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(71) Applicants (for all designated States except US): WIS-CONSIN ALUMNI RESEARCH FOUNDATION [US/US]; P.O. Box 7365, Madison, WI 53707 (US). STEMI-NA BIOMARKER DISCOVERY, INC. [BR/US]; 504 S. Rosa Road, Suite 150, Madison, WI 53719 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): CEZAR, Gabriela, Gebrin [BR/US]; 6924 Ramsey Road, Middleton, WI 53563 (US). SMITH, Alan, M. [US/US]; 1146 Erin Street, Madison, WI 53715 (US).

(74) Agent: MCMULLEN-TACK, Michelle, L.; Mcdonnell Boehnen Hulbert & Berghoff LLP, 300 South Wacker Drive, Suite 3100, Chicago, IL 60606 (US).

(54) Title: METABOLIC BIOMARKERS OF AUTISM

(57) Abstract: This invention provides methods and biomarkers for diagnosing autism by identifying cellular metabolites differentially produced in autistic patient samples versus non-autistic controls. Methods for identifying a unique profile of metabolites present of secreted in brain tissue, cerebrospinal fluid, plasma, or biofluids of autistic samples are described herein. The individual metabolites or a pattern of secreted metabolites provide metabolic signatures of autism, which can be used to provide a diagnosis thereof.

METABOLIC BIOMARKERS OF AUTISM

Field of the Invention

This invention relates to autism and diagnosing autism and autism spectrum disorders. Specifically, the invention provides methods and biomarkers for identifying individuals, particularly children, having autism and autism spectrum disorders. More particularly, the invention provides methods for identifying metabolites secreted by brain tissues and into biofluids of individuals with autism, wherein such metabolites have a molecular weight from about 10 Daltons to about 1500 Daltons. The invention provides multiple collections or spectra that comprise one or a plurality of such metabolites that are present in autistic individuals that are diagnostically and significantly different from levels found in non-autistic individuals. Collections of one or more differentially secreted metabolites provided herein comprise metabolic signatures diagnostic for autism. Additionally, specific biomarkers for autism are identified herein.

Background of the Invention

Autism is a neurological disorder characterized by alterations in social interaction, language development, repetitive movements and patterns of behavior. Its prevalence has increased from 1 in 2,325 births prior to the 1980's to an alarming 1 in 101 births today (Blaylock *et al.*, 2009, *Curr. Med. Chem.* 16:157-170). Autism is a very complex neurological disorder that does not follow strictly genetic or deterministic etiology (Lander *et al.*, 1994, *Science*, 265:2037-48; Baron-Cohen *et al.*, 2005, *Science*, 310:819-23; Santangelo *et al.*, 2005, *Am. J. Pharmacogenomic.* 5:71-92). Accordingly, defining a role for metabolism in the pathogenesis of autism is important to developing an

understanding of the disorder and providing an accurate diagnosis and patient management of the disease, because strictly genetic causes account for only approximately 10% of autism cases. Multiple candidate susceptibility genes have been identified, such as the serotonin transporter gene (5HTT), the GABA β receptor subunit (GABRB3), ubiquitin ligase 3 (UBE3A), wingless type MMTV integration site family member 2 (WNT2), and reelin (RELN) (Folstein et al., 2001, Nat. Rev. Genet. 2:943-955; Buxbaum et al., 2004, Mol. Psychiatry, 9:144-150; Devlin et al., 2005, Mol. Psych. 10: 1110-1116). Despite the genetic diversity underlying mutations and patient symptoms (McCracken et al., 2002, N. Engl. J Med. 347:314-321; Moore et al., 2004, Ann. Pharmacother. 38:1515-1519; Zhao et al., 2005, Intern. J Neuroscience, 115:1183-1191) there seems to be a converging metabolic dysfunction across cases (Bryson et al., 1998, Mental Retard. Dev. Disabil. Res. Rev. 4:97-103; Betancur et al., 2002, Mol. Psychiatry, 7:67-71; Moore et al., 2004, Ann. Pharmacotherapy. 38: 1515-19). Metabolism and metabolic alterations can play a significant role during neurodevelopment and autism pathogenesis (Pardo et al., 2007, Brain Pathol. 17:434- 447). Thus, comparative studies to examine global biochemical differences between autistic and non-autistic brain can identify specific metabolic pathways that contribute to autism onset and progress. (Moore et al., 2004, Pharmacother. 38:1515-1519; Rasalam et al., 2005, Dev. Med. Child Neural. 47 (8):551-555).

Summary of the Invention

In a first aspect, the present invention provides a method for identifying a human having a metabolic signature characteristic for autism, the method comprising the steps of: a. isolating a biosample comprising cerebrospinal fluid, brain tissue, blood, serum, or plasma from an individual; and b. assaying the biosample for a plurality of cellular metabolites wherein the cellular metabolites are N-acetylaspartylglutamic acid, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl) imidazole-4-carboxamido]succinate),

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wherein step (b) further comprises physically separating the plurality of cellular metabolites that are present in the biosample, wherein the individual is identified as having a metabolic signature characteristic of autism when a plurality of the cellular metabolites in the individual's biosample are differentially produced compared to the plurality of cellular metabolites in non-autistic biosamples.

In a second aspect, the present invention provides method for diagnosing autism in a human fetus in utero, the method comprising the steps of: a. assaying a fetal sample for a plurality of cellular metabolites wherein the cellular metabolites are N-acetylaspartylglutamic acid, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-Sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate); b.detecting the plurality of cellular metabolites; and c. diagnosing the fetus with autism when a metabolic signature for autism is detected in the fetal sample, wherein the signature comprises a significant increase in production of any of N-acetylaspartylglutamic acid, 5-hydroxylysine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-Sialyllactosamine, 3,4-dihydroxybenzylamine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate) and a significant decrease in production of any of 2-aminooctanoic acid, vinylacetylglycine, proline betaine, or dipalmitoyl-phosphatidylcholine.

This invention provides reagents and methods for identifying a plurality of metabolite compounds differentially produced in autistic patients, as well as methods for

using one or a plurality of such identified metabolite compounds for providing a diagnosis of autism or autistic spectrum disorder. Said metabolites are found using the methods set forth herein differentially secreted in patient tissues or biofluids, particularly brain-associated biofluids such as cerebrospinal fluid. These metabolites are found in either greater or lesser amounts in autistic as compared to non-autistic individuals. Additionally, the invention provides biomarkers, either individually or in collections of spectra of a plurality of said biomarkers, comprising the metabolites differentially detected in biofluids from autistic individuals, particularly children. Said collections or spectra are useful in the practice of the methods of the invention for diagnosing of autism.

As set forth herein, multiple neurodevelopmental disorders including autism can produce alterations in common metabolic pathways. It will be understood by those skilled in the art that metabolites (*e.g.*, small molecules from about 10 Daltons to about 1500 Daltons) present in autistic samples versus controls can be detected at varying relative levels, which provides information regarding the biochemical spectrum of compounds present in both diseased and healthy tissue. Such spectra provide "biochemical fingerprints" comprising combinations of metabolites whose changes, together, can serve as specific indicators of autism. As shown herein, certain metabolites, including those involved in glutamate, cysteine, methionine and γ-amino butyric acid (GABA) metabolism can have a synergistic role on the cerebellar pathogenesis of autism. Additionally, non-annotated or unreported metabolites were found to be present in autistic brains in a statistically-significant manner and also provide useful candidate biomarkers for autism and autism spectrum disorders. Metabolites identified herein

provide sensitive and unique biochemical signatures of autism that are useful as

diagnostic biomarkers for the disorder.

This disclosure herein of these metabolic signatures of autism and autistic

spectrum disorders is the first to apply metabolomics on postmortem autistic brains and to

5   provide a fingerprint of metabolites and their changes, between autistic and non-autistic

individuals. The studies provided herein identified metabolites that were significantly

altered in the brains of autistic subjects as compared to non-autistic controls. In addition,

subsets of metabolites that were commonly altered in autistic tissues were identified.

As set forth in more detail below, differentially-secreted metabolites having a molecular

10   weight from between about 10 Daltons to about 1500 Daltons were identified by liquid

chromatography electrospray ionization time-of-flight mass spectroscopy (LC-ESI-TOF-

MS) and/or hydrophilic interaction chromatography (HILIC), although the skilled worker

will recognize that these methods are non-limiting and that other methods for detecting

metabolites in biofluids from autistic patients can be utilized in the practice of the

15   methods of this invention. However, the skilled worker will also recognize that this

modality of mass spectrometry as applied to metabolomic analysis of fresh frozen brain

tissue was highly sensitive (*e.g.*, resolution of 3 ppm) and capable of detecting metabolites

at very low abundances (*i.e.*, micromolar to picomolar concentrations). In addition, other

analytical chemistry platforms known in the art for practicing metabolomic methods, such

20   as nuclear magnetic resonance (NMR) are less sensitive than mass spectrometry,

requiring larger amounts of biological samples to detect metabolites at significantly higher

concentrations (reviewed in Glish *et al.,* 2003, *Nat. Rev. Drug Discov.* 2:140-150);

nevertheless, the invention expressly envisions using such methods, and other methods

known in the art, under appropriate circumstances.

Specific embodiments of this invention will become evident from the following

more detailed description of certain preferred embodiments and the claims.

5

Brief Description of the Drawings

These and other objects and features of this invention will be better understood

from the following detailed description taken in conjunction with the drawings, wherein:

Figures 1 (A) and (B) are total ion chromatograms of all postmortem brain samples.

10   Figure 1 (A) represents HILIC chromatography and Figures 1 (B) represents C18

chromatography. Autistic samples are designated by gray lines and non-autistic controls

are designated by black lines.

Figures 2 (A) and Figure 2 (B) are ion chromatogram controls that verified the

chemical identities of test sample metabolites by comparative ion fragmentation pattern

15   (Figure 2 (A)) and retention time (Figure 2 (B)) in the presence of standard chemical

controls.

Figure 3 (A) - Figure 3 (C) are extracted ion chromatograms (EICs) of L-

cystathionine, and Figure 3 (D) is a plot of the relative abundance of L-cystathionine in

autistic and control brain samples. Figure 3 (A) shows the results of metabolomic

20   analysis of fresh frozen postmortem brain samples of autistic (gray lines) and non-autistic

controls (black lines) showing a significant increase in the abundance of L-cystathionine

(exact mass 222.0628) in the post vermis and lateral hemispheres of autistic brains.

Figure 3 (B) shows EIC having the retention time of a chemical standard. Figure 3 (C)

shows EIC of brain samples run at the same time as chemical standard, which matched

retention time of chemical standard shown in Figure 3 (B). Figure 3 (D) is a mean plot of

the abundance of L-cystathionine in autistic and control brain samples. An ANOVA on

the difference of the means was statistically significant (p= 0.019). Error bars are

standard error of the mean.

Figure 4 (A) - Figure 4 (D) are extracted ion chromatograms of L-cystathionine

and N-acetylaspartylglutamic acid. Liquid chromatography electrospray ionization time-

of-flight-mass spectrometry (LC-ESI-TOF-MS)/MS fragmentation of L-cystathionine in

brain samples (Figure 4 (A)) and chemical standards (Figure 4 (B)) have matching

fragmentation patterns, confirming the identity of L-cystathionine. The identity of N-

acetylaspartylglutamic acid was confirmed by a matching MS/MS fragmentation pattern

of brain (Figure 4 (C)) and chemical standard (Figure 4 (D)).

Figure 5 (A) - Figure 5 (C) are extracted ion chromatograms (EICs) of 2-

aminooctanoic acid, and Figure 5 (D) is a plot of the relative abundance of 2-

aminooctanoic acid in autistic and control brain samples. Figure 5 (A) shows the results

of metabolomic analysis of fresh frozen postmortem brain samples of autistic (gray lines)

and non-autistic controls (black lines), revealing a significant decrease in the abundance

of 2-aminooctanoic acid (exact mass 159.1253) in the post vermis and lateral

hemispheres of autistic brains, as shown by extracted ion chromatograms (EICs). Figure

5 (B) is an EIC showing the retention time of a chemical standard. Figure 5 (C) are EICs

of brain samples run at same time as chemical standard, which match retention time of

control shown in Figure 5 (B). Figure 5 (D) is a mean plot of the abundance of 2-

aminooctanoic acid in autistic and control brain samples. An ANOVA on the difference

of the means was statistically significant (p= 0.027). Error bars are standard error of the mean.

Figure 6 (A) - Figure 6 (C) are extracted ion chromatograms (EICs) of N-acetylaspartylglutamic acid (NAAG), and Figure 6 (D) is a plot of the relative abundance of N-acetylaspartylglutamic acid in autistic and control brain samples. Figure 6 (A) shows the results of metabolomic analysis of fresh frozen postmortem brain samples of autistic (gray lines) and non-autistic controls (black lines) of N-acetylaspartylglutamic acid (exact mass 304.0909) in the post vermis and lateral hemispheres. Figure 6 (B) is an EIC showing retention times of chemical standards. Figure 6 (C) are EICs of brain samples run at the same time as the chemical standard, which matched retention time shown in Figure 6 (B). Figure 6 (D) is a mean plot of the abundance of N-acetylaspartylglutamic acid in autistic and control brain samples. An ANOVA on the difference of the means was not statistically significant at p less than 0.05 (p= 0.054), but was at a p-value less than 0.1. Error bars are standard error of the mean.

Figure 7 (A) and Figure 7 (B) are extracted ion chromatograms (EICs) of yet to be identified metabolites. Multiple unknown compounds were observed to be significantly different between autistic and control brain samples. The EICs of two such metabolites are shown for molecules with the exact mass of 366.1426 (Figure 7 (A)) and 422.2482 (Figure 7 (B)).

Detailed Description of the Preferred Embodiments

This invention is more particularly described below and the Examples set forth herein are intended as illustrative only, as numerous modifications and variations therein will be apparent to those skilled in the art. As used in the description herein and throughout the claims that follow, the meaning of "a", "an", and "the" includes plural

5   reference unless the context clearly dictates otherwise. The terms used in the specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Some terms have been more specifically defined below to provide additional guidance to the practitioner regarding the description of the invention.

