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Recent and potential developments in the analysis of urine

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\textbf{Abstract}
Analysis of urine is a widely used diagnostic tool that traditionally measured one or, at most, a few metabolites. However, the recognition of the need for an holistic approach to metabolism led to the application of metabolomics to urine for disease diagnostics. This review looks at various aspects of urinalysis including sampling and traditional approaches before reviewing recent developments using metabolomics. Spectrometric approaches are covered briefly since there is already a number of very good reviews on NMR spectroscopy and mass spectrometry and other spectrometries are not as highly developed in their applications to metabolomics. On the other hand, there has been a recent surge in chromatographic applications dedicated to characterising the human urinary metabolome. While developments in the analysis of urine encompassing both classical approaches of urinalysis and metabolomics are covered, it must be emphasized that these approaches are not orthogonal – they both have their uses and are complementary. Regardless, the need to normalise analytical data remains an important impediment.

1. Introduction

The first line of defence for any medical condition is diagnosis. There are many different ways that a disease state can be recognised including those that manifest themselves visually (an abrasion) or those that rely on an indirect diagnosis as, for example, in the measurement of glucose in diabetes. Metabolite changes have long been observed in diseased individuals whether as a primary cause or a secondary indicator. Thus, the measurement of metabolites has been an integral part of clinical practice for over a century. The diagnostic potential of body fluids such as serum, urine and bile is underscored by the number of publications devoted to this area. Despite the significant advances in analytical technologies, the discovery of
biomarkers in biological fluids remains a significant challenge. Whilst such studies employ the full range of biofluids including saliva [1,2], serum [3], plasma and whole blood [4-6], semen [7] and breath [8], urine has a number of advantages as identified in Section 1.1. These advantages have ensured the widespread use of urine as a diagnostic tool in clinical practice.

Traditionally, tests on urine measured one or two components such as the measurement of urinary glucose and these remain an important part of the clinician’s hardware to this day. However, these one- or two-component chemical tests are distinguishable from more recent developments which aim for complete characterization of the entire metabolome regardless of molecular size. This represents a significant challenge. Various terms are used [9] to encompass this more modern approach although metabolomics is probably most common in this and other application areas. Metabolomics has been applied to an understanding of the health and disease continuum [10] and its benefits have been demonstrated in diverse clinical areas [11-15].

Various meanings are attached to the term metabolomics and the relative merits of the different meanings have been discussed [16-18]. It is important to establish the precise meaning of metabolomics in the current context. We lean towards a definition that encompasses characterization and quantitation of all metabolites within a sample but with the qualification that it considers both spatial resolution (due to cellular compartmentalization of metabolism) and temporal changes in response to environmental stimuli. Thus, the measured metabolome represents a snapshot of catabolic and anabolic processes at a particular point in time for the specific sample as
the sample defines different aspects of an organism’s metabolome [11]. For instance, both concentrations and the nature of metabolites will differ between the metabolome as measured in urine and that measured in say blood.

The human urinary metabolome thus defined is potentially vast if one includes all endogenous metabolites and exogenous entities that might be encountered. Thus, urinary metabolomics currently remains an impossible task owing to the complexity of the metabolome both in the diversity of analytes and its wide dynamic range covering at least mM in the case of glucose to nM for compounds such as acylcarnitines [19] in urine. Our ability to measure the urinary metabolome is related to analytical sensitivity and specificity and thus the measured metabolome becomes analytically-determined. However, any analytical restrictions to achieving urinary metabolomics are artefactual representing our current limitations and the goal must be to seek the development of analytical techniques (including sample preparation) that are consistent with the definition. This requires a paradigm-shift in analytical approach as we now require techniques that are both sensitive and specific yet capable of measuring all analytes even at trace levels.

A number of previous reviews have examined various aspects of urine analysis ranging from an historical view [20] of clinical applications to identification of urinary markers of specific pathological conditions [21,22]. This paper examines developments in the analysis of urine encompassing both classical approaches of urinalysis and metabolomics. It must be emphasized that these approaches are not orthogonal – they both have their uses and are complementary.
1.1 Urine Sampling

The use of urine as an analytical tool has a number of advantages over other biofluids. It can be obtained in large quantities by non-invasive sampling and repeat sampling is not a problem. Analytical advantages claimed for urine relative to say serum include the need for less complex sample pre-treatment due to lower protein content, the relatively small size and higher thermodynamic stability of urinary peptides/proteins, and the lower sample complexity including less intermolecular interaction [23]. Some of these advantages such as lower complexity may be seen as counter-productive to metabolomics.

Urine samples are normally collected [24] as random samples [25,26], timed samples [27,28] or 24-hour samples [29] although much longer time series sampling may be used in some areas such as pharmacokinetics [30]. There are advantages and disadvantages of the use of these techniques of urine collection.

Random samples can be taken at any time of day, but do not take into account diurnal variation of excretion. Furthermore, random sampling cannot give direct measures of volume of urine excreted and to correct for this in biomarker studies measurement of creatinine is often used. However, this correction is useless if the biomarker being monitored is variable in its time of excretion (e.g. glycosaminoglycans) [31]. Although 24 h samples are cumbersome, they provide a more complete picture of excretion. For example, random spot urine collection and measurement of protein/creatinine ratio predicts actual 24 h protein excretion with reasonable accuracy in patients with lower levels of protein excretion but is unreliable in patients with high protein excretion [32]. When urine collection is required for research, it is
recommended that a marker such as PABA (p-aminobenzoic acid) be used to cross check completeness of urine collection in conjunction with self reporting of amounts of urine lost and timing of collected samples [33]. The incorporation of such steps obviously has significant budgetary implications. Some metabolites are related to circadian rhythms and these metabolites plus dietary components have differentiated time of day urine collection [34] and this detail is lost with 24-hour sampling. The latter is more demanding of subject compliance and strategies for improving collection of these samples have been devised [35].

