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Metabolic fingerprinting as a diagnostic tool

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Within the framework of systems biology, functional analyses at all 'omic levels have seen an intense level of activity during the first decade of the twenty-first century. These include genomics, transcriptomics, proteomics, metabolomics and lipidomics. It could be said that metabolomics offers some unique advantages over the other 'omics disciplines and one of the core approaches of metabolomics for disease diagnostics is metabolic fingerprinting. This review provides an overview of the main metabolic fingerprinting approaches used for disease diagnostics and includes: infrared and Raman spectroscopy, Nuclear magnetic resonance (NMR) spectroscopy, followed by an introduction to a wide range of novel mass spectrometry-based methods, which are currently under intense investigation and developmental activity in laboratories worldwide. It is hoped that this review will act as a springboard for researchers and clinicians across a wide range of disciplines in this exciting era of multidisciplinary and novel approaches to disease diagnostics.

Within the systems biology framework, functional analyses at all 'omic levels have seen an intense level of activity during the first decade of the twenty-first century. These include transcriptomics (the measurement of mRNA levels for quantifying gene expression), proteomics (protein translation, including post-translational modifications) and metabolomics. In a metabolomics experiment, one aims to use an array of analytical platforms [1] to quantify as many metabolites in a cellular system (cell or tissue) under a set of defined states and at different time points (see Table 1 for a definition of terms used in this review). This approach not only allows one to separate subjects with disease from healthy matched controls, but also permits the dynamics of any biotic, abiotic or genetic perturbation to be accurately assessed.

Figure 1 highlights how the area of metabolomics is becoming increasingly popular, and this is in part due to the ability to measure multiple metabolites directly from complex biological systems with excellent accuracy and precision. In addition, metabolism is closer to the organism's phenotype, and will be affected by disease, and so it makes sense to measure the metabolites themselves. As a single metabolite can be a substrate for a number of different enzymes, this linkage of metabolites through complex metabolic neighborhoods [2,3] makes unraveling changes in mRNA products and proteins very difficult, especially when hierarchical and metabolic control are evident [4]. Thus, measuring the metabolome is more attractive,

especially when one considers that only 2766 metabolites are estimated to be derived from man [5] compared with the 31,897 genes found in man [301], and the 10⁶ different proteins estimated from gene expression, alternative splicing and post-translational modifications [6,7].

For disease diagnostic purposes, one would ideally want a system that was easy to use, gave probabilistic outcomes, was quantitative and produced results that could be readily interpreted in biological terms. As highlighted above, we believe that metabolomics offers some unique advantages over the other 'omics disciplines [8], and one of the core approaches of metabolomics for disease diagnostics is metabolic fingerprinting. In this approach, a rapid biochemical snapshot [9] from a human body fluid or tissue under disease perturbation (Figure 2) is measured and changes from 'normality' are detected and correlated with disease progression or remission; the latter may be nutritional, pharmacological or surgical intervention.

This review provides an overview of the main metabolomics approaches that have been applied to disease diagnostics. This starts with Fourier transform infrared spectroscopy and Raman microspectroscopy, which have a long history in this field. This is followed by a discussion of nuclear magnetic resonance (NMR)-based metabolomics for disease diagnostics. Finally, a wide range of contemporary mass spectrometry (MS)-based methods are introduced that are currently under intense investigation within many laboratories. This is because

Table 1. Definitions of terms used in metabolomics and related approaches.

Term	Definition
Metabolome	The complete set of all low-molecular-weight metabolites (i.e., metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) to be found in a biological sample, such as a single organism, which are the end products of gene expression
Metabolomics	The nonbiased identification and quantification of all metabolites in a biological system
Metabolic profiling	Identification and quantification of a selective number of predefined metabolites, which are generally related to a specific metabolic pathway
Metabolic fingerprinting	Global, high-throughput, rapid analysis to provide sample classification. Also utilized as a screening tool to discriminate between samples from different biological status or origin (i.e., case/control, disease/healthy)
Metabolic footprinting	Analysis of the (exo)metabolites secreted/excreted by an organism; if the organism is growing in culture this will include its environmental and growth substances
Metabolite target analysis	Qualitative and quantitative analysis of one, or several, metabolites related to a specific metabolic reaction
Metabonomics	Quantitative analysis of metabolites in response to biological perturbation (i.e., disease or therapeutic treatment) or genetic modification
Lipidomics	Analysis of all lipids, and the molecules with which they interact, and their function within biological systems

MS offers exquisite sensitivity and the ability to readily identify many hundreds of metabolites in a single analytical run.

Vibrational spectroscopy

Fourier transform infrared spectroscopy

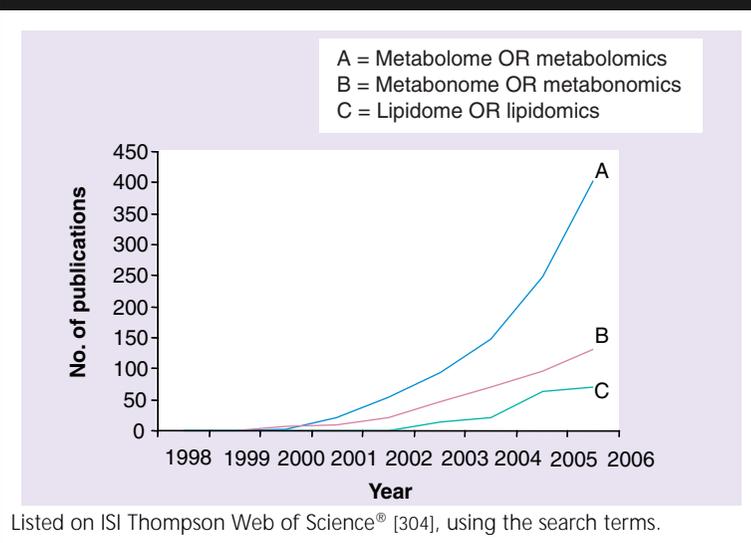
Fourier transform infrared (FT-IR) spectroscopy has a significant and ongoing history of research into its potential as a diagnostic tool and allows for the extremely rapid, high-throughput and nondestructive analysis of a wide range of sample types. The technique is based on the principle that when a sample is interrogated with an infrared beam, functional groups within the sample will absorb this radiation and vibrate in one of a number of recognized ways (such as stretching or bending vibrations) [10], and these vibrations/absorptions can be correlated directly with (bio)chemical species. The resultant infrared absorbance spectrum can therefore be described as a 'fingerprint', as it is characteristic of the particular sample under analysis and, hence, every chemical or biochemical substance will have its own unique infrared 'fingerprint'.

In terms of disease diagnosis, much of the work undertaken has concentrated on the mid-infrared (IR) part of the electromagnetic spectrum (from 4000–600 cm), as this part of the spectrum is where the fundamental vibration is seen, as opposed to the overtone or harmonic in near-IR (for reviews on the diagnostic potential of near-IR see [11–13]), and is particularly information rich. With relevance to biological

applications the mid-IR can be further broken down into regions, or windows, of biological interest such as CH₂ and CH₃ stretching vibrations from fatty acids (3050–2800 cm), C=O, NH and C-N from proteins and peptides (1750–1500 cm) and C-O, C-O-C from polysaccharides (1200–900 cm).

While it has been recognized that FT-IR is not as specific and sensitive as some of the hyphenated chromatographic techniques, such as gas chromatography/time-of-flight mass spectrometry (GC-TOF-MS) [14–18], the rapidity and reproducibility of FT-IR is demonstrable through the large body of research published using this technology. Furthermore, owing to its holistic nature, FT-IR has been recognized as a valuable tool for metabolic fingerprinting as it is able to analyze carbohydrates, amino acids, fatty acids, lipids, proteins, nucleic acids and polysaccharides rapidly and simultaneously with a minimum amount of sample preparation [1,19–22]. One of the potential limitations of FT-IR is that the absorption of water is very intense but this problem can be overcome either by dehydration of samples, subtraction of the water signal, or using attenuated total reflectance (ATR) as a sampling method [19,23–25]. Alternatively, one may use the related vibrational technique, Raman spectroscopy (*vide infra*). Another perceived disadvantage is that as a holistic measurement is made with biochemical information spread across the whole of the IR (or Raman) spectrum, validated and robust chemometrics must be used in order to turn data into information [12,26,27].

