US 20110240842A1

(19) **United States**

(12) **Patent Application Publication**

Grant et al.

(10) **Pub. No.: US 2011/0240842 A1**

(43) **Pub. Date: Oct. 6, 2011**

(54) **METHODS AND SYSTEMS FOR THE QUANTITATIVE ANALYSIS OF BIOMARKERS**

(75) Inventors: **Russell Philip Grant**, Chapel Hill, NC (US); **Andrew Dennis Wagner**, New Haven, CT (US); **Mary Katherine Morr**, Thousand Oaks, CA (US); **Milan Rameshkumar Patel**, West Hills, CA (US)

(73) Assignee: **Laboratory Corporation of America Holdings**

(21) Appl. No.: **13/080,014**

(22) Filed: **Apr. 5, 2011**

Related U.S. Application Data

(62) Division of application No. 11/805,985, filed on May 25, 2007, now Pat. No. 7,935,921.

(60) Provisional application No. 60/808,812, filed on May 26, 2006.

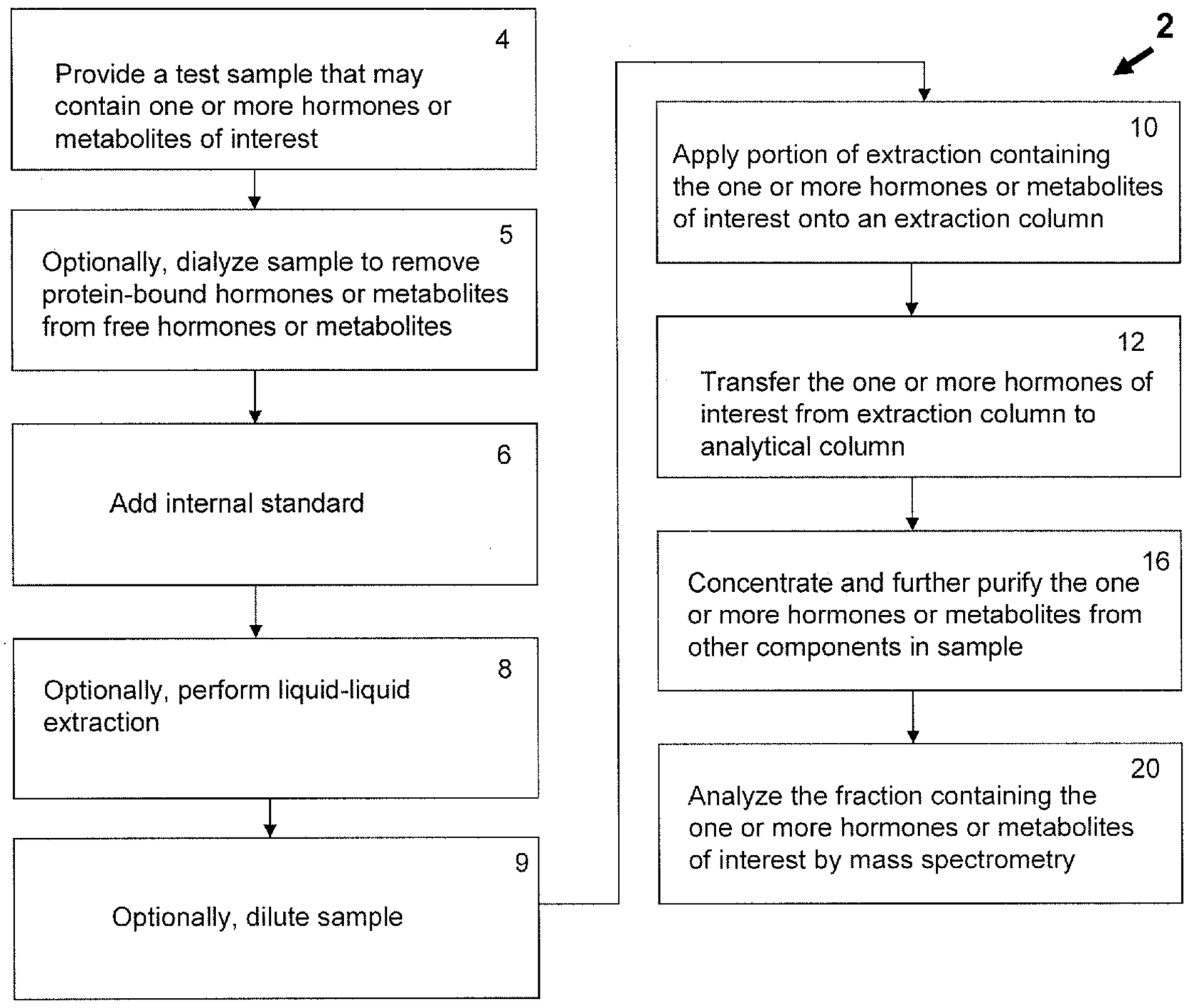
Publication Classification

(51) **Int. Cl.**
H01J 49/26 (2006.01)

(52) **U.S. Cl.** **250/282**; 250/288

(57) **ABSTRACT**

Disclosed are methods and systems using liquid chromatography/tandem mass spectrometry (LC-MS/MS and 2D-LC-MS/MS) for the analysis of endogenous biomarkers, including steroid hormones, such as estrone and estradiol, thyroid hormones, such as free thyroxine, and metabolites, such as 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, in biological samples.



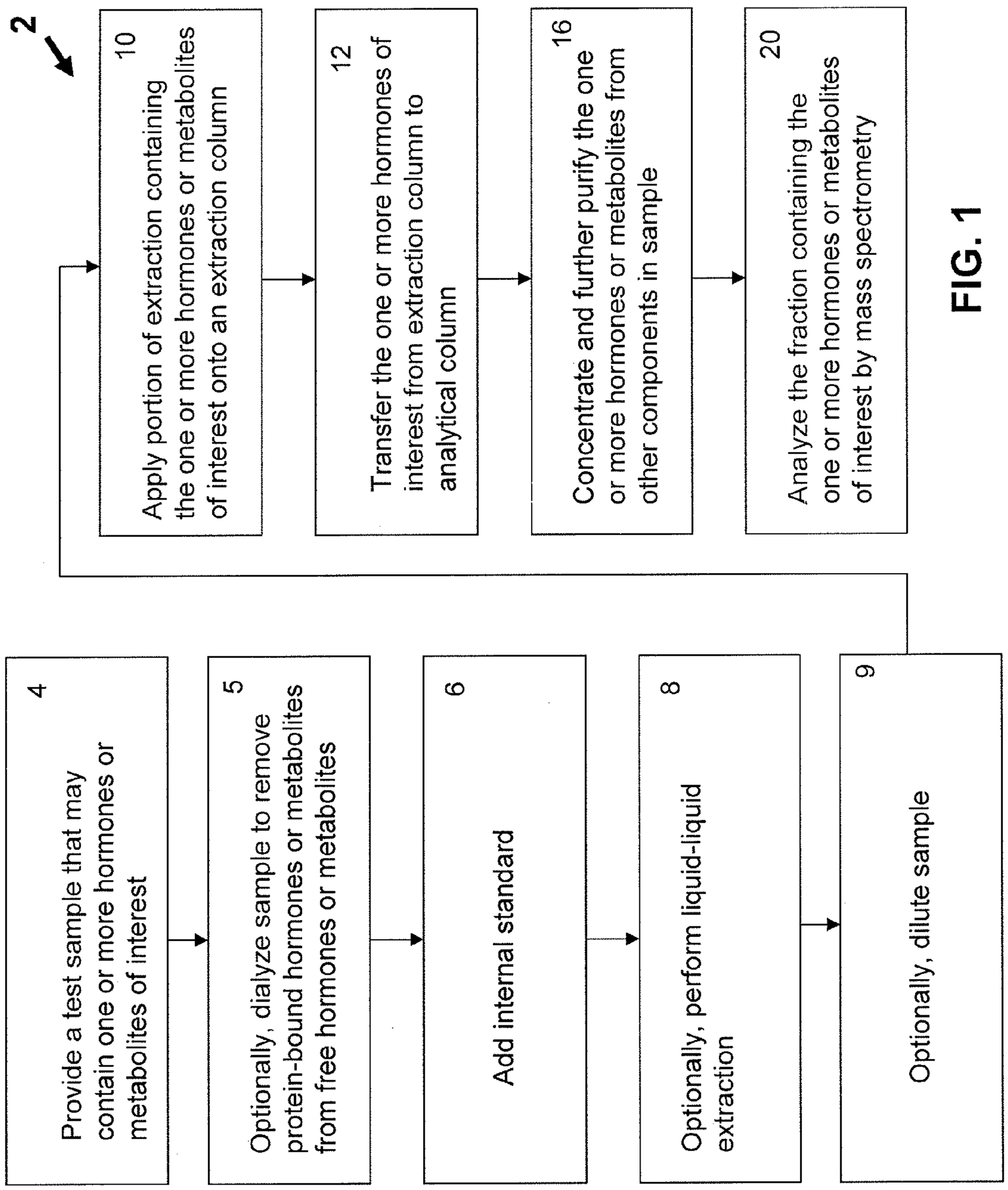

2
4
Provide a test sample that may contain one or more hormones or metabolites of interest
5
Optionally, dialyze sample to remove protein-bound hormones or metabolites from free hormones or metabolites
6
Add internal standard
8
Optionally, perform liquid-liquid extraction
9
Optionally, dilute sample
10
Apply portion of extraction containing the one or more hormones or metabolites of interest onto an extraction column
12
Transfer the one or more hormones of interest from extraction column to analytical column
16
Concentrate and further purify the one or more hormones or metabolites from other components in sample
20
Analyze the fraction containing the one or more hormones or metabolites of interest by mass spectrometry

FIG. 1

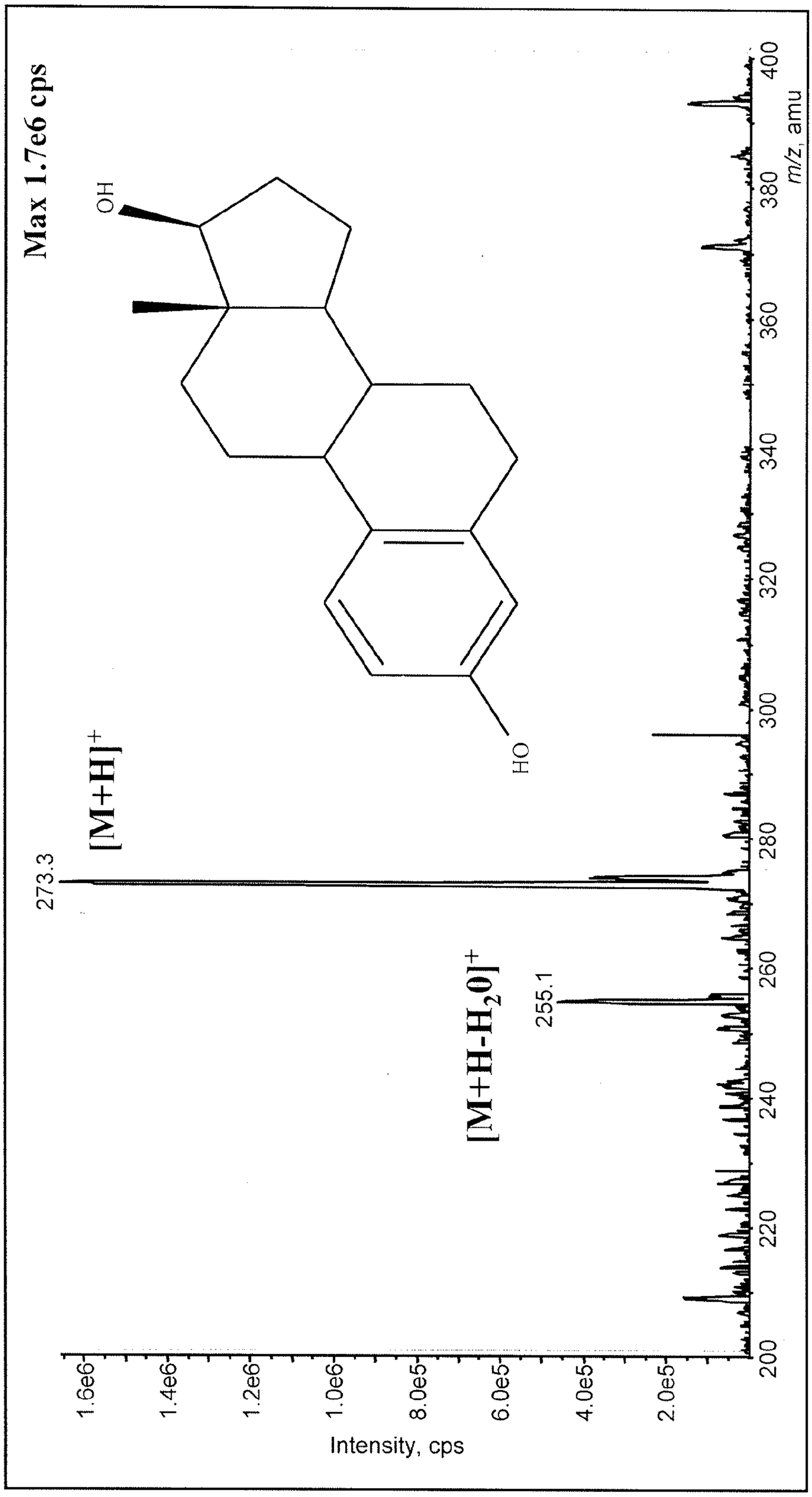

Estradiol Q1 Scan (FIA) 400°C
Max 1.7e6 cps
OH
HO
273.3
[M+H]+
255.1
[M+H-H2O]+
Intensity, cps
m/z, amu

FIG. 2A

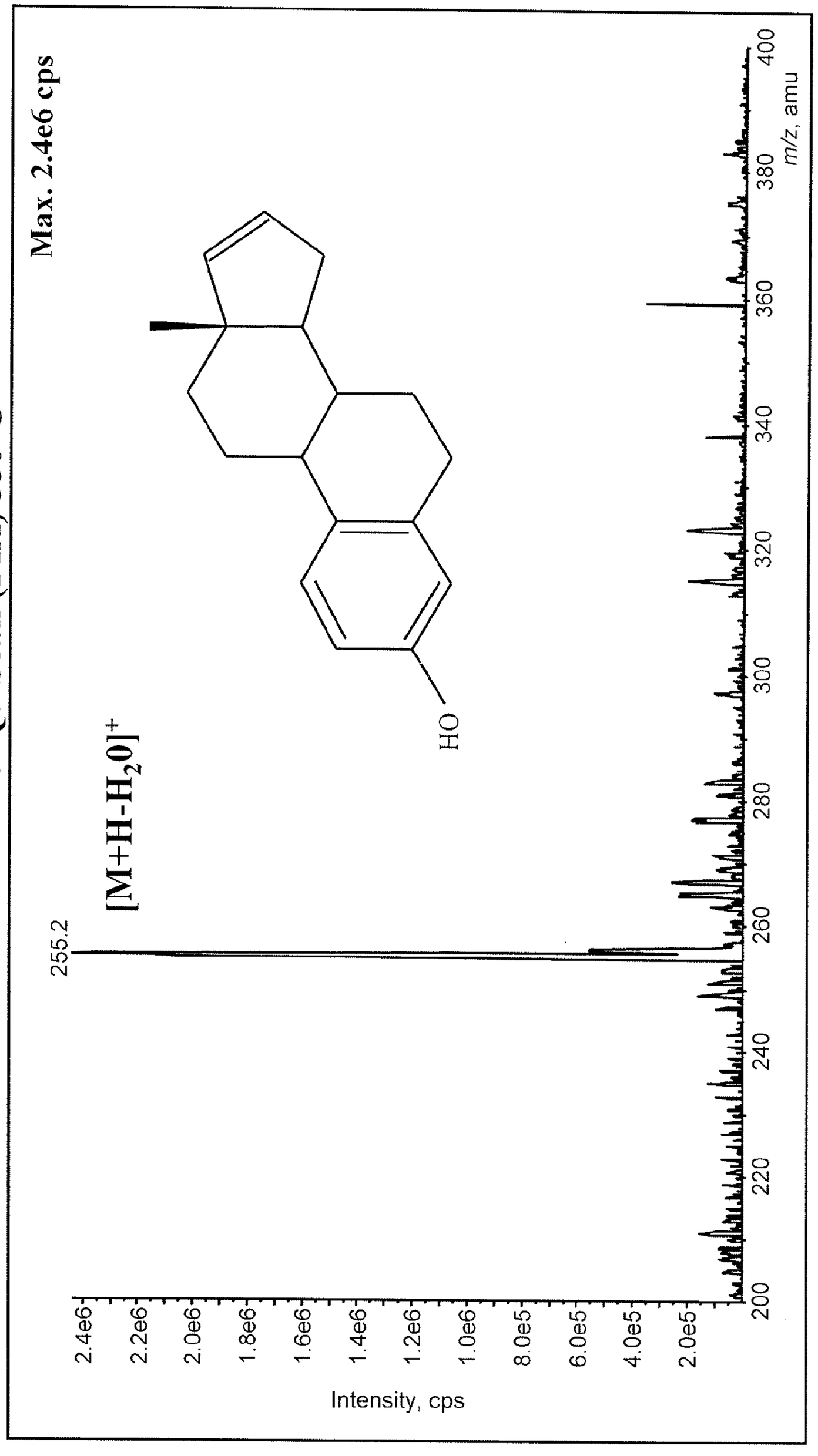

Estradiol Q1 Scan (FIA) 550°C
Max. 2.4e6 cps
[M+H-H2O]+
255.2
HO
Intensity, cps
m/z, amu
2.4e6
2.2e6
2.0e6
1.8e6
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
200
220
240
260
280
300
320
340
360
380
400

FIG. 2B

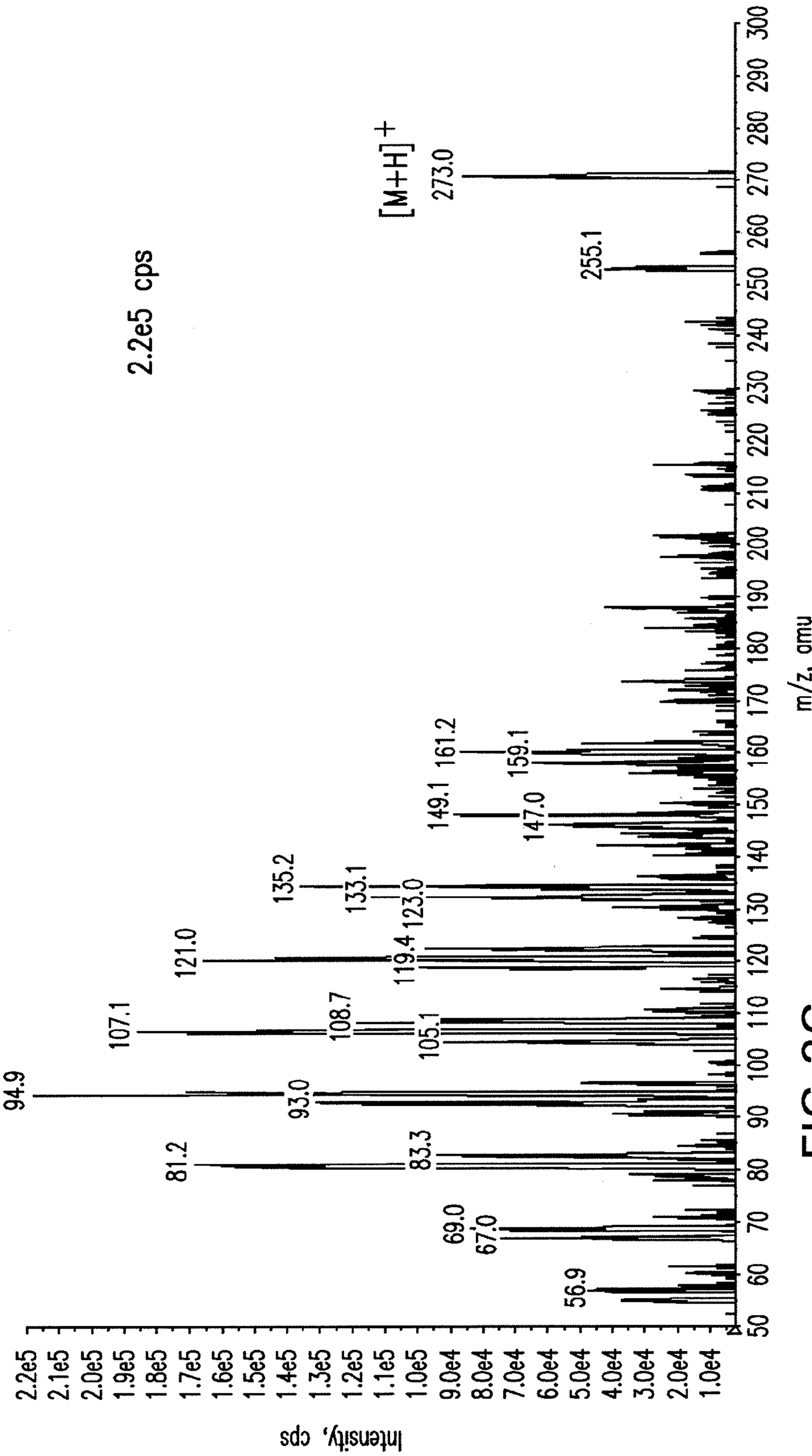

Product Ion Scan of m/z 271
2.2e5 cps
[M+H]+
273.0
255.1
121.0
135.2
133.1
119.4
123.0
149.1
161.2
159.1
147.0
107.1
108.7
105.1
94.9
93.0
81.2
83.3
69.0
67.0
56.9
m/z, amu
Intensity, cps

FIG.2C

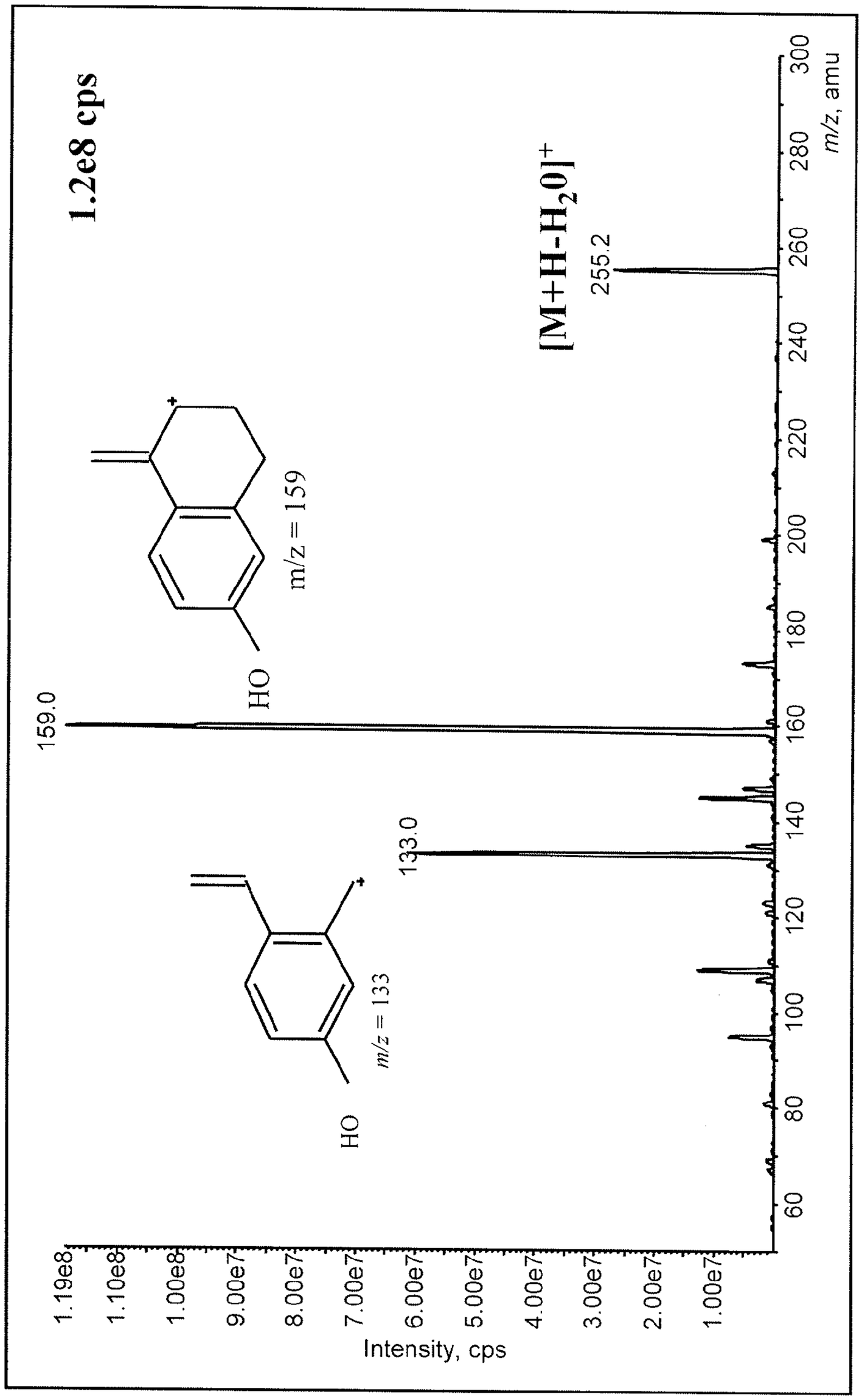

Product Ion Scan of m/z 255
1.2e8 cps
[M+H-H2O]+
255.2
m/z, amu
159.0
HO
m/z = 159
133.0
m/z = 133
HO
Intensity, cps

FIG. 2D

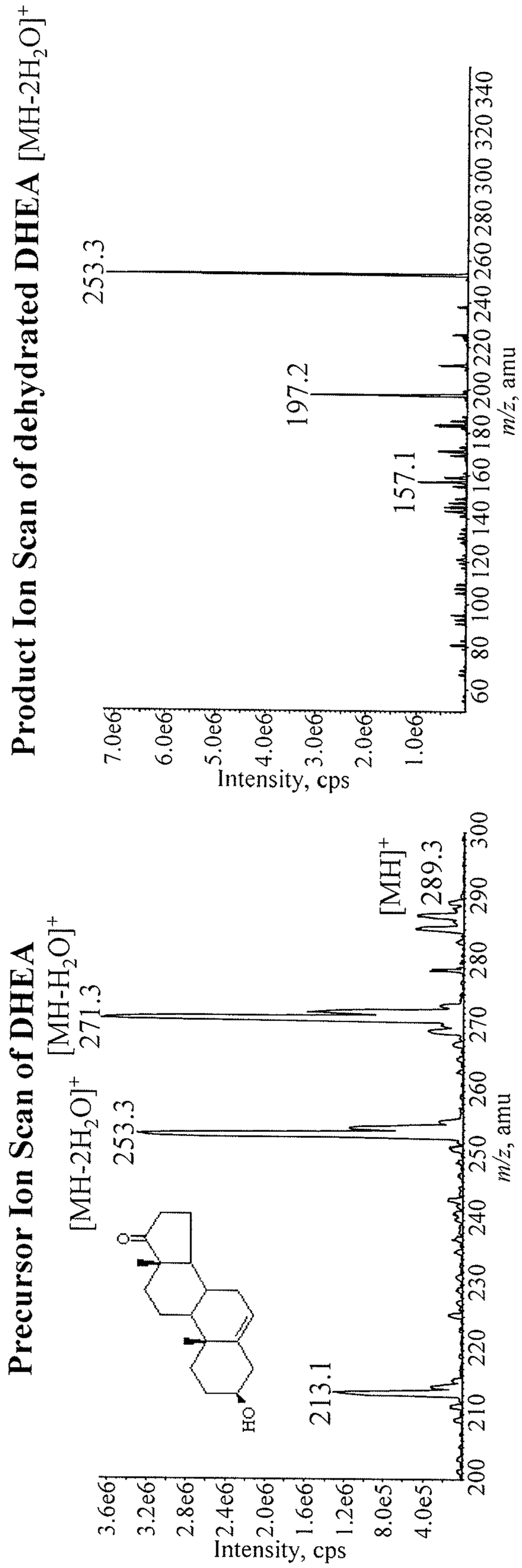

Product Ion Scan of dehydrated DHEA [MH-2H2O]+
253.3
197.2
157.1
Intensity, cps
m/z, amu
Product Ion Scan of DHEA
[MH-H2O]+
271.3
[MH]+
289.3
Precursor Ion Scan of
[MH-2H2O]+
253.3
213.1
HO
Intensity, cps
m/z, amu

FIG. 3

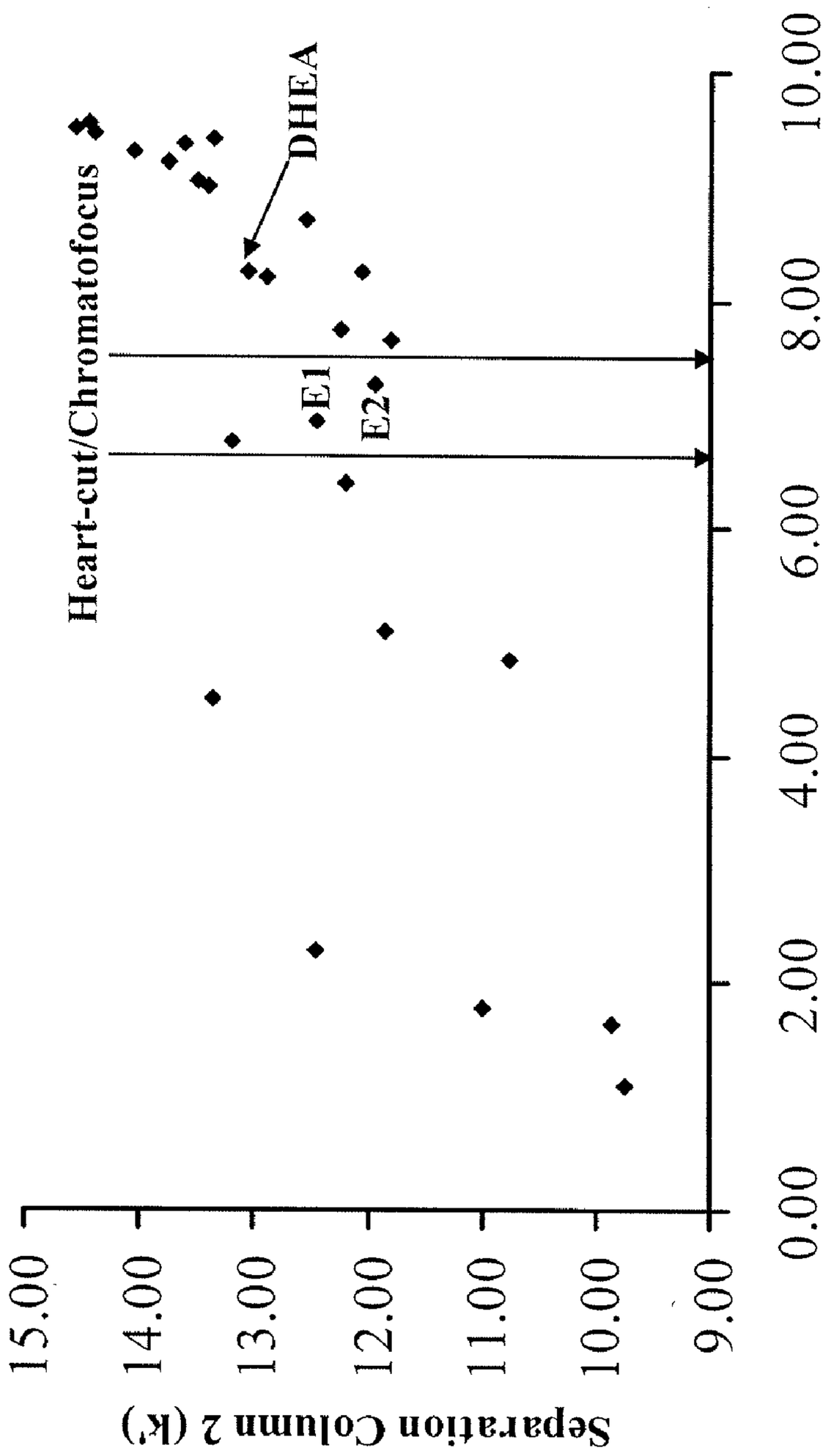

Heart-cut/Chromatofocus
DHEA
E1
E2
Separation Column 1 (k')
2.00
4.00
6.00
8.00
10.00
Separation Column 2 (k')
9.00
10.00
11.00
12.00
13.00
14.00
15.00
0.00

FIG. 4

FIG. 5

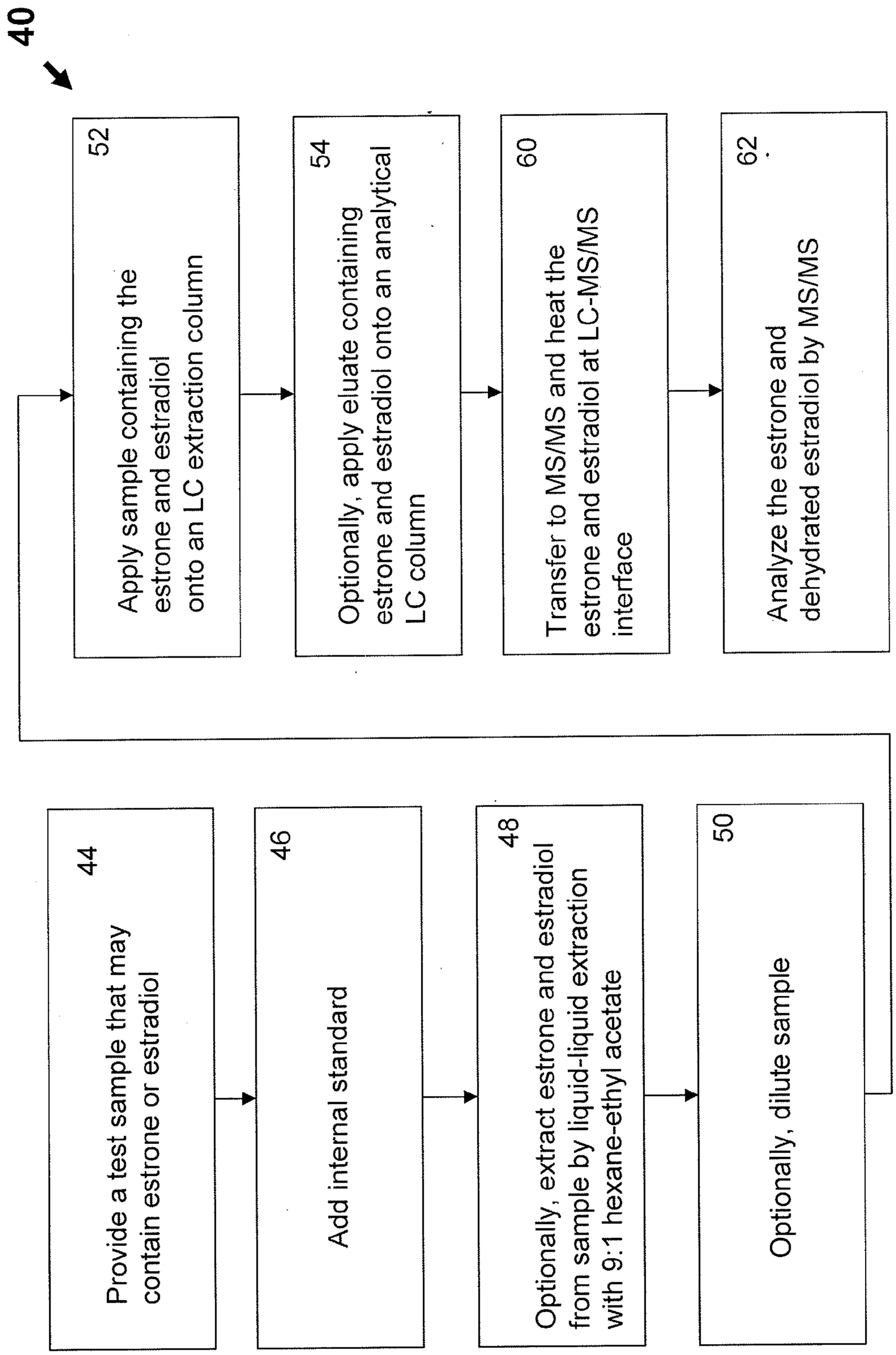
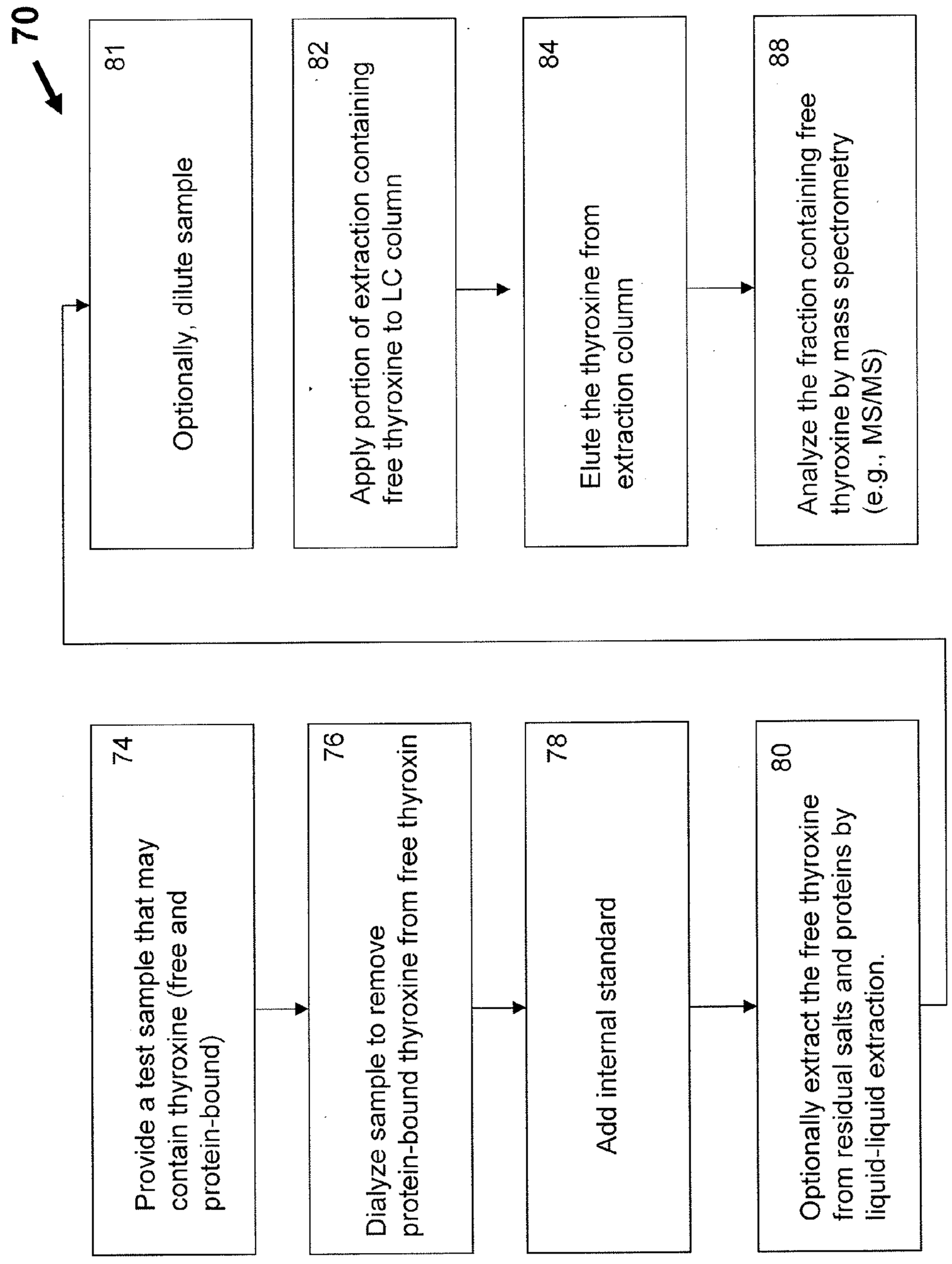

40
44
Provide a test sample that may contain estrone or estradiol
46
Add internal standard
48
Optionally, extract estrone and estradiol from sample by liquid-liquid extraction with 9:1 hexane-ethyl acetate
50
Optionally, dilute sample
52
Apply sample containing the estrone and estradiol onto an LC extraction column
54
Optionally, apply eluate containing estrone and estradiol onto an analytical LC column
60
Transfer to MS/MS and heat the estrone and estradiol at LC-MS/MS interface
62
Analyze the estrone and dehydrated estradiol by MS/MS

FIG. 6


70
74
Provide a test sample that may contain thyroxine (free and protein-bound)
76
Dialyze sample to remove protein-bound thyroxine from free thyroxin
78
Add internal standard
80
Optionally extract the free thyroxine from residual salts and proteins by liquid-liquid extraction.
81
Optionally, dilute sample
82
Apply portion of extraction containing free thyroxine to LC column
84
Elute the thyroxine from extraction column
88
Analyze the fraction containing free thyroxine by mass spectrometry (e.g., MS/MS)

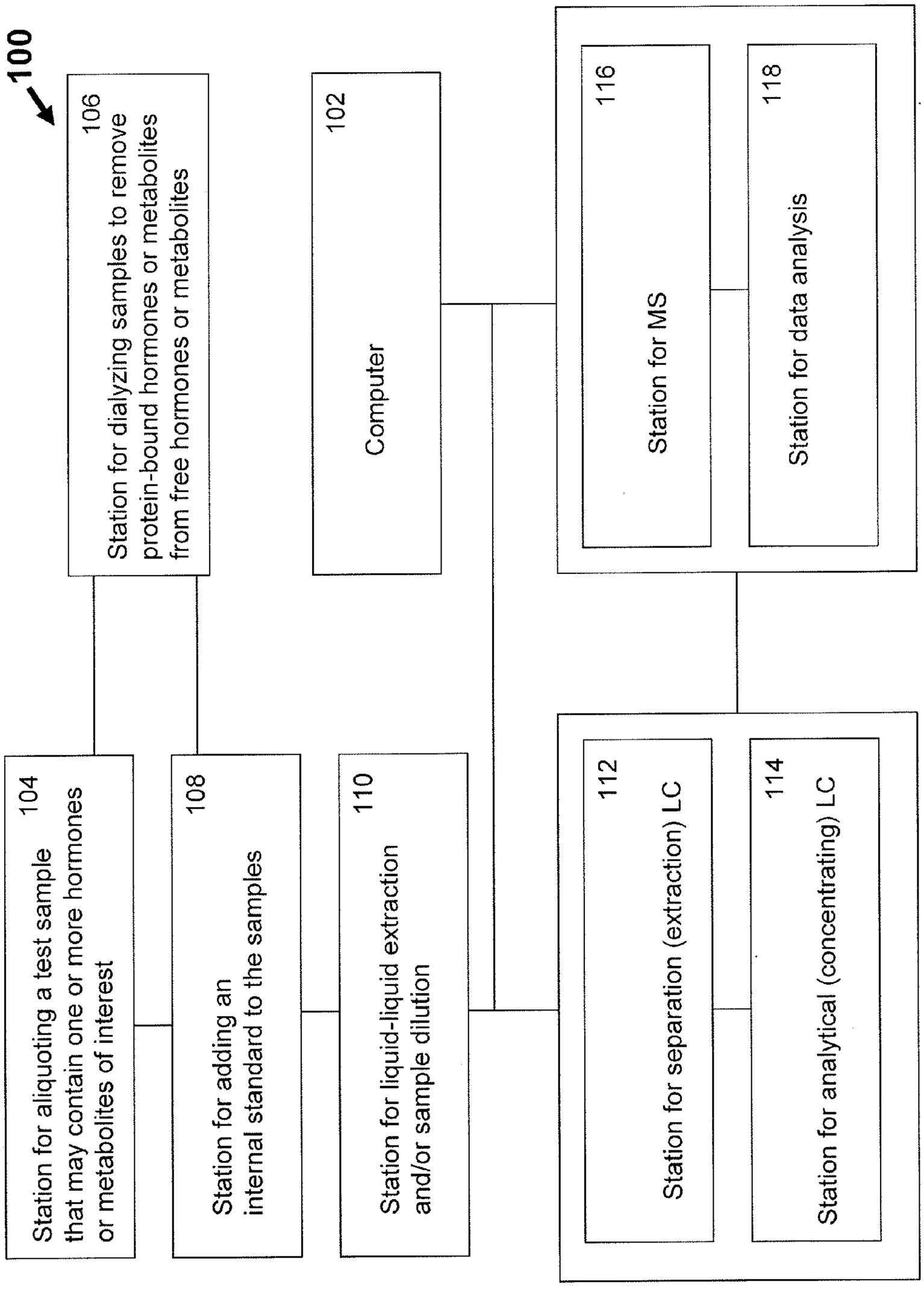

100
106
Station for dialyzing samples to remove protein-bound hormones or metabolites from free hormones or metabolites
102
Computer
116
Station for MS or MS/MS
118
Station for data analysis
104
Station for aliquoting a test sample that may contain one or more hormones or metabolites of interest
108
Station for adding an internal standard to the samples
110
Station for liquid-liquid extraction and/or sample dilution
112
Station for separation (extraction) LC
114
Station for analytical (concentrating) LC

