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Quantitated Effects of Nutritional Supplementation on Exercise Induced Sweat

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QUANTITATED EFFECTS OF NUTRITIONAL SUPPLEMENTATION ON EXERCISE INDUCED SWEAT

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Andrew Blake Austin Browder ENTITLED Quantitated Effects of Nutritional Supplementation on Exercise Induced Sweat BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Discovery studies have identified many metabolites contained in human sweat. However, quantitative analysis of the sweat metabolome content remains mostly unknown. Furthermore several attributes, including rate, have been defined to affect sweat metabolite content, while other effectors, like diet, remain unknown. This study works to quantitatively define the metabolite impact caused by nutritional supplementation.

To better understand the effect diet plays, a LC-MS method was developed focusing on improving resolution and peak width. While the literature provided examples of how diet affected sweat metabolite concentrations, the long-term effects of diet have not been explored. The experiment described here attempts to fill that gap.

Partial data separation was found among groups ingesting high and low nutritional supplementation. Several subjects given the high nutritional supplementation had decreased sweat metabolite concentrations after twelve weeks. These results suggest nutritional supplementation can impact the sweat metabolome, and diet should be considered in biomarker discovery experimentation.

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Hypothesis and Aims

The impact of diet on sweat metabolomics has been minimally investigated and holds the potential to drastically impact biomarker discovery. The hypothesis is by ingesting a dietary supplementation that contains proteins and vitamins sharing metabolic pathways with the metabolites of interest, the amount of those metabolites will be quantifiably affected in sweat. In order to test this hypothesis, a new analytical method will need to be developed to adequately separate the metabolites of interest. After determining a separation method, calibration curves for each metabolite, in both mass and dynamic range, must be developed. Application of the developed methodology will be applied to samples acquired from subjects before ingesting a high nutritional supplement and then again after 6 and 12 weeks of dietary supplementation. To account for other sources of change in sweat metabolites, a control group will be administered a low nutritional supplement and undergo the same testing as the high nutritional supplement group.

AIM1: Improve upon the liquid chromatography and mass spectrometry analytical methods used to analyze sweat for quantitative analysis.

AIM2: Analyze sweat samples from volunteers who were administered a dietary supplement or placebo to determine the effects of dietary supplementation on sweat metabolite content.

Chapter 1: Optimization of Liquid Chromatography Methods for Sweat Metabolite Quantitation

Introduction

Significance

The mission of the United States Air Force "to fly, fight and win in air, space, and cyberspace" is negatively impacted by diminished airman performance. In the field, commanders making mission personnel selections consider training as well as the current performance state of the airman. Additionally, airman serving at home, such as security officers and drone operators, can develop fatigue degrading job performance. Therefore, the Air Force has a significant investment in attempting to predict human performance and to enable commanders to choose "the right person at the right time" for missions both in the field and at home. To accomplish this overall goal, bio-fluids that can be collected without hindering job functions (non-invasive, on demand, and field applicable collections) must be evaluated. While other bio-media have been evaluated, such as saliva, to meet this goal, novel fluids must be continually evaluated to meet the Air Force's ever-changing demands.

Sweat: A Non-Invasively Collected Bio-fluid for Performance Monitoring

Several potential bio-fluids that could be utilized to predict a subject's performance exist including blood, saliva, urine, and sweat. The most often studied bio-fluid for human performance prediction is blood based, such as whole blood, serum, or plasma (36, 37). For example, potassium (K^+) ions have been shown to increase in serum after exercise (36). However, while holding potential, the Air Force requires a bio-source that does not inhibit job duty, i.e., non-invasive. In the field obtaining a blood sample from every soldier prior to every mission is logistically unrealistic and dangerous. Additionally, complications to the solider and the overall operation from poor sterility or fainting from blood draws cause serious concerns.

A second alternative to sweat is saliva. While saliva is a viable option, strict protocols in the time prior to testing to reduce the influence of food and drink on the detectable compounds are substantial drawbacks (3, 19). For example, xylitol, which is present in some chewing gums, can be detected in saliva for over an hour post chewing (19). Additionally, a significant fasting time, i.e., time between eating, drinking or other oral activities such as using toothpaste or mouthwash, chewing gum, smoking and smokeless tobacco, is required prior to sampling (3, 19). These attributes of saliva sampling are often difficult to control during military operations thereby precluding saliva from being the sample of choice for the Air Force's performance analysis.

Urine, a frequently analyzed bio-fluid, is the third alternative bio-media with potential to be used to predict performance. For example, Gorski et al. found that uric

acid is reduced in participants' urine following short term high intensity workouts (21). In contrast, Saat et al. illustrated that heat acclimation has no significant effect on urine ion content (36). Urine analysis has difficulty meeting the long-term goals of the Air Force. The primary issue in utilizing urine to predict performance is that urine cannot be produced on demand. For performance prediction it is imperative to the Air Force that a bio-fluid is available at any given moment because a mission assignment could present itself at any time. Urine's inability to be produced on demand precludes it from selection for performance analysis.

As a result of the shortcomings of the above mentioned bio-sources, the Air Force is continually investigating non-invasive bio-media for potential real-time continuous human performance monitoring. Currently, focus has transitioned to the investigation of sweat to predict human performance. Sweat glands, small diameter tubes that lead to the surface of the skin, are located all over the body (41). There are two types of sweat glands, apocrine and eccrine. Apocrine sweat glands develop during puberty and are located in hairy places. Apocrine glands function as scent glands (41). In contrast, eccrine sweat glands function in temperature regulation. Sweat and sweat glands mentioned throughout this paper are referring to eccrine sweat and sweat glands unless apocrine is specified. The autonomic nervous system controls the eccrine sweat glands, as the body senses an increase in temperature the glands are stimulated to release sweat. In addition to temperature, stress and fear and also cause the body to release sweat (41). While the primary components are water and salt, proteins and metabolites have also been detected in sweat (23, 41).

In contrast to the previously mentioned bio-sources, sweat samples can be acquired directly from the outer epidermal layer of the skin there by avoiding invasive contact. Additionally, sweat can be produced on demand in most any situation through iontophoretic stimulation or light exercise (30). Iontophoresis is the process of delivering a transdermal drug, such as pilocarpine, to the dermal layer utilizing a voltage gradient on the skin thereby inducing sweat. For instance clinical cystic fibrosis testing uses this pilocarpine aided stimulation on infants to generate sweat for chloride ion determination, an estimation of a genotypic modification of the cystic fibrosis transmembrane conductance regulator protein (30). Accurate calculation of sweat chloride is important for clinical diagnosis of cystic fibrosis. For example, a concentration of sweat chloride $\geq$60 mmol L^{-1} is indicative of a positive diagnosis and values ranging between 30-59 mmol L^{-1} are inconclusive (30), while values under 30 mmol L^{-1} are indicative of a negative diagnosis.

For initial analytical development a greater volume of sweat than iontophoresis produces (~30-40 µL) is required. Light exercise can produce significant amounts of sweat (1-2 mL) (10, 23). In addition, exercise can also be used to link sweat to physical performance. The Air Force has developed a list of factors, including aerobic and anaerobic measurements, to analyze performance during exercise. Measurements of sweat analytes paired with these physical performance factors could hold the potential to link sweat content and performance.

Not only does sweat allow for the sampling attributes the Air Force desires, sweat has been illustrated to have potential as a diagnostic bio-fluid. For example, a group of twenty-six immune response and auxiliary protein transport proteins, not usually found in

human sweat, have been found in the sweat of tuberculosis patients (1) suggesting that sweat composition can change based on the individual's health. Additionally, five sweat metabolites have been used to differentiate between lung cancer patient risk groups (9) further supporting the hypothesis that sweat composition can be used as a predictor of human health. While only a few examples exist, sweat shows early promise as a diagnostic bio-fluid.

As with any human biological sample, many factors, known and unknown, surround sweat content including sampling and analysis methods. These factors must be defined to understand what can affect the concentrations of sweat analytes. For example personal care products have been implicated in sweat metabolite abundance with links to approximately 54% of metabolites frequently found in sweat (23). These results provide evidence for the impact of exogenous metabolite contamination in sweat analysis. Furthermore, sweat rate has also been recently connected to variable concentration of sweat molecules. For instance, sweat ion variability has been illustrated to be reduced significantly by normalizing to sweat rate (24). The concentration of sodium increases linearly with sweat rate (2), such that subjects with higher sweat rates will have a higher concentration of sodium in their sweat regardless of current performance ability. In addition to the previously mentioned attributes that may impact sweat metabolite content, several methods for sweat sampling exist, each with its own advantages and disadvantages, and potential to influence sweat content (5, 22, 39).

Sweat Collection Methods

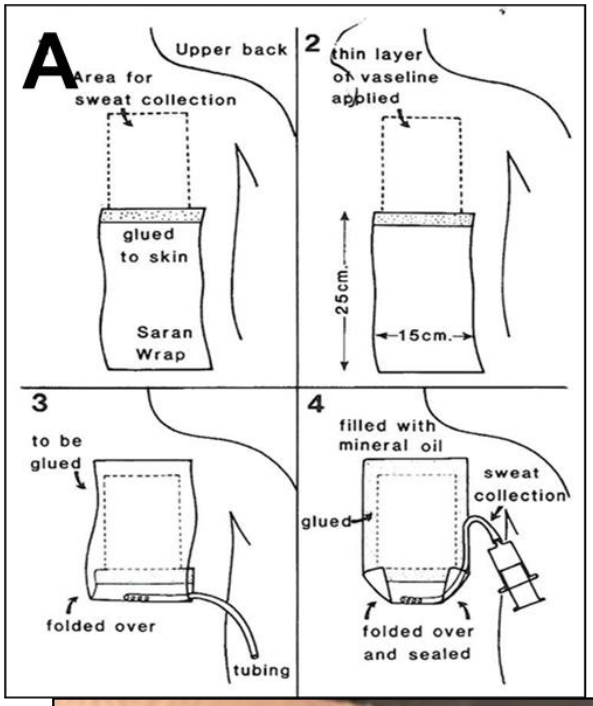
Several sweat collection techniques have been developed over the years both simple and complex. The techniques to be discussed include sweat scraping, filter paper absorption, bag collection, Macroduct collection, and the patch. First, on the simplistic side, sweat scraping (scraping sweat from the skin into a container) and filter paper absorption (porous paper applied to the skin to absorb sweat) are among the most common. However, these techniques have been shown to lead to significant contamination of the resulting samples from the epidermal layer of skin (5).

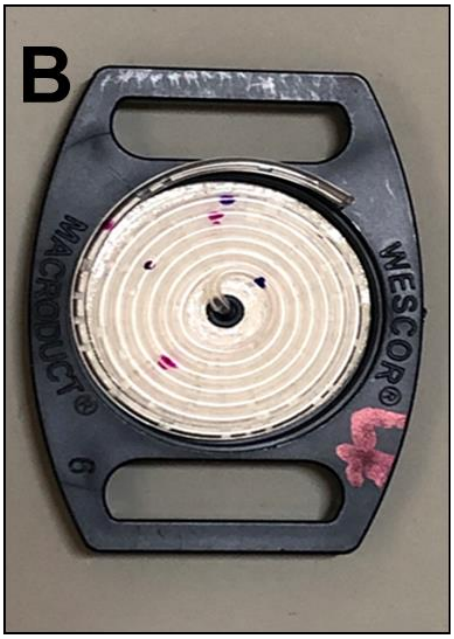
Conversely, a more complex method pioneered by Boysen et al, creates a pouch on the subject's back to collect samples (5). Briefly, the area is cleaned and a layer of Vaseline is applied. Plastic wrap is glued to the edges of the cleaned and lubricated sample area to create a bag. The bag is filled with mineral oil to induce sweat pooling at the bottom. Tubing is inserted in the bottom of the bag for removal as sweat pools (Figure 1A) (5). Many of the molecules analyzed by Boysen et al. including sodium, potassium, lactate, and glucose, showed little concentration differences when collected using the mineral bag collection approach compared to simplistic collection methods (5). However, other molecule concentrations, including calcium, urea and proteins were found in significantly higher amounts in the simplistic methods during the first ten minutes of the trial (5). These results were hypothesized to be a consequence of epidermal contamination (5, 16). The collection method, by Boysen et al., allows for multiple collections and removes epidermal contaminates. While advantageous, the setup put forth by Boysen et al. is too complex to be the model for a field collection.

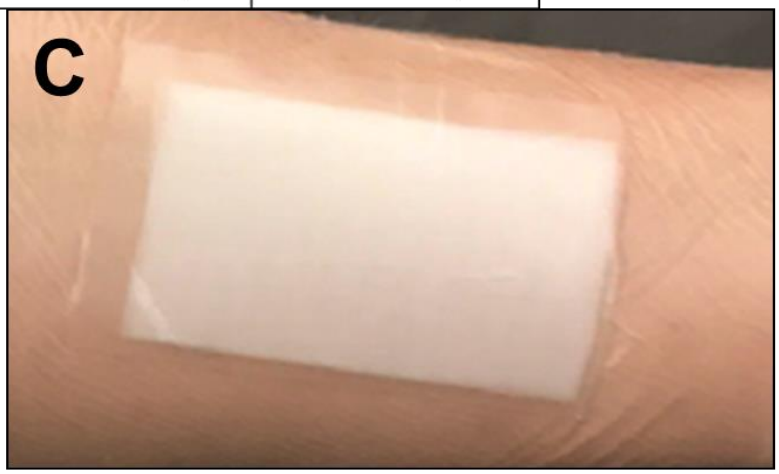
As an alternative to the bag collection method, the Macroduct is a well-accepted standardized sweat collection technique utilizing microbore tubing attached to a hole in a concave surface placed on the skin. The microbore tubing coil collects the sweat as it is pushed through the hole of the collector. This approach, using the Macroduct sweat collection system, is currently utilized to aide in the diagnosis of infants with cystic fibrosis (Figure 1B) (30). The advantages of using the Macroduct to collect sweat include reduced evaporation (leading to increased accuracy of concentration measurements), the ability to calculate sweat rate (24), and commercial availability. However, the microbore tubing of the Macroduct collection system yields small sample volumes (80-90 µL) limiting analytical approaches. Therefore, while advantageous for future collections, analytical development requires larger volumes. As a result, alternative collection methods need to be investigated.

Finally, the most frequently utilized sweat collection technique, for discovery applications, is the patch (Figure 1C). A patch collector is a packet of nylon mesh covered by a polyethylene layer attached to the epidermis by a bandage. For this experiment the advantages of the patch collection setup outweigh the negatives. For instance, the patch can collect relatively large sample collection sizes of 1-2mL (22), can be utilized at versatile localized-collection sites, and has a simplistic affordable setup (39). However, a downside to the patch collection method includes possible epidermal contamination (5) leading to the need for the samples to be filtered after collection (22). However, as analytical development currently requires large volumes, the patch method was utilized for sweat collection in the experiment outlined here.

Figure 1: Selection of sweat collection devices **A:** Pouch (figure modified from Boysen et al. 5) **B:** Wescor Macroduct **C:** Sweat Patch







Sweat Content: Ions

Human sweat has been shown to contain many ions including sodium, chloride, and urea (18). Sweat ions are often studied using ion chromatography. Ion analysis could be useful in linking performance to sweat as sweat ions have been shown to change depending on the sweat induction method (17, 18). For example, in a physical exertion model, sweat sodium and chloride ions increase in response to running when compared to heat induced sweat (18). In heat stress, sweat potassium ions as well as urea nitrogen increase when compared to sweat induced by running (18). Based on these few examples sweat ion content could possibly be used as an indicator of changes in physical performance. While holding potential and a source of future experimentation, for this experiment ion analysis was not performed due to current instrumentation availability.

Sweat Content: Proteins

The sweat proteome is defined as the identification of all proteins found in sweat samples. Proteomic analysis of sweat has revealed a great deal about sweat makeup and purpose. For example, a group of 26 proteins were identified in sweat from active tuberculosis patients suggesting the sweat proteome may change in response to illnesses (1). Some proteins have also been shown to decrease with illnesses, such as those linked with ectodermal dysplasia, a skin condition characterized by abnormalities of the skin and other ectoderm derivatives (7). One of the abnormalities often associated with ectodermal dysplasia is reduced sweating. Reduced sweat precludes a reduction of the sweat proteins in the human immune system (7, 11). These proteins act as antimicrobials, killing off many of the infections that could attack the epidermal or dermal layers of the skin (7). Illness is likely not the only source of systemic changes that illustrate

variations in the sweat proteome, but further research is required to understand the links between human performance and sweat protein content.

While understanding sweat proteomics is a current research area, proteins that have been identified in sweat serve a plethora of other functions as well, including chaperone proteins metabolic proteins, and apoptosis proteins (7, 23). Unfortunately, many of these proteins are found in very low concentrations. The low concentrations combined with the large dynamic range of proteins within sweat leads to the need for increased sample handling and state-of-the-art analytical tools (23). For examples, nanoflow liquid chromatography (nLC) allows for greater sensitivity and high-end mass spectrometers allow for the greatest depth of protein analysis at the expense of great monetary cost (12). While the proteomics analysis of sweat may hold potential for performance monitoring, illustrated by the links of illnesses to sweat content, the properties of sweat proteins will require substantial analytical development, beyond the scope of this project to achieve this goal.

Sweat Content: Metabolites

Similar to the sweat proteome, the sweat metabolome is the complete set of all small-molecules that can be found in sweat samples. Several methods for analyzing metabolites exist including nuclear magnetic resonance (NMR), gas chromatography mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). While LC-MS was chosen for the analysis performed here, each method has advantages and disadvantages.

Methods for Sweat Metabolite Analysis: NMR

NMR analysis has been used to map sweat composition (29, 40). For example, an experiment by Kutyshenko et al. using NMR analysis found the main constituents of the sweat metabolic profile are lactate, glycerol, pyruvate, and serine (29). Sauza et al. utilized NMR to discover 34 different metabolites in sweat samples, including lactate and several amino acids (40). This experiment also found that sweat stimulation by pilocarpine and physical exercise both produce similar results (40). Although utilized with sweat, NMR has both advantages and disadvantages as an analytical approach.

Advantages of NMR analysis compared to mass spectrometry analyses include reusability of samples, short analysis time, and high reproducibility (27). Conversely the disadvantages of NMR analysis compared to mass spectrometry analysis include decreased sensitivity and need of larger sample size. Both these disadvantages generally lead to decreased biomarker detection (27). Lastly, and most importantly, our lab does not have NMR instrumentation available. While a viable method for sweat analysis, due to the dilute nature of human sweat paired with NMR's decreased sensitivity and our lack of instrumentation, NMR analysis was not utilized for this study.

Methods for Sweat Metabolite Analysis: Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) has been utilized in sweat analysis (14, 20). Gentili et al. developed a procedure to detect drugs of abuse in sweat collected from individuals' foreheads. Additionally, 60 drivers displaying intoxicated behaviors tested positive for one or more drugs of abuse when headspace solid-phase

microextraction (HS-SPME) was utilized with GC-MS. Furthermore, Delgado et al. utilized GC-MS to tentatively identify 135 compounds in sweat including lipids, benzenoids, and alkaloids (14). While GC-MS has been utilized to analyze sweat, similar to NMR analysis, advantages and disadvantages to utilizing GC-MS as an analytical tool have been illustrated.

An advantage of GC-MS analysis compared to other analytical methods utilizing chromatography is an improved ability to separate volatile compounds. However the compounds selected for this experiment are non-volatile and require derivatization (attaching a large volatile molecule to a non-volatile compound) for analysis by GC-MS. Derivatization alone has several analytical challenges including reduced throughput, compared to other methods such as LC-MS, and the need to check derivatization efficiency associated with it. Due to the non-volatility of the metabolites selected for analysis GC-MS was precluded from our study.

