RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2006; **20**: 1192–1198
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.2434

Matrix-suppressed laser desorption/ionisation mass spectrometry and its suitability for metabolome analyses

Seetharaman Vaidyanathan*, Simon Gaskell and Royston Goodacre
School of Chemistry, The University of Manchester, P.O. Box 88, Sackville Street, Manchester M60 1QD, UK

Received 1 November 2005; Revised 10 February 2006; Accepted 13 February 2006

Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry was investigated for the simultaneous detection of several metabolites, as applicable to global metabolite analysis (metabolomics). The commonly employed organic matrices α-cyano-4-hydroxycinnamic acid and 3,5-dihydroxybenzoic acid, in both the crystalline and ionic liquid forms, were investigated. The employment of a low matrix-to-analyte molar ratio suppressed matrix peaks and was effective in detecting all the metabolites with a unique mass in a 30-metabolite synthetic cocktail, albeit to varying degrees. These matrix-suppressed laser desorption/ionisation (MSLDI) analyses were performed in the positive ion mode, and metabolites were detected as the protonated $[M+H]^+$, sodiated $[M+Na]^+$ or potassiated $[M+K]^+$ species. The spectral signals were dominated by basic metabolites. It was possible to detect components of a synthetic cocktail when it was spiked quantitatively into a microbial extract, demonstrating the feasibility of using the technique for detecting metabolite signals in a complex biological matrix. However, analyte suppression effects were noted when the relative proportion of one analyte was allowed to increasingly dominate the others in a mixture. The implications of the findings with respect to applications in metabolomic investigations are discussed.

Profiling of the transcriptomic, proteomic and metabolomic expression forms an integral part of functional genomic investigations, and is increasingly becoming relevant in understanding the behaviour of biological systems. While transcriptomic and proteomic analyses have been actively pursued to enhance our understanding of gene functions,[1–4] only relatively recently have metabolome analyses emerged as a complementary route in functional genomics and systems biology.[5–7] Consequently, there is considerable interest in developing approaches for analysing metabolomes, including methods based on hyphenated techniques using separations based on chromatography (GC, LC) or electrophoresis (CE) followed by mass spectrometry, 2D-thin layer chromatography, NMR spectroscopy and vibrational spectroscopic techniques including Fourier transform infrared (FT-IR) and Raman.[7–9]

Matrix-assisted laser desorption/ionisation (MALDI) time-of-flight mass spectrometry (ToFMS) is a very popular technique for the analysis of biopolymers, such as peptides, proteins and oligonucleotides. The key factor in its success is the 'soft' ionisation characteristic of MALDI combined with the huge mass range of ToFMS that enables even large biomolecules to be detected, predominantly as singly charged species. Although not as popular, its application has also been extended to the analysis of lower molecular weight compounds.[10] Given the soft ionisation characteristic, the propensity to generate singly charged species, better tolerance than electrospray ionisation (ESI)-MS to interference from salts and buffers, and simplicity of sample preparation, MALDI-MS is ideally suited for simultaneous, rapid, high-throughput analyses of metabolites in mixtures, such as would be encountered in metabolomic investigations.

Metabolome analyses involve measurements (ideally quantitative) of the metabolite complement (the low molecular weight components and intermediates of metabolism) of a cell, tissue or organ in a particular physiological state.[5,8,11] Metabolomics, metabonomics, metabolite profiling, metabolic fingerprinting and metabolic footprinting are some of the terminologies in current use, depending on the type of analysis and the area of application.[8,12] Fingerprinting (in which changes in intracellular components are captured) and footprinting[13] (where changes in extracellular components secreted into the immediate environment of organisms, cells or tissues are monitored) ideally involve minimal separation or sample clean-up operations, enabling simple protocols to be developed. Such analyses are useful in instances where high-throughput measurements need to be made. These include, but are not limited to, the screening of samples/mutants for more elaborate investigations and identification/monitoring of biomarkers representative of specific physiological or disease states. MALDI-MS is ideally

*Correspondence to: S. Vaidyanathan, School of Chemistry, The University of Manchester, P.O. Box 88, Sackville Street, Manchester M60 1QD, UK.
E-mail: s.vaidyanathan@manchester.ac.uk
Contract/grant sponsor: UK BBSRC.

suited to such analyses if prominent changes can be captured in a reproducible manner. It can also be applied in conjunction with separation and sample clean-up techniques when more comprehensive information is required. The requirement in all these cases, especially with fingerprinting or footprinting, is to obtain consistent signals for many metabolites simultaneously, ideally in a quantitative manner. Despite the popular perception of the unreliability of the MALDI technique for quantitative determinations, the quantitative analysis of individual small molecular weight components have been demonstrated.[14–17]

Conventional MALDI-MS with organic matrices (like α-cyano-4-hydroxycinnamic acid, HCCA) result in peaks from matrix ions and their adducts in the low mass range where low molecular weight (<1000 Da) compounds give signals. In order to counteract these interferences, several strategies have been adopted, including the use of high molecular weight compounds as matrices,[18] derivatisation of analytes,[19] and employment of post-source decay to reduce interferences from matrix ions.[20] In addition, laser desorption/ionisation on inorganic matrices such as porous silicon[21–23] have also been developed. However, employing a lower than conventional matrix-to-analyte molar ratio has been shown to be an effective means of suppressing matrix signals, while still utilising matrix properties for effective desorption and ionisation.[15,24] In traditional MALDI-MS, matrix excess conditions with matrix-to-analyte molar ratios above 1000:1 are required to detect large molecular weight biopolymers such as proteins effectively. However, for the analyses of low molecular weight compounds, matrix-to-analyte molar ratios of 100:1 and 10:1 have been successfully employed.[15,25] In addition, it has also been shown that ionic liquid-based matrices in the same molar ratios are effective in obtaining reproducible signals from MALDI-MS of low mass compounds.[17]