FIG. 7A

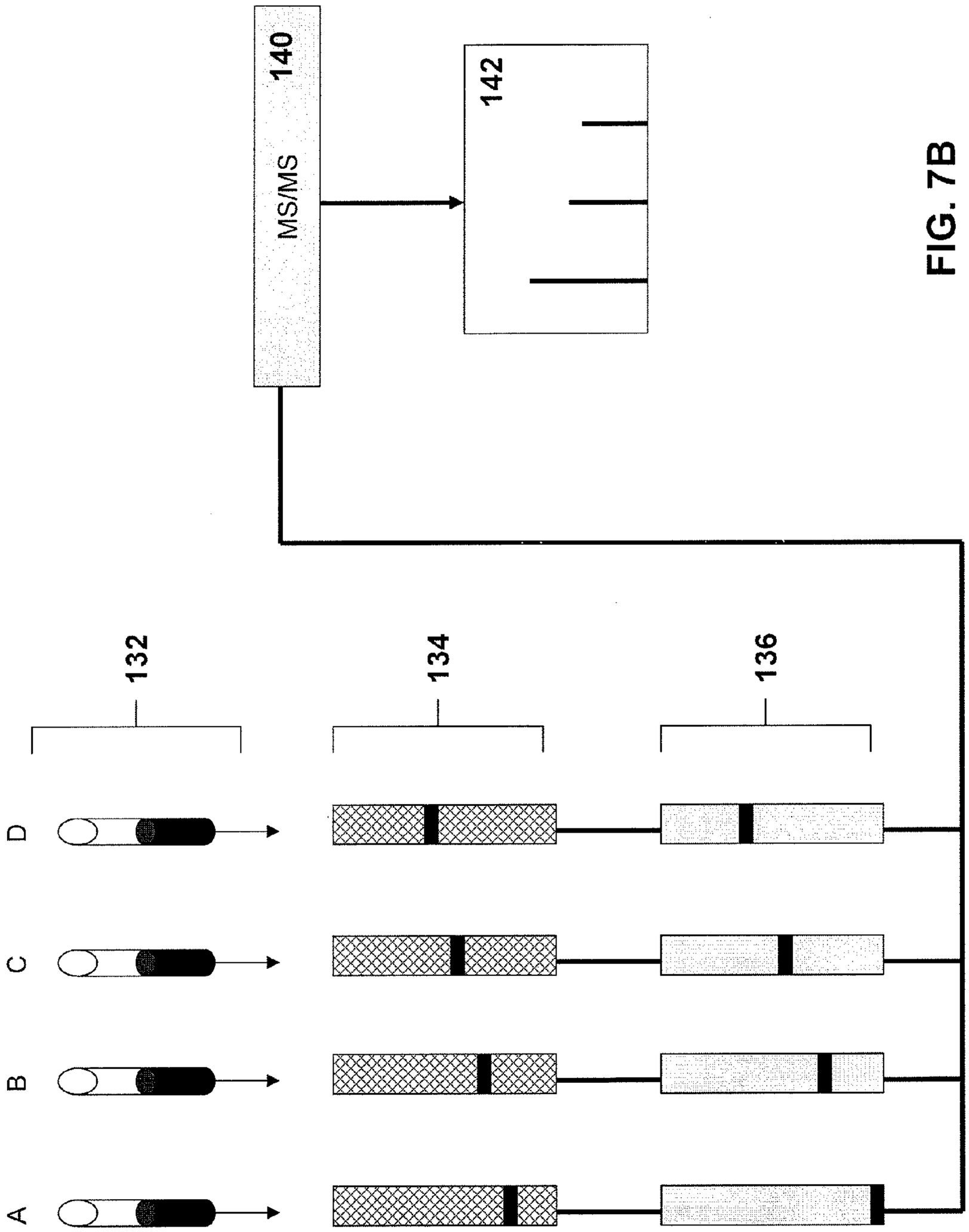


FIG. 7B

FIG. 8

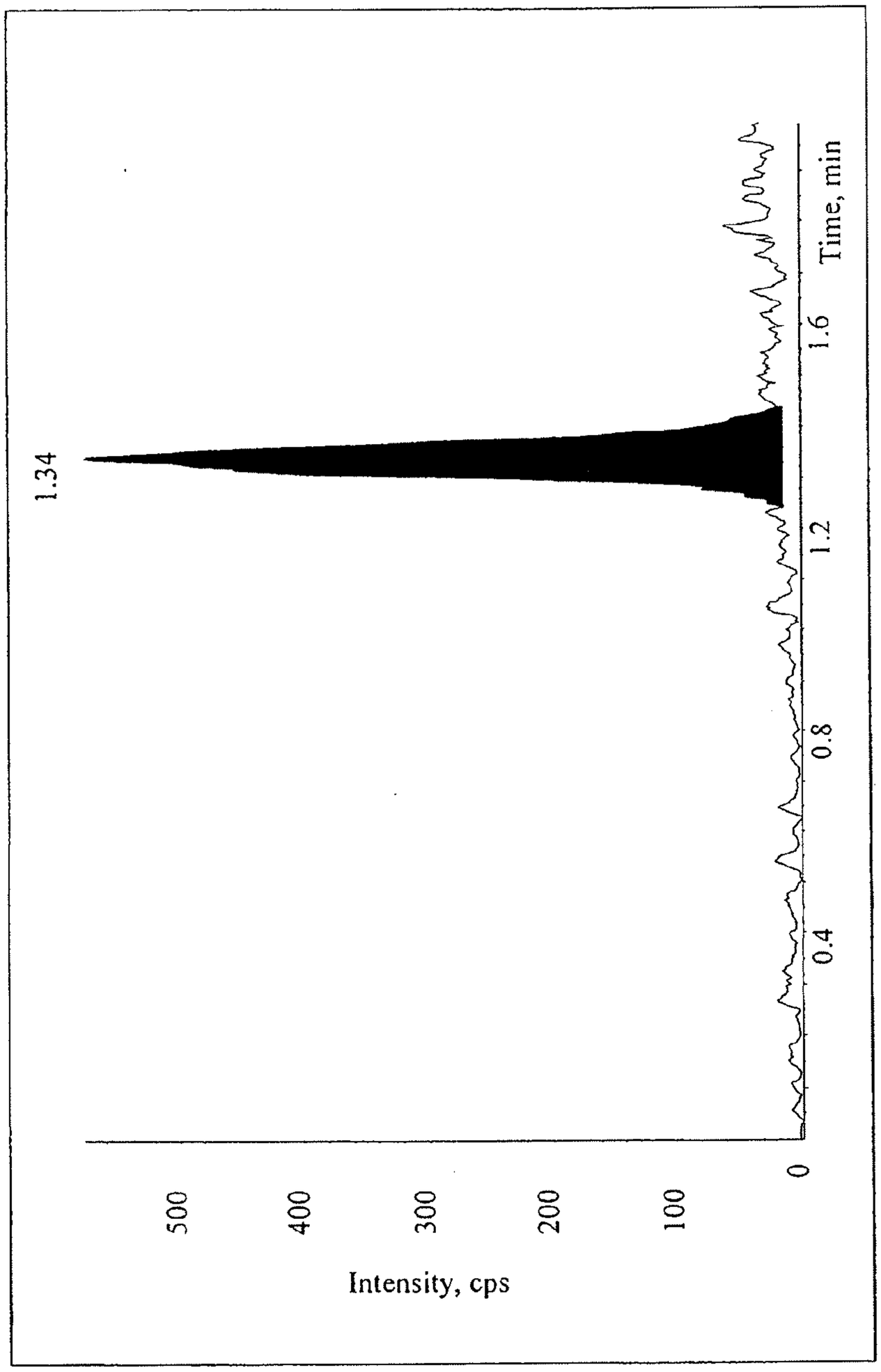

1.34
0.4
0.8
1.2
1.6
Time, min
0
100
200
300
400
500
Intensity, cps
Estrone Sulfate
LOQ (100 pg/mL)

FIG. 9

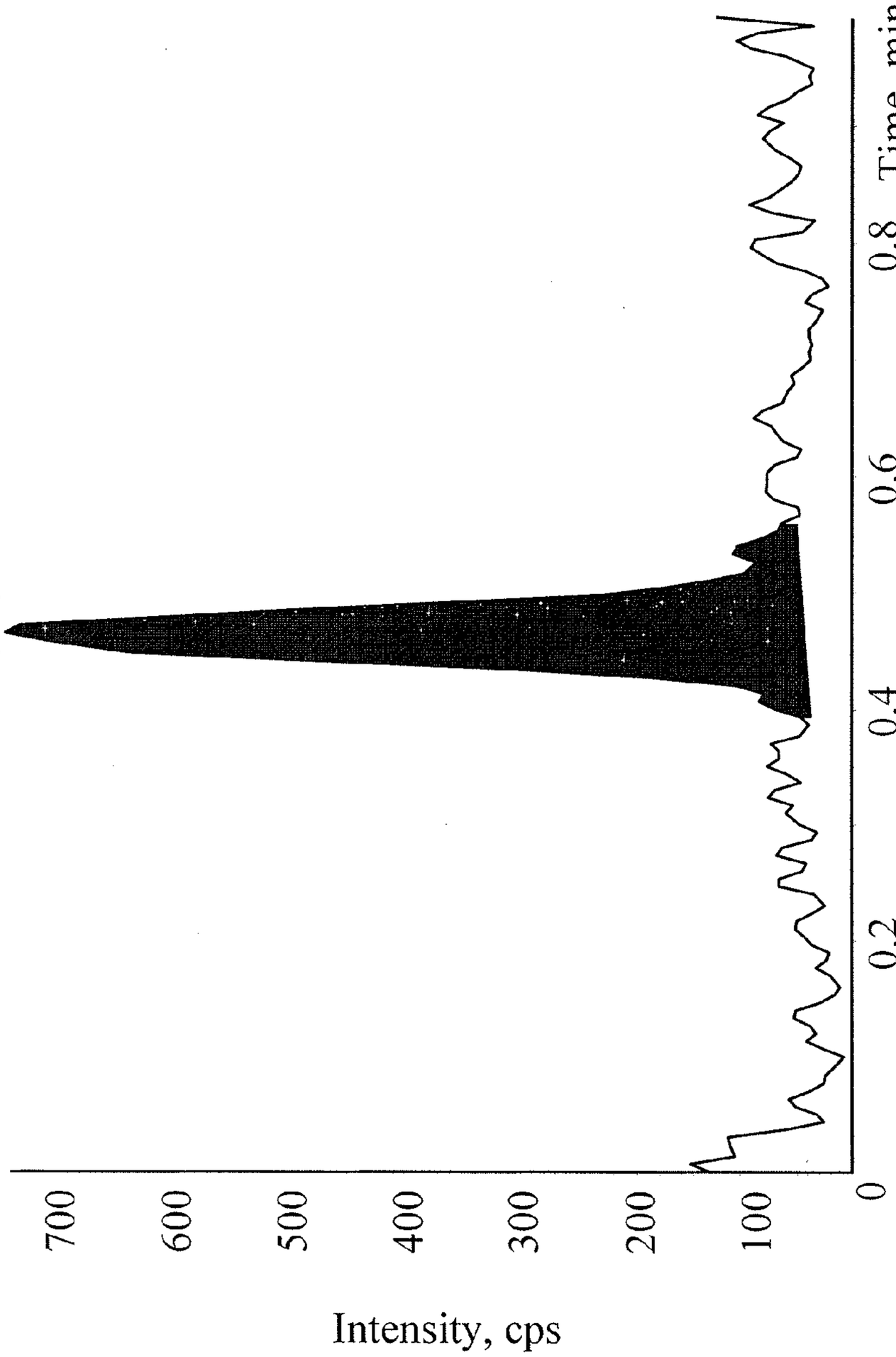

0.46
0.2
0.4
0.6
0.8
Time, min
Free Thyroxine
LOQ 2 pg/mL
700
600
500
400
300
200
100
0
Intensity, cps

FIG. 10

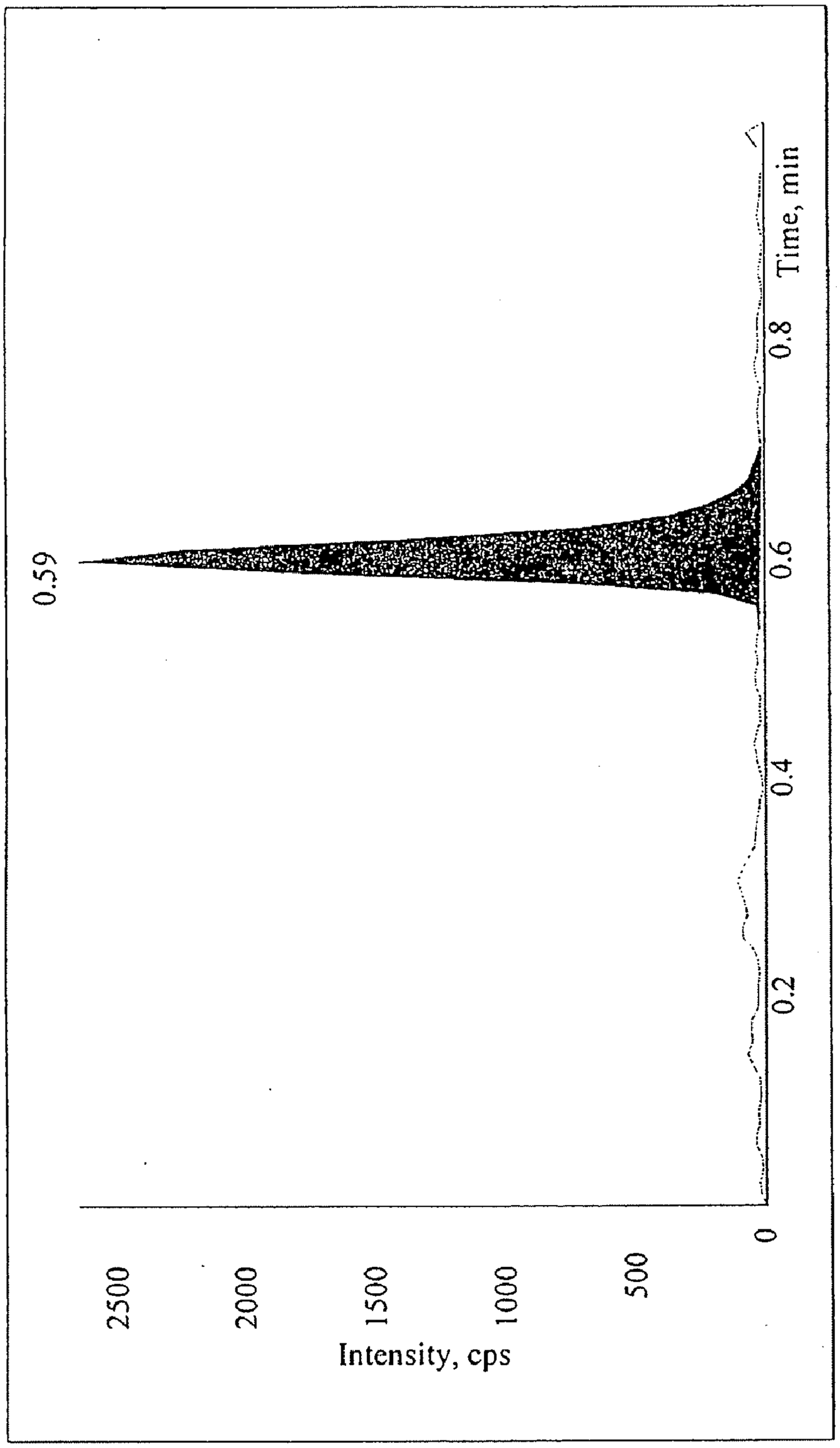

25-Hydroxyvitamin D2
LOQ (1 ng/mL)
Time, min
0.59
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
2500
Intensity, cps

FIG. 11

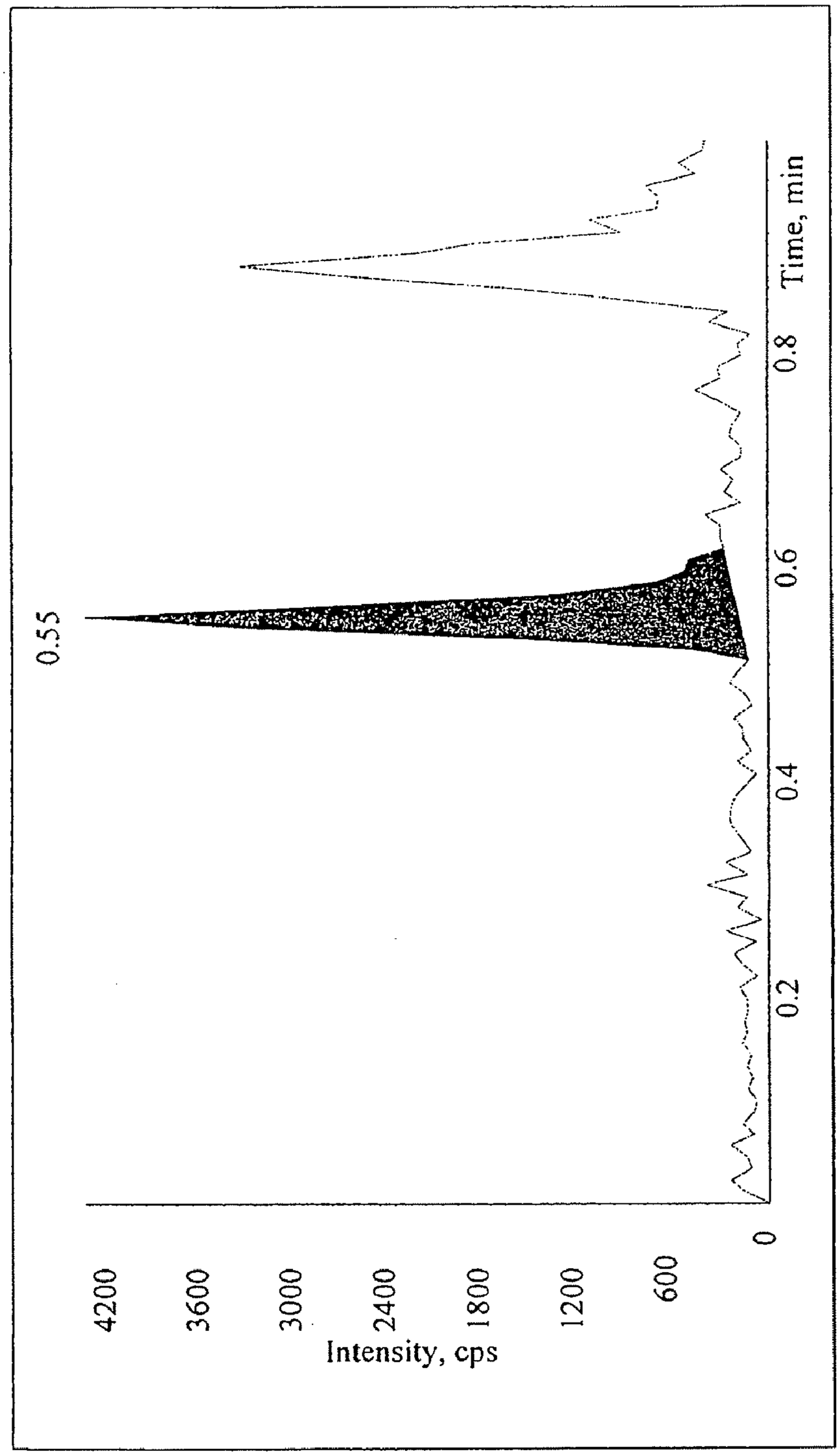

Time, min
0.8
0.6
0.4
0.2
0
0.55
25-Hydroxyvitamin D3
LOQ (1 ng/mL)
Intensity, cps
4200
3600
3000
2400
1800
1200
600

FIG. 12

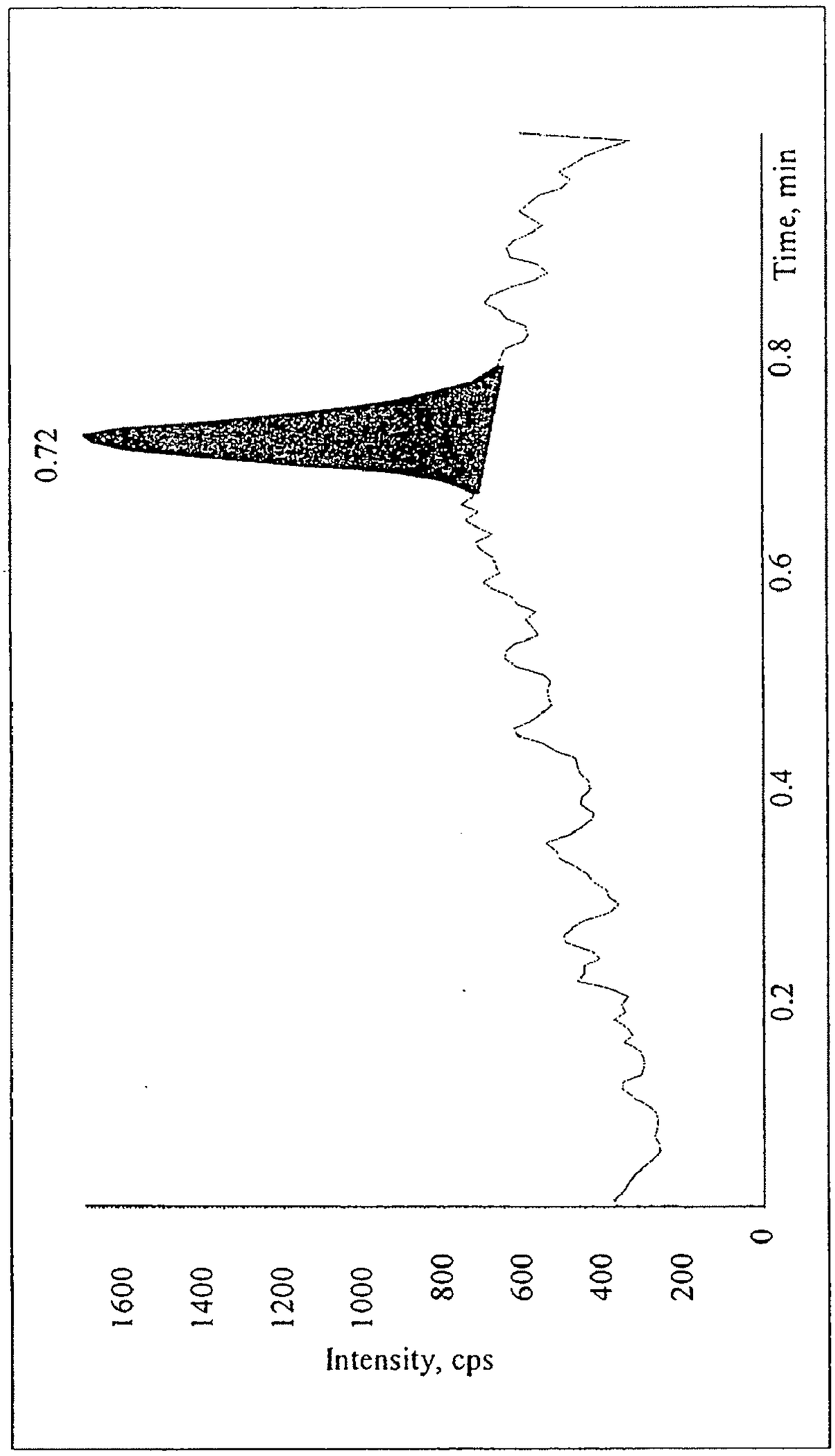

0.72
Time, min
0.2
0.4
0.6
0.8
Estrone
LOQ (2.5 pg/mL)
0
200
400
600
800
1000
1200
1400
1600
Intensity, cps

FIG. 13

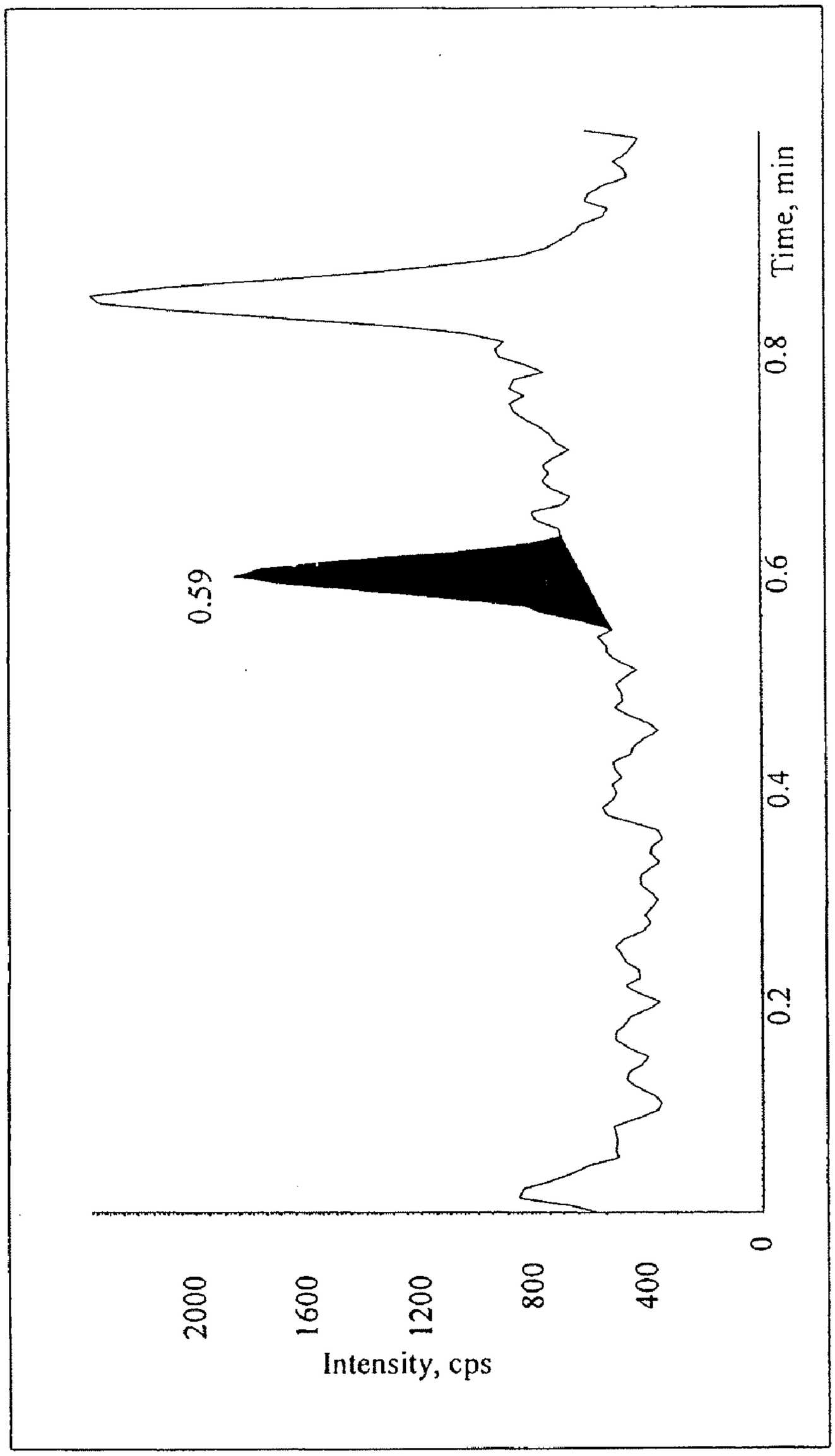

Time, min
0.8
0.6
0.4
0.2
0
0.59
Estradiol
LOQ (1 pg/mL)
2000
1600
1200
800
400
Intensity, cps

FIG. 14

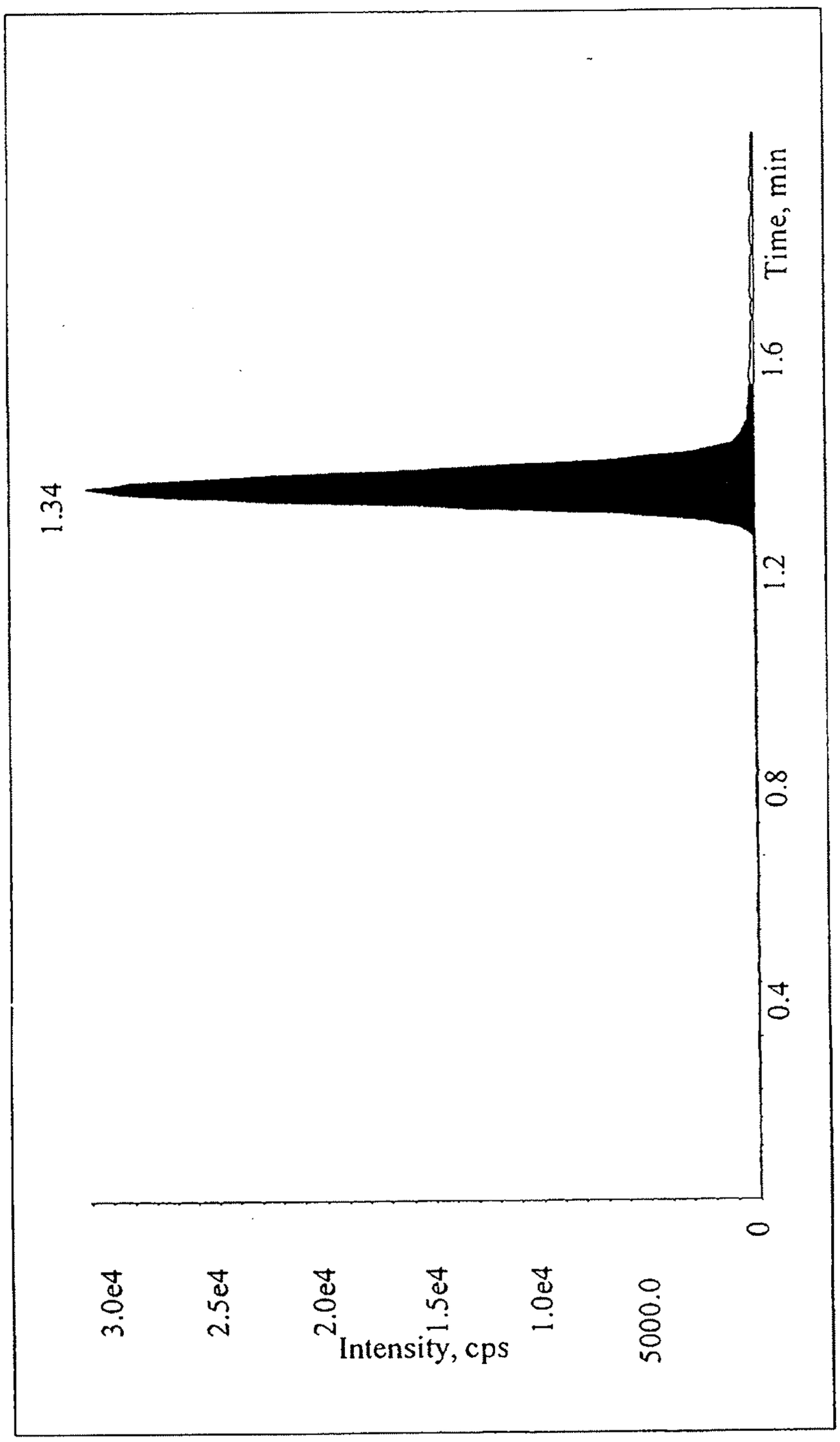

Time, min
1.34
1.6
1.2
0.8
0.4
0
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
Intensity, cps
Estrone Sulfate
ULOQ (50 ng/mL)

FIG. 15

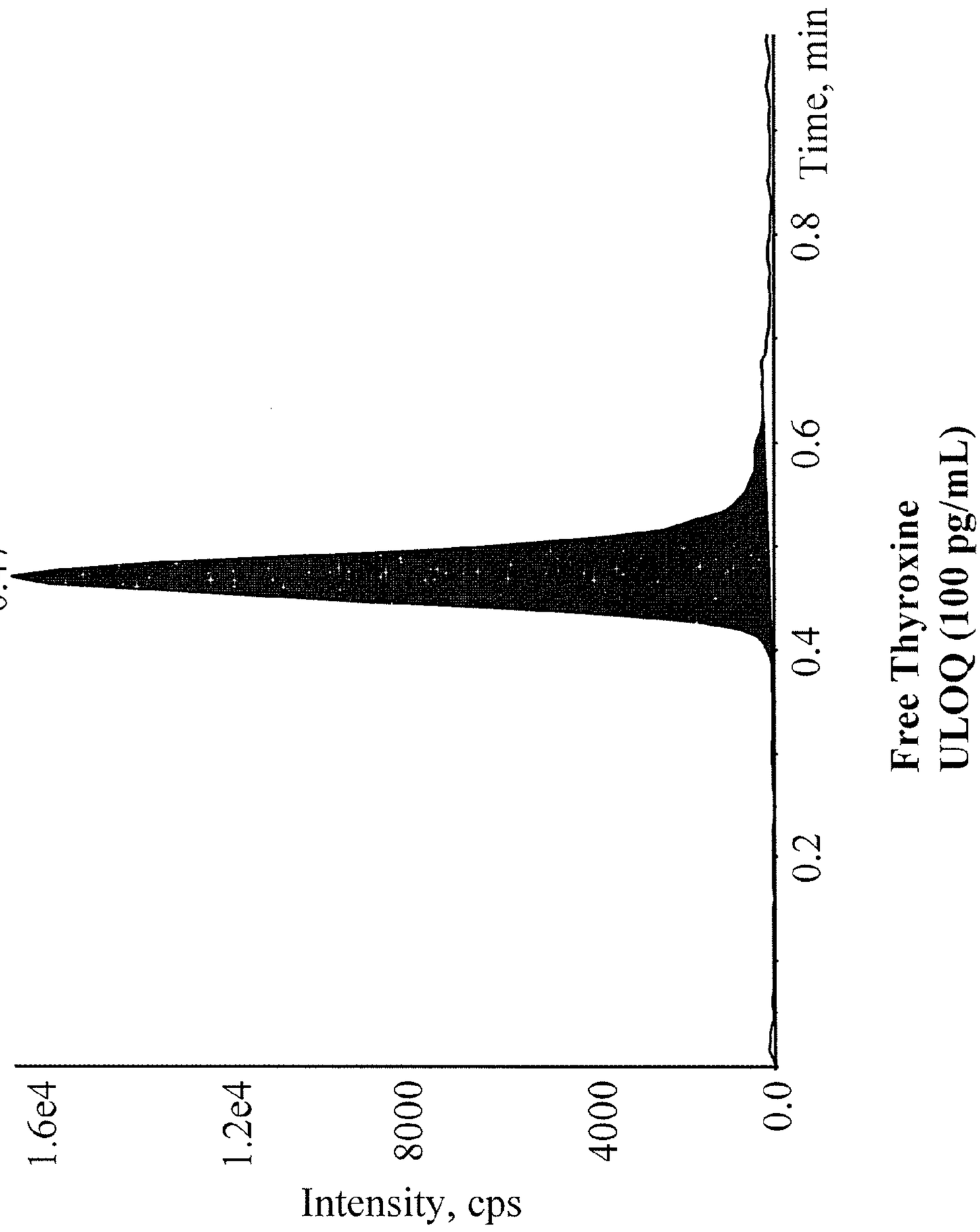

Free Thyroxine
ULOQ (100 pg/mL)
0.47
0.2
0.4
0.6
0.8
Time, min
1.6e4
1.2e4
8000
4000
0.0
Intensity, cps

FIG. 16

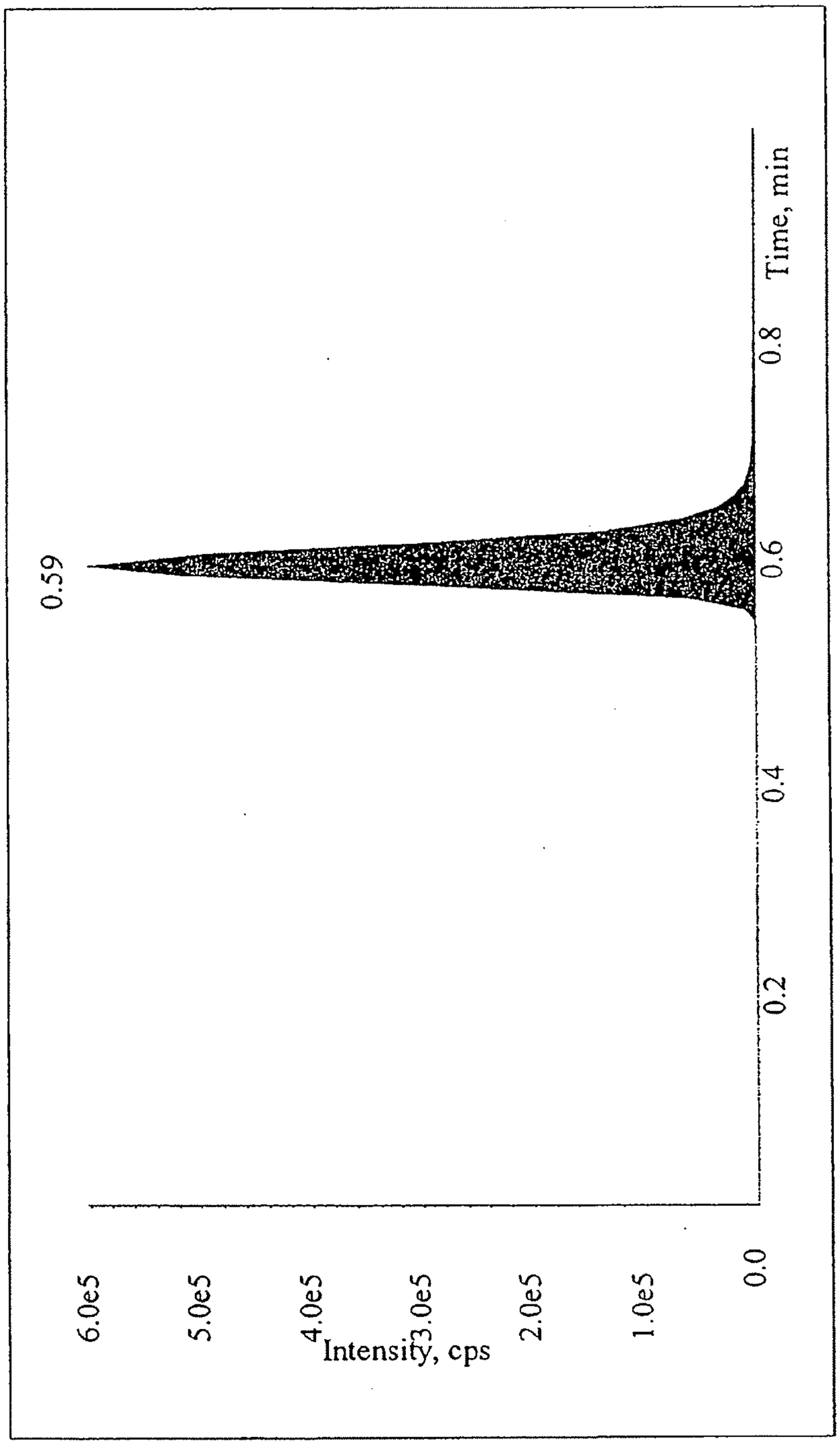

0.59
0.2
0.4
0.6
0.8
Time, min
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
Intensity, cps
25-Hydroxyvitamin D2
ULOQ (250ng/mL)

FIG. 17

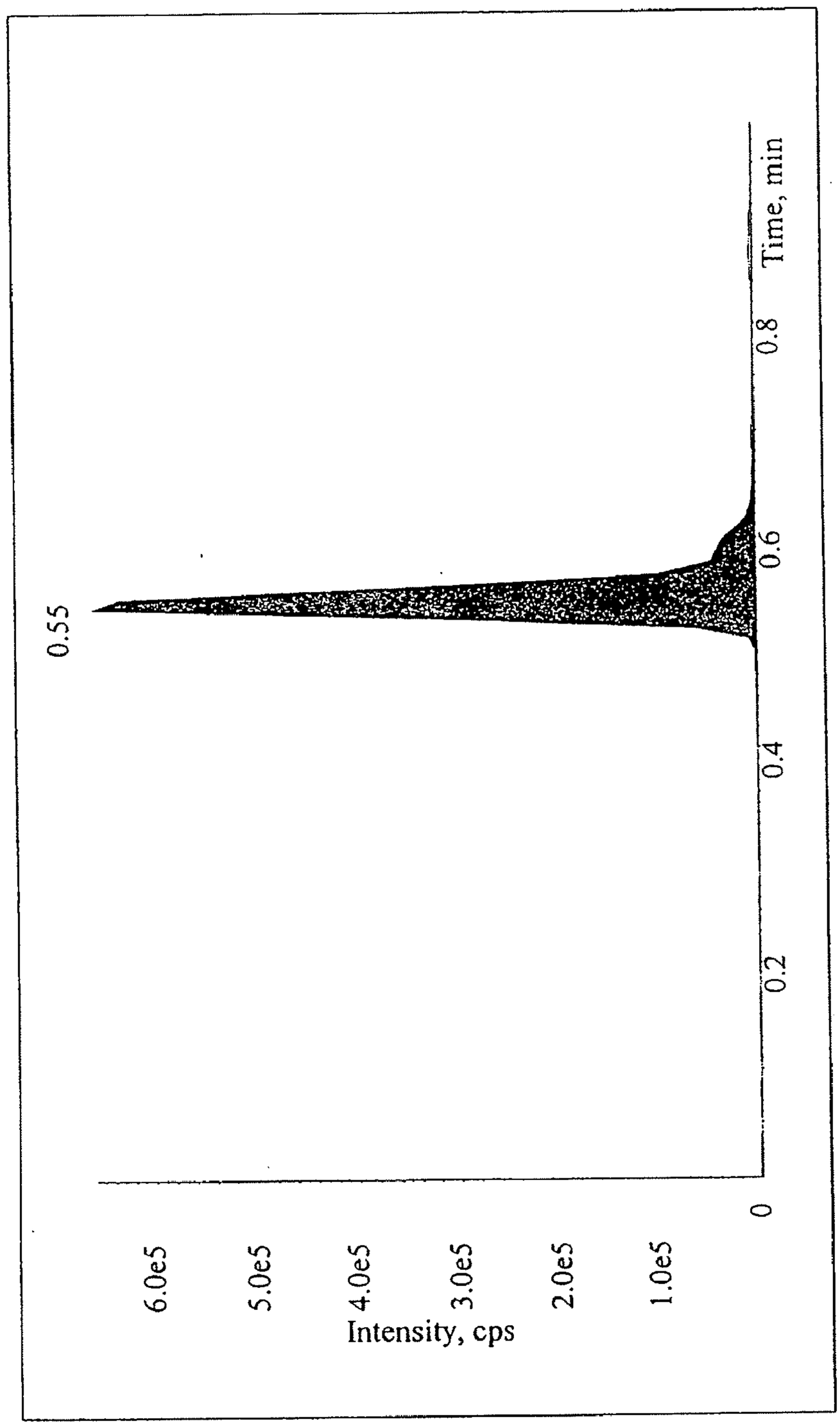

Time, min
0.55
0.2
0.4
0.6
0.8
0
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
Intensity, cps
25-Hydroxyvitamin D3
ULOQ (250 ng/mL)

