PILOT STUDY: IDENTIFICATION OF ANTHOCYANIN METABOLITES IN THE MICE FED PURPLE-FLESHED SWEETPOTATO

by

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Abstract

Anthocyanins may prevent chronic diseases such as cancer and cardiovascular disease, however, the anthocyanin metabolites are not well elucidated. We previously selected a purple-fleshed sweet potato clone P40 that contained anthocyanins at up to 7.5 g/kg dry matter, most of which are cyanidin and peonidin derivatives. The main objective of this study is to identify anthocyanin metabolites in the mice fed 20-30% of purple sweet potato P40 (287 mg and 430 mg peonidin-3-glucoside equivalent /kg body weight) diet for 6 weeks. Plasma, liver, and feces were analyzed for anthocyanin metabolites using HPLC/MS and MALDI-TOF-MS. Fifteen hours after consumption of P40 diet, we identified 4 anthocyanin metabolites cyanidin 3,5- diglucoside; cyanidin 3-sophoroside-5-glucoside; cyanidin3-p-hydroxybenzoylsophroside-5-glucoside; and peonidin 3-p-hydroxybenzoylsophroside-5-glucoside in fecal samples. No anthocyanin metabolites were detected in plasma or liver extracts by HPLC/MS or MALDI-TOF-MS. The results indicate that anthocyanin metabolites in fecal samples might provide health benefits for colonic mucosal cells. However, the lack metabolites in both plasma and liver samples suggest a continuous intake of the anthocyanins may be required for systemic benefits due to their quick degradation and low bioavailability.

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Chapter 1 - Literature Review

Source and Chemistry of Anthocyanins

Anthocyanins are phytochemicals in vaccinium species such as blueberries, chokeberries, and cherries that are responsible for pigmentation; making colors such as red, purple, and blue (1). Anthocyanins are glycosides of anthocyanidins, but aglycone anthocyanidins are rare in nature (2). Moreover, the color of anthocyanins is influenced by pH. In acidic solutions, anthocyanins form red flavylium cations. When pH increases, anthocyanins lose a proton to form the blue quinonoidal structure and become a colorless, hydrated kemiketal form (1).

The chemical structure of anthocyanins are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium (3). Six of the most common anthocyanidins are: cyanidin, delphinidin, peonidin, pelarogonidin, malvidin, and petunidin (Fig.1-1). Furthermore, anthocyanins often contain β-3-0-glycoside or β-3,5-0-diglycoside bonds with sugars such as glucose, galactose, arabinose, or xylose (1, 2). In addition, there are also acylated forms in nature, resulting in more than 500 anthocyanin derivatives (4). While most flavonoids are neutral, anthocyanins contain a positive charge in an acidic solution (5). This positive charge enables them to absorb light and have color, while the glycoside contributes to their water-solubility.

Anthocyanins, Health, and Disease

Anthocyanins are the largest group of flavonoids, and are widespread in plant foods with daily consumption in the United States estimated at 180-255 mg/day. This is 9-fold higher than any of the other flavonoids (6). Anthocyanins are believed to be antioxidants, anti-proliferative, anti-mutagenic, and anti-inflammatory (1).

Antioxidant:

The hydroxyl groups in their phenolic structure, allows anthocyanins to act as antioxidants scavenging some reactive oxygen species (ROS) (7). Anthocyanins have been demonstrated to reduce superoxide ($O2^-$), peroxide (ROO^-), hydroxylradical (OH^-), and hydrogen peroxide (H_2O_2, 8). By acting as a free radical scavenger, anthocyanins can inhibit lipid peroxidation of cell membranes in the microsomal system (9). In the liposomal system, anthocyanins reduce the

formation of malondialdehyde from UVB radiation (10). In low-density lipoproteins (LDL), anthocyanins can act as antioxidants by donating hydrogen and chelating metal to decrease their oxidization (11). Oxidized LDL contributes to the development of atherosclerosis; thus, anthocyanins intake decrease the risk of cardiovascular disease. Moreover, anthocyanins could stimulate phase II detoxification enzymes by increasing antioxidant and detoxifying enzymes like glutathione reductase and glutathione peroxidase (12).

Anti-inflammatory:

A common inflammatory signaling pathway is the nuclear factor-kappa B (NF-kB) pathway. NF-kB is a stress-sensitive transcription factor like tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) (1). Anthocyanin-rich extracts may prevent the degradation of inhibitor of nuclear factor-kappa B α (IkB-α) that suppresses nuclear NF-kB and IL-6 expression (13). In addition, anthocyanin extracts decrease the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are involved in inflammation pathways and activated by NF-kB (14). Consequently, anthocyanins may diminish inflammation by inhibiting the NF-kB pathway.

Anti-carcinogenic:

Anthocyanins attenuate cellular proliferation and induce apoptosis in breast cancer cells and colon cancer cells, suggesting that anthocyanins may have anti-carcinogenic properties (15-18). Anthocyanins induce dephosphorylation of protein kinases in the extracellular signal regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways, which are main pathways within the mitogen-activated protein kinase (MAPK) pathway (19). Anthocyanins may also regulate various regulator proteins (e.g., p53, p21, p27, cyclin A, cyclin D1 etc.) to inhibit the cell cycle. Another very important anti-carcinogenic function is induction of apoptosis in cancer cells (18). Anthocyanins may activate p38 MAPK and JNK, which are mitogen-active protein kinases to regulate apoptosis. In addition, anthocyanins promote apoptosis by decreasing anti-apoptosis proteins like Bim and increasing the expression of pro-apoptosis proteins like Bcl-2 and Bcl-xl (20).

Other Disease Prevention:

Some studies have shown that consumption of anthocyanin-rich products significantly attenuate postprandial insulin response and increase insulin sensitivity (21). Moreover, anthocyanin-rich extracts appear to benefit vision by increasing retinal pigment, thereby decreasing the risk of developing macular degeneration (21).

Bioavailability of Anthocyanins

Bioavailability

Bioavailability is defined as the proportion of consumed dietary compounds absorbed into the body (22). The bioavailability of anthocyanins is less than 1% (23-27). Anthocyanins containing different glycosides have different bioavailabilities, for instance, non-acylated anthocyanins are absorbed better than acylated anthocyanins (10,18). Furthermore, the food matrix may affect anthocyanin absorption as well, for example, alcohol does not affect anthocyanin absorption, while cream and fiber delay the time of absorption into the blood (27-29). All studies indicate that anthocyanins are readily degraded and excreted in vivo.

Absorption, Transport, Metabolites

Anthocyanins naturally exist in glycosylated and acylated forms (30). After ingestion salivary enzymes can modify anthocyanins, one study suggests that β-glucosidase in the surface oral epithelia and terminal salivary ducts deglycosylate anthocyanins and generates aglycone forms (31).

After anthocyanins arrive in the stomach, the low pH value makes anthocyanins stable and maintains the flavylium cation (32). The t-max in plasma is 15-30 minutes, suggesting that anthocyanins may be absorbed in the stomach. Previous research also indicates that anthocyanins are absorbed in glycoside forms present in food (26, 33, 34). While in the stomach, anthocyanins could permeate through the gastric mucosa to circulation blood via a bilitranslocase-mediated mechanism (35). Bilitranslocase is a plasma membrane protein involved in organic anion uptake expressed in the gastric mucosa epithelium, liver plasma membrane, and kidney tubular cells. Bilitranslocase acts as a carrier to transport anthocyanins back and forth across the membrane of cells (36).

After absorption, anthocyanins enter the circulatory system and are transported to the liver and kidneys, where they undergo major biotransformations such as methylation and glucuronidation facilitated by multiple phase-II detoxification enzymes (37, 38). Following incubation in liver microsomes, anthocyanins could form glucuronide conjugates by UDP glucose dehydrogenase (39, 40).

Anthocyanins that are not absorbed are transported to lower gastrointestinal tract, where the higher pH makes anthocyanins unstable and converts them to a combination of hemiketal, chalcon, and quinonoidal forms (1). Because of their poor bioavailability, the concentration of anthocyanins in the lumen of lower gastrointestinal tract is much higher than in plasma and urine (25, 41-43). Finally, unabsorbed anthocyanins pass through the colon and are excreted in feces. Previous studies have indicated that anthocyanins may prevent colon cancer (44). The gut microflora in the colon could deglycosylate by cleaving glycosidic linkages and breaking down the anthocyanidin heterocycle (45). In addition, colonic bacteria may demethylate anthocyanins and in turn convert them to aglycone forms. The neutral pH of the colon makes anthocyanidins easier to be degraded into phenolic acids and aldehydes by C-ring cleavage (1, 45, 46).

Excretion

Anthocyanins are prepared for excretion by the liver and kidney (37). In the kidney, anthocyanins may be pumped out from kidney tubular cells to urine by the primary efflux transporter (47). In addition, anthocyanin metabolites may be secreted from the liver through bile and excreted in feces. The anthocyanins which are not absorbed are excreted in feces by passing through the gastrointestinal tract (44).

Detection Methods

HPLC

Conventionally, anthocyanins are characterized and quantified using high performance liquid chromatography (HPLC) with an ultraviolet/visible (UV-vis) detector. Anthocyanin fractions elute based on their polarities and can be further quantified (48). The ideal method to interpret HPLC results is using standard compounds for their retention time and quantification. Some limitations of this method are restricted sensitivity, cost, time, and co-elution of contaminated compounds (49).