Stability and sample integrity during storage are important considerations as in any analysis. The urine samples must be stable to provide valid data. Urine stability was examined in a pilot metabolomic study of urine (and serum) samples from 40 separate healthy volunteers [36]. Samples were either processed immediately by freezing at –80°C or stored at 4°C for 24 h before being frozen, and then thawed and profiled by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) revealing more than 700 unique metabolite peaks. Over 200 peaks were detected in any one sample. Differences between samples stored in the two ways were assessed by Wilcoxon rank sum test and Principal Components Analysis (PCA) methods. In general, there was no statistically significant difference in the metabolic profile of samples stored under the two conditions (Figure 1). Moreover, analytical variance (from replicate analyses of the same sample) was of the same magnitude as the variance between samples stored under the two conditions. This study should be extended to analysis of fresh samples. It is curious that storing and transporting of urine samples for doping analysis does not include a specific protocol for cooled transport from the place of urine sampling to the analytical testing laboratory [37].
2. Urinalysis

Urine has been an important marker of health and disease since 4000 BC. Hippocrates (460-355 BC) hypothesized that urine was a filtrate of the humors and began using urine as a tool to diagnose disease. Galen (AD 129-200) further went on to describe urine as a filtrate from blood, and began to use urine to describe diseases now recognised as diabetes mellitus and renal failure. ‘Uroscopy’, the examination of urine, became a primary diagnostic tool until the Victorian era. Strict rules were adhered to on the inspection of urine including the use of a matula (glass vessel in the shape of a bladder), and a urine wheel that described possible colors, smells and tastes of urine and corresponding diseases. However, the importance of uroscopy became increasingly exaggerated; physicians began to diagnose urine samples without consultation with the patient and uroscopy began to be practiced by charlatans.

More recently, urine analysis (urinalysis) has been firmly established as a diagnostic tool that is an important aspect of the physical examination. Urinalysis involves physical, chemical and microscopic analysis of the urine and in a comprehensive review [38] it was noted that clean mid-stream sample collection is adequate in most situations although testing should be conducted within 2 h of collection. Indeed, the stability of common analytes in urine has been examined [39]. The classical approach to urinalysis as applied to clinical practice may involve near-patient testing or laboratory testing. The former invariably employs reagent strip or dipstick testing, which is routinely performed and recommended [40] before more complex, comprehensive and expensive laboratory techniques are employed.
Dipstick testing is widely used because of its speed and simplicity coupled with ability to perform near-patient testing and a number of studies have examined its diagnostic accuracy for a variety of measurements [41-47]. The modern era of dipstick testing began in 1956 [48] and now represents a simple screening technique available to the clinician at low cost which yields very fast results. Data are at best semi-quantitative but dipsticks can give almost instantaneous feedback on a number of excreted products that allows clinicians to formulate a differential diagnosis and order more conclusive quantitative tests [49,50]. Typical applications of dipsticks (and associated condition) include measurement of urine specific gravity or osmolarity (diabetes insipidus), urinary pH (e.g., renal tubular acidosis), haematuria (e.g., glomerulonephritis), glycosuria (e.g., diabetes mellitus), ketonuria nitrites (e.g., urinary tract infection) [51], bilirubin/urobilinogen (e.g., biliary obstruction), and hormones (e.g., human chorionic gonadotropin indicating pregnancy) [38]. Urine dipstick testing is routinely used in pre-natal care [52] and, in Korea, all school age children must have an annual urinalysis. This is performed using dipstick analysis of the first early morning urine specimen [53] and it has aided in detection and prognosis of chronic renal disease progression. A similar dipstick screening process has been proposed elsewhere [54].

Care is necessary in the application of dipstick testing as it may be inadequate in some situations [55] and this was demonstrated in a study of the use of multireagent strip dipsticks in detection of urinary tract infection in the long-term care setting [56]. The high false positive rates for some tests made the strips unsuitable for routine detection. Dipstick testing failed to detect pathological proteinuria in a number of patients and urinary protein/creatinine ratio was preferable to dipstick testing for measurement of
proteinuria at least in nephrology units [57]. Similar conclusions were made in assessment of hypertensive disorders in pregnancy using proteinuria measurement by dipstick with the recommended procedure involving 24 h urine protein measurement [58]. Interpretation of results can be complicated by a number of factors. For example, sexual intercourse is a benign cause of proteinuria in men and should be avoided for at least 12 h before urinary dipstick testing [59]. Major health organizations do not support screening for hematuria by dipstick testing because of the high false-positive rate [60]. Nevertheless, millions of asymptomatic patients are tested annually. Thus, there is ample scope for improvement in dipstick technology by broadening the range of analytes to include a broader range of species such as β-hydroxybutyrate [61] and/or by improving sensitivity [62].

Laboratory testing involves routine measurement of metabolites to obtain quantitative data useful for diagnostic purposes [63]. Less routine analyses are also performed to obtain information on: metabolite concentrations [64] and metabolic pathways [65,66]; errors of metabolism; drug interactions and monitoring of therapeutic dosage [67] including anaesthesia [68]; exposure to exogenous chemicals [69-72] including antibiotics [73] and measurement of background exposure levels [74]; intake of illicit drugs [37,75,76]; doping control [77,78] and measurement of drug metabolites [79,80]; and nutrition and dietary intake [81] including food contaminants [82,83]. These analyses are generally characterised by selective sample preparation coupled with highly specific methods of detection [84,85]. Analytes are typically limited to one or a very small number of chemically related components although there are some notable exceptions as in drug screening procedures [84].
3. Recent developments - Metabolomics

The traditional approach to urine analysis is inconsistent with the modern holistic view of metabolism in which the link between all metabolic pathways is recognised. Approaches to this that are now historical but still widely practised are metabolite profiling [86-88], metabolite fingerprinting [89-91] and target analysis [76,92]. The aims of these approaches have been delineated elsewhere [93] and are summarized in Table 1 but by their nature, they provide a restricted non-comprehensive view of the metabolome. Metabolite profiling can be considered the precursor for metabolomics which alone has the potential to provide fully integrated data on the metabolome [94-96]. Molecular biomarkers that differentiate environmental, pathogenic or toxicological insult to individuals [97-99] have been used to confirm or aid in diagnosis of a disease and to provide early warning of a disease at the pre-clinical stage as in the case of diabetes. Such procedures may be regarded as metabolomics when they involve completely untargeted analysis that leads to identification of a suitable marker. However, two general approaches that are more limited in scope have generally been used. One is based on knowledge of the biochemistry of a substance to look for predicted metabolites or the parent compound in urine. Alternatively, the urine may be screened for compounds related to a compound of interest. Biomarkers must generally meet high standards for acceptance by physicians and patients and this can delay their widespread implementation [100].

Biomarker structure and identification are important to an holistic understanding of metabolism. Structural identification is frequently based on limited data as noted by Bedair and Sumner [101] who stated “it is generally believed that a single chemical shift, m/z value, or other singular chemical parameter is insufficient for validating
non-novel metabolite identifications.” This has led to the proposed guideline [101] for the identification of non-novel metabolites in which “a minimum of two independent and orthogonal data relative to an authentic standard compound analyzed under identical experimental conditions are proposed as necessary for metabolite identification. Examples would include retention time/index, mass or NMR spectrum, accurate mass and tandem MS, accurate mass and isotope pattern, 1H and/or 13C NMR spectra, and 2D NMR spectra. Identifications performed without authentic standard compounds and based upon spectral similarity with public/commercial spectral libraries, or published literatures are generally believed insufficient to validate a confident and rigorous identification. Thus, such identifications should be regarded as putatively annotated compounds.”