While the detection of low molecular weight compounds has been demonstrated, this has typically involved the detection of one or two analytes. In metabolomics the challenge is to be able to detect simultaneously many analytes in a complex milieu in a quantitative manner, with hopefully simple protocols. In this study we investigated the employment of a low matrix-to-analyte molar ratio, and the use of ionic liquid-based matrices, for the simultaneous detection of several analytes, and discuss the suitability of these approaches for metabolomic investigations.

EXPERIMENTAL

Materials

Two metabolite cocktail stock solutions, each containing 30 metabolites, were used in the study. Cocktail A consisted of the amino acids (in L-form) alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, valine (all at 30 mM), aspartic acid, glutamic acid, tryptophan (all at 3 mM), tyrosine (0.3 mM), the organic acids fumaric (3 mM), citric, malic, lactic, pyruvic, succinic, oxaloacetic (all at 30 mM), and the metabolites 4-aminobutyric acid, putrescine and D-glucose (all at 30 mM). Cocktail B consisted of the above 30 metabolites at an equimolar concentration of 0.1 mM each. All the metabolites were obtained from Sigma-Aldrich (Dorset, UK).

Escherichia coli (MG1655) cells were grown in Lauria-Bertini broth in shake flasks (37°C incubation, orbital shaker at 200 rpm) to an optical density (at 600 nm) of 3. Intracellular extracts of the cell were prepared by rapidly plunging the cell culture (0.4 ml) in 0.6 mL of cold methanol (stored at −80°C) to rapidly quench metabolism. The cells were harvested by centrifugation, washed and sonicated after re-suspension in 60% aqueous methanol. The sonicated cells were centrifuged and the supernatant used as a biological matrix to assess the feasibility of detecting metabolites in this mixture of biological origin. An appropriate dilution of cocktail A was spiked into the microbial extract so that the final concentrations of the spiked metabolites in the mixture before spotting were 0.03 mM each. DHB-pyridine was used as the matrix at matrix-to-total analyte molar ratios ($M:A_T$) ranging from 40:1 to 0.01:1 (measured relative to the spiked analyte concentrations).

In order to study ion suppression effects, two additional metabolic cocktails consisting of the 20 amino acids listed above, one excluding serine and the other excluding only histidine, were prepared. The cocktails were serially spiked with increasing concentrations of these two amino acids such that the final concentration of each of the spiked metabolites before spotting ranged from 10 μM to 1 mM, while maintaining the concentration of all the other metabolites at 50 μM. The matrix used was DHB-pyridine, and the preparations were made such that $M:A_T$ was maintained between 0.4:1 and 1.7:1 (i.e., matrix-reduced conditions prevailed).

Sample preparation and mass spectrometry

α-Cyano-4-hydroxycinnamic acid (HCCA) and 3,5-dihydroxybenzoic acid (DHB) were used as the solid organic matrices in the study. Ionic liquid-based organic matrices (ILMs) HCCA-pyridine and DHB-pyridine (both at 1:1 molar ratio) were prepared as detailed elsewhere;[17] briefly, an equimolar mixture of HCCA (or DHB) and pyridine in aqueous acetonitrile (1:1 containing 0.1% trifluoracetic acid, TFA) was mixed and sonicated for 3–4 min to give the respective ILMs. Stock solutions of the matrices (30 mM) were prepared and appropriately diluted in aqueous acetonitrile to give $M:A_T$ ratios of 40:1, 4:1, 0.4:1 (1:2.5) and 0.04:1 (1:25) in the mixed solutions. The metabolite cocktail (A or B) was diluted to appropriate concentrations in the matrix solution. Then, 1 μL of a mixed solution was spotted onto a stainless steel MALDI-MS target plate, air dried (dried-droplet method) and analysed using a Voyager DE-STR (Applied Biosystems, Framingham, MA, USA) MALDI-ToF mass spectrometer, in the positive ion mode. Each spectrum consisted of an average of three acquisitions from the same sample, with 100 shots/acquisition. For all measurements, the reflectron mode in the range m/z 10–500 was employed, typically with an acceleration voltage of 20 kV, a grid ratio of 76% and a delay time of 325 ns.

The mass spectral data were imported into MATLAB (The Math Works, Natick, MA, USA) and processed for analysis. Typically, the data were normalised to total ion counts, peak areas were calculated based on Simpson's method[26] and

DOI: 10.1002/rcm

RCM

expressed as a percent of the total of all peak areas; peaks at the relevant *m/z* corresponding to those of the protonated $[M+H]^+$, sodiated $[M+Na]^+$, or potassiated $[M+K]^+$ forms, in the range *m/z* 50–250, were analysed.