HPLC-MS-MS

HPLC connected with tandem mass spectrometry is a method to overcome some of the shortcomings of HPLC. HPLC-MS can measure the mass-to-charge ratio of charged particles to determine the molecular weight of particles and elucidate the chemicals for identification. After compounds have been separated by HPLC, they become charged particles or molecular fragments by electrospray ionization, chemical ionization, or laser desorption/ionization. The ions are then separated according to their mass-to-charge ratio in the electromagnetic field of the mass analyzer (e.g. quadrupole, time-of-flight, and quadrupole time-of-flight) (50). After ions pass through the first dimension MS, the parent mass fragment is formed and selectively monitored by passing through the second dimension of MS to provide the unique daughter fragment. This technique can be utilized for both qualitative and quantitative analyses. However, a compatible mobile phase with MS interface and ion suppression by a different ion sources are needed for successful HPLC-MS (51).

MALDI

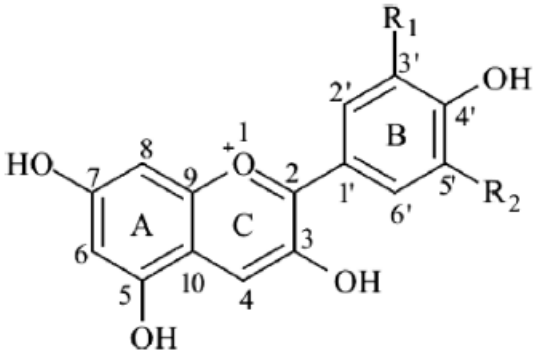
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) can also be used to analyze anthocyanins. Originally, MALDI was developed for large molecule analysis. By mixing the analyte with suitable matrix, the analyte and matrix are ablated and ionized during laser irradiation (52). MALDI coupled with TOF is a highly sensitive and efficient technique, which is easy to perform quickly, with good tolerance of contaminants, and unlimited mass range detection (53). One limitation is that MALDI-TOF-MS cannot distinguish between isomers with the same mass.

Anthocyanin-rich Purple-fleshed Sweet Potato

Purple-fleshed sweet potatoes are a special cultivar that contains high and complex anthocyanins like non-acylated or acylated cyanidin and peonidin (54). Previous studies by us and others indicate that purple sweet potatoes may have health benefits due to their antioxidant, anti-proliferation, apoptosis induction, anti-fibrosis action (14, 55-58). Most of these benefits were associated with purple sweet potato's high anthocyanin contents. We previously selected a purple sweet potato clone P40 that significantly inhibited cell proliferation in human colonic SW480 cancer cells (58). Moreover, we found that 10-30% P40 diets inhibited azoxymethane

induced aberrant crypt foci formation, a pre-neoplastic lesion of colorectal cancer, in CF-1 mice (58). In this study we focused on identifying the anthocyanins metabolites from these mice. A better understanding of anthocyanin metabolism and bioavailability may help us understand how the purple-fleshed anthocyanin-enriched sweet potato prevented colon cancer.

Figure 1-1 Chemical structures of anthocyanins (from Ref 19)



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH_3	H
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH

Chapter 2 - Pilot Study: Metabolites of Anthocyanins in the Mice fed Purple Sweet Potato P40

Introduction

Anthocyanins are unique flavonoids whose structure contains a positive charge that allows them to serve as pigments in fruits and vegetables (5). Additionally, like other flavonoids, anthocyanins may provide health benefits due to their antioxidant, anti-inflammatory, anti-proliferative, anti-carcinogenesis and possibly other properties (1, 7, 14, 16). Anthocyanin enriched foods thus may prevent chronic diseases.

In the US, daily consumption of anthocyanins is about 150-220 mg, which is significantly higher than other types of flavonoids (26). In addition, maximal blood anthocyanin concentrations occur 15-30 min after intake (59). Previous research suggests that anthocyanins are mainly absorbed into the circulatory system through the gastric mucosa and are further modified by the liver and kidneys. While anthocyanin consumption is high, anthocyanin bioavailability is low, less than 1% of intake (24-27). Despite their low bioavailability, in vivo studies have found health benefits from anthocyanin intake (59-63). One explanation for how anthocyanins can provide such health benefits despite their low bioavailability is that bioactive metabolites might be produced that are efficacious at low concentrations. Another possible explanation for the low bioavailability observed with anthocyanin intake is that we do not have the proper equipment or knowledge, to collect and accurately analyze all the possible anthocyanin metabolites in vivo.

Recently, several studies have reported the bioavailability of anthocyanin and identification of anthocyanin metabolites (59-64). Most studies have utilized one or a few purified anthocyanin derivatives instead of using an anthocyanin-rich food. In addition, most studies identified anthocyanin metabolites after a single dose, while our study provided a diet with a consistent supply of anthocyanins.

The purple sweet potato P40 is a purple-fleshed sweet potato that contains 7.5 g/kg anthocyanins in dry matter. Our previous study found that the P40 purple sweet potato diet prevented cancer in azoxymethane-induced murine colorectal cancer model. In this study, we want to identify the potential anthocyanin metabolites in the mice fed P40 containing diets for six weeks.

Materials and Methods

Preparation of Purple Sweet Potato Diet

Purple sweet potato root tubers were randomly chosen, washed with running tap water, and cut into 0.5cm cubes. Freeze-dried cuts were then prepared using a general purpose freeze dryer (VirTis, Gardiner, NY, USA), ground with a food processor (Retsch, Haan, Germany) and stored in a -80℃ freezer until incorporated into diets. The control AIN-93M powder diet was purchased from Harlan Laboratories (Madison, WI, USA). The experimental powdered diet contained 20-30% purple sweet potato powder, and the nutrition content of protein, lipid, and carbohydrate was mixed with single ingredients of AIN-93M diet (Harlan Laboratories, Madison, WI, USA) to be similar to the control diet (Table 2-1, 2-2). All purple sweet potato diets were stored in zip bags and all diets were kept in 4℃ in a refrigerator in the dark.

Animal Studies

Female CF-1 mice at 6-7 weeks of age were purchased from Charles River (Wilmington, MA, USA) and kept in plastic shoebox cages (23± 0.5℃, 20-25% humidity) on a 12:12 hour light-dark cycle. Diet and water were provided ad libitum during the whole experiment. Food intakes and body weights were recorded daily and weekly, respectively. During the first week, all mice were fed the AIN-93M diet to allow the mice to adjust to the new environment. In the second week, mice were randomized into a control group and P40-fed group. In the P40-fed group, mice were intraperitonially (i.p.) injected with azoxymethane in a saline vehicle at 10 mg/kg body weight (65). At the same time, the control group received saline injection at 10mg/kg body weight. After six weeks on their experimental diets, the mice were fasted for 15 hours and sacrificed by decapitation. Blood was drawn from the carotid into heparinized tubes and centrifuged at 3,000g for 5 min in 4℃ to collect plasma. Liver was collected and washed by saline to remove residue blood, and fecal samples were collected from cages. All samples were stored in a -80℃ freezer until analysis.

Analytical Sample Preparation

The extraction of anthocyanin metabolites from plasma was performed according to the procedure of Suda et al. (20) with slight modification. Plasma (1mL) was acidified with 200 µl of 1% trifluoroacetic acid (TFA) solution, centrifuged at 4,000g for 15 min at 4℃ and the

supernatant removed. An octadecylsilane (ODS) solid phase extraction cartridge (Sep-Pak C18, waters, Milford, MA) was washed with 10 mL of methanol and 10 mL of 1% TFA aqueous solution to activate the cartridge, before adding the supernatant. The unwanted water-soluble compounds, polar lipids, and neutral lipid were eluted from the sample using 10 mL of 7% formic acid aqueous solution, 15 mL of dicholoromethane (DCM), and 15 mL of benzene respectively. The cartridge was dried with nitrogen gas and anthocyanins were eluted from the cartridge by adding 5 mL of methanol containing 1% TFA. TFA is used to adjust the pH value and stabilize anthocyanins. The eluate was evaporated to dryness with nitrogen gas at 25℃. The dried extract was dissolved in 200 μl of 15 % acetonitrile and 1% formic acid in water. The sample was then passed through a syringe filter (0.45 μm, 25 mm dia., Nylon Acrodisc, Gelman Sciences) before HPLC analysis.

A 500 mg liver sample was mixed with 2.5 mL of 1% TFA aqueous solution and homogenized on ice using a general laboratory homogenizer (Omni, Kennesaw, GA, USA) for 5 min. The homogenized liver sample was centrifuged at 12,500 rpm for 30 min at 4℃ to collect clear supernatant. For extracting anthocyanins, the supernatant was applied through the Sep-Pak C18 cartridge. The extraction steps were the same extraction methods as described for plasma. The recovery rate of anthocyanin internal standard is about 52% in liver samples.

Fecal samples were defrosted and ground to a powder using a mortar and pestle. A 0.1g sample of fecal powder was mixed with 20 mL of extraction solution containing methanol:water (V:V= 60:40) and 1% TFA for homogenization. The mixture was homogenized using a general laboratory homogenizer (Omni, Kennesaw, GA, USA.) for 5 min and centrifuged at 3,500 g for 10 min at 4℃. The supernatant was collected and the pellet re-extracted twice with 10 mL of extraction solution through the same process as described before. The combined supernatants were evaporated to dryness under 25℃ by nitrogen gas, and then redissolved in 1 mL of 1% TFA aqueous solution. A Sep-Pak C18 cartridge was washed with 10 mL of methanol and 10 mL of 1% TFA aqueous solution before the supernatant was added. Unwanted water-soluble compounds, polar lipids, and neutral lipids were removed using the same procedure as mentioned above in the plasma. After washing, 5mL of methanol in 1% TFA was used to elute anthocyanin metabolites from the cartridge which were collected for HPLC analysis.

