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Metabolomic Analysis of Eukaryotic Tissue and Prokaryotes Using Negative Mode MALDI Time-of-Flight Mass Spectrometry

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Metabolites in islets of Langerhans and *Escherichia coli* strain DH5-α were analyzed using negative-mode, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For analysis of anionic metabolites by MALDI, 9-aminoacridine as the matrix yielded a far superior signal in comparison to α-cyano-4-hydroxycinnamic acid, 2,5-dihydrobenzoic acid, 2,4,6,-trihydroxyacetophenone, and 3-hydroxypicolinic acid. Limits of detection for metabolite standards were as low as 15 nM for GDP, GTP, ADP, and ATP and as high as 1 μM for succinate in 1-μL samples. Analysis of islet extracts allowed detection of 44 metabolites, 29 of which were tentatively identified by matching molecular weight to compounds in METLIN and KEGG databases. Relative quantification was demonstrated by comparing the ratio of selected di- and triphosphorylated nucleotides for islets incubated with different concentrations of glucose. For islets at 3 mM glucose, concentration ratios of ATP/ADP, GTP/GDP, and UTP/UDP were 1.9 ± 1.39, 1.12 ± 0.50, and 0.79 ± 0.35 respectively, and at 20 mM glucose stimulation, the ratios increased to 4.13 ± 1.89, 5.62 ± 4.48, and 4.30 ± 4.07 (n = 3). Analysis was also performed by placing individual, intact islets on a MALDI target plate with matrix and impinging the laser directly on the dried islet. Direct analysis of single islets allowed detection of 43 metabolites, 28 of which were database identifiable. A total of 43% of detected metabolites from direct islet analysis were different from those detected in islet extracts. The method was extended to prokaryotic cells by analysis of extracts from *E. coli*. Sixty metabolites were detected, 39 of which matched compounds in the MetaCyc database. A total of 27% of the metabolites detected from prokaryotes overlapped those found in islets. These results show that MALDI can be used for detection of metabolites in complex biological samples.

Metabolomics, or large-scale metabolite profiling, has a potentially important role as a complement to functional genomics and proteomics in discovery of biomarkers, signaling pathway relationships, disease mechanisms, drugs, and drug effects. It has already been demonstrated that metabolite profiling can distinguish phenotypes where gene expression profiling has failed, indicating that the metabolome may be more indicative of phenotype than the transcriptome.[1,2] Metabolomics has also been used to identify "silent mutations" that result in identical phenotypes but different metabolite content.[3]

Analysis of the metabolome is, in some ways, simpler than genomic expression or proteomic analysis because it is thought to consist of 2000−3000 low molecular mass (<1500 Da) compounds, a much smaller set of compounds than the transcriptome or proteome. Methods used for metabolomic and metabonomic analysis include direct infusion electrospray ionization mass spectrometry (ESI-MS),[4] GC/MS,[5,6] NMR,[7] and LC NMR.[8] ESI-MS allows for detection of many ionizable metabolites; however, if not combined with a chemical fractionation method, it is susceptible to interference from salts and competitive ionization. GC/MS is a powerful method for analysis of volatile organics, but it is not readily adapted to some nonvolatile compounds such as phosphorylated metabolites. NMR allows the detection of most compounds, though it is hindered by low sensitivity. Thus, while each of these methods has clear benefits, no single method is suitable for all metabolites. New methods that can detect or identify many metabolites are of interest.

In this work, the use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for metabolomic analysis was explored. While MALDI-MS has become a workhorse of proteomics methods, it has yet to be applied to metabolomics. This paucity of applications can be attributed in part to the strong interference from matrix ions in detection of low molecular weight compounds. Furthermore, intracellular metabolites are predomi-

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(1) Goodacre, R.; Vaidyanathan, S.; Dunn, W. B.; Harrigan, G. G.; Kell, D. B. *Trends Biotechnol.* **2004**, *22*, 245−252.

(2) Urbanczyk-Wochniak, E.; Luedemann, A.; Kopka, J.; Selbig, J.; Roessner-Tunali, U.; Willmitzer, L.; Fernie, A. R. *EMBO Rep.* **2004**, *4*, 989−993.

(3) Raamsdonk, L. M.; Teusink, B.; Broadhurst, D.; Zhang, N. S.; Hayes, A.; Walsh, M. C.; Berden, J. A.; Brindle, K. M.; Kell, D. B.; Rowland, J. J.; Westerhoff, H. V.; van Dam, K.; Oliver, S. G. *Nat. Biotechnol.* **2001** *19*, 45−50.

(4) Allen, J.; Davey, H. M.; Broadhurst, D.; Heald, J. K.; Rowland, J. J.; Oliver, S. G.; Kell, D. B. *Nat. Biotech*nol. **2003**, *21*, 692−696.

(5) Szopa, J.; Wilczynski, G.; Fiehn, O.; Wenczel, A.; Willmitzer, L. *Phytochemistry.* **2001** *58*, 315−320.

(6) Fiehn, O.; Kopka, J.; Dormann, P.; Altmann, T.; Trethewey, RN.; Willmitzer, L. *Nat. Biotechnol.* **2000**, *18*, 1157−1161.

(7) Wang, Y. L.; Holmes, E.; Nicholson, J. K.; Cloarec, O.; Chollet, J.; Tanner, M.; Singer, B. H.; Ultzinger, J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12676−12681.

(8) Connelly, J. C.; Connor, S. C.; Monte, S.; Bailey, N. J. C.; Borgeaud, N.; Holmes, E.; Troke, J.; Nicholson, J. K.; Gavaghan, C. L. *Drug Met. Disp.* **2002**, *30*, 1357−1363.

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nantly organic acids or phosphorylated moeities, making them anionic over a wide pH range, and detection of such compounds with positive ionization mode is plagued by adduct formation. Negative ionization mode has rarely been utilized due to poor sensitivity with commonly used matrixes.[9]

Recent advances in MALDI have begun to make analysis of small molecules, including anions, more feasible by this approach. A novel sample deposition method has allowed semiquantitative determination of amino acids by positive mode MALDI-MS.[10] Desorption ionization on silicon (DIOS) has been demonstrated in both positive and negative modes with exceptionally low background spectra allowing small-molecule detection.[11] A drawback of this approach is that commercially available DIOS plates are not recommended for reuse and therefore is not as cost-effective as matrix-compatible reusable plates. The use of 9-aminoacridine (9-AA), a compound containing one basic amine moiety on a conjugated ring system, as a matrix for MALDI has been shown to yield low background and good sensitivity for selected low molecular weight anions, such as bile acids, in negative mode MS.[12−14]

