RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2007; **21**: 2072–2078
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3063

Quantitative detection of metabolites using matrix-assisted laser desorption/ionization mass spectrometry with 9-aminoacridine as the matrix

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Received 2 March 2007; Accepted 29 April 2007

Quantitative detection of metabolites is a highly desirable feature in metabolome analyses. Recently, the successful detection of multiple metabolites using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in the negative ion mode employing 9-aminoacridine as the organic matrix was reported (Edwards JL, Kennedy RT. *Anal. Chem.* 2005; 77: 2201–2209). However, there is little information available on quantitative detection of multiple metabolites using MALDI-MS and in particular the influence changes in metabolite levels have on such detections. We investigated this aspect by spiking a synthetic metabolite cocktail (consisting of 39 metabolites including amino acids, organic acids and phospho-metabolites) with five representative metabolites at increasing concentrations, one metabolite at a time, and assessed the signals from replicate determinations. It was possible to detect quantitative changes in the spiked metabolites. Although analyte suppression was observed, it was possible to observe scenarios where the spiked metabolite had little or no influence on the quantitative detection of some metabolites. It appears that the mass spectral response of the metabolite is suppressed only when the spiked chemical species are relatively similar in chemical terms. This suggests that quantitation is possible in scenarios where changes in a specific metabolite or a class of metabolites are monitored following appropriate analyte separation strategies, and that careful interpretations must be made when using the technique for quantitative analysis in unbiased metabolomic approaches. Copyright © 2007 John Wiley & Sons, Ltd.

There is considerable interest in developing strategies towards quantitative detection of metabolites in biological matrices using simplistic and rapid protocols. This stems from the relatively recent emergence of analyses at the metabolome level as a complementary route in functional genomics and systems biology.[1–3] Metabolomic analyses involve the detection (ideally in a quantitative manner) of changes in the metabolite complement (the low molecular weight components and intermediates of metabolism) of a cell, tissue or organ in a particular physiological state. The analyses range from detecting global metabolic changes in the system to monitor deviations from a normal or control state.

Several approaches are being developed to monitor metabolomic changes in biological systems.[3–5] These involve combination of separation strategies, often multidimensional, followed by appropriate detection typically with mass spectrometry (MS) or nuclear magnetic resonance (NMR) which is also popular. In developing these techniques an appropriate balance between ease of operation and sufficient information resolution is desirable, for applications in a practical environment. In this regard, simple protocols with minimal sample intervention are desirable for profiling changes at the metabolome level.

Approaches based on MS offer the spectral resolution and sensitivity required for simultaneous monitoring of multiple analytes. The advent of 'soft' ionisation methods like matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) opens the possibility of using desorption/ionisation MS-based methods for such analyses. Given the simplicity of sample preparation, MALDI-MS, in particular, is well suited for simultaneous, rapid, high-throughput analyses of multiple metabolites in mixtures, such as would be encountered in metabolomic investigations. It can be applied in conjunction with separation and sample clean-up techniques when comprehensive information is required. More importantly, where prominent changes can be detected in a reproducible manner, there is considerable potential for direct mass spectral analysis without the employment of separation techniques and hence more simplistic, rapid, high-throughput analyses.

Whilst MALDI-MS has been popular for the analysis of biomacromolecules like peptides, proteins and nucleic acids, its application to the detection of low molecular weight components has largely been limited due to interference from

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Contract/grant sponsor: UK BBSRC.

matrix peaks in the low-mass range (typically <500 *m/z*). Several strategies have been investigated to counter this problem. These include the use of high molecular weight compounds as matrices,[6,7] derivatisation of analytes,[8] matrix-suppressed laser desorption/ionization methods,[9,10] and desorption/ionization on porous silicon.[11,12]

