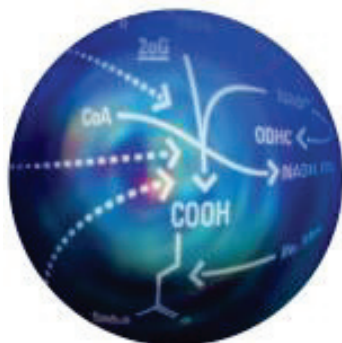


Agilent in Metabolomics

A list of abstracts from peer-reviewed metabolomics publications using Agilent LC/MS and GC/MS systems.



Mass spectrometry (MS)-based metabolomics is a technique that can be applied to many biological problems. Because of the vast chemical diversity of metabolites, and their wide variation in abundance, metabolomics research usually requires multiple techniques; certain classes of samples are more amenable to one analysis technique than others. The two most commonly used techniques are GC/MS and LC/MS. Comprehensive metabolomics laboratories frequently incorporate both of these approaches.

Discovery metabolomics involves the comparison of metabolomes (the full metabolite complement of an organism) between control and test groups to find differences in their profiles. Identification is the next step to determine the chemical structure of these metabolites followed by interpretation, the last step in the workflow, makes connections between the metabolites discovered and the biological processes or conditions studied.

The keys to success in **targeted metabolomics** are accuracy, high throughput and reliability. While these analyses are more routine than profiling, very large numbers of samples must be processed. Generally, these studies are performed by selected ion monitoring (SIM) with a GC/MS system, or by targeted MS/MS using multiple reaction monitoring (MRM) with a triple quadrupole LC/MS system.

LC/MS

Discovery Metabolomics

TOF

1. Identification of an overabundant cholesterol precursor in hepatitis B virus replicating cells by untargeted lipid metabolite profiling

Rodgers MA, Saghatelian A, Yang PL
J Am Chem Soc. 2009 Apr 15;131(14):5030-1

Viruses rely upon host lipid metabolic pathways for successful replication, and there is increasing interest in these pathways as novel therapeutic targets for antiviral drug discovery. Despite this, relatively little is known about the impact of viral infection on cellular lipid metabolism, and the specific lipid metabolites utilized by viruses have not yet been examined. We have applied liquid chromatography-mass spectroscopy (LC-MS) based untargeted metabolite profiling to identify lipid metabolites whose steady-state abundance is significantly altered by replication of hepatitis B virus (HBV), a major human pathogen. Untargeted metabolite profiling indicated that although major lipid classes were unaffected by HBV, an ion of 367 m/z was overabundant in HBV+ cells by 18-fold. As shown by ion fragmentation mass spectrometry and co-injection with standard, the identity of this ion is 7-dehydrocholesterol (7-DHC), an immediate dehydrogenated precursor to cholesterol. While cholesterol has previously been demonstrated to be essential in the replication of many viruses, this is the first to show that viral replication is associated with the selective accumulation of 7-DHC. Most virological studies to date have relied upon methods that deplete all sterols and preclude the observation of any selectivity in sterol utilization by viral pathogens. Our study suggests that HBV may selectively utilize 7-DHC versus other sterols and prompts experiments investigating the functional significance of this enrichment and the elucidation of the mechanism by which it is achieved. The results also highlight the value of untargeted metabolite profiling as a method for identifying critical metabolites for viral infection.

2. Analysis of the hibernation cycle using LC-MS-based metabolomics in ground squirrel liver.

Nelson CJ, Otis JP, Martin SL, Carey HV.
Physiol Genomics. 2009 Mar 3;37(1):43-51. Epub 2008 Dec 23.

A hallmark of hibernation in mammals is metabolic flexibility, which is typified by reversible bouts of metabolic depression (torpor) and the seasonal shift from predominantly carbohydrate to lipid metabolism from summer to winter. To provide new insight into the control and consequences of hibernation, we used LC/MS-based metabolomics to measure differences in small molecules in ground squirrel liver in five activity states: summer, entering torpor, late torpor, arousing from torpor, and interbout arousal. There were significant alterations both seasonally and within torpor-arousal cycles in enzyme cofactor metabolism, amino acid catabolism, and purine and pyrimidine metabolism, with observed metabolites reduced during torpor and increased upon arousal. Multiple lipids also changed, including 1-oleoyllysophosphatidylcholine, cholesterol sulfate, and sphingosine, which tended to be lowest during torpor, and hexadecanedioic acid, which accumulated during a torpor bout. The results reveal the dramatic alterations that occur in several classes of metabolites, highlighting the value of metabolomic analyses in deciphering the hibernation phenotype.

3. A global metabolite profiling approach to identify protein-metabolite interactions

Tagore R, Thomas HR, Homan EA, Munawar A, Saghatelian A
J Am Chem Soc. 2008 Oct 29;130(43):14111-3. Epub 2008 Oct 3

Understanding the biochemical functions of proteins is an important factor in elucidating their cellular and physiological functions. Due to the predominance of biopolymer interactions in biology, many methods have been designed to interrogate and identify biologically relevant interactions that proteins make to DNA, RNA, and other proteins. Complementary approaches that can elucidate binding interactions between proteins and small molecule metabolites will impact the understanding of protein-metabolite interactions and fill a need that is outside the scope of current methods. Here, we demonstrate the ability to identify natural protein-metabolite interactions from complex metabolite mixtures by combining a protein-mediated small molecule enrichment step with a global metabolite profiling platform.

4. Molecular formula and METLIN Personal Metabolite Database matching applied to the identification of compounds generated by LC/TOF-MS.

Sana TR, Roark JC, Li X, Waddell K, Fischer SM.
J Biomol Tech. 2008 Sep;19(4):258-66.

In an effort to simplify and streamline compound identification from metabolomics data generated by liquid chromatography time-of-flight mass spectrometry, we have created software for constructing Personalized Metabolite Databases with content from over 15,000 compounds pulled from the public METLIN database (<http://metlin.scripps.edu/>). Moreover, we have added extra functionalities to the database that (a) permit the addition of user-defined retention times as an orthogonal searchable parameter to complement accurate mass data; and (b) allow interfacing to separate software, a Molecular Formula Generator (MFG), that facilitates reliable interpretation of any database matches from the accurate mass spectral data. To test the utility of this identification strategy, we added retention times to a subset of masses in this database, representing a mixture of 78 synthetic urine standards. The synthetic mixture was analyzed and screened against this METLIN urine database, resulting in 46 accurate mass and retention time matches. Human urine samples were subsequently analyzed under the same analytical conditions and screened against this database. A total of 1387 ions were detected in human urine; 16 of these ions matched both accurate mass and retention time parameters for the 78 urine standards in the database. Another 374 had only an accurate mass match to the database, with 163 of those masses also having the highest MFG score. Furthermore, MFG calculated a formula for a further 849 ions that had no match to the database. Taken together, these results suggest that the METLIN Personal Metabolite database and MFG software offer a robust strategy for confirming the formula of database matches. In the event of no database match, it also suggests possible formulas that may be helpful in interpreting the experimental results.

5. Analysis of hydrophilic metabolites by high-performance liquid chromatography-mass spectrometry using a silica hydride-based stationary phase.

Pesek JJ, Matyska MT, Fischer SM, Sana TR.
J Chromatogr A. 2008 Sep 12;1204(1):48-55. Epub 2008 Jul 31.

A novel silica hydride-based stationary phase was used to evaluate the retention behavior in the aqueous normal-phase (ANP) mode of standards representing three classes of metabolites. The effects on retention behavior of amino acids, carbohydrates and small organic acids were examined by altering the column temperature, and by adding different additives to both the mobile phase and sample solvent. Gradient mode results revealed the repeatability of retention times to be very stable for these compound classes. At both 15 and 30 degrees C, excellent RSD values were obtained with less than 1% variation for over 50 injections of an amino acid mixture. The ability to separate the 19 nonderivatized amino acid standards, organic acids and carbohydrates was demonstrated as well as the potential for this material to separate polar metabolites in complex fluids such as urine.

6. Rapid analysis of fungal cultures and dried figs for secondary metabolites by LC/TOF-MS.

Senyuva HZ, Gilbert J, Oztürkoğlu S.
Anal Chim Acta. 2008 Jun 9; 617(1-2):97-106. Epub 2008 Jan 16.