The previous success of 9-AA with negative ionization suggested that it may be a useful and simple approach for metabolomic analysis. The use of this matrix with MALDI time-of-flight mass spectrometry (TOF-MS) in negative ionization mode for detection of low molecular weight metabolites has been investigated here. The method is used for analysis of extracts of islets of Langerhans as well as direct analysis of single islets. Islets are an intriguing target for metabolomic analysis because of the complex metabolic networks involved in regulating their function. Islets are pancreatic microorgans, containing ~2000 cells each that secrete hormones involved in regulating glucose homeostasis. The most abundant cell type in islets is the insulin-secreting β-cell. When β-cells are exposed to elevated glucose concentration, their ATP/ADP ratio will increase via an increase in glycolytic flux and oxidative phosphorylation. The increase in ATP/ADP ratio evokes membrane depolarization that results in secretion of insulin.[15] Numerous other pathways and second messengers derived from metabolism or receptor activation also regulate insulin secretion. Some of these signals are known, such as cyclic-AMP,[16] but other metabolic signals believed to exist have not yet been identified.[17] Thus, considerable insight into islet function is expected from metabolomic analysis. The versatility of this method is demonstrated by extending it to prokaryotes through analysis of *Escherichia coli* extracts. In all, over 100 metabolites were detected by this approach.

EXPERIMENTAL SECTION

Reagents. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All solvents were purchased from Burdick & Jackson (Muskegon, MI). RPMI media, fetal bovine serum, and penicillin−streptomycin were purchased from Invitrogen Corp. (Carlsbad, CA). Eppendorf Safelock Biopur tubes were purchased from Fisher Scientific (Fairland, NJ).

Islet Isolation. Islets of Langerhans were isolated from CD-1 mice (20−30 g) as previously described[18] with slight modifications. Briefly, collagenase IX (0.5 mg/mL) was infused through the pancreatic duct. The pancreas was then removed and incubated in 5 mL of collagenase IX (0.5 mg/mL) at 37 °C for 7 min. Islets were isolated by passing digested pancreas through a 100-μm pore diameter nylon cell strainer (Fisher Scientific) with 10 mL of Krebs ringer buffer. This procedure collected islets that were greater than 100 μm in diameter. Islets were transferred to a Petri dish by inverting the filter, which was rinsed with 5 mL of buffer. Islets were manually selected using a pipet and transferred to a Petri dish containing cell culture media (10 mM glucose RPMI media 1640 with L-glutamine) supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin. Islets were incubated in this medium at 37 °C, 5% CO_2 for 2−7 days before use.

Metabolite Extraction from Islets. Thirty islets were removed from the media and transferred to RPMI media supplemented with either 20 mM glucose (incubation for 5 min) or 3 mM glucose under 5% CO_2 at 37 °C. After incubation, islets were rinsed of cell culture media by removing them in a pipet and quickly depositing them in water containing the same glucose concentration as incubation media. Islets were then immediately transferred in 10 μL of glucose solution to a 0.5-mL sample tube (Biopure). This rinse step was performed to remove salts present in media that were found to suppress analyte signals. The sample tube was tapped to congregate islets to tube bottom. For cold methanol extraction,[19] 5 μL of glucose water was removed and replaced with 5 μL of methanol chilled to −15 °C. Islets were triturated in methanol to assist lysis. The entire process from deposition into glucose water to trituration was performed in <60 s. Blank samples were prepared by removing 5 μL of glucose water after islet had been removed. Blank was henceforth treated exactly as sample. Where indicated, some islets were lysed by heating them to 100 °C for 5 min[20] and then rapidly immersing the tube in ice water. After lysing, islets were placed on dry ice for 30 min and then thawed in ice water. Lysed islets were centrifuged at 1200*g* for 3 min, and supernatant was analyzed.

Direct Analysis of Single Islet. Single islets were incubated in 20 mM glucose media for 5 min as described above. The islet was rinsed of salts from media by placing it in 5 mL of aqueous glucose solution and then rapidly removing the islet with 1 μL of solution using a pipet. The islet was immediately transferred onto a single well of a MALDI plate. A 1-μL droplet of matrix (9 mg/mL 9-AA in 1:1 methanol/acetone) was deposited onto the islet-

(9) Lavanant, H.; Lange, C. *Rapid Commun. Mass Spetrom.* **2002**, *16*, 1928−1933.
(10) Dally, J. E.; Gorniak, J.; Bowie, R.; Bentzley, C. M. *Anal. Chem.* **2003**, *75*, 5046−5053.
(11) Shen, Z. X.; Thomas, J. J.; Averbuj, C.; Broo, K. M.; Englehard, M.; Crowell, J. E.; Finn, M. G.; Siuzdak, G. *Anal. Chem.* **2001**, *73*, 612−619.
(12) Vermillion-Salsbury, R. L.; Hercules, D. M. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1575−1581.
(13) Mims, D.; Hercules, D. *Anal. Bioanal. Chem.* **2003**, *375*, 609−616.
(14) Mims, D.; Hercules, D. *Anal. Bioanal. Chem.* **2004**, *378*, 1322−1326.
(15) Detimary, P.; Jonas, J. C.; Henquin, J. C. *J. Clin. Invest.* **1995**, *96*, 1738−1745.
(16) Renstrom, E.; Barg, S.; Galvanovskis, J.; Ivarsson, R.; Jing, X. J.; Rorsman, P.; Rutter, G.; Seino, S. *Diabetologia* **2001**, *44*, A62−A62 233.
(17) Rutter, G. A. *Mol. Aspects Med.* **2001**, *22*, 247−284.
(18) Roper, MG.; Shackman, J. G.; Dahlgren, G. M.; Kennedy, R. T. *Anal. Chem.* **2003**, *75*, 4711−4717.
(19) Buchholz, A.; Takors, R.; Wandrey, C. *Anal. Biochem.* **2001**, *295*, 129−137.
(20) Deng, Q.; German, I.; Buchanan, D.; Kennedy, R. T. *Anal. Chem.* **2001**, *73*, 5415−5421.

containing droplet, and the mixture was allowed to dry. Blanks for this experiment used 1 μL of glucose solution without islet present.

Escherichia coli Sample Preparation**.** *E. coli* DH5-α was grown in Lauria-Bertani broth (LB) until reaching stationary phase. A 5 mL aliquot of cells in LB media were centrifuged at 1200*g* and rinsed 3 times with 5 mL of deionized water. After the third rinse, the *E. coli* suspension was centrifuged and 100 μL of supernatant was removed as blank; the remaining supernatant was decanted and replaced with 500 μL of cold methanol. Cells were lysed using a sonic dismembrator (Fischer Scientific, model 60) set to a power of 9 W for 10 s. The cell lysate was centrifuged at 1200*g* for 10 min. Supernatant was removed and analyzed as described below.