In contrast to the restricted approaches, metabolomics presents a unique opportunity to “identify” new biomarkers by use of chromatographic or spectroscopic techniques coupled with chemometrics [24]. Potential applications are the identification of a healthy urinary profile, to establish impacts of diet on the metabolome, to act as a general toxicity screening aid, to aid in early diagnosis of disease [21], to provide evidence of novel biochemical pathways or confirm existing metabolic pathways [102], to monitor donor organ viability or rejection [13,103] and to relate the metabolome to the genome. However, the INTERSALT Study [104] showed that “accurate estimates of associations ...... required large population-based samples, high-quality dietary information, control for multiple confounding variables, and modern multivariate methods of data analyses, including correction of observed associations for within-person variation in intake”.
The human metabolome is influenced by a number of phenotypic, physiological and external factors, that include (but are not limited to) gender, age, BMI, diet, stress, medications, exercise, fasting, and consumption of alcohol 24 h prior to collection [105], which can potentially be distinguished via urinary analysis. This can be an advantage in many applications or a confounding factor in clinical practice. Consumption of dietary phytochemicals is one confounding variable that results in acute changes in urinary metabolite profiles [106]. Apart from the analytical problems associated with metabolome measurement, this further complicates metabolomics. For instance, daidzein, genistein, and total isoflavonoids were elevated in urine samples following soya consumption [107]. Urinary excretion of genistein and total parent isoflavones plus metabolites was decreased during antibiotic use versus when healthy [108]. In contrast, O-desmethylandolensin production increased during antibiotic treatment. The reduction in urinary isoflavone appearance was attributed to changes of intestinal bacteria by antibiotic treatment and/or to processes related to the infection.

The use of metabolomics in discovery of new biomarkers of phytochemical intake has been reviewed [109] and such studies will help elucidate the complex interactions between human health and dietary intake of phytochemicals. Alternatively, diversity in phytochemical intakes [110] may be reduced by dietary restrictions in the 24 h before urine sample collection that may reduce variation and improve data interpretation in metabolomics studies using urine. This raises the issue of what constitutes the human metabolome per se and the possibility of normalizing the dietary metabolome via a standardized diet. In this connection, the human
metabolome has been classified [109] as comprising the endogenous metabolome, the microbial metabolome and the xenobiotic metabolome.

### 3.1 Non-hyphenated spectrometric platforms

Spectrometric analyses provide a composite picture of the ensemble of molecules in a sample. In many ways, pre-instrumental urine analysis with eye, nose and tongue, was a forerunner to modern instrumental methods, in that our senses provide a collective response to a mixture. Interestingly in other settings sensory analysis is considered superior to instrumental methods e.g. in wine or olive oil tasting.

In principle, any form of spectrometry may be applied to metabolomic investigations of urine, including ultra-violet/visible (UV-Vis), infrared, Raman, nuclear magnetic resonance (NMR) and mass spectrometry (MS) [111]. In practice, the majority of metabolomic studies, based purely on spectrometric analyses, have employed NMR spectroscopy. Other spectrometries (especially UV-Vis and MS) are rarely used in non-hyphenated mode, but when coupled to a chromatographic system, provide quantification/identification of metabolites subsequent to separation (see below). As far as the authors are aware, there are no reports on the use of UV-Vis spectroscopy in metabolomics studies and only one for the application of FT-IR spectroscopy [112]. In this study rat urine from a group treated with an inflammatory agent could be distinguished from a control group.

New developments in sample introduction to a mass spectrometer have contributed to a growing number of metabolomics applications by mass spectrometry. Techniques such as desorption electrospray ionization (DESI) and extractive electrospray
ionization (EESI) are methods of directly introducing a sample to the mass spectrometer that overcome matrix effects that are problematic for urine analysis. Applications of these techniques have included: distinguishing between healthy mice and those with lung cancer (Figure 2) [113]; distinguishing between rats fed three different diets [114]; and the detection of inborn errors of metabolism in humans [115]. In each of these studies, $^1$H-NMR spectroscopy was used to validate the results from MS. Both techniques gave similar clusterings on principal component analysis (PCA) plots.

While not a direct injection application of a whole sample into the mass spectrometer, the report by Bullinger et al. [116] is interesting in that ribose containing metabolites were selectively enhanced using cis-diol specific affinity chromatography and new metabolites were identified via ESI-FTICR mass spectrometry. A total of 22 compounds were identified, including modified nucleosides and ribosylated metabolites, associated with at least 5 different metabolic pathways. These 22 compounds are not typically reported in NMR spectroscopic studies, possibly due to very low concentrations and overlapping signals. Nevertheless, they represent a potentially useful range of metabolites for diagnosis of various diseases. This study highlights some of the issues that make true metabolomics problematic in that these ribosylated metabolites would not have been detected without selective enrichment, yet selective enrichment is counter to true metabolomics.

By far the majority of purely spectrometric approaches to metabolomics have used one-dimensional (1D) $^1$H NMR spectroscopy as the technique of choice. Various reviews have appeared highlighting the range of current applications such as
physiological evaluation, drug safety assessment, diagnosis of human disease, drug therapy monitoring [24,117], detection of inborn errors of metabolism [118], and diet [27]. As well, the advantages of NMR spectroscopy have been well documented and they include “little or no sample preparation, [is] rapid and non-destructive, and uses small sample sizes” [117]. Further, NMR spectroscopy has been put forward as intrinsically better for quantitative work because “variable detection responses, such as differential volatilization or ionization effects as in MS, are not an issue for NMR spectroscopy” [117]. However, more recent studies by Law et al. [119] challenge this assertion and in fact the authors conclude that “The close agreements of LC/MS with 1H NMR data showed that the effects of ion suppression in LC/MS for early eluting metabolites were not significant.”

NMR spectroscopic analysis of the metabolic composition of urine (in experimental animals) under various normal physiological conditions has been assessed [120]. NMR spectroscopy of urine samples of rats was used to construct a substantial metabolic and pathological database as part of the Consortium on Metabonomic Toxicology (COMET) project [121]. The COMET project clearly established the potential benefits of NMR in urine profiling and metabolomics [122] especially in the area of in vivo screening of drug toxicity. In one part of the COMET study [121] 12935 urine samples from 1652 laboratory rats were screened by 1H NMR spectroscopy following treatment of the rats with one of 80 different drugs or toxins (e.g. acetaminophen, carbon tetrachloride, etc.). Damage to target organs could be identified i.e. whether the drugs/toxins preferentially altered liver or kidney metabolism, even at sub-toxic levels. Sensitivity and specificity of the approach was also reported e.g. the sensitivity to kidney toxicity was 41% whereas the specificity
was 100%. One of the goals of this study is to assist in drug discovery and development where kidney/liver toxicity can be a major cause of drug recall, hence early detection of the potential for such toxicity is advantageous. Recently, biomarkers of pancreatic toxicity have been reported [123] with 2′-deoxyctydine emerging as a possible marker for the apoptotic mechanism of toxicity (subject to ongoing verification).