Recently, Edwards and Kennedy[13] reported the efficient detection of metabolites using 9-aminoacridine as the organic matrix in the negative ion mode. They showed that metabolite molecular ions could be efficiently detected with little or no interference from matrix ions and that the resulting spectra can be used to generate useful metabolic information. In metabolomics, the challenge is to be able to detect simultaneously many analytes in a complex milieu ideally in a quantitative manner. In MS, relative quantitation is often accomplished using isotope ratio analysis. However, this is not a universally suitable method due to the requirement of introducing isotopic species in the biological sample analysed. The ability to derive quantitative information with simplistic protocols is very desirable. However, there is little information available on the quantitative detection of metabolites using MALDI-MS and in particular the influence changes in metabolite levels have on the quantitative detection of multiple metabolites. Despite the popular perception of the unreliability of the MALDI technique for quantitative determinations, the quantitative analysis of individual small molecular weight components has been demonstrated.[14–17] Whilst the behaviour of individual analytes (metabolites) can be predicted based on their gas-phase characteristics, there is little information available on the behaviour of analytes in mixtures, as would be encountered in metabolome analyses. In this study we investigated MALDI-MS using 9-aminoacridine for quantitative detection of metabolites. We studied the changes in the mass spectral responses of the metabolites in a synthetic cocktail to observe the behaviour of analytes in mixtures and assess the suitability of the technique in metabolome analyses.

EXPERIMENTAL

A metabolite cocktail consisting of 39 metabolites, including 20 amino acids, 6 organic acids associated with primary metabolism, 9 phospho-metabolites, the metabolites glucose, putrescine and 4-aminobutyric acid, and a deuteriated metabolite, namely d_4-succinate, were employed. The metabolites used are listed in Table 1. All the metabolites were obtained from Sigma-Aldrich (Dorset, UK) and were dissolved in methanol/water mixture containing 65–95% methanol. AMP and G6P were obtained as monosodium salts, UMP, ATP and CTP as disodium salts, TTP as trisodium salt and FPP as the tetra(cyclohexylammonium) salt.

Five metabolites representative of the different types of chemical species that occur in metabolism from the cocktail were chosen (*viz.*, histidine, tryptophan, malic acid, glucose and ATP). The cocktail was serially spiked with increasing concentrations of these five metabolites such that the final concentration of each of the spiked metabolites ranged from 10 to 1000 pmol/μL, while maintaining the concentration of all the other metabolites at 50 pmol/μL.

For mass spectrometry, 9-aminoacridine (9AA) was used as the matrix, dissolved in acetone to a concentration of 9 mg/mL. Equal volumes of the metabolite cocktail and the matrix solution were mixed and 1 μL of this mixture spotted on a stainless steel MALDI-MS target plate and air dried. The spots were then analysed using an Axima CFR+ MALDI-ToF mass spectrometer (Shimadzu Biotech, Manchester, UK) in the negative ion mode. A total of 150 shots were averaged per spot, and typical spectral collection times were 2 min per sample. Each sample was analysed on nine different spots (technical replicates). The mass spectral data were imported into MATLAB (The Math Works, Natick, MA, USA) and processed for analysis. The data were normalised to total ion counts and the peak areas at *m/z* corresponding to those of the deprotonated metabolite ions $[M-H]^-$ were used to assess the spectral response.

Box-whisker plots were employed to compare the spectral responses. The lower and upper lines of the 'box' are the 25th and the 75th percentiles of the sample; the box limits therefore indicate the interquartile range, with the horizontal bar representing the median of replicate determinations. The 'whiskers' (lines extending above and below the box) show the range (the maximum and minimum values), excluding outliers (values of >1.5 times the interquartile range). A star sign outside the whiskers indicates the outlier(s) in the data. The notches in the box are a graphic confidence interval about the median. A side-by-side comparison of two notched plots can be considered as a graphical equivalent of a t-test.

RESULTS AND DISCUSSION

The objective of the investigation was to assess MALDI-MS using 9-aminoacridine as the matrix for the quantitative detection of metabolites in mixtures. If successful this would give confidence that this approach would be a useful technique for metabolomics, where multiple biochemical species need to be measured. Rather than start with a complex biological sample, of unknown metabolite composition, the approach adopted was to spike a synthetic metabolite cocktail with five representative metabolites at increasing concentrations, one metabolite at a time, and assess the signals from replicate determinations to study changes in the response of the metabolites.