Mixture and Deposition of Sample with Matrix. Standards and extracts (islet and *E. coli*) in methanol were mixed 1:1 with matrix (9 mg/mL 9-AA) in acetone for deposition. Standards were dissolved in 1:1 (v/v) methanol/water at the concentrations indicated. For islets lysed by boiling, supernatant was mixed 1:1 (v/v) with methanol, and this mixture mixed 1:1 (v/v) with 9-AA matrix for deposition. A 1−μL aliquot of the sample−matrix mixture was deposited onto a single well of a 96-well, stainless steel, MALDI target plate using the dried-droplet method.[21]

For tests of other matrixes, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 2,4,6-trihydroxyacetophenone (THAB) were dissolved in methanol at both 10 and 20 mg/mL. 3-Hydroxypicolinic acid (3-HPA) was dissolved with 1:1 methanol/water. All matrixes were tested with and without the addition of 40 mM triammonium citrate as described elsewhere.[10,22] These matrixes were mixed with samples and deposited as described above.

Mass Spectrometry. A MALDI TOF-2E (Micromass, Milford, MA) mass spectrometer equipped with a 337-nm pulsed nitrogen laser (4 ns) operated in reflectron mode was used for all experiments. Unless otherwise noted, mass spectra were obtained in negative ionization mode with the following settings: source voltage of −20 000 V, extraction pulse voltage of 2200 kV, extraction voltage of 19 950 V, and focus voltage of 16 000 V. Ion suppression was set to 50 *m*/*z* and the scan extended to 3000 *m*/*z*. External calibration was performed on all spectra. Data were acquired with 5 shots/scan and 20 scans/spectrum. Data were treated with a nine-point Savitsky−Golay smooth.

Metabolite Identification by Database Searching. Metabolites were identified by matching molecular weight obtained from MS to one of three databases: KEGG,[23] METLIN (http://metlin.scripps.edu/), and MetaCyc.[24] Matching was performed using the detected mass plus 1 Da with a range of ±0.3 Da. For each search in KEGG and MetaCyc, the molecular formulas of possible compounds were transferred to an isotope calculator and monoisotopic mass was compared with peak. Metabolites within 0.1 Da were considered confirmation of identity.

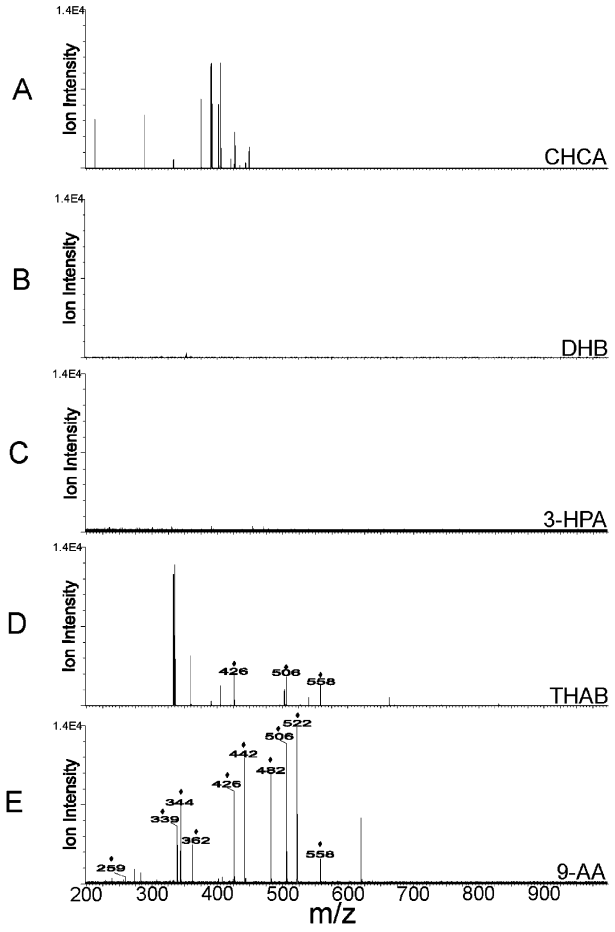

Figure 1. Comparison of metabolite signals using different matrixes. (A), (B), and (D) matrixes were 20 mg/mL in methanol and (C) 20 mg/mL in 50:50 methanol/water. 40 mM ammonium citrate was added to matrixes A−D. (E) Matrix concentration 9 mg/mL in acetone. Metabolite standards detected are indicated by ◆. All samples contained 25 μM ADP ribose (558 *m*/*z*), 27 μM GTP (522 *m*/*z*), 26 μM ATP (506 *m*/*z*), 25 μM CTP (482 *m*/*z*), 26 μM GDP (442 *m*/*z*), 22 μM ADP (426 *m*/*z*), 24 μM GMP (362 *m*/*z*), 29 μM cyclic GMP (344 *m*/*z*), 25 μM fructose 1,6 bisphosphate (339 *m*/*z*), and 29 μM glucose 6-phosphate (259 *m*/*z*).

RESULTS AND DISCUSSION

Metabolite Standards. Initial investigations explored the utility of 5 different matrixes (CHCA, DHB, 3-HPA, THAP, 9-AA) for detection of 10 phosphorylated metabolites at concentrations of 20−30 μM. In positive mode, no strong analyte signals were detected, although acidic matrixes did yield some weak adduct signals (data not shown). In negative mode, 9-AA provided a far superior analyte signal, allowing all 10 metabolites to be detected while yielding a spectrum with few interfering matrix peaks (Figure 1). In contrast, CHCA and DHB yielded no noticeable analyte peaks while 3-HPA and THAP showed weak signal for 4 and 3 of the 10 metabolites, respectively. Varying the concentration of matrixes from 10 to 20 mg/mL did not significantly alter either signal intensities or the number of metabolites detected. Addition of triammonium citrate, sometimes used as a co-matrix to reduce sodium adducts,[12] yielded a modest increase in signal intensity. Besides this lack of effectiveness, citrate is not desirable as a co-matrix for metabolite analysis given that citrate and isocitrate are analytes of interest and would be masked by its use.

(21) Wei, H.; Nolkrantz, K.; Powell, D. H.; Woods, J. H.; Ko, M. C.; Kennedy, R. T. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1193−1200.
(22) Zehl, M.; Allmaier, G. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1931−1940.
(23) Kanehisa, M.; Goto, S.; Kawashima, S.; Okuno, Y,.; Hattori, M. *Nucleic Acid Res.* **2004**, *32*, D277−D280.
(24) Karp, P. D.; Riley, M..; Paley, S. M.; Pelligrino-Toole, A. *Nucleic Acid Res.* **2002**, *30*, 59−61.