10   Autism remains a poorly-understood disease, particularly with regard to its etiology and the significance of any detectable alteration in brain chemistry to its etiology. A large number of changes in brain chemistry have been detected in autistic brains (all post-mortem), but without any consistent understanding of whether such changes are fundamentally related to development or progression of the disease or

15   produced as a consequence thereof. The metabolomic signatures provided herein, on the other hand, reflect consistent differences in the autistic brains studied and identify particular metabolic pathways consistently associated with the disease.

The metabolome, defined as the total dynamic set of cellular metabolites present

20   in cells or their surrounding matrix, is a product of health or disease/insult states. Metabolomics is particularly sensitive to environmental effects in comparison to other "omic" areas of study, such as genomics and proteomics. Cellular metabolites include but are not limited to sugars, organic acids, amino acids and fatty acids, particularly those

species secreted, excreted or otherwise released from cells (*e.g.*, as the result, *inter alia*, of chemical or physical trauma), that participate in functional mechanisms of cellular response to pathological or chemical insult. Such cellular metabolites, properly appreciated, can serve as biomarkers of disease or toxic response and can be detected in

5   biological fluids (Soga *et al.*, 2006, *J Biol Chem* 281:16768-78; Zhao *et al.*, 2006, *Birth Defects Res A Clin Mol Teratol* 76:230-6). Within the "omics" sciences, it is postulated that metabolomics is the platform that is closest to the phenotype in comparison to other "omics," as it measures the end products of metabolism (Dettmer *et al.*, 2007, *Mass Spectrom*. Rev. 26:51-78).

10   A metabolomic signature (*i.e.*, a population of cellular metabolites) differentially produced by autistic patient biofluids provides a reliable diagnostic marker for detecting autism. Certain aspects of the invention provide assays of cellular metabolites using physical separation methods, including but not limited to liquid chromatography electrospray ionization time-of-flight mass spectrometry (ESI-TOF), for example.

15   Metabolites can be identified using their exact molecular mass, as well as mass spectrometry fragmentation patterns of the metabolites. The sensitivity of applying such methods to detecting cellular metabolites produced by autistic patients provides improved identification of autistic disorders compared with less robust methods in the art, which have focused on psychological, developmental and behavioral assessments rather than

20   metabolic biochemistry. Using a metabolomics diagnostic approach is a more reliable indicator of autistic phenotype versus gene specific diagnostics given the fact that not all changes in gene transcription correlate with a phenotype.

Additional embodiments of the invention include a metabolomic signature unique to autism. In one embodiment, a metabolic signature comprising one or more cellular metabolites, and in a particular embodiment, one or more cellular metabolites of the serotonin, cysteine, tryptophan, methionine, glutamate or GABA metabolic pathways, is provided herein. In certain embodiments, the metabolites comprising a metabolomic signature set forth herein comprise one or more of N-acetylaspartylglutamic acid, L-cystathionine, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, caffeine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate), glutamate, or GABA intermediates. (*e.g.*, metabolites involved in the anabolism or catabolism of GABA such as L-glutamine, L-glutamate, alpha-ketogluratate, succinic acid semialdehyde, 4-aminobutyraldehyde, 4-guanidinobutanoate, or L-ornithine

Methods and reagents for identifying and measuring cellular and particularly biochemical effects of autism, are provided, including methods for diagnosing autism. The term "metabolite," "cellular metabolite" or the plural form, "cellular metabolites," as used herein refers to any molecule or mass feature in the range of about 10 Daltons to about 1500 Daltons secreted by a cell and present in a tissue sample or biofluid. A cellular metabolite can include but is not limited to the following types of molecules: acids, bases, lipids, sugars, glycosides, amines, organic acids, lipids, amino acids, oximes, esters, dipeptides, tripeptides, fatty acids, cholesterols, oxysterols, glycerols, steroids, and/or hormones. In one embodiment, the cellular metabolites can include but are not limited to N-acetylaspartylglutamic acid, L-cystathionine, 2-aminooctanoic acid,

5-hydroxylysine, vinylacetylglycine, proline betaine, caffeine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate), glutamate, or GABA intermediates

5   The phrases "identifying one or a plurality of cellular metabolites . . . differentially produced" and "differentially produces" as used herein include but are not limited to comparisons of cells or tissues from autistic humans with cells or tissues from non-autistic humans. Detection or measurement of variations in metabolite populations or mass features between autistic and non-autistic control samples are included in this
10   definition. In a preferred embodiment, alterations in production of various metabolites are measured by determining a profile of changes in metabolite molecules in autistic versus control samples.

The term "physical separation method" as used herein refers to any method known to those with skill in the art sufficient to detect a profile of changes and
15   differences in metabolites produced in the tissue or biofluid (*e.g.,* lateral cerebellum, and post vermis brain, cerebrospinal fluid, blood, or plasma) of autistic humans according to the methods of this invention. In a preferred embodiment, physical separation methods permit detection of cellular metabolites including but not limited to sugars, organic acids, amino acids, fatty acids, hormones, vitamins, and peptides, as well as ionic fragments
20   thereof and other cellular metabolites (preferably having a molecular weight less than 3000 Daltons, more particularly between 10 and 1500 Daltons, and even more particularly between 100 and 1000 Daltons). In certain embodiments, the physical separation method is liquid chromatography/electrospray ionization time of flight mass

spectrometry (LC/ESI-TOF-MS) and/or hydrophilic interaction chromatography (HILIC), however it will be understood that cellular metabolites as set forth herein can be detected using alternative spectrometry methods or other methods known in the art for analyzing these types of cellular compounds in this size range.

5   Data for statistical analysis can be extracted from chromatograms (*i.e.*, spectra of mass signals) using statistical analysis packages such as Agilent Mass Hunter software (Product No. G3297AA, Agilent Technologies, Inc. Santa Clara, CA); it will be understood that alternative statistical analysis methods can be used instead. Masses are advantageously binned together if they are within 10 ppm and eluted within a 2 minutes

10   retention time window. A binned mass can be considered to be the same molecule across different LC/ESI-TOF-MS analyses (referred to herein as an "exact mass") if the detected masses are within ±10ppm. Binning of the data is required for statistical analysis and comparison of masses across experiments. If multiple peaks with the same mass at the same retention time within a single sample were detected, for example, by Mass Hunter,

15   they can be averaged to assist data analysis. Masses lacking a natural isotopic distribution or with a signal-to-noise ratio of less than 3 are removed from the data prior to analysis. The results from these assays provided relative values that were assessed according to annotated values within 20 ppm and provided a putative identity for the molecular weight detected according to chemical databases. Thus, a mass shift within 20

20   ppm was considered consistent with determining the identity of a specific annotated cellular metabolite known in the art due to differences in ionization source and instrumentation, (*e.g.*, between different experiments or using different instruments).

As used herein, a mass can be considered to be the same across separate LC/ESI-TOF-MS injections using an algorithm that first sorts the data by mass and retention time. After sorting, a compound can be considered unique if it had a retention time difference of less than or equal to three minutes and a mass difference less than or equal to a weighted formula (0.00002 x mass). If a series of measurements from different separations fit this definition said measurements are considered to arise from separation of the same compound. If either the mass or the retention time is found to vary by more than the limits listed above, the mass is considered to be a different compound and given its own unique designation.

Significance tests such as ANOVAs on the log base 2 can be used to transform abundance values of unique compounds present in autistic versus not-autistic samplea at each time point. A randomized complete block design using the ANOVA model including diagnosis, experiments, and a residual term, can be expressed using the following formula:

$$Log_2(abundance_{tb}) = diagnosis_t + section_b + error_{tb}.$$

Missing data are omitted from the test, changing the degrees of freedom (rather than assuming the missing data were absent). This assumption was made because the extensive filtering performed by the Mass Hunter software can miss or filter certain peaks because they are below a certain abundance threshold and not zero. The ANOVA F-test was considered significant if its p-value was less than 0.05. Fold changes were calculated using the least squared means for a given time and treatment.

The terms "metabolic signature" and "biomarker profile" as used herein refer to one or a plurality of metabolites identified by the inventive methods. Metabolic

signatures and biomarker profiles according to the invention can provide a molecular "fingerprint" of autism spectral disorders and identify one or preferably a population of cellular metabolites significantly altered in individuals with autism spectral disorders. In preferred embodiments, metabolic signatures or biomarker profiles can be used to

5   diagnose autism in an individual.

The term "biomarker" as used herein refers to cellular metabolites that exhibit significant alterations between diseased and controls. In preferred embodiments, biomarkers are identified as set forth above, by methods including LC/ESI-TOF-MS. Metabolomic biomarkers were identified by their unique molecular mass and

10   consistency, thus the actual identity of the underlying compound that corresponds to the biomarker is not required for the practice of this invention. Alternatively, certain biomarkers can be identified by, *for example*, gene expression analysis, including real-time PCR, RT-PCR, Northern analysis, and *in situ* hybridization, but these will not generally fall within the definition of the term "cellular metabolites" as set forth herein.

15   Metabolite profiling as set forth herein was conducted on postmortem tissue as opposed to samples collected from live patients. Ultimately, biomarkers discovered *in vivo* are expected to be useful for analyzing samples such as biofluids including for example, cerebrospinal fluid, blood, plasma, amniotic fluid and urine, *i.e.*, complex mixtures of extracellular biomolecules. This inventive methods are advantageous over

20   invasive procedures such as tissue biopsies because metabolites in biofluids can be detected non-invasively (in contrast to intracellular compounds). In addition, processing cellular supernatant for mass spectrometry is more robust and less laborious than cellular extracts. However, cellular extracts from, for example, tissue biopsies or lysed cells, are

encompassed in the methods of the invention. The terms "samples" or "biosamples" or "patient samples" include but are not limited to cerebrospinal fluid, brain tissue, amniotic fluid, blood, or plasma.

The identification of differential metabolites in the organ that is primarily affected

5   in autism (brain) prior to biofluid analysis provides a targeted approach towards the development of novel diagnostics. Identifying specific biomarkers for autism in biofluids, such as cerebrospinal fluid, urine or plasma, without a defined set of candidates, would be particularly arduous. Biofluids are complex mixtures of systemic byproducts influenced by both endogenous and exogenous factors (genetics, diet, the environment).

10   Using the methods of the current invention, specific analytical chemistry protocols can be used for assessing biofluids for defined exact mass and retention times of specific candidate biomarkers.

As set forth in more detail in the Examples below, analysis of fifteen fresh frozen samples from the Autism Tissue Program (ATP) (six autism and nine controls) using the

15   methods described herein detected statistically-significant differences in the abundance of multiple metabolites between postmortem autistic and non-autistic brain samples. Preliminary experiments using hydrophilic interaction chromatography (HILIC) followed by positive mode ESI-TOF ionization identified a total of 98 metabolites that were differentially produced between autistic and non-autistic brains. For example, 5-

20   hydroxylysine (exact mass 162.0981) displayed significant changes ($p<0.05$). Parallel preliminary experiments using C18 chromatography followed by positive mode ESI-TOF ionization, generated 47 statistically significant metabolites ($p <0.05$). The chemical

identity of these metabolites can be definitively confirmed by tandem mass spectrometry (MS-MS).

Metabolites from fresh frozen postmortem brain were initially separated by liquid chromatography (LC) and then ionized and detected by electrospray ionization time of

5   flight mass spectrometry (ESI-TOF-MS). This mass spectrometry modality was chosen because it is particularly suited for metabolomics of fresh frozen brain tissue: it is highly sensitive, detecting small molecule metabolites at very low abundances (*e.g.,* micromolar to picomolar concentrations) and providing highly accurate measurements of the exact mass of metabolites with a resolution of 3 ppm. Other analytical chemistry platforms

10   employed in metabolomics, such as NMR (nuclear magnetic resonance) are less sensitive than mass spectrometry, requiring larger amounts of biological samples to detect metabolites at significantly higher concentrations (reviewed in Glish *et al.,* 2003, *Nat. Rev. Drug Discov.* 2:140-150). As set forth herein, metabolomics analysis of postmortem brain samples revealed a plurality of biochemical differences between

15   autistic and non-autistic brains. The identification of cellular metabolites differentially secreted by autistic brain provide biomarkers for diagnosing autism, as set forth with particularity herein.

In certain embodiments, the invention provides the following metabolites, taken alone, as a population, or in any informative combination, as biomarkers of autism: N-

20   acetylaspartylglutamic acid, L-cystathionine, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, caffeine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-

phosphatidylcholine, SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate), glutamate, or GABA intermediates.

Abnormalities in neurochemical pathways have been hypothesized as a potential basis for the pathogenesis and/or clinical manifestation of autism. It has been speculated

5   with varying amounts of supporting, generally anecdotal evidence that perturbations to serotonin, kynurenine, glutamate, cysteine, and methionine metabolism may play a role in autism. The methods provided herein and the biomarkers provided according to the practice of these methods in contrast, provides an experimental evidentiary basis for associating detection of one or a plurality of said biomarkers with the existence of autism

10   or an autism spectrum disorder in an individual.

Examples

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and

15   are not to be taken as limiting the invention.

Example 1

Preparation of Postmortem Brain Tissue

20   Postmortem brain tissue from autistic patients and age-matched controls was obtained from the Autism Tissue Program (ATP). Altogether, 6 autistic and 9 non-autistic postmortem brain samples from the University of Maryland Brain and Tissue Bank for Developmental Disorders were studied (*See* Table 1). Metabolomics was performed with fresh frozen sections of the lateral cerebellum and post vermis from the

25   above-mentioned samples. The cerebellum was selected because it is a region where

abnormal neuroanatomical findings most consistent in autistic patients are localized

(Kemper *et al.,* 1998, *J. Neuropathol. Exp. Neurol.* 57:645-652). Differences in age,

PMI (postmortem interval) and degree of clinical manifestation were acknowledged to be

a source of variability in the abundance and nature of metabolites measured by

5   metabolomics.