RESULTS AND DISCUSSION

Matrix type and the influence of matrix-to-analyte molar ratios

Matrix signals dominate the MALDI mass spectra in the low mass range (m/z 500) under matrix-excess conditions (matrix-to-analyte molar ratios >1000:1) that are typically employed in MALDI-MS of macromolecules. However, it is known[15,25] that reducing the matrix-to-analyte molar ratio to 100:1 or 10:1 can improve the detection of low molecular weight (<500 Da) species due to matrix suppression effects.[24,27] It is also known that analyte suppression effects operate when two or more analytes are present.[24,28] We examined the signals under low matrix-to-analyte molar ratios with the objective of analysing metabolite mixtures, as would be applicable in metabolomics. In addition, room temperature ionic liquids[29] have been suggested as matrices for effective MALDI-MS, owing to their higher stability under vacuum conditions and to the homogeneity of sample distribution in such matrices. Subsequently, ILMs based on traditional matrices such as HCCA and DHB have been shown to be useful for the analysis of low molecular weight compounds.[17] Therefore, the popular MALDI matrices, crystalline HCCA and DHB and their ionic liquid forms, HCCA-pyridine and DHB-pyridine, were examined.

The average spectra of five replicates for the analysis of an equimolar mixture of 30 metabolites using the four matrices are shown in Fig. 1. Cocktail B, diluted to give a final concentration of 0.03 mM of each metabolite before spotting, was used; this is equivalent to a mixture containing 30 pmol of each metabolite on the target plate. The analysis was performed under a matrix-reduced condition, where the $M:A_T$ molar ratio was 0.4:1 and the corresponding matrix-to-maximum analyte molar ratio ($M:A_M$) was 10:1. We term the MALDI-MS analysis under such matrix-reduced conditions as matrix-*suppressed* laser desorption/ionisation mass spectrometry (MSLDI-MS). Under these conditions, the analyte signals (labeled 1 to 5 in Fig. 1) dominate the spectral response compared to the matrix peaks (indicated by asterisks), for all four matrices. A striking feature of the observed analyte peaks is the heterogeneity in their relative detectability, despite the presence of the analytes in the mixture at equimolar levels. This is not unexpected as the analytes differ in their chemical nature and ionisability and can be expected to have different mass spectral responses.

Although the spectra observed using the crystalline HCCA and DHB matrices (under matrix-reduced conditions) show dominant analyte peaks with few or no dominant matrix peaks, several less dominant analyte peaks have interfering contributions from matrix ions. This makes it difficult to interpret the spectrum when using such matrices. The problem is to some extent minimised with the application of room temperature ionic liquids, where the basic component (pyridine in this case) minimises the observation of matrix

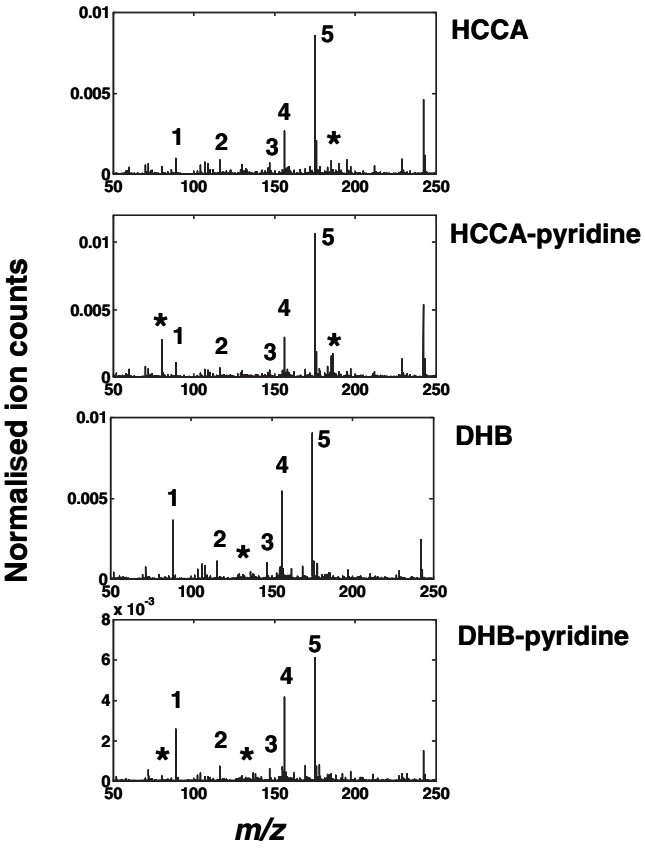

Figure 1. Matrix-suppressed laser desorption/ionisation (MSLDI) mass spectra of an equimolar mixture of 30 metabolites (refer to the text for details) in the positive ion mode. The spectra shown were obtained using the matrices HCCA, DHB and the ionic liquid matrices HCCA-pyridine and DHB-pyridine, under matrix-reduced conditions such that the matrix-to-total analyte molar ratio $M:A_T$ was 0.4:1 and the corresponding matrix-to-maximum analyte molar ratio ($M:A_M$) was 10:1. The average of five replicate spectra is shown for each matrix, and five representative ions observed are labeled for ease of comparison: (1) putrescine $[Put+H]^+$; (2) proline $[Pro+H]^+$; (3) glutamine $[Gln+H]^+$ or lysine $[Lys+H]^+$; (4) histidine $[His+H]^+$; and (5) arginine $[Arg+H]^+$. The matrix peaks are indicated by asterisks.

ions, resulting in 'cleaner' spectra. While HCCA and DHB are crystalline, HCCA-pyridine is solid and DHB-pyridine is liquid under the analytical conditions. As has been noted earlier,[17,29] such a characteristic is helpful in enhancing signal response due to a more homogeneous distribution of analytes. However, the laser power required for the detection of the analytes was higher for analysis with the ionic liquid-based matrices compared with the solid matrices, consistent with earlier observations.[17] Due to the low concentrations employed, the dried matrix-sample spots tended to concentrate on tiny areas in the well. Therefore, manually controlled accumulation was resorted to. Nevertheless, it is noteworthy that signal responses were observed for most metabolites even when the matrix concentration was lower than the total analyte concentration.