HPLC-ESI-MS

Anthocyanins were determined with HPLC-ESI-MS performed with an HPLC coupled with an UV detector (Agilent 1100 system, Massy, France), using a Waters Symmetry C18 column (250 mm length, 4.6 mm inner diameter, 5 µm particle diameter Waters, Milford, MA) connected with an electrospray ionization (ESI) source (Bruker Daltonics, Billerica, MA), and Bruker Esquire 3000 ion trap MS (Bruker Daltonics, Billerica, MA).Ten µl of sample was injected, column temperature was 35 ℃ and the flow rate was 0.3 mL/min. The mobile phase consisted of a mixture of solvents A and B; solvent A was 95% water/5% formic acid, solvent B was 95% acetonitrile/5% formic acid. A gradient method was used for separation, which consisted of 95% solvent A at 0 min, 90% solvent A at 20 min, 78% solvent A at 50 min, and returned to 95% solvent A at 55 min during the 55 minute run time. A UV-vis detector was set at 520 nm. The electrospray ionization was carried out in positive ion mode.

The MS conditions were as follows: nebulizing pressure was 10 psi, capillary voltage was 3,500 V, skimmer voltage was 40 V, dry temperature was 350℃, dry gas flow rate was 5 L/min, and the scanning range was 200-1200 m/z. Data was analyzed using Bruker Hystar Post Processing (Bruker, Coventry, United Kingdom).

MALDI – TOF

The MALDI-TOF was performed using Autoflex III Bruker Analytical system Inc. (Billerica, MA). The matrix was created by adding 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in acetonitrile (V:V= 1:1) containing 0.1% TFA. The matrix DHB was used to crystallize samples. One µl of matrix solution and 1 µl of sample were spotted on the target to co-crystallize and air dried for analysis. The MALDI conditions were as follows: the sample rate was 2.0 G S/S and the samples were ionized by nitrogen laser pulse (λ=337nm, 66.7 Hz). One thousand consecutive laser shots were conducted in positive ion mode in the range of 300-1200m/z were analyzed. The laser power was adjusted between 30-50% of the maximal intensity.

Results

Potential Anthocyanin Metabolites in Plasma sample

HPLC chromatograms from the plasma of control and a 30% P40-fedmouseshowed a similar pattern at 520 nm for HPLC and MS peaks (Fig. 2-1 a-d). Therefore, five 30% P40-fedmouse samples were pooled to increase our likelihood of detecting metabolites, and we increased the run time from 35 min to 60 min to get optimal component separation. HPLC results still showed a similar pattern between the control and 30% P40-fedsample with only two peaks at approximately 14.5 min and 17.4 min (Fig. 2-2 a, b). In addition, there are similar MS peaks in P40-fed and control diet-fed samples, and no native anthocyanin metabolite peaks matched previous published m/z results (Fig. 2-2 c, d). MALDI-TOF-MS was then used to try to detect anthocyanin metabolites in the plasma samples, because it is a more sensitive technique. However, patterns between 30% P40-fed and control diet-fed samples were also similar in MALDI-TOF-MS results (Fig. 2-3). Based upon previously published data (66), the most likely anthocyanin fragments from the purple sweet potato would be at 449 m/z (cyaniding-3-glucoside) and 463 m/z (peonidin-3-glucoside). However, no 449 m/z (cyaniding-3-glucoside) and 463 m/z (peonidin-3-glucoside) detected by MALDI-TOF-MS (Fig. 2-3). In order to determine whether the 30% P40-fed samples contained anthocyanin metabolites, these 449 m/z and 463 m/z fragments were further confirmed by MS/MS analysis. However, both 449 m/z and 463 m/z fragments did not ionize into 287 m/z (cyanidin) and 301 m/z (peonidin), respectively, after MALDI-TOF-MS-MS (Fig. 2-4). Thus, anthocyanin fragments in the 30% P40-fed pooled plasma sample were not detected.

Potential Anthocyanin Metabolites in Liver sample

There were not different peaks in control, 20%, and 30% P40-fed liver samples analyzed by HPLC at 525 nm (Fig. 2-5). The 4 peaks detected in P40-fed samples by MS were: 611 m/z, 860 m/z, 924 m/z, 1056 m/z (Fig. 2-6). However, those peaks did not match the molecular weight of any reported anthocyanin metabolites, although the molecular weights were in the range of anthocyanin derivatives. The intensity of the peaks was too low to further analyze by HPLC/MS/MS for identification. Thus, we used MALDI-TOF-MS to identify anthocyanin metabolites from liver samples. No different peaks were found between the control and P40-fed

samples (Fig. 2-7). No aglycone anthocyanin fragments like 287 m/z and 301 m/z were further identified at the molecular weights of 449 m/z and 463 m/z (Fig. 2-8). Therefore, no anthocyanin metabolites in either P40-fed liver sample were detected.

Potential Anthocyanin Metabolites in Feces sample

HPLC analysis of control diet-fed feces sample showed 2 peaks between 15-17 min (Fig. 2-9 a), but these peaks did not match native anthocyanin metabolite peaks m/z's published previously (Fig. 2-9 b,c, 66). HPLC/MS of P40-fed feces sample resulted in 4 peaks at 28.4, 31.8, 33.2, and 35.6 min, respectively (Fig. 2-10). Moreover, the m/z's of the peaks: 773 m/z, 611 m/z, 893 m/z, and 907 m/z, matched those in a previous publication (66). After identification using HPLC/MS/MS, the peaks were exactly matched with the following respective anthocyanin metabolites: cyanidin 3-sophoroside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-p-hydroxybenzoylsophroside-5-glucoside, and peonidin 3-p-hydroxybenzoylsophoroside-5-glucoside (Fig. 2-11). MALDI-TOF-MS was further used to identify anthocyanin metabolites in fecal samples. As a result of the different ionizations in HPLC/MS and MALDI-TOF-MS, the result from MALDI-TOF-MS showed a peak at 611 m/z which is cyanidin 3,5-diglucoside. Powerful ionization was further used to break anthocyanin metabolites into fragments by choosing 449 m/z and 463 m/z for MALDI-TOF-MS/MS analysis where 287 m/z (cyanidin) and 301 m/z (peonidin) fragments were identified in the P40-fed feces sample (Fig. 2-12). The anthocyanin content in 30% P40 feces sample was about 0.17g of peonidin-3-glucoside equivalent/kg.

Discussion

Four anthocyanin metabolites were detected in 30% P40-fed feces, but not in plasma or liver samples. One possible explanation for not finding metabolites in vivo is that the mice were fasted 15 hours before termination. Mice were fasted before sacrifice to prevent the possible interference of glucose, insulin, and chylomicrons with anthocyanin extraction in plasma samples (67). However, during this fast anthocyanins may have been excreted. Previous research has shown that plasma concentrations reach a maximum in 15-30 minutes after consumption, are barely detectable after two hours, and no longer detectable three hours after one time consumption (59, 63, 64, 68). Even though P40 diets were fed for six weeks, no detectable

anthocyanins were detected in plasma; this is likely a result of how quickly they excreted from the body.

Another potential explanation is that the anthocyanins were not absorbed. In purple sweet potatoes, more than 95% of anthocyanins are acylated (67) which are less bioavailable than non-acylated anthocyanins (4, 6, 66, 68, 69). This fact may explain why we could not detect anthocyanins in plasma or liver.

The liver is the metabolic organ for most phytochemicals; this includes anthocyanins, which are modified by the liver and excreted (25, 34, 37, 43). Previous studies have reported barely detectable anthocyanins in liver two hours after consumption (33, 34, 41, 68). In P40-fed liver samples, there were some unidentified peaks, but their intensity was too low for further identification. We tried to identify the peaks using MALDI-TOF-MS/MS, which is more sensitive than HPLC/MS. However, there still were no matched anthocyanin fragments after MS/MS.

The P40-fed fecal samples showed four anthocyanin metabolites. Low amounts of anthocyanins found in fecal samples may indicate that they were degraded or absorbed in the large intestine. It has been suggested that fecal samples should be collected as quickly as possible and stored at -80℃ to minimize enzymatic degradation by the microbiota (70). We collected fecal samples quickly and stored at -80℃.However, anthocyains in fecal samples might have degraded before collecting, defrosting samples, and grounding them to a powder. Typically, finding a water-soluble compound such as an anthocyanin metabolite in the feces would indicate that is was not bioavailable and simply passed through the gastrointestinal tract without being absorbed. However, previous studies have shown that anthocyanin metabolites were present in bile, indicating that they must have been absorbed (37, 43, 68, 71). Bile is secreted into the gastrointestinal tract later, so part of absorbed anthocyanins may still be excreted via feces. Therefore, it is possible that some anthocyanin metabolites found in the feces may have originated from bile acid excretion.

Anthocyanins have previously been shown to have low bioavailability (25-27). So far, most research has detected anthocyanins or anthocyanin metabolites in flavylium cation forms that present a red color at a low pH. However, anthocyanins pH values change as they form chalcons, and quinonoidals, which are unable to revert back to flavylium cation forms during sample

preparation. Moreover, these limitations may also restrict further identification of anthocyanin metabolites. Thus, this method might underestimate real anthocyanin bioavailability.