FIG. 18

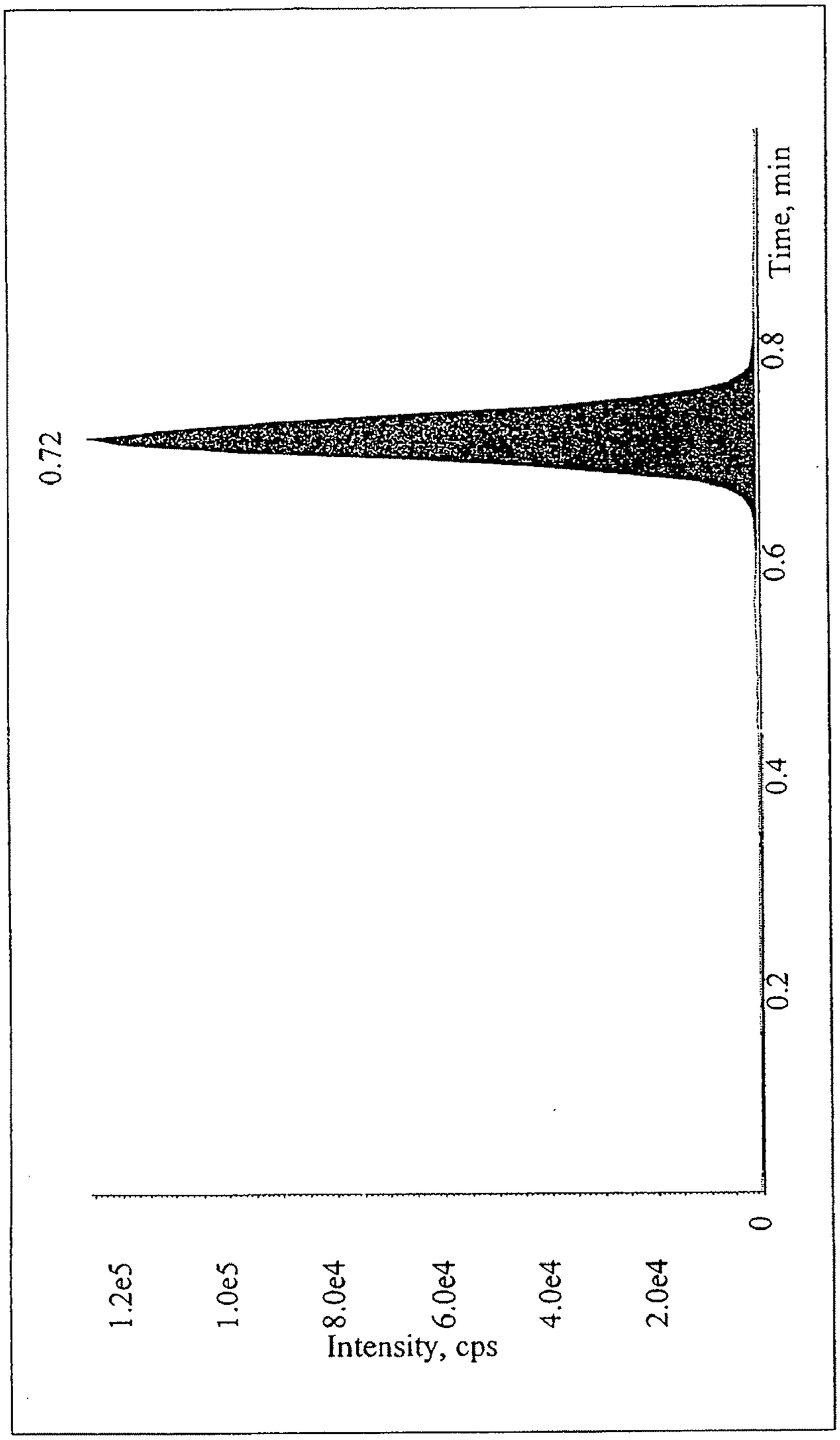

Time, min
0.72
0.2
0.4
0.6
0.8
0
1.2e5
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
Intensity, cps
Estrone
ULOQ (500 pg/mL)

FIG. 19

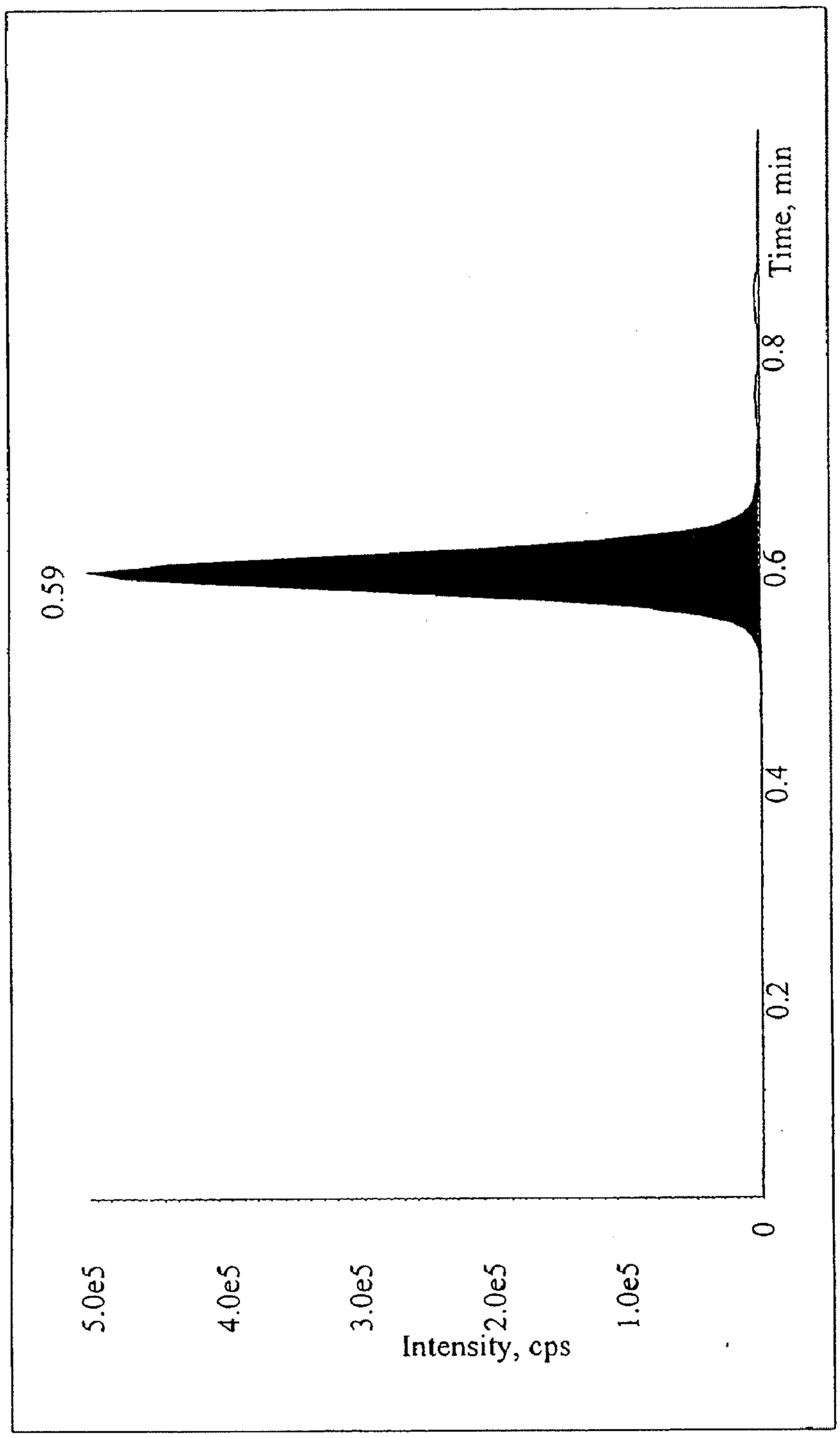

Estradiol
ULOQ (500 pg/mL)
0.59
Time, min
0.2
0.4
0.6
0.8
0
1.0e5
2.0e5
3.0e5
4.0e5
5.0e5
Intensity, cps

FIG. 20

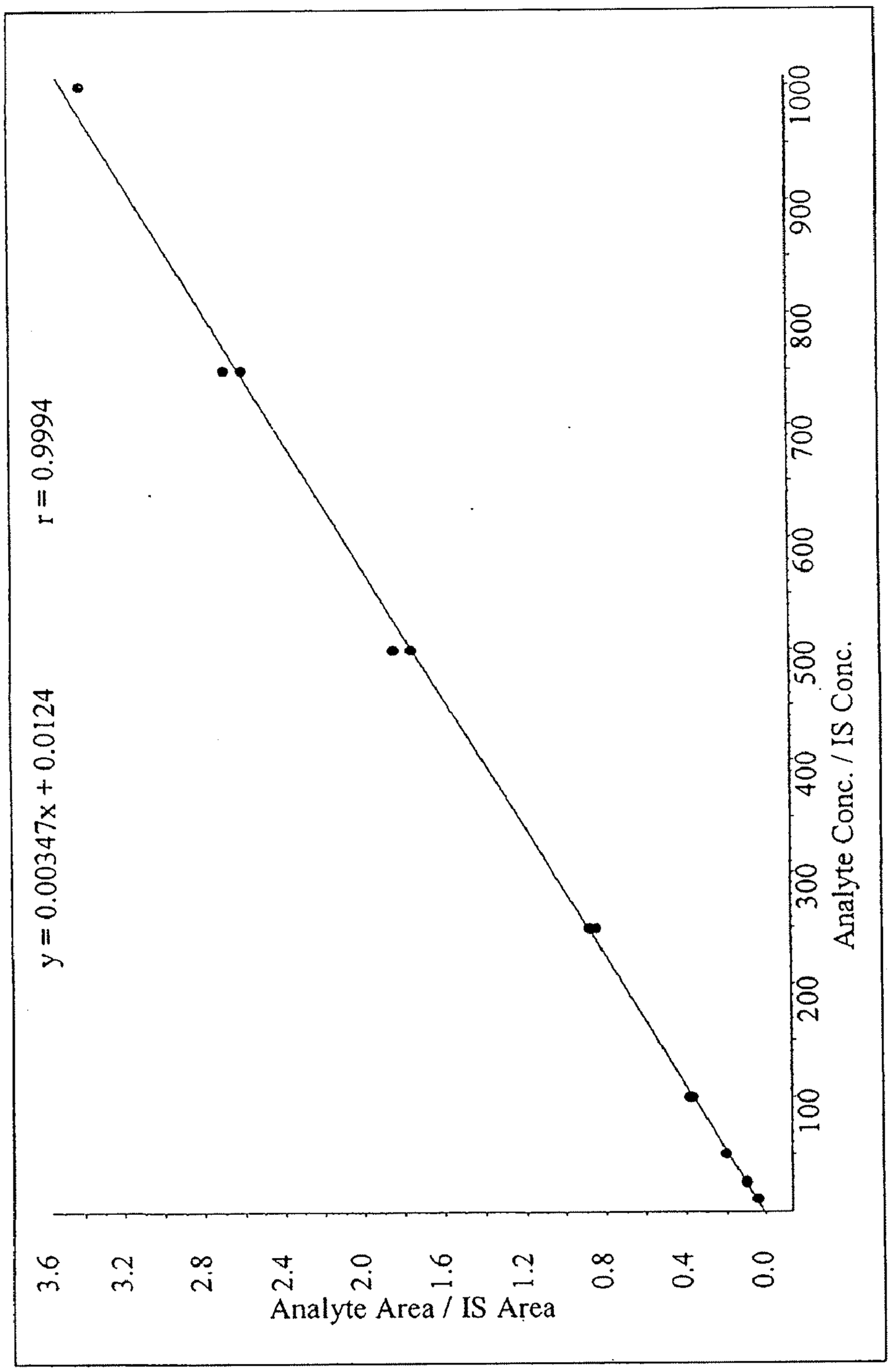
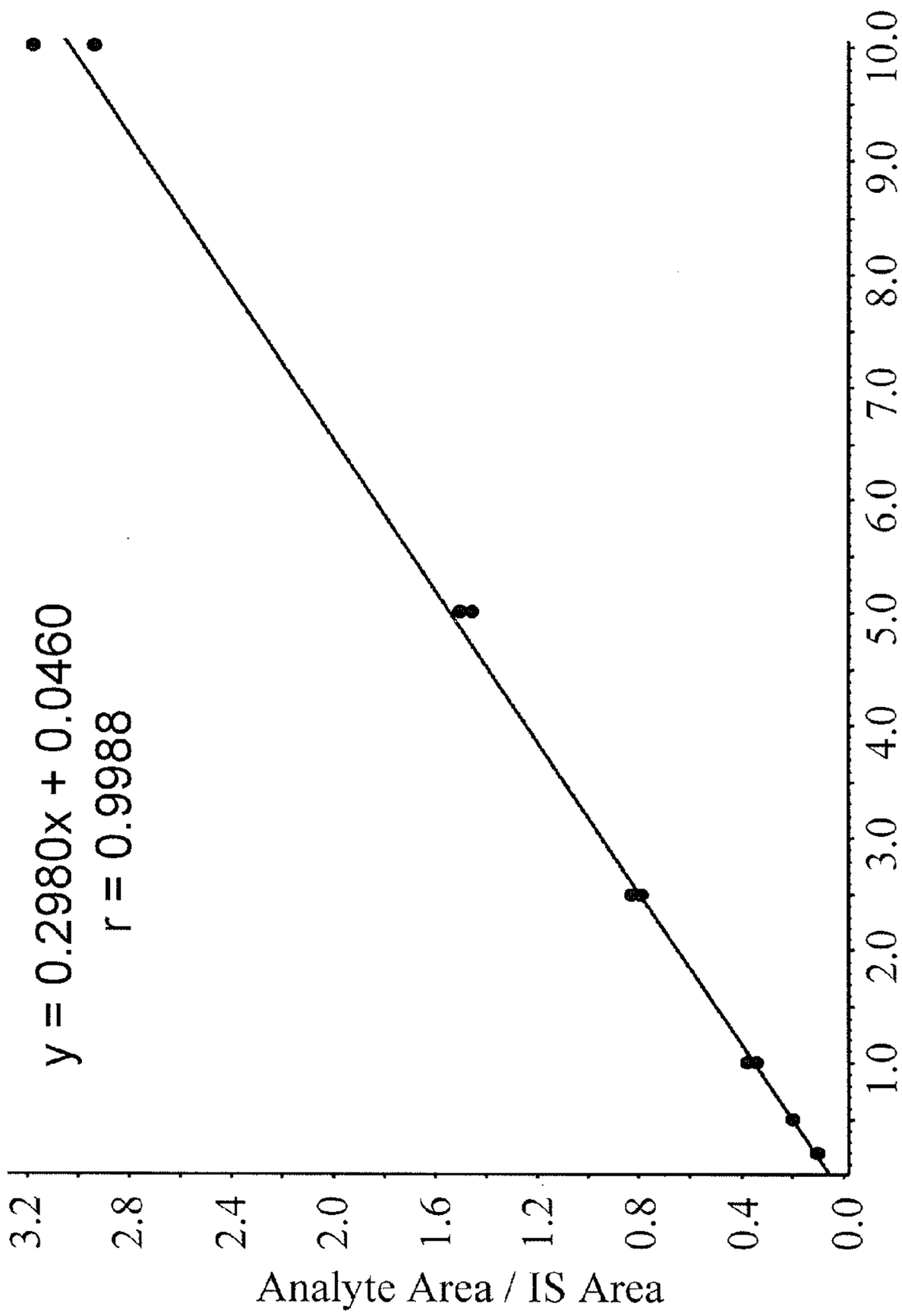
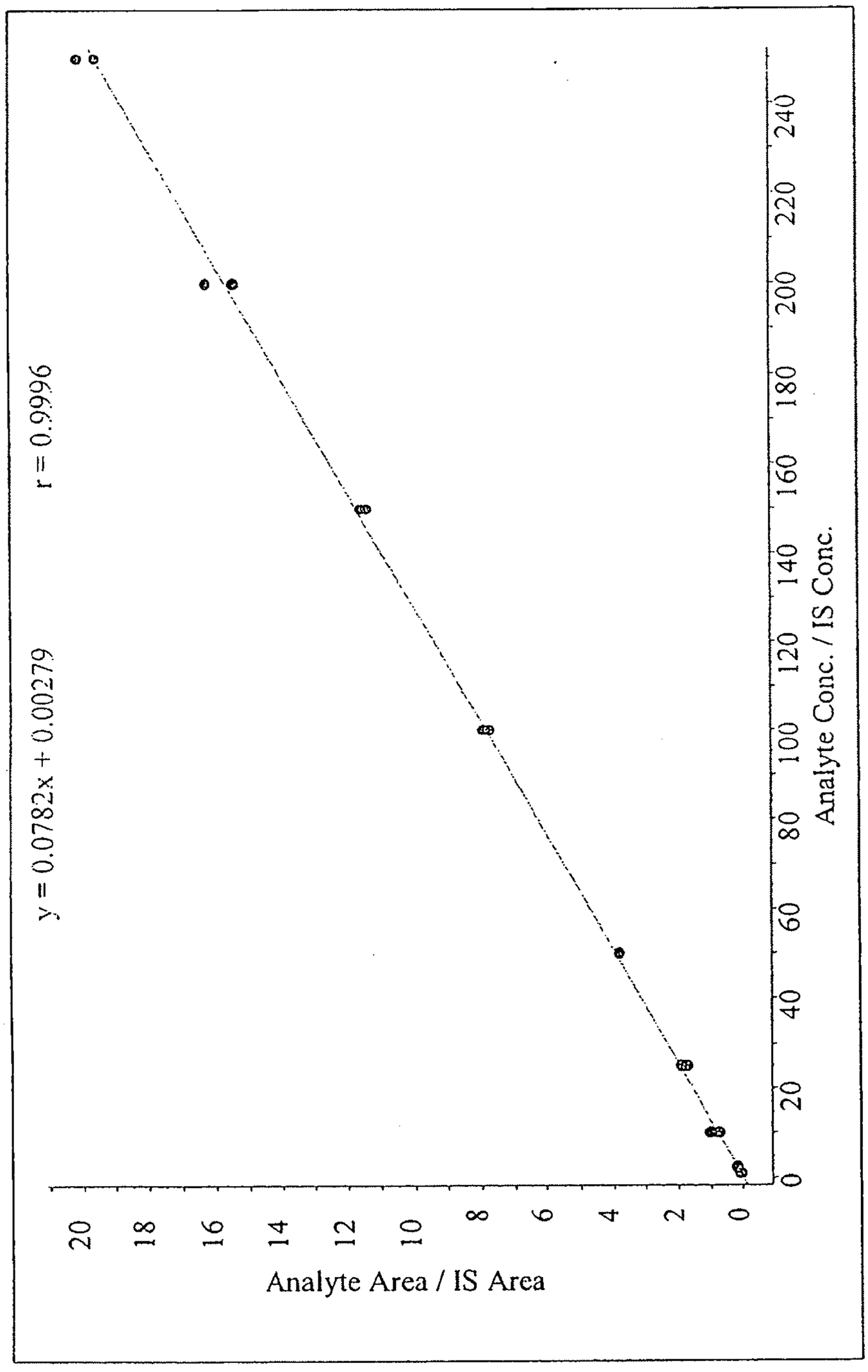

y = 0.00347x + 0.0124
r = 0.9994
Analyte Area / IS Area
Analyte Conc. / IS Conc.
Estrone Sulfate

FIG. 21



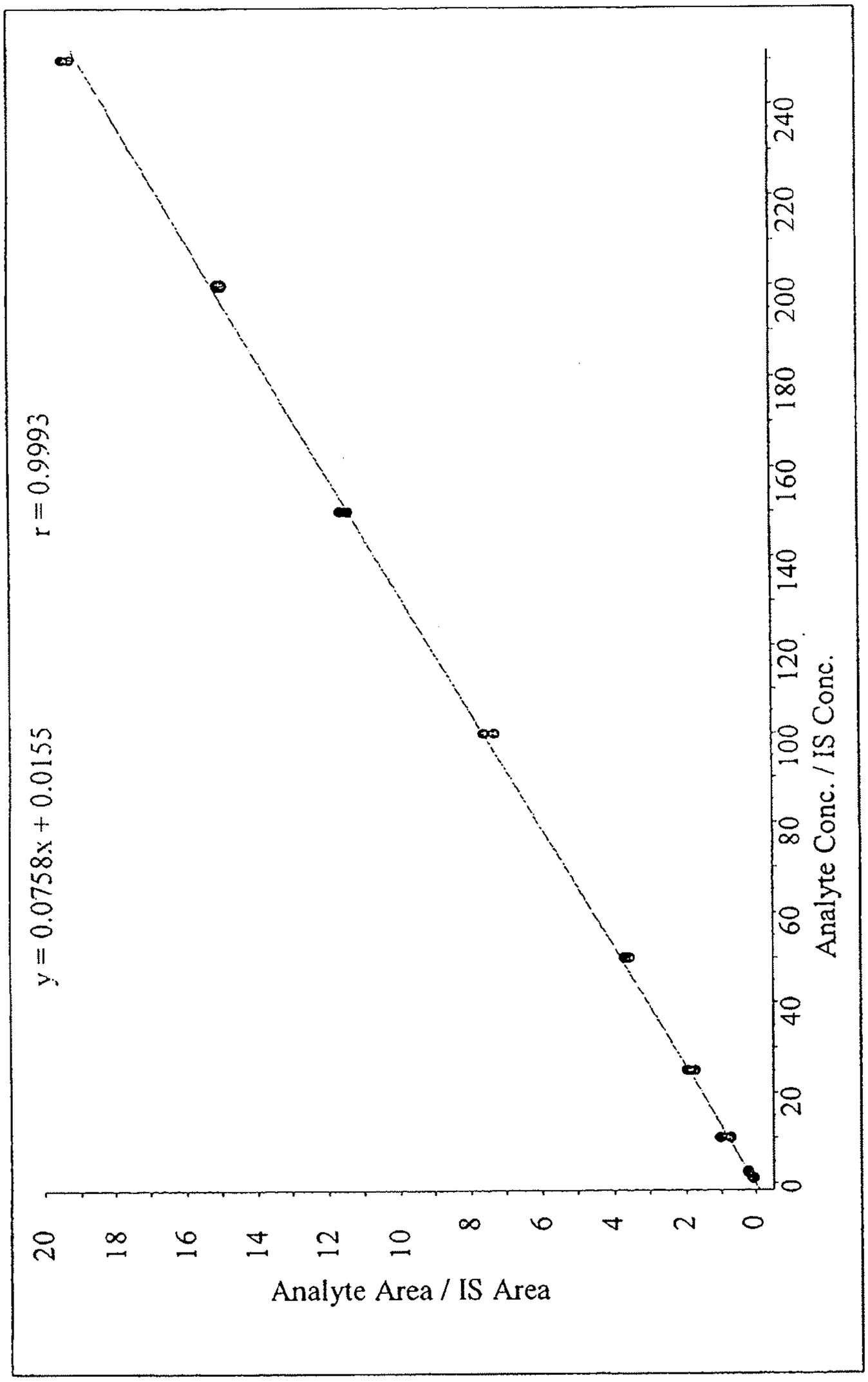
Free Thyroxine

FIG. 22



25-Hydroxyvitamin D2

FIG. 23



25-Hydroxyvitamin D3

FIG. 24

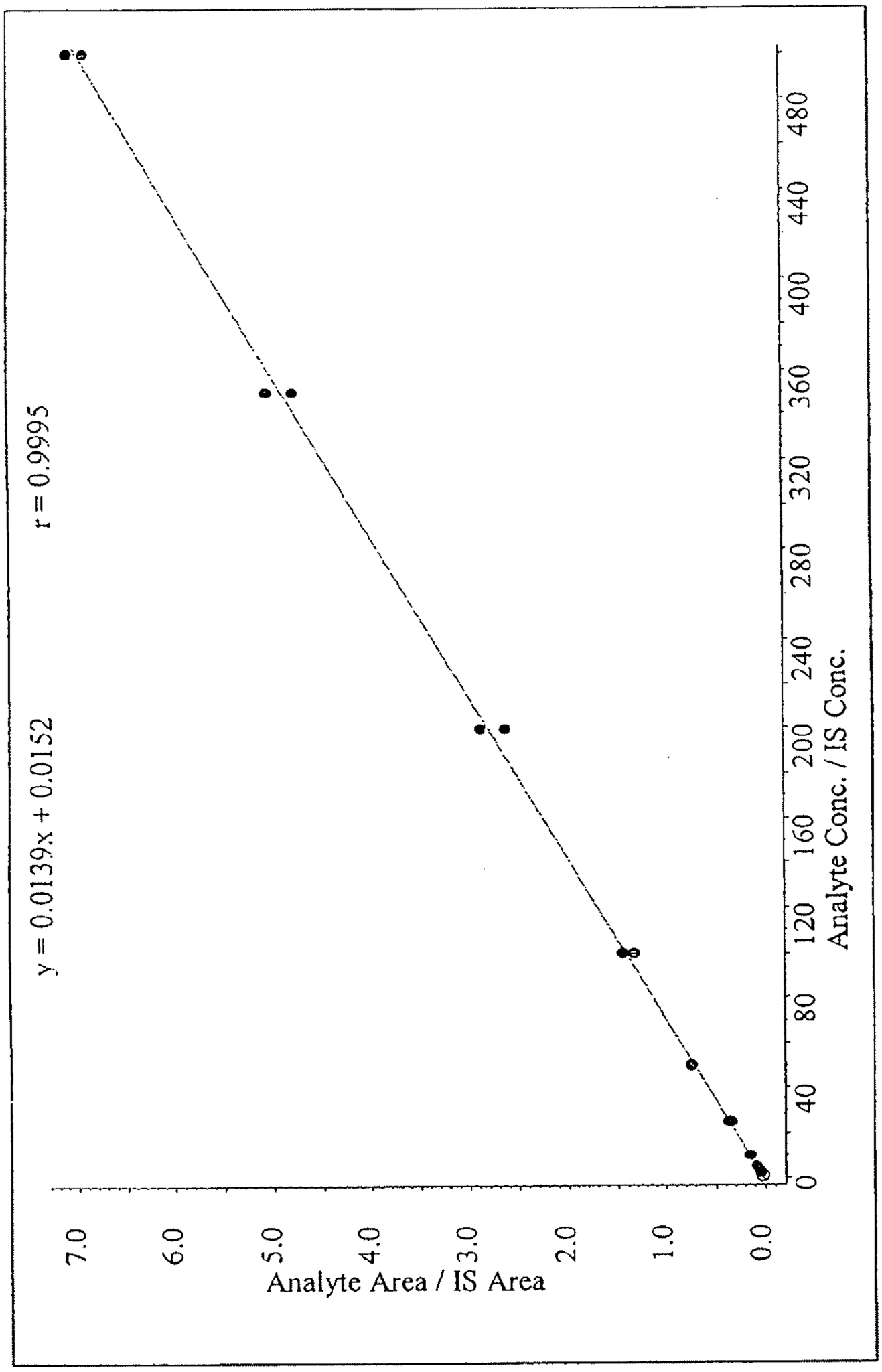

y = 0.0139x + 0.0152
r = 0.9995
Analyte Area / IS Area
Analyte Conc. / IS Conc.
Estrone

FIG. 25

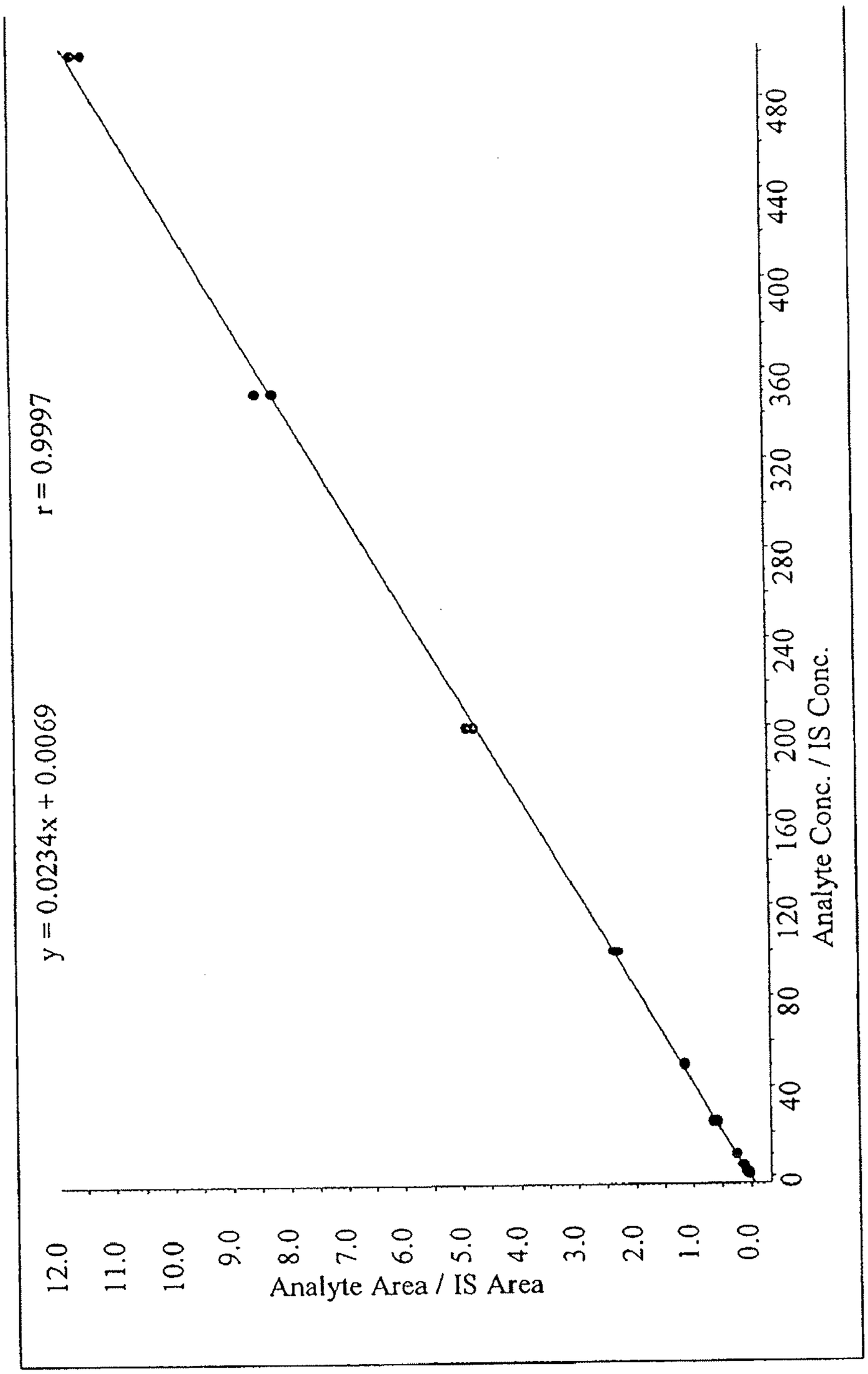

y = 0.0234x + 0.0069
r = 0.9997
Analyte Area / IS Area
Analyte Conc. / IS Conc.
Estradiol

FIG. 26

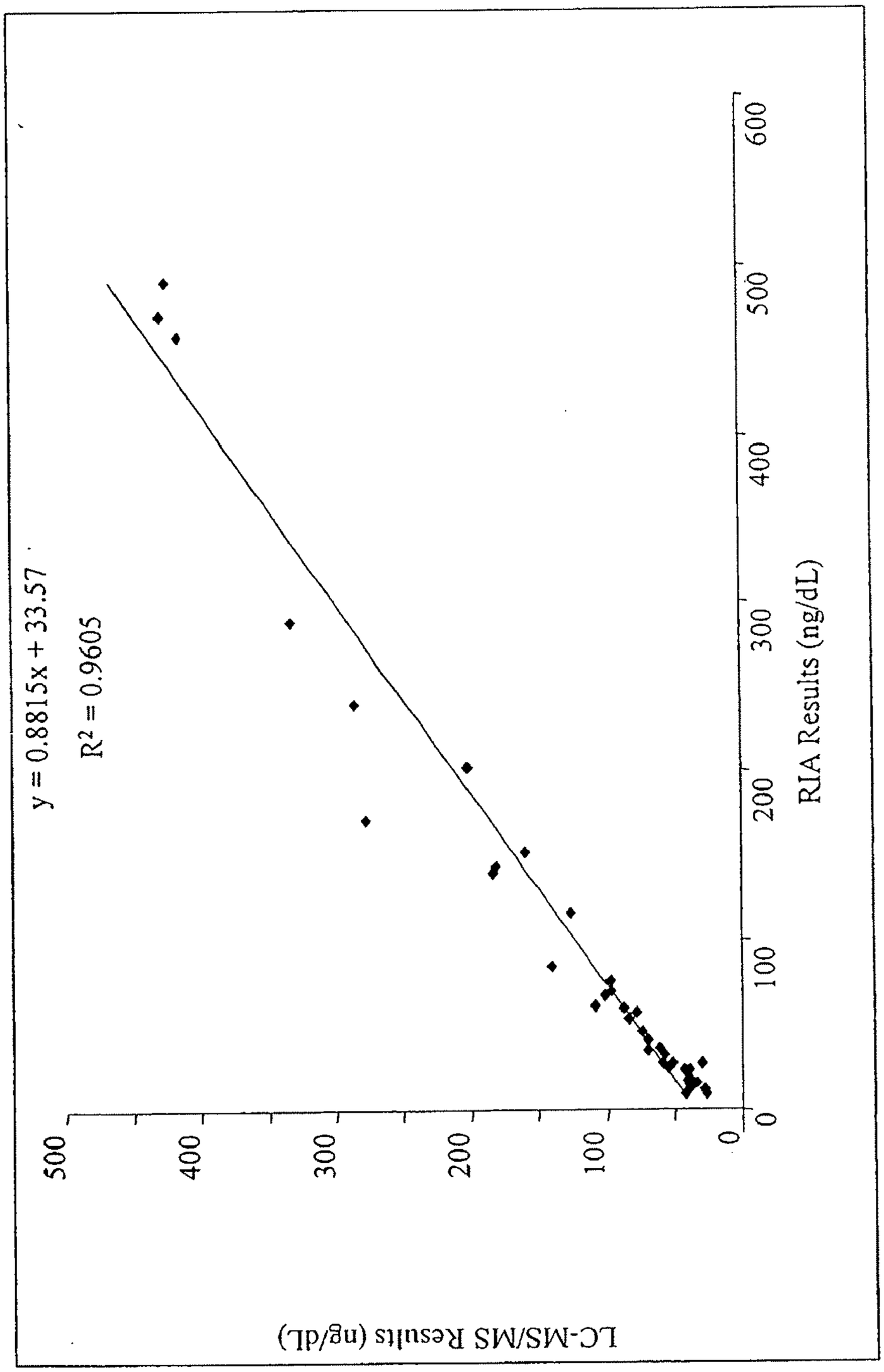
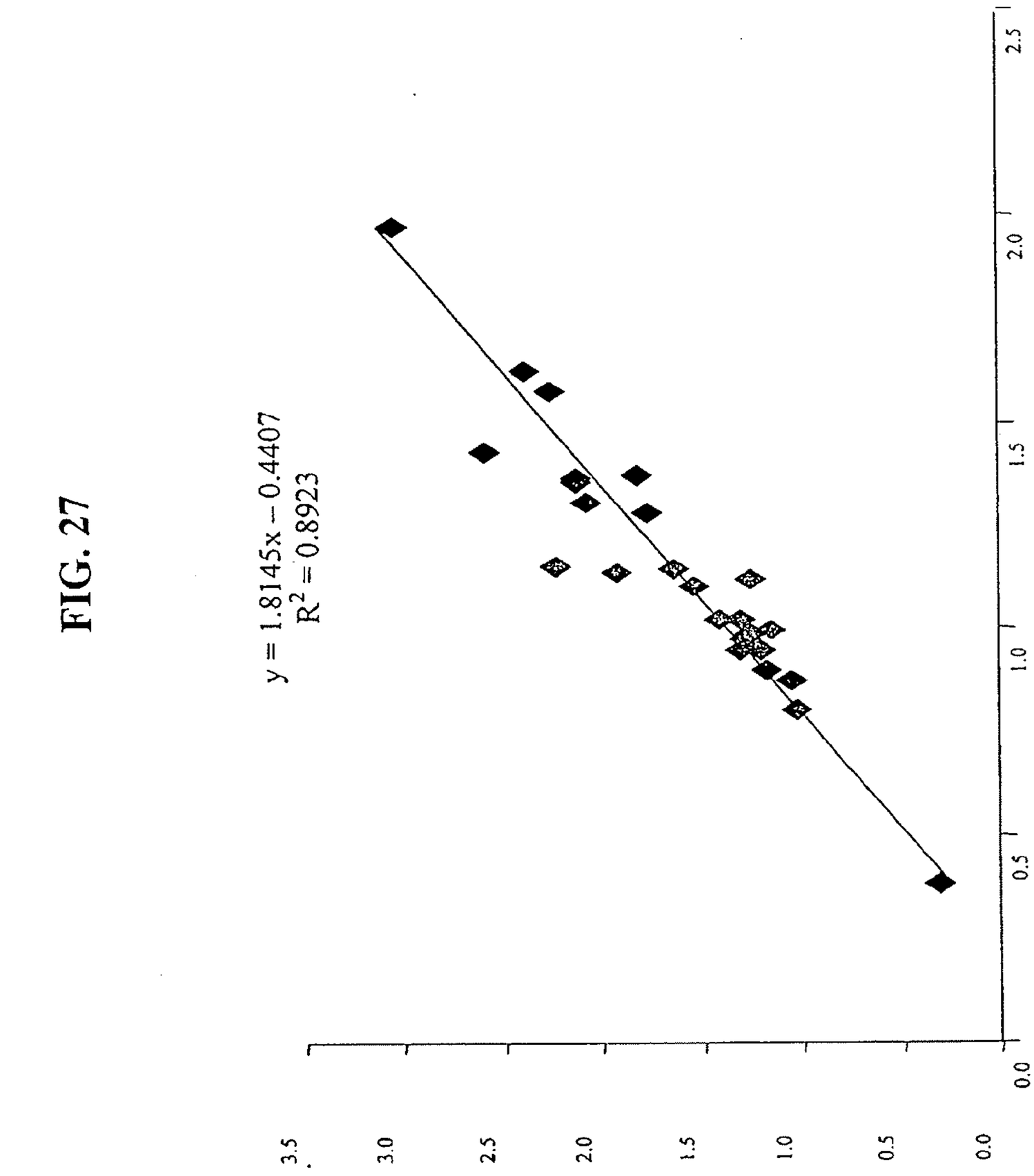
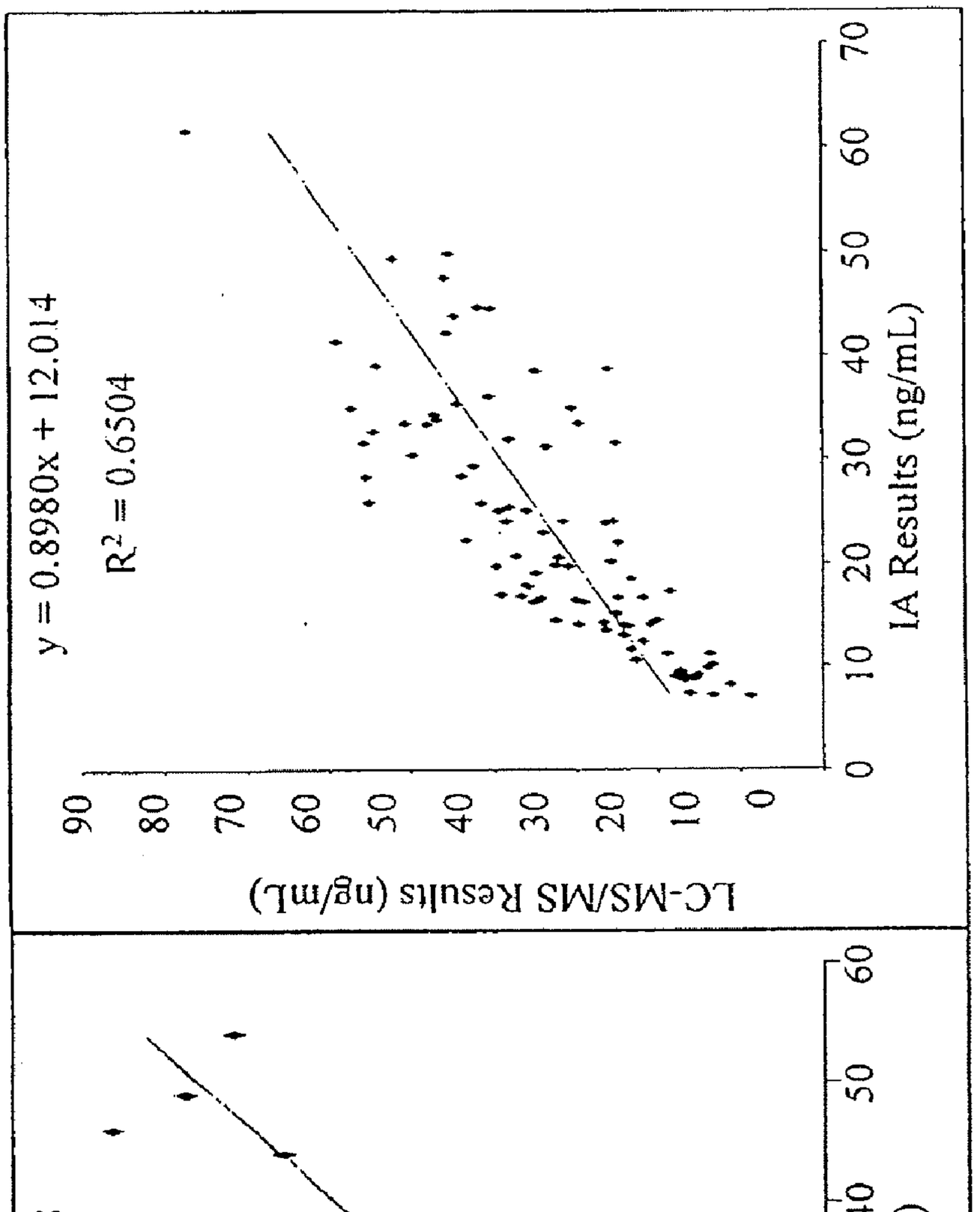

y = 0.8815x + 33.57
R² = 0.9605
LC-MS/MS Results (ng/dL)
RIA Results (ng/dL)
Estrone Sulfate
0
100
200
300
400
500
600