A liquid chromatography-time-of-flight mass spectrometry (LC/TOF-MS) method has been developed for profiling fungal metabolites. The performance of the procedure in terms of mass accuracy, selectivity (specificity) and repeatability was established by spiking aflatoxins, ochratoxins, trichothecenes and other metabolites into blank growth media. After extracting, and carrying out LC/TOF-MS analysis, the standards were correctly identified by searching a specially constructed database of 465 secondary metabolites. To demonstrate the viability of this approach 11 toxigenic and four non-toxicogenic fungi from reference collections were grown on various media, for 7-14 days. The method was also applied to two toxigenic fungi, *A. flavus* (200-138) and *A. parasiticus* (2999-465) grown on gamma radiation sterilised dried figs, for 7-14 days. The fungal hyphae plus a portion of growth media or portions of dried figs were solvent extracted and analysed by LC/TOF-MS using a rapid resolution microbore LC column. Data processing based on cluster analysis, showed that electrospray ionization (ESI)-TOF-MS could be used to unequivocally identify metabolites in crude extracts. Using the elemental metabolite database, it was demonstrated that from culture collection isolates, anticipated metabolites. The speed and simplicity of the method has meant that levels of these metabolites could be monitored daily in sterilised figs. Over a 14-day period, levels of aflatoxins and kojic acid maximised at 5-6 days, whilst levels of 5-methoxysterigmatocystin remained relatively constant. In addition to the known metabolites expected to be produced by these fungi, roquefortine A, fumagillin, fumigaclavine B, malformins (peptides), aspergillilic acid, nigragillin, terrein, terrestric acid and penicillilic acid were also identified.

7. Multiple ionization mass spectrometry strategy used to reveal the complexity of metabolomics.

Nordström A, Want E, Northen T, Lehtiö J, Siuzdak G.
Anal Chem. 2008 Jan 15; 80(2):421-9. Epub 2007 Dec 18.

A multiple ionization mass spectrometry strategy is presented based on the analysis of human serum extracts. Chromatographic separation was interfaced inline with the atmospheric pressure ionization techniques electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in both positive (+) and negative (-) ionization modes. Furthermore, surface-based matrix-assisted laser desorption/ionization (MALDI) and desorption ionization on silicon (DIOS) mass spectrometry were also integrated with the separation through fraction collection and offline mass spectrometry. Processing of raw data using the XCMS software resulted in time-aligned ion features, which are defined as a unique m/z at a unique retention time. The ion feature lists obtained through LC-MS with ESI and APCI interfaces in both +/- ionization modes were compared, and unique ion tables were generated. Nonredundant, unique ion features, were defined as mass numbers for which no mass numbers corresponding to $[M + H]^+$, $[M - H]^-$, or $[M + Na]^+$ were observed in the other ionization methods at the same retention time. Analysis of the extracted serum using ESI for both (+) and (-) ions resulted in >90% additional unique ions being detected in the (-) ESI mode. Complementing the ESI analysis with APCI resulted in an additional approximately 20% increase in unique ions. Finally, ESI/APCI ionization was combined with fraction collection and offline-MALDI and DIOS mass spectrometry. The parts of the total ion current chromatograms in the LC-MS acquired data corresponding to collected fractions were summed, and m/z lists were compiled and compared to the m/z lists obtained from the DIOS/MALDI spectra. It was observed that, for each fraction, DIOS accounted for approximately 50% of the unique ions detected. These results suggest that true global metabolomics will require multiple ionization technologies to address the inherent metabolite diversity and therefore the complexity in and of metabolomics studies.

8 Metabolomics identifies perturbations in human disorders of propionate metabolism.

Wikoff WR, Gangoiti JA, Barshop BA, Siuzdak G.
Clin Chem. 2007 Dec; 53(12):2169-76. Epub 2007 Oct 19.

We applied untargeted mass spectrometry-based metabolomics to the diseases methylmalonic acidemia (MMA) and propionic acidemia (PA). METHODS: We used a screening platform that used untargeted, mass-based metabolomics of methanol-extracted plasma to find significantly different molecular features in human plasma samples from MMA and PA patients and from healthy individuals. Capillary reverse phase liquid chromatography (4 μ L/min) was interfaced to a TOF mass spectrometer, and data were processed using nonlinear alignment software (XCMS) and an online database (METLIN) to find and identify metabolites differentially regulated in disease. RESULTS: Of the approximately 3500 features measured, propionyl carnitine was easily identified as the best biomarker of disease (P value 1.3×10^{-18}), demonstrating the proof-of-concept use of untargeted metabolomics in clinical chemistry discovery. Five additional acylcarnitine metabolites showed significant differentiation between plasma from patients and healthy individuals, and gamma-butyrobetaine was highly increased in a subset of patients. Two acylcarnitine metabolites and numerous unidentified species differentiate MMA and PA. Many metabolites that do not appear in any public database, and that remain unidentified, varied significantly between normal, MMA, and PA, underscoring the complex downstream metabolic effects resulting from the defect in a single enzyme. CONCLUSIONS: This proof-of-concept study demonstrates that metabolomics can expand the range of metabolites associated with human disease and shows that this method may be useful for disease diagnosis and patient clinical evaluation.

9. Stable isotope assisted assignment of elemental compositions for metabolomics.

Hegeman AD, Schulte CF, Cui Q, Lewis IA, Huttlin EL, Eghbalnia H, Harms AC, Ulrich EL, Markley JL, Sussman MR.
Anal Chem. 2007 Sep 15; 79(18):6912-21. Epub 2007 Aug 21.

Assignment of individual compound identities within mixtures of thousands of metabolites in biological extracts is a major challenge for metabolomic technology. Mass spectrometry offers high sensitivity over a large dynamic range of abundances and molecular weights but is limited in its capacity to discriminate isobaric compounds. In this article, we have extended earlier studies using isotopic labeling for elemental composition elucidation (Rodgers, R. P.; Blumer, E. N.; Hendrickson, C. L.; Marshall, A. G. J. Am. Soc. Mass Spectrom. 2000, 11, 835-40) to limit the formulas consistent with any exact mass measurement by comparing observations of metabolites extracted from *Arabidopsis thaliana* plants grown with (I) ^{12}C and ^{14}N (natural abundance), (II) ^{12}C and ^{15}N , (III) ^{13}C and ^{14}N , or (IV) ^{13}C and ^{15}N . Unique elemental compositions were determined over a dramatically enhanced mass range by analyzing exact mass measurement data from the four extracts using two methods. In the first, metabolite masses were matched with a library of 11,000 compounds known to be present in living cells by using values calculated for each of the four isotopic conditions. In the second method, metabolite masses were searched against masses calculated for a constrained subset of possible atomic combinations in all four isotopic regimes. In both methods, the lists of elemental compositions from each labeling regime were compared to find common formulas with similar retention properties by HPLC in at least three of the four regimes. These results demonstrate that metabolic labeling can be used to provide additional constraints for higher confidence formula assignments over an extended mass range.

QTOF

1. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites.

Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G.
Proc Natl Acad Sci U S A. 2009 Mar 10; 106(10):3698-703. Epub 2009 Feb 20.

Although it has long been recognized that the enteric community of bacteria that inhabit the human distal intestinal track broadly impacts human health, the biochemical details that underlie these effects remain largely undefined. Here, we report a broad MS-based metabolomics study that demonstrates a surprisingly large effect of the gut "microbiome" on mammalian blood metabolites. Plasma extracts from germ-free mice were compared with samples from conventional (conv) animals by using various MS-based methods. Hundreds of features were detected in only 1 sample set, with the majority of these being unique to the conv animals, whereas approximately 10% of all features observed in both sample sets showed significant changes in their relative signal intensity. Amino acid metabolites were particularly affected. For example, the bacterial-mediated production of bioactive indole-containing metabolites derived from tryptophan such as indoxyl sulfate and the antioxidant indole-3-propionic acid (IPA) was impacted. Production of IPA was shown to be completely dependent on the presence of gut microflora and could be established by colonization with the bacterium *Clostridium sporogenes*. Multiple organic acids containing phenyl groups were also greatly increased in the presence of gut microbes. A broad, drug-like phase II metabolic response of the host to metabolites generated by the microbiome was observed, suggesting that the gut microflora has a direct impact on the drug metabolism capacity of the host. Together, these results suggest a significant interplay between bacterial and mammalian metabolism.

2. Identification of a new endogenous metabolite and the characterization of its protein interactions through an immobilization approach.

Kalisiak J, Trauger SA, Kalisiak E, Morita H, Fokin VV, Adams MW, Sharpless KB, Siuzdak G.
J Am Chem Soc. 2009 Jan 14; 131(1):378-86.

The emerging field of global mass-based metabolomics provides a platform for discovering unknown metabolites and their specific biochemical pathways. We report the identification of a new endogenous metabolite, N (4)-(N-acetylaminopropyl) spermidine and the use of a novel proteomics based method for the investigation of its protein interaction using metabolite immobilization on agarose beads. The metabolite was isolated from the organism *Pyrococcus furiosus*, and structurally characterized through an iterative process of synthesizing candidate molecules and comparative analysis using accurate mass LC-MS/MS. An approach developed for the selective preparation of N(1)-acetylthermospermine, one of the possible structures of the unknown metabolite, provides a convenient route to new polyamine derivatives through methylation on the N(8) and N(4) of the thermospermine scaffold. The biochemical role of the novel metabolite as well as that of two other polyamines: spermidine and agmatine is investigated through metabolite immobilization and incubation with native proteins. The identification of eleven proteins that uniquely bind with N(4)-(N-acetylaminopropyl)spermidine, provides information on the role of this novel metabolite in the native organism. Identified proteins included hypothetical ones such as PF0607 and PF1199, and those involved in translation, DNA synthesis and the urea cycle like translation initiation factor IF-2, 50S ribosomal protein L14e, DNA-directed RNA polymerase, and ornithine carbamoyltransferase. The immobilization approach demonstrated here has the potential for application to other newly discovered endogenous metabolites found through untargeted metabolomics, as a preliminary screen for generating a list of proteins that could be further investigated for specific activity.

