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Metabonomic Study with a High Performance Liquid Chromatography Coupling to a Triple Quadruple Mass Spectrometer to Identify Biomarkers from Urine of High-fat Fed and Streptozotocin Treated Rats

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ABSTRACT

High performance liquid chromatography coupled with an electrospray ionization (ESI) tandem mass (MS/MS) detector was employed to scan the urinary metabolites of Wistar rats (n=5 each group) fed with either control diet or high fat diet with a single dose of streptozotocin (STZ) for 14 weeks. Principle component analysis (PCA) of the LC-MS data was performed for pattern re-cognition and it clearly showed that the dosed animals displayed different metabolic responses even though they received the same treatment. Components responsible for the separation were identified with MS/MS. Glucose and malonic acid were among some other components in Krebs cycle to show high loading strength in PCA. It was therefore postulated that the high fat diets have driven the animals toward the metabolic flux to gluconeogenesis and then energy production through Krebs cycle. Excess energy was also stored through fatty acid biosynthesis with the observation of high loading strength of malonic acid. This experiment demonstrated that a triple quadruple mass spectrophotometer is capable of the metabonomic study on diet modulation of animals. Since metabonomics is a congruous part of nutrigenomics that can provide information on nutritional status, toxicity and gene function of an organism, it is hoped that this technique be extended to study the efficacy of functional foods and the development of personalized foods.

Key words: biomarkers, high performance liquid chromatography, mass spectrometry, metabolic profiling, metabonomics

INTRODUCTION

Metabonomics is a rapidly evolving research discipline that is complementary to genomics and proteomics for a systematic study of a living organism following the perturbation of diseases, chemicals or gene defects[1]. The discipline consists of a metabolic profiling of biological fluids or tissues followed by a mathematic calculation for pattern recognition to highlight both subtle and gross differences in the samples[2]. As a consequence, metabonomics not only serves as a platform to indicate holistic changes in a living organism but also identify compounds responsible for the metabolic changes. Application of metabonomics has been observed mainly in pharmaceutical researches for toxicity evaluation and drug development[3-5]. Biological study with metabonomics is also a usual practice in deciphering diversed phenotypes resulted from different genetic or environmental origins of plants[6-8] and microorganisms[9-12]. Food and nutrition researches also found metabonomics a valuable tool to study biochemical effects of dietary components[13,14] and to characterize vegetable oils by their polar components[15] or amino acid profiles[16].

High-field proton nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the techniques of choice in the detection and identification of metabolites. Both showed their effectiveness and high-throughput in profiling for pattern recognition. However, the sensitivity of MS is higher than NMR in characterizing the responsible compounds for the change in the profile, both quantitative and qualitative[2]. It is therefore not surprised to observe more and more metabonomic researches taking advantage of MS over NMR across different laboratories[17-22]. Time of flight (TOF) MS has been prevalently used because of its good sensitivity and high resolution[18,23,24]. Exact mass data of the metabolites obtained from TOF MS can be annotated by consulting several open databases, e.g. the Kyoto Encyclopedia of Genes and Genomes (KEGG)[25]. This approach was proved to be successful except for isomeric metabolites, which are not uncommon among endogenous metabolites. Further structural analysis with triple quadruple MS may be performed to resolve

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the chemical identities of the metabolites. Accordingly, a metabonomics study may need TOF MS for pattern recognition and triple quadruple MS for component identification. However, not many laboratories have the opportunity to possess both instruments. To date, a triple quadruple instrument is still prevalent and affordable. In this study, we tried to use a triple quadruple instrument for both pattern recognition and component identification in a metabonomics study. Animals, fed with high fat diet and received streptozotocin (STZ) treatment, were used as a model for diabetes and obesity study. It was anticipated that a triple quadruple instrument is able to work ambidextrously in both pattern recognition and component identification for metabonomics.

MATERIALS AND METHODS

I. *Materials*

Streptozotocin, nicotinamide, glucose and malonic acid were products of Sigma (Darmstadt, Germany). Formic acid was purchased from Riedel-de Haen (Seelze, Germany) and acetonitrile (HPLC grade) was from Tedia (Fairfield, OH, USA).

