-	-	No
1-OH-D_2 (Hectoral)	-	-	No
$24,25(OH)_2D_3$	-	-	No
$25,26(OH)_2D_3$	-	-	No
3-epi-$25OHD_3$	-	-	No
3-epi-$1,25(OH)_2D_3$	-	**33.3%**	Yes
Dihydrotachysterol	-	-	No
$1,25(OH)_2D_3$-26,23-lactone	-	-	No
Paracalcitol (Zemplar)	-	-	No
Calcipotriene (Dovonex)	-	-	No
7-Dehydrocholesterol	-	-	No

Example 7: Reproducibility

[00120] Six standards at 5, 15, 30, 60, 90, and 120 ng/mL for each analyte were run in every assay as a means as quantitating reproducibility. The day-to-day reproducibility was determined using calibration curves from 19 assays. The data from these 19 assays are presented in Tables 6A (for $25OHD_2$) and 6B (for $25OHD_3$).

Table 6A. Standard curves demonstrate reproducibility of PTAD-$25OHD_2$ determination

	Concentration
Assay	

	5 ng/mL	15 ng/mL	30 ng/mL	60 ng/mL	90 ng/mL	120 ng/mL
1	0.06	0.16	0.36	0.68	0.92	1.23
2	0.08	0.17	0.36	0.61	0.94	1.18
3	0.07	0.17	0.32	0.66	0.92	1.19
4	0.06	0.19	0.29	0.69	0.98	1.16
5	0.07	0.15	0.37	0.60	0.85	1.13
6	0.07	0.16	0.32	0.64	0.95	1.20
7	0.07	0.16	0.35	0.63	0.99	1.18
8	0.06	0.16	0.35	0.60	0.98	1.31
9	0.06	0.18	0.32	0.66	0.96	1.10
10	0.06	0.15	0.35	0.62	0.89	1.22
11	0.05	0.17	0.33	0.65	0.96	1.17
12	0.04	0.17	0.32	0.61	0.97	1.12
13	0.05	0.16	0.34	0.62	0.97	1.30
14	0.06	0.17	0.31	0.61	0.95	1.21
15	0.07	0.16	0.34	0.70	0.94	1.30
16	0.08	0.17	0.39	0.70	1.06	1.27
17	0.06	0.15	0.36	0.65	1.03	1.20
18	0.05	0.18	0.34	0.81	0.91	1.33
19	0.06	0.17	0.30	0.62	1.06	1.21
Avg	0.06	0.16	0.34	0.65	0.96	1.21
SD	0.01	0.01	0.02	0.05	0.05	0.07
CV%	15.4	6.3	7.4	8.0	5.6	5.4

Table 6B. Standard curves demonstrate reproducibility of PTAD-25OHD$_3$ determination

Assay	Concentration					
	5 ng/mL	15 ng/mL	30 ng/mL	60 ng/mL	90 ng/mL	120 ng/mL
1	0.07	0.16	0.36	0.61	0.95	1.19
2	0.07	0.17	0.32	0.66	1.01	1.12
3	0.06	0.16	0.32	0.60	1.00	1.16
4	0.06	0.17	0.31	0.60	0.94	1.09
5	0.05	0.16	0.33	0.65	0.96	1.11
6	0.07	0.17	0.34	0.65	0.87	1.13
7	0.07	0.17	0.31	0.61	0.95	1.21
8	0.06	0.15	0.29	0.58	0.90	1.21
9	0.07	0.17	0.32	0.65	0.88	1.15
10	0.06	0.14	0.30	0.57	1.05	1.16
11	0.06	0.15	0.30	0.56	0.87	1.15
12	0.05	0.15	0.31	0.64	0.85	1.06
13	0.06	0.16	0.33	0.60	0.88	1.08
14	0.06	0.17	0.31	0.61	0.91	1.22
15	0.06	0.18	0.34	0.66	0.96	1.18
16	0.06	0.17	0.35	0.65	0.94	1.21
17	0.06	0.17	0.36	0.64	0.94	1.17
18	0.07	0.17	0.34	0.66	0.98	1.18
19	0.07	0.16	0.34	0.68	0.84	1.27
Avg	0.06	0.16	0.33	0.63	0.93	1.16
SD	0.00	0.01	0.02	0.03	0.06	0.05
CV%	7.9	5.8	5.9	5.5	6.1	4.6

Example 8: Intra-assay and Inter-assay Variation Studies

[00121] Intra-assay variation is defined as the reproducibility of results for a sample within a single assay. To assess intra-assay variation, twenty replicates from each of four quality control (QC) pools covering the reportable range of the assay were prepared and measured from pooled serum with 25OHD$_2$ and 25OHD$_3$ at arbitrary ultralow, low, medium, and high concentrations for each analyte. Acceptable levels for the coefficient of variation (CV) are less then 15% for the three higher concentration, and less than 20% for the lowest concentration (at or near the LOQ for the assay).

[00122] The results of the intra-assay variation studies indicate that the CV for the four QC pools are 9.1%, 6.4%, 5.0%, and 5.9% with mean concentrations of 13.7 ng/mL, 30.0 ng/mL,

52.4 ng/mL, and 106.9 ng/mL, respectively, for PTAD-25OHD$_2$, and 3.5%, 4.9%, 5.1%, and 3.3% with mean concentrations of 32.8 ng/mL, 15.0 ng/mL, 75.4 ng/mL, and 102.3 ng/mL, respectively, for PTAD-25OHD$_3$. The data from analysis of these replicates is shown in Tables 7A and 7B.

Table 7A. PTAD-25OHD$_2$ Intra-assay variation studies

Replicate	QC (U)	QC (L)	QC (M)	QC (H)
	Lot # 090837	Lot # 090838	Lot # 090839	Lot # 090840
	ng/mL	ng/mL	ng/mL	ng/mL
1	15.2	31.4	49.5	108.9
2	12.3	29.7	53.2	109.3
3	13.8	30.8	50.9	98.9
4	12.4	30.1	50.4	111.5
5	14.6	27.2	49.7	109.0
6	14.6	29.1	47.6	110.3
7	13.6	33.0	53.3	95.6
8	11.4	29.9	53.3	98.5
9	14.0	31.5	55.2	110.7
10	13.7	29.1	49.0	113.5
11	13.7	29.5	56.8	100.4
12	13.0	25.5	54.1	105.4
13	15.6	34.2	53.6	102.0
14	11.7	28.7	52.9	103.2
15	13.5	28.1	49.4	121.0
16	13.6	29.8	52.0	102.9
17	13.1	29.4	56.8	113.4
18	14.4	30.6	54.5	103.3
19	16.2	31.6	53.1	110.8
20	12.7	30.7	-	110.4
Avg	0.06	0.16	0.33	0.63
SD	0.00	0.01	0.02	0.03
CV%	7.9	5.8	5.9	5.5

Table 7B. PTAD-25OHD$_3$ Intra-assay variation studies

Replicate	QC (U)	QC (L)	QC (M)	QC (H)
	Lot # 090837	Lot # 090838	Lot # 090839	Lot # 090840
	ng/mL	ng/mL	ng/mL	ng/mL
1	34.4	13.7	75.7	101.7
2	35.0	14.2	78.7	101.8
3	33.2	14.7	73.1	103.2
4	34.4	14.9	83.7	104.1
5	32.4	14.5	72.7	107.0
6	33.3	14.3	73.6	107.6
7	33.8	15.0	79.1	97.5
8	32.1	15.8	73.1	98.7
9	32.4	15.5	74.2	106.5
10	31.4	15.4	74.5	106.1
11	31.8	14.7	69.3	105.9
12	31.2	16.8	73.5	97.7
13	34.1	15.4	72.7	104.9
14	33.8	15.3	75.1	99.8
15	32.0	15.7	76.2	102.2
16	33.2	14.7	74.2	102.2
17	32.6	14.7	85.0	100.5
18	31.6	13.9	75.5	101.8
19	31.3	15.6	73.6	99.9
20	32.5	15.3	-	96.3
Avg	32.8	15.0	75.4	102.3
SD	1.1	0.7	3.8	3.4
CV%	3.5	4.9	5.1	3.3

[00123] Five aliquots of each of the same four QC pools were assayed over six days to determine the coefficient of variation (CV) between assays. The results of the intra-assay variation studies indicate that the inter-assay CV for the four QC pools are 8.3%, 6.2%, 8.1%, and 6.4% with mean concentrations of 13.1 ng/mL, 29.8 ng/mL, 51.9 ng/mL, and 107.8 ng/mL, respectively, for PTAD-25OHD$_2$, and 4.8%, 6.7%, 4.7%, and 6.7% with mean concentrations of 31.1 ng/mL, 14.5 ng/mL, 75.1 ng/mL, and 108.4 ng/mL, respectively, for PTAD-25OHD$_3$. The data from analysis of these replicates is shown in Tables 8A and 8B.

Table 8A. PTAD-25OHD$_2$ Inter-assay variation studies

Assay	QC (U)	QC (L)	QC (M)	QC (H)
	Lot # 090837	Lot # 090838	Lot # 090839	Lot # 090840
	ng/mL	ng/mL	ng/mL	ng/mL
1	13.6	28.1	51.7	119.6
	12.8	30.1	49.4	117.9
	14.6	32.0	49.7	105.1
	13.0	30.8	52.3	100.2
	13.0	29.2	56.6	110.3
2	12.9	31.3	46.3	108.1
	13.5	30.3	52.1	117.8
	10.9	29.7	46.9	105.8
	11.2	30.6	43.6	105.2
	12.8	28.7	50.3	104.9
3	12.6	28.8	56.5	115.3
	16.4	29.3	63.8	103.0
	13.2	26.2	45.5	103.2
	11.5	30.8	53.8	113.2
	12.4	33.7	51.6	106.9
4	12.1	28.5	58.5	97.0
	13.9	26.2	51.8	115.1
	14.4	29.6	48.9	112.2
	13.1	32.1	52.3	97.9
	12.6	30.5	52.2	104.2
5	12.7	29.9	54.5	101.3
	14.3	28.3	46.3	102.2
	13.9	30.0	56.1	111.4
	13.1	32.6	51.2	123.1
	12.4	26.2	51.2	98.3
6	12.5	30.6	50.1	104.6
	12.9	32.6	51.8	104.8
	14.0	28.6	53.7	108.9
	14.3	29.1	51.0	113.8
	12.9	29.1	56.4	102.2
Avg	13.1	29.8	51.9	107.8
SD	1.1	1.8	4.2	6.8
CV%	8.3	6.2	8.1	6.4

Table 8B. PTAD-25OHD$_3$ Inter-assay variation studies

Assay	QC (U)	QC (L)	QC (M)	QC (H)
	Lot # 090837	Lot # 090838	Lot # 090839	Lot # 090840
	ng/mL	ng/mL	ng/mL	ng/mL
1	32.6	13.4	76.7	104.9
	30.0	12.7	77.6	107.0
	34.1	15.4	78.4	107.1
	34.0	14.8	76.6	105.1
	30.2	15.5	74.8	110.2
2	33.5	13.2	69.8	109.8
	32.4	14.3	75.0	106.4
	30.2	16.2	73.4	112.1
	31.4	16.1	71.9	97.0
	31.4	13.7	75.2	117.5
3	31.5	13.3	70.2	112.4
	32.1	14.6	82.6	101.5
	31.0	15.4	70.8	99.8
	28.7	15.6	74.3	103.6
	30.7	15.1	79.8	99.1
4	31.9	14.5	76.3	124.2
	27.5	14.0	70.5	113.6
	27.9	14.8	74.5	112.5
	32.1	16.1	74.3	108.8
	31.0	14.4	74.5	110.1
5	31.2	13.1	76.7	96.5
	31.5	13.5	82.9	106.1
	31.5	14.7	70.9	112.9
	30.9	14.5	77.6	117.7
	31.0	13.9	73.1	101.9
6	29.8	15.6	73.3	110.1
	30.5	13.5	71.5	99.3
	31.0	13.9	72.6	120.5
	30.5	14.6	74.2	109.4
	30.7	13.6	81.8	115.9
Avg	31.1	14.5	75.1	108.4
SD	1.5	1.0	3.6	6.9
CV%	4.8	6.7	4.7	6.4

Example 9: Recovery Studies

[00124] Two recovery studies were performed. The first was performed using six specimens, spiked with two different concentrations each of 25OHD$_2$ and 25OHD$_3$. These spiked specimens were analyzed in quadruplicate. The spiked concentrations were within the

workable range of the assay. The six pools yielded an average accuracy of 89% at spiked levels of grater than 44 ng/mL and 92% at spiked levels of greater than 73 ng/mL. Only two of the 24 experimental recoveries were less than 85%; the remaining 22 assays were within the acceptable accuracy range of 85-115%. The results of the spiked specimen recovery studies are presented in Table 9, below.

Table 9. Spiked Specimen Recovery Studies

Pool	*Spike Level*	**25OHD$_2$**		**25OHD$_3$**	
		ng/mL	(% Recovery)	ng/mL	(% Recovery)
1	-	12.0	-	10.8	-
	44 ng/ml 25OHD$_2$	48.0	81.2	10.7	-
	73 ng/mL 25OHD$_2$	79.0	91.6	10.7	-
	44 ng/mL 25OHD$_3$	12.7	-	51.9	92.9
	73 ng/mL 25OHD$_3$	11.5	-	76.5	89.9
2	-	11.9	-	10.8	-
	44 ng/mL 25OHD$_2$	48.0	81.4	10.6	-
	73 ng/mL 25OHD$_2$	75.6	87.1	11.0	-
	44 ng/mL 25OHD$_3$	10.0	-	48.8	85.6
	73 ng/mL 25OHD$_3$	11.6	-	76.4	89.7
3	-	13.6	-	6	-
	44 ng/mL 25OHD$_2$	52.5	87.8	10.9	-
	73 ng/mL 25OHD$_2$	76.8	86.4	10.5	-
	44 ng/mL 25OHD$_3$	13.2	-	49.6	88.0
	73 ng/mL 25OHD$_3$	12.3	-	78.0	92.2
4	-	9.0	-	12.7	-
	44 ng/mL 25OHD$_2$	50.3	93.1	13.5	-
	73 ng/mL 25OHD$_2$	77.6	93.8	13.2	-
	44 ng/mL 25OHD$_3$	10.0	-	52.1	89.0
	73 ng/mL 25OHD$_3$	9.5	-	83.6	97.0
5	-	21.8	-	14.0	-
	44 ng/mL 25OHD$_2$	68.0	104.2	13.3	-
	73 ng/mL 25OHD$_2$	91.1	94.8	13.6	-
	44 ng/mL 25OHD$_3$	23.3	-	53.5	89.1
	73 ng/mL 25OHD$_3$	22.2	-	86.4	99.1
6	-	13.8	-	9.3	-
	44 ng/mL 25OHD$_2$	50.6	83.0	9.2	-
	73 ng/mL 25OHD$_2$	83.9	95.9	9.5	-
	44 ng/mL 25OHD$_3$	13.5	-	48.6	88.6
	73 ng/mL 25OHD$_3$	13.2	-	76.5	91.9

[00125] The second recovery study was performed again using six specimens. Of these six specimens, three had high endogenous concentration of $25OHD_2$ and three had high endogenous concentrations of $25OHD_3$. The specimens were paired and mixed at ratios of 4:1, 1:1, and 1:4, and the resulting mixtures analyzed in quadruplicate. These experiments yielded an average accuracy of 98% for $25OHD_2$ and 93% for $25OHD_3$. All individual results were within the acceptable accuracy range of 85-115%. The results of the mixed specimen recovery studies are presented in Table 10, below.

Table 10. Mixed Specimen Recovery Studies

Specimen Mixture	**$25OHD_2$**			**$25OHD_3$**		
	Measured ng/mL	Expected ng/mL	Recovery (%)	Measured ng/mL	Expected ng/mL	Recovery (%)
100% A	45.2	-	-	5.5	-	-
4:1 A:B	37.1	37.0	100	11.6	13.1	88
1:1 A:B	26.4	24.6	107	24.4	24.4	100
1:4 A:B	12.6	12.3	102	33.9	35.7	95
100% B	4.1	-	-	43.3	-	-
100% C	46.8	-	-	8.3	-	-
4:1 C:D	38.1	38.7	98	17.7	18.3	97
1:1 C:D	25.0	26.6	94	32.0	33.4	96
1:4 C:D	14.4	14.4	100	46.5	48.4	96
100% D	6.3	-	-	58.5	-	-
100% E	38.7	-	-	7.4	-	-
4:1 E:F	33.4	34.3	97	15.7	17.5	89
1:1 E:F	27.1	27.7	98	27.8	32.6	85
1:4 E:F	18.3	21.0	87	44.0	47.7	92
100% F	16.6	-	-	57.8	-	-

*Measured values are averages of analysis of four aliquots.

Example 10: Method Correlation Study

[00126] The method of detecting vitamin D metabolites following PTAD-derivatization was compared to a mass spectrometric method in which the vitamin D metabolites are not derivatized prior to analysis. Such a method is described in the published U.S. Patent Application 2006/0228808 (Caulfield, et al.). Eight specimens were split and analyzed according to both methods. The correlation between the two methods was assessed with

linear regression, deming regression, and Bland-Altman analysis for complete data sets (including calibration samples, QC pools, and unknowns), as well as for unknowns only.

[00127] Plots of the linear regression analysis and the Deming regression analysis are shown in Figures 4A-B (for $25OHD_2$) and Figures 5A-B (for $25OHD_3$).

Example 11: Hemolysis, Lipemia, and Icteria Studies

[00128] The effect hemolysis, lipemia, and icteria have on the assay was also investigated.

[00129] Hemolysis. The effect of hemolysis was evaluated by pooling patient samples with known endogenous concentrations of both $25OHD_2$ and $25OHD_3$ to create five different pools with concentrations across the dynamic range of the assay. Then, lysed whole blood was spiked into the pools to generate lightly and moderately hemolyzed samples.

[00130] The lightly and moderately hemolyzed samples were analyzed in quadruplicate and the results were compared to levels of samples without whole blood spikes. The resulting comparison indicated a % difference of less than 15% for both $25OHD_2$ and $25OHD_3$. Therefore, light to moderately hemolyzed specimens are acceptable for analysis.

[00131] Lipemia. The effect of lipemia was evaluated by pooling patient samples with known endogenous concentrations of both $25OHD_2$ and $25OHD_3$ to create five different pools with concentrations across the dynamic range of the assay. Then, powdered lipid extract was added to the pools to generate lightly and grossly lipemic specimens. Specimens were run in quadruplicate and results were compared to the non-lipemic pool result and the accuracy was calculated. The data shows that determination of $25OHD_2$ is unaffected by lipemia (all values were within an acceptable accuracy range of 85-115%), however, $25OHD_3$ is affected by lipemia, resulting in determination in lower than expected values. The degree of variance increased with the degree of lipemia. Therefore, light but not grossly lipemic specimens are acceptable.

[00132] Icteria. The effect of icteria was evaluated by pooling patient samples with known endogenous concentrations of both $25OHD_2$ and $25OHD_3$ to create five different pools with concentrations across the dynamic range of the assay. Then, a concentrated solution of Bilirubin was spiked into the pools to generate lightly and grossly icteric specimens. Specimens were run in quadruplicate and results were compared to the non-icteric pool result and the accuracy was calculated. The data showed that $25OHD_2$ and $25OHD_3$ are unaffected

by icteria (with all values within an acceptable accuracy range of 85-115%). Therefore, icteric specimens are acceptable.

Example 12: Injector Carryover Studies

[00133] Blank matrices were run immediately after a specimen with a high concentration of $25OHD_2$ and $25OHD_3$ in order to evaluate carryover between samples. These studies indicated that the response at the retention time of analyte or internal standard was not large enough to compromise the integrity of the assay. Data from these studies is presented in Table 11, below.

Table 11. Injector Carryover Study Results

Injection	Specimen Type	$25OHD_2$ (ng/mL)	$25OHD_3$ (ng/mL)
1	Blank	0.9	1.6
2	High	292.6	356.8
3	Blank	1.0	0.9
4	Blank	-0.1	0.5
5	High	290.1	360.1
6	High	299.9	350.5
7	Blank	1.0	1.5
8	Blank	0.6	1.4
9	Blank	1.3	1.4
10	High	285.8	352.1
11	High	303.1	312.1
12	High	293.8	295.1
13	Blank	0.9	0.8
14	Blank	1.0	1.8
15	Blank	1.1	1.4
16	Blank	1.0	1.6
17	High	291.7	371.6
18	High	334.2	360.1
19	High	301.7	328.5
20	High	283.1	382.1
21	Blank	0.6	1.1
22	Blank	0.6	1.3
23	Blank	0.7	1.4
24	Blank	0.4	1.9
25	Blank	0.4	0.9
26	High	300.7	311.7
27	High	279.5	302.0
28	High	317.5	341.0
29	High	261.5	403.4
30	High	288.3	362.6

Injection	Specimen Type	$25OHD_2$ (ng/mL)	$25OHD_3$ (ng/mL)
41	High	291.5	322.3
42	High	278.9	336.5
43	Blank	2.1	2.5
44	Blank	0.6	1.6
45	Blank	0.7	1.4
46	Blank	0.7	1.5
47	Blank	0.1	1.0
48	Blank	0.7	1.1
49	Blank	1.3	1.0
50	High	281.2	345.6
51	High	312.5	348.3
52	High	304.8	329.1
53	High	290.5	353.9
54	High	286.4	344.9
55	High	302.5	330.6
56	High	292.2	388.5
57	Blank	0.8	1.5
58	Blank	1.3	1.4
59	Blank	3.5	2.6
60	Blank	0.4	1.8
61	Blank	1.0	1.4
62	Blank	1.0	1.2
63	Blank	0.7	1.0
64	Blank	1.1	1.4
65	High	285.4	355.4
66	High	318.0	355.0
67	High	285.5	345.7
68	High	303.0	317.1
69	High	276.3	351.4
70	High	321.8	350.4

31	Blank	2.7	1.6
32	Blank	1.7	1.2
33	Blank	0.5	1.3
34	Blank	1.3	1.7
35	Blank	0.3	1.6
36	Blank	0.6	1.4
37	High	311.7	366.2
38	High	314.1	342.0
39	High	325.7	349.1
40	High	289.6	326.6

71	High	279.4	329.6
72	High	299.1	337.9
73	Blank	0.9	1.6
74	Blank	1.7	1.6
75	Blank	1.0	1.1
76	Blank	1.8	2.7
77	Blank	1.0	1.9
78	Blank	0.6	1.1
79	Blank	0.9	0.9
80	Blank	1.2	2.2

Example 13: Suitable Specimen Types

[00134] The assay was conducted on various specimen types. Human serum and Gel-Barrier Serum (i.e., serum from Serum Separator Tubes), as well as EDTA Plasma and Heparin were established as acceptable sample types. In these studies, sets of human serum (serum), Gel-Barrier Serum (SST), EDTA Plasma (EDTA), and heparin (Na Hep) drawn at the same time from the same patient were tested for $25OHD_2$ (40 specimen sets) and $25OHD_3$ (6 specimen sets). Due to the limitations with clot detection/sensing in existing automated pipetting systems, plasma was not tested for automated procedures.

[00135] The results of the specimen type studies are presented in Tables 12A and B for $25OHD_2$ and $25OHD_3$, respectively.

Table 12A. Results from Specimen Type Studies for $25OHD_2$

Specimen Set	CC ID#	Measured Concentration $25OHD_2$ (ng/mL)			
		Serum	SST	EDTA	Na Hep
2	5207	6.6	6.9	7.1	7.2
6	5339	5.8	5.2	4.5	5.6
11	5355	7.8	8.2	8.8	8.2
20	5493	3.9	4.2	4.3	4.2
37	5650	3.7	4.5	4.6	5.2
39	5653	4.7	5.1	4.6	4.7

Table 12B. Results from Specimen Type Studies for $25OHD_3$

Specimen Set	CC ID#	Measured Concentration $25OHD_3$ (ng/mL)			
		Serum	SST	EDTA	Na Hep
1	5804	26.8	25.7	24.3	26.8
2	5207	16.1	17.6	16.1	16.5
3	5235	17.4	17.7	16.8	17.2

4	5333	62.9	62.7	63.7	57.4
5	5336	33.0	32.4	28.8	28.8
6	5339	17.2	17.6	17.8	17.8
7	5340	16.7	17.1	16.8	16.5
8	5342	28.6	27.9	26.9	30.5
9	5344	23.3	23.8	22.3	22.9
10	5351	19.4	20.0	20.4	21.4
11	5355	17.6	16.7	19.4	18.3
12	5362	25.3	25.2	23.5	24.0
13	5365	40.9	44.7	46.8	42.9
14	5406	23.1	20.3	21.5	20.5
15	5408	31.7	33.9	31.6	32.3
16	5414	21.1	21.8	21.2	20.4
17	5422	44.0	47.7	45.5	47.3
18	5432	13.6	14.2	12.3	13.8
19	5463	15.1	15.4	15.6	14.5
20	5493	38.6	42.2	40.1	36.8
21	5366	47.5	48.1	46.7	45.1
22	5368	23.0	23.6	22.3	22.3
23	5392	34.1	33.4	34.4	27.6
24	5451	36.4	42.1	40.0	38.3
25	5455	27.3	29.9	25.1	27.9
26	5476	16.7	17.9	15.8	16.6
27	5483	30.4	28.2	26.5	28.1
28	5484	38.2	37.7	37.2	36.0
29	5537	30.5	30.3	27.2	27.0
30	5547	9.2	9.0	8.7	8.2
31	5560	9.4	10.9	9.8	8.6
32	5571	30.9	31.7	29.6	29.2
33	5572	47.6	50.3	47.7	48.6
34	5577	11.2	11.7	10.4	9.2
35	5606	39.3	38.8	41.0	37.7
36	5611	21.9	25.3	20.7	21.1
37	5650	38.0	34.3	34.6	36.2
38	5651	34.8	32.8	32.4	32.4
39	5653	29.4	32.3	28.1	27.0
40	5668	11.4	12.8	14.2	13.1

Example 14: Exemplary spectra from LDTD-MS/MS analysis of PTAD derivatized 25-hydroxyvitamin D_2 and PTAD derivatized 25-hydroxyvitamin D_3

[00136] PTAD derivatives of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$, 25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and 25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$

were prepared as described above. The resulting solutions were analyzed by LDTD-MS/MS. Results of these analyses are presented below.

[00137] Exemplary Q1 scan spectra from the analysis of samples containing PTAD-25-hydroxyvitamin D_2, PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$, and PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ are shown in Figures 6A, 7A, and 8A, respectively. These spectra were collected by scanning Q1 across a m/z range of about 520 to 620.

[00138] Exemplary product ion scans from each of these species are presented in Figures 6B, 7B, and 8B, respectively. The precursor ions selected in Q1, and collision energies used in fragmenting the precursors are indicated in Table 13.

[00139] A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_2 is fragmenting a precursor ion with a m/z of about 570.3 to a product ion with a m/z of about 298.1. A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$ is fragmenting a precursor ion with a m/z of about 573.3 to a product ion with a m/z of about 301.1. A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ is fragmenting a precursor ion with a m/z of about 576.3 to a product ion with a m/z of about 298.1. However, as can be seen in the product ion scans in Figures 6B, 7B, and 8B, additional product ions may be selected to replace or augment the preferred fragment ion.