Our identified anthocyanin metabolites in feces may come into direct contact with colonic epithelia and thus potentially might have cancer preventive activity. Some studies indicated that the anthocyanins might provide health benefits through direct contact with tissues without being absorbed, which might be especially applicable to colon cancer (18, 66).

In conclusion, the anthocyanin metabolites found in the fecal samples might provide health benefits for colonic mucosal cells. On the other hand, the lack metabolites in both plasma and liver samples suggest a continuous intake of the anthocyanins may be required for systemic benefits due to their quick degradation and low bioavailability. Future anthocyanin bioavailability and metabolite identification studies should have no fasting period. In addition, urine and bile samples should be collected over a time course to evaluate absorption, distribution, and excretion.

Table 2-1 Nutrient composition of purple sweet potato P40

Diet	P40
Dry matter **	29.01 %
Protein***	1.5 %
Fat	0.17 %
Starch	14.36 %
Fiber	0.96 %
Phosphorus	0.04 %
Calcium	0.08 %
Potassium	0.44 %
Magnesium	0.03 %

*Data are reported on a 100% fresh matter basis.

**Dry matter = 100 - %moisture

***Protein is calculated using a 6.25 conversion factor

Table 2-2 AIN-93M based diet formulations

g/kg total diet	Control	20% P40	30% P40
Purple sweet potato powder	0	200.0	300.0
Casein	140.0	129.7	124.6
L-cystine	1.8	1.8	1.8
Corn starch	465.7	367.0	317.7
Maltodextrin	155.0	71.7	30.0
Sucrose	100.0	100.0	100.0
Soybean Oil	40.0	38.9	38.3
Cellulose	50.0	43.3	40.1
Mineral Mix ¥	35.0	35.0	35.0
Vitamin Mix ¶	10.0	10.0	10.0

* Each gram of P40 powder contains 51.7 mg of protein, 5.7 mg of fat, 493.4 mg of starch, and 32.9 mg of fiber.

¥ AIN-93M mineral mix formulation

¶ AIN-93M mineral mix formulation

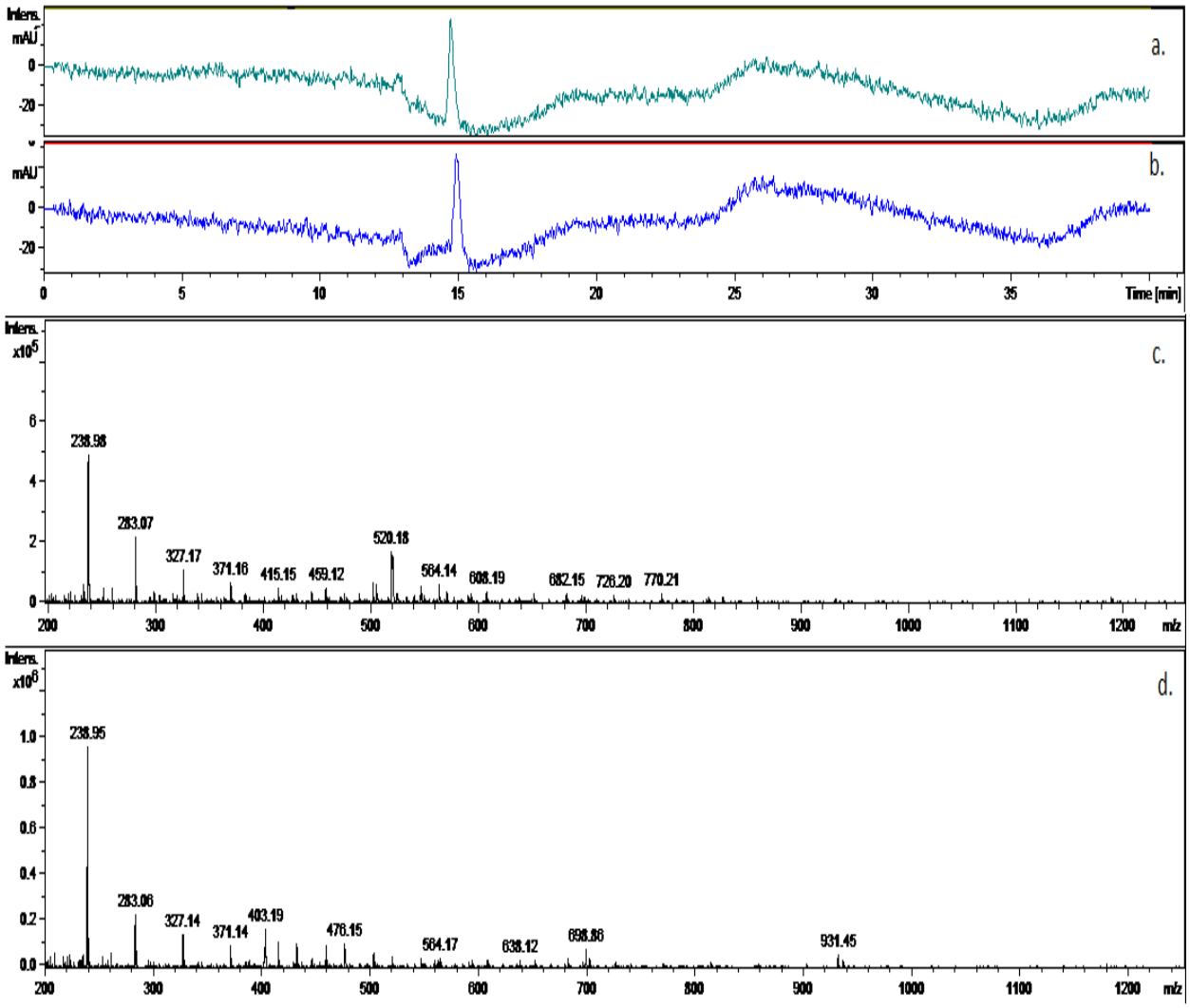


Figure 2-1 UV-HPLC chromatograms at 520 nm of mice plasma (a) control group and (b) single 30% P40 sample have similar patterns. Positive ion MS data in 10-30 mins of mice plasma (c) control group, (d) 30% P40 group.

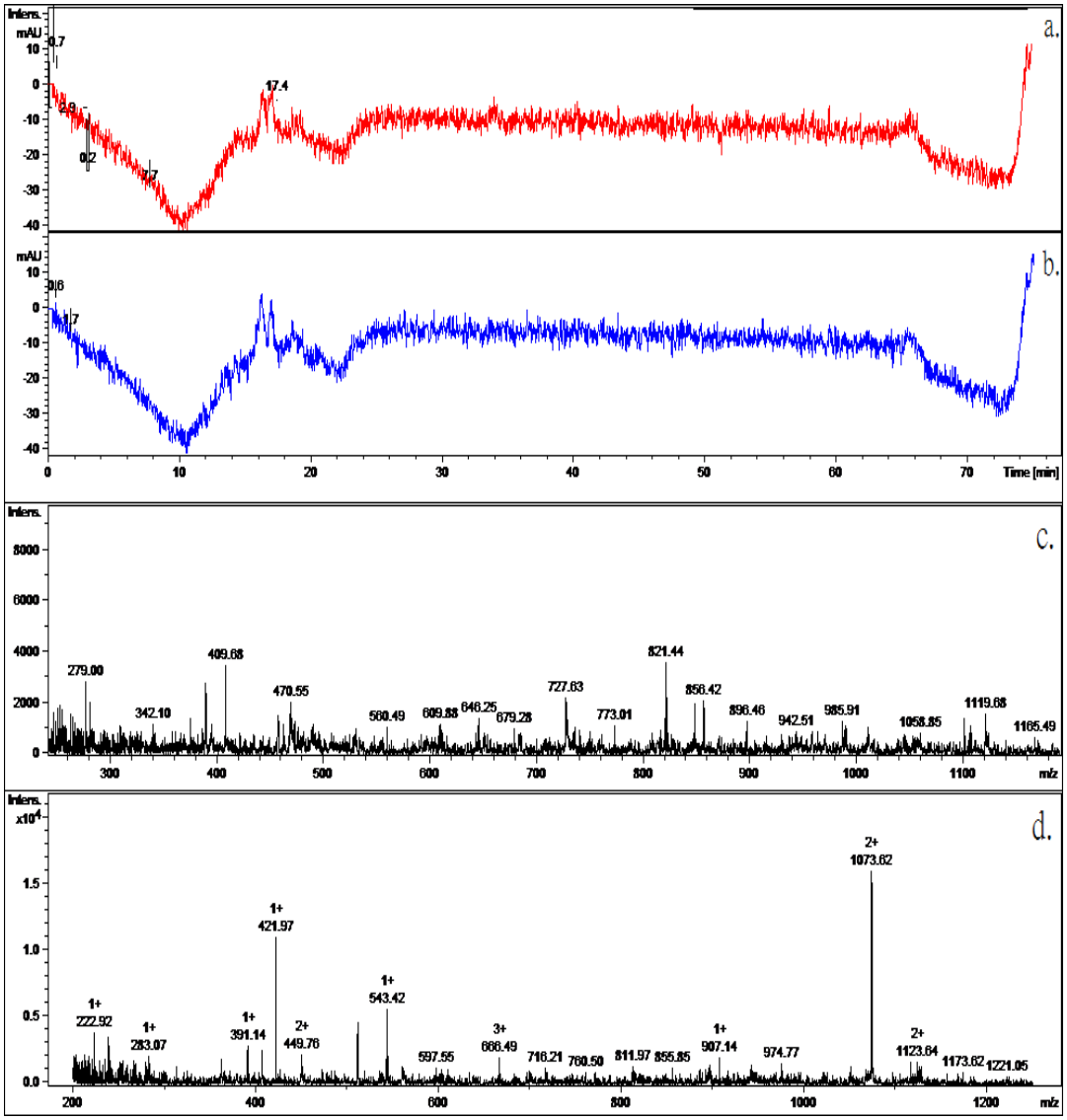


Figure 2-3 UV-HPLC chromatograms at 520 nm of mice plasma (a) control group and (b) 30% P40 pooled sample have similar patterns. Positive ion MS data in 15-17 mins of mice plasma (c) control group, (d) P40 group.