3. Cross-platform Q-TOF validation of global exo-metabolomic analysis: Application to human glioblastoma cells treated with the standard PI 3-Kinase inhibitor LY294002.

Pandher R, Ducruix C, Eccles SA, Raynaud FI.
J Chromatogr B Analyt Technol Biomed Life Sci. 2008 Dec 6. [Epub ahead of print]

The reproducibility of a metabolomics method has been assessed to identify changes in tumour cell metabolites. Tissue culture media extracts were analyzed by reverse phase chromatography on a Waters Acquity T3 column with a 13min 0.1% formic acid: acetonitrile gradient on Agilent and Waters LC-Q-TOF instruments. Features (m/z, RT) were extracted by MarkerLynxtrade mark (Waters) and Molecular Feature Extractor (Agilent) in positive and negative ionization modes. The number of features were similar on both instruments and the reproducibility of ten replicates was <35% signal variability for approximately 50% and 40% of all ions detected in positive and negative ionization modes, respectively. External standards spiked to the matrix showed CVs <25% in peak areas within and between days. U87MG glioblastoma cells exposed to the PI 3-Kinase inhibitor LY294002 showed significant alterations of several confirmed features. These included glycerophosphocholine, already shown by NMR to be modulated by LY294002, highlighting the power of this technology for biomarker discovery.

4. A sample extraction and chromatographic strategy for increasing LC/MS detection coverage of the erythrocyte metabolome.

Sana TR, Waddell K, Fischer SM.

J Chromatogr B Analyt Technol Biomed Life Sci. 2008 Aug 15; 871(2):314-21. Epub 2008 Apr 26.

Reproducible and comprehensive sample extraction and detection of metabolites with a broad range of physico-chemical properties from biological matrices can be a highly challenging process. A single LC/MS separation method was developed for a 2.1 mm x 100 mm, 1.8 micron ZORBAX SB-Aq column that was used to separate human erythrocyte metabolites extracted under sample extraction solvent conditions where the pH was neutral or had been adjusted to either, pH 2, 6 or 9. Internal standards were included and evaluated for tracking sample extraction efficiency. Through the combination of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques in both positive (+) and negative (-) ion modes, a total of 2370 features (compounds and associated compound related components: isotopes, adducts and dimers) were detected across all pHs. Broader coverage of the detected metabolome was achieved by observing that (1) performing extractions at pH 2 and 9, leads to a combined 92% increase in detected features over pH 7 alone; and (2) including APCI in the analysis results in a 34% increase in detected features, across all pHs, than the total number detected by ESI. A significant dependency of extraction solvent pH on the recovery of heme and other compounds was observed in erythrocytes and underscores the need for a comprehensive sample extraction strategy and LC/MS analysis in metabolomics profiling experiments.

5. XCMS2: processing tandem mass spectrometry data for metabolite identification and structural characterization.

Benton HP, Wong DM, Trauger SA, Siuzdak G.

Anal Chem. 2008 Aug 15; 80(16):6382-9. Epub 2008 Jul 16.

Mass spectrometry based metabolomics represents a new area for bioinformatics technology development. While the computational tools currently available such as XCMS statistically assess and rank LC-MS features, they do not provide information about their structural identity. XCMS(2) is an open source software package which has been developed to automatically search tandem mass spectrometry (MS/MS) data against high quality experimental MS/MS data from known metabolites contained in a reference library (METLIN). Scoring of hits is based on a "shared peak count" method that identifies masses of fragment ions shared between the analytical and reference MS/MS spectra. Another functional component of XCMS(2) is the capability of providing structural information for unknown metabolites, which are not in the METLIN database. This "similarity search" algorithm has been developed to detect possible structural motifs in the unknown metabolite which may produce characteristic fragment ions and neutral losses to related reference compounds contained in METLIN, even if the precursor masses are not the same.

6. Metabolomic analysis of the cerebrospinal fluid reveals changes in phospholipase expression in the CNS of SIV-infected macaques.

Wikoff WR, Pendyala G, Siuzdak G, Fox HS.

J Clin Invest. 2008 Jul; 118(7):2661-9.

HIV infiltrates the CNS soon after an individual has become infected with the virus, and can cause dementia and encephalitis in late-stage disease. Here, a global metabolomics approach was used to find and identify metabolites differentially regulated in the cerebrospinal fluid (CSF) of rhesus macaques with SIV-induced CNS disease, as we hypothesized that this might provide biomarkers of virus-induced CNS damage. The screening platform used a non-targeted, mass-based metabolomics approach beginning with capillary reverse phase chromatography and electrospray ionization with accurate mass determination, followed by novel, nonlinear data alignment and online database screening to identify metabolites. CSF was compared before and after viral infection. Significant changes in the metabolome specific to SIV-induced encephalitis were observed. Metabolites that were increased during infection-induced encephalitis included carnitine, acyl-carnitines, fatty acids, and phospholipid molecules. The elevation in free fatty acids and lysophospholipids correlated with increased expression of specific phospholipases in the brains of animals with encephalitis. One of these, a phospholipase A2 isoenzyme, is capable of releasing a number of the fatty acids identified. It was expressed in different areas of the brain in conjunction with glial activation, rather than linked to regions of SIV infection and inflammation, indicating widespread alterations in infected brains. The identification of specific metabolites as well as mechanisms of their increase illustrates the potential of mass-based metabolomics to address problems in CNS biochemistry and neurovirology, as well as neurodegenerative diseases.

7. Correlating the transcriptome, proteome, and metabolome in the environmental adaptation of a hyperthermophile.

Trauger SA, Kalisak E, Kalisiak J, Morita H, Weinberg MV, Menon AL, Poole FL 2nd, Adams MW, Siuzdak G.
J Proteome Res. 2008 Mar;7(3):1027-35. Epub 2008 Feb 2.

We have performed a comprehensive characterization of global molecular changes for a model organism *Pyrococcus furiosus* using transcriptomic (DNA microarray), proteomic, and metabolomic analysis as it undergoes a cold adaptation response from its optimal 95 to 72 degrees C. Metabolic profiling on the same set of samples shows the down-regulation of many metabolites. However, some metabolites are found to be strongly up-regulated. An approach using accurate mass, isotopic pattern, database searching, and retention time is used to putatively identify several metabolites of interest. Many of the up-regulated metabolites are part of an alternative polyamine biosynthesis pathway previously established in a thermophilic bacterium *Thermus thermophilus*. Arginine, agmatine, spermidine, and branched polyamines N4-aminopropylspermidine and N4-(N-acetylaminopropyl)spermidine were unambiguously identified based on their accurate mass, isotopic pattern, and matching of MS/MS data acquired under identical conditions for the natural metabolite and a high purity standard. Both DNA microarray and semiquantitative proteomic analysis using a label-free spectral counting approach indicate the down-regulation of a large majority of genes with diverse predicted functions related to growth such as transcription, amino acid biosynthesis, and translation. Some genes are, however, found to be up-regulated through the measurement of their relative mRNA and protein levels. The complimentary information obtained by the various "omics" techniques is used to catalogue and correlate the overall molecular changes.

8 .DNA immunization perturbs lipid metabolites and increases risk of atherogenesis.

Yang F, Yan S, Wang F, He Y, Guo Y, Zhou Q, Wang Y, Zhang X, Zhang W, Sun S.
J Proteome Res. 2008 Feb;7(2):741-8. Epub 2008 Jan 1.

In addition to conventional vaccination, DNA-mediated immunization has been developed as an alternative approach in the prevention and treatment of different infectious diseases, including hepatitis B. To define sets of serum protein and metabolite biomarkers that could be employed to determine the efficacy and safety of DNA vaccines, an integrated multiple systems biology approach was undertaken on mice immunized with DNA vaccine, recombinant protein, plasmid vector, and phosphate-buffered solution. Their sera were analyzed by two-dimensional electrophoresis and HPLC coupled with time-of-flight mass spectrometry. We detected an increase in phytosphingosine, dihydrosphingosine, palmitoylcarnitine, and ceramide in the sera of DNA-vaccinated mice. Several protein molecules were found to be altered in DNA-vaccinated mice, including apolipoprotein A-I precursor. Taken together, these results indicated that DNA vaccine stimulated hepatic sphingolipid synthesis, which may have altered the structure of circulating lipoproteins and promoted atherogenesis. This study also underscores the power of metabolomics and proteomics in the definition of DNA-vaccine-mediated metabolic phenotypes.