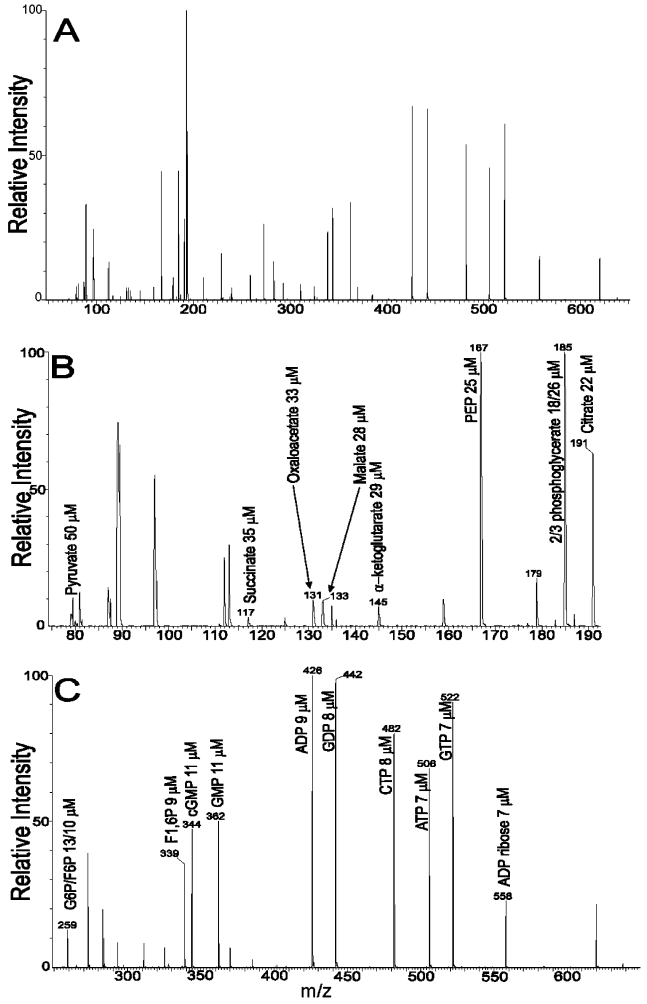

Figure 2. MALDI-TOF-MS of 18 metabolite standards at 4 μg/mL in 50:50 MeOH/water. Samples were mixed 1:1 with matrix solution (9 mg/mL 9-AA in acetone) before deposition by the dried-droplet method. A full spectrum (A), zoom of spectrum from 70 to 192 *m*/*z* (B), and zoom of spectrum A from 250 to 630 *m*/*z* (C) are shown. Abbreviations are phosphoenolpyruvate (PEP), fructose 1,6, bisphosphate (F1,6P), glucose 6-phosphate (G6P), fructose-6-phosphate (F6P), and cyclic GMP (cGMP). NAD was in the sample but not detected.

Based on these promising initial results, 9-AA was further investigated as matrix for a more diverse set of 19 metabolite standards including nucleotides, glycolytic intermediates, and citric acid cycle metabolites. In these more complex mixtures, 18 of the 19 metabolic standards were readily detected (Figure 2). NAD was not detected, presumably because of the positive charge from the quaternary amine. Limits of detection, calculated by taking 3 times the peak-to-peak noise of the blank (measured at analyte mass) divided by the slope of the calibration curve, were 15 nM (nucleotides) to 1 μM (succinate) for individual compounds.

Islet Extract Analysis. Use of this method for analysis of extracts from islets of Langerhans was next investigated. As cell lysis is critical for achieving good extraction, multiple methods were evaluated. Cell lysis using a sonic dismembrator is advantageous because it gives rapid lysis with definite start time; however, it is limited to sample volumes greater than 50 μL. Other lysing methods evaluated include boiling and trituration in cold methanol, digitonin, and a combination of cold methanol and digitonin. These

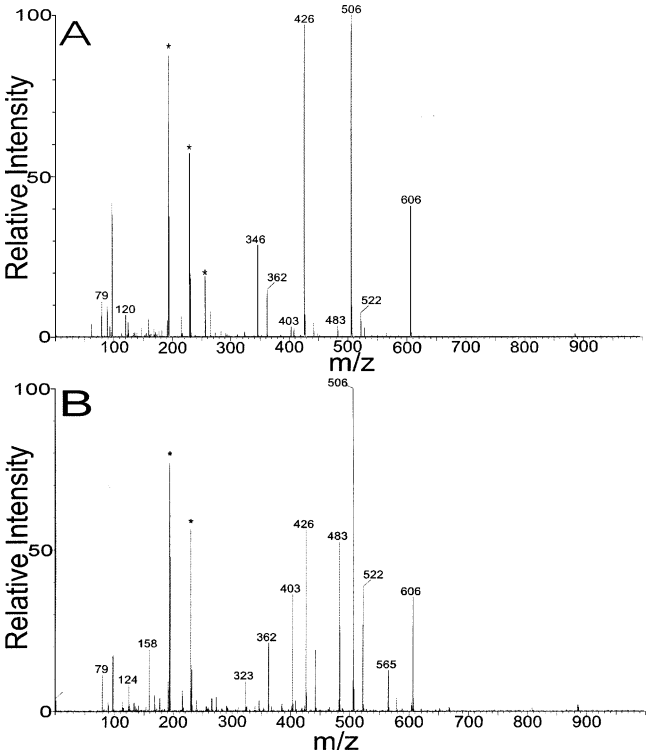

Figure 3. MALDI-TOF mass spectrum of extract from 30 islets preincubated in 3 mM glucose (A) or 20 mM glucose (B). Peaks with *m*/*z* label were identified by matching mass to chemical in database (see Table 1).

methods all gave equivalent results (data not shown) so trituration in cold methanol was used for all further experiments because it was simple and compatible with hydrophobic matrix.

Metabolite Detection. Figure 3 compares MALDI-TOF-MS spectra from an extract of 30 islets preincubated in 3 mM glucose media (A) or 20 mM glucose (B). Peaks detected in the spectra were considered to be the metabolite signal if the peak intensity was 3 times that of the blank in the same m/z region. Peaks were eliminated if they appeared to be adducts based on analysis of isotopic ratios. As chloride is the most prevalent anionic salt in physiological solution, it is the most likely adduct to form with metabolites in negative mode with 9-AA as the matrix. (This expectation was confirmed by experiments in which the cell culture medium was not rinsed from samples prior to extraction.) Chloride has an isotopic ratio of 3:1 and differential spacing of 2 Da between isotopes; therefore, peaks meeting this criterion were discarded as they could not be differentiated between a single metabolite−chloride adduct or two distinct metabolites that happen to be separated by 2 m/z and at the 3:1 ratio. A total of three pairs of peaks were eliminated this way. Using these criteria, 33 of the peaks detected from islets incubated in 3 mM glucose and 39 peaks from islets incubated in 20 mM glucose were considered metabolites. Figure 4A illustrates the overlap of occurrence of the metabolites detected. A total of 72% of metabolites detected in islets incubated in 20 mM glucose were also detected in islets incubated in 3 mM glucose.