Detailed protocols for biofluid (urine, serum/plasma) and tissue sample collection and preparation have been reported elsewhere [24]. Quantification of metabolites can be achieved through the use of the chemical shift reference, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid. Unlike quantification in GC-MS or LC-MS studies (see below), this standard is sufficient to quantify all metabolites because the response of every proton is essentially uniform (as long as there is sufficient delay to allow all protons to relax). Quantification of low abundance metabolites can be problematic due to overlapping signals [124]. Greater precision can be achieved through 2D NMR techniques, but the trade-off is significantly longer acquisition times [124], which are not compatible with high-throughput applications.

Interferences in the 1H NMR spectrum are water, which can be suppressed via an appropriate pulse sequence, and urea (when urine is the target matrix). While NMR spectroscopy has a number of advantages (as per above) and is able to detect many molecular classes, including organic acids, ketones, sugars, and amines, nevertheless there are some disadvantages that need to be recognised. These include a general failure to detect low abundance metabolites (< 5 μM) (e.g. nucleosides or neurotransmitters) or hydrophobic species (steroids, fatty acids, etc), which occur in
urine in higher abundance [125]. Possible reasons may include that with larger, hydrophobic molecules such as lipids, there would be many overlapping peaks (due to large numbers of C-H and C-H₂ groups) leading to broad signals. Hydrophobicity may also lead to molecules aggregating in aqueous solution, affecting relaxation times and consequently broadening the signal. This highlights again the complementary nature of NMR spectroscopy with that of GC-MS or LC-MS techniques.

Spectral binning [126] has been the predominant approach in analysing NMR spectroscopic data [34] for metabolomic studies [127]. An alternate approach is targeted profiling [34], also known as “quantitative metabolite profiling” [125], which is fundamentally different to spectral binning. In spectral binning, chemometrics techniques are first utilised to find regions of the spectrum that differentiate between groups. Subsequently, metabolites with NMR spectra consistent with those regions are identified and quantified. On the other hand, targeted profiling seeks to build the spectrum of interest based on the spectra of known metabolites and thus identification and quantification are incorporated from the beginning. Slupsky et al. [34] demonstrated the differences between the two approaches by looking at gender differences in urinary metabolite profiles. They also showed that age and diurnal variation could also be distinguished by targeted profiling. Despite these advantages, targeted profiling does have some limitations e.g. with its need for comprehensive databases and specialized curve-fitting software, it is not yet as automated or as unbiased as current chemometric approaches [125]. Furthermore, only known compounds can be identified and quantified, removing the possibility of identifying new biomarkers.
3.2 Chromatographic platforms

Chromatographic platforms have exploited the full range of chromatographic techniques for urine analysis but are biased towards liquid chromatography in more recent publications and hence such LC platforms will be discussed in more detail.

Sample pre-treatment has been necessary particularly for GC and it has two goals; to selectively enhance concentration and eliminate interferences including sample components that may reduce column lifetimes. Thus, it is incompatible with the goal of metabolomics, to measure all metabolites in their naturally occurring state. The only qualification would be to allow non-selective enhancement of all analyte concentrations although selective enhancement of a broad class of compounds has probably been accepted as a compromise. This is a severe restriction as there is a wide range in analyte concentrations and chemical nature from low molecular size to large biomolecules with limited volatility. For instance, urine has a high urea concentration and hence treatment of the sample with urease has been recommended to reduce possible column overloading, matrix effects and, in the case of mass spectrometric detection, ion suppression [128] (cited in [105]). The first report of the use of urease in urine analysis was by Shoemaker and Elliot (cited in [129]) in 1991 as a way to address major chromatographic interference by urea and masking of many low intensity metabolite peaks. However, urease treatment is known to interfere with at least some metabolites including acotonic acid, hypoxanthine and tricarboxylic acid intermediates (for example citrate, succinate, tyrosine, ascorbate) which are greatly diminished following such treatment [128] (cited in [130]). Instead, chromatographic conditions should be optimised to account for high concentrations of urea either
through column selectivity, or ensuring excess urea elutes with the column void volume [105] which is generally achievable under reversed phase LC conditions.

3.2.1 Gas chromatography

GC and GC-MS approaches dominated early ‘metabolomics’ experiments (as cited in [131]) as a result of their successful application in metabolite profiling studies as illustrated by the use of GC to profile phenolic derivatives [132,133]. Issaq et al. [105] state that GC-MS is well suited to the analysis of stable, volatile metabolites including fatty acids, steroids, and flavonoids whilst HPLC is well suited to the analysis of lipids, peptides, nucleotides, and ionic species. In terms of cost, they state that GC-MS systems are much more affordable than HPLC-MS systems, with GC-MS being preferred for targeted metabolomics, namely target analysis.

Whilst the application of GC is limited to volatile, thermally stable and non-polar compounds, derivatization can be successfully used to improve the volatility of some analytes [134], and is common practice in the analysis of urinary metabolites (see below). The fact that established GC-MS metabolite libraries are commercially available combined with the somewhat qualitative nature of retention indices and retention time locking certainly make GC and GC-MS methods highly attractive, since such libraries and retention indices are not available in LC-MS methods.

Pasikanti et al [129] have used GC-quadrupole MS for the global profiling of human urine samples in light of the technique’s sensitivity, peak resolution and reproducibility. Various derivatization agents were used and compared, namely $N,O$-bis(trimethylsilyl)trifluoroacetamide (BSTFA), $N$-methyl-$N$-
(trimethylsilyl)trifluoroacetamide (MSTFA) and methyl bis(trifluoroacetamide (MBTFA). BSTFA and MSTFA demonstrated similar derivatization efficiencies with respect to the number of detected peaks, peak intensity and reproducibility. MBTFA was investigated in combination with BSTFA, since the former is known to be more efficient in derivatising secondary and tertiary amine groups. More amino acids were detected when BSTFA derivatization was preceded by MBTFA derivatization, however the integrated peak area data was not found to be reproducible. The authors thus chose BSTFA as the derivatization agent in their optimised GC-MS method.