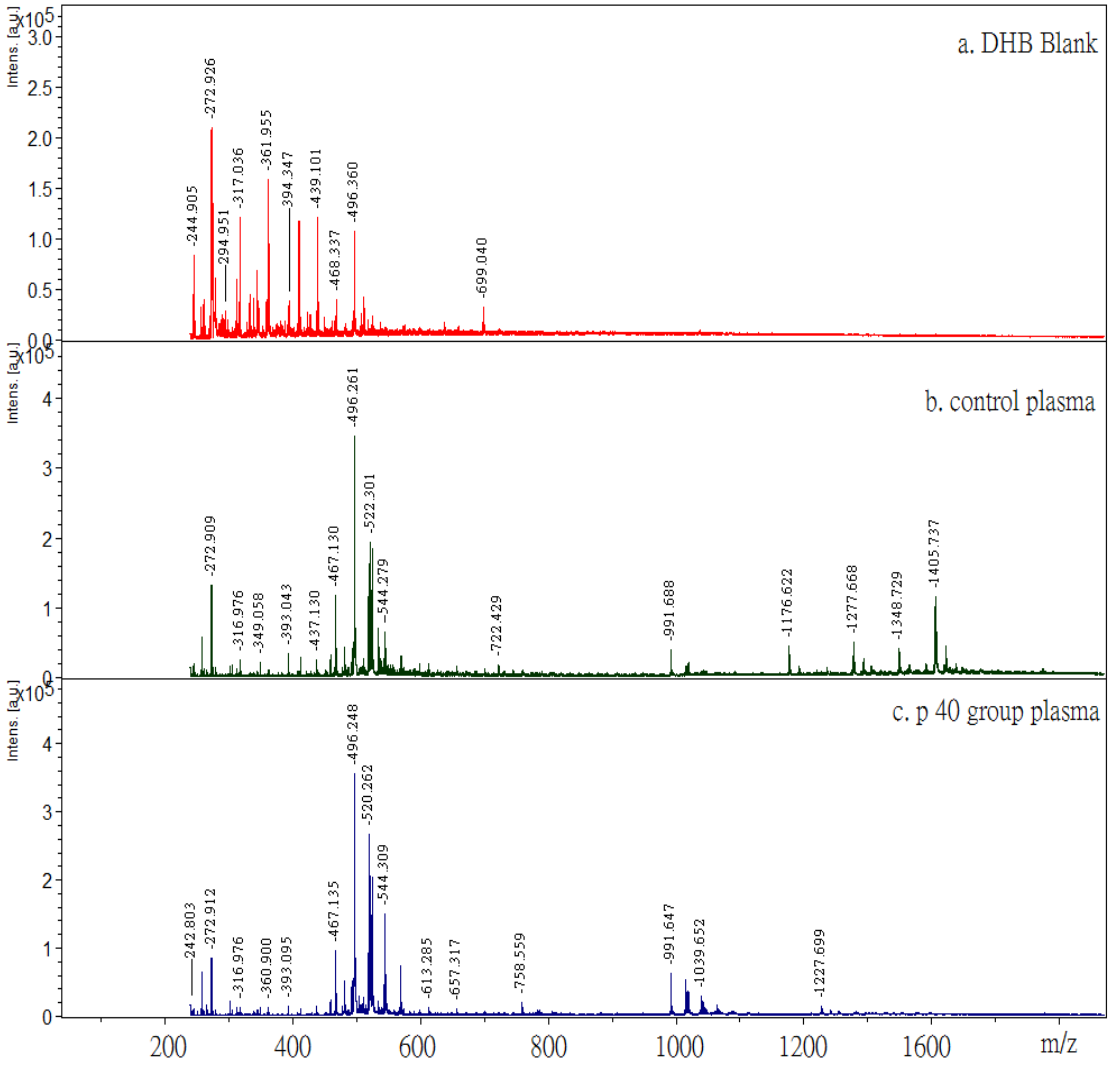


Figure 2-4 MALDI-TOF-MS data of mice plasma sample (a) DHB blank, (b) control group, (c) 30% P40 group contain similar patterns.

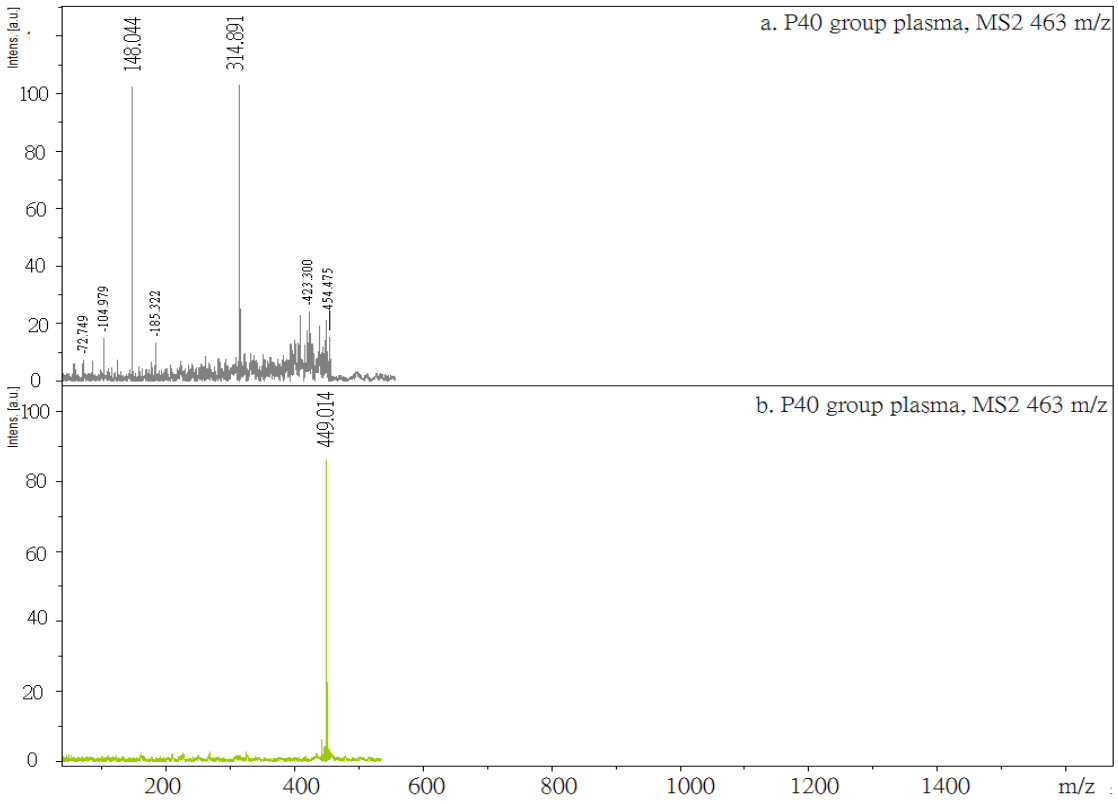


Figure 2-5 MALDI-TOF/MS/MS data of mice plasma in 30% P40 group monitored at the extracted ion of m/z=463.0 (a) and m/z=449.0 (b), but did not find these two fragments in sample.