Not all of the peaks detected were observed in each sample, and the number of times a peak was detected depended on the glucose concentration (Figure 4B). For islets incubated in 3 mM glucose, only 18% were detected in all extracts and the majority of metabolites were detected once for three samples. Incubating

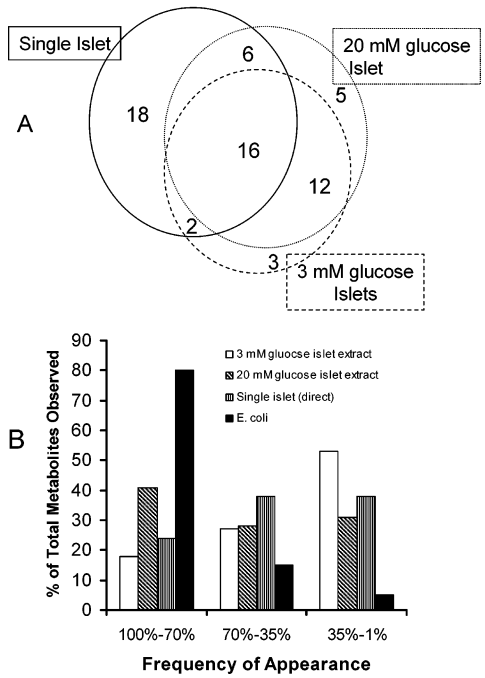

Figure 4. Summary of overlap and frequency of detection of metabolites by MALDI-TOF-MS. (A) Venn diagram illustrating the overlap of detected metabolites in islet extracts preincubated in 3 mM glucose, 20 mM glucose, and direct analysis of single islets. Area is indicative of number of metabolites with actual number of metabolites as indicated. (B) Frequency of metabolite detection under differing conditions. For islet extracts, three different extracts were analyzed for each glucose concentration, for direct analysis, six individual islets were analyzed, and for *E. coli*, three separate extracts were analyzed.

islets in 20 mM glucose resulted in more than doubling the percentage of metabolites found with high frequency so that 41% of the metabolites were observed 100% of the time.

As β-cells use glucose exclusively by glycolysis, these differences reflect the effect of enhanced glyolytic flux on the metabolome. These results suggest the potential to detect changes in metabolic patterns associated with environmental conditions based upon the metabolites that are detected and their frequency of detection.

Metabolite Identification. An accurate monoisotopic molecular mass can be calculated by adding 1 Da to the first isotopic peak of detected mass. These molecular weights were entered into a database search (KEGG or METLIN) to obtain tentative identification of peaks based on matching mass. Most masses that were successfully assigned from the islet samples were matched using KEGG. (KEGG is an extensive database containing over 11 000 compounds; however, many of the compounds are pharmaceuticals that had to be eliminated when producing matches in this experiment.) METLIN was used as a supplement to KEGG to confirmation matches. METLIN also provided identification for ~10% of metabolites that did not have matches in KEGG. MetaCyc was not used for islet identification as its search is species dependent and mouse was not a species included in the database. A total of 29 of the 44 total metabolite peaks detected in islet extracts could be matched using this method. Table 1 lists all the masses detected, their identification, and frequency of detection for the different islet incubation conditions. These identifications should be considered tentative because they are based only on molecular mass. More chemical information, such as is obtainable by tandem MS may be necessary to increase confidence in the assignments. An increase in chemical information, as well as improved databases, may also be useful identifying the 15 unknown peaks that were detected.

Many compounds that were identified in islets were expected such as nucleotides and the energy metabolism intermediates, e.g., fructose 1,6,-bisphosphate and citrate. Unsuspected metabolites were also identified. The most intense of these was UDP−*N*-acetylglucosamine (606 m/z), which is an intermediate of the hexosamine pathway initiated by glutamine:fructose-6-phosphate amidotransferase (GFAT).[25] This pathway, which is activated at elevated glucose concentrations, has been implicated in damage to β-cells associated with hyperglycemia in diabetics.[26] The presence of UDP−*N*-acetylglucosamine indicates that the GFAT pathway is activated under the conditions used here. The relative importance of islet culture conditions (10 mM glucose) and preincubation conditions (3 or 20 mM glucose in media) on the activation of this pathway are unclear from these initial experiments as the metabolite was detected under all conditions. Nevertheless, these results demonstrate the ability of this approach to detect the activation of key metabolic pathways.

Semiquantitative Analysis. MALDI is not suitable for quantitative analysis; however, ratiometric measurements may be used to determine relative amounts of analytes.[27] To explore this possibility, the ratio of di- and triphosphorylated nucleotides as a function of glucose concentration was evaluated. For standards, the signal ratio for ATP/ADP was 1.11 ± 0.07 ($n = 6$) when the concentration ratio was 1.0, suggesting comparable sensitivity for these compounds. As shown in Table 2, repeated analysis of a given sample of islet extract (different spots of the same sample) yielded a reproducible ratio of ATP/ADP with relative standard deviations (RSDs) of 8−12%; however, for different samples, the reproducibility was much worse with RSDs of 46 and 73%. The difference in reproducibility is also reflected in the observation that the increase in ATP/ADP ratio with increasing glucose concentration was statistically significant for intrasample comparisons but not for the intersample comparison (Table 2). Similar results were found for GTP and UTP. The intersample variability could represent differences in the islet preparations due to factors such as specimen age and time in culture or variability in the extraction procedure.

The signal ratio of ATP/ADP increased an average of 217% with an increase in glucose concentration from 3 to 20 mM. This increase is in good agreement with previous experiments that used a luminescence assay and detected a 227% increase in ATP/ADP ratio upon increasing glucose to 20 mM.[28] While the percent change reported here is similar to this previous study, the actual ratio of ATP to ADP measured previously was 5.1−11.6 while in this case these values were almost 3-fold lower (see Table 2). This difference in absolute ratio of ATP to ADP could be due to differences in extraction (ATP is bound in many subcellular organelles making extraction critical), incubation time, or the sensitivity of ATP to ADP by MALDI-TOF-MS in the samples, although such differences with standards was not observed.

(25) Brownlee, M. *Nature* **2001**, *414*, 813−820.
(26) Kaneto, H.; Xu, G.; Song, K. H.; Suzuma, K.; Bonner-Weir, S.; Sharma, A.; Weir, G. C. *J. Biol. Chem.* **2001**, *276*, 31009−31104.
(27) Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6591−6596.
(28) Detimary, P.; Gilon, P.; Henquin, J. C. *Biochem. J.* **1998**, *333*, 269−274.