Table 13. Precursor Ions and Collision Cell Energies for Fragmentation of PTAD-25-hydroxyvitamin D_2, PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$, and PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$

Analyte	*Precursor Ion (m/z)*	*Collision Cell Energy (V)*
PTAD-25-hydroxyvitamin D_2	570.3	15
PTAD-25-hydroxyvitamin D_2–[6, 19, 19]-$^2H_3$	573.3	15
PTAD-25-hydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$	576.3	15

[00140] Exemplary Q1 scan spectra from the analysis of PTAD-25-hydroxyvitamin D_3, PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and PTAD-25-hydroxyvitamin D_3–[26, 26, 26,

27, 27, 27]-$^2H_6$ are shown in Figures 9A, 10A, and 11A, respectively. These spectra were collected by scanning Q1 across a m/z range of about 520 to 620.

[00141] Exemplary product ion scans from each of these species are presented in Figures 9B, 10B, and 11B, respectively. The precursor ions selected in Q1 and the collision energies used to generate these product ion spectra are indicated in Table 14.

[00142] A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_3 is fragmenting a precursor ion with a m/z of about 558.3 to a product ion with a m/z of about 298.1. A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ is fragmenting a precursor ion with a m/z of about 561.3 to a product ion with a m/z of about 301.1. A preferred MRM transition for the quantitation of PTAD-25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ is fragmenting a precursor ion with a m/z of about 564.3 to a product ion with a m/z of about 298.1. However, as can be seen in the product ion scans in Figures 9B, 10B, and 11B, additional product ions may be selected to replace or augment the preferred fragment ion.

Table 14. Precursor Ions and Collision Cell Energies for Fragmentation of PTAD-25-hydroxyvitamin D_3, PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and PTAD-25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$

Analyte	*Precursor Ion (m/z)*	*Collision Cell Energy (V)*
PTAD-25-hydroxyvitamin D_3	558.3	15
PTAD-25-hydroxyvitamin D_3–[6, 19, 19]-$^2H_3$	561.3	15
PTAD-25-hydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$	564.3	15

Example 15: Exemplary spectra from LDTD-MS/MS analysis of PTAD derivatized 1α,25-dihydroxyvitamin D_2 and 1α,25-dihydroxyvitamin D_3

[00143] PTAD derivatives of 1α,25-dihydroxyvitamin D_2, 1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$, 1α,25-dihydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and 1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ were prepared by treating aliquots of stock solutions of each analyte with PTAD in acetonitrile. The derivatization reactions was allowed to proceed for approximately one hour, and were quenched by adding

water to the reaction mixture. The derivatized analytes were then analyzed according to the procedure outlined above in Example 1.

[00144] Exemplary Q1 scan spectra from the analysis of samples containing PTAD-1α,25-dihydroxyvitamin D_2 and PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ are shown in Figures 12A, and 13A, respectively. These spectra were collected by scanning Q1 across a m/z range of about 520 to 620.

[00145] Exemplary product ion scans generated from three different precursor ions for each of PTAD-1α,25-dihydroxyvitamin D_2 and PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ are presented in Figures 12B-D, and 13B-D, respectively. The precursor ions selected in Q1 and the collision energies used to generate these product ion spectra are indicated in Table 15.

[00146] Exemplary MRM transitions for the quantitation of PTAD-1α,25-dihydroxyvitamin D_2 include fragmenting a precursor ion with a m/z of about 550.4 to a product ion with a m/z of about 277.9; fragmenting a precursor ion with a m/z of about 568.4 to a product ion with a m/z of about 298.0; and fragmenting a precursor ion with a m/z of about 586.4 to a product ion with a m/z of about 314.2. Exemplary MRM transitions for the quantitation of PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ include fragmenting a precursor ion with a m/z of about 556.4 to a product ion with a m/z of about 278.1; fragmenting a precursor ion with a m/z of about 574.4 to a product ion with a m/z of about 298.1; and fragmenting a precursor ion with a m/z of about 592.4 to a product ion with a m/z of about 313.9. However, as can be seen in the product ion scans in Figures 12B-D and 13B-D, several other product ions are generated upon fragmentation of the precursor ions. Additional product ions may be selected from those indicated in Figures 12B-D and 13B-D to replace or augment the exemplary fragment ions.

Table 15. Precursor Ions and Collision Cell Energies for Fragmentation of PTAD-1α,25-dihydroxyvitamin D_2 and PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$

Analyte	*Precursor Ion (m/z)*	*Collision Cell Energy (V)*
PTAD-1α,25-dihydroxyvitamin D_2	550.4, 568.4, 586.4	15
PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$	556.3, 574.4, 592.4	15

[00147] Exemplary Q1 scan spectra from the analysis of PTAD-1α,25-hydroxyvitamin D_3, PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ are shown in Figures 14A, 15A, and 16A, respectively. These spectra were collected by scanning Q1 across a m/z range of about 520 to 620.

[00148] Exemplary product ion scans generated from three different precursor ions for each of PTAD-1α,25-hydroxyvitamin D_3, PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$ are presented in Figures 14B-D, 15A-D, and 16B-D, respectively. The precursor ions selected in Q1 and the collision energies used to generate these product ion spectra are indicated in Table 16.

[00149] Exemplary MRM transitions for the quantitation of PTAD-1α,25-hydroxyvitamin D_3 include fragmenting a precursor ion with a m/z of about 538.4 to a product ion with a m/z of about 278.1; fragmenting a precursor ion with a m/z of about 556.4 to a product ion with a m/z of about 298.0; and fragmenting a precursor ion with a m/z of about 574.4 to a product ion with a m/z of about 313.0. Exemplary MRM transitions for the quantitation of PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$ include fragmenting a precursor ion with a m/z of about 541.4 to a product ion with a m/z of about 280.9; fragmenting a precursor ion with a m/z of about 559.4 to a product ion with a m/z of about 301.1; and fragmenting a precursor ion with a m/z of about 577.4 to a product ion with a m/z of about 317.3. Exemplary MRM transitions for the quantitation of PTAD-1α,25-dihydroxyvitamin D_2–[26, 26, 26, 27, 27, 27]-$^2H_6$ include fragmenting a precursor ion with a m/z of about 544.4 to a product ion with a m/z of about 278.0; fragmenting a precursor ion with a m/z of about 562.4 to a product ion with a m/z of about 298.2; and fragmenting a precursor ion with a m/z of about 580.4 to a product ion with a m/z of about 314.0. However, as can be seen in the product ion scans in Figures 14B-D, 15B-D, and 25B-D, several other product ions are generated upon fragmentation of the precursor ions. Additional product ions may be selected from those indicated in Figures 14B-D, 15B-D, and 16B-D to replace or augment the exemplary fragment ions.

Table 16. Precursor Ions and Collision Cell Energies for Fragmentation of PTAD-1α,25-dihydroxyvitamin D_3, PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$, and PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$

Analyte	*Precursor Ion (m/z)*	*Collision Cell Energy (V)*
PTAD-1α,25-dihydroxyvitamin D_3	538.4, 556.4, 574.4	15
PTAD-1α,25-dihydroxyvitamin D_3–[6, 19, 19]-$^2H_3$	541.4, 559.4, 577.4	15
PTAD-1α,25-dihydroxyvitamin D_3–[26, 26, 26, 27, 27, 27]-$^2H_6$	544.4, 562.4, 580.4	15

[00150] The contents of the articles, patents, and 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.

[00151] 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. 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.

[00152] 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.

[00153] 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 one or more vitamin D metabolites in a sample by mass spectrometry, the method comprising the steps of:

(i) subjecting one or more 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)-derivatized vitamin D metabolites in the sample to an extraction column and an analytical column;

(ii) subjecting one or more PTAD-derivatized vitamin D metabolites in the sample to an ionization source to generate one or more ions detectable by mass spectrometry;

(iii) determining the amount of one or more of the PTAD-derivatized vitamin D metabolite ions by mass spectrometry; and

(iv) relating the amount of PTAD-derivatized vitamin D metabolite ions determined in step (iii) to the amount of a vitamin D metabolite in the sample,

wherein the sample is subjected to PTAD under conditions sufficient to generate one or more PTAD-derivatized vitamin D metabolites prior to step (i).

2. The method of claim 1, wherein the extraction column of step (i) is a solid-phase extraction (SPE) column.

3. The method of claim 1, wherein the extraction column of step (i) is a turbulent flow liquid chromatography (TFLC) column.

4. The method of any one of claims 1-3, wherein the analytical column of step (i) is a high performance liquid chromatography (HPLC) column.

5. The method of any one of claims 1-4, wherein the extraction and analytical columns of step (i) and the ionization source of step (ii) are connected in an on-line fashion.

6. The method of any one of claims 1-5, wherein mass spectrometry is tandem mass spectrometry.

7. The method of claim 6, wherein tandem mass spectrometry is conducted as multiple reaction monitoring, precursor ion scanning, or product ion scanning.

8. The method of any one of claims 1-7, wherein the one or more vitamin D metabolites comprise one or more vitamin D metabolites selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

9. The method of claim 8, wherein the one or more vitamin D metabolites comprise 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$).

10. The method of claim 8, wherein the one or more vitamin D metabolites comprise 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

11. The method of any one of claims 1-7, wherein the one or more PTAD-derivatized vitamin D metabolites comprises PTAD-derivatized 25-hydroxyvitamin D_2 (PTAD-$25OHD_2$).

12. The method of claim 11, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-$25OHD_2$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 570.3 ± 0.5 and 298.1 ± 0.5.

13. The method of claim 11, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-$25OHD_2$ parent ion with a mass/charge ratio (m/z) of 570.3 ± 0.5 and a PTAD-$25OHD_2$ fragment ion with m/z of 298.1 ± 0.5.

14. The method of any one of claims 1-7, wherein the one or more PTAD-derivatized vitamin D metabolites comprises PTAD-derivatized 25-hydroxyvitamin D_3 (PTAD-$25OHD_3$).

15. The method of claim 14, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-$25OHD_3$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 558.3 ± 0.5 and 298.1 ± 0.5.

16. The method of claim 14, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-$25OHD_3$ parent ion with a mass/charge ratio (m/z) of 558.3 ± 0.5 and a PTAD-$25OHD_3$ fragment ion with m/z of 298.1 ± 0.5.

17. The method of any one of claims 1-7, wherein the one or more PTAD-derivatized vitamin D metabolites comprises PTAD-derivatized 1,25-dihydroxyvitamin D_2 (PTAD-1α,25$(OH)_2D_2$).

18. The method of claim 17, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-1α,25$(OH)_2D_2$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5.

19. The method of claim 17, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_2$ parent ion with a mass/charge ratio (m/z) of 550.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_2$ fragment ion with a mass/charge ratio (m/z) of 277.9 ± 0.5.

20. The method of claim 17, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_2$ parent ion with a mass/charge ratio (m/z) of 568.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_2$ fragment ion with m/z of 298.0 ± 0.5.

21. The method of any one of claims 1-7, wherein the one or more PTAD-derivatized vitamin D metabolites comprises PTAD-derivatized 1,25-dihydroxyvitamin D_3 (PTAD-1α,25$(OH)_2D_3$).

22. The method of claim 21, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-1α,25$(OH)_2D_3$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

23. The method of claim 21, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 538.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with m/z of 278.1 ± 0.5.

24. The method of claim 21, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 556.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with m/z of 298.0 ± 0.5.

25. The method of any one of claims 1-24, wherein the sample comprises a biological sample.

26. The method of any one of claims 1-24, wherein the sample comprises plasma or serum.

27. The method of any one of claims 25-26, wherein the sample is from a human, and the determined amount of a vitamin D metabolite is the amount present in the sample when taken from a human.

28. The method of any one of claims 1-27, wherein the ionization source comprises atmospheric pressure chemical ionization (APCI).

29. The method of claim 28, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprises one or more selected from the group of PTAD-1α,25$(OH)_2D_2$ ions with mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5, and PTAD-1α,25$(OH)_2D_3$ ions with m/z of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

30. A method for determining the amount of one or more vitamin D metabolites in a sample by mass spectrometry, the method comprising the steps of:

(i) subjecting the sample to turbulent flow liquid chromatography (TFLC);

(ii) ionizing one or more Cookson-type-derivatized vitamin D metabolites in the sample to generate one or more Cookson-type-derivatized vitamin D metabolite ions detectable by mass spectrometry;

(iii) determining the amount of one or more of the Cookson-type-derivatized vitamin D metabolite ions by mass spectrometry; and

(iv) relating the amount of Cookson-type-derivatized vitamin D metabolite ions determined in step (iv) to the amount of a vitamin D metabolite in the sample,

wherein the sample is subjected to a Cookson-type derivatizing reagent under conditions sufficient to generate one or more Cookson-type-derivatized vitamin D metabolites prior to step (i).

31. The method of claim 30, wherein the sample is subjected to high performance liquid chromatography (HPLC) after step (i) but prior to step (ii).

32. The method of claim 31, wherein the TFLC of step (i), the HPLC, and the ionization

of step (ii) are conducted in an on-line fashion.

33. The method of any one of claims 30-32, wherein the Cookson-type derivatizing reagent is selected from the group consisting of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD), 4-(4-nitrophenyl)-1,2,4-triazoline-3,5- dione (NPTAD), and 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD), and isotopically labeled variants thereof.

34. The method of any one of claims 30-32, wherein the Cookson-type derivatizing reagent is 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) or an isotopically labeled variant thereof.

35. The method of any one of claims 30-34, wherein mass spectrometry is tandem mass spectrometry.

36. The method of claim 35, wherein tandem mass spectrometry is conducted as multiple reaction monitoring, precursor ion scanning, or product ion scanning.

37. The method of any one of claims 30-36, wherein the one or more vitamin D metabolites comprise one or more vitamin D metabolites selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

38. The method of claim 37, wherein the one or more vitamin D metabolites comprise 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$).

39. The method of claim 37, wherein the one or more vitamin D metabolites comprise 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$) and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

40. The method of any one of claims 30-36, wherein the one or more Cookson-type-derivatized vitamin D metabolites comprise PTAD-derivatized 25-hydroxyvitamin D_2 (PTAD-$25OHD_2$).

41. The method of claim 40, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise PTAD-25OHD$_2$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 570.3 ± 0.5 and 298.1 ± 0.5.

42. The method of claim 40, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-25OHD$_2$ parent ion with a mass/charge ratio (m/z) of 570.3 ± 0.5 and a PTAD-25OHD$_2$ fragment ion with m/z of 298.1 ± 0.5.

43. The method of any one of claims 30-36, wherein the one or more Cookson-type derivatized vitamin D metabolites comprises PTAD-derivatized 25-hydroxyvitamin D$_3$ (PTAD-25OHD$_3$).

44. The method of claim 43, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise PTAD-25OHD$_3$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 558.3 ± 0.5 and 298.1 ± 0.5.

45. The method of claim 43, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-25OHD$_3$ parent ion with a mass/charge ratio (m/z) of 558.3 ± 0.5 and a PTAD-25OHD$_3$ fragment ion with m/z of 298.1 ± 0.5.

46. The method of any one of claims 30-36, wherein the one or more Cookson-type-derivatized vitamin D metabolites comprises PTAD-derivatized 1α,25-dihydroxyvitamin D$_2$ (PTAD-1α,25(OH)$_2$D$_2$).

47. The method of claim 46, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise PTAD-1α,25(OH)$_2$D$_2$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5.

48. The method of claim 46, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-1α,25(OH)$_2$D$_2$ parent ion with a mass/charge ratio (m/z) of 550.4 ± 0.5 and a PTAD-1α,25(OH)$_2$D$_2$ fragment ion with m/z of 277.9 ± 0.5.

49. The method of claim 46, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-1α,25(OH)$_2$D$_2$ parent ion with a mass/charge ratio (m/z) of 568.4 ± 0.5 and a PTAD-1α,25(OH)$_2$D$_2$ fragment ion with m/z of 298.0 ± 0.5.

50. The method of any one of claims 30-36, wherein the one or more Cookson-type-derivatized vitamin D metabolites comprises PTAD-derivatized 1α,25-dihydroxyvitamin D_3 (PTAD-1α,25$(OH)_2D_3$).

51. The method of claim 50, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise PTAD-1α,25$(OH)_2D_3$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

52. The method of claim 50, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 538.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with m/z of 278.1 ± 0.5.

53. The method of claim 50, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 556.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with a mass/charge ratio (m/z) of 298.0 ± 0.5.

54. The method of any one of claims 30-53, wherein the sample comprises a biological sample.

55. The method of any one of claims 30-53, wherein the sample comprises plasma or serum.

56. The method of any one of claims 54-55, wherein the sample is from a human, and the determined amount of a vitamin D metabolite is the amount present in the sample when taken from a human.

57. The method of any one of claims 30-56, wherein the ionization source comprises atmospheric pressure chemical ionization (APCI).

58. The method of claim 57, wherein the one or more Cookson-type-derivatized vitamin D metabolite ions comprises one or more selected from the group of PTAD-1α,25$(OH)_2D_2$ ions with mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5, and PTAD-1α,25$(OH)_2D_3$ ions with m/z of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

59. A method for determining the amount of one or more vitamin D metabolites in a sample by mass spectrometry, the method comprising the steps of:

(i) subjecting the sample to 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) under conditions sufficient to generate one or more PTAD-derivatized vitamin D metabolites;

(ii) ionizing one or more PTAD-derivatized vitamin D metabolites in the sample to generate one or more PTAD-derivatized vitamin D metabolite ions detectable by mass spectrometry, wherein said ionizing comprises volatilizing said one or more PTAD-derivatized vitamin D metabolites by laser diode thermal desorption (LDTD);

(iii) determining the amount of one or more of the PTAD-derivatized vitamin D metabolite ions by mass spectrometry; and

(v) relating the amount of PTAD-derivatized vitamin D metabolite ions determined in step (iv) to the amount of one or more vitamin D metabolite in the sample;

wherein said one or more vitamin D metabolites are selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$).

60. The method of claim 59, wherein the one or more vitamin D metabolites comprise two or more selected from the group consisting of 25-hydroxyvitamin D_2 ($25OHD_2$), 25-hydroxyvitamin D_3 ($25OHD_3$), 1α,25-dihydroxyvitamin D_2 ($1\alpha,25(OH)_2D_2$), and 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$); and wherein step (i) generates two or more PTAD-derivatized vitamin D metabolites.

61. The method of claim 60, wherein the two or more PTAD-derivatized vitamin D metabolites are volatilized simultaneously.

62. The method of any one of claims 59-61, wherein the sample is not subjected to chromatography.

63. The method of any one of claims 59-61, wherein the sample is not subjected to liquid chromatography.

64. The method of any one of claims 59-63, wherein the PTAD-derivatized vitamin D metabolites comprise PTAD-derivatized 25-hydroxyvitamin D_2 (PTAD-25OHD_2).

65. The method of claim 64, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-25OHD_2 ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 570.3 ± 0.5 and 298.1 ± 0.5.

66. The method of claim 64, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-25OHD_2 parent ion with a mass/charge ratio (m/z) of 570.3 ± 0.5 and a PTAD-25OHD_2 fragment ion with m/z of 298.1 ± 0.5.

67. The method of any one of claims 59-63, wherein the PTAD-derivatized vitamin D metabolites comprise PTAD-derivatized 25-hydroxyvitamin D_3 (PTAD-25OHD_3).

68. The method of claim 67, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-25OHD_3 ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 558.3 ± 0.5 and 298.1 ± 0.5.

69. The method of claim 67, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-25OHD_3 parent ion with a mass/charge ratio (m/z) of 558.3 ± 0.5 and a PTAD-25OHD_3 fragment ion with m/z of 298.1 ± 0.5.

70. The method of any one of claims 59-63, wherein the PTAD-derivatized vitamin D metabolites comprises PTAD-derivatized 1α,25-dihydroxyvitamin D_2 (PTAD-1α,25$(OH)_2D_2$).

71. The method of claim 70, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-1α,25$(OH)_2D_2$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5.

72. The method of claim 70, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_2$ parent ion with a mass/charge ratio (m/z) of 550.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_2$ fragment ion with m/z of 277.9 ± 0.5.

73. The method of claim 70, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_2$ parent ion with a mass/charge ratio (m/z) of 568.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_2$ fragment ion with m/z of 298.0 ± 0.5.

74. The method of any one of claims 59-63, wherein the one or more PTAD-derivatized vitamin D metabolites comprise PTAD-derivatized 1α,25-dihydroxyvitamin D_3 (PTAD-1α,25$(OH)_2D_3$).

75. The method of claim 74, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise PTAD-1α,25$(OH)_2D_3$ ions selected from the group consisting of ions with a mass/charge ratio (m/z) of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

76. The method of claim 74, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 538.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with m/z of 278.1 ± 0.5.

77. The method of claim 74, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprise a PTAD-1α,25$(OH)_2D_3$ parent ion with a mass/charge ratio (m/z) of 556.4 ± 0.5 and a PTAD-1α,25$(OH)_2D_3$ fragment ion with m/z of 298.0 ± 0.5.

78. The method of any one of claims 59-77, wherein the sample comprises a biological sample.

79. The method of any one of claims 59-77, wherein the sample comprises plasma or serum.

80. The method of any one of claims 78-79, wherein the sample is from a human, and the determined amount of a vitamin D metabolite is the amount present in the sample when taken from a human.

81. The method of any one of claims 59-80, wherein the ionization source comprises atmospheric pressure chemical ionization (APCI).

82. The method of claim 81, wherein the one or more PTAD-derivatized vitamin D metabolite ions comprises one or more ions selected from the group of PTAD-1α,25$(OH)_2D_2$ ions with mass/charge ratio (m/z) of 550.4 ± 0.5, 568.4 ± 0.5, 277.9 ± 0.5, and 298.0 ± 0.5, and PTAD-1α,25$(OH)_2D_3$ ions with a mass/charge ratio (m/z) of 538.4 ± 0.5, 556.4 ± 0.5, 278.1 ± 0.5, and 298.0 ± 0.5.

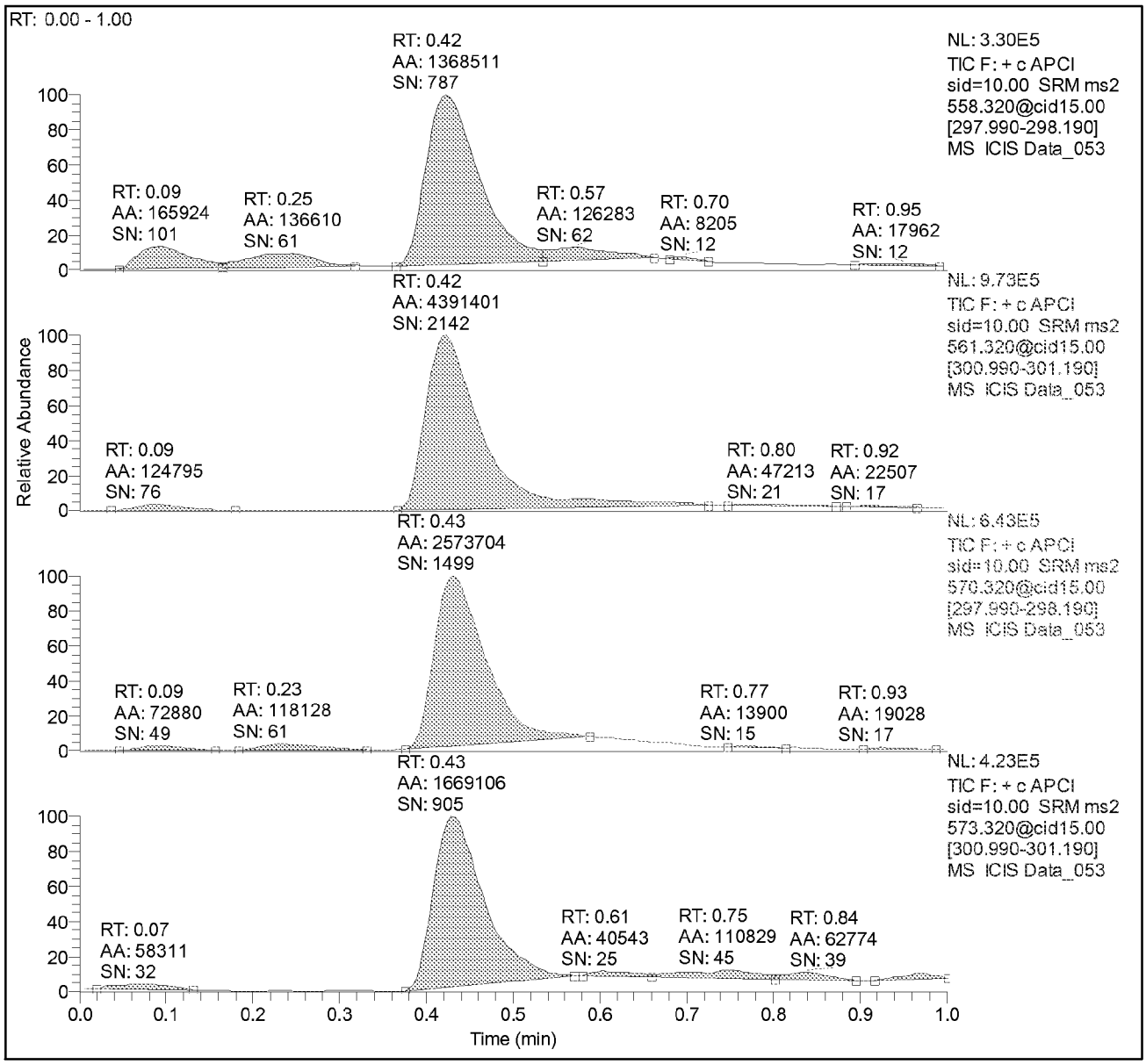

RT: 0.00 - 1.00
A
RT: 0.42
AA: 1368511
SN: 787
NL: 3.30E5
TIC F: + c APCI
sid=10.00 SRM ms2
558.320@cid15.00
[297.990-298.190]
MS ICIS Data_053
RT: 0.09
AA: 165924
SN: 101
RT: 0.25
AA: 136610
SN: 61
RT: 0.57
AA: 126283
SN: 62
RT: 0.70
AA: 8205
SN: 12
RT: 0.95
AA: 17962
SN: 12
B
RT: 0.42
AA: 4391401
SN: 2142
NL: 9.73E5
TIC F: + c APCI
sid=10.00 SRM ms2
561.320@cid15.00
[300.990-301.190]
MS ICIS Data_053
Relative Abundance
RT: 0.09
AA: 124795
SN: 76
RT: 0.80
AA: 47213
SN: 21
RT: 0.92
AA: 22507
SN: 17
C
RT: 0.43
AA: 2573704
SN: 1499
NL: 6.43E5
TIC F: + c APCI
sid=10.00 SRM ms2
570.320@cid15.00
[297.990-298.190]
MS ICIS Data_053
RT: 0.09
AA: 72880
SN: 49
RT: 0.23
AA: 118128
SN: 61
RT: 0.77
AA: 13900
SN: 15
RT: 0.93
AA: 19028
SN: 17
D
RT: 0.43
AA: 1669106
SN: 905
NL: 4.23E5
TIC F: + c APCI
sid=10.00 SRM ms2
573.320@cid15.00
[300.990-301.190]
MS ICIS Data_053
RT: 0.07
AA: 58311
SN: 32
RT: 0.61
AA: 40543
SN: 25
RT: 0.75
AA: 110829
SN: 45
RT: 0.84
AA: 62774
SN: 39
Time (min)