FIG. 27



Free Thyroxine

FIG. 28A



Total 25-Hydroxyvitamin D (25-Hydroxyvitamin D2+D3

FIG. 28B

FIG. 29

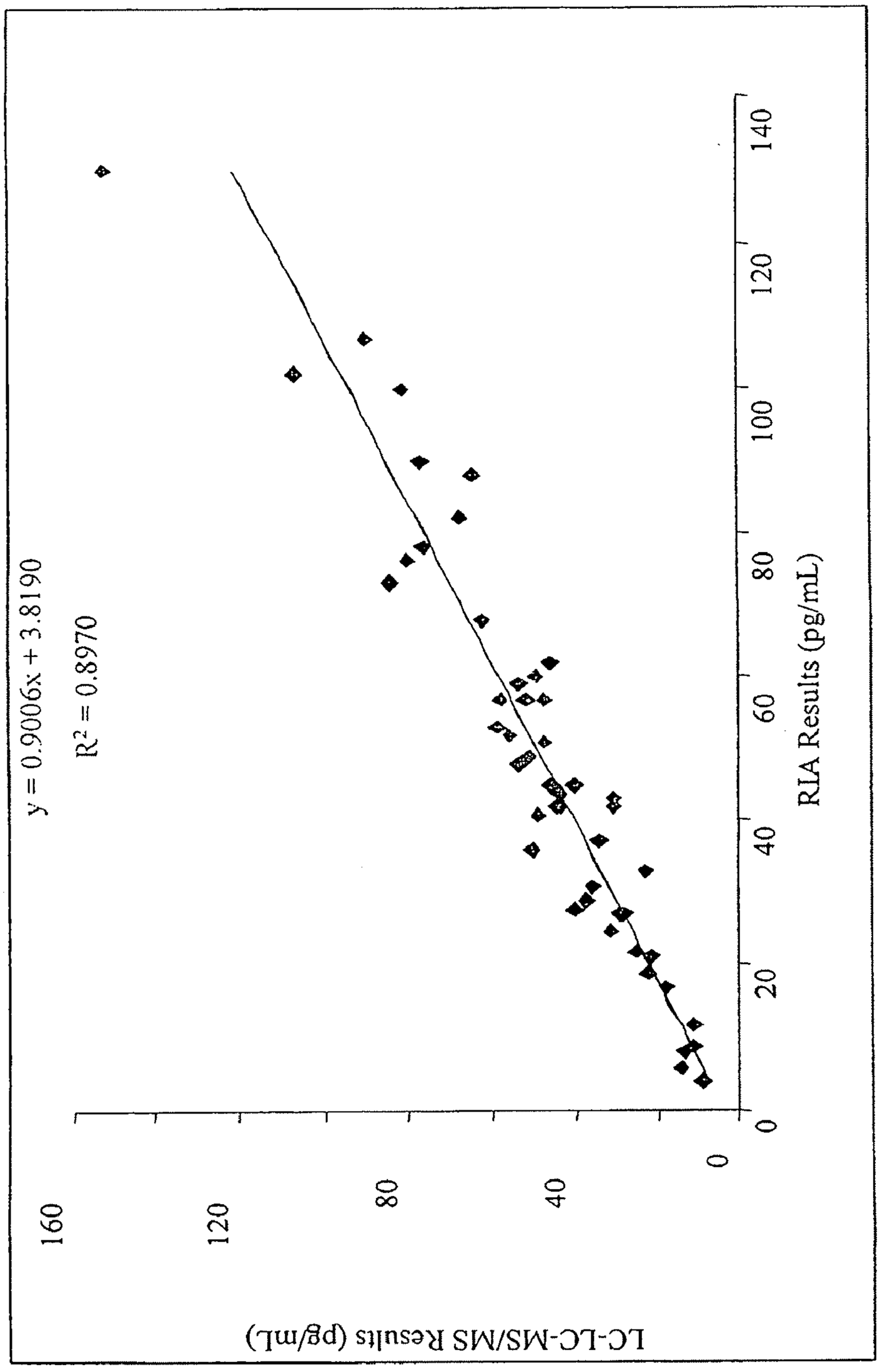

y = 0.9006x + 3.8190
R² = 0.8970
RIA Results (pg/mL)
LC-LC-MS/MS Results (pg/mL)
Estrone
0
20
40
60
80
100
120
140
160

FIG. 30

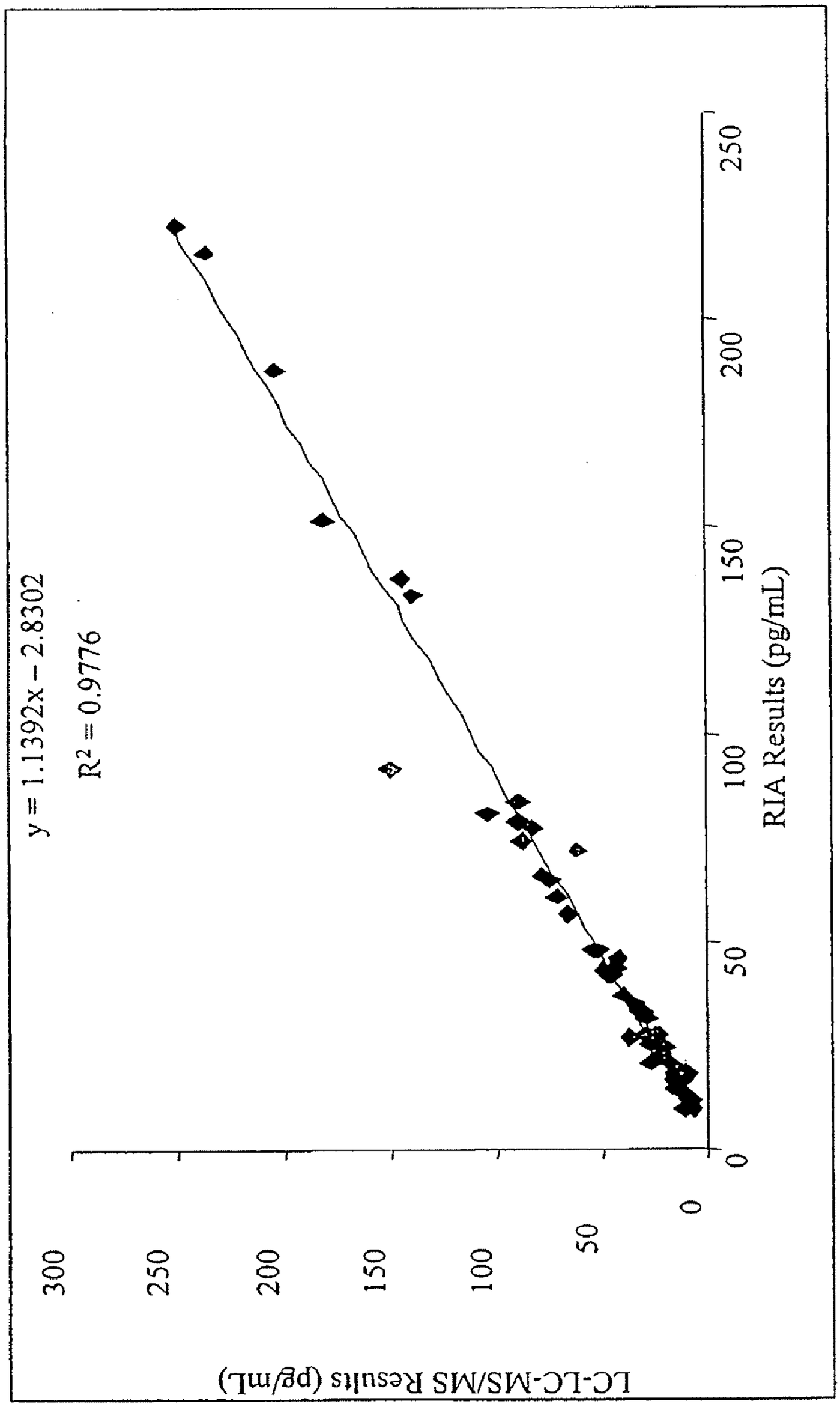

y = 1.1392x – 2.8302
R² = 0.9776
LC-LC-MS/MS Results (pg/mL)
RIA Results (pg/mL)
0
50
100
150
200
250
300
Estradiol

FIG. 31

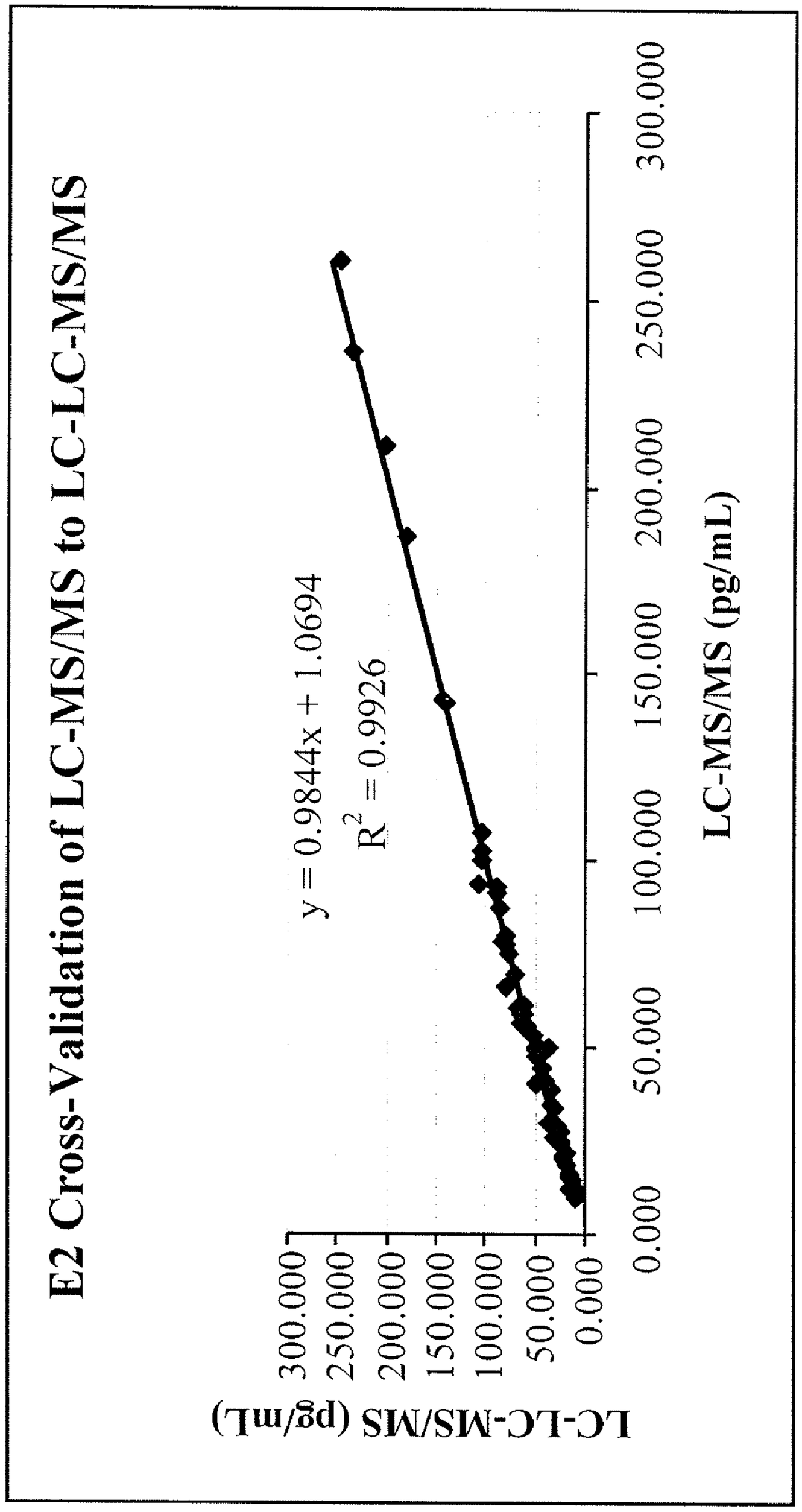
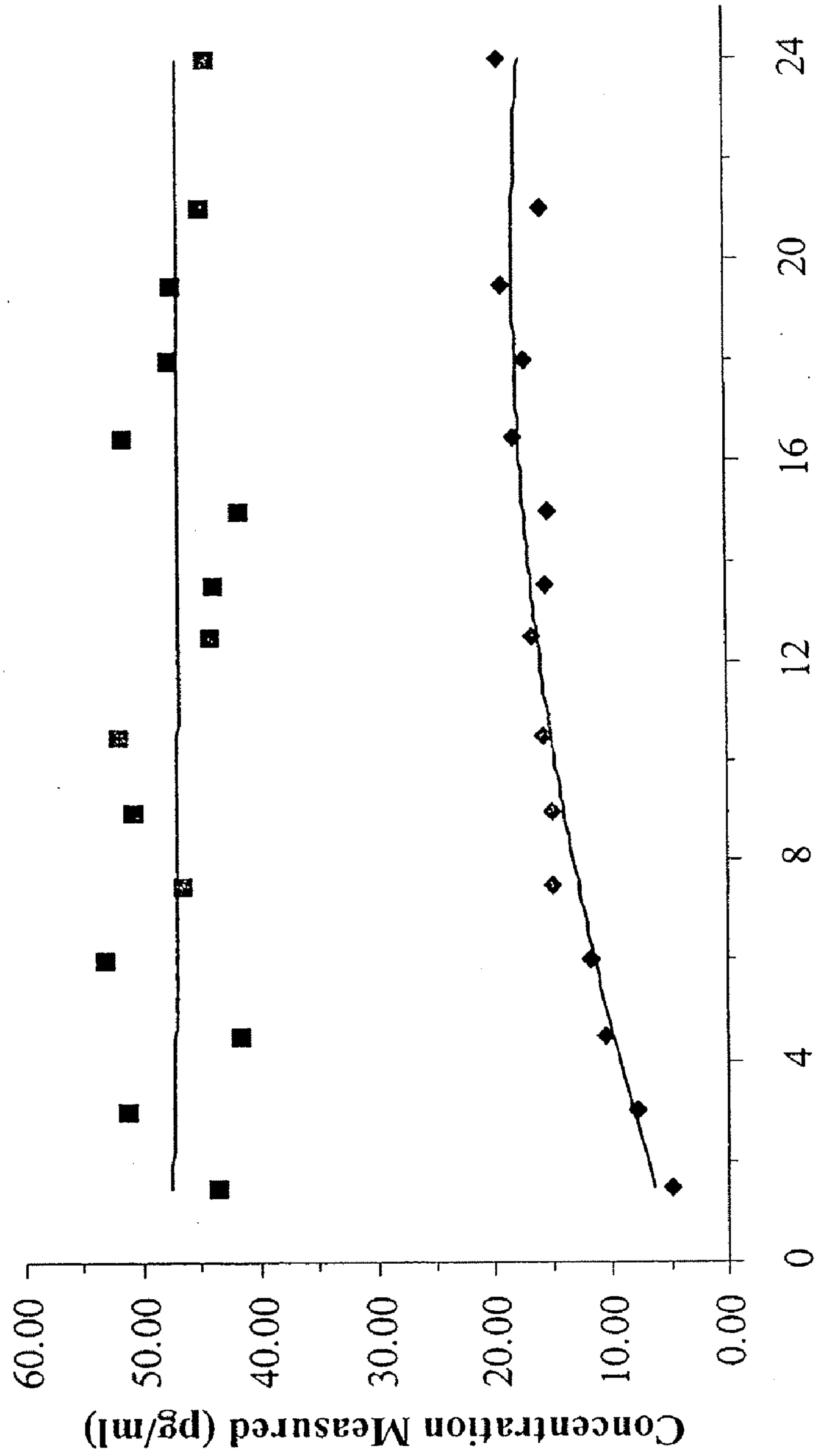

E2 Cross-Validation of LC-MS/MS to LC-LC-MS/MS
y = 0.9844x + 1.0694
R2 = 0.9926
LC-LC-MS/MS (pg/mL)
LC-MS/MS (pg/mL)
0.000
50.000
100.000
150.000
200.000
250.000
300.000

FIG. 32


Concentration Measured (pg/ml)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Dialysis Time (hours)
0
4
8
12
16
20
24

METHODS AND SYSTEMS FOR THE QUANTITATIVE ANALYSIS OF BIOMARKERS

PRIORITY CLAIM TO RELATED APPLICATIONS

[0001] The present application is a divisional application of allowed U.S. patent application Ser. No. 11/805,985, filed May 25, 2007, entitled "Methods and Systems for the Quantitative Analysis of Biomarkers which claims priority under 35 U.S.C. §119(e) from U.S. Provisional Patent Application No. 60/808,812, filed May 26, 2006. The disclosure of U.S. patent application Ser. No. 11/805,985 and U.S. Provisional Patent Application No. 60/808,812 are incorporated by reference in their entireties herein.

FIELD OF INVENTION

[0002] The presently disclosed subject matter relates to methods and systems for the analysis of biomarkers. In certain embodiments, the biomarkers are endogenous to human subjects such that the measurement may be used for clinical diagnosis.

BACKGROUND

[0003] Biomarkers, such as hormones, vitamins, metabolites, can be used for the clinical diagnosis of multiple disorders and as endogenous biomarkers in endocrinology. For example, the measurement of estrogen compounds, such as estrone and estradiol can be used to evaluate ovarian function and to evaluate excess or diminished estrogen levels in a patient. Also, measurement of thyroxine can be used to quantify thyroid function.

[0004] Requirements for the clinical diagnostic testing of endogenous biomarkers in endocrinology may include highly sensitive and specific assays, the ability to analyze small sample volumes (e.g., pediatric sample volumes can be limited to less than about 200 μL), and the ability to screen for multiple analytes to accurately diagnose a disease state, e.g., an endocrine disorder. Historically, radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) methods have been used in such clinical diagnostic testing. Immunoassay methods (IA), such as RIA and EIA, however, may suffer from low throughput, antibody cross-reactivity, which can require extra preparation for specificity, and poor scalability. Also, the analysis of endogenous biomarkers by RIA may require multiple serial dilutions for the analysis of each individual marker, which can lead to the need to make multiple adjustments to normalize sample volumes and/or the need for multiple separate tests. Also, immunoassay testing is not particularly conducive to the analysis of multiple biomarkers in each sample. The analysis for multiple analytes in a single assay can allow for using samples of reduced size which results in assays of increased sensitivity and efficiency per sample.

[0005] An important class of hormones are the steroid hormones, such as testosterone and estrogens. Testosterone develops and maintains the male secondary sex characteristics, and promotes growth and development of sperm. Estrogen is the term used for a group of hormones of which there are three principle forms, estrone, estradiol, and estriol.

[0006] For example, relatively small variations in estrogen levels may be clinically significant. Generally, the level of estrogen in post-menopausal women, adult males, and prepubescent children is ≦10 pg/mL. Elevated estrogen levels in children may lead to precocious puberty (and short stature). In post-menopausal women, low estrogen levels may require replacement, where as levels greater than 5 pg/mL may be prognostic for certain cancers. In adult males, elevated estrogen levels may be indicative of certain disease states (testicular cancer). In adult females, reduced or elevated levels may also be indicative of certain cancers (e.g., ovarian cancer). A level of serum estrogen of 15 pg/mL is clinically different from 10 pg/mL and thus, measurement of estrogen compounds (e.g., estradiol and estrone) requires an LLOQ of 1-5 pg/mL irrespective of sample type, patient age, gender and diet.

[0007] Another important class of hormones are the thyroid hormones. Thyroxine (T4) and triiodothyronine (T3) are examples of thyroid hormones. T4 and T3 enter cells and bind to intracellular receptors. T4 and T3 are important in regulation of a number of factors including growth and development, carbohydrate metabolism, oxygen consumption, and protein synthesis. T4 acts as a prohormone, as the bulk of T3 present in blood is produced by monodeiodination of T4 by intracellular enzymes. Thyroid hormone concentrations in blood are essential tests for the assessment of thyroid function.

[0008] Thus, there is a need to develop analytical techniques that can be used for the measurement of endogenous biomarkers, and for methods that provide more sensitivity and higher throughput than RIA. Until recently, however, only GC-MS or LC-MS/MS with derivatization has been successful for small sample volumes. Thus, there is a need in the art for LC-MS/MS techniques for the analysis of endogenous biomarkers for clinical diagnosis in endocrinology capable of providing detection limits at acceptable levels, without the need for the cumbersome derivatization processes.

SUMMARY

[0009] In some embodiments, the presently disclosed subject matter provides methods and systems for the quantitative analysis of endocrine biomarkers in a test sample. The quantification of such markers may, in certain embodiments, be used for clinical diagnosis in endocrinology. For example, in some embodiments, the methods and systems of the present invention may be used for the quantitative analysis of total levels of certain hormones, including steroid hormones, such as estrone and estradiol, and their metabolites, such as estrone sulfate. In other embodiments, the methods and systems of the present invention provide for the quantitative analysis of biomarkers that can be difficult to detect in their active state. For example, the systems and methods of the present invention may be used to quantify free (i.e., not bound to protein) serum hormones, such as free thyroxine (T4) in biological samples. Or, in other embodiments, the systems and methods of the present invention may be used to quantify free triiodothyronine (T3) or testosterone. In an embodiment, the methods and systems of the present invention allow for measurement of such hormones without the need for derivation processes.

[0010] In some embodiments, the biomarkers of interest are estradiol and/or estrone. Thus, in one embodiment, the present invention comprises a method for determining the presence or amount of estradiol in a sample by tandem mass spectrometry, comprising: (a) generating a dehydrated precursor ion of the estradiol; (b) generating one or more frag-

ment ions of the precursor ion; and (c) detecting the presence or amount of one or more of the ions generated in step (a) or (b) or both, and relating the detected ions to the presence or amount of the estradiol in the sample. In an embodiment, the sample comprises a mixture of estradiol and estrone.

[0011] In other embodiments, the biomarker comprises free thyroxine (T4) or triiodothyronine (T3). In certain embodiments, the present invention provides a high-throughput assay for free thyroxine (T4). Thus, in one embodiment, the present invention comprises a method for determining the presence or amount of free thyroxine in a plurality of samples by tandem mass spectrometry, comprising: (a) dialyzing the plurality of samples to separate the free thyroxine from the protein-bound thyroxine in the samples; (b) generating a precursor ion of the thyroxine; (b) generating one or more fragment ions of the thyroxine; and (c) detecting the presence or amount of one or more of the ions generated in step (b) or (c) or both, and relating the detected ions to the presence or amount of the free thyroxine in the plurality of samples.

[0012] In some embodiments, the methods and systems of the present invention comprise liquid chromatography (LC) methods in combination with other analytical techniques as a means to measure such biomarkers with high sensitivity and high throughput. In certain embodiments, the present invention comprises quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of endocrine biomarkers in a test sample. In some embodiments, two-dimensional or tandem LC is used. The method may include, in alternate embodiments, liquid-liquid extractions, dialysis, sample dilution, and/or sample dehydration steps prior to analysis by tandem mass spectrometry.

[0013] Accordingly, embodiments of the present invention may provide methods for the quantitative LC-MS/MS and 2D-LC-MS/MS analysis of hormones, including steroid hormones, such as estrone and estradiol. Additionally or alternatively, embodiments of the present invention may provide methods for the quantitative determination of a free (i.e., non-protein bound) hormone or metabolite using dialysis in combination with LC-MS/MS analysis for hormones that in biological samples, may be predominantly protein-bound. Such hormones may include free thyroxine (T4), free triiodothyronine (3), or free testosterone. Certain objects of the present invention, having been stated hereinabove, will become further evident as the description proceeds when taken in connection with the accompanying figures and examples as described herein below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Having thus described the invention in general terms, reference will now be made to the accompanying drawings, which are not necessarily drawn to scale.

[0015] FIG. **1** shows a flow chart of a method for quantitative analysis of a biomarker of interest in accordance with one embodiment of the present invention.

[0016] FIG. **2** shows dehydration of estradiol and the effect on mass spectrometry (MS) analysis in accordance with an embodiment of the present invention.

[0017] FIG. **3** shows potential isobaric interferences for measurement of estrone and estradiol due to dehydration of dehydroepiandrosterone (DHEA) in accordance with one embodiment of the present invention.

[0018] FIG. **4** shows an example of heart-cutting from a primary separation gradient to remove compounds that comprise isobaric interference in the analysis of estrone and estradiol in accordance with one embodiment of the present invention.

[0019] FIG. **5** shows a method for the quantification of estrone and estradiol in accordance with an embodiment of the present invention.

[0020] FIG. **6** shows a method for the quantification of free thyroxine (T4) in accordance with an embodiment of the present invention.

[0021] FIG. **7** shows a system for quantitative analysis of a metabolite in accordance with one embodiment of the present invention (Panel A), and a system for multiplex analysis (Panel B) in accordance with alternate embodiments of the present invention.

[0022] FIG. **8** shows a LC-MS/MS chromatogram of estrone sulfate at a limit of quantification of 100 pg/mL in accordance with one embodiment of the present invention.

[0023] FIG. **9** shows a LC-MS/MS chromatogram of free thyroxine at a limit of quantification of 2 pg/mL in accordance with one embodiment of the present invention.

[0024] FIG. **10** shows a 2D-LC-MS/MS chromatogram of 25-hydroxyvitamin D2 at a limit of quantification of 1 ng/mL in accordance with one embodiment of the present invention.

[0025] FIG. **11** shows a 2D-LC-MS/MS chromatogram of 25-hydroxyvitamin D3 at a limit of quantification of 1 ng/mL in accordance with one embodiment of the present invention.

[0026] FIG. **12** shows a 2D-LC-MS/MS chromatogram of estrone at a limit of quantification of 2.5 pg/mL in accordance with one embodiment of the present invention.

[0027] FIG. **13** shows a 2D-LC-MS/MS chromatogram of estradiol at a limit of quantification of 1 pg/mL in accordance with one embodiment of the present invention.

[0028] FIG. **14** shows a LC-MS/MS chromatogram of estrone sulfate at an upper limit of quantification of 50 ng/mL in accordance with one embodiment of the present invention.

[0029] FIG. **15** shows a LC-MS/MS chromatogram of free thyroxine at an upper limit of quantification of 100 pg/dL in accordance with one embodiment of the present invention.

[0030] FIG. **16** shows a 2D-LC-MS/MS chromatogram of 25-hydroxyvitamin D2 at an upper limit of quantification of 250 ng/mL in accordance with one embodiment of the present invention.

[0031] FIG. **17** shows a 2D-LC-MS/MS chromatogram of 25-hydroxyvitamin D3 at an upper limit of quantification of 250 ng/mL in accordance with one embodiment of the present invention.

[0032] FIG. **18** shows a 2D-LC-MS/MS chromatogram of estrone at an upper limit of quantification of 500 pg/mL in accordance with one embodiment of the present invention.

[0033] FIG. **19** shows a 2D-LC-MS/MS chromatogram of estradiol at an upper limit of quantification of 500 pg/mL in accordance with one embodiment of the present invention.

[0034] FIG. **20** shows a calibration curve obtained by LC-MS/MS for estrone sulfate in accordance with one embodiment of the present invention.

[0035] FIG. **21** shows a calibration curve obtained by LC-MS/MS for free thyroxine in accordance with one embodiment of the present invention.

[0036] FIG. **22** shows a calibration curve obtained by 2D-LC-MS/MS for 25-hydroxyvitamin D2 in accordance with one embodiment of the present invention.

[0037] FIG. **23** shows a calibration curve obtained by 2D-LC-MS/MS for 25-hydroxyvitamin D3 in accordance with one embodiment of the present invention.

[0038] FIG. **24** shows a calibration curve obtained by 2D-LC-MS/MS for estrone in accordance with one embodiment of the present invention.

[0039] FIG. **25** shows a calibration curve obtained by 2D-LC-MS/MS for estradiol in accordance with one embodiment of the present invention.

[0040] FIG. **26** shows cross-validation data for LC-MS/MS as compared to radioimmunoassay (RIA) for estrone sulfate in accordance with one embodiment of the present invention.

[0041] FIG. **27** shows cross-validation data for LC-MS/MS as compared to immunoassay (IA) for free thyroxine in accordance with one embodiment of the present invention.

[0042] FIG. **28** shows cross-validation data for 2D-LC-MS/MS as compared to a competitive binding protein assay (CBP) (Panel A) or immunoassay (IA) (Panel B) for total 25-hydroxyvitamin D (25-hydroxyvitamin D2+D3) in accordance with alternate embodiments of the present invention.

[0043] FIG. **29** shows cross-validation data for 2D-LC-MS/MS as compared to RIA for Estrone in accordance with one embodiment of the present invention.

[0044] FIG. **30** shows cross-validation data for 2D-LC-MS/MS as compared to RIA for Estradiol in accordance with one embodiment of the present invention.

[0045] FIG. **31** shows a comparison of Estradiol (E2) cross-validation of LC-MS/MS with derivatization to 2D-LC-MS/MS without derivatization in accordance with an embodiment of the present invention.

[0046] FIG. **32** shows the measured concentration (pg/mL) of free thyroxine vs. dialysis time (hours). The squares (■) show dialysis losses and the diamonds (♦) show effective dialysis for free thyroxine using 96-well equilibrium dialysis plates in accordance with one embodiment of the present invention.

DETAILED DESCRIPTION

[0047] The presently disclosed subject matter now will be described more fully hereinafter with reference to the accompanying description and drawings, in which some, but not all embodiments of the presently disclosed subject matter are shown. The presently disclosed subject matter can be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

[0048] Many modifications and other embodiments of the presently disclosed subject matter set forth herein will come to mind to one skilled in the art to which the presently disclosed subject matter pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the presently disclosed subject matter is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation. The disclosure utilizes the abbreviations shown below.

ABBREVIATIONS

[0049] APCI=atmospheric pressure chemical ionization
CBP=competitive binding protein

E1=Estrone

ABBREVIATIONS

E2=17β-Estradiol or Estradiol

FT4=Free Thryoxine

[0050] HTLC=high turbulence (throughput) liquid chromatography
HPLC=high performance liquid chromatography
LLE=liquid-liquid extraction
LOQ=limits of quantification
LLOQ=lower limit of quantification
IA=immunoassay
ELISA=enzyme linked immunoassay
RIA=radioimmunoassay
SST=system suitability test
ULOQ=upper limit of quantification
2D-LC-MS/MS=two-dimensional liquid chromatography hyphenated to tandem mass spectrometry
(LC)-LC-MS/MS=two-dimensional liquid chromatography tandem hyphenated to mass spectrometry
(LC)-MS/MS=liquid chromatography hyphenated to tandem mass spectrometry

Definitions

[0051] While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter. Other definitions are found throughout the specification. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this presently described subject matter belongs.

[0052] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. Moreover, all ranges disclosed herein are to be understood to encompass any and all subranges subsumed therein. For example, a stated range of "1 to 10" should be considered to include any and all subranges between (and inclusive of) the minimum value of 1 and the maximum value of 10; that is, all subranges beginning with a minimum value of 1 or more, e.g. 1 to 6.1, and ending with a maximum value of 10 or less, e.g., 5.5 to 10. Additionally, any reference referred to as being "incorporated herein" is to be understood as being incorporated in its entirety.

[0053] The terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, for example, reference to "a cell" includes a plurality of such cells, unless the context clearly is to the contrary (e.g., a plurality of cells), and so forth.

[0054] As used herein, the term "biomarker" is any biomolecule that may provide biological information about the physiological state of an organism. In certain embodiments, the presence or absence of the biomarker may be informative. In other embodiments, the level of the biomarker may be

informative. A biomarker may be a hormone, such as an estrogen (e.g., estradiol, estrone), testosterone, thyroxine (T4), triiodothyronine (T3), or a metabolite of a hormone (estrogen sulfate). A biomarker may also be a vitamin or a metabolite of a vitamin. For example, in one embodiment, the measured biomarker may comprise a vitamin D compound such as 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3.

[0055] As used herein, the terms "purify" or "separate" or derivations thereof do not necessarily refer to the removal of all materials other than the analyte(s) of interest from a sample matrix. Instead, in some embodiments, the terms "purify" or "separate" refer to a procedure that enriches the amount of one or more analytes of interest relative to one or more other components present in the sample matrix. In some embodiments, a "purification" or "separation" procedure can be used to remove one or more components of a sample that could interfere with the detection of the analyte, for example, one or more components that could interfere with detection of an analyte by mass spectrometry.

[0056] As used herein, "derivatizing" means reacting two molecules to form a new molecule. Derivatizing agents may include isothiocyanate groups, dansyl groups, dinitro-fluorophenyl groups, nitrophenoxycarbonyl groups, and/or phthalaldehyde groups.

[0057] As used herein, "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.

[0058] As used herein, "liquid chromatography" (LC) means a process of selective retardation of one or more components of a fluid solution as the fluid uniformly percolates through a column of a finely divided substance, or through capillary passageways. The retardation results from the distribution of the components of the mixture between one or more stationary phases and the bulk fluid, (i.e., mobile phase), as this fluid moves relative to the stationary phase(s). "Liquid chromatography" includes reverse phase liquid chromatography (RPLC), high performance liquid chromatography (HPLC) and high turbulence liquid chromatography (HTLC).

[0059] As used herein, the term "HPLC" or "high performance 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. The chromatographic column typically includes a medium (i.e., a packing material) to facilitate separation of chemical moieties (i.e., fractionation). The medium may include minute particles. The particles include a bonded surface that interacts with the various chemical moieties to facilitate separation of the chemical moieties such as the biomarker analytes quantified in the experiments herein. One suitable bonded surface is a hydrophobic bonded surface such as an alkyl bonded surface. Alkyl bonded surfaces may include C-4, C-8, or C-18 bonded alkyl groups, preferably C-18 bonded groups. The chromatographic column includes an inlet port for receiving a sample and an outlet port for discharging an effluent that includes the fractionated sample. In the method, the sample (or pre-purified sample) may be applied to the column at the inlet port, eluted with a solvent or solvent mixture, and discharged at the outlet port. Different solvent modes may be selected for eluting different analytes of interest. For example, liquid chromatography may be performed using a gradient mode, an isocratic mode, or a polytyptic (i.e. mixed) mode. In one embodiment, HPLC may performed on a multiplexed analytical HPLC system with a C18 solid phase using isocratic separation with water:methanol as the mobile phase.

[0060] As used herein, the term "analytical column" refers to a chromatography column having sufficient chromatographic plates to effect a separation of the components of a test sample matrix. Preferably, the components eluted from the analytical column are separated in such a way to allow the presence or amount of an analyte(s) of interest to be determined. In some embodiments, the analytical column comprises particles having an average diameter of about 5 μm. In some embodiments, the analytical column is a functionalized silica or polymer-silica hybrid, or a polymeric particle or monolithic silica stationary phase, such as a phenyl-hexyl functionalized analytical column.

[0061] Analytical columns can be distinguished from "extraction columns," which typically are used to separate or extract retained materials from non-retained materials to obtained a "purified" sample for further purification or analysis. In some embodiments, the extraction column is a functionalized silica or polymer-silica hybrid or polymeric particle or monolithic silica stationary phase, such as a Poroshell SBC-18 column.

[0062] The term "heart-cutting" refers to the selection of a region of interest in a chromatogram and subjecting the analytes eluting within that region of interest to a second separation, e.g., a separation in a second dimension.

[0063] The term "electron ionization" as used herein 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.

[0064] The term "chemical ionization" as used herein 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.

[0065] The term "field desorption" as used herein 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.

[0066] The term "matrix-assisted laser desorption ionization," or "MALDI" as used herein 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.

[0067] The term "surface enhanced laser desorption ionization," or "SELDI" as used herein 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.

[0068] The term "electrospray ionization," or "ESI," as used herein 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. Upon reaching the end of the tube, the solution may be vaporized (nebulized) into a jet or spray of very small droplets of solu-

tion in solvent vapor. This mist of droplet can flow 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.

[0069] The term "Atmospheric Pressure Chemical Ionization," or "APCI," as used herein refers to mass spectroscopy 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 a 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.

[0070] The term "Atmospheric Pressure Photoionization" ("APPI") as used herein refers to the form of mass spectroscopy where the mechanism for the photoionization of molecule M is photon absorption and electron ejection to form the molecular M+. Because the photon energy typically is just above the ionization potential, the molecular ion is less susceptible to dissociation. In many cases it may be possible to analyze samples without the need for chromatography, thus saving significant time and expense. In the presence of water vapor or protic solvents, the molecular ion can extract H to form MH+. This tends to occur if M has a high proton affinity. This does not affect quantitation accuracy because the sum of M+ and MH+ is constant. Drug compounds in protic solvents are usually observed as MH+, whereas nonpolar compounds such as naphthalene or testosterone usually form M+ (see e.g., Robb et al., 2000, Anal. Chem. 72(15): 3653-3659).

[0071] The term "inductively coupled plasma" as used herein refers to methods in which a sample is interacted with a partially ionized gas at a sufficiently high temperature to atomize and ionize most elements.

[0072] The term "ionization" and "ionizing" as used herein 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 ions having a net negative charge of one or more electron units, while positive ions are those ions having a net positive charge of one or more electron units.

[0073] The term "desorption" as used herein refers to the removal of an analyte from a surface and/or the entry of an analyte into a gaseous phase.

[0074] As used herein, the term "immunoassay" (IA) refers to a method for measuring the amount of an analyte of interest by quantifying the binding, or the inhibition of binding, of a substance to an antibody. Where an enzyme is used to detect the amount of binding of the substance (e.g. antigen) to an antibody, the assay is an enzyme-linked immunoassay (ELISA). As used herein, the term "radioimmunoassay" (RIA) refers to a method for measuring the amount of an analyte of interest by quantifying the binding, or the inhibition, of binding, of a radiolabeled substance to an antibody.

[0075] As used herein, the term "hemolysed" refers to the rupturing of the red blood cell membrane, which results in the release of hemoglobin and other cellular contents into the plasma or serum and the term "lipemic" refers to an excess of fats or lipids in blood.

[0076] Analysis of Biomarkers by LC-MS/MS

[0077] Thus, embodiments of the present invention relate to methods and systems for the quantitative analysis of endogenous biomarkers for clinical diagnosis. The present invention may be embodied in a variety of ways.

[0078] In one embodiment, the present invention comprises a method for determining the presence or amount of at least one biomarker of interest in a biological sample, the method comprising: providing a biological sample believed to contain at least one biomarker of interest; chromatographically separating the at least one biomarker of interest from other components in the sample; and analyzing the chromatographically separated at least one biomarker of interest by mass spectrometry to determine the presence or amount of the at least one biomarker of interest in the sample.

[0079] In an embodiment, the at least one biomarker comprises a steroid hormone or a thyroid hormone. For example, in one embodiment, the at least one biomarker comprises estradiol and estrone. Or, the at least one biomarker may comprise free thyroxine (T4) or triiodothyronine (T3).

[0080] In certain embodiments, the chromatography may comprise high performance liquid chromatography (HPLC). In an embodiment, the chromatography may comprises extraction and/or analytical liquid chromatography.

[0081] In an embodiment, the method may comprise purifying the biomarker of interest prior to chromatography. For example, the sample may be partially purified by at least one of liquid-liquid extraction. Also, the method may comprise the step of diluting the sample into a solvent or solvents used for LS and/or MS.

[0082] In some embodiments, the method may comprise the use of two liquid chromatography steps. For example, in certain embodiments, the method for determining the presence or amount of one or more biomarkers in a test sample may comprise the steps of: (a) providing a sample suspected of containing one or more biomarkers of interest; (b) partially purifying the one or more biomarkers of interest from other components in the sample by at least one of liquid-liquid extraction or by diluting the sample; (c) transferring the extracted one or more hormones or metabolites onto an extraction column (i.e., on-line or off-line); (d) transferring the one or more biomarkers of interest from the extraction column onto an analytical column and chromatographically separating the one or more biomarkers of interest from other components in the sample; and (e) analyzing the chromatographically separated biomarkers of interest by mass spectrometry to determine the presence or amount of the one or more biomarkers in the test sample.

[0083] In certain embodiments, the present invention comprises methods for measuring at least one of estradiol and/or estrone in a sample. In certain embodiments, the estradiol is dehydrated to reduce the complexity of the MS/MS spectrum, such that the sensitivity of estradiol detection is increased. For example, in one embodiment, the present invention comprises a method for determining the presence or amount of estradiol in a sample by tandem mass spectrometry, comprising: (a) generating a dehydrated precursor ion of the estradiol; (b) generating one or more fragment ions of the precursor ion; and (c) detecting the presence or amount of one or more of the ions generated in step (a) or (b) or both, and relating the detected ions to the presence or amount of the estradiol in the sample.

[0084] In an embodiment, the sample may be subjected to a purification step prior to ionization. For example, in certain embodiments, the purification step may comprises chromatography. As discussed herein, in certain embodiments, the chromatography comprises high performance liquid chromatography (HPLC). The LC step may comprise one LC separation, or multiple LC separations. In one embodiment, the

chromatographic separation comprises extraction and analytical liquid chromatography. Additionally or alternatively, high turbulence liquid chromatography (HTLC) (also known as high throughput liquid chromatography) may be used.

[0085] The purification may comprise steps in addition to HPLC or other types of chromatographic separation techniques. In alternate embodiments, the method may comprise at least one of liquid-liquid extraction or dilution. In one embodiment, the sample is diluted into a solvent or solvent mixture that may be used for LC and/or MS (e.g., LC-MS/MS or 2D-LC-MS/MS).

[0086] In an embodiment, the treatment of estradiol to form a dehydrated form of the compound reduces the molecular weight of the estradiol by about 18 mass units. Thus, in an embodiment, the precursor ion has a mass/charge ratio (m/z) of about 255.2. Also, in an embodiment, treatment of estradiol to form a dehydrated form of the compound reduces the complexity of the mass spectrum. Thus, in a embodiment the fragment ions comprise ions having a mass/charge ratio (m/z) of about 159.0 and 133.0. By reducing the complexity of the spectrum, the sensitivity of the procedure may be increased. The method may comprise detection of estradiol over a range of from a LOQ of about 1 pg/ml to an ULOQ of about 500 pg/mL as a single assay (i.e., as a linear assay without multiple dilution of the samples). Also, the method may comprise detection of estrone over a range of from a LOQ of about 2.5 pg/mL to and ULOQ of about 500 pg/mL as a single assay (i.e., as a linear assay without multiple dilution of the samples).

[0087] Also, since the spectrum of the estradiol is simplified, the analysis may further comprise a determination of the amount of other estrogens, such as estrone, in the sample.

[0088] The sample may only require heating for a relatively brief period of time to form the dehydrated estradiol. For example, the sample may be heated within the range of 300° C. to 1000° C. In an embodiment, the sample is heated in the interface where the sample is transferred to the mass spectrometer. In alternate embodiments, the heating step is done for less than 1 second, or less than 100 milliseconds (msec), or less than 10 msec, or less than 1 msec, or less than 0.1 msec, or less than 0.01 msec, or less than 0.001 msec.

[0089] In other embodiments, the present invention comprises methods for determining the presence or amount of a free thyroxine in a sample or a plurality of samples. In an embodiment, the present invention may comprise a method for determining the presence or amount of free thyroxine in a plurality of samples by tandem mass spectrometry, comprising: (a) dialyzing the plurality of samples to separate the free thyroxine from the protein-bound thyroxine in the samples; (b) generating a precursor ion of the thyroxine in each sample; (b) generating one or more fragment ions of the thyroxine in each sample; and (c) detecting the presence or amount of one or more of the ions generated in step (b) or (c) or both in each sample, and relating the detected ions to the presence or amount of the free thyroxine in the plurality of samples.

[0090] In an embodiment, the method may comprise detection of thyroxine over a range of from a LLOQ of about 2.0 pg/mL to an ULOQ of about 100 pg/mL as a single assay (i.e., without dilution of the samples). In an embodiment, and as described in more detail herein, the dialysing step may comprise the use of a buffer, and wherein the buffer comprises and sufficient salts such that the buffer is isotonic.

[0091] In an embodiment, the sample may be subjected to a purification step prior to ionization. For example, in certain embodiments, the purification step may comprises chromatography. As discussed herein, in certain embodiments, the chromatography comprises high performance liquid chromatography (HPLC). The LC step may comprise one LC separation, or multiple LC separations. In one embodiment, the chromatographic separation comprises extraction and analytical liquid chromatography. Additionally or alternatively, high turbulence liquid chromatography (HTLC) may be used.