Methods for Sweat Metabolite Analysis: Liquid Chromatography-Mass Spectrometry

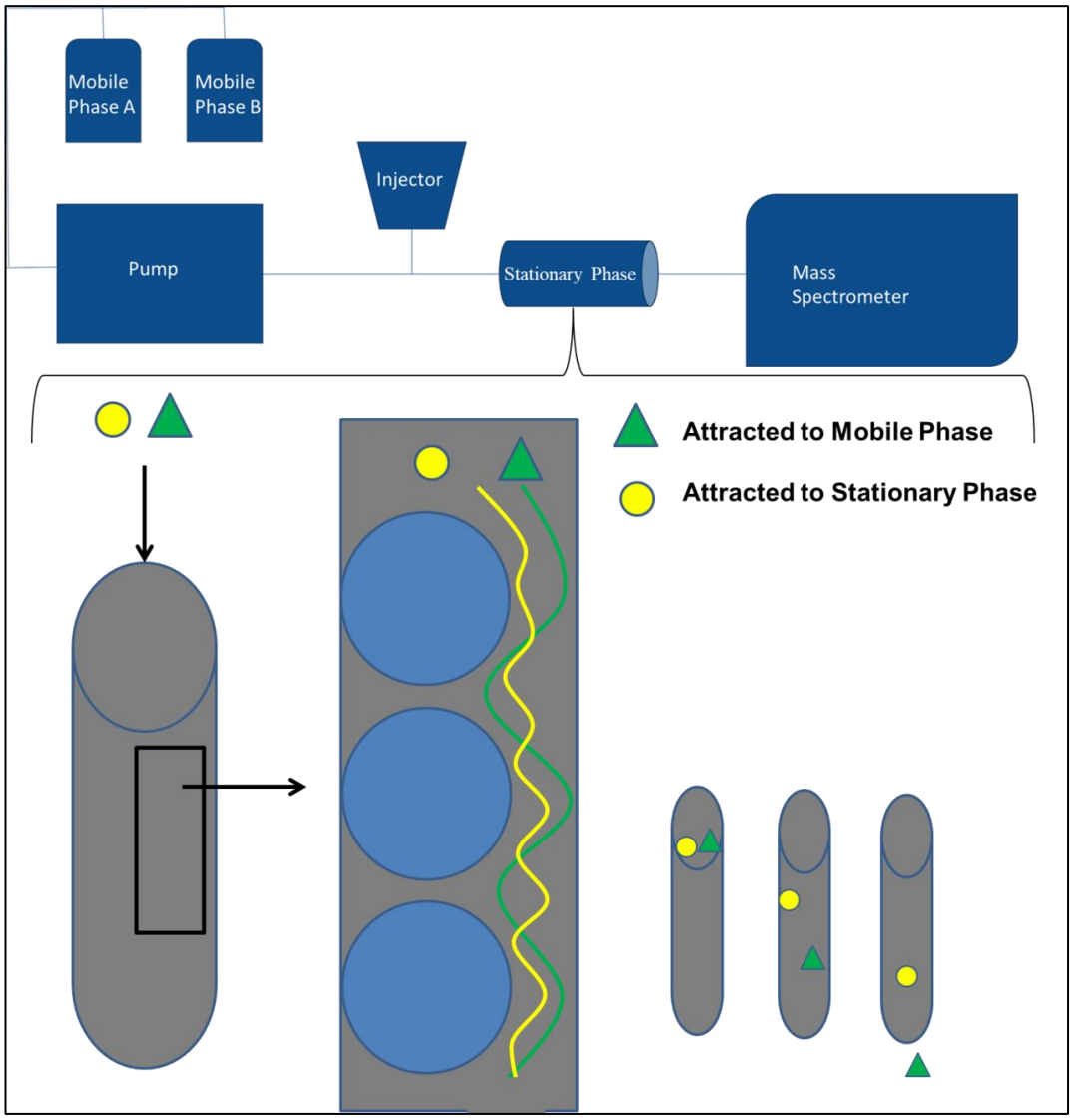
While other discovery analytical methodologies have been applied to sweat, such as NMR and GC-MS, the most frequently utilized technique to analyze sweat, and the analytical technique utilized in the experiments outlined here, is liquid chromatography-mass spectrometry (LC-MS) (1, 8, 9, 11, 15, 22- 24, 26, 35). Discovery studies utilizing LC-MS have tentatively identified several small molecules in human sweat (8, 9, 15, 22-24, 26). For example, Hooton et al. identified 83 metabolites while Delgado-Povedano et al identified 118 compounds in human sweat (15, 26). While only a few examples are

discussed here, the advantages associated with LC-MS analysis, as well as the instrument availability within our lab make it the analytical method of choice.

An LC-MS experiment utilizes high pressure liquid chromatography (HPLC) or liquid chromatography (LC) to separate analytes to allow for decreased complexity when introduced into the detector. HPLC involves pumping a sample through a piece of tubing filled with a liquid mobile phase. Typically two mobile phases are being pumped through the system under variable compositions. The mixture of mobile phase and sample is consistently pushed on to a column, a separate piece of tubing filled with a stationary phase, allowing the sample to interact with the stationary phase based on the chemical properties of the analytes in the sample (Figure 2). For example, different molecules within a sample will have variable affinities for the stationary phase or the mobile phase allowing separation over time, so that molecules with higher affinity for the stationary phase will be detected after those with a higher affinity for the mobile phase (Figure 2).

Liquid chromatography will only yield separated compounds, leaving the analytes with the need to be detected. HPLC separations can be detected by many types of detectors such as UV/Vis, photodiode array, and conductivity. However, the detection type utilized in the experiments described here uses mass spectrometry (MS). Mass spectrometry is the most frequent discovery detection method used in conjunction with HPLC. A mass spectrometer generally has two specific parts, the ion source and the mass detector. Following separation, the molecules are ionized within the ion source, frequently performed using electrospray ionization (ESI).

Figure 2: Liquid chromatography map with an extended view of the column (stationary phase). Mobile phase is drawn through the tubing by the pump. An injector adds the sample to the mobile phase. The sample is separated by the stationary phase in the column. The separated sample is then sent to the detector, in this case a mass spectrometer.



This soft ionization technique involves running a high voltage across the sample as it leaves the LC instrument aerosolizing and charging the analytes producing analyte ions. Then, the ions are transferred to the mass analyzer. Several types of mass analyzers available, such as a quadrupole or an ion trap. All these analyzers have pros and cons. However, the detector utilized in the experiments described here is an Orbitrap mass analyzer. The Orbitrap mass analyzer uses electric fields to separate ions based on their mass to charge (m/z) ratio by Fourier transformation of frequency of ion oscillations about a central ring electrode. While an Orbitrap mass spectrometer has high resolution and accurate mass measurements, drawbacks include low sensitivity and scan speed compared to other mass analyzers. Although there are several drawbacks to utilizing LC-MS and an Orbitrap detector, it is an effective analytical tool to for complex samples like sweat.

Due to metabolite separation by LC, metabolites in a complex sample are detected by the mass spectrometer at different times. A combination of repetitive scans from the mass spectrometer can be plotted to determine a chromatographic peak over time. As a result, a link between the metabolite abundance and the chromatographic peak area can be established. While analyzing several compounds simultaneously the high resolution mass/charge measurement afforded by the Orbitrap instrument can allow for differentiation of analytes of similar m/z ratio. Through the use of mass spectrometry software, the abundance of hundreds of analytes, based on m/z values can be determined across a chromatographic run. Therefore, the complexity of sweat samples can be broken down and analyzed after LC-MS analysis.

LC-MS based metabolomics of sweat, utilizing high resolution accurate mass instrumentation appears very promising for studying correlation between performance and sweat. However, significant analytical development is still necessary to accurately determine sweat concentrations of amino acids and several other metabolites to meet the human performance predictive goals of the United States Air Force (14, 15, 22-24).

Summary

The long-term goal of the US Air Force is to develop a wearable sensor to predict an individual's performance state. A downstream vision of a sensor could be something integrated onto a bracelet or smart watch with inexpensive and easily deployable components. Additional significant research is required to evaluate the potential to obtain this goal.

Of all the bio-fluids sweat may be useful in detecting changes in a human subject's performance state. Although several of the analyte types found within sweat show promise, the metabolome will be the focus of this study. While some initial studies have been performed on the analysis of sweat metabolites much is still unknown. Additionally, only a few LC-MS studies have looked quantitatively at sweat metabolites (26).

Analyzing quantitatively is important for several reasons. First, if a sensor was created to monitor human performance (the long term goal) the sensor would need to have a defined working range. Second, while many things are known to affect sweat metabolite content, including sweat rate, personal hygiene, drugs, and topical products the extent of the effects are unknown. Further, the extent of unknown sweat content

affecters will need to be defined. So, while the ultimate goal for the Air Force is to link sweat to performance, this study strives to define the metabolite deviations caused by diet, by quantitating selected sweat metabolites in subjects consuming different diets.

Methods

Sweat Collection

The sweat collection for the experiments throughout this document was performed previously as part of another experiment (22). Briefly, the forearms of each subject were wiped with isopropyl alcohol wipes (one per arm) and rinsed with tap water. After the subject's forearms were given ample time to air dry a sweat patch, as described above, was placed on each forearm. The sweat patch consisted of a packet of four pieces of heat fused 100 um nylon mesh covered with a virgin polyethylene layer. An Opsite adhesive bandage was placed over the mesh and polyethylene to hold everything in place. This combination of materials was collectively called the "Patch" (Figure 1C). Patches were placed on the hairless section of the upper forearm of the 14 subjects prior to periods of exercise.

Once the exercise protocol was complete, the sweat contained in the patch was collected with a blunt tipped needle into a 5mL syringe and placed into separate (one per arm) Ultrafree-CL 0.1 µm PVDF membrane centrifugal filters. The nylon mesh was removed from each patch with forceps and added to the respective filters to insure all sweat was collected. After the sweat and mesh were placed in the filters they were individually centrifuged at 3000 ×g for 10 min at 4 °C. After filtration sweat samples from each subject's arms were combined and vortexed. The pooled samples were

aliquoted into 250 µL aliquots and lyophilized (Labconco, Kansas City, MO, USA) overnight to insure dryness. The samples were then stored at −80 °C until analysis.

Optimization of the Liquid Chromatography with an Amino Acid Standard

Previous work within our lab utilized an LC-MS method to aid in sweat metabolite discovery (23). From these data, twenty four metabolites (Table 1) were chosen to be investigated further by quantitative approaches. In order to achieve this goal, evaluation and optimization of the liquid chromatography method was needed to improve separation and ultimately quantitation by mass spectrometry, which was the objective and focus of this thesis.

Standards Preparation

The Amino Acid Standard H by Thermo Scientific (Waltham, MA, USA) was used to perform all optimization of the LC separation experiments. The standard is a mixture of 17 amino acids with a concentration of 2.5±0.1 µM mL^{-1} with an additional amino acid (cysteine) concentrated at 1.25±0.1 µM mL^{-1}. Of the 17 amino acids contained in the Amino Acid Standard H, 12 can be effectively detected by positive ionization mass spectrometry (denoted with an * in Table 2). The neat standard solution as provided by the manufacturer was diluted to a 1000 µM stock solution using 50% acetonitrile (aq) (water, acetonitrile Optima Grade, Thermo Scientific) and stored at 4ºC until use. Additionally, a second dilution of 10µM was made using 50% acetonitrile (aq) and stored at 4ºC until use.

Table 1: General information for metabolites of interest and other compounds utilized throughout this study

Compound	Manufacturer	CAS#	Purity	Accurate Mass (amu)
Phenylalanine	Thermo Fisher	63-91-2	98%	165.0790
Phenylalanine 13C9 15N	Cambridge Isotope Laboratories	N/A	98%	175.1062
Leucine	Thermo Fisher	61-90-5	98%	131.0946
Leucine 13C6 15N	Cambridge Isotope Laboratories	202406-52-8	98%	137.1147
Isoleucine	Thermo Fisher	73-32-5	98%	131.0946
Isoleucine 13C6 15N	Cambridge Isotope Laboratories	N/A	98%	137.1147
Methionine	Thermo Fisher	63-68-3	98%	149.0511
Methionine 13C5 15N	Cambridge Isotope Laboratories	N/A	98%	163.1151
Valine	Thermo Fisher	72-18-4	98%	117.0790
Valine 13C5 15N	Cambridge Isotope Laboratories	N/A	98%	123.0927
Proline	Thermo Fisher	147-85-3	98%	115.0633
Proline 13C5 15N	Cambridge Isotope Laboratories	N/A	98%	121.0771
Tyrosine	Thermo Fisher	60-18-4	98%	181.0739
Tyrosine 13C9 15N	Cambridge Isotope Laboratories	202407-26-9	98%	190.1041
Alanine	Thermo Fisher	56-41-7	98%	89.0477
Alanine 13C3 15N	Cambridge Isotope Laboratories	312623-85-1	98%	92.0577
Glutamic Acid	Thermo Fisher	56-86-0	98%	147.0532
Glutamic Acid 13C5 15N	Cambridge Isotope Laboratories	N/A	98%	152.0699
Histidine	Thermo Fisher	71-00-1	98%	110.0718
Histidine:HCL:H2O 13C6 15N3	Cambridge Isotope Laboratories	N/A	98%	119.0718
Arginine	Thermo Fisher	74-79-3	98%	174.1117
Arginine:HCL 13C6 15N4	Cambridge Isotope Laboratories	202468-25-5	98%	184.1117
Lysine	Thermo Fisher	70-54-2	98%	146.1055
Lysine:2HCL 13C6 15N2	Cambridge Isotope Laboratories	N/A	98%	154.1055
Ornithine	Sigma Aldritch	70-26-8	98%	132.0899
Citrulline	Sigma Aldritch	372-75-8	98%	175.0957
Citrulline 2,3,3,4,4,5,5-D7	CDN Isotopes	N/A	98%	182.2300
5-Hydroxyectoine	Sigma Aldritch	165542-15-4	95%	158.0691
Carnitine	Sigma Aldritch	6645-46-1	98%	161.1052
Pyrrolidine	Sigma Aldritch	123-75-1	99%	71.0735
Pyrrolidine 2,2,3,3,4,4,5,5-D8	Cambridge Isotope Laboratories	212625-79-1	98%	79.1230
Taurine	Sigma Aldritch	107-35-7	99%	125.0147
Taurine 13C2 15N	Cambridge Isotope Laboratories	N/A	98%	127.1420
Diethanolamine	Sigma Aldritch	111-42-2	98%	105.0790
Dimethylethanolamine	Sigma Aldritch	108-01-0	99%	89.0840
Trolamine	Sigma Aldritch	102-71-6	99%	149.1052
Choline	Sigma Aldritch	67-48-1	99%	104.1075
Choline 12C2 15N	Sigma Aldritch	1219388-71-2	99%	106.1730
Creatinine	Sigma Aldritch	60-27-5	98%	113.0589
Creatinine- D3 (Methyl-D3)	CDN Isotopes	143827-20-7	99%	116.1400
Prolinamide	Sigma Aldritch	7531-52-4	98%	114.0793

Table 2: Metabolites contained in the Amino Acid Standard H (* indicates metabolite analyzed in this study)

Amino Acid Standard H Metabolites
L-Alanine*
L-Arginine*
L-Aspartic Acid
L-Cystine
L-Glutamic Acid*
Glycine
L-Histidine*
L-Isoleucine*
L-Leucine*
L-Lysine HCl*
L-Methionine*
L-Phenylalanine*
L-Proline*
L-Serine
L-Threonine
L-Tyrosine*
L-Valine*

Mass Spectrometry Settings

A Thermo Scientific LTQ Orbitrap XL mass spectrometer affixed with a heated ESI source was used for all experiments performed in chapter one. The ESI source was operated in positive ionization mode at 3.5 kV with a capillary temperature of 280 °C, a sheath gas of 30(no units), and aux gas of 10 (no units). Scans were made at a resolution of 60,000 (no units) across 65-400 mass over charge (m/z).

High Performance Liquid Chromatography: Initial Settings

All analyses were performed on a Vanquish Ultra High Pressure Liquid Chromatography (UPLC) system from Thermo Fisher Scientific. The analysis used a 2µL injection of the 1000µM Amino Acid Standard H solution prepared as described in the previous section unless otherwise noted.

The Vanquish system was setup with a flow rate of 1mL min^{-1} affixed with a Phenomenex Luna Hydrophilic Interaction Chromatography (HILIC) column (3µm, 200 Å, 100 x 3mm, Torrance, CA, USA). The mobile phase A consisted of 10 mM Ammonium Formate (≥99.0%, Sigma Aldritch, St. Louis, MO, USA) in 4.5% acetonitrile (aq). Mobile Phase B contained 10mM Ammonium Formate in 95.5% acetonitrile (aq). The initial starting conditions were 97% B mobile phase held for five minutes. From five minutes to 18.5 minutes the percent of mobile phase B transitioned to 65% with a five minute hold at 65% (23.5 minutes).The mobile phase composition returned to the initial conditions (97% B) from 23.5 minutes to 24.5 minutes and held at 97% B for an additional 5.5 minutes for a 30 minute total analysis time. The resulting data was

evaluated for chromatographic peak shape and resolution as described in the following section.

Data Analysis

Raw data (samples and blanks) were collected and analyzed using Xcalibur (2.2 Thermo Fisher) software. For the optimization of the liquid chromatography the peak shape was defined by full width at half maximum (FWHM) (Figure 3). Resolution between two peaks is defined as the difference between the two peaks retention time (RT) divided by the average FWHM (W) of the two peaks or

$$R = (RT2-RT1) / (½(W1+W2).$$

Figure 3 shows an example for the calculation of FWHM and resolution. Both the peak shape and resolution were determined using the Xcalibur software.

For the development of the calibration curves scatter plot graphs were generated in Microsoft Excel (v. 2007, Remond, WA). Each calibration curve graph used the theoretical concentration of each calibrant (x value) and the peak area of the given compound (instrument response, y value) as the coordinates of the scatterplot points. Raw data analysis for peak area determination was performed with the Xcalibur software. Excel was used to provide a line of best fit for the y scatter points. Using the equation of the line of best fit and the unknown peak area corresponding to calibrated compounds from the sweat samples, the concentration of the specific metabolites in the sweat were approximated. The sweat sample's peak area for a given metabolite was entered as the y value in the line of best fit, (y=mx+b) and the found value of x yielded the approximated metabolite concentration of the sample.

Figure 3: Example for calculating full width at half maximum (FWHM) and resolution between peaks (R)

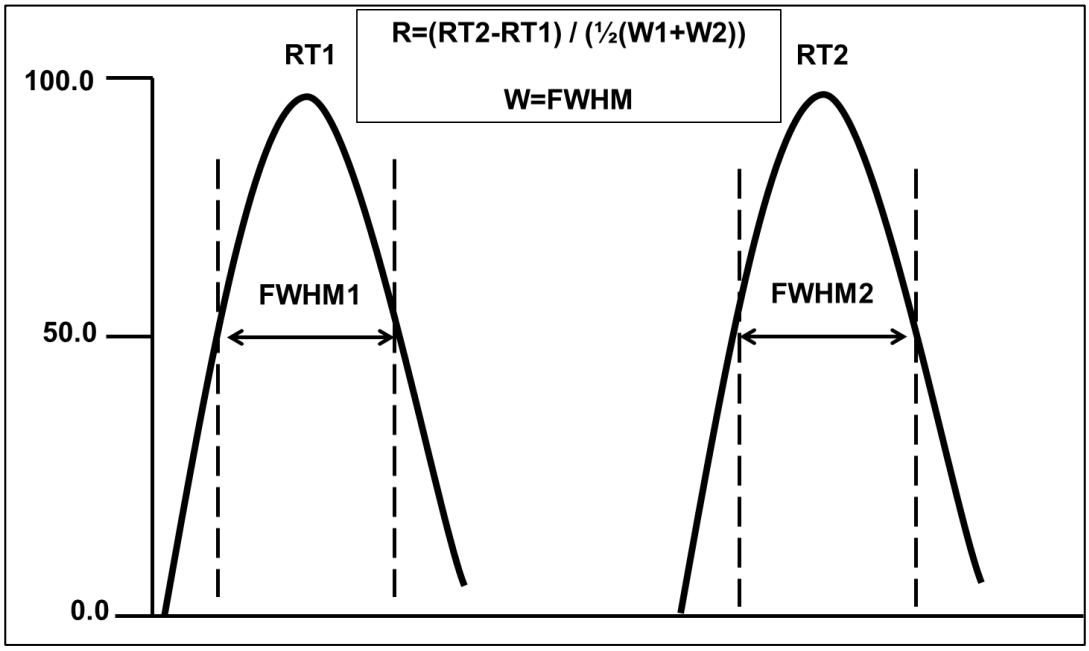


Table 3: Initial and optimized liquid chromatography parameters

Parameter	Initial Settings	Optimized Settings
Run Time	30min	30min
Flow Rate	1000µL/min	170µL/min
Stationary Phase	Luna HILIC	BEH Amide
Moblie Phase	HILIC	HILIC
Mobile Phase Gradient	97%	90%

Optimization of the Liquid Chromatography

All HPLC optimization experiments utilized the Amino Acid Standard H dilutions, as described previously, 1000 µM or 10 µM. The flow rate was evaluated to improve the liquid chromatography methodology to achieve optimized separations. Flow rate was evaluated on two stationary phases, the Luna HILIC Column and the Waters Acquity BEH Amide Column (130 Å, 1.7 µm, 2.1mm x 100mm, Water Corp., Milford, MA, USA). To further optimize separations, the mobile phase gradient was evaluated on the Waters Acquity BEH Amide Column. Three parameters were evaluated to improve the liquid chromatography methodology (flow rate, stationary phase, and mobile phase gradient) to achieve optimized separations (Table 3).

Flow Rate Optimization

To begin, utilizing the initial conditions described above, several mobile phase flow rates (1.000mL min^{-1}, 0.500 mL min^{-1}, 0.300 mL min^{-1}, 0.170 mL min^{-1}, and 0.100 mL min^{-1}) were evaluated with the Luna HILIC column. A 2 µL injection of the 1000 µM Amino Acid Standard H was evaluated with each flow rate for improvements, as compared to similar injections performed under the initial conditions, in peak shape (narrower individual peaks) and resolution (peaks better separated). Additionally, as described in the previous section, the Waters Acquity BEH Amide column was assessed. As a result, the flow rate was reevaluated with the alternative column at 0.300 mL min^{-1}, 0.170 mL min^{-1}, and 0.100 mL min^{-1}. The data were evaluated for peak shape and resolution as described previously.

Mobile Phase Gradient Optimization

Once the optimized flow rate was decided upon (0.170 mL min^{-1}) two mobile phase gradient initial conditions (97% B and 90% B) were evaluated with the Waters Acquity BEH Amide Column. A two µL injection of both the 1000 µM and 10 µM Amino Acid Standard H were evaluated with each mobile phase gradient initial condition for narrowing of the peak shapes and an increase of the resolution with the goal of increasing separation among the metabolite peaks.

Final Optimized LC Conditions

The optimized settings were utilized by the Vanquish system affixed with a Waters Acquity BEH Amide column. The flow rate was 0.170 mL min^{-1} (Table 3). Mobile phases A and B remained unchanged. The initial starting conditions were 90% B mobile phase held for five minutes. From five minutes to 18.5 minutes the percent of mobile phase B transitioned to 65% with a five minute hold at 65% (23.5 minutes).The mobile phase composition returned to the initial conditions (90% B) from 23.5 minutes to 24.5 minutes and held at 90% B for an additional 5.5 minutes for a 30 minute total analysis time.

Validation of the Liquid Chromatography

Optimization of the HPLC parameters was performed with only the Amino Acid Standard H solutions (Table 2). However, the target compound list, determined from a previous experiment, contained several additional metabolites (ornithine, 5-hydroxyectoine, carnitine, pyrrolidine, taurine, diolamine, dimethylethanolamide, trolamine, choline, creatinine, and prolinamide) that require evaluation for analytical

performance in conjunction with the Amino Acid Sandard H solution (Table 1). Therefore, further analysis of these additional compounds, is required to fully validate the optimized parameters for use with sweat samples.