Figure 1

Figure 2A

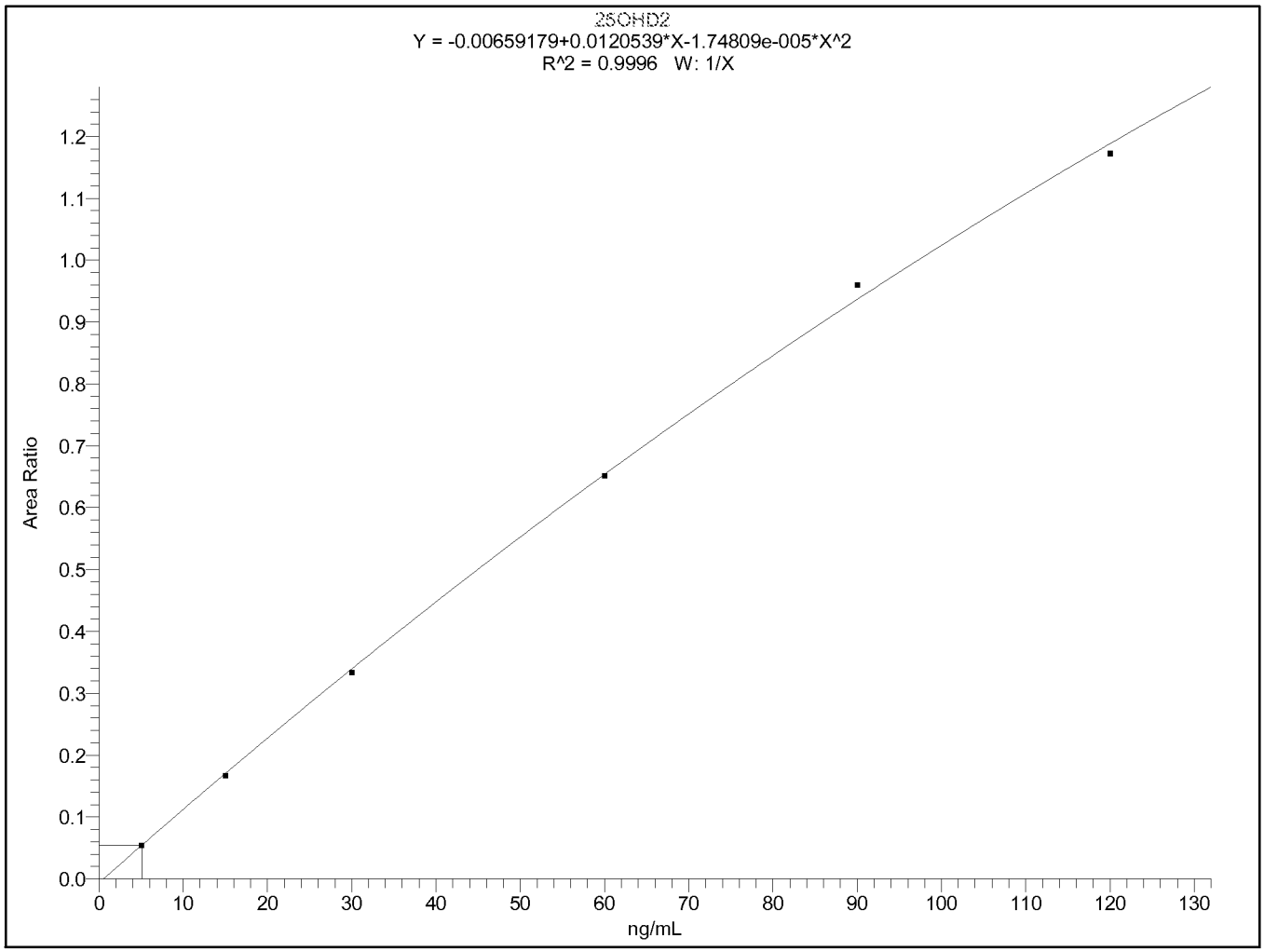

25OHD2
Y = -0.00659179+0.0120539*X-1.74809e-005*X^2
R^2 = 0.9996 W: 1/X
Area Ratio
ng/mL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
0
10
20
30
40
50
60
70
80
90
100
110
120
130

Figure 2B

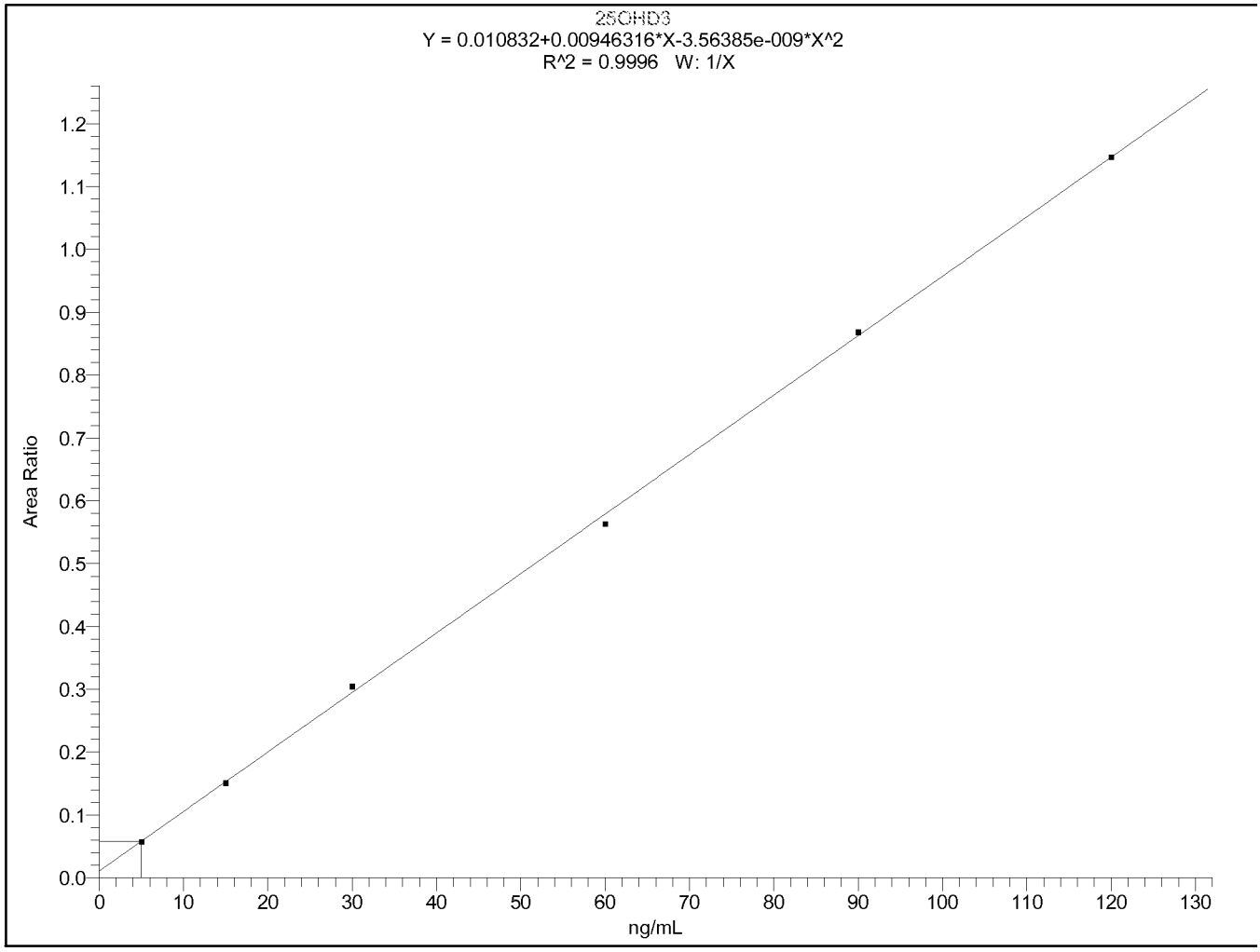

25OHD3
Y = 0.010832+0.00946316*X-3.56385e-009*X^2
R^2 = 0.9996 W: 1/X
Area Ratio
ng/mL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
0
10
20
30
40
50
60
70
80
90
100
110
120
130

Figure 3A

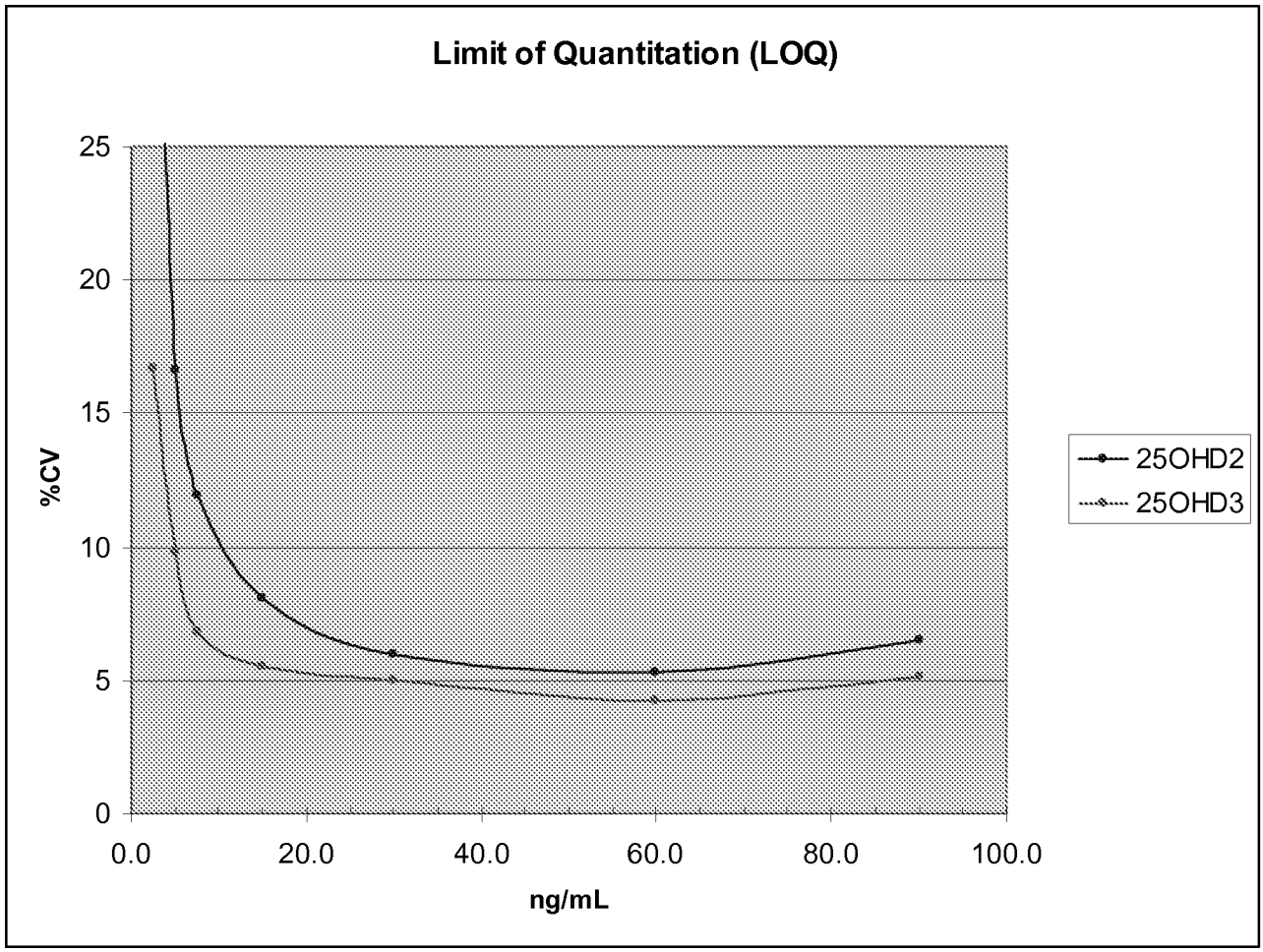

Limit of Quantitation (LOQ)
25
20
15
10
5
0
%CV
0.0
20.0
40.0
60.0
80.0
100.0
ng/mL
25OHD2
25OHD3

Figure 3B

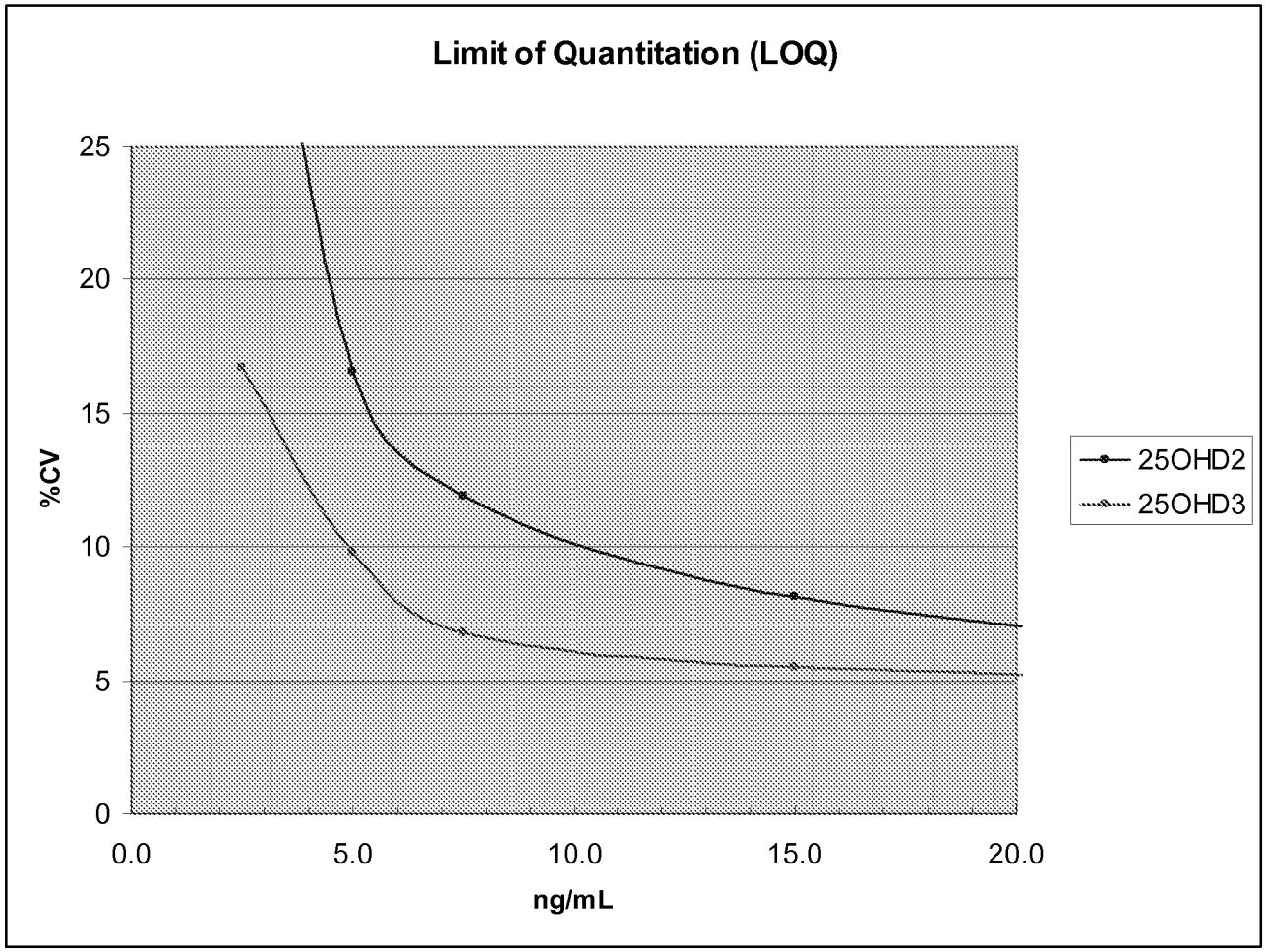

Limit of Quantitation (LOQ)
25
20
15
10
5
0
%CV
0.0
5.0
10.0
15.0
20.0
ng/mL
25OHD2
25OHD3

Figure 4A

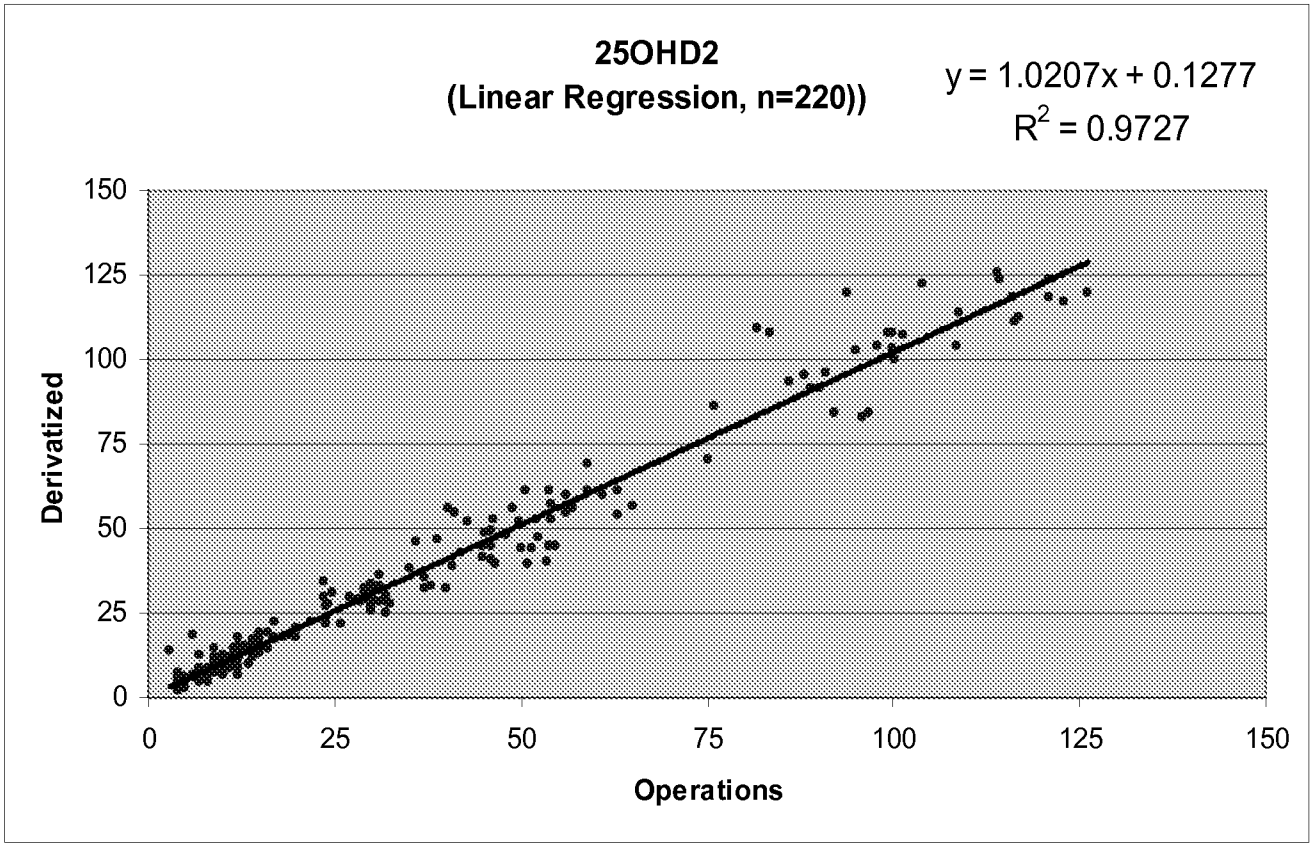

25OHD2
(Linear Regression, n=220))
y = 1.0207x + 0.1277
R2 = 0.9727
Derivatized
Operations
0
25
50
75
100
125
150

Figure 4B

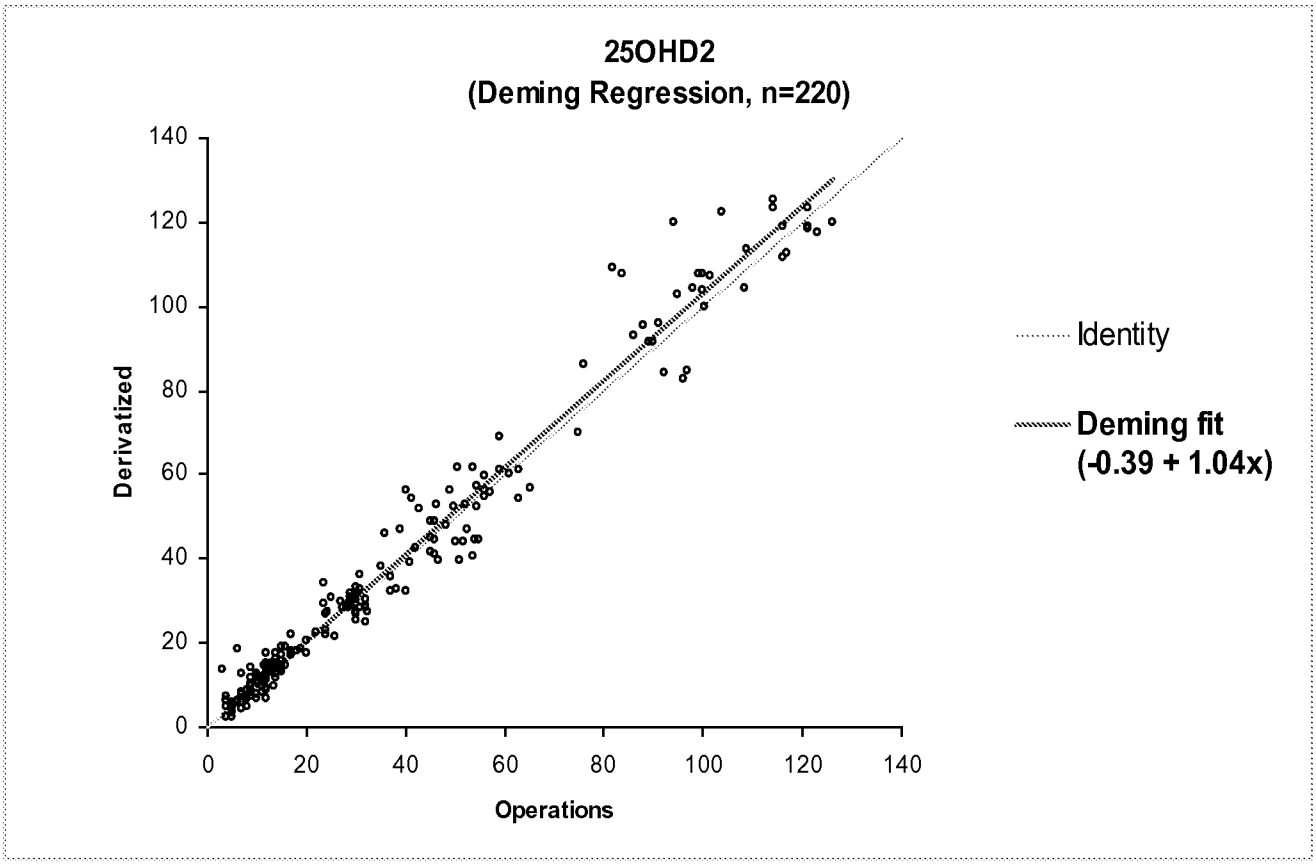

25OHD2
(Deming Regression, n=220)
Derivatized
Operations
0
20
40
60
80
100
120
140
Identity
Deming fit
(-0.39 + 1.04x)

Figure 5A

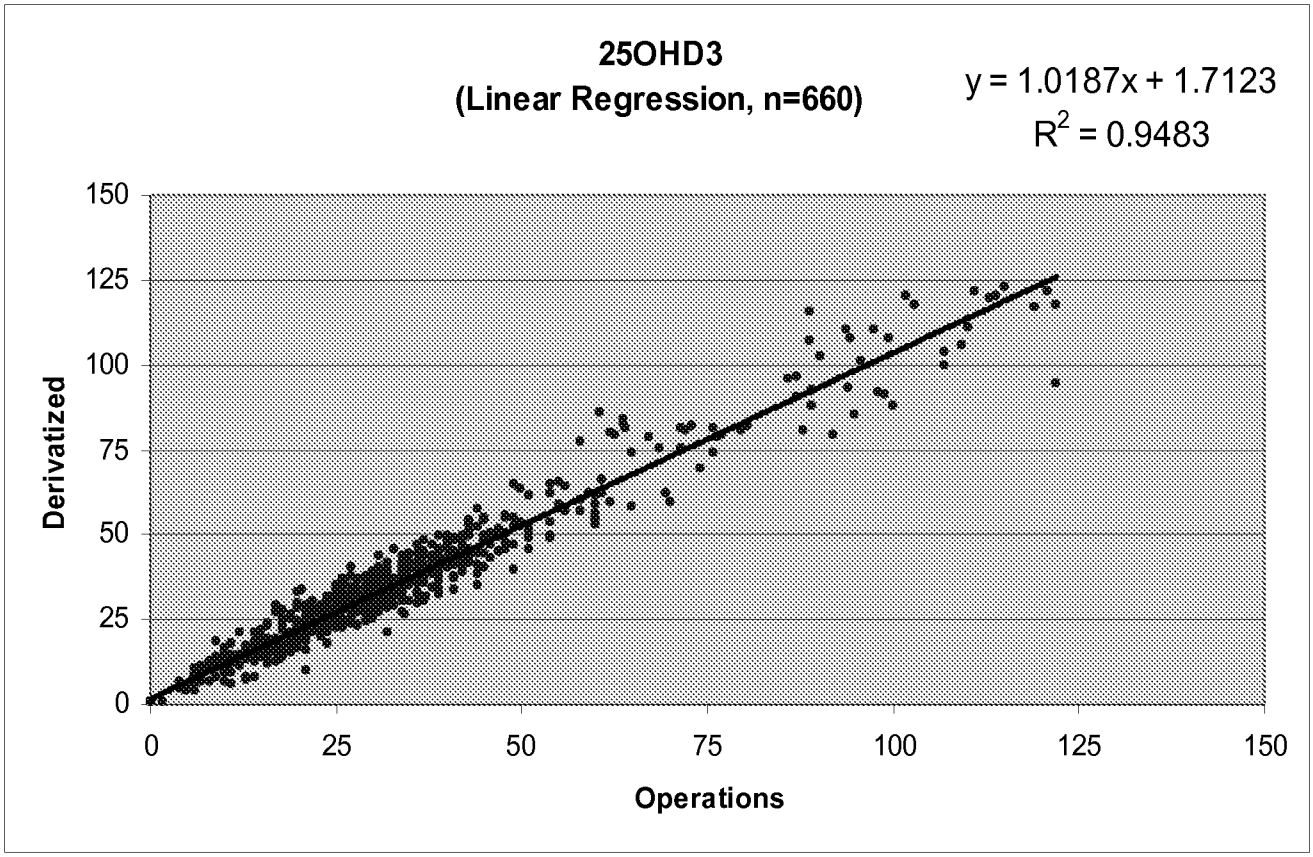

25OHD3
(Linear Regression, n=660)
y = 1.0187x + 1.7123
R2 = 0.9483
Derivatized
Operations
0
25
50
75
100
125
150