II. *Animal Studies*

Ten male Wistar rats of 8-week old were obtained from National Laboratory Animal Center (Taipei, Taiwan). The animals were housed individually and maintained on a 12 h light/dark cycle (21±1°C). Diet and water were provided *ad libitum*. The animals were fed with normal diet (LabDiet 5001; PMI Nutrition International, Richmond, IN, USA) for 6 weeks and then randomly divided into two groups. One group still received normal diet for 14 weeks while the other group received the high fat diet (TestDiet 47332; PMI Nutrition International, Richmond, IN, USA) for the same duration and a single dose of STZ/nicotinamide (65/125 mg/kg body weight) orally in the middle of the high fat feeding period. The TestDiet 47332 is a modified version of LabDiet 5001 with the addition of 40% total fat. Chemical composition of both diets is shown in Table 1. At the end of the experiment, the animals were moved to grid-bottomed urine collection cages. Urine samples were collected over 0-24 h and stored at -20°C until analysis by LC/MS. The urine samples were allowed to thaw at room temperature and filtered through 0.45 μm membrane prior to analysis.

Table 1. Chemical composition of experimental dietsab

Chemical composition	LabDiet 5001	TestDiet 47332
Protein	23.9	15.0
Fat (ether extract)	5.0	38.6
Fiber (crude)	5.1	3.3
Mineral (ash)	7.0	4.5
Nitrogen-free extract (by difference)	48.7	28.7

[a]Data provided by PMI Nutrition International (Richmond, IN, U.S.A.)

[b]Nutrients expressed as percent of ration. Moisture content is assumed to be 10.0% for the purpose of calculations.

III. *MS Analysis*

An LC-MS/MS system including a Waters 600E multi-solvent delivery pump, a Waters 2487 UV/VIS detector (Milford, MA, USA) and a Quattro LC MS/MS detector (Wythenshawe, UK) with electrospray ionization (ESI) in negative ion mode was used. The capillary voltage was 3 kV. Desolvation temperature was 300ºC. Extraction cone voltage was 20 V. Samples were resolved in a gradient elution over a LiChroCART 125-2 Superspher 100 RP-18 column (Merck, Darmstadt, Germany) before entering the MS/MS detector for total ion scanning or product ion scanning. The total ions were scanned from m/z 100 to 800 with a scan time of 500 ms and an interscan delay time of 100 ms, whose resolving power provided unit-mass resolution across the entire range of scanning[(2)]. The product ions of each interested m/z value were scanned under different collision energy settings to obtain better results for chemical structure elucidation. Sample size was 10 μL. Flow rate was 0.2 mL/min. Both solvent A (H_2O) and solvent B (acetonitrile) contained 0.1% (v/v) formic acid. The gradient was programmed as following: 0% B for 3 min, linear gradient from 0% to 100% B for 37 min, and stayed at 100% B for 10 min.

IV. *Data Analysis*

The method of Plumb *et al.*[(2)] for data arrangement was followed. Briefly, the mass spectra were combined for each individual base peak intensity (BPI) chromatogram of total ion scanning. The peak list and ion abundances were then exported to an Excel (Microsoft, Seattle, WA, USA) file and then aligned in order with zeros inserted as intensity values where no ion signal was observed, so that each line of data had one intensity value for each m/z integer between 100 and 800. The data sets were then transferred to STATISTICA software (StatSoft, Inc., Tulsa, OK, USA) for principle component analysis (PCA) using the factor analysis module.

RESULTS AND DISCUSSION

I. *LC-MS*

Preliminary scanning of the urine samples with both

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positive and negative ion modes revealed that the negative ion mode gave more information than the positive ion mode. The negative ion ESI-MS BPI chromatograms were, therefore, compared among samples. Samples of the control group shared similar chromatographic profile with stronger responses at the hydrophobic region (RT 15-25 min) than those at the hydrophilic region (RT 0-7 min) (Figure 1a). On the other hand, those of high fat-STZ dosed rats showed stronger responses at the hydrophilic region. However, none of the chromatographic profiles with those of subjects 3 and 5 were similar (Figure 1b) and are distinctly different from those of the other high fat-STZ dosed rats, which shared another similarity (Figure 1c). The variation among samples implied that the compounds associated with the animals' metabolism maybe different, both qualitatively and quantitatively. The spectra of each chromatogram were therefore summated to obtain the intensity for each m/z value (Figure 2). Differences between the control and the dosed samples were observed. The high-fat STZ dosed 3 and 5 samples show elevated responses at m/z 173, 179 and 191, while the high-fat STZ dosed 1, 2 and 4 samples have the highest response at m/z 103. These results suggested that there were metabolic variations among animals even they have received the same treatment.