Standard Preparation

For the remaining metabolites of interest, 10 mL of 200 mM solutions were prepared in water (Optima, Thermo Scientific). These solutions were then combined with the neat Amino Acid Standard H (2.5 mM) and 50% acetonitrile (aq) to yield an overall concentration of 1000 µM of all compounds. The mixture of all the metabolites of interest was created to be analyzed in subsequent experiments. The overall mixture was further diluted to 10 µM for LC-MS analysis utilizing the optimized parameters along with the 1000 µM mixture.

Sweat Metabolite Calibration Curve Development

In order to determine the accurate range of each metabolite of interest in sweat, three calibration curves were created and analyzed. Each calibration curve was created with increasingly refined ranges of concentration to find the most accurate concentration range for the selected metabolites.

Standards Development

The standard prepared for the LC validation, containing the 24 selected metabolites (Table 1) and concentrations at 1000 µM was used for this experiment. The standard was used as a stock and diluted to create the different calibration points used in the calibration curve tests.

Calibration Curve Test 1

The stock of all 24 metabolites was diluted into several aliquots with several concentrations ranging from 10 µM-1000 µM (Table 4). To check to see if this range was accurate for sweat samples, five lyophilized sweat samples (250 µL before being lyophilized) were re-suspended in 250 µL of 50% aqueous acetonitrile. The calibration curve and samples were analyzed by LC-MS using the optimized HPLC method with the peak areas determined using Xcalibur software as described previously. As described previously, a scatter plot graph was developed in Microsoft Excel to find the line of best fit. The concentration of the metabolites in the sweat samples was approximated using the peak area and the line of best fit equation.

Calibration Curve Test 2

In the first calibration curve test the different selected metabolites had widely varying concentrations compared to the calibrated range. To better capture selected metabolites within a calibration curve, two separate curves, a low 5-50 µM and a high 5-500 µM curve (Table 5) were developed from the mixture containing the 24 metabolites. The two calibration ranges were chosen based on the outcomes of the sweat results from the first calibration test. The two calibration curves were analyzed with ten new sweat samples in the same manner as described previously. Data were evaluated as described in the previous section.

Calibration Curve Test 3

Calibration curve tests showed that the different metabolite concentrations were too diverse for one or several grouped calibration curves.

Table 4: Test 1 results. Calibration curve and observed sample ranges for twenty-four selected metabolites of interest from sweat. The dynamic range among the selected sweat metabolites was too great for a universal calibration curve.

Compound	Calibration Points (µM)	Sample Range (µM)
Ornithine	10;20;40;100;200;500;1000	40-130
Citruline	10;20;40;100;200;500;1000	<10-180
5-Hydroxyectone	10;20;40;100;200;500;1000	<10
Carnitine	10;20;40;100;200;500;100	<10
Pyrrolidine	10;20;40;100;200;500;1000	<10
Taurine	10;20;40;100;200;500;1000	<10
Diolamine	10;20;40;100;200;500;1000	<10
Dimethylethanolmide	10;20;40;100;200;500;1000	<10
Trolamine	10;20;40;100;200;500;1000	<10-20
Choline	10;20;40;100;200;500;1000	<10
Creatinine	10;20;40;100;200;500;1000	<10
Prolinamide	10;20;40;100;200;500;1000	<10
Phenylalaine	10;20;40;100;200;500;1000	<10-30
Alanine	10;20;40;100;200;500;1000	40-220
Arginine	10;20;40;100;200;500;1000	<10-50
Glutamic Acid	10;20;40;100;200;500;1000	<10-80
Histidine	10;20;40;100;200;500;1000	<10-120
Isoleucine	10;20;40;100;200;500;1000	<10-50
Leucine	10;20;40;100;200;500;1000	10-160
Lysine	10;20;40;100;200;500;1000	<10-30
Methionine	10;20;40;100;200;500;1000	<10-30
Proline	10;20;40;100;200;500;1000	<10-40
Tyrosine	10;20;40;100;200;500;1000	<10-20
Valine	10;20;40;100;200;500;1000	<10-60

Table 5: Test 2 results. Calibration curves and observed sample ranges for twenty-four selected metabolites of interest from sweat. The dynamic range among the selected sweat metabolites was too great for only two calibration curves.

Compound	Calibration Points (µM)	Sample Range (µM)
Ornithine	5;50;150;250;350;450;500	33-106
Citruline	5;50;150;250;350;450;500	<5-188
5-Hydroxyectone	5;10;15;25;35;45;50	<5
Carnitine	5;10;15;25;35;45;50	<5
Pyrrolidine	5;10;15;25;35;45;50	<5
Taurine	5;10;15;25;35;45;50	<5
Diolamine	5;10;15;25;35;45;50	<5
Dimethylethanolmide	5;10;15;25;35;45;50	<5
Trolamine	5;10;15;25;35;45;50	<5-16
Choline	5;10;15;25;35;45;50	<5
Creatinine	5;10;15;25;35;45;50	<5
Prolinamide	5;10;15;25;35;45;50	<5
Phenylalaine	5;10;15;25;35;45;50	6-18
Alanine	5;50;150;250;350;450;500	47-236
Arginine	5;50;150;250;350;450;500	<5-45
Glutamic Acid	5;50;150;250;350;450;500	9-82
Histidine	5;50;150;250;350;450;500	7-110
Isoleucine	5;10;15;25;35;45;50	5-48
Leucine	5;50;150;250;350;450;500	13-174
Lysine	5;50;150;250;350;450;500	<5-22
Methionine	5;50;150;250;350;450;500	<5-27
Proline	5;10;15;25;35;45;50	<5-30
Tyrosine	5;10;15;25;35;45;50	<5-19
Valine	5;50;150;250;350;450;500	11-50

Table 6: Test 3 results. Calibration curves, observed sample ranges, and limit of detection (LOD) determination for twenty-four selected metabolites of interest from sweat. Sweat metabolites required individualized calibration curves, however the concentration of some metabolites was too low for quantification.

Compound	Calibration Points (µM)	Sample Range (µM)	LOD (µM) = 5x noise
Ornithine	5;50;100;150;200;250;300	42-127	2.0
Citruline	5;50;100;150;200;250	<5-176	1.5
5-Hydroxyectone	5;10;15;20;25	<5	3.0
Carnitine	5;10;15;20;25	<5	2.5
Pyrrolidine	5;10;15;20;25	<5	1.0
Taurine	5;10;15;20;25	<5	1.5
Diolamine	5;10;15;20;25	<5	2.0
Dimethylethanolmide	5;10;15;20;25	<5	3.0
Trolamine	5;10;15;20;25	<5-12	1.5
Choline	5;10;15;20;25	<5	2.5
Creatinine	5;10;15;20;25	<5	1.0
Prolinamide	5;10;15;20;25	<5	2.5
Phenylalaine	5;10;15;20;25	8-21	3.0
Alanine	5;50;100;150;200;250;300	43-218	10.0
Arginine	5;15;25;35;45;55	<5-47	2.0
Glutamic Acid	5;15;25;35;45;55;65	7-76	1.0
Histidine	5;50;100;150;200	10-117	8.0
Isoleucine	5;15;25;35;45;55	9-41	1.5
Leucine	5;50;100;150;200	9-153	1.5
Lysine	5;10;15;20;25;30	<5-25	1.5
Methionine	5;10;15;20;25;30	<5-23	2.0
Proline	5;10;15;20;25;30	<5-38	1.5
Tyrosine	5;10;15;20;25	<5-16	2.0
Valine	5;15;25;35;45;55	<5-54	3.0

Therefore individual calibration curves were developed for each selected metabolite (Table 6). The curves were generated based on the ranges of the samples from the previous two calibration curve tests. Ten new sweat samples were analyzed with the refined calibration ranges in the same manner performed as described for the previous calibration curve tests.

Limit of Detection Tests

The 1000 µM stock of all the metabolites of interest was diluted to concentrations at every 0.5 µM from 0 µM to 5 µM. Each of these samples was analyzed using the optimized LC settings previously developed. Using Excalibur software each metabolite of interest at each concentration was compared to the surrounding noise discovering, the ratio of signal to noise based on peak height. The limit of detection was determined by finding the lowest concentration at which the metabolite peak height was at least five times the surrounding noise height, thus the minimum S/N ratio was five (Table 6).

Results and Discussion

Optimization of the Liquid Chromatography

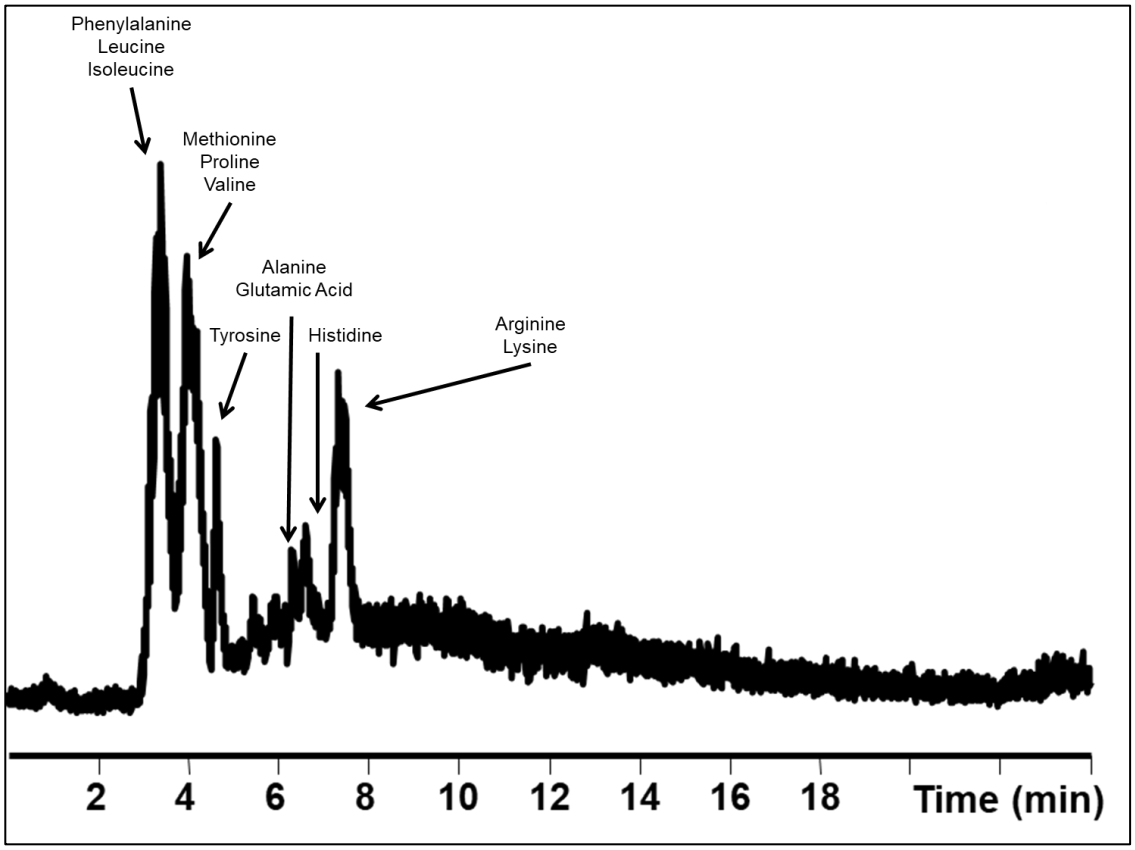
In a previous discovery experiment within our lab, 48 metabolites were tentatively identified from human sweat with 29 verified utilizing retention time comparisons with neat standards and MS^n fragmentation patterns (23). As the methodologies were evaluated to transition from discovery to quantitative approaches, the need for LC methodology optimization became evident. For example, the LC method, utilized by Harshman et al., was unable to separate high abundant metabolites such as phenylalanine,

isoleucine and leucine. One possible reason for the inability to separate phenylalanine, leucine and isoleucine is the oxygen tails on these metabolites. Additionally, co-elution of several of the other metabolites, including methionine, proline, and valine was observed in the study (Figure 4). To address this problem, chromatographic parameters influencing peak shape and peak resolution, such as flow rate and analytical column stationary phase, were evaluated for optimization. As a result, a much improved liquid chromatographic method for the detection of sweat metabolites was determined.

Standard Selection

Several discovery studies have tentatively identified a variety of small molecules in human sweat encompassing a wide variety of chemical families including lipids, benzene derivatives, amino acids, carboxylic acids, organoheterocyclic compounds, alkaloids, and carbohydrates (6, 8, 9, 15-18, 21- 24, 26, 29, 30- 32). For example, in a 2016 study by Hooton et. al. 83 metabolites were identified from human sweat while a more recent study conducted by Delgado-Povedano et. al identified 118 compounds (15, 26). Of the many discovery investigations into sweat metabolite content, individual amino acids were found most frequently among the highest abundant metabolites (8, 9, 15, 16, 22-24, 26, 29- 31). As a result, focus has been placed on amino acids for biomarker discovery applications. For instance, a study by Calderon-Santiago et.al. successfully used select sweat amino acid content, among other metabolites, to screen for lung cancer (9). Furthermore, research conducted in our lab evaluating sweat metabolite abundance from airman illustrated that several amino acids were some of the most abundant metabolites in exercise induced sweat (22-24).

Figure 4: Chromatogram of the Amino Acid Standard H when analyzed utilizing the original LC-MS method. Poor peak shape and resolution lead to peak co-elution. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)



From these studies, alanine was identified as the most abundant amino acid in sweat with an approximate concentration range of 99µM to 355µM (22-24). It is possible alanine sweat concentrations are linked to the high alanine concentrations in blood. While the amino acids have been found most frequently in sweat, other compounds have been routinely identified from human sweat discovery experimentation (8, 14, 15, 23, 26, 30). For example, citrulline, choline and creatinine are frequently identified in sweat discovery experimentation (15, 23, 26). As a result, 24 total compounds were selected for further quantitative investigation within sweat based on data generated from our lab and literature reports (Table 1) (9, 14-16, 22, 23, 26, 29).

In addition to overall frequency of identification within sweat discovery experiments, the metabolites selected for quantitative analysis were also chosen for their participation in specific metabolic pathways. As the Air Force has a unique interest in prediction of performance, metabolite participation in energy metabolism are considered interesting candidates. For example, during prolonged exercise anaerobic metabolism becomes inefficient for the energy expenditure. Aerobic metabolism utilizing glucose, pyruvic acid, free fatty acids from adipose tissue, and amino acids from protein catabolism are used (34). Small molecules related to protein catabolism, for instance alanine, and small molecules related to oxidation of fatty acids, particularly carnitine, were among the candidates chosen (Table 7) (34). The urea cycle is used to process waste from protein catabolism (34). Metabolites in the urea cycle, such as ornithine, were also considered for analysis (Table 7) (34). While these are only a few examples of potential pathways associated with metabolites selected, further mechanistic studies for the source of sweat metabolites are warranted.

Table 7: A sample of metabolic pathways represented by the metabolites of interest provides a possible link between sweat content and human performance. Pathways were determined from the Human Metabolome Database (https://hmdb.ca)

Metabolite	Pathways
Phenylalanine	Phenylalanine and Tyrosine Metabolism
Leucine	Valine Leucine and Isoleucine Degradation, Transcription/ Translation
Isoleucine	Valine Leucine and Isoleucine Degradation,
Methionine	Betaine Metabolism, Methionine Metabolism, Glycine and Serine Metabolism, Transcription and Translation
Valine	Transcription/ Translation, Arginine and Proline Metabolism
Proline	Valine Leucine and Isoleucine Degradation, Propanoate Metabolism, Transcription/ Translation
Tyrosine	Phenylananine and Tyrosine Metabolism, Transcription/ Translation
Alanine	Transcription/ Translation, Urea Cycle, Glucose-Alanine Cycle, Alanine Metabolism
Glutamice Acid	Alanine Metabolism, Histidine Metabolism, Malate-Aspartate Shutle, Ammonia Recycling, Cysteine Metabolism
Histidine	Histidine Metabolism, Ammonia Recycling, Transcription/ Translation
Arginine	Urea Cycle, Transcription/ Translation, Arginine and Proline Metabolism, Glycine and Serine Metabolism
Lysine	Transcription/ Translation, Biotin Metabolism, Carnitine Synthesis
Ornithine	Arginine and Proline Metabolism, Glycine and Serine Metabolism, Urea Cycle
Citrulline	Arginine and Proline Metabolism, Aspartate Metabolism, Urea Cycle
5-Hydroxyestrone	N/A
Carnitine	Beta Oxidation of Long Chain and Branched Chain Fatty Acids
Pyrrolidine	N/A
Taurine	Taurine and Hypotaurine Metabolism, Bile Acid Biosynthisis
Dimethylethanolamine	N/A
Trolamine	N/A
Choline	Betaine Metabolism, Methionine Metabolism, Phospholipid Biosynthesis
Creatinine	N/A
Prolinamide	N/A
Diolamine	N/A

Method optimization requires a consistent representative sample to adequately determine improvements. For this reason, the Thermo Scientific Amino Acid Standard H, a neat mixture of 17 amino acids, was chosen for chromatographic optimization experiments. Interestingly, the isobars (compounds that have the same exact mass), leucine and isoleucine, contained in the Amino Acid Standard H cannot be separated by mass spectrometry and must be chromatographically separated for detection. The Amino Acid Standard H was utilized at both 1000µM, a concentration to allow for optimum signal within the mass spectrometer, and 10µM, a representative concentration similar to that observed in excreted human sweat.

HPLC Parameters

The liquid chromatographic settings utilized in previous discovery experiments within our lab have illustrated poor separation, low chromatographic peak resolution, and broad peaks with standard injections of the Amino Standard H (Figure 4) (22- 24). Peak separation is the ability to separate metabolites into individual peaks. Chromatographic peak resolution is the ability to adequately distinguish between two peaks. Peak width is the length of time needed for a peak to complete elution. For example, the first two peaks in Figure 4 contain phenylalanine, leucine, isoleucine, methionine, proline and valine.

Breaking the poor chromatogram down further the phenylalanine peak had poor shape as characterized by a full width at half height (FWHM; lower FWHM= narrower peak) of 0.25 minutes, while the FWHM for the isoleucine peak was 0.35 minutes (FWHM is rounded to nearest half of a tenth of a minute). The resolution between the two peaks was 0.30 (no units) suggesting they are heavily overlapping (larger resolution=

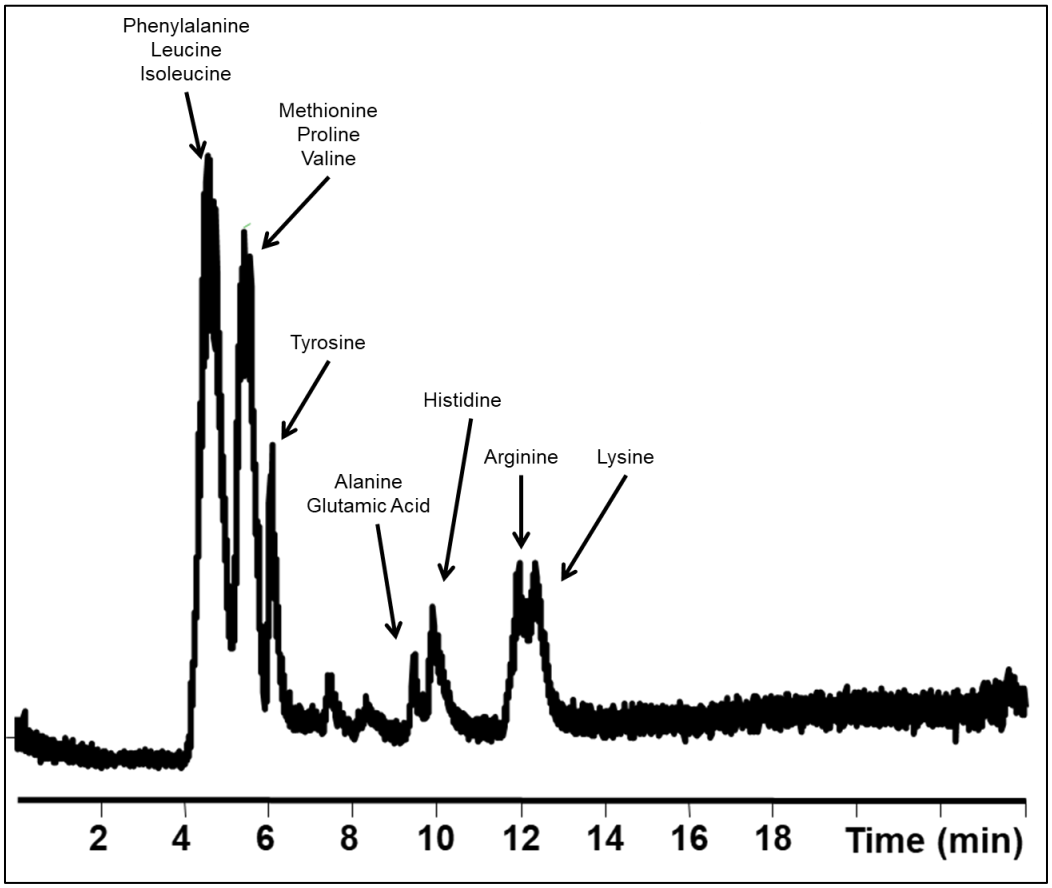
better separation). Each of these attributes, peak width and resolution, can lead to poor instrumental sensitivity resulting from dynamic range limitations of the mass spectrometer.

As a result, the chromatographic methodology required optimization to improve overall sensitivity (intensity of signal recorded) for quantitation. Method optimization began with varying the mobile phase flow rate to allow compounds more time to interact with the stationary phase of the column yielding greater separation (Figure 5).