Figure 5B

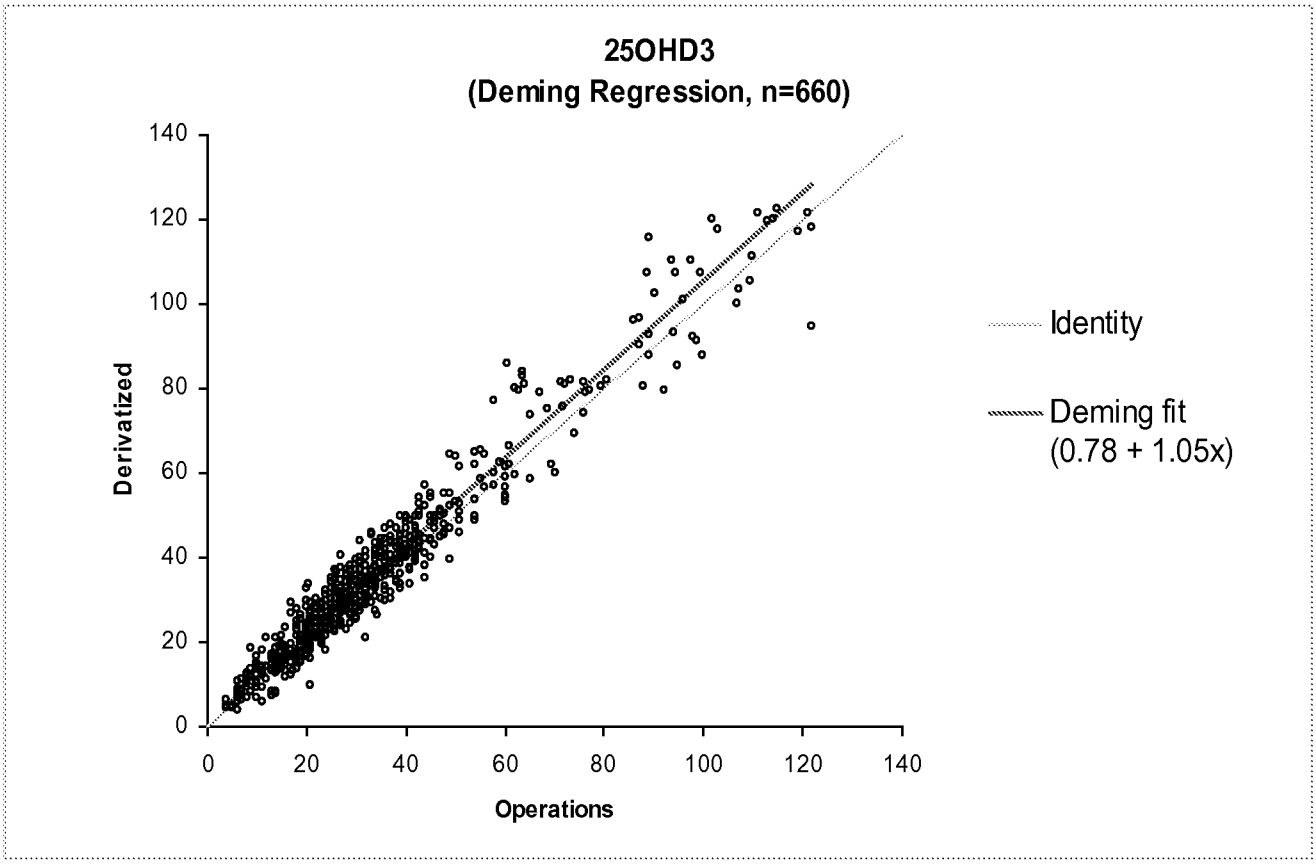

25OHD3
(Deming Regression, n=660)
Derivatized
Operations
0
20
40
60
80
100
120
140
Identity
Deming fit
(0.78 + 1.05x)

Figure 6A

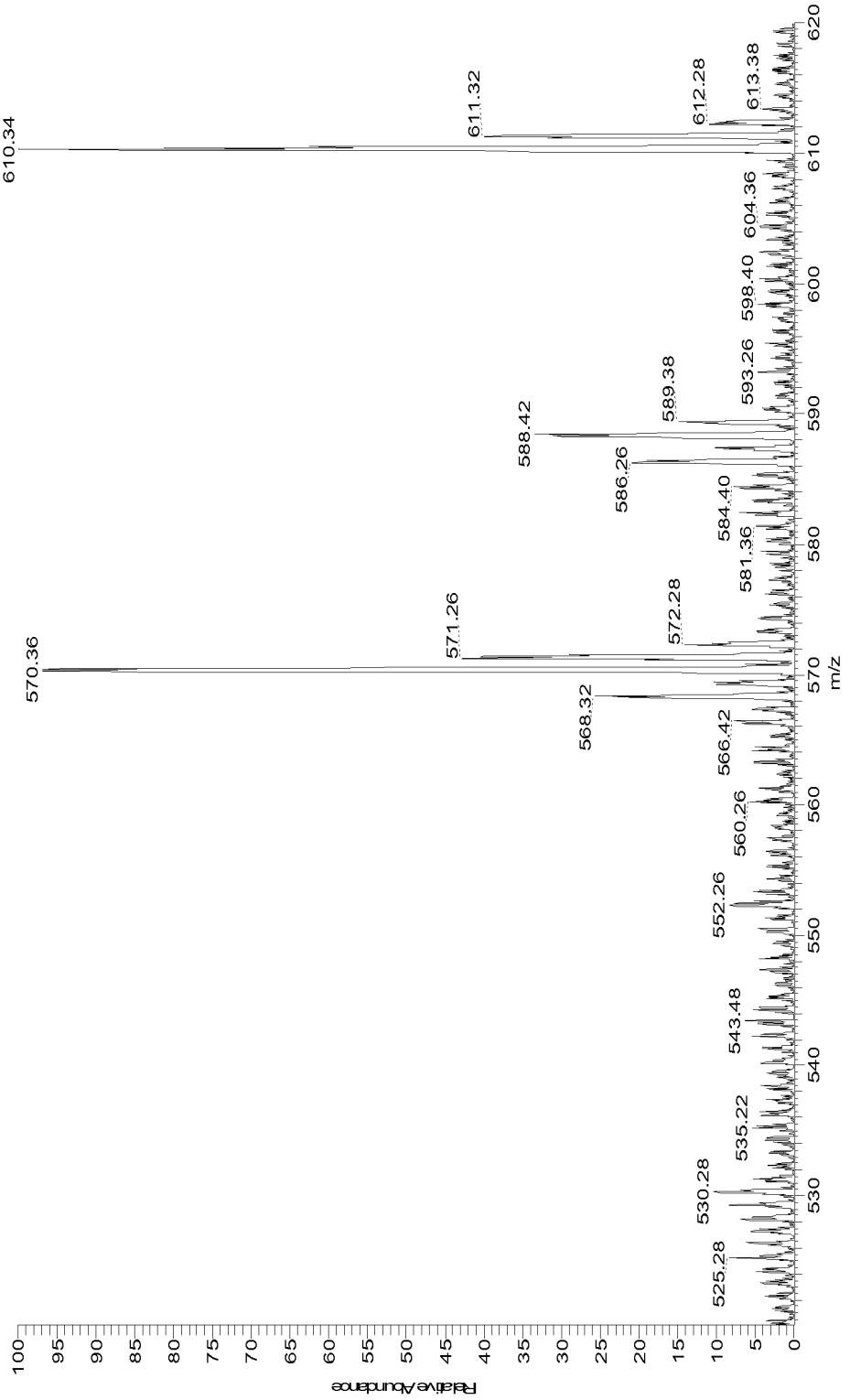

Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
530
540
550
560
570
580
590
600
610
620
525.28
530.28
535.22
543.48
552.26
560.26
566.42
568.32
570.36
571.26
572.28
581.36
584.40
586.26
588.42
589.38
593.26
598.40
604.36
610.34
611.32
612.28
613.38

Figure 6B

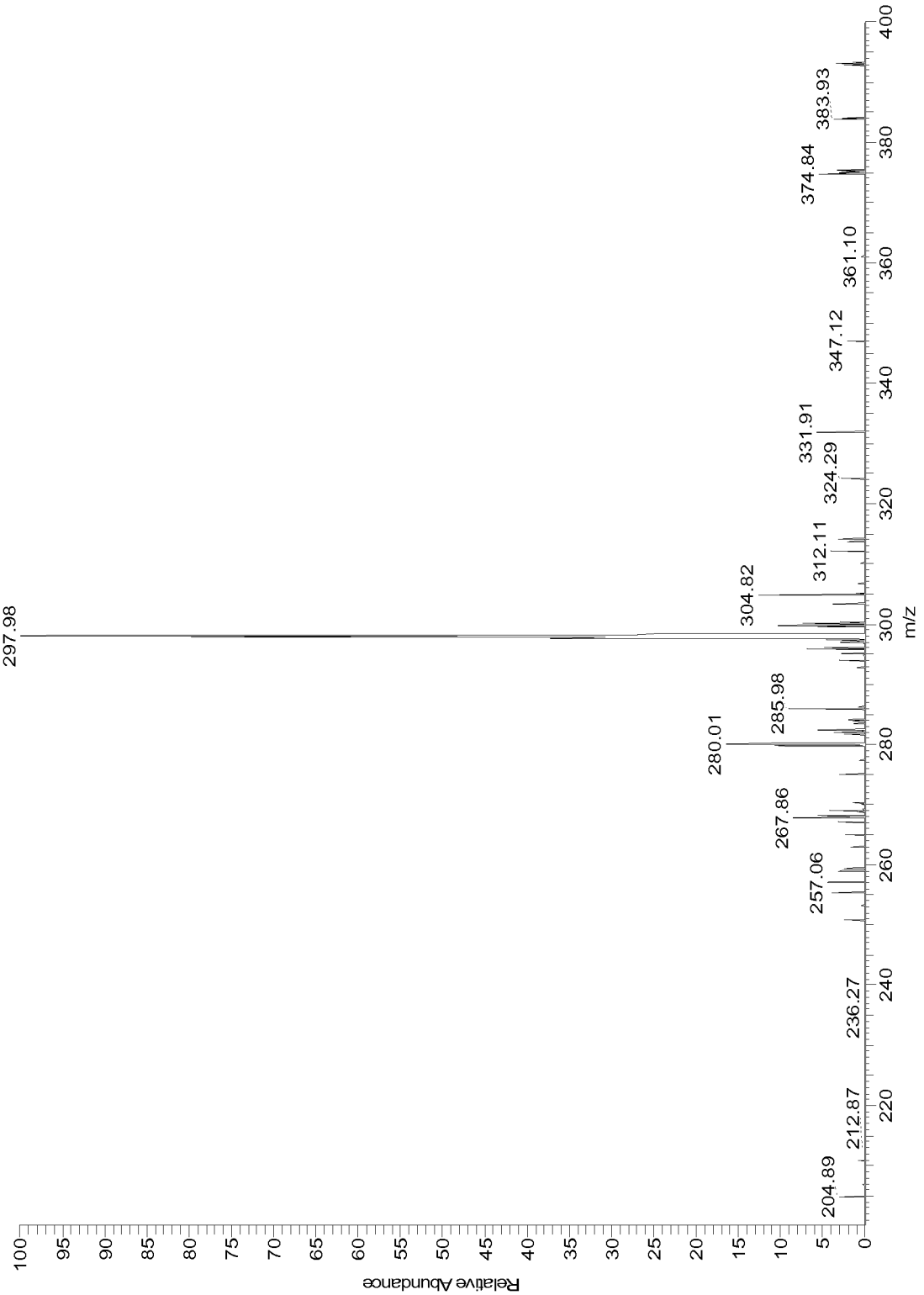

Relative Abundance
m/z
297.98
304.82
312.11
324.29
331.91
347.12
361.10
374.84
383.93
280.01
285.98
267.86
257.06
204.89
212.87
236.27

Figure 7A

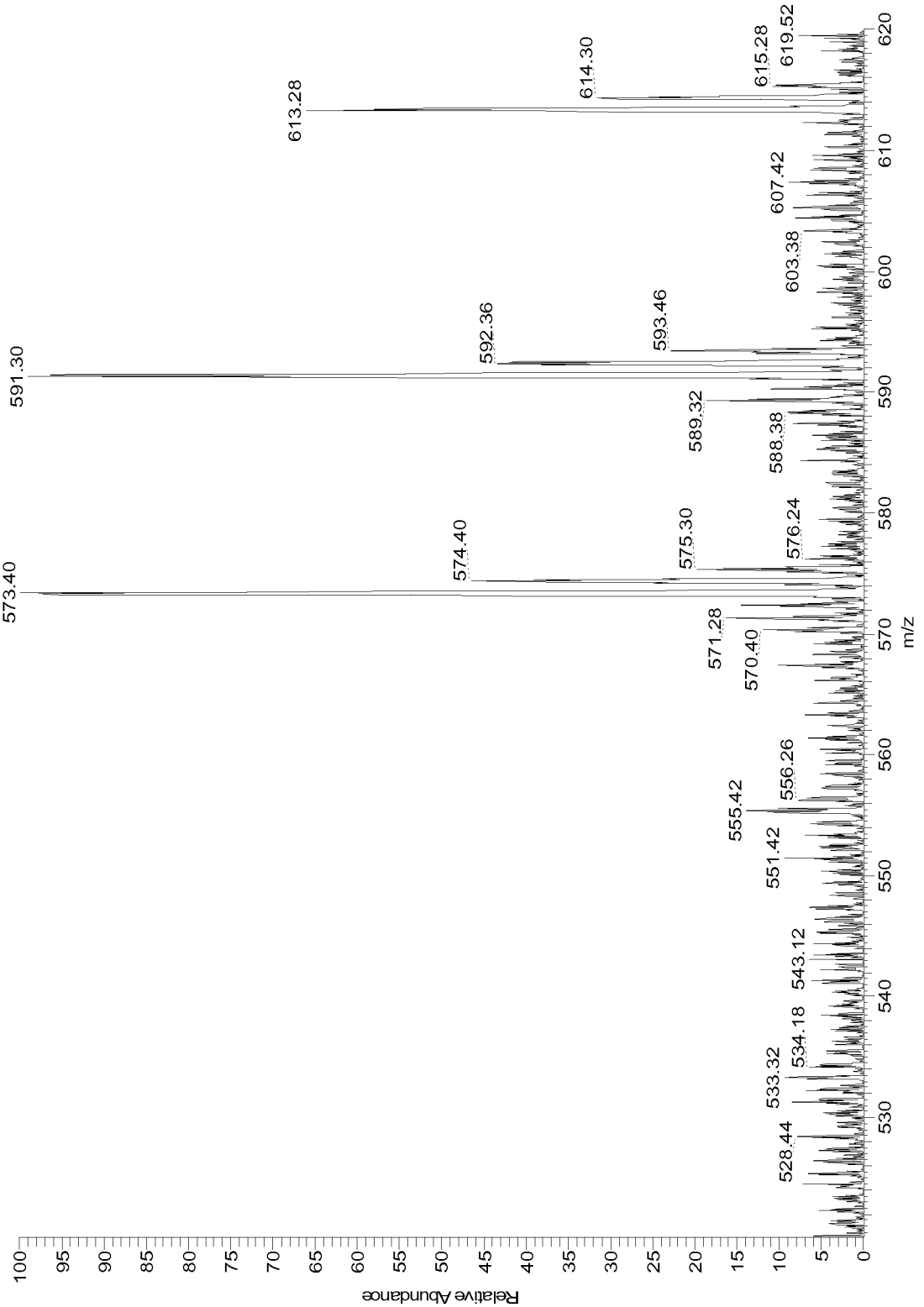

Relative Abundance
m/z
573.40
591.30
613.28
574.40
592.36
614.30
575.30
593.46
615.28
576.24
619.52
571.28
589.32
607.42
570.40
588.38
603.38
555.42
556.26
551.42
543.12
534.18
533.32
528.44

Figure 7B

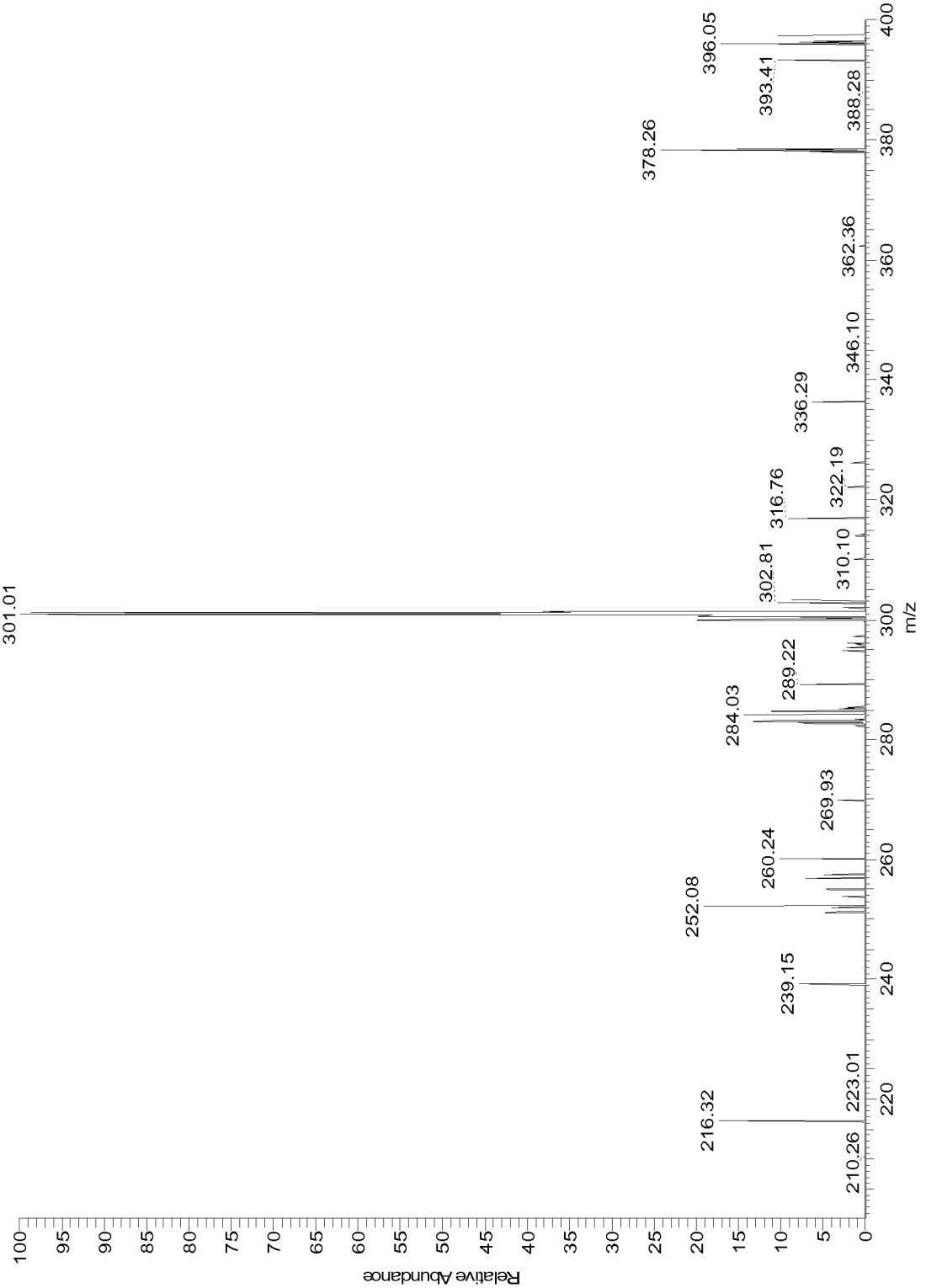

Relative Abundance
m/z
301.01
378.26
396.05
393.41
388.28
362.36
346.10
336.29
322.19
316.76
310.10
302.81
289.22
284.03
269.93
260.24
252.08
239.15
223.01
216.32
210.26

Figure 8A

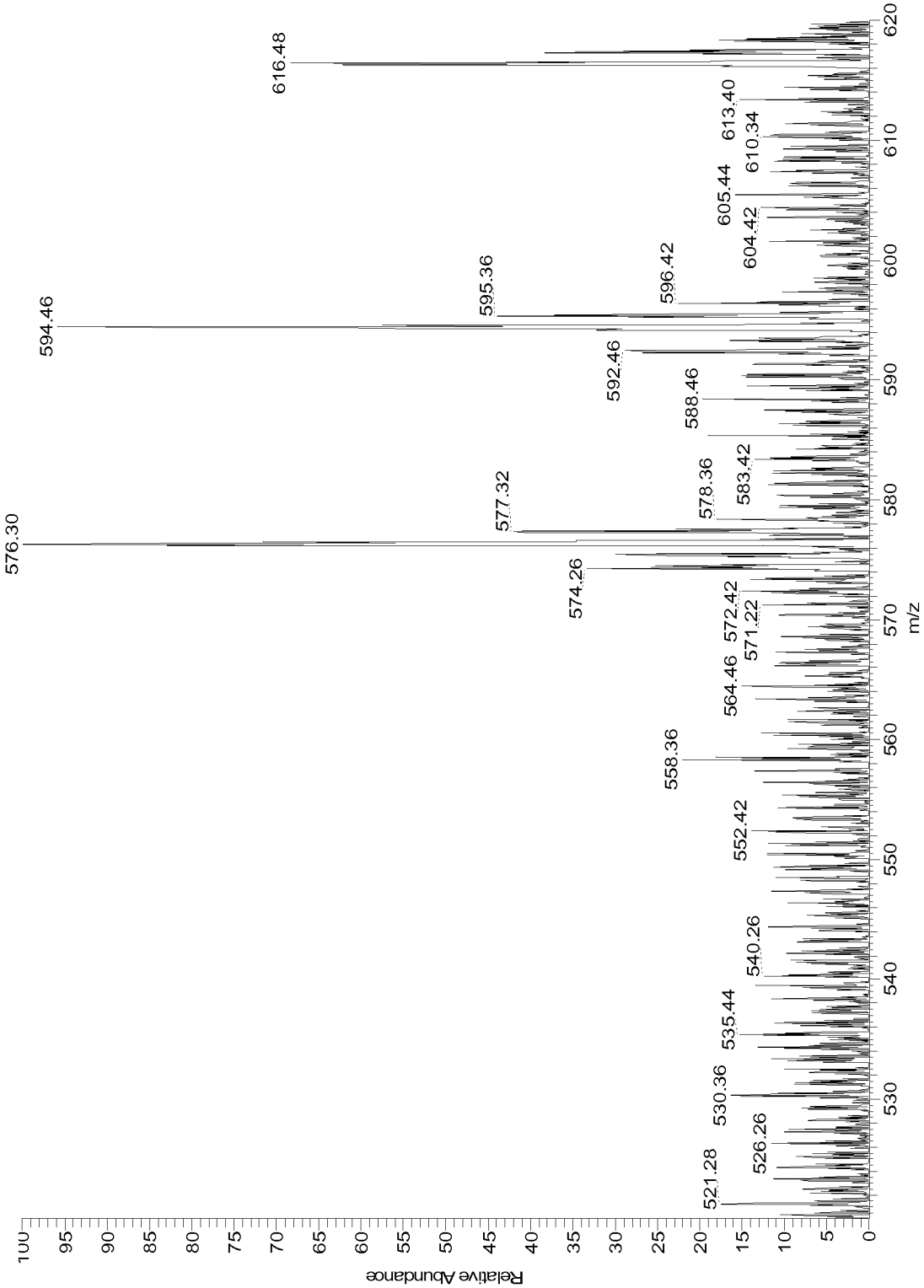

Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
530
540
550
560
570
580
590
600
610
620
521.28
526.26
530.36
535.44
540.26
552.42
558.36
564.46
571.22
572.42
574.26
576.30
577.32
578.36
583.42
588.46
592.46
594.46
595.36
596.42
604.42
605.44
610.34
613.40
616.48

Figure 8B

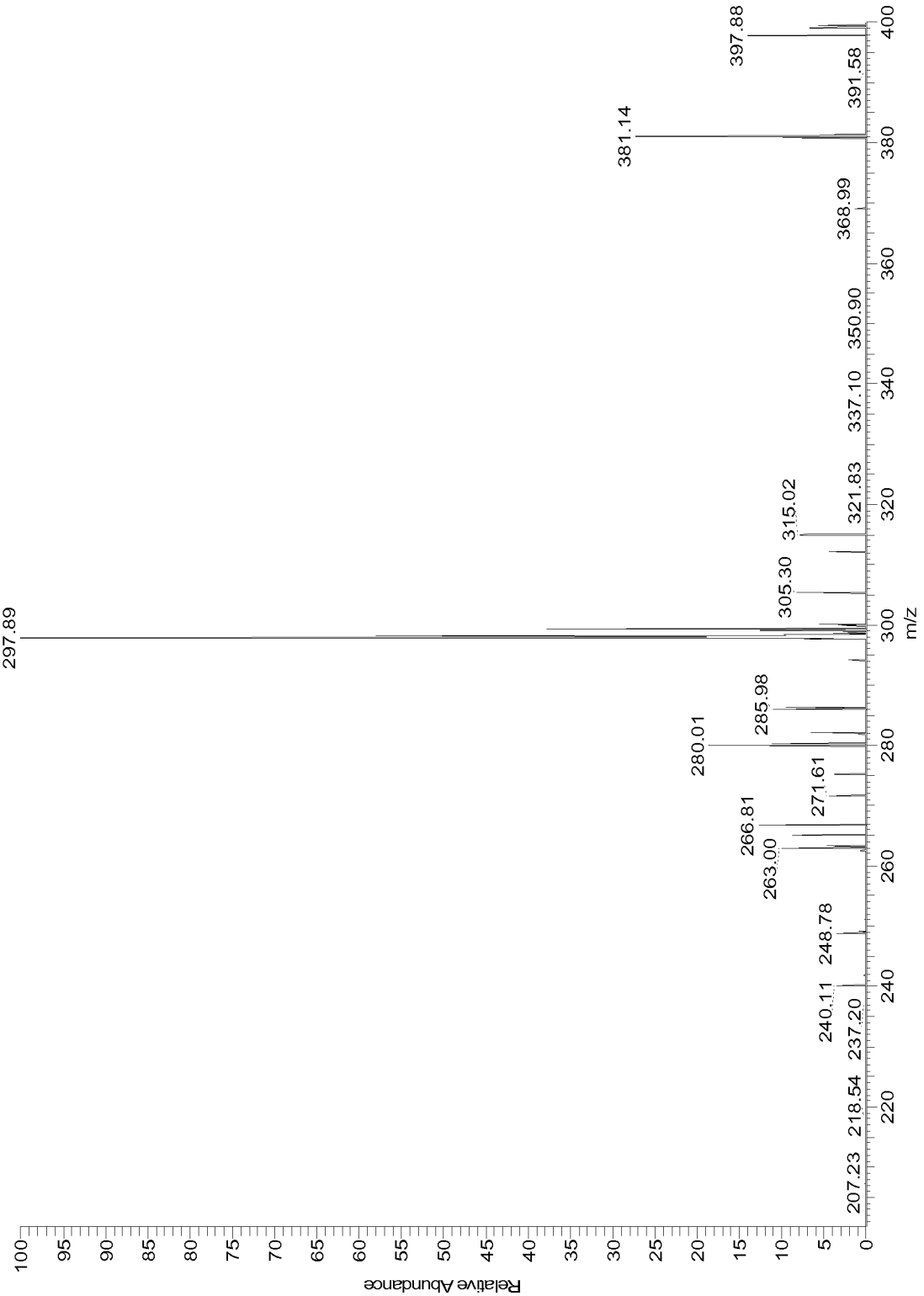

Relative Abundance
m/z
297.89
381.14
397.88
280.01
285.98
305.30
315.02
263.00
266.81
271.61
240.11
248.78
207.23
218.54
237.20
321.83
337.10
350.90
368.99
391.58