II. *Principle Component Analysis (PCA)*

The PCA performed by using the whole data set of responsed intensities and m/z values clearly shows the variation among dosed samples. The control samples gave very consistent results as are observed in a very confined area on the score plot (Figure 3). The high-

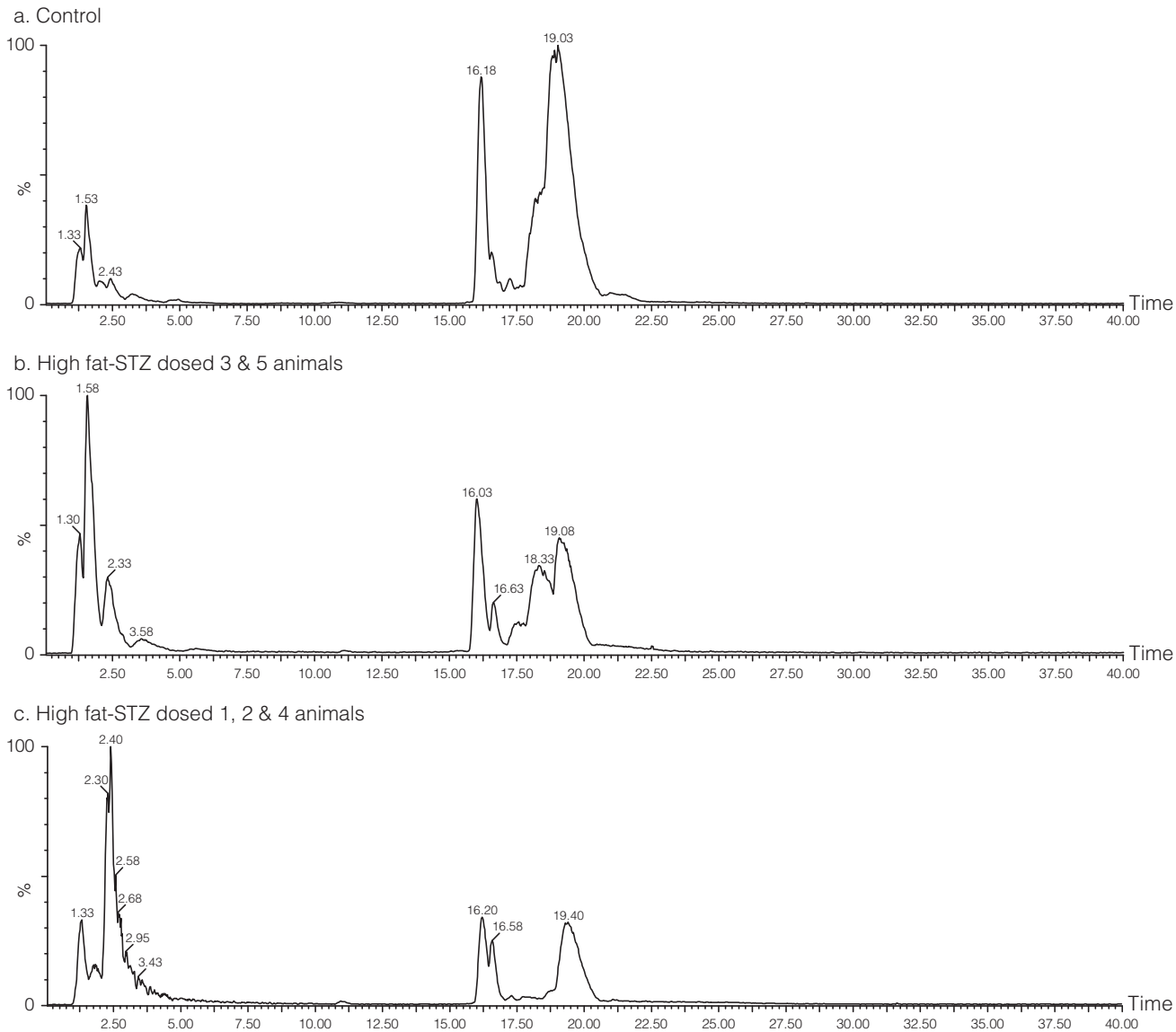

Figure 1. Negative ion MS BPI chromatograms of the urine samples from rats of (a) control, (b) high fat-STZ dosed 3 & 5 animals, and (c) high fat-STZ dosed 1, 2 & 4 animals.