Figure 1. Results of bibliometric analysis of the number of publications per year



Raman spectroscopy

Whereas FT-IR measures the absorption of energy, Raman spectroscopy measures the exchange of energy with electromagnetic (EM) radiation of a particular wavelength, usually provided by a laser in the visible to near-IR part of the EM. From the exchange in EM energy, a measurable Raman shift in the wavelength of incident laser light is observed; this is also referred to as the inelastic light scattering effect [28–30], and one usually measures the Stokes shift as this has a higher probability of occurring compared with anti-Stokes. Significantly, it should be noted that this shift is complementary to IR absorption, according to various selection rules (see for details [28,30]) and a metabolic ‘fingerprint’ of the same sample analyzed by FT-IR can be constructed via Raman spectroscopy. Whilst the absorption of water is very intense with FT-IR, this is not generally the case with Raman spectroscopy as visible lasers are used (so no light is absorbed), and in all events water is a very weak scatterer [12]. Importantly, this allows for Raman measurements to be collected directly from biofluids, and further, there are several reports of *in vivo* measurements collected from both organs and blood vessels such as the cervix [31–33], bladder and prostate [34], esophagus [35–37], skin [38–41] and arteries [42].

What could be described as one of the limitations of Raman spectroscopy is the fact that the Raman effect is relatively weak with typically only as little as 1 in 10^6 – 10^8 photons undergoing an inelastic light scattering event, with the result that collection times can be in the order of

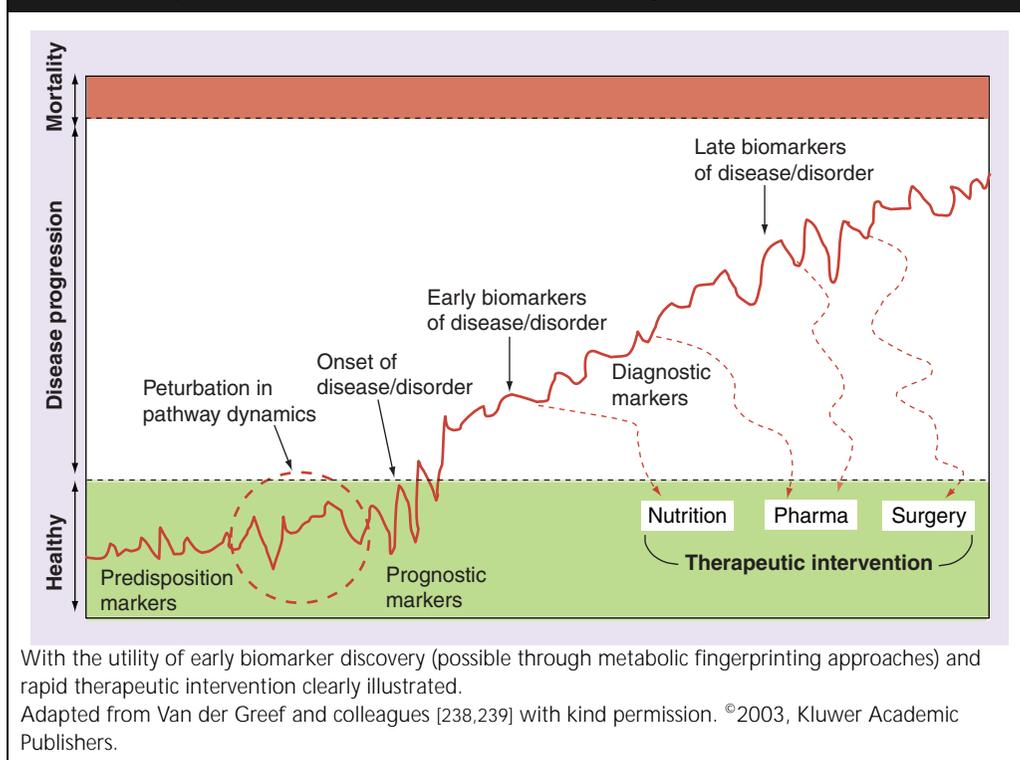
several minutes. One other limitation is that Raman spectra collected from samples of biological origin can contain significant amounts of fluorescence, which, as they are much broader, can detract from sharp Raman peaks, and therefore frequently need to be removed mathematically, or by moving the laser wavelength to the near infrared part of the EM. However, it is possible to enhance the Raman signal and this can typically be achieved by one of two methods. The first of these methods is based on resonance enhancement, which occurs owing to the fact that the laser wavelength used to excite the Raman spectrum lies beneath an intense electronic absorption band of a chromophore [12,43,44]. This can result in an enhancement of Raman scattering, to the point where some of the band intensities are increased by a factor of 10^3 – 10^5 . As this method uses chromophores, this knowledge can be used to the researcher’s advantage, whereby resonance Raman spectroscopy can be targeted to selected chemical species. Examples of this include the deep UV, 227 nm, which is used to enhance selectively aromatic amino acids, and 244 nm, predominantly leading to enhancement from nucleic acids [12,43,45].

The second method to enhance the Raman signal is surface-enhanced Raman spectroscopy (SERS) [46], which is reliant on the absorption or close proximity of the analyte to a roughened metal surface, colloidal solution or roughened electrode [47–51], with the coinage metals Ag, Au and Cu being most popular. Using SERS can result in an enhancement of the order of 10^3 – 10^6 (with some claims up to 10^{14} which allow single molecule detection [52]), and this can also be combined with a chromophore to effect surface-enhanced resonance Raman spectroscopy (SERRS) [53,54].

Disease diagnostics with vibrational spectroscopy

In terms of mortality, cancers are the most important range of diseases where rapid and/or early diagnosis would seem to be the most beneficial. This would include the correct identification and early diagnosis of (pre)cancer, enabling prompt therapeutic intervention, leading to a desirable prognosis. A significant number of studies have been undertaken using vibrational spectroscopic techniques to detect several forms of cancers with varying degrees of success. However, it must be stated that it is extremely important that if these studies are to be taken seriously then they should be supported with sufficiently validated

Figure 2. The development and progression of disease from healthy state, to homeostatic perturbation, to onset of disease and beyond.



histopathological data on the assayed biological samples [12]. Furthermore, it is also of paramount importance that characteristic wavenumber absorptions/shifts are assigned correctly, as at least one previous study has shown how characteristic absorptions of collagen could potentially be misassigned as DNA phosphate absorptions [55].

Whilst widespread screening programs exist for cervical cancer, it is still a significant public health problem worldwide and a major cause of death in females, with mortality rates estimated at approximately 30% [56,57]. Cervical cancer is also the second most prevalent cancer in females after skin cancer, and in terms of the present screening method, it has been stated that the Papanicolaou (PAP) smear and histopathology is tedious, and prone to human error [58]. Moreover, it is prohibitively expensive, labor-intensive and subject to inaccuracies that give rise to significant numbers of false negatives [25].

Several spectroscopic studies have been undertaken on cervical cancer, and within the field of clinical obstetrics and gynecology, spectroscopy has been identified as an emergent technology in cervical cancer screening [59]. Cohenford's group has undertaken a number of studies, including the analysis of cervical smears, and demonstrated that in addition to aiding in the diagnosis of

cervical cancer, FT-IR spectroscopy could also result in information into its pathogenesis [60,61]. Results from the analysis of spectra from one study of over 2000 cells showed a continuum of changes, which, the authors stated, paralleled the transition from normalcy to malignancy. This was illustrated by the fact that the ratio of the peak of cancerous samples at 1026 cm (ascribable to glycogen) was less than half that of the control group, and these ratios were observed to decrease progressively from normal (1.77) through dysplasia (1.17) to cancer (0.77) [60,62]. Other studies of interest include: the analysis of cell maturation in cervical tissue resulting in the observation that the different stages of cell maturation could be associated with changes in glycogen concentration [63–65], microspectroscopic analyses of individual cultured cervical cancer cells [66,67], and spectral mapping of the squamous and glandular cervical epithelium and the cervical transformation zone [68].