The study identified 150 endogenous compounds, 144 of which were assigned names based on retention indices and mass spectral matching (> 70 %) with the NIST mass spectral library. Of these metabolites, 29% were classified as organic acids, 23% as sugars, 10% as amino acids, 3% as aromatics, 3% as unknown compounds, 2% as fatty acids, 1% as glycerols, and 29% as other chemical classes. In their validation experiments using a pooled human urine sample analysed at 0, 7, 14 and 90 days, intra- and inter-day precision values were less than 15% for 95% of the 150 putative metabolites identified in human urine. The optimised GC/MS method was applied to distinguishing between urine samples from male and female volunteers (Figure 3). A total of 53 subjects (31 male and 22 female) and 150 variables (peak areas) were the input in the PCA analysis. PCA was then followed by orthogonal partial least squares (OPLS) to distinguish class information. A clear discrimination between male and female groups was found. Concentrations of succinic acid, 3-hydroxyhippuric acid, fumaric acid and fucose were higher in female urine samples, whilst concentrations of 2,4-dihydroxybutanoic acid and 2,3-butandiol were higher in male urine samples.
What is interesting in this study is that despite the occurrence of overlapping peaks in the GC-MS chromatogram, deconvolution of the mass spectral data was not undertaken. The authors cite that the repeatability of most deconvolution software programs is such that it is likely to generate a number of false positive and false negative results [135] which may jeopardise distinguishing between analytical variation and variation due to deconvolution errors. Because of this, the application of their GC-MS method is limited to the detection of only major components contrary to their conclusion that their GC-MS “metabonomic platform is suitable for global profiling of urinary metabolites”.

Michell et al. [136] have investigated biomarkers of Parkinson’s disease by profiling urine and serum samples using GC-TOFMS. Urine samples from 46 participants (including 23 age and sex-matched control subjects) were spiked with an internal standard solution incorporating succinic d4 acid, malonic d2 acid and glycine d5 prior to urease treatment to reduce the high amounts of urea present in samples. Extracts were necessarily derivatised, firstly using O-methylhydroxylamine (in pyridine) to generate oxime derivatives, and finally with MSTFA to give trimethylsilyl derivatives. The final solutions were spiked with an alkane retention index solution prior to analysis. In urine approximately 900 unique mass spectral signatures were distinguished. Unsupervised principal components analysis using a combination of peak intensity and spectral data was used for the initial treatment of the data to identify clustering and remove extreme outliers, followed by projection to latent structures discriminant analysis (PLS-DA).
The results showed that any metabolic disturbance associated with Parkinson’s disease is relatively subtle; subtle difference in urinary metabolite profiles was observed, however this signature was highly multivariate (i.e. caused by many metabolites “each making a small, and individually insignificant, contribution to discrimination”) rather than related to specific biomarkers. Interestingly, no such signature was identified in serum samples, however, this may be attributed to the reduced number of unique mass spectral signatures (approximately 700) identified in serum¹ compared to urine. In light of these results, the authors suggest that rather than a tool to search for individual biochemical markers, it is possible that the overall metabolic profile of body fluids may represent a powerful biomarker in itself [136]. This clearly supports and emphasises the desire and need for a true metabolomics approach to urinary analysis as opposed to classical metabolite profiling and targeted analyses, which are used in the absence of the availability of such an approach.

3.2.2 Liquid chromatography

Analysis of urine using LC platforms generally employs reversed phase conditions using C18 columns, with typical mobile phases of water + formic acid (Solvent A) and either methanol or acetonitrile + formic acid combinations (Solvent B) [137,138]. Detection is a key element in the application of LC to urinary metabolomics. Common detection methods used in metabolomics (incorporating metabolite profiling) include UV/Vis absorption and MS [139]. Both methods have their advantages and limitations [139],[140] however MS is preferred for quantitation (generally due to enhanced sensitivity of MS detection compared to UV/Vis [140],

¹ Alternatively, it may be a result of greater homeostatic control in serum.
and the provision of structural fragmentation data for qualitative identification). MS is also preferred since not all compounds in a mixture will have the same response factor at the same wavelength, i.e. extinction coefficients will be vastly different for different metabolites. However, not all compounds ionise to the same extent, which is problematic for global metabolomics studies but can be accommodated in targeted analyses (not true metabolomics) since the metabolites of interest will have similar chemical properties [139] or, at the very least, have well understood properties such that quantitation could be achieved [140]. Nevertheless, with electrospray ionisation MS detection, ion suppression or enhancement may occur as a result of coeluting analyte and matrix components competing for ionization at the electrospray interface [141]. Such phenomena are detrimental for quantitative analyses and can obscure the identification of minor metabolites. The interested reader is referred to the following references for a more detailed discussion of ionization suppression/enhancement effects in metabolomic applications [141,142]. For quantitation of individual targeted analytes UV detection is suitable. In all applications involving UV detection, a photodiode array detector (PDA) is recommended over a single wavelength UV/Vis detector, since multiple detection wavelengths can be monitored simultaneously.

The use of internal standards in traditional targeted analytical applications is encouraged, particularly for quantitative analyses, and the use of isotopically labelled analogues is recommended since such analogues behave in the closest possible manner to the analyte of interest i.e. with respect to relative response factors and ionisation efficiency. Of course, the use of isotopically labelled internal standards requires that the identity of the analytes is known [143]. In metabolomics however, this is rarely the case in that the range of compounds being analysed is maximal, and
analyte diversity in terms of functionality and polarity is great. This implies that numerous internal standards must be used in a single sample to accommodate the multiple biochemical classes of compounds within the sample. Hodson et. al. [138] warn that the addition of an increasing number of internal standards has the potential to obscure and alter the detection of analytes of interest in an already complex matrix. For example, ionization suppression of important ions or metabolites can occur when mass spectrometric detection is employed.

Whilst not a replacement for the use of internal standards, alternative approaches to quality control for metabolomics applications have been recommended; Gika et al. [89] advocate the use of a pooled urine sample, as a “mean” representative of all of the analytes which will be encountered during the analysis. The implementation of a pooled sample allows standardization of the LC-MS system performance, particularly with respect to reproducibility in that Gika et al. [144] found that a number of injections of urine were required to equilibrate the system prior to the commencement of the analytical run to ensure retention time reproducibility. Hodson et al. [138] also endorse the use of a pooled sample in addition to the use of an artificial urine sample composed of common analytes found in urine for quality control. The latter serves as a convenient means to assess chromatographic and mass spectral performance to facilitate analytical method optimisation. Collectively, the use of an artificial urine mix and a pooled sample should provide a “degree of confidence in the performance of the system for the purposes of an initial semi-quantitative open metabolomic screening of any sample set” [138].

3.2.3 Future directions in LC for achieving urinary metabolomics
Successful application of chromatography to achieve true metabolomics will require enhanced column resolution or ‘improved’ detection capability. It is hard to envisage what the latter might involve but it must encompass the ability to detect and isolate signals from simultaneously eluted metabolites. MS offers some potential here but is limited by ionization effects.