The chromatogram in Figure 5 of a 1000 µM injection of Amino Acid Standard H showed reducing the flow rate from 1.000 mL min^{-1} to 0.300 mL min^{-1} had a slight positive effect on the resolution of the first two peaks containing phenylalanine, leucine, isoleucine, methionine, proline, valine, and tyrosine (Figure 5). However, many of the metabolites continued to co-elute. For example, the resolution between phenylalanine and isoleucine increased from 0.30 to 0.40. In contrast, the peak width of the compounds worsened with the FWHM of phenylalanine and isoleucine increased from 0.25 minutes to 0.30 minutes and from 0.35 minutes to 0.55 minutes respectively.

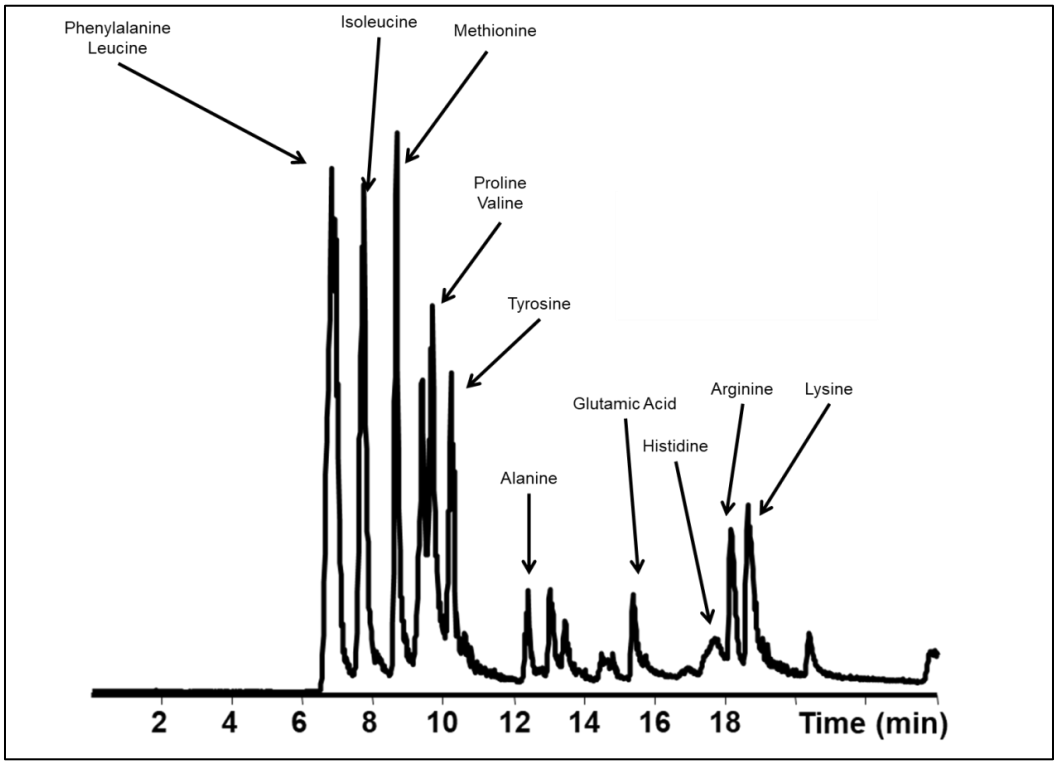
While reducing the flow rate provided some positive results, the resolution was still poor with heavy overlapping between peaks (Figure 5). Later eluting compounds showed improvement in their resolution. For example, arginine and lysine which were unresolved with the original flow rate (1.000 mL min^{-1}) began to separate (compare Figures 4 and 5). While a slight improvement in resolution was observed by reducing the flow rate, many peaks remained unresolved and peak shape remained poor or worse (Figures 4 and 5).

Figure 5: Chromatogram of the Amino Acid Standard H when analyzed utilizing the original LC-MS method except, with the flow rate decreased from 1.000 mL min^{-1} to 0.300 mL min^{-1}. While an improvement from the original methods, greater separation is needed. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)



Since reducing the flow rate showed minimal improvement in chromatographic separations, an evaluation for an alternative analytical column was conducted. The initial experimentation, performed by Harshman et al., used a silica based HILIC Luna column. As an alternative, a BEH Amide column, which utilizes the chemically stable, tri-functionally bonded amide phase for separation was identified. A bridged ethylene hybrid technology with no net charge makes up the remainder of the column packing, allowing the amide phase to separate without interference from the remainder of the column. As a result, the BEH Amide column was evaluated to determine the efficiency of separation utilizing the Amino Acid Standard H solution. Figure 6 shows a 1000 µM injection of the Amino Acid Standard H on the BEH Amide column. Compared to the Luna HILIC column, where many compounds co-eluted with poor chromatographic resolution, the BEH Amide column separated many of the compounds with baseline resolution among peaks (Figures 6). For instance, the phenylalanine peak and isoleucine peak were not separated by the Luna HILIC column with the best resolution achieved being 0.40. The two metabolite peaks were separated by the BEH Amide column with a resolution of 4.90. However, not all compounds were separated. For example, phenylalanine and leucine continue to co-elute with the BEH Amide column (Figure 6). The BEH Amide column also improved peak shape. The FWHM of phenylalanine and isoleucine were respectively 0.30 minutes and 0.55 minutes when analyzed at a flow rate of 0.300 mL min^{-1} on the Luna HILIC column. When analyzed under the same conditions using the BEH Amide column the FWHM improved for phenylalanine and isoleucine to 0.20 minutes and 0.20 minutes respectively. Although the BEH Amide column showed

Figure 6: Chromatogram of the Amino Acid Standard H when analyzed utilizing the original LC-MS method, except with the flow rate decreased from 1.000 mL min^{-1} to 0.300 mL min^{-1} and the BEH Amide analytical column in place of the Luna HILIC column. The separation from the BEH Amide column is significantly improved over the Luna HILIC column. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)



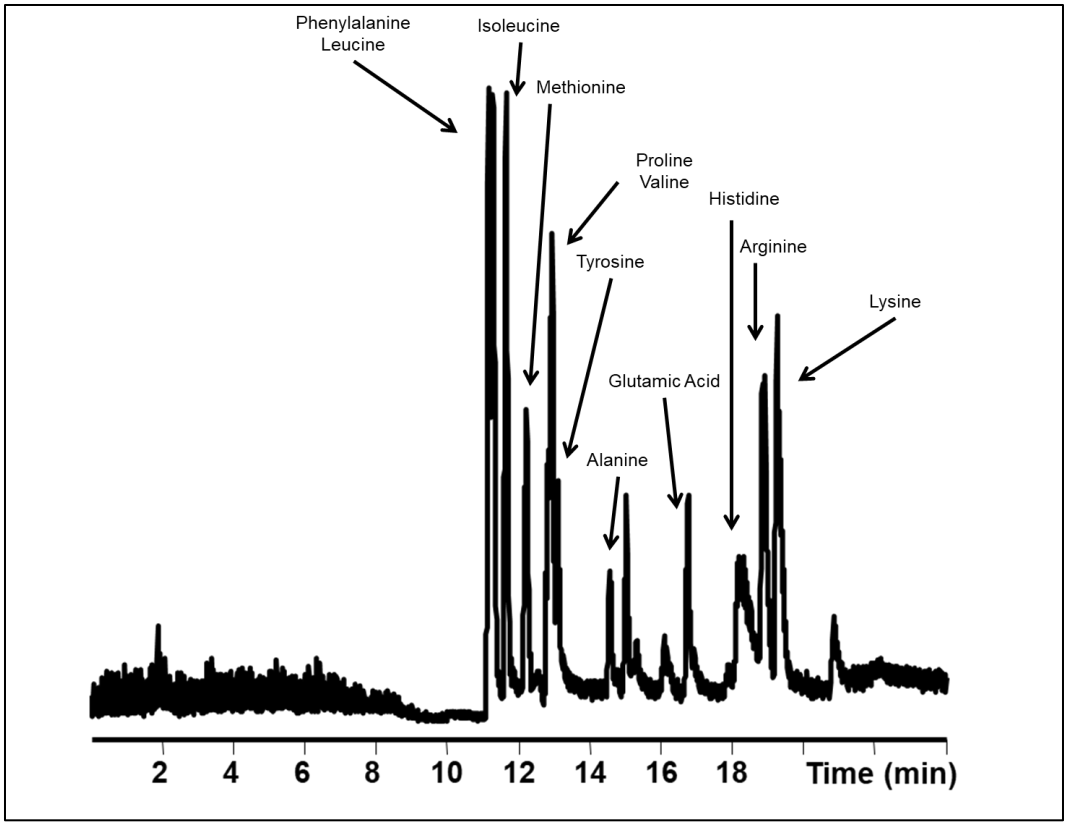
improved separations and peak widths, additional parameters were required for full optimization to separate co-eluting compounds.

Similar to the flow rate changes performed with the HILIC Luna column a reduced flow rate was evaluated on the BEH Amide column with a 1000 µM injection of the Amino Acid Standard H. The change in flow rate from 0.300 mL min^{-1} to 0.170 mL min^{-1} illustrated significant improvement in peak width. For example, the FWHM of phenylalanine and isoleucine both decreased from 0.20 minutes with 0.300mL min^{-1} flow rate to 0.10 minutes at 0.170 mL min^{-1} column flow. In contrast, the resolution had improvements and deteriorations with the resolution between the example phenylalanine and isoleucine peaks decreasing from 4.90 to 4.80, but resolution improving between the phenylalanine and the leucine peaks leading to separation (Figure 7). The overall effect of reducing the flow rate was an improvement. However, one more parameter remained to be optimized.

The initial mobile phase composition of 97% mobile phase B was a residual parameter from experimentation performed with the Luna HILIC column. As the literature provided with the BEH Amide column recommended higher aqueous phase (mobile phase A) compositions, the final parameter assessed was evaluating the mobile phase gradient. To this end, the percentage of mobile phase B (with mobile phase A completing the total mobile phase) was lowered from 97% to 90% at initial conditions.

Utilizing both the 1000 µM and 10 µM standards the higher initial mobile phase B percentage (97%) illustrated increased background noise, compared to the lower percent mobile phase B (90%) in the lower concentration injections. As a result of the reduction

Figure 7: Chromatogram of the Amino Acid Standard H when analyzed utilizing the original LC-MS method except, with the flow rate decreased from 1.000 mL min^{-1} to 0.170 mL min^{-1} and the BEH Amide analytical column in place of the Luna HILIC column. By reducing the flow rate with the BEH Amide column separation is improved. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)



in background noise, the lower initial mobile phase B percentage (90%) allowed for improved signal to noise ultimately leading to the potential for quantitation of low abundance metabolites. The improvements outweighed the increase in peak width at the lower concentration (Figure 8). Utilizing the higher concentration not only was the signal to noise improved, resolution also improved. For example, the resolution between the phenylalanine and isoleucine peaks increased from 4.80 to 5.4. The FWHM for phenylalanine remained at 0.10 minutes while it decreased for isoleucine from 0.10 minutes to 0.05 minutes (Figure 9). Changing the initial conditions of the mobile phase gradient as suggested in the column literature improved the quantitation potential of the analysis.

Using the optimized LC settings the resolution between the phenylalanine and isoleucine peaks was 5.47 compared to 0.30 when utilizing the original settings used by Harshman et.al. (23). The FWHM of both phenylalanine (0.25 minute to 0.10 minute) and isoleucine (0.35 minute to 0.05 minute) were also improved (Figure 4 & 9). The improvements as illustrated by the phenylalanine and isoleucine examples, to resolution and peak shape increased instrumental sensitivity and quantitation potential.

This investigation represents the first study to optimize an LCMS method for quantitation of sweat metabolites. Previous studies have relied on Luna HILIC columns much like Harshman et. al. (8, 22- 24), or reverse phase columns (9, 15, 26). The resolution provided by the BEH Amide column greatly enhances the sensitivity and quantitation potential for sweat metabolomics. The lower flow rate associated with the optimization (0.170 mL min^{-1}) provided increased resolution and sensitivity over the higher flow rates used previously (1.000 mL min^{-1} and 0.600 mL min^{-1}).

Figure 8: Comparison of initial conditions of mobile phase gradient (97% B vs 90%B). Chromatograms of the Amino Acid Standard H at 10µM were analyzed utilizing the original LC-MS method, except with the flow rate decreased from 1.000 mL min^{-1} to 0.170 mL min^{-1} and the BEH Amide analytical column in place of the Luna HILIC column. The ratio between metabolite peak height and noise is improved utilizing higher aqueous phase, as suggested in the column literature. At the 90% B concentration the mobile phase is more attractive to the metabolites at the start of the analysis, leading to quicker elution times. The chromatograms were set to the same scale for the comparison.

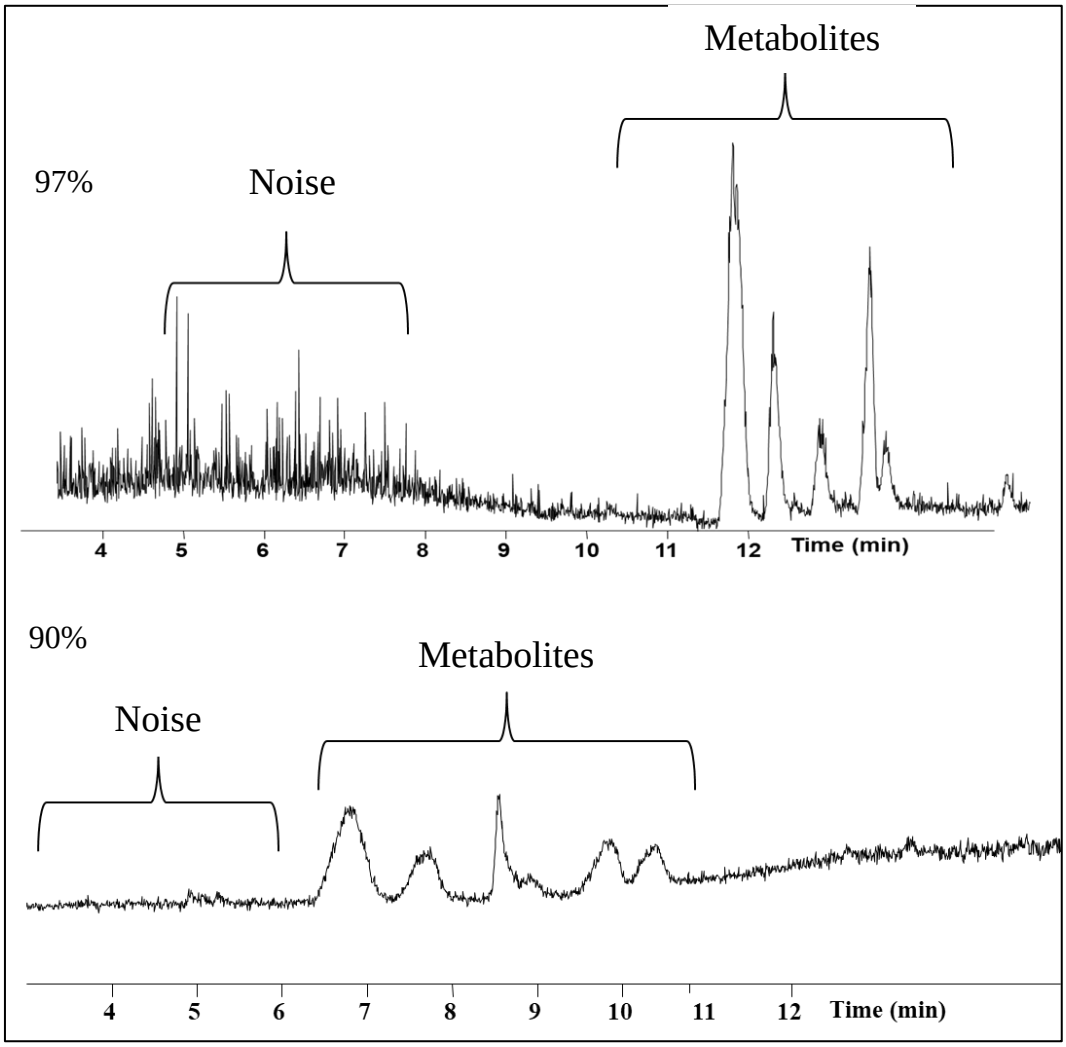
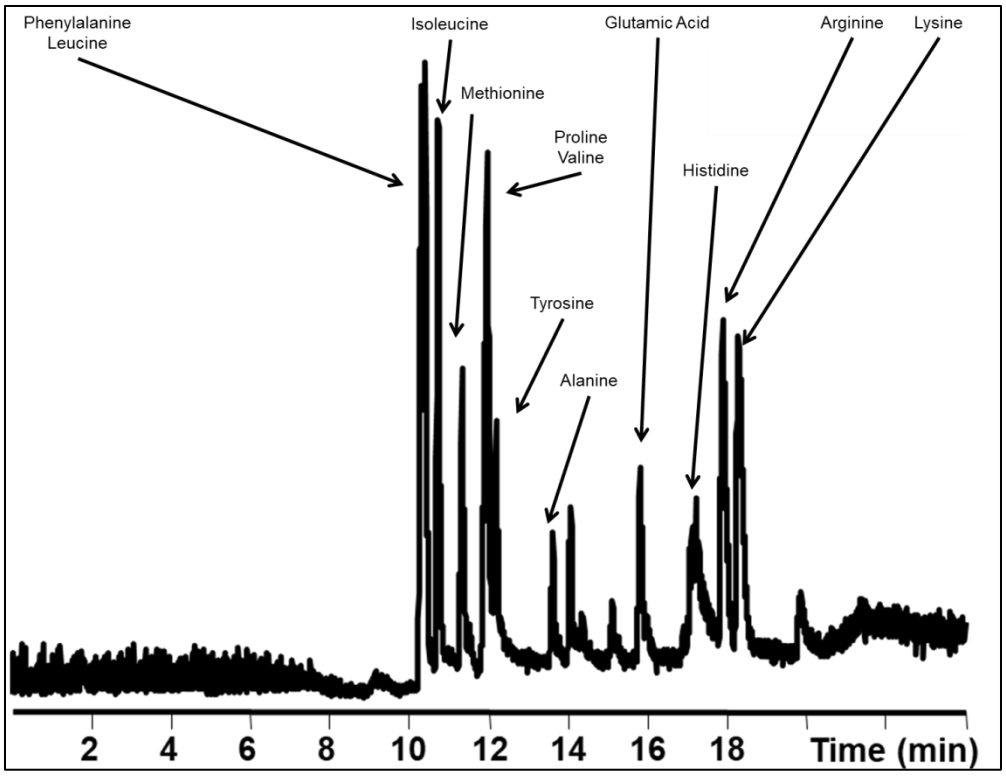


Figure 9: Chromatogram of the Amino Acid Standard H when analyzed utilizing optimized LC-MS settings. Optimal separation and peak shape was achieved using the optimized settings. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)

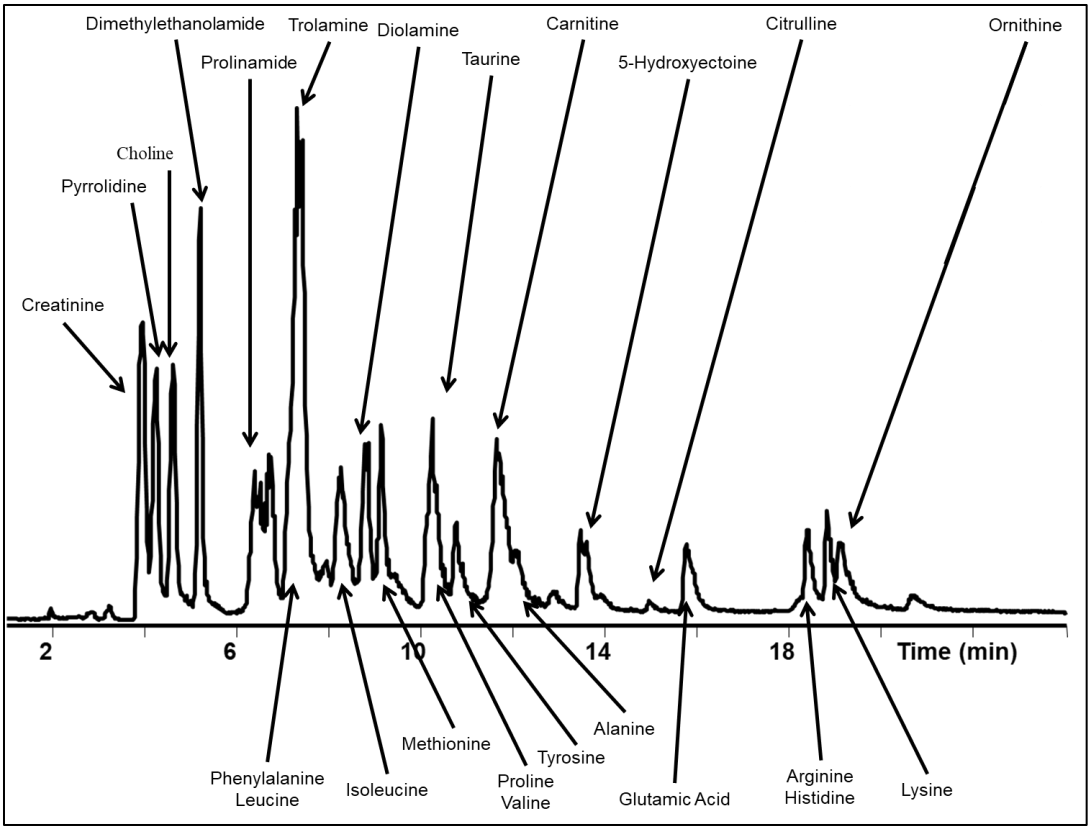


Since the original method we utilized was the method utilized by Harshman et al. (23) a direct comparison between the LC methods can be made by both looking at the chromatograms (Figure 4 and 9) and by the calculated resolution between the phenylalanine and isoleucine peaks (0.30 to5.47). By developing a LC method with low flow rate specifically for improved quantitation potential the resolution and sensitivity of the mass spectrometer were maximized allowing for better quantitation as well as discovery of low abundance metabolites in sweat.