[0092] The purification may comprise steps in addition to HPLC or other types of chromatographic separation techniques. In alternate embodiment, the purification may comprise at least one of liquid-liquid extraction or dilution. In alternate embodiment, the sample may diluted in a solvent or solvents used for LC or MS, rather than undergoing LLE.

[0093] In other embodiments, the present invention comprises a system for determining the presence or amount of one or more biomarkers in a sample. In an embodiment, the system for determining the presence or amount of one or more biomarkers in a sample may comprise a station for chromatographically separating the one or more biomarkers from other components in the sample. For example, in some embodiments, the present invention may comprise system for determining the presence or amount of at least one biomarker of interest in a sample, the system comprising: a station for providing a sample believed to contain at least one biomarker of interest; a station for chromatographically separating the at least one biomarker of interest from other components in the sample; and a station for analyzing the chromatographically separated at least one biomarker of interest by mass spectrometry to determine the presence or amount of the one or more biomarkers in the sample. In an embodiment, the system may comprise a station for partially purifying the at least one biomarker of interest from other components in the sample. In an embodiment, the mass spectrometry is operated in an atmospheric pressure chemical ionization (APCI) mode. In an embodiment, the system may further comprise a station for dialyzing a plurality of samples as a means to separate the at least one biomarker of interest that is bound to proteins in the sample from the portion of the biomarker of interest that is free in solution (i.e., "free"). Also in certain embodiments, at least one of the stations is automated and/or controlled by a computer. For example, as described herein, in certain embodiments, at least some of the steps are automated such that little to no manual intervention is required.

[0094] In one embodiment, the station for chromatographic separation comprises at least one apparatus to perform liquid chromatography (LC). In one embodiment, the station for liquid chromatography comprises a column for extraction chromatography. Additionally or alternatively, the station for liquid chromatography comprises a column for analytical chromatography. In certain embodiments, the column for extraction chromatography and analytical chromatography comprise a single station or single column. For example, in one embodiment, liquid chromatography is used to purify the biomarker of interest from other components in the sample that co-purify with the biomarker of interest after extraction or dilution of the sample.

[0095] The system may also include a station for analyzing the chromatographically separated one or more biomarkers of interest by mass spectrometry to determine the presence or amount of the one or more biomarkers in the test sample. In certain embodiments, tandem mass spectrometry is used (MS/MS). For example, in certain embodiments, the station for tandem mass spectrometry comprises an Applied Biosys-

tems API4000 or API5000 or thermo quantum or Agilent 7000 triple quadrupole mass spectrometer.

[0096] The system may also comprise a station for extracting the one or more hormones or metabolites from the test sample and/or diluting the sample. In an embodiment, the station for extraction comprises a station for liquid-liquid extraction. The station for liquid-liquid extraction may comprise equipment and reagents for addition of solvents to the sample and removal of waste fractions. In some cases a isotopically-labeled internal standard is used to standardize losses of the biomarker that may occur during the procedures. Thus, the station for liquid-liquid extraction may comprise a hood or other safety features required for working with solvents.

[0097] Additionally, the system may comprise a station for dialyzing sample as a means to separate the free hormone or metabolite from a sample that comprises free and protein-bound hormone or metabolite for measurement. The station for dialysis may comprise equipment for aliquoting samples into dialysis chambers. Also, the station for dialysis may comprise a mixing chamber to effect dialysis of the free analyte (e.g., free hormone) from the sample.

[0098] In embodiments of the methods and systems of the present invention, the biomarker is a hormone or a metabolite. The methods and systems of the present invention may be used to measure the amount of either total and/or free biomarkers of intersect in serum. In an embodiment, the hormone may comprise a steroid hormone. Or, the hormone may comprise a thyroid hormone. Or, the hormone may comprise a protein or peptide hormone. For example, in alternate embodiments, the steroid hormone may comprise an estrogen, androgen, mineralcorticoid, or glucocorticoid hormone. In certain embodiments, the hormone may comprise at least one of estrone or estradiol. In other embodiments, the hormone may comprise an estrogen metabolite. For example, the hormone may comprise estrone sulfate and/or glucoronidated and sulphated metabolites of estradiol, estrone or estriol. Or, other steroid hormones or steroid hormone metabolites may be measured. For example, the hormone may comprise testosterone. Or, non-steroid hormones may be measured. For example, in certain embodiments, the methods and systems may be used to measure a thyroid hormone, such as free thyroxine (T4) or triiodothyronine (T3). Or, pre-hormones (such as 25 hydroxyvitamin D) may be measured. For examples, the methods and systems of the present invention may be used to measure vitamins or other metabolites. In some embodiments, the metabolite may comprise a vitamin D compound such as 25-hydroxyvitamin D2 or 25-hydroxyvitamin D3, 1,25 dihydroxyvitamin D2 and 1,25 dihydroxyvitamin D3. In yet other embodiments, the methods and systems of the present invention may be used to measure a non-hormone compound.

[0099] In certain embodiments, the test samples suitable for analysis by the methods and systems of the present invention can include any liquid sample that can contain one or more target analytes of interest. In an embodiment, the biomarker is endogenous to a subject. For example, in some embodiments, the test sample comprises a biological sample. As used herein, the term "biological sample" refers to a sample obtained from a biological source, including, but not limited to, an animal, a cell culture, an organ culture, and the like. Suitable samples include blood, plasma, serum, urine, saliva, tear, cerebrospinal fluid, organ, hair, muscle, or other tissue sample.

[0100] As used herein, a subject may comprise an animal. Thus, in some embodiments, the biological sample is obtained from a mammalian animal, including, but not limited to a dog, a cat, a horse, a rat, a monkey, and the like. In some embodiments, the biological sample is obtained from a human subject. In some embodiments, the subject is a patient, that is, a living person presenting themselves in a clinical setting for diagnosis, prognosis, or treatment of a disease or condition. In some embodiments, the test sample is not a biological sample, but comprises a non-biological sample, e.g., obtained during the manufacture or laboratory analysis of a synthetic steroid, which can be analyzed to determine the composition and/or yield of the manufacturing and/or analysis process.

[0101] A variety of methods may be used to extract the biomarker of interest from the sample. In certain embodiments, extracting the one or more hormones or metabolites from the test sample comprises a liquid-liquid extraction procedure. For example, for the analysis of estrone and estradiol in serum, a hexane:ethyl acetate is used for extraction. For example, in one embodiment, a 9:1 hexane:ethyl acetate solution may be used.

[0102] In certain embodiments, purifying the at least one biomarker of interest from the test sample may also comprise the use of a liquid chromatography extraction column. In one embodiment, the column is on-line. In an embodiment, purification of the biomarker of interest using a extraction column may comprises the steps of: (i) transferring the test sample on an extraction column; and (ii) eluting the biomarker of interest from the extraction column.

[0103] In certain embodiments, the methods and systems of the present invention may comprise multiple liquid chromatography steps. Thus, in certain embodiments, a two-dimensional liquid chromatography (LC) procedure is used. For example, in one embodiment, the method and systems of the present invention may comprise transferring the biomarker of interest from the LC extraction column to an analytical column. In one embodiment, the transferring of the at least one biomarker of interest from the extraction column to an analytical column is done by a heart-cutting technique. In another embodiment, the biomarker of interest is transferred from the extraction column to an analytical column by a chromatofocusing technique. Alternatively, the biomarker of interest is transferred from the extraction column to an analytical column by a column switching technique. These transfer steps may be done manually, or may be part of an on-line system.

[0104] Various columns comprising stationary phases and mobile phases that may be used for extraction or analytical liquid chromatography are described herein. The column used for extraction liquid chromatography may be varied depending on the biomarker of interest. In some embodiments, the extraction column is a functionalized silica or polymer-silica hybrid or polymeric particle or monolithic silica stationary phase, such as a Poroshell SBC-18 column. The column used for analytical liquid chromatography may be varied depending on the biomarker of interest and/or the column that was used for the extraction liquid chromatography step. For example, in certain embodiments, the analytical column comprises particles having an average diameter of about 5 μm. In some embodiments, the analytical column is a functionalized silica or polymer-silica hybrid, or a polymeric particle or monolithic silica stationary phase, such as a phenyl-hexyl functionalized analytical column.

[0105] A variety of methods may be used to quantify the at least one biomarker of interest once the biomarker of interest has been substantially purified (i.e., substantially separated away from other components that may have been present in the sample). In some embodiments, mass spectrometry is used to quantify the at least one biomarker of interest. In certain embodiments, the mass spectrometer may comprise a tandem mass spectrometer (MS/MS). For example, in one embodiment of the methods and systems of the present invention, the tandem MS/MS spectrometry comprises a triple quadrupole tandem mass spectrometer.

[0106] The tandem MS/MS may be operated in a variety of modes. In one embodiment, the tandem MS/MS spectrometer is operated in an atmospheric pressure chemical ionization (APCI) mode. In some embodiments, the quantification of the analytes and internal standards is performed in the selected reaction monitoring mode (SRM).

[0107] Thus, embodiments of the present invention comprise methods and systems for applying liquid chromatography and mass spectrometry as a means to separate a biomarker analyte of interest from other components that may be present in a biological sample. In certain embodiments, two liquid chromatography (LC) steps are used in tandem. Also, the method may comprise an off-line liquid-liquid extraction and/or sample dilution step as a means to partially purify the sample prior to liquid chromatography. In some embodiments, tandem mass spectrometry is used to quantify the analyte of interest. The methods and systems may be used for clinical diagnosis.

[0108] The systems and methods of the present invention may, in certain embodiments, provide for a multiplexed assay. For example, certain embodiments of the present invention may comprise a multiplexed liquid chromatography tandem mass spectrometry (LC-MS/MS) or two-dimensional or tandem liquid chromatography-tandem mass spectrometry (LC)-LC-MS/MS) methods for the quantitative analysis of one or more analytes, including steroid hormones, such as estrone and estradiol and/or thyroid hormones, such as free thyroxine (T4) or triiodothyroine (T3) in biological samples.

[0109] An example of a method (**2**) of the present invention is shown in FIG. **1**. Thus, in an embodiment, the method may include a step of providing a biological sample, for example, a serum sample believed to contain one or more analytes of interest (**4**). In some embodiments, an appropriate internal standard is added to the sample (**6**). For example, in some embodiments of the presently disclosed method for analyzing estrone and estradiol in serum samples, deuterated D_4-estrone and D_5-estradiol are added as internal standards. Or, C_{13}-estrone and C_{13}-estradiol stable labeled isotopes may be used. Or, for thyroxine, a deuterated or C_{13} derivative may be used. For example, in one embodiment, Thyroxine Ring-$^{13}C_6$ may be used. In yet other embodiments, structural analogues of the biomarker of interest may be used. For example, such structural analogues may comprise compounds wherein a first chemical group is replaced with a second chemical group. In general, the groups are of similar chemical reactivity, but different mass, as for example, the replacement of a methyl (—CH_3) group with an ethyl (—CH_2CH_3) group.

[0110] In some embodiments, the analytes of interests are partially purified by liquid-liquid extraction of the sample (**8**). Or, the sample may be diluted (**9**) in a solvent that can be used for LC or MS in subsequent purification steps.

[0111] In an embodiment, the liquid-liquid extraction is used to concentrate and partially purify the analyte. For example, for estradiol/estrone analysis, the liquid extraction may be used to remove conjugated estrogens, such as sulfated and glucoronidated estrogens. Also, the liquid extraction may remove lipids and/or fibrinogen from the samples. In some embodiments, estrone and estradiol can be extracted from a serum sample with an organic solvent that can separate estrone and estradiol from conjugated estrogens. For example, in an embodiment, an alkane mixed with a more polar solvent is used. For example, in certain embodiments, hexane is mixed with a more polar solvent. In an embodiment, the polar solvent comprises ethyl acetate or a similar solvent. In an embodiment, 9:1 hexane:ethyl acetate is used.

[0112] Or, other solvents may be used. As is known in the art, the solvents employed may be optimized to separate the analyte of interest from the sample. For example, the solvents used to extract estrone and estradiol from serum may not be the same solvent or solvent mix as used to extract estrone and estradiol from urine. Or, the solvents used to extract estrone and estradiol from serum may not be the same solvent or solvent mix as used to extract thyroxine (T4), triiodothyronine (T3), or vitamin-D compounds from serum. For example, in certain embodiments, acetonitrile is used for liquid extraction of vitamin-D compounds, and ethyl acetate: hexane:methanol is used for extraction of T4.

[0113] Certain biomolecules may have a propensity to non-specifically bind to proteins or other biomolecules. For example, thyroid hormones can non-specifically bind to proteins such as serum albumin, sex hormone binding globulin, and the like. For determination of free thyroxine (T4), the sample may be treated to separate the free thyroxine from thyroxine that is bound to proteins in the biological sample (e.g., serum).

[0114] In one embodiment, the sample may initially be dialyzed to separate the free hormone or metabolite from a mixture of free and protein-bound hormone or metabolite (**5**). In certain embodiments, multiple samples may be processed concurrently. For example, the dialysis may be performed using a multiwell dialysis plate which allows for the dialysis of multiple samples at one time. In certain embodiments 96 well plates are used. In this way, multiple samples are processed to comprise a high throughput assay.

[0115] For example, samples of serum that may contain free thyroxine and protein-bound thyroxine may be introduced into the individual sample chambers which are on one side of the membrane and a buffer solution introduced into the diluent chambers on the other side of the membrane from the sample. The 96 well plate is then positioned vertically and rotated to facilitate transfer of the free thyroxine across the membrane.

[0116] The dialysis buffer may, in certain embodiments, be isotonic and contain gelatin. The gelatin may be used over a range of concentrations depending upon the nature of the membranes and hardware used for dialysis. In alternate embodiments, the gelatin may be in a range of from about 0.1 to 10 mg/mL. In an embodiment, the gelatin is at about 1 mg/mL. In certain embodiments, the buffer used for dialysis comprises multiple endogenous salts to provide a buffer that is isotonic with the serum sample to thereby negate any potential dilution effects and/or disruptions to the ratio of bound thyroxine to free thyroxine in the sample. Also, gelatin may be include to prevent adsorptive losses of free thyroxine onto the dialysis membrane or the sample chamber. Gelatin may act as a carrier on the dialysate side of the 96-well plate to ensure free thyroxine remains in the dialysate solution. Gela-

tin does not bind free thyroxine and thus, does not affect the ratio of bound thyroxine to free thyroxine in the sample on the sample side of the membrane.

[0117] For the analysis of free thyroxine, a liquid extraction step may be performed after the dialysis. The liquid extraction may be designed to remove residual salts and/or other additives which are used in the dialysis solution and/or remain from the sample, but that may interfere with the MS analysis. Thus, in one embodiment, the dialysate comprising free thyroxine is extracted with 71.25:23.75:5 ethyl acetate:hexane: methanol. In another embodiment, the dialysate may be diluted with a solution of methanol containing a stable labeled internal standard and directly injected onto the LC-MS/MS system for analysis.

[0118] Where the sample is extracted, the internal standard addition may include a protein to prevent the free thyroxin from sticking to the walls of the sample container. Addition of protein (e.g., bovine serum albumin) can minimize losses in extraction and recovery for liquid-liquid extraction. Where extraction is not performed, the internal standard may be added in methanol or a similar solvent used for LC.

[0119] As is known in the art, in some embodiments, the organic extract may be transferred to a fresh tube and then back-washed. For example, in an embodiment where the analyte of interest is estradiol and/or estrone, the solvent may be back-washed with aqueous sodium hydroxide (pH of about 12) to further purify the sample. Or, for extraction of other biomarkers, back-extraction may employ other solvents. The back-wash may, in certain embodiments, remove additional lipids or interfering analytes from the sample.

[0120] The extract supernatant may then be evaporated and the sample reconstituted. For example, for analysis of estradiol and/or estrone, the sample may be reconstituted in 70:30 water:methanol. Or, for analysis of thyroxine, the solvent used for liquid-liquid extraction may be evaporated and the sample reconstituted in 50:50 water:methanol.

[0121] Still referring to FIG. **1**, the method may further include liquid chromatography as a means to separate the analyte of interest from other components in the sample. In an embodiment, two liquid chromatography steps are used. For example, the method may comprise a first extraction column liquid chromatography (**10**), transfer of the biomarker of interest to a second analytical column (**12**), and an analytical column liquid chromatography (**16**). In other embodiments, only one liquid chromatography step is used.

[0122] The first extraction liquid chromatography column may, in certain embodiments, comprise a step whereby the analytes of interest are separated from a majority of contaminants. Thus, in certain embodiments, the first column provides the majority of selectivity for the procedure. The second analytical liquid chromatography column may, in certain embodiments, comprise a step whereby the analytes of interest are concentrated, to thereby increase sensitivity for analysis by mass spectrometry (MS).

[0123] For example, the reconstituted extract may be applied onto a high performance liquid chromatography (HPLC) system, wherein the analytes are eluted using an isocratic separation through an extraction column. In certain embodiments, the mobile phase that is used comprises a gradient. For example, in an embodiment for the separation of estradiol and estrone from other components in serum, the stationary phase comprises a Poroshell 300SBC-18 column. Thus, the inventors have found that surprisingly, a stationary phase designed for large molecules such as proteins may be used to separate smaller molecules such as estrone and estradiol. The mobile phase may comprise methanol and water.

[0124] Depending upon the biomarker of interest, a variety of analytical columns known in the art may be used as needed to provide good purification. In certain embodiments, the analytical column may comprise particles having an average diameter of about 5 μm. In some embodiments, the analytical column is a functionalized silica or polymer-silica hybrid, or a polymeric particle or monolithic silica stationary phase, such as a phenyl-hexyl functionalized analytical column.

[0125] For example, in one embodiment, estrone and estradiol are separated from isobaric substances by separation using a Poroshell 300SBC10 column (7.5 mm by 2.1 mm) with 5 micron particle size using a gradient separation using methanol and water for elution at 1 mL per minute flow rate. Estrone and estradiol are transferred from the extraction column after 2.5 minutes and chromatofocused onto a phenyl-hexyl column (50 mm by 2.1 mm) with 5 micron particles using water for 45 seconds. The transferred and purified analytes are chromatographed using an accelerated gradient employing methanol and water to improve sensitivity prior to introduction into the mass spectrometer interface and subsequent detection.

[0126] For liquid chromatography of thyroxin, a single liquid chromatography step may be used. Thus, for liquid chromatography of thyroxin, a phenyl hexyl column (50 mm by 2.1 mm) with 5 micron particle size may be used. Thus, following either: (a) liquid-liquid extraction, evaporation and reconstitution; or (b) post-dialysis sample dilution with internal standard solution; samples are injected onto the liquid chromatography column. The transferred analyte and internal standard are chromatographed using a methanol:water gradient separation at 1 mL per minute. To enable further sensitivity gains, a post-separation additional flow of 90:10 methanol:water containing ammonium carbonate (1 mM) is introduced at 200 microliters per minute prior to introduction into the mass spectrometer (MS) electrospray interface.

[0127] If two liquid chromatography steps are employed, the eluted analytes may be transferred to the analytical column in a manner such that the sample is concentrated upon application to the analytical column. In some embodiments, the eluted analytes are transferred to the analytical column via a heart-cutting technique. In some embodiments, a chromatofocusing procedure is used to transfer and focus the analytes on the analytical column. Also in some embodiments, a column-switching procedure is used to transfer the analytes to the analytical column. The analytes may then be separated on the analytical column (**16**) and the fraction containing the analyte of interest is eluted. In an embodiment, the second column in run in a manner to maximize throughput, and to provide the sample in a reduced volume.

[0128] The separated analytes are then introduced into a mass spectrometer (MS) system (**20**). In some embodiments, a tandem MS/MS system is used. As is known by those of skill in the art, in tandem MS spectrometry, the precursor ion is selected following ionization, and that precursor ion is subjected to fragmentation to generate product (i.e., fragment) ions, whereby one or more product ions are selected for detection. A sample may therefore be analyzed for both estradiol and estrone since the compounds have different precursor and product ions in tandem mass spectrometric methodologies (i.e., different transitions).

[0129] The analyte of interest may then be quantified based upon the amount of the characteristic transitions measured by

tandem MS. In some embodiments, the tandem mass spectrometer comprises a triple quadrupole mass spectrometer. In some embodiments, the tandem mass spectrometer is operated in a positive ion Atmospheric Pressure Chemical Ionization (APCI) mode. In some embodiments, the quantification of the analytes and internal standards is performed in the selected reaction monitoring mode (SRM). Or, other methods of ionization such as the use of inductively coupled plasma, or MALDI, or SELDI, ESI, or APPI may be used for ionization.

[0130] In some embodiments, the back-calculated amount of each analyte in each sample may determined by comparison of unknown sample response or response ratio when employing internal standardization to calibration curves generated by spiking a known amount of purified analyte material into a standard test sample, e.g., charcoal stripped human serum. In one embodiment, calibrators are prepared at known concentrations and analyzed as per the biomarker methodology to generate a response or response ratio when employing internal standardization versus concentration calibration curve.

[0131] In one embodiment, the sample may be treated so as to chemically modify the analyte of interest to allow for improved detection in the MS system. For example, in one embodiment, a sample being analyzed for estrone and/or estradiol may be heated to the extent that the estradiol loses a molecule of water thereby converting the estradiol to a dehydrated form of the compound (FIG. **2**, Panels A and B, respectively). This conversion can reduce the number of major product ion peaks seen for estradiol from about 60 to 3 (FIG. **2**, panels C and D). For MS analysis, the sensitivity of the analysis is generally inversely proportional to the number of product ion peaks. Thus, with fewer peaks, the sensitivity of detection using tandem mass spectrometry is increased. For example, as illustrated in FIG. **2**, estradiol may be quantified by measuring the transition from the precursor ion at a mass to charge (m/z) 255.3±0.5 mass units to the two product (fragment) ions at a mass to charge (m/z) of 159.0±0.5 mass units and 133.0±0.5.

[0132] In alternate embodiments, the sensitivity obtained for measurement of estradiol is increased more than 10 fold, or more than 20 fold, or more than 50 fold, or more than 100 fold, or more than 150 fold, or more than 200 fold, or more than 500 fold, or more than 1,000 fold. For example, in alternate embodiments, the sensitivity is increased by about 5-1,000 fold, or a by about 20-500 fold, or by about 50-150 fold, or by about 100 fold.

[0133] The temperature for heating the sample may, in alternate embodiments range from 300° C. to about 1000° C. and includes all ranges therein. In an embodiment, the dehydration step is performed within the interface of the mass spectrometer employed in APCI or electrospray mode at 500 degrees C.±100 degrees. In an embodiment, the sample is heated for several microseconds at the interface for dehydration to occur. In alternate embodiments, the heating step is done for less than 1 second, or less than 100 milliseconds (msec), or less than 10 msec, or less than 1 msec, or less than 0.1 msec, or less than 0.01 msec, or less than 0.001 msec.

[0134] In an embodiment, the tandem liquid chromatography (LC) steps help reduce isobaric interferences. For example, in one embodiment, there are 24 potential isobaric interferences in estradiol (transition m/z 255->159, 133), and **16** potential interferences for estrone (transition m/z 273->159, 133). For example, dehydroepiandrosterone (DHEA) undergoes thermal dehydration forming MH-H20]+ and MH-2H_2O]+ (FIG. **3**). There may be DHEA concentrations that are about 300-1,500 times the levels of estrone and estradiol in healthy patients. Thus, the M+2 isotopic overlap of dehydrated DHEA may become an isobaric interference. Heart cutting from the primary separation using isocratic or gradient separation resolves most isobaric interferences (FIG. **4**). Thus, as shown in FIG. **4**, heart cutting combined with chromatofocusing may be used to separate estradiol (E2) and estrone (E1) from all but one potential isobaric contaminant which is separated within the analytical (second) liquid chromatography separation dimension.

[0135] An example of a method for measuring estradiol and estrone is provided in FIG. **5**. For example, in an embodiment, a method (**40**) of measuring estrone and estradiol comprise providing a sample believed to contain at least one of estrone and estradiol (**44**). The method may also comprise adding an internal standard of D_4-estrone and D_5-estradiol to the sample (**46**).

[0136] Also, the method may optionally comprise partial purification of the estrone and estradiol by liquid-liquid extraction of the estrone and/or estradiol from the serum with 9:1 hexane-ethyl acetate (**48**). Or, the sample may be diluted (**50**) as a means to improve sensitivity in subsequent purification and/or analysis steps (e.g. LC and/or MS).

[0137] After initial purification by liquid-liquid extraction or dilution, the sample may be further purified by liquid chromatography. Thus, in one embodiment, the solvent is evaporated and the extracted estrone/estradiol is reconstituted in 30:70 methanol water for application to a liquid chromatography extraction column (**52**). The estradiol/estrone may be eluted from the extraction column. For example, in alternate embodiments, the estradiol/estrone may be eluted by heart cutting, chromatofocusing or column switching. Next, the fraction containing the estrone/estradiol may, in certain embodiments, be applied to an analytical LC column (**54**). The fraction containing the estrone/estradiol may then be transferred to the LC-MS/MS interface to undergo ionization and dehydration of the estradiol (**60**) prior to MS/MS detection in SRM mode (**62**). In an embodiment, heating the estradiol removes a molecule of water, and changes the resultant MS/MS profile such that it comprises only three major product ions.

[0138] Thus, the methods provide the ability to quantify estrone and/or estradiol at physiologically relevant levels. As discussed herein, the difference between a serum level of 10 pg/mL and 15 pg/mL may be clinically relevant. In one embodiment, the method is able to measure estrone and/or estradiol at levels of about 2.5 pg/mL and 1 pg/mL respectively.

[0139] An example of a method for measuring free thyroxine (T4) (**70**) is provided in FIG. **6**. In an embodiment, the method may comprise providing a sample that includes thyroxine (both free and protein-bound) (**74**). The method may also comprise dialyzing the sample (**76**) to separate the free thyroxine from the protein bound thyroxine. Also, the method may comprise adding an internal standard such as $^{6}C_{13}$-thyroxine (**78**) to allow for the measured amount of thyroxine to be correlated to the actual amount present in the sample (i.e., to quantify the amount lost during the extraction and measurement procedures).

[0140] The method may also comprise an optional step whereby the free thyroxine present in the dialysate is extracted by liquid-liquid extraction (**80**). Alternatively, the sample may be diluted into the solvent used for LC-MS/MS

as a means to reduce interference from non-T4 or non-T3 analytes (**81**). At this point, the solvent used for extraction may be evaporated, and the extracted thyroxine reconstituted in 50:50 methanol:water for application to an LC column (**82**). The free thyroxine may then be eluted from the column (**84**) and then quantified by MS/MS (**86**).

[0141] Thus, the methods provide the ability to quantify free thyroxine at physiologically relevant levels. The difference between a serum level of 8 pg/mL and 12 pg/mL T4 may be clinically relevant. The method is able to measure free thyroxine (T4) at levels of 2 pg/mL.

[0142] Systems for Quantification of Endogenous Biomarkers

[0143] FIG. **7**A shows an embodiment of a system of the present invention. As shown in FIG. **7**, the system may comprise a station for aliquoting a sample (**104**) that may comprise a biomarker of interest into sampling containers. In one embodiment, the sample is aliquoted into a container or containers to facilitate liquid-liquid extraction or sample dilution. The station for aliquoting may comprise receptacles to discard the portion of the biological sample that is not used in the analysis.

[0144] Alternatively or additionally, the sample may be aliquoted into a container for dialysis. As described above, the container for dialysis may comprise a multi-well plate. Thus, in addition to the station for aliquoting, the system may comprise a station for dialysis (**106**). The station for dialysis may comprise a rotator oven, multi-chamber pipettes for sample transfer, as well as receptacles to discard the portion of the biological sample that is not used in the analysis.

[0145] The system may further comprise a station for adding an internal standard to the sample (**108**). In an embodiment, the internal standard comprises the biomarker of interest labeled with a non-natural isotope. Thus, the station for adding an internal standard may comprise safety features to facilitate adding an isotopically labeled internal standard solutions to the sample. The system may also, in some embodiments, comprise a station (**110**) for liquid-liquid extraction and/or dilution of the sample.

[0146] The system may also comprise a station for liquid chromatography (LC) of the sample. As described herein, in an embodiment, the station for liquid chromatography may comprise an extraction liquid chromatography column (**112**). The station for liquid chromatography may comprise a column comprising the stationary phase, as well as containers or receptacles comprising solvents that are used as the mobile phase. In an embodiment, the mobile phase comprises a gradient of methanol and water, acetonitrile and water, or other miscible solvents with aqueous volatile buffer solutions. Thus, in one embodiment, the station may comprise the appropriate lines and valves to adjust the amounts of individual solvents being applied to the column or columns. Also, the station may comprise a means to remove and discard those fractions from the LC that do not comprise the biomarker of interest. In an embodiment, the fractions that do not contain the biomarker of interest are continuously removed from the column and sent to a waste receptacle for decontamination and to be discarded.

[0147] A variety of extraction LC systems may be used. For example, in the embodiment where the system is being used to measure estrone or estradiol, a Poroshell 300SBC18 extraction column with a phenyl hexyl analytical column, with mobile phases comprising a gradient of methanol and water are used. Or, for measurement of thyroxine, a phenyl hexyl column, with a mobile phase of methanol:water is used with post-column addition of a methanol:water solution containing ammonium carbonate. Or, for vitamin D metabolites, a Fluophase WP extraction column, with a mobile phase of methanol:water is used and an Extent C18 analytical column is used with a mobile phase of methanol:water is used.

[0148] The system may also comprise an analytical LC column (**114**). The analytical column may facilitate further purification and concentration of the biomarker of interest as may be required for further characterization and quantification.

[0149] Also, the system may comprise a station for characterization and quantification of the biomarker of interest. In one embodiment, the system may comprise a station for mass spectrometry (MS) of the biomarker. In an embodiment, the station for mass spectrometry comprises a station for tandem mass spectrometry (MS/MS). Also, the station for characterization and quantification may comprise a computer and software for analysis of the MS/MS results. In an embodiment, the analysis comprises both identification and quantification of the biomarker of interest.

[0150] In some embodiments, one or more of the purification or separation steps can be preformed "on-line." As used herein, the term "on-line" refers to purification or separation steps that are performed in such a way that the test sample is disposed, e.g., injected, into a system in which the various components of the system are operationally connected and, in some embodiments, in fluid communication with one another. The on-line system may comprise an autosampler for removing aliquots of the sample from one container and transferring such aliquots into another container. For example, an autosampler may be used to transfer the sample after extraction onto an LC extraction column. Additionally or alternatively, the on-line system may comprise one or more injection ports for injecting the fractions isolated from the LC extraction columns onto the LC analytical column. Additionally or alternatively, the on-line system may comprise one or more injection ports for injecting the LC purified sample into the MS system. Thus, the on-line system may comprise one or more columns, including but not limited to, an extraction column, including an HTLC extraction column, and in some embodiments, an analytical column. Additionally or alternatively, the system may comprise a detection system, e.g., a mass spectrometer system. The on-line system may also comprise one or more pumps; one or more valves; and necessary plumbing. In such "on-line" systems, the test sample and/or analytes of interest can be passed from one component of the system to another without exiting the system, e.g., without having to be collected and then disposed into another component of the system.

[0151] In some embodiments, the on-line purification or separation method can be automated. In such embodiments, the steps can be performed without the need for operator intervention once the process is set-up and initiated. For example, in one embodiment, the system, or portions of the system may be controlled by a computer or computers (**102**). Thus, in certain embodiments, the present invention may comprise software for controlling the various components of the system, including pumps, valves, autosamplers, and the like. Such software can be used to optimize the extraction process through the precise timing of sample and solute additions and flow rate.

[0152] Although some or all of the steps in the method and the stations comprising the system may be on-line, in certain

embodiments, some or all of the steps may be performed "off-line." In contrast to the term "on-line", the term "off-line" refers to a purification, separation, or extraction procedure that is performed separately from previous and/or subsequent purification or separation steps and/or analysis steps. In such off-line procedures, the analytes of interests typically are separated, for example, on an extraction column or by liquid/liquid extraction, from the other components in the sample matrix and then collected for subsequent introduction into another chromatographic or detector system. Off-line procedures typically require manual intervention on the part of the operator.

[0153] Liquid chromatography may, in certain embodiments, comprise high turbulence liquid chromatography or high throughput liquid chromatography (HTLC). See, e.g., Zimmer et al., J. Chromatogr. A 854:23-35 (1999); see also, U.S. Pat. Nos. 5,968,367; 5,919,368; 5,795,469; and 5,772,874. Traditional HPLC analysis relies on column packings in which laminar flow of the sample through the column is the basis for separation of the analyte of interest from the sample. In such columns, separation is a diffusional process. Turbulent flow, such as that provided by HTLC columns and methods, may enhance the rate of mass transfer, improving the separation characteristics provided. In some embodiments, high turbulence liquid chromatography (HTLC), alone or in combination with one or more purification methods, may be used to purify the biomarker of interest prior to mass spectrometry. In such embodiments, samples may be extracted using an HTLC extraction cartridge which captures the analyte, then eluted and chromatographed on a second HTLC column or onto an analytical HPLC column prior to ionization. Because the steps involved in these chromatography procedures can be linked in an automated fashion, the requirement for operator involvement during the purification of the analyte can be minimized. Also, in some embodiments, the use of a high turbulence liquid chromatography sample preparation method can eliminate the need for other sample preparation methods including liquid-liquid extraction. Thus, in some embodiments, the test sample, e.g., a biological fluid, can be disposed, e.g., injected, directly onto a high turbulence liquid chromatography system.

[0154] For example, in a typical high turbulence or turbulent liquid chromatography system, the sample may be injected directly onto a narrow (e.g., 0.5 mm to 2 mm internal diameter by 20 to 50 mm long) column packed with large (e.g., >25 micron) particles. When a flow rate (e.g., 3-500 mL per minute) is applied to the column, the relatively narrow width of the column causes an increase in the velocity of the mobile phase. The large particles present in the column can prevent the increased velocity from causing back pressure and promote the formation of vacillating eddies between the particles, thereby creating turbulence within the column.

[0155] In high turbulence liquid chromatography, the analyte molecules may bind quickly to the particles and typically do not spread out, or diffuse, along the length of the column. This lessened longitudinal diffusion typically provides better, and more rapid, separation of the analytes of interest from the sample matrix. Further, the turbulence within the column reduces the friction on molecules that typically occurs as they travel past the particles. For example, in traditional HPLC, the molecules traveling closest to the particle move along the column more slowly than those flowing through the center of the path between the particles. This difference in flow rate causes the analyte molecules to spread out along the length of the column. When turbulence is introduced into a column, the friction on the molecules from the particle is negligible, reducing longitudinal diffusion.

[0156] The methods and systems of the present invention may use mass spectrometry to detect and quantify the biomarker of interest. The terms "mass spectrometry" or "MS" as used herein generally refer to methods of filtering, detecting, and measuring ions based on their mass-to-charge ratio, or "m/z." In MS techniques, one or more molecules of interest are ionized, and the ions are subsequently introduced into a mass spectrometer where, due to a combination of electric fields, the ions follow a path in space that is dependent upon mass ("m") and charge ("z").

[0157] In certain embodiments, the mass spectrometer uses a "quadrupole" system. In a "quadrupole" or "quadrupole ion trap" mass spectrometer, ions in an oscillating radio frequency (RF) field experience a force proportional to the direct current (DC) potential applied between electrodes, the amplitude of the RF signal, and m/z. The voltage and amplitude can be selected so that only ions having a particular m/z travel the length of the quadrupole, while all other ions are deflected. Thus, quadrupole instruments can act as both a "mass filter" and as a "mass detector" for the ions injected into the instrument.

[0158] In certain embodiments, tandem mass spectrometry is used. See, e.g., U.S. Pat. No. 6,107,623, entitled "Methods and Apparatus for Tandem Mass Spectrometry," which is hereby incorporated by reference in its entirety. Further, the selectivity of the MS technique can be enhanced by using "tandem mass spectrometry," or "MS/MS." Tandem mass spectrometry (MS/MS) is the name given to a group of mass spectrometric methods wherein "parent or precursor" ions generated from a sample are fragmented to yield one or more "fragment or product" ions, which are subsequently mass analyzed by a second MS procedure. MS/MS methods are useful for the analysis of complex mixtures, especially biological samples, in part because the selectivity of MS/MS can minimize the need for extensive sample clean-up prior to analysis. In an example of an MS/MS method, precursor ions are generated from a sample and passed through a first mass filter to select those ions having a particular mass-to-charge ratio. These ions are then fragmented, typically by collisions with neutral gas molecules in a suitable ion containment device, to yield product (fragment) ions, the mass spectrum of which is recorded by a electron multiplier detector. The product ion spectra so produced are indicative of the structure of the precursor ion, and the two stages of mass filtering can eliminate ions from interfering species present in the conventional mass spectrum of a complex mixture.

[0159] In an embodiment, the methods and systems of the present invention use a triple quadrupole MS/MS (see e.g., Yost, Enke in Ch. 8 of Tandem Mass Spectrometry, Ed. McLafferty, pub. John Wiley and Sons, 1983). Triple quadrupole MS/MS instruments typically consist of two quadrupole mass filters separated by a fragmentation means. In one embodiment, the instrument may comprise a quadrupole mass filter operated in the RF only mode as an ion containment or transmission device. In an embodiment, the quadrupole may further comprise a collision gas at a pressure of between 1 and 10 millitorr. Many other types of "hybrid" tandem mass spectrometers are also known, and can be used in the methods and systems of the present invention including various combinations of magnetic sector analyzers and quadrupole filters. These hybrid instruments often comprise high

resolution magnetic sector analyzers (i.e., analyzers comprising both magnetic and electrostatic sectors arranged in a double-focusing combination) as either or both of the mass filters. Use of high resolution mass filters may be highly effective in reducing chemical noise to very low levels.

[0160] For the methods and systems of the present invention, ions can be produced using a variety of methods including, but not limited to, electron ionization, chemical ionization, fast atom bombardment, field desorption, and matrix-assisted laser desorption ionization ("MALDI"), surface enhanced laser desorption ionization ("SELDI"), photon ionization, electrospray ionization, and inductively coupled plasma.

[0161] In those embodiments, such as MS/MS, where precursor ions are isolated for further fragmentation, collision-induced dissociation ("CID") may be used to generate the fragment ions for further detection. In CID, 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.

[0162] In some embodiments, to attain the required analytical selectivity and sensitivity, the presently disclosed 2D-LC-MS/MS methods include multiplexed sample preparation procedures. For example, in certain embodiments dialysis of the sample is performed using a 96 well plate having a dialysis membrane in each well or multiple sample tubes (FIG. **7**B). Additionally or alternatively, the multiplex system may comprise staggered multiplexed LC and MS sample inlet systems. Also, the methods and systems of the present invention may comprise multiple column switching protocols, and/or heart-cutting (LC-LC or 2D-LC) techniques, and/or LC separations prior to MS detection. In some embodiments, the methods and systems of the present invention may include a multiplexed two-dimensional liquid chromatographic system coupled with a tandem mass spectrometer (MS/MS) system, for example a triple quadrupole MS/MS system. Such embodiments provide for staggered, parallel sample input into the MS system.

[0163] Thus, as shown in FIG. **7**B, four samples (**132** A-D) may each be applied to individual extraction columns (**134** A-D). Once the samples have each run through the extraction column, they may each be transferred directly (e.g., by column switching) to a second set of analytical columns (**136** A-D). As each sample elutes from the analytical column, it may be transferred (**138**) to the mass spectrometer (**140**) for identification and quantification.

[0164] A plurality of analytes can be analyzed simultaneously or sequentially by the presently disclosed LC-MS/MS and 2D-LC-MS/MS methods. Exemplary analytes amenable to analysis by the presently disclosed methods include, but are not limited to, steroid hormones, such as estradiol, estrone, and metabolites, such as estrone sulfate. In other embodiments, thyroid hormones, such as free thyroxine (T4) and triiodothyronine (T3) can be measured. In the other embodiments, metabolites, such as 25-Hydroxyvitamin D2,25-Hydroxyvitamin D3, may be measured. One of ordinary skill in the art would recognize after a review of the presently disclosed subject matter that other similar analytes could be analyzed by the methods and systems disclosed herein. Thus, in alternate embodiments, the methods and systems may be used to quantify steroid hormones, protein and peptide hormones, peptide and protein biomarkers, drugs of abuse and therapeutic drugs. For example, optimization of key parameters for each analyte can be performed using a modular method development strategy to provide highly tuned bioanalytical assays. Thus, certain steps may be varied depending upon the analyte being measured as disclosed herein.

[0165] Also, embodiments of the methods and systems of the present invention may provide greater sensitivity than the sensitivities previously attainable for many of the analytes being measured. For example, through using this optimization procedure, an LOQ of about 1 picogram per milliliter (pg/mL), or less than 5 pg/mL, or less that 10 pg/mL, or less than 25 pg/mL is attained for the analysis of at least one of estradiol, estrone or free thyroxine without the cumbersome derivatization processes historically required for LC-MS/MS analyses of steroids. Importantly, the low levels of detection allow for the analysis of small sample volumes, for example 100 μL, 200 μL, 500 μL, or less than 1 mL, which can be necessary to analyze pediatric sample volumes. Thus, the presently disclosed LC-MS/MS and (LC)-LC-MS/MS methods can be used to measure levels of steroid hormones, such as estrone and estradiol, or other hormones or metabolites (e.g., free thyroxine, vitamin D metabolites and the like) in serum or plasma samples from children, women, and men.

[0166] Embodiments of the present invention may provide certain advantages. In certain embodiments, the methods and systems of the present invention may provide greater sensitivity than the sensitivities previously attainable for many of the analytes being measured.

[0167] Also, embodiments of the methods and systems of the present invention may provide for rapid throughput that has previously not been attainable for many of the analytes being measured. For example, using the methods and systems of the present invention, multiple samples may be analysed for free thyroxine using 96 well plates and a multiplex system of four LC-MS/MS systems, significantly increasing the throughput.

[0168] As another advantage, the specificity and sensitivity provided by the methods and systems of the present invention may allow for the analysis of analytes from a variety of biological materials. For example, the 2D-LC-MS/MS methods of the present invention can be applied to the quantification of analytes of interest in complex sample biological matrices, including, but not limited to, blood, serum, plasma, urine, saliva, and the like. Thus, the methods and systems of the present invention are suitable for clinical applications and/or clinical trials.

[0169] As additional potential advantages, in certain embodiments, the systems and methods of the present invention provide approaches for addressing isobaric interferences, varied sample content, including hemolysed and lipemic samples, while attaining low pg/mL limits of quantification (LOQ) of the target analytes. Accordingly, embodiments of the methods and systems of the present invention may provide for the quantitative, sensitive, and specific detection of clinical biomarkers used in the clinical diagnosis of endocrine disorders.

Validation of LC-MS/MS and 2D-LC-MS/MS Assays for Endogenous Biomarkers

[0170] A general strategy for the validation of the presently disclosed LC-MS/MS and 2D-LC-MS/MS methods for endogenous biomarkers is provided in Scheme 1. Thus, Scheme 1 shows the different tests that were used to validate

the procedures. Matrix specificity testing was performed by analyzing 6 different lots of charcoal stripped matrix in quadruplicate for the presence of residual analyte, absence of analyte enables the charcoal stripped matrix to be spiked with known concentrations of target analytes to generate calibration curves. Internal standard specificity was performed by spiking the stable labeled internal standard into analyte-free charcoal stripped matrix and measuring for the presence of analyte in quadruplicate. Absence of unlabeled analyte confirms the purity of internal standard materials. Endogenous (hormones) and exogenous (drugs) are spiked into analyte free matrix to confirm the selectivity of the method.

[0171] Accuracy and precision was determined using 6 replicates per level in spiked charcoal stripped serum at the LLOQ, 2 levels within the analytical range and the ULOQ in 3 different batches. Precision was determined using 6 replicates in 3 separate runs of pooled matrix samples at concentrations of approximately 3 to 10 times the LLOQ, the mid point of the analytical range and approximately 80% of the ULOQ. Accuracy was determined in pooled matrix samples using spike and recovery (standard addition) at 3 different concentrations throughout the analytical range using 4 replicates per level.