A typical MALDI mass spectrum of the metabolite cocktail using 9AA as the matrix is shown in Fig. 1(A). As can be seen, molecular ions of the metabolites (at *m/z* corresponding to their deprotonated form) can be predominantly detected, with minimal interference from the matrix, as reported by Edwards and Kennedy.[13] The median response for each metabolite in the mixture, obtained from replicate determinations, is shown as a box-whisker plot in Fig. 1(B) (four of the metabolites have identical *m/z* as four others in the mixture (Table 1) and could not be resolved with certainty). A noticeable feature here is the difference in the median response for the different metabolites despite their presence in the mixture at equimolar concentrations. This is not unexpected as the metabolites differ in their chemical nature and ionisability and can be expected to have different mass

DOI: 10.1002/rcm

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Table 1. List of metabolites used in the study along with their respective deprotonated *m/z* values in the negative ion mode

No.	Metabolite	Shorthand identifier	Identifier on plots	$[M-H]^-$ (*m/z*)
1	Glycine	Gly	17	74.2
2	Pyruvic acid	Pyr	27	87.1
3	Putrescine	Put	27	87.1
4	L-Alanine	Ala	2	88.1
5	Lactic acid	Lac	20	89.1
6	4-Aminobutyric acid	Aba	1	102.1
7	L-Serine	Ser	28	104.1
8	L-Proline	Pro	24	114.1
9	Fumaric acid	Fum	12	115.1
10	L-Valine	Val	35	116.1
11	L-Threonine	Thr	30	118.1
12	L-Cysteine	Cys	10	120.1
13	d_4-Succinic acid	d_4-suc	29	121.1
14	L-Leucine	Leu	19	130.1
15	L-Isoleucine	Ile	19	130.1
16	Oxaloacetic acid	Oaa	5	131.1
17	L-Asparagine	Asn	5	131.1
18	L-Aspartic acid	Asp	6	132.1
19	Malic acid	Mal	21	133.1
20	L-Glutamine	Gln	14	145.1
21	L-Lysine	Lys	14	145.1
22	L-Glutamic acid	Glu	16	146.1
23	L-Methionine	Met	22	148.2
24	L-Histidine	His	18	154.1
25	L-Phenylalanine	Phe	23	164.2
26	L-Arginine	Arg	4	173.2
27	Glucose	Gl	15	179.1
28	L-Tyrosine	Tyr	33	180.2
29	Citric acid	Cit	8	191.1
30	Phospho-threonine	pThr	25	198.1
31	L-Tryptophan	Trp	31	203.2
32	Glucose-6-phosphate	G6P	13	259.1
33	Phospho-tyrosine	pTyr	26	260.2
34	Uridine monophosphate	UMP	34	323.1
35	Fructose-1,6-bisphosphate	FPP	11	339.1
36	Adenosine 5′-monophosphate	AMP	3	346.1
37	Thymidine triphosphate	TTP	32	481.0
38	Cytosine triphosphate	CTP	9	482.0
39	Adenosine 5′-triphosphate	ATP	7	506.0

spectral responses. We have made a similar observation while analysing such metabolite cocktails using laser desorption/ionization mass spectrometry on porous silicon[12] and matrix-suppressed laser desorption/ionization mass spectrometry.[10]

Another noticeable feature from Fig. 1(B) (in conjunction with Table 1) is the spread in the response of replicate determinations for some metabolites (e.g., AMP, ATP, FPP, etc.). This arises from the heterogeneity of analyte distributions on the surface that is analysed, being typical of MALDI-MS determinations arising from dried-droplet depositions. However, the application of electrospray deposition[18,19] is likely to minimise this spread and result in tighter responses from replicate measurements that will be more suitable for quantitative estimations.

Influence of changes in concentration

The five metabolites that were spiked at various concentrations were chosen to represent different metabolite classes that show different mass spectral responses. Histidine and tryptophan are amino acids that give a moderate mass spectral response, malic acid is an organic acid with a strong response, ATP is a phospho-metabolite with a strong response, and glucose is a carbohydrate with a poor response. The decreasing order of mass spectral response of the spiked metabolites as depicted in Fig. 1 is: ATP>malate>histidine>tryptophan>glucose.

The influence of these spiked metabolites on the quantitative response of the other spiked metabolites is shown in Fig. 2. As can be seen, increasing the concentrations of the spiked metabolites shows a proportional increase in their respective mass spectral response (plots along the diagonal – top left to bottom right – of Fig. 2). Whilst histidine, tryptophan and glucose show an exponential increase in response, malic acid and ATP show a near linear response. Histidine and tryptophan are affected by changes in the other spiked metabolites (other than glucose), especially at concentrations above which the metabolite is present in the mixture (i.e., 50 pmol/μL). Both malate and ATP are the least affected by changes in the spiked concentrations, whilst the glucose response is too low to show any significant differences. It is also noteworthy that the spiked metabolites are least affected by glucose spikes, even when the glucose concentration is an order of magnitude higher than the rest.