Raman spectroscopy has also been used to investigate cervical cancer, and with linear discriminant analysis was able to differentiate between cervical precancers from normal tissues, as well as diagnosing low-grade from high-grade precancers [31,32,69]. In relation to one of these studies, Utzinger and colleagues showed

that the ratios of Raman peak intensities at 1454 cm:1656 cm (ascribable to collagen and phospholipids) were greater for squamous dysplasia than all other tissue types, whilst the ratio of peak intensities at 1330 cm to 1454 cm (a region associated with DNA) was lower for samples with squamous dysplasia than all other cell types [32]. Furthermore, this group applied an algorithm based on these two peak intensities ratios with the result that they were able to discriminate high-grade squamous dysplasia from all other sample types, with only one sample being misclassified from a total of 312 *in vivo* measurements. More recently, Krishna and colleagues [70] have used Raman spectroscopy to discriminate between normal and malignant cervical tissue and reported very high sensitivity and specificity (99.5%) when using their multiparametric approach.

Epidemiologically, prostate cancer is the second most common cancer in the Western world after skin cancer [71], with mortality rates in the UK, for example, of over 10,000 recorded deaths in 2003 [302]. The main diagnostic method for prostate cancer is the prostate-specific antigen (PSA) test, which has received considerable attention in the literature as to its efficacy [71–73] and its routine use has been questioned due to a lack of specificity [74]. A significant number of studies have been undertaken on tissues, cell lines and DNA from subjects with normal and malignant prostates, and benign prostate hyperplasia (BPH) using FT-IR [75–80] and Raman spectroscopy [34,80–86,302].

Of these studies, Gazi and colleagues analyzed prostate cancer cell lines from a variety of metastatic sites, as well as tissue samples from both BPH and Gleason-graded malignant prostate tissue [87]. They reported that the ratio of peak areas ascribed to glycogen and phosphate vibrations (1030 cm and 1080 cm), suggested a potential method for the differentiation of benign and malignant cells. Furthermore, they demonstrated that the use of this ratio, in association with FT-IR imaging, could also provide a basis for the estimation of malignant tissue within defined regions of a specimen [87]. More in-depth studies revealed that the extent to which the clusters were separated suggested that this may be associated with the invasive properties of each cell line [76]. It was also suggested that the cluster plots could be used to elucidate whether inorganic ions had an effect on invasiveness as a consequence of this ion uptake, and that this could be subsequently confirmed and

quantified using imaging time-of-flight secondary ion mass spectrometry (TOF-SIMS) [76]. This same group has also demonstrated for the first time that FT-IR spectra of prostate cancer tissue could be used to predict the Gleason score [88] and the clinical stage of the tumor at the time of biopsy, concluding that there is a correlation between tissue architecture using Gleason score with tissue biochemistry using FT-IR and linear discriminant analysis (LDA) [89]. A very recent study, also from this group, has applied FT-IR photoacoustic spectroscopy and principal components analysis (PCA) to differentiate prostate cancer cell lines [90].

Analysis of prostate DNA by FT-IR has inferred that progression from normal prostate tissue to BPH to malignancy involves structural alterations. The separation of these sample groups was said to be possible using two regions of the infrared spectra, namely 1174–1000 cm (assigned to strong stretching vibrations of the PO² and C-O groups of the phosphodiester-deoxyribose structure) and 1499–1310 cm (assigned to weak NH vibrations and CH in-plane deformations of nucleic acids) and that these mutagenic alterations were due to the hydroxyl radical [78]. More recent work by this same group demonstrated with FT-IR that a cancer DNA phenotype is produced well in advance of palpable tumors and that this is a potential early indicator of tumor formation, and further postulated that agents capable of inhibiting this phenotype may delay or prevent carcinogenesis [91]. Fernandez and colleagues coupled FT-IR imaging with statistical pattern recognition and demonstrated the potential for automated histopathologic characterization of prostatic tissue, without any requirement for dyes or molecular probes (which would normally be used for histology), which was able to differentiate benign from malignant prostatic epithelium [75].

Raman spectroscopy has also been used to investigate both prostate and bladder cancer [83,86]. Indeed, Stone's group have been particularly active within the area of disease diagnostics using Raman spectroscopy, with a significant number of studies undertaken [34,80–86,92,93]. For the investigation of bladder and prostate cancer a total of 220 and 197 Raman spectra were collected with a fiberoptic probe from the bladder and prostate samples, respectively, using a 785 nm diode laser for excitation. The 785 nm wavelength was used as it was said to provide a compromise among the Raman signal strength,

detector sensitivity and fluorescence intensity. The spectra were correlated with histological features and used to construct separate diagnostic algorithms for the bladder (benign [normal and cystitis]) versus malignant (transitional cell carcinoma) and prostate (benign [BPH and prostatitis]) versus malignant (prostate cancer). Results showed an overall accuracy of 84% for bladder samples and 86% for prostate, with the authors concluding that this study demonstrates that a clinical Raman system provides an accurate and objective method to diagnose prostate and bladder cancer *in vivo* [83]. Other studies by this group have centered on the differentiation between different cell lines using PCA and LDA [34,80,82], as well as an investigation of the ability of Raman spectroscopy to grade cancers, achieving an overall accuracy of 89% [84].

The most frequent form of cancer in children and adults below the age of 30 is leukemia, and investigations into the diagnostic potential of FT-IR for leukemia have been undertaken for over a decade, through the analysis of normal and leukemic lymphocytes [94–99], with only a few studies applying Raman spectroscopy [100–103]. Extensive investigations of chronic lymphocytic leukemia (CLL) have been undertaken by Schultz and colleagues, who compared CLL cells to normal cells and observed differences in the amide region, as well as a reduction in lipid content, and major spectral differences were observed primarily from absorptions ascribed to the DNA backbone region (900–1300 cm) [98]. It was further reported that it was possible to separate the CLL cells further into subclusters, based on their different DNA content, and suggested that this may provide the basis for a diagnostic tool for staging (disease progression) and multiple clone detection [99].

Investigations of the effect of chemotherapeutic agents such as etoposide have been undertaken with results suggesting that FT-IR has the potential as a clinical utility for the rapid and reagent-free assessment of chemotherapeutic efficacy in leukemia patients [95,96,104]. Several features were observed in the difference spectra [105], which discriminated between control cells and those treated with etoposide; these included a shift from 1635 cm in control cells to 1657 cm in treated cells, and significant changes in the region of the amide I band (shift from β -sheet to unordered), the amide II band (~1545 cm) and the band at 1517 cm, arising from tyrosine in protein side chains [96]. Another study used FT-IR in an

attempt to discriminate between sensitive and multiresistant K562 cells with results as accurate as 93% [106]. Whilst leukemia results in the unrestrained proliferation of white blood cells, a group of diseases known as myelodysplastic syndrome (MDS) involves the disruption of any (and in the worst cases all) blood cells by the bone marrow. Malins and colleagues have employed statistical models based on FT-IR spectra to discriminate between the DNA of normal granulocytes and samples from MDS patients. These models were said to allow for the high sensitivity and specificity prediction of case and control granulocytes with the authors proposing that this method could be used as the basis for the development of a diagnostic blood test for MDS, for which there is currently a paucity of molecular markers [107].

Whilst few studies have used Raman spectroscopy to investigate leukemia [100], one very recent study has used both FT-IR and Raman to analyze normal, benign and malignant ovarian tissue with some success. It was stated that spectra from normal and benign tissue contained more proteins and less DNA and lipids in comparison with spectra from malignant tissue. The first derivative Raman spectra in the 700–1700 cm range resulted in two clear groups and second derivative FT-IR spectra from two combined regions (1540–1680 and 1720–1780 cm) had similar results to the Raman cluster plots [108].