Validation of the Liquid Chromatography

While optimization of chromatographic parameters was performed with the Amino Acid Standard H, amino acids were not the only compounds identified for further quantitative exploration in sweat. Therefore, it was necessary to insure the optimized parameters would sufficiently separate the remaining metabolites of interest (Table 1). An injection of a 1000 µM mixture of the 24 total metabolites was performed on the BEH Amide column utilizing the optimized method settings (Figure 10). The addition of the remaining 12 selected metabolites to the amino acids mildly reduced the efficiency of the separation. For example, resolution of the phenylalanine and isoleucine peaks decreased from 5.47 to 5.43 (Figure 9, 10). Additionally, several of the added metabolites co-eluted with previous peaks (Figure 10). The peak width of all the metabolites remained narrow. For example the FWHM of phenylalanine and isoleucine was 0.15 minutes and 0.20 minutes respectively. Although the efficiency of the separations was reduced by the addition of the 12 additional compounds, the data illustrated sufficient peak shape improvements and enough resolution between the metabolites of interest for the mass

Figure 10: Chromatogram of all selected metabolites of interest when analyzed utilizing optimized LC-MS settings. The optimized settings achieved acceptable resolution and peak shape even when all 24 selected metabolites are analyzed. (Unlabeled peaks are made of amino acids from the Standard H that were not of interest in this study.)



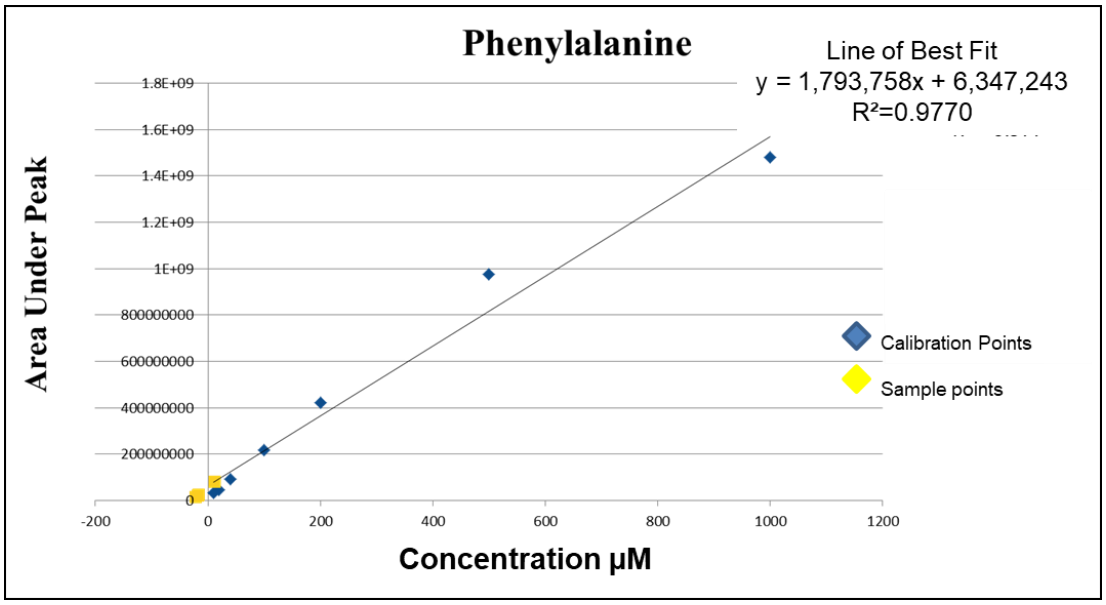
spectrometer to perform quantitation. As a result, the optimized liquid chromatography method was further utilized to define the metabolites present in excreted human sweat.

Sweat Metabolite Calibration Curve Development

A goal of the project presented here is to quantitate specific metabolites from human sweat samples. Following liquid chromatographic optimization, developing calibration curves was necessary to quantitate the specific metabolites of interest. An accurate calibration curve will be centered on the data range, with calibration points outside the overall range of the data. Five calibration points are minimally required to fully define a linear range associated with the line of best fit. The range 10µM to 1000µM was utilized by Harshman et al. in a previous experiment (23). In light of this, the 10 µM- 1000 µM range was utilized for the first calibration curve test.

While the 10 µM to 1000 µM range had been effective for Harshman et. al.'s exploratory study it was insufficient for this study. A 2 µL injection of the samples was analyzed alongside the 24 metabolite standard calibration range (10 µM to 1000 µM) required utilizing the optimized LC-MS method. The concentration of the sample metabolites was estimated based on the line of best fit. The dynamic range of the samples was smaller than the calibration curve (Table 4). For example, in the test samples the phenylalanine concentration was between 0-30 µM (Figure 11) and taurine was concentrated below 10 µM (Table 4), while sample alanine concentration was approximated near 300 µM (Table 4). Therefore the 10-1000µM calibration curve did not match the dynamic range of the individual selected metabolites from the sweat samples (Table 4). In order to improve the calibration curves ranges, patterns in the data

Figure 11: Calibration Curve Test 1; a representative example of data illustrated by the amino acid phenylalanine showing sample data points and the line of best fit from the calibration. The calibration range is not centered on the sample points.

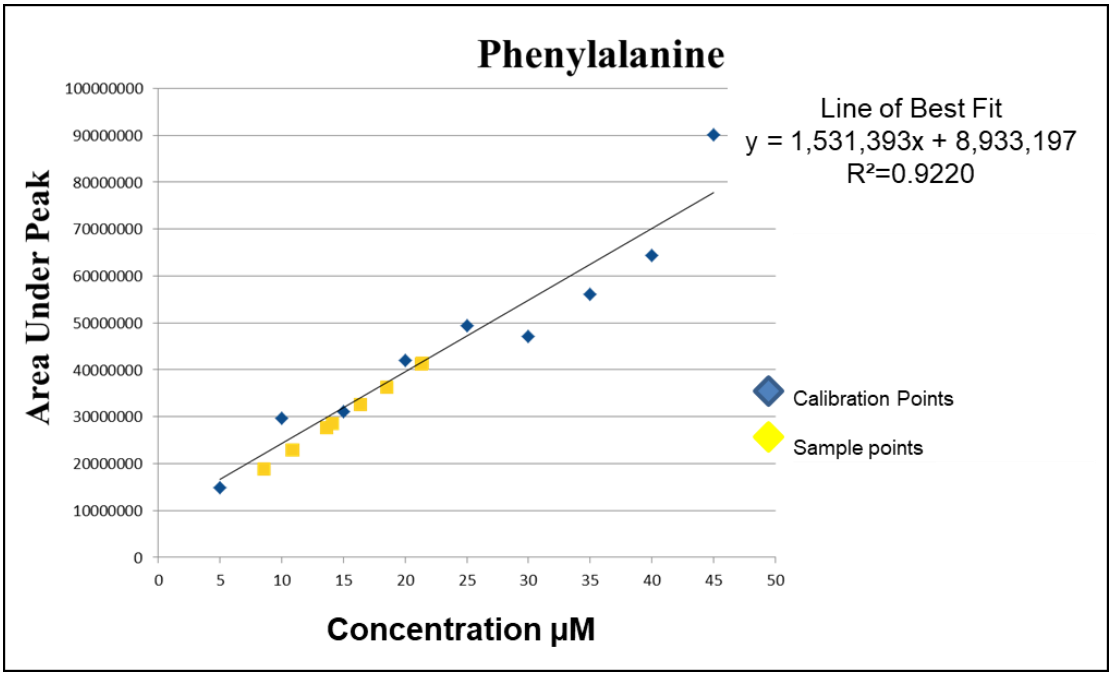


where analyzed. Two categories of metabolite concentration were observed in the sweat samples, a high concentration group (including alanine) and a low concentration group (including taurine).

The calibration range performed in Trial 1 was determined to not adequately represent dynamic range of the test sweat samples. As a result, two new calibration ranges were developed for the metabolites of interest, a high range 5-500 µM and a low range 5-50 µM. The low point of the calibration curves was placed at 5µM to insure the concentration was detectable by the mass spectrometer. While the calibration ranges were an improved from Trial 1 the dynamic range of the samples was not matched by the two, low and high, calibration curves (Table 4, 5). For example, phenylalanine was placed in the low (5-50µM) calibration range and had a sample range of 6µM to 18µM (Figure 12).

By decreasing the lowest point of the calibration curve from 10 µM to 5 µM additional low abundant metabolites were fit into the calibration range when compared to the first trial. For example, one sample had a phenylalanine concentration of 6 µM, another had a histidine concentration of 7µM, and yet another had an isoleucine concentration of 5µM (Table 5). These data suggest lowering of the bottom point of the calibration range should be considered for accurate quantitation. While lowering the range to 5µM was an improvement upon Trial 1, many of the concentrations of the selected metabolites remained below the calibration range (Table 5). For example, taurine sample concentration never approached 5 µM (Table 5).

Figure 12: Calibration Curve Test 2; A representative example of data illustrated by the amino acid phenylalanine showing sample data points and the line of best fit from the calibration. While closer the calibration range can be reduced further to better center the sample points within the range.

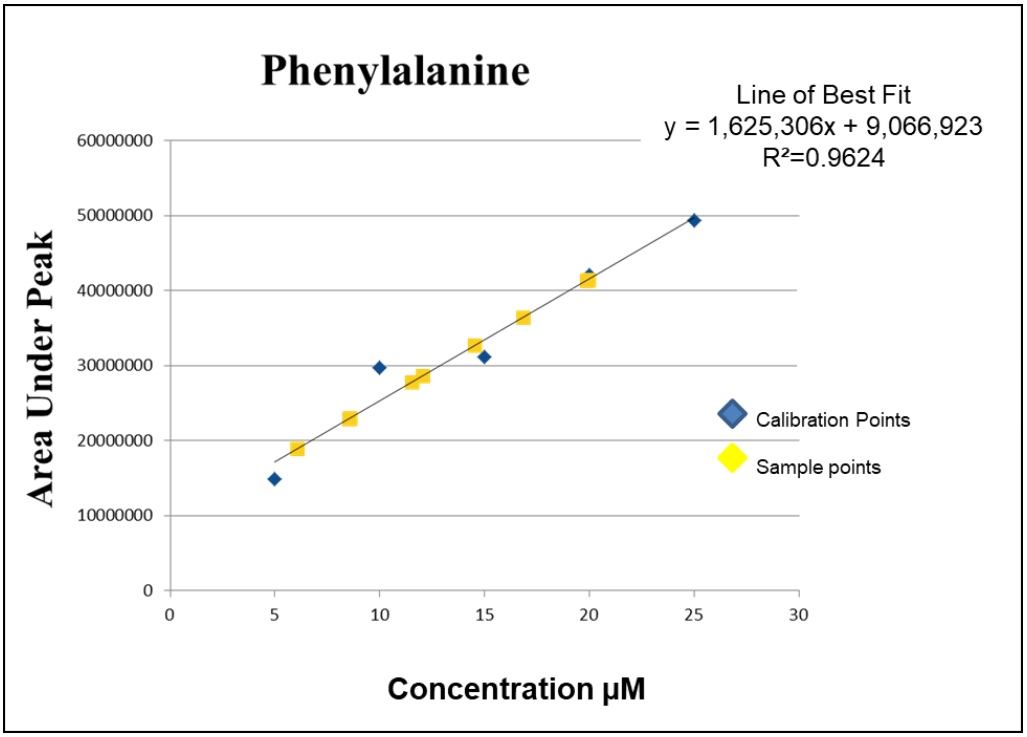


Additionally, the calibration range of both categories was not centered for several metabolites. For instance, ornithine had a samples range of 33-106 µM while the calibration range was 5-500 µM (Table 5). Due to the high dynamic range of sweat metabolites and in order to provide the best calibration fit for all the selected metabolites individual calibration curve ranges needed to be developed.

After the second trial was unable to account for the differences in dynamic range among the specific metabolites, each metabolite was given an individual calibration range. By looking at the approximated concentration of the tested samples from Trials 1 and 2 (Tables 4, 5) the range of each metabolite was approximated to develop the individual calibration range for Trial 3 (Table 6). These ranges were introduced to center the metabolites within the calibration range and better account for the dynamic range in sweat metabolites. For example, phenylalanine was given a range of 5-25µM (Figure 13) and ornithine was given a range of 5-300 µM (Table 6). In contrast, several metabolites including citrulline, taurine, and choline had sample concentrations below the calibration curve suggesting these compounds were approaching the limit of detection and limit of quantitation for the instrument (Table 6). Overall, the individualized calibration curves better represented the metabolites' ranges in the sweat samples compared to trials 1 and 2 (Table 4, 5, 6). However, many metabolites had lower sample concentrations than the lowest point of their calibration curves. Defining the sweat concentration ranges of the select metabolites will improve sweat quantitative analysis.

Very little quantitative work has been performed on the sweat metabolome (18, 26, 29). Hooton et al. pooled and labeled a portion of all their samples. They spiked the labeled sample back into all the samples allowing for direct comparison between labeled

Figure 13: Calibration Curve Test 3; A representative example of data illustrated by the amino acid phenylalanine showing sample data points and the line of best fit from the calibration. The individualized calibration range was well centered on the sample points allowing for improved accuracy when quantitating.



and unlabeled samples (26). While metabolite ratios and relative concentrations could prove important, the method from Hooten et al. only provides relative quantitation not absolute concentrations which are needed for sensor development. Creatinine and urea nitrogen were both quantitated by Fukumoto et. al. utilizing an AutoAnalyzer (18). Liappis et al. utilized an amino acid analyzer for the analysis of the concentration amino acids in sweat (29). The LC-MS method is able to corroborate the findings as well as be utilized for the quantification of additional metabolites found in human sweat. The development of an LC-MS method to quantitate sweat metabolites allows for improved understanding of how not only diet or exercise change sweat composition, but also sweat composition to predict performance. These values are in line with the overall goal of, and having concentration ranges for the development of sweat metabolite sensor devices.

Limit of Detection Tests

In order to quantitate the less abundant metabolites the limit of quantification (lowest concentration of analyte that can be quantified within defined limits of certainty) needed to be decreased. As the concentration of the metabolites is reduced a point is reached where the metabolite peaks become indistinguishable from the background noise, called the limit of detection, placing a lower limit on the ability to quantitate (limit of quantification). Therefore, an experiment to define the limit of detection for the selected metabolites was performed. For this experiment, the limit of detection was defined as five times the height of the surrounding noise. The 1000 µM mixture of the 24 total metabolites was diluted to 10 µM, 8µM, 5 µM, 4.5 µM, 4.0 µM, 3.5 µM, 3.0 µM, 2.5 µM, 2.0 µM, 1.5 µM, and 1.0 µM. A 2 µL injection of the diluted mixtures were analyzed using the optimized LC-MS methodology to determine the limit of detection for

each metabolite (Table 6). For example, the limit of detection for ornithine was determined to be 2 µM as the peak height of ornithine was five times greater than the height of the surrounding noise but less when the 1.5 µM concentration was evaluated. Of note alanine was only analyzed at 10 µM due to the high concentration observed in sweat. Once the limit of detection was found for each selected metabolite (Table 6), the data was evaluated to determine if the lower limit of the calibration range could be reduced.

In this experiment the limit of quantitation concentration is required to be higher than the concentration for the limit of detection to achieve the lowest defined limits of the calibration range. For example, the calibration curves used to quantitate the sweat in the samples utilize peak area, which is reliant on peak shape. The peak tails define the area of the peak near the background noise. When quantitating at concentrations near the limit of detection, differentiation is difficult between the background noise and the peak tails. Once the concentration is too low background noise can contribute significantly to the peak area. Therefore, in this experiment as the limit of detection for all the selected compounds was only slightly lower than the 5µM, further lowering the lowest calibration point would have a high chance for inaccuracy. For this reason, the lowest calibration point was left at 5 µM. In future experimentation an instrument with greater sensitivity (the smallest signal that can be detected) could be used to reduce the limit of detection and likely the limit of quantitation. Many sweat metabolites, outside of the 24 being analyzed in this study, are very dilute. Quantitating them will require instrumentation with greater sensitivity or signal to noise. The dilute nature of sweat is one of the reasons

it has not been as well studied as other bio-fluids. As new technologies allow for greater sensitivity the ability of sweat to be used as a bio-marker will increase.

Conclusion

The previous liquid chromatography methodology within our lab, as utilized by Harshman et. al., required optimization to transition from discovery to quantitative approaches (23). A collection of 24 metabolites were chosen for quantitative analysis based on data generated in our lab and found in the literature. A subset of these metabolites, found in the Amino Acid Standard H, was utilized to optimize the LC methodology. Three settings were altered to the previous LC methodology, flow rate, column, and the initial settings of the mobile phase gradient. Once the LC method was optimized an injection of the 24 selected metabolites of interest was analyzed utilizing the optimized methods to ensure sufficient separation. The optimized methodology showed improvement in peak shape, peak resolution, and instrumental sensitivity.

A sweat metabolite calibration curve was developed for each of the 24 metabolites to facilitate the project goal of quantification. Optimized curves for each individual metabolite were developed over a period of three trials. While successful calibration curves were developed, several sample metabolite concentrations remained below the limit of quantitation.

The development of a LC-MS method for quantitating sweat metabolites may unlock the potential of sweat bio-markers. By quantitating the sweat metabolites the full effects diet and exercise have on the sweat metabolome can be examined. Quantitation also allows for finding specific concentration ranges for the metabolites of interest.

Having concentration ranges for sweat metabolites allows for the development of sensor technology that could predict performance or alert the user to possible health issues.

Chapter 2: Application of Optimized Method for Sweat Metabolite Determination

Introduction

Sweat Biomarker Discovery

Several sweat biomarker discovery experiments have been performed utilizing different metabolomics analytical techniques. As a result of these experiments many metabolites have been identified in human sweat, including amino acids and the other metabolites selected for analysis in this study (6, 8, 9, 14-18, 21- 23, 25, 26, 29, 30- 32). For example, Liappis et al. identified 20 amino acids in exercise induced human sweat (29). Some of the most often identified metabolites in discovery investigations into sweat metabolite content are amino acids (8, 9, 15, 16, 22, 23, 26, 29-31). Other metabolites are also often found in sweat research including ammonia (6) and sugars (14). While many metabolites can be found in sweat, amino acids are often among the most abundant (22, 23). For this reason amino acids are often selected for biomarker discovery experiments, including this experiment.

Sweat Quantitation

Only a few studies have been performed where sweat metabolites were quantified (18, 26, 29, 32, 37). Sweat lactate is one of the metabolites that have been quantified (37).

Sakharov et al. found that by measuring sweat lactate concentration the blood concentration could be predicted allowing for non-invasive monitoring for oxygen deficiency in athletes (37). Planar electrodes were used to measure the lactate concentration; however the measurements were not accurate (37). A study utilizing LC-MS to quantitate a large selection of sweat metabolites was performed by Hooton et al. This study utilized labeled metabolites to compare between sweat samples (26). However, the study was not designed to produce an exact concentration of each metabolite in the samples. While a few studies have been performed concentration ranges for the metabolites need to be developed for the Air Force's overall goal to develop a sensor that can link performance to sweat content.

Sweat Composition Effectors

The goal of the Air Force is to use excreted human sweat to predict airman performance levels. To achieve this goal, sources of sweat alteration must be analyzed. It has been shown that sampling method, medication, and sweat rate can all affect sweat composition (4, 5, 24, 33). For example, anticholinergics and antidepressants can cause hypohydrosis, a condition in which the ability of the sweat glands to make sweat is reduced (4). Additionally, normalizing for sweat rate has been shown to greatly reduce the variability of sweat ions among samples (24). Furthermore, sampling methods have also been shown to have an effect on perceived sweat composition (5). Based on these few examples, further investigation in to factors that influence sweat composition is necessary for accurate biomarker discovery.

Effects of Diet on Sweat

Understanding dietary effects on sweat metabolites is necessary to remove factors that can influence biomarker discovery results. For example, a study by Boysen et al. has shown that sweat glucose levels can be affected by diet (5). In this study, thermally induced sweat and plasma were collected from two subjects before and after oral administration of glucose. A concurrent rise in glucose concentrations was observed in both the sweat and plasma. Additionally, sweat ammonia concentrations have been linked to diet. In a study by Czarnowski et. al. sweat ammonia concentrations were measured from a control group as well as a group that ingested ammonium chloride(13). The subjects that ingested the ammonium chloride had increased sweat ammonia concentrations. Interestingly, no increase in sweat urea (the product of ammonia breakdown) concentrations was observed (13). While sweat glucose and ammonia have been illustrated having a correlation with diet the same cannot be said of amino acids in sweat.

As stated previously, amino acids are typically the most frequently identified and most abundant metabolites in sweat (8, 9, 14-16, 22, 23, 26, 29-31). Surprisingly, only one experiment correlating sweat amino acids with diet was found in the literature. The experiment by Hier et al. found no correlation between diet and sweat amino acid content (25). However, the experiment only analyzed the differences between pre-breakfast sweat and post-lunch sweat, failing to account for long term dietary differences. As a result, the long-term impact of diet on sweat metabolite content remains unknown.

Summary

To address the gaps in the literature surrounding the quantitative long-term dietary impact on sweat metabolite concentrations, here, the optimized method determined within Chapter 1 was applied to analyze 42 sweat samples. These sweat samples were analyzed to quantitatively assess sweat metabolite concentrations from individuals who were given a high or low nutritional supplement twice daily over a 12 week period. The data from this experiment shows that the concentrations of the chosen sweat metabolites from the subjects who received high nutritional dietary supplementation were increased over the 12 week period. Therefore, these data suggest dietary intake must be considered in future sweat biomarker discovery studies.

