ABSTRACT

FLETCHER, KELLY ANN. Metabolomic Analysis of *Escherichia coli* O157:H7 under Acid Stress using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry. (Under the direction of Suzanne Johanningsmeier and David Muddiman).

Escherichia coli O157:H7 is a human pathogen noted for its ability to resist acidic environments. The acid resistance of this serotype is understood through two hypotheses. Organic acids that cross the cell membrane of *E. coli* at pH 3.2 decrease the intracellular pH upon dissociating inside; to counteract this, the cell expends ATP to run the primary proton pump in reverse of the proton motive force and transport protons out of the cytoplasm. *E. coli* also has four acid resistance (AR) mechanisms, three of which rely on the decarboxylation of amino acids. Researchers have presented genomic, transcriptomic, and proteomic evidence to support these mechanisms in *Escherichia coli* O157:H7, but a metabolomic approach has not yet been used to study acid resistance in this serotype. The objective of this work was to use a GCxGC-ToFMS metabolomic platform to discover the metabolic changes in *E. coli* O157:H7 under organic acid and acid pH stress.

A microbial metabolomics experiment was designed to test the properties of an acid-resistant strain of *E. coli* O157:H7, B241 (28RC1). Cells were exposed to acid pH (3.2) in the presence and absence of acetic acid, a commonly used food acidulant, in order to induce changes in the intracellular metabolite pool. Cells in identical buffer at unstressed pH (7) were used as a control. After acid-stressing the cells, they were filtered from the treatment medium and quenched. Metabolites were extracted and chemically derivatized for analysis by two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS).

Work was undertaken to ensure the conditions used in the experiment were sufficient to induce acid stress without resulting in cell death. It was found that strain B241 survived acid pH conditions with and without 40 mM acetic acid as an additional stressor, at 99 % survival over a thirty minute exposure, eliminating the concern of detection of metabolites from dead cells. The quantity of cells to be treated, collected, and analyzed in order to detect cellular metabolites was optimized. Experimental measurement of cell dry weights and the visual analysis of chromatograms indicated that the ideal amount of cells for this work was equivalent to approximately 3 mg dry weight. Derivatization and GCxGC-ToF-MS analysis of pure standard compounds was conducted to confirm the identities of metabolites. Furthermore, measurement of time and temperature conditions for the halting of cellular metabolism ("quenching") was performed from which a method was selected which quenched the cells at -40 °C in 9.33 ± 2.50 seconds.

An experiment was carried out which compared the intracellular metabolite pools of *E. coli* O157:H7 which had been exposed to neutral pH, acid pH, or acid pH plus 40 mM acetic acid for thirty minutes. Seven independent biological replicates were measured and sample order was randomized. Acid pH decreased the concentration of glutamate and increased the concentration of GABA, supporting current theories explaining acid resistance in O157:H7. Hierarchical clustering analysis uncovered 27 metabolites that decreased significantly (including amino acids and nitrogenous metabolites) and 20 that increased significantly, solely in response to acetic acid stress ($p < 0.001$). These results represent a promising first look into the metabolomics of acid-stressed *E. coli* O157:H7, which directly contributes to enhanced knowledge about the acid resistance properties of this serotype.

Metabolomic Analysis of *Escherichia coli* O157:H7 under Acid Stress using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Food Science

Raleigh, North Carolina
2012

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DEDICATION

To Chief, Fletch, and Treewhacker.

BIOGRAPHY

Kelly Ann Fletcher was born in Abington, Pennsylvania and raised in Cherry Hill, New Jersey. Her family was scientifically-oriented and encouraged her to be intellectually curious. In high school, her chemistry teacher sparked her interest in the subject and also served as a mentor and role model. As an undergraduate at Roanoke College, she quickly discovered a love for organic and analytical chemistry and a desire for a nontraditional chemistry career. Two different undergraduate research projects, the first studying surface plasmon resonance spectroscopy to monitor self-assembled monolayer deposition, and the second on synthesis and characterization of bisamine boron cations, fostered her interest in instrumentation as a research tool. Her foray into food science began in her senior year, when a lecture on flavor chemistry opened her eyes to the possibility of experimenting on edible substrates. She subsequently enrolled in graduate school at North Carolina State University in order to further investigate this interest by studying the biochemistry of *Escherichia coli* O157:H7 using two-dimensional gas chromatography-time-of-flight mass spectrometry as presented herein. After graduation, she hopes to begin working in the food industry. In addition to scientific pursuits, Kelly enjoys running, swimming, playing violin, dining, traveling, video games, and spending quality time with her family and friends.

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CHAPTER ONE

Detection and Interpretation of Metabolic Changes in *Escherichia coli* O157:H7 due to pH and Organic Acid Stress: A Review

Abstract

Escherichia coli O157:H7 is a bacterial strain which has gained notoriety in the last few decades due to its causatory role in several high-profile foodborne illness outbreaks. Research has uncovered its noteworthy acid resistance properties, which contribute both to its virulence and to its ability to survive in acid pH food environments. There is a wealth of knowledge published on the acid resistance mechanisms responsible, but little is known about the changes induced in the intracellular metabolite pool of *E. coli* O157:H7 in response to acid stress.