DOI: 10.1002/rcm

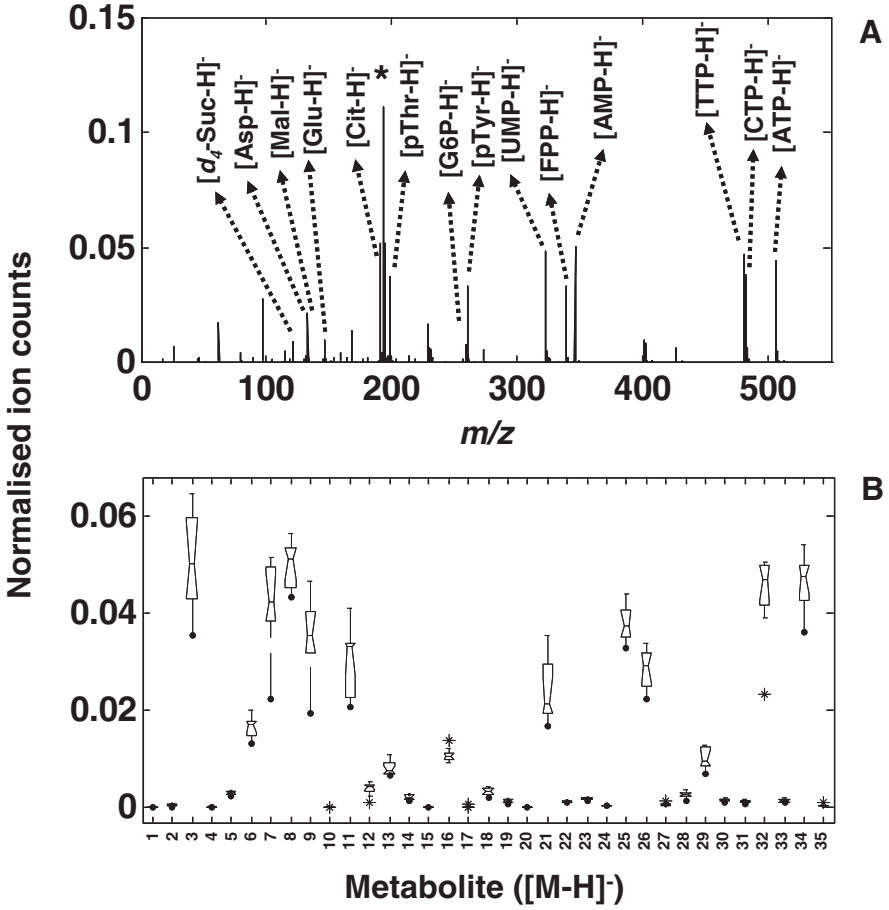

Figure 1. (A) A typical MALDI mass spectrum of the metabolite cocktail in the negative ion mode. Some prominent peaks are labeled, and the matrix peak indicated by an asterisk. (B) Median response of the metabolites in the mixture (refer to text for details of the box-whisker plot; the metabolite identities are listed in Table 1). The matrix used was 9-aminoacridine and the cocktail consisted of 39 metabolites (see Table 1 for details) at equimolar concentrations of 50 pmol/μL each.

The influence of the spiked metabolites on the quantitative response of some of the other representative metabolites is shown in Fig. 3. Proline, aspartate, phenylalanine (all amino acids), citrate (an organic acid), AMP and CTP (both nucleotide phosphates) were studied. Proline and aspartate responses are suppressed by all the spiked metabolites (other than glucose), the order of influence being Mal>His>Trp> ATP (for proline) and Mal>His>ATP>Trp (for aspartate). Although malate, histidine and tryptophan spikes suppress phenylalanine response (in the order Mal>His>Trp), ATP spikes do not. Citrate is suppressed only by malate and the responses of the nucleotide phosphates AMP and CTP are suppressed only by ATP spikes. The decreasing order of mass spectral response of the representative metabolites (plotted in Fig. 3) is: AMP∼CTP>Cit>Asp>Phe>Pro.

The quantitative response of other phospho-metabolites to the metabolite spikes is illustrated in Fig. 4. G6P is the only phospho-metabolite studied whose response is suppressed by all the spiked metabolites (other than glucose), the order of influence being ATP>His>Mal>Trp. The response of UMP is suppressed by malate and ATP spikes. TTP, FPP and pThr responses are suppressed by ATP spikes only and pTyr does not appear to be influenced by any of the metabolite spikes. In fact, in some cases, as with ATP, TTP and CTP, the response increases with increase in the spiked histidine or malate concentrations. The decreasing order of mass spectral response of the phospho-metabolites (plotted in Fig. 4) is: UMP∼TTP>FPP∼pThr∼pTyr>G6P.