Triple Quad

1. A multi-analytical approach for metabolomic profiling of zebrafish (*Danio rerio*) livers.

Ong ES, Chor CF, Zou L, Ong CN.
Mol Biosyst. 2009 Mar;5(3):288-98. Epub 2008 Dec 24.

A metabolomic study was performed to investigate the biochemical profiles of livers from male and female zebrafish (*Danio rerio*), using a multiple platform approach, incorporating ¹H NMR, GC/MS and LC/MS. The reproducibility and reliability of the three methods were validated prior to the assays. Major biomolecules detected using one method were also cross examined using the other techniques. These metabolites included carbohydrates, lipids, amino acids detected using ¹H NMR and GC/MS, and acetylcarnitine, choline and various phospholipids determined using ¹H NMR and LC/MS. Our findings suggest that ¹H NMR provided comprehensive information on glucose, amino acids, pyruvate and other smaller biochemical constituents of the zebrafish liver. On the other hand, GC/MS was able to assay cholesterol, saturated and unsaturated fatty acids, and LC/MS was ideal for the analysis of lipids/phospholipids. These techniques revealed that there are significant differences in the biochemical profiles of male and female zebrafish liver tissue extracts. Specifically, we noted that although there were no significant differences observed for the carbohydrate profile, the amino acid profile was rather different in male and female zebrafish liver. Furthermore, data from all three techniques revealed that although the saturated fatty acid profile was similar, the compositions of unsaturated fatty acids were different in the two phenotypes. The overall findings suggested that this multiplatform approach offers comprehensive coverage of a metabolome as well as provides valuable insight towards understanding the different biochemical profiles of a biosystem.

2. Combination of ¹H Nuclear Magnetic Resonance Spectroscopy and Liquid Chromatography/Mass Spectrometry with Pattern Recognition Techniques for Evaluation of Metabolic Profile Associated with Albuminuria.

Law W, Huang P, Ong ES, Sethi S, Saw S, Ong CN, Li S.
J Proteome Res. 2009 Feb 2. [Epub ahead of print]

A method using ¹H NMR and LC/MS with pattern recognition tools such as principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (O-PLS-DA) was used to study the urinary metabolic profiles associated with an increase in urinary albumin in a general population. The normalized peak intensities obtained from ¹H NMR and LC/MS with non parametric two-tailed Mann-Whitney analysis was used for the identification of network of potential biomarkers corresponding to the increase of albumin in urine. The specificity of detecting the stated metabolites by ¹H NMR and LC/MS was demonstrated. Our preliminary data obtained demonstrated that LC/MS may produce more distinctive metabolic profiles. For the patient group, changes in alanine, kynurenic acid and xanthurenic acid might be associated with changes in the tryptophan metabolism. At the same time, other metabolites that were involved in citric acid cycle, amino acid metabolism and cellular functions were affected in the patient group. The proposed approach provided a comprehensive picture of the metabolic changes induced by the increase of protein in urine and demonstrated the advantages of using multiple diagnostic biomarkers. At the same time, the current work demonstrated as a potential cost effective solution of high throughput analytics with pattern recognition tools as applied here in a real clinical situation.

Targeted Metabolomics

Triple Quad

1. Lithospermic acid B is more responsive to silver ion (Ag⁺) than rosmarinic acid in *Salvia miltiorrhiza* hairy root cultures.

Xiao Y, Gao S, Di P, Chen J, Chen W, Zhang L.
Biosci Rep. 2009 Feb 11. [Epub ahead of print]

Lithospermic acid B (LAB) is a dimer of rosmarinic acid (RA), and has been suggested to be derived from RA, but the detailed biosynthesis process has not yet been identified. The accumulation of RA has been intensively investigated in the plant species of Boraginaceae and Lamiaceae. Our study reported an abiotic elicitor, silver ion (Ag⁺, 15 microM) did not stimulate RA accumulation but dramatically enhanced LAB from approx. 5.4% to 18.8% of dry weight in *Salvia miltiorrhiza* hairy root cultures, and their content was found perfectly competitive at each time point after treatment. Meanwhile, profiling analysis of genes and metabolites (intermediates) involved in RA synthesis pathway was performed, the result indicated several gene transcripts and metabolite accumulations showed temporal changes in abundance consistent with LAB production. Thus, a potential (putative) biosynthetic route from RA to LAB was presumed, which was suggested to be fantastically activated by Ag⁺ in *Salvia miltiorrhiza* hairy root cultures. Further intermediates monitoring and compound feeding experiments were applied to rank the strength of this hypothesis. Our study, for the first time, provides the evidence for that RA is precursor for leading to LAB synthesis.

2. Liquid chromatography/triple quadrupole tandem mass spectrometry with multiple reaction monitoring for optimal selection of transitions to evaluate nutraceuticals from olive-tree materials.

Luján RJ, Capote FP, Marinas A, de Castro MD.
Rapid Commun Mass Spectrom. 2008;22(6):855-64.

Optimal transitions have been selected for the identification and quantitation of the most interesting hydrophilic biophenols in extracts from olive-tree materials, which are of interest because of their nutraceutical properties. The tested materials were extra virgin olive oil, waste from oil production (known as alperujo), and olive-tree materials such as leaves, small branches and fruit stones. The identification and determination steps of the target biophenols are based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) with a triple quadrupole (QQQ) mass detector. The interface between the chromatograph and the QQQ was an electrospray ionization source operated in the negative ion mode. Highly selective identification of the biophenols was confirmed by multiple reaction monitoring (MRM) using the most representative transitions from the precursor ion to the different product ions. Quantitative MS/MS analysis was carried out by optimization and selection of the most sensitive transition for each analyte, which resulted in estimated detection limits of 5.10 to 11.65 ng/mL for the extracts. The biophenols were extracted from the tested samples by different methods: liquid-liquid extraction for virgin olive oil, microwave-assisted leaching for olive leaves, branches and stones, and pressurized liquid leaching for alperujo. This study provides valuable information about the most suitable source for the isolation of each nutraceutical biophenol and enables us to obtain a complete profile of them in *Olea Europaea*.

3. Rapid-resolution liquid chromatography/mass spectrometry for determination and quantitation of polyphenols in grape berries.

Cavaliere C, Foglia P, Gubbiotti R, Sacchetti P, Samperi R, Laganà A.
Rapid Commun Mass Spectrom. 2008 Oct; 22(20):3089-99.

A rapid-resolution liquid chromatography/mass spectrometric (RRLC/MS) method for detection and quantitation of polyphenols in grape berry skins and seeds has been developed. Pulp-free berry skins were treated with liquid nitrogen and ground; seeds were also ground. Then, 3 g of samples were extracted with 30 mL of a mixture of methanol/water/formic acid 70:30:1 (v/v/v) under sonication and 1 microL of the final extract was injected into two 100 x 2.1 mm i.d., 1.8 microm Zorbax Eclipse plus C18 columns connected in series. Compounds were fractionated using a gradient elution of acidified acetonitrile/methanol 50:50 (v/v)/water. Columns were thermostatted at 70 degrees C. MS was carried out on an Agilent 6410 QqQ instrument equipped with an electrospray ionization source. Positive and negative MS/MS product ion scans were used for compound identification, whereas positive full scan MS in the m/z range 200-1400 was used for quantitation. By means of mass spectra comparison, various flavonols, flavan-3-ols, anthocyanins and stilbenes were identified. Quantitation was performed by external calibration, and concentration values were corrected for matrix effect that was evaluated in separate experiments. Semi-quantitative estimation was performed for compounds for which standards were not commercially available. Recoveries ranged from 90-102% with relative standard deviation (RSD) <5%, whereas the between samples RSD was in the range 4-12%. Two surrogate standards were used for quality control. The developed method was applied to analyze the polyphenol content of three *Vitis vinifera* table cultivars at physiological maturity and after proper preservation for 6 weeks. Results demonstrated that during preservation about half of the polyphenol content was lost.

4. Rosiglitazone reduces renal and plasma markers of oxidative injury and reverses urinary metabolite abnormalities in the amelioration of diabetic nephropathy.

Zhang H, Saha J, Byun J, Schin M, Lorenz M, Kennedy RT, Kretzler M, Feldman EL, Pennathur S, Brosius FC 3rd. Am J Physiol Renal Physiol. 2008 Oct;295(4):F1071-81. Epub 2008 Jul 30.