Microbial metabolomics is a field that studies intracellular and/or extracellular metabolite pools in order to obtain a biological fingerprint of the cells and gain insight into changes that occur as part of metabolism at the cellular level. This approach can be used to observe changes in metabolite concentrations due to changes in the environment or genetic makeup of the organism. Many analytical instruments exist which are well-suited to this sort of non-targeted analysis, but one of the better choices is two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS). Its advantages include a more complete chromatographic separation (relative to traditional GC/MS) due to the different stationary phase polarities, as well as excellent detection limits and high mass resolution from the time-of-flight detector. Currently, there is a gap in the scientific knowledge concerning the effect of pH and organic acid stress on the intracellular metabolite pool of *Escherichia coli* O157:H7. The hypothesis is that the use of GCxGC-ToF-MS to address this gap will result in the observation of biologically significant metabolic changes resulting

from acid pH and organic acid conditions. The currently published scientific literature states that there are genes being upregulated and downregulated and changes in transcription as a direct result of those conditions, and the metabolites resulting from those pathways should be detectable by this method.

1.1 Introduction

Escherichia coli is a species of gram-negative, mesophilic, oxygen-facultative, rod-shaped, non-spore-forming bacteria that belong to the Enterobacteriaceae family. Bacteria in this family ferment both glucose and lactose. There are several classes of *E. coli* which are categorized by their virulence properties. These virotypes include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC). One specific subtype, or serovar, of *E. coli* is O157:H7, which is a member of the EHEC virotype and is commonly referred to as "pathogenic *E. coli*." Overall, this species tends to tolerate environmental stressors poorly; it is quickly inactivated by heat and ionizing radiation and generally prefers a neutral pH. However, some strains of *E. coli*, including O157:H7, have adapted to be able to tolerate acidic surroundings (Gorden and Small, 1993; Small *et al.*, 1994).

Pathogenic *E. coli* is primarily associated with bovine fecal material; however, it can also be found in the feces of other ruminant animals such as deer or wild boar (Ryu *et al.*, 1999; Tosun *et al.*, 2007). The bacteria colonize the gastrointestinal tract of the animal while it is young, without causing disease symptoms. The animal then sheds the bacteria in its feces, at even higher levels when it is exposed to stressors such as weaning, transport, or relocation (Bach *et al.*, 2003). A secondary source of the bacteria is via the slaughter process of cattle, if ground beef is allowed to comingle and comes in contact with the gut microflora in the slaughtered cow. Thirdly, contamination by *E. coli* can occur in fresh produce that has been washed with contaminated water, handled improperly, or has been in contact with animal feces (Tosun *et al.*, 2007). Other less common routes of infection are via cross-contamination or person-to-person spread. These ways that food may become contaminated with *E. coli* make it a challenge for food preparers and consumers.

Though the bacteria tend to have little resistance to most environmental stressors, recent outbreaks of pathogenic *E. coli* in foods with an acid pH have illustrated the robust acid-tolerance response that this species of bacteria can have under acidic conditions (Leyer *et al.*, 1995). One example of an *E. coli* outbreak in an acid food is an apple cider outbreak that occurred in 1991, which resulted in twenty-three confirmed infections: sixteen of these developed hemorrhagic colitis, and four cases progressed to a dangerous complication known as hemolytic uremic syndrome (HUS) (Besser *et al.*, 1993). A larger outbreak later occurred in a similar acid food vector: After drinking Odwalla apple juice, sixty-six people were infected, fourteen developed HUS, and one died (CDC, 1996). The fact that *E. coli* can persist in these acid pH environments at levels significant enough to cause foodborne disease makes its control very important in acidic foods (Griffin and Tauxe, 1991). This review will examine the nature of pathogenic *E. coli* and its acid-survival mechanisms, as well as an experimental method which can be used to gain a better understanding of how the organism's acid-tolerance response functions.

1.2 *Escherichia coli* O157:H7 Serotype

The *E. coli* O157:H7 strain differs in certain key ways from generic strains of *E. coli*, so that O157:H7 must be cultured in the laboratory using different methods than those developed for other fecal coliforms. What makes O157:H7 especially dangerous, however, is the Shiga toxin genes it is believed to have acquired by horizontal transfer from a bacterial species known as *Shigella* (O'Brien *et al.*, 1983). This allows it to cause a toxicoinfection in people who consume it in doses of ten colony forming units (CFU) or even less (Griffin and Tauxe, 1991). The possible symptoms of this toxicoinfection are hemorrhagic colitis (bloody diarrhea), hemolytic uremic syndrome (HUS), which causes the kidneys to fail, and thrombotic thrombocytopenic purpura (TTP), a rare blood disease for

which the elderly are at increased risk (Bhagwat *et al.* 2005, Cheng and Chou 2001, Cheng *et al.* 2003, Deng *et al.* 1999, House *et al.* 2009, Huang *et al.* 2007, Ryu and Beuchat 1998). The low infectious dose is also connected to the ability of the bacteria to survive gastric acid of around pH 2 for up to three hours during digestion (Barua *et al.* 2002, Booth *et al.* 2002, House *et al.* 2009, Huang *et al.* 2007). Less acid-resistant pathogens would not survive those conditions, so they have higher infectious doses to compensate.

