

metabomeeting 2008

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Poster 1

Introducing Biological Indeterminacy In Mutant Path Modeling As A Gene Specific Irreducible Pattern Of Chemical Bonds Confounded In The NIR-Spectral Phenome

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There is a need for reconceptualization of the last 50 years of experience in classical and molecular genetics, when new high throughput physical chemical descriptors are introduced at different –ome levels. The classical geneticist S.S. Chetverikov's vision from 1926, that all active genes are both contributing to and dependent on the *internal "genetic milieu"* of the cell, is recently revived in literature by emphasizing the fine-tuning and robustness of metabolic fingerprints. By analyzing barley endosperm mutants by NIR in an isogenic background, we have observed gene specific, remarkably finely tuned highly reproducible MSC log1/R spectra that cannot be accurately modelled by chemometric data compression. The mutant gene *lys5.f* is a structural mutant with a lesion in a gene for an ADP-glucose pyrophosphorylase (AGPase) transporter in starch synthesis. It was revealed by NIR that the *lys5.f* gene changes the synthesis from starch (from 55 to 30% d.m) to β -glucan (from 5 to 20% d.m). Consistent gene specific spectra were identified in developing seeds from 9 days post anthesis (dpa). A peak in β -glucan synthesis was observed at day 20 concomitantly with an increase in water content compared to the control of 7-9% persisting up to day 39 dpa. It was concluded that the increase of the water-absorbing β -glucan is likely to change water activity in the cell in relation to the control from 16 to 39 dpa. In principle all active enzymes could be affected. The change in water activity will derail a classical hard path way model following the *lys5.f* effect from alpha-glucan to β -glucan and onwards. BIOLOGICAL INDETERMINACY is now introduced as a probabilistic component of the internal cell milieu here mainly induced by water activity. The new kind of probability is finely tuned by the self-organisation of the barley endosperm cell "as a narrow path between determinism and pure chance" (I. Prigogine). It cannot be modelled by mathematics. However, the result can be overviewed as a whole spectral phenomenological pattern that by large is irreducible. The reproducible differential NIR pattern between the *lys5.f* spectrum and that of the control summarizes on the level of chemical bonds both the primary (deficient AGPase transporter) and the secondary pleiotropic gene effects. Large pleiotropic effects of the *lys5.f* mutant were verified in the proteome pattern, in seed fat content (+50%) and by a change in tocopherol pattern. The NIR spectral definitions on the phenome, pleiotropy and Biological Indeterminacy of general significance in Systems Biology is discussed by L. Munck in *J. Chemometrics* 21:406-426 (2007). Before data compression all kinds of omics data should now be graphically inspected and further visualised by computer programmes (see www.Latentix.com) to reveal the finely tuned genetic patterns that may already be computed by the "cell computer".

Poster 2

Bio-Spectroscopy And Functional Genomics For Optimized Milk And Meat Quality

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This is a summary status report of a new 3-year research project, financed by the Norwegian Research Council, which was recently started at Campus Ås in cooperation with the companies TINE, GILDE and GENO. It is coordinated with several other on-going Norwegian research projects within functional genomics, bio-chemometrics and bio-spectroscopy.

The project is intended to yield better understanding of genetic and management-related variations in milk quality. This includes storing and analyzing FTIR Foss Milkoscan spectra of one million milk samples per year. Meat quality variations will likewise be studied.

Large amounts of data on milk and meat quality, obtained by modern measurement techniques of various kinds, will be related to large amounts of genomic measurements, pedigree and management data, using new multivariate data-modeling techniques from bio-chemometrics and genetics.

Some preliminary quantitative results will be reported, for predicting the fatty acid profile of individual milk samples from high-speed FTIR bio-spectroscopy. Multivariate calibration was done with cross-model-validated Power-PLSR relating about 1000 FTIR channels (X) to about 30 GC-MS- determined fatty acids and their aggregates, via about 20 latent variables, based on measurements in several hundred samples from an animal feeding experiment.

The purpose of this presentation is to inform colleagues about the new opportunities that modern bio-spectroscopy and bio-chemometrics can offer in conjunction with modern genetics and genomics.

Poster 3

Cold Acclimation Duration And Freezing Temperatures – Distinct Intra-Specific Metabolic Phenotypes Of Two Geographically Isolated Plant Populations

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Plant populations growing at the margin of their range may exhibit traits that indicate genetic differentiation and adaptation to their local abiotic environment. *Arabidopsis lyrata* subspecies *petraea*, a close relative to the model species *A. thaliana*, is sparsely distributed across Europe. Small populations occur in Wales, Ireland, Scotland, Norway and Sweden with larger populations in Iceland. Our goal as a project consortium (<http://www.petraea.shef.ac.uk/>) is to measure the similarities and differences between geographically isolated populations of this species using ecological, genomic, transcriptomic, proteomic and metabolomic data. This aims to provide a clearer understanding of the mechanisms involved in limiting plant distribution, especially in relation to varied temperatures among the sites. We have found that the survival of young *A. petraea* after exposure to sub-zero temperatures is dependent on the duration of pre-shock cold acclimation time. This duration is population specific as some populations respond faster to cold acclimation than other populations. Therefore, we investigated whether such differences in cold acclimation duration and survival can be detected at the global metabolic level. Seeds of European *A. l. petraea* were obtained from populations along a latitudinal gradient (High altitude Norwegian and Lowland Irish) followed by germination and growth in a controlled cabinet environment. Young Helin (Norway) and Leitrim (Ireland) plants were subjected to various conditions. These were: Control (20/15 °C day night temperature 12/12 hours); 2 and 14 day cold 2 °C acclimation; 2 day cold acclimation followed by a -9 °C shock or a 14 day cold acclimation followed by -9 °C shock. Global metabolite fingerprints were measured at the same initial developmental stage. Metabolite fingerprints were obtained for populations of *A. l. petraea* by non-targeted direct-injection mass spectrometry (DIMS) using a MicroMass LCT ESI-ToF mass spectrometer. Metabolite fingerprints of each population were assessed using principal component analysis (PCA). A list of the *m/z* values, and possible compounds based on monoisotopic mass that discriminate between populations, was obtained using our in-house binning and metabolite identification software. Principal component analysis of metabolite fingerprints revealed global metabolic phenotypes for each population, cold acclimation duration and freezing temperature. Cold acclimation had a clear effect on the metabolic fingerprints of the Helin samples, with clear clustering of control; 2 day acclimation and 14 day acclimation samples. However, the difference between control and acclimation times in the metabolic fingerprints of the Leitrim samples was less distinct. These results suggest that the Leitrim control plants are metabolically similar to cold acclimated plants, especially after 2 days acclimation. However, Helin plants are metabolically different after cold acclimation. As the acclimated Leitrim plants were metabolically similar to control plants, this may help explain why after a 2 day acclimation period prior to freezing shocks, the Leitrim plants perform better in terms of percent survival after freezing at -9 °C than Helin plants. These combined results suggest that Leitrim (Irish) plants are 'slow acclimators', adapted to shorter cold spells prior to freezing or frosts. However, the Helin (Norwegian) plants are 'long acclimators', taking longer to adapt to cold spells before freezing conditions. These results suggest that there is significant natural variation in metabolism among these populations of *A. l. petraea*. This will ultimately aid our understanding of the complex genetic and environmental factors underlying plant metabolism and plant distribution.

Poster 4

Assessing Of Complexity And Reliability Of Urinary Nucleosides Patterns By Their Metabonomic Analysis

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Urinary nucleosides are an interesting object of biomedical analyses because their elevated level could be observed in some cancer diseases. Nowadays, however, there is limited information about their relationship with current health status and internal variability of nucleosides patterns. Metabonomic studies can be of utmost help in describing of nucleosides patterns complexity and defining biological reliability of conducted studies. Application of selective analytical procedures and advances chemometric tools may lead to better understanding of relations of metabolites on an organism level. Our study is concentrated on development and implementation of an appropriate strategy to analyze nucleosides profiles from urine of healthy and urogenital cancer patients. This strategy comprises selective solid phase extraction (SPE), validated capillary electrophoresis (CE) method and different chemometric tools such as preprocessing and pattern recognition ones. A metabolic variable could be a level of a single metabolite e.g. pseudouridine as well as a single point of fingerprint-like trace obtained by capillary electrophoresis e.g. point #240 corresponding to maximum of electrophoretic peak of that compound. In this study, three parallel approaches of metabolic data analysis were compared by means of data informativity. Firstly, peaks of twenty nucleosides were detected and their levels calculated. Secondly, the electrophoretic peaks were treated with multivariate curve resolution techniques (such as multivariate curve resolution-alternating least squares method (MCR-ALS)), to triple the number of analyzed nucleosides. Lastly, the whole electrophoretic profiles were used after application of warping to eliminate peaks shift. Then, obtained data were explored by pattern recognition methods such as principal component analysis (PCA) and cluster analysis (CA). This allowed assessment of data structure and the relations of analyzed metabolites. In each case, analyzed data expressed different information about studied urine samples and nucleosides, that together enhanced the interpretation of the results. That supports the idea of reliability of metabonomic studies, which combine numerous data and performed analyses.

Poster 5

Investigating The Metabolomic Response Of *Lolium Perenne* To A PEG Induced Drought Stress

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Perennial ryegrass is the principal forage grass species used in temperate grassland systems. Predictions for changing climate suggest a shift towards warmer and dryer summers across the British Isles with greater temperature extremes, making drought tolerance an important target trait in breeding programmes. A study was conducted to investigate the genetic basis of phenotypic and metabolic plasticity to drought for a set of perennial ryegrass lines. An ecotype, 'NZ02', documented as having drought tolerance was obtained from the USDA seed bank collection. The phenotypic response of this line to an osmotic PEG induced drought stress was compared to a commercial variety and was found to exhibit superior drought tolerance/avoidance. The metabolomic profiles of stressed vs. non stressed material (above and below ground biomass) were compared for both lines in an attempt to identify metabolites (and pathways) responsible for the increased drought tolerance/avoidance of the 'NZ02' ecotype. Both polar and non-polar metabolites were analyzed by GC-TOF-MS. The results were subsequently related to data generated from a transcriptomic differential expression study carried out in the 'NZ02' ecotype in order to understand the underlying mechanisms of drought tolerance/avoidance in this ecotype.

Poster 6

Urinary Metabolic Profiles: Possible Relationship To Blood Pressure Differences Between Northern And Southern Chinese, The INTERMAP Study

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Background: Epidemiological data show that higher average blood pressure (BP) for northern than southern Chinese are attributable to multiple dietary factors. Data are lacking on metabolic pathways involved, and relations among dietary factors, diet-related metabolites and BP.

Methods: Chinese participants from the INTERMAP Study (an international study on macronutrients and BP conducted in 1996-99) were from northern (Beijing, n=272, and Shanxi, n=289) and southern (Guangxi, n=278) areas, ages 40 to 59 years, 50% women, with 8 BP measurements, 4 detailed 24-hour dietary recalls, 2 timed 24-hour urine specimens and data on multiple possible confounders. Proton nuclear magnetic resonance (¹H-NMR) spectroscopy was done on all urine samples (including splits at source as external standards). Multivariate statistical analyses were used to identify patterns/groupings within the NMR spectra.

Results: Metabonomics was used to classify the urine samples and to assess the technique as a metabolic profiling tool in molecular epidemiology. Urinary metabolic profiles for northern and southern Chinese were different: northern Chinese showed high urinary branch-chain amino acids, lactate, guanidoacetate and hippurate; southern Chinese showed higher urinary creatine and three currently unknown metabolites. Clear differences could be seen within northern Chinese populations: Shanxi population showed higher urinary dimethylglycine, N-acetylglycoproteins and 2-methylerythritol (2ME) level; the Beijing showed higher level of trimethylamine N-oxide (TMAO), glycine, guanidoacetate and hippurate. These metabolite differences were in part due to dietary differences. The main source of TMAO is marine fish and 2ME is a common constituent in herbaceous plants. Different levels of hippurate excretion suggested differences in gut microbiota composition between northern and southern Chinese.

Conclusions: ¹H-NMR spectroscopy identified urinary metabolites possibly related to BP differences between northern and southern Chinese. Urinary metabolomic profiles may enhance understanding of the complex interrelationships among dietary factors, mammalian microbial metabolism, endogenous metabolic pathways, and BP.

Poster 7

Discrimination Between Structural And Non-Structural Correlations In NMR Statistical Total Spectroscopy

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Metabolic Profiling research is underpinned by analytical techniques such as NMR spectroscopy for the identification and measurement of metabolite concentration. Many resonances in ^1H NMR metabolic profiles cannot be immediately identified and thus peak assignment is a major bottleneck in metabolic profiling. Statistical Total Correlation Spectroscopy (STOCSY) is a tool based on the Pearson correlation developed to aid metabolite identification. At present, there is no guideline for the correlation level that discriminates a structural from a non structural correlation. We have studied the relevant factors that influence the correlation space of NMR metabolic profiles and assessed the ability of the method to identify structural correlations. Using a large sample of 1048 ^1H NMR spectra of urine from normal laboratory rats, a rigorous analysis demonstrated a significant difference between structural and non structural correlation distributions. The choice of metabolites used to access the structural correlation distribution has an important effect on the uncertainty of the estimations. There is a proportional relationship between sample size and the predictive power of the correlation level. The spectral variables selected to represent a metabolite, e.g. peak apex or peak range also have impact on the predictive power of the Pearson correlation to discriminate structural correlations. Peak position variation, caused by differences in pH and ionic concentration, was shown to attenuate structural correlations and to distort the correlation space since it produces spurious correlations between spectral variables. It is possible to establish a threshold on the correlation level for which only structural correlations are attained, but the sensitivity at this level is low. Total area and probabilistic quotient spectral normalization methods were compared but this choice did not have a great impact on the predictive power of correlations on samples from control animals. This study validates the use of STOCSY as a practical tool to aid the identification of unknown signals in metabolic profiles. The strategy here proposed can be applied to improve the discrimination of structurally related signal from others, thus, providing the means to better identify and characterise new biomarkers.