Figure 9A

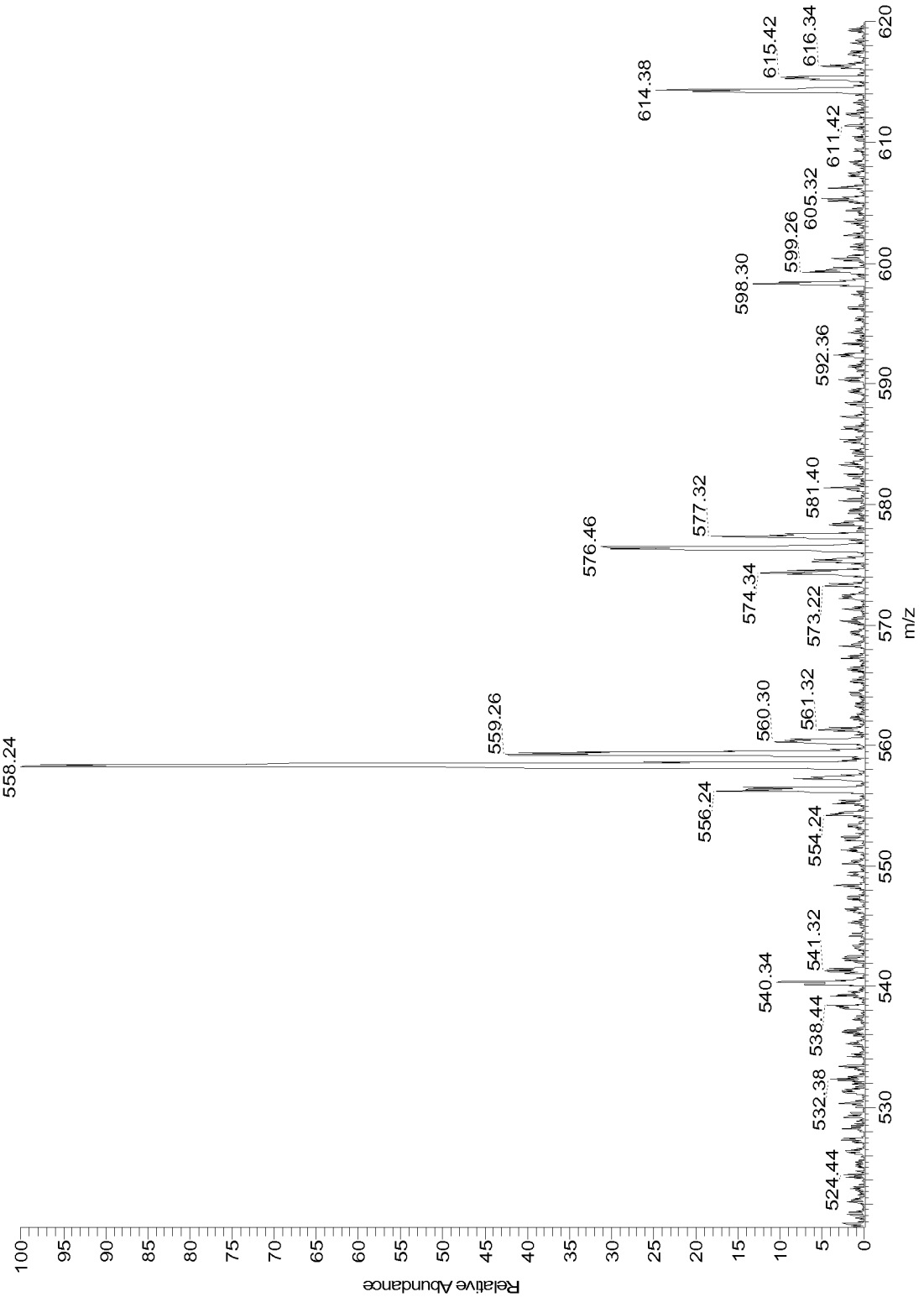

Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
530
540
550
560
570
580
590
600
610
620
524.44
532.38
538.44
540.34
541.32
554.24
556.24
558.24
559.26
560.30
561.32
573.22
574.34
576.46
577.32
581.40
592.36
598.30
599.26
605.32
611.42
614.38
615.42
616.34

Figure 9B

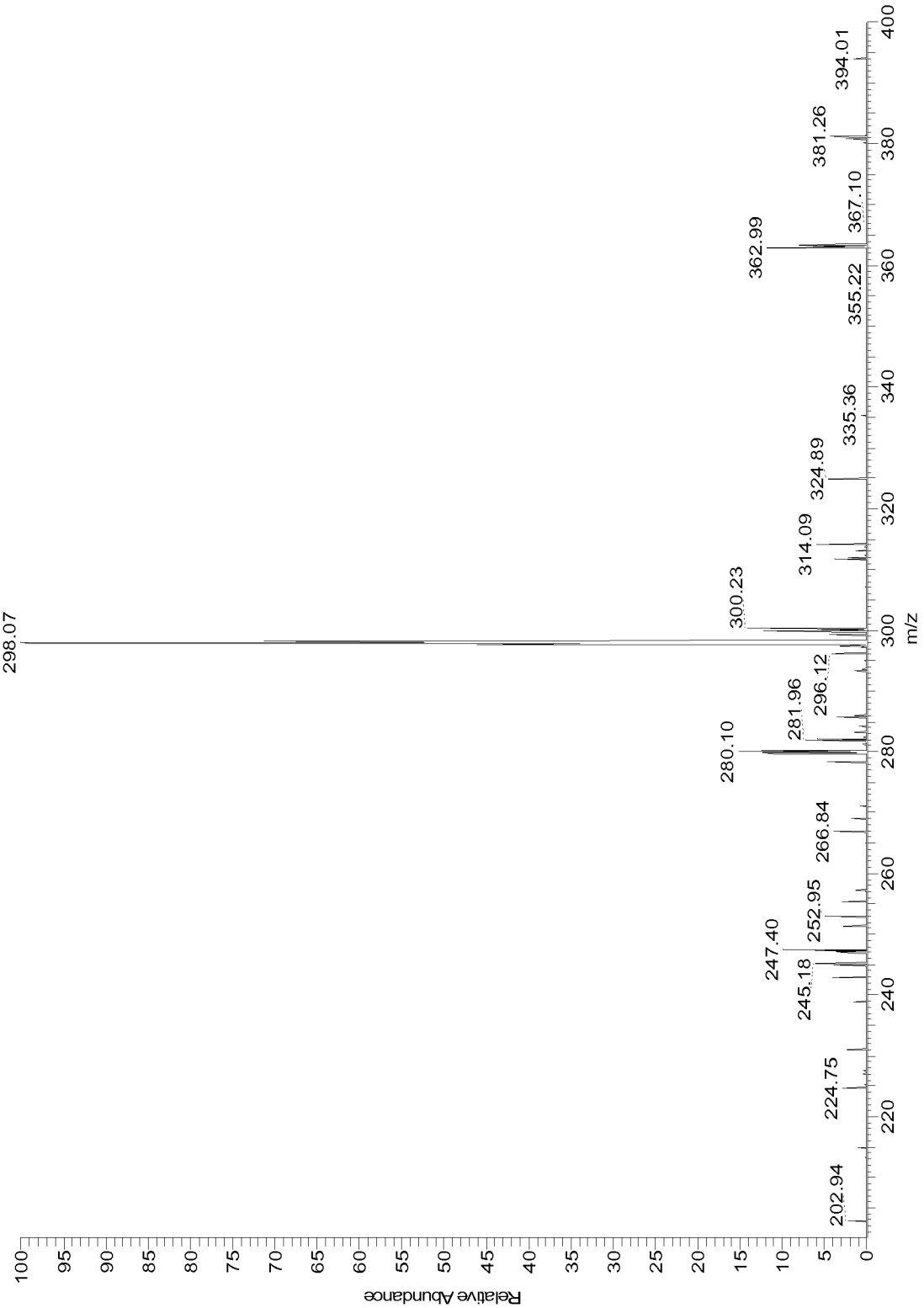

Relative Abundance
m/z
298.07
300.23
314.09
324.89
335.36
355.22
362.99
367.10
381.26
394.01
280.10
281.96
296.12
247.40
245.18
252.95
266.84
224.75
202.94

Figure 10A

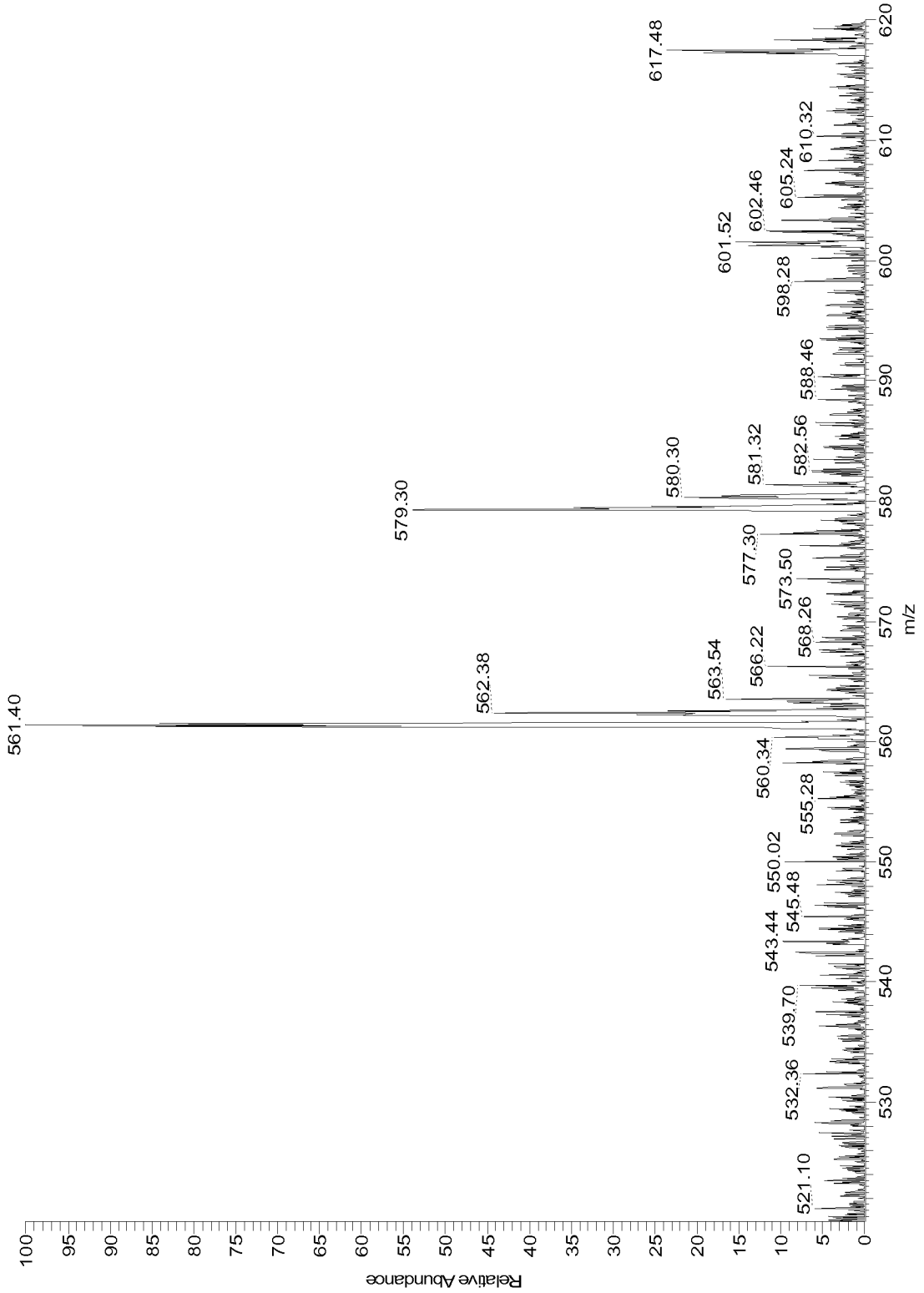

Relative Abundance
m/z
561.40
562.38
563.54
566.22
568.26
573.50
577.30
579.30
580.30
581.32
582.56
588.46
598.28
601.52
602.46
605.24
610.32
617.48
521.10
532.36
539.70
543.44
545.48
550.02
555.28
560.34

Figure 10B

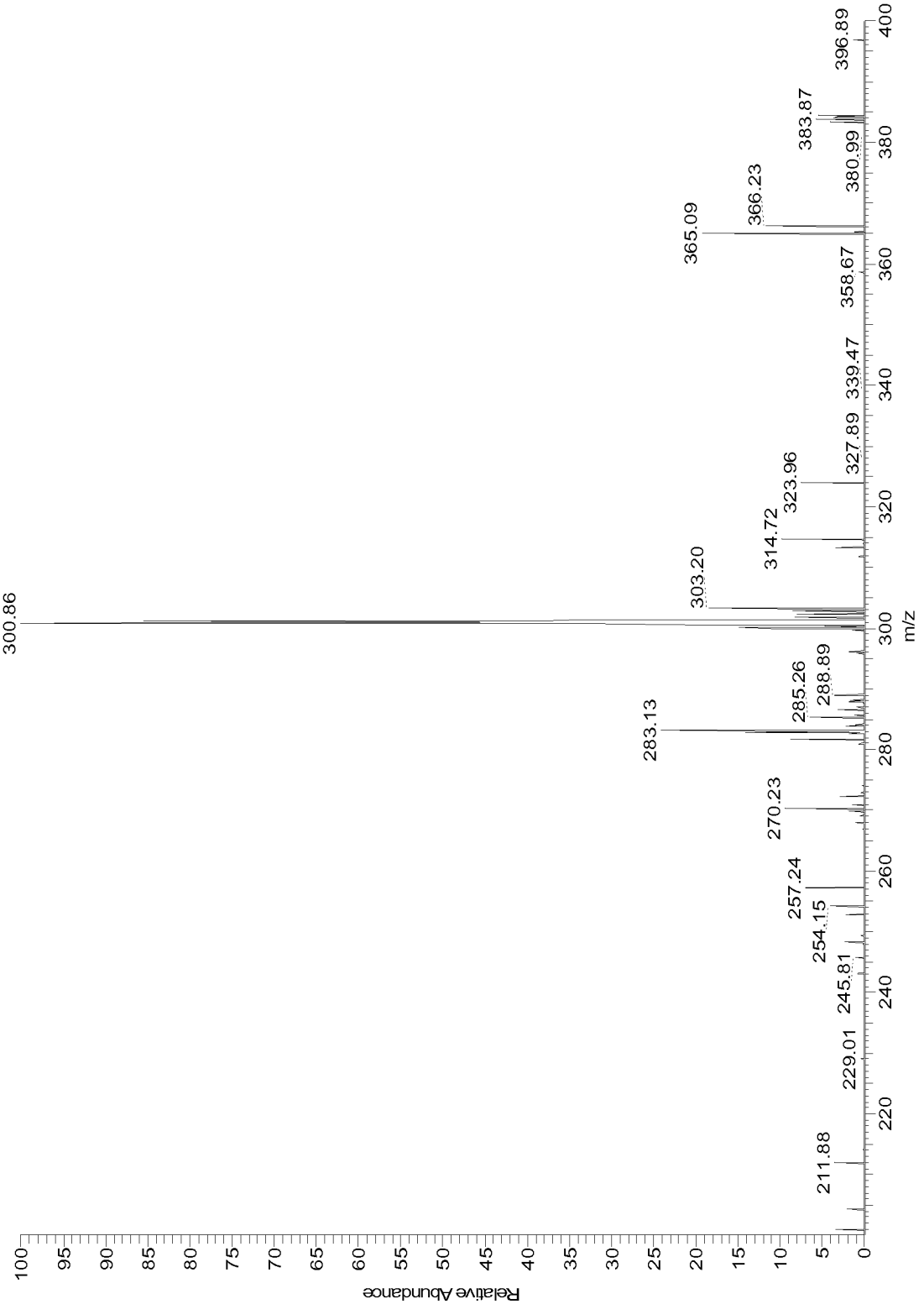

Relative Abundance
m/z
300.86
303.20
314.72
323.96
327.89
339.47
358.67
365.09
366.23
380.99
383.87
396.89
283.13
285.26
288.89
270.23
257.24
254.15
245.81
229.01
211.88

Figure 11A

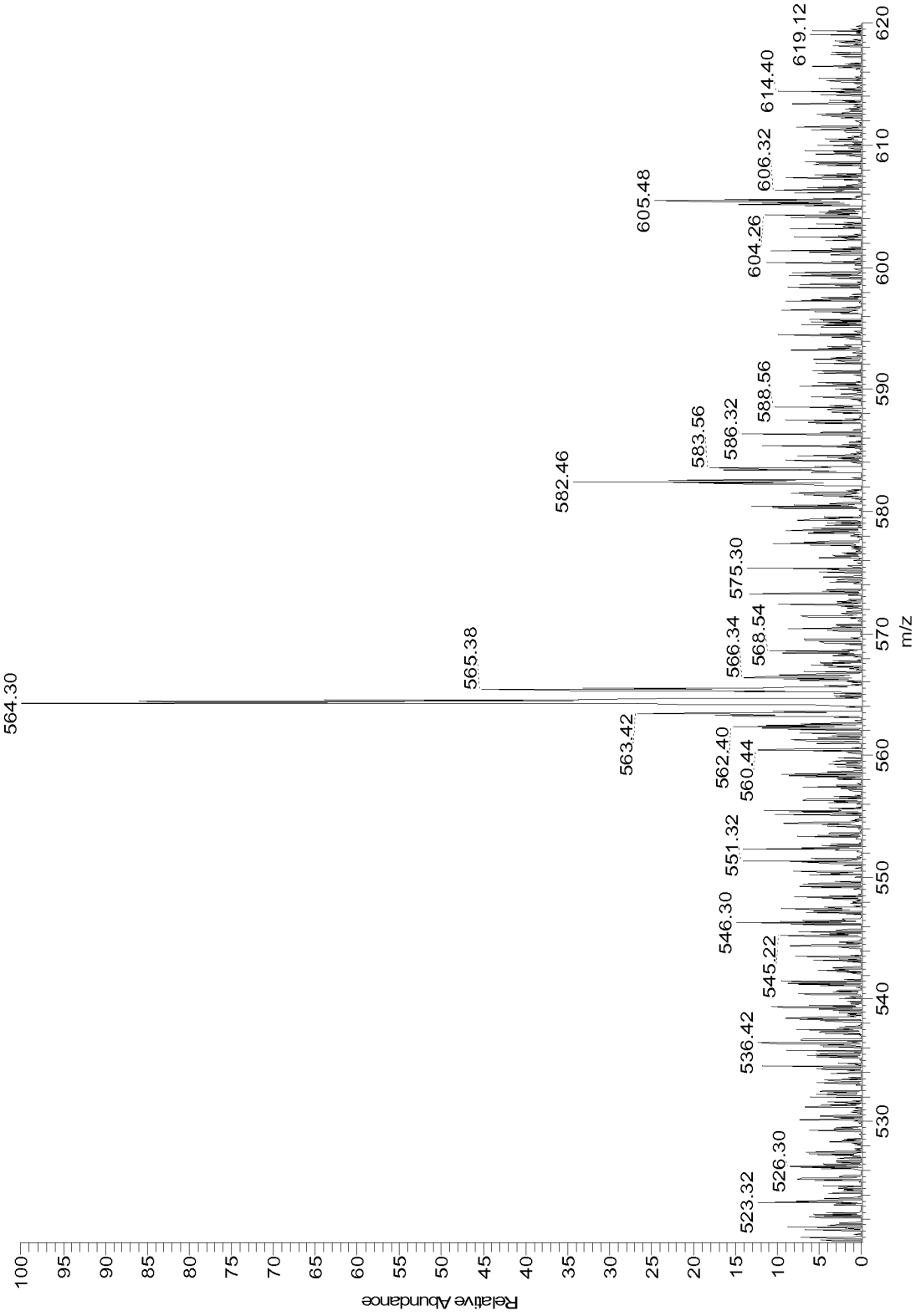

Relative Abundance
m/z
564.30
565.38
563.42
582.46
605.48
523.32
526.30
536.42
545.22
546.30
551.32
560.44
562.40
566.34
568.54
575.30
583.56
586.32
588.56
604.26
606.32
614.40
619.12

Figure 11B

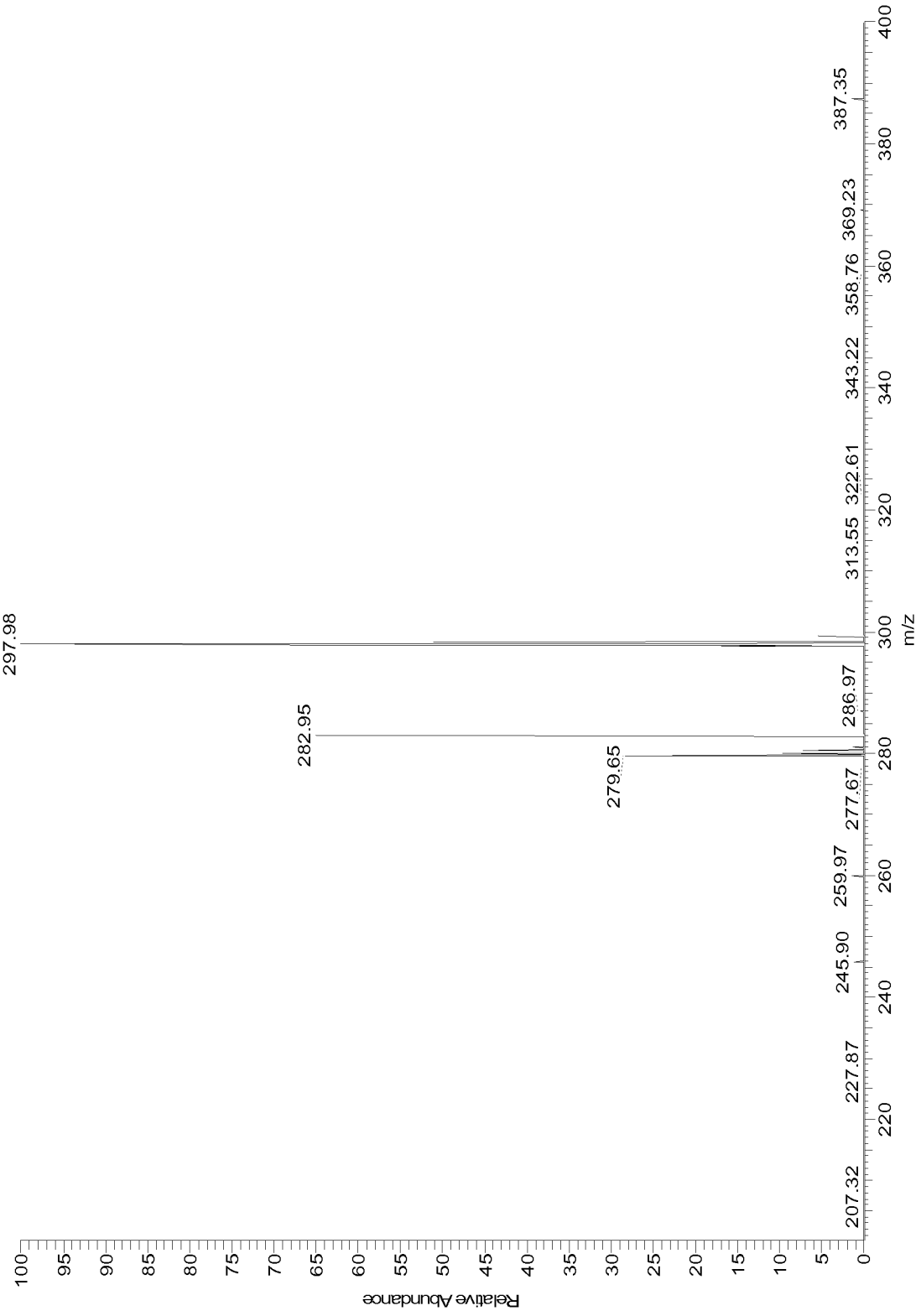

297.98
282.95
279.65
286.97
313.55
322.61
343.22
358.76
369.23
387.35
207.32
227.87
245.90
259.97
277.67
m/z
Relative Abundance

Figure 12A

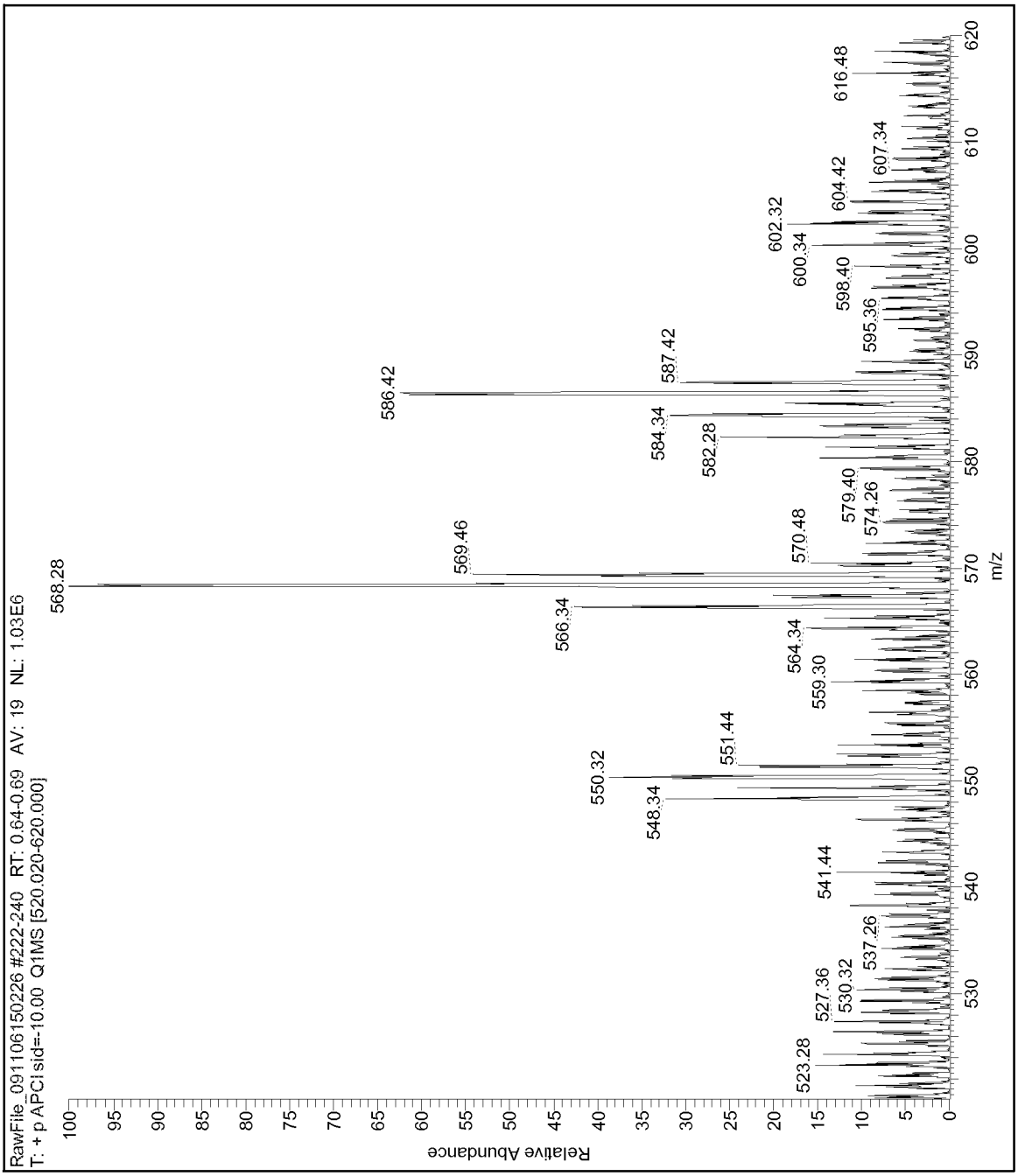

RawFile_09110615022 6 #222-240 RT: 0.64-0.69 AV: 19 NL: 1.03E6
T: + p APCI sid=-10.00 Q1MS [520.020-620.000]
Relative Abundance
m/z
568.28
569.46
566.34
586.42
587.42
584.34
582.28
570.48
579.40
574.26
564.34
559.30
550.32
551.44
548.34
541.44
537.26
530.32
527.36
523.28
595.36
598.40
600.34
602.32
604.42
607.34
616.48