Some food vectors that have been associated with *E. coli* O157:H7 infection include ground beef; fresh raw produce such as sprouts, lettuce, and spinach; unpasteurized fruit juices and dairy products; fermented products like sausage and yogurt; and condiments such as mayonnaise and salad dressing (Cheng and Chou 2001, Cheng *et al.* 2003, Deng *et al.* 1999, Erdoğrul and Erbilìr 2005, Huang *et al.* 2007, Leyer *et al.* 1995, Ryu and Beuchat 1998, Tosun *et al.* 2007). Of these, the products with low pH values likely to cause acid stress in *E. coli* O157:H7 cells were fresh produce, juices, yogurt, and mayonnaise, to highlight a few of the major foods of concern (Cheng and Chou 2001, Erdoğrul and Erbilìr 2005, Leyer *et al.* 1995, Ryu and Beuchat 1998, Tosun *et al.* 2007). Foods where pH is the only barrier to pathogens may pose an increased risk to consumers.

1.3 *Escherichia coli* O157:H7 and Acid Stress

The ability of *E. coli* O157:H7 to tolerate acidic environments makes it one of the most dangerous foodborne pathogens. The basic mechanism for how it does this involves some simple chemistry. The Henderson-Hasselbalch equation describes the relationship between pH and pK_a in aqueous solutions of organic acids: When the pH of the environment is greater than the pK_a of the acid, the majority of the acid is in its dissociated form, and when the pH of the environment is less than the pK_a of the acid, the majority of the acid is in its undissociated form (Henderson, 1908;

Hasselbalch, 1917). Thus, outside of the cell, when the pH is acidic, weak undissociated organic acids with relatively high pK_a values can diffuse through the cell membrane into the cell. Once inside the cell, the pH is greater than the pK_a of these acids and they dissociate into hydrogen ions and their respective anions. The cell uses energy in the form of adenosine triphosphate (ATP) to run its primary proton pump and remove hydrogen ions from the cell, re-equilibrating its internal pH (Booth, 1985). The cytoplasm accumulates anions, which inhibit metabolism and increase the pH gradient across the cell membrane, leading to cell death (Russell, 1992; Diez-Gonzalez *et al.*, 1997).

Axe and Bailey found that some organic acids such as acetic acid and lactic acid can cross the cell membrane in either the dissociated or undissociated form, which allows for significant accumulation of acetate and lactate anions in the cytoplasm when the external solution is at a pH below the pKa values of the acids (1995). The charged ions are able to cross the cell membrane because acetate acts as a classical uncoupling agent. However, Barua *et al.* theorized that in acid-adapted cells of O157:H7, protons do not flow into the cell as easily as unadapted cells (Barua *et al.* 2002). This possibility would mean that *E. coli* would not be required to expend as much ATP to keep its pH around neutral and maintain homeostasis. Cells of O157:H7 that have been sublethally injured by acid can be completely repaired in a few hours in nutrient-rich, pH-neutral conditions, making acid-killing difficult (Wesche *et al.* 2009).

When considering acid stress in *E. coli*, however, it is important to note that the effects cannot be attributed solely to the activity of the protons, but also in part to the presence of anions that build up in the cytoplasm. A study by Diez-Gonzalez *et al.* found that the presence of acetate anion decreased the intracellular pH (1999), although acetate can also have an effect independent of pH (Schellhorn and Stones, 1992; Diez-Gonzalez *et al.*, 1997). Schellhorn and Stones found that acetate induced *rpoS*, the gene encoding for transcription of sigma factor 38, which regulates the

expression of stress-response genes (1992). Another study by Diez-Gonzalez and coworkers found that acetate accumulation increased glucose consumption, for which the authors propose the explanation of decreased ATP production (1997). Aside from these clues, little else is known about the ways in which acetate anion buildup contributes to eventual cell death.

Prior to acid-challenging *E. coli*, cells can also become acid habituated during the log phase. Chung *et al.* explained that exposing log-phase cells to a mildly acidic pH for a short time causes them to subsequently survive better in stronger acids with a lower pH (2006; Buchanan and Edelson, 1999; Brudzinski and Harrison 1998; Rowbury and Goodson 1998a). Factors that were found to affect this phenomenon include whether the cells have previously been acid-adapted; the nature of the acid stress including pH and identity of the acid; the duration of the acid habituation; and the strain of *E. coli* (Buchanan and Edelson, 1999; Samelis *et al.* 2003, Brown *et al.* 1997). A number of compounds, including glucose, glutamate, aspartate, and L-proline, can induce acid habituation during cell growth in certain media (Chung *et al.* 2006). The optimal pH range for the acid habituation has been reported to be between 4 and 5.5, with pH 5.0 being the optimum (Koutsoumanis and Sofos 2004; Rowbury and Goodson 1998b). Specifically, Jordan *et al.* reported that briefly exposing the pathogenic cells to mildly acidic conditions, ranging from approximately pH 6.0 to pH 4.5, elicits the expression of genes whose specific purpose is to resist the acid stress (1999). Buchanan *et al.* explained this notion of acid habituation and how it leads to the concept of acid tolerance, the increased survival of bacterial cells at pH 4.0 to pH 2.5, following a period of adjustment in the more moderate pH (1999). Brudzinski *et al.* concurred and suggested that the acid tolerance response in cells is a very different one than the acid shock response, which is implicated in the survival of bacteria in gastric acid (1998). The acid tolerance response in *E. coli* refers to the behavior of cells grown in a mildly acidic pH and then exposed to a much lower pH; the acid shock