The overall decreasing order of mass spectral response of the metabolites (those plotted in Figs. 2–4) when present in the mixture at 50 pmol/μL is: ATP∼CTP∼TTP∼AMP∼UMP >FPP∼pThr∼pTyr>Cit∼Mal>Asp>G6P>His>Phe>Trp> Pro>Gl.

The decrease in mass spectral response of the amino acids (His, Phe, Trp and Pro) is in line with their reported gas-phase acidities (GA)[20] (ΔG^0_{acid} in kJ mol^{-1} 1356, 1379, 1381 and 1395 for His, Phe, Trp and Pro, respectively); the lower the GA value, the greater the tendency for deprotonation. The poor mass spectral response of glucose in the mixture is also in line with its reported GA value of 1398 kJ mol^{-1},[21] which is relatively higher than the rest. Spiking the mixture with glucose therefore does not influence the mass spectral response of the other metabolites, even at high concentrations. Tryptophan, which also has a relatively high GA value, does not significantly influence the mass spectral response of the other metabolites. The amino acids proline, phenylalanine, histidine and aspartate are marginally suppressed at higher (>100 pmol/μL) spiked tryptophan

DOI: 10.1002/rcm

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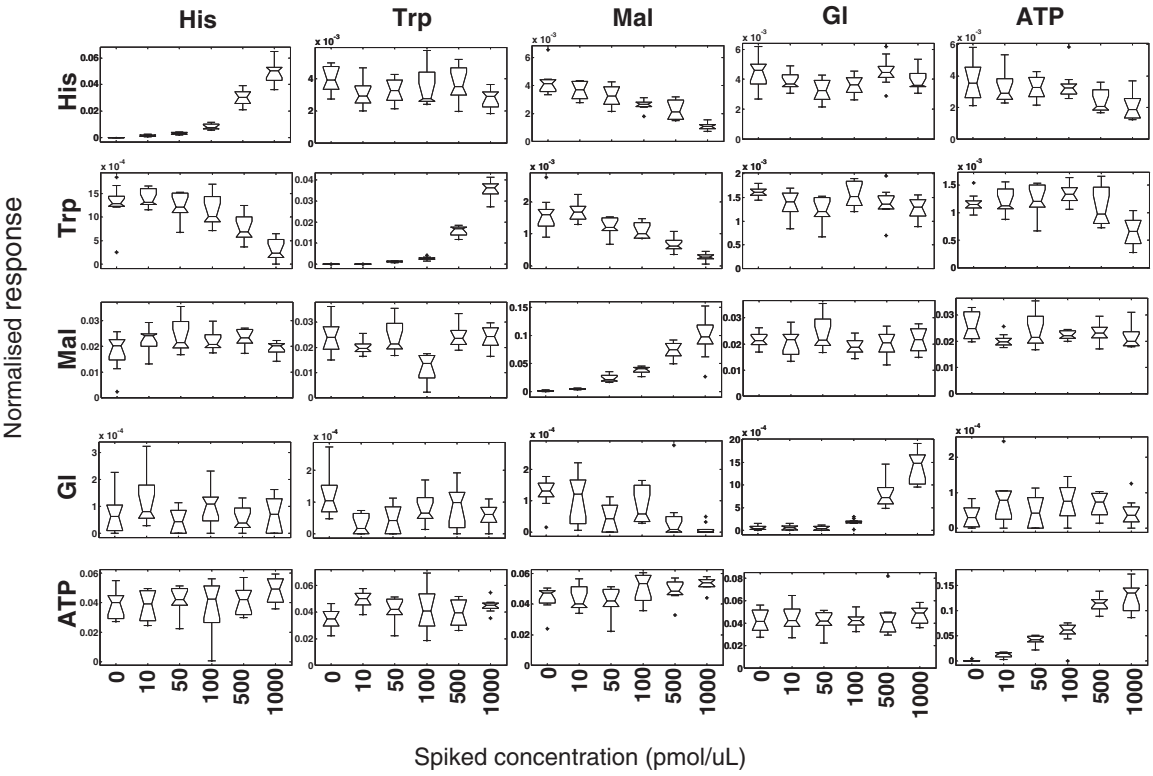

Figure 2. Influence of concentration variations in five spiked metabolites (His, Trp, Mal, Gl, ATP) on the quantitative detection of the other spiked metabolites. The normalised median response (from 9 replicate measurements) at the *m/z* corresponding to the deprotonated molecular ion for the representative metabolites (along rows) at different concentrations of the spiked metabolite (along columns) are plotted as box-whisker plots.