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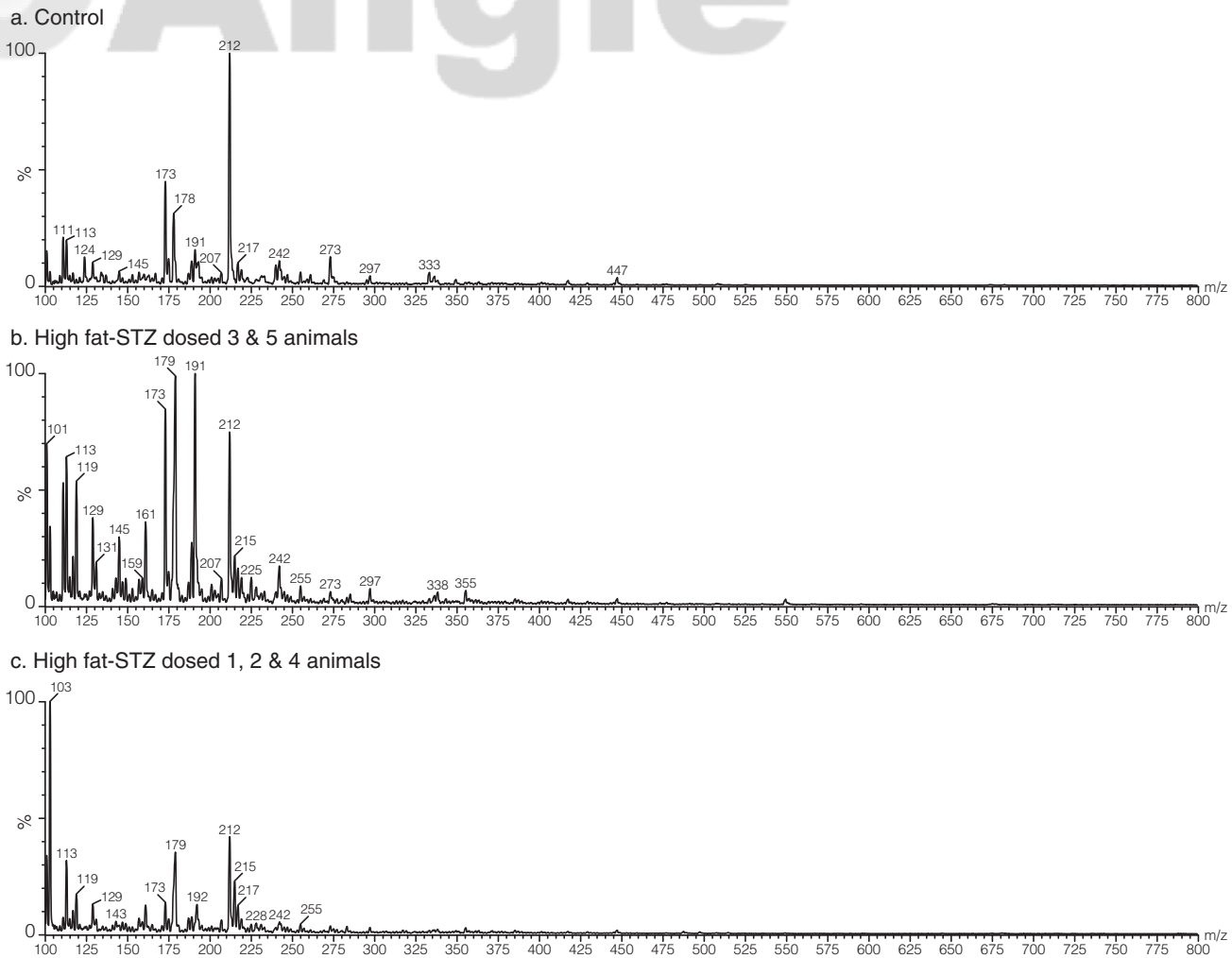