response differs in that cells are typically at a near-neutral pH initially, before exposure to the acid pH (Brudzinski *et al.*, 1998). Bhagwat *et al.* also presented this distinction, noting that its ability to resist acid pH contributes to the virulence of *E. coli* O157:H7 (2005). Acid habituation is a known contributor to the survival of O157:H7 in acid pH environments.

The mechanism for acid tolerance of *E. coli* O157:H7 involves the RNA polymerase sigma factor RpoS, which controls the expression of genes involved in the general stress response of *E. coli* O157:H7 and other gram-negative bacteria (Allen *et al.* 2008, Barua *et al.* 2002, Bhagwat *et al.* 2005, Booth *et al.* 2002, Brudzinski and Harrison 1998, Buchanan and Edelson 1999, Cui *et al.* 2001, Garren *et al.* 1997, Huang *et al.* 2007, Jordan *et al.* 1999, Leenanon *et al.* 2003, Tosun *et al.* 2007). Sigma factors are proteins involved in transcription initiation that allow RNA polymerase to bind to a specific gene promoter, resulting in the expression of various genes, depending on which sigma factor is activated by the environment. In *E. coli*, the *rpoS* gene codes for the protein sigma 38 (σ^{38}), allowing the cells to withstand their current environment and providing cross-protection for future environmental stresses. Hall and coworkers noted that many of the proteins observed to be present in response to acid stress and also present in response to other kinds of stress as well (1995).

Many studies currently support this mechanism. Buchanan *et al.* found that both RpoS gene and pH-dependent resistance systems are active in stationary phase cells of *E. coli* (1999). The RpoS gene encodes proteins responsible for imparting acid tolerance to O157:H7, whereas the pH-dependent systems rely on decreasing the pH gradient across the cell membrane (Cheville *et al.*, 1996; Small *et al.*, 1994; Arnold and Kaspar, 1995). Brudzinski *et al.* stated that this mechanism governed by RpoS-regulated proteins provided cross-protection against acidity and heat stress in *E. coli* O157:H7 (1998). Allen *et al.* observed that genes related to acid shock were significantly upregulated in cells that had also been exposed to cold shock, in another instance of cross-

protection (Allen *et al.*, 2008). Booth *et al.* explained in greater depth how strains of *E. coli* without the HdeA protein in the periplasm of the cell have a survival rate of ten thousand times less than strains with this protein at pH 3 (2002). The HdeA protein, one of the most abundant in the periplasm, is one regulated by the RpoS sigma factor, specifically expressed from the hdeAB operon of the genetic sequence (Booth *et al.* 2002). It is responsible for preventing acid-induced aggregation of proteins in the cell (Booth *et al.*, 2002).

Foster and coworkers have done extensive work to describe the acid resistance mechanisms at work in *Escherichia coli* O157:H7. The first system, AR1, is induced when cells are grown in Luria-Bertani (LB) medium at pH 5.5. As the cells grow, they ferment the glucose and reduce the extracellular pH, gradually habituating themselves to the presence of acid (Foster, 1994). However, the enzymes glutamate decarboxylase and arginine decarboxylase are also an important part of the acid tolerance response of *E. coli* O157:H7. These are only observed when the cells are glucose-repressed and the appropriate amino acid (either glutamate or arginine) is supplied in the extracellular medium (Foster, 1994). The glutamate-dependent acid resistance system is known as AR2, and the arginine-dependent system is called AR3. Booth *et al.* reported that these decarboxylases, as well as lysine decarboxylase, were the most abundant decarboxylases when *E. coli* was exhibiting its acid-tolerance response (Booth *et al.* 2002). House *et al.* found that the genes coding for glutamate and arginine decarboxylases, as well as the gene which codes for the enzyme succinate dehydrogenase, were significantly upregulated by a factor of two or more following a period of acid adaptation using real-time polymerase chain reaction (PCR) methods (2009). It has been further specified that the action of glutamate decarboxylase is due to the gadABC operon, and that adiA codes for arginine decarboxylase (Cui *et al.* 2001; Bearson *et al.* 2009). Cui and others hypothesized that these acid resistance mechanisms function by increasing the alkalinity inside the

cell (Cui *et al*. 2001). The presence of these amino acids in the external environment of the cell enhances their survival in the acidic environment because the decarboxylation reaction consumes a proton in a 1:1 molar ratio with the amino acids, helping increase the pH (Booth *et al.* 2002). This mechanism is a good explanation for the survival of *E. coli* O157:H7 in acidic environments because it is in accordance with the idea of the cell not having to expend ATP to run its primary proton pump in reverse of the proton motive force.