Analysis of individual analytes using ILMs has been found to be optimal at matrix-to-analyte molar ratios of 1:1 to 10:1,[17]

DOI: 10.1002/rcm

an order of magnitude lower than those employed for the corresponding solid matrix.[15,25] We studied different matrix-to-analyte molar ratios, including conditions where analyte concentrations are relatively high and the matrix suppression effect is expected to be operable, i.e., M:A_T of 40:1 to 0.01:1, corresponding to a M:A_M of 1000:1 to 0.25:1. The influence of the matrix-to-analyte molar ratio under low matrix and low analyte conditions is demonstrated in Fig. 2 for the ionic liquid matrix DHB-pyridine. The metabolite mixture used was cocktail B (an equimolar mixture of analytes), diluted to give a final concentration of 0.03 mM of each metabolite before spotting. Matrix peaks, which dominated the spectrum when a M:A_T of 40:1 (analyte less condition) was used, are suppressed and the analyte peaks dominate the spectrum when a low M:A_T of 0.4:1 (matrix reduced condition) was employed.

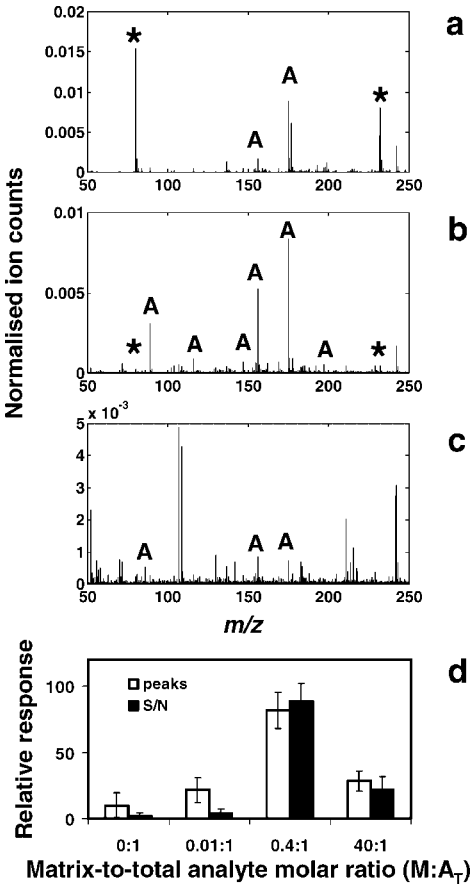

Figure 2. Positive ion MALDI and MSLDI mass spectra of a cocktail of 30 metabolites (refer to the text for details) under (a) analyte-less conditions (M:A_T = 40:1; M:A_M = 1000:1) and matrix-reduced conditions (b) M:A_T = 0.4:1; M:A_M = 10:1 and (c) M:A_T = 0.01:1; M:A_M = 0.25:1. The matrix used was the ionic liquid-based DHB-pyridine. Matrix peaks are indicated by asterisks and analyte peaks as 'A'. Notice the suppression of the matrix peaks under the matrix-reduced condition (hence the term matrix-*suppressed* laser desorption/ionisation). (d) Influence of M:A_T ratio on the overall signal response for the ionic liquid-based matrix DHB-pyridine. The signal-to-noise (S/N) and the number of species detected (relative to the maximum) are shown. Peaks observed at the relevant *m/z* values and contributing a value greater than 1% of the total peak area were considered in the calculation. Mean values of five replicates with standard error bars are shown.

In the positive ion mode the analyte spectral information was dominated by the basic amino acid arginine, followed by other basic metabolites. The relative signal strength of the protonated ions in MALDI-MS has been correlated with analyte basicity.[28] The observed analyte signals for the metabolites in the mixture also correlated with their gas-phase basicities (data not shown). Prominent signals were detected (in decreasing order of prominence) at *m/z* values corresponding to the protonated forms of arginine, histidine, putrescine, glutamine, lysine, 4-aminobutyric acid, proline, and to sodium or potassium ion adducts of tryptophan and of malic, succinic, glutamic, aspartic, and oxaloacetic acids. Although the total analyte concentration is relatively high under matrix-reduced conditions, it is possible that dominant analytes with high gas-phase basicities (like arginine in this study) dictate proton transfer from the matrix. They abstract most of the available protons, leaving few protons for the less basic analytes. At progressively matrix-reduced conditions only the dominant analyte signals are observed. It has been noted[24] that a component that yields a sufficiently stable $[M+H]^+$ ion can effectively abstract most of the available charge at the expense of the other coupled species (matrix and analytes) provided that enough of the relevant compound is present and is homogeneously distributed. As a result, although the M:A_T ratio at which signal response is high appears to be under matrix-reduced conditions, the effective M:A_T may well be closer to a ratio of 1:1.