Poster 8

Robust Automated Calibration For ^1H -NMR Of Serum

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One of the most valuable features of NMR spectroscopy is that observed resonance frequencies of nuclei are extremely sensitive to the local chemical environment. It is this property that causes dispersion of the resonances in a molecule across the spectrum, facilitating structural characterisation and the differentiation between different compounds in a mixture. However this also means that resonance frequencies are affected by even minor fluctuations in temperature, pH, and the external magnetic field and so, precise comparison from one sample to another requires frequencies being presented as relative to some internal standard. In high resolution metabolic profiling studies, hundreds or even thousands of individual spectra must be compared and typically such studies require automated sample handling and spectral acquisition. While the first sample in a run may be manually calibrated, subtle variations in sample temperature often causes variations in the frequency of the lock solvent that in turn introduce small errors in the alignment for each individual spectrum. Proton NMR spectra of aqueous samples are typically referenced to the single peak of either 3-(trimethylsilyl)- propionic acid- D_4 (TSP) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Both TSP and DSS produce a singlet resonance that occupies a sparsely inhabited region of the ^1H spectrum, it is therefore algorithmically trivial to locate and reference to either compound by searching for the highest intensity within a defined chemical shift range. However in some routinely analysed biofluids such as blood plasma or serum, the chemical shift of the TSP or DSS resonance becomes highly variable and unpredictable due to interaction with proteins, preventing their use as an internal reference compound. In these sample types, the anomeric doublet resonance of glucose is often used as a substitute reference due to its abundance in serum and positional stability. In normal mammalian blood-serum the alpha-anomeric glucose doublet is partially overlapped by broad lipid olefinic resonances. Depending on the precise composition of the sample, the lipid or glucose peak may appear more intense, thus removing the utility of an automated search for the peak of greatest intensity in locating the alpha-glucose doublet Here we present a novel automated method of detecting and referencing to alpha-glucose, a comparison to manual calibration methods demonstrate the robustness of this method. Based on a second-derivative transform this provides a computationally inexpensive method of referencing spectra and is applicable to many other biofluids.

Poster 9

Rapid And Robust Compound Identification For Metabolomics Analysis Using A Retention Time/ Accurate Mass Database And Molecular Formula Generator

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Untargeted metabolomics workflows typically include sample preparation, data collection, feature finding, statistical analysis and compound identification. In an effort to simplify and streamline the compound identification step in a high throughput manner we have collaborated with the Scripps Research Institute to create a customizable personal metabolite database using content from their web-based METLIN Metabolite Database. This is the largest database in the world and includes masses, chemical formulas, and structure information for over 15,000 metabolites. Retention time (RT) and an automated Molecular Formula Generation (MFG) capability has been added that uses isotopic pattern matching to enable higher confidence identification of compounds from accurate mass data. It can be used concurrently with database searches, generating a list of masses with a specific RT, a metabolite compound match and a putative formula(s). Thus MFG provides additional confirmation of the compound identification generated by the database search. Our personalized database also enables batch search querying of an unlimited number of stored masses and associated RTs. For masses that do not have a database match the molecular formula generated can still be used to guide further experiments for compound identification. The software has the capability of creating custom databases to which new compounds can be added. A urine database was developed from a mixture of standards representing the most abundant compounds normally found in urine, to generate a list of masses and associated RTs using LC/ESI- time-of-flight (TOF) mass spectrometry (MS) in positive ion mode. After the database was populated, actual urine samples were analyzed under the same analytical conditions and screened for mass and RT matches against the urine database of standards. We used MFG to confirm the results of the database matches. Identified metabolites were annotated with a chemical formula, structure and other metadata, including CAS and KEGG identifiers

Poster 10

Simple Classification Models For Metabonomics Using A Genetic Algorithm For Simultaneous Variable And Sample Selection

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Metabolic profiles are complex and information rich, and mathematical models are often used to gain insight into the systems being studied. One way of simplifying models, and thus aiding their interpretation, is to focus on subsets of the original samples and variables. Here we put forward a method, based on a genetic algorithm (GA), for simultaneously selecting samples and variables to build informative classifiers of liver and kidney toxicity. Many of the thousands of variables monitored in metabolic experiments have little relevance to the effect being studied, and this can confound conventional statistical algorithms. Even high dimensional techniques, such as principal component analysis and partial least squares, can have their predictive ability improved through the use of variable selection. Crucially, variable selection makes interpretation of the resulting model much more straightforward since those metabolites important for class distinction are highlighted. In many studies, it is also possible to select a small number of prototypical samples which delineate the metabolic effect of interest and thus methods for sample selection are important. The selected samples may subsequently be investigated more closely using more in-depth data and chemical analytic methods. Additionally, automatic sample selection can help with the detection of outliers which are a common feature of biological data and not easily identified. A further motivation for sample selection is to reduce the amount of time taken to build models, since calculations rely on a reduced sample set. Overall, selection of both samples and variables will simplify the model and aid biological interpretation. We evaluate our GA method on data from the Consortium for Metabonomic Toxicity (COMET) which built a large temporal metabolic database of ^1H NMR spectra of urine from rats treated with model toxins and stressors. The training set comprised of 205 variables (with some missing data points due to removal of drug metabolites) and 209 samples from 6 treatments. We evaluated the predictive accuracy of our classifiers based on an independent test set (23 treatments). Simultaneous sample and variable selection improved performance relative to either method alone (average predictive accuracy 60.97%). Simultaneous selection also decreased computation time by 69.6% compared to sequential selection. A range of visualisations were employed to interpret the evolved models. Visualisation of variables frequently selected by different models found that a small subset of variables were important in the class distinction, including isoleucine, valine and 2-oxoglutarate. A novel method for visualization of frequently selected samples was developed, using a kernel density approach, which identified a surprisingly small subset of samples prototypical of the problem. Further, visualization of the classifier solutions over the evolutionary process indicated interesting evolutionary dynamics, including long periods of small improvements and neutral mutations, punctuated by large jumps to radically different and fitter solutions. Overall, this work shows that selection of both samples and variables can be of benefit in building classification models with metabolic profile data and that simultaneous selection is superior to other methods. The methodology could help to improve the simplicity and interpretability of diverse types of models used throughout the post-genomic sciences.

Poster 11

Targeted Quantitative Metabolomics Of Diabetes In Mice And Human

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Recent advances in high precision high throughput mass spectrometry have brought the field of metabolomics to a point where quantitative targeted metabolomic measurements with ready-to-use kits allow for the automated in-house screening for hundreds of different metabolites in large sets of biological samples. Metabolic disorders, such as type II diabetes, are among the prime candidate diseases for which a largely improved understanding can be expected to be gained from a truly holistic metabolomics approach. Here we present results from two studies, where targeted metabolite profiling by electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was performed at Biocrates life sciences GmbH, Austria. A targeted profiling scheme is used to quantitatively screen for known small molecule metabolites using multiple reaction monitoring, neutral loss and precursor ion scans. The quantification of the metabolites of the biological sample is achieved by reference to appropriate internal standards. In the first study, we perform a systematic analysis of a targeted quantitative characterization of more than 800 metabolites in blood plasma samples from healthy and diabetes mice under rosiglitazone treatment. We show that known and new metabolic phenotypes of diabetes and medication can be recovered in a statistically objective manner. Analyzing ratios between metabolite concentrations dramatically reduces the noise in the dataset, allowing for the discovery of new potential biomarkers of diabetes. Using a hierarchical clustering technique on partial eta squared values, we identify functionally related groups of metabolites. The bioinformatics data analysis approach presented here can be readily generalized to other drug testing scenarios and other medical disorders. In the second study, we apply the same technology, this time addressing the question of whether results found in a batch of monoclonal mice can be reproduced in a human population. The main challenge here resides in the overall heterogeneity of the human metabolome, which is a superposition of different environmental factors, such as life style, nutrition, medication, and of genetic background and health state. Based on a case-control design with 40 diabetes and 256 healthy male individuals above the age of 55, issued from the KORA population study, we show that this goal can indeed be achieved using the present technology.

Poster 12

Metabolic Profiling Investigation Of Cisplatin Sensitive And Resistant Ovarian Cancer Cell Lines By ^1H NMR Spectroscopy.

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Ovarian cancer is a devastating disease affecting millions of women worldwide. Initial successful platinum therapy of ovarian cancer is often followed by a relapse after which platinum therapy is ineffective and prognosis poor. The development of such drug resistance as well as its reversal is of major interest in modern medical oncology. Using NMR-based metabonomics we were able to identify baseline differences between platinum-sensitive and platinum-resistant isogenic pairs of ovarian cancer cells isolated from patients before and after the clinical appearance of resistance. Our findings included differences in glutathione, which showed increased intracellular levels, and formate, which showed increased extracellular levels and decreased intracellular levels in resistant cells. To support these findings the cell sensitivity to the platinum drug (cisplatin) was tested and was found to be increased in resistant cells after glutathione depletion. While glutathione metabolism is of known importance to the outcome of platinum therapy, the role of formate metabolism in resistance was less clear. Using an NMR approach we also investigated if formate excretion specifically decreased with inhibition of DNA synthesis. In future work, platinum sensitive and resistant isogenic ovarian cancer cells will be treated with either platinum, modulating agents or a combination of both, in order to generate novel metabolic biomarkers of either prediction, response or resistance reversal, and eventually generate a model of resistance to platinum.

Poster 13

Metabolomic Study Of GPR40 Knockout Mice: From Analytics To Biology

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The long chain free fatty acid receptor GPR40 (FFAR1) is a G protein-coupled receptor expressed in pancreatic beta-cells which participates in glucose-stimulated insulin secretion. Inactivation of GPR40 by gene targeting protects mice on high fat diet (HFD) from obesity-induced hyperinsulinemia, hypertriglyceridemia, and hyperglycemia. In order to explore the physiological mechanism of action we have conducted a mass spectrometry based metabolomic study comparing samples from GPR40(-/-) and wild type (WT) mice on HFD and normal chow diet. Plasma samples were analyzed with HPLC/NanoMate/Orbitrap mass spectrometer after pretreatment. Principal component analysis followed by discriminant analysis were performed after MS data pretreatment, including normalization, smoothing, peak picking, mean centering, and variance scaling. Differential metabolic profiling discovered hundreds of highly significant HFD-dependent metabolic changes (up- and down-regulated mass peaks) specific to GPR40(-/-) samples. The peak list of the differential mass spectrometric profile was deisotoped by isotope pattern matching and adducts removed. This list was input to a unified multi-database search tool. Triglycerides, phosphocholines, and glycerophosphoethanolamines, among others, were found in the hit list. In addition to candidate substances, scored pathway associations are proposed.

Poster 14

Metabolic Profiling For Biomarkers Of Colorectal Cancer

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Capecitabine is currently used as a monotherapy and an adjuvant therapy for colorectal cancer and it is the main treatment for patients with advanced metastatic colorectal cancer. However, it has been associated with toxicity in some patients. Individuals undergoing chemotherapy are particularly susceptible to adverse effects due to low safety margins and dosing inaccuracies. NMR-based metabolic profiling of biological samples has the potential to identify metabolic biomarkers of disease status and progression. We have conducted a metabolic profiling experiment in order to define such biomarkers for a group of patients undergoing a phase II trial of capecitabine chemotherapy. We were able to identify NMR-detectable differences in the pre-treatment serum profiles of patients that experienced a severe toxic response compared to those that experienced, mild, moderate, or no toxicity. With further analysis into the specific mechanism of toxicity this result has the potential to provide a clinically useful predictive biomarker of capecitabine toxicity. Predictive and surrogate response markers would be able to aid identification of populations of patients that are likely to respond to a particular therapy or dose, decreasing the likelihood of adverse effects, and improving the quality of life and the survival odds of a patient undergoing chemotherapy.

Poster 15

Comparative Microbial Metabolomics Across Different Times And Conditions

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Metabolomic profiling of bacteria has immense promise for helping understand the connections between the bacterial genotype and the actual expressed biochemical phenotype. It is particularly important to understand to what degree metabolic profiles are dominated by either phylogeny (e.g. bacterial 'species'/accepted taxonomic groupings) or by external environmental factors. However, to date very little attention has been paid to comparative bacterial metabolomics under different conditions. The metabolome of any given cell or compartment is the results of the integration of its genetic background and the conditions it is presented with, and therefore the results obtained by measuring the metabolome will be highly context-dependent. We aimed to investigate this context-dependency for the exo- and endo-metabolome and simultaneously test the potential of metabolic profiling as a taxonomic tool for a group of very closely related bacteria. As test species, we chose bacteria of the *Burkholderia cepacia* complex, a closely-related group of ten bacterial species. Often found in cystic fibrosis lung infections, they are associated with severe worsening of prognosis for patients. We employed NMR-based metabolic profiling to examine the metabolome under different conditions and at different time-points. We were interested if any stable contributors to strain/species separation could be found or if additional information could be gained by sampling at more than one time-point. The growth conditions as well as the growth phase were major factors influencing the observed metabolic phenotype. The endo-metabolome analyses revealed very different strain clustering under different sampling approaches, and the metabolites responsible for group separation also changed. The exo-metabolome (metabolic footprinting) was sampled over an entire growth curve. Using exo-metabolome data from the final time point only, some species discrimination was seen. Alternatively, metabolism throughout growth was modelled by fitting non-linear equations. This approach leads to a much clearer strain separation, reflecting phylogeny. More importantly, the metabolites contributing to this separation changed over time, and compound uptake was tightly and differentially regulated for different metabolites by the bacteria, even to the extent of reversing the order in which different species took up specific metabolites. These results show that modelling the depletion of the growth medium over a time-course contains more information than single time point metabolic profiling. In the future, it could serve as a tool to characterise the differences in physiology caused by mutations or to compare the metabolic phenotypes of a bacterial strain growing in different growth media.