Traditionally, improving selectivity has been the best approach to enhancing resolution. Hydrophilic interaction chromatography (HILIC) and aqueous normal phase (ANP) chromatography offer different selectivity relative to reversed phase systems for highly polar analytes including urinary metabolites such as creatinine [145,146]. This offers an alternative analysis scheme for the separation and retention of urea. Indeed, uric acid and methyl uric acids have been successfully separated and retained using a diol column in HILIC [147]. Although HILIC and ANP are often used synonymously, a clear distinction between the two modes of retention and types of columns used in each approach exists. Briefly, HILIC stationary phases are selective for polar-ionic compounds, since reverse phase stationary phases do not effectively retain these compounds, and separation is achieved via partitioning of the solute into and out of the hydrated surface of the negatively charged silica stationary phase surface [148]. Typical stationary phases are based on silica and polar bonded organic groups (e.g. amino, cyano, diol, or zwitterionic) on silica [146]. For ANP, columns used are silica hydride-based which offers dual retention mechanisms, namely a “reversed phase” mechanism due to the aqueous nature of the mobile phase, and a “normal phase” mechanism whereby increasing analyte retention is achieved as the mobile phase becomes more non polar [149]. Because of this, ANP with silica hydride columns affords retention of both polar (hydrophilic) and non polar
(hydrophobic) compounds. ANP is preferable to HILIC separations for high-throughput metabolomic applications, since HILIC columns are often slow to re-equilibrate after gradient elution, and resolution and efficiency are reduced compared to reversed phase separations [146]. Furthermore, ANP columns are selective to a more diverse range of analytes [149].

Callahan et. al. [146] and Pesek et al. [150] have used ANP with silica hydride based stationary phases for the LC-MS analysis of urine samples. In the former case, the separation power of a diamond hydride column (MicroSolv Technology; 100 × 2.1 mm; 4 μm dₜ) was compared to that of a standard commercially available reversed phase column (Agilent Zorbax Eclipse XBD-C18; 100 × 2.1 mm; 1.8 μm dₜ). Despite the particle size differences, the two columns detected similar numbers of features with similar reproducibility, however broad irreproducible peak shapes were observed for some metabolites, including citric acid, using the diamond hydride column. For the analysis of urine samples, more than 1000 compounds, although not fully resolved, were detected in a single run. Comparison of urine samples taken before and after ingestion of both coffee and a multivitamin supplement showed 144 unique mass features in samples in the latter, including those relating to riboflavin and proline betaine. Interestingly, caffeine and its metabolites could not be retained using the diamond hydride column; however, caffeine was easily detected using reversed phase conditions. The authors concluded that no single column and ionization mode enables the simultaneous separation and detection of all metabolites and thus deem their ANP method as suitable for the analysis of complex mixtures of polar compounds for metabolomic studies [146].
Pesek et. al. [145] have used ANP and MS detection for the analysis of metabolites in human urine and saliva samples. Successful separation of polar isobaric compounds was achieved (Figure 4) [150], and retention and peak shape were controlled by variation of the additive in the mobile phase and gradient conditions. Creatinine in urine was successfully detected using an optimised gradient yielding a narrow symmetrical peak, and creatinine, creatine and 4-hydroxyproline were detected in synthetic urine. The authors stated that future work to evaluate different mobile phase compositions will be conducted to establish if a single method can be developed to accommodate most, if not all, hydrophilic metabolites.

Enhanced resolution necessary for true metabolomics may also be approached by improving column efficiency. As the majority of analytes remain unknown in metabolomics, traditional approaches for HPLC optimization must be reconsidered, and a “pragmatic approach” to method development is necessary [138]. This requires a “best guess” of the expected number of analytes, and an estimate of the representative biochemical classes in order to develop an appropriate elution strategy [138]. In some respects, this task has been simplified via the enhanced separation power afforded by Ultra performance liquid chromatography (UPLC). Columns used for UPLC are packed with sub-2μm particles and operating pressures of 8000-12000 psi are commonly used (compared to 2000-4000 psi typically used in HPLC). Efficiency and resolution are significantly increased in UPLC facilitating greater opportunities for analyte separation and detection with the added advantages of reduced run times and solvent usage. In a direct comparison, sub-2 μm particles can generate 30000 plates/15 cm column length compared to 22000 and 12000 plates/15 cm for 3-3.5 μm and 5 μm particles, respectively [151]. Direct comparison of UPLC
and HPLC has not been reported for urine samples but data from UPLC-MS and HPLC-MS based metabolomics of human serum demonstrated the superiority of [137]. UPLC which generated around 20% more “features” than HPLC, (features or peaks are defined as a unique m/z at a unique time point), and higher signal-to-noise ratios as a result of narrower elution profiles.

Apart from increasing column efficiency, high throughput metabolomics of rat urine was facilitated by UPLC-TOFMS [138] with orthogonal acceleration time of flight mass spectrometry (oa-TOFMS) [152]. Run times were merely 1.5 min, and a reversed phase gradient separation was employed [152]. The authors found that their UPLC-oaTOFMS approach was similar to, or better than their established urine analysis performed via HPLC-MS with a 10 min runtime [153] with respect to peak capacity and detected marker ions. For the latter, 3900 marker ions were detected via UPLC-oaTOFMS, compared to 1000 ions detected via conventional HPLC-MS. From their UPLC-oaTOFMS results, rapid discrimination between mice age, strain, gender and diurnal variation could be achieved. Interestingly, the authors caution that their rapid screening method should be used to establish gross biochemical differences in samples, and that once differences have been established, a smaller subset of samples “can then be correctly selected and subjected to more comprehensive analysis to determine the entire biomarker set” [152].

Compared to high throughput metabolomics [138,152], Guy et al. [154] have developed an optimised and validated global metabolite profiling method for urine analysis using UPLC-TOFMS, with specific focus on nutritional metabolomics applications. The authors’ state that for the latter, a high analytical quality standard is
essential since for nutritional applications, subtle changes in metabolic profiles are expected as compared to toxicological applications. Three UPLC gradient programs were investigated with increasing run times from 10 min (gradient 1), 26 min (gradient 2) and 31 min (gradient 3). Figure 5 compares the 3 gradients, and shows that gradient 3, a three-step linear gradient produced the best chromatographic separation with respect to resolved peaks compared to gradients 1 and 2, particularly in the second half of the run (Fig. 5c). This is numerically supported by the total retention time/mass pairs obtained for each gradient, namely 2378, 8743 and 21,862 for gradients 1, 2 and 3, respectively. With respect to quality control, repeatability was best using gradient 3, hence this gradient was used for further investigations. Fluctuation of the ion response was found to be the most variable parameter, and coefficients of variation (CV) of the area measured for the four spiked compounds ranged from 3% (hippuric acid) up to 22% (nortriptyline) in positive ionization mode, and from 7% (hippuric acid) up to 16% (succinate) in negative ionization mode. Based upon their findings, and knowledge of the variation inherent in urine samples due to time of collection, the authors recommend a maximum CV value of ± 25% for ion response (CV calculated from the mean value of spiked compounds) in metabolomics investigations of urine.