The overall signal responses for the 30 metabolites in the mixture were studied for the four matrices. The responses in terms of signal-to-noise (S/N) and the number of species detected (both parameters calculated for peaks whose peak areas contribute >1% of the total) are shown in Fig. 2(d), for the ionic liquid matrix DHB-pyridine at the four M:A_T ratios 40:1, 0.4:1 and 0.01:1 and 0:1. It is clear from Fig. 2(d) that the highest signal response is observed for the matrix-reduced condition (i.e., when M:A_T is 0.4:1). Some analyte signals are observed even when no matrix is used (M:A_T = 0:1). However, the response in this case is much weaker than when the matrix is used. Even here the response was dominated by the basic metabolites, arginine and histidine. It is possible that the UV-absorbing aromatic amino acids tyrosine, tryptophan and phenyalanine present in the mixture may assist in desorption/ionisation of the basic arginine and histidine, even though they themselves showed little or no signal response. Nevertheless, the presence of matrix enhances analyte signals, albeit at a specific concentration. Analyte fragmentation is expected when little (M:A_T = 0.01:1) or no matrix is used. The mass spectra obtained under these conditions indeed showed additional peaks, but it is difficult to confirm if these were a result of fragmentation. However, at an M:A_T ratio of 0.4:1, the $[M+H]^+$ (or $[M+Na]^+$, $[M+K]^+$) signals significantly dominated the spectral response compared to those of the (possible) fragments.

In order to simulate a real-life sample in which the concentrations of metabolites vary, cocktail A (in which the concentrations of fumaric, glutamic, and aspartic acids, and of tryptophan, were an order of magnitude less than the

DOI: 10.1002/rcm

RCM

others, and that of tyrosine was two orders less) was studied in comparison to that of cocktail B (all metabolites at equimolar concentrations). The relative change between the two spectra, obtained using the liquid matrix DHB-pyridine at a M:A_T ratio of 0.4:1, is shown in Fig. 3(a). Although changes in signals corresponding to four of the five low-level metabolites, namely aspartic acid, glutamic acid, tyrosine and tryptophan, are observable, the most noticeable difference is that of tryptophan. Signals corresponding to some of the other metabolites, notably threonine $[Thr+K]^+$, citrate $[cit+K]^+$ and oxaloacetate $[OAA+Na]^+$, also appeared to be enhanced in cocktail B as compared with the spectra of cocktail A.

Overall, excluding the four metabolite pairs that have the same nominal mass (i.e., putrescine/pyruvate, glycine/lysine, oxaloacetic acid/asparagine and leucine/isoleucine), for which an unambiguous peak assignment is difficult, it is possible to observe a spectral response for most of the other metabolites, albeit to varying degrees. The ionic liquid matrix (DHB-pyridine) appears to be a useful matrix for the detection of most metabolites in the mixture. It has also been noted to be effective in detecting most of the amino acids used in the mixture when they were analysed individually.[17]

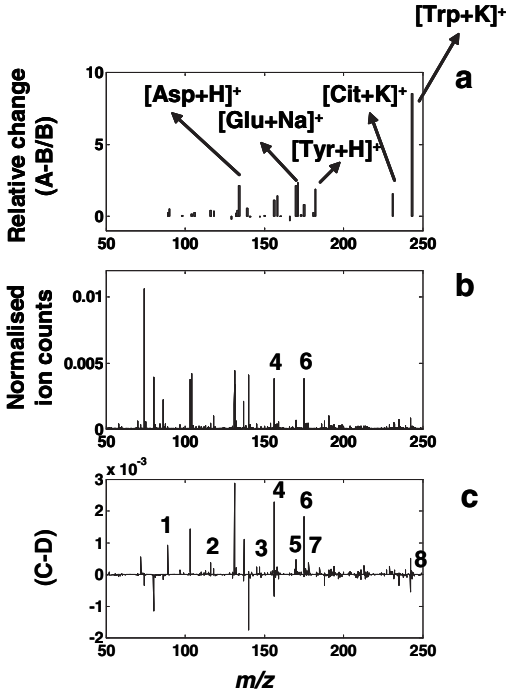

Figure 3. (a) Relative change in the MSLDI mass spectral response to changes in the concentration of metabolites in a 30-metabolite cocktail. The normalised responses at the *m/z* values corresponding to those of the metabolites in the cocktail were used in the calculation. A and B indicate responses for cocktails A and B, respectively. Relative change was calculated by subtracting the responses for A from B and dividing by those of A. (b) A typical MSLDI positive ion mass spectrum of *E. coli* cell extract, using DHB-pyridine. (c) The mean difference spectrum (C–D) of the *E. coli* cell extract and the extract spiked with cocktail B. C denotes the average spectrum of the extract spiked with cocktail B, and D that of the raw cell extract. Prominent metabolites recovered are labeled: (1) putrescine $[Put+H]^+$; (2) proline $[Pro+H]^+$; (3) glutamine $[Gln+H]^+$ or lysine $[Lys+H]^+$; (4) histidine $[His+H]^+$; (5) glutamate $[Glu+Na]^+$; (6) arginine $[Arg+H]^+$; (7) histidine $[His+Na]^+$; and (8) tryptophan $[Trp+K]^+$. DHB-pyridine was the matrix used at a M:A_T ratio of 4:1.