       Prior to metabolomics, frozen brain specimens were mounted on the pre-cooled

stage of a Leica SM200R microtome using 30% sucrose and sectioned to create uniform

samples weighing ~50 mg. Metabolites were extracted in 80% methanol/20% 0.5%

formic acid on a volume per wet tissue weight ratio by homogenizing the sample in a

10   conical glass homogenizer. The samples were then stored at -20° C for 30 minutes to

precipitate proteins and insoluble material. Thereafter, the cold incubation samples were

centrifuged at 16,000 x g for 25 minutes at 4° C to pellet cell debris, proteins, and other

insoluble material. Supernatent was then transferred to a new tube, and re-extracted with

300 µl of extraction buffer. Samples were dried in a rotary evaporator for 6-7 hours until

15   dry to remove the extraction buffer. Extracts were then dissolved in 500 µl of 20%

acetonitrile/80% 0.1% formic acid and centrifuged through a Millipore 3kDa Microcon

column (Millipore) for three hours at 13,000 x g to remove large molecular weight

(>3kDa) biomolecules. Flowthrough was dried in a rotary evaporator and dissolved in 2

µl/mg 95% 0.1% formic acid/5% acetonitrile and stored at -80° C prior to analysis by

20   LC-MS.

       Liquid chromatography electrospray-ionization time-of-flight mass spectrometry

(LC-ESI-TOF-MS) was performed on these extracts using an Agilent QTOF LC/MS

system consisting of a G6520AA QTOF high resolution mass spectrometer capable of

exact mass MS and MS/MS ion fragmentation. The polar metabolite fraction of each

sample was analyzed using two different chromatographic methods for maximal

separation and small molecule resolution: a) 3 x 100mm Phenomenex 3μm Luna

hydrophilic interaction chromatography (HILIC) and b) 2.1 x 50mm Zorbax 1.8 μm C18-

5   SB columns (C18). This approach maximized the number of metabolites that were

separated and subsequently ionized and measured. Ultra high pressure liquid

chromatography (UPLC) analysis was performed with the Zorbax C18 column using a 15

minute gradient from 5% acetonitrile/95% water/0.1% formic acid to 100%

acetonitrile/0.1% formic acid at a flow rate of 400 μl/min. The HILIC HPLC method was

10   performed using a 16 minute gradient from 95% acetonitrile/5% water/0.1% formic acid

to 60% acetonitrile/40% water/0.1% formic acid with a 500 μl/min flow rate.

Electrospray ionization was employed using a dual ESI source, with an Agilent isocratic

pump continuously delivering an internal mass reference solution into the source at

approx. 0.01 ml/min. The mass range of the instrument was set at 75-1500 Da and data

15   were acquired in 2 GHz mode to maximize dynamic range. Data acquisition was

performed with Agilent MassHunter version B.02.00 using high-resolution exact mass

conditions.

20

Example 2

Identification of Metabolites Differentially Produced in Autistic Brain

Prior to data analysis, total ion chromatogram (TIC) of each sample was carefully inspected for quality and reproducibility of the MS signal. For those samples wherein the TIC abundance deviated by more than 25% from the median across the LC-MS gradient, LC-MS analysis was repeated (Figure 1). Data was deisotoped and then converted into the open source mzData format.

Data analysis was performed using the open source statistical programming and analysis software R. The XCMS package (Smith *et al.,* 2006, Anal., Chem. 78:779-787) was used to analyze the LC-ESI-TOF-MS resulting files using the Centwave algorithm for peak peaking (Tautenhahn *et al.,* 2008, BMC Bioinf. 9:504). Retention time deviations across LC-MS samples were corrected using rector (retention time correction) loess regression of features common to all LC-MS samples and the features grouped using the density based functions in XCMS. The maximum observed deviation for HILIC chromatography was 15 seconds and 4 seconds for C18 chromatography. After the grouping function was performed features missing in LC-MS samples were iteratively integrated using m/z and retention time windows based on the range of the feature group. The peak intensity tables were evaluated using both univariate and multivariate statistical analysis. Contaminant ions were removed by comparing brain tissue extracts to mock extraction blanks. Features present in both the brain tissue extracts and the mock extraction blanks were removed from the data set if their abundance was less than five-fold greater than the extraction blank. Statistical significance of individual mass features was performed under the null hypothesis that no difference in their abundance existed between control and autistic post mortem brains. Differential metabolites, or features, were determined using an incomplete block design ANOVA with the model

log2(abundance) ~ section + diagnosis on the combined brain regions. Features were

considered statistically significant if they exhibited a fold change greater than 25% and a

p value less than 0.05 in the diagnosis factor of the ANOVA model. The extracted ion

chromatogram of each statistically significant feature was further evaluated to confirm an

5   observable difference between autistic and normal extracts to reduce the inclusion of

spurious results.

Direct injections were analyzed using the Continuous Wavelet Transform (Du *et al.*, 2006, *Bioinformatics,* 22:2059-2065) peak picking algorithm in XCMS. Agilent Mass

Qualitative Analysis Software Version 2.00B was used to convert vendor specific raw

10   data files into open source mzData files. Features with a height of equal to or greater

than 1000 were included for analysis and then deisotoped prior to identifying mass

features. The generation of mass features (putative metabolites) was implemented using

methods present in the *XCMS* (Smith *et al., 2006, Anal., Chem.* 78:779-787) library from

R/Bioconductor. Each ESI polarity was processed independently. The centwave

15   algorithm (Tautenhahn *et al.,* 2008, BMC Bioinf. 9:504) designed for the detection of

peaks in high resolution high mass accuracy data was utilized for peak picking. The

input variables for the centwave algorithm were based on QTOF settings and the

chromatography method. Following peak picking deviations in retention times were

corrected using loess regression. Mass feature bins or groups were generated using the

20   density based grouping algorithm using parameters optimized for LC-MS gradients.

After data had been grouped into mass features, missing features were integrated based

on retention time and mass range of a feature using the iterative peak filling method in

XCMS.

21

Binning of mass features detected in individual LC-MS was used to identify features that were present at the same retention time across injections. A binning algorithm based on both exact mass and retention time was used to consider a mass feature to be the same across different LC/ESI-MS-QTOF experiments. Binning criteria

5   were based on both a sliding mass difference scale that allowed for larger mass differences at lower molecular weights and a constant retention time window based on the reproducibility of the chromatography.

Statistical significance testing of individual mass features was performed under the null hypothesis that no difference in their abundance existed between control and

10   autistic vermis samples using a permutation based test statistic. This analysis was performed by exploring different combinations of metadata in the loading and scores plots using the pcaMethods library in R (Stacklies *et al.*, 2007. PcaMethods: A collection of PCA methods. R package version 1.12.0. Available from CRAN.R-project.org/). General differences between control and autism postmortem brain samples were

15   evaluated using Welch t-tests. Bayesian principal component analysis was performed because this method can tolerate missing data (Oba *et al.*, 2003, *Bioinformatics*, 19:2088-2096) when determining whether global changes in metabolism existed between control and autistic brain tissue. This analysis was performed by exploring different combinations of metadata in the loading and scores plots using the pcaMethods library in

20   R (Stacklies *et al.*, 2007. Pca Methods: A collection of PCA methods. R package version 1.12.0. Available from CRAN.R-project.org/).

Statistically significant features were also modeled using Principal Cluster Analysis (PCA)-based methods and hierarchical clustering to examine their ability to

classify the samples in an unsupervised manner. As a result, a comprehensive list of low

metabolites that were statistically significantly different between postmortem autistic

brains and non-autistic controls were identified following statistical analysis of an

average of 6,000 features per experiment. Mass spectrometry based metabolomic

profiling, or metabolomics, revealed significant changes (p-value < 0.05) in autistic

brains in 98 metabolites using HILIC and 47 metabolites using C18 chromatography.

Table 1: Autism Tissue Program (ATP) approved postmortem samples subject to LC-ESI-TOF-MS metabolomics for identifying metabolites and pathways that are statistically significantly altered in autistic brain. UMB# represents archival sample number.

UMB#	Frozen							
	Vermis	Lateral Hem	Diagnosis	Sex	Age Yrs	Days	Age	PMI (Hours)
1182	+	+	Autism	F	9	354	9.97	24
1185	+	+	Control	M	4	258	4.71	17
1349	+	+	Autism	M	5	220	5.60	39
1407	+	-	Control	F	9	46	9.13	20
1500	+	+	Control	M	6	320	6.88	18
1541	+	+	Control	F	20	228	20.62	19
1638	+	+	Autism	F	20	277	20.76	50
1674	+	+	Control	M	8	339	8.93	36
1706	+	+	Control	F	8	214	8.59	20
1708	+	+	Control	F	8	50	8.14	20
1860	-	+	Control	M	8	2	8.01	5
4231	+	+	Autism	M	8	300	8.82	12
4671	+	+	Autism	F	4	165	4.45	13
4721	+	+	Autism	M	8	304	8.83	16
4898	+	+	Control	M	7	272	7.75	12
15	14	14	Autism = 6	8 Male				
			Control = 9	7 Female			9.19	18.56

Example 3

Chemical Annotation of Autism-Specific Metabolites

Following identification of metabolites differentially secreted in autistic cerebelli as described in Example 2, chemical annotation of these molecules was further refined by ion fragmentation pattern analysis (MS-MS). Specifically, fragmentation patterns and retention times of each small molecule identified above were compared to analytical

5   grade chemical standards. While confirmation of the chemical identity of each small molecule is not required for autistic biomarker determination, annotated identity is advantageous in that it provides more uniform nomenclature for each small molecule biomarker. Subsequent chemical identification of the differentially produced metabolites also permits *in silico* mapping of metabolites onto specific pathways via bioinformatics

10   (GeneGo software). This information thus revealed systematic metabolic pathways and/or networks that underlie biochemical differences between postmortem brains of autistic and non-autistic age matched controls.

The neutral exact mass of each compound was queried against the public searchable databases METLIN (metlin.scripps.edu), The Human Metabolome Database

15   (www.hmdb.ca), and the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/), or the Biological Magnetic Resonance Bank (www.bmrb.wisc.edu/metabolomics/) for candidate identities. Measured mass features are considered to match a metabolite present in the databases if their exact masses were within 20 parts per million of the annotated database molecule (0.00002 x mass).

20   The chemical identity of the biomarker candidates was confirmed using one or more of the following three criteria: (1) molecular exact mass, (2) MS/MS fragmentation pattern and (3) chromatographic retention time. The exact mass of each candidate biomarker (*i.e.*, mass feature), as determined by interpretation of electrospray ionization time of

flight mass spectrometry (LC-ESI-TOF MS/MS) (*i.e.*, tandem mass spectrometry) data

was queried against the various public searchable databases set forth above for putative

molecular identities. Annotated candidates were then validated by acquiring MS/MS

product ion spectra for the candidate molecular ion, then matching the MS/MS

5 fragmentation pattern for product ion spectra obtained for a known analytical grade

standard reference compound (Cummings *et al.*, 2009, *J Chromatogr B Analyt Technol*

Biomed Life Sci., 877:1221).

Analytical grade chemical standards for L-cystathionine, 2-aminooctanoic acid

and N-acetylaspartylglutamic acid were purchased from Sigma Chemical Co., St. Louis,

10 MO for comparative mass spectrometry. Chemical references were evaluated using

identical chromatographic methods used in the metabolomic analysis of the original

samples. Additionally, seven of the original samples (4 autistic and 3 control) were re-

analyzed for comparison with the chemical standards, to ensure retention time and ion

fragmentation match. Chemical references were dissolved in appropriate buffers and 100

15 μM standards were prepared by diluting into 95% 0.1% formic acid/ 5% acetonitrile. The

experimental samples and chemical standards were fragmented using the following

formula to determine collision energy : Collision Energy = (m/z of precursor-

100)/100*3V + 10V. A putative annotation was considered correct if the retention time

and fragmentation pattern in the original sample matched the chemical references. If the

20 putative metabolite was insufficiently abundant in the sample extract to be fragmented,

the retention time alone was used to confirm annotation.

The third confirmation criterion was established by co-injecting (*i.e.*, spiking)

endogenous metabolites with a known reference compound to determine whether the

chromatographic retention time and ionization fragmentation patterns (Figure 2) of the

chemical standard matched the retention time of the biomarker candidate.   Tandem mass

spectrometry, or MS-MS, was used to confirm the identity of an endogenous metabolite

in these experiments. After data analysis was completed, a subset of statistically

5   significant metabolites was selected for confirmation against known chemical standards.

In these assays, fresh frozen brain extracts used to identify mass features as

discussed above were spiked with 1 mM of the chemical standards and then analyzed by

MS-MS. Remaining unused samples or spiked samples were processed by LC-ESI-

MS/MS to confirm the structure and abundance of the ions of the putatively-identified

10   compounds. Standards used as controls in these experiments had known retention times.

Parallel retention times observed in spiked samples provided additional confirmation of

metabolite identity. Final confirmation of chemical identity of candidate biomarkers was

made upon both detection of fragment ions (Figure 2, A) and retention time (Figure 2, B).

Classification of hits was based on a "shared peak count" method that identified masses

15   of fragment ions shared between the analytical (fresh frozen brain extract) and chemical

reference MS/MS spectra.

Statistically significant differences were detected in the abundance of multiple

metabolites between postmortem autistic and non-autistic brain samples (Table 2). To

focus on meatbolites that have a high probability of being significant, and to eliminate

20   potential spurious artifacts, each data set (HILIC and C18 chromatography) was filtered

using a moderate filter of a 25% fold change in abundance between control and autistic

brain samples. HILIC chromatography followed by positive mode ESI-QTOF ionization

identified 988 mass features exhibiting a fold change greater than 25%, of which 98

metabolites were statistically significantly different (p<0.05) between autistic and non-autistic brains. Additionally, C18 chromatography followed by positive mode ESI-QTOF ionization measured 938 mass features with at least a 25% fold change, generating 47 statistically significant metabolites (p <0.05). Table 2 shows a subset of the measured

5   significant metabolites that have at least a 25% change in abundance between autistic and control postmortem brain samples with a corresponding extracted ion chromatogram (EIC) displaying a marked difference between autistic and control brain samples. One of the most challenging aspects of metabolomics studies is that many of the small molecules measured are yet not annotated in public databases. While current methods allow the

10   identification of small molecule metabolites by unique mass, the identification by compound name is more challenging. The metabolites identified represent novel biomarkers, and importantly point to additional pathways contributing to the etiology of autism.