Maximum peak capacities using standard HPLC columns (ie 250 mm × 4.6 mm i.d), which have been widely used for metabolome analyses, are approximately 300 [155], however, in practice, this value is seldom achieved and more realistic peak capacities are between 100 and 200 [156]. This capacity is enhanced with single dimension UPLC separations using sub-2μm particles. However, more significantly, in a complex chromatogram with random peak spacing, one can never expect to see more
than 37% of the peaks theoretically possible with uniform spacing in a one-dimensional chromatographic separation [157]. Even more disheartening is that the number of single-component (singlet) peaks cannot exceed about 18% of the peak capacity even under optimised conditions. Multidimensional and comprehensive techniques (e.g., LC-LC and LC×LC, respectively) offer significantly increased gains in separation power compared to their single dimension counterparts, and offer unmatched potential for metabolite resolution. Some have postulated that through multidimensional techniques it may one day be possible to separate any sample on a generic multidimensional system without method development [158]. Such a hypothesis fits well with the fundamental goals of metabolomics. In comprehensive multidimensional approaches, theoretical peak capacities are equal to the product of the peak capacities of the individual dimensions [159] although practical comprehensive LC×LC peak capacities are substantially reduced due to various parameters [160, 161]. Such techniques have not been reported for urinary metabolomics but it is clear that the inherent separation power of comprehensive LC×LC approaches will ensure such techniques will play a critical role in the future of urinary metabolomics as well as other applications.

4. Standardization

The examples that we have quoted illustrate the complexity and other issues associated with urine analysis. However, standardization represents one of the biggest challenges regardless of the analytical approach and we hope that we can draw attention to its importance and the need for further investigations. There are many aspects of standardization and some have been treated comprehensively (see, for
example, [162] which provides guidelines for metabolomics in *plants*) and this includes standardization of the analytical method.

The purpose of the analysis is an important consideration. This may include functional genomics, investigation of physiological status, classification of disease and toxicity, monitoring efficacy of therapeutic intervention and nutritional studies. Factors that required standardization in human urine nutritional metabolomics [163] were diet, culture, cohabitation and gender. In other instances, knowledge of the healthy state is critical to determine normal or acceptable ranges for metabolites. Thus, careful consideration must be given to the selection of a healthy or normal group with well characterized metabolite homeostasis. The definition of ‘normal’ and ‘healthy’ for subjects chosen to participate in the control group, as well as choosing an ‘idealised’ versus a ‘normal’ population [164] is not simple. The philosophical definition of a normal or healthy population may raise several questions. In most studies, however, this involves selection of individuals with no underlying chronic illness although there is a clear distinction between healthy and the lack of clinical symptoms. This can be illustrated by the early-stage development of diabetes.

A certain degree of homeostasis is assumed in the excretion of metabolites in normal urine. However, a major challenge in the use of urine for metabolomics is the large biological variation in its composition. Thus, it becomes important to define the inter- and intra-individual metabolite variance within the normal or control group. The Geigy Scientific Tables is the standard reference for normal and diseased human metabolite levels. However, data regarding normal metabolite concentrations and degrees of variance found in urine are severely limited relative to the number of
potential analytes [164-166]. Most studies involve limited size cohorts of typically 30-50 individuals with a normal or control group of similar size [167]. Factors such as gender, age, ethnicity and exercise level can be controlled whereas factors of a cultural and dietary nature are much more complex [34]. Poor subject compliance with experimental protocol may compound the problems of limited cohort size.

The lack of standardization in other aspects with particular reference to urine requires more efforts specifically from analytical chemists. The most significant unresolved issue relates to variance in the volume of urine within the same person day-to-day and person-to-person due to hydration status, which is affected by factors such as level of water lost through perspiration, respiration, and defecation. This variance (600-2500 mL/d) [168] further complicates data comparisons when concentration data are expressed as mass per unit volume e.g., µg/L. Urinary metabolite concentrations are governed by the quantity of a chemical present in the blood, the rate at which the chemical is excreted from the blood, and the amount of fluid excreted by the kidney [169,170]. Thus, changes in the amount of fluid excreted will result in variability in urinary metabolite concentrations, irrespective of exposure to the chemical. To account for this variability, the renal elimination rate, that is the rate at which the kidney filters the blood, needs to be measured. However, as this rate is not readily accessible, alternative measures are often used to adjust for the urinary concentration of metabolites. For a measure to be useful as a correction factor, it must not systematically vary across demographic groups of interest, such as age or sex, or across study factors, such as season or time of day. Otherwise, comparisons among individuals that differ across these factors may be artifically set apart, irrespective of
actual exposure levels [170]. Therefore, it is very important that we have methods to normalise for volume in urinary metabolite studies.

Various approaches are used including flow rate correction [171], electrical conductivity, specific gravity or osmolarity [172-174] and, most commonly, creatinine normalisation [165,175,176]. Other normalisation strategies include statistical normalisation e.g. probabilistic quotient normalisation [177] and histogram normalisation for NMR data [178].

4.1 Use of creatinine to normalise urinary metabolite data
Creatinine is present in serum, erythrocytes, sweat, bile, gastrointestinal fluids, cerebrospinal fluid and urine and concentrations of endogenous metabolites are most commonly expressed as ratios relative to creatinine [164,165]. Its presence in urine was identified as early as 1901 by Folin Otto, and its measurement is now a widely accepted and common measure for controlling for urine volume. Excretion of creatinine in urine represents the end-point of endogenous energy transfer from stored adenosine triphosphate in skeletal and cardiac muscle. In the absence of kidney disease, the urinary creatinine is excreted in relatively constant amounts [164] representing glomerular filtration and active tubular excretion of the kidney. Thus, there are expected normal values for creatinine in human urine although these are highly dependent on the age, gender and lean body mass of the individual. Although creatinine concentration is proportional to urine flow at flows below 1 mL/min it remains constant for flows above this value [168]. It is generally accepted that there is little metabolic or excretory variance in healthy individuals [164] with no circadian rhythmicity [179] although this has been challenged [180-182]. The underlying theory
for creatinine correction is that the blood ratio of creatinine to the chemical of interest is maintained in urine because both compounds are filtered at the same rate by the kidney [170], however, caution should be used as this may not always hold true. For example, renal excretion mechanisms are xenobiotic-specific and creatinine was inferior as a normalising technique for measurement of xenobiotic biomarkers in smokers [173].