Recent studies suggest that thiazolidinediones ameliorate diabetic nephropathy (DN) independently of their effect on hyperglycemia. In the current study, we confirm and extend these findings by showing that rosiglitazone treatment prevented the development of DN and reversed multiple markers of oxidative injury in DBA/2J mice made diabetic by low-dose streptozotocin. These diabetic mice developed a 14.2-fold increase in albuminuria and a 53% expansion of renal glomerular extracellular matrix after 12 wk of diabetes. These changes were largely abrogated by administration of rosiglitazone beginning 2 wk after the completion of streptozotocin injections. Rosiglitazone had no effect on glycemic control. Rosiglitazone had similar effects on insulin-treated diabetic mice after 24 wk of diabetes. Podocyte loss and glomerular fibronectin accumulation, other markers of early DN, were prevented by rosiglitazone in both 12- and 24-wk diabetic models. Surprisingly, glomerular GLUT1 levels did not increase and nephrin levels did not decrease in the diabetic animals; neither changed with rosiglitazone. Plasma and kidney markers of protein oxidation and lipid peroxidation were significantly elevated in the 24-wk diabetic animals despite insulin treatment and were reduced to near-normal levels by rosiglitazone. Finally, urinary metabolites were markedly altered by diabetes. Of 1,988 metabolite features identified by electrospray ionization time of flight mass spectrometry, levels of 56 were altered more than twofold in the urine of diabetic mice. Of these, 21 were returned to normal by rosiglitazone. Thus rosiglitazone has direct effects on the renal glomerulus to reduce reactive oxygen species accumulation to prevent type 1 diabetic mice from development of DN.

5. Metabonomics investigation of human urine after ingestion of green tea with gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry and (1)H NMR spectroscopy.

Law WS, Huang PY, Ong ES, Ong CN, Li SF, Pasikanti KK, Chan EC. Rapid Commun Mass Spectrom. 2008 Aug; 22(16):2436-46.

A method using gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and (1)H NMR with pattern recognition tools such as principle components analysis (PCA) was used to study the human urinary metabolic profiles after the intake of green tea. From the normalized peak areas obtained from GC/MS and LC/MS and peak heights from (1)H NMR, statistical analyses were used in the identification of potential biomarkers. Metabolic profiling by GC/MS provided a different set of quantitative signatures of metabolites that can be used to characterize the molecular changes in human urine samples. A comparison of normalized metabonomics data for selected metabolites in human urine samples in the presence of potential overlapping peaks after tea ingestion from LC/MS and (1)H NMR showed the reliability of the current approach and method of normalization. The close agreements of LC/MS with (1)H NMR data showed that the effects of ion suppression in LC/MS for early eluting metabolites were not significant. Concurrently, the specificity of detecting the stated metabolites by (1)H NMR and LC/MS was demonstrated. Our data showed that a number of metabolites involved in glucose metabolism, citric acid cycle and amino acid metabolism were affected immediately after the intake of green tea. The proposed approach provided a more comprehensive picture of the metabolic changes after intake of green tea in human urine. The multiple analytical approach together with pattern recognition tools is a useful platform to study metabolic profiles after ingestion of botanicals and medicinal plants.

6. Soluble epoxide hydrolase is a susceptibility factor for heart failure in a rat model of human disease.

Monti J, Fischer J, Paskas S, Heinig M, Schulz H, Gösele C, Heuser A, Fischer R, Schmidt C, Schirdewan A, Gross V, Hummel O, Maatz H, Patone G, Saar K, Vingron M, Weldon SM, Lindpaintner K, Hammock BD, Rohde K, Dietz R, Cook SA, Schunck WH, Luft FC, Hubner N. Nat Genet. 2008 May; 40(5):529-37.

We aimed to identify genetic variants associated with heart failure by using a rat model of the human disease. We performed invasive cardiac hemodynamic measurements in F2 crosses between spontaneously hypertensive heart failure (SHHF) rats and reference strains. We combined linkage analyses with genome-wide expression profiling and identified Ephx2 as a heart failure susceptibility gene in SHHF rats. Specifically, we found that cis variation at Ephx2 segregated with heart failure and with increased transcript expression, protein expression and enzyme activity, leading to a more rapid hydrolysis of cardioprotective epoxyeicosatrienoic acids. To confirm our results, we tested the role of Ephx2 in heart failure using knockout mice. Ephx2 gene ablation protected from pressure overload-induced heart failure and cardiac arrhythmias. We further demonstrated differential regulation of EPHX2 in human heart failure, suggesting a cross-species role for Ephx2 in this complex disease.

7. Quantification of acetylcholine in microdialysate of subcutaneous tissue by hydrophilic interaction chromatography/tandem mass spectrometry.

Fu B, Gao X, Zhang SP, Cai Z, Shen J.
Rapid Commun Mass Spectrom. 2008 May;22(10):1497-502.

It has recently been suggested that acetylcholine plays an important role in the modulation of tissue inflammation. In order to further understand the newly discovered cholinergic anti-inflammatory pathway, tracking the concentration changes of acetylcholine in tissue is required. This paper describes the development of a method coupling hydrophilic interaction chromatography with electrospray ionization tandem mass spectrometry (HILIC/ESI-MS/MS) for the separation and quantification of acetylcholine in microdialysis samples of normal rats and of rats with local inflammation. The separation of acetylcholine from interferential endogenous compounds and inorganic cations was achieved with a zwitterionic stationary phase column using isocratic elution. Low-energy collision-induced dissociation tandem mass spectrometric (CID-MS/MS) analysis was carried out in the positive ion mode using multiple reaction monitoring (MRM) of the following mass transitions: m/z 146 \rightarrow 87 for acetylcholine and m/z 155 \rightarrow 87 for the internal standard acetylcholine-D9. The limit of detection for acetylcholine was found to be 0.075 fmol on-column with a signal-to-noise ratio of 3:1. The lower limit of quantification was 0.25 fmol on-column. The calibration curves obtained for acetylcholine in blank microdialysates were linear in the ranges of 0.025-50 nM and 0.025-0.5 nM, with correlation coefficients equal to or greater than 0.9994 and 0.9969, respectively. The recoveries of acetylcholine for high (2 nM) and low (0.5 nM) concentrations were in the ranges of 90-96% and 95-109%, respectively. The coefficients of variation for intra-day and inter-day reproducibility were equal to or less than 7.3% and 10.4%, respectively. The method has been successfully applied in the measurement of acetylcholine in microdialysates from normal and inflamed rat tissue.

8. Uterine vascular function in a transgenic preeclampsia rat model.

Verlohren S, Niehoff M, Hering L, Geusens N, Herse F, Tintu AN, Plagemann A, LeNoble F, Pijnenborg R, Muller DN, Luft FC, Dudenhausen JW, Gollasch M, Dechend R.
Hypertension. 2008 Feb;51(2):547-53. Epub 2008 Jan 14.

We investigated intrauterine growth restriction, endothelial function, and uterine artery blood flow characteristics in a transgenic preeclampsia rat model with an activated renin-angiotensin system. We compared preeclamptic Sprague-Dawley (SD-PE) rats with normal pregnant Sprague-Dawley and nonpregnant Sprague-Dawley rats. We used transabdominal ultrasound and found that SD-PE rat embryos developed intrauterine growth restriction. Isolated uterine arteries from SD-PE rats incubated with phenylephrine exhibited an increased contractile response, whereas a single high dose of acetylcholine resulted in an impaired vasorelaxation compared with controls. Incremental acetylcholine doses increased relaxation of SD-PE vessels at low acetylcholine doses but caused a paradoxical contraction at higher acetylcholine doses. Indomethacin and a thromboxane-receptor antagonist (SQ 29,548) blocked this effect, suggesting maternal prostanoid-dependent endothelial dysfunction. SD-PE rats had a decreased prostacyclin (6-keto-prostaglandin F1 α):thromboxane ratio in the serum compared with normal pregnant Sprague-Dawley rats or nonpregnant Sprague-Dawley. Surprisingly, the Doppler resistance index decreased during pregnancy in SD-PE compared with normal pregnant Sprague-Dawley rats, suggesting unimpaired uteroplacental flow in the uterine artery. Umbilical flow was unchanged with absent end-diastolic flow in all of the groups. Renin-angiotensin system activation-induced preeclampsia is associated with altered placentation, modified resistance index, and endothelial dysfunction. A disturbed prostacyclin:thromboxane ratio could be an important mediator.

9. Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quadrupole mass spectrometry with multiple reaction monitoring.

Priego Capote F, Jiménez JR, Granados JM, de Castro MD.
Rapid Commun Mass Spectrom. 2007; 21(11):1745-54.

A method for determination of fat-soluble vitamins K(1), K(3), A, D(2), D(3) and E (as α - and δ -tocopherol) and metabolites 25-hydroxyvitamin D(2) and D(3) and 1,25-dihydroxyvitamin D(3) in human serum by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) in positive mode is proposed. Highly selective identification of the target compounds in serum was confirmed by the most representative transitions from precursor ion to product ion. Quantitative MS/MS analysis was carried out by multiple reaction monitoring optimizing the most sensitive transition for each analyte in order to achieve low detection limits (from 0.012 to 0.3 ng/mL estimated with serum). The analysis was performed with 1 mL of serum, which was subjected to protein precipitation, liquid-liquid extraction to an organic phase, evaporation to dryness and reconstitution with methanol. The precision of the overall method ranged from 3.17-6.76% as intra-day variability and from 5.07-11.53% as inter-day variability. The method, validated by the standard addition method, provides complete information on the fat-soluble vitamins profile, which is of interest in clinical and metabolomics studies.