15   Table 2: Metabolites that exhibited significantly different levels between postmortem autistic and non-autistic brain samples.

Exact Neutral Mass	Retention time (minutes)	Alteration in autistic brain samples	Putative Annotation	Biochemical Pathway	p-value
C18 Chromatography					
136.5265	1.48	↑	Unspecified		0.049
143.0939	0.33	↓	Vinylacetylglycine / Proline Betaine	Homocysteine	0.01
159.1253	2.72	↓	DL-2-Aminooctanoic acid (Confirmed)		0.02
173.0308	0.33	↓	Unspecified		0.04
180.0640	0.93	↓	Unspecified		0.0005
194.0805	3.62	↓	Caffeine		0.034
279.1636	0.4	↓	Unspecified		0.03
286.1751	2.06	↑	Unspecified		0.012

321.0561	1.5	↑	Unspecified		0.02
356.2055	3.16	↑	Unspecified		0.05
363.1455	1.33	↑	Unspecified		0.05
366.1421	0.58	↑	Unspecified		0.016
382.8678	1.22	↑	Unspecified		0.035
408.2181	5.58	↓	Unspecified		0.029
422.2482	4.83	↑	Unspecified		0.023
426.1936	4.67	↑	Unspecified		0.039
531.2841	1.14	↑	Unspecified		0.049
597.2905	6.98	↓	Unspecified		0.012
599.3340	1.07	↑	Unspecified		0.032
632.2266	0.56	↑	3-carboxy-1-hydroxypropylthiamine diphosphate / 3'-Sialyllactosamine		0.037
806.0834	5.18	↓	Unspecified		0.027
823.0550	6.85	↑	Unspecified		0.016
823.7238	6.85	↑	Unspecified		0.034
HILIC Chromatography					
87.0139	8.74	↑	3,4-Dihydroxybenzylamine		0.02
133.0168	8.74	↑	Unspecified		0.008
133.0195	8.98	↑	Unspecified		0.016
139.0609	1.33	↓	Unspecified		0.03
143.0922	2.55	↓	Unspecified		0.049
162.0981	9.37	↑	5-hydroxylysine		0.008
222.0628	8.74	↑	L-Cystathionine (Confirmed)	Tryptophan	0.019
241.1764	5.76	↓	Unspecified		0.019
255.0853	5.16	↑	Unspecified		0.039
304.0909	1.23	↑	N-Acetylaspartylglutamic acid (Confirmed)	Neuropeptide	0.054
309.9921	5.68	↓	Unspecified		0.046
317.1924	5.87	↑	Unspecified		0.002
364.0987	4.07	↑	Unspecified		0.042
418.0277	3.37	↑	Unspecified		0.018
430.1120	7.66	↑	Unspecified		0.047
438.0307	2.58	↑	Unspecified		0.013
454.0045	2.6	↑	Unspecified		0.02
454.0692	4.04	↑	SAICAR		0.042
464.0335	3.36	↑	Unspecified		0.018
473.2109	4.06	↑	Unspecified		0.025
501.9891	3.34	↑	Unspecified		0.012
517.1560	4.09	↑	Unspecified		0.027

522.0543	4.02	↑	Unspecified		0.012
527.0686	2.63	↑	3-carboxy-1-hydroxypropylthiamine diphosphate		0.014
538.0278	3.96	↑	Unspecified		0.007
543.0427	2.89	↑	Unspecified		0.017
547.9951	3.41	↑	Unspecified		0.008
568.0602	4.03	↑	Unspecified		0.004
585.1485	4.10	↑	Unspecified		0.035
590.0422	4.02	↑	Unspecified		0.001
616.9752	2.61	↑	Unspecified		0.015
636.0475	4.07	↑	Unspecified		0.032
645.1668	7.67	↑	Unspecified		0.041
658.0298	4.06	↑	Unspecified		0.031
668.0565	2.79	↑	Unspecified		0.044
674.0040	4.01	↑	Unspecified		0.015
733.5650	0.79	↓	Dipalmitoyl-phosphatidylcholine		0.044
811.0869	4.15	↑	Unspecified		0.038
860.2212	7.68	↑	Unspecified		0.042
882.1976	7.67	↑	Unspecified		0.049

Chemical Annotation of Differentially Secreted Metabolites

Chemical annotations were confirmed for the following subset of differentially secreted metabolites.

L-cystathionine

Several metabolites in the cysteine and methionine metabolic pathway were significantly elevated in autistic brains in comparison to controls. Using HILIC chromatography, a statistically significant difference was detected for L-cystathionine (exact mass 222.067) in both the post vermis and lateral cerebellum of autistic brain samples (p=0.019, Figure 3(A) and 3(D)). The chemical identity of L-cystathionine was secondarily confirmed by comparison of the retention time (Figure 3(B) and 3(C)) and

ion fragmentation pattern between the postmortem brain samples (Figure 4(A)) and the chemical standard (Figure 4(B)).

2-Aminooctanoic Acid

5   In contrast to L-cystathionine , the abundance of the metabolite 2-aminooctanoic acid (exact mass 159.125) was significantly reduced in both the post vermis and lateral cerebellum of autistic patients (p=0.027, Figure 5(A) and 5(D)). The global brain decrease in 2-aminooctanoic acid was 144% between autistic and control samples. 2-aminooctanoic acid has not been previously studied in brain samples, and thus the first

10   evidence that alterations in 2-aminooctanoic acid concentration is related to autism is provided herein.   Previous studies have only reported and measured this compound in urine (Parry *et al.*, 1957, *Clinica Chimica Acta*, 2:115-125). Comparative mass spectrometry between the chemical standard (Figure 5(B)) and postmortem brain samples (Figure 5(C)) demonstrated a match in metabolite retention time. The low abundance

15   range of 2-aminooctanioc acid in postmortem brain samples was an impediment to obtaining its ion fragmentation according to the standard MS-MS method employed for other metabolites in this study.

N-Acetylaspartylglutamic Acid (NAAG)

         The abundance of N-acetylaspartylglutamic acid (exact mass 304.091) was

20   elevated in post-mortem autistic brains in comparison to normal controls. These results are consistent with previously reported perturbations in the glutamate pathway for a human *in vitro* model of autism (Cezar *et al.*, 2007, Stem Cells Dev. 16:869-882), as well as other clinical studies (Blaylock *et al.*, 2009, *Curr. Med. Chem.* 16:157-170;

Friedman *et al.*, 2006, *Arch. Gen. Psychiatry*, 63:786-794; Kleinhans *et al.*, 2007, *Brain Res.* 1162, 85-97; Pardo *et al.*, 2007, *Brain Pathol.* 17:434-447; Shinohe *et al.*, 2006, *Prog. Neuro-Psychopharmacol. Biol. Psychiatry*, 30:1472-1477). However, unlike previous reports, the results set forth herein show a previously unappreciated direct

5   perturbation to the glutamate metabolism intermediate, N-acetylaspartylglutamic acid. Showing statistical significance ($p=0.054$, Figure 6(A) and 6(D)), the chemical identity of N-acetylaspartylglutamic acid was confirmed by comparative mass spectrometry, revealing both a retention time (Figure 6(B) and 6(C)) and ion fragmentation pattern between the postmortem brain samples (Figure 4(C)) and the chemical standard (Figure

10   4(D)).

<u>Unspecified Compounds</u>

In addition to those metabolites described above, the metabolomics analyses described herein identified alterations in multiple non-annotated metabolites (*i.e.*, not

15   annotated in public databases). Abundance of these metabolites was significantly increased or decreased in autistic brain samples as compared to controls ($p<0.05$). The extracted ion chromatograms of two such unspecified small molecule metabolites are shown in Figure 7(A) and 7(B) (exact masses 366.1426 and 422.2482, respectively). Both of these non-annotated endogenous metabolites were significantly increased in autistic

20   brain regions. Feature A (exact mass 366.1426) had a 42% increase in abundance between autistic and control brain samples. The abundance of feature B (exact mass 422.2482) was increased 181% in autistic brain samples. Lack of current annotation of these metabolites in public databases does not exclude them as candidate diagnostic

biomarkers for autism. The highly sensitive, quantitative nature of the analytical

chemistry detection system employed herein provides a measurable endpoint (*i.e.*, exact

mass), which can be measured in patient samples (*e.g.*, tissues or biofluids) from autistic

patients. In addition, other analytical platforms, sch as NMR, can be used to confirm the

5   chemical formula of these compounds. An absence in chemical annotation merely

prevents mapping of the molecules onto a specific biochemical pathway.

         In summary, statistically significant differences were detected in the abundance of

multiple metabolites ranging in size from about 10 to about 1500 Daltons between

postmortem autistic and non-autistic brain samples (Table 2). HILIC chromatography

10   followed by positive mode ESI-QTOF ionization identified a total of 98 metbolites that

were statistically significantly different ($p<0.05$) between autistic brain and non-autistic

controls. Additionally, C18 chromatography followed by positive mode ESI-QTOF

ionization, generated 47 statistically significant metabolites ($p <0.05$). This

comprehensive signature provides immediate means for translational approaches to

15   examine if perturbations to these endogenous chemicals are also measured in a

quantitative manner in biofluids.

Example 4

Identification and Assessment of Metabolites Secreted in Patient Biofluids

20   While the assessment of brain tissue, the organ primarily affected by autism,

permits the identification of autistic biomarkers as described herein, the inventive

methods are not limited to the examination of brain tissue. Analyzing patient samples

such as biofluids, including for example, cerebrospinal fluid, blood, plasma, amniotic

fluid and urine, *i.e.*, complex mixtures of extracellular biomolecules will also enable the identification and assessment of differentially secreted metabolites. This method is advantageous over invasive procedures such as tissue biopsies because small molecules present in biofluids can be detected non-invasively (in contrast to intracellular

5   compounds). In addition, processing cellular supernatant for mass spectrometry is more robust and less laborious than cellular extracts. Cellular extracts from, for example, tissue biopsies or lysed cells, are encompassed in the methods of the invention.

Furthermore, one of skill would appreciate the secretion of brain metabolites into surrounding cerebrospinal fluid. In turn, the collection and assessment of cerebrospinal

10   fluid by the described methods (i.e., metabolomics analysis) will permit the analysis and identification of differentially expressed metabolites. Using the methods of the current invention, specific analytical chemistry protocols can be used for assessing cerebrospinal fluid and other biofluids for defined exact mass and retention times of specific candidate biomarkers.

15   Thus, in these aspects of the inventive methods a biofluid is obtained, such as cerebrospinal fluid, using conventional techniques (e.g., lumbar puncture) and the biofluid processed as set forth herein to remove proteins and other biological materials having a molecular weight greater than about 3000 Daltons. Separation methods, for example LC-TOF mass spectrometry, are applied to the processes biofluid and

20   metabolites detected therein. Detection of one or a plurality of differentially produced metabolites characteristic for autism is used to identify a subject having or at risk for autism, and to indicate that further behavioral and other diagnostic methods are indicated.

Similarly, one of skill would appreciate the secretion of brain small metabolites

across the blood-brain barrier. Metabolomic analysis of blood plasma has been

performed on blood samples obtained from mammals as described: Thaw plasma and

add 450 ul of ice cold MeOH:H2O (8:1 v/v) and agitate for 10 minutes at 2-8 C. Then

centrifuge the samples at 18,400 x g for 20 minutes at 4 C and transfer the supernatant to

5   a tube and repeat steps 3 and 4 until no pellet is observed. Dry samples and then re-

solublize in 50 ul of 50:50 ACN: 0.1% formic acid for subsequent metabolic analysis. In

summary, the inventive methods are applied to biofluids including cerebrospinal fluid

and plasma as two examples.

10   **Example 5**

Identification of Biochemical and Metabolic Pathways Altered in Autistic Brain

        Statistically significant metabolites whose chemical identity was confirmed as

described above were compared and mapped *in silico* to the Kyoto Encyclopedia of

Genes and Genomes (KEGG) pathway database. A Fisher's exact test was used find

15   biochemical pathways that were statistically overrepresented. Groupings of features that

show a common trend across samples also were evaluated using the KEGG database to

determine if any biochemical interactions exist between the molecules that are not

represented on a canonical KEGG pathway. This approach allowed mechanistic

pathways that underlie metabolic changes observed *in vivo* to be identified. Mapping

20   differential metabolomic profiles to their respective biochemical pathways as outlined in

the Kyoto Encyclopedia of Genes and Genomes (KEGG, release 41.1,

www.genome.jp/kegg) revealed specific metabolic pathways that were upregulated or

downregulated in the cerebellum of autistic subjects in comparison to non-autistic

controls. Significant changes to glutamate, cysteine, methionine, tryptophan and GABA metabolic pathways were detected in autistic brains in comparison to non-autistic controls.

The results of the metabolomic methods set forth herein measured simultaneous

5   changes in multiple biochemical pathways and networks, providing a quantitative *de facto* biochemical signature of specific autistic brain regions. Comparative metabolomics between postmortem brains of autistic patients and non-autistic controls revealed robust biomarkers as well as biochemical changes that provide means for the early diagnosis of autism. The differentially secreted metabolites provided herein comprise several

10   candidate diagnostic biomarkers for autism.

The invention is not intended to be limited to the disclosed embodiments of the invention. It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the

15   appended claims.