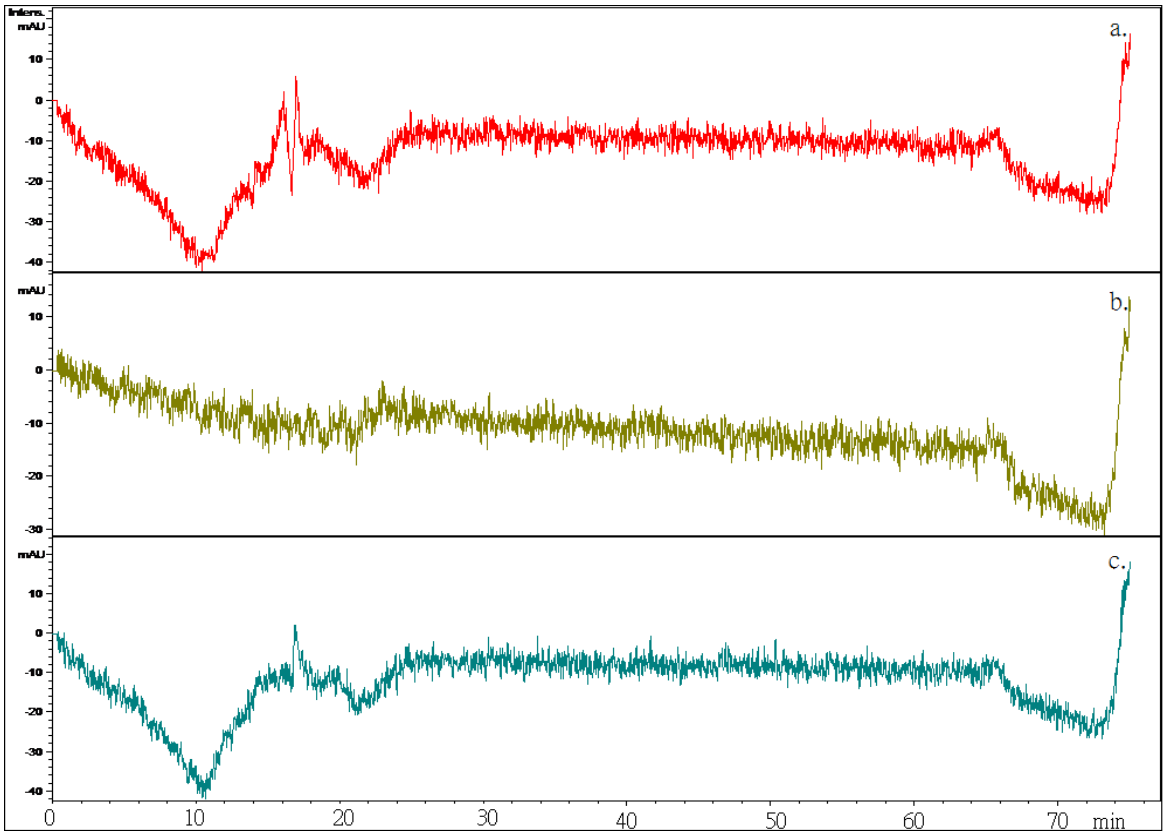


Figure 2-6 UV-HPLC chromatograms at 520 nm of mice liver (a) control group, (b) 20% P40 group and (c) 30% P40 group have similar patterns.

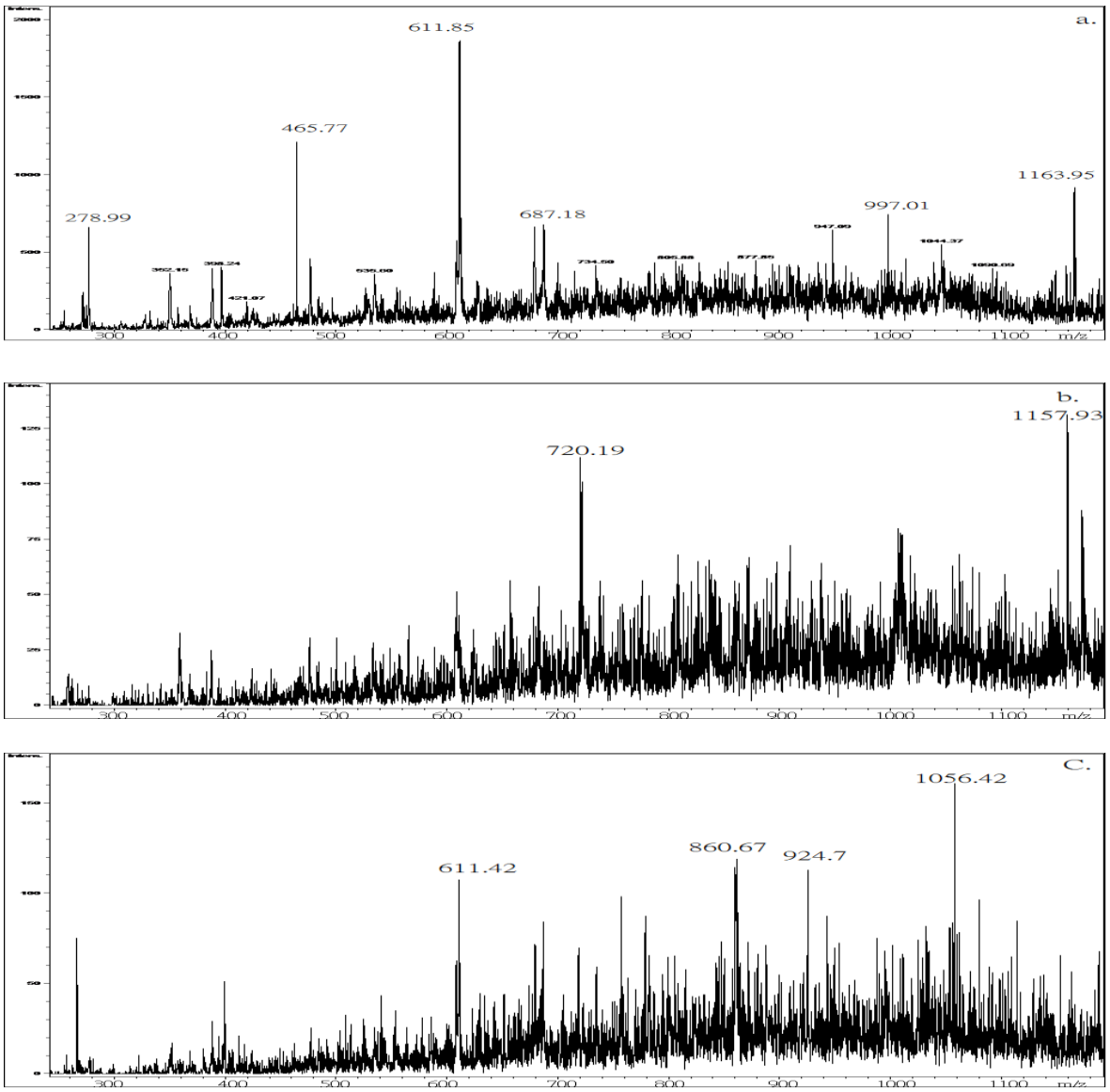


Figure 2-7 Positive-ion MS spectra data in mice liver (a) control group, (b) 20% P40 group and (c) 30% P40 group, showed 611 m/z, 860 m/z, 924 m/z, 1056 m/z in P40 group.

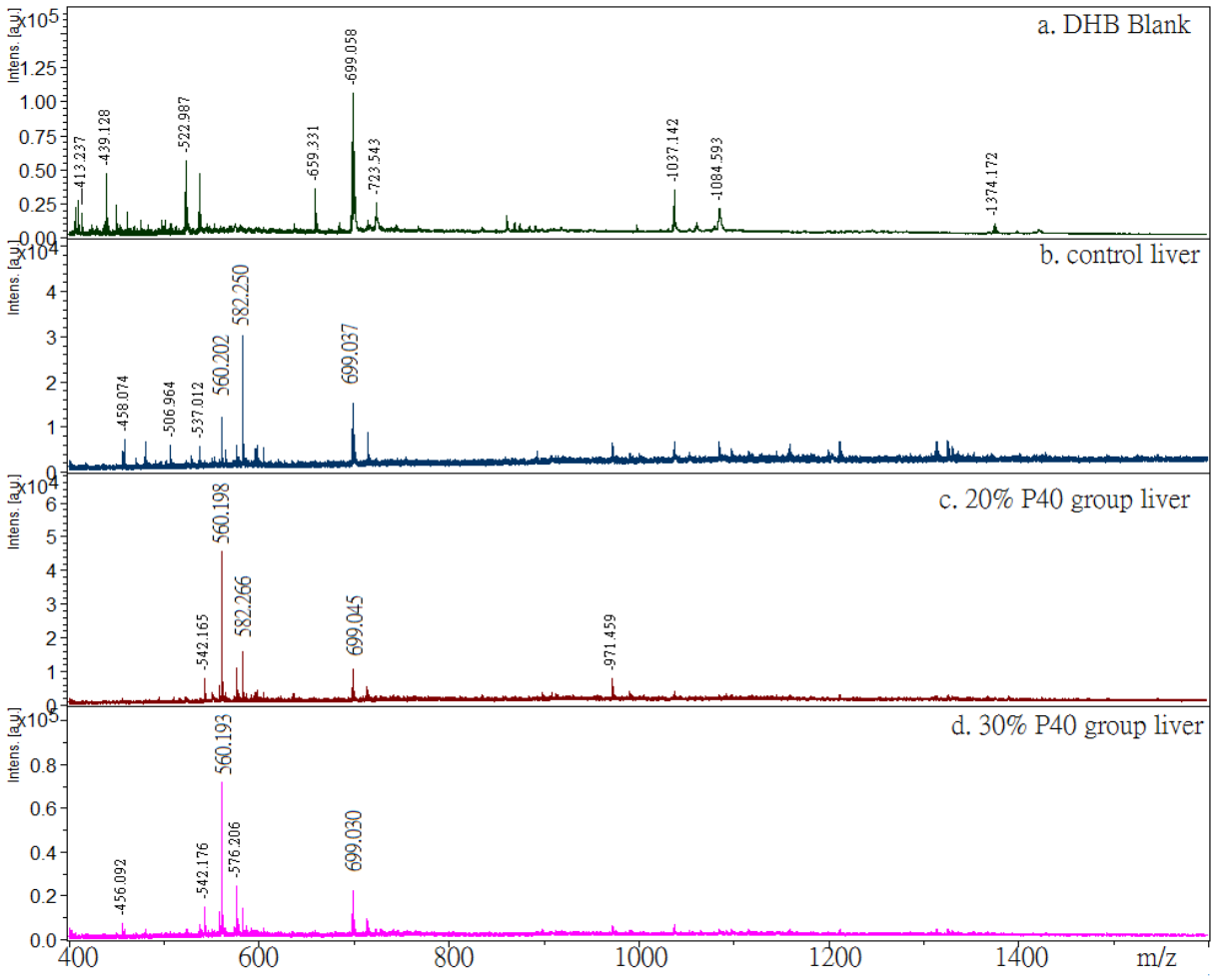


Figure 2-8 MALDI/MS spectrum of mice liver sample in (a) DHB blank, (b) control group, (c) 20% P40 group, and (d) 30% P40 group.