Though widely accepted that glutamine and arginine decarboxylases are involved in the survival of *E. coli* under acid stress, another amino acid decarboxylase has also been found to play a noteworthy role. Researchers have found that lysine decarboxylase is active in many O157:H7 strains and that it does help increase the intracellular pH, to a lesser extent than arginine decarboxylase and glutamine decarboxylase (Iyer *et al.*, 2003; Diez-Gonzalez, 2004). It has been proposed that this acid resistance mechanism be termed AR4 (Diez-Gonzalez, 2004).

While acid resistance is beneficial for the survival of *E. coli* O157:H7 cells, it poses some serious problems for humans. A study by Cheng *et al.* found that acid adaptation of O157:H7 increased its survival rate by about 1 log CFU/mL in acidic mango and asparagus juices and in a fermented milk product called Yakult after several hours (2001). The implications for this study are significant because if acid-adapted *E. coli* can survive in fruit and vegetable juices and dairy products, it may also survive in acidic products consumed by many Americans, such as fruit juice, acidified vegetables, and yogurt.

1.4 Metabolomics

The chemical reactions involved in metabolism allow the cell to obtain and use oxygen, break down molecules for energy, convert waste products into useful molecules, and many other

functions necessary for the survival of the cell. Microbial metabolomics is the field that examines these biochemicals within cells and observes changes in chemicals or their concentrations in response to environmental perturbations or genetic alterations (Barsch *et al.*, 2004; Rabinowitz, 2007). This approach is complementary to those of transcriptomics and proteomics, which deal specifically with the expression of specific genes and the synthesis of proteins (Lockhart *et al.*, 1996; Shevchenko *et al.*, 1996). This information provides insight into the regulatory processes of the cell, both under typical conditions and atypical ones, such as acid stress. Metabolomic studies have been completed on samples ranging in diversity from human urine to Granny Smith apple peels to various strains of *Escherichia coli* (Pasikanti *et al.* 2008; Rudell *et al.* 2008; Maharjan and Ferenci 2003; Bennett *et al.* 2009; Bolten *et al.* 2007; Jozefczuk *et al.* 2010). Information on human metabolism can contribute to our understanding of digestion and nutrition. In an analogous way, metabolomic studies of human foodborne pathogens can uncover methods to deter or eliminate these pathogens in food by better understanding how the cells' metabolism affects their survival.

There are several publications in the scientific literature detailing the proteomic and transcriptomic analysis of *E. coli* O157:H7. One study identified several protein biomarkers that differentiated pathogenic O157:H7 from nonpathogenic *E. coli* strains, but not from genetically similar non-O157 pathogenic strain O55:H7 (Fagerquist *et al.* 2010). A recent transcriptomic analysis discovered strain- and acidulant-specific responses to acid stress in both K-12 and O157 which involved up-regulation in genes related to oxidative stress, iron and manganese uptake, and cold shock (King *et al.* 2010). Interestingly, that study observed activity of lysine decarboxylase, but none of the other amino acid decarboxylases, in the presence of inorganic acid. The authors were unable to hypothesize a reason for this observation. Exposure to acetic acid, an organic acid, resulted in up-regulation of several genes and intergenic regions across both strains; however, most of the

transcriptomic changes observed were common to different acidulants (King *et al.* 2010). Parallel proteomic and transcriptomic studies have also been carried out on *E. coli* O157:H7, which demonstrated the changes in gene expression and proteins of the RpoS regulator due to decreased temperature and water activity (Kocharunchitt *et al.* 2012). These conditions would be sufficient to induce cross protection against acid stress within the cell.

The literature describes several metabolomic analyses of *Escherichia coli* K-12, and a few proteomic or transcriptomic analyses of O157:H7. In *E. coli* K-12, King and coworkers found transcriptomic evidence for the cellular response to inorganic acid (hydrochloric) and organic acids (acetic and lactic); the *gadE* gene responsible for glutamic acid decarboxylase was actually downregulated in K-12 and upregulated in O157:H7 for all acidulants (King *et al.*, 2010). Several other studies examined non-O157 strains of *E. coli*. Almstetter and others were able to differentiate two non-O157 strains from each other by 48 statistically significant peaks using GCxGC-ToF-MS (Almstetter *et al.*, 2009). Of these 48 peaks, those identified were tricarboxylic acid cycle intermediates, glycolysis intermediates, amino acids, sugars, and several other compounds (Almstetter *et al.*, 2009). Jozefczuk and coworkers metabolomically analyzed a non-O157 strain for its stress responses to cold, heat, oxidation, and glucose-lactose shift, and found numerous metabolites changing in response to each condition, including many of the same classes of compounds found by Almstetter and coworkers. (Jozefczuk *et al.*, 2010). Studies have also been undertaken on O157:H7; Fagerquist and others took a proteomic approach to identify the acid stress protein HdeA in *E. coli* O157:H7 using matrix-assisted laser desorption ionization coupled to tandem time-of-flight mass spectrometry (MALDI-ToF-ToF) (2010). More recently, King and coworkers did not see any upregulation of any acid resistance mechanisms in O157:H7 except for the gene encoding the lysine-dependent system (*cadB*), in response to hydrochloric acid as the acidulant