CLAIMS

1.   A method for identifying a human having a metabolic signature characteristic for autism, the method comprising the steps of:

    a.   isolating a biosample comprising cerebrospinal fluid, brain tissue, blood, serum, or plasma from an individual; and

    b.   assaying the biosample for a plurality of cellular metabolites wherein the cellular metabolites are N-acetylaspartylglutamic acid, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, 3-carboxy-l-hydroxypropylthiamine diphosphate, 3'-sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, or SAICAR ((S)-2-[5-Amino-l-(5-phospho-D-ribosyl) imidazole-4-carboxamido]succinate),

        wherein step (b) further comprises physically separating the plurality of cellular metabolites that are present in the biosample,

        wherein the individual is identified as having a-metabolic signature characteristic of autism when a plurality of the cellular metabolites in the individual's biosample are differentially produced compared to the plurality of cellular metabolites in non-autistic biosamples.

2.   The method of claim 1, wherein the physical separation is by liquid chromatography electrospray-ionization time of flight mass spectrometry.

3.   A method for diagnosing autism in a human fetus in utero, the method comprising the steps of:

    a.   assaying a fetal sample for a plurality of cellular metabolites wherein the cellular metabolites are N-acetylaspartylglutamic acid, 2-aminooctanoic acid, 5-hydroxylysine, vinylacetylglycine, proline betaine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-

Sialyllactosamine, 3,4-dihydroxybenzylamine, dipalmitoyl-phosphatidylcholine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate);

    b.   detecting the plurality of cellular metabolites; and

    c.   diagnosing the fetus with autism when a metabolic signature for autism is detected in the fetal sample,

wherein the signature comprises a significant increase in production of any of N-acetylaspartylglutamic acid, 5-hydroxylysine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-Sialyllactosamine, 3,4-dihydroxybenzylamine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate) and a significant decrease in production of any of 2-aminooctanoic acid, vinylacetylglycine, proline betaine, or dipalmitoyl-phosphatidylcholine.

4.   The method of claim 1 or claim 2, wherein the metabolic signature characteristic of autism comprises a significant increase in production of any of N-acetylaspartylglutamic acid, 5-hydroxylysine, 3-carboxy-1-hydroxypropylthiamine diphosphate, 3'-Sialyllactosamine, 3,4-dihydroxybenzylamine, or SAICAR ((S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate) and a significant decrease in production of any of 2-aminooctanoic acid, vinylacetylglycine, proline betaine, or dipalmitoyl-phosphatidylcholine.

Wisconsin Alumni Research Foundation
and
Stemina Biomarker Discovery, Inc.
Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

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FIG. 1A

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FIG. 1B

AMENDED SHEET

Counts vs. Mass-to-Charge (m/z)

FIG. 2A

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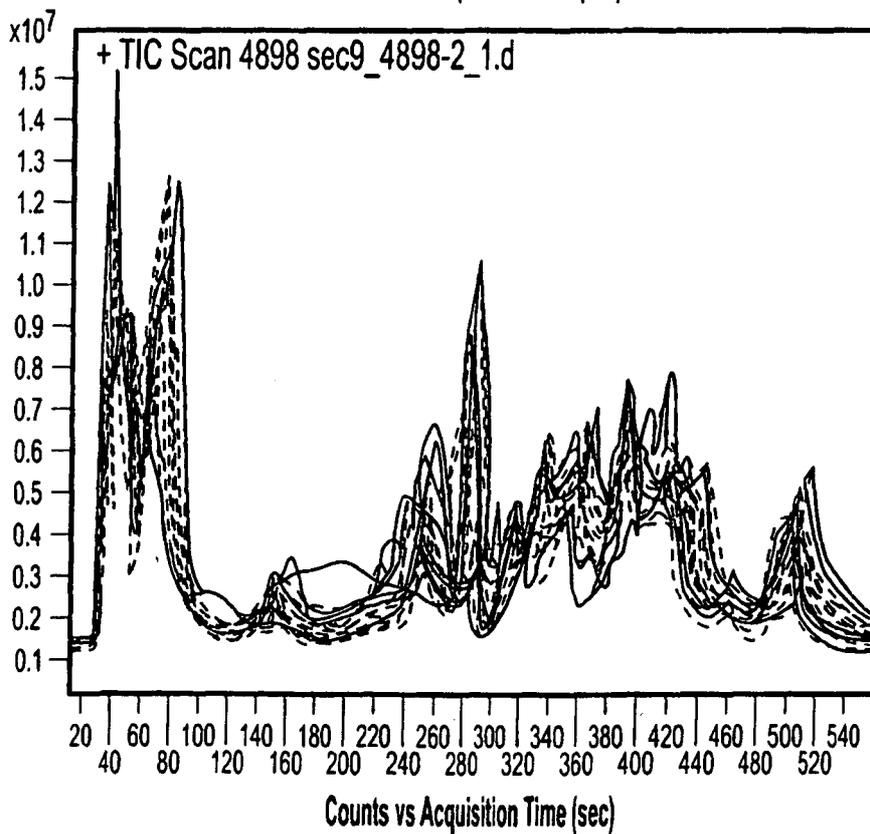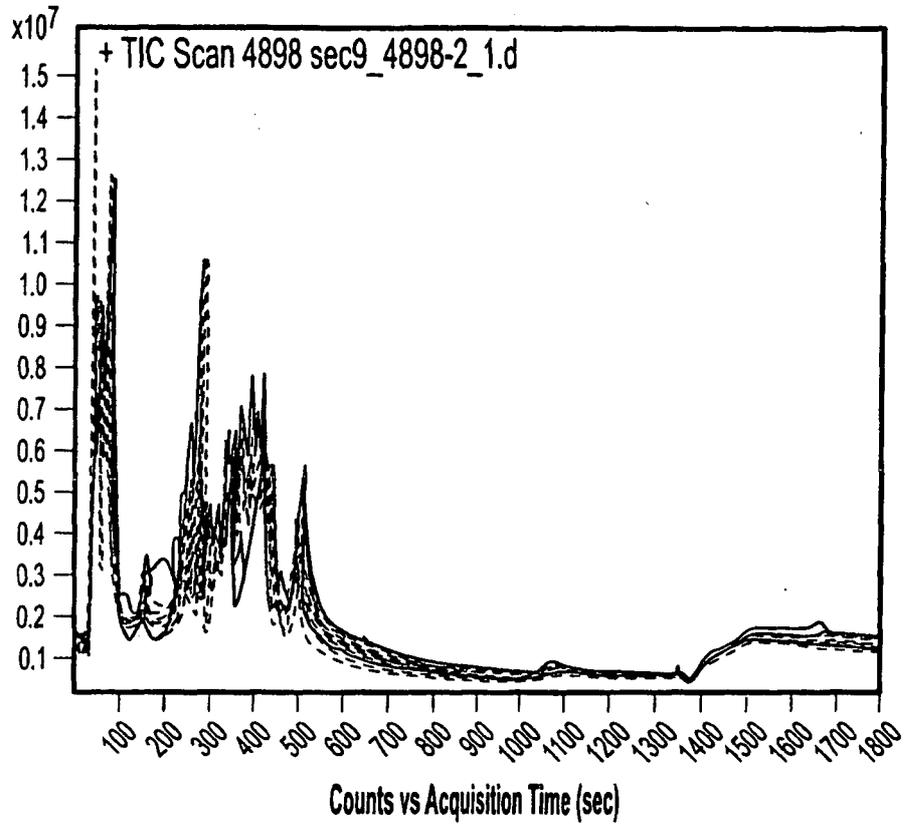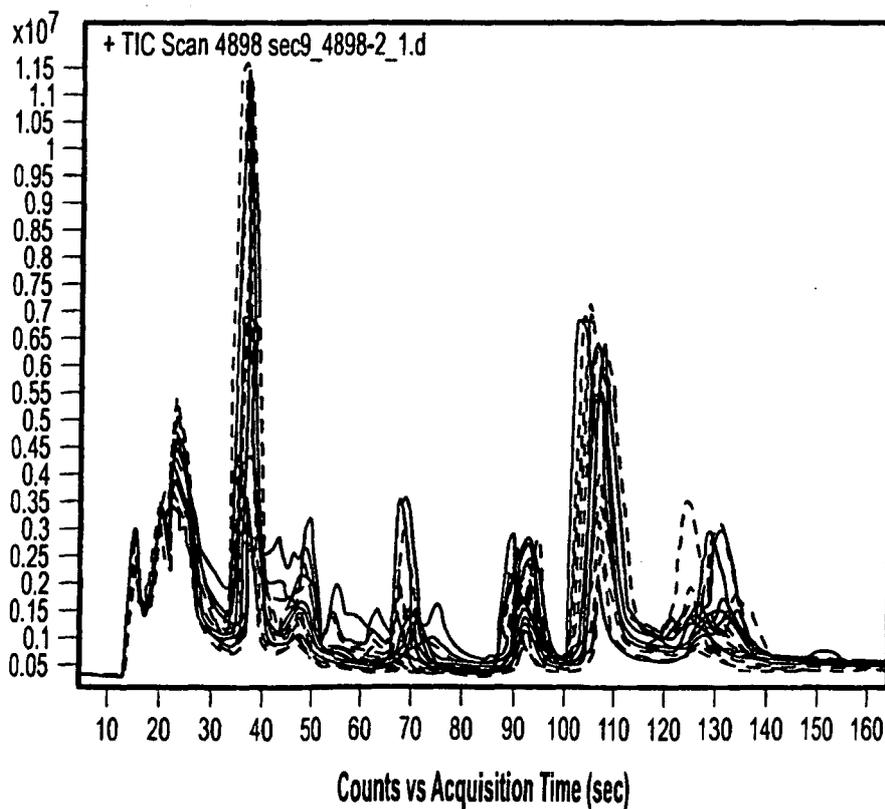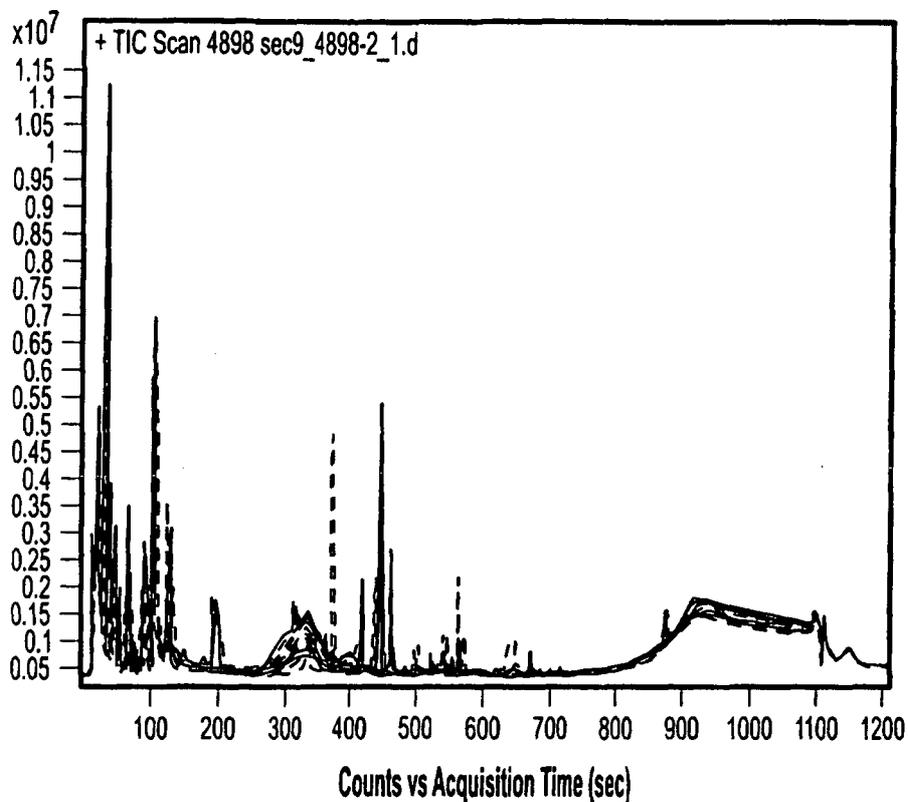
Counts (%) vs. Acquisition Time (min)
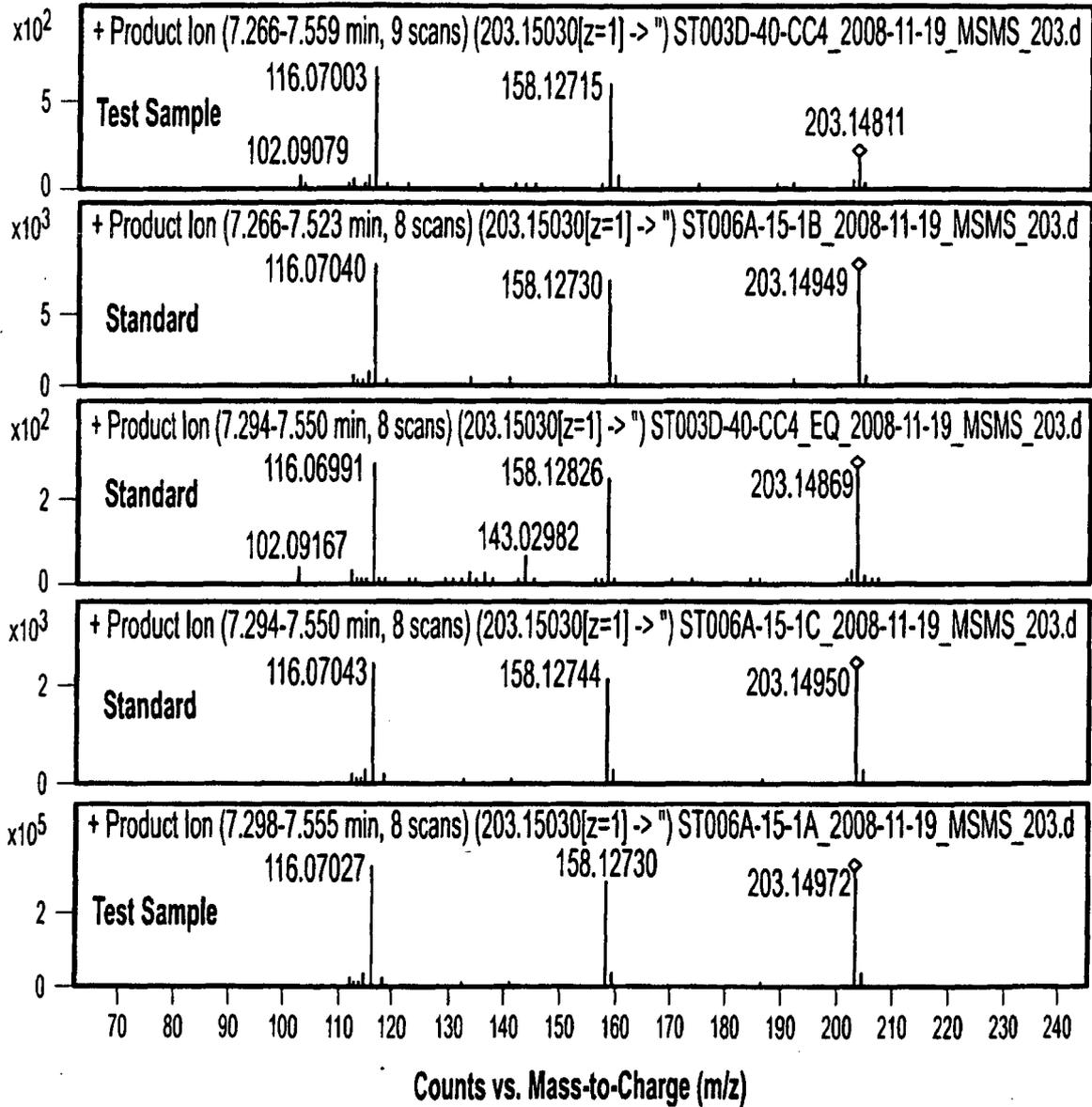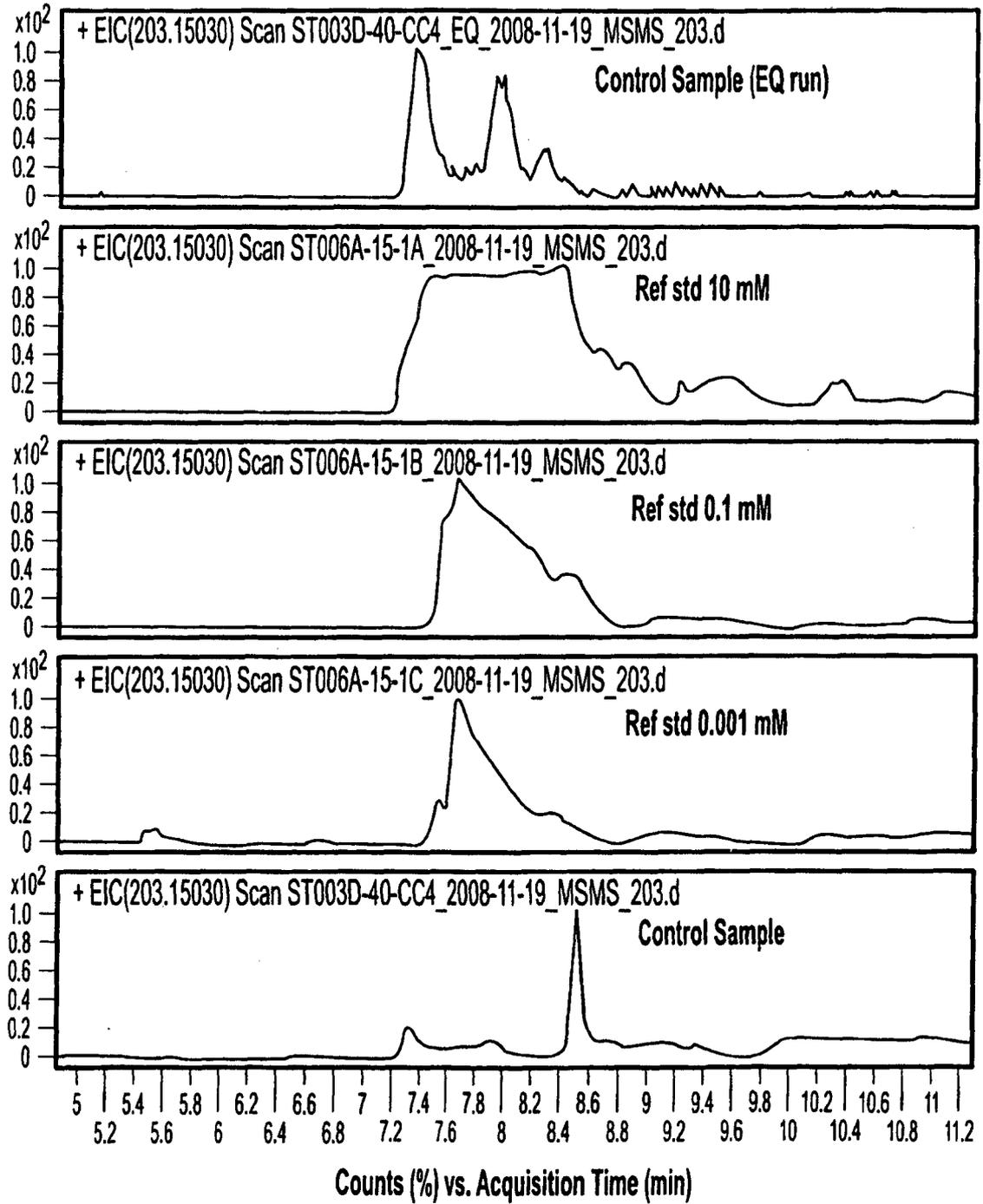
FIG. 2B