These discussions on vibrational spectroscopic investigations of cancers only highlight a small part of the intense activity in this and other biological areas in terms of disease diagnosis. With perhaps the most exciting aspect being the ability to use FT-IR and Raman spectrometry to generate chemical images from tissue slices. In addition to those already noted, further studies have been undertaken on a range of cancers including: breast [109–114], brain [115], bladder [116], thyroid tumors [117–119], colorectal adenocarcinoma sections [120–122], gastric cancer [123–125], *in situ* measurements for Barrett's esophagus (a preindicator for esophageal cancer) [35,85], and studies of precancers and cancers of the larynx [92,93,126]. Outside of the field of cancer diagnostics a wealth of other diseases/disorders have also been investigated, including diabetes [127–129], arthritis [130–132], reproductive biology [133] and transmissible spongiform encephalopathies (TSEs) [134–138], amongst many others. For further and perhaps more detailed background into the biomedical applications of vibrational spectroscopy, the reader is directed to the following reviews [12,20,139–142].

Advances in measurement technology

Continual advances in optical measurement science and technology have resulted in the development of portable, hand-held and micro-devices which are already commercially available. These devices include both infrared and Raman mobile spectrometers [143–148] and infrared filters [149,150]. Theoretically, once robustly validated research has been undertaken as to which wavenumbers, wavenumber regions or Raman shifts are relevant to the metabolic fingerprint of a specific disease or disorder, then such miniaturized devices can be calibrated with this information. It would then be possible to obtain rapid measurements with these portable diagnostic tools wherever required, such as hospital wards, clinics, doctor's surgeries or even field hospitals. In addition, FT-IR, owing to its rapidity (<10 s per sample is readily achievable), is an ideal candidate technology for high-throughput screening, as it is possible to measure thousands of samples per day and obtain information-rich spectra using as little as 0.5 µl of a biofluid (i.e., plasma) per sample.

Nuclear magnetic resonance spectroscopy

NMR spectroscopy is an ideal analytical tool in many respects for metabolomics. Most elements possess at least one isotope that has a magnetic spin number greater than zero necessary for the NMR effect. The NMR transitions detected when a sample is placed in a magnetic field are induced by radiation in the radiofrequency region of the EM spectrum. This radiation can readily travel through material, including soft biological tissues, with often minimal heating effects and thus providing a noninvasive analytical tool. The technique is also exquisitely sensitive to different chemical environments, allowing the easy distinction of a wide range of chemicals in a mixture. Furthermore, lacking moving parts and relying on the observation of one physical effect in a highly regulated environment (the centre of a magnet), the approach is also highly robust, reproducing the same spectrum for a given sample across extended time periods. However, perhaps the biggest bugbear of any NMR spectroscopist is the inherent lack of sensitivity of the approach. Relying on nuclear transitions, that have an energy difference of the order of thermal energy at room temperature, mean that for most nuclei the population difference that is observed is very small, and typically less than one part in 100,000.

Of the most common nuclei that are observed by NMR spectroscopy, ¹H NMR spectroscopy is the most sensitive, and as a result it is also the most common nuclei used in NMR-based metabolomics. The proton is an ideal nucleus for metabolomics, as by definition all organic molecules must possess a proton and, hence, can be observed by ¹H NMR spectroscopy, which has made it a powerful functional genomic tool. Raamsdonk and colleagues used NMR spectroscopy to monitor the metabolic fingerprints of yeast extracts, and demonstrated that this approach could be used to distinguish different yeast mutants according to the functional role of the gene, clustering mutants associated with oxidative phosphorylation and glycolysis [151]. This approach has since been extended by Bundy and colleagues to examine the structure of metabolic pathways and the network of metabolism in yeast [152].

Samples are usually prepared in the solution state to allow metabolites to freely rotate, with this tumbling producing sharp resonances in the NMR spectrum. For the analysis of urinary metabolites and tissue extracts this is easily achieved, but for blood plasma and sera, metabolites may be bound to lipoprotein particles, producing line broadening, which in the extreme can make metabolites 'NMR invisible' unless the metabolites are extracted. Slow tumbling metabolites produce NMR resonances that decay relatively quickly and can be 'edited' out by specific pulse sequences such as the Carr Purcell Meiboom and Gill (CPMG) pulse sequence. Thus, for many metabolomic studies of blood plasma; a range of pulse sequences may be used to optimize the observation of different metabolites depending on their size and environment [153,154].

NMR spectroscopy, in vitro, in vivo & in situ metabolomics

The robustness and relative cheapness on a per-sample basis of NMR spectroscopy as an analytical tool has allowed the acquisition of relatively large datasets in metabolomics from its inception. With improvements in automation, sample throughput for metabolite rich fluids, such as urine and blood plasma, is as high as 300 samples per day, with no significant costs or time associated with sample preparation. Hence, the tool has been widely used in the drug safety assessment area of toxicology [155]. Using such an approach, the consortium for metabolomic toxicology (COMET) consisting of Imperial College London, UK, Bristol-Myers Squibb, Eli Lilly

and Company, Hoffman-LaRoche, NovoNordisk, Pfizer Incorporated and the Pharmacia Corporation, investigated approximately 150 model liver and kidney toxins through NMR analysis of urinary metabolites over a 3-year period [156,157]. It is hoped that such an approach will allow the generation of expert systems where liver and kidney toxicity can be predicted for model drug compounds, with the databases being easily transferable between laboratories. It has even been suggested that NMR-based metabolomics could be used to predict an individual's response to a given drug. Clayton and colleagues recently showed that the use of an individual rat metabolic profile could provide a strong correlation with the extent of acetaminophen-metabolizing capability and the associated liver toxicity [158]. This preliminary study also showed the outcome of galactosamine toxicity (known to show a high degree of variability) had a strong correlation with the pre-dose metabolome.

In addition, the approach has also been used to screen human populations to understand disease processes. Brindle and colleagues used ^1H NMR spectroscopy to follow changes in the composition of blood plasma to predict the occurrence and severity of coronary artery disease [159]. Their work suggested that such a diagnostic test could be more than 90% successful in terms of detecting coronary artery disease. However, this study had a definite gender bias, as well as some of the patients being treated by statins, widely used lipid-lowering drugs used to treat atherosclerosis. In a more recent study, Kirschenlohr and colleagues demonstrated that gender and statin treatment are both confounding factors for pattern recognition models to detect the presence and severity of coronary artery disease [160]. They showed that even in stratified datasets, where classification of disease status or presence was confined to one gender and treatment, predictions were poorer, being as low as approximately 60%.

The high-throughput capability of NMR spectroscopy for the analysis of urinary metabolites has also been used to monitor changes in animal models of Type 2 diabetes and compare these changes with human sufferers. Salek and colleagues used a range of univariate and multivariate statistics to cross compare metabolite changes in the db/db mouse and the Zucker fatty rat, both animal models that lack the leptin receptor, with humans who had Type 2 diabetes but controlled their blood glucose concentration by largely dietary control [161]. In addition to a

range of metabolic perturbations associated with glucose metabolism, glycolysis and the citric acid cycle, nucleotide metabolism was also profoundly altered (Figure 3).

Others have used the NMR-based metabolomic approach to monitor the effects of diet, either to understand biological variation in clinical studies, or as a means to understand the impact that different diets have on human health. Lenz and colleagues compared the variability between urine samples across a group of British and Swedish volunteers [162]. The effects of diet and lifestyle were noted to have the greatest influence on the urinary metabolomic profile, to such an extent as to obscure the variability due to gender. Indeed, some diets, such as the Atkins diet, meant those volunteers were classed as outliers in the subsequent pattern recognition.