Methods

Human Subjects

Volunteer subjects were taken from of an ongoing unrelated exercise and nutrition intervention study being performed within the Air Force Research Laboratory. Subjects were given the protocol and permitted to ask questions. The subjects provided informed written consent to participate. The subjects (n=14) were male volunteers from the United States Air Force, stationed at Wright-Patterson Air Force Base in Dayton, Ohio, of variable age (19-35) and rank. Prior to the start of the study permission for human subject research was obtained from Wright-Patterson AFB Institutional Review Board (IRB# FWR20150032H).

Sweat Stimulation and Sample Collection

The sweat used in this study was collected from participants taking part in the ongoing exercise and nutrition study being performed within the Air Force Research Laboratory mentioned previously (42). Participants were randomly selected to receive either a high nutritional supplementation drink (HNSD) or a low nutritional supplementation drink (LNSD), produced by Abbot Nutrition for the ongoing study, twice daily for the duration of the 12 week experiment. Table 8 shows the contents of both high and low nutritional supplementation drinks. Six subjects were given the low nutritional supplementation drink and eight subjects were given the high nutritional supplementation drink. Both supplements were taken orally as 8oz of liquid immediately prior to or immediately following the subjects' exercise and sweat collection protocols. The supplements were taken from weeks 1 to 12 of the ongoing experiment. Throughout the 12 weeks the unrelated experiment prescribed metabolic and resistance circuits as well as cardiovascular exercise routines for the subjects each weekday. Sweat was collected from each participant three times, at the start of week 1, in the middle of week 6, and at the end of week 12. Between the six LNSD subjects and the eight HNSD subjects, samples from 14 subjects were available. These subjects had sweat samples taken at week one, six, and twelve making a total of 42 samples (Figure 14).

For this study, subjects' exercise induced sweat was sampled during cardiovascular training routines from the larger unrelated study. The subjects were given identical heart rate zone prescriptions to attempt to homogenize exhaustion levels, and subjects exercise was performed in the same room to maintain environmental conditions. The training routines gave the subject the choice of stationary bike, treadmill, or elliptical

to perform continuous cardiovascular exercise. During these exercises the subjects had their intensity regulated by percent max heart rate (HR). Max HR had been determined prior using a VO_2 max running test.

The sweat samples were taken from the subjects' forearms using sweat patches as described in Chapter 1. Briefly, the samples from each individual's forearms were collected with needle and syringe, filtered, centrifuged, and pooled. An individual's combined sample was then separated into 250µL aliquots. The aliquots were next frozen using liquid nitrogen and lyophilized to dryness. Finally, the sweat aliquots were stored at -80ºC until needed for analysis as described in Chapter 1.

Resuspension Buffer, Standards, and Sample Preparation

Twenty mL of a 1000 µM stock of each isotopically labeled compound was developed. A 50% acetonitrile (aq) solution was supplemented with the 1000µM stock of the previously described isotopically labeled standards for taurine, choline, creatinine, citrulline, pyrrolidine, alanine, arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine and valine resulting in a final concentration of 30µM for each labeled compound (called Resuspension Buffer). Isotopically labeled standards for the remaining selected compounds of interest were unavailable (see Table 1 for labels). The samples were re-suspended to the starting volume of sweat (250 µL) in the resuspension buffer. Following re-suspension 10 µL of each sample (Figure 14) was taken and pooled to provide a standard. The pooled sample was used to help account for ionization drift during the analysis.

Table 8: Contents of the high nutritional supplementation drink and the low nutritional supplementation drink produced by Abbott Nutrition adapted from Zwilling et al. (42). While further breakdown of the amino acid content from the protein could be useful the information was unavailable.

		Abbott Nutritional Supplements					
			High Nutrient			Low Nutrient	
Active Nutrient	Unit / serving (8 fl oz)*	Target	Minimum	Maximum	Target	Minimum	Maximum
Carbohydrates	g	38	35	43	18	15	23
Protein	g	13	12.5	14	2	1.5	2.5
CaHMB	g	1.5	1.3	2		---	---
Fat	g	7	6.3	8	2	1.75	2.75
Lecithin	g	0.5	0.4	0.8	0.008	0.01	0.12
Choline	mg	100	83	237	---	---	---
DHA	mg	125	100	200	---	---	---
Lutein	mg	6	5	7	---	---	---
Nat Vitamin E 9IU	mg	6	5	12	---	---	---
Vitamin D (480 IU)	mcg	12	11.5	27.5	---	---	---
Vitamin C	mg	36	30	166	---	---	---
Vitamin B1	mg	0.38	0.35	1.42	---	---	---
Vitamin B2	mg	0.43	0.4	1.19	---	---	---
Niacin	mg	5	4.74	8.3	---	---	---
Pantothenic Acid	mg	2.51	2.37	5.45	---	---	---
Vitamin B6	mg	0.8	0.5	1.3	---	---	---
Vitamin B12	mcg	1.5	1.4	32	---	---	---
Folic Acid	mcg	100	95	237	---	---	---
Zinc	mg	4.2	3.25	8.3	0.1	0.08	0.8
Magnesium	mg	100	83	140	2.5	1.5	5
Selenium	mcg	21	15.4	56.9	---	---	---
Ash	g	2.3	2.3	3.1	0.2	0.1	0.3
Calories	Kcal	250	250	270	100	90	110
Total Solids/ serving	g	59.9	56.1	63.8	24	22	26.7
Total Solids g / 100g	%	23.5	22	25.0	9.9	9	10.9

Figure 14: A figure illustrating the breakdown of sweat samples for analysis.

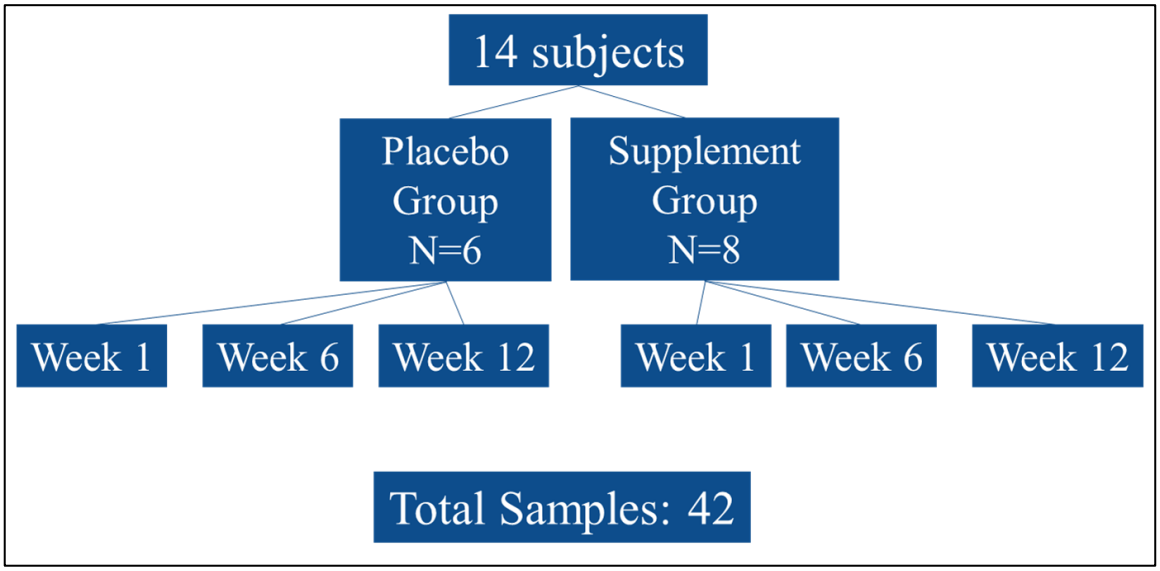


Table 9: Finalized calibration curves for the metabolites with sample concentration ranges. Of note 5-hydroxyectone, carnitine, pyrrolidine, taurine, diolamine, dimethylethanolamine, trolamine, choline, prolinamide, and methionine compounds were removed from the analysis as the concentrations were below 5 µM and approach the limit of detection.

Compound	Calibration Points (µM)	Sample Range (µM)
Ornithine	5;50;100;150;200;250;300	32.81-352.42
Citruline	5;50;100;150;200;250;300	43.70-967.48
Creatinine	5;50;100;150;200	70.23-547.66
Phenylalaine	5;50;100;150;200	20.39-117.79
Alanine	50;100;150;200;250;300	62.53-1362.46
Arginine	5;50;100;150;200;300	19.05-308.95
Glutamic Acid	5;50;100;150;200	15.68-178.15
Histidine	5;50;100;150;200;300	57.36-298.57
Isoleucine	5;35;55;100;150;200	17.66-162.81
Leucine	5;50;100;150;200	33.53-184.54
Lysine	5;50;100;200;300	20.67-241.64
Proline	5;50;100;150;200;250	24.30-202.00
Tyrosine	5;50;100;150;200	10.89-145.84
Valine	5;55;100;200;300	26.28-272.75

A custom calibration curve for each compound was made of unlabeled standards as defined in a previous section (Table 6). These calibration curves consist of at least five different known concentrations of unlabeled standard. The calibration curve standards were then dried down, and re-suspended to concentration using the resuspension buffer. An additional calibration range of 5-300 µM was made for each metabolite standard in the same manner. The calibration curves were finalized using both groups of concentrations after analyzing the metabolite concentration of the samples (Table 9).

High Performance Liquid Chromatography

The optimized LC method as discussed in chapter one was used for this experiment. Briefly, the optimized settings utilized a Vanquish system with a flow rate of 0.170 mL-min^{-1} affixed with a Waters Acquity BEH Amide Column. A 30 minute analytical gradient was used with a mobile phase composition starting at 90% mobile phase B. Please refer to Table 3 for additional details surrounding the optimized parameters.

Mass Spectrometry

A Q-Exactive HF – Orbitrap mass spectrometer utilizing a heated electrospray ionization source was used in all chapter two experiments. To ensure mass accuracy, the instrument was calibrated within 24 hours of the experimental analysis. The instrument was used in positive ionization mode with the following settings: spray voltage 3.5 kV, capillary temperature 280 ºC, sheath gas 30, and the aux gas 10. The resolution was set to 60,000 over a scan range of 65-400 m/z.

Three calibration curves were analyzed, at the beginning middle and end of the overall analysis. Samples were analyzed randomly, with a pooled sample run approximately every tenth sample analysis. In order to eliminate crossover contamination a blank of 50% aqueous acetonitrile was used to flush the instruments after each sample and pooled sample.

Data and Statistical Analysis

Sample metabolites were confirmed based on retention time comparison to the metabolite standard and/or labeled metabolite in the resuspension buffer if available. The Xcalibur software package was used for collecting the raw data (samples, and blanks) and peak area determination as describedin Chapter 1 (v. 2.2. Thermo Scientific). Briefly, unknown sample concentrations were determined utilizing the line of best fit equation from the calibration of the known concentrations to peak area ratios plotted in Microsoft Excel (Figure 13). The area of an unknown sample metabolite peak area can then be fit into y value of the line of best fit to estimate the metabolite concentration (x value) of the samples from a y=mx+b equation.

For the selected metabolites with a labeled version available (taurine, choline, creatinine, citrulline, pyrrolidine, alanine, arginine, glutamic acid, histidine, isolucine, lucine, lysine, methionine, phenylalanine, proline, tyrosine and valine) (Table 1 for labels) the method was supplemented to improve accuracy related to ionization fluctuations. For these compounds the ratio of the area of the metabolite peak divided by the peak of the labeled version of the same metabolite and the known concentration of the standards were plotted in Excel, rather than the peak area of the metabolite, to determine

the line of best fit. Therefore, a similar ratio was calculated (unlabeled/ labeled) for each sample to determine the unknown concentrations from the line of best fit equation.

Statistical analyses of calculated metabolite concentrations were performed using the RStudio software suite. The ggplot2 package was used to generate the PCA plots and the heatmaps, while the ropls package was used for Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA)

Results and Discussion

Quantification of the Selected Metabolites

Quantitation for this experiment utilized calibration curves formed from standards of the selected metabolites as illustrated in the previous chapter. During the development of the calibration curves twenty sweat samples were analyzed (five in Test 1, five in Test 2 and 10 in Test 3) to insure acceptable calibration range for all compounds. However, as found previously, several of the selected metabolites were not abundant enough in the sweat samples to allow for accurate quantitation. For example, pyrrolidine yielded concentration values less than zero in every sample (Table 6). Quantitation utilizing calibration curves relies on the line of best fit for the calibration points. In the case of pyrrolidine choline, methionine, hydroxyectoine, carnitine, taurine, diolamine, dimethylethanolamide, trolamine and prolinamide the line had a negative y intercept, leading to negative x values (concentration) when y values (area under the metabolite peak) are small. While several metabolites were below quantifiable levels (pyrrolidine, choline, methionine, hydroxyectoine, carnitine, taurine, diolamine, dimethylethanolamide, trolamine and prolinamide), other metabolites were well within

quantifiable ranges. Although accurate calibration curves were developed, removing the ten metabolites with quantified values frequently below calibrated ranges from the final analysis was decided to be the best course of action due to the impact of noise on the values. Removing these metabolites allowed focus to be placed on those compounds (citruline, ornithine, creatinine, phenylalanine, alanine, arginine, glutamic acid, histidine, isoleucine, leucine, lycine, proline, tyrosine, and valine) accurately quantified within the method.

While the initial 20 samples from the calibration curve tests test suggested the calibration curve ranges from Test 3 would be appropriate for the larger analysis, the dynamic range of several metabolites exceeded the calibration curve limits. However all metabolites were analyzed from 5- 300 µM within the analysis. Therefore, expansion of the calibration curves to accommodate many of the samples was performed (Table 6, 9). The concentrations in some samples were above the upper point in the calibration curve (300 µM) (Table 9). While the sample concentrations below the lower calibration point were removed, the metabolite concentrations above the upper calibration curve limit were utilized but considered estimated values. The range of the high metabolite concentrations was limited by the line of best fit. In LC-MS detection, as the instrument approaches saturation the linear relationship between peak area and concentration are reduced. Manual inspection of the data suggested instrumental saturation had not occurred. Therefore, while not as accurate as concentrations within the calibration curve, the estimations are unlikely to lead to false conclusions.

The dynamic ranges of the sweat metabolites are larger than was illustrated by the test samples from the calibration development (Chapter 1). For example, in most samples

alanine concentration was between 50 µM and 300 µM however one individual had alanine concentrations over 1000 µM (Table 9). While accurate quantitation for these samples could not be achieved directly with the developed methods, they were left in the analysis as estimations. For future experimentation, additional samples need to be analyzed before defining the range of the calibration curve. Alternatively, to achieve an accurate calibration range a wide range of standard concentrations could be analyzed with the samples and then calibration ranges could be determined based on the unknown values. While ten of the selected metabolites were removed from the analysis due to low abundance, the remaining 14 selected metabolites provided sufficient quantitative data for analysis.

Principal Component Analysis (PCA)

Exploratory data analysis often utilizes principle component analysis (PCA) to visualize large data sets. Data sets with multiple variability dimensions can be difficult to visualize. The PCA model reduces the variable dimensionality while preserving the variation of the data to the greatest degree possible. PCA allows for visualization of data subsets but does not determine factors that differentiate the subsets. Instead, PCA allows focus to be placed on what best separates the explained variability of the data as a whole (38). If the subsets are well defined by the PCA, the variable being analyzed is likely a major factor in the separation of data points.

A PCA was used to visualize variability among the sweat samples. To reduce interpersonal variation among the data, the week 6 and week 12 data were normalized to the week one results. The data was then transformed (log_2) and the fold change among

the data points was determined. Figure15 illustrates the first two principal components (PC1 & PC2) from the week 1 normalized $\log_2$ fold change of all the data. The data show PC1 and PC2 account for 86.7% of the variability within the data. PC1 shows partial separation between the samples of the subjects who took the high nutritional supplement (red) and those taking the low nutritional supplement (blue). For example the six points representing subjects 9, 11, and 12 (low nutritional supplement) at both week six and week 12 remained outside the confidence ellipse for the high nutritional supplement subject points. Three of the 16 points for subjects who took the high nutritional supplement, are found outside the confidence ellipse for the low nutritional supplement subject points (Figure 15). These data provide evidence supporting the hypothesis that low and/or high nutritional supplementation may alter the sweat metabolome concentration including the selected metabolites.

To further investigate the data PCAs were generated for each time point separately (i.e. separated by week). This analysis yielded similar results to the analysis of all data points (Figures 15- 17). For instance, in the week 6 data, the variability explained by PC1 is 80.5 compared to 80.9 when all the data is analyzed (Figures 15, 16). Similarly, the week 12 PCA shows the variance explained by PC1 is increased from 80.9% to only 83.8% when comparing only week 12 data to the whole data (Figures 15, 17). Interestingly, the high nutritional supplementation data points of week 12 show increased clustering compared to the data points of week 6. These results suggest the effects of the nutritional supplementation will have mostly taken affect after six weeks.

The PC2 and PC3 graph of the whole data set and of both single time points were also examined. Little was expected from PCAs 2 and 3as greater than 80% of the

variance was previously explained by PC1 in all three cases. Supplemental Figures 1, 2, and 3 show PC2 and PC3 only account for around 10% of the overall explained variability of each data set. Furthermore, PC2 and PC3 were unable to separate the subjects based on high or low nutritional supplementation for all scenarios. Therefore while evaluated, these PCAs do not reveal much separation for this analysis and account for low amounts of overall variability.

The PCAs illustrate a contradiction with the idea put forth by Hier et al. that diet and sweat amino acid content have no correlation (25). The experiment by Hier et al. attempted to analyze the effects of diet on sweat amino acid content by sampling sweat from students before they ate breakfast and after eating lunch. The data in the experiment by Hier et al. showed what appeared to be random fluctuations in amino acid concentrations from pre-breakfast to post-lunch (25). The subjects in this experiment who were given the high nutritional supplement for 12 weeks showed partial separation from the subjects given the low nutritional supplement. The data in this experiment suggests amino acids, as well as the other selected sweat metabolite concentrations may be affected by diet over a period of weeks in contrast to the idea put forth by Hier et al.

The partial separation in the PCA illustrates that the nutritional supplements could be influencing the sweat metabolome (Figure 15- 17). One of the weaknesses of this study is the small number of subjects. It is possible true separation or lack of separation of the data between the high and low nutritional supplement groups could be observed in a study involving more participants. While the number of subjects was out of our control for various reasons, such as participant attendance, the data established evidence that accounting for diet in sweat metabolite biomarker studies is necessary.

Figure 15: PCA of the week 1 normalized log_2 fold change of all data shows the partial separation of the high and low nutrition supplementation groups. Supporting the hypothesis that dietary supplementation does affect sweat metabolite concentrations.

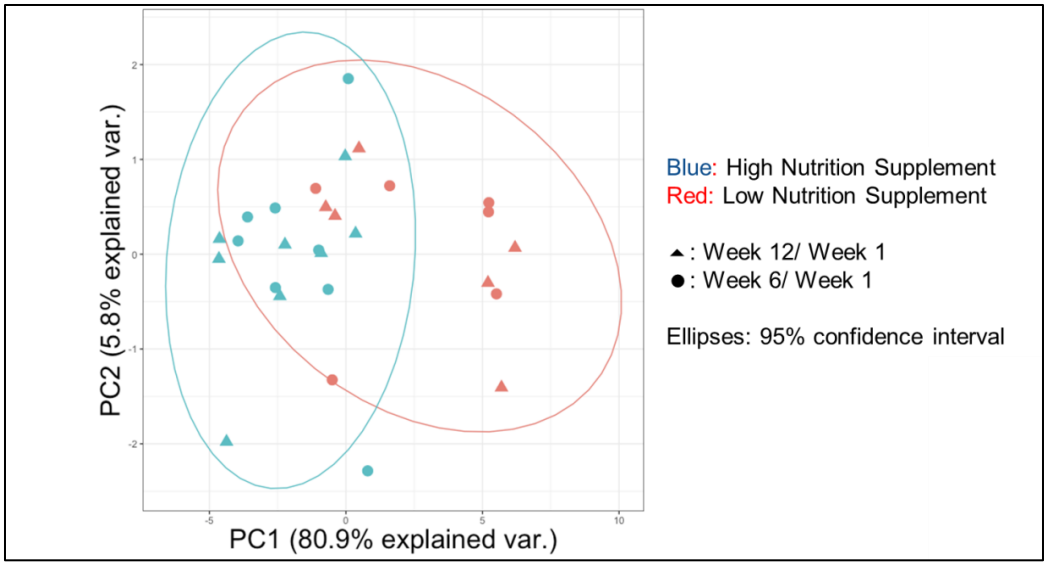


Figure 16: PCA of the week 1 normalized log_2 fold change of week six data shows a slight decrease in separation of the high and low nutritional supplementation groups in comparison to the whole data. Suggesting that the effects of supplementation have taken affect by week 6.