10. Determination of the ubiquinol-10 and ubiquinone-10 (coenzyme Q10) in human serum by liquid chromatography tandem mass spectrometry to evaluate the oxidative stress.

Ruiz-Jiménez J, Priego-Capote F, Mata-Granados JM, Quesada JM, Luque de Castro MD.
J Chromatogr A. 2007 Dec 21;1175(2):242-8. Epub 2007 Oct 25.

A method for the rapid and simultaneous determination of ubiquinone-10 (coenzyme Q10, CoQ(10)) and the reduced form ubiquinol-10 (CoQ(10)H(2)) in human serum by LC-MS-MS with electrospray ionization (ESI) in the positive mode is here proposed. High selective identification and sensitive quantitation of both analytes have been carried out by monitoring the transition from the corresponding precursor ion to the product ion. Prior to the chromatographic analysis, serum samples (100 microl) were subject to a conventional pre-treatment based on protein precipitation, liquid-liquid extraction, evaporation to dryness and reconstitution with 95:5 methanol/hexane (v/v). The overall method has enabled to achieve low detection limits--5.49 and 15.8 ng/ml for CoQ(10) and CoQ(10)H(2), respectively--which were estimated with serum. The accuracy and potential matrix effects have been studied with spiked serum resulting recoveries between 92.82 and 106.97%. The proposed method has been applied to serum samples from healthy middle-age women, in which the CoQ(10)H(2)/CoQ(10) ratio has been used as marker of oxidative stress.

11. Analysis of underivatized polyamines by reversed phase liquid chromatography with electrospray tandem mass spectrometry.

Häkkinen MR, Keinänen TA, Vepsäläinen J, Khomutov AR, Alhonen L, Jänne J, Auriola S.
J Pharm Biomed Anal. 2007 Nov 30;45(4):625-34. Epub 2007 Sep 7.

A reversed phase liquid chromatography-electrospray ionization-tandem mass spectrometric method (RP-LC-ESI-MS/MS) was developed to separate and detect polyamines with minimal sample pre-treatment and without any derivatization. Prior to MS/MS analysis, a complete chromatographic separation of polyamines was achieved by a linear gradient elution using heptafluorobutyric acid as a volatile ion-pair modifier, and signal suppression was prevented by post-column addition of 75% propionic acid in isopropanol to the column flow. The developed method was successfully applied to the identification of metabolites formed from N(1), N(12)-diethylspermine in the reaction catalyzed by recombinant human polyamine oxidase and to the detection of eight different polyamines in a standard mixture.

12. Saturated very-long-chain fatty acids promote cotton fiber and Arabidopsis cell elongation by activating ethylene biosynthesis.

Qin YM, Hu CY, Pang Y, Kastaniotis AJ, Hiltunen JK, Zhu YX.
Plant Cell. 2007 Nov;19(11):3692-704. Epub 2007 Nov 9.

Fatty acids are essential for membrane biosynthesis in all organisms and serve as signaling molecules in many animals. Here, we found that saturated very-long-chain fatty acids (VLCFAs; C20:0 to C30:0) exogenously applied in ovule culture medium significantly promoted cotton (*Gossypium hirsutum*) fiber cell elongation, whereas acetochlor (2-chloro-N-[ethoxymethyl]-N-[2-ethyl-6-methyl-phenyl]-acetamide; ACE), which inhibits VLCFA biosynthesis, abolished fiber growth. This inhibition was overcome by lignoceric acid (C24:0). Elongating fibers contained significantly higher amounts of VLCFAs than those of wild-type or fuzzless-lintless mutant ovules. Ethylene nullified inhibition by ACE, whereas C24:0 was inactive in the presence of the ethylene biosynthesis inhibitor (l-[2-aminoethoxyvinyl]-glycine), indicating that VLCFAs may act upstream of ethylene. C24:0 induced a rapid and significant increase in ACO (for 1-aminocyclopropane-1-carboxylic acid oxidase) transcript levels that resulted in substantial ethylene production. C24:0 also promoted Ser palmitoyltransferase expression at a later stage, resulting in increased sphingolipid biosynthesis. Application of C24:0 not only stimulated *Arabidopsis thaliana* root cell growth but also complemented the cut1 phenotype. Transgenic expression of Gh KCS13/CER6, encoding the cotton 3-ketoacyl-CoA synthase, in the cut1 background produced similar results. Promotion of *Arabidopsis* stem elongation was accompanied by increased ACO transcript levels. Thus, VLCFAs may be involved in maximizing the extensibility of cotton fibers and multiple *Arabidopsis* cell types, possibly by activating ethylene biosynthesis.

13. Simultaneous microwave-assisted solid-liquid extraction of polar and nonpolar compounds from alperujo.

Pérez-Serradilla JA, Japón-Luján R, Luque de Castro MD.
Anal Chim Acta. 2007 Oct 17;602(1):82-8. Epub 2007 Sep 14.

Microwave-assisted extraction (MAE) has been used for the simultaneous isolation of polar and nonpolar compounds from alperujo using methanol-water and n-hexane as extractant system. Multivariate methodology has been used to establish the optimum extraction conditions. The target fractions (phenol compounds and fatty acids) were quantitatively extracted within 14 min. Following leaching and separation of the two phases by centrifugation, the polar and nonpolar fractions were analysed by HPLC-MS-MS and GC-MS, respectively. The proposed method was compared with the reference method for the isolation of each fraction (Folch method and stirring-based method for fatty acids and biophenols extraction, respectively) in terms of efficiency and extract composition. The paramount importance of both fractions, the simplicity of the MAE approach and the low costs of the raw material make advisable the implementation of the proposed method at an industrial scale.

TOF

1. Simultaneous Quantification of Metabolites Involved in Central Carbon and Energy Metabolism Using Reversed-Phase Liquid Chromatography-Mass Spectrometry and in Vitro (13) C Labeling.

Yang WC, Sedlak M, Regnier FE, Mosier N, Ho N, Adamec J.
Anal Chem. 2008 Nov 14. [Epub ahead of print]

Comprehensive analysis of intracellular metabolites is a critical component of elucidating cellular processes. Although the resolution and flexibility of reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) makes it one of the most powerful analytical tools for metabolite analysis, the structural diversity of even the simplest metabolome provides a formidable analytical challenge. Here we describe a robust RPLC-MS method for identification and quantification of a diverse group of metabolites ranging from sugars, phosphosugars, and carboxylic acids to phosphocarboxylic acids, nucleotides, and coenzymes. This method is based on in vitro derivatization with a (13)C-labeled tag that allows internal standard based quantification and enables separation of structural isomer pairs like glucose 6-phosphate and fructose 6-phosphate in a single chromatographic run. Calibration curves for individual metabolites showed linearity ranging over more than 2 orders of magnitude with correlation coefficients of $R^2 > 0.9975$. The detection limits at a signal-to-noise ratio of 3 were below 1.0 μM (20 pmol) for most compounds. Thirty common metabolites involved in glycolysis, the pentose phosphate pathway, and tricarboxylic acid cycle were identified and quantified from yeast lysate with a relative standard deviation of less than 10%.

GC/MS

Discovery Metabolomics

1. Integrated analysis of serum and liver metabolome in liver transplanted rats by gas chromatography coupled with mass spectrometry.

Wang Y, Tao Y, Lin Y, Liang L, Wu Y, Qu H, Liang T, Cheng Y.
Anal Chim Acta. 2009 Feb 2;633(1):65-70. Epub 2008 Nov 25.

In this paper, we present a metabolomic method for the investigation of abnormal metabolic process in both serum and liver tissue of liver transplanted rats. Syngeneic transplantation was performed on male Lewis rats. The serum and grafted liver on day 1, 3, and 7 post-transplant were collected to analyze endogenous metabolites using gas chromatography coupled with mass spectrometry (GC-MS). The method was validated with acceptable linearity, precision, and repeatability. Thirty-four metabolites in serum and 29 metabolites in liver were identified. Results of correlation analysis illustrated metabolites with similar function exhibited similar variations in liver and serum. The data processed by principle component analysis (PCA) showed time-dependent biochemical variations. As a consequence, the present study may offer specific putative pathways in the pathophysiological mechanism of orthotopic liver transplantation.

2. Hydroxylated metabolites of polybrominated diphenyl ethers in human blood samples from the United States.

Qiu X, Bigsby RM, Hites RA.
Environ Health Perspect. 2009 Jan;117(1):93-8. Epub 2008 Aug 1.