Poster 16

Heteronuclear ^{19}F - ^1H Statistical Total Correlation Spectroscopy As A Tool In Metabolic Profiling And Drug Metabolism Studies

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The utility of statistical total correlation spectroscopy (STOCSY) for enhanced information recovery and simplified interpretation of analytical spectra has been demonstrated previously and can be applied with relative ease to datasets containing a sufficient number of high quality spectra. The use of spectral correlations to expedite the process of drug metabolite identification is of particular interest to those in drug discovery and development as determining the metabolic fate of pipeline compounds is costly in terms of analyst time. STOCSY analysis is also useful to those conducting metabonomic experiments that wish to distinguish endogenous and xenobiotic entities prior to chemometric treatment of spectroscopic datasets. It is common for pharmaceutical agents to contain constituent atoms that are largely absent from the endogenous metabolic profile of experimental animals and humans but are NMR-active (e.g. fluorine). In the context of drug metabolism studies, compounds such as these allow the collection of NMR spectra that contain features relating solely to drug metabolites. Although typically well-resolved and largely free from confounding features, these spectra contain little structural information, and they are commonly used in conjunction with ^1H spectra which may contain far more, but are complex in the case of biofluids. In this work, we exemplify the use of heteronuclear statistical total correlation spectroscopy (HET-STOCSY) to integrate parallel ^{19}F and ^1H NMR spectra in the context of drug metabolite profiling. Urine samples from healthy human volunteers ($n = 6$) were collected at various intervals in the 24 hours following oral dosing of 500 mg flucloxacillin, a fluorinated antibiotic. Paired one-dimensional ^1H and $^{19}\text{F}\{^1\text{H}\}$ NMR spectra ($n = 21$) were obtained at 18.81 T for each sample and a variety of statistical relationships describing correlations within and between these datasets were investigated. ^1H - ^{19}F STOCSY, ^{19}F - ^1H HET-STOCSY, ^{19}F - ^{19}F STOCSY and ^{19}F -edited ^1H - ^1H STOCSY spectroscopic maps were generated without loss of high spectroscopic resolution. ^{19}F - ^1H HET-STOCSY was used to guide interrogation of the complex ^1H spectra obtained for each sample to identify peaks relating to metabolites of flucloxacillin. It was shown that peaks in the ^1H spectra relating to the parent flucloxacillin and several flucloxacillin metabolites could be well differentiated using a 1D HET-STOCSY approach. The specificity of the 1D HET-STOCSY could be rationalized by consideration of the intermetabolite correlations, which were summarized using hierarchical cluster analysis. It was possible to relate these intermetabolite correlations to the metabolism scheme of flucloxacillin. In principle, this approach allows the likelihood of biochemical interconversions to be assessed, and might find utility in reconstructing metabolic pathways. Statistical equivalents of 3D heteronuclear experiments (e.g. 3D HSQC-TOCSY) were generated by way of editing 2D ^1H - ^1H correlation spectra according to correlations with particular ^{19}F peak intensities (X-STOCSY). This process greatly simplified data visualization and selectively highlighted metabolite-specific ^1H - ^1H cross-peaks. These approaches have general applicability to the metabolism study of other fluorine-containing drugs, and the HET-STOCSY strategy for integrating parallel NMR datasets is extendible to any drug metabolism study in which an NMR-detectable heteroatom is present.

Poster 17

The Construction Of Mass Spectral Libraries For Applications In GC-MS And UPLC-MS Focused Metabolic Profiling

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The development of analytical platforms and methodologies in metabolic profiling applications has resulted in an avalanche of valid and robust data. Typically, hundreds or thousands of peaks or features are detected in GC-MS and LC-MS analyses, defining a wide range of metabolite classes. However, to convert this data to biological knowledge it is necessary in many applications to describe these peaks or features as the associated metabolite. Currently this is a large bottleneck in metabolic profiling investigations where a large proportion of metabolites are 'unidentified'. Work at the University of Manchester has focussed on the development of mass spectral libraries for both GC-MS and LC-MS. GC-MS mass spectral/retention index libraries have been constructed for two different instrumental methodologies [1], employing the retention index and EI mass spectrum as two orthogonal properties for appropriate metabolite identification. UPLC-MS mass spectral/retention time libraries have been constructed for one instrumental method and employs the retention time, accurate mass and CID-induced MS/MS mass spectrum for appropriate metabolite identification. The construction, short- and long-term reproducibility and applicability will be discussed.

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Poster 18

Comparative Time-Course Metabolite Profiles Of Murine TNF- α Secreting And Non-Expressing *Streptomyces Lividans* TK24 Strains By GC-TOF-MS

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A time-course metabolite profiles of *Streptomyces lividans* TK24 was investigated using wild-type, empty pIJ486 plasmid and mTNF- α expressing strains. About 15 mg biomass (n=72) consisting of six biological replicates per strain and four sampling points (48, 72, 96 and 120 h) was quenched in 60% methanol 10 mM HEPES (-40°C) and the intracellular metabolites were extracted in 100% methanol (-40°C) for GC-MS analysis. All data were normalized to 0.16 mg/ml succinic d₄ acid internal standard by peak area and peak ratio. Averages of 126 metabolites were detected and 54 of these were positively identified from the in-house mass spectral/retention index library. Forty-seven (47) metabolites were significantly different (ANOVA test FDR \leq 5%) between non-producing (wild-type and empty pIJ486) and mTNF- α expressing strains or with sampling time. Analysis of the peak ratio of the detected metabolites shows no significant differences between the non-producing wild-type and empty pIJ486 strains. This suggests that the pIJ486 plasmid does not significantly affect the metabolism of *S. lividans* TK24 as measured by GC-MS and thus strains carrying empty pIJ486 can be used as single biological control for GC-MS experiments. Within those metabolites that were positively identified, the levels of trehalose, glycerol, glutamic acid, pyroglutamic acid, aspartic acid, 2, 6-diaminopimelic acid and a variety of sugars were significantly higher in non-producing wild-type and empty pIJ486 strains compared to the mTNF- α expressing strains. Benzoic acid and fumaric acid were lower in mTNF- α secreting strains. The time effect on the differences between mTNF- α secretion and non-expressing strains was most significant up to 72 h. The concentration of several metabolites changes in three stages with time, showing distinctive stable levels between 72 and 96 h that corresponds to the late exponential stage of the growth cycle. The levels of benzoic acid, fumaric acid, 2, 6-diaminopimelic acid, glutamic acids and aspartic acid show overall decreasing levels with time in all three strains. On the other hand the levels of sugars, phosphate, trehalose and pyroglutamic acids also decreased with time in non-secreting strains but increasing levels were detected in mTNF- α strains. This study validates the use of non-secreting *S. lividans* with empty pIJ486 as sole biological control for GC-MS experiments. It also elucidates some of the metabolite changes during the expression mTNF- α in *S. lividans* TK24.

Poster 19

Metabolic Markers Of Dietary Protein Source

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The aim of this study was to investigate the ability of NMR-based metabonomics, applied to serum and urine, to explore and identify overall exogenous and endogenous biochemical effects of a short time high intake of milk protein or meat protein given to prepubertal children. A total of 24 8-year old boys were asked to take 53 g protein as milk (n=12) or meat daily (n=12). At baseline and after 7 days, urine and serum samples were collected and high-resolution ¹H NMR spectra were acquired on these using a 800 MHz spectrometer. We observed that the milk diet reduced the urinary excretion of hippurate, while the meat diet increased the urinary excretion of creatine, histidine and urea. The NMR measurements on serum revealed minor changes in the lipid profile, which most probably should be ascribed to an increase in the content of short chain fatty acids in the blood upon the milk diet. The meat diet had no effect on the metabolic profile of serum. In conclusion, the study for the first time demonstrates the capability of proton NMR-based metabonomics to identify molecular markers related to a high intake of different animal proteins.

Metabolomic Approach In Studying Biochemical Effects Of Alpha-Tocopherol In Rats

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Metabolomics is a new approach in which profiles of metabolites in different tissues and/or biofluids are investigated to understand the changes, which is induced in the profile of metabolites, following an induced modulation. We have used this approach to investigate the biochemical activities of α -tocopherol in the liver using a rat model. 21-day Rats were either fed a sufficient control (n=10) or a deficient diet (n=12) for two month before sacrifice. Their livers were collected and extracted with chloroform-methanol-water. The extracts were analyzed using $^1\text{H-NMR}$ to profile the metabolites and the data was analyzed using multivariate statistical method (PCA and PLS). The statistical analysis revealed the α -tocopherol impact on the metabolism in rat liver.

Poster 21

Analysis Of Green Tea And Black Tea Extracts Using A High-Speed U-HPLC Coupled To A LTQ Orbitrap XL™ Hybrid Linear Ion Trap Mass Spectrometer With HC

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Metabolomics is the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples and marks promising new territory in “omics” research. These numerous analytes have very diverse chemistries and polarities occurring at different abundance levels within complex matrices. Consequently, comprehensive metabolomics investigations offer up many challenges that can be surmounted with chromatographic mass spectrometry methods. The analysis of tea represents a significant analytical challenge. Tea contains a wide range of components including vitamins, amino acids, and catechins many of which are structurally similar and may differ only in the type and location of a side chain. The use of high resolution chromatography is essential for the analysis of such a complex mixture. Acquisition of accurate mass data in both full scan and MS_n mode enables complete structural characterization. Here, we highlight an untargeted metabolomic workflow from data acquisition through unambiguous metabolite ID. The study included differential and structural characterization of polyphenolic anti-cancer components of Green Tea. Several catechins were able to be identified including epigallocatechin gallate, EGCG. Chromatography was performed using an Accela U-HPLC equipped with a 2.1 mm id Hypersil Gold C18 column packed with 1.0 μm particles. Data dependent analysis was performed on a LTQ Orbitrap XL with full scan data acquired at a resolving power 30,000 and MS_n data acquired at a resolving power 7,500 with HCD fragmentation. The analysis focused on the detection and quantification of low MW components of green tea and black tea extracts, focusing on the polyphenolic compounds. U-HPLC coupled with a small particle column afforded a fast analysis time while maintaining very high chromatographic resolution (peak width 4 seconds at half height). The high mass accuracy data (mass difference less than 3 ppm with external calibration) was used to confirm elemental composition and identification of compounds while also enabling precise quantitation. The fast cycle time of the LTQ Orbitrap XL mass spectrometer enabled HCD fragmentation at high resolution which provided detailed elucidation of fragmentation pathways, as demonstrated with the example of EGCG. The study also included a comparative analysis of green tea and black tea using differential analysis software for identification of compositional variations between the two teas.

Poster 22

MCF7 profiling by HRMAS-¹H-NMR identify myoinositol and phospholipid metabolism as respectively, a biomarker response and a target of kahalalide-F

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PURPOSE Metabolic profiling by HRMAS-¹H-MRS is a powerful tool for identification of drug response pathways and biomarkers (1,2). In this study, HRMAS-¹H-MRS was applied to human MCF7 breast carcinoma cells treated by the marine depsipeptide Kahalalide F (KF). This anticancer drug promotes cell oncosis and presents the advantage of antitumor activity independent of multidrug resistance (MDR) and of p53 status. The hepatocellular carcinoma, NSCLC and melanoma phase II clinical trials have finished in 2006. Because it modulates lysosomal function, we hypothesised that KF could strongly affect phospholipid metabolism.

METHODS To test this hypothesis, exponentially growing human breast MCF7 carcinoma cells were treated by KF (5 µM) in kinetic mode. The cell survival was determined with the resazurin reduction test which determines the amount of fluorescent resorufin produced by living cells. Treated and control cells were submitted to ¹H-NMR spectroscopy with a Bruker DX500 equipped with a high resolution magic angle spinning (HRMAS) probe. Sequences were used after water signal suppression. Spectra were recorded in 1D mode for qualitative response profiles and in 2D mode ([¹H, ¹H]-TOCSY) for quantification of cell metabolites.

RESULTS. 31 metabolites were identified and quantified. As expected, strong and unusual alterations of phospholipid metabolism leading to rapid blockade of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEth) remodeling pathways were observed 6h after MCF7 treatment. These alterations were correlated with Krebs cycle and glycolysis disturbance. Significant changes in the content of the sodium transporter myoinositol (involved in protection of cell homeostasis) and, irreversible accumulation of phosphoethanolamine were further observed.

CONCLUSION The present data demonstrate that in vitro, human breast carcinoma response to KF involves early and profound alterations of phospholipid metabolism leading to progressive krebs-cycle and glycolysis blockade and to myoinositol accumulation. In MCF7 cultures, myoinositol is a biomarker of KF response. Further studies are needed to determine its value as biomarker of clinical response.

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Poster 23

Metabolomics: The Way Forward For Accelerated Nutritional Enhancement In Soft Fruit.

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Plant polyphenolics continue to be the focus of attention with regard to their putative impact on human health. An increasing and ageing human population means that the focus on nutrition and nutritional enhancement, or optimization of our foodstuffs, is paramount. Using raspberry as a model we have shown how modern metabolic profiling approaches can be used to identify the changes in the level of beneficial polyphenolics in fruit breeding segregating populations and how the level of these components are to what degree these are determined by genetic and/or environmental control. Interestingly, the Vitamin C content appeared to be significantly influenced by environment (growth conditions) whilst the content of the polyphenols such as cyanidin, pelargonidin and quercetin glycosides appeared to much more tightly regulated suggesting a rigorous genetic control. Preliminary metabolic profiling showed that the fruit polyphenolic profiles divided into two gross groups segregating on the basis of relative levels of cyanidin-3-sophoroside and cyaniding-3-rutinoside, compounds implicated as conferring human health benefits.