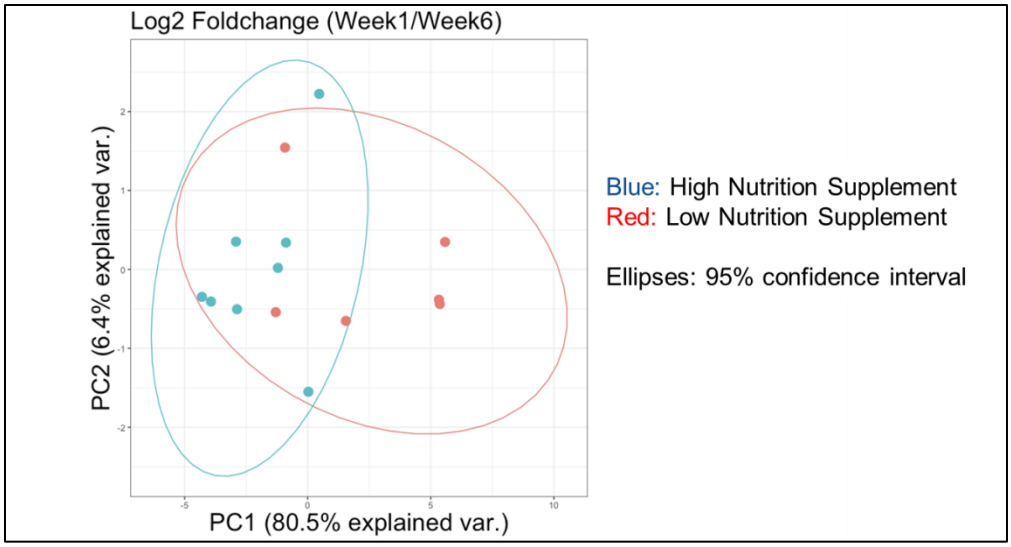
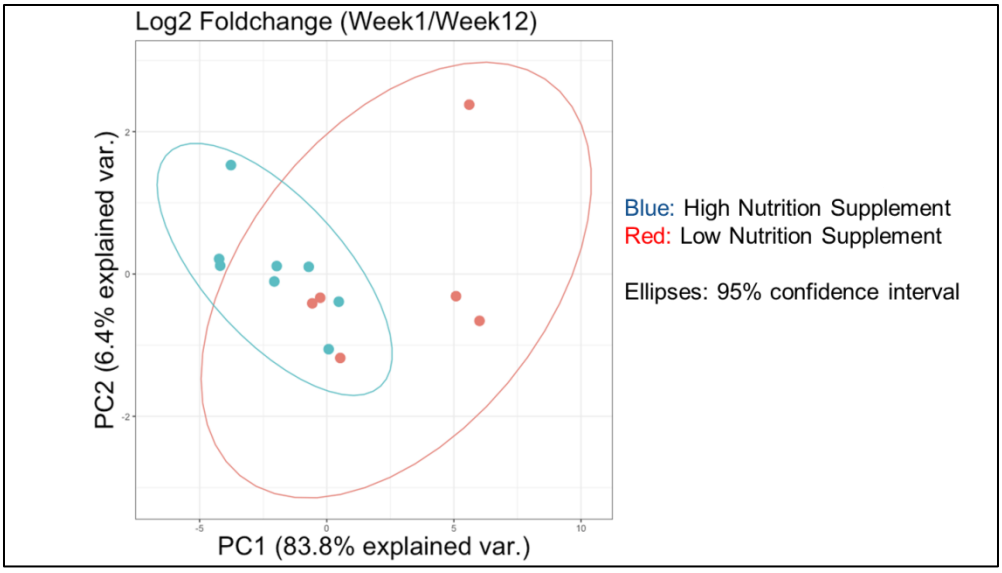


Figure 17: PCA of the week 1 normalized log_2 fold change of week 12 data shows greater clustering among the high nutritional supplementation points than at six weeks. Suggesting that while the majority of the supplementation's effects have occurred by 6 weeks it may have greater effects if supplementation continues.



For the Air Force to utilize the sweat metabolite abundance to predict human performance developing a way to account or control dietary intake will be necessary.

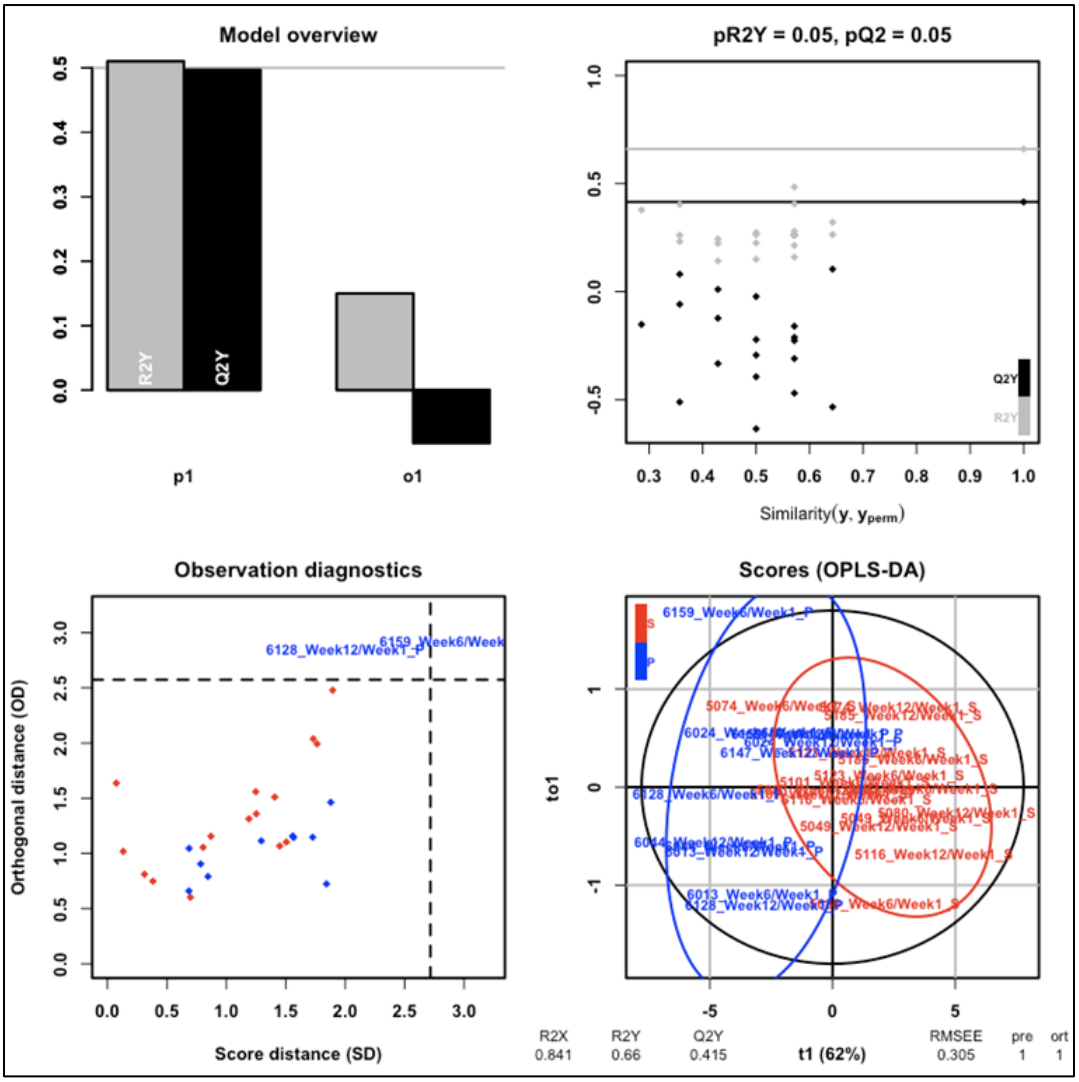
Discriminate Analysis

Similar to PCA, discriminate analysis is often used in making predictive models for exploratory data analysis. However, discriminant analysis allows the user to define subsets within the data (38). The analysis then maximizes the separation between the defined groupings instead of the data as a whole. However the variable that best separates the data groupings may not separate the whole data as well as other variables. For example, a selection of individuals could be grouped and separated into two groups by eye color (brown and blue) utilizing a discriminate analysis while more separation among the individuals could be found by separating them by height.

The discriminate analysis, applied to the sweat results, was better able to separate the samples based on high (red) and low (blue) nutritional supplementation than the PCA (Figure 15, 18). However, the 95% confidence ellipses overlapped, similar to the PCA with several samples remaining in the overlapping space of the two ellipses (Figure 18).

Along with the partial separation, the discriminate analysis did not well represent the data. The Q2Y value, a number between 0 and 1, tells how well the model fits the data, with one being a perfect fit. The Q2Y value for these data was only 0.415 most likely due to the low n-value (Figure 18). Furthermore the model was over fit, likely due to the data containing a large number of features (metabolites) and very few observations (subjects). The overfitting of the data is worse when performed on single time points only (i.e. separated by week Supplemental Figures 4, 5).

Figure 18: Discriminate analysis of the week 1 normalized $\log_2$ fold change of all data shows better separation of the high (red) and low (blue) nutritional supplementation groups than the PCA (bottom right) further supporting the hypothesis that nutritional supplementation affects sweat metabolite concentration. Interestingly the model shows low nutritional supplementation produced outliers while high nutritional supplementation did not (bottom left). However, the model was over fit to the data reducing its potential impact achieving a Q2Y value, for the only known factor, of less than 0.5 (top left) and an overall Q2Y value of only 0.415. The top right section shows randomly permuted y responses plotted with the x value being the observed value. The loose clustering shows the model's poor predictive capability while the points being below their lines show that the model makes significant calculations.



The poor representation of the data through overfitting by the discriminate analysis reduces its usefulness to the analysis. Additional samples will be required to fully utilize the analytical approach.

The discriminate analysis model achieved slightly better separation than the PCA having, i.e. fewer subjects within the overlapping sections of the confidence ellipses. However, the separation improvement does not justify utilizing the model when it has a low Q2Y value suggesting the data has been over fit. The discriminate analysis further emphasizes the need for testing with a larger population to verify the findings. However, evidence that accounting for subjects dietary differences continues to be established. The need to develop a method to account for dietary intake remains important to the goal of the Air Force to utilize sweat metabolites to predict human performance.

Hierarchical Cluster Map Analysis

The PCA results suggest potential separation among the data as illustrated by Figure 15. To better visualize the results a hierarchical cluster map was utilized. Figure 19 illustrates the clustering of the samples into three groups based on relative metabolite fold change to week 1. The data suggest three groupings of samples- those with reduced metabolite concentrations, increased metabolite concentrations, and those with little to no change in metabolite concentration over time (Figure 19).

Further evaluation of the data show the samples with increased relative metabolite concentrations (bottom) are composed completely of subjects who took the low nutritional supplement. Conversely, the samples showing decreased metabolite concentrations (top) are entirely made up of subjects who took the high nutritional

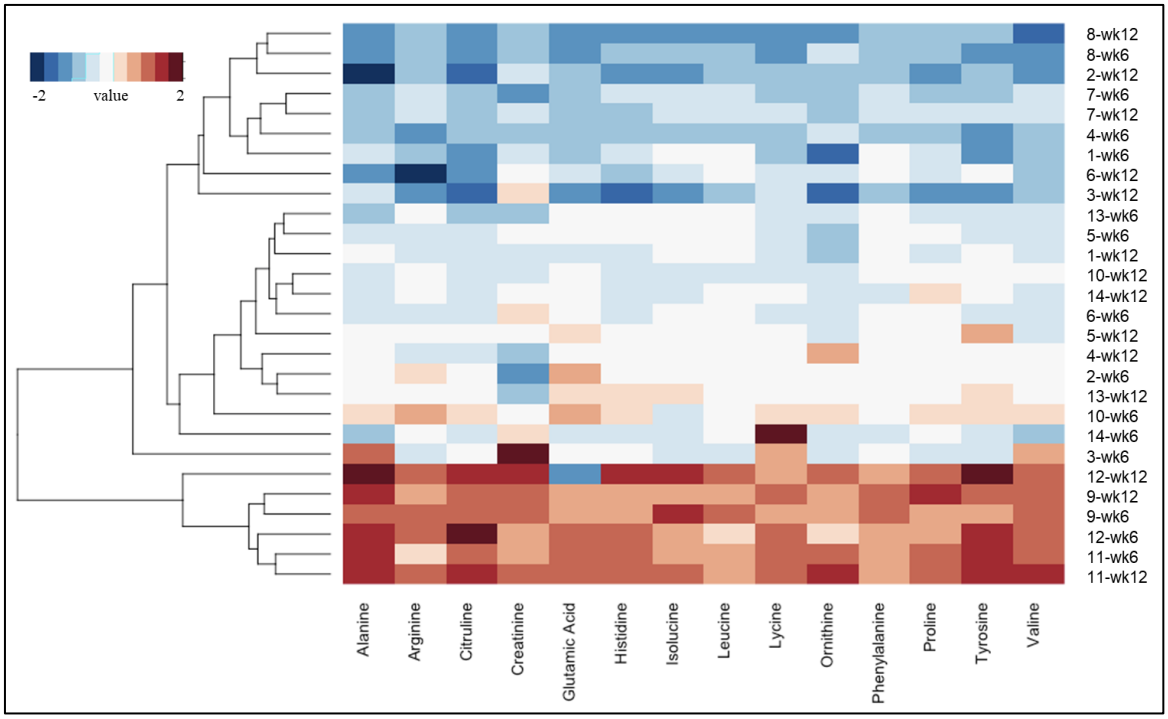
supplement. Finally, the samples illustrating little to no change to the relative metabolite concentration (middle) contain a mixture of both nutritional supplement groups (Figure 19).

Of the eight participants given the high nutritional supplement two subjects were in the decreased relative abundance grouping both weeks while three did not achieve the observed trend of decreased relative abundance grouping until week 12 (Figure 19). Collectively, these five subjects support the hypothesis that long-term ingestion of a high nutritional supplement decreases the relative sweat metabolite composition over time. However, two subjects that received the high nutritional supplement were in the decreased relative abundance grouping at week 6 and then dropped to the little to no change in relative abundance grouping at week 12 (Figure 19). Additionally, one subject who was given the high nutritional supplement remained in the little to no change in relative abundance grouping for both the week 6 and the week 12 analysis (Figure 19). These subjects highlight the variability in human studies and a greater number of subjects is required to truly confirm the results that high nutritional supplementation decreased the relative abundance of sweat metabolites.

Other hierarchical cluster maps of single time points (i.e. separated by week) and of high or low nutritional supplementation only were also analyzed. These figures show similar grouping to the hierarchical cluster map of the whole data. No new separations or information could be deduced from the partial cluster maps. Please refer to Supplemental Figures 6 to 9 for the individual cluster maps.

Figure 19: The hierarchical cluster map of the week 1 normalized $\log_2$ fold change of all data shows separation into 3 groupings, reduced metabolite concentrations, increased metabolite concentrations, and those with little to no change in metabolite concentration over time. The reduced and increased metabolite concentration groupings show separation in sweat metabolite concentration between the low and high nutritional supplementation subjects.

Subjects 1- 8 received high nutritional dietary supplementation. Subjects 9-14 received low nutritional dietary supplementation.



The data shown in the hierarchical cluster map further illustrates the contradiction between the data collected in this experiment and the conclusions of Hier et al. that diet and sweat amino acid content have no correlation (25). However, the random daily fluctuations in sweat amino acid concentrations illustrated in the data collected by Hier et. al. (25) could explain the inability of the high nutritional supplement to universally decrease sweat metabolite content. The fluctuations could also be an explanation for the subjects who received low nutritional supplementation and had an increase in their metabolite concentrations. This is only one possible explanation for the observed inconsistencies in nutritional supplementation.

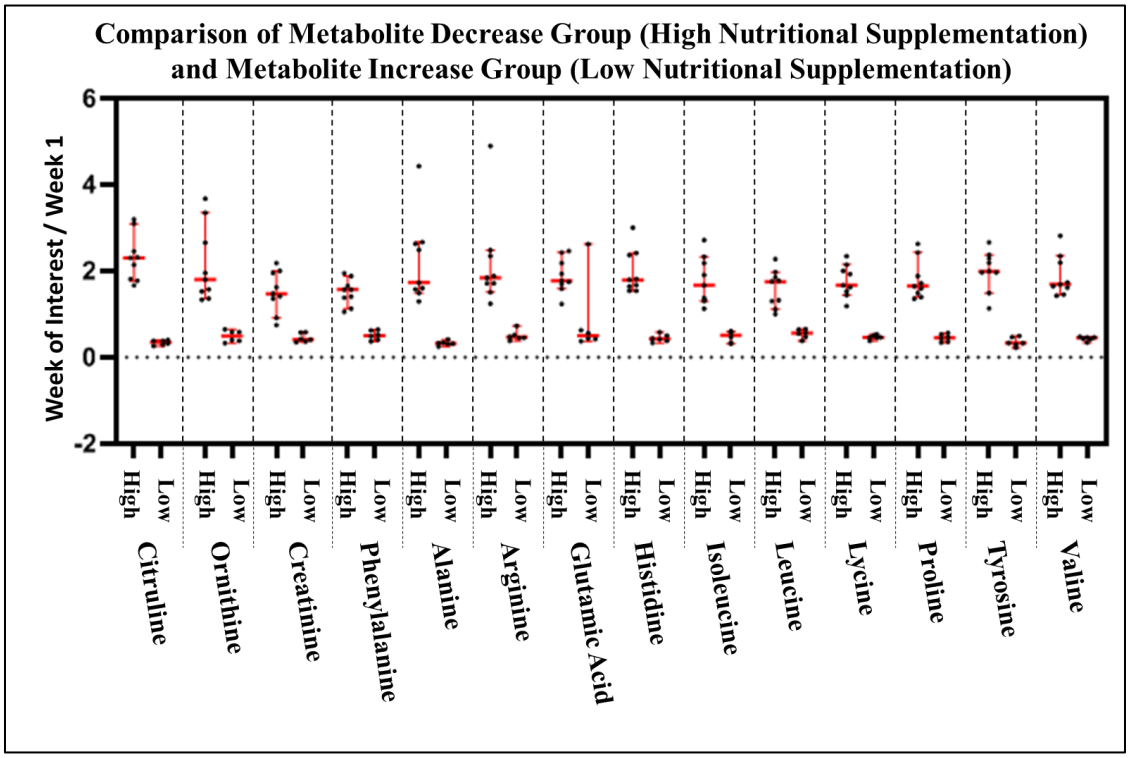
As mentioned previously, high nutritional supplementation was not shown to uniformly decrease subjects sweat metabolites (Figure 19). Taking the supplement did not account for the remainder of the subjects' diet. A hypothesis for why the high nutritional supplementation did not uniformly decrease sweat metabolites among subjects is that some subjects who had little change to their metabolite concentrations may have had a decrease in normal dietary protein intake during the experiment. Another hypothesis is the subjects' diet was already high in proteinbefore the experiment providing the same nutritional benefits as the supplement. Two subjects had a decrease in their metabolite concentrations in week six and then rose back to the week one metabolite concentrations for the week twelve test. I hypothesize the variable effects of the high nutritional supplementation was caused by a changes in diet during the procedure. Similar to subjects with high nutritional supplementation, the subjects with low nutritional supplementation did not illustrate uniform relative sweat concentration in their metabolite compositions.

As expected, several subjects who took the low nutritional supplement had little to no change to their sweat metabolite concentration (Figure 19). However, some subjects' sweat metabolite concentration increased (Figure 19). A possible reason for the concentration increase could be that these subjects decreased their daily caloric intake to compensate for what they thought was a high calorie supplement. The inconsistences between the subjects who took the same supplement could also be explained by differing physical fitness, levels of hydration, etc. Since many uncontrolled variables still exist, further testing with a larger number of subjects is needed to confirm that long-term nutritional supplementation can influence the sweat metabolite concentrations.

Comparison of Metabolite Subsets

The hierarchical cluster map analysis divided the samples into three subsets (metabolites reduced, little to no change, or increased). The increased metabolite subset (consisting of subjects given the low nutritional supplement) and the reduced metabolite subset (consisting of subjects given the high nutritional supplement) were then taken and the individual metabolite concentrations were compared. Creatinine and glutamic acid were the only two metabolites with overlap within the concentrations of the two subsets (Figure 20). Even though there was slight overlap between the two groups in creatinine and glutamic acid both did show significance in separation with glutamic acid's p-value being the largest at 0.0113. Significance of $p<0.05$ was determined utilizing an unpaired t-test with Welch's correction. The next highest p-value was isoleucine at 0.0014 and several metabolites (alanine, arginine, lycine, and valine) had p-values <0.0001 with the other metabolites within that p-value range.

Figure 20: By only comparing the increased and decreased subsets from the Hierarcical cluster map significant separation can be found between 13 of the 14 metabolites of interest ($p<0.05$) utilizing a unpaired t-test with Welch's correction. The high nutritional supplementation resulted in decreased metabolite concentrations while the low nutritional supplementation resulted in increased metabolite concentrations from week 1 to week 6 and 12. The vertical red bars encompass the 95% confidence intervals for each dataset while the horizontal red bars represent the median of each dataset.



Using only the two subsets, complete separation was achieved among 12 of the selected metabolites (Figure 20). Focusing on the two groupings from the hierarchical cluster map hopefully allows for the removal of the human variation and shows a more accurate depiction of how the dietary supplementation effected the sweat metabolite composition. It is more clearly shown that the high nutritional supplementation reduced the metabolite concentration in sweat while the low nutritional supplementation increased the metabolite concentration of sweat. By comparing just the two data subsets the hypothesis that dietary supplementation effects the metabolite concentration is strongly supported. The analysis confirms the need for continued study with a larger subject pool on how diet can affect metabolite concentration in sweat as well as confirming the need to account for dietary supplementation in other sweat studies.

The data from this experiment are in line with data found in the literature on several sweat metabolites. For example, when dietary glucose is supplemented in diet sweat glucose concentration increases (5). In addition to sweat glucose concentration another sweat metabolite, ammonia, has been observed increasing when supplemented in the diet in the form of ammonium chloride (13). Curiously, sweat urea (the product of ammonia breakdown) concentrations remained consistent with the ammonia supplementation (13). The results presented here in addition to these few example studies on the impact of diet on sweat metabolite abundance provide further justification for continued research into dietary effects on sweat metabolites.