(2010). With acetic acid as the acidulant, they observed upregulation of three genes (*yojI*, a multidrug transporter membrane component and ATP-binding component; *yfdX*, which codes for a hypothetical protein; and *yqgA*, which encodes a predicted inner membrane transport protein) and three intergenic regions, and the fold-increase of these were similar to those observed in nonpathogenic *E. coli* K-12 (King *et al.*, 2010). In a transcriptomic and proteomic study of the effects of low water activity and cold stress, researchers found activation of the stress regulator RpoS, but observed downregulation in processes involved with protein synthesis (Kocharunchitt *et al.*, 2012). Despite the available knowledge, there is little information pertaining to the intracellular metabolite pool of *Escherichia coli* O157:H7 cells. Its metabolome under stress conditions such as acid pH and weak organic acids is even less well understood. The absence of published literature on this subject, coupled with the importance of food safety to people everywhere, highlights the need for more inquiry into metabolic analysis of foodborne pathogens such as *E. coli* O157:H7. This research is necessary for the understanding of how O157:H7 responds biochemically to acid stress; knowledge in this area is readily applicable to the safety of acid and acidified foods and those who consume them.

1.5 GCxGC-ToF-MS for Metabolomic Analysis

The instrumentation used in metabolomics research varies, but generally involves a chromatographic separation such as liquid or gas chromatography coupled to a mass spectrometer for detection and identification of the resulting metabolites. Two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS) is an excellent choice for this type of analysis due to its excellent resolving capabilities, sensitivity, throughput, and numerous other merits which have been described elsewhere (Adahchour *et al.*, 2004, Dalluge *et al.*, 2003; Bertsch, 1999; Gorecki *et al.*,

2004). The separation is achieved when analytes partition between the stationary phase column lining and the mobile phase carrier gas. The addition of a second column allows for different polarities of the stationary phase, which helps further separate the analytes. While one dimensional GC has been employed in many metabolomic studies (e.g., Barsch *et al.* 2004; Börner *et al.* 2007; Coucheney *et al.* 2008; Koek *et al.* 2006; Lisec *et al.* 2006; Pasikanti *et al.* 2008; Plassmeier *et al.* 2007; Roessner *et al.* 2000; Rudell *et al.* 2008; Smilde *et al.* 2008; Villas-Bôas *et al.* 2005), the second dimension of separation often allows for the resolution of two or more analytes which would co-elute on a one-dimensional gas chromatograph. Adahchour *et al.* demonstrated this principle with the example analysis of the spleen of an obese mouse; without the second dimension of separation, the number of analytes detected decreases from 563 to 79 (Adahchour *et al.* 2006).

The method of detection for this technique is time-of-flight mass spectroscopy (ToF-MS). The detector identifies fragments by their mass-to-charge (m/z) ratio. The National Institute of Standards and Technology (NIST, 2011) maintains a mass spectral library database for identification of compounds. Other libraries such as the Fiehn library (Kind *et al.*, 2009) or Planck library (Wagner *et al.*, 2003) have been specially developed for metabolomics research, and take into account the derivatized forms of the analytes for library identification. These libraries are able to provide a tentative identification for detected peaks with mass spectra that match the library spectra; identities are confirmed by comparison to GCxGC-ToF-MS of pure standard compounds and by matching retention indices.

1.6 Implications

The complexity of *E. coli* O157:H7 makes its control both a challenge and a necessity. Very little is known about the chemicals and reactions inside the cytoplasm of the cell when it is exposed

to the acidic environment. Current knowledge of the metabolic changes in *E. coli* O157:H7 during acid stress is limited. Two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-ToF-MS) is an excellent tool by which to study the metabolites of acid-challenged *E. coli*. This study will shed much-needed light on the subject of how *E. coli* O157:H7 resists acid so well and what researchers can do about it to ensure the safety of the food supply.

1.7 Proposed Research

The following research is proposed in order to address missing information in the scientific literature. This study aims to use a non-targeted approach to fill this knowledge gap. The hypothesis of this research is that metabolomic analysis of *E. coli* O157:H7 using GCxGC-ToF-MS will allow observation of metabolic changes resulting from acid pH and organic acid stresses. The specific objectives are (1) to establish the necessary conditions required to optimize metabolomic analysis of *E. coli* O157:H7 (*e.g.*, acid concentration, duration of acid challenge, amount of cells to sample, and quenching temperature) and (2) to determine the changes in intracellular metabolites due to acid stress.

1.8 Acknowledgements

The author thanks Frederick Breidt, Jr., Roger F. McFeeters, Suzanne D. Johanningsmeier, Sophia Kathariou, and Lee-Ann Jaykus for helpful discussions of this review and Sandra Parker for excellent administrative assistance.