Figure 12B

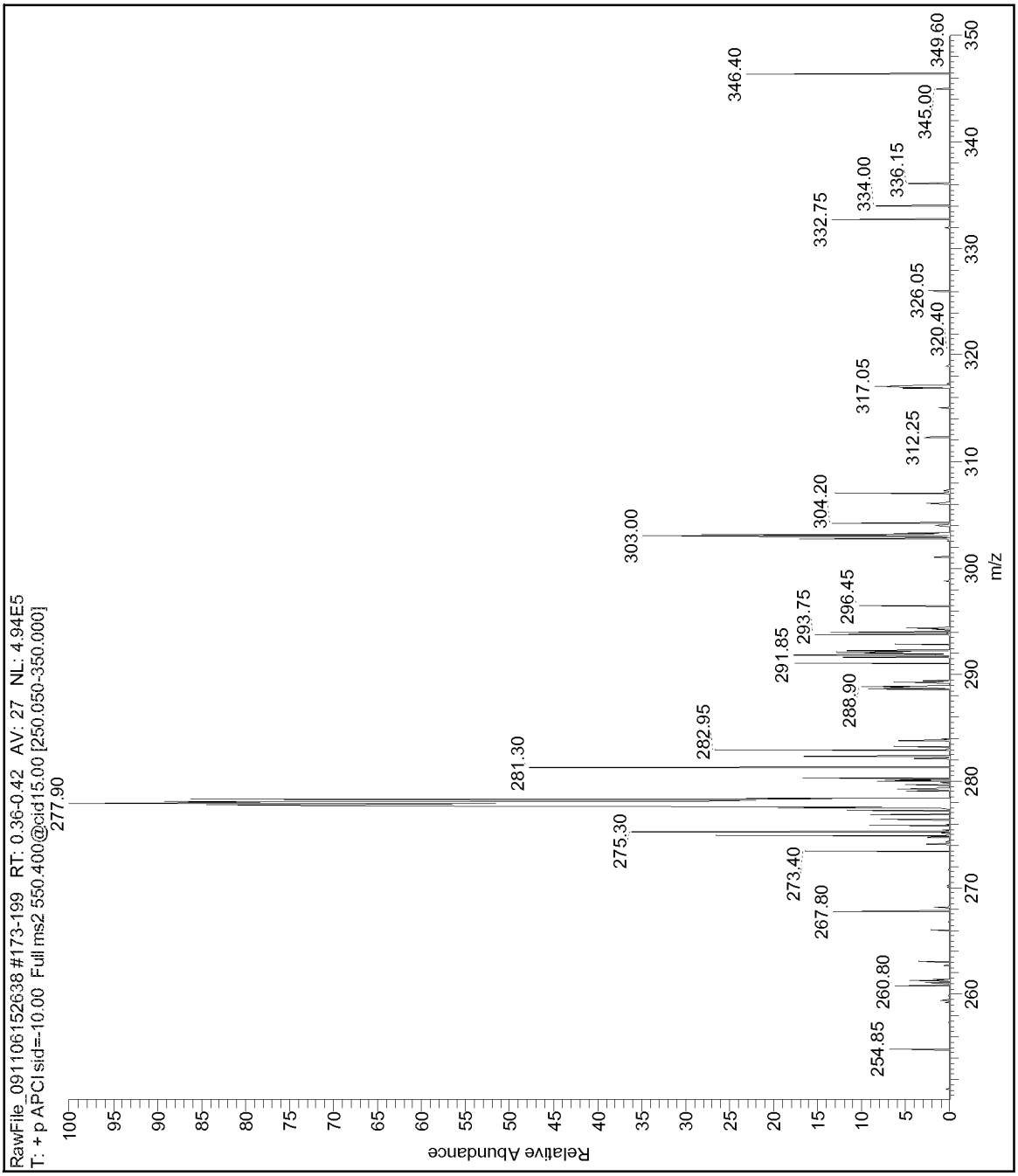

RawFile_091106152638 #173-199 RT: 0.36-0.42 AV: 27 NL: 4.94E5
T: + p APCI sid=-10.00 Full ms2 550.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
277.90
281.30
275.30
282.95
303.00
346.40
254.85
260.80
267.80
273.40
288.90
291.85
293.75
296.45
304.20
312.25
317.05
320.40
326.05
332.75
334.00
336.15
345.00
349.60

Figure 12C

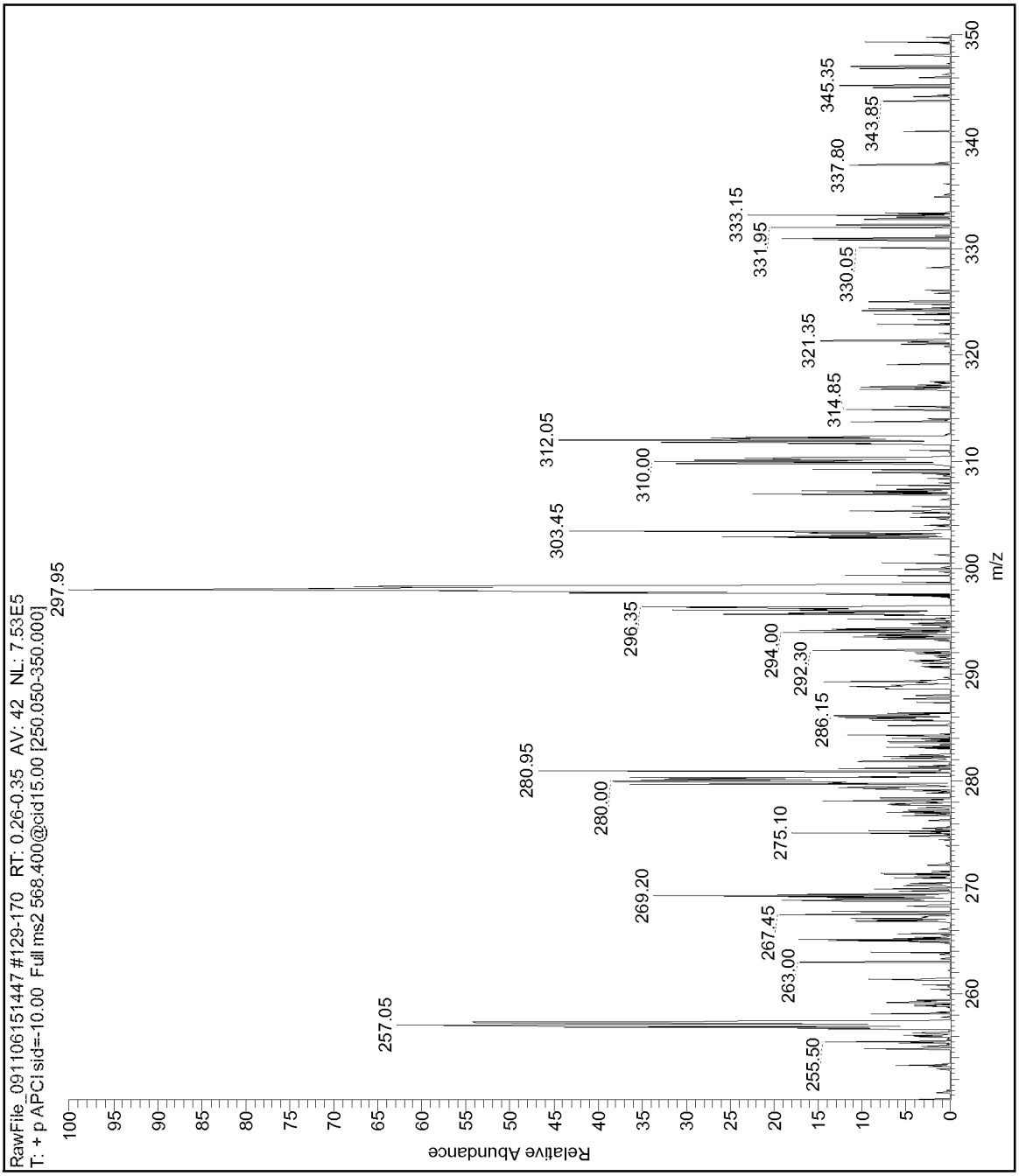

RawFile_091106151447 #129-170 RT: 0.26-0.35 AV: 42 NL: 7.53E5
T: + p APCI sid=-10.00 Full ms2 568.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
297.95
257.05
312.05
303.45
310.00
280.95
280.00
296.35
269.20
333.15
331.95
345.35
337.80
343.85
321.35
314.85
330.05
294.00
292.30
286.15
275.10
267.45
263.00
255.50

Figure 12D

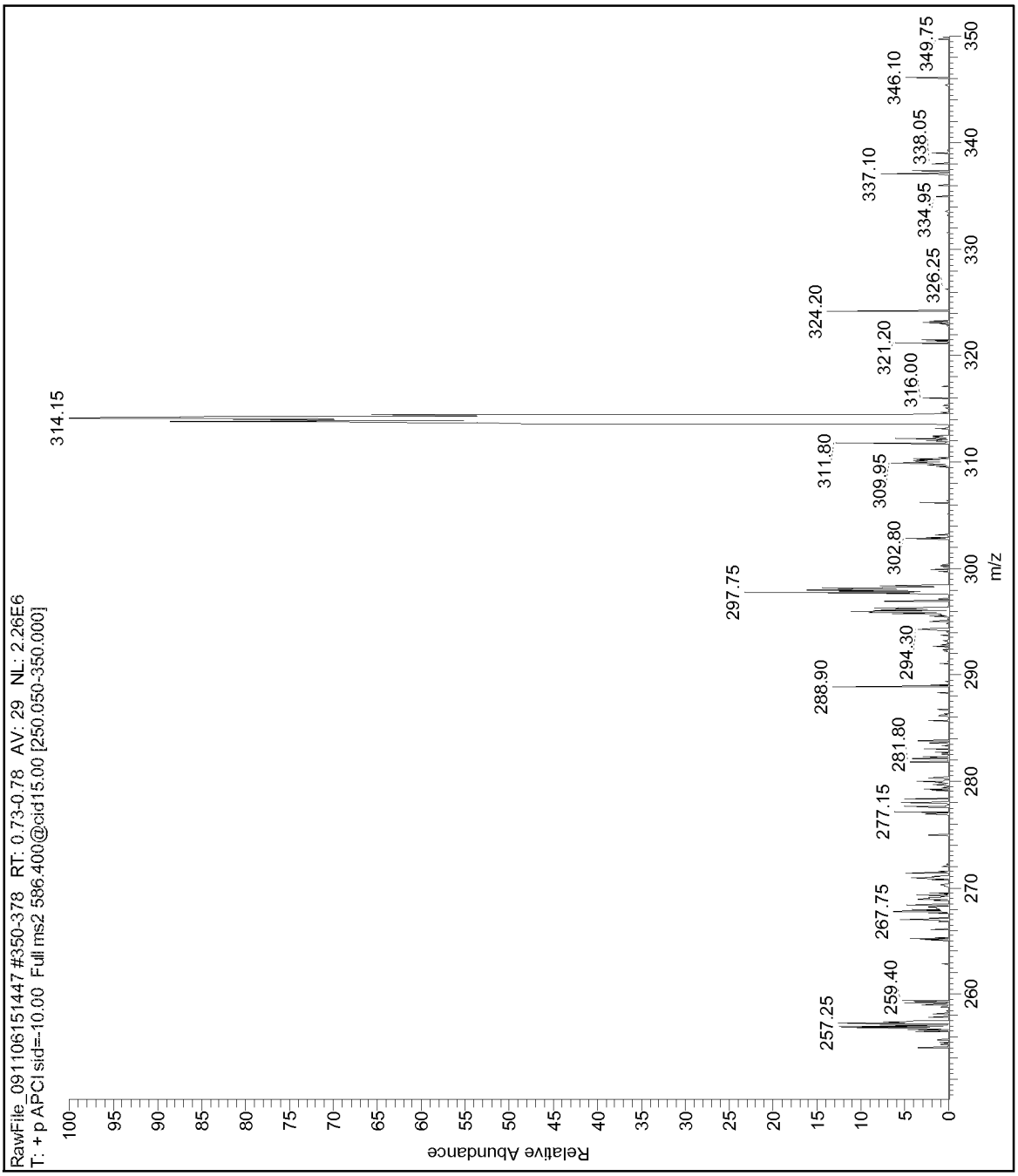

RawFile_091106151447 #350-378 RT: 0.73-0.78 AV: 29 NL: 2.26E6
T: + p APCI sid=-10.00 Full ms2 586.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
314.15
297.75
288.90
257.25
324.20
337.10
346.10
349.75

Figure 13A

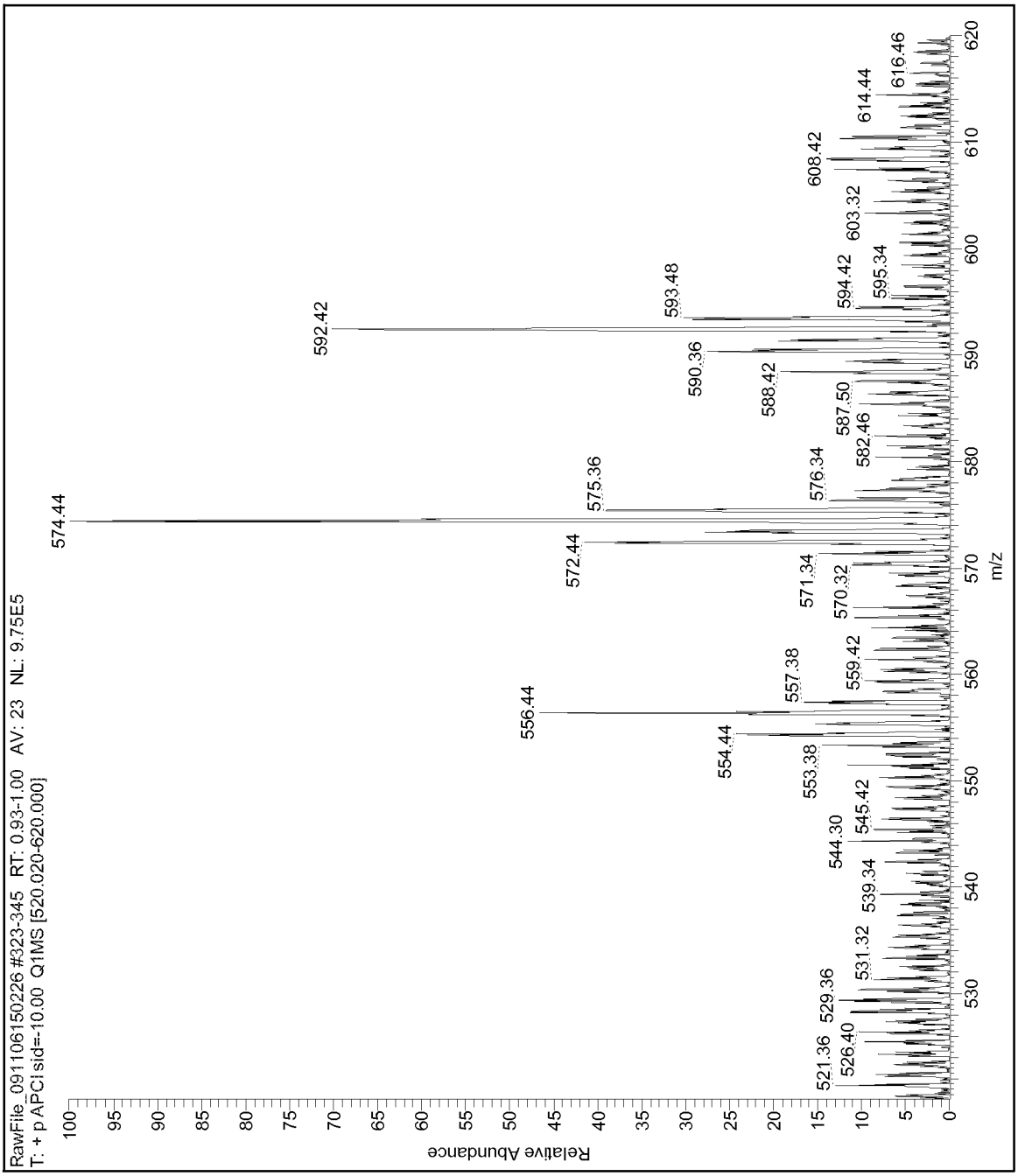

RawFile_09110615022 6 #323-345 RT: 0.93-1.00 AV: 23 NL: 9.75E5
T: + p APCI sid=-10.00 Q1MS [520.020-620.000]
Relative Abundance
m/z
574.44
592.42
556.44
572.44
575.36
593.48
554.44
590.36
608.42
614.44
616.46
588.42
603.32
594.42
595.34
587.50
582.46
576.34
571.34
570.32
559.42
557.38
553.38
544.30
545.42
539.34
531.32
529.36
526.40
521.36

Figure 13B

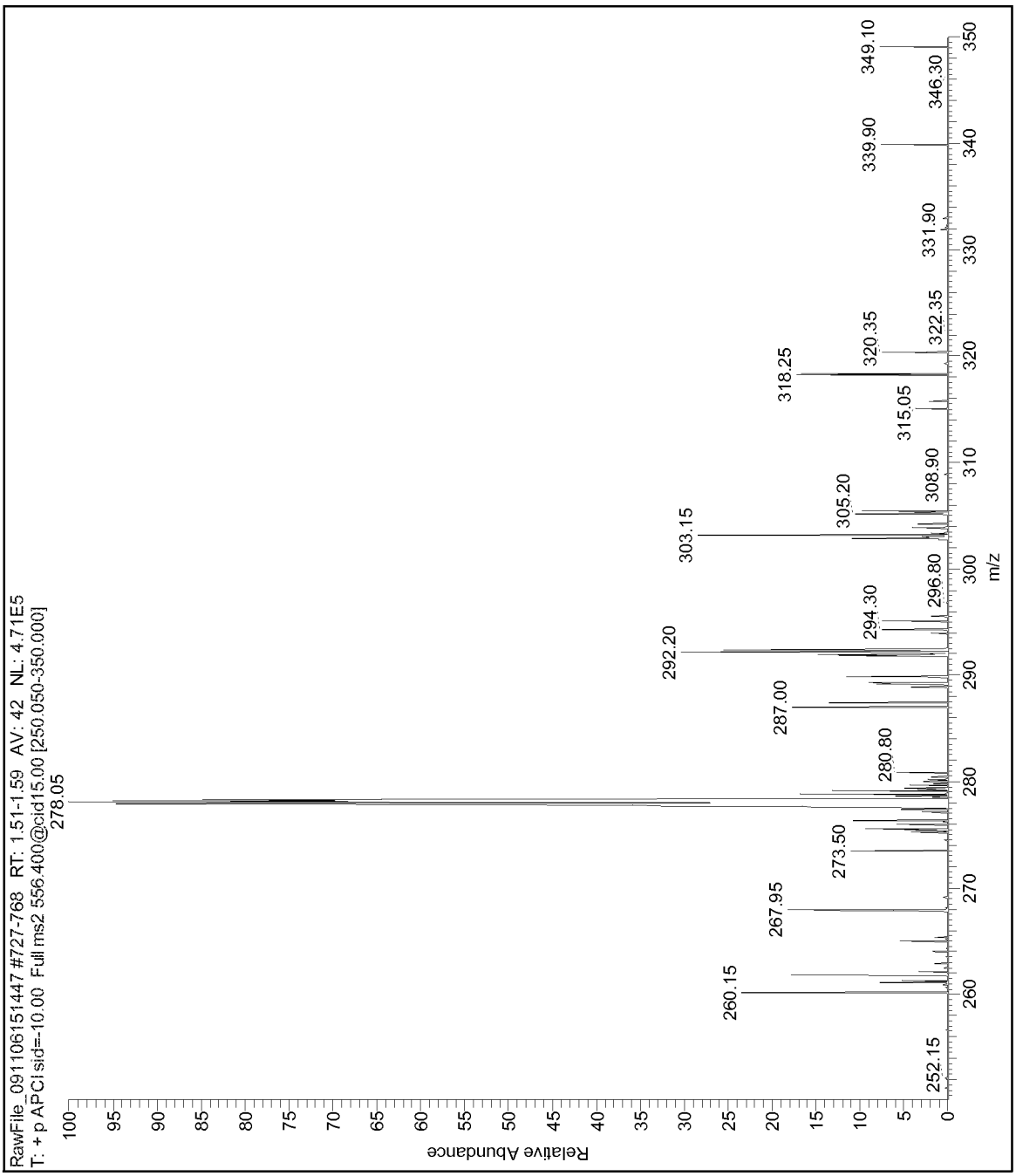

RawFile_091106151447 #727-768 RT: 1.51-1.59 AV: 42 NL: 4.71E5
T: + p APCI sid=-10.00 Full ms2 556.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
278.05
292.20
303.15
260.15
318.25
287.00
267.95
273.50
280.80
294.30
296.80
305.20
308.90
315.05
320.35
322.35
331.90
339.90
346.30
349.10
252.15

Figure 13C

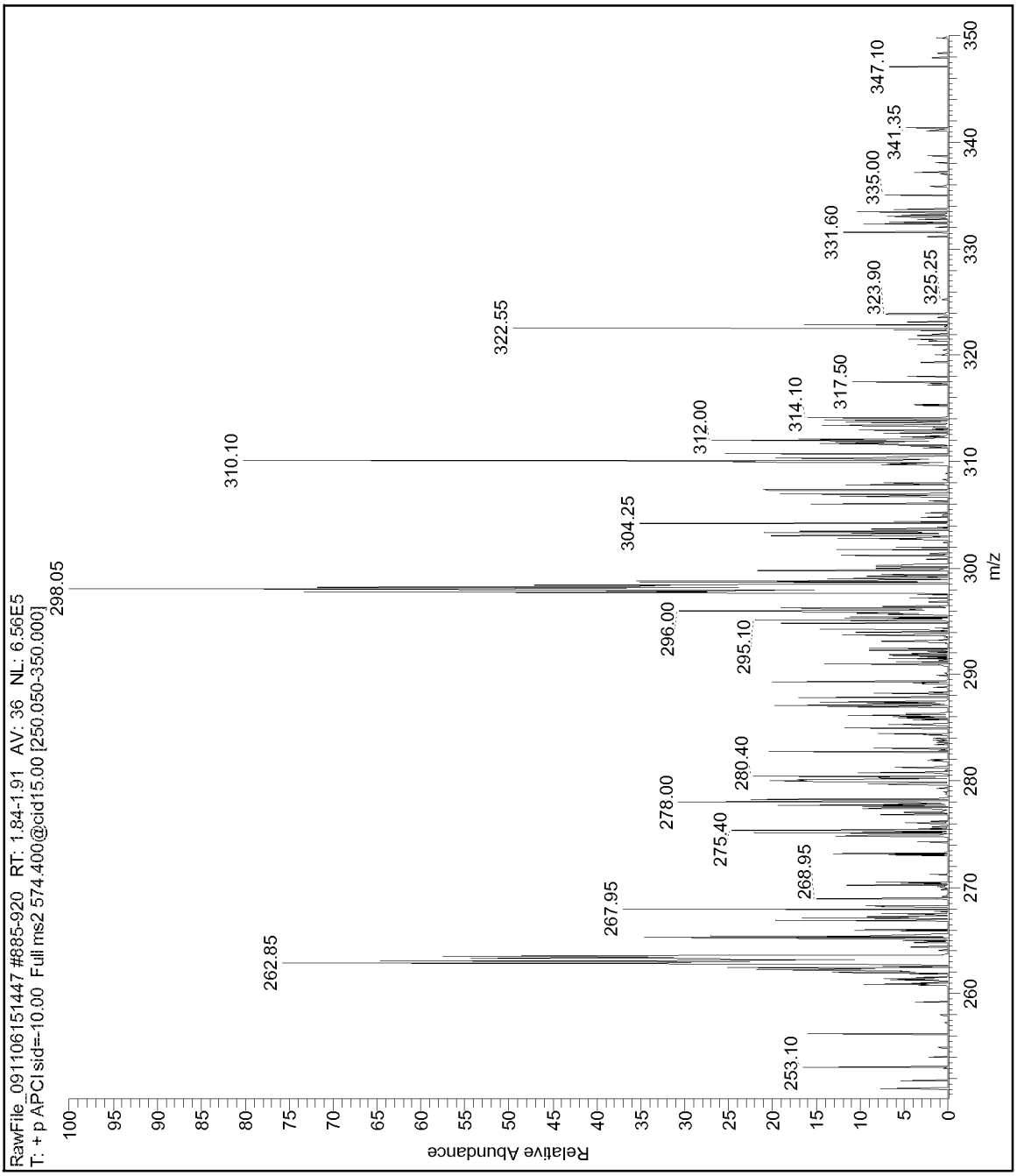

RawFile_09110615l447 #885-920 RT: 1.84-1.91 AV: 36 NL: 6.56E5
T: + p APCI sid=-10.00 Full ms2 574.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
298.05
310.10
262.85
322.55
304.25
312.00
314.10
317.50
331.60
335.00
341.35
347.10
323.90
325.25
253.10
267.95
268.95
275.40
278.00
280.40
296.00
295.10