Direct observation of metabolites within tissues is impaired by a number of physical processes that serve to broaden spectral resonances. Relaxation times are often short giving rise to broader lines, and anisotropic NMR parameters are not averaged completely to zero, also causing line broadening. For ^1H NMR spectroscopy, dipolar coupling and diamagnetic susceptibility effects are the two processes that cause line-broadening. Both effects vary in magnitude according to the angle the sample makes within the magnetic field, and at the magic angle (54.7°), these terms are averaged to zero if the sample is spun (typically faster than 1 kHz), resulting in high-resolution spectra, comparable with the solution state [163].

This approach, referred to as high-resolution magic-angle spinning (HRMAS) ^1H NMR spectroscopy, has been used to examine a range of intact tissues, and in particular tumors. Cheng and colleagues demonstrated that this approach could produce high-resolution spectra from intact tissues that allowed the classification of different types of brain tumors [164]. By measuring the concentration of 11 metabolites and the relaxation properties for the resonances associated with these metabolites, they were able to distinguish glioblastomas, schwannomas and meningiomas from normal tissue. In addition, Cheng and colleagues have also demonstrated that this approach is highly complementary to histopathology within tumour tissue, capable of monitoring microheterogeneity in human glioblastoma [165].

Griffin and colleagues have used the ability to measure both aqueous and lipid metabolites simultaneously by HRMAS ^1H NMR spectroscopy to monitor the accumulation of polyunsaturated

lipids during apoptosis in glioma obtained from rats [166]. By examining the line widths of these lipids at different temperatures and spinning rates, and measuring diffusion rates for the lipids, it was established that these metabolites were found in cytoplasmic vesicles seen by electron microscopy.

HRMAS ^1H NMR spectroscopy has also been used to follow the progression of fatty liver disease in rats exposed to orotic acid [167,168]. While it is known that orotic acid supplementation disrupts the production of various Apo proteins by the liver, the exact mechanism by which orotic acid exposure results in this disruption of lipid transport is unknown. Griffin and colleagues used a combined metabolomic and transcriptomic approach to understand the metabolic changes that occur during the development of fatty liver disease [168]. In particular, HRMAS ^1H NMR spectroscopy was used to monitor the increase in concentration of mobile, metabolically active lipids found in the cytosol. This approach not only identified changes associated with the exposure time to orotic acid, but it also distinguished the two rat strains examined. Kyoto rats possessed more 'HRMAS observable' lipids than Wistar rats, suggesting that the reason that Kyoto rats are predisposed to fatty liver disease relates to the animals possessing higher baseline concentrations of cytosolic lipids.

If one is willing to forgo some sensitivity and resolution, NMR metabolomics can even be performed *in vivo*. NMR is often referred to as magnetic resonance spectroscopy (MRS) when performed *in vivo*. The approach has proven to be particularly useful at following cancer metabolism. In one such example, the effects of hypoxia-induced factor-1 β (HIF-1 β , a constituent of the transcription factor HIF-1) deficiency on tumor metabolism and growth have been analyzed *in vivo* and *in vitro* using MRS in liver tumors [169,170]. HIF-1 β is upregulated in several cancer types as a consequence of hypoxia, resulting in the increased expression of proteins involved in glucose transport, glycolysis and growth factors to counteract hypoxia. The *in vivo* approach demonstrated that tumors with HIF-1 β knocked out had reduced ATP content, reduced glycolysis and reduced nucleotide synthesis. Metabolomic studies of this type could be used to identify metabolic pathways that could be targeted therapeutically to undermine the bioenergetic status of the tumor.

The application of *in vivo* MRS has also been applied to human sufferers of brain cancer. Hagberg and colleagues proposed a set of

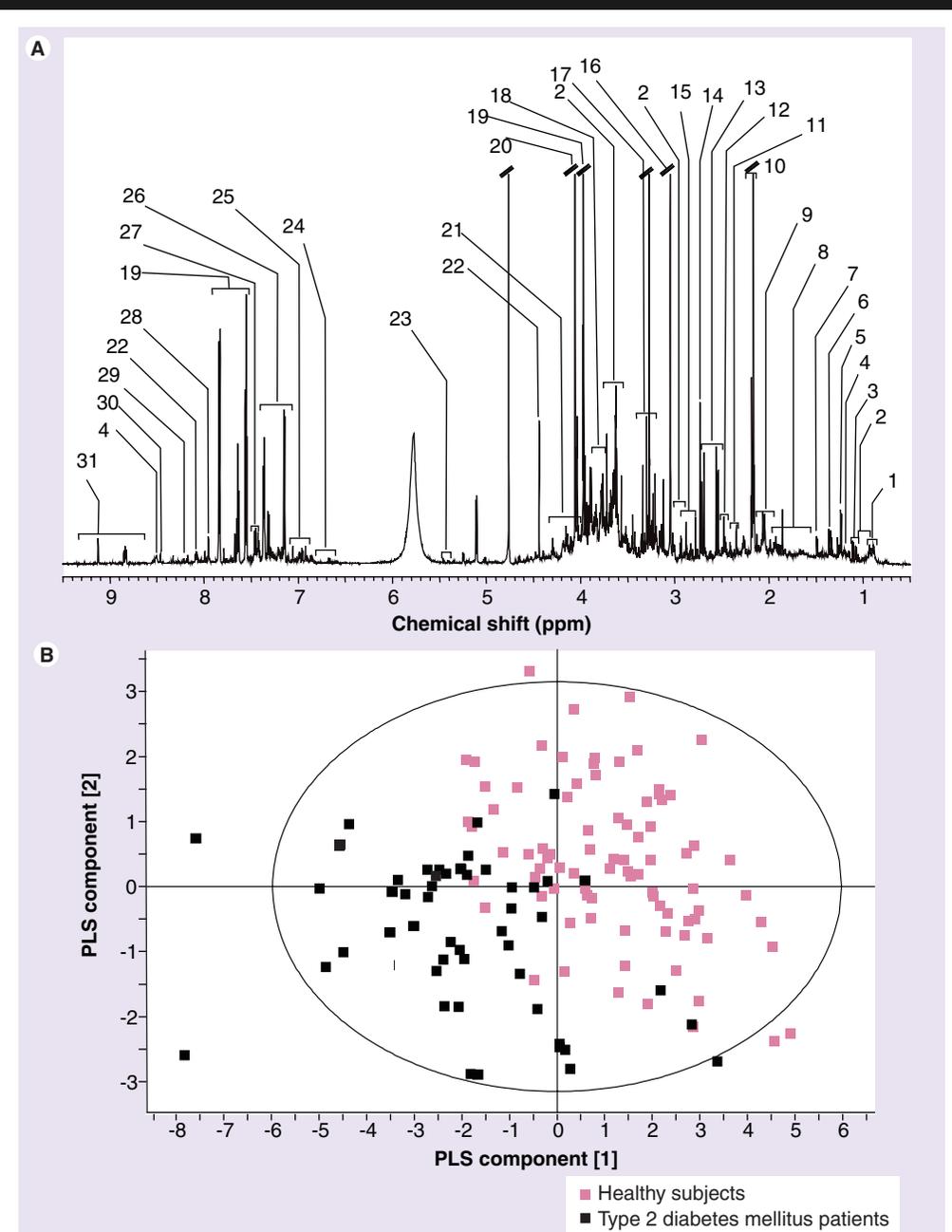
multidimensional statistical methods for processing *in vivo* ^1H NMR spectra to classify human glial tumors [171]. Usenius and colleagues coined the term MRS metabolic phenotype [172,173] after using simplified ^1H NMR spectra from healthy brain and tumors comprising of six metabolites (choline-containing metabolites, creatine, *N*-acetyl aspartate, alanine, lactate and lipid resonances) in conjunction with an artificial neural network to classify the tumor types and grades. This approach has shown a high degree of accuracy, predicting 104 out of 105 cases correctly [172]. This has been further demonstrated by a number of other *in vivo* studies [174–176].