The applicability of the technique to a real biological sample was also tested by attempting to monitor the metabolites in the synthetic cocktail after spiking it in a microbial extract, a matrix that would be encountered when assessing the intracellular metabolite levels. The spectrum of the unspiked extract, and the difference spectrum between the spiked and the unspiked samples, are shown in Figs. 3(b) and 3(c), respectively. The matrix used was DHB-pyridine and the microbial extract was spiked with cocktail B. Since the native levels of the metabolites in the microbial extract are unknown, different matrix concentrations were assessed. The result shown is for a matrix concentration that yielded the highest S/N, and corresponds to a matrix-to-total spiked analyte molar ratio of 4:1; the corresponding result for the unspiked sample used the same matrix concentration. The spectrum of the unspiked microbial extract has contributions at *m/z* values corresponding to those in the spiked cocktail. For example, the peaks marked 4 and 6 in Fig. 3(b) correspond to those of protonated histidine and arginine. As can be seen from Fig. 3(c), the prominent metabolites in the spiked cocktail are recovered in the difference spectrum, demonstrating that it is possible to detect multiple metabolites in a complex biological matrix typical of those applicable in metabolomic investigations.

Ion suppression and response to changes in analyte concentrations

In reality, intracellular metabolite concentrations vary over several orders of magnitude. In addition to being able to detect multiple metabolites in a mixture, it is imperative that the response from the metabolites is consistent and is not significantly influenced by changes in the concentrations of other components in the mixture. To test this aspect, a mixture of 19 metabolites (all but one of the amino acids) was spiked with the missing amino acid, serially increasing the concentration of the spiked amino acid while keeping the concentrations of the others unchanged at 0.05 mM each. Histidine, which gives a good response, and serine with a relatively poor response, were chosen as the amino acids to be spiked. The result is demonstrated in Fig. 4 for serine spikes; the protonated serine peak in the mixture increases in proportion to the change in the concentration of the metabolite up to a certain concentration, but remains unchanged above this concentration. It is also clear that ionisation of other amino acids is suppressed with increasing concentrations of serine.

This is illustrated more clearly in Fig. 5, where the difference in response between the control (unspiked) and spiked mixture (both serine and histidine) is plotted for all the components. Negative values suggest suppression, while positive values imply enhancement in signal compared to the control. It is clear from Fig. 5 that, for both serine and histidine spikes, the other analyte peaks are suppressed as the concentration of the spiked amino acid is increased. Also,

DOI: 10.1002/rcm

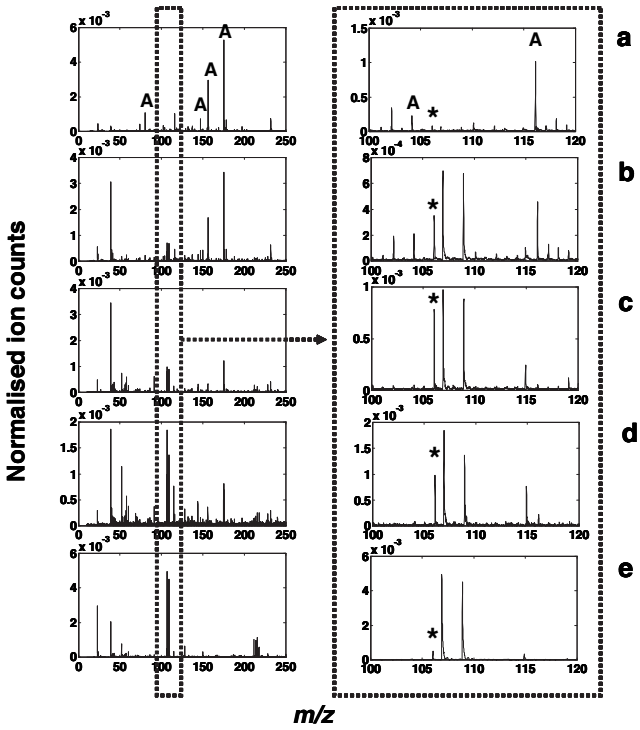

Figure 4. Influence of the concentration of one metabolite on the detection of others. A cocktail of 20 amino acids minus serine was spiked with varying concentrations of serine (10 μM (a), 100 μM (b), 500 μM (c), 1 mM (d), and 10 mM (e)). The resulting MSLDI mass spectra in the positive ion mode using DHB-pyridine matrix, in the relevant range *m/z* 0–250, is shown on the left, and an expanded view for *m/z* 100–120 around the protonated serine peak at *m/z* 106 (indicated by a box for clarification) is shown on the right. The serine peak is highlighted by an asterisk, and prominent peaks attributable to other metabolites are highlighted as 'A'. Note the changes in the analyte peaks marked 'A' and the serine peak as the serine concentration in the mixture is increased.