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CHAPTER TWO

Survival of *Escherichia coli* O157:H7 Grown in Complex and Minimal Growth Media During Acid pH and Acetic Acid Stress

Abstract

Escherichia coli O157:H7 is a foodborne pathogen noted for its resistance to acidic environments. In order to determine the intracellular metabolite changes related to acid stress using two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS), the survival of ninety percent or greater of the cells in the test conditions must be established. Strain B241 was grown statically at 37 °C for 15 hours in complex medium or for 18 hours in minimal medium and inoculated into solutions of pH 3.2 buffer and pH 3.2 buffer with 40 mM acetic acid for up to 6 hours. Survival was determined by plating onto Luria-Bertani (LB) agar in 15 to 60 minute intervals. Cells grown in complex media survived no better than cells grown in minimal media when acid stressed at pH 3.2 or in the presence or absence of acetic acid ($p = 0.504$). All cells exhibited at least 94 % survival for at least sixty minutes. Growth of *E.coli* O157:H7 in minimal media supplemented with thiamin for 18 hrs at 37 °C followed by a thirty minute acid challenge at pH 3.2 or pH 3.2 with 40 mM acetic acid were selected as suitable conditions to observe the effects of acid stress on the changes in intracellular metabolites of *E. coli* O157:H7.

2.1 Introduction

Escherichia coli O157:H7 is a bacterial pathogen notorious for its virulence, especially because of its resistance to acidic environments. Estimates suggest that this strain of Shiga toxin-producing *E. coli* is responsible for 63153 infections, 2183 hospitalizations, and 20 deaths annually (Scallan *et al.*, 2011). It is able to survive in the presence of acid pH and various acids due to several acid tolerance responses (ATR) it possesses. Three of these acid resistance systems, described by Castanie-Cornet *et al.*, are the oxidative, glutamate-dependent, and arginine-dependent systems (1999). A fourth acid resistance system proposed by Diez-Gonzalez *et al.* is the lysine-dependent system (2004). The oxidative, or glucose-repressed, system requires the alternative sigma factor RpoS, as well as cyclic AMP and its receptor protein (Castanie-Cornet *et al.*, 1999). However, growth in the presence of glucose suppresses this system, and the way in which it functions is currently not well characterized (Castanie-Cornet *et al.*, 1999). The amino acid decarboxylase systems, which can function in the presence of glucose, involve structural genes which enable *E. coli* to survive acid stress by increasing the alkalinity within the cell (Cui *et al.*, 2001). Evidence published by House *et al.* supports this theory, having observed that the genes encoding the amino acid decarboxylases were significantly upregulated in response to acid stress using real-time PCR (2009).

Acetic acid is a common, generally recognized as safe, acidulant used to inhibit spoilage and pathogenic microbes in acidified food products such as pickles (Breidt, 2006). Products like these could have a pH as low as 3.2. The antimicrobial effect of acetic acid has been well documented (Axe and Bailey, 1995; Brudzinski and Harrison, 1998; Oh *et al.*, 2009). Acetic acid also imparts the sour vinegar taste that consumers expect in both fermented and fresh pack pickle products (Neta *et al.*, 2007). Therefore, it is often selected to serve as a hurdle to the growth of pathogens, especially such acid resistant ones as *E. coli* O157:H7, in acid pH foods.

The medium in which cells are grown may affect the behavior and metabolism of cells. McQuestin and coworkers found that slight differences in the composition of minimal and complex growth media changed the inactivation characteristics of *E. coli* by acid and osmotic stress (2006). *Escherichia coli* O157:H7 grown in complex medium has more nutrients available to it than *E. coli* grown in minimal growth medium. The additional nutrients can diffuse into the cell and undergo metabolism, utilizing pathways that are present in minimal medium-grown cells, but not in use due to the absence of the substrate. However, complex growth medium has a high quantity of components therein that may result in analytical interference with other peaks in the chromatogram. Using a minimal growth medium may reduce background interference.

The purpose of this experiment was to establish a set of conditions and a time frame for which the survival of 90 % or greater of a population of *Escherichia coli* O157:H7 cells could be guaranteed. These conditions will be used to treat cells in preparation for analysis of their intracellular metabolites using GCxGC-ToF-MS. The treatments were designed to induce observable differences in the metabolite pools of the exposed cells. However, greater than 90 % of the cells should remain alive after the acid challenge treatment to ensure that only the metabolite pools of live cells are under analysis.

2.2 Materials and Methods

Chemicals

D-glucose, sodium chloride, magnesium sulfate, disodium phosphate hydrate, MES hydrate, MOPS, gluconic acid, and potassium phosphate monobasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) agar, hydrochloric acid, and acetic acid were purchased from Fisher (Fair Lawn, NJ, USA). LB broth was purchased from BD (Sparks, MD, USA). Ammonium

chloride was purchased from Fluka (St. Louis, MO, USA). Calcium chloride and sodium hydroxide were purchased from Aldrich (Milwaukee, WI, USA).

Media Preparation

Complex medium ("LBG") was prepared as follows: Luria-Bertani broth was prepared according to the manufacturer's directions, with the addition of 1 % D-glucose. Minimal medium ("M9-GT") was prepared according to recipe for M9 medium (Sambrook *et al.*, 1989), but supplemented to 1 % total D-glucose and 0.005 % thiamine.