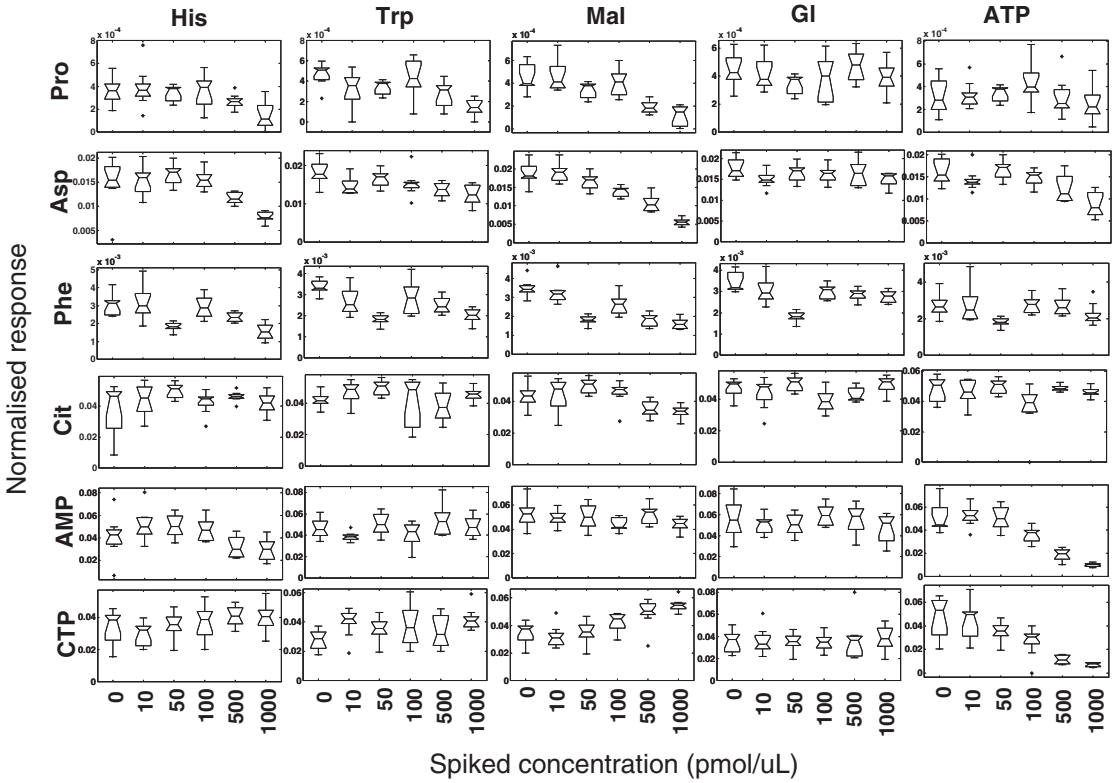

Figure 3. Influence of concentration variations in five spiked metabolites (His, Trp, Mal, Gl, ATP) on the quantitative detection of other representative metabolites. The normalised median response (from 9 replicate measurements) at the *m/z* corresponding to the deprotonated molecular ion for the representative metabolites (along rows) at different concentrations of the spiked metabolite (along columns) are plotted as box-whisker plots.