Poster 24

DEVELONUTRI – Development Of High Throughput Approaches To Optimise The Nutritional Value Of Crops And Crop-Based Foods

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Micronutrients, such as vitamins, antioxidants and minerals, are essential components of our diet. They are present in food often in trace amounts and are thus difficult to quantify. Highly sensitive methods for their quantification are available, but not yet routine. These methods can help assessing which treatments, before and after harvesting, help preserving the nutritional value of food. The DEVELONUTRI project will develop and validate state-of-the-art analytical techniques for rapid quantification of micronutrients. It will analyse three widely consumed crops (potato, wheat and tomato) throughout the production, processing and transportation chain. Both traditional and GM varieties will be analysed. The project involves partners from Europe, emerging member states and INCO countries and aims to cross compare standard analytical approaches with the metabolomics (LC-MSn & GC-ToF-MSn) and emergent technologies, such as MALDI-ToF-MS, FT-MS, LC-NMR etc, to establish what level of detail can be obtained, or indeed is necessary, as part of food compositional and nutrient database construction. The majority of these technologies will be ring tested and validated in different labs, and the results will be made available in plain language through a public web-site, for the information of EU citizens and policymakers.

Poster 25

OPLS Model Visualization For Biomarker Identification Of Multi Class Metabolomic Data

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Metabolomics studies tend to generate increasingly complex data tables which are hard to summarize and visualize without appropriate tools. The use of chemometrics tools e.g. principal component analysis (PCA), partial least squares to latent structures (PLS) and orthogonal PLS (OPLS), are therefore of great importance as these includes efficient, validated and robust methods for modeling of information rich chemical and biological data. The advantage with OPLS compared to PCA or PLS is that the model is rotated so that a two class separation is found in the first component t_1 and variation not related to class separation is seen in additional components. This improves the model diagnostics and facilitates model interpretation. A strategy is presented which is easy and straight forward for model diagnostics and visualization. For interpretation and visualization we propose the S-plot as a tool for multivariate classification models, e.g. OPLS-DA, having two or more classes. The S-plot visualizes both the covariance and correlation between the metabolites and the class designation. Thereby the S-plot helps identifying putative biomarkers based both on contributions to the model as well as their reliability. The S-plot can be compared with the STOCY plot which is often used in NMR metabolomics studies. An extension of the S-plot, the SUS-plot (Shared and Unique Structure), is applied to compare the outcome of multiple classification models with a common reference. The example used is a GC-MS based metabolomics study for identification of putative biomarkers in two different transgenic poplar lines compared to wild type poplar.

Poster 26

Practical Information Extraction Process In NMR-Based Metabolomics

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We have successfully applied various computational methods to the NMR-based biomarker research. We found the optimal information extraction process would be: spectral signal enhancement, initial multivariate space exploration, most likely to be followed by PLS-DA. Spectral normalization and alignment are critical steps to enhance the signal consistency among samples in a collection. Spectral binning may be used to suppress slight experimental variation. PCA is used as an initial step to explore "natural" grouping patterns. When classes are separable by PCA, the strong signals in the loadings plot can be used to correlate to NMR peaks of individual chemicals. Alternatively, the scores plot can be used as a space to project known chemicals in order to find the relationship of a known compound with a chemical class. However, PCA is not always successful in determining what causes class separation. The PLS-DA method can be applied to enforce the separation of sample classes. Because it is a sensitive statistical algorithm, full resolution spectra work the best with it and statistical knowledge is needed to fine-tune parameters and interpret the results. However, for researchers who want to have a quick view of the difference among classes, we force class separation simply by finding the difference between two classes' spectra. This report showcases our strategy of analyzing body fluids, plant fluids, cellular, and animal fluids.

Poster 27

Selective Extraction Of Polyphenols For Enhanced Discrimination Between Commercially Available Grape Juices

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In modern society there is an increasing interest in health improvement through dietary alterations, i.e. prevention of potential health problems rather than cure. However, with very few exceptions, the molecular composition of foods cannot as yet be fully described. Molecular diversity and complexity within foods presents a daunting challenge to the derivation of predictive structure/function relationships for nutrition and most work currently being carried out on understanding the nutritional value of foods at a mechanistic level is achieved by studying the effects of single, potentially bioactive components of the food. This is not an effective route, in particular for phytochemicals, which typically occur in complex mixtures. Therefore, approaches which allow the analysis of the whole food product, such as metabolomics are becoming increasingly indispensable tools in food research. Grape juice and related products, such as grapes and red wine have previously been associated with many health benefits, such as protection against cardiovascular disease, [1-4]. Current consensus is that the polyphenols are the bioactive species in these products [1-4]. Within grapes, a large range of structurally diverse polyphenols can be present, and their characterisation stands as a challenge. ¹H NMR spectroscopy in principle would provide a rapid, non-destructive and straightforward method for profiling of polyphenols. However, polyphenol profiling and identification in grape juices is hindered due to signals of prevailing carbohydrates, causing spectral overlap and compromising dynamic range. A pre-extraction prior to analysis by NMR spectroscopy can therefore significantly aid both the number of detectable polyphenols and their identification, by reduction of signal overlap and selective removal of heavily dominating compounds such as sugars. The work presented here illustrates the ability of several different methods to selectively extract groups of compounds present within commercially available grape juices. This enabled better NMR discrimination between grape juices differing in both type and label. Within the selective extracts, the discriminating polyphenolic species could be identified in a more straight forward manner.

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Poster 28

NMR Metabolomic Analysis Of Tomato Fruits: Characterization Of Transformants Affected In Fruit Vitamin C Content.

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Vitamin C (L-ascorbic acid) is one of the essential vitamins for human health. Humans are unable to synthesize this vitamin because of the lack of the L-gulonolactone oxidase, the enzyme catalysing the final step of vitamin C biosynthesis. Thus, in humans ascorbic acid has to be supplemented through food and or as tablets. Vitamin C is widely distributed in fresh fruit and vegetables. Tomato is not only a major crop but also the model plant for fleshy fruit development. Its content in vitamin C varies from 61 to 314 µg/g fresh weight (Stevens *et al.*, 2007). Commercial varieties contain up to 5 times less ascorbic acid than wild varieties. Comprehension of the mechanisms underlying these differences for improvement of vitamin C content in these species is of agronomical interest. Three vitamin C biosynthesis pathways are described in plants up to now. In this study, two anti-sens transformants, one affected in the vitamin C synthesis pathway at the GDP-mannose epimerase (GME) node and one affected in the vitamin C recycling pathway at the monodehydroascorbate reductase (MDHAR) node, were chosen and compared to the cherry wild type (WT) tomato (*Solanum lycopersicum* 'West Virginia 106'). Vitamin C content was determined by spectrophotometric method (Stevens *et al.*, 2007). Soluble metabolites were extracted with hot ethanol/water from orange fruit pericarp. ¹H NMR metabolomic profiling of these extracts (three biological and two technological replicates) followed by Principal Component Analysis of the entire spectral signatures showed a clear differentiation of the 3 groups (GME, MDHAR and WT) on the first two components (explaining 82% of total variability). The "loading plots", associated to NMR spectral areas, responsible for the group separation contained the resonances of citrate, malate, fructose, glucose, glutamate or GABA. Each of these metabolites was quantified along with 16 other identified metabolites and 10 unknowns by means of quantitative ¹H NMR metabolomic analysis (Mounet *et al.*, 2006). This metabolome study suggests that, in the transformants, not only the vitamin C content was altered but also the primary metabolism. Transcriptomic changes of the same fruits are under investigation. The resulting metabolic and transcriptomic data will be combined to identify the regulatory networks that control fruit development under different ascorbic acid contents.

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Stevens *et al.*, 2007 *Plant Physiol.*143:1943-1953

Poster 29

Using Fourier Transform Mass Spectrometry To Elucidate Metabolic Changes In Trypanosomatids

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The trypanosomatids – *Trypanosoma brucei* and *Leishmania mexicana* – are responsible for the neglected diseases human African trypanosomiasis and cutaneous leishmaniasis. Previous research has shown a metabolic shift that accompanies differentiation between the insect and human infectious forms of *T. brucei*. To mimic this shift, procyclic trypanosomes were grown in the presence of either proline or glucose. Proteomic analysis of these cells revealed no significant modulation in proteins associated with energy metabolism despite clear changes in metabolism. Using an Orbitrap FT Mass Spectrometer we have analysed global metabolomes of trypanosomes. Using customised software that is able to de-convolute the raw data gained from the Orbitrap MS we identified thousands of metabolites within the trypanosomatids. Advanced visualisation tools were employed to explore *ab initio* networks based on the de-convoluted mass lists and known biochemical transformations. This enabled us to outline a large number of condition-specific differences in levels of metabolites between cells grown in different carbon sources.

Poster 30

A Metabolomic Analysis Of Non-Genotoxic Hepatocarcinogens In The Rat

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Non-genotoxic hepatocarcinogens (NGHCs) are particularly difficult to screen for in toxicology as there are no reliable in vitro screens and in vivo screens typically last 2-3 years. Furthermore, NGHCs are difficult to monitor at a biochemical level as their mechanisms of action are largely poorly understood. In this study we have used metabolomics to study the early stage biochemical changes associated with non-genotoxic hepatocarcinogenicity in rats. Plasma, urine and liver extracts were profiled using a combination of ¹H nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS). Multivariate statistics, including principle component analysis and partial least squares discriminant analysis, were used to look for trends and clusters in the data and to identify metabolites responsible for differences. In this study ten compounds were chosen to cover a range of biological effects in the liver. These were phenobarbital Na salt, chlorendic acid, diethylhexyl phthalate, monuron and methapyrilene HCl which are NGHCs, cinnamyl anthranilate, diethylhexyl adipate, benzophenone and diethylthiourea which are non-mutagenic non-hepatocarcinogens (NMNHCs) and 2-acetyl aminofluorene which is a genotoxic hepatocarcinogen. The biggest effect in the data set was the response of rats exposed to peroxisome proliferator-activated receptor (PPAR) agonists. These rats had distinct metabolic profiles and showed a large increase in the relative concentration of betaine and changes in unsaturated fatty acid synthesis. For all the compounds investigated unique metabolic profiles could be associated with an individual compound allowing good classification. In conjunction with transcriptomics we are currently pursuing the different mechanisms represented by these compounds to identify what is unique to NGHCs as a class of compound.

Poster 31

Metabolic Profiling As A Non-Invasive Tool For Non-Alcoholic Steatohepatitis Diagnosis

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Non-alcoholic steatohepatitis (NASH) is a critical stage in the process that spans from hepatic steatosis to cirrhosis and eventual liver failure. A potentially painful and hazardous biopsy is the only widely accepted test for distinguishing NASH from other forms of disease, with assessment being subjective and prone to sampling error. Here, a metabolomics approach using ultraperformance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (UPLC-TOFMS) is evaluated as a possible non-invasive alternative for NASH diagnosis. Serum samples taken from a set of patients were grouped according to the result of liver biopsy – grade of steatosis (1, 2 or 3) or NASH – and analysed together with a series of healthy volunteers. Multivariate statistical analysis of the data shows clear distinction between NASH and other samples. Additionally, data mining techniques were used to highlight a number of key biomarkers differentially expressed with respect to disease progression.

Poster 32

Identification Of Lactate As A Biomarker For Consumption Of Acidified Milk Products – A ¹H-NMR-Based Metabonomic Study Of IBS Patients

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Acidified milk products enriched with probiotic bacteria have developed into one of the most successful categories of functional foods. This success is based on a growing body of evidence that these products sustain a healthy flora inside our gastrointestinal tract and/or have immunoregulatory activity, which promote our general wellbeing. Furthermore, research suggests that consumption of acidified milk products containing probiotics can ameliorate symptoms in patients suffering from Irritable Bowel Syndrome (IBS). To further understand the beneficial effects of these products both with regard to the effect of delivering food matrix and the probiotics individually and in combination, solid analytical tools to explore biological responses need to be employed. In this context metabonomics holds great potential. In this study we have investigated the effect of a daily intake of 0.5 L acidified milk products on the plasma metabolite profile in two separate groups of IBS patients. One group was administered a non-probiotic acidified milk product, while the other group was given a fermented milk product containing probiotics. A proton nuclear magnetic resonance (¹H NMR) metabonomic approach was employed to analyse blood plasma collected before and after an eight- week intervention period. Comparison of the overall blood plasma metabolite composition using multivariate data analysis showed a clear discrimination between samples collected before and after the trial. Elevated levels of blood plasma lactate and 3-hydroxybutyrate were identified as the primary cause of the discrimination. In conclusion, using a proton NMR-based metabonomic approach, it was established that lactate is a strong biomarker for consumption of acidified milk products. This shows the potential for metabonomics in the evaluation of nutrition studies.

Poster 33

Identification Of Myb Transcription Factor Functions Belonging To The Sub-Family IV In *Arabidopsis Thaliana* By Metabolomics.

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Generally, plant transcription factors belong to large families of proteins which share domains for DNA binding and protein-protein interaction. In the frame of our project, the transcription factors belong to the R2R3Myb gene family which is divided into sub-families sharing common biological functions dictated by their recognition of common DNA binding sites and their common interactions with other proteins. These transcription factors are involved in a wide range of processing including the control of secondary metabolism which is our area of interest. We propose to use metabolite profiling techniques (NMR, LC/MS and LC/NMR) in order to determine the precise regulatory roles that these transcription factors play in *Arabidopsis* and to define the similarities and differences for the genes within a particular sub-family. In this poster, we show the methodology used for the study of the transcription factors belonging to sub-family IV (SF IV) starting with the analysis of the AtMyb4 gene, known to be a negative regulator of general phenylpropanoid metabolism, targeting in particular the expression of the gene encoding cinnamate 4-hydroxylase (C4H) [1]. This methodology consisted in the study of NMR and LC/MS profiles of transgenic and control lines. Statistical analyses (PCA, PLS-DA) were used to characterise the metabolites responsible of the discrimination between the two groups. The identification of several discriminant metabolites was performed using in-house databases. However, many interesting compounds remained unknown and we present the LC/NMR method used in order to help in their identification. The functions of other SF IV Myb transcription factors were also investigated by NMR.