Figure 2. Summated spectra from the negative ion MS BPI chromatograms of the urine samples from rats of (a) control, (b) high fat-STZ dosed 3 & 5 animals, and (c) high fat-STZ dosed 1, 2 & 4 animals.

fat STZ dose samples were clearly separated into two regions with No. 3 and 5 samples closed to each other but were distanced from the No. 1, 2 and 4 samples where the latter three also closed to each other. The close of No. 3 and 5 samples toward the control samples suggested that these two high-fat STZ dosed animals did not have much metabolic difference from the control animals.

The loading plot (Figure 4) reveals the mass ions that are responsible for the mapping of the high-fat STZ dosed and the control samples to different regions on the score plot. The first two principal components explained a total 94.80% (69.04% + 25.76%) of the original variability. Several mass ions, ex: m/z 191, 179, 207, etc., show heavy loading strength along the component 1 axis, while only m/z 103 has a substantial loading strength along both the component 1 and 2 axes. These results suggest that the mass ions mentioned were much more responsible for the metabolic difference between the dosed and the control animals. Resolving the chemical structures for the mass ions provides the possibility to interpret the metabolic changes due to the administration on the dosed animals.

III. *MS/MS*

Before MS/MS analysis on the ion of an interested m/z value to decipher its chemical structure, prediction is always performed by consulting online databases such as KEGG, METLIN or NIST Chemistry WebBook, etc. Most of the metabonomic studies relied on fascinating TOF mass spectrometers to provide mass accuracy of an interested metabolite down to the third digit after the point, which helps in the database searching process by reducing the number of possible hits for an interested m/z value. Even though, biological outcome prediction, if possible, on the dosed animals is still valuable[26]. In this study, the dosed animals were fed high fat diets together with a single dose of STZ/nicotinamide, which was an animal model to the study metabolic syndrome and diabetes. We expected to observe higher concentrations

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of glucose and fatty acid related metabolites in the urine samples of dosed animals than of the control animals. The m/z 179 ion which has heavy loading strength along the component 1 axis (Figure 4) was therefore suspected to be a hexose moiety. Its identity was verified by comparing its product ion spectrum with that of glucose. Product ion spectrum of the m/z 179 ion in a urine sample of high-fat STZ dosed animal was almost identical to that of glucose (Figure 5). The identity of m/z 103 ion was also verified. It was postulated to be a compound with a nominal mass of 104, and 17 results were found in the online KEGG database. Malonic acid was the best guess among all since the product ion spectrum of the m/z 103 ion in high-fat STZ dosed 5 sample gave two major breakdown ions at m/z 59.6 and 103.0, and was also verified with the product ion spectrum of malonic acid (Figure 6). Some other ions with high loading strength along the component 1 axis of the loading plot (Figure 4) were also identified with the same manner. The m/z 173, 145 and 117 ions were postulated after consulting the KEGG database and then verified to be aconitic, 2-oxoglutaric and succinic acids, respectively. The m/z 191 ion was postulated to be citric acid but turned out to be isocitric acid after observing the signified fragments of m/z 73 and 117 in both product ion spectra of sample and isocitric acid, but not that of citric acid (data not shown). Other ions, such as m/z 207, 161, 212, etc., were also interesting but their product ion spectra did not give explicit fragment profiles to support the component identification.

From the MS/MS results, it was possible to depict the biochemical differences between dosed and control animals. Glucose was obvious the biomarker for STZ treatment which may result from the necrosis of