Table 1. Summary of Peaks Detected and Tentative Assignments from Databases[a]

		no. of observations for sample type[b]						no. of observations for sample type[b]			
m/z	chemical match of *m/z* from metabolite database	islet extract (3 mM glucose)	islet extract (20 mM glucose)	direct analysis of single islet	*E. coli*	*m/z*	chemical match of *m/z* from metabolite database	islet extract (3 mM glucose)	islet extract (20 mM glucose)	direct analysis of single islet	*E. coli*
109		1	2			545	TDP-4-oxo-6-deoxy-D-glucose				3
111	uracil		1	2		546		1			
121		1	2			547	deox-TDP-L-rhamnose				3
124	taurine	2	3	6	–	549	UDP-deoxyglucose		1		
131	aspartate			2	–	565	UDP-glucose	2	3	2	3
133	malate	2	3		–	579	inositol pentaphosphate or UDP-glucuronate				3
135	oxalate	2	3		–	588	guanosine diphosphate fucose				3
137				4	–	602	guanosine 5′-diphosphate-3′-diphosphate				3
145	α-ketoglutarate/adipate				2	606	UDP−*N*-acetyl glucosamine	3	3	6	
149				4	–	618	GDP-D-mannuronate				3
151			2	3	–	620	UDP−*N*-acetyl mannosaminourate				3
153		1	2	–	–	626	tetrahydropteroyltri-l-glutamate			2	
159		1	3	4	3	632		1	1		3
161	4-hydroxy-2-ketoglutarate			3	–	638					3
163				3	–	678	UDP−*N*-acetyl muramate				3
167	phosphoenolpyruvate	2	2	3	1	686	dephospho-CoA				3
171	glycerol phosphate	2	3	6	–	688				2	
177	pyrophosphate				1	698				2	
179	glucose		1	3		703					2
182			1	3		719	phosphoribosyl ATP				3
184	3-phosphoserine		1			733					3
188	*N*-acetyl glutamate	1				744	NADPH			2	–
191	citrate	1	3	5	2	747					3
215	glucose + Cl^-	1	3			756					3
288	*N*-succinyl-2 amino-6-ketoimelate				3	766	coenzyme A			2	3
293			3			774					3
323	UMP	1	3		1	784	FAD				3
322	CMP				2	786	$FADH_2$				3
337	5′-phosphoribosyl-5-amino 4-imidazole carboxamide	2	2	4		791	UDP-3-*O*-3-hydroxymyristol-*N*-acetylglucosamine			3	
339	fructose 1,6-bisphosphate or inositol biphosphate	1	2	2		811				2	
346	AMP	1		2	3	814					2
362	GMP	1	2			826				1	
367	orotidine phosphate		2	6		833				1	
383	adenosyl homocysteine			2		866	succinyl-CoA			2	
385			1		3	878					2
389	phosphoribosyl diphosphate	2		3	–	886	hydroxybenzoyl-CoA			1	
401	4 phospho-*N*-pantothenoylcysteine or TDP				3	945					3
403	UDP	3	3	6	3	991	cobinamide				3
408		1	2		3	1004	palmityl-CoA				3
426	ADP	3	3	6	3	1050	UDP−*N*-acetylmuramoyl-L-alanyl-γ-D-glutamyl-meso-diaminopimelate				3
442	GDP	3	3	5	3	1064					3
452		1			–	1072	CoA-glutathione				3
456	methylene tetrahydrofolate	1	1		–	1146					3
464			1	2	–	1192	UDP−*N*-acetylmuramoyl-L-alanyl-D-glutamyl-6-carboxy-L-lysyl-D-alanyl-D-alanine				3
481	TTP				3	1371					2
483	UTP	3	3	4	3	1451					3
487		1	1			1721					2
490	deoxy-ATP				3	1845	KDO_2-lipid IV_A				3
500			1			2007					3
506	ATP	3	3	6	3	2428					3
513		1	1	3		2470				3	2
522	GTP	2	2	5	3	2576				3	
540					3						

[a] A total of 105 metabolites found in various cells under different conditions are reported. *m/z* for peaks were rounded to nearest integer for clarity. Identification was made using KEGG and METLIN for islet experiments and MetaCyc for *E. coli* experiments. For compounds with two possible identifications, both are given. [b] Experiment was replicated 3 times for the islet extract samples and the *E. coli* sample. Experiment was replicated 6 times for the direct analysis of single islets. Number in column indicates how many of these samples contained the metabolite or *m/z* indicated. Islets were incubated in 3 or 20 mM glucose prior to the extraction procedure as indicated at the column heading.

The GTP/GDP ratio has also been reported to increase in response to increased glucose concentration.[29] As seen in Table 2, this ratio changes by 501% when comparing islets incubated in 3 mM glucose to 20 mM glucose, a value in reasonable agreement

Table 2. Islet Nucleotide Ratios under 3 and 20 mM Incubation[a]

	ATP/ADP		GTP/GDP		UTP/UDP	
sample	3 mM glucose	20 mM glucose	3mM glucose	20 mM glucose	3 mM glucose	20 mM glucose
1	1.14 ± 0.14	2.42 ± 0.21**	1.66 ± 0.18	1.97 ± 0.23*	1.11 ± 0.28	1.77 ± 0.25**
2	3.50 ± 0.29	6.16 ± 0.87	1.04 ± 0.21	4.29 ± 0.52**	0.84 ± 0.25	2.14 ± 0.34**
3	1.06 ± 0.08	3.82 ± 0.50*	0.66 ± 0.17	10.62 ± 2.45**	0.41 ± 0.15	9.00 ± 3.30**
av	1.90 ± 1.39	4.13 ± 1.89	1.12 ± 0.50	5.62 ± 4.48	0.79 ± 0.35	4.30 ± 4.07

[a] Individual samples were analyzed from four different spots. Each sample is a separate extract of 30 islets. Ratios were determined using peak height corresponding to nucleotide. Statistical significance is indicated between 3 mM and 20 mM glucose incubated islets as follows: * $p < 0.05$ and **$p < 0.01$.