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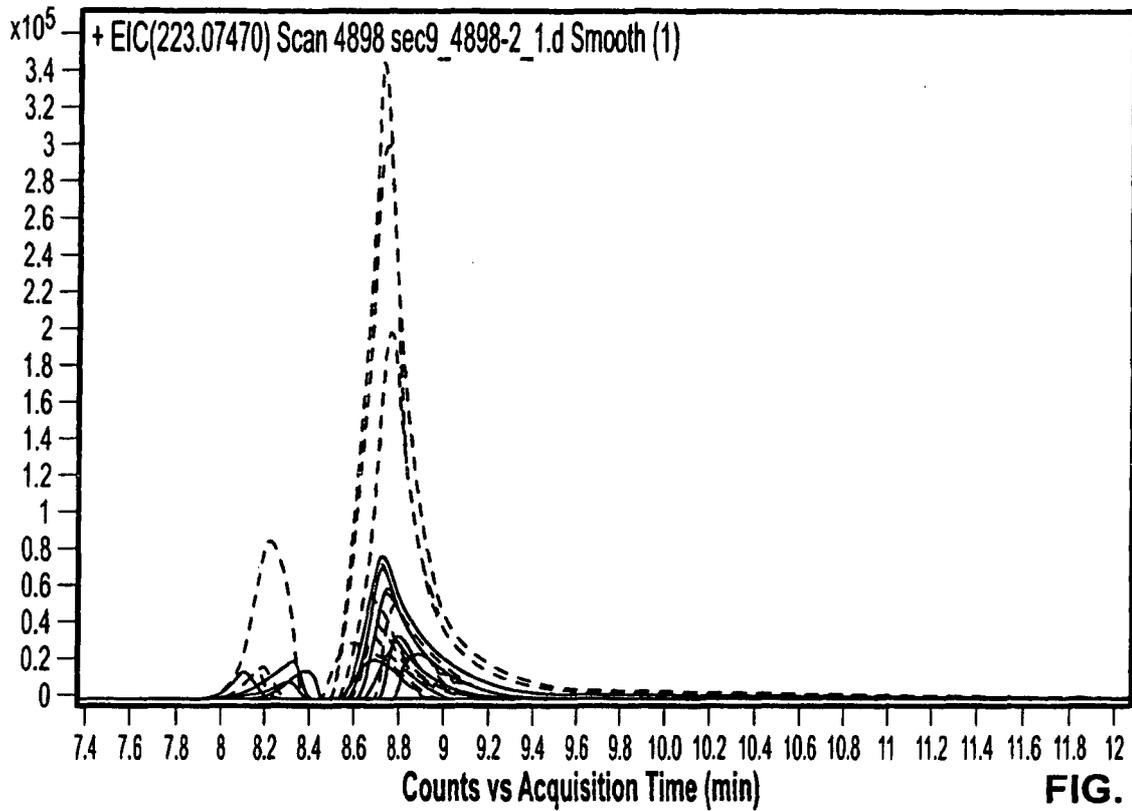
FIG. 3A
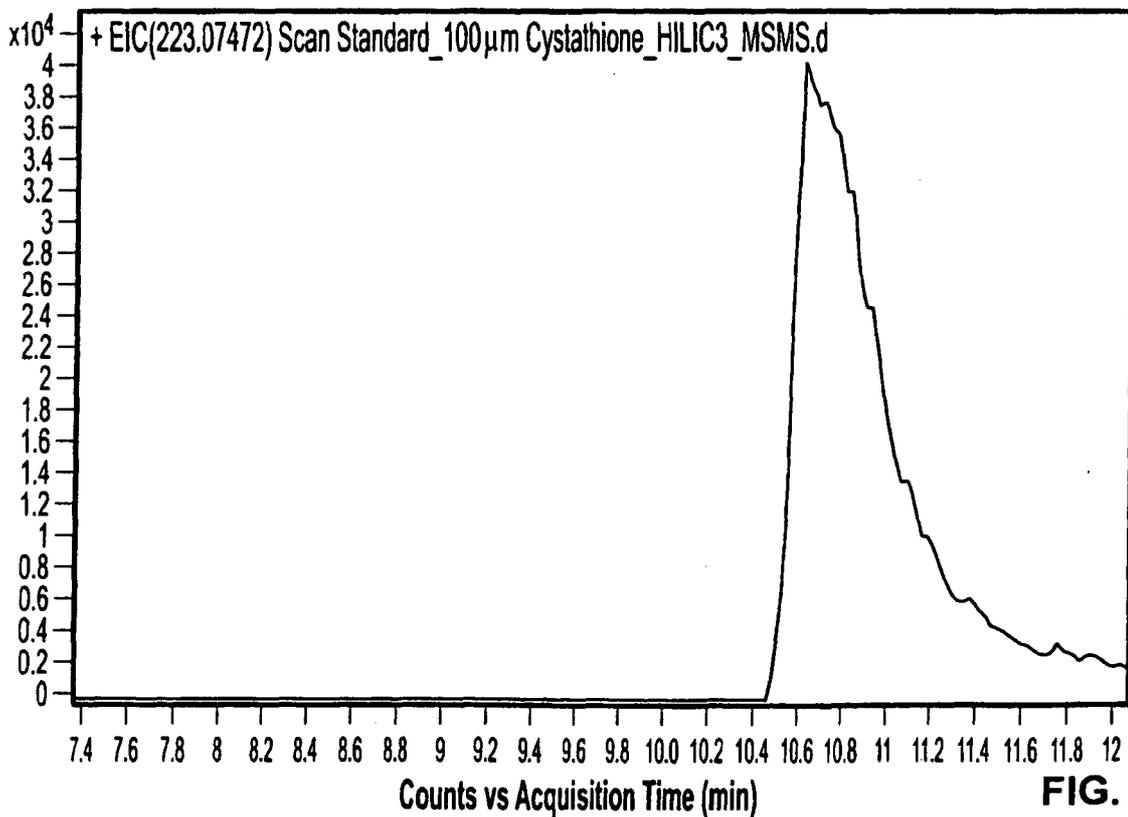
FIG. 3B
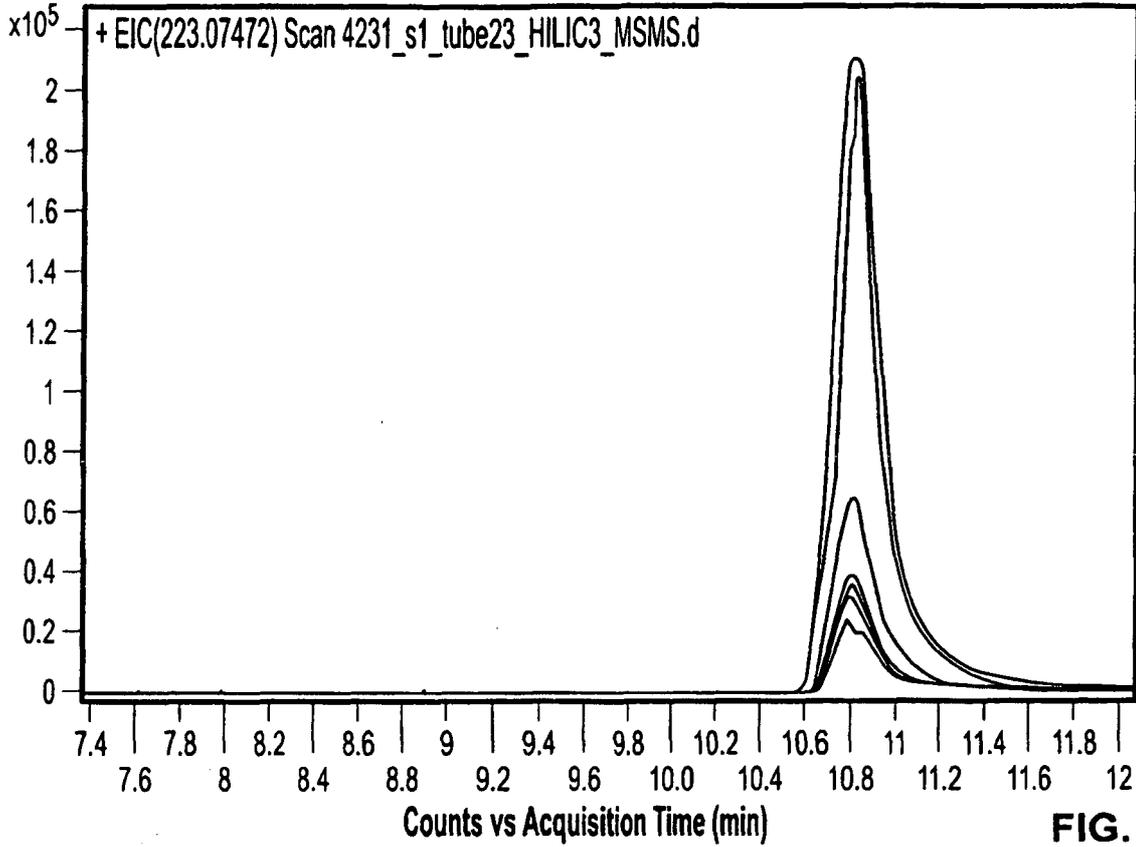
FIG. 3C