[1] Jin *et al.*, EMBO J., 2000, 19:6150-6161.

Poster 34

NMR Spectroscopic Analysis Of Anoxia Resistance In Fish: Metabolomics Of Brains And Heart That Never Stop

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The capacity to tolerate extended anoxia is restricted to only a few vertebrates. These include some North American freshwater turtles and two cyprinid fishes, the crucian carp (*Carassius carassius L.*) and the congeneric goldfish (*Carassius auratus L.*). The crucian carp can survive days of anoxia at room temperature and even several months in ice covered ponds during the winter. In contrast, its cousin, the common carp (*Cyprinus carpio*) survives only 24 hours of severe hypoxia. During periods of anoxia, the crucian carp can retain fully functional cardiac and brain performance. Additionally, to avoid lactate self-poisoning, these fish have an unique anaerobic pathway producing ethanol. To investigate the 'metabolomics' of the main organs of the crucian carp during acute and chronic anoxia, nuclear magnetic resonance (NMR) will be used. This spectroscopic method has already proven to be useful for the quantification and identification of metabolites in vertebrates. Metabolomic processes of the anoxia resistant crucian carp will be compared with the changes in metabolism of the closely related, anoxia-intolerant common carp. By studying these animals, a better understanding of the underlying mechanisms of anoxia resistance can be gained.

Poster 35

The Comparative Metabolomics Of Strain-Related Changes In The Urinary Composition Of Goto-Kakizaki, Zucker (Fa/Fa) And Wistar-Derived Rats

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The application of global endogenous metabolite profiling methods is an emerging approach in both clinical and experimental diabetes investigations. Among commonly used experimental models of diabetes, Zucker (fa/fa) rats represent obesity related disease state, whereas Goto-Kakizaki (G-K) rats are characterized by impaired glucose tolerance. In present study, we used metabolomic analysis strategy based on analytical methods of nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass spectrometry (LC/MS) to obtain metabolite profiles for urine samples excreted by male G-K, Zucker (fa/fa) with those of age matched Wistar rats. Multivariate statistical analysis was applied to handle the large amount of data generated. Two software packages for LC/MS data processing, MZmine and xcms, were compared for efficiency in extracting the information. The extracted data from both analytical methods were subjected to statistical analysis by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to identify the disease-related metabolic changes. PLS-DA models showed good separation between Wistar - G-K and Wistar -Zucker (fa/fa) animal strains, whereas G-K and Zucker (fa/fa) rat samples were characterized by similar changes in metabolite concentrations, including hippurate and allantoin. In conclusion, the metabolomic studies of urine samples from experimental diabetes animals provide further insights concerning experimental methodologies for data generation and processing, as well as possible markers for diabetes research.

Poster 36

HRMAS ¹H-NMR Spectroscopy-Based Profiling Of Docetaxel-Treated MCF7 Tumor Cells

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Docetaxel (Taxotère®) is a microtubule-stabilizing agent with antitumour activity in various tumor types, including advanced and metastatic breast cancer. Docetaxel induces apoptosis through decreased protein kinase C (PKC) activity and cell cycle blockade with decreased structural and antioxidative gene expression. Metabolic changes in response to taxanes were previously shown to involve lipid accumulation. However, the cytotoxic mechanisms of docetaxel remain partially unknown. The identification of metabolic pathways and biomarkers of the response is an important challenge for cancer treatment. We thus used high resolution magic angle spinning (HRMAS) ¹H-NMR spectroscopy, a well known metabolomics tool, in an attempt to obtain such information in human MCF7 breast tumor cells treated by docetaxel. MCF7 cells were exposed to docetaxel at clinical dose (5 μM) and followed at various intervals of time (12h, 36h and 72h). Cytotoxicity was assayed using Hoescht 33342. Proton 1D saturation-recovery and 2D TOCSY spectra were acquired on untreated and treated intact cell pellets. A new quantitative procedure, applied to 2D NMR data, permitted, in combination with 1D data, to quantify variations of thirty metabolites. A 72h-exposition to docetaxel decreased MCF7 cell survival to 8.3% (P < 0.01). Among the quantified metabolites, the most significant variations were: a decrease of total glutathione pool by 39% (P < 0.05) and of lactate by 53% (P < 0.05). We observed strong variations of phospholipid metabolism derivatives involving an increase of polyunsaturated fatty acids by 1600 % (P < 0.01), phosphoethanolamine by 119 % (P < 0.01) and cytidinediphosphocholine by 470 % (P < 0.01), and a decrease of glycerophosphoethanolamine by 44 % (P < 0.01), glycerophosphocholine by 54 % (P < 0.01) and phosphatidylcholine (PtdCho) by 74 % (P < 0.05). Taken together our data support a down-regulation of PtdCho biosynthesis, at the cholinephosphotransferase (CPT) level, and glycolysis disturbances thus reducing diacylglycerol (DAG) biosynthesis. This suggests that docetaxel induces apoptosis through a reduction of DAG level and a down-regulation of PKC activity, as was previously reported for farnesol-induced apoptosis. Moreover, since PtdCho is involved in the removal of ceramides, its strong decrease may promote an accumulation of ceramides that may contribute to induce apoptosis.

Poster 37

Benefits Of Metabonomics To Reveal Subclinic Metabolic Disruptions: Investigation Of *Hypochoeris Radicata* Toxicity Using The Mouse Model

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The power of Metabonomics is tested for investigating diseases without working on target species and without having any prior knowledge about physiopathological events observed in an orphan disease. In this regard, *Hypochoeris radicata* is suspected to be the main plant incriminated in a neurological horse's disease (Australian Stringhalt) observed for the first time in Australia in the XIX century and which occurred in France in 2003 after the dry-prolonged summer. No direct proof has been yet shown between this plant and the disease. Moreover, physiopathology of the disease in horse and toxic composition of the plant have been kept ignored for more than 150 years. So Indeed, in this context, the challenge was to explore dose-response metabolic profiles obtained in mice fed with *Hypochoeris radicata* (complex mixture with unknown composition), this laboratory model does not represent the target species, but may present analogous metabolic disruptive events. 72 mice C57BL/6J (36 males, 36 females) were used and separated into groups according to sacrifice dates (8th, 15th and 21st day) and doses (0, 3 and 9 %) of *Hypochoeris radicata* incorporated in commercial rodent feed after nutritional adjustment. Urine has been individually collected over the course of the study. Brain extracts and urinary ^1H NMR spectra were acquired at 300K on a Bruker DRX-600 Avance NMR spectrometer operating at 600.13 MHz with cryoprobe. The data have been obtained after data-reduction by applying multivariate statistical analysis techniques. Zootechnic, clinical and histopathological results did not show any significant difference between control and treated animals. Urinary and aqueous brain extracts ^1H NMR profiles revealed a unidirectional metabolic dose-response with scyllo-inositol as the main metabolic biomarker and brain is the main organ displaying this metabolic disruption. Data have been confirmed by Magnetic Resonance Imaging in vivo. Scyllo-inositol is one of the nine stereoisomers of inositol, whose physiological concentration is low in peripheral and central nervous system, compared to myo-inositol. Even if the functions of scyllo-inositol in brain are unknown, contrary to myo-inositol, many studies have already shown that changes in scyllo-inositol concentration are observed in several neuropathologies. In fact, a high cerebral concentration in scyllo-inositol was shown as a new marker of brain metabolism disturbances induced by chronic alcoholism and significant increases in this metabolite have been found in brain of patients with Alzheimer's disease. Moreover, in diabetic peripheral neuropathy, changes in myo-inositol and scyllo-inositol content in nerve cells alter nerve conduction velocity and neuropathological features of the peripheral nervous system in this disease are also observed in affected horses. So, for the first time, our study showed that metabonomics can be used to explore the subtoxicity of a complex matrix in a non-target animal even if this model does not allow to reproduce the disease. The main metabolic biomarker in brain corresponds to scyllo-inositol, a metabolite widespread found in many neurological diseases and disorders.

Poster 38

Multi-Spectroscopic Metabolic Profiling

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Nuclear magnetic resonance (NMR) spectroscopy and gas chromatography – mass spectrometry (GC-MS) are two important technology platforms for metabolite profiling in biological samples. Varying physico-chemical properties and diverse abundance level of metabolites as well as sensitivity and resolution of current analytical techniques make it almost impossible to have comprehensive coverage of all metabolites during profiling in a single analysis. Due to detection limit of high-resolution ^1H NMR, it has been observed that almost 10-30% of NMR spectral region in bio-fluids remains unidentified using current Chenomx NMR software (due to spectral overlap, concentration differences, etc.). Thus the aim of the present GC-MS based metabolic profiling studies has been to identify hitherto non-identified metabolites and also to re-confirm the already NMR identified metabolites. At first, the cell extract and serum samples have been profiled by NMR and then subsequently derivatized and profiled using GC-MS technique. Initially cell extract sample has been evaluated for different dilution factors (1:2 versus 1:10 dilution with heptane during GC sample preparation) and its effect on mass spectral quality has been compared. Total of 180 and 189 mass peaks have been detected for serum and cell extract TIC respectively. Mass spectral database matching has clearly identified 67 (compared to 51 by NMR) and 62 (compared to 50 by NMR) metabolites respectively for serum and cell extract, of which almost 33% re-confirmed with previous NMR based metabolite identifications. Thus, the combined NMR and GC-MS experimental approach has unambiguously identified more than 90 metabolites each in serum and cell extract samples. It can therefore be concluded that concurrent analysis of metabolites using complimentary multi-spectroscopic methods has huge prospect for wider profiling coverage.

Poster 39

Metabolite Profiling From The Viewpoint Of Stochastic Systems Theory: The Tool For Realistic Data Evaluation

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In many situations, system biology depends on metabolite profiling which, due to its experimental nature, usually does not provide the generality and uniformity of data needed for pathway models. This paper analyzes LC-MS based the metabolite profiling experiment in general, and describes it in a manner which is mathematically correct and instrument independent. This has two advantages: (a) Signal-probability pairs for each signal allow to compare data sets and help in stochastic modeling; (b) comparing to the usual threshold or blank experiment subtraction, the interpretation of data entirely automatic.

Poster 40

Diagnosis Of SLE Severity By MS Metabolomics In Mouse Models: Biomarker Discovery And Comparison Of Kidney Pathology Scoring Systems

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Systemic Lupus Erythematosus (Lupus) is a chronic, inflammatory, systemic autoimmune disease of unknown origin. The disease develops in an irregular fashion both in human and in animal models. Because of this the disease is difficult to reliably diagnose prior to sacrifice and organ pathohistology. Here, we present metabolomics investigations directed toward discovery of combinatorial markers for disease diagnosis based on small molecules in blood. The animal model was a strain of mice which spontaneously develops lupus nephritis. Plasma samples were analyzed with HPLC/nanospray and high resolution mass spectrometry (MS). Principal component analysis followed by discriminant analysis was performed after MS data pretreatment. Disease severity in sacrificed animals was assessed by using the Glomerulonephritis (GL) score of kidney biopsy. A framework of the metabolic state was established using healthy control and highly diseased animals. Projection of mildly diseased animals fell between these two groups, validating the framework. Time series data obtained from a number of animals exhibited continuous progression from healthy to diseased state. Consequently, we were able to define a disease severity score from the metabolic pattern in the blood sample which corresponds well with post-mortem GL scores. Such a score, based on metabolomic blood markers and validated for human lupus diagnosis, could prove highly useful as a clinical tool.

Poster 41

Metabolomics Tools And Applications By Small Molecule Biology Laboratory, Singapore

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Our laboratory has been working on the development of metabolomics as a research platform as well as applying it to understand fundamental properties of metabolic networks and cellular responses to perturbations. Our efforts are mainly in the following five areas. 1. Pathway discovery using comparative metabolomics: We described novel flavonoid degradation pathways in *Pseudomonas* bacteria by comparing wild type and flavonoid auxotrophic strains (Pillai and Swarup, 2002). 2. Rhizosphere metabolomics: Using *Arabidopsis* as plant model, we identified greater than 125 metabolites and showed that *Pseudomonas* strains that can utilize major constituents of root exudates (flavonoids) have competitive advantage for survival in rhizosphere (Narasimhan *et al.*, 2003). 3. Metabolic networks in *Arabidopsis*: We have characterized the responses to perturbation of *Arabidopsis* by expressing *Pseudomonas* phenylpropanoid oxidoreductase and performing metabolomics. A number of short range and long range effects in phenylpropanoid pathway were uncovered by this approach. 4. Metabolite biomarkers of heat stress response in rat model: More recently, in collaboration with Defence Laboratories, we have performed biomarker and metabolic pathway identification for thermotolerance in rats. These studies have revealed early effects of thermotolerance in the liver and heart tissue. 5. Development of software tools for metabolomics: We have developed two tools to improve the efficiency of metabolic profiling data and its biological interpretation:

A. METDAT tool (<http://smbl.nus.edu.sg/METDAT/>). This online tool allows researchers to submit chromatograms and mass spectral data, perform several preprocessing and statistical analysis and finally visualize results as graphs or plots. Its unique feature is that users can create workflows for data analysis and is meant for small scale projects.

B. Dragon Plant Biology Explorer (DPBE) (<http://research.i2r.a-star.edu.sg/DRAGON/ME2/>). This online text mining tool is based on PubMed abstracts. Users can input PubMed texts files and visualize networks of terms from lists of gene ontology, metabolites, enzymes and pathways.

In this presentation, we will provide brief glimpse of the major features of some of these metabolomics studies and tools developed at the Small Molecule Biology Laboratory, (<http://smbl.nus.edu.sg/>) National University of Singapore.