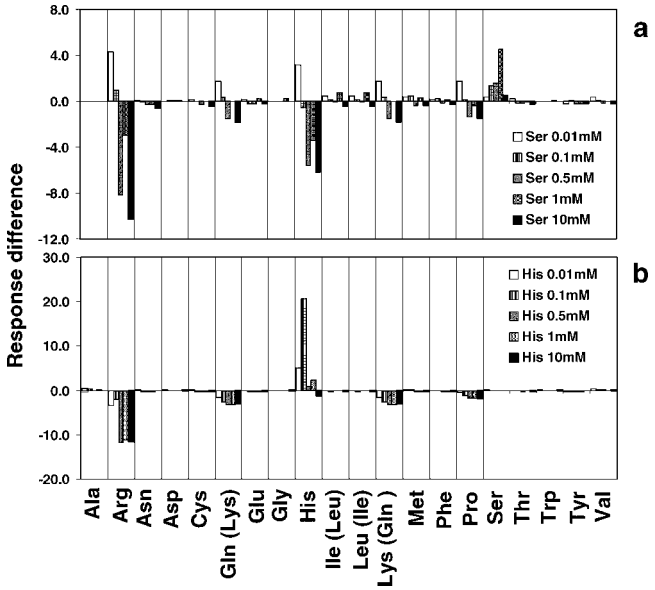

Figure 5. Typical variation in signal response for the 20 metabolites when the mixture containing them is spiked with serially increasing concentrations of serine (a) and histidine (b). The signal response is the difference between control (19 amino acid mixture without the spiked amino acid) and the spiked responses.

for both amino acids at high concentrations the proportionality between response and concentration disappears. Whilst histidine spikes at all concentrations result in complete suppression of response of the other amino acids, serine spikes at a low concentration result in signal enhancement for some of the amino acid signals, such as those for arginine, histidine, proline, etc. It is possible that the difference in the ease of ionisation between serine and histidine is reflected in the observations made at the lower concentrations of the spiked amino acid. The M:A_T ratios for the corresponding spikes were as follows: 1:2.5 (0.4:1) for the 10 μM spike, 1:3.3 (0.3:1) for the 100 μM and 10 mM spikes, 1:5 (0.2:1) for the 500 μM spike and 1:0.6 (1.7:1) for the 1 mM spike. Thus in all cases matrix-reduced conditions were maintained.

The results discussed above thus clearly demonstrate analyte suppression effects, even with a relatively poorly ionisable analyte (serine), suggesting that the concentration of the analytes present in the mixture is a key component that influences the resulting spectrum. It has been proposed that ionisation in MALDI is often thermodynamically controlled and influenced by secondary ion-molecule reactions in the plume.[24] It has also been suggested that, following the generation of primary ions, secondary ion-molecule reactions in the ensuing desorption plume expansion convert the primary ions into the most thermodynamically favourable secondary ion products that are those detected.[30] Matrix suppression effects (MSEs) occur when enough analyte is present to react with all the primary ions. This is observed when matrix-reduced conditions are employed, as demonstrated in the current study. In an analogous manner, it has also been noted that analyte-analyte and matrix-analyte reactions leading to analyte suppression effects (ASEs) are operational in MALDI.[28,30] It has been hypothesised that ASEs are a function of the total matrix/analyte ratio and of the ratios between analytes.[31] The extent of the suppression is also a function of the nature of the analytes. Model predictions[31] suggest that, at increasingly higher concentrations of the less reactive analyte in a mixture, larger deviations in concentration dependence of the analyte signal can be expected. Accordingly, serine (or histidine), that would be less efficient in reacting with the matrix for proton abstraction and hence ionisation than arginine (a dominant component of the mixture), shows deviations in concentration dependence of its $[M+H]^+$ ion signal beyond a certain concentration. At lower serine concentrations there is even apparent enhancement in the signal response for most metabolites, suggesting the possible existence of secondary ion-molecule reactions.

As has been noted here and by other investigators,[31] it appears that the concentrations of the components of the mixture have to be similar to obtain detectable signals for all (or most of) the analytes. As long as the analytes in the mixture are of similar concentrations their detection can be predicted, in simplistic terms, according to their ionisability

DOI: 10.1002/rcm

based on their gas-phase basicities or proton affinities, even in mixtures. Clearly, a better understanding of ionisation when analysing mixtures is required. This would enable the development of strategies for the prediction of signal response in mixtures containing components that have a range of concentration, as would be ideally desired in metabolomic investigations.

CONCLUSIONS

The application of low matrix-to-analyte molar ratios (lower than previously published, i.e., matrix reduced conditions), in combination with use of ionic liquid-based matrices, enabled the detection of several metabolites in a synthetic mixture in the positive ion mode; the most basic metabolites dominated the spectral response. While both solid and ionic liquid matrices gave detectable responses for the analytes, DHB-pyridine gave cleaner spectra, possibly a result of more homogeneous sample distribution in this ionic liquid matrix. The possibility of detecting multiple metabolites in a biological matrix was also demonstrated by spiking a metabolite mixture of known composition into a microbial extract and detecting the prominent metabolite signals. However, in another experiment the signal responses for the analytes were inconsistent when the concentration of one analyte was varied while keeping the others the same, suggesting the operation of analyte suppression effects. In metabolomic applications where multiple metabolites need to be monitored, but where the concentration of the analytes of interest do not vary considerably, matrix-suppressed laser desorption/ionisation mass spectrometry (MSLDI-MS) offers a rapid and high-throughput option for analysis. However, in cases where the concentrations of the analytes of interest vary over orders of magnitude, it may be difficult to interpret the spectral information in absolute quantitative terms, and chromatographic separation prior to MSLDI-MS would be advantageous. Further understanding of the ionisation process of small chemically diverse molecules would be required to derive rules for systematic interpretations or develop strategies for efficient analysis.

Acknowledgements

The authors are grateful to the UK BBSRC for financial assistance. We thank Prof. Douglas Kell for reading the manuscript and for his comments, and acknowledge the contribution of the referees for their invaluable comments.