[0172] Linearity was confirmed using multi-level calibrators over 5 separate runs. Sample mixing experiments were also undertaken mixing pooled matrix samples with fortified stripped matrix samples to ensure the assays were free of matrix interferences in quadruplicate. Recovery was undertaken using both spiked stripped matrix and pooled matrix samples in quadruplicate as confirmation of linearity and also further proof that the assay was free of matrix effects. The effect of matrix content on measurement was also tested following post-column infusion, addition of lipemia and hemolysis content, alternate sample types (e.g. serum and or plasma) and sample draw-tubes in quadruplicate. Sample stability was undertaken using both spiked stripped matrix samples and pooled matrix samples at storage conditions expected from sample collection to final analysis. Each condition was compared against baseline samples drawn and frozen at −70° C. for comparison and analyzed in quadruplicate at each concentration.

[0173] Inter-assay comparison was performed using at least 50 samples representing physiological range during comparison of LC-MS/MS and LC-MS/MS assays with alternate techniques. Reference range generation and/or transference was undertaken using guidance from the National Committee on Clinical Laboratory Standards (NCCLS).

Scheme 1. Bioanalytical Validation Strategy

Specificity Testing:	Matrix, Internal Standard, Endogenous/ Exogenous Analytes
Accuracy and Precision:	Stripped Matrix: LLOQ, Mid levels (×2) ULOQ; Pooled Matrix: 3× LLOQ, mid and 80% of ULOQ; Spike and Recovery at 3 levels
Linearity:	Stripped Matrix: 7-10 point duplicate curves, 5 batches; Pooled Matrix: Sample dilution (mixing) at 4 levels
Recovery:	Stripped and Pooled Matrix: Spike and recovery at 3 levels
Ionization Effect:	Stripped and Pooled Matrix: Post-column infusion, post-extraction spiking, Heparin and EDTA anticoagulants, Lipemic and Hemolysis additives

-continued

Scheme 1. Bioanalytical Validation Strategy

Specificity Testing:	Matrix, Internal Standard, Endogenous/ Exogenous Analytes
Sample Stability:	Stripped and Pooled Matrix: Pre and Post processing stability compared against −70° C. baseline samples, Whole blood stability and stock solutions
Inter-Assay Comparison:	Pooled Matrix samples: At least 50 samples spanning normal and abnormal range
Reference Range:	Transference based upon 90% CI for at least 20 samples per range

[0174] Representative LC-MS/MS and 2D-LC-MS/MS chromatograms of selected analytes at the limit of quantification (LOQ) obtained by using the LC-MS/MS and 2D-LC-MS/MS methods of the present invention are shown in FIGS. **8-13**. In these figures the X axis is time, and the Y axis corresponds to the amount of material (i.e., the response). Thus, it can be seen that estrone sulfate was detected at 100 pg/mL (FIG. **8**); free thyroxine was detected at 2 pg/mL (FIG. **9**); 25-hydroxyvitamin D2 was detected at 1 ng/mL (FIG. **10**); 25-hydroxyvitamin D3 was detected at 1 ng/mL (FIG. **11**); estrone was detected at 2.5 pg/mL (FIG. **12**); and estradiol was detected at 1 pg/mL (FIG. **13**).

[0175] Similarly, representative LC-MS/MS and 2D-LC-MS/MS chromatograms of selected analytes at the upper limit of quantification (ULOQ) (the level of analyte above which the assay is outside of linear range) obtained by using the LC-MS/MS and 2D-LC-MS/MS methods and systems of the present invention are shown in FIGS. **14-19**. Thus, it can be seen that estrone sulfate has an ULOQ of 50 ng/mL (FIG. **14**); free thyroxine has an ULOQ of 100 pg/mL (FIG. **15**); 25-hydroxyvitamin D2 has an ULOQ of 250 ng/mL (FIG. **16**); 25-hydroxyvitamin D3 has an ULOQ of 250 ng/mL (FIG. **17**); estrone has an ULOQ of 500 pg/mL (FIG. **18**); and estradiol has an ULOQ of 500 pg/mL (FIG. **19**).

[0176] Representative LC-MS/MS and 2D-LC-MS/MS calibration curves for selected analytes are shown in FIGS. **20-25**. FIG. **20** shows a calibration curve for estrone sulfate where it is seen that the assay is linear over a 1000-fold range. Calibration curves for thyroxine (FIG. **21**), 25-hydroxyvitamin D2 (FIG. **22**), 25-hydroxyvitamin D3 (FIG. **23**), estrone (FIG. **24**) and estradiol (FIG. **25**) also show linearity over at 100 to 250 fold range. In these figures, the X axis is the concentration, and the Y axis is the ratio of the analyte to an internal standard response.

[0177] Representative inter-assay comparison results for the presently disclosed LC-MS/MS and 2D-LC-MS/MS methods versus RIA, CBP, or IA methods are shown in FIGS. **26-30**. Thus, it can be seen that using LC-MS/MS provides excellent correlation with RIA for measurement of estrone sulfate (FIG. **26**); good correlation but a bias (slope offset) with IA for measurement of free thyroxine (FIG. **27**), excellent correlation but a bias (slope offset) for measurement of total 25-hydroxyvitamin D by CBP and average correlation with IA (FIG. **28**, panels A and B, respectively); excellent correlation with RIA for measurement of estrone (FIG. **29**); and excellent correlation with RIA for measurement of estradiol (FIG. **30**). Also, FIG. **31** shows good correlation between the 2D-LC-MS/MS assays described herein for estradiol with an alternate LC-MS/MS strategy involving derivatization.

Excellent correlation is observed for samples within the higher analytical range of the derivatization assay (10 pg/mL LLOQ).

[0178] FIG. **32** shows results of dialysis losses for thyroxine. The squares (■) show dialysis losses and the diamonds (♦) show effective dialysis for free thyroxine using 96-well equilibrium dialysis plates in accordance with one embodiment of the present invention. This indicates that during the dialysis experiments, free T4 does not degrade or bind to the 96-well plate apparatus (i.e. losses). Further, FIG. **31** indicates that dialysis is complete after approximately 16 hours.

[0179] Data showing the validation bias due to ionization effect and recovery for selected analytes are provided in Tables 1 through 7, below. Data showing the accuracy and precision of the presently disclosed LC-MS/MS and 2D-LC-MS/MS methods are provided in Tables 8 through 13. As known by those in the art, acceptable values based on the FDA and CLIA regulations are ≦20% bias or imprecision (% CV) at the LLOQ and ≦15% over the remainder of the assay. See e.g., FDA Guidance: 1.1 Guidance for Industry, Bioanalytical Method Validation, FDA, May 2001, BP, and CLIA Regulation: 42 CFR 493.1253 Standard: Establishment and verification of performance specifications. Subart K, Quality System for Non-waived Testing. Thus, as used herein, "acceptable" or "good" indicates that the assay or aspect of the method being measured meets the NCCLS, FDA and CLIA criteria.

[0180] Each of the presently disclosed LC-MS/MS and 2D-LC-MS/MS methods was evaluated for specificity against multiple steroids and/or other potential interferences for a total of up to 60 different analytes (see Examples 1 and 2) at excessive concentrations, for example, 100 μg/dL, to ensure accurate measurement in each assay independent of clinical levels of endogenous and therapeutic agents, such as steroids, which either cross-react in RIA assays or are not discriminated using the specificity provided by MS/MS detection. Additionally, analyte stability was evaluated for all conditions expected from original patient sampling to final result. Proven stability includes sample shipment (−20° C.), sample processing (20° C.; >3 freeze/thaw cycles) and post processing (20° C. and autosampler at 10° C.) to ensure accurate and precise determination of analytes, such as hormone steroids, derived from patient samples (see Examples 1 and 2).

TABLE 1

Estrone Sulfate

Validation	Bias (%)
Serum	−3.8-2.6
Lipemia	−0.2
Hemolysis	0.7
Recovery	85.0-97.1

TABLE 2

Free Thyroxine

Validation	Bias (%)
Serum	−6.9-4.8
Lipemia	1.0
Hemolysis	12.8
Recovery	90.5-95.8

TABLE 3

25-Hydroxyvitamin D2

Validation	Bias (%)
Serum	−3.8-2.2
Lipemia	−5.0
Hemolysis	−5.4
Recovery	93.2-100.5

TABLE 4

25-Hydroxyvitamin D3

Validation	Bias (%)
Serum	−8.8-11.9
Lipemia	−0.3
Hemolysis	−1.6
Recovery	88.8-94.1

TABLE 5

Estrone

Validation	Bias (%)
Serum	−11.9-7.8
Lipemia	8.3
Hemolysis	2.8
Recovery	97.2-106.9

TABLE 6

Estradiol

Validation	Bias (%)
Serum	−4.5-4.0
Lipemia	−5.9
Hemolysis	−2.9
Recovery	90.0-95.7

TABLE 7

Estrone Sulfate

	Accuracy (%)				Precision (%)			
Conc. (ng/dL)	10	150	2500	5000	10	150	2500	5000
Intra - 1	−13.1	−1.6	3.0	2.1	3.2	5.4	7.3	8.2
Intra - 2	−18.9	−5.7	4.7	3.2	14.1	3.8	2.0	8.2
Intra - 3	−7.5	−9.3	8.6	−0.4	3.6	4.6	6.0	7.8
Inter	−13.2	−5.5	5.4	0.5	9.4	5.5	5.7	7.9

TABLE 8

Free Thyroxine

	Accuracy (%)			Precision (%)		
Conc. (ng/dL)	0.2	5	10	0.2	5	10
Intra - 1	−3.4	−7.0	3.0	5.0	6.2	5.5
Intra - 2	0.1	−1.2	−0.3	5.1	5.2	2.2
Intra - 3	−0.4	0.6	3.7	4.4	4.1	5.5
Inter	−1.3	−2.5	2.1	4.8	6.0	4.8

TABLE 9

	25-Hydroxyvitamin D2							
	Accuracy (%)				Precision (%)			
Conc. (ng/mL)	1.0	2.5	1.00	250	1.0	2.5	100	250
Intra - 1	6.3	−8.1	−2.0	−0.4	4.6	5.6	0.9	2.2
Intra - 2	13.9	8.6	−2.2	1.7	3.3	6.0	2.6	4.5
Intra - 3	8.7	4.2	−0.1	4.2	6.8	5.2	4.4	3.7
Inter	11.3	1.6	−1.4	1.3	6.9	8.9	3.0	3.9

TABLE 10

	25-Hydroxyvitamin D3							
	Accuracy (%)				Precision (%)			
Conc. (ng/mL)	1.0	2.5	1.00	250	1.0	2.5	100	250
Intra - 1	−3.9	−4.0	−2.8	−0.4	5.8	5.7	4.6	2.1
Intra - 2	6.2	4.8	−1.4	1.5	11.2	4.6	3.9	5.5
Intra - 3	5.9	3.7	−2.1	−0.4	3.0	9.9	3.0	3.9
Inter	2.7	1.5	−2.1	0.3	8.5	7.8	3.7	3.9

TABLE 11

	Estrone					
	Accuracy (%)			Precision (%)		
Conc. (pg/mL)	2.5	250	500	2.5	250	500
Intra - 1	12.4	1.1	−1.4	2.9	5.5	3.3
Intra - 2	−0.3	9.2	4.0	7.3	2.8	2.4
Intra - 3	3.6	6.3	5.1	6.3	3.8	1.6
Inter	4.8	5.5	2.6	7.4	5.1	3.7

TABLE 12

	Estradiol							
	Accuracy (%)				Precision (%)			
Conc. (pg/mL)	1.0	2.5	1.00	250	1.0	2.5	100	250
Intra - 1	−7.1	1.1	−1.1	0.4	4.7	4.8	2.8	1.2
Intra - 2	−7.4	1.1	6.4	6.5	4.4	6.3	1.6	4.3
Intra - 3	4.7	5.6	0.6	1.4	4.7	5.6	2.6	3.5
Inter	−3.3	2.7	1.9	2.8	7.4	5.2	3.9	4.1

EXAMPLES

[0181] Additional data from the analytical validation and standard operating procedures for the presently disclosed method for the quantification of estrone and estradiol by liquid-liquid extraction and 2D-LC-MS/MS, or free thyroxine by dialysis, an optional liquid-liquid extraction, and LC-MS/MS are set forth in the following Examples.

[0182] The following Examples have been included to provide guidance to one of ordinary skill in the art for practicing representative embodiments of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill can appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently disclosed subject matter.

Example 1

2D-LC-MS/MS Analysis for Estrone, Estradiol, and Estrone Sulfate

[0183] Estrone and estradiol were validated to 2.5 pg/mL and 1 pg/mL, respectively, from a 1-mL serum sample. Sensitivity and selectivity were generated using heat assisted and guided fragmentation of estradiol to optimize sensitivity. Analytical specificity was generated via 2D LC using gradients in both LC dimensions, heart-cutting and chromatofocusing prior to MS/MS detection. Optimum selectivity was generated following titration of buffer pH and chromatography to enable separation from co-extracted isobaric endogenous interferences, while retaining necessary sensitivity (S/N>20) for accurate quantification in estrone sulfate analysis using LC-MS/MS.

[0184] More particularly, estrone (E1) and estradiol (E2) were measured by two-dimensional (2D) liquid chromatography with tandem mass spectrometry detection (2D-LC-MS/MS) after liquid-liquid extraction (LLE). Deuterated D_4-Estrone and D_5-Estradiol were added as internal standards to serum aliquots. Estrone and Estradiol were extracted from 1 mL serum samples with 8 mL of 9:1 Hexane:Ethyl Acetate. The organic extract was transferred to a fresh tube and back washed with 1 mL aqueous sodium hydroxide solution (pH of about 12), then evaporated and reconstituted in 70:30 Water: Methanol.

[0185] Duplicate sets of stripped serum calibrators were analyzed in each batch. All injections were made in singlicate. All samples are injected onto the ARIA TX4 or Transcend TX4 HTLC system (Thermo Fisher, Franklin, Mass., United States of America), where the analytes were first eluted using a separation gradient through an extraction column. Analytes of interest were then transferred onto the analytical column, where chromatographic separation was continued via a gradient. An API5000 triple quadrupole mass spectrometer (MDS-SCIEX, Concord, Ontario, Canada), operating in positive ion Atmospheric Pressure Chemical Ionization (APCI) mode was used for detection.

[0186] Quantification of analyte and internal standard was performed in selected reaction monitoring mode (SRM). The back-calculated amount of each analyte in each sample was determined from duplicate calibration curves generated by spiking known amounts of purified estrone and estradiol into charcoal stripped human serum.

[0187] Measurement of estrone and estradiol was used to evaluate ovarian function and to evaluate excess or diminished estrogen levels. Analysis of estrone and estradiol by 2D-LC-MS/MS detection was developed to measure levels in serum or plasma samples from children, women and men.

[0188] The lower limit of detection using the default sample aliquot of 1 mL was 1.0 pg/mL for Estradiol and 2.5 pg/mL for Estrone.

Definitions

[0189] APCI—Atmospheric Pressure Chemical Ionizationl LLE—Liquid-Liquid Extraction; SST—System Suitability Test; 2D-LC-MS/MS—Two-dimensional liquid chromatography with tandem mass spectrometry detection;

E1—Estrone; E2-17β-Estradiol or Estradiol; HTLC—High Throughput Liquid Chromatography

Specimens

[0190] A recommended sample is 1.5 mL serum or plasma. Separate within one hour. Store and ship frozen in plastic vial.

Adult:	1.5 mL serum or plasma
Pediatric:	1.5 mL serum or plasma
Minimum:	1 mL serum or plasma

[0191] Draw into red top vacutainer tube. Allow clotting to occur for 20 minutes at room temperature (or until clot has retracted). Spin and remove serum to labeled plastic vial. Freeze immediately. Storage is for short term storage (2 weeks): Frozen (≦−20° C.); for long term storage (6 months): Frozen (≦−20° C.). Shipping of specimens is: frozen (≦−20° C.)—on dry ice. Generally, samples drawn into SST tubes are unacceptable for this procedure.

Equipment & Materials

[0192] The following materials were used: Standard manual pipetting devices; 1.2 ml MBlock Polypropylene 96 Well Collection Plate (SPE Ware, Inc. Product No. SPE0210); Heat Sealing Foil (SPE Ware, Inc. Product No. AB-0589); Mechanical Shaker, Eberbach Inc.; Rotary Evaporator (rotovap), Speed Vac SC 200, Savant (or equivalent); Class A Volumetric Glass Containers, various sizes; API 5000 Tandem Mass Spectrometer, Sciex, (Toronto, Canada); Turbo V™ Ion Source with APCI probe, Sciex, (Toronto, Canada); Aria TX4 HTLC System, Cohesive Technologies, (MA, USA) consisting of 4 each: 1100 Series Quaternary Pump, 1100 Series Binary Pump, 1100 Series Vacuum Degasser; HTS Twin PAL System Autosampler, CTC Analytics AG (Switzerland); Luna Phenyl-Hexyl Analytical Column, 50×2.0 mm, 5 μm particle size, Phenomenex, (USA) Product No. 00B-4257-B0; Poroshell 300SB-C18 Column, 2.1×75 mm, 5 μm particle size, Agilent Inc., (USA) Product No. 660750-902; 16 mm Flange Caps, Stockwell Scientific, (AZ, USA) Product No. 8558; Vortex Mixer, VWR or equivalent; Combi-thermo Heat Sealer, Abgene Inc., Product No. AB-0559; 96-Well Centrifuge 5804R Eppendorf or equivalent; 16×100 mm borosilicate glass tubes; Thermo Hot pocket column oven, Thermo Electron Corp. (USA) Product No. 92016-150; Analyst Version 1.4 or greater. Applied Biosystems, (CA, USA); Aria OS Version 1.4 or greater, Cohesive Technologies (MA, USA).

Reagents

[0193] The following reagents were used. Water—Type II, Millipore MilliQ or equivalent; Charcoal Stripped Human Serum (Bioreclamation, Inc); Ethyl alcohol USP (AAPER Alcohol, USA); Acetonitrile HPLC grade (EM Science AX0142-1); Methanol (Reagent A.C.S., Fisher Scientific); Isopropanol HPLC Grade (Fisher Scientific, Catalog #A451-4); 17β-Estradiol, Sigma-Aldrich, (USA) Product # E8875 or USP, (USA) Product #1250008; Estrone, Sigma-Aldrich, (USA) Product # E1274 or USP, (USA) Product #1255001; 17β-Estradiol-2,4,16,16,17-D_5, CDN Isotopes, (USA) Product # D-5403; Estrone-2,4,16,16-D_4, CDN Isotopes, (USA) Product # D-3650; Acetone HPLC Grade (Burdick & Jackson, Catalog #AH010-4); Total Estrogen Stock Solution (10 μg/mL in ethanol) prepared by serial dilution.

[0194] The following solvents were used as the mobile phases for liquid chromatography. Eluting and Loading Pump A Mobile Phase (90% Water and 10% Methanol); Loading Pump B Mobile Phase (90% Methanol and 10% Water); and Loading Pump C Mobile Phase (60:30:10 Acetonitrile:Isopropanol:Acetone).

[0195] Internal Standard Solution (1 ng/mL D_5-Estradiol and 2 ng/mL D_4-Estrone) was prepared in 99:1 water:acetonitrile solution. The reconstitution solution (for reconstituting the sample after liquid-liquid extraction) was 70:30 Millipore Water: Methanol. Two needle wash solutions were used: Needle Wash Solution 1 (Aqueous 1% Formic Acid); and Needle Wash Solution 2 (70:30 Acetonitrile: 1N Ammonium Hydroxide).

[0196] Total Estrogen Stock Solution (10 μg/mL)—5 μg/mL estrone and 5 μg/mL estradiol was used to prepare intermediate stock solutions for preparation of calibrators. To prepare standards, 0.5 mL of Total Estrogen Stock (10 μg/mL) was diluted to 100 mL with stripped human serum to yield 25 ng/mL E1E2 solution, and 2 mL of the 25 ng/mL solution was diluted to 100 mL in charcoal stripped human serum to yield a 0.5 ng/mL E1E2 solution. The diluted stocks (0.5 ng/mL and 25 ng/mL) were stored at −20° C. Calibration Standards were then prepared as shown in the following table. All standards were prepared in charcoal stripped human serum. The standard curve back-fit data should be 80-120% at LLOQ, 85-115% at other concentrations of expected curve values.

Standard Preparation Procedure

Standard Number	Standard Concentration (pg/mL)	Stock Solution. Concentration (ng/mL)	Stock Volume (mL)	Final Volume (mL)	Final Concentration (pg/mL)
S1	1.0	0.5	1	500	1.0
S2	2.5	0.5	2.5	500	2.5
S3	5.0	0.5	5	500	5.0
S4	10	0.5	10	500	10
S5	25	25	0.5	500	25
S6	50	25	1	500	50
S7	100	25	2	500	100
S8	200	25	4	500	200
S9	350	25	7	500	350
S10	500	25	10	500	500

Quality Control

[0197] Control pools are prepared in human serum and introduced into use according to the analytically robust procedures. Each run contained duplicates of four control pools, each with a nominal target value.

Quality Control Concentrations

Control	Target concentration (pg/mL)	
Name	Estrone	Estradiol
Pool 1	10	10
Pool 2	25	25
Pool 3	115	115
Pool 4	300	300

Assay Procedure

[0198] The assay was performed as follows. Pipetted 1.0 ml standard, control or patient into the tube with an Eppendorf pipette (or equivalent). Using an Eppendorf Plus repeating pipette with 5 ml tip (or equivalent), added 50 μl Internal Standard Solution to each tube except double blanks. Mix all samples on multi-vortexer for 30 seconds. Added 8 mL 9:1 Hexane:Ethyl Acetate extraction solvent to all tubes and shook on mechanical shaker for 10 minutes. Removed tubes from shaker and spun down all samples in centrifuge up to 2000 rpm. Labeled a separate set of 16×100 mm test tubes, and add 1 mL redistilled water and 1 drop of 1N NaOH to each. Froze aqueous layer of extracted samples and poured into the tubes containing the water and NaOH. Capped and shook on mechanical shaker for 10 minutes. Removed from shaker and spin down all samples in centrifuge up to 2000 rpm. Froze aqueous layer of extracted samples and poured into new tubes. Placed all tubes into a Rotovap (or equivalent) and allow solvent to evaporate for 45 to 60 minutes. Once there was no trace of solvent left in the tubes, remove from Rotovap and reconstitute each with 120 μL E1E2 Reconstitution solution. Covered tubes with parafilm and mixed on multivortexer, 4×30 seconds.

[0199] Using an Eppendorf pipette, or equivalent, transferred the reconstituted tubes to a 96-well plate. Placed a heat-sealing foil over the plate and seal the plate with the heated plate sealer. Centrifuged plate at 3700 rpm at 10° C. for 10 minutes and placed 96-well plate in LC-MS/MS Autosampler.

Liquid Chromatography Procedures

[0200] After liquid-liquid extraction and reconstitution in 100 μL, 80 μL of extracted sample was injected into the HTLC system using methanol:water in the mobile phase. The HTLC system is logically divided into two functions: (1) first dimension extraction/separation using a highly selective LC column using a binary gradient; and (2) second dimension separation using an sharper binary gradient of methanol and water and a 5 μm reverse phase analytical column. In this example, a Poroshell 300SBC18 75×2.1, 5 μm column was used for extraction.

[0201] In the extraction mode of the HTLC system, the sample was first pumped through the extraction column at a 1 ml/min flow rate using the HTLC loading pump. This separation ensures optimized separation of isobaric interferences and the passage of unwanted coextracted analytes to waste.

[0202] After the first dimension separation step, the flow rate was reduced to 0.5 mL/min and combined with 0.5 mL/min of water during transfer and chromatofocusing onto the second dimension phenyl hexyl column. Such HPLC columns are commercially available (e.g., Thermo Hypersil Phenyl hexyl, Phenomenex Luna Phenyl Hexyl)). In the analytical mode of the HTLC, after the sample was chromatofocused onto the analytical column. A binary gradient from 0% to 90% methanol at 1 mL/min was used, resulting in the separation and increase in peak concentration of estrone and estradiol from other analytes contained in the sample. The separated sample was then transferred to the MS/MS for quantitation. The 2D-LC method is summarized below. Estrone, estradiol, D_4-estrone and D_5-estradiol elute from the second column at approximately 5.2 minutes (+ or −1 minute).

Estrone and Estradiol 2D-LC Method

STEP	Start Time (minutes)	Step Duration (seconds)	Loading Pump** (% B)	Flow Rate (mL/minute)	Eluting Pump** (% B)	Flow Rate (mL/minute)	Eluting Pump Gradient Type
1	0.00	20	20	0.7	0.0	1.0	Isocratic
2	0.33	124	36	0.7	0.0	1.0	Isocratic
3*	2.40	42	41	0.5	0.0	0.5	Isocratic
4	3.10	30	100	1.0	23	1.0	Gradient
5	3.60	40	100	1.0	43	1.0	Gradient
6	4.27	70	100	1.0	70	1.0	Gradient
7	5.43	41	20	1.0	90	1.0	Gradient
8	6.12	45	20	0.7	100	1.0	Gradient
9	6.87	10	20	0.7	0.0	1.0	Isocratic

*Step 3: Transfer of eluent from the extraction column at 0.5 mL/minute and chromatofocus with 0.5 mL/Mminute of millipore water provided by the eluting pump.
**Loading and Eluting Buffers: Loading Pump Buffer A 90:10 Millipore Water:Methanol; Loading Pump Buffer B 10:90 Millipore Water:Methanol; Eluting Pump Buffer A Millipore Water; Eluting Pump Buffer B 10:90 Millipore Water:Methanol

Mass Spectrometry

[0203] The flow of liquid solvent from the HTLC system entered the heated nebulizer (APCI) interface of the MS/MS analyzer. The solvent/analyte mixture was first converted to vapor in the heated tubing of the interface. The analytes, contained in the nebulized solvent, were ionized and a positive charge added by the corona discharge needle of the interface, which applies a large voltage to the nebulized solvent/analyte mixture. During heating and ionization of estradiol (E2), the selected interface heater settings of 500° C. enable dehydration of estradiol to the dehydrated moiety at m/z 255.3 that enables the sensitivity gains observed within the underivatized assay described here. The ions passed through the orifice of the instrument and entered the first quadrupole. Quadrupoles 1 and 3 (Q1 and Q3) were the mass filters, allowing selection of ions based on their mass to charge ratio (m/z). Quadrupole 2 (Q2) was the collision cell, where ions were fragmented.

[0204] The first quadrupole of the MS/MS (Q1) selected for molecules with the mass to charge ratio of estrone/estradiol (271.3/255.3±0.5 m/z or mass units). Ions with these m/z passed to the collision chamber (Q2), while ions with any

other m/z collided with the sides of the quadrupole and were fragmented. Ions entering Q2 collided with neutral gas molecules and fragment. This process is called Collisionally Activated Dissociation (CAD). The CAD gas used in this example was nitrogen resulting in the generation of fragment (products). The fragment ions generated were passed into quadrupole 3 (Q3), where the two fragment ions of estradiol to be measured (m/z 159.0±.0.5 m/z and 133.0±0.5 m/z or mass units) and 2 ions for estrone (m/z 159.0±0.5 m/z and 133.0±+−0.5 m/z or mass units) were selected for, while other ions were screened out. The selected fragment ions were collected by the detector. The same process was carried out for an internal standards, which were 5-deuterated estradiol and 4-deuterated estrone molecules. Thus, the selected MS/MS transitions (nominal masses) measured were as follows: Estradiol m/z 255 to 159 and 133; Estrone m/z 271 to 159 and 133; D_5-Estradiol m/z 260 to 161; and D_4-Estrone m/z 275 to 161.

[0205] Selected MS/MS parameters were as follows: Dwell time: 100 msec for each estradiol and estrone transition, and 50 msec for each internal standard transition; Unit mass resolution in both resolving g quadrupoles (Q1 and Q3); Curtain Gas: 10; CADGas: 6; NC: μA; Temp: 500° C.; GS1: 20; GS2: 0; CE: 27 for estradiol and internal standards and 35 for estrone and internal standards.

[0206] As ions collide with the detector, they produce a pulse of electrons. The pulse was converted to a digital signal, which was counted to provide an ion count. The acquired data was relayed to the computer, which plotted counts of the ions collected vs. time. Areas of the chromatographic peaks generated were computer-measured, response factors (ratio of analyte to internal standard responses) were generated from calibration materials spiked into stripped matrix calibrators at known concentrations, and estradiol and estrone concentrations in unknown samples were thereby quantitated by back-calculating the area response ratios of analyte to internal standards against the constructed calibration curves.

[0207] The HTLC system can be operated with 1 to 4 channels in parallel, with each channel incorporating 1 or more columns. Given that a single assay requires about 3-6 minutes to traverse the column or columns, by staggering the start time on each column, a 4-fold multiplexed system can inject four times as many test samples into the MS/MS instrument than with a single column. Thus, a set of 1000 samples may be assayed for estrone and estradiol in 1 day using HTLC 4 channel multiplexing, as opposed to 4 days when using a single channel system. Furthermore, following transfer of samples to the autosampler, no further operator handling of samples is required, as the HTLC may be computer-controlled to perform the subsequent purification and analysis steps in a fully on-line configuration.

Calculations

[0208] Calibration curves are constructed using the software system (Analyst) that controls the mass spectrometer. Calibration curves are generated by assigning the known concentrations to calibrators to generate a response ratio of analyte to internal standard versus concentration of analyte added [FIG. **20-26**]. The concentrations of unknown samples are automatically calculated by comparing the response ratio of analyte to internal standard observed in measuring unknown samples to the calibration curve generated above. Samples that have an initial value greater than 500 pg/mL are diluted and re-extracted/analyzed to provide a result within the linear range of the assay.

Assay Performance Characteristics

[0209] A quantitative bioanalytical method for the determination of estrone and estradiol in human serum using LLE and 2D LC-MS/MS detection was developed and validated. The assay accuracy and precision was shown to be specific for the analysis of estrone and estradiol.

[0210] Spiked standard samples at nine (estrone) and ten (estradiol) concentrations were used to generate a weighted (1/x) linear regression calibration curve, which covered the range from 2.5 to 500 pg/mL for estrone and 1 to 500 pg/mL for estradiol. Average inaccuracies and imprecision<20% at the LLOQ and <15% throughout the remainder of the range were observed. The correlation coefficients of the curves were greater than 0.98. Sample dilution was evaluated for both estrone and estradiol. Acceptable bias was observed for all samples within the analytical ranges of both assays.

Selectivity

Blank Matrix Interference

[0211] Quadruplicate injections of stripped serum were injected to determine the degree of blank matrix interference for each analyte. Matrix responses were <20% of the mean LLOQ for estrone in all 6 lots tested and <20% of the mean LLOQ for estradiol in 5 of 6 lots tested.

Anticoagulant Effect on Matrix.

[0212] The effect of EDTA and heparin as anticoagulants was tested by drawing four healthy volunteers using red top serum collection tubes and vacutainers containing Sodium Heparin and EDTA anticoagulants. The results of the heparin and EDTA tubes were compared to the results from the serum collection tubes. Variation of sample type and anticoagulant exhibited a bias<±15% for measurement of estrone and estradiol. Thus, plasma samples collected with Heparin and EDTA are acceptable specimen types.

Anticoagulant Effect on Estrone and Estradiol Measurement

	Concentration (pg/mL)		
Sample	Serum	Heparin	EDTA
Estrone	32.200	27.953	28.590
	20.213	19.387	20.425
	162.481	151.423	156.020
	17.925	17.841	16.521
Mean	58.205	54.151	55.389
Mean Matrix Effect (%)	NA	−6.96	−4.84
n	4	4	4
Estradiol	22.475	21.226	20.300
	40.975	42.657	42.183
	181.435	184.180	193.202
	13.833	13.635	13.092
Mean	64.680	65.425	67.194
Mean Matrix Effect (%)	NA	1.15	3.89
n	4	4	4

Effect of Lipemia and Hemolysis in the Matrix.

[0213] The effect of lipemia and hemolysis on the quantitative result was determined by spiking pooled patient serum with 5% by volume of lipid solution or lysed red blood cells. The samples were run in quadruplicate and the results compared to the results of the pool before contamination. Bias was <±15% following addition of lipemic or hemolyzed material was observed for both estrone and estradiol. Thus, lipemic and hemolyzed samples may be processed using this assay.

Internal Standard Interference.

[0214] Internal standards (D_4-estrone and D_5-estradiol) working solution was spiked into charcoal stripped serum and tested in quadruplicate to evaluate the presence of unlabeled analyte. Internal standards (D_4-estrone and D_5-estradiol) interference responses were less than 20% of the mean LLOQ response for both estrone and estradiol.

Matrix Effect.

[0215] The matrix effect was calculated at low, mid and high level concentrations. The matrix effect for the internal standard was measured at a single concentration. A minimum of 4 samples per QC level were analyzed for both analytes to determine matrix effect on quantitative result. Matrix effects were less than 15% for all analytes (estrone, estradiol and D_4-estrone and D_5-estradiol internal standards) tested when comparing water and pooled samples.

Effect of Lipemia and Hemolysis on Estrone and Estradiol

	Concentration (pg/mL)		
Sample	Serum	Lipemic	Hemolyzed
Estradiol	118.286	118.877	113.430
	108.600	120.709	114.765
	108.772	120.159	114.246
	113.406	126.448	119.172
Mean	112.266	121.548	115.403
Mean Matrix Effect (%)	NA	8.27	2.79
n	4	4	4
Estrone	108.159	97.741	114.344
	108.131	103.348	96.851
	105.072	98.358	104.242
	102.476	99.496	96.149
Mean	105.960	99.736	102.897
Mean Matrix Effect (%)	NA	−5.87	−2.89
n	4	4	4

Estrone Matrix Effect

	Sample Matrix					
	Millipore Water			Pooled Human Serum		
Post-Column Infusion Level	Analyte Response (cps)	Internal Standard Response (cps)	Ratio	Analyte Response (cps)	Internal Standard Response (cps)	Ratio
Low Infusion	13704.285	13282.465	1.032	12977.458	14357.420	0.904
	11983.207	12280.880	0.976	12726.749	15154.084	0.840
	13447.370	14129.109	0.952	13866.153	14011.588	0.990
	12912.166	13543.690	0.953	12798.316	14614.364	0.876
MARR per Conc.	NA	NA	0.978	NA	NA	0.902
% Change	NA	NA	NA	NA	NA	−7.76
Mid Infusion	34680.714	12444.598	2.787	37110.060	13962.366	2.658
	34353.842	11496.604	2.988	35363.380	13567.318	2.607
	38284.618	13119.045	2.918	37102.053	14459.360	2.566
	36175.359	12448.411	2.906	37599.353	15799.305	2.380
MARR per Conc.	NA	NA	2.900	NA	NA	2.553
% Change	NA	NA	NA	NA	NA	−11.98
High Infusion	123737.835	11465.898	10.792	132777.319	14635.791	9.072
	109476.758	11070.122	9.889	123422.614	14005.516	8.812
	130085.379	12808.997	10.156	142380.844	14729.975	9.666
	124071.128	11506.322	10.783	133162.192	13750.007	9.685
MARR per Conc.	NA	NA	10.405	NA	NA	9.309
% Change	NA	NA	NA	NA	NA	−10.54

Estradiol Matrix Effect

	Sample Matrix					
	Millipore Water			Low Pooled Human Serum		
Post-Column Infusion Level	Analyte Response (cps)	Internal Standard Response (cps)	Ratio	Analyte Response (cps)	Internal Standard Response (cps)	Ratio
Low Infusion	9289.430	15134.202	0.614	10756.094	16273.794	0.661
	9142.882	14970.197	0.611	9623.939	15742.986	0.611
	9178.822	14245.804	0.644	10323.741	15672.658	0.659
	9115.187	14769.048	0.617	10657.311	16270.173	0.655
MARR per Conc.	NA	NA	0.622	NA	NA	0.646
% Change	NA	NA	NA	NA	NA	4.02
Mid Infusion	32753.289	14367.449	2.280	34773.500	15118.142	2.300
	33992.565	14615.840	2.326	33326.110	14131.532	2.358
	34038.400	14387.971	2.366	34970.558	14653.828	2.386
	33191.028	13699.972	2.423	33447.218	14509.296	2.305
MARR per Conc.	NA	NA	2.348	NA	NA	2.338
% Change	NA	NA	NA	NA	NA	−0.47
High Infusion	123906.209	13814.999	8.969	147602.652	15918.071	9.273
	129674.464	13636.345	9.509	140981.287	16008.385	8.807
	126576.356	13173.822	9.608	136601.192	15250.626	8.957
	128043.106	13263.078	9.654	149499.158	16614.989	8.998
MARR per Conc.	NA	NA	9.435	NA	NA	9.009
% Change	NA	NA	NA	NA	NA	−4.52

For both the estrone and estradiol matrix effect tables, matrix effect = [(Mean Analyte to Internal standard ratio in pooled serum)/Mean Analyte to Internal standard ratio in water)] − 1, expressed as a percentage

[0216] A. Intra-Assay Precision

[0217] The intra-assay precision of the analytical method was calculated for three assays using patient pools (Pool 1 at 10 pg/mL, Pool 2 at 25 pg/mL, Pool 3 at 115 pg/mL, and Pool 4 at 300 pg/mL, all concentrations are approximate). The following tables show the data for these pools, as well as, the data from quality controls made from spiked charcoal stripped human serum at four concentrations (1.0 pg/mL, 2.5 pg/mL, 250 pg/mL, and 500 pg/mL).

Estrone Intra-assay Precision

	Intra-Assay % CV							
Assay No.	Pool 1	Pool 2	Pool 3	Pool 4	1.0	2.5	250	500
1	4.50	5.03	4.42	2.69	15.35	2.91	5.52	3.30
2	5.21	4.71	3.64	4.30	19.56	7.16	2.76	2.39
3	3.47	2.91	1.87	2.95	29.91	6.30	3.81	1.63

Estradiol Intra-assay Precision

	Intra-Assay % CV							
Assay No.	Pool 1	Pool 2	Pool 3	Pool 4	1.0	2.5	250	500
1	2.27	3.43	1.80	3.23	4.72	4.77	2.81	1.15
2	4.31	3.04	1.34	2.56	4.44	6.26	1.60	4.33
3	5.59	1.35	3.64	4.86	4.70	3.92	2.61	3.46

[0218] Reproducibility

[0219] The inter-assay precision was calculated from the overall data from the precision assays for each of the QC samples. As shown in the inter-assay tables, the method has good inter-assay precision.

Estrone Inter-Assay Precision

	Pool 1	Pool 2	Pool 3	Pool 4	1.0	2.5	250	500
Average	10.34	26.16	116.91	309.26	1.22	2.62	263.79	512.80
% CV	4.88	4.59	4.65	5.37	29.23	7.39	5.08	3.67
N	18	18	18	18	18	18	18	18

Estradiol Inter-Assay Precision

	Pool 1	Pool 2	Pool 3	Pool 4	1.0	2.5	250	500
Average	10.83	23.94	114.18	293.45	0.97	2.57	254.82	513.87
% CV	4.36	3.53	3.33	4.86	7.39	5.22	3.94	4.10
N	18	18	18	18	18	18	18	18

[0220] Accuracy

[0221] The inter-assay accuracy was determined by calculating the percent bias for samples of known concentrations. Stripped human serum was spiked to 1.0 pg/mL, 2.5 pg/mL, 250 pg/mL, and 500 pg/mL and then assayed 6 times in three different runs. As shown in the intra-assay tables, the method has good inter-assay accuracy.

Estrone Inter-assay Accuracy Data

System Precision	1.0 pg/mL	2.5 pg/mL	250 pg/mL	500 pg/mL
Expected Result	1.0	2.5	250	500
Average Result	1.22	2.62	263.79	512.80
% Bias	22.13	4.79	5.51	2.56

Estradiol Inter-assay Accuracy Data

System Precision	1.0 pg/mL	2.5 pg/mL	250 pg/mL	500 pg/mL
Expected Result	1.0	2.5	250	500
Average Result	0.97	2.57	254.82	513.87
% Bias	−3.28	2.70	1.93	2.77

Spike and Recovery

[0222] A patient pool and stripped human serum calibrator were spiked with Estrone and Estradiol. Recovery was performed by comparing the measured results of samples spiked with 50, 200 and 400 pg/mL of standard material against expected values. Samples were analyzed in quadruplicate. Both estrone and estradiol exhibited recoveries>85% and <115%.

Specificity

[0223] Specificity was tested by adding 1 μg of the steroids listed below to 1 mL (equivalent to 100 μg/dL) of water before extraction and injection. Where specificity is being further evaluated, lower concentrations of analytes are used (i.e. 100 ng/mL or 10 ng/mL). Acceptability Criteria: Response less than the LLOQ at the appropriate retention time in excess of physiologically significant amounts of potential interfering analytes.

[0224] It was found that estrone analysis is not affected by the presence of circulating hormones or drugs at physiological concentrations in the testing. The liquid-liquid extraction step employed is known to exclude extraction of estrone sulfate during sample processing, thus, it is apparent that the estrone sulfate material tested contains estrone as an impurity at approximately 0.03% and thus, the assay is considered specific for the measurement of estrone. Also, estradiol analysis was not affected by the presence of the circulating hormones and drugs tested at relevant physiological levels.