Poster 42

Targeted Metabolomics – Simultaneous And Quantitative LC/MS/MS Analysis Of Energy Metabolism Intermediates In Biological Samples

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Introduction: The energy metabolism pathways glycolysis/glucogoneosis, urea cycle, pentose phosphate pathway, and krebs cycle play a key role in the endogenous metabolism. The investigation of the energy metabolism would help to improve the understanding of diseases, (e.g. cancer and diabetes), on the metabolic level.

Method: A multiparametric, highly robust, sensitive and high-throughput targeted metabolomic LC/MS/MS method for the simultaneous quantification of energy metabolism intermediates in biological samples was developed including minimized sample preparation for low volume biological samples enabling the simultaneous quantification of a broad range of energy metabolism intermediates.

Results: The solution of the analytical challenges (e.g. chemical properties, MS/MS selectivity, and lack of information about expected concentrations in real biological samples) for this group of intermediates will be presented. Two liquid chromatography strategies (ion-pairing (IP)-HPLC and hydrophobic interaction liquid chromatography (HILIC-HPLC) were investigated to find the optimal chromatographic conditions for all intermediates of interest using API-MS/MS (API4000 QTrap) detection. The method optimization and the sample preparation (even for small sample volumes in low μL -range) will be presented for different biological matrices including plasma, whole blood, and tissue. Validation of the method for human plasma was performed according to FDA guidelines adapted for endogenous metabolites.

Discussion: Quantitative targeted metabolomics for a simultaneous analysis of a broad range of energy metabolism intermediates (in low μL -range biological samples) will be presented and discussed. The method validation will be presented and the endogenous concentrations of various endogenous matrices will be displayed.

Poster 43

Targeted And Robust Analysis Of The Plasma Metabolome Using Automated Sample Preparation And Fast FIA-MS Detection

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Introduction: Metabolomics has started applying profiling techniques (either by NMR- or MS-based techniques) to investigate the overall composition of biological samples or to identify biological markers. As all “-omics” sciences strive for targeted and quantitative measures during the process of maturation, we sought to create an integrated sample preparation and detection system to foster targeted metabolomics.

Methods: A filter-based sample preparation and extraction process was combined with subsequent FIA-MS detection. Not more than 10µl of human blood plasma was processed for each sample point. The whole procedure was automated using liquid handling robotics. An integrated software system was developed to monitor and control the complete assay process, and to calculate concentration values out of the acquired mass spectrometric data.

Results: We conceived and developed an assay using on-filter sample preparation, derivatization, and extraction of biological metabolites. This system, in combination with flow-injection mass spectrometry, is able to generate robust data on more than 150 metabolites from blood plasma samples. The quantified analytes are representatives of these four biologically-interesting classes: amino acids, acylcarnitines, phospho- and sphingolipids and hexoses. Powerful software was used to perform the complex conversion of spectrometric data into concentration values.

Discussion: The major advantage of the analytical method we have developed is that it enables high-throughput targeted metabolomics in an (optionally) automated fashion. High-throughput targeted metabolomics is vital for the acquisition of reproducible quantitative data. This data is needed to perform routine analyses and to pave the way for metabolomic biomarkers to be used in diagnostics. Although some of the analyzed metabolites did not meet all analytical validation criteria, the overall robustness and reproducibility provided convincing data during field testing. Therefore, this assay could be applied to routine analyses in biomarker-related and pharmaceutical research. Also, studies using other matrices beside plasma have shown promise and we feel this metabolomics method has great potential when applied to other biological systems.

Poster 44

Data Mining And Integration Tools For Ecotoxicogenomics

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In order to understand and predict the impact of environment change (chemical pollutant exposure) on aquatic and terrestrial ecosystems, it is of critical importance to be able to characterise the biological responses to pollutants and conduct comparisons of effects across different chemicals and species. The NERC-funded project 'data mining and integration tools for ecotoxicogenomics' will develop new statistical integration methods for multi-omics data and interpretable visualisations for the integrated models. The data sets used in this project were generated from the metabolomics, transcriptomics, and proteomics studies with metals, non-polar organic compounds, pesticides and endocrine disruptors in species including *L. rubellus*, *C. elegans*, *Daphnia magna*, *Platichthys flesus*, *Gasterosteus aculeatus*, *Danio rerio*, and *R. Rutilus*. Several techniques are being investigated to improve information recovery from these diverse data sets including mutual information to identify linear and nonlinear relationships and latent variable techniques to build coupled multivariate models. The statistical relationships discovered between metabolites, genes, and life cycle end points will be visualised using the concept of spring embedding. This work will result in a suite of software which will be made publicly available for use by the ecotoxicology community.

Poster 45

Characterization Of Early Stage Metabolic Perturbations In A Rat Model Of Hepatocellular Carcinogenesis

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Hepatocellular carcinoma is one of the leading causes of cancer-related death, and is characterized by the late onset of symptoms in most patients. Dietary choline deficiency in rodents is a common animal model to study hepatocarcinoma, which is preceded by fatty liver in the early stages. GC-1 is a thyroid hormone receptor-beta agonist that has a triglyceride lowering effect and has been shown to mitigate fatty liver pathology. Here we investigate rat liver metabolic profiles with the aim of following biomarkers of early stage disease and the impact of subsequent therapeutic intervention. Liver tissue was collected from rats on choline-deficient (CD), choline-sufficient (CS), CD supplemented with GC-1 (CDGC), and control diets. Tissue was examined for the CD and CDGC groups after 3, 7, and 14 days (n=5 for each) and 14 days for the CS (n=3) and control (n=5) groups. Metabolic profiles of the aqueous and organic fractions from were obtained by a combination of high-resolution NMR spectroscopy, GC-MS, and LC-MS analysis. The data were analyzed by appropriate pre-processing methods followed by unsupervised and supervised multivariate analysis projection methods such as PCA and OPLS-DA. Pattern recognition models indicate significant alterations in metabolic profiles of the aqueous metabolites as detected by NMR and GC-MS, primarily in energy-related (malate, glycerol 3-phosphate, fumarate, creatine, succinate) and sugar-derived metabolites (hexonate, gluconate, maltose or sucrose, myo-inositol, glycogen). This large-scale metabolic perturbation is also reflected by lipid analysis of the organic fraction using LC-MS, which demonstrates unique profiles for each of the dietary groups, with an abundance of triglycerides in the CD animals. Interestingly, GC-MS analysis of fatty acid methyl esters from the organic fraction suggests that total fatty acid content is perturbed to a lesser extent, although differences were observed in essential fatty acid pathways. Analysis is ongoing to integrate the metabolic information with the goal of providing a comprehensive picture of the mechanisms of disease initiation.

Poster 46

Metabolomic Biomarker Identification Via Statistical Modelling Of LC-MS Data

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The efficient identification of biomarkers of disease is central to faster drug development and improved screening techniques. Such identification is often carried out by using mass spectrometers to obtain the exact masses not only of the biomarker but also of the ions into which it typically fragments when ionised with high-energy collisions. Manual interpretation of the data derived from such high-throughput experiments is cumbersome and impractical and so an automated procedure for reliably relating fragment ions to their parents is highly desirable. While Liquid Chromatography-Mass Spectrometry provides excellent sensitivity and mass accuracy, it suffers from poor reproducibility in the time dimension and is often complicated by the co-elution of very similar molecules. Moreover, chemical reactions such as adduct and dimer formation may take place in the chromatogram, adding to the complexity of the data. Although these factors make quantitative analysis more challenging, certain aspects of them may be exploited in order to better relate fragment ions to their parents. In particular, an approximately linear relationship between the concentrations of adducts, dimers, isotopes and fragments, all derived from the same underlying parent molecule, facilitates the detection of their interconnectedness through statistical methods. We present the preliminary results of our investigation of methods for relating fragment ions to their parents through statistical modelling of the LC-MS data.

Poster 47

Comparison Of Different Solvents By NMR For Bacterial Metabolite Extraction And Precipitation

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The metabolites present in a bacterial cell at any time are a representation of the cell's metabolic activity in that particular growth medium. Robust, reproducible and reliable biological data sets are needed to accurately study these cells. There are many protocols validated and written for the study of metabolism, many of which use organic solvents to extract metabolites. Surprisingly there is very little known about the loss of metabolites due to precipitation in these solvents. Metabolites have diverse chemical properties dictating their interaction with the solvents used in the extraction procedure. Hence we tested a range of solvents at different concentrations to see at what point different solvent concentrations lead to metabolite losses. Previous studies have not used NMR spectroscopy for validation; NMR spectroscopy is a near-universal detector that is widely used for metabolic profiling studies. In this study *Pseudomonas aeruginosa* was grown until stationary phase and extracted using four different solvents (acetonitrile, acetone, isopropanol and methanol) at six different concentrations from 50%-98%. The biological metabolite data were also compared to a model defined metabolite mixture made up of pure compounds with a range of chemical properties. Metabolite profiling was carried out using 600 MHz ¹H NMR spectroscopy. Solvent choice was shown to strongly effect which metabolites were lost at which concentrations. This information is important in planning a metabolome analysis, and shows that extraction protocols cannot be validated based just on the diversity and number of metabolites seen from biological extractions alone.

Poster 48

Identification, Bioavailability And Pharmacokinetics Of Fermentative Metabolites

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Polyphenols are plant secondary metabolites characterized by the presence of more than one phenol group per molecule. These compounds are present in fruits, vegetables, cereals, chocolate, and beverages, such as tea, coffee, or wine. Epidemiological studies and experiments in laboratory animals support a role of polyphenols in the prevention of e.g. cardiovascular diseases, malignancies, neurodegenerative disorders, metabolic syndrome, etc. Recent data indicate however that the bioavailability of the intact polyphenols is very limited. The hypothesis is that the bulk of the dietary polyphenols end up in the large bowel where bacteria split some of the aromatic rings of complex polyphenols. The resulting breakdown products consist of smaller and simpler phenolics that are absorbed into the human body. The knowledge about the bacterial metabolites generated from dietary polyphenols is very limited and in fact only the metabolites of a few well characterised flavonoids have been described. The intestinal microflora is probably also influenced by complex dietary polyphenols. Bacteria not only metabolise the complex polyphenols but also produce simpler phenolics that have specific growth inhibiting properties (e.g. benzoic acid). Both the bacterial polyphenol metabolites that are absorbed into the body and the changes in intestinal bacterial flora may be responsible for the health effects attributed to complex polyphenols. To investigate (a) the bioconversion of polyphenolic compounds in the gut, (b) the bioavailability of polyphenolic metabolites and (c) the role of microbiota diversity upon polyphenolic metabolism, a pharmacokinetic intervention study has been designed and executed. In this crossover-designed study 20 human subjects participated. These volunteers consumed a (single-dose) placebo, and two natural extracts with high polyphenol levels. During 48 hours, urine samples were collected and measured by means of ^1H NMR and gas-chromatography (GC-MS). Based on the NMR spectra, the GC-MS chromatograms and multilevel data analysis, three pharmacokinetic parameters were estimated for a series of discriminating metabolites, i.e. (1) total urinary clearance, (2) elimination rate constant and (3) lag time. These parameters were then used to examine the intrinsic variability and consistency between-subjects in terms of "High and low producers", "fast and slow metabolizers" and "fast and slow responders".

Poster 49

Metabolic Profiling Of Uropathies By ¹H-Nuclear Magnetic Resonance Spectroscopy On Human Amniotic Fluid And Paediatric Urine

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The use of ¹H-NMR spectroscopy in metabolic profiling was proved to be relevant in studying the biochemical composition of various human biofluid: amniotic fluid - opening the perspective to investigate physiological disorders of the foetus[1], and urine - bringing information on the renal function and the excretion pathways. Our goal is to provide statistically significant and mechanistically relevant metabolic markers of uropathies presented by fetuses and children, complementary to common diagnosis methods based on ultrasound scanning, MRI. The aim of this preliminary study is to compare the metabolic profiles of amniotic fluids obtained by amniocentesis (16-25 weeks of gestation) from 12 control fetuses and 7 fetuses presenting different renal ultrasound abnormalities (megacystis, renal hypertrophy, renal hyperechogenicity, polymalformation syndrome and Dandy-Walker syndrome) in order to identify a common metabolic signature for these several uropathies. ¹H spectra were acquired using a 500 MHz Bruker spectrometer and analysed using multivariate statistical methods (PCA and OPLS). We observed a variation of lactate peaks between samples, uncorrelated with the pathophysiological outcome. Once these peaks were removed from the analysis, a significant discrimination was found between the two subpopulations, probably due to a strong protein baseline variation in uropathy samples in comparison to control samples, a situation suggestive of foetal proteinuria. Glucose, alanine, leucine, valine and trimethylamine-N-oxide were significantly associated with control samples. On the other hand, we studied the biochemical variations observed in abnormalities of the urine flow. 2 groups of 4 and 5 children with pyelo-ureteral dilatation were recruited and surgical treatment was performed in one of them. Urines were collected 12 months after. Preliminary results show a signature associated with the effect of surgery, which will need further investigation.

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Poster 50

Investigation Of Metabolic Changes In A Full GDH Knock Out Mutant *Arabidopsis Thaliana* By ¹H-NMR Metabolomics

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If it is well established that the GS/GOGAT cycle is the major mechanism of ammonium assimilation in higher plants it has often been argued that other enzymes have the capacity to assimilate ammonium, leading to the hypothesis that alternative pathways might operate under particular physiological conditions. In higher plants, the enzyme glutamate dehydrogenase (GDH; EC 1.4.1.2) catalyses *in vitro* the reversible amination of 2-oxoglutarate to glutamate. Recently, we showed in several species that the GDH was strongly induced by ammonium and appeared only in the mitochondria of companion cells (CCs) of vascularized organs such as stems, petioles and midribs (Tercet *et al.*, 2004; Fontaine *et al.*, 2006). This novel finding revealed that the role of this enzyme in a whole plant context is not as simplistic as it has already been proposed. In order to elucidate the physiological role of GDH and taking advantage of the disponibility of several insertional mutants lines in *Arabidopsis thaliana* we have undertaken a study on this model plant. The GDH in *Arabidopsis thaliana* is encoded by two distinct genes, GDH1 and GDH2 producing an α - or a β -subunit respectively (Turano *et al.* 1997). More recently an *in silico* study searching for additional GDH genes has identified a third putative GDH (At3g03910) encoding a β -subunit-like (Purnell *et al.* 2005, The Arabidopsis genome initiative, 2000). In order to precise the function of GDH, full GDH knock out mutants were obtained and their metabolic changes investigated by ¹H-NMR metabolomics. These experiments were performed on roots, leaves and stems. Statistical treatment (Principal Component Analysis and Partial Least Square - Discriminant Analysis) of the data generated after binnings of the NMR spectra showed discrimination between the organs and between mutants and controls. The identification of the discriminant compounds is currently under investigation.