REFERENCES

1. Giaever G, Chu AM, Ni L, Connelly C, Riles L, Veronneau S, Dow S, Lucau-Danila A, Anderson K, Andre B, Arkin AP, Astromoff A, El-Bakkoury M, Bangham R, Benito R, Brachat S, Campanaro S, Curtiss M, Davis K, Deutschbauer A, Entian KD, Flaherty P, Foury F, Garfinkel DJ, Gerstein M, Gotte D, Guldener U, Hegemann JH, Hempel S, Herman Z, Jaramillo DF, Kelly DE, Kelly SL, Kotter P, LaBonte D, Lamb DC, Lan N, Liang H, Liao H, Liu L, Luo C, Lussier M, Mao R, Menard P, Ooi SL, Revuelta JL, Roberts CJ, Rose M, Ross-Macdonald P, Scherens B, Schimmack G, Shafer B, Shoemaker DD, Sookhai-Mahadeo S, Storms RK, Strathern JN, Valle G, Voet M, Volckaert G, Wang CY, Ward TR, Wilhelmy J, Winzeler EA, Yang Y, Yen G, Youngman E, Yu K, Bussey H, Boeke JD, Snyder M, Philippsen P, Davis RW, Johnston M. *Nature* 2002; **418**: 387.
2. Bader GD, Heilbut A, Andrews B, Tyers M, Hughes T, Boone C. *Trends Cell. Biol*. 2003; **13**: 344.
3. Jones DL, Petty J, Hoyle DC, Hayes A, Ragni E, Popolo L, Oliver SG, Stateva LI. *Physiol. Genomics* 2003; **16**: 107.
4. Vazquez A, Flammini A, Maritan A, Vespignani A. *Nat. Biotechnol*. 2003; **21**: 697.
5. Oliver SG, Winson MK, Kell DB, Baganz F. *Trends Biotechnol*. 1998; **16**: 373.
6. Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ, Westerhoff HV, van Dam K, Oliver SG. *Nat. Biotechnol*. 2001; **19**: 45.
7. Vaidyanathan S, Harrigan GG, Goodacre R (eds). *Metabolome Analyses: Strategies for Systems Biology*. Springer: New York, 2005.
8. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. *Trends Biotechnol*. 2004; **22**: 245.
9. Dunn WB, Ellis DI. *Trends Anal. Chem*. 2005; **24**: 285.
10. Cohen LH, Gusev AI. *Anal. Bioanal. Chem*. 2002; **373**: 571.
11. Tweeddale H, Notley-McRobb L, Ferenci T. *J. Bacteriol*. 1998; **180**: 5109.
12. Vaidyanathan S. *Metabolomics* 2005; **1**: 17.
13. Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. *Nat. Rev. Microbiol*. 2005; **3**: 557.
14. Ling YC, Lin L, Chen YT. *Rapid Commun. Mass Spectrom*. 1998; **12**: 317.
15. Kang MJ, Tholey A, Heinzle E. *Rapid Commun. Mass Spectrom*. 2000; **14**: 1972.
16. Hatsis P, Brombacher S, Corr J, Kovarik P, Volmer DA. *Rapid Commun. Mass Spectrom*. 2003; **17**: 2303.
17. Zabet-Moghaddam M, Heinzle E, Tholey A. *Rapid Commun. Mass Spectrom*. 2004; **18**: 141.
18. Ayorinde FO, Hambright P, Porter TN, Keith QL Jr. *Rapid Commun. Mass Spectrom*. 1999; **13**: 2474.
19. Tholey A, Wittmann C, Kang MJ, Bungert D, Hollemeyer K, Heinzle E. *J. Mass Spectrom*. 2002; **37**: 963.
20. Crecelius A, Clench MR, Richards DS, Evason D, Parr V. *J. Chromatogr. Sci*. 2002; **40**: 614.
21. Wei J, Buriak JM, Siuzdak G. *Nature* 1999; **399**: 243.
22. Go EP, Prenni JE, Wei J, Jones A, Hall SC, Witkowska HE, Shen Z, Siuzdak G. *Anal. Chem*. 2003; **75**: 2504.
23. Kraj A, Dylag T, Gorecka-Drzazga A, Bargiel S, Dziubanand J, Silberring J. *Acta Biochim. Pol*. 2003; **50**: 783.
24. Knochenmuss R, Stortelder A, Breuker K, Zenobi R. *J. Mass Spectrom*. 2000; **35**: 1237.
25. Goheen SC, Wahl KL, Campbell JA, Hess WP. *J. Mass Spectrom*. 1997; **32**: 820.
26. Carley AF, Morgan PH. *Computational Methods in the Chemical Sciences*. Ellis Horwood: Chichester, UK, 1989.
27. McCombie G, Knochenmuss R. *Anal. Chem*. 2004; **76**: 4990.
28. Zhu YF, Lee KL, Tang K, Allman SL, Taranenko NI, Chen CH. *Rapid Commun. Mass Spectrom*. 1995; **9**: 1315.
29. Armstrong DW, Zhang LK, He L, Gross ML. *Anal. Chem*. 2001; **73**: 3679.
30. Knochenmuss R, Zenobi R. *Chem. Rev*. 2003; **103**: 441.
31. Knochenmuss R. *Anal. Chem*. 2003; **75**: 2199.

DOI: 10.1002/rcm