While measurement of urinary creatinine is used to correct for the effects of urine volume on total urinary concentration, variation in creatinine excretion in the urine has been a topic of debate since Otto Folin (in 1905) reported little day-to-day variation in creatinine excretion for healthy individuals. While it is widely accepted to use creatinine to normalise volume of urine output, there are potential problems with the use of creatinine with regard to its variability in excretion day-to-day based on exogenous factors. Abnormal results of urine creatinine and creatinine clearance are often non-specific, but may be due to any of the following variables: diet especially meat and creatine intake [183,184], sleep deprivation [185], time of day, age, sex, fat versus fat-free mass, level of physical exercise, pregnancy, mental state [184] and disease states including urinary tract obstruction, kidney failure, reduced kidney blood flow (resulting from shock or congestive heart failure), glomerulonephritis, late stages of muscular dystrophy, myasthenia gravis, pre-renal azotemia, pyelonephritis, and rhabdomyolysis. Moreover, a number of drugs are known to affect creatinine measurements including aminoglycosides (gentamicin), cimetidine, heavy metal chemotherapeutic agents (cisplatin), and nephrotoxic drugs such as cephalosporins (cefoxitin). Clinically, experienced physicians understand the variability both in creatinine excretion and endogenous and exogenous factors that effect its excretion,
and as such will (consciously or unconsciously) interpret known and/or observed variation accordingly. In the clinical setting, analysis is more targeted to a particular diagnosis whereas metabolomics research seeks to identify subtle changes in fine detail across the metabolome.

In metabolomics research, there is a shift to view excretion products holistically and, in this environment, it cannot be assumed that creatinine is a suitable agent for normalisation of all metabolites. For instance, with pervasive development disorders, it was concluded that efforts should be directed to utilizing other internal (or external) ratio standards when attempting to quantify urinary compounds [186]. Others have questioned the validity of using a single reference standard and have proposed use of a broader molecular basis using a multi-compound normalisation strategy [23]. Callahan et al. [146] concluded “To date we have found normalisation to be one of the greatest challenges when comparing chromatograms from biological samples. In order to increase confidence, larger datasets with more replication are required”. A normalisation strategy based upon the concept of mass spectrometer total useful signal (MSTUS) has been proposed by Warrack et al. [187] for metabonomic analysis of urine as an alternative to common normalisation approaches including creatinine and osmolality. This approach is analogous to that used in proton NMR whereby each spectrum is normalised to the total integrated proton signal after exclusion of xenobiotic and artefact prone regions (for example water and urea regions). MSTUS incorporates only those peaks that are present in all samples, thereby limiting contributions from xenobiotics and artefacts: integrals for all peaks which were common among the sample set are summed to generate the base peak chromatogram necessary for comparative purposes. Based upon their results, the authors recommend
two different normalisation strategies to facilitate detection of statistically significant changes in endogenous metabolite profiles when dealing with urine samples, namely MSTUS and osmolality. The former is specifically recommended when osmolality measurements are neither convenient nor valid.

The validity of creatinine for normalisation of the entire metabolome as opposed to single metabolites requires further testing and intra-individual variation of creatinine excretion in this context has not been systematically investigated. There is clearly a need to address this gap in urinary metabolomics research using a prospective, single centre, feasibility study to test the intra-individual variation of creatinine excretion. This would involve a cohort of healthy and disease free individuals - as assessed by a physician (normal weight, taking no regular medications, no known chronic disease states) - and aged 18-30 years. These parameters – health and age - can be controlled for in recruitment, however controlling for genetics and gut microflora are as yet impossible. The collection of moment-by-moment information on the excretion of urinary creatinine would require direct access to the bladder so urine would not accumulate and mix over the time period until the next void. Such samples would require introduction of a catheter but this could compromise the sample [188,189] due to introduction of microbes.

**Concluding remarks**

Metabolomics offers potential advantages that classical approaches do not. For example, earlier pre-symptom diagnosis of a greater range of diseases becomes possible. The discovery of new biomarkers has been demonstrated but the vast potential has not yet been unleashed. This does raise some issues about routine
clinical practice; following discovery of a new clinically relevant biomarker, does one continue to use metabolomics for its measurement or revert to classical targeted approaches which are probably more accurate. On the other hand, metabolomics provides the opportunity to diagnose based on a pattern of biomarkers, not a single compound, and this would be useful where the disease is not characterised by a single compound but by a suite of metabolites that are simultaneously affected by the disease. It is this application that will probably drive urinary metabolomics and provide greatest benefits at least in the short-term.
Reference List


[185] R.T. Rubin, Psychosomatic Medicine, 33 (1971) 539-.


Table 1
Comparison of analytical approaches to urine analysis

Figure 1.
Principal Components Analysis plots for urine for six randomly chosen volunteers illustrating sample similarity independent of storage conditions. Red triangles depict $T = 0$ h and blue circles depicts $T = 24$ h storage. PCs 1 and 2 represent, 19 and 8% of the variance. Labels represent subject identifiers. Each sample was analysed in triplicate and all three replicate analyses are shown. Reproduced from [36].

Figure 2.
PCA plot of (a) $^1$H NMR spectroscopic data and (b) DESI-MS data derived from rat urine of healthy rats (C1 and C3) and those with lung cancer (T2 and T4). Both data sets show similar separation among the samples. (Adapted from reference [113]).

Figure 3.
An OPLS scores plot obtained from the urine analysis of healthy male and female volunteers showing a clear distinction based on sex of the subject. Reproduced from [129].

Figure 4.
Extracted ion chromatograms from synthetic urine sample. (A) m/z 145 negative ion mode. (B) m/z 176 positive ion mode. Column: DH 2.16150 mm. Flow rate 0.4
mL/min. Injection volume = 1 µL. Mobile Phase, A = water + 0.1% formic acid; B =
acetonitrile + 0.1% formic acid. Gradient: 0–0.2 min 95% B; 0.2–30 min to 50% B;
30–35 min 50% B. Solute: 1 = adipic acid (145.0506); 2 = 2-oxoglutaric acid
(145.0142); 3 = several possible identities; 4 = citrulline; 5 = arginine. Reproduced
from [150].

Figure 5.
Typical chromatograms obtained from the analysis of a male urine sample analysed
by UPLC–TOFMS in positive ESI mode. The UPLC gradient was realised within (a)
a 10-min, (b) a 26-min, and (c) a 31-min gradient conditions. All other analytical
parameters were kept the same. The gradient conditions used for the three
experiments are reported as insert. Reproduced from [154]
Figure 2.
Figure 3.
Figure 4.
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Table 1