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Poster 51

NMR Metabonomic Approach For Characterising Biomarkers Of Vascular Calcification

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Vascular calcifications are a common complication in chronic kidney disease (CKD) patients, and are associated with an increased cardiovascular morbidity and mortality (London *et al.* 2003). Present detection and quantification methods of this pathology are expensive, difficult to elaborate and currently non-predictive (Ferramosca *et al.*, 2005). Hence, the determination of vascular calcifications related biomarkers could be an attractive approach. Different techniques have been established in the identification for these biomarkers, including ¹H-NMR metabonomics (Nicholson *et al.*, 1999; Brindle *et al.*, 2002). We used the Carr Purcell Meiboom Gill sequence associated with freeze-dried serum after testing different analysis conditions. This final protocol was validated by PCA which allowed the discrimination of the two study populations (CKD patients and control patients with normal renal function) and according the calcification score in CKD patients. Creatinine, identified in this analysis, was used as a criterion for the validation of this methodology.

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Ferramosca *et al.* (2005) Curr Med Chem – Cardiovascular & Hematological Agents, 3: 165-171

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Poster 52

Detecting Potential Metabolic Biomarkers For Exercise And Recovery In Well-Trained Athletes By NMR-Based Metabolomics On Biofluids.

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Up to now, studies of human exercise metabolism in athletes have been performed by measuring the concentration of a few metabolites in blood plasma or muscle tissue pre- and post-exercise on the basis of well established physiological hypotheses. However, a global methodological approach able to simultaneously handle the concentrations of a large number of metabolites to yield novel potential biomarkers and/or detect metabolic perturbations in absence of *a priori* hypotheses may be usefully applied to this specific field, whereas the raw measurement of lactate concentrations is currently being assumed as a standard indicator of the rest/exercise/recovery metabolic conditions. In this respect, an interesting GC/TOFMS-based methodological approach has been proposed by Antti H. *et al.* on serum samples of healthy and regularly trained male subjects (1). For this purpose, we have applied an NMR-based metabolomic strategy for metabolite screening on both blood plasma and urine samples in well trained rowers in pre-exercise (plasma, urine), post-exercise (plasma) and recovery (plasma, urine) states. Forty male athletes (age between 18 and 35) of the Italian Olympic Rowing Team performed a 20 minutes row ergometer warm-up, a 1000 meters maximal exercise and a 50 minutes sub-maximal exercise. Blood samples were collected pre- and post-exercise and after rehydration with water. Urine samples were collected pre- and post- exercise. Plasma samples were deproteinized by ultrafiltration (10kD filter) and ¹H-NMR spectra were acquired by a Bruker Avance 400 Spectrometer. Urine samples were sterile filtered and pH was adjusted to 2.5; spectra were acquired by a Bruker Avance 700 Spectrometer. Selected signals areas from NMR spectra have been measured by either integration or curve deconvolution. Partial least squares discriminant analysis (PLS-DA) has been applied to the data matrices to model the systematic variation related to the acute effect of exercise, and valid multivariate models for the differences between pre-, post-exercise and recovery subjects were obtained for plasma and urine, on the basis of the relative concentrations of the resolved metabolites. The proposed experimental protocol has been selected because it allows the evaluation of metabolic shifts in relation to exercise and recovery after rehydration, giving information on the physiologic adaptation of the whole system and not only of the muscular component. In particular, the proposed approach may be applied to the evaluation of the impact of nutritional interventions on the physiology of athletes.

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Poster 53

Discriminant Q2

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Classifying groups of individuals based on their metabolic profile is one of the main topics in metabolomics research. Due to the low number of individuals compared to the large number of variables, this is not an easy task. PLS-DA is one of the data analysis methods that is often used for the classification. Unfortunately this method eagerly overfits the data and rigorous validation is necessary. Cross validation is the standard validation procedure and the Q² statistic is often used as the figure of merit to quantify the performance of the classification model. The Q² value as a measure for classification performance however has some non-intuitive properties, one of them being the group homogeneity requirement. In metabolomics research this requirement is often not met. We have adjusted the Q²-statistic by removing the homogeneity requirement and call it the Discriminant Q² statistic (DQ²). Using a rigorous Monte Carlo simulation study we show that the power of the DQ² statistic is larger than the power of the normal Q² statistic. This means that when using DQ², a smaller difference between groups (treatment effect) can be found statistically significant than when the Q² statistic is used.

Poster 54

Metabonomic Analysis Of Cadmium Exposure In Human Volunteers Living In Proximity To A Smelter Source.

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Among metal pollutants, Cadmium (Cd) presents one of the worst health risks to humans, due to its long biological half life and propensity to accumulate in various tissues. Cd has a biological half life of 10-30 years in the kidney but also accumulates in the liver, bone and lung. The kidney is generally accepted as the organ that exhibits the first adverse effects in humans. Cd is classified as a human carcinogen by the International Agency for Research on Cancer and the US National Toxicology Program. Avonmouth, a small town near Bristol, UK, was the site of one of the world's largest heavy metal smelters and significant amounts of Cd, lead and zinc were released into the local environment. Approximately 50,000 people live within a 5 km radius of the smelter, and certain features (e.g. geographical) have caused a clear radiation of Cd deposition. We aim to define the link between Cd pollution and health effects by incorporating field measurements and metabonomic analysis into a human volunteer study at the Avonmouth site. The study also includes measurements of urinary Cd and existing validated molecular biomarkers of nephropathy (N-acetyl- β -glucosaminidase (NAG), α 1- μ -globulin and retinol binding protein). Specifically, the project was designed to provide novel geospatially-referenced data relevant to assessing Cd exposure and effects in humans (e.g. nephropathy), as well as providing a "proof-of-principle" experiment for the use of metabonomics in human population toxicology. Urinary metabolic profiles were obtained from human urine samples (180 volunteers) using high resolution flow injection 1D ^1H NMR. There was a significant ($p < 0.0001$) negative correlation ($r = -0.3602$) between 3-hydroxyisovaleric acid and urinary Cd concentration. 3-hydroxyisovaleric acid levels in urine are known to be affected by renal impairment as a consequence of effects on biotin metabolism. Further correlation ($r = -0.3378$, $p < 0.001$) to urinary Cd concentration was demonstrated for an, as yet, unassigned metabolite. In addition, a PLS regression model was developed that demonstrated significant correlation of urinary metabolites to NAG, a urinary biomarker of renal tubular damage. NAG was shown to be significantly correlated to urinary Cd concentration ($r = 0.3167$, $p = 0.0003$). In summary, we demonstrated that a metabonomic approach can detect the effects of Cd exposure and toxicity in a human population at environmentally relevant levels of Cd contamination. The results serve as a proof-of-principle for the use of metabolic profiling in epidemiological studies of the impact of environmental pollution on human health.

Poster 55

Standardization Of Sample Preparation Protocol For Large-Scale NMR-Based Metabonomic Screening Of *C. Elegans* Mutants.

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Our group recently demonstrated that ^1H high-resolution magic angle spinning nuclear magnetic resonance spectroscopy (^1H HRMAS-NMR) is particularly well adapted to the characterization of *Caenorhabditis elegans* metabotypes associated with different mutants. In particular, it was demonstrated that this approach enables the characterization of strains carrying mutations that do not produce any visible phenotype. Our goal is to extend this approach to the comparison of approximately 1000 different mutants and thus contribute to the functional analysis of *Caenorhabditis elegans* genome. However, the experimental protocol that has been validated so far for the comparison of a few strains has to be adapted before scaling up. Transposing a simple protocol performed on a weekly basis into a robust standardized protocol performed over several years represents an analytical and statistical challenge. One has to ascertain for long-term storage issues, users and batch effects as well as other biological variables such as age.

Poster 56

Metabolic Profiling Of Longevity In *Caenorhabditis Elegans*

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The nematode *Caenorhabditis elegans* is widely used as a model organism for a number of biological questions, one of which is ageing. The extensive genetic resources available for *C. elegans* mean that many genes have been identified that increase longevity when deleted, in several different molecular pathways. One of the most widely studied is that of insulin-like signalling (ILS): deletions of the gene *daf-2* (tyrosine kinase and homologue of the human insulin receptor) are lethal, but partial loss-of-function mutants exhibit an increase in longevity by up to several hundred per cent. However the interaction of metabolism with longevity and ageing has not been widely studied. Metabolomics could provide additional information about molecular changes associated both with ageing and with resistance to ageing (i.e. increased longevity). We used proton NMR to obtain metabolic profiles of several mutant strains with gene deletions relevant to ageing, including ILS mutants and mutants in a gene related to protein translation. We also looked at how the worms' metabolism changed throughout their lifespan as they aged. Very distinct metabolic changes were seen not only as the worms passed through the different larval stages, but also as they aged from young (approx 70 hours old) to old adults (approx 200 hours old). The metabolic changes as a result of ageing during adulthood were not the same as the changes that occurred throughout larval development. The long-lived and stress-resistant dauer larval stage showed a unique metabolic phenotype of its own. We profiled long-lived mutants at 3 ages: L1 larval stage, 6 days old and 10 days old. The mutants had distinctive metabolic profiles even as larvae and these differences become more pronounced as the worms aged. To investigate the possibility that long-lived mutants are metabolically similar to younger wild-type worms, we compared 10-day old mutants with both 10-day old and 6-day old wild-type worms. In general the mutants were more similar to wild-type worms of the same age than to younger worms, indicating that long-lived mutants do not simply resemble chronologically younger wild-type worms. Furthermore, long-lived mutants did not simply resemble dauer larvae, and so it is not the case that these mutants can be characterized as being either retarded adult or dauer-like.

Poster 57

Examining The Metabolic Effects Of WWOX Transfection In The Ovarian Cancer Cell Line PEO1 Using Metabonomics

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WW domain containing oxidoreductase (WWOX) protein is found to be lost or significantly reduced in numerous cancers and WWOX knockout mice produce multiple tumour types. As such the WWOX gene is a recognised tumour suppressor gene. To help elucidate the significance of WWOX in ovarian cancer, we investigated the metabolic changes associated with expression of the WWOX gene. The PEO1 ovarian cancer cell line, known to be null for the WWOX gene, was used to generate different cell lines that were either stably transfected with a WWOX expressing plasmid (PEO1 H clones) or were stably transfected with an empty vector (PEO1 F clones). The aqueous cell extracts of both sets of clones were analysed through ^1H NMR spectroscopy and pattern recognition. The most significant spectral differences were decreased phosphocholine and glycerophosphocholine in the WWOX-positive cell lines (H clones). Levels of phosphocholine and glycerophosphocholine are already known to be increased in numerous cancers including ovarian cancer. Iorio *et al.* (2005) observed 3- to 8-fold higher phosphocholine in human epithelial ovarian cancer carcinoma cell lines (EOC) when compared to normal or immortalised ovary epithelial cells (EONT). These data suggest WWOX decreases the biosynthesis and catabolism of phosphatidylcholine, effectively reversing malignancy. This is consistent with the proposed role of WWOX as a tumour suppressor gene. Previously, Iorio *et al.* (2005) observed higher enzyme activities of choline kinase, phospholipase C and phospholipase D in EOC cells relative to EONT cells. Expression microarray data performed on our transfected PEO1 clones revealed the gene expression of phospholipase C epsilon 1 was down regulated in WWOX-positive cell lines (H clones), which is highly likely to be related to the decreased phosphocholine levels observed through metabonomics. This work clearly demonstrates that metabolic effects defined by metabonomics can be integrated with effects at the transcriptional level by pathway-directed analysis and supports the hypothesis that WWOX expression reverses the tumour-associated choline metabolic phenotype.

Iorio, E; Mezzanzanica, D; Alberti, P, *et al.* (2005) Cancer Research, 65:20, 9369-9376

Poster 58

The Application Of UPLC-ToF-MS In The Search For Low Level Wound-Biomarkers In *A. Thaliana*

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In the study described, the model plant *Arabidopsis thaliana* was selected to evaluate the potential of a metabolomic strategy based on ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC-TOF-MS) for studying the significant metabolome variations that are related to stress caused by wounding. UPLC-TOF-MS offers the advantages of excellent sensitivity and reproducibility in both chromatographic and spectrometric dimensions, providing robust datasets for data mining, making it a powerful platform for metabolomics. MarkerLynx, a program which incorporates a peak deconvolution package and collects data into a single matrix by aligning peaks with the same exact mass/retention time along with their normalised intensities, was used to identify the monoisotopic mass of the constituent components and perform PCA. The data table was exported to advanced statistical packages to enable univariate and multivariate statistical analyses to be performed. Studying the wound-response provides important information on the defence mechanisms of plants against herbivores and this study shows that wounded and unwounded *Arabidopsis* specimens can be effectively discriminated based on the induction of minor key biomarkers. Examination of the metabolites contributing to the main differences between the two sets of samples enabled the detection of various wound-induced oxylipins. This study demonstrates that compounds that play a key role in defence signalling can be efficiently highlighted in the crude biological matrix without the need for specific sample preparation. The generic character of the method provides a broad survey of all metabolome changes that are related to wounding and makes the identification of new wound biomarkers possible. This may contribute to a better understanding of the complex plant response to herbivore attack.