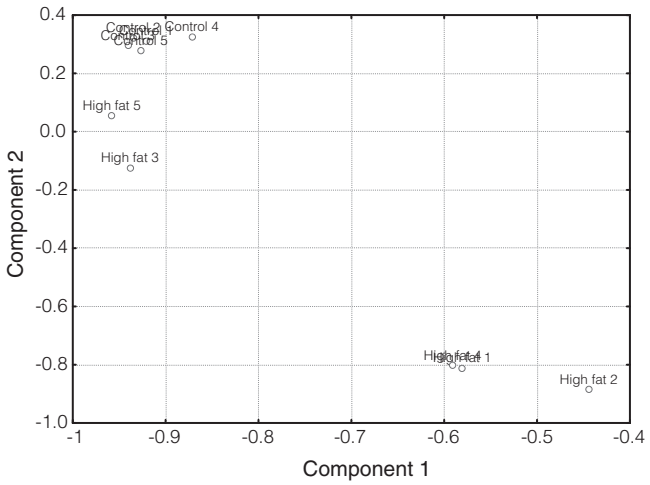

Figure 3. Score plot of the PCA

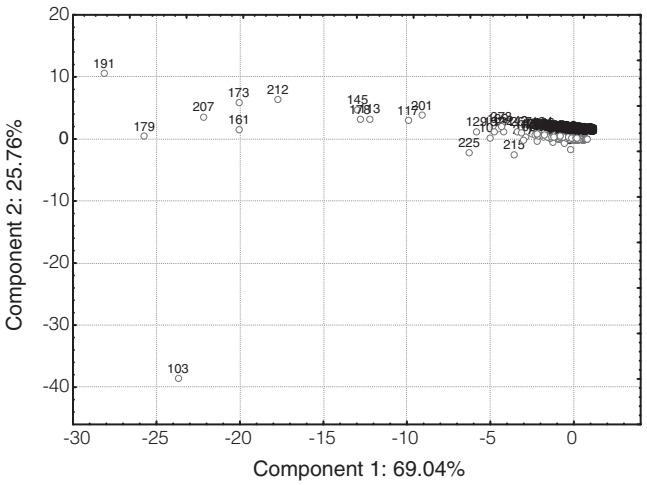

Figure 4. Loading plot of the PCA

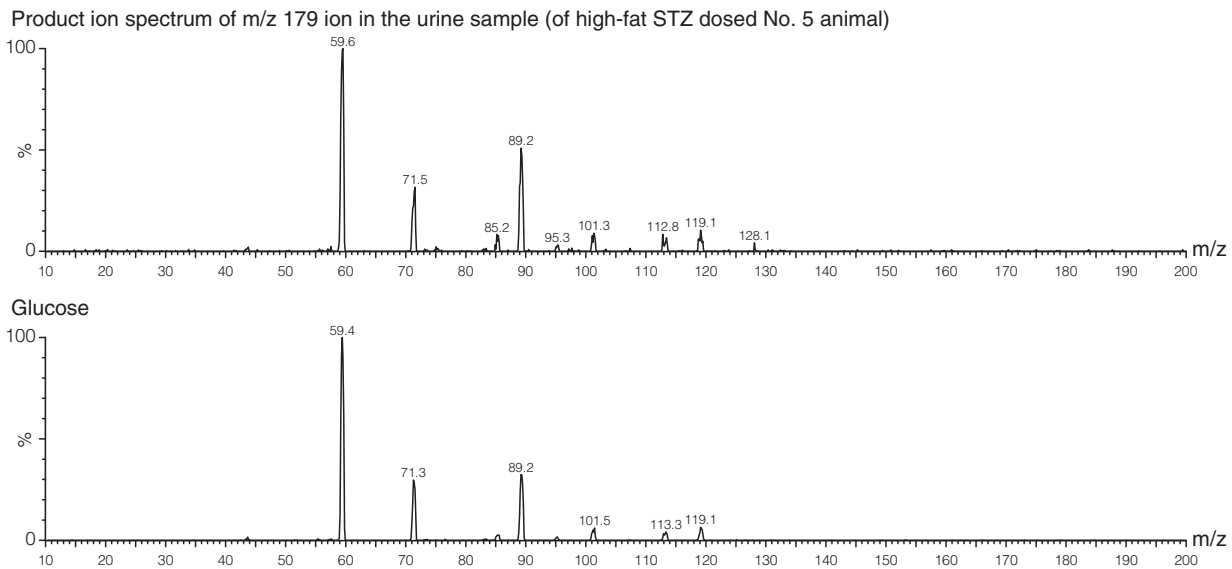

Figure 5. Product ion spectra of the m/z 179 ion in the urine sample of high-fat STZ dosed No. 5 animal and of glucose