DOI: 10.1002/rcm

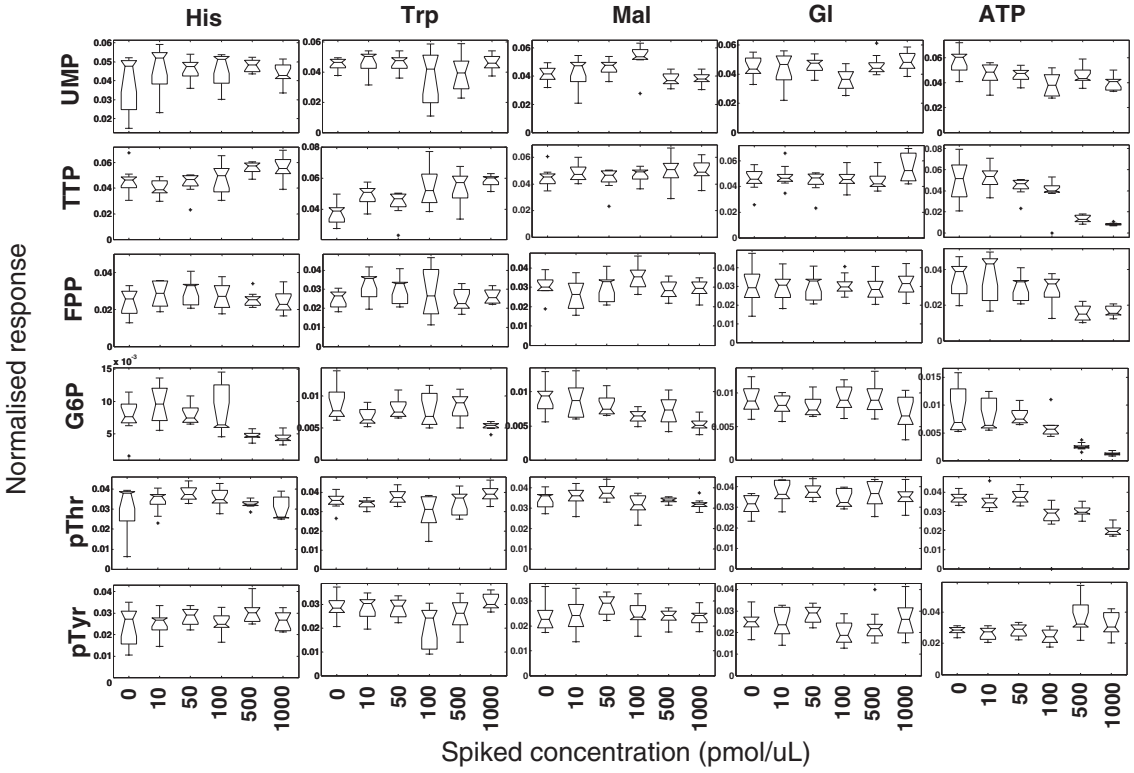

Figure 4. Influence of concentration variations in five spiked metabolites (His, Trp, Mal, Gl, ATP) on the quantitative detection of phospho-metabolites. The normalised median response (from 9 replicate measurements) at the *m/z* corresponding to the deprotonated molecular ion for the representative metabolites (along rows) at different concentrations of the spiked metabolite (along columns) are plotted as box-whisker plots.

concentrations. However, the other metabolites are not influenced by changes in tryptophan introduced in the mixture.

The amino acids proline, tryptophan and phenylalanine are all suppressed when the mixture is spiked with higher concentrations of histidine, which is in line with their reported GA values. In addition, histidine spikes above 100 pmol/μL suppress the mass spectral response of aspartate, AMP and G6P, but have little or no suppressive effect on the response of the other metabolites in the mixture. In contrast, histidine spikes lead to a marginal enhancement in the response of the nucleotide triphosphates ATP, CTP and TTP, suggesting that the increased presence of histidine possibly aids deprotonation of these species. Malic acid spikes suppress the spectral response from all but the phospho-metabolites. UMP and G6P show marginal suppression at higher (>500 pmol/μL) concentrations of malic acid in the mixture. However, the nucleotide triphosphates again show an enhanced response to malic acid spikes.

The phospho-metabolites can be expected to have a high acidity attributed to the phosphate moiety (the theoretical[22] ΔG^0_{acid} of orthophosphoric acid being 1343 kJ mol^{-1}). *Ab initio* calculations suggest that the phosphate group is the most acidic site within nucleotide phosphates (one with the smallest GA value) and experiments based on ion-molecule reaction equilibria suggest the nucleotide monophosphates AMP and UMP would have GA values in the range of 1360 kJ mol^{-1}.[23] In phospho-metabolites, the negative charges localised primarily on the acidic phosphate oxygen atoms are stabilised by charge delocalisation among the phosphate oxygen atoms and by intramolecular hydrogen-bonding interactions.[24,25] Accordingly, the phospho-metabolites give a strong mass spectral response and dominate the spectral information.