L-Cystathionine xcms Areas
Both Regions
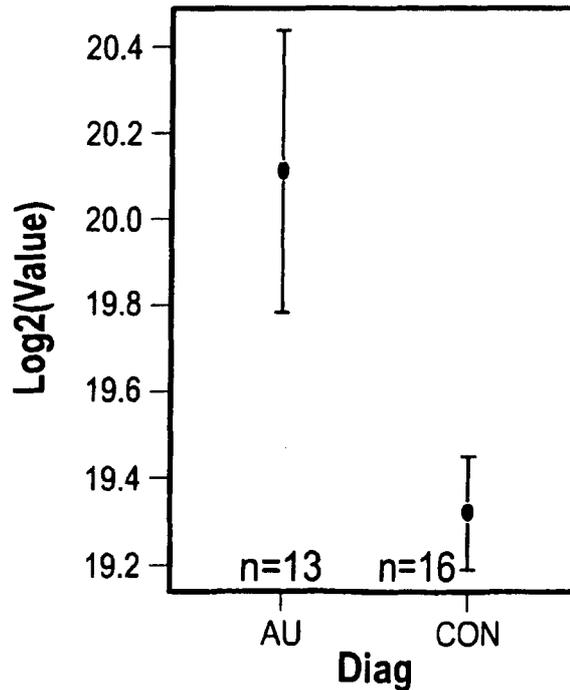
FIG. 3D

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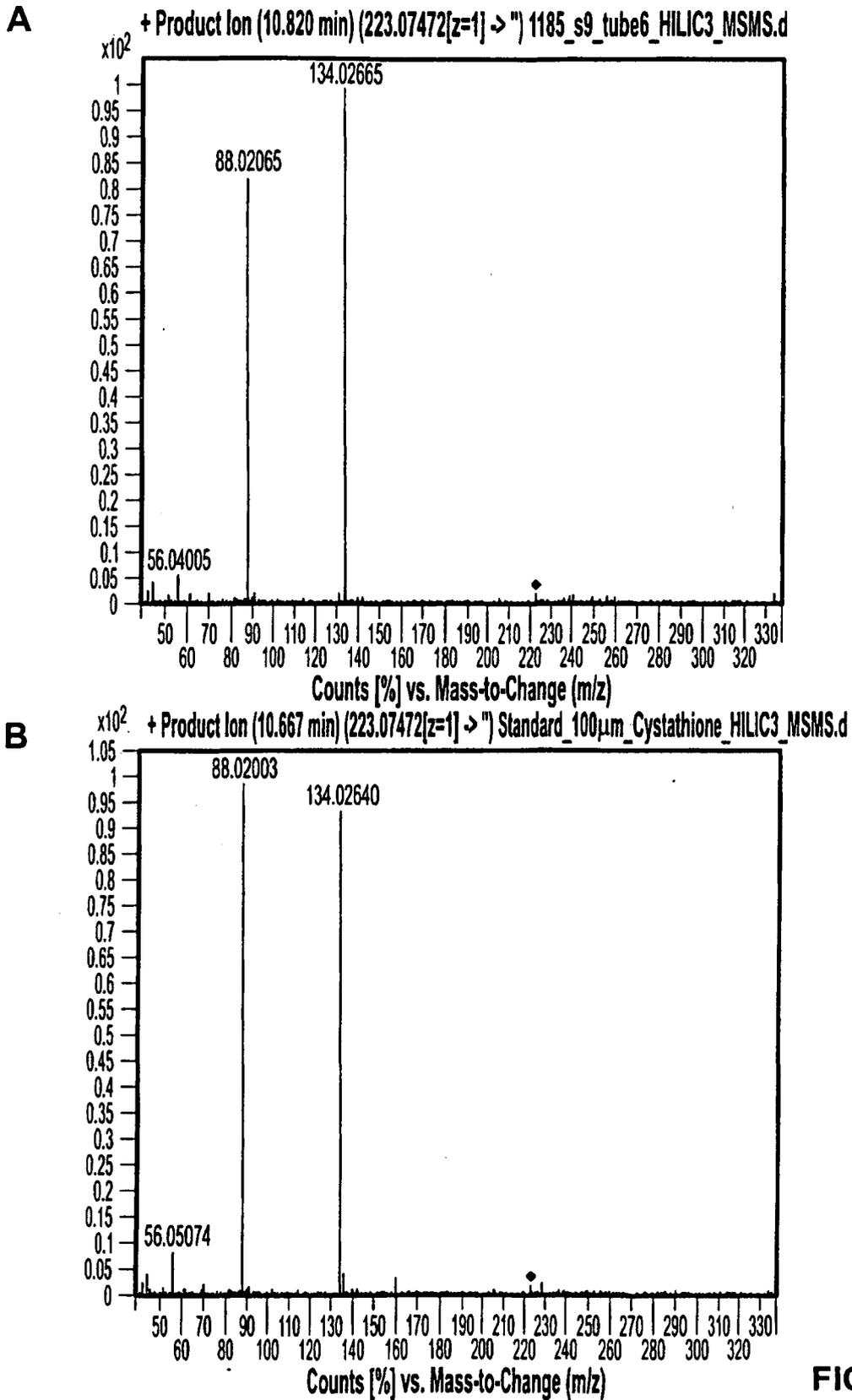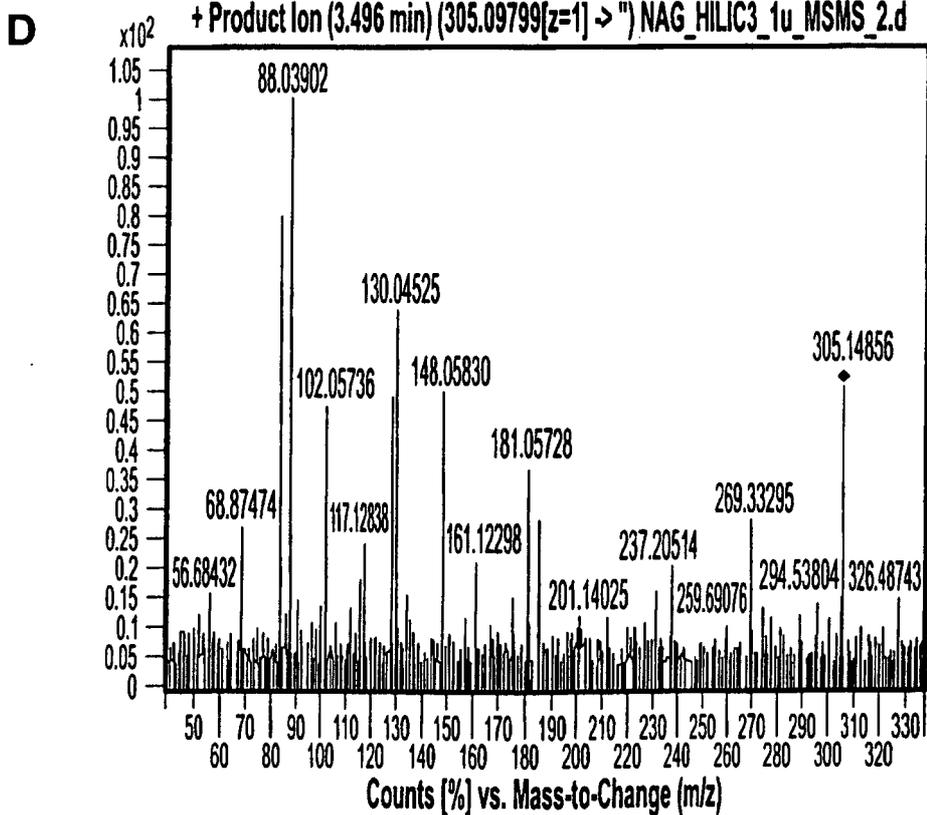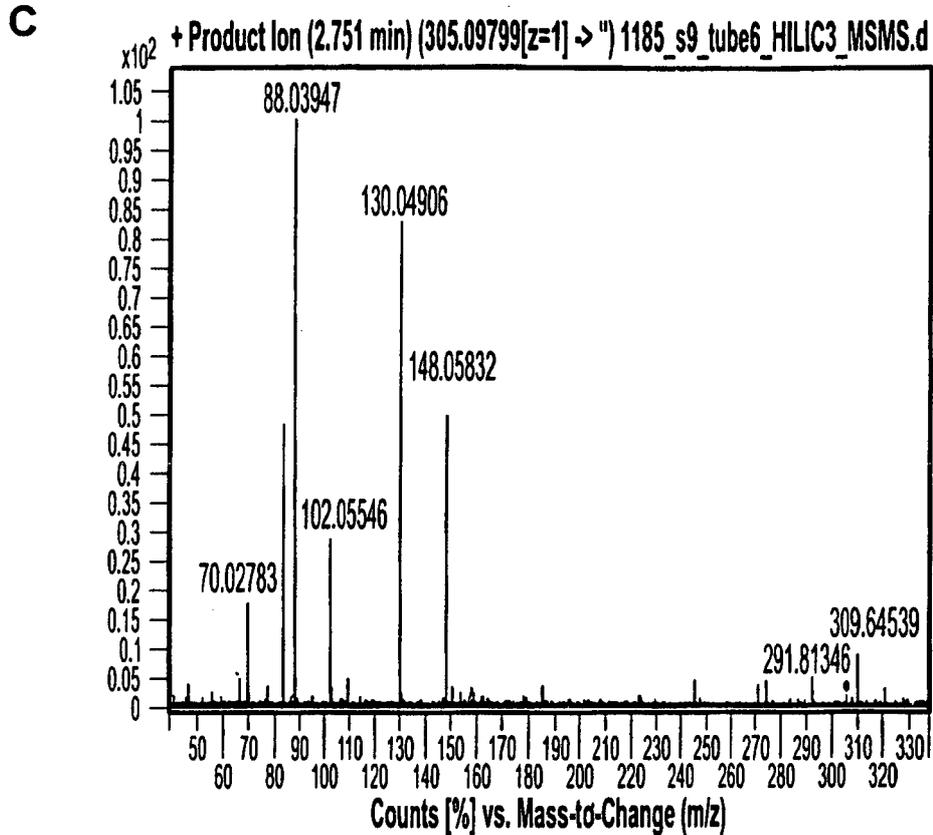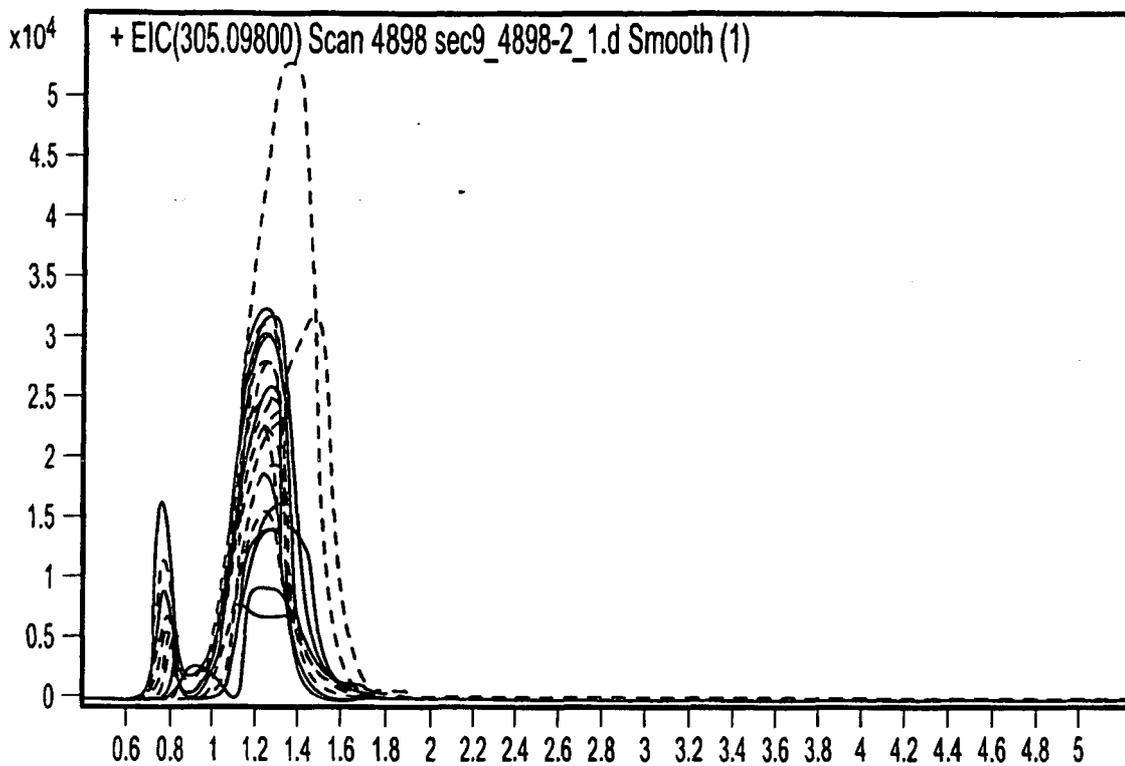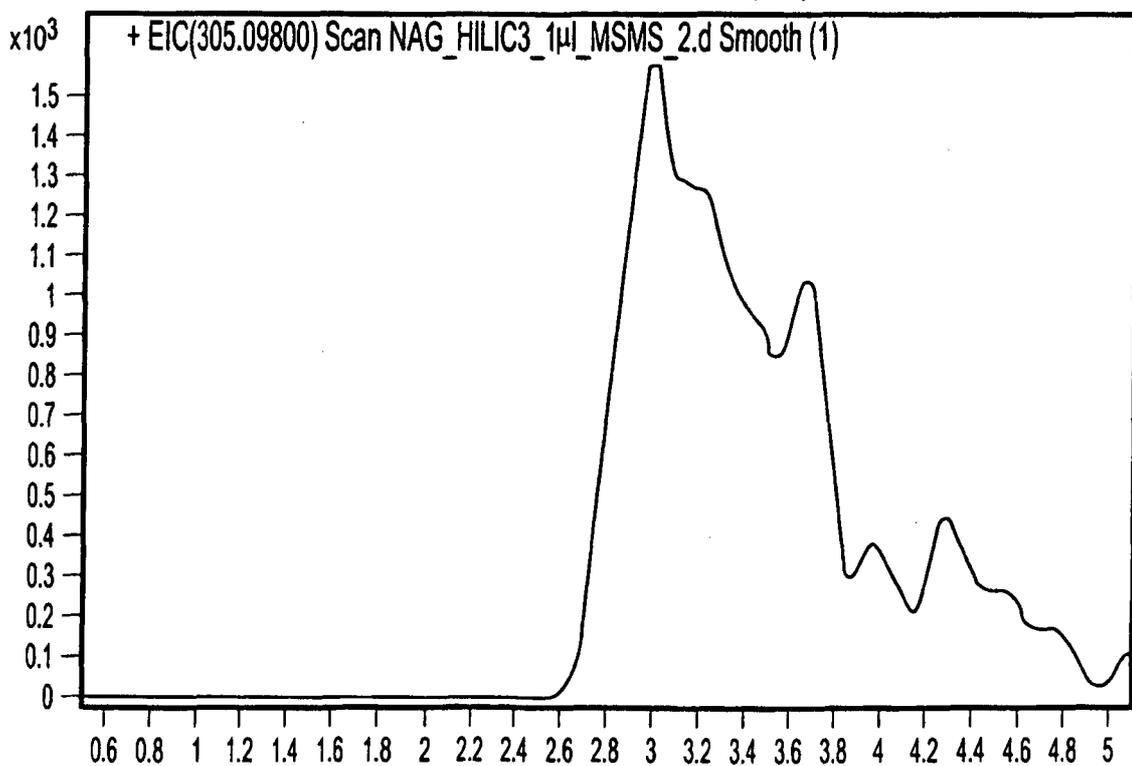
A