Recent technological developments have significantly improved the sensitivity of the NMR experiment. Cryogenic probes improve sensitivity by reducing the contribution that electronic and thermal noise make by cooling the receiver/transmitter coil in liquid helium, resulting in an approximate four-times improvement in sensitivity as determined by signal/noise ratios. Such increased sensitivity has the added benefit of allowing the use of nuclei with a lower gyromagnetic ratio than ^1H , which would normally be prohibited owing to the time required to acquire sufficient signal [177]. For small-sized samples, miniature NMR probe coils can be used to allow the analysis of volumes as low as 1–3 μl , such as mouse cerebrospinal fluid [178].

Direct-injection mass spectrometry

Mass spectrometry is a complex analytical technique focused on the measurement of mass and is employed for quantification and identification of biological or chemical entities. The molecular weight of intact metabolites (correlated to the elemental composition) and the mass pattern of fragmented metabolites (correlated to chemical structure) are experimentally determined. The array of mass spectrometers commercially available operate with analogous experimental approaches [179–181]: sample introduction, ion formation in an ion source, separation of ions according to mass:charge ratio (m/z) in a mass analyzer, and detection of spatially or temporally separated ions. The technique provides high sensitivity to allow detection of metabolites at physiological concentrations (typically $\mu\text{M/l}$). High selectivity allows the specific detection and, more importantly, confirmation of identification, of metabolites in complex biological systems without extensive sample preparation. Selectivity is normally provided by tandem mass spectrometry [180–182] and accurate

Figure 3. High-resolution 700 MHz ¹H NMR spectrum of an aqueous urine sample from a healthy control volunteer with the relevant resonance assignments shown.



(A) Each resonance corresponds to a chemical moiety within a particular metabolite, with the intensity proportional to the concentration of that metabolite. 1: g-hydroxybutyrate/valerate; 2: Amino acids; 3: Valerate; 4: Unassigned; 5: γ -hydroxybutyrate; 6: Lactate; 7: Alanine; 8: Amino acids/ornithine; 9: *N*-acetyl groups/aspartate/glutamate; 10: Methionine; 11: Oxalacetate/pyruvate; 12: γ -hydroxybutyrate/glutamine/glutamate; 13: Citrate; 14: Dimethylamine; 15: Trimethylamine/dimethylglycine; 16: Creatine/creatinine; 17: Taurine; 18: Polyalkylene glycol; 19: Hippurate; 20: Creatine/creatinine; 21: Uridine bases; 22: nmN acid; 23: Allantoin; 24: Unassigned pyrimidine; 25: 3-hydroxypropionic acid/tyrosine?; 26: mHPPA sulphate/indoxyl sulfate; 27: PAG; 28: 2PY; 29: nmN amide; 30: Formate; 31: nmN amide/NMN acid. **(B)** Partial least square discriminant analysis score plot of the healthy subjects compared with the Type 2 diabetes mellitus patients following analysis of urine from individuals in a clinical trial. All diabetics had well controlled blood glucose concentrations according to dietary, rather than drug, intervention and the glucose containing region of the spectrum was excluded from the analysis. Adapted from [161].

mass measurements [183]. These factors can be achieved with and without the requirement for chromatographic separations prior to detection [184] and resulted in the development of direct infusion (or injection) mass spectrometry (DIMS). Here, samples are directly introduced to electrospray ionization mass spectrometers in a rapid and automated manner using flow-injection technologies [185].

Many developments in biology correlate to advances in analytical instrumentation. Clinical diagnostic capabilities have progressed from the development of electrospray ionization [186,187] and its combination with tandem mass spectrometry [182]. The latter two advances were the drivers for the development of high-throughput (HT) screening programs. These developments enabled the application of metabolic fingerprinting, and DIMS is a perfect tool for screening of samples for disease diagnostics, or classification studies, as it lends itself to full-automation, is rapid, allows quantification and identification of metabolites and is applicable for screening of large (>10000) sample sets. Difficulties arising from matrix effects in complex samples [188] and the nonuniversal detection of all metabolites can be easily overcome with the correct experimental design.

The application of DIMS to clinical diagnosis

Today, mass spectrometry is the most frequently applied of all the technologies discussed in this review to the diagnosis of single or multidisorder diseases, the majority of screens being performed on neonates. The application of DIMS for newborn screening has expanded rapidly worldwide in the previous decade (study numbers in parentheses) including in the USA (700,000) [189], Australia (362,000) [190], Germany (382,000) [191] and Japan (23,000) [192]. With its widescale uptake, new diagnostic tests are being developed for a number of disorders, including galactosemia, peroxisomal disorders and creatine deficiency, as well as other specific metabolite classes such as bile acids [193].

Inborn metabolic disorders (IMD), otherwise referred to as inborn errors of metabolism (IEM), are a collection of more than 200 single-gene disorders that are normally, but not exclusively, inherited from parents as autosomal recessive traits [194,195]. **Box 1** highlights a wide range of these disorders, although this is not comprehensive. These include the most frequently screened disorders of fatty acid oxidation

defects, organic acidemias, urea cycle defects and disorders of amino acid metabolism, which all result in abnormal catabolism. The disorders are caused by the impaired activity of enzymes, transporters or cofactors resulting in accumulation of abnormal metabolites proximal to the metabolic block.

Alternatively, the reduction or accumulation of byproducts produced by utilization of alternative pathways can result from these impaired activities. Metabolic symptoms are a result of increasing metabolite concentrations rising to toxic levels because of the loss of their routes for elimination from the body. Clinical symptoms (presenting hours to months after birth) include lethargy, nausea, failure to thrive, acute and progressive encephalopathy (seizures, mental retardation and physical handicaps) and organ failure and can ultimately lead to fatality. Toxicity is not normally observed in prenatales, as most of the toxic metabolites traverse the placenta and are therefore cleared maternally during gestation, resulting in infants appearing normal at birth.

Measurement of metabolites (amino acids and organic acids) directly or as conjugates with carnitine (acylcarnitines) is the basis of high-throughput, multidisorder screening of newborns today [194–199]. The objective is to diagnose in a presymptomatic or early symptomatic stage (before severe clinical outcome) to reduce morbidity and mortality, to provide proper therapeutic or dietary intervention and enable a favorable prognosis (Figure 2). It has been statistically shown that detection rates have increased since the introduction of screening programs when compared with clinical diagnosis, especially for fatty acid oxidation disorders (FAODs) [200–202]. Commonly, the detection of up to 65 metabolites for the screening of more than 40 metabolic disorders is performed on average [193,196], with rapid analysis times (less than 3 min). Furthermore, in excess of 500 samples/day can be automatically prepared, with more than 1000 samples analyzed between instrument maintenance [195].

Guthrie produced the seminal work of newborn screening in the 1960s, employing bacterial inhibition assays for diagnosis of phenylketonuria [203]. Millington and colleagues first suggested using tandem mass spectrometry for neonatal analyses, and this has been the most striking recent advance in newborn screening [204]. Initial developments in the 1990s were centered around the Millington and Chace groups at Duke University in the USA, who employed fast atom bombardment (FAB)

Box 1. Overview of many inherited metabolic disorders associated with amino acid and urea cycle, organic acid and fatty acid oxidation disorders.