Figure 13D

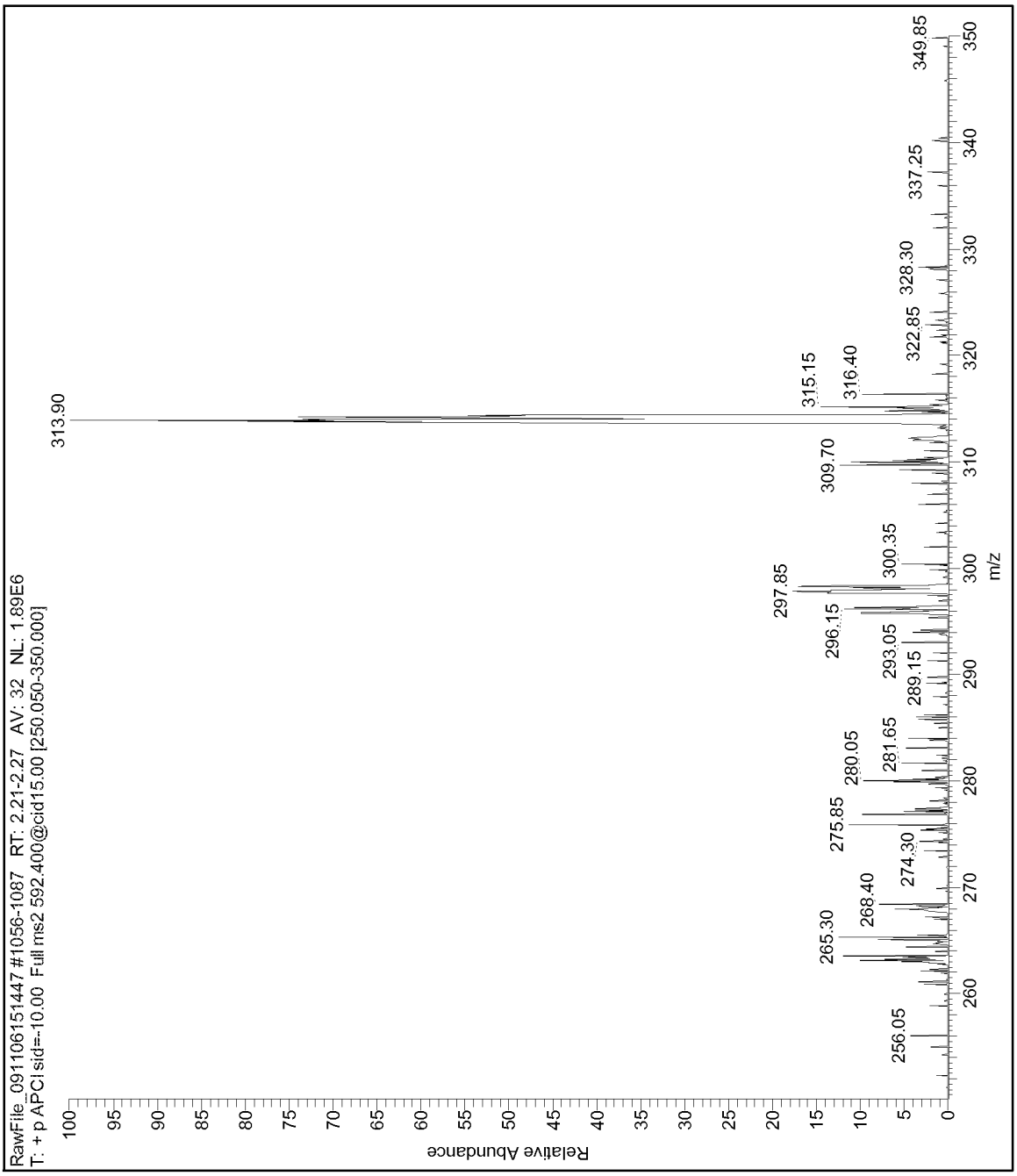

RawFile_091106151447 #1056-1087 RT: 2.21-2.27 AV: 32 NL: 1.89E6
T: + p APCI sid=-10.00 Full ms2 592.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
313.90
315.15
316.40
309.70
297.85
296.15
300.35
293.05
289.15
281.65
280.05
275.85
274.30
268.40
265.30
256.05
322.85
328.30
337.25
349.85

Figure 14A

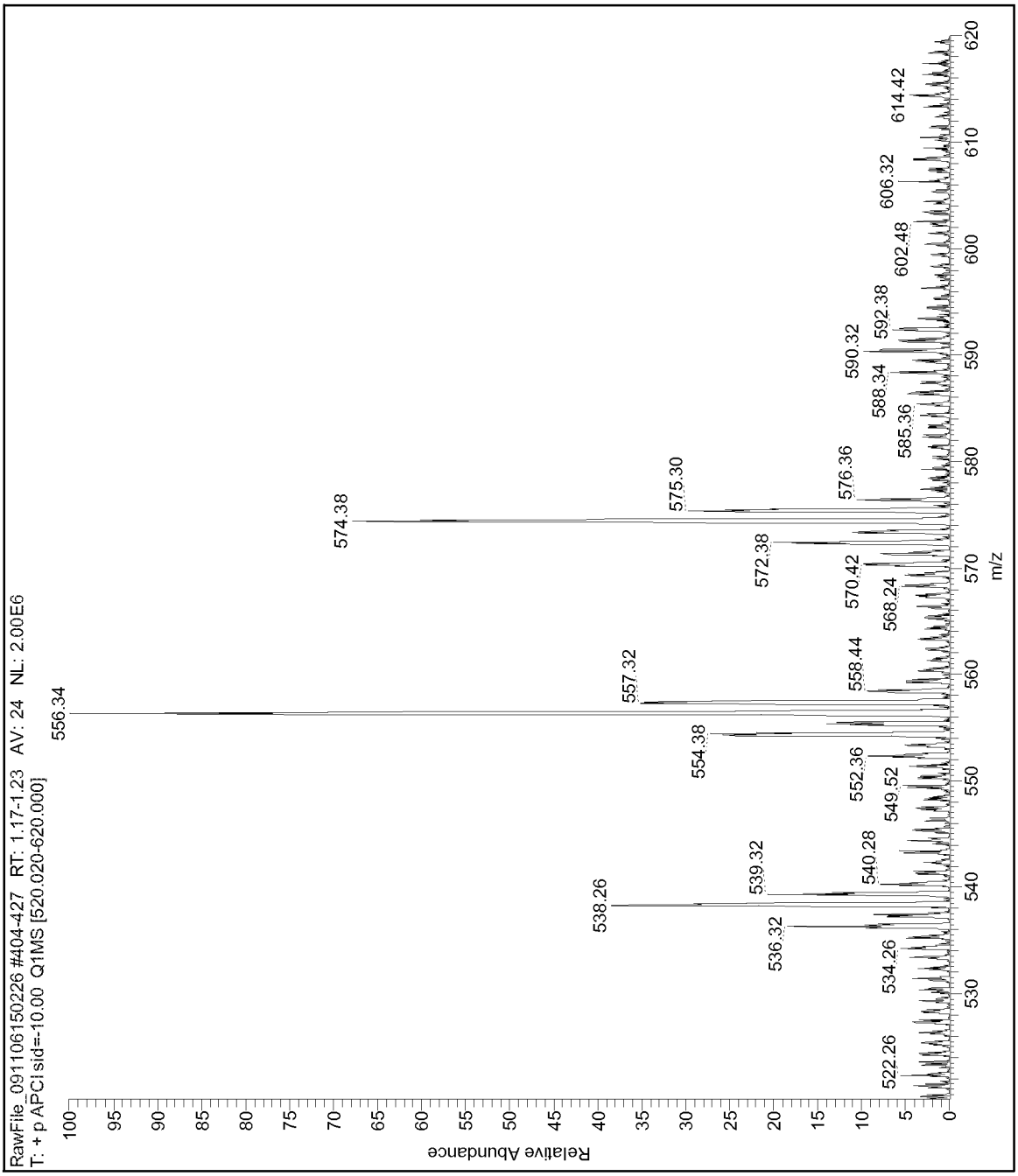

RawFile_09110615022 6 #404-427 RT: 1.17-1.23 AV: 24 NL: 2.00E6
T: + p APCI sid=-10.00 Q1MS [520.020-620.000]
Relative Abundance
m/z
556.34
574.38
557.32
575.30
538.26
554.38
572.38
539.32
536.32
558.44
576.36
552.36
570.42
590.32
540.28
549.52
568.24
588.34
592.38
602.48
606.32
614.42
522.26
534.26
585.36

Figure 14B

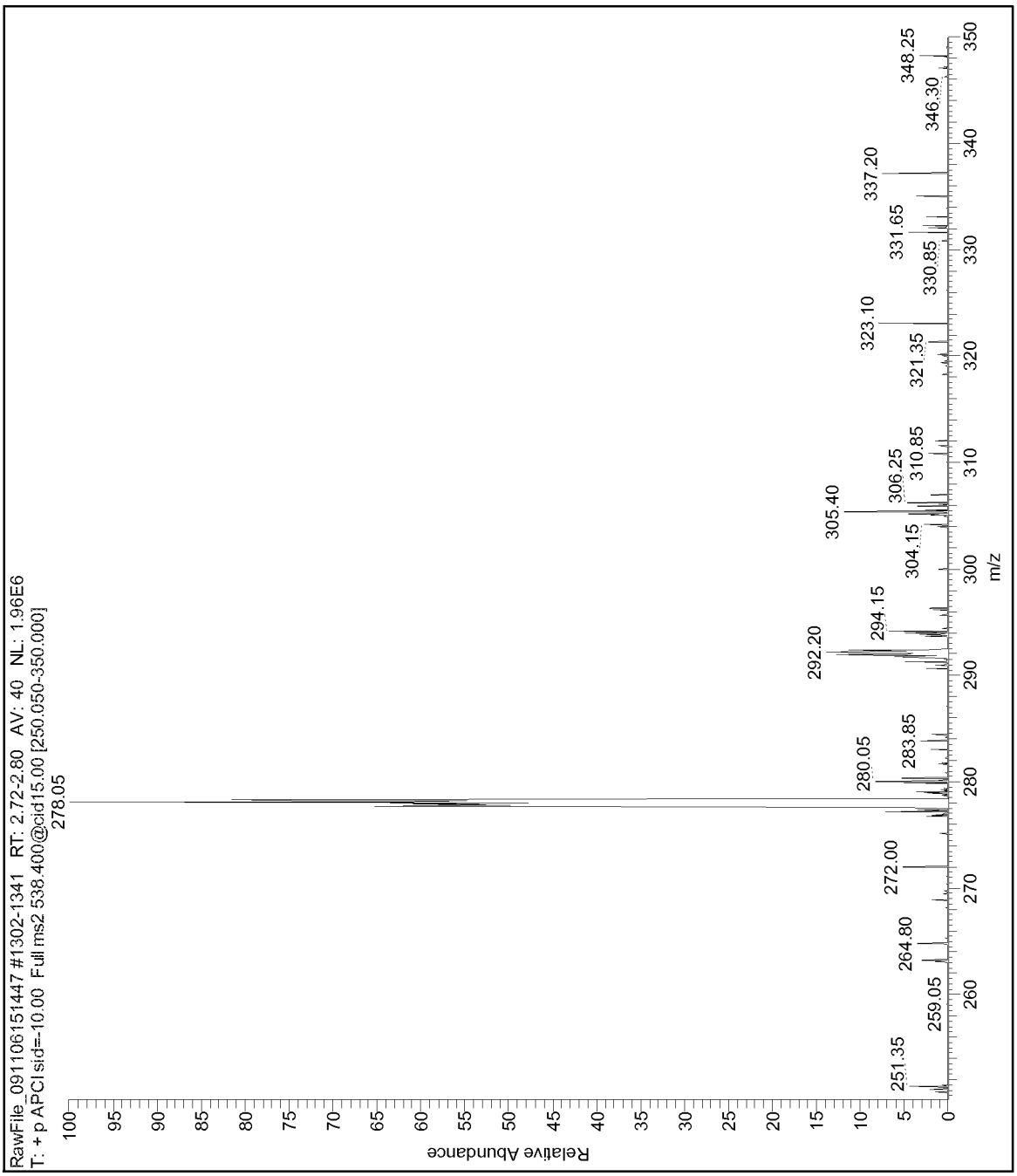

RawFile_091106151447 #1302-1341 RT: 2.72-2.80 AV: 40 NL: 1.96E6
T: + p APCI sid=-10.00 Full ms2 538.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
278.05
280.05
283.85
292.20
294.15
304.15
305.40
306.25
310.85
321.35
323.10
330.85
331.65
337.20
346.30
348.25
251.35
259.05
264.80
272.00

Figure 14C

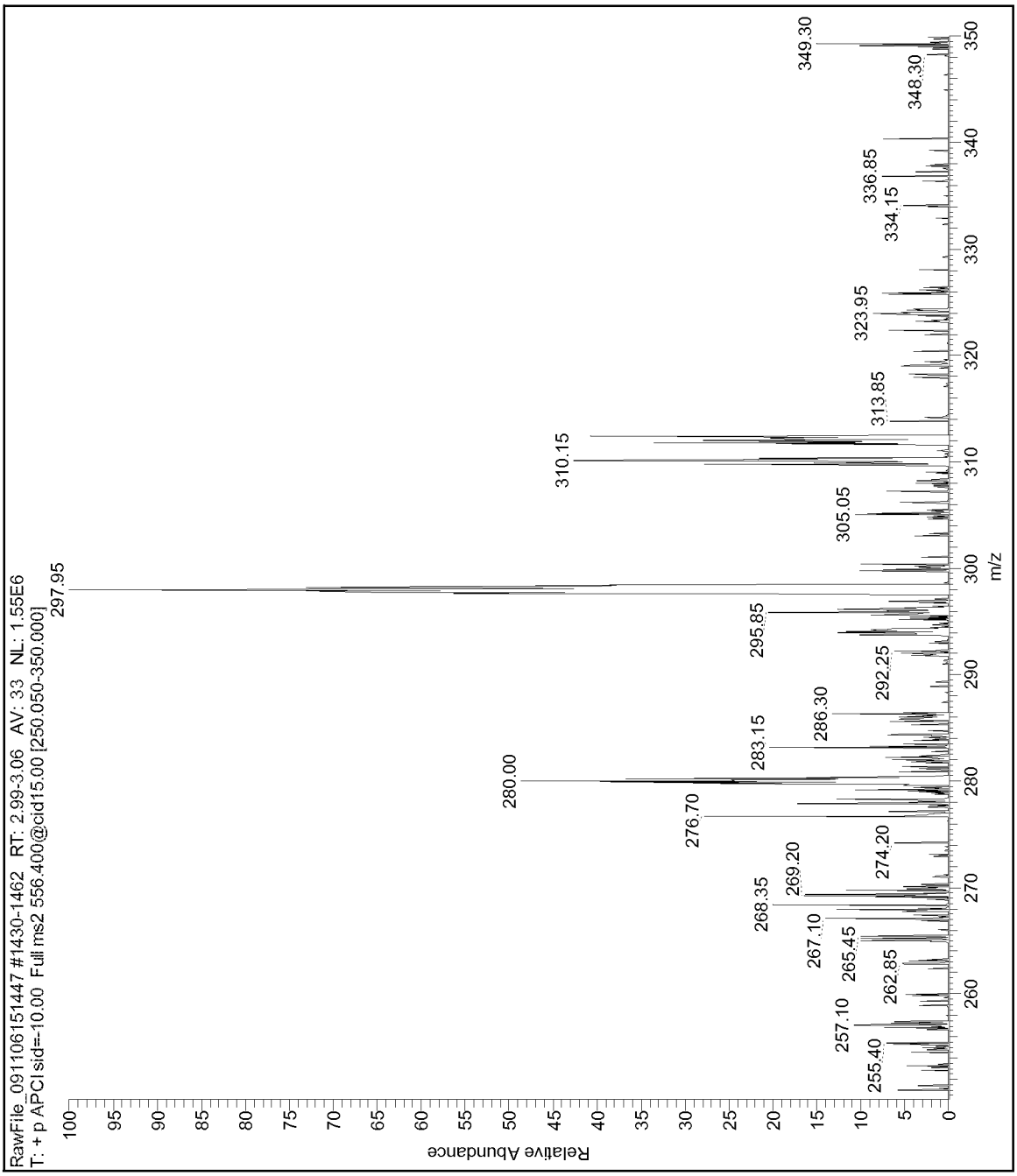

RawFile_09110615144 7 #1430-1462 RT: 2.99-3.06 AV: 33 NL: 1.55E6
T: + p APCI sid=-10.00 Full ms2 556.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
297.95
280.00
310.15
349.30
276.70
283.15
295.85
286.30
292.25
305.05
313.85
323.95
334.15
336.85
348.30
255.40
257.10
262.85
265.45
267.10
268.35
269.20
274.20

Figure 14D

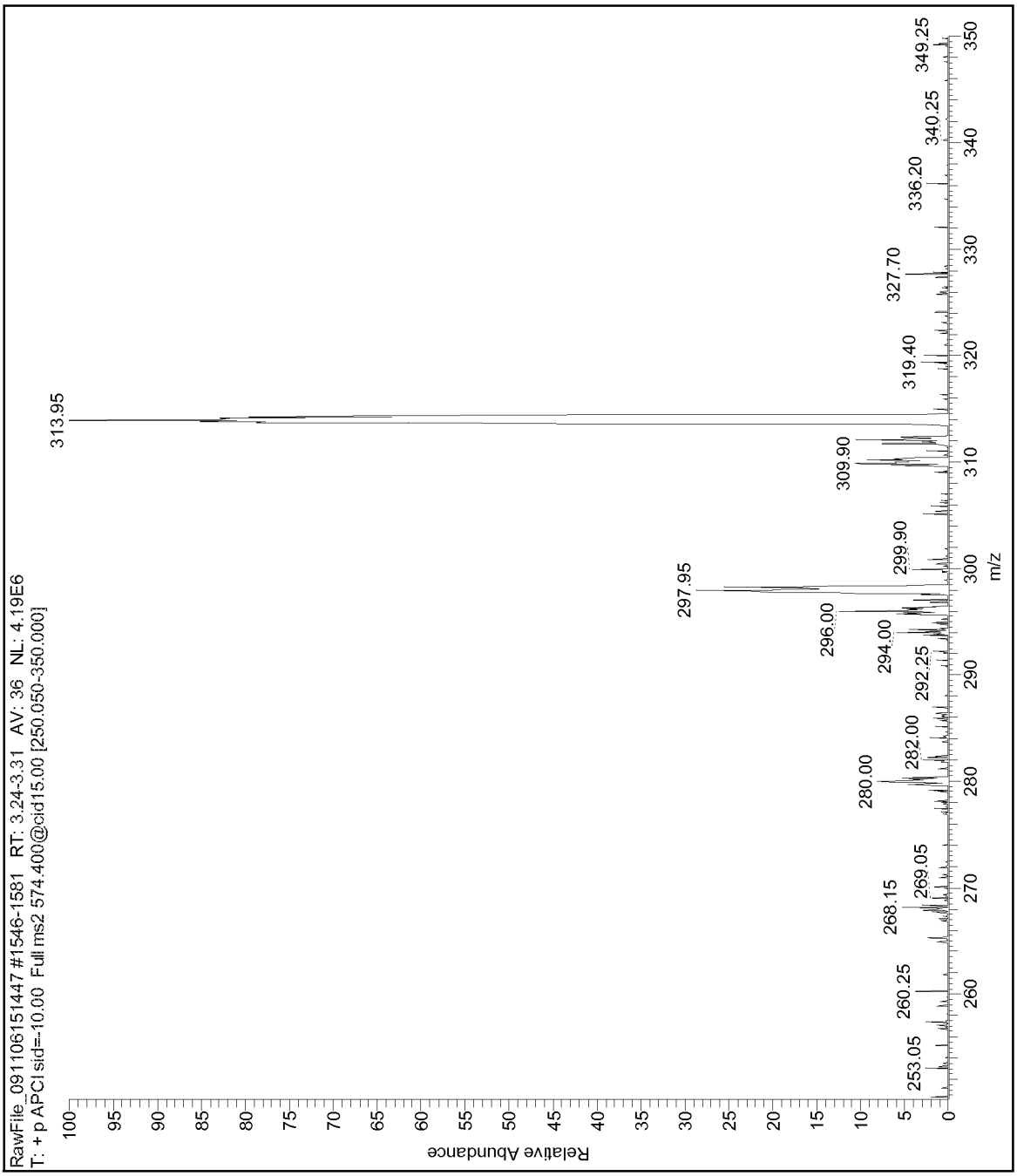

RawFile_091106151447 #1546-1581 RT: 3.24-3.31 AV: 36 NL: 4.19E6
T: + p APCI sid=-10.00 Full ms2 574.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
313.95
297.95
309.90
319.40
327.70
336.20
340.25
349.25
296.00
299.90
294.00
292.25
280.00
282.00
268.15
269.05
253.05
260.25

Figure 15A

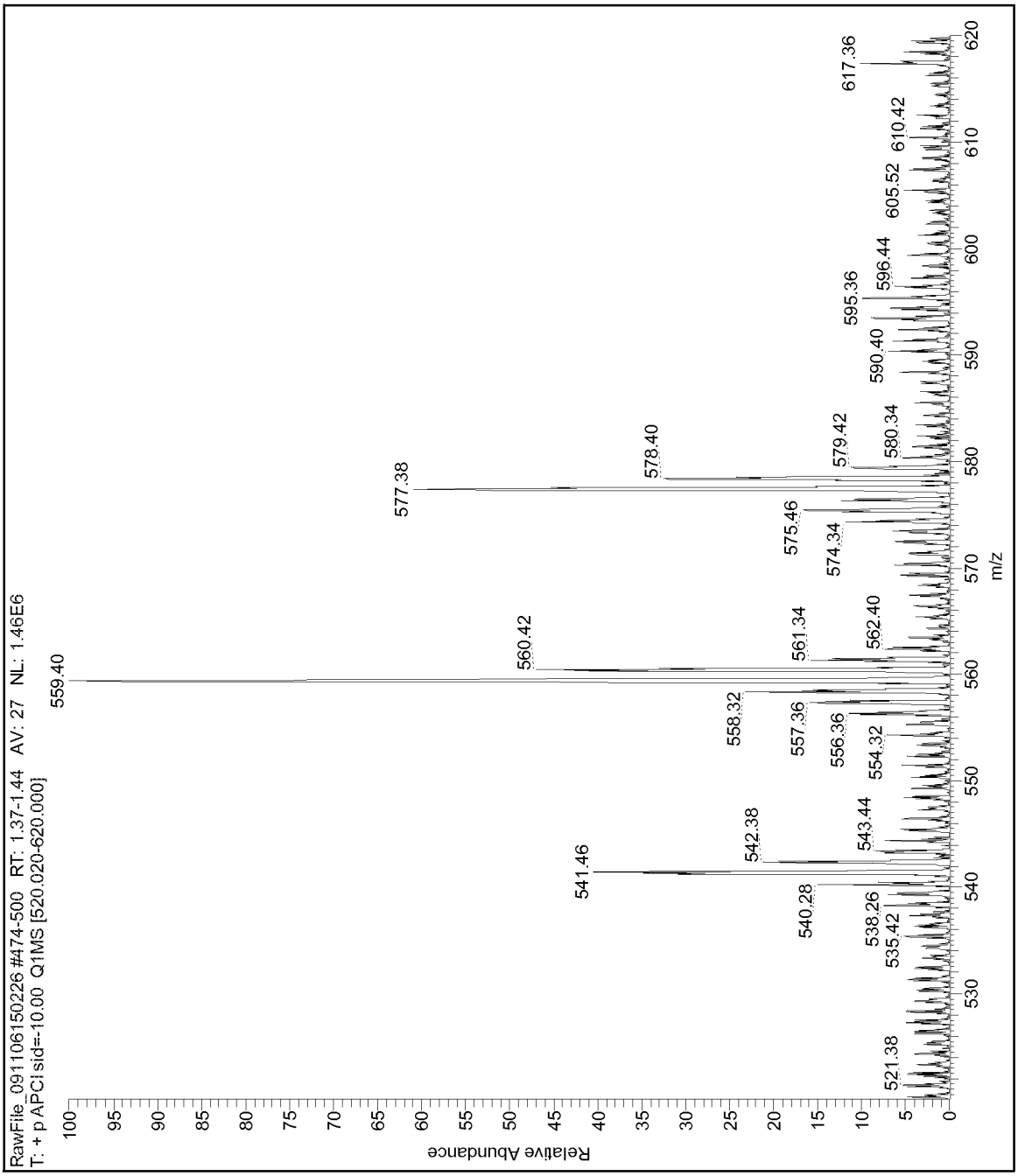

RawFile_09110615022 6 #474-500 RT: 1.37-1.44 AV: 27 NL: 1.46E6
T: + p APCI sid=-10.00 Q1MS [520.020-620.000]
Relative Abundance
m/z
559.40
560.42
577.38
578.40
541.46
542.38
617.36
610.42
605.52
596.44
595.36
590.40
580.34
579.42
575.46
574.34
561.34
562.40
558.32
557.36
556.36
554.32
543.44
540.28
538.26
535.42
521.38

Figure 15B

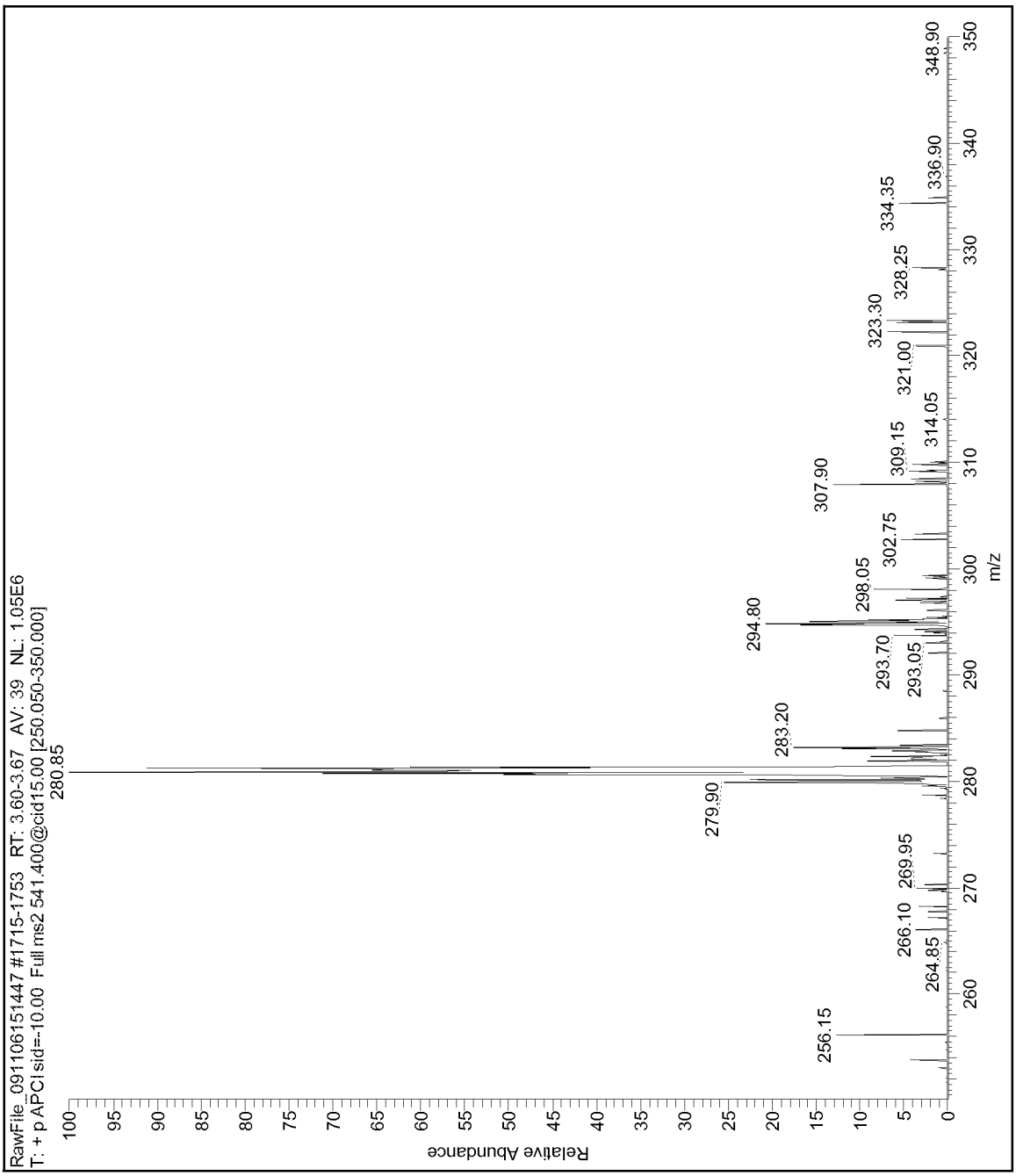

RawFile_09110615447 #1715-1753 RT: 3.60-3.67 AV: 39 NL: 1.05E6
T: + p APCI sid=-10.00 Full ms2 541.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
280.85
279.90
294.80
283.20
307.90
256.15
264.85
266.10
269.95
293.70
293.05
298.05
302.75
309.15
314.05
321.00
323.30
328.25
334.35
336.90
348.90