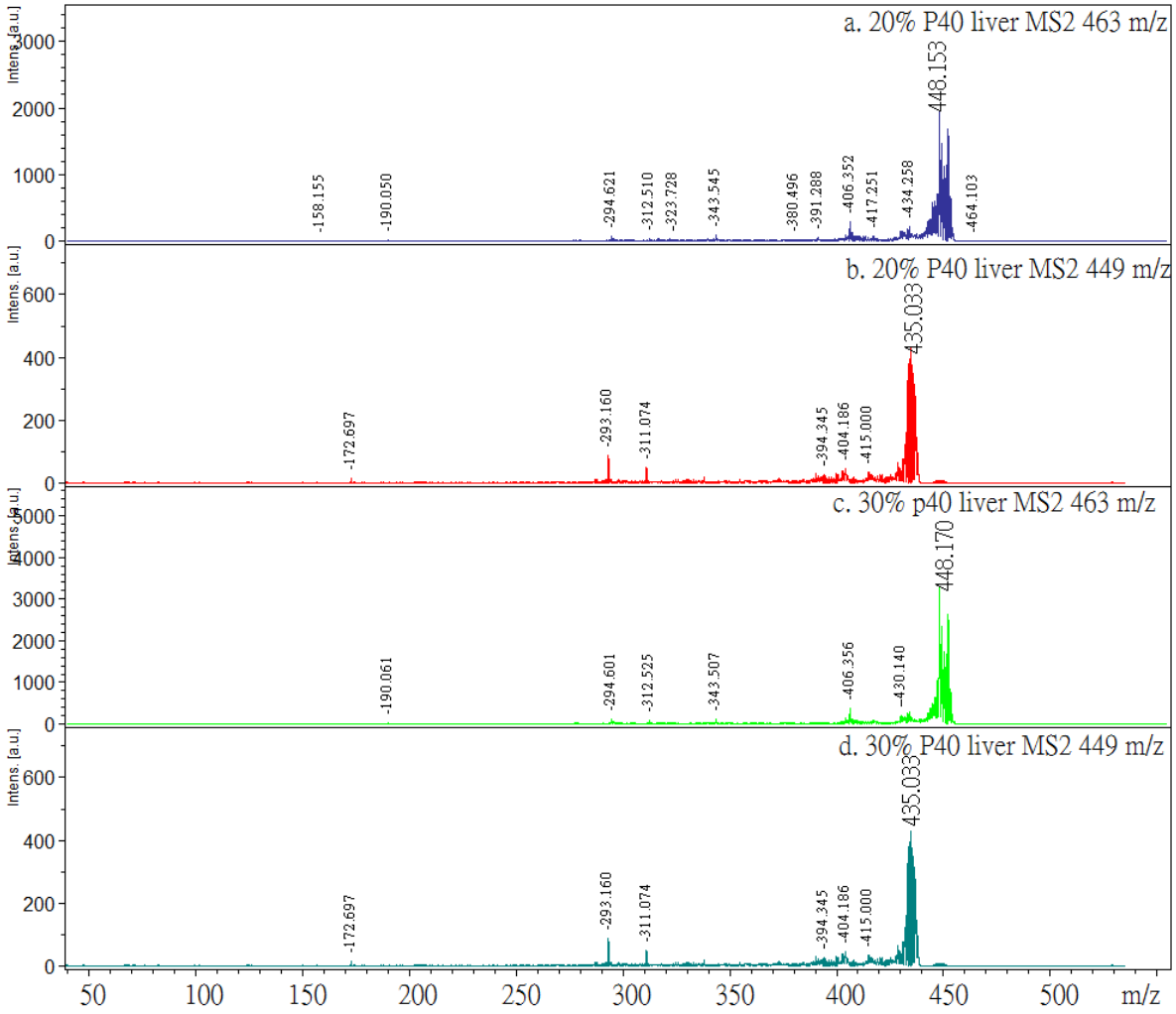


Figure 2-9 MALDI-TOF/MS/MS data of mice liver sample monitored at the extracted ion of m/z= 463.0 in 20% P40 group (a) and in 30% P40 group (c); m/z= 449.0 in 20% P40 group (b) and in 30% P40 (d) group but did not find their daughter fragment 301 m/z and 287 m/z after second MS.

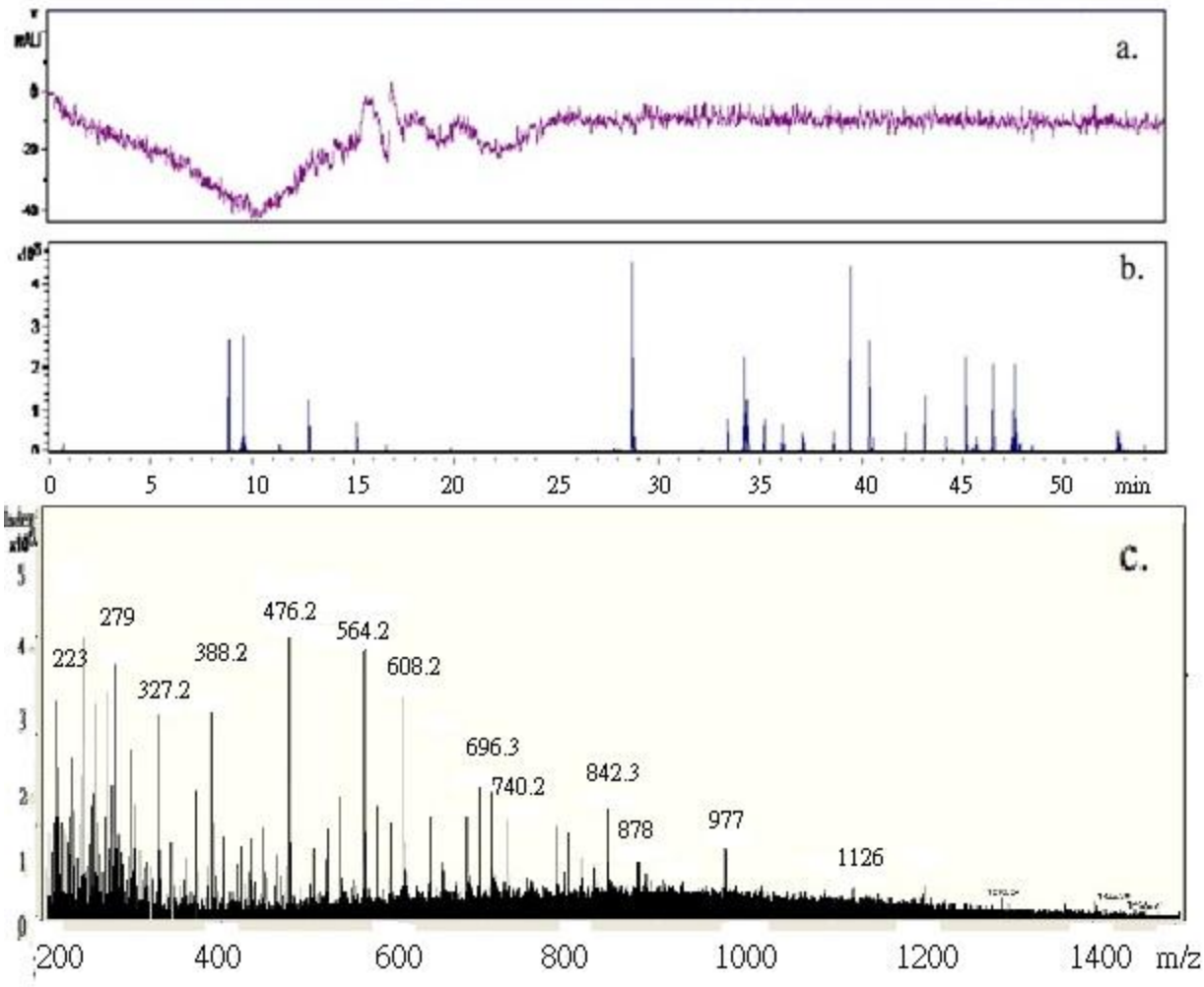


Figure 2-10 UV-HPLC chromatogram of control group mice feces sample (a). The total positive-ion mass spectrometry spectra of control group mice feces sample (b), and in 10-37 mins (c).