Amino acid/urea cycle disorders

- Argininemia
- Arginosuccinic aciduria
- Citrullinemia
- Homocystinuria
- Maple syrup urine disease
- Phenylketonuria
- Tyrosinemia (Types 1 and 2)
- Hyperprolinemia (Types 1 and 2)

Organic acid acidemias

- Glutaric aciduria
- 3-hydroxy-3-methyl-glutaryl-CoA lyase deficiency
- Isovaleric aciduria
- 3-oxothiolase deficiency
- 3-methylcrotonyl-coa carboxylase deficiency
- 2-methylbutyryl-CoA dehydrogenase deficiency
- 3-methylglutanoyl-CoA hydratase deficiency
- Methylmalonic aciduria
- Multiple CoA carboxylase deficiency
- Propionic aciduria
- 3-methylcrotonyl-CoA carboxylase deficiency

Fatty acid oxidation disorders

- Carnitine–acylcarnitine translocase deficiency
- Carnitine–palmitoyl transferase Type I and I0I deficiency
- Carnitine uptake/transporter defects
- 3-hydroxyl long-chain acyl-CoA dehydrogenase deficiency (LHCHAD/MTP)
- Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)
- Multiple acyl-CoA dehydrogenase deficiency
- Short-chain acyl-CoA dehydrogenase deficiency (SCAD)
- Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD)

Further information can be found at the National Newborn Screening and Genetics Resource Center [305].

for profiling acylcarnitines (ACs), and subsequently amino acids (AAs; acidic, basic and neutral amino acids) in dried blood spots on filter paper or plasma [205,206]. However, difficulties arose from the use of glycerol. The commercial introduction of electrospray ionization has overcome these problems with up to 1000 samples being analyzed between source cleaning [195]. The most common approach is that of screening acylcarnitines and amino acids from one blood spot using electrospray ionization and tandem mass spectrometry for the screening of amino acid, organic acid (OA) and fatty acid (FA) disorders in one analysis. To expand screening programs, the use of urine is required and has been discussed for both AAs and ACs in positive ion mode and OAs in negative ion mode [207], with a turnaround time of 2.1 min.

Typically, triple quadrupole instruments (QQQ) are employed to undertake tandem mass spectrometry to obtain suitable sensitivity and selectivity [179–181,193,196]. Normally, a blood sample taken from a heel prick is used traditionally followed by derivatization according to Millington's protocol [204] to synthesize butyl esters, though derivatization is not necessarily essential [208]. Quantification is undertaken using isotope-dilution techniques. Automated disease recognition using advanced automated algorithms has also increased throughput and accuracy [192,209]. Diagnosis of diseases can be performed by a single concentration falling outside the 'normal range', or by the relative ratios (molar ratios) of different metabolites, for example phenylketourinuria, where tyrosine/phenylalanine ratios are assessed [210].

The objective of this review is not to discuss all disorders, and so the reader is referred to a number of excellent reviews for further information [193–197,199]. The IMDs related to FAODs will be discussed in more detail as a typical example. A further diagrammatic illustration is shown (Figure 4) detailing phenylketonuria. Fatty acid oxidation plays a major role in energy production in increased-demand states (stress, fasting and disease) where glycogen and glucose levels are low and result in mobilization of lipids from adipose tissues and its use in the liver, heart and muscle for energy production via ketone-body formation. Fatty acid β -oxidation in mitochondria involves more than 20 steps, of which not all are enzymatic. Short- (<C10) and medium-chain (C10–C14) fatty acids freely traverse the mitochondrial membrane, whilst long-chain fatty acids (C16–C20) require conjugation with carnitine. Mitochondrial fatty acid oxidation defects result in the accumulation of toxic levels of acyl-coenzyme esters (acyl-CoA) in the mitochondria.

In both these disorders, carnitine acts as a route for acyl-CoA elimination via the formation of AC esters, thus releasing CoA and providing homeostasis in the mitochondria. Problems with the metabolism [193,194,196] of these conjugated or free fatty acids in the mitochondria results in increased acylcarnitine concentrations in the circulatory system and urine, especially long-chain fatty acid acylcarnitines. This allows for a method for newborn screening. Plasma ACs and carnitine, concomitant with an increase of certain organic acids in urine are typical phenotypes and are used to evaluate patients presenting with hypoglycemia. This includes

times, equivalent to the DIMS applications described above. New applications are expected in the future [218,219].

Laser desorption ionization mass spectrometry

The roots of laser desorption ionization (LDI) stem from matrix-assisted laser desorption ionization (MALDI). MALDI is a soft ionization mass spectrometry technique, which has become an extremely popular method for the analysis of a wide variety of compounds, most commonly proteins, polymers and nucleic acids [220]. The greatest hindrance to widespread use of MALDI is that it is extremely difficult to analyze low-molecular-weight compounds (<500 m/z) with any confidence. This is due to the matrices applied in typical MALDI experiments, that facilitate the samples initial absorption and subsequent ionization. Most matrices are organic acids which produce low m/z analytes (e.g., H⁺, Na⁺ or K⁺ cation adducts) that interfere with the low-mass range of the analytes of interest, metabolites being the prime example. One strategy to open up the analysis of low-mass species has been to apply a high-molecular-weight matrix; however, problems occurred relating to the matrix not co-crystallizing efficiently with the sample (efficient co-crystallization being a prerequisite for successful ionization) [221]. Consequently, in recent years there have been considerable efforts put towards developing 'matrix-free' LDI techniques that are more applicable to the analysis of metabolites [222]. However, further developments in MALDI matrices have also proven fruitful for both qualitative and quantitative metabolite analysis, a prime example being the 9-aminoacridine matrix [223].

Both LDI and MALDI function by interrogating either the sample alone (LDI) or co-crystallized sample and matrix (MALDI) with a laser (commonly a UV nitrogen 337 nm laser, although IR variants are also available). In the case of MALDI, the laser transfers energy to the matrix, resulting in it and the sample's transition from solid to gas phase; the matrix in turn transfers the energy to the gaseous sample molecules, thus leading to their ionization [224,225]. By contrast, in LDI the plate surface must be capable of transferring the laser energy directly to the sample for the molecules to become gaseous and ionized. Figure 5 illustrates the LDI ionization process. Within both techniques, ionization is usually

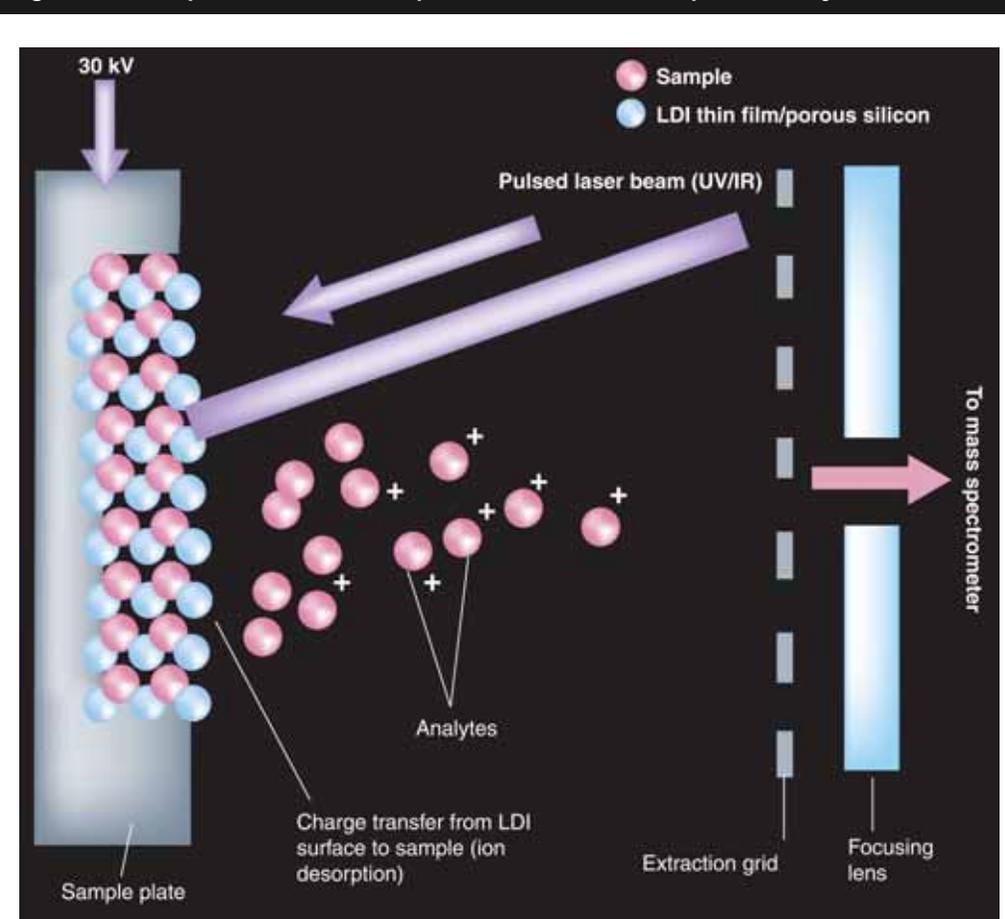
achieved by proton transfer. Once the samples molecules have been ionized it is possible to analyze them via mass spectrometry.