Estrone and Estradiol Spike and Recovery

	Concentration Added (pg/mL)			
	0.000	50.000	200.000	400.000
	Concentration Measured (pg/mL)			
Estrone Stripped	10.716	61.395	199.547	394.156
Serum	11.578	58.841	198.228	423.264
	12.133	65.149	216.259	400.486
	10.381	59.695	206.891	431.507
Mean	11.202	61.270	205.231	412.353
Expected Conc.	NA	61.202	211.202	411.202
Recovery (%)	NA	100.1	97.2	100.3
n	4	4	4	4
Estrone Pooled	24.513	83.123	234.620	434.374
Serum	27.357	82.876	228.706	431.772
	27.574	81.036	230.123	434.208
	25.478	78.861	231.405	433.010
Mean	26.231	81.474	231.214	433.341
Expected Conc.	NA	76.231	226.231	426.231
Recovery (%)	NA	106.9	102.2	101.7
n	4	4	4	4
Estradiol Stripped	10.029	54.400	197.858	362.120
Serum	9.950	56.014	197.599	373.907
	10.542	59.706	200.453	373.998
	9.757	56.920	204.747	366.630

-continued

Estrone and Estradiol Spike and Recovery

	Concentration Added (pg/mL)			
	0.000	50.000	200.000	400.000
	Concentration Measured (pg/mL)			
Mean	10.070	56.760	200.164	369.164
Expected Conc.	NA	60.070	210.070	410.070
Recovery (%)	NA	94.5	95.3	90.0
n	4	4	4	4
Estradiol Pooled	21.627	67.816	207.658	375.399
Serum	23.183	70.755	202.194	390.105
	24.317	70.378	206.603	411.400
	23.876	71.583	221.472	400.773
Mean	23.251	70.133	209.482	394.419
Expected Conc.	NA	73.251	223.251	423.251
Recovery (%)	NA	95.7	93.8	93.2
n	4	4	4	4

Estrone Hormone Specificity

Steroid	Amount added (pg/mL)	Measured Concentration (pg/mL)	Relative Response (%) (LLOQ of 2.5 pg/mL = 0.00025%)
Dihydrotestosterone	1000000	6.234	0.00062
Androstenediol	1000000	0.000	0.00000
5-androsten-3,11,17-trione	1000000	0.000	0.00000
Androstenedione	1000000	0.000	0.00000
17a-Methyltestosterone	1000000	0.000	0.00000
Cortisone	1000000	0.000	0.00000
Epitestosterone	1000000	0.000	0.00000
Dehydroepiandrostenedione	1000000	0.000	0.00000
Dexamethasone	1000000	0.000	0.00000
5a-androstan-3b,17b-diol	1000000	0.000	0.00000
5b-androstan-3a,17b-diol	1000000	0.000	0.00000
Epiandrosterone	1000000	0.000	0.00000
17a-Hydroxyprogesterone	1000000	0.000	0.00000
11-Desoxycortisol	1000000	7.741	0.00077
Prednisone	1000000	0.000	0.00000
Estriol	1000000	0.385	0.00004
Corticosterone	1000000	0.000	0.00000
Androsterone	1000000	0.000	0.00000
Prednisolone	1000000	0.000	0.00000
17-Hydroxypregnenolone	1000000	0.000	0.00000
Progesterone	1000000	0.000	0.00000
20a-Hydroxy-progesterone	1000000	0.000	0.00000
20b-Hydroxy-progesterone	1000000	0.000	0.00000
Beclomethasone	1000000	0.000	0.00000
Triamcinolone Acetonide	1000000	0.000	0.00000
Fluticasone Propionate	1000000	0.000	0.00000
Pregnanetriol	1000000	2.385	0.00024
Tetrahydrocortisol	1000000	0.000	0.00000
Tetrahydrocortisone	1000000	0.000	0.00000
Pregnenolone sulphate	1000000	0.000	0.00000
Ethinyl Estradiol	100000	0.845	0.00085
Budesonide	1000000	0.000	0.00000
Pregnanediol	1000000	1.828	0.00018
Desoxycorticosterone	1000000	0.951	0.00010
Cortisol	1000000	0.000	0.00000
21-Desoxycortisol	1000000	0.000	0.00000
Pregnenolone	1000000	0.000	0.00000
Andrenosterone	1000000	0.000	0.00000
Aldosterone	1000000	0.000	0.00000
Dihydroandrosterone	1000000	0.000	0.00000
11a Hydroxy-Progesterone	1000000	0.000	0.00000
Testosterone	1000000	0.000	0.00000
Estrone-3-Sulfate	1000000	339.301	0.03393

Estradiol Hormone Specificity

Steroid	Amount added (pg/mL)	Measured Concentration (pg/mL)	Relative Response (%) (LLOQ of 1 pg/mL = 0.00010%)
Dihydrotestosterone	1000000	0.000	0.00000
Androstenediol	100000	2.763	0.00276
5-androsten-3,11,17-trione	1000000	0.000	0.00000
Androstenedione	1000000	0.000	0.00000
17a-Methyltestosterone	1000000	0.000	0.00000
Cortisone	1000000	0.000	0.00000
Epitestosterone	1000000	0.000	0.00000
Dehydroepiandrostenedione	1000000	0.000	0.00000
Dexamethasone	1000000	0.000	0.00000
5a-androstan-3b,17b-diol	1000000	0.000	0.00000
5b-androstan-3a,17b-diol	1000000	0.000	0.00000
Epiandrosterone	1000000	0.000	0.00000
17a-Hydroxyprogesterone	1000000	0.000	0.00000
11-Desoxycortisol	1000000	1.739	0.00017
Prednisone	1000000	0.000	0.00000
Estriol	10000	2.937	0.02937
Corticosterone	1000000	0.000	0.00000
Androsterone	1000000	0.000	0.00000
Prednisolone	1000000	0.000	0.00000
17-Hydroxypregnenolone	1000000	4.560	0.00046
Progesterone	1000000	0.000	0.00000
20a-Hydroxy-progesterone	1000000	0.000	0.00000
20b-Hydroxy-progesterone	1000000	0.000	0.00000
Beclomethasone	1000000	0.000	0.00000
Triamcinolone Acetonide	1000000	0.611	0.00006
Fluticasone Propionate	1000000	0.000	0.00000
Pregnanetriol	1000000	1.434	0.00014
Tetrahydrocortisol	1000000	9.511	0.00095
Tetrahydrocortisone	1000000	0.000	0.00000
Pregnenolone sulphate	1000000	0.000	0.00000
Ethinyl Estradiol	100000	0.937	0.00094
Budesonide	1000000	0.417	0.00004
Pregnanediol	1000000	0.000	0.00000
Desoxycorticosterone	1000000	0.000	0.00000
Cortisol	1000000	0.000	0.00000
21-Desoxycortisol	1000000	0.000	0.00000
Pregnenolone	1000000	0.000	0.00000
Andrenosterone	1000000	0.000	0.00000
Aldosterone	1000000	0.000	0.00000
Dihydroandrosterone	1000000	0.000	0.00000
11a Hydroxy-Progesterone	1000000	0.000	0.00000
Testosterone	1000000	0.000	0.00000
Estrone-3-Sulfate	100000	0.000	0.00000

[0225] Stability

[0226] Stability in human serum was demonstrated at the following conditions for the times shown below.

Storage Condition	Estrone	Estradiol
Room Temperature	6 days	6 days
Refrigerated (4° C.)	48 hours	48 hours
Frozen (−20° C.)	33 months	32 months
Freeze Thaw	3 cycles	3 cycles
Whole Blood	48 hours	48 hours
Autosampler	72 hours	72 hours

[0227] Sensitivity

[0228] The lower limit of quantitation was determined to be 1.0 pg/mL for estradiol and 2.5 pg/mL for estrone using a sample size of 1 mL with 804 being injected into the 2D-LC-MS/MS system.

Inter-Assay Comparison

A. Radioimmunoassay Compared to 2D LC-MS/MS.

[0229] A minimum of 50 routine samples representing the physiological range were analyzed by 2D LC-MS/MS and RIA following extraction/off-line chromatographic separation for estrone and estradiol and LC-MS/MS with derivatization for estradiol.

[0230] The inter-assay comparison of estrone RIA to LC-LC-MS/MS yielded an average bias of 5.46% for samples within the analytical range of both assays. Comparison of data throughout the range generated a slope of 0.9005 with a correlation coefficient of 0.8962; thus, estrone assay-to-assay cross-validation was successful (FIG. **29**). Inter-assay comparison of estradiol RIA to LC-LC-MS/MS yielded an average bias of 2.22% for samples within the analytical range of both assays. Comparison of data throughout the range generated a slope of 1.1392 with a correlation coefficient of 0.9776; thus, estradiol inter-assay comparison was successful (FIG. **30**). Inter-assay comparison of LC-MS/MS with derivatization compared to LC-LC-MS/MS yielded an average bias of −0.51% (combined data for both Tables below) for samples within the analytical range of both assays. Comparison of data throughout the range generated a slope of 0.9844 with a correlation coefficient of 0.9926 (FIG. **31**).

-continued

Estrone Inter-assay Comparison of Radioimmunoassay to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	RIA	LC-LC-MS/MS	Bias (%)
Sample 73	28	39.740	41.93
Sample 74	25	30.986	23.94
Sample 75	51	47.044	−7.76
Sample 76	42	43.604	3.82
Sample 77	9	11.251	25.01
Sample 78	37	33.795	−8.66
Sample 79	42	44.081	4.95
Sample 80	19	21.918	15.36
Sample 81	88	64.153	−27.10
Sample 82	78	75.737	−2.90
Sample 83	107	90.226	−15.68
Sample 84	57	51.286	−10.02
			Average Bias = 5.46

Bias (%) = (Total LC-LC-MS/MS result − RIA result)/RIA result, expressed as a percentage

Samples were selected in sequential order following cross-validation of Estradiol LC-MS/MS to LC-LC-MS/MS to coincide with anticipated measurable levels in the Estrone RIA assay. BLQ = below limit of quantification.

Estrone Inter-assay Comparison of Radioimmunoassay to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	RIA	LC-LC-MS/MS	Bias (%)
Sample 12	53	58.387	10.16
Sample 14	45	40.046	−11.01
Sample 15	60	49.222	−17.96
Sample 16	12	11.171	−6.91
Sample 17	82	67.171	−18.08
Sample 19	BLQ	8.561	NA
Sample 20	8	12.399	54.99
Sample 25	59	52.737	−10.62
Sample 31	8	13.281	66.01
Sample 33	44	43.813	−0.42
Sample 38	45	45.408	0.91
Sample 39	6	13.919	131.98
Sample 42	52	54.994	5.76
Sample 44	49	51.138	4.36
Sample 46	22	25.341	15.19
Sample 47	31	35.076	13.15
Sample 48	BLQ	15.587	NA
Sample 51	90	76.809	−14.66
Sample 52	57	57.380	0.67
Sample 53	27	28.932	7.16
Sample 54	43	30.371	−29.37
Sample 55	21	21.693	3.30
Sample 56	42	30.132	−28.26
Sample 57	27	28.123	4.16
Sample 58	48	53.190	10.81
Sample 59	68	62.183	−8.55
Sample 60	36	49.805	38.35
Sample 61	130	151.848	16.81
Sample 62	76	79.513	4.62
Sample 63	73	84.343	15.54
Sample 64	102	106.993	4.90
Sample 66	33	23.136	−29.89
Sample 67	100	80.956	−19.04
Sample 68	17	17.714	4.20
Sample 69	62	46.039	−25.74
Sample 70	41	48.489	18.27
Sample 71	29	36.534	25.98
Sample 72	57	47.071	−17.42

Estradiol Inter-assay Comparison of Radioimmunoassay to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	RIA	LC-LC-MS/MS	Bias (%)
Sample 12	14	13.512	−3.49
Sample 14	19	11.612	−38.88
Sample 15	18	9.950	−44.72
Sample 16	18	17.069	−5.17
Sample 17	188	203.458	8.22
Sample 19	25	21.031	−15.88
Sample 20	10	10.688	6.88
Sample 25	92	149.479	62.48
Sample 31	12	8.816	−26.53
Sample 33	28	24.187	−13.62
Sample 38	66	79.085	19.83
Sample 39	10	8.092	−19.08
Sample 42	78	82.430	5.68
Sample 44	74	86.914	17.45
Sample 46	23	22.228	−3.36
Sample 47	28	25.616	−8.51
Sample 48	17	16.038	−5.66
Sample 51	152	181.717	19.55
Sample 52	84	89.387	6.41
Sample 53	42	45.092	7.36
Sample 54	48	50.658	5.54
Sample 55	17	12.738	−25.07
Sample 56	32	30.153	−5.77
Sample 57	34	33.255	−2.19
Sample 58	27	25.110	−7.00
Sample 59	44	44.229	0.52
Sample 60	26	27.235	4.75
Sample 61	223	248.650	11.50
Sample 62	134	139.889	4.39
Sample 63	138	144.874	4.98
Sample 64	216	235.982	9.25
Sample 66	12	7.893	−34.23
Sample 67	46	41.866	−8.99
Sample 68	21	18.206	−13.30
Sample 69	61	70.482	15.54
Sample 70	57	65.890	15.60
Sample 71	27	36.189	34.03
Sample 72	43	50.018	16.32

-continued

Estradiol Inter-assay Comparison of Radioimmunoassay to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	RIA	LC-LC-MS/MS	Bias (%)
Sample 73	37	40.562	9.63
Sample 74	72	61.767	−14.21
Sample 75	81	104.333	28.81
Sample 76	28	28.832	2.97
Sample 77	42	47.134	12.22
Sample 78	33	31.675	−4.02
Sample 79	48	55.149	14.89
Sample 80	15	15.629	4.19
Sample 81	65	75.748	16.54
Sample 82	79	89.239	12.96
Sample 83	35	35.169	0.48
Sample 84	21	27.692	31.87
			Average Bias = 2.22

Bias (%) = (Total LC-LC-MS/MS result − RIA result)/RIA result, expressed as a percentage

Samples were selected in sequential order following cross-validation of Estradiol LC-MS/MS to LC-LC-MS/MS to coincide with measurable levels in the Estradiol RIA assay.

-continued

Estradiol Inter-assay Comparison of LC-MS/MS to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	LC-MS/MS	LC-LC-MS/MS	Bias (%)
Sample 42	78.66	82.430	4.79
Sample 43	12.37	11.371	−8.10
Sample 44	87.28	86.914	−0.42
Sample 45	BLQ	1.043	NA
Sample 46	21.21	22.228	4.80
Sample 47	25.18	25.616	1.74
Sample 48	15.81	16.038	1.43
Sample 49	BLQ	4.677	NA
Sample 50	ALQ	ALQ	NA
			Average Bias (samples 1-50) = −3.30

Bias (%) = (Total LC-LC-MS/MS result − LC-MS/MS result)/LC-MS/MS result, expressed as a percentage

BLQ = Below limit of quantification (10 pg/mL for LC-MS/MS, 1 pg/mL for LC-LC-MS/MS)

ALQ = Above limit of quantification (1000 pg/mLfor LC-MS/MS, 500 pg/mL for LC-LC-MS/MS)

Estradiol Inter-assay Comparison of LC-MS/MS to 2D LC-MS/MS

	Concentration (pg/mL)		
Sample #	LC-MS/MS	LC-LC-MS/MS	Bias (%)
Sample 1	BLQ	5.166	NA
Sample 2	BLQ	1.754	NA
Sample 3	BLQ	6.888	NA
Sample 4	BLQ	1.697	NA
Sample 5	BLQ	2.488	NA
Sample 6	ALQ	ALQ	NA
Sample 7	BLQ	1.911	NA
Sample 8	BLQ	8.004	NA
Sample 9	BLQ	3.811	NA
Sample 10	BLQ	1.115	NA
Sample 11	BLQ	3.387	NA
Sample 12	14.56	13.512	−7.22
Sample 13	ALQ	ALQ	NA
Sample 14	12.06	11.612	−3.73
Sample 15	10.04	9.950	−0.86
Sample 16	18.56	17.069	−8.01
Sample 17	210.69	203.458	−3.43
Sample 18	BLQ	3.202	NA
Sample 19	21.13	21.031	−0.46
Sample 20	11.05	10.688	−3.23
Sample 21	BLQ	1.906	NA
Sample 22	BLQ	BLQ	NA
Sample 23	BLQ	2.716	NA
Sample 24	BLQ	BLQ	NA
Sample 25	143.77	149.479	3.97
Sample 26	BLQ	BLQ	NA
Sample 27	BLQ	BLQ	NA
Sample 28	BLQ	BLQ	NA
Sample 29	BLQ	BLQ	NA
Sample 30	BLQ	6.283	NA
Sample 31	10.22	8.816	−13.75
Sample 32	BLQ	1.110	NA
Sample 33	23.94	24.187	1.02
Sample 34	BLQ	6.641	NA
Sample 35	BLQ	8.473	NA
Sample 36	BLQ	BLQ	NA
Sample 37	BLQ	BLQ	NA
Sample 38	79.90	79.085	−1.02
Sample 39	11.06	8.092	−26.86
Sample 40	BLQ	BLQ	NA
Sample 41	BLQ	4.344	NA

Estradiol Cross-Validation of LC-MS/MS to 2D LC-MS/MS Continued

	Concentration (pg/mL)		
Sample #	LC-MS/MS	LC-LC-MS/MS	Bias (%)
Sample 51	187.11	181.717	−2.88
Sample 52	91.41	89.387	−2.21
Sample 53	47.76	45.092	−5.59
Sample 54	53.73	50.658	−5.71
Sample 55	14.32	12.738	−11.07
Sample 56	26.19	30.153	15.14
Sample 57	34.38	33.255	−3.28
Sample 58	27.69	25.110	−9.30
Sample 59	41.44	44.229	6.73
Sample 60	27.36	27.235	−0.46
Sample 61	260.11	248.650	−4.41
Sample 62	142.53	139.889	−1.85
Sample 63	142.95	144.874	1.34
Sample 64	236.23	235.982	−0.11
Sample 65	ALQ	ALQ	NA
Sample 66	12.14	7.893	−34.99
Sample 67	44.69	41.866	−6.31
Sample 68	21.81	18.206	−16.51
Sample 69	69.20	70.482	1.85
Sample 70	60.36	65.890	9.16
Sample 71	50.51	36.189	−28.35
Sample 72	47.80	50.018	4.65
Sample 73	41.42	40.562	−2.08
Sample 74	58.87	61.767	4.92
Sample 75	100.37	104.333	3.95
Sample 76	28.66	28.832	0.59
Sample 77	50.36	47.134	−6.41
Sample 78	34.31	31.675	−7.68
Sample 79	54.11	55.149	1.91
Sample 80	15.43	15.629	1.28
Sample 81	75.17	75.748	0.78
Sample 82	92.92	89.239	−3.96
Sample 83	34.64	35.169	1.52
Sample 84	28.92	27.692	−4.23
Sample 85	107.78	105.278	−2.32
Sample 86	77.28	80.854	4.62
Sample 87	29.52	36.282	22.92
Sample 88	61.28	60.413	−1.42
Sample 89	56.01	58.909	5.18
Sample 90	49.78	49.144	−1.27
Sample 91	56.81	65.677	15.62

-continued

Estradiol Cross-Validation of LC-MS/MS to 2D LC-MS/MS Continued

Sample #	Concentration (pg/mL) LC-MS/MS	LC-LC-MS/MS	Bias (%)
Sample 92	102.40	104.305	1.86
Sample 93	39.18	34.476	−12.00
Sample 94	58.75	63.487	8.06
Sample 95	66.04	79.660	20.63
Sample 96	20.45	20.995	2.65
Sample 97	49.59	47.858	−3.49
Sample 98	12.34	14.914	20.88
Sample 99	40.30	49.841	23.69
Sample 100	93.42	108.173	15.79
			Average Bias (samples 51-100) = 0.36

Bias (%) = (Total LC-LC-MS/MS result − LC-MS/MS result)/LC-MS/MS result, expressed as a percentage
BLQ = Below limit of quantification (10 pg/mL for LC-MS/MS, 1 pg/mL for LC-LC-MS/MS)
ALQ = Above limit of quantification (1000 pg/mL for LC-MS/MS, 500 pg/mL for LC-LC-MS/MS)

Reference Interval

[0231] A. Reference Range Sample Groups and Results

[0232] Reference range transfer for estrone, estradiol and total estrogens was evaluated using NCCLS guidance (see references). Transfer of the reference range was established using the samples listed below.

	Normal Patient Serum Reference Sample Groups			
	Children [1]	Adult Males	Adult Females	Post-menopausal Females
Sample Number	50	25	25	50

[1] Children samples will include 25 boys <10 years old and 25 girls <9 years old.

[0233] B. Reference Interval of Patient Test Results

Estrone Reference Ranges

Reference Population	Reference Range (pg/mL)
Adult Female (luteal)	30-100 pg/mL
Adult Female (follicular)	90-160 pg/mL
Adult Male	10-50 pg/mL
Prepubertal Children	<15 pg/mL
Post-menopausal Female	<40 pg/mL

Estradiol Reference Ranges

Reference Population	Reference Range (pg/mL)
Adult Female	30-100 pg/mL
Adult Female (follicular)	70-300 pg/mL
Adult Male	8-35 pg/mL
Prepubertal Children	<15 pg/mL
Post-menopausal Female	<15 pg/mL

[0234] C. Reference Range Transfer

[0235] Guidance provided by NCCLS allows reference range transfer where no more than 2 out of 20 (10%) of samples fall outside the original reference range. A total of 22 out of 23 normal adult female samples were within range for reference range transfer of estrone, estradiol and total estrogens. Adult female reference ranges are transferable. All normal adult male samples were within range for reference range transfer of estrone and estradiol. A total of 22 out of 23 normal adult male samples were within range for reference range transfer of total estrogens. Adult male reference ranges are transferable. All 50 pre-pubertal reference samples were within range for reference range transfer for estrone, estradiol and total estrogens. Pre-pubertal reference ranges are transferable. A total of 24 out of 25 normal post-menopausal female samples were within range for reference range transfer of estrone and total estrogens. A total of 23 out of 25 normal post-menopausal female samples were within range for reference range transfer of estradiol. Post-menopausal female reference ranges are transferable.

Estrone and Estradiol Adult Reference Range Verification

Adults	Concentration (pg/mL) Estrone	Estradiol	Total
Female 1	70.883	95.809	166.692
Female 2	76.600	127.096	203.696
Female 3*	13306.414	14156.104	27462.518
Female 4	66.318	60.373	126.691
Female 5	65.789	132.145	197.934
Female 6	95.624	109.358	204.982
Female 7	81.402	114.658	196.060
Female 8	45.846	33.105	78.951
Female 9	37.735	39.985	77.720
Female 10	30.071	38.714	68.785
Female 11	58.483	65.622	124.105
Female 12	119.945	345.081	465.026
Female 13	112.298	246.219	358.517
Female 14	103.689	51.824	155.513
Female 15	86.195	109.157	195.352
Female 16	43.546	30.348	73.894
Female 17	83.344	96.485	179.829
Female 18	31.321	31.050	62.371
Female 19	64.664	158.151	222.815
Female 20	48.442	30.094	78.536
Female 21	45.145	30.001	75.146
Female 22*	46.019	11.005	57.024
Female 23	26.042	35.425	61.467
Female 24	52.566	64.966	117.532
Female 25	44.511	142.452	186.963
Male 1	30.575	21.764	52.339
Male 2	28.002	20.025	48.027
Male 3	32.190	22.324	54.514
Male 4	28.509	31.160	59.669
Male 5	29.666	19.525	49.191
Male 6	40.293	18.999	59.292
Male 7	20.721	16.842	37.563
Male 8	46.467	19.506	65.973
Male 9*	77.912	48.095	126.007
Male 10	24.086	20.251	44.337
Male 11	30.402	14.473	44.875
Male 12	40.782	23.593	64.375
Male 13	48.644	34.843	83.487
Male 14	29.029	19.883	48.912
Male 15	38.322	34.511	72.833
Male 16	26.460	34.060	60.520
Male 17	38.597	28.584	67.181
Male 18*	71.676	55.531	127.207
Male 19	49.944	26.688	76.632
Male 20	37.529	24.837	62.366

-continued

Estrone and Estradiol Adult Reference Range Verification

	Concentration (pg/mL)		
Adults	Estrone	Estradiol	Total
Male 21	33.808	19.570	53.378
Male 22	33.282	25.675	58.957
Male 23	18.813	14.025	32.838
Male 24	26.792	17.124	43.916
Male 25	30.602	33.692	64.294

*= Abnormal results using alternate assay, excluded from reference range calculations.
Estrone Female reference range = 30-100 pg/mL (luteal), 90-160 pg/mL (follicular)
Estrone Male reference range = 10-50 pg/mL.
Estradiol Female reference range = 30-100 pg/mL (luteal), 70-300 pg/mL (follicular)
Estrone Male reference range = 8-35 pg/mL
Total Estrogens Female reference range = 60-200 pg/mL (luteal), 160-400 pg/mL (follicular)
Total Estrogens Male reference range = 20-80 pg/mL

Estrone and Estradiol Prepubertal Reference Range Verification

	Concentration (pg/mL)		
Children	Estrone	Estradiol	Total
Female 1	BLQ	BLQ	BLQ
Female 2	BLQ	BLQ	BLQ
Female 3	BLQ	BLQ	BLQ
Female 4	BLQ	BLQ	BLQ
Female 5	BLQ	1.052	1.052
Female 6	BLQ	BLQ	BLQ
Female 7	BLQ	BLQ	BLQ
Female 8	BLQ	BLQ	BLQ
Female 9	BLQ	BLQ	BLQ
Female 10	BLQ	BLQ	BLQ
Female 11	BLQ	BLQ	BLQ
Female 12	BLQ	BLQ	BLQ
Female 13	BLQ	BLQ	BLQ
Female 14	BLQ	BLQ	BLQ
Female 15	BLQ	1.718	1.718
Female 16	BLQ	BLQ	BLQ
Female 17	BLQ	BLQ	BLQ
Female 18	BLQ	BLQ	BLQ
Female 19	BLQ	BLQ	BLQ
Female 20	BLQ	BLQ	BLQ
Female 21	BLQ	BLQ	BLQ
Female 22	BLQ	BLQ	BLQ
Female 23	BLQ	BLQ	BLQ
Female 24	BLQ	BLQ	BLQ
Female 25	BLQ	BLQ	BLQ
Male 1	BLQ	BLQ	BLQ
Male 2	BLQ	BLQ	BLQ
Male 3	BLQ	BLQ	BLQ
Male 4	BLQ	BLQ	BLQ
Male 5	BLQ	BLQ	BLQ
Male 6	BLQ	BLQ	BLQ
Male 7	BLQ	BLQ	BLQ
Male 8	BLQ	BLQ	BLQ
Male 9	BLQ	BLQ	BLQ
Male 10	BLQ	BLQ	BLQ
Male 11	BLQ	BLQ	BLQ
Male 12	BLQ	BLQ	BLQ
Male 13	BLQ	BLQ	BLQ
Male 14	BLQ	BLQ	BLQ
Male 15	BLQ	BLQ	BLQ
Male 16	BLQ	BLQ	BLQ
Male 17	2.577	BLQ	2.577
Male 18	BLQ	BLQ	BLQ
Male 19	BLQ	BLQ	BLQ
Male 20	BLQ	BLQ	BLQ
Male 21	BLQ	BLQ	BLQ

-continued

Estrone and Estradiol Prepubertal Reference Range Verification

	Concentration (pg/mL)		
Children	Estrone	Estradiol	Total
Male 22	BLQ	BLQ	BLQ
Male 23	BLQ	BLQ	BLQ
Male 24	BLQ	BLQ	BLQ
Male 25	BLQ	BLQ	BLQ

Estrone Pre-pubertal reference range = <15 pg/mL
Estradiol Pre-pubertal reference range = <15 pg/mL
Total Estrogens Pre-pubertal reference range = <25 pg/mL

Estrone and Estradiol Post-menopausal Reference Range Verification

	Concentration (pg/mL)		
Post Menopausal	Estrone	Estradiol	Total Estrogens
Post Menopausal Female 1	15.512	6.174	21.686
Post Menopausal Female 2	26.081	14.535	40.616
Post Menopausal Female 3	17.392	5.680	23.072
Post Menopausal Female 4	11.037	4.297	15.334
Post Menopausal Female 5	26.321	12.717	39.038
Post Menopausal Female 6	22.282	4.653	26.935
Post Menopausal Female 7	10.769	5.123	15.892
Post Menopausal Female 8	34.193	14.398	48.591
Post Menopausal Female 9	8.257	4.016	12.273
Post Menopausal Female 10	25.306	11.681	36.987
Post Menopausal Female 11	22.873	10.268	33.141
Post Menopausal Female 12	7.838	14.060	21.898
Post Menopausal Female 13	12.848	3.788	16.636
Post Menopausal Female 14	8.348	21.916	30.264
Post Menopausal Female 15	8.502	3.173	11.675
Post Menopausal Female 16	17.502	11.371	28.873
Post Menopausal Female 17	9.837	5.903	15.740
Post Menopausal Female 18	34.756	14.946	49.702
Post Menopausal Female 19	24.933	6.502	31.435
Post Menopausal Female 20	10.280	6.123	16.403
Post Menopausal Female 21	30.275	19.200	49.475
Post Menopausal Female 22	15.434	4.680	20.114
Post Menopausal Female 23	8.889	9.670	18.559
Post Menopausal Female 24	7.437	5.139	12.576
Post Menopausal Female 25	44.117	14.607	58.724

Estrone Post-menopausal female reference range = <40 pg/mL
Estradiol Post-menopausal female reference range = <15 pg/mL
Total Estrogens Post-menopausal female reference range = <50 pg/mL

Standard Curve Fitting and Reproducibility

[0236] The reproducibility of the standard curve was evaluated by comparing the back-calculated concentrations to the theoretical concentration of the standard in five analytical runs using the concentrations listed below. Calibrator Concentrations for estrone and estradiol (pg/mL) were as follows: 1; 2.5; 5; 10; 25; 50; 100; 200; 350; and 500.

[0237] The reproducibility of the standard curve was evaluated by comparing the back-calculated concentrations to the actual concentration of the standard in five analytical runs. The curve was fit with a straight line with weighted 1/x fit, as established during method development. Acceptability Criteria: Imprecision of ≦20% at the LLOQ and less than ≦15% at other concentrations. Correlation coefficient (r) greater than 0.98. It was found that estrone calibration curves exhibited mean imprecision<15% for all concentrations between

2.5 pg/mL and 500 pg/mL. Correlation coefficients were greater than 0.98. Estradiol calibration curves exhibited mean imprecision<15% for all concentrations between 1.0 pg/mL and 500 pg/mL. Correlation coefficients were greater than 0.98.

Analytical Reportable Range

A. LLOQ (Lower Limit of Quantification)

[0238] The lower limit of quantification for estrone using this assay was 2.5 pg/mL as determined during evaluation of inaccuracy, imprecision and calibration curve reproducibility. The lower limit of quantification for estradiol using this assay was 1.0 pg/mL as determined during evaluation of inaccuracy, imprecision and calibration curve reproducibility.

B. ULOQ (Upper Limit of Quantification)

[0239] The upper limit of quantification using this assay was 500 pg/mL for both estrone and estradiol, as determined during evaluation of inaccuracy, imprecision and calibration curve reproducibility.

Estrone Standard Curve Fitting and Reproducibility Continued

Batch	K_0 (Y-intercept)	K_1 (slope)	Correlation Coefficient (R)
1	0.0071	0.0148	0.9973
2	0.0227	0.0126	0.9989
3	0.0168	0.0134	0.9998
4	0.0210	0.0135	0.9996
5	0.0153	0.0136	0.9995
Mean	0.0166	0.0136	0.9990
Precision (% RSD)	NA	5.81	0.10
n	5	5	5

Estrone Standard Curve Fitting and Reproducibility

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	Std 10
	Actual Concentration (pg/mL)									
Batch	1.000	2.500	5.000	10.000	25.000	50.000	100.000	200.000	350.000	500.000
1	1.925	2.768	5.648	9.711	23.467	46.373	94.259	185.715	349.442	469.565
	1.390	2.754	4.873	11.121	23.507	47.062	92.564	191.601	366.589	557.980
2	0.461	2.173	4.906	9.681	24.933	52.284	97.879	208.321	373.433	466.693
	1.511	2.321	5.407	10.333	24.749	52.497	103.488	200.953	355.922	489.025
3	0.886	2.613	4.448	10.294	25.019	50.470	97.538	203.637	352.701	495.023
	1.897	2.896	4.602	9.475	24.639	50.202	99.235	209.807	340.983	501.419
4	0.110	2.514	5.357	9.913	24.999	50.807	100.802	202.390	354.388	473.363
	1.146	2.371	5.183	9.199	24.605	48.966	101.284	204.313	354.972	509.573
5	IE	2.728	5.174	9.417	23.121	46.892	98.169	188.782	344.288	500.983
	1.204	2.372	5.230	10.185	24.417	54.467	102.299	203.899	363.129	499.448
Mean	1.170	2.551	5.083	9.933	24.346	50.002	98.752	199.942	355.585	496.307
Accuracy (% RE)	17.00	2.04	1.66	−0.67	−2.62	0.00	−1.25	−0.03	1.60	−0.74
Precision (% RSD)	52.09	9.28	7.33	5.71	2.91	5.37	3.49	4.15	2.79	5.29
n	9	10	10	10	10	10	10	10	10	10

IE = Injection error

Estradiol Standard Curve Fitting and Reproducibility

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	Std 10
	Actual Concentration (pg/mL)									
Batch	1.000	2.500	5.000	10.000	25.000	50.000	100.000	200.000	350.000	500.000
1	0.957	2.550	5.264	10.062	24.338	48.037	96.655	208.854	349.140	495.339
	0.939	2.590	4.946	10.258	26.356	48.204	100.102	202.014	362.357	488.038
2	0.948	2.281	5.005	10.065	26.660	51.180	100.076	203.386	361.785	493.088
	0.838	2.168	5.524	10.089	25.499	51.815	110.920	228.732	329.844	467.097
3	0.998	2.550	5.306	10.203	23.766	51.117	100.742	206.641	341.105	516.785
	0.951	2.500	5.171	9.725	24.066	50.488	98.928	195.879	349.439	490.639
4	1.083	2.494	5.032	9.294	22.005	44.878	95.496	190.745	348.234	477.884
	1.073	2.463	5.346	9.807	25.939	51.655	105.187	208.906	365.338	514.143
5	IE	2.685	4.853	9.247	25.130	47.971	97.174	196.876	349.441	500.232
	0.995	2.517	5.178	9.999	25.929	49.433	102.279	203.003	355.375	497.684
Mean	0.976	2.480	5.163	9.875	24.969	49.478	100.756	204.504	351.206	494.093
Accuracy (% RE)	−2.42	−0.81	3.25	−1.25	−0.12	−1.04	0.76	2.25	0.34	−1.18
Precision (% RSD)	7.59	6.06	3.98	3.61	5.73	4.47	4.52	5.05	3.05	3.02
n	9	10	10	10	10	10	10	10	10	10

IE = Injection error

Estradiol Standard Curve Fitting and Reproducibility Continued

Batch	K_0 (Y-intercept)	K_1 (slope)	Correlation Coefficient (R)
1	0.0069	0.0234	0.9997
2	0.0167	0.0217	0.9980
3	0.0058	0.0229	0.9997
4	0.0035	0.0223	0.9991
5	0.0014	0.0226	0.9999
Mean	0.0088	0.0226	0.9990
Precision (% RSD)	NA	2.83	0.08
n	5	5	5

Example 2

96-Well Equilibrium Dialysis Followed by Isotope Dilution LC-MS/MS for Free Thyroxine

[0240] Development and validation of free thyroxine (FT4) was developed using a high throughput 96-well based equilibrium dialysis techniques to provide a generational improvement in assay performance over historical radioimmunoassays. Equilibrium dialysis of free thyroxine was chosen over ultrafiltration during method development. Significant variance between ultrafiltration and equilibrium dialysis with RIA detection was observed at 37° C. Further, filtrate yield (and analytical sensitivity) was limited for sample types containing significant lipemic content, resulting in blockage of the filtration membrane. Optimization of dialysis parameters (rotator speed, dialysate buffer composition and dialysis time) was undertaken using pooled normal calibrators, controls of known free thyroxine concentration and spiked dialysis buffer (50% yield of original result when completely dialysed). Dialysis losses were evaluated using spiked dialysis buffer. Referring now to FIG. **31**, the measured concentration of free thyroxine versus dialysis time is shown.

[0241] Free thyroxine (FT4) was measured by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) after 96-well plate based equilibrium dialysis and either sample dilution or liquid-liquid extraction (ED-LLE). Thyroxine Ring-$^{6}C_{13}$ is added as internal standard to post dialysis aliquots. For liquid liquid extraction protocols, free thyroxine was extracted from dialysate and calibrator samples with 71.25:23.75:5 Ethyl Acetate:Hexane:Methanol. The organic extract was transferred to a fresh tube and then evaporated and reconstituted in 50:50 Water:Methanol. For the sample dilution preparative protocols, dialysate duffer without gelatin was prepared and only internal standard solution in methanol was added, prior to LC-MS/MS analysis.

[0242] Duplicate sets of FT4 calibrators were analyzed in each batch. All injections were made in singlicate. All samples were injected onto the ARIA TX4 or Transcend TX4 system where the analyte of interest was chromatographed through an analytical column via a gradient separation. An MDS-SCIEX API5000 triple quadrupole mass spectrometer, operating in negative ion electrospray ionization (ESI) mode (Turboionspray) was used for detection.

[0243] Quantification of analyte and internal standard was performed in selected reaction monitoring mode (SRM). The back-calculated amount of each analyte in each sample was determined from duplicate calibration curves generated by spiking known amounts of purified thyroxine into dialysis buffer or methanol:water solutions.

[0244] Measurement of free thyroxine is used to evaluate hyperthyroidism and hypothyroidism, to differentiate and evaluate disorders involving variance in levels of circulating proteins, such as, familial dysalbuminemic hyperthyroximenia, euthyroid hypothyroxemias, transthyretin excess and variant thyroxine binding globulin. Analysis of free thyroxine by ED-LLE-LC-MS/MS or ED-LC-MS/MS detection was developed to measure levels in serum samples from children, women and men. The lower limit of detection using the default sample aliquot of 200 μL is 0.2 ng/dL (2 pg/mL) for free thyroxine.

Definitions

[0245] T4—Thryroxine, ED—Equilibrium Dialysis, LC-MS/MS—Liquid Chromatography tandem mass spectrom-

etry detection, ESI—Electrospray Ionization (Turboionspray), L-Thyroxine, Sigma-Aldrich, (USA) Product # T2376 or USP, (USA) Product #044K1436; Stable Labeled Thyroxine, Tyrosine Ring-$^{13}C_6$, CDN Isotopes, (USA) Product # CLM-6725-O.

Specimen Requirements

[0246] Recommended: 0.5 mL serum or plasma. Separate within one hour. Store and ship frozen in a plastic vial.

Adult:	0.5 mL serum preferred or plasma
Pediatric:	0.5 mL serum preferred or plasma
Minimum:	0.5 mL serum preferred or plasma

[0247] Blood was drawn into red top vacutainer tube and clotting allowed to occur for 20 minutes at room temperature (or until clot had retracted). The sample was then centrifuged and the serum transferred to a labeled plastic vial, and immediately frozen. Storage is short term storage (2 weeks); frozen (≦−20° C.). Shipping is Frozen (≦−20° C.)—on dry ice.

Equipment & Materials

[0248] The following materials were used: Standard manual pipetting devices; Big Shot II Hybridization Oven (Rotator, Boekel Scientific Model No. 230401); 96-Well Equilibrium 10 kD Dialyzer Plates (Harvard Apparatus, Product No. 74-2331); 1.2 ml MBlock Polypropylene 96 Well Collection Plate (SPE Ware, Inc. Product No. SPE0210); Heat Sealing Foil (SPE Ware, Inc. Product No. AB-0589); Mechanical Shaker, Eberbach Inc.; Rotary Evaporator (rotovap), Speed Vac SC 200, Savant; Volumetric Glass Bottles for mobile phases, various sizes; API 5000 Tandem Mass Spectrometer, Sciex, (Toronto, Canada); Turbo V™ Ion source with ESI probe, Sciex, (Toronto, Canada); Aria TX4 HTLC System, Cohesive Technologies, (MA, USA) consisting of 4 each: 1100 Series Quaternary Pump, 1100 Series Binary Pump, 1100 Series Vacuum Degasser; HTS Twin PAL System Autosampler, CTC Analytics AG (Switzerland); BETASIL Phenyl-Hexyl Analytical Column, 50×2.1 mm, 5 μm particle size, Thermo Electron Corporation, (USA) Product No. 73005-052130; Vortex Mixer, VWR or equivalent; Combi-thermo Heat Sealer, Abgene Inc., Product No. AB-0559; 96-Well Centrifuge 5804R Eppendorf or equivalent; Solvent washed 12×75 mm borosilicate glass tubes with polypropylene snap caps; Analyst Version 1.4 or greater. Applied Biosystems, (CA, USA); Aria OS Version 1.4 or greater, Cohesive Technologies (MA, USA).

Reagents

[0249] Water—Type II, Millipore MilliQ or equivalent; Water—Glass Distilled; Acetonitrile HPLC Grade (EM Science, Catalog#AX0142-1); Methanol HPLC Grade (Fisher Scientific, Catalog#A452-4); Hexane HPLC Grade (Fisher Scientific, Catalog #H302-4); Ethyl Acetate OPTIMA Grade (Fisher Scientific, Catalog #E196-4); Ammonium Hydroxide 28.0-30.0% (JT Baker, Catalog#9721-04); Formic Acid 88% (Fisher Scientific, Catalog#A118-500); 1 mg/mL stock L-Thyroxine (Sigma, Catalog#T2376) (Dissolve appropriate amount of L-Thyroxine powder into the appropriate volume of 25% Ammonium Hydroxide in Methanol); Blue Dextran Dye Marker (18/mg/mL) in millipore water. Ammonium Carbonate (100 mM) in millipore water. Extraction solvent for liquid-liquid extraction (71.25% Ethyl Acetate, 23.75% Hexane, and 5% Methanol).

[0250] 1 mg/mL stock Tyrosine Ring-$^{13}C_6$ (Cambridge Isotope Labs, Catalog#CLM-6725-0) Dissolve appropriate amount of Tyrosine Ring-$^{13}C_6$ powder into the appropriate volume of 25% Ammonium Hydroxide in Methanol).

[0251] The following protocol was used to prepare FT4 Dialysis Buffer (with and without gelatin). Mix 800 mL of Millipore Water or equivalent, add 1 mL Sodium DL-Lactate 60% (w/w) (Sigma-Aldrich, Catalog#L1375), 5.265 g Sodium Chloride (EMD Science, Catalog#SX0420-1), 0.224 g Potassium Chloride (EMD Science, Catalog#PX1450-1), 0.180 g Potassium Phosphate (EMD Science, Catalog#PX1565-1), 0.246 g Magnesium Sulfate $7H_2O$ (Sigma-Aldrich, Catalog#M1880), 0.300 g Urea (Sigma-Aldrich, Catalog#U0631). Dissolved 0.275 g of Calcium Chloride (EMD Science, Catalog#SX0130-1) into 5 mL of Millipore Water in a separate glass vial and added to the above solution. Next (Step A) is performed: The buffer solution was heated to 50° C. and while mixing, slowly added 1.0 g Laboratory Grade Gelatin Type A (Fisher, Catalog#G8-500). The buffer solution was then allowed to. Step B: added 5.891 g HEPES Sodium Salt (Sigma-Aldrich, Catalog#H7006). In a separate glass bottle dissolved 7.190 g of HEPES Acid (Sigma-Aldrich, Catalog#H3375) into 200 mL of Millipore Water. Added this to above solution slowly until the pH reached 7.4 and QS to 1000 mL with Millipore Water. The buffer is then aliquoted into glass vials and stored frozen at −20° C. for up to 3 months. To prepare FT4 Dialysis Buffer without gelatin, the buffer is prepared as above except excluding step A.

[0252] To prepare internal standard diluent 1, two Liters Millipore Water or equivalent are mixed with 0.200 g Calcium Chloride (EMD Science, Catalog#SX0130-1), 16.37 g Sodium Chloride (EMD Science, Catalog#SX0420-1), 2.0 g Sodium Azide (Sigma-Aldrich, Catalog#S8032), 4.30 g Sodium Phosphate Dibasic (EMD Science, Catalog#SX0715), 0.380 g Sodium Phosphate Monobasic (J. T. Baker, Catalog#3818-0), 26 mL 10% BSA (Sigma-Aldrich, Catalog# A3803). The lot is tested to ensure minimal analyte response from buffer and stored refrigerated at 4° C. for up to 6 months. The internal standard diluent 2 is methanol.

[0253] To prepare an internal standard solution for liquid-liquid extraction (100 pg/mL $^{13}C_6$-Thyroxine) added 0.1 mL of 100 ng/ml $^{13}C_6$-Thyroxine to 100 mL internal standard diluent 1, and mixed well. The standard was then aliquoted into 20 mL glass scintillation vials. To prepare an internal standard solution for diluting and inject (100 pg/mL $^{13}C_6$-Thyroxine), added 0.1 mL of 100 ng/ml $^{13}C_6$-Thyroxine to 100 mL methanol.

[0254] The FT4 Reconstitution Solution was 50:50 Millipore Water:Methanol. The following mobile phases were used: Eluting Pump A Mobile Phase (90% Water and 10% Methanol); Eluting Pump B Mobile Phase (90% Methanol and 10% Water); Loading Pump A Mobile Phase for post-column addition (90% Methanol and 10% Water with 1 mM Ammonium Carbonate).

[0255] Two needle wash solutions were used: Needle Wash Solution 1 (Aqueous 1% Formic Acid), Needle Wash Solution 2 (70:30 Acetonitrile: 1N Ammonium Hydroxide). The needle wash solutions were stored at room temperature for up to 6 months.