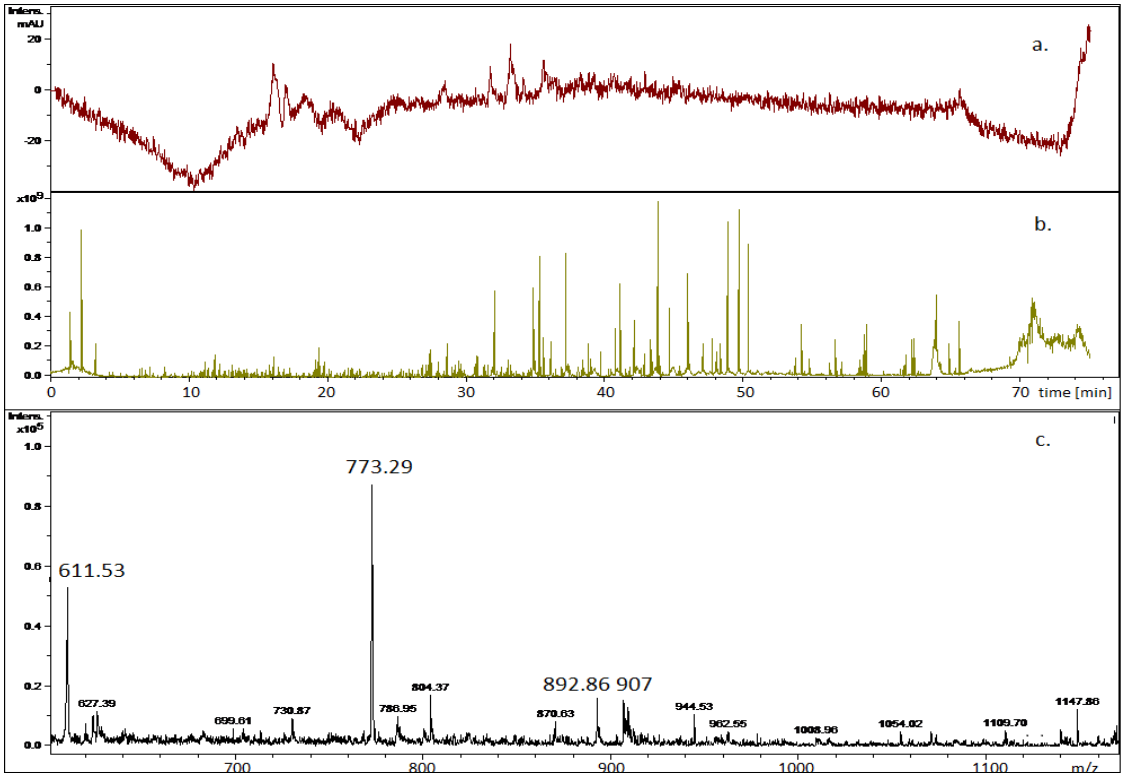


Figure 2-11 UV-HPLC chromatogram of 30% P40 group mice feces sample (a). The total positive-ion mass spectrometry spectra of 30% P40 group mice feces sample (b), and in 27-37 mins (c).

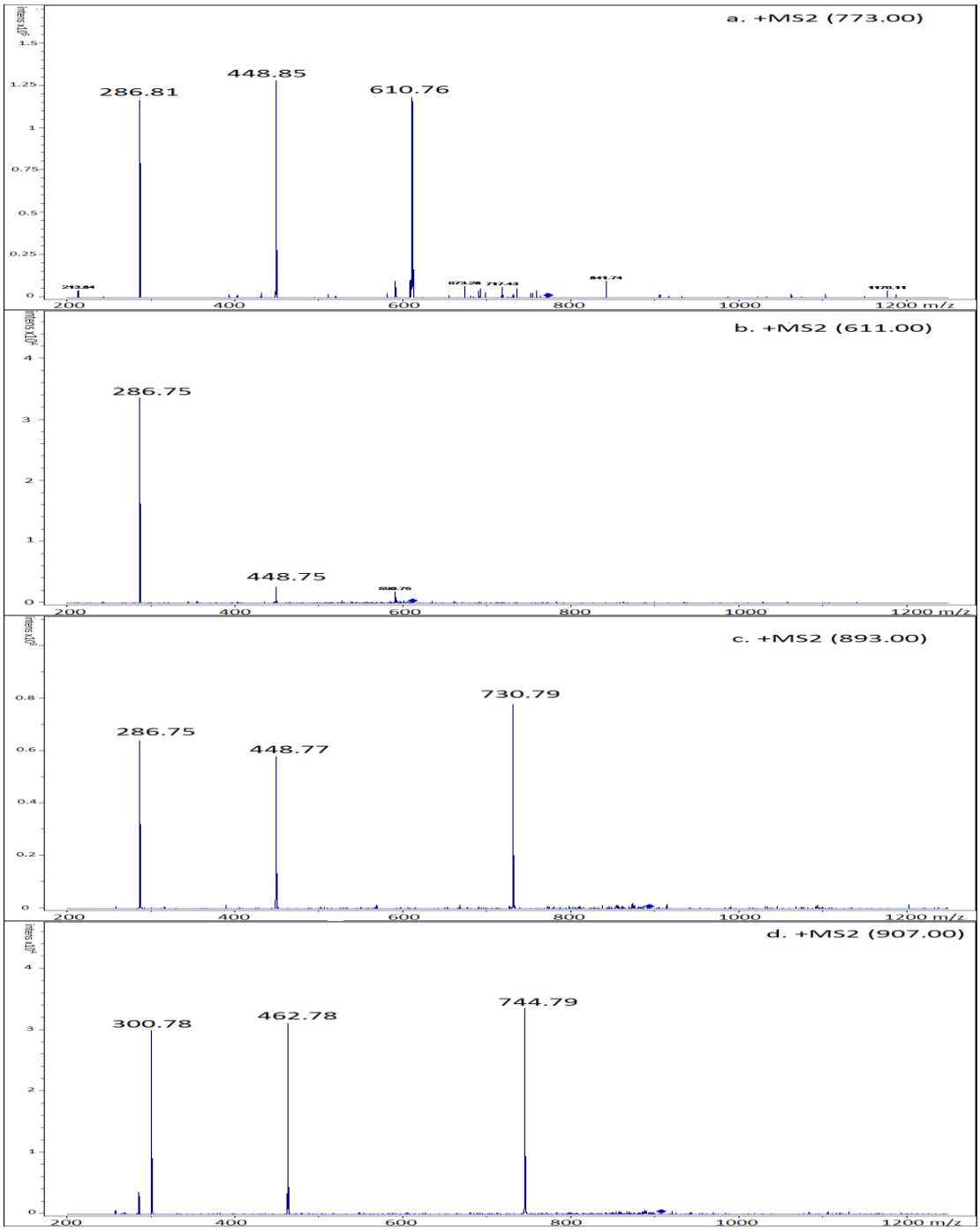


Figure 2-12 HPLC/MS/MS data of mice feces sample in 30% P40 group monitored at the fragment ion of m/z=773.0 (a), m/z= 611.0 (b), m/z=893.0 (c), and m/z=907 (d).

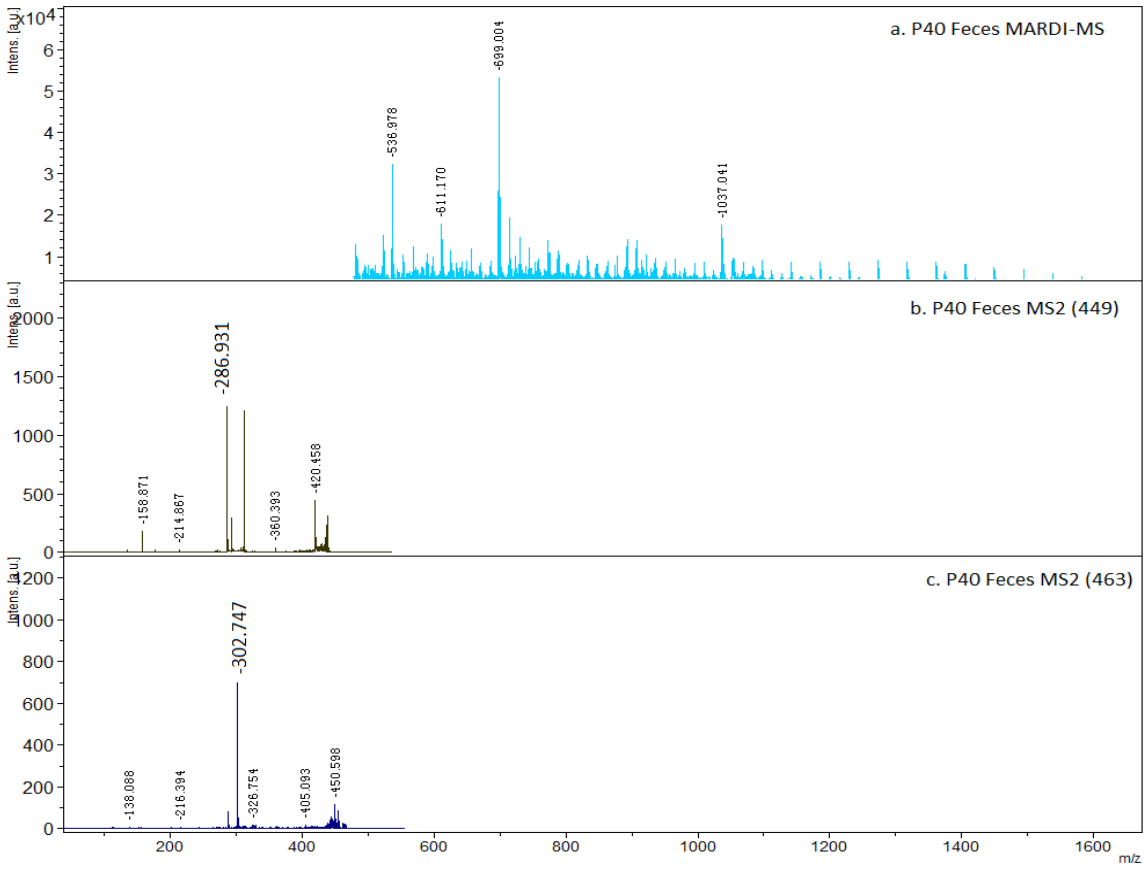


Figure 2-13 MALDI/MS data of mice feces sample in 30% P40 group (a) and monitored at the extracted ion of m/z=449.0 (b) and 463.0 (c) and shown m/z=287 (b) and m/z=301 (c) which arethe unique daughter fragments of cyanidin-3-glucose and peonidin-3-glucose, respectively.

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