BACKGROUND: A previous study from our laboratory showed that polybrominated diphenyl ethers (PBDEs) were metabolized to hydroxylated PBDEs (HO-PBDEs) in mice and that para-HO-PBDEs were the most abundant and, potentially, the most toxic metabolites. **OBJECTIVE:** The goal of this study was to determine the concentrations of HO-PBDEs in blood from pregnant women, who had not been intentionally or occupationally exposed to these flame retardants, and from their newborn babies. **METHODS:** Twenty human blood samples were obtained from a hospital in Indianapolis, Indiana, and analyzed for both PBDEs and HO-PBDEs using electron-capture negative-ionization gas chromatographic mass spectrometry. **RESULTS:** The metabolite pattern of HO-PBDEs in human blood was quite different from that found in mice; 5-HO-BDE-47 and 6 HO-BDE-47 were the most abundant metabolites of BDE-47, and 5'-HO-BDE-99 and 6'-HO-BDE-99 were the most abundant metabolites of BDE-99. The relative concentrations between precursor and corresponding metabolites indicated that BDE-99 was more likely to be metabolized than BDE-47 and BDE-100. In addition, three bromophenols were also detected as products of the cleavage of the diphenyl ether bond. The ratio of total hydroxylated metabolites relative to their PBDE precursors ranged from 0.10 to 2.8, indicating that hydroxylated metabolites of PBDEs were accumulated in human blood. **CONCLUSIONS:** The quite different PBDE metabolite pattern observed in humans versus mice indicates that different enzymes might be involved in the metabolic process. Although the levels of HO-PBDE metabolites found in human blood were low, these metabolites seemed to be accumulating.

3. A serum metabolomic investigation on hepatocellular carcinoma patients by chemical derivatization followed by gas chromatography/mass spectrometry.

Xue R, Lin Z, Deng C, Dong L, Liu T, Wang J, Shen X.
Rapid Commun Mass Spectrom. 2008 Oct;22(19):3061-8.

The purpose of this study was to investigate the serum metabolic difference between hepatocellular carcinoma (HCC, n = 20) male patients and normal male subjects (n = 20). Serum metabolome was detected through chemical derivatization followed by gas chromatography/mass spectrometry (GC/MS). The acquired GC/MS data was analyzed by stepwise discriminant analysis (SDA) and support vector machine (SVM). The metabolites including butanoic acid, ethanimidic acid, glycerol, L-isoleucine, L-valine, aminomalonic acid, D-erythrose, hexadecanoic acid, octadecanoic acid, and 9,12-octadecadienoic acid in combination with each other gave the strongest segregation between the two groups. By applying these variables, our method provided a diagnostic model that could well discriminate between HCC patients and normal subjects. More importantly, the error count estimate for each group was 0%. The total classifying accuracy of the discriminant function tested by SVM 20-fold cross validation was 75%. This technique is different from traditional ones and appears to be a useful tool in the area of HCC diagnosis.

4. Arabidopsis 10-formyl tetrahydrofolate deformylases are essential for photorespiration.

Collakova E, Goyer A, Naponelli V, Krassovskaya I, Gregory JF 3rd, Hanson AD, Shachar-Hill Y. *Plant Cell*. 2008 Jul; 20(7):1818-32. Epub 2008 Jul 15.

In prokaryotes, PurU (10-formyl tetrahydrofolate [THF] deformylase) metabolizes 10-formyl THF to formate and THF for purine and Gly biosyntheses. The *Arabidopsis thaliana* genome contains two putative purU genes, At4g17360 and At5g47435. Knocking out these genes simultaneously results in plants that are smaller and paler than the wild type. These double knockout (dKO) mutant plants show a 70-fold increase in Gly levels and accumulate elevated levels of 5- and 10-formyl THF. Embryo development in dKO mutants arrests between heart and early bent cotyledon stages. Mature seeds are shriveled, accumulate low amounts of lipids, and fail to germinate. However, the dKO mutant is only conditionally lethal and is rescued by growth under nonphotorespiratory conditions. In addition, culturing dKO siliques in the presence of sucrose restores normal embryo development and seed viability, suggesting that the seed and embryo development phenotypes are a result of a maternal effect. Our findings are consistent with the involvement of At4g17360 and At5g47435 proteins in photorespiration, which is to prevent excessive accumulation of 5-formyl THF, a potent inhibitor of the Gly decarboxylase/Ser hydroxymethyltransferase complex. Supporting this role, deletion of the At2g38660 gene that encodes the bifunctional 5,10-methylene THF dehydrogenase/5,10-methenyl THF cyclohydrolase that acts upstream of 5-formyl THF formation restored the wild-type phenotype in dKO plants.

5. Application of dissimilarity indices, principal coordinates analysis, and rank tests to peak tables in metabolomics of the gas chromatography/mass spectrometry of human sweat.

Xu Y, Gong F, Dixon SJ, Brereton RG, Soini HA, Novotny MV, Oberzaucher E, Grammer K, Penn DJ. *Anal Chem*. 2007 Aug 1; 79(15):5633-41. Epub 2007 Jun 30.

The majority of works in metabolomics employ approaches based on principal components analysis (PCA) and partial least-squares, primarily to determine whether samples fall within large groups. However, analytical chemists rarely tackle the problem of individual fingerprinting, and in order to do this effectively, it is necessary to study a large number of small groups rather than a small number of large groups and different approaches are required, as described in this paper. Furthermore, many metabolomic studies on mammals and humans involve analyzing compounds (or peaks) that are present in only a certain portion of samples, and conventional approaches of PCA do not cope well with sparse matrices where there may be many 0s. There is, however, a large number of qualitative similarity measures available for this purpose that can be exploited via principal coordinates analysis (PCO). It can be shown that PCA scores are a specific case of PCO scores, using a quantitative similarity measure. A large-scale study of human sweat consisting of nearly 1000 gas chromatography/mass spectrometry analyses from the sweat of an isolated population of 200 individuals in Carinthia (Southern Austria) sampled once per fortnight over 10 weeks was employed in this study and grouped into families. The first step was to produce a peak table requiring peak detection, alignment, and integration. Peaks were reduced from 5080 to 373 that occurred in at least 1 individual over 4 out of 5 fortnights. Both qualitative (presence/absence) and quantitative (equivalent to PCA) similarity measures can be computed. PCO and the Kolomorgov-Smirnoff (KS) rank test are applied to these similarity matrices. It is shown that for this data set there is a reproducible individual fingerprint, which is best represented using the qualitative similarity measure as assessed both by the Hotelling t² statistic as applied to PCO scores and the probabilities associated with the KS rank test.

6. Systematic identification of conserved metabolites in GC/MS data for metabolomics and biomarker discovery.

Styczynski MP, Moxley JF, Tong LV, Walther JL, Jensen KL, Stephanopoulos GN. *Anal Chem*. 2007 Feb 1; 79(3):966-73.

Analysis of metabolomic profiling data from gas chromatography-mass spectrometry (GC/MS) measurements usually relies upon reference libraries of metabolite mass spectra to structurally identify and track metabolites. In general, techniques to enumerate and track unidentified metabolites are nonsystematic and require manual curation. We present a method and software implementation, freely available at <http://spectconnect.mit.edu>, that can systematically detect components that are conserved across samples without the need for a reference library or manual curation. We validate this approach by correctly identifying the components in a known mixture and the discriminating components in a spiked mixture. Finally, we demonstrate an application of this approach with a brief analysis of the *Escherichia coli* metabolome. By systematically cataloguing conserved metabolite peaks prior to data analysis methods, our approach broadens the scope of metabolomics and facilitates biomarker discovery.

7. Metabolite profiling studies in *Saccharomyces cerevisiae*: an assisting tool to prioritize host targets for antiviral drug screening.

Schneider K, Krömer JO, Wittmann C, Alves-Rodrigues I, Meyerhans A, Diez J, Heinzle E.
Microb Cell Fact. 2009 Jan 30;8:12.

The cellular proteins Pat1p, Lsm1p, and Dhh1p are required for the replication of some positive-strand viruses and therefore are potential targets for new antiviral drugs. To prioritize host targets for antiviral drug screening a comparative metabolome analysis in *Saccharomyces cerevisiae* reference strain BY4742 *his3Delta1 leu2Delta0 lys2Delta0 ura3Delta0* and deletion strains *pat1Delta*, *lsm1Delta* and *dhh1Delta* was performed. RESULTS: GC/MS analysis permitted the quantification of 47 polar metabolites and the identification of 41 of them. Metabolites with significant variation between the strains were identified using partial least squares to latent structures discriminate analysis (PLS-DA). The analysis revealed least differences of *pat1Delta* to the reference strain as characterized by Euclidian distance of normalized peak areas. The growth rate and specific production rates of ethanol and glycerol were also most similar with this strain. CONCLUSION: From these results we hypothesize that the human analog of yeast Pat1p is most likely the best drug target candidate.

8. Urinary markers of adrenarche: reference values in healthy subjects, aged 3-18 years.

Remer T, Boye KR, Hartmann MF, Wudy SA.
J Clin Endocrinol Metab. 2005 Apr;90(4):2015-21. Epub 2005 Jan 25.