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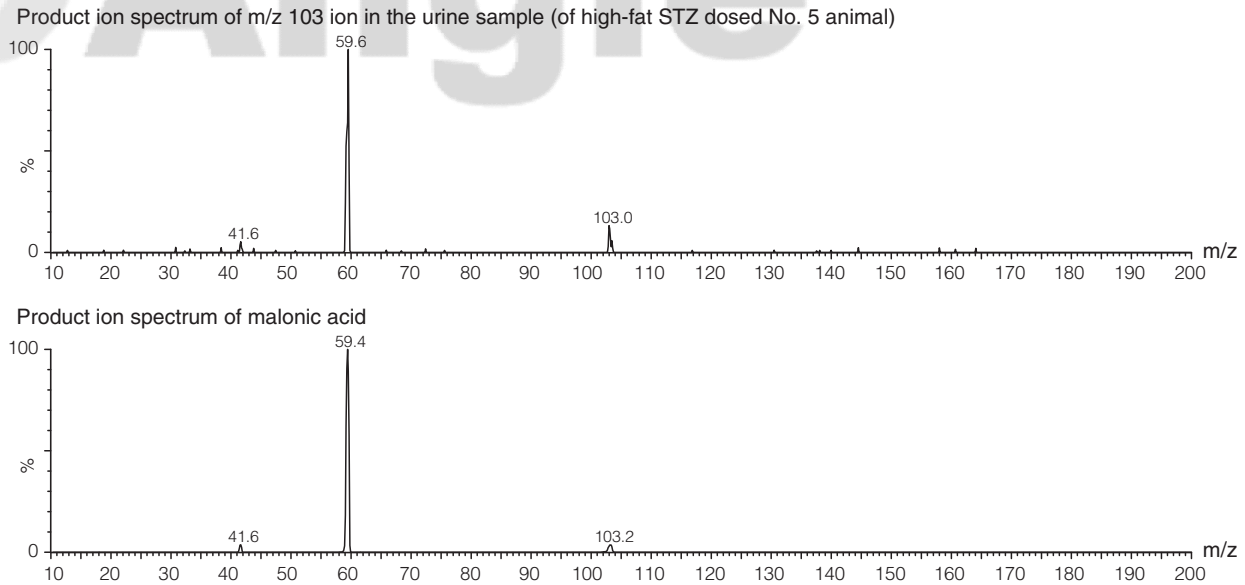

Figure 6. Product ion spectra of the m/z 103 ion in the urine sample of high-fat STZ dosed No. 5 animal and of malonic acid

pancreatic β cells and insulitis in the dosed animals. Aconitic, isocitric, 2-oxoglutaric and succinic acids are all participating compounds in Krebs cycle (KEGG pathway 00020), which signified the difference in energy production between high-fat STZ dosed and control animals. The high loading strength of malonic acid also suggested that the metabolic flux toward fatty acid biosynthesis was an important factor to differentiate the dosed from control animals. Another interesting observation is the negative loading of malonic acid along factor 2 axis as opposite to those of the Krebs cycle components (Figure 4). This result suggests a counteract effect of malonic acid toward Krebs cycle, which is already a well-known biochemical phenomenon[27]. It is therefore concluded that animals fed with high fat diets together with STZ infusion have an effect on gluconeogenesis and energy disposition through Krebs cycle and fatty acid biosynthesis.

CONCLUSIONS

Among all the spectroscopic techniques in metabonomics study, LC-MS is gaining popularity because of its widespread availability and robustness. TOF MS is the detector of choice in metabolic profiling because its mass accuracy facilitates the follow-up provisional annotation of the responsible components by database consultation. However, component identification will not be completed until using a validated, compound specific method[28], such as MS/MS analysis for structural elucidation by triple quadruple detector. We demonstrate here a successful metabonomics study by using a triple quadruple detector for both metabolic profiling and component identification. There may be more laborious annotation works than those by using a TOF detector, but a single detector throughout the whole metabonomics study is welcome. As the food industry continue to see the opportunity in nutrigenomics, metabonomics study with LC-MS does provide some sort of information to interpret the biological effects of food components. However, we also understand that metabonomics should not work alone, but should ideally form part of system biology-based strategy with the integration of other omics technologies such as genomics and proteomics[28].

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