Poster 59

Metabolomics In Brassica Vegetable Nutrigenomics

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Brassica vegetables contain a wide variety of secondary metabolites, including glucosinolates, flavonoids and carotenoids that are potentially healthy to human. The assumed health effect of these phytonutrients has stimulated interest in breeding for new Brassica vegetable varieties with improved nutritional traits. In order to identify genes and genomic loci regulating these phytonutrients, we perform a comprehensive metabolic profiling of *Brassica rapa* and *B. oleracea*. For this profiling both targeted and untargeted metabolomics approaches based on accurate mass LC-QTOF MS, ¹H-NMR and HPLC-UV/Vis-fluorescence are applied. In this research we use *Arabidopsis thaliana* as a model species. By correlating metabolomics data to transcriptomics and genetic markers, we aim to identify novel quantitative trait loci (QTLs) involved in the regulating of phytonutrients in Brassica vegetable crops. These genetic markers can be used for selection of and breeding for crops with specific metabolite composition and other quality traits.

Poster 60

¹H NMR And LC-MS Metabolomic Analysis Of Tomato Leaf

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Metabolomics can rely on several methods to produce robust data and give unambiguous results. This study presents two methods, one based on proton NMR (¹H NMR) and the other on LC-MS, to reveal expected and unexpected differences between wild type and transformant lines. Tomato plants (*Solanum lycopersicum cv. Ferum*) were transformed, following an antisens strategy and targeting a gene coding for a fruit-specific phosphoenolpyruvate carboxylase. Leaves of the wild type and three transformant lines (T2) were harvested, frozen, ground and freeze-dried. ¹H NMR analyses were carried out on hot hydroethanolic extracts adapted from Moing *et al.* (2004) and 21 metabolites were quantified. LC-MS analyses were performed on hydromethanolic extracts, using reversed phase chromatography coupled to Q-TOF mass spectrometer. LC-MS data were deconvoluted and areas of ions were calculated using XCMS software (Smith *et al.*, 2006). Principal Component Analysis was applied to ¹H NMR and LC-MS processed data. Both methods revealed compositional differences of leaves after transformation. ¹H NMR data showed a slight difference between lines due to identified metabolites, which was confirmed by univariate statistical test. LC-MS data showed clear differences between lines, but metabolites remain to be identified. Both methods were usefully applied to the same samples and were complementary in terms of analysed metabolites, provided information and analysis time.

Moing A., Maucourt M., Renaud C., Gaudillère M., Brouquisse R., Lebouteiller B., Gousset-Dupont A., Vidal J., Granot D., Denoyes-Rothan B. and Rolin. D., 2004, *Functional Plant Biology*, 31, 1-15.

Smith C.A., Want E.J., O'Maille G., Abagyan R., Siuzdak G., 2006, *Analytical Chemistry*, 78, 779-787.

Poster 61

A Metabolomic Study Of Transgenic Mouse Model Of Alzheimer Disease

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Metabolomics has been used to identify perturbations in biochemical pathways associated with a transgenic (TgCRND8) mouse model of Alzheimer disease (AD), which encodes a mutant form of the amyloid precursor protein 695 (APP695). The aqueous metabolic profile of various brain regions (cortex, frontal cortex, cerebellum, hippocampus, olfactory bulb, pons, midbrain and striatum) from affected and control mice were examined by ¹H NMR spectroscopy following extraction using a chloroform/methanol. This approach identified and quantified ~30 metabolites per tissue region including N-acetyl-L-aspartate (NAA), glutamate, glutamine, taurine, choline, phosphocholine, creatine/phosphocreatine, γ -amino butyric acid (GABA) and various other acids. Following data collection, multivariate and univariate pattern recognition techniques and clustering analysis methods were applied to the data set. The results provide us with a metabolic profile of the affected animal model at different disease stages. Analysis of the NMR spectrum from the cortex, frontal cortex, hippocampus, cerebellum and midbrain discriminated control from APP695 samples, with cortical and hippocampal regions being most affected. Histopathology on the brain samples revealed the presence of amyloid deposition in the hippocampus and the cortex (both anterior and posterior) as well as the cerebellum for the TgCRND8 APP695 mice. The presence of plaques was apparent from 2-3 months of age (except in the cerebellum) with increasing frequency as the mice aged with dystrophic neurons in older mice despite the absence of significant neuronal loss. Metabolite profiling demonstrated a decrease in NAA in the hippocampus, cortex and the frontal cortex in both young and older mice. In the midbrain no significant change was observed in the young mice but in older mice NAA was decreased. NAA changes in the affected brain regions suggest neuronal dysfunction, similar to the histology results, across the cortical and hippocampal brain regions. Other metabolites found to be decreased in the Alzheimer's mouse model were glutamate and GABA in hippocampus and cortical brain regions. These metabolites, with the exception of GABA, were also found to be decreased in the midbrain and cerebellum of the older mice only. A decrease in glutamate/glutamine has been reported in AD patients suggesting an impaired glutamate-glutamine cycle; we also see similar changes in the TgCRND8 mouse model of AD. Using metabolomics techniques we were able to detect early changes in the metabolic profile of affected brain regions before accumulation of plaques and at an early stage of the disease. Metabolomic studies on neurological disorders have begun to define the metabolic consequences of disease better. While some of these changes may not be primary to the disease, these pathways may still produce beneficial effects when targeted by drug intervention and provide useful prognostic information from other pharmacological interventions.

Poster 62

Probabilistic Methods And Tools In Metabolomics And Systems Biology

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The identification and quantitation of small-molecular-weight metabolites is considered to be a prerequisite for the understanding of reaction pathways and dynamics that define the underlying biological system. Metabolite fingerprinting as well as compound-specific profiling are among the strategies that have been pursued, sometimes in combination, as approaches that enable a systems-based analysis. While fingerprinting identifies the overall pattern of change in the metabolome without identifying specific metabolites, profiling requires specific identification. Fingerprinting utilizes chemometric strategies such as principal component analysis (PCA) to extract relevant variations in the datasets. In contrast, profiling is seen as “just” analytical chemistry involving multi-analytes; where biochemists have excelled for decades. Fingerprinting and profiling are two approaches along the spectrum of data collection and analysis methods that could yield complementary information and be used to implement key steps toward assembling a suite of quantitative methodologies. A deeper understanding of biological systems will leverage the emerging experimental approaches, which in turn will play a key role in establishing new guideposts for interpretation of data. The resulting pool of heterogeneous data obtained using multiple approaches must be analyzed for key metabolites from different bio-chemical pathways. The complementarity of data can restrain the state space for the underlying system and ultimately enable the transformation of individual data points in the pool of experiments to biologically informative patterns. The generically robust and realistic biochemical pathways can manifest as highly individualized and unique pattern of metabolites that exhibit variability along a number of dimensions. Therefore, a host of novel mathematical and computational tools that can translate the pool of data to predictive system behavior will be needed. For example, these tools should be able to extract information about dynamics from the multiple data modalities, address the heterogeneity of observation noise, and deal with the intrinsic variability present in living biological system. One modality of data collection, nuclear magnetic resonance (NMR) spectroscopy, offers a unique potential in metabolomics because of the relative ease of sample preparation, non-destructive analysis, the prospect of identifying a broad range of molecules, capacity for definitive compound identification, and the provision of structural information for compounds. Spectral complexity, overlapping resonance peaks, and the lack of a comprehensive spectral library of standard compounds could make profiling of specific compounds challenging. In fingerprinting, NMR signals can be dominated by highly abundant metabolites that may be difficult to interpret within a context of known biochemical pathways. As new experimental approaches emerge, the probabilistic paradigm appears to be uniquely suited to the task of robust and flexible data interpretation. We have embarked on the development of a set of novel probabilistic models that can be readily adapted to the interpretation of new experimental data. These tools include, new sample preparation strategies to increase sensitivity, refined pulse sequences that lead to better-resolved spectra, a centralized effort to provide isotopic-labeled standards for a wide range of metabolites (MMCD), enhanced methods for fingerprint analysis of 1D and 2D data, and a formalism for the development of reduced chemical pathway models. The work presented will illustrate these ideas by discussing representative examples and results that highlight our approach.

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Metabolomic Investigation Of Diseases Of Papaya Fruit (*Carica Papaya L.*) By Nuclear Magnetic Resonance And Principal Component Analysis.

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Papaya (*Carica papaya L.*) is a plant originating from Central and South America which is now cultivated throughout the tropics. Brazil is the major producer of papaya fruits (1.7 million tons in 2005). A disease, known as gelification, is observed which changes the aspect of the fruit flesh, changing its aspect to more rigid and translucent, also turning it more dark. This problem of the fruit is not easily identified because the external appearance of the fruit is not changed. However, it leads to a lower quality of the fruit and represents a problem in the major papaya producing areas of Brazil. The origin of the disease is unknown, but there seems to be no viral or bacterial involvement. A metabolomic investigation of the juice of affected and non-affected fruits revealed that the affected parts of the fruit contained higher concentrations of a number of organic acids, including lactic acid, acetic acid and pyruvic acid. Also higher concentrations of ethanol were encountered. These data point to a fermentative process going on in the affected fruits, which might be caused by local lack of oxygen. Further studies are going on.

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Discrimination between GH treated and non treated animals: Untargeted fingerprints of urine and plasma samples by LC-MSⁿ-HRMS measurements.

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Growth hormone (GH), also known as somatotropin, exhibits growth promoter effects. In spite of regulation which stipulates the prohibition of this molecule in Europe, GH is thought to be abused in sport to improve performances and in meat producing animals as a general growth promoter. In this context of anti-doping fight, analytical methods for GH detection have recently been developed. However, method performances ran up against the short half time of GH which does not allow long term control. A new strategy aimed at screening animals according to their biological matrices fingerprints was envisaged as a global approach. Untargeted fingerprints are established by LC-MSⁿ-HRMS analysis of urine or plasma samples collected on both GH-treated and non treated animals and subsequent appropriate statistical retreatment. Overall, the repeatability of the fingerprinting process is probably one of the most crucial points for the strategy efficiency. As a consequence, high attention must be paid to these analytical aspects which directly influence the quality of the data produced (sample collection, sample preparation, data acquisition). Indeed, sample collection has to minimize variability between animals; sample preparation has to be reproducible and exact mass measurements by LC-MSⁿ-HRMS are necessary to elucidate chemical structures.

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Development and application of metabolomic approaches based on LC-ESI-LTQ-Orbitrap™ for controlling the illegal use of growth promoters in cattle.

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In the field of chemical food safety, the main interest of metabolomics is to establish a link between a given exposure and the potential physiological impact associated to this exposure. The development of such approaches at LABERCA is first motivated by its status of National Reference Laboratory (NRL) for the control of various classes of residues and contaminants in foodstuff of animal origin. Indeed, the development of new strategies with large scale and high throughput capabilities appears today as a necessity. From this point of view, the identification of cases of abuse of growth promoters in cattle using an indirect measurement of metabolic profiles by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) is one of our objectives. Another objective is to identify some specific biomarkers of interest permitting to sign an exposure to residue and contaminant. In this scope our strategy is in a first step to paid special attention to the analytical tools and methods employed. Indeed, a huge number of analytical choices have to be made at each stage of the procedure which has crucial importance on the final quality of the generated data. The type of sample preparation, ionisation mode, mass filter, signal acquisition mode, and data processing, are example of such issues to be controlled. In this context the laboratory have acquired a LTQ-Orbitrap™ instrument which authorises both the generation of ultra-high resolution metabolic fingerprints and the possibility of multistage MS for structural elucidation purpose. Then, several research projects have been initiated in order to fix several analytical rules and suitable working frame for this approach. One of first application support consisted to differentiate tissue samples collected from control bovine animals *versus* animals treated with anabolic steroids. Besides this demonstration of feasibility, this study have underlined the influence of the sample preparation procedure on the obtained results, and more precisely the complementarities of different techniques that can be used simultaneously in order to maximise the information of the generated metabolic fingerprints.

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Plant And Food Metabolomics: Fingerprint Of Phytochemicals As Authenticity Markers For Medicinal Plant Extracts And Functional Oils

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Advanced methods (LC-MS, GC-MS, NMR, FTIR, Raman spectroscopy, isotopic analysis) are applied nowadays to characterize the most relevant secondary metabolites of medicinal plants (i.e. terpenoids, phenolics, pigments, hormones) or food/feed/botanical products, as authenticity or quality fingerprints. Our studies aim the characterization of the specific profile of some representative medicinal plants cultivated in Romania or other geographical areas, as well as functional edible oils. We used mature flowers and leaves of *Echinaceea sp. (palida, augustifolia)* and *Melissa off.* originating from different geographical areas (Romania, Germany, Czech Republic) and their phenolic compositions were fingerprinted by HPLC and FTIR. Quantitative data were obtained using spectrometry methods (Folin Ciocalteu) as well as calibrations from pure standards. Different extracts (methanol, ethanol or glycerin-ethanol mixtures) were compared in order to find the best extraction yield. Some extracts are used in nutraceutical recipes where the authenticity and a correct estimation of phenolics is needed. Such examples of nutraceuticals based on these plant extracts are presented. The target molecules used to fingerprint their metabolome were phenolic derivatives: phenolic acids, flavonoids and catechins, as free or conjugated forms. We were able to identify as principal components (by HPLC) the rosmarinic acid, cichoric acid, echinacoside, as markers of quality and authenticity. FTIR analysis was correlated with HPLC pattern and found to be a useful technique to identify distinctive patterns of plant phenolics in their specific fingerprint regions. Different oils (linseed, hemp, pumpkin) were compared for their content in phytosterols (using GC-FID) as well by FTIR, in order to find authenticity markers. Chemometrics and advanced statistics will be used for the appropriate interpretation of the data. These methods have good results for the determination of biological and geographical origin, in both taxonomic studies on plants as well the characterization of the nutraceutical preparations (herbal supplements, functional oils) with biomedical applications.