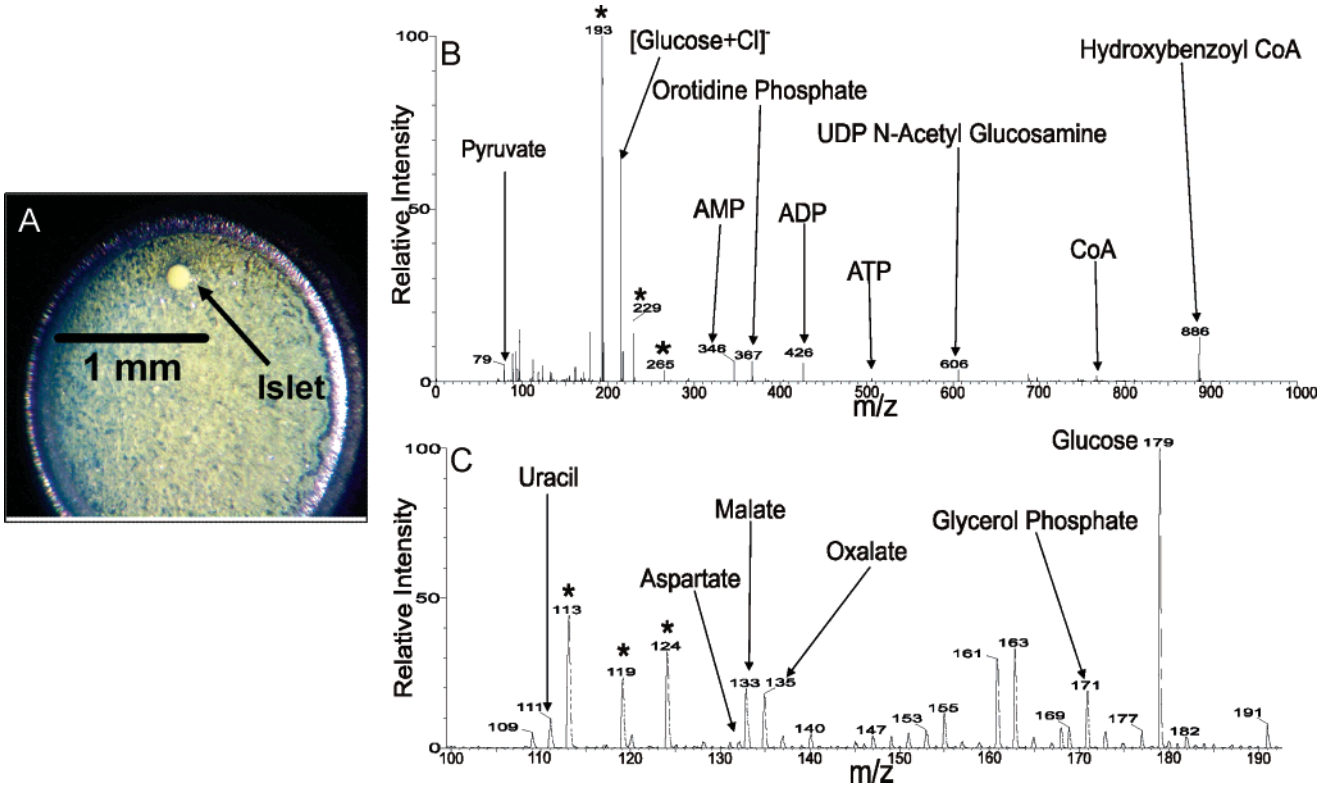

Figure 5. Direct analysis of single islets by MALDI-TOF-MS. (A) Photograph of islet in matrix on stainless steel MALDI target. (B) Spectrum of single islet with matches of detected masses from database shown. * indicates peaks due to matrix and blank. The full spectrum from 0 to 1000 *m*/*z* is shown in (B) and an expansion from 100 to 192 *m*/*z* is shown in (C).

with previous observations of a 320% increase. The effect of glucose on the UTP/UDP ratio has not been reported, though increases in UTP under stimulatory glucose concentrations have been observed.[30] UTP is used primarily for complex carbohydrate synthesis while GTP is used for protein synthesis. The increases in these nucleotide ratios under glucose stimulation may reflect the increase in availability of energy for synthesis of proteins and carbohydrates.

Direct Analysis of Single Islet. Recent work has demonstrated the feasibility of direct analysis of tissue coated with matrix by MALDI-MS allowing the peptide content of single neurons and vesicles to be determined.[30,31] This prior work, combined with the successful detection of metabolites here, suggested the possibility of direct analysis of tissue for metabolites. To evaluate this possibility, single islets were rinsed of salts and deposited onto a target plate with matrix for MALDI-TOF-MS analysis (Figure 5A). For these experiments, matrix solution was increased to 50% methanol as it led to slower drying and better crystallization of matrix with islet, compared to 100% acetone. Single islets were found to maintain shape and integrity with this sample preparation (see Figure 5A).

Good quality spectra could be obtained from single islets with this procedure (Figure 5B). Using the criteria outlined above for islet extracts, a total of 42 metabolites were detected in 6 islets. A total of 28 of the metabolites were identified using the KEGG and METLIN databases (see Table 1). Though direct analysis of islets yielded a similar number of detected metabolites as islet extracts (42 and 45, respectively), only 47% of the detected metabolites overlapped (Figure 4A) indicating that direct analysis accesses a different pool of metabolites than the extraction method. This may be because of the difference in dilution and chemical environment

(29) Detimary, P.; Van den Berghe, G.; Henquin, J. C. *J. Biol. Chem.* **1996**, *271*, 20559−20565.
(30) Garden, R. W.; Moroz, L. L.; Shippy, S. A., Sweedler, J. V. *J. Mass Spectrom.* **1996**, *31*, 1126−1130.
(31) Li, L.; Garden, R. W.; Sweedler, J. V. *Trends Biotechnol.* **2000**, *18*, 151−160.