The Siuzdak group were the first to develop a truly 'matrix-free' LDI-MS method, which involved placing the sample on a porous silicon plate, produced by electrochemical etching of silicon wafers in HF to generate a nanocrystalline surface [226]. They successfully demonstrated the method by analyzing antiviral drugs, des-arg-bradykin, and several small peptides. The technique was subsequently coined desorption ionization on porous silicon (DIOS). The performance of DIOS is determined by the type of silicon (p or n) and the etching conditions that are used [227], as well as the conditions that the porous silicon is produced under [228].

Since the emergence of DIOS, several other 'matrix-free' LDI surfaces have been developed. These include inorganic sol gels that are polymeric in structure and are formed from micellar concentrations of 'pore-filling' alkyl quaternary ammonium cations with subsequent condensation and polymerization with a silica precursor, resulting in a siloxane (SiO) backbone with hydrophilic Si-OH headgroups. The first application of sol gels to LDI, involved the incorporation of an immobilized matrix compound into the gel, thus producing a surface that was capable of directly transferring laser energy to the sample molecules, leading to their ionization [229]. A range of carbon-based polymers and porous polymer monoliths have also been applied as LDI surfaces, with varying degrees of success [222].

Mesoporous nanostructures (with pore sizes ranging from 2–50 nm) are also ever increasing in popularity for use as 'matrix-free' surfaces for LDI. Primarily, research focused upon carbon nanotube and silicon wire-based surfaces. These were found to produce minimal background S/N interference within spectra and required much lower laser powers to induce sample ionization when compared to DIOS and MALDI, as well as permitting the routine detection of metabolites at attomole concentrations [230,231]. However, the generation of these nanostructure surfaces unfortunately requires highly experienced synthetic chemists. Alternative approaches have been to use mesoporous 'acid-base' pairing of tungsten and titanium oxide precursors, yielding structures with a well-defined 2D hexagonal mesostructure, which again showed high sample ionization at low laser powers. An even more recent approach has applied the use of thiol

Figure 5. Desorption in laser desorption ionization-mass spectrometry.



The LDI thin film/porous silicon is prepared upon a sample plate, to which the sample is spotted on top. A 30 kV charge is applied to the sample plate, which is simultaneously exposed to a pulsed laser beam. The LDI thin film/porous silicon then transfers the charge from the laser to the sample, resulting in the ionization and desorption of the sample's metabolites. The analytes are then channelled through the extraction grid and focusing lens before entering the mass spectrometer.

stabilized gold nanoparticles to produce 10^{-6} m thick mesoporous thin films with evenly distributed particles. Gold particles of 2–5 nm have been shown to induce ionization at much greater levels than the larger-sized carbon and silicon nanoparticles [232].

LDI-MS & its potential for medical diagnostics

Published literature supporting biological applications of nanoporous materials have been limited in LDI-MS due to most studies concentrating upon the development of new and improved surfaces. Despite this, Vaidyanathan and colleagues applied DIOS-MS to identify 26 metabolites in a cocktail of 30 metabolites over both positive and negative ion modes. They then went on to analyze and metabolically distinguish cell-free supernatants from cultures of

Saccharomyces cerevisiae haploid single-gene deletants [233]. More recently, Vaidyanathan and colleagues analyzed a 30-metabolite cocktail on both oxidized and nonoxidized DIOS surfaces by LDI-MS. Surface oxidation appeared to influence mass spectral responses, with the signal intensities of the hydrophobic amino acids being noticeably reduced. It was revealed that quantitative changes of the individual analytes could be detected, although ion suppression effects were seen to interfere when the levels of a single analyte were significantly altered, thus potentially restricting the instrument's dynamic range [223]. DIOS-MS has also been applied to forensics, where a range of low-molecular-weight polymers, including an ethoxylate polymer, were identified without fragmentation to aid during a criminal investigation [234]. Chen and Wu applied DIOS-MS to identify a range of small

Table 2. Comparison of the various vibrational, NMR and mass spectrometry approaches.

	FT-IR	Raman	NMR	DIMS	LDI
Sample preparation	Direct analysis	Direct analysis	Dissolved in suitable solvent	Extracted into suitable solvent	Extracted into suitable solvent
Rapidity (spectral acquisition)	10 s–1 min	10 s–3 min	Depends on strength of magnet and 1D versus 2D experiments. Can be as rapid as 8 min	1–3 min	10 s–1 min
Reproducibility (1 = poor to 5 = high)	5	4	5	4	3
Sensitivity (1 = poor to 5 = high)	2	2	2 (unless hyperpolarizability is used)	4 (5 when applying MS ⁿ)	4
Specificity (1 = poor to 5 = high)	1	1	3 (or higher when 2D methods are used) Co-resonant peaks	4 (or higher with MS ⁿ)	4 (or higher with MS ⁿ)
Data analysis	All very similar. Very little data preprocessing is needed, in comparison with the extensive deconvolution needed for GC-MS and LC-MS				
Portability/miniturisation	Already in existence	Already in existence	Given magnetic field strengths needed, this is currently not portable or likely to be in the near future	Not currently, but miniaturization in progress	Not currently, but miniaturization in progress
Instrument cost (1 = low to 5 = high)	1	2	5	4/5	3
Cost per sample (1 = low to 5 = high)	1 (reagentless)	1 (reagentless)	2 (some solvents needed)	2 (some solvents needed)	2 (some solvents needed)
Metabolite ID (1 = poor to 5 = high)	2	2	3 (or higher with 2D approaches)	4 (or higher with MS ⁿ)	4 (or higher with MS ⁿ)

organic molecules such as methylphenine, cytosine, and small PEG polymers [235]. The large range of metabolites detected in these studies highlight the potential of DIOS-MS to be applied to medical diagnostics.

Finally, two recent studies have actually gone on to analyze material of medical significance. Su and Tseng analyzed plasma and urine samples derivatized using sodium borohydride to convert disulphides into thiols [236]. The derivatized samples were analyzed directly on steel plates by LDI-MS in order to successfully determine their homocysteine concentrations. The second study employed an oxidized carbon nanotube-based surface [231] that had been subsequently immobilized by a polyurethane adhesive [237]. The immobilized carbon nanotube surface was primarily applied to the analysis of a range of sugars, prior to further analyses where urine

from healthy and diabetic patients was distinguished by the presence of glucose in the diabetic sample [237]. These analyses have indicated that LDI-MS is particularly appropriate for the analysis of low-molecular-weight metabolites of biological significance. With the imminent further development of 'on-chip' nanopore-based LDI surfaces, its suitability for metabolic fingerprinting will be greatly enhanced and many more examples of its application to medical diagnostics will be reported.

Conclusion

Metabolomics is the functional analysis method aimed at acquiring robust and reproducible quantitative information on cellular metabolites. Clearly, there are many technical challenges that need to be addressed in order to generate comprehensive metabolomics data,

and one needs to use a combination of orthogonal technologies to increase metabolite coverage as much as possible (see Table 2 for a comparison of the technological approaches discussed in this review). Notwithstanding this, as discussed above, metabolic fingerprinting is gaining considerable interest across a wide variety of disciplines, with the current main focus, as reviewed here, being on biomarker discovery for disease prognoses, diagnoses and therapy monitoring. Not only will the discovery of multiple metabolite markers be beneficial for patient stratification and assessing therapeutic intervention, but the identification of key metabolites and their spatial distribution

within tissues will likely lead to a greater understanding of the physiology of the disease process that will serve as a hypothesis generator for novel drug targets.

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