Detection of the deprotonated form of the metabolites, as would be expected in the negative ion mode, can be attributed to analyte acidity. However, the trend in suppression of some of the analyte responses cannot be entirely explained in terms of acidity of the analyte detected in the negative ion mode. When spiked in the mixture to concentrations above 50 pmol/μL, the nucleotide phosphates, represented by ATP, suppress the response of the other phospho-metabolites, suggesting that there is competition for deprotonation, and that the most charge stabilised negative ion survives. However, ATP spikes do not influence the spectral response of malic acid or citric acid, which are relatively less dominant and would also be expected to be suppressed. Even the amino acids whose responses are significantly lower than that of ATP are only marginally influenced, when compared to the response of the phospho-metabolites. Interestingly, G6P is influenced, albeit marginally, by all the spiked metabolites, except glucose. Given that there is bound to be an excess of the basic matrix ions for deprotonation (matrix ion concentration in the preparation is at least ten times more than the maximum total metabolite concentration in the cocktail), it is not straightforward to explain the suppression effects observed. Further experimentation would be required to understand the behaviour of multiple analytes in mixtures.

From our observations, it appears that the mass spectral response of the metabolite is suppressed only if the changes

DOI: 10.1002/rcm

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are in biochemical species that are relatively similar in chemical terms. More importantly, chemically dissimilar species appear to be immune to changes in the mixture, suggesting that quantitation is possible in scenarios where changes in a specific metabolite or a class of metabolites are monitored following appropriate analyte separation strategies. Clearly, careful interpretations must be made when using the technique for quantitative analysis in unbiased metabolomic approaches.

Acknowledgements

The authors are grateful to the UK BBSRC for financial support.

REFERENCES

1. Oliver SG, Winson MK, Kell DB, Baganz F. *Trends Biotechnol.* 1998; **16**: 373.
2. 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.
3. Vaidyanathan S, Harrigan GG, Goodacre R. (eds). *Metabolome Analyses: Strategies for Systems Biology*. Springer: New York, 2005.
4. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. *Trends Biotechnol.* 2004; **22**: 245.
5. Dunn WB, Ellis DI. *Trends Anal. Chem.* 2005; **24**: 285.
6. Ayorinde FO, Hambright P, Porter TN, Keith QL Jr. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2474.
7. Woldegiorgis A, von Kieseritzky F, Dahlstedt E, Hellberg J, Brinck T, Roeraade J. *Rapid Commun. Mass Spectrom.* 2004; **18**: 841.
8. Tholey A, Wittmann C, Kang MJ, Bungert D, Hollemeyer K, Heinzle E. *J. Mass Spectrom.* 2002; **37**: 963.
9. McCombie G, Knochenmuss R. *Anal. Chem.* 2004; **76**: 4990.
10. Vaidyanathan S, Gaskell S, Goodacre R. *Rapid Commun. Mass Spectrom.* 2006; **20**: 1192.
11. Wei J, Buriak JM, Siuzdak G. *Nature* 1999; **399**: 243.
12. Vaidyanathan S, Jones D, Broadhurst DI, Ellis J, Jenkins T, Dunn WB, Hayes A, Burton N, Oliver SG, Kell DB, Goodacre R. *Metabolomics* 2005; **1**: 243.
13. Edwards JL, Kennedy RT. *Anal. Chem.* 2005; **77**: 2201.
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. Hensel RR, King RC, Owens KG. *Rapid Commun. Mass Spectrom.* 1997; **11**: 1785.
19. Wei H, Nolkrantz K, Powell DH, Woods JH, Ko MC, Kennedy RT. *Rapid Commun. Mass Spectrom.* 2004; **18**: 1193.
20. O'Hair RAJ, Bowie JH, Gronert S. *Int. J. Mass Spectrom. Ion Processes* 1992; **117**: 23.
21. Salpin JY, Tortajada J. *J. Mass Spectrom.* 2004; **39**: 930.
22. Morris RA, Knighton WB, Viggiano AA, Hoffman BC, Schaefer HF. *J. Chem. Phys.* 1997; **106**: 3545.
23. Freitas MA, Shi SDH, Hendrickson CL, Marshall AG. *J. Am. Chem. Soc.* 1998; **120**: 10187.
24. Blades AT, Ho YH, Kebarle P. *J. Phys. Chem.* 1996; **100**: 2443.
25. Burke RM, Pearce JK, Boxford WE, Bruckmann A, Dessent CEH. *J. Phys. Chem. A* 2005; **109**: 9775.

DOI: 10.1002/rcm