Figure 15C

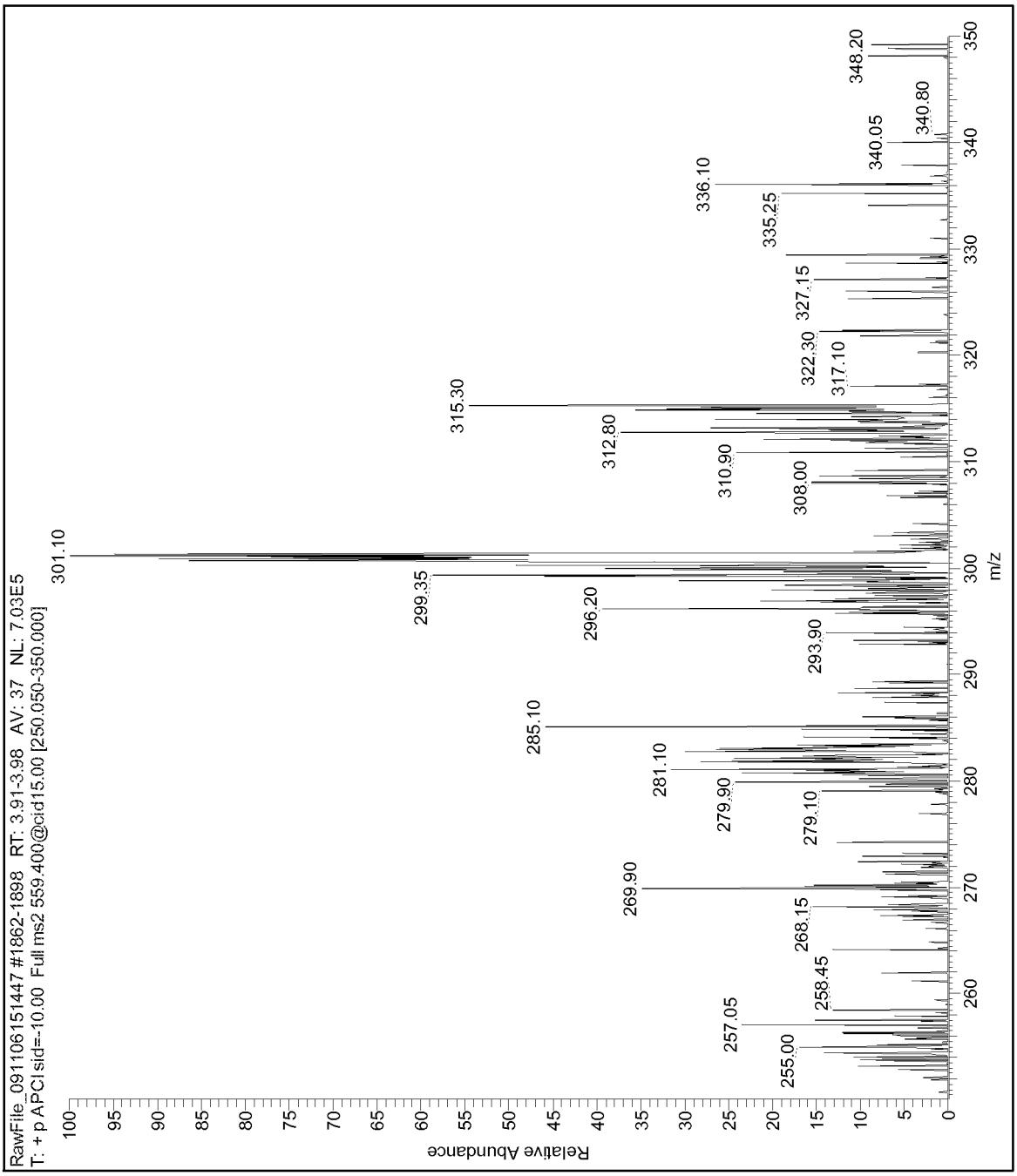

RawFile_09110615144 7#1862-1898 RT: 3.91-3.98 AV: 37 NL: 7.03E5
T: + p APCI sid=-10.00 Full ms2 559.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
301.10
299.35
296.20
293.90
285.10
281.10
279.90
279.10
269.90
268.15
258.45
257.05
255.00
315.30
312.80
310.90
308.00
322.30
317.10
327.15
335.25
336.10
340.05
340.80
348.20

Figure 15D

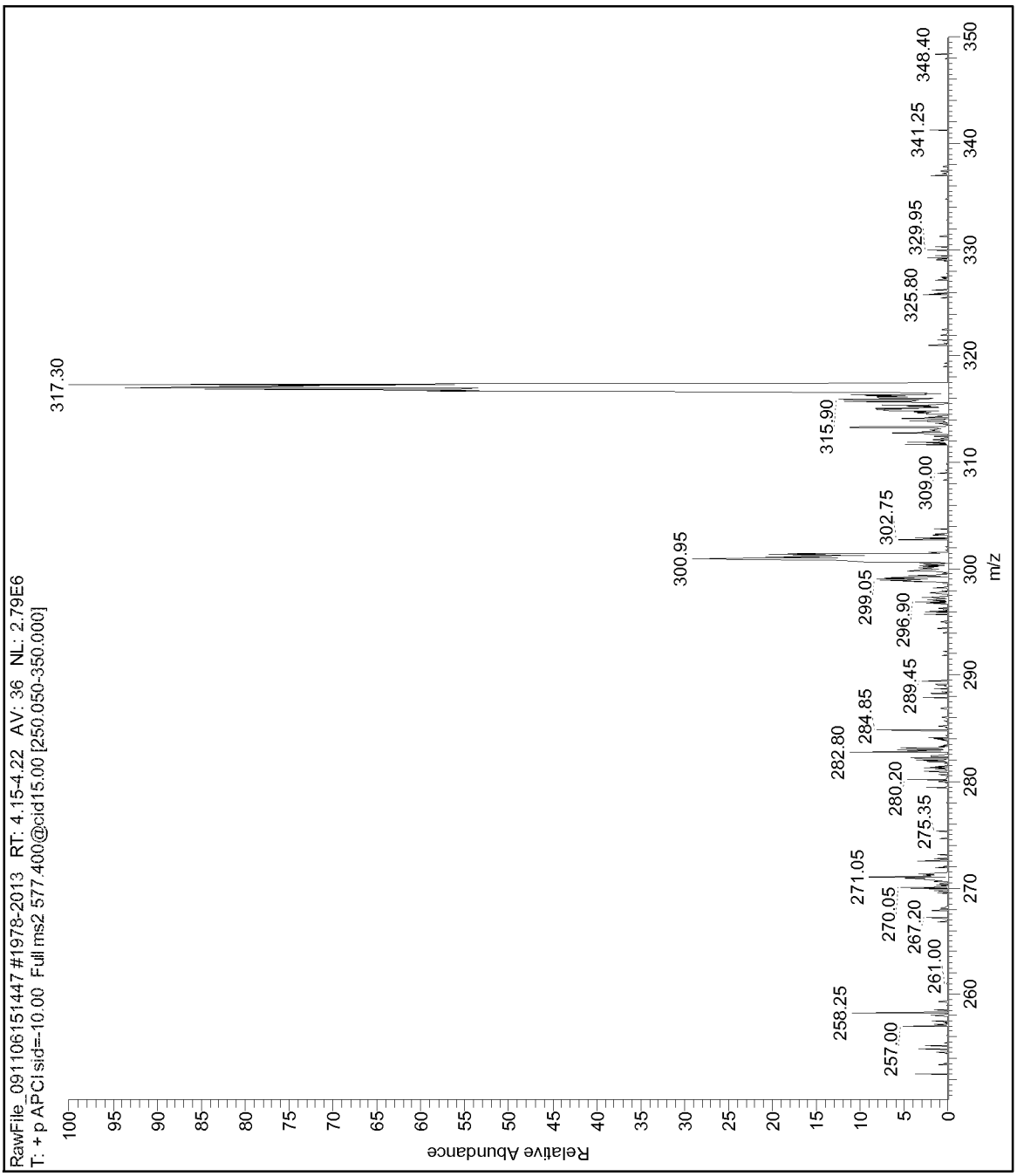

RawFile_091106151447 #1978-2013 RT: 4.15-4.22 AV: 36 NL: 2.79E6
T: + p APCI sid=-10.00 Full ms2 577.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
317.30
300.95
315.90
302.75
309.00
299.05
296.90
289.45
284.85
282.80
280.20
275.35
271.05
270.05
267.20
261.00
258.25
257.00
325.80
329.95
341.25
348.40

Figure 16A

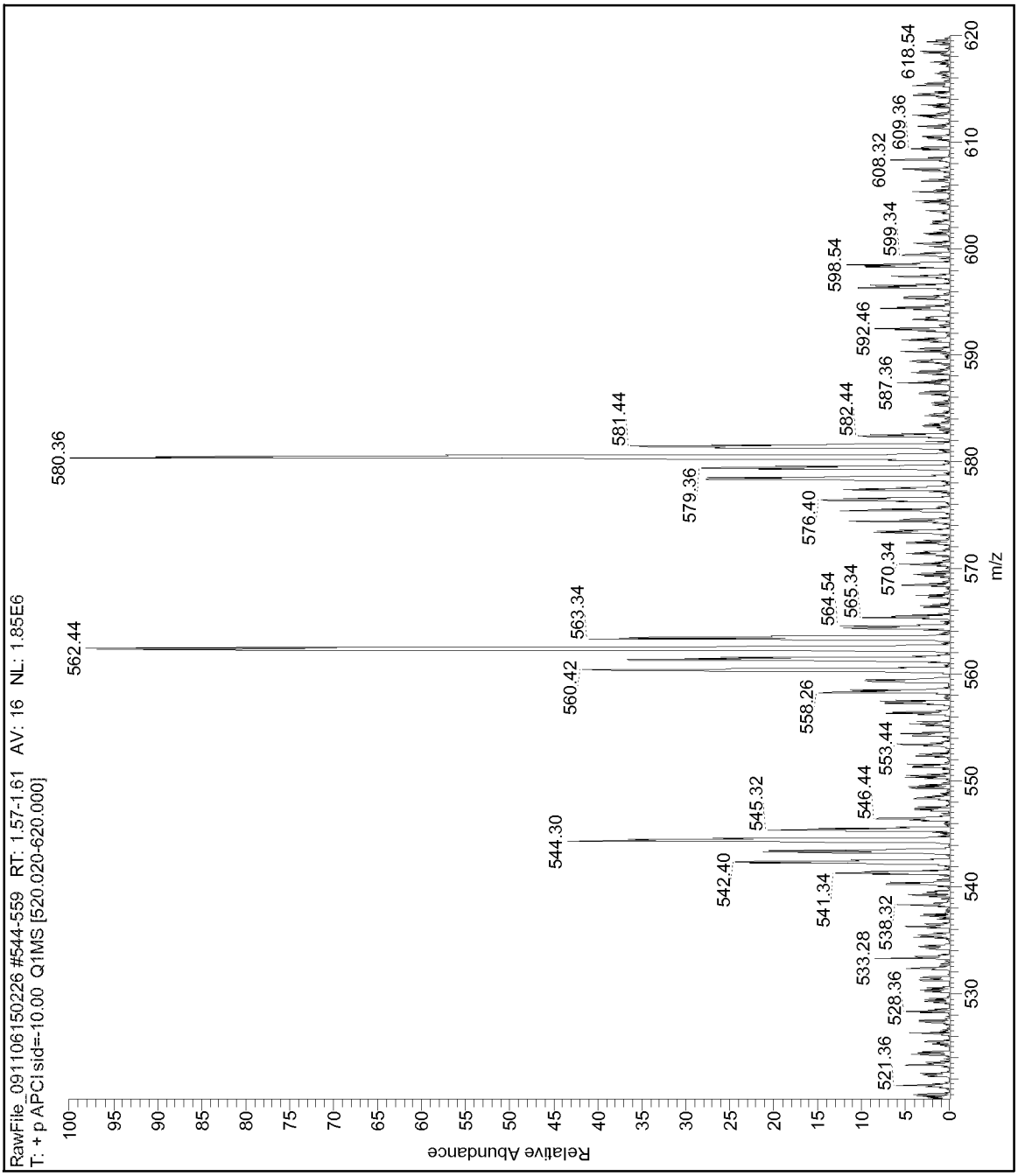

RawFile_09110615022 6 #544-559 RT: 1.57-1.61 AV: 16 NL: 1.85E6
T: + p APCI sid=-10.00 Q1MS [520.020-620.000]
Relative Abundance
m/z
580.36
562.44
581.44
563.34
579.36
560.42
576.40
564.54
565.34
570.34
558.26
544.30
545.32
553.44
546.44
542.40
541.34
533.28
538.32
528.36
521.36
582.44
587.36
592.46
598.54
599.34
608.32
609.36
618.54

Figure 16B

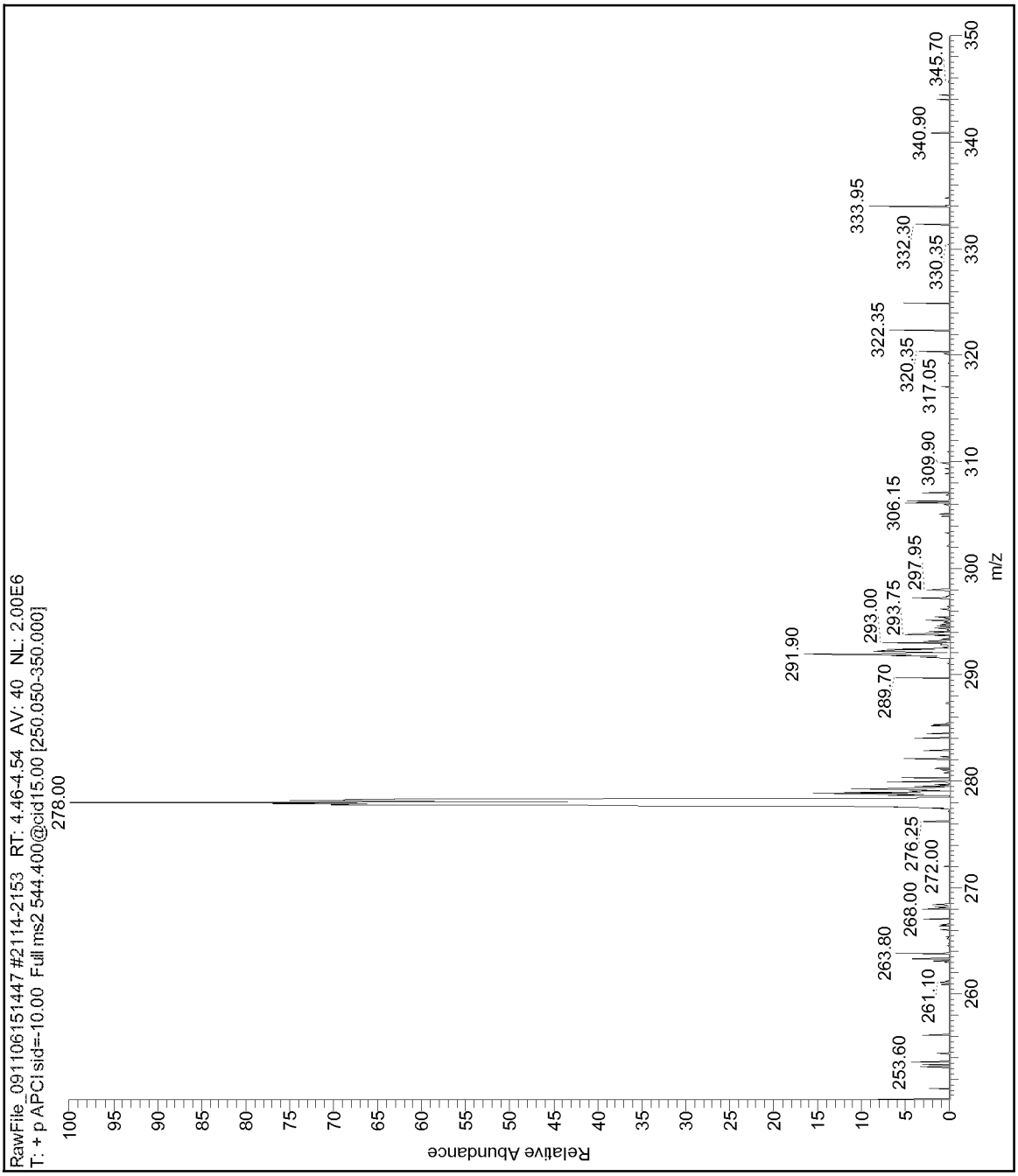

RawFile_091106151447 #2114-2153 RT: 4.46-4.54 AV: 40 NL: 2.00E6
T: + p APCI sid=-10.00 Full ms2 544.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
278.00
291.90
289.70
293.00
293.75
297.95
306.15
309.90
317.05
320.35
322.35
330.35
332.30
333.95
340.90
345.70
253.60
261.10
263.80
268.00
272.00
276.25

Figure 16C

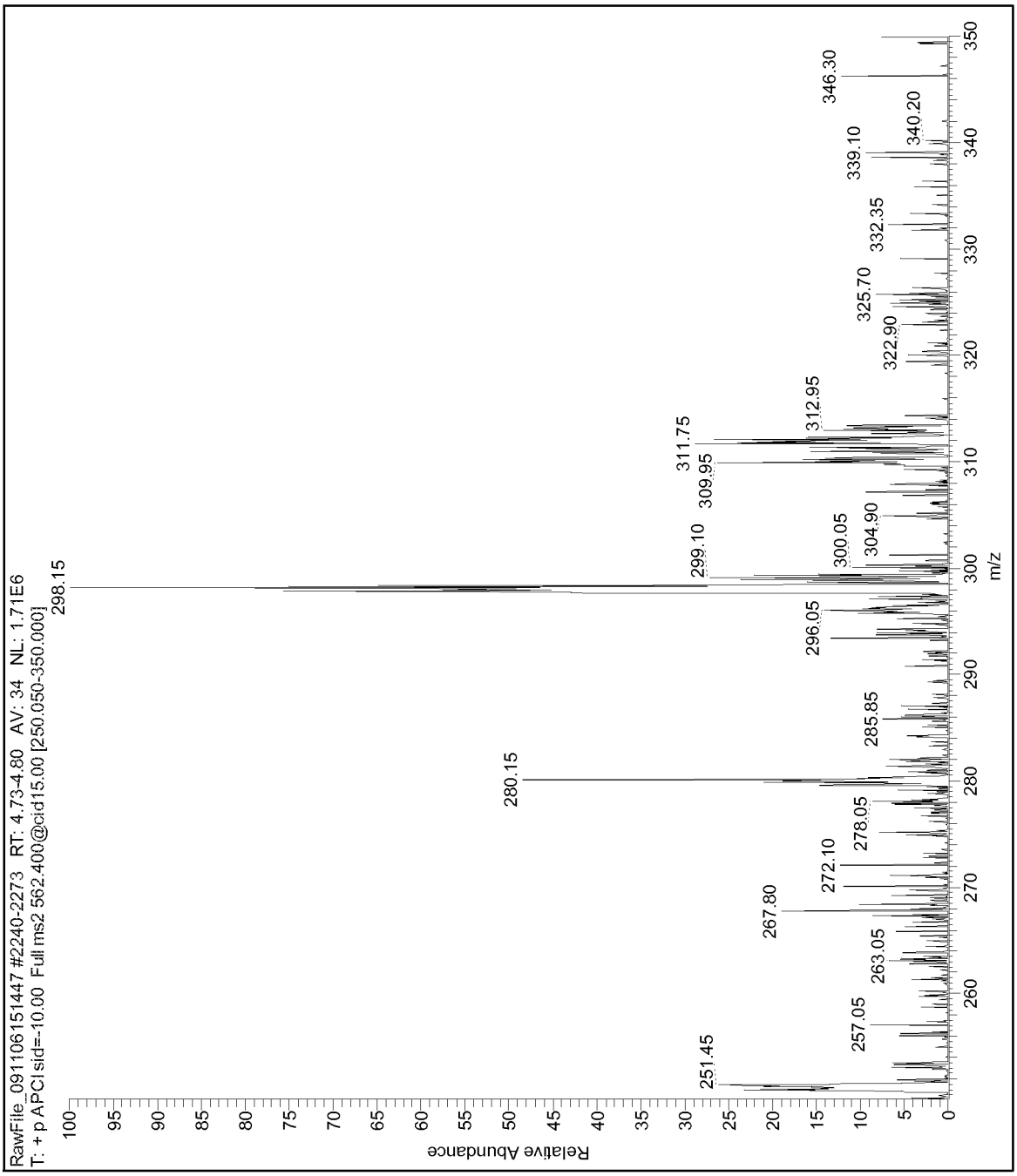
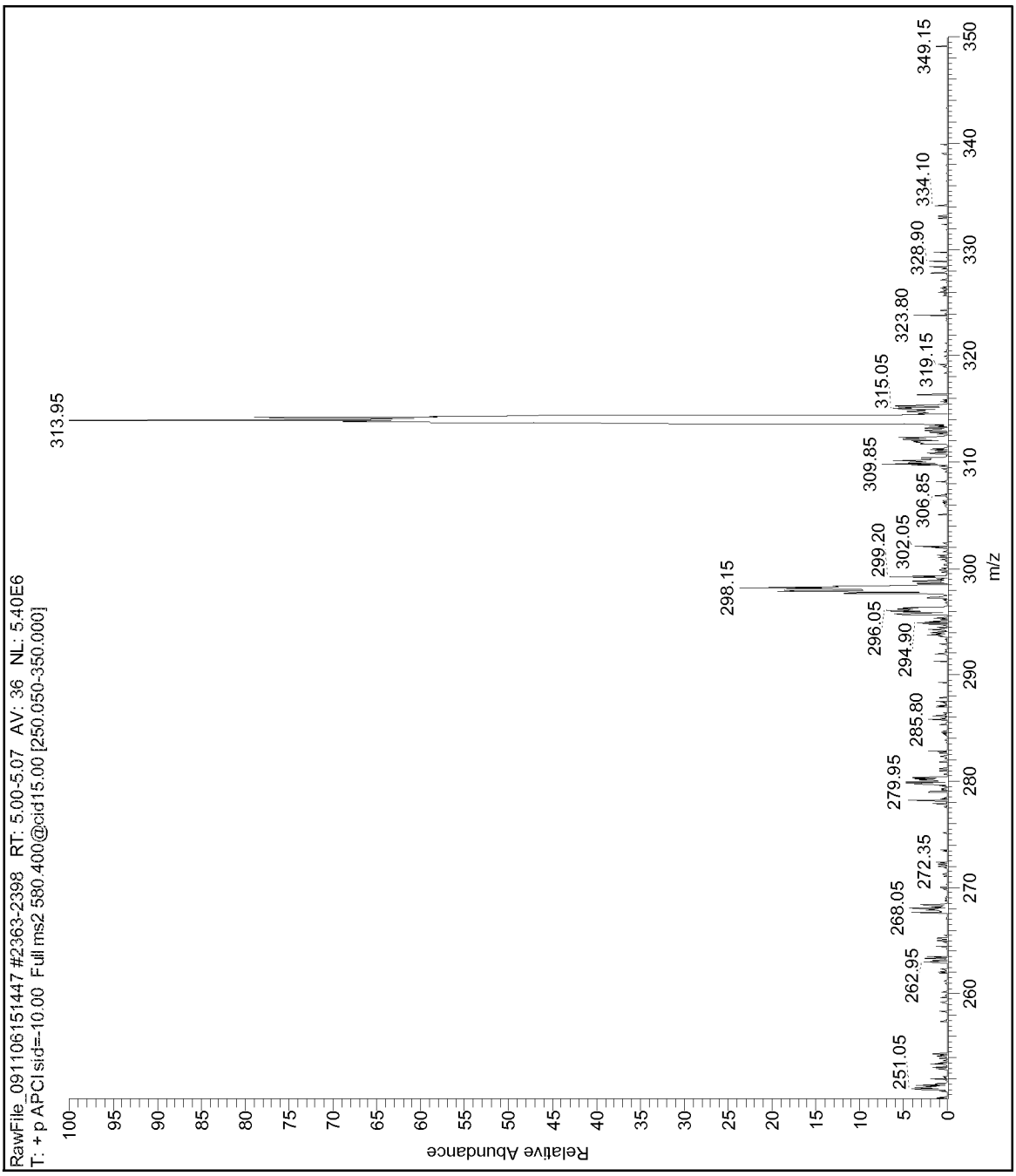

RawFile_091106151447 #2240-2273 RT: 4.73-4.80 AV: 34 NL: 1.71E6
T: + p APCI sid=-10.00 Full ms2 562.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
298.15
280.15
251.45
267.80
311.75
346.30
339.10
340.20
332.35
325.70
322.90
312.95
309.95
299.10
300.05
304.90
296.05
285.85
278.05
272.10
263.05
257.05

Figure 16D


RawFile_091106151447 #2363-2398 RT: 5.00-5.07 AV: 36 NL: 5.40E6
T: + p APCI sid=-10.00 Full ms2 580.400@cid15.00 [250.050-350.000]
Relative Abundance
m/z
313.95
298.15
315.05
309.85
299.20
296.05
323.80
328.90
334.10
349.15
319.15
302.05
306.85
294.90
279.95
285.80
268.05
272.35
262.95
251.05

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/57627

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/00 (2010.01)
USPC - 436/127
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 33/00 (2010.01)
USPC: 436/127

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8): G01N 33/00 (2010.01)
USPC: 436/127 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google Scholar
Search terms used: mass spectrometry vitamin D metabolite ptad solid phase extraction urbulent flow liquid chromatography HPLC TFLC SPE LDTD laser diode extraction column

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0137056 A1 (HOLMQUIST et al.) 28 May 2009 (28.05.2009), entire document, especially: Abstract, para [0008]-[0011], [0019], [0037], [0040], [0051]-[0059], [0064]	1-4, 30-34 --- 59-63
Y	US 2006/0054807 A1 (PICARD et al.) 16 March 2006 (16.03.2006), para [0019]	59-63
X --- Y	ARONOV et al., "Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry," Anal Bioanal Chem, 391:1917-1930, 2008, (2008), entire document, especially: Abstract; pg. 1917, col. 2, para 2 - pg. 1918, col. 2, para 1; pg. 1919, col. 2, para 4 - pg. 1920, col. 2, para 1; pg. 1929, col. 1, para 1	1-4, 30-34 --- 59-63

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 January 2011 (06.01.2011)	27 JAN 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/57627

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-29, 35-58, 64-82
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.