Calibration Procedures With Liquid-Liquid Extraction

[0256] Duplicate standard curves, as described in this procedure, are included with each analytical batch. An L-Thyroxine Stock Solution (100 ng/mL)—100 ng/ml L-Thyroxine stock, made by serially diluting the 1 mg/mL L-Thyroxine

stock in methanol, is used to prepare intermediate stock solutions for preparation of calibrators. Dilute 1.0 mL of L-Throxine Stock (100 ng/mL) to 100 mL with FT4 Dialysis Buffer to yield 1 ng/mL thyroxine solution. This stock is stable when stored at −70° C. Next, dilute 10 mL of the 1 ng/mL solution to 100 mL in FT4 Dialysis Buffer to yield a 100 pg/mL free thyroxine solution. Stable when stored at −70° C. Using these stock solutions, calibration standards of 0.2, 0.5, 1.0, 2.5, 5.0, and 10 ng/dL were made. All standards were prepared in FT4 Dialysis Buffer with or without gelatin if performing liquid-liquid extraction. The calibration standards were then transferred into appropriately labeled glass vials in 1 mL aliquots, capped and stored frozen at −20° C.

Calibration Procedures With Sample Dilution

[0257] Duplicate standard curves, as described in this procedure, are included with each analytical batch. An L-Thyroxine Stock Solution (100 ng/mL)—100 ng/ml L-Thyroxine stock, made by serially diluting the 1 mg/mL L-Thyroxine stock in methanol, was used to prepare intermediate stock solutions for preparation of calibrators. To make the intermediate stock solutions, diluted 1.0 mL of L-Throxine Stock (100 ng/mL) to 100 mL with methanol to yield 1 ng/mL thyroxine solution. This stock is stable when stored at −70° C. Next, diluted 10 mL of the 1 ng/mL solution to 100 mL in methanol to yield a 100 pg/mL thyroxine solution. This stock is also stable when stored at −70° C. Using these stock solutions, calibration standards of 0.2, 0.5, 1.0, 2.5, 5.0, and 10 ng/dL were made. All standards were prepared in 1:1 methanol:water. The calibration standards were transferred into appropriately labeled glass vials in 1 mL aliquots, capped and stored frozen at −20° C.

Quality Control

[0258] Control pools are prepared in human serum as shown below and introduced into use according to analytically robust procedures.

Quality Control Concentrations

Control Name	Target concentration (ng/dL) Free Thyroxine
QC 1	1.0
QC 2	1.5
QC 3	2.5
QC 4	8.0

[0259] The control data was recorded for each run on Levy-Jennings charts. Points were plotted and connected. The control chart is reviewed for shifts or trends. A warning situation may exists if one control falls within the ±2 SD and ±3 SD interval; all test results may be released.

Test Procedure

[0260] A. Assay Procedure

[0261] Dialysis of samples: Dialysis was performed as follows. Warmed the Big Shot II hybridization oven to reach 37° C. Capped sample side (blue side) of the dialysis plate. Turned the plate over and added 300 μL of FT4 Dialysis Buffer either with or without gelatin using an Eppendorf Plus repeating pipette, or equivalent, with a 10 mL tip to the buffer side (clear side) of the dialysis plate. Added the buffer gently to stop bubble formation. Capped the buffer side. Turned the plate over and uncapped the sample side of the dialysis plate. Pipetted 10 μl of 18 mg/mL blue dextran dye marker solution into each well. Pipette controls and patient samples (0.2 mL) into the wells with an Eppendorf pipette (or equivalent). Added the patient and QC samples gently to stop bubble formation. Recapped the sample side of the dialysis plate, and place the dialysis plate into the oven and turn on the rotator with the speed set at 15 RPM. Let rotate overnight for 16 hours±1 hour.

[0262] After the dialysis procedure was complete, thawed and mix the internal standard solution to add to the samples for liquid-liquid extraction. Labeled a solvent washed 12×75 glass tube for each dialysis sample, duplicate tubes for each standard point, and four tubes for the double blanks. After dialysis was complete checked for any trace of blue dextran in the buffer side (clear side) of the plate. Noted any membrane leakage and did not transfer the dialysate from the wells with faulty membranes. Pipetted 200 μL of dialysate from the buffer side (clear side) of the dialysis plate for each sample into the corresponding 12×75 glass tube. Pipetted 200 μL of standard into the appropriate labeled tubes. Pipetted 200 μL of FT4 Dialysis Buffer into the double blanks.

[0263] Liquid-liquid Extraction Procedure: Liquid-liquid extraction was done as follows. 50 μL of 100 pg/mL FT4 Internal Standard 1 was added to all tubes, except the double blanks to which 50 μL of water was added. Tubes were mixed 10 times up and down (e.g., on a multi-tube vortexer) and let stand for 10 minutes.

[0264] For extraction, 2 mL of extraction solvent was added to all tubes. Tubes were capped and mixed on a multi-tube vortexer, 4 times for 1 minute intervals. The tubes were removed from the shaker and spun down in centrifuge at 3000 rpm for 10 minutes. A separate set of 12×75 solvent (methanol) washed glass tubes was labeled, and after freezing the aqueous layer, the extract was poured into the labeled tubes. The tubes were placed in a Rotovap to allow solvent to evaporate for at least 45 minutes.

[0265] Once there was no trace of solvent left in the tubes, the samples were reconstituted with 100 μL reconstitution solution. Tubes were then covered with parafilm and mixed on multivortexer, 4 times for 30 second intervals. Using an Eppendorf pipette, a robotic liquid handler, or equivalent, the reconstituted sample was transferred from the 12×75 tubes into a 96-well plate. A heat-sealing foil was placed over the plate and the plate sealed with a heated plate sealer. The sealed plate was then centrifuged at 3700 rpm (approximately 2000 g) at 10° C. for 10 minutes. 80 μL was used for injection. Dilute and Inject Procedure: For injection, 50 μL of 100 pg/mL FT4 Internal Standard solution 2 (methanol) was added to all tubes, except the double blanks to which 50 μL of water was added. Tubes were mixed (e.g. 10 times up and down on a multi-tube vortexer) and allowed to stand for 10 minutes. Place a heat-sealing foil over the plate and seal the plate with the heated plate sealer. Centrifuge plate at 3700 rpm (approximately 2000 g) at 10° C. for 10 minutes. Inject 100 μL.

LC-MS/MS Procedures: For LC-MS/MS, the 96-well plate is placed in LC-MS/MS Autosampler and the system filled with LC system reagents. After liquid-liquid extraction or sample dilution, 80 to 100 μL of processed sample was injected into the HTLC system using methanol:water in the mobile phase. The HTLC system comprises two HPLC pumps per channel that can be employed in two functions: (1) Post HPLC-column addition of solvents to improve sensitivity; and (2) HPLC chromatography using a binary gradient and a 5 μm reverse phase analytical column. In this example a phenyl hexyl column was used for chromatography, which had a 5-μm particle size. Such HPLC columns are commercially

available (e.g., Thermo Hypersil Phenyl Hexyl, Luna Phenyl Hexyl). In the analytical mode of the HTLC, the sample was first loaded onto the analytical column. A binary gradient of from 40% to 90% methanol at 1 mL per minute over 2 minutes was used, resulting in the separation of thyroxine and internal standard from matrix interferences and other analytes contained in the sample. Ionization efficiency and thus detection limits were enhanced by post-column addition of a 90:10 Methanol:water solution containing 1 mM ammonium carbonate at 200 microliters per minute. The separated sample was then transferred to the MS/MS for quantitation. The LC method is summarized in the table below. Thyroxine and internal standard elute at approximately 2.5 minutes from the start of the method (+ or −0.5 minutes)

Free Thyroxine LC Method

STEP	Start Time (minutes)	Step Duration (seconds)	Post-column Loading pump Flow Rate (mL/minute)	Eluting Pump* (% B)	Flow Rate (mL/minute)	Eluting Pump Gradient Type
1	0.00	40	0.2	40	1.0	Isocratic
2	0.67	120	0.2	100	1.0	Gradient
3	2.67	25	0.2	100	1.0	Isocratic
4	3.08	45	0.2	100	1.2	Isocratic
5	3.83	30	0.2	40	1.0	Isocratic

*Loading Pump Buffer 10:90 Millipore Water: Methanol with 1 mM Ammonium Carbonate
Eluting Pump Buffer A Millipore Water
Eluting Pump Buffer B 10:90 Millipore Water: Methanol

Mass Spectrometry: The flow of combined liquid solvents from the HTLC entered the turboionspray (ESI) interface of the MS/MS analyzer. The solvent/charged analyte mixture was first electrosprayed and converted to fine droplets after exiting the electrospray capillary. The residual solvent is removed from the charged analytes through a combination of heating and nitrogen gas flow to eventually yield gas phase analyte ions. The ions passed through the orifice of the instrument and entered the first quadrupole. Quadrupoles 1 and 3 (Q1 and Q3) were the mass filters, allowing selection of ions based on their mass to charge ratio (m/z). Quadrupole 2 (Q2) was the collision cell, where ions were fragmented.

[0266] The first quadrupole of the MS/MS (Q1) selected for molecules with the mass to charge ratio of thyroxine (775.5±1.0 m/z or mass units). Ions with these m/z passed to the collision chamber (Q2), while ions with any other m/z collided with the sides of the quadrupole and were fragmented. Ions entering Q2 collided with neutral gas molecules and fragmented. This process is called Collisionally Activated Dissociation (CAD). The CAD gas used in this example was nitrogen resulting in the generation of fragments (product). The fragment ions generated were passed into quadrupole 3 (Q3), where the two fragment ions of thyroxine to be measured (m/z 574.6+−.1.0 m/z & 126.8+−1.0 m/z or mass units) were selected for, while other ions were screened out. The selected fragment ions were measured by the detector. The same process was carried out for the internal standard, which was $^{13}C_6$-Thyroxine. Thus, the selected MS/MS transitions (nominal masses) measured were as follows: Thyroxine m/z 775 to 574 and 127, $^{13}C_6$-Thyroxine m/z 781 to 580 and 127.

[0267] Selected MS/MS parameters were as follows: Dwell time: 100 msec for each transition, Unit mass resolution in both resolving quadrupoles (Q1 and Q3) Curtain Gas: 20 CADgas: 6, Temp.: 450.degree. C. Gas1: 80, GS2: 40 CE: −80 for m/z 127 product ions, −45 for m/z 574 and 580 product ions. Electrospray voltage=−4500V.

[0268] As ions collide with the detector, they produce a pulse of electrons. The pulse was converted to a digital signal, which was counted to provide an ion count. The acquired data was relayed to the computer, which plotted counts of the ions collected vs. time.

Calculations

[0269] Calibration curves are constructed using the software system (Analyst) that controls the mass spectrometer. Calibration curves are generated by assigning the known concentrations to calibrators to generate a response ratio of analyte to internal standard versus concentration of analyte added [FIG. **20-26**]. The concentrations of unknown samples are automatically calculated by comparing the response ratio of analyte to internal standard observed in measuring unknown samples to the calibration curve generated above.

[0270] Only results within the measurable range (2-100 pg/ml, 0.2-10 ng/dL) are valid.

Assay Performance Characteristics

[0271] A quantitative bioanalytical method for the determination of free thyroxine (FT4) in human serum using ED, LLE or sample dilution, and LC-MS/MS detection was developed and validated.

[0272] The assay was shown to be specific for the analysis of free thyroxine. Quantitative interference was not observed in the FT4 Dialysis Buffer. The matrix effect for free thyroxine and its internal standard was within acceptable limits for all post-dialysis sample types including pooled serum, plasma containing heparin and EDTA anticoagulants, hemolyzed and lipemic samples. Assay specificity was shown for $^{13}C_6$-Thyroxine internal standard.

[0273] The method was assayed and validated using four analytical batches. Replicates of spiked quality control (QC) samples at approximately 1.0, 1.5, 2.5 and 8.0 ng/dL were prepared in pooled serum to determine imprecision for free thyroxine. Replicates of calibrator samples at approximately 0.1, 0.2, 5.0 and 10.0 ng/dL were prepared in FT4 Dialysis Buffer to determine inaccuracy and imprecision for free thyroxine. The intra- and inter-assay inaccuracy (% bias) and imprecision (% CV) for the calibrators and QC (imprecision only) samples were ≦20% at the LLOQ and ≦15% at all

other concentrations. The analytical range of the assay was validated between 0.2 ng/dL (LLOQ) and 10.0 ng/dL (ULOQ) for free thyroxine.

[0274] Spike and recovery experiments were performed using a post-dialysis serum pool and a FT4 Dialysis Buffer Calibrator containing low levels of thyroxine. The dialysate from the pool and the calibrator were spiked with pure thyroxine standard material. Mean recoveries of between 85% and 115% were observed for 1.0 ng/dL, 2.0 ng/dL and 8.0 ng/dL spikes respectively.

[0275] Hormone specificity interference less than the LLOQ response was observed during evaluation of free thyroxine for all analytes tested at relevant circulating levels.

[0276] Free thyroxine whole blood stability has been shown for up to 48 hours at room temperature. Free thyroxine calibrator stability has been shown for up to 16 hours at room temperature. Free thyroxine QC sample stability has been shown for up to 24 hours at room temperature. Free thyroxine calibrator and QC stability has been shown for up to 14 days when stored at −20° C. Free thyroxine calibrator and QC stability has been shown for up to three freeze/thaw cycles. Free thyroxine stock solution stability has been shown for up to 10 days frozen at −70° C. Free thyroxine working stock solution stability has been shown for up to 6 days when stored refrigerated at 4° C. $^{13}C_6$-Thyroxine internal standard solution stability has been shown for up to 7 days refrigerated at 4° C. Free thyroxine autosampler stability has been shown for 24 hours at 10° C.

[0277] Cross-validation of samples analyzed by Centaur (Free thyroxine) against ED-LLE followed by LC-MS/MS indicates acceptable bias. Comparison using scatter plots (FIG. **27**) produces acceptable comparison for Centaur and ED-LLE followed by LC-MS/MS for Free thyroxine. Reference range transfer was successful for free thyroxine in adults and pre-pubertal children ages 2-8 years.

[0278] Standard curve fitting and reproducibility was evaluated using spiked standard samples at six concentrations. The standards were used to generate a weighted (1/x) linear regression calibration curve, which covered the range from 0.2 to 10.0 ng/dL for free thyroxine. Average inaccuracies and imprecision≦20% at the LLOQ and ≦15% throughout the remainder of the range were observed. The correlation coefficients of the curves were greater than 0.98 (Tables 29-30).

Selectivity

Blank Matrix Interference.

[0279] Quadruplicate injections of FT4 Dialysis Buffer and 1:1 methanol:water were injected to determine the degree of blank matrix interference for thyroxine. For each assay, matrix analyte responses were less than the mean LLOQ responses.

Anticoagulant Effect on Measurement.

[0280] The effect of EDTA and heparin as anticoagulants was tested by drawing healthy volunteers using red top serum collection tubes and vacutainers containing sodium heparin and potassium EDTA anticoagulants. The results of the heparin and EDTA tubes were compared to the results from the serum collection tubes. It was found that variation of sample type and anticoagulant exhibited a bias<±15% for measurement of free thyroxine. Thus, plasma samples collected with heparin and EDTA are acceptable specimen types.

Anticoagulant Effect on Free Thyroxine Measurement

	Concentration (ng/dL)		
Sample	Serum	Heparin	EDTA
FT4	273.094*	1.536	1.570
	1.746	1.754	1.669
	1.745	1.413	1.553
	1.651	1.521	1.527
Mean	1.714	1.556	1.580
Mean Matrix Effect (%)	NA	−9.22	−7.83
n	4	4	4

*Dialysis error, excluded from calculations; NA = Not Applicable.

Effect of Lipemia and Hemolysis in the Matrix.

[0281] The effect of lipemia and hemolysis on the quantitative result was determined by spiking pooled patient serum with either a lipid solution or lysed red blood cells at a concentration of 5% by volume. The samples were run in quadruplicate and the results were compared to the results of the pool before contamination. A bias≦±15% following addition of lipemic or hemolyzed material was observed for free thyroxine. Thus, lipemic and hemolyzed samples may be processed using this assay.

Effect of Lipemia and Hemolysis on Free Thyroxine Measurement

	Concentration (ng/dL)		
Sample	Serum	Lipemic	Hemolyzed
FT4	1.417	1.463	2.157*
	1.403	1.385	1.684
	1.696	3.021*	1.621
	1.285	1.547	1.602
Mean	1.450	1.465	1.636
Mean Matrix Effect (%)	NA	1.02	12.79
n	4	4	4

*= Data point outside 3SD from mean, excluded from calculations.
NA = Not Applicable.

Internal Standard Interference.

[0282] Internal standard ($^{13}C_6$-Thyroxine) working solution was spiked into FT4 Dialysis Buffer and tested in quadruplicate to evaluate the presence of unlabeled analyte. Internal standard ($^{13}C_6$-Thyroxine) interference response≦LLOQ response was observed. Thus, $^{13}C_6$-Thyroxine interference was considered acceptable.

Matrix Effect.

[0283] Matrix effect was calculated at low, mid, and high level concentrations. Matrix effect for internal standard was measured at a single concentration. A minimum of 4 samples per QC level were analyzed for FT4 to determine matrix effect on quantitative result. Matrix effects were less than 15% for free thyroxine and $^{13}C_6$-Thyroxine Internal Standard when comparing water and dialysate from pooled samples.

Free Thyroxine Matrix Effect

Sample Matrix	Millipore Water			Post-Dialysis Pooled Serum		
Post-Column Infusion Level	Analyte Peak Height (cps)	IS Peak Height (cps)	Ratio	Analyte Peak Height (cps)	IS Baseline Height (cps)	Ratio
Low Infusion	24299.421	16100.000	1.509	26033.955	17600.000	1.479
	24027.876	15400.000	1.560	25844.532	16800.000	1.538
	25823.905	15900.000	1.624	25012.124	17100.000	1.463
	24917.386	15900.000	1.567	24618.647	15900.000	1.548
MARK per Conc.	NA	NA	1.565	NA	NA	1.507
% Change	NA	NA	NA	NA	NA	−3.71
Mid Infusion	173144.290	24400.000	7.096	164420.482	21200.000	7.756
	181732.088	23100.000	7.867	164337.907	20800.000	7.901
	175921.645	23900.000	7.361	155907.875	20200.000	7.718
	178237.273	23400.000	7.617	156578.143	20000.000	7.829
MARK per Conc.	NA	NA	7.485	NA	NA	7.801
% Change	NA	NA	NA	NA	NA	4.22
High Infusion	303787.094	23000.000	13.208	264941.287	19500.000	13.587
	312136.060	21600.000	14.451	266588.958	19000.000	14.031
	329631.304	24400.000	13.509	272643.856	19100.000	14.275
	311677.872	22300.000	13.977	269085.967	19300.000	13.942
MARR per Conc.	NA	NA	13.786	NA	NA	13.959
% Change	NA	NA	NA	NA	NA	1.25

Matrix effect = [(Mean Analyte to Internal standard ratio in pooled serum)/Mean Analyte to Internal standard ratio in water)] − 1, expressed as a percentage

Inaccuracy and Imprecision

[0284] A. Intra-Assay and Inter-Assay Imprecision.

[0285] Intra-assay imprecision was calculated with replicate samples of spiked FT4 Buffer solutions (data from 4 runs) and replicate samples at different concentrations in post-dialysis pooled human serum (data from 4 runs). Inter-assay imprecision was calculated using data from each of the assay runs (n≧18). Free thyroxine exhibited intra and inter-assay imprecision≦20% at the LLOQ, which was established to be 0.2 ng/dL, and ≦15% throughout the remainder of the linear range (0.2 to 10.0 ng/dL) in both post-dialysis pooled serum and FT4 Dialysis Buffer.

[0286] B. Intra-Assay and Inter-Assay Inaccuracy.

[0287] Intra-assay inaccuracy was calculated in 4 assay runs with replicates at 4 different concentrations in FT4 dialysis buffer spiked with known amounts of analyte. Inter-assay inaccuracy was calculated using data from each of 4 assay runs (n≧18). Free thyroxine exhibited intra and inter-assay inaccuracy≦±20% at the LLOQ, which was established to be 0.2 ng/dL, and ≦±15% throughout the remainder of the linear range (0.2 to 10.0 ng/dL) in FT4 Dialysis Buffer.

[0288] A. Intra-Assay Precision

[0289] The intra-assay precision of the analytical method was calculated for four assays using patient pools (QC **1**, QC **2**, QC **3**, and QC **4**). The following tables show the data for these pools, as well as, the data from quality controls made from spiked FT4 Dialysis Buffer at four concentrations (0.1 ng/dL, 0.2 ng/dL, 5.0 ng/dL, and 10.0 ng/dL).

Free Thyroxine Intra-assay Precision

Batch	Intra-day % CV							
Number	QC1	QC2	QC3	QC4	0.1	0.2	5	10
1	9.61	4.75	3.89	3.79	153.4	4.98	6.20	5.53
2	12.16	9.55	5.53	4.41	39.65	5.12	5.24	2.19
3	4.96	2.31	5.44	6.42	22.20	4.37	4.05	5.47
4	3.92	3.51	4.29	5.89	117.7	2.48	0.53	3.90

[0290] B. Reproducibility

[0291] The inter-assay precision was calculated from the overall data from the precision assays for each of the QC samples. As shown in the table below, the method has acceptable inter-assay precision.

Free Thyroxine Inter-Assay Precision

	QC 1	QC 2	QC 3	QC 4	0.1	0.2	5.0	10.0
Average	1.12	1.52	2.51	7.67	0.19	0.20	4.88	10.18
% CV	9.86	8.41	6.79	6.34	144.44	4.61	5.66	4.67
N	23	22	21	24	20	19	20	20

0.1 ng/dL standard failed inter-assay precision requirements, therefore 0.2 ng/dL standard determined to be the LLOQ.

Accuracy

[0292] The inter-assay accuracy was determined by calculating the percent bias for samples of known concentrations. FT4 Dialysis Buffer was spiked to 0.1 ng/dL, 0.2 ng/dL, 5.0 ng/dL, and 10.0 ng/dL and then assayed a total of at least 18 times in 4 different runs.

Free Thyroxine Accuracy Data

Concentration	0.1 ng/dL	0.2 ng/dL	5.0 ng/dL	10.0 ng/dL
Expected Result	0.1	0.2	5.0	10.0
Average Result	0.102	0.198	4.884	10.184
% Bias	2.22	−1.18	−2.31	1.84

0.1 ng/dL standard failed Inter-Assay precision, therefore 0.2 ng/dL standard determined to be the LLOQ.

Spike and Recovery

[0293] Spike and Recovery Preparation

[0294] A post dialysis low level QC sample and a FT4 dialysis buffer calibrator were spiked with thyroxine. Recov-

ery was performed by comparing the measured results of samples spiked with 1.0, 2.0, and 8.0 ng/dL of standard material against expected values. Samples were analyzed in quadruplicate. Free thyroxine assay exhibited recoveries>85% and <115%.

Thyroxine Spike and Recovery

	Concentration Added (ng/dL)			
	0.000	1.000	2.000	8.000
	Concentration Measured (ng/dL)			
FT4 Calibrator	0.949	1.885	2.760	8.691
	1.034	1.887	2.746	7.761
	0.972	1.873	2.900	8.867
	1.030	1.855	2.844	8.666
Mean	0.996	1.875	2.813	8.496
Expected Conc.	NA	1.996	2.996	8.996
Recovery (%)	NA	93.9	93.9	94.4
n	4	4	4	4
Post-Dialysis	1.072	2.043	2.657	8.600
Pooled Serum	1.084	1.963	2.905	8.285
	1.088	1.991	2.898	8.515
	1.097	1.992	2.761	8.124
Mean	1.085	1.997	2.805	8.381
Expected Conc.	NA	2.085	3.085	9.085
Recovery (%)	NA	95.8	90.9	92.2
n	4	4	4	4

NA = Not Applicable

Selectivity

[0295] Selectivity was tested by spiking 1 ng of the analytes listed below into 1 mL (equivalent to 100 ng/dL) of FT4 dialysis buffer before extraction and injection. Acceptability Criteria: Response less than the LLOQ at the appropriate retention time in excess of physiologically significant amounts of potential interfering substance.

[0296] The circulating concentrations of the cross reactants above are all at physiological concentrations less than 3 ng/dL. This concentration would give a response equal to 0.03 ng/dL of FT4 at most in the case of RT3. This is well below the level of detection for free thyroxine. Free thyroxine analysis is not affected by the presence of circulating hormones or drugs at physiological concentrations in the testing performed. Interfering substances are removed through sample purification, chromatography and selected reaction monitoring.

Free Thyroxine Hormone Specificity

Steroid	Amount added (ng/dL)	Measured Concentration (ng/dL)	Mean Relative Response (%)
Cross Reactant_T3	100	0.377	0.40
Cross Reactant_T3	100	0.336	
Cross Reactant_T3	100	0.519	
Cross Reactant_T3	100	0.365	
Cross Reactant_RT3	100	1.123	1.07
Cross Reactant_RT3	100	1.032	
Cross Reactant_RT3	100	1.082	
Cross Reactant_RT3	100	1.025	
Cross Reactant_3,5-DIT	100	0.162	0.09
Cross Reactant_3,5-DIT	100	0.043	
Cross Reactant_3,5-DIT	100	0.062	
Cross Reactant_3,5-DIT	100	0.108	

-continued

Free Thyroxine Hormone Specificity

Steroid	Amount added (ng/dL)	Measured Concentration (ng/dL)	Mean Relative Response (%)
Cross Reactant_3-IT	100	0.104	0.17
Cross Reactant_3-IT	100	0.183	
Cross Reactant_3-IT	100	0.176	
Cross Reactant_3-IT	100	0.208	

T3 = 3,5,3'-Triiodo-L-Thyronine; RT3 = 3,3',5'-Triiodo-L-Thyronine; 3,5-DIT = 3,5-Diiodo-L-Thyronine; 3-IT = 3-Iodo-L-Thyronine.

Stability

[0297] Stability was demonstrated at the following conditions for the listed times.

Storage Condition	Free thyroxine
Room Temperature	1 day
Frozen (−20° C.)	14 days
Freeze Thaw	3 cycles
Whole Blood	48 hours
Autosampler	24 hours
Refrigerated (4° C.)	7 days

Inter-Assay Comparison

A. Bayer Centaur Immunoassay Compared to ED-LLE-LC-MS/MS.

[0298] A minimum of 25 routine samples representing the physiological range, were analyzed by ED-LLE-LC-MS/MS and Bayer Centaur for assay-to-assay comparison (see FIG. **27**).

Free Thyroxine Cross-Validation of Centaur to ED-LLE-LC-MS/MS

	Concentration (ng/dL)		
Sample #	Centaur	ED LC-MS/MS	Bias (%)
Sample 1	1.16	2.231	92.33
Sample 2	1.29	1.781	38.06
Sample 3	1.31	2.072	58.17
Sample 4	0.99	1.265	27.78
Sample 5	1.37	2.128	55.33
Sample 6	1.58	2.274	43.92
Sample 7	0.95	1.325	39.47
Sample 8	1.12	1.272	13.57
Sample 9	0.90	1.191	32.33
Sample 10	1.03	1.305	26.70
Sample 11	0.95	1.212	27.58
Sample 12	1.63	2.399	47.18
Sample 13	1.03	1.417	37.57
Sample 14	1.11	1.545	39.19
Sample 15	0.81	1.035	27.78
Sample 16	1.15	1.647	43.22
Sample 17	0.98	1.288	31.43
Sample 18	1.36	2.145	57.72
Sample 19	1.38	1.817	31.67
Sample 20	1.43	2.591	81.19
Sample 21	1.14	1.927	69.04
Sample 22	1.00	1.159	15.90

-continued

Free Thyroxine Cross-Validation of Centaur to ED-LLE-LC-MS/MS			
	Concentration (ng/dL)		
Sample #	Centaur	ED LC-MS/MS	Bias (%)
Sample 23	1.98	3.066	54.85
Sample 24	0.38	0.297	−21.84
Sample 25	0.88	1.052	19.55
	Average bias (%)		39.59

Bias (%) = (ED-LLE-LC-MS/MS result − Centaur result)/Centaur result, expressed as a percentage.

Inter-Assay Comparison of Centaur to LC-MS/MS:

[0299] Cross-validation of free thyroxine Centaur analysis to ED-LLE-LC-MS/MS yielded an average bias of 39.59% for samples within the analytical range. Comparison of data throughout the range generated a slope of 1.8145 with a correlation coefficient of 0.8923 (FIG. **27**).

Reference Interval

A. Reference Range Sample Groups

[0300] Reference range transfer for free thyroxine was evaluated using NCCLS guidance (see references). Transfer of the reference range was established using the samples listed below.

	Normal Patient Serum Reference Sample Groups		
	Children [1]	Adult Males	Adult Females
Sample Number	50	25	25

[1] Children samples will include 25 boys <10 years old and 25 girls <9 years old.

B. Reference Interval of Patient Test Results

[0301]

Reference Intervals Range (ng/dL)	
Premature Infants:	
26-30 Weeks, 3-4 Days	0.4-2.8
Full-Term Infants:	
3 Days:	2.0-4.9
1-11 Months	0.9-2.6
Prepubertal Children:	0.8-2.2
Pubertal Children and Adults:	0.8-2.3

C. Reference Range Transfer

[0302] Guidance provided by NCCLS allows reference range transfer where 2 out of 20 (10%) of samples fall outside the original reference range.

[0303] It was found that all normal adult female samples were within range for reference range transfer of FT4. Adult female reference ranges are transferable. All normal adult male samples were within range for reference range transfer of FT4. Adult male reference ranges are transferable. There was one pre-pubertal reference sample that was outside the normal reference range, and another sample that was potentially out of range. Both samples initially produced high results and upon repeat one sample was still high outside of normal range, while the other was within normal range. The reference sample that repeated within range was not run a third time, which would be in accordance with sample repeat requirements, due to the fact that the pre-pubertal reference range passes with or without this sample being in the normal reference range. A total of 48 out of 50 pre-pubertal reference samples were within range for reference range transfer of FT4. Pre-pubertal reference ranges are transferable. Thus, the free thyroxine reference range transfer acceptance criteria were met.

Free Thyroxine Adult Reference Range Verification			
Adults	Concentration (ng/dL) FT4 Result	Adults	Concentration (ng/dL) FT4 Result
Female 1	1.039	Male 1	1.474
Female 2	1.074	Male 2	1.566
Female 3	1.311	Male 3	1.631
Female 4	1.383	Male 4	1.210
Female 5	0.977	Male 5	1.520
Female 6	1.120	Male 6	1.226
Female 7	1.501	Male 7	1.417
Female 8	1.010	Male 8	1.432
Female 9	1.236	Male 9	1.538
Female 10	1.277	Male 10	1.430
Female 11	1.263	Male 11	1.292
Female 12	1.317	Male 12	1.153
Female 13	1.452	Male 13	1.268
Female 14	0.983	Male 14	1.107
Female 15	1.432	Male 15	1.528
Female 16	1.270	Male 16	1.479
Female 17	1.005	Male 17	1.338
Female 18	1.071	Male 18	1.411
Female 19	1.132	Male 19	1.470
Female 20	1.414	Male 20	1.597
Female 21	1.405	Male 21	1.660
Female 22	1.355	Male 22	1.312
Female 23	1.767	Male 23	1.150
Female 24	1.086	Male 24	1.188
Female 25	1.566	Male 25	1.780

Male and Female samples are from an in house draw and are considered to be a healthy reference population. Free thyroxine adult reference range is 0.8 to 2.3 ng/dL.

Free Thyroxine Pre-pubertal Reference Range Verification			
Children	Concentration (ng/dL) FT4 Result	Children	Concentration (ng/dL) FT4 Result
Female 1	1.848	Male 1	1.845
Female 2	1.543	Male 2	1.740
Female 3	1.561	Male 3	1.569
Female 4	1.604	Male 4	1.753
Female 5	1.974	Male 5	1.849
Female 6	1.826	Male 6	2.090
Female 7	1.485	Male 7	1.624
Female 8	1.846	Male 8	1.704
Female 9	1.528	Male 9	2.047
Female 10	1.612	Male 10	2.754 RPT = 2.474

-continued

Free Thyroxine Pre-pubertal Reference Range Verification

Children	Concentration (ng/dL) FT4 Result	Children	Concentration (ng/dL) FT4 Result
Female 11	1.800	Male 11	1.765
Female 12	1.585	Male 12	2.54 RPT = 1.799
Female 13	1.812	Male 13	2.167
Female 14	1.801	Male 14	1.304
Female 15	1.471	Male 15	1.656
Female 16	1.853	Male 16	1.860
Female 17	1.617	Male 17	1.063
Female 18	1.443	Male 18	1.536
Female 19	1.619	Male 19	1.494
Female 20	1.553	Male 20	1.875
Female 21	1.827	Male 21	1.629
Female 22	1.956	Male 22	1.966
Female 23	1.549	Male 23	1.583
Female 24	1.820	Male 24	1.508
Female 25	1.886	Male 25	1.902

Pre-pubertal children samples were previously tested in Allergy screens and assumed as normal. Children ranging from ages 2-8 years for both males and females were selected. Free thyroxine pre-pubertal reference range is 0.8 to 2.2 ng/dL RPT is the repeat value for the sample after being run a second time for verification of initial high result.

Standard Curve Fitting and Reproducibility

[0304] The reproducibility of the standard curve was evaluated by comparing the back-calculated concentrations to the theoretical concentration of the standard in 5 analytical runs. The calibrator concentrations for free thyroxine (ng/dL) were as follows: 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0.

[0305] The reproducibility of the standard curve was evaluated, using standards 2-7, by comparing the back-calculated concentrations to the actual concentration of the standard in five analytical runs. The curve was fit with a straight line with weighted 1/x fit, as established during method development. Free thyroxine calibration curves exhibited mean imprecision<15% for all concentrations between 0.2 ng/dL and 10.0 ng/dL. Correlation coefficients were greater than 0.98.

Analytical Reportable Range

A. LLOQ (Lower Limit of Quantification)

[0306] The lower limit of quantification for free thyroxine using this assay was 0.2 ng/dL as determined during evaluation of inaccuracy, imprecision and calibration curve reproducibility.

B. ULOQ (Upper Limit of Quantification)

[0307] The upper limit of quantification using this assay was 10.0 ng/dL for free thyroxine, as determined during evaluation of inaccuracy, imprecision and calibration curve reproducibility.

Dye Marker Analysis

[0308] A. Dye Marker Correlation

[0309] Blue dextran was used as a visual dye marker to indicate membrane leakage during dialysis. Various concentrations of blue dextran (18 mg/mL, 9 mg/mL, and 4.5 mg/mL in water) were added to the sample side of the dialysis plate, and the results were compared to the same samples that were run with no dye added to see if the dye had any effect on dialysis of FT4 All concentrations of blue dextran tested correlated to the results obtained when no dye was used. The highest concentration of blue dextran tested, 18.0 mg/mL, will be used as an indicator of membrane leakage during the dialysis step. This concentration of blue dextran was easily noted when membrane leakage occurred.

Free Thyroxine Standard Curve Fitting and Reproducibility

	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
	Actual Concentration (ng/dL)					
Batch	0.200	0.500	1.000	2.500	5.000	10.000
1	0.231	0.489	0.960	2.511	4.824	10.642
	0.190	0.480	0.989	2.536	5.023	9.524
2	0.181	0.518	0.976	2.501	4.728	10.478
	0.190	0.516	1.106	2.623	4.881	9.701
3	0.484*	0.525	0.999	2.451	4.556	10.816
	0.227	0.473	0.938	2.432	5.070	9.713
4	0.188	0.509	1.030	2.565	4.936	10.796
	2.113*	0.498	0.980	2.580	5.007	9.111
5	0.207	0.508	0.992	2.531	5.061	9.878
	0.184	0.549	0.923	2.412	5.228	9.927
Mean	0.200	0.507	0.989	2.514	4.931	10.059
Accuracy (% RE)	−0.12	1.30	−1.07	0.57	−1.37	0.59
Precision (% RSD)	9.83	4.46	5.17	2.68	3.91	5.85
n	8	10	10	10	10	10

*Outliers excluded due to preparative error

Free Thyroxine Standard Curve Fitting and Reproducibility Continued

Batch	K_0 (Y-intercept)	K_1 (slope)	Correlation Coefficient
1	0.0489	0.2840	0.9987
2	0.0457	0.2980	0.9988
3	0.0344	0.3070	0.9978
4	0.0268	0.3110	0.9974
5	0.0408	0.2880	0.9995
Mean	0.0393	0.2976	0.9984
Precision (% RSD)	NA	3.92	0.08
n	5	5	5

NA = Not Applicable.
Data calculated using Standards 2-7.

Free Thyroxine Dye Marker Correlation

Blue Dextran Concentration	Concentration (ng/dL) QC1	QC2	QC3	QC4	Patient 3	Average Bias (%)
0 mg/mL Dye	1.23	1.90	2.49	7.00	0.93	
0 mg/mL Dye	1.09	1.75	2.71	7.06	N/A	
Average Concentration	1.16	1.83	2.60	7.03	0.93	
4.5 mg/mL Dye	1.11	1.87	2.53	7.71	0.94	
4.5 mg/mL Dye	1.14	1.59	2.69	7.53	0.88	
Average Concentration	1.13	1.73	2.61	7.62	0.91	
Bias (%)	−3.02	−5.23	0.33	8.45	−1.51	−0.20
9.0 mg/mL Dye	1.14	1.70	2.69	7.09	0.94	
9.0 mg/mL Dye	1.06	1.70	2.74	6.55	0.99	
Average Concentration	1.10	1.70	2.72	6.82	0.97	
Bias (%)	−5.39	−6.90	4.48	−2.96	4.37	−1.28

-continued

Free Thyroxine Dye Marker Correlation

Blue Dextran	Concentration (ng/dL)					Average
Concentration	QC1	QC2	QC3	QC4	Patient 3	Bias (%)
18.0 mg/mL Dye	1.14	1.90	2.79	6.95	0.99	
18.0 mg/mL Dye	1.16	1.58	2.85	7.45	1.02	
Average Concentration	1.15	1.74	2.82	7.20	1.00	
Bias (%)	−0.95	−4.52	8.42	2.50	8.48	2.79

N/A = Not Applicable; only 1 duplicate run.

Bias (%) = (Average concentration dye added − Average concentration no dye added)/Average concentration no dye added, expressed as a percentage.

Example 3

[0310] For dialysis of samples to remove free thyroxine or other free hormones from hormones that are bound to proteins in the sample, a 200 microliter (μL) sample and 10 μL of an 18 mg/mL dextran blue are added to one side of a 5 or 10 kilodalton molecular weight cut-off cellulose dialysis membrane in a 96-well equilibrium dialysis plate and capped. Then, 300 microliters of a dialyzing buffer (described in Example 2) is added to the other side of the plate and the wells are capped. The plate is placed vertically within a temperature controlled (37° C.) rotating oven and rotated at 15 cycles per minute for 16 hours. The 96-well plate may then be removed from the rotating oven and the dialysate buffer side is uncapped. A sample of 200 μL may be removed for processing by either liquid liquid extraction using the isotope dilution LC-MS/MS method.

[0311] In some cases, a liquid extraction step is performed after the dialysis to remove residual salts and/or other additives which are used in the dialysis solution and/or remain from the sample, but that may interfere with the MS/MS analysis. The dialysate is extracted with 71.25:23.75:5 ethyl acetate:hexane:methanol. Alternatively, the dialysate is diluted with a solution of 1:1 methanol:water containing stable labeled internal standard and directly injected onto the LC-MS/MS system for analysis.

Example 4

2D-LC-MS/MS Analysis for 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3

[0312] 25-Hydroxyvitamin D3 (Native) and 25-Hydroxyvitamin D2 (Supplemented) analysis was validated to 1 ng/mL using 200-4, of sample. Optimum analytical specificity and sensitivity was generated using 2D LC using gradient separations in both LC dimensions, heart-cutting and chromato-focusing prior to MS/MS detection.

Abbreviations for Examples

[0313] ALQ: Above limit of quantification; BLQ: Below limit of quantification; CAP: College of American Pathologists; CLIA: Clinical Laboratory Improvement Act; CPS: Counts per scan; CV: Coefficient of variance; ED: Equilibrium Dialysis; EDTA: Ethylenediamminotetraacetic acid; FT4: Free Thyroxine; IE: Injection Error; IS: Internal Standard; LC: Liquid Chromatography; LLE: Liquid-Liquid Extraction; LLOQ: Lower Limit of Quantitation; MARR: Mean average response ratio; MS/MS: Tandem MS/MS detection; N: Number of replicates; NA: Not Applicable; QC: Quality Control; R: Correlation Coefficient; RIA: Radioimmunoassay; SD: Standard Deviation; ULOQ: Upper Limit of Quantitation

[0314] All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

That which is claimed:

1. A method for determining the presence or amount of free thyroxine in a plurality of samples by tandem mass spectrometry, comprising: (a) dialyzing the plurality of samples to separate the free thyroxine from the protein-bound thyroxine in the samples; (b) generating a precursor ion of the thyroxine in each sample; (b) generating one or more fragment ions of the thyroxine in each sample; and (c) detecting the presence or amount of one or more of the ions generated in step (b) or (c) or both, and relating the detected ions in each sample to the presence or amount of the free thyroxine in the plurality of samples.

2. The method of claim **1**, wherein the samples are subjected to a purification step prior to ionization.

3. The method of claim **1**, wherein the purification step comprises chromatography.

4. The method of claim **3**, wherein the chromatography comprises high performance liquid chromatography (HPLC).

5. The method of claim **1**, further comprising at least one of liquid-liquid extraction or dilution of the plurality of samples prior to mass spectrometry.

6. The method of claim **1**, further comprising detection of thyroxine over a range of from about 2.0 pg/mL to about 100 pg/mL.

7. The method of claim **1**, wherein the dialysing step comprises the use of a buffer, and wherein the buffer comprises and sufficient salts such that the buffer is isotonic.

8. A method for determining the presence or amount of at least one biomarker of interest in a biological sample, the method comprising:

providing a biological sample believed to contain at least one biomarker of interest;

chromatographically separating the at least one biomarker of interest from other components in the sample; and

analyzing the chromatographically separated at least one biomarker of interest by mass spectrometry to determine the presence or amount of the at least one biomarker of interest in the sample.

9. The method of claim **8**, further comprising partially purifying the biomarker of interest prior to chromatography.

10. The method of claim **8**, wherein the at least one biomarker comprises at least one of steroid hormone or a thyroid hormone.

11. The method of claim **8**, wherein at least one biomarker comprises free thyroxine.

12. The method of claim **11**, wherein the method comprises dialyzing a plurality of samples to separate the free thyroxine from the protein-bound thyroxine in the samples and isolating

the portion of the samples comprising the majority of the free thyroxine prior to chromatographically separating the free thyroxine from other components extracted from the plurality of samples.

13. The method of claim **12**, further comprising detection of thyroxine over a range of from about 2.0 pg/mL to about 100 pg/mL.

14. The method of claim **12**, wherein the dialysing step comprises the use of a buffer, and wherein the buffer comprises and sufficient salts such that the buffer is isotonic.

15. The method of claim **8**, wherein the biological sample comprises blood, serum, plasma, urine, or saliva.

16. The method of claim **8**, wherein the chromatography comprises high performance liquid chromatography (HPLC).

17. The method of claim **8**, wherein the chromatography comprises extraction and analytical liquid chromatography.

18. The method of claim **9**, wherein the partial purification comprises liquid-liquid extraction.

19. The method of claim **8**, further comprising diluting the sample into a solvent used for liquid chromatography or mass spectrometry.

20. The method of claim **8**, wherein the tandem MS/MS spectrometer is operated in an atmospheric pressure chemical ionization (APCI) mode.

21. A system for determining the presence or amount of at least one biomarker of interest in a test sample, the system comprising:

a station for providing a test sample suspected of containing one or more hormones or metabolites;

a station for partially purifying the at least one biomarker of interest from other components in the sample;

a station for chromatographically separating the at least one biomarker of interest from other components in the sample; and

a station for analyzing the chromatographically separated one or more hormones or metabolites by mass spectrometry to determine the presence or amount of the one or more hormones or metabolites in the test sample.

22. The system of claim **21**, further comprising a station for dialyzing a plurality of samples as a means to separate the hormone or metabolite from protein-bound hormone or metabolite.

23. The system of claim **21**, wherein at least one of the stations is controlled by a computer.

* * * * *