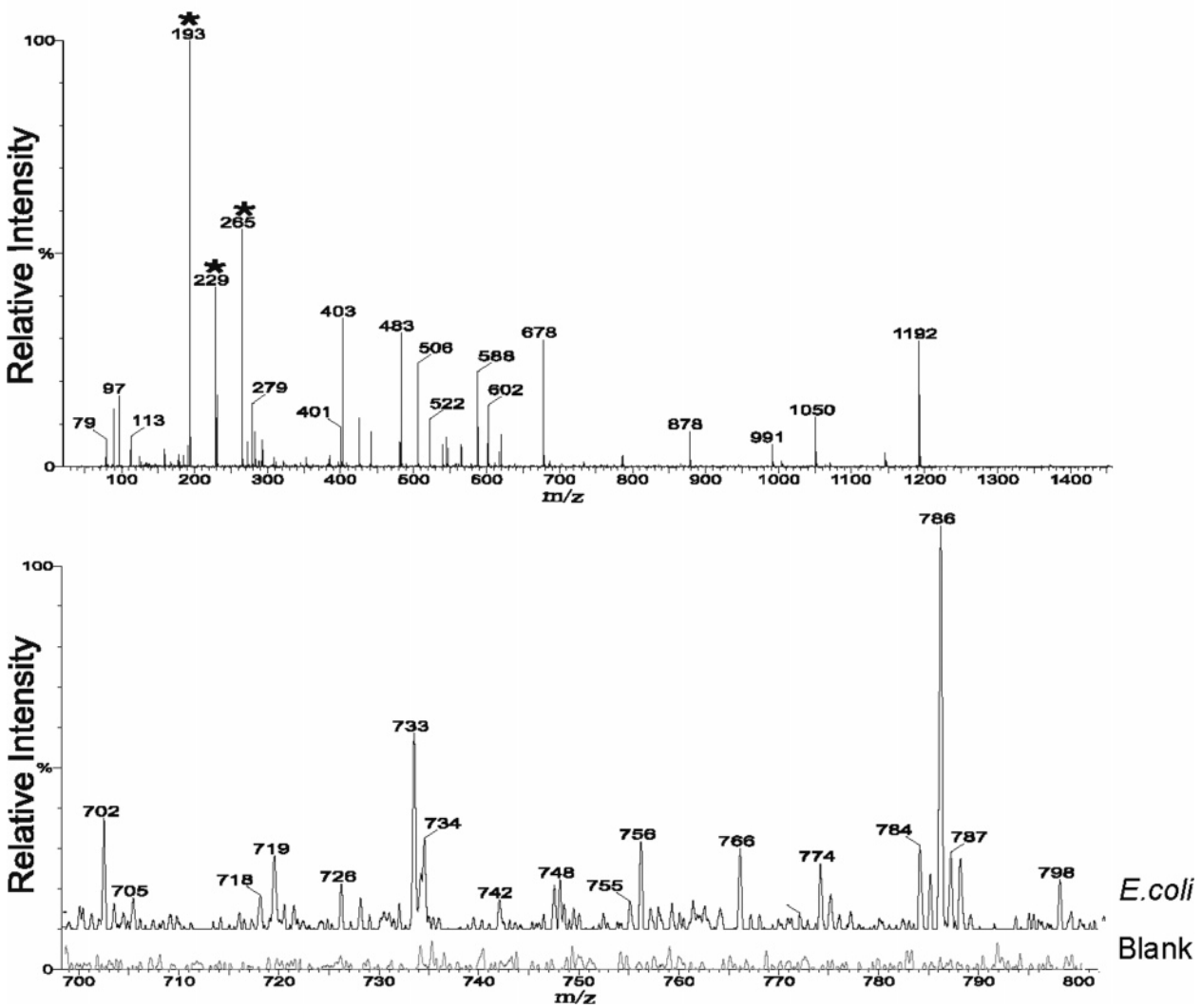

Figure 6. Metabolite analysis *E. coli* extract by MALDI-TOF-MS. Top: mass spectrum of methanol lysed *E. coli* strain DH5-α. Bottom: magnification from 700 to 800 *m*/*z*. Lower trace is blank. Upper trace is *E. coli* sample, offset 10% for clarity. * indicates peaks in matrix or blank.

for MALDI between the two methods. Furthermore, in the direct analysis method, metabolites have not been partitioned between two phases (methanol and lipid/cell debris) as in the extraction method.

The direct analysis method was less reproducible than the extraction method in terms of the overlap of metabolites detected between different analyses (Figure 4B). This is reflected in the observation that only 17% of the metabolites were observed in all samples. The variability could be due to several factors including variation in matrix crystallization around the islet, laser positioning relative to the islet, and islet variability (i.e., single-islet analysis compared to pooled islet analysis).

E. coli Analysis. Metabolite profiling of bacteria may prove useful for discovery of biochemical pathways and novel connections between preexisting pathways. Therefore, the suitability of this technique for analysis of *E. coli* extracts was examined. As seen in Figure 6, MALDI-TOF-MS analysis of *E. coli* extract yields strong signal intensity for multiple analytes across almost the entire range of 50−1500 m/z. The lower traces of Figure 6 illustrate the numerous low-intensity peaks that are distinct and detectable compared to blank in the 695−805 m/z range.

Though cells were triply rinsed, the blank still contained more interfering peaks, albeit low intensity, than observed with the islet extracts. These minor peaks are thought to arise either from media contamination (the LB media *E. coli* are grown in yeast extract) or from lysis of some cells during centrifugation. Because of this background, a more stringent method was used to identify signals. Peaks for *E. coli* were referenced to blank and determined to be *E. coli* metabolites when signal intensity was at least 10× greater than blank at a given m/z. Using this criterion, a total of 60 peaks were detected in the three *E. coli* samples. These peaks had only 27% overlap with those detected in the islet extracts, illustrating the large difference in metabolite profile for these cell types.

The detection of a given metabolite was more reproducible than with the islet extracts and direct islet analysis as shown in Figure 4B. For *E. coli*, 80% of the detected metabolites were observed in all three samples. The reason for this more reproducible response was not investigated, but it could be due to less variability among the *E. coli* samples than the islets. Furthermore, the large mass of cells available for *E. coli* analysis allowed

preconcentration, which may have enabled more consistent detection of the metabolites amenable to this approach.

For identification of peaks, MetaCyc was particularly useful in *E. coli* studies as it allows for specific searching by organism. *E. coli* DH5-α was not in the database; however, *E. coli* strain K-12, another nonvirulent strain, was available. Using MetaCyc with the K-12 *E. coli* database, 39 of the 60 detected metabolites could be assigned an identity as shown in Table 1. The ability to detect and identify metabolites in bacteria may prove useful not only for studying metabolic pathways but as a complement to protein measurements currently being developed to identify strains of bacteria.[32,33]

CONCLUSIONS

This work demonstrates the utility of negative mode MALDI-TOF-MS with 9-AA as the matrix for metabolic profiling. Detection limits for standards were in the nanomolar to micromolar concentration range, indicating suitable sensitivity for many metabolites. A total of 105 different metabolites were detected in complex biological samples including single, intact islets of Langerhans and crude extracts of islets and *E. coli*. Effects of environment (glucose concentration) and sample type revealed the ability to detect different metabolic states. Identification of peaks was attempted based on matching the mass detected to databases. While good matches were found, more chemical information, such as that obtained by tandem mass spectrometry (e.g., TOF-TOF-MS), may be necessary to achieve confident identification. In addition, as numerous peaks could not be assigned, improved databases are needed. Identification was also limited by inability to distinguish isomers such as citrate and isocitrate. The combination of this approach with high-resolution separations may be necessary to ameliorate isomer overlap. The use of separations may also improve the number of compounds detected and detection limits by minimizing competitive ionization. Ratiometric quantification was demonstrated for the nucleotides. Based upon these initial results and possibilities for improvement, negative mode MALDI-TOF-MS appears to be a promising addition to the armamentarium of metabolomic techniques.

(32) Ryzhov, V.; Fenselau, C. *Anal. Chem.* **2001**. *73*, 746−750.
(33) Bundy, J. L.; Fenselau, C. *Anal. Chem.* **2001**. *73*, 751−757.

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