Information on the urinary excretion of dehydroepiandrosterone (DHEA) and its direct metabolites is scarce for healthy subjects during growth. We used gas chromatography-mass spectrometry urinary steroid profiling to noninvasively study adrenarchal metabolome in 400 healthy subjects, aged 3-18 yr. Urinary 24-h excretion rates of DHEA did not increase significantly before age 7-8 yr. However, DHEA together with its 16 α -hydroxylated downstream metabolites, 16 α -hydroxy-DHEA and 3 β ,16 α ,17 β -androstetriol (DHEA&M), as well as the DHEA metabolite, 5 α -androstene-3 β ,17 β -diol (ADIOL), and the sum of major urinary androgen metabolites (C19) rose consistently from the youngest to the oldest age group. The significant increases ($P < 0.01$) observed for 24-h excretion rates of C19, ADIOL, and DHEA&M were 2- to 4-fold in boys and girls between age 3 and 8 yr. DHEA&M, for example, rose from about 20 to 80 microg/d ($P < 0.0001$) during this period. Until the age of 16 yr, DHEA&M excretion also increased to nearly 1000 microg/d. Patterns of steroidogenic enzyme activities were assessed (from definite ratios of urinary steroid metabolites) for 21-hydroxylase, 3 β -hydroxysteroid dehydrogenase, 17 β -hydroxysteroid dehydrogenase, and 5 α -reductase. Our results indicate for healthy boys and girls that adrenarche is a gradual process starting much earlier than hitherto believed. Efficient metabolism of DHEA, especially to 16-hydroxylated steroids, may explain the almost constant levels seen for this steroid until age 7-8 yr. The established reference values for DHEA, DHEA&M, ADIOL, C19 (including androsterone and etiocholanolone), and urinary parameters of steroidogenic enzyme activities could be useful to identify nutritional, environmental, and pathophysiological interrelations with the progressive maturational process of adrenarche. Our data may also be used as reference data for the diagnosis of steroid-related disorders.

9. Identification of 19 new metabolites induced by abnormal amino acid conjugation in isovaleric acidemia.

Loots DT, Erasmus E, Mienie LJ.
Clin Chem. 2005 Aug;51(8):1510-2.

10. Metabolic profiling of the sink-to-source transition in developing leaves of quaking aspen.

Jeong ML, Jiang H, Chen HS, Tsai CJ, Harding SA.
Plant Physiol. 2004 Oct; 136(2):3364-75. Epub 2004 Sep 24.

Profiles of small polar metabolites from aspen (*Populus tremuloides* Michx.) leaves spanning the sink-to-source transition zone were compared. Approximately 25% of 250 to 300 routinely resolved peaks were identified, with carbohydrates, organic acids, and amino acids being most abundant. Two-thirds of identified metabolites exhibited greater than 4-fold changes in abundance during leaf ontogeny. In the context of photosynthetic and respiratory measurements, profile data yielded information consistent with expected developmental trends in carbon-heterotrophic and carbon-autotrophic metabolism. Suc concentration increased throughout leaf expansion, while hexose sugar concentrations peaked at mid-expansion and decreased sharply thereafter. Amino acid contents generally decreased during leaf expansion, but an early increase in Phe and a later one in Gly and Ser reflected growing commitments to secondary metabolism and photorespiration, respectively. The assimilation of nitrate and utilization of stored Asn appeared to be marked by sequential changes in malate concentration and Asn transaminase activity. Principal component and hierarchical clustering analysis facilitated the grouping of cell wall maturation (pectins, hemicelluloses, and oxalate) and membrane biogenesis markers in relation to developmental changes in carbon and nitrogen assimilation. Metabolite profiling will facilitate investigation of nitrogen use and cellular development in *Populus* sp. varying widely in their growth and pattern of carbon allocation during sink-to-source development and in response to stress.

Targeted Metabolomics

1. 1H NMR and GC/MS metabolomics of earthworm responses to sub-lethal DDT and endosulfan exposure

McKelvie JR, Yuk J, Xu Y, Simpson AJ and Simpson MJ
Metabolomics 2009 5:84–94

The metabolic response of the earthworm *Eisenia fetida* to two pesticides, dichlorodiphenyltrichloroethane (DDT) and endosulfan, was characterized in contact tests using proton nuclear magnetic resonance (¹H NMR) and principal component analysis (PCA). PCA loading plots suggested that maltose, leucine and alanine were important metabolites contributing to the differences in dosed and control earthworms for both compounds at doses of 0.5, 1.0 and 2.0 µg/cm². Gas chromatography/mass spectrometry (GC/MS) was used to quantify the metabolites identified in *E. fetida* and determine if the changes in maltose, leucine and alanine following exposure to DDT and endosulfan (at 0.5 and 1.0 µg/cm²) were reproducible and greater than the natural variability. Quantification by GC/MS suggested that maltose was not a reliable biomarker since it both increased and decreased in earthworms exposed to DDT and increased by just 3% with exposure to endosulfan. Leucine was not stable with the GC/MS derivatization method used in this study and could not be confirmed as a reliable biomarker. However, alanine consistently increased for both DDT and endosulfan exposed *E. fetida*. Alanine showed considerable variability in control earthworms (±41.6%), yet the variability in alanine to glycine ratios was just ±10.5%. Increases in the alanine to glycine ratio were statistically significant at the $P = 0.05$ level for the 1.0 µg/cm² DDT dose and both the 0.5 and 1.0 µg/cm² endosulfan doses, suggesting that deviations from the normal homeostatic ratio of 1.5 for alanine to glycine is a potential biomarker of DDT and endosulfan exposure warranting further study.

2. Microbial metabolomics with gas chromatography/mass spectrometry.

Koek MM, Muilwijk B, van der Werf MJ, Hankemeier T.
Anal Chem. 2006 Feb 15;78(4):1272-81.

An analytical method was set up suitable for the analysis of microbial metabolomes, consisting of an oximation and silylation derivatization reaction and subsequent analysis by gas chromatography coupled to mass spectrometry. Microbial matrixes contain many compounds that potentially interfere with either the derivatization procedure or analysis, such as high concentrations of salts, complex media or buffer components, or extremely high substrate and product concentrations. The developed method was extensively validated using different microorganisms, i.e., *Bacillus subtilis*, *Propionibacterium freudenreichii*, and *Escherichia coli*. Many metabolite classes could be analyzed with the method: alcohols, aldehydes, amino acids, amines, fatty acids, (phospho-) organic acids, sugars, sugar acids, (acyl-) sugar amines, sugar phosphate, purines, pyrimidines, and aromatic compounds. The derivatization reaction proved to be efficient (>50% transferred to derivatized form) and repeatable (relative standard deviations <10%). Linearity for most metabolites was satisfactory with regression coefficients better than 0.996. Quantification limits were 40-500 pg on-column or 0.1-0.7 mmol/g of microbial cells (dry weight). Generally, intrabatch precision (repeatability) and interbatch precision (reproducibility) for the analysis of metabolites in cell extracts was better than 10 and 15%, respectively. Notwithstanding the nontargeted character of the method and complex microbial matrix, analytical performance for most metabolites fit the requirements for target analysis in bioanalysis. The suitability of the method was demonstrated by analysis of *E. coli* samples harvested at different growth phases.

3. A metabolomic study of substantial equivalence of field-grown genetically modified wheat.

Baker JM, Hawkins ND, Ward JL, Lovegrove A, Napier JA, Shewry PR, Beale MH.
Plant Biotechnol J. 2006 Jul;4(4):381-92.

The 'substantial equivalence' of three transgenic wheats expressing additional high-molecular-weight subunit genes and the corresponding parental lines (two lines plus a null transformant) was examined using metabolite profiling of samples grown in replicate field trials on two UK sites (Rothamsted, Hertfordshire and Long Ashton, near Bristol) for 3 years. Multivariate comparison of the proton nuclear magnetic resonance spectra of polar metabolites extracted with deuterated methanol-water showed a stronger influence of site and year than of genotype. Nevertheless, some separation between the transgenic and parental lines was observed, notably between the transgenic line B73-6-1 (which had the highest level of transgene expression) and its parental line L88-6. Comparison of the spectra showed that this separation resulted from increased levels of maltose and/or sucrose in this transgenic line, and that differences in free amino acids were also apparent. More detailed studies of the amino acid composition of material grown in 2000 were carried out using gas chromatography-mass spectrometry. The most noticeable difference was that the samples grown at Rothamsted consistently contained larger amounts of acidic amino acids (glutamic, aspartic) and their amides (glutamine, asparagine). In addition, the related lines, L88-6 and B73-6-1, both contained larger amounts of proline and gamma-aminobutyric acid when grown at Long Ashton than at Rothamsted. The results clearly demonstrate that the environment affects the metabolome and that any differences between the control and transgenic lines are generally within the same range as the differences observed between the control lines grown on different sites and in different years.

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