Cell Culture

Escherichia coli O157:H7 strain B241 (28RC1), a bovine isolate chosen for its acid resistance, was obtained from the USDA-ARS culture collection (USDA-ARS Food Science Research Unit, Raleigh, NC, USA). The culture was streaked on LB agar and incubated overnight. Isolated colonies were picked from three separate sites on the plate to comprise independent biological replicates. Cells were inoculated into either LBG and incubated at 37 °C statically for 15 hours, or into M9-GT and incubated at the same temperature for 18 hours. Cells were centrifuged and resuspended in sterile 0.85 % sodium chloride at a tenfold concentration.

Survival Curves

Buffer ("MG") was made containing 20 mM MES (pK_a = 6.15)and 20 mM gluconic acid (pK_a = 3.60) as noninhibitory buffers (Good *et al.*, 1966; Breidt *et al.*, 2004), and 0.85 % sodium chloride, and adjusted to a final pH of 3.2 with hydrochloric acid or sodium hydroxide as necessary. An identical buffer was made which also contained 40 mM acetic acid and was adjusted to pH 3.2 with hydrochloric acid or sodium hydroxide as necessary. Cells were tenfold diluted in each buffer and incubated at 30 °C aerobically for several hours. For the pH 3.2 buffer, samples were taken hourly for a total of six hours. For the pH 3.2 buffer with 40 mM acetic acid, samples were taken every

fifteen minutes for the first hour of incubation, and every twenty minutes for the remaining two hours. To measure survival of cells grown in minimal medium, cells were tenfold diluted in pH 3.2 MG buffer with no acetic acid or 40 mM acetic acid and incubated at 30 °C for three hours. Samples were taken every thirty minutes for the first two hours of incubation and once at the third hour. Samples were serially diluted and spiral plated (model 4000; Spiral Biotech, Inc., Norwood, MA) on LB agar plates to obtain the number of live cells using an automated plate reader (QCount model 510; Spiral Biotech). The 10^{-1} dilution was made into 0.1 M MOPS at pH 7.2; all further serial dilutions were into 0.85 % sodium chloride. Data were plotted in SigmaPlot 10 (Systat, Chicago, IL, USA). Error bars were calculated using the standard deviation of the plate counts for three biological replicates.

Statistical Analysis

Means and standard deviations for cell counts were calculated. A one-tailed student's t test was performed on cell counts in Excel (Microsoft, Redmond, WA, USA). Values were reported in log CFU/mL.

2.3 Results and Discussion

Survival of E. coli O157:H7 Grown in Complex Medium

Escherichia coli O157:H7 grown in complex medium reached a concentration of 9.27 ± 0.01 log CFU/mL when incubated at 37 °C for 15 hours (Table 2.1). When the cells were subjected to the low-pH buffer, their numbers decreased by 1.37 log CFU/mL over about six hours (Figure 2.1). The small amount of cell death was indicative of the acid resistance of this particular strain. For the first hour of the acid challenge, the cell counts did not change significantly ($p= 0.09$). This indicates that greater than 99 % of the cells remained viable during that portion of the acid challenge.

Cells exposed to pH 3.2 in the presence of 40 mM total concentration of acetic acid decreased in numbers more rapidly. The initial cell count was 9.59 log CFU/mL after 15 hours of incubation at 37 °C (Table 2.1). In the three-hour acid challenge, the cell count decreased by 4.25 log CFU/mL, demonstrating the bactericidal effect of acetic acid at this concentration (Figure 2.1). However, the decrease in cell count through the first sixty minutes was statistically insignificant ($p = 0.08$), decreasing by only 0.17 log CFU/mL. The majority (greater than 98 %) of *E. coli* cells survived that period of acid challenge.

Survival of E. coli O157:H7 Grown in Minimal Medium

E. coli grown in minimal medium exhibited different survival characteristics than the same strain grown in complex medium (Bhagwat and Bhagwat, 2004; Castanie-Cornet *et al*., 1999; Lin *et al*., 1995). Cells were grown for 18 hours at 37 °C to obtain an initial inoculum of 9.83 log CFU/mL in the pH 3.2 MG buffer (Table 2.1). After being added to the buffer, the cell counts stayed roughly constant for the first hour, decreasing by 0.55 log CFU/mL (Figure 2.1). However, greater than 94 % of the cells survived that period of the acid challenge. The overall decrease in cell numbers throughout the three-hour acid challenge was 0.96 log CFU/mL.

The cells used in the pH 3.2 acid challenge in the presence of 40 mM acetic acid began at a concentration of 9.80 log CFU/mL after 18 hours of growth at 37 °C (Table 2.1) and decreased by 1.65 log CFU/mL over the three-hour period, suggesting that these cells survived the acid challenge better in the presence of a low concentration of acetic acid than cells grown in complex media (Figure 2.1). The cells began to die off more rapidly in the presence of the additional acid than at acid pH alone, decreasing by 0.25 log CFU/mL over the first hour of the acid challenge ($p = 0.04$). Greater than 97 % of the cells survived