+ Product Ion (10.820 min) (223.07472[z=1] → ") 1185_s9_tube6_HILIC3_MSMS.d

B

+ Product Ion (10.667 min) (223.07472[z=1] → ") Standard_100µm_Cystathione_HILIC3_MSMS.d

FIG. 4A

FIG. 4B

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FIG. 5A

FIG. 5B

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+ EIC(305.09800) Scan 4231_s1_tube23_HILIC3_MSMS.d Smooth (1)

FIG. 5C

Counts vs. Acquisition Time (min)

NAAG xcms Areas Both Regions

FIG. 5D

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+ EIC(305.09800) Scan 4898 sec9_4898-2_1.d Smooth (1)

Counts vs. Acquisition Time (min)

FIG. 6A

+ EIC(305.09800) Scan NAG_HILIC3_1µl_MSMS_2.d Smooth (1)
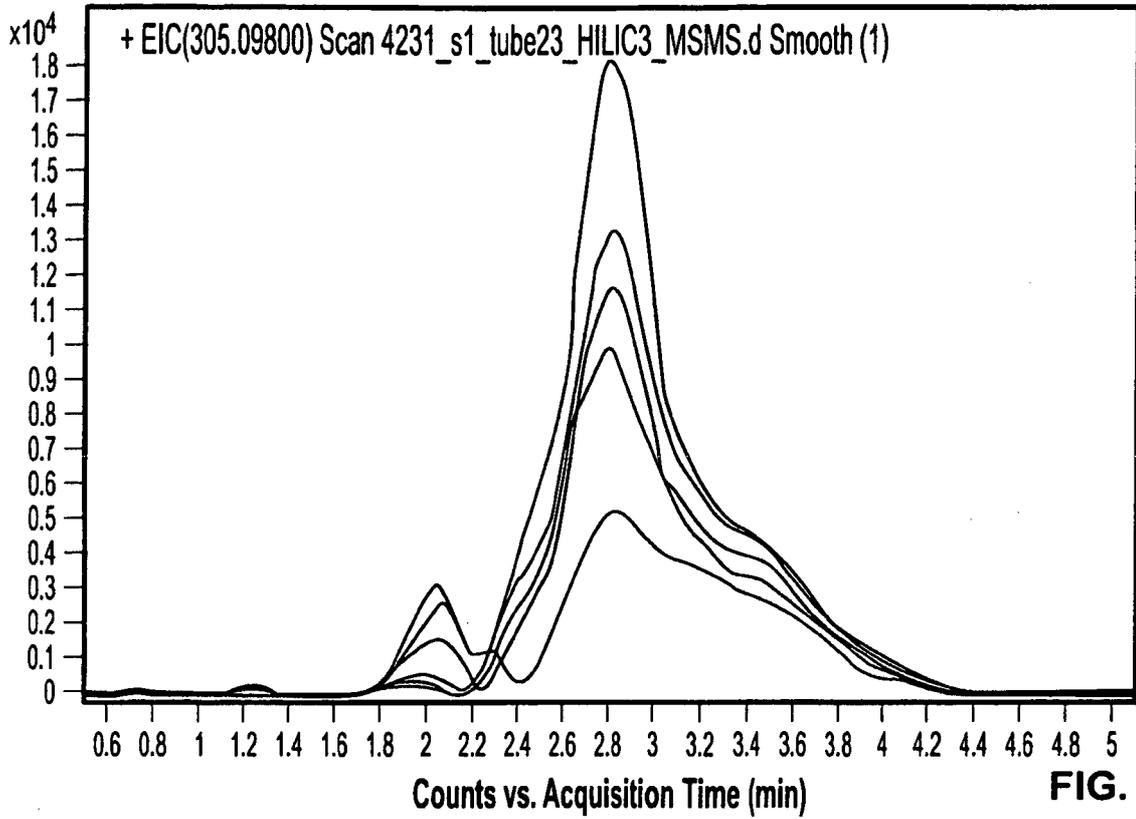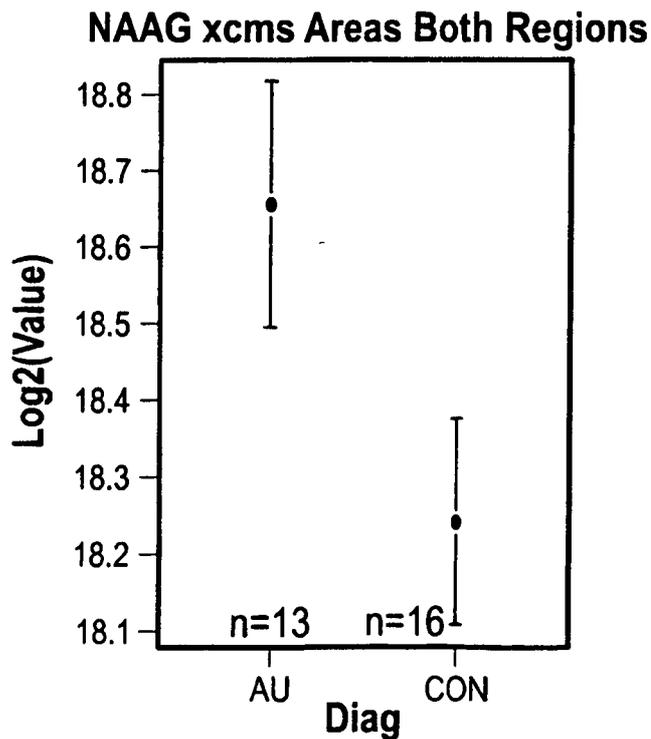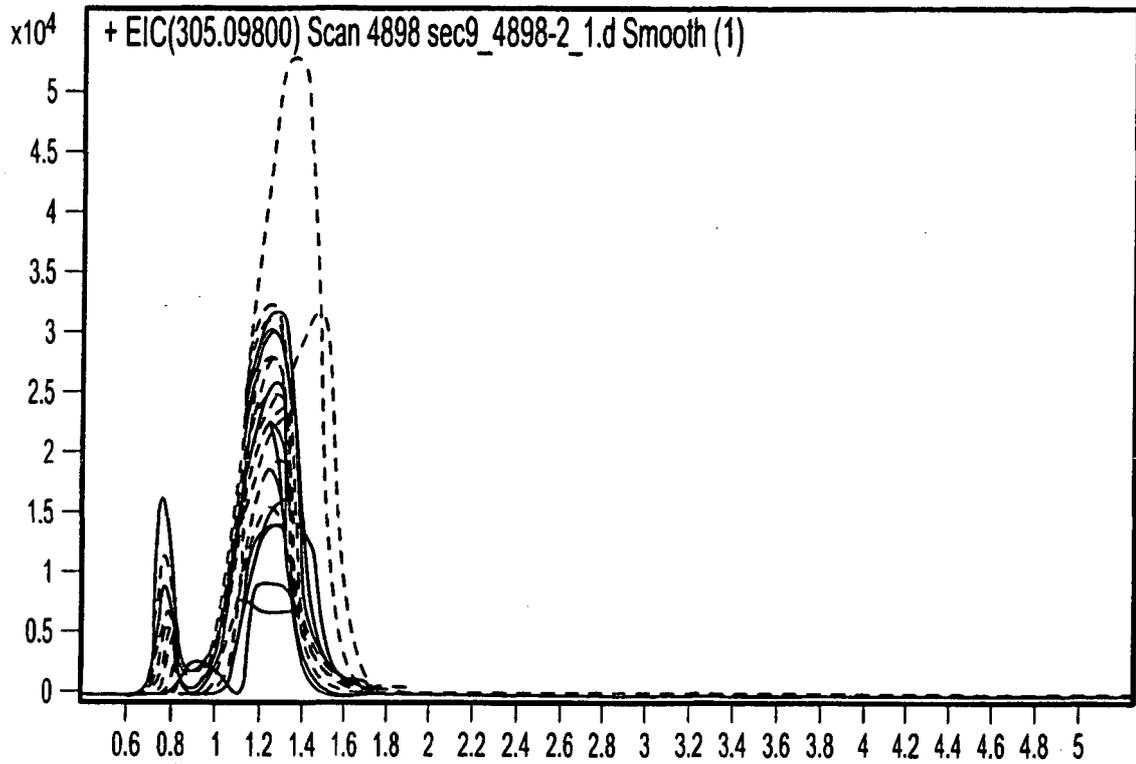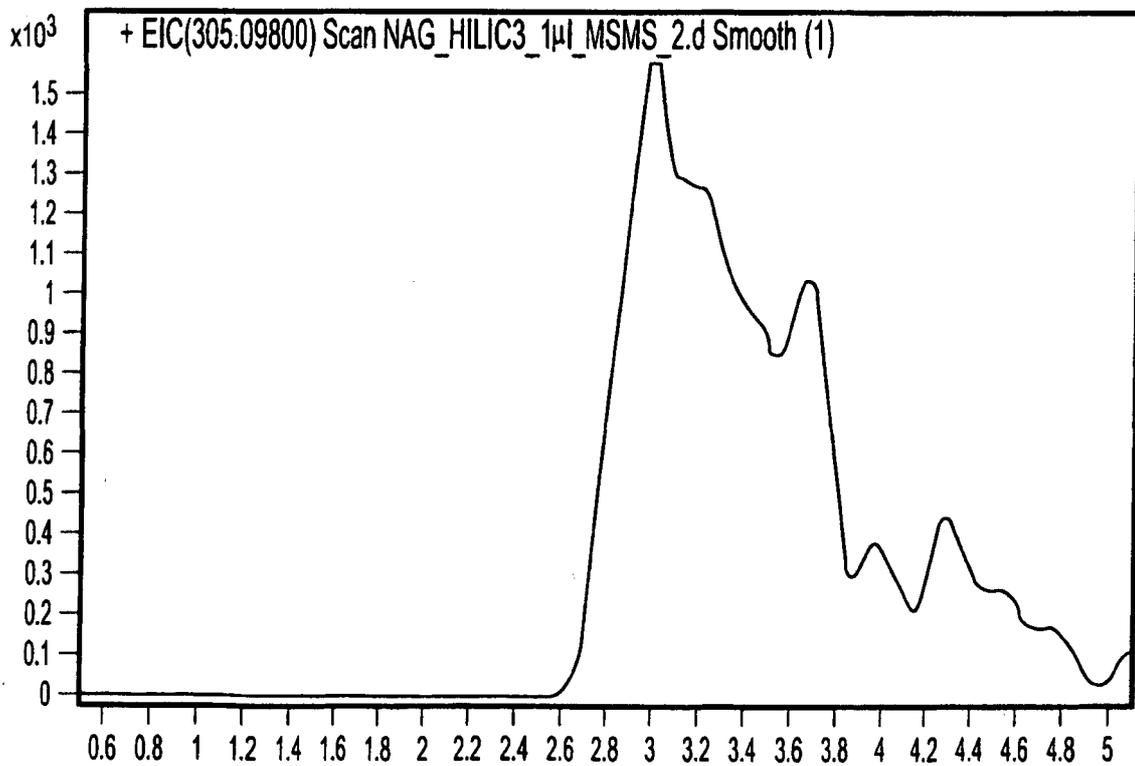
Counts vs. Acquisition Time (min)

FIG. 6B

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FIG. 6C

FIG. 6D

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+ EIC(423.25524) Scan 4898 sec3_4898-2_1.d Smooth (1)

Counts vs. Acquisition Time (min)

FIG. 7B

+ EIC(367.14957) Scan 4898 sec3_4898-2_1.d Smooth (1)

Counts vs. Acquisition Time (min)

FIG. 7A