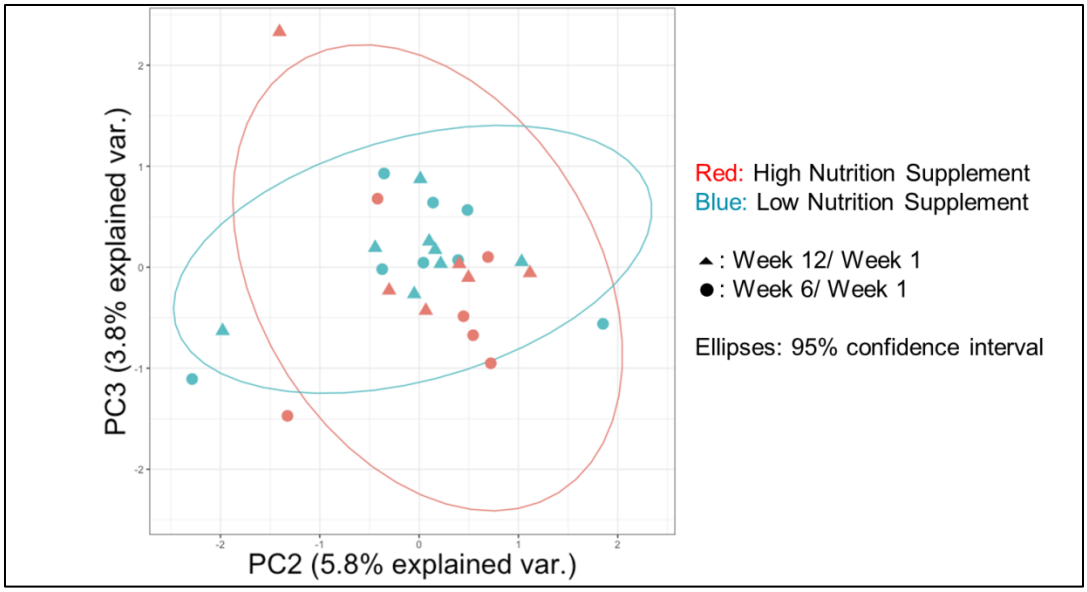
Conclusions

Ten of the chosen metabolites had to be removed from the analyses performed in this experiment. Metabolites with concentrations under 5 µM were unable to be quantitated with the method and instrumentation available. A more sensitive mass spectrometer must be utilized to analyze low abundant metabolites in sweat.

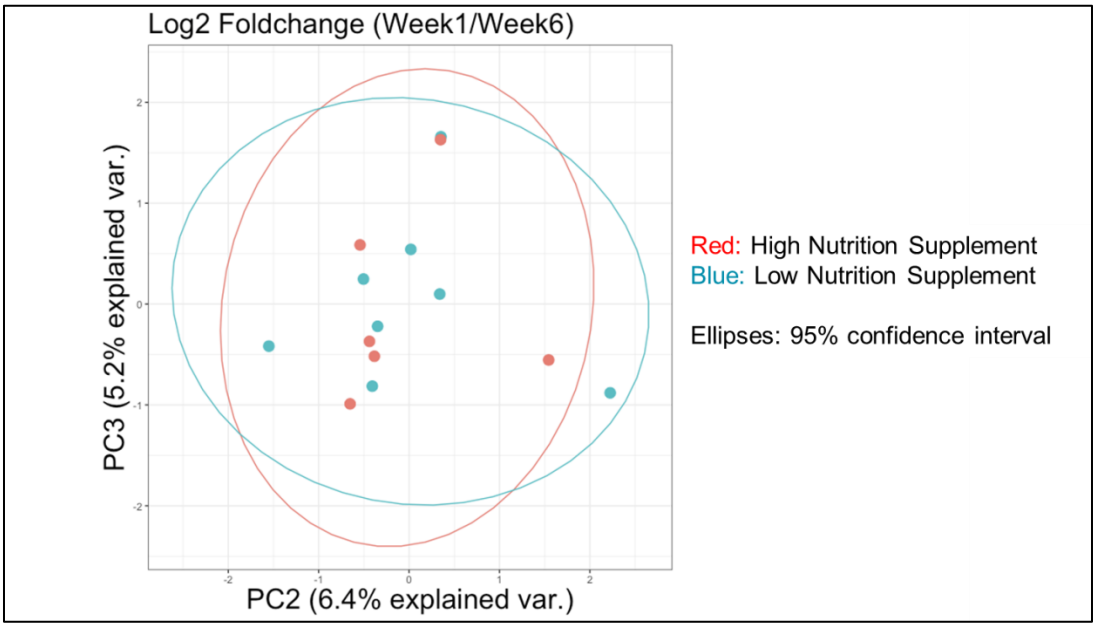
Although the goal of the experiment in Chapter 2 was to apply the experimental methods determined in Chapter 1 to evaluate the influence of diet on sweat content the statistical analysis performed in this study would have benefited from a larger subject population to ensure a more representative population. While the study started with a greater number of subjects, throughout the 12 weeks attendance was variable, precluding samples from inclusion in the analysis. Despite the low sample number, the data suggests diet could impact sweat metabolite content.

Despite the setbacks in sample number, this study was able to advance the understanding of the sweat metabolome. Past research had shown that concentration of amino acids in sweat had no correlation to dietary intake (25). However, no quantitative studies on the effects of long term diet supplementation on sweat metabolites had been attempted. This study provides data supporting the hypothesis that high nutritional supplementation can affect the concentration of metabolites including amino acids in human sweat. These results support the need for diet to be considered in biomarker discovery experimentation.

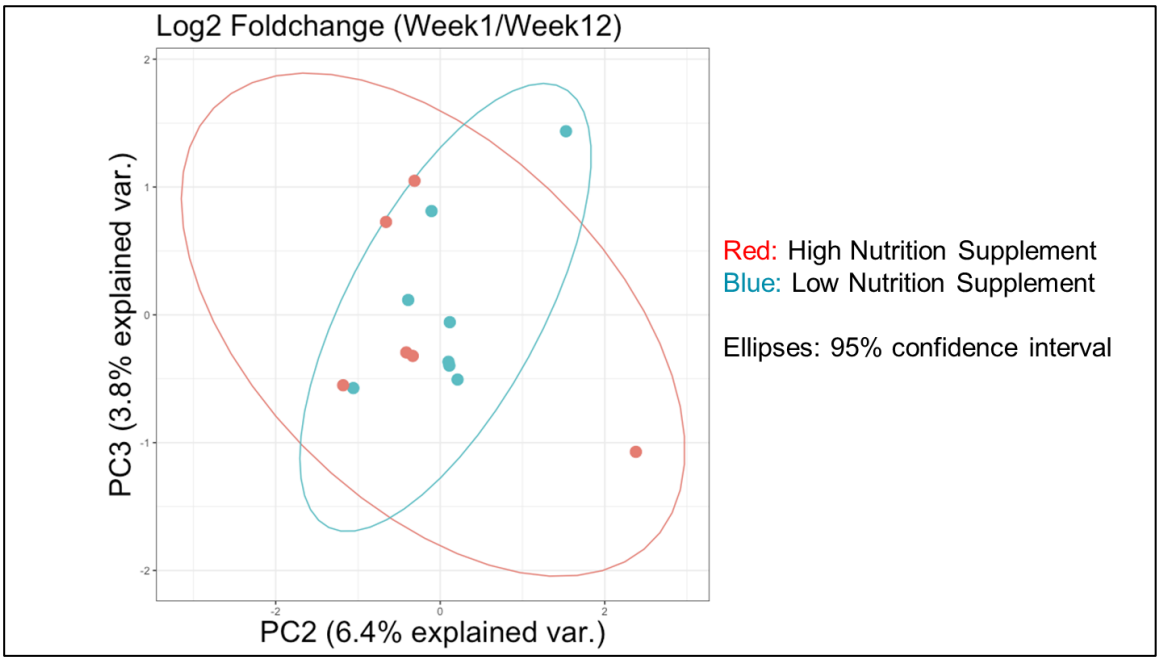
Supplemental Figure 1: PC2 & 3 of all data



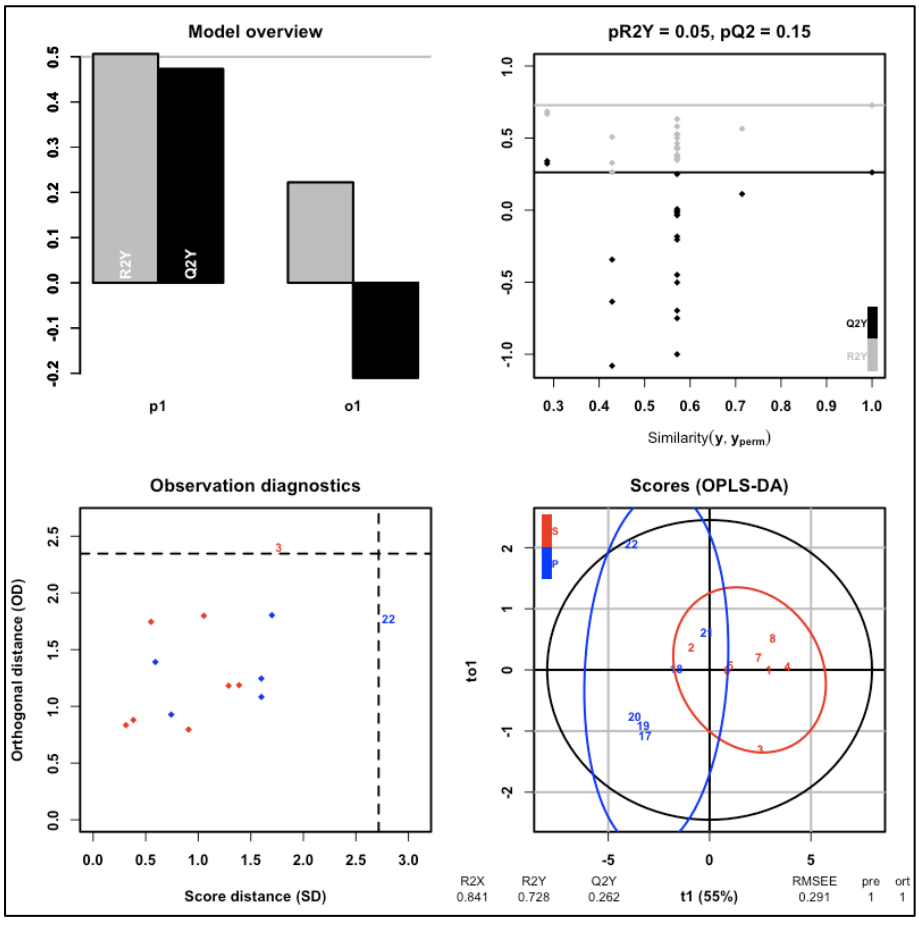
Supplemental Figure 2: PC2 & 3 of week 6 data



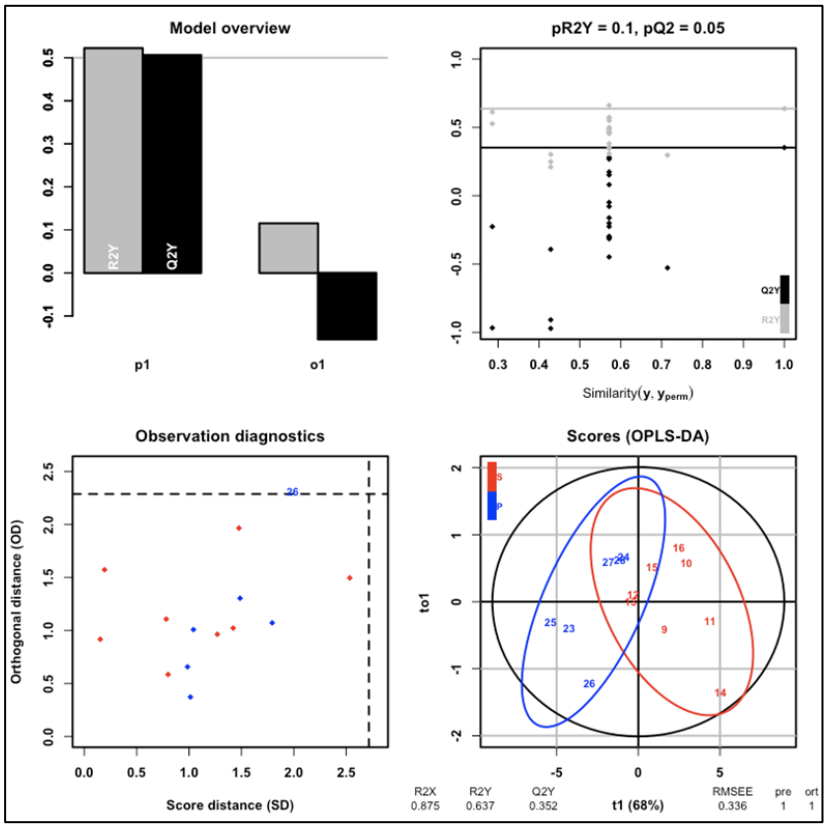
Supplemental Figure 3: PC2 & 3 of week 12 data



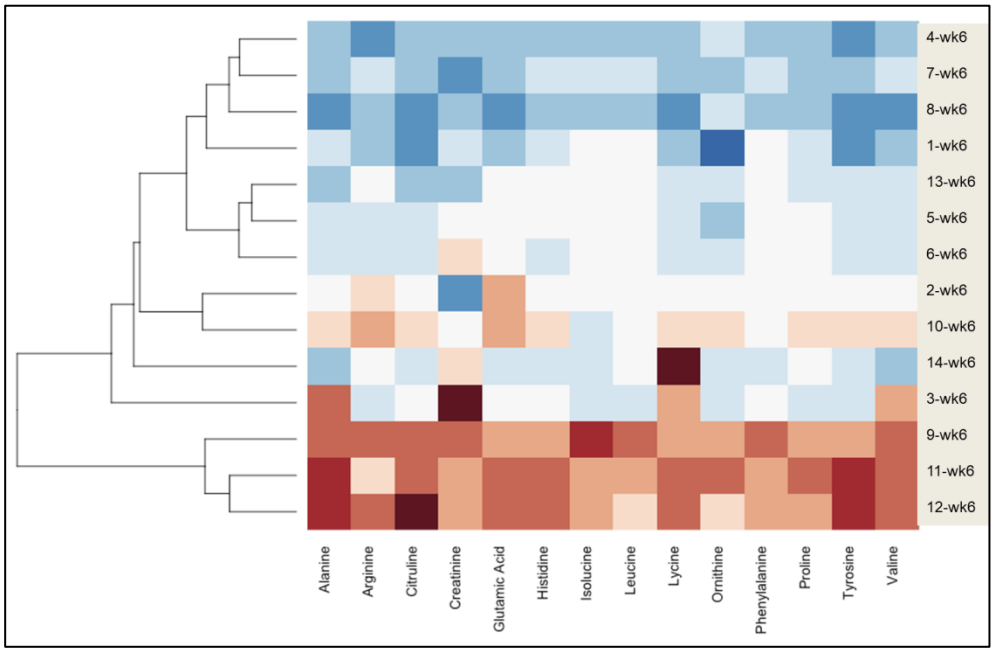
Supplemental Figure 4: Discriminate analysis of week 6 data



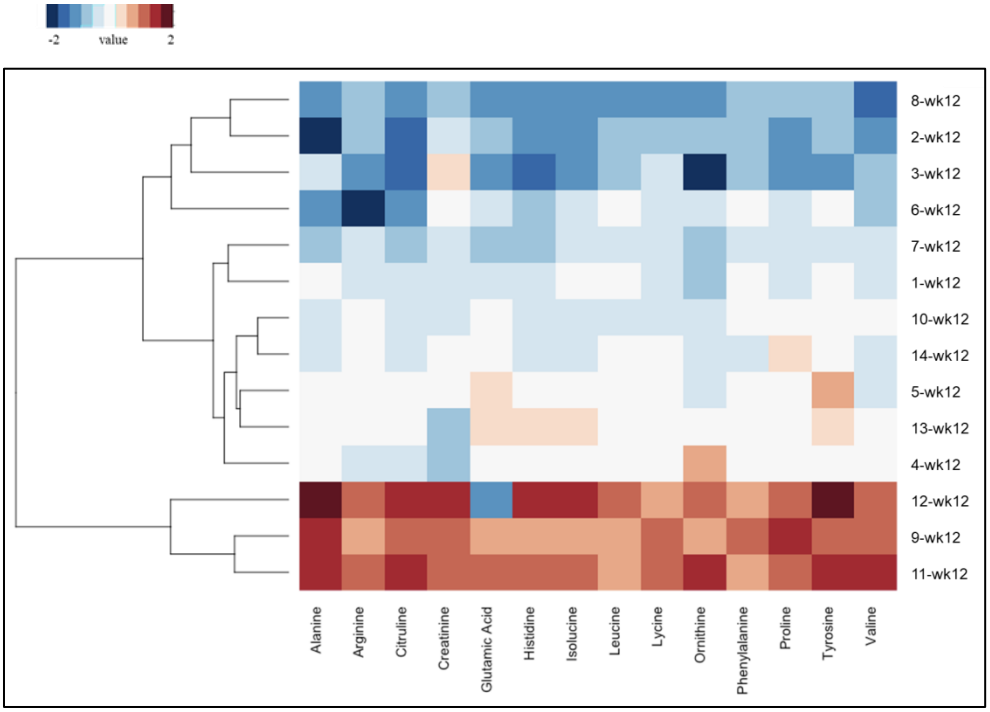
Supplemental Figure 5: Discriminate analysis of week 12 data



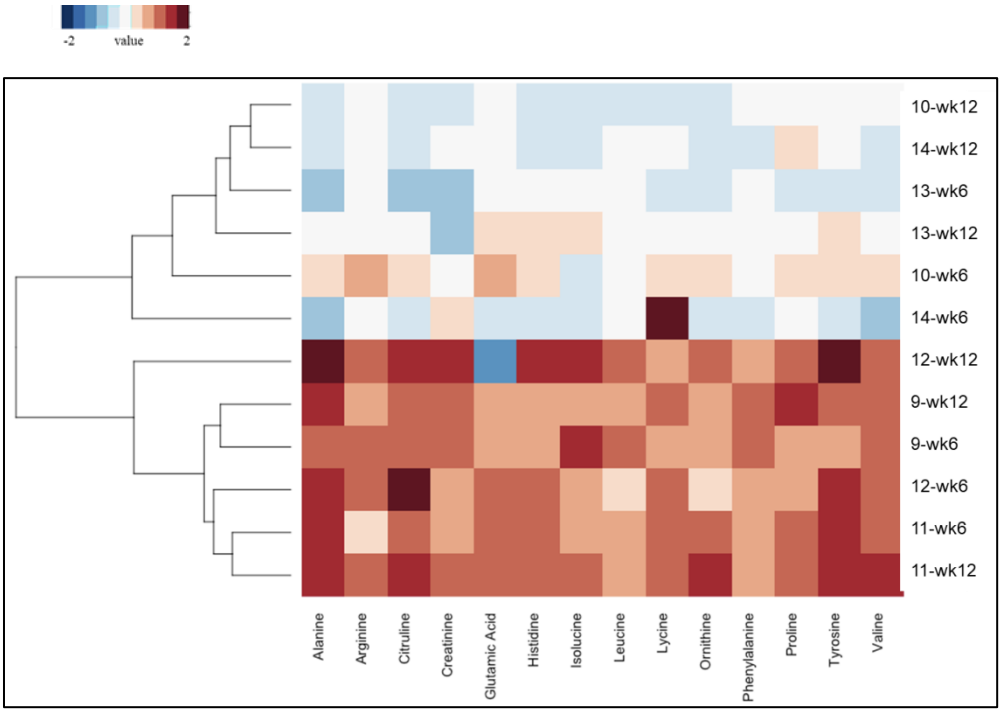
Supplemental Figure 6: Hierarchical cluster map of week 6 data



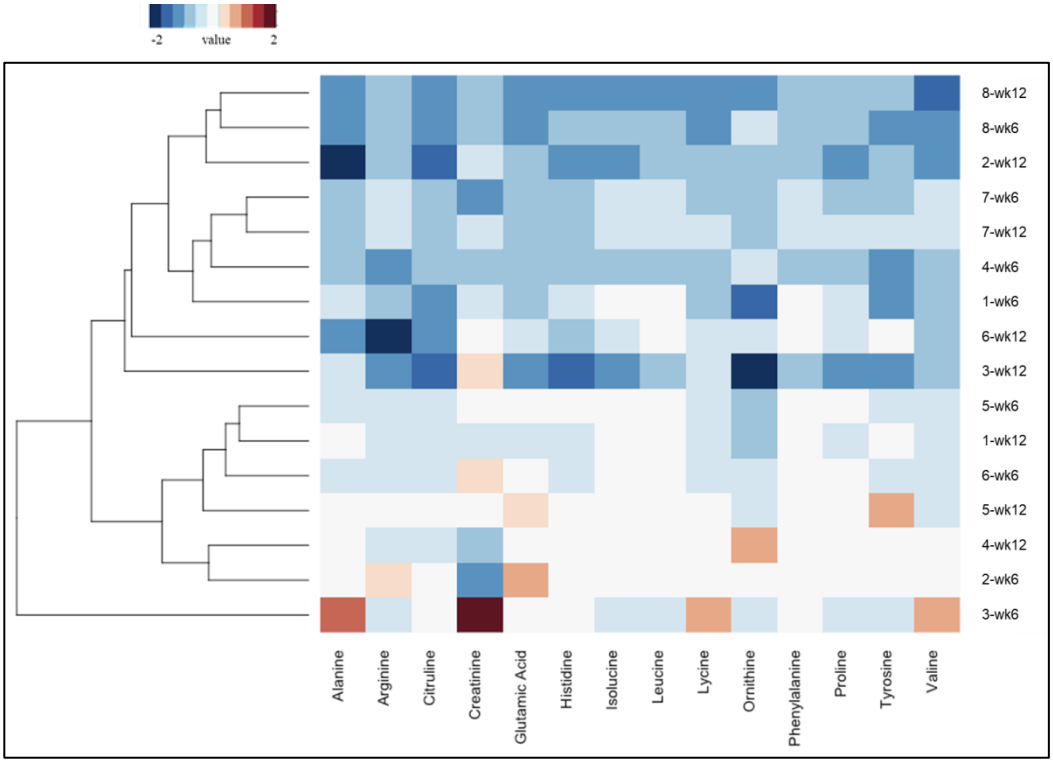
Supplemental Figure 7: Hierarchical cluster map of week 12 data



Supplemental Figure 8: Hierarchical cluster map of low nutritional supplementation subjects



Supplemental Figure 9: Hierarchical cluster map of high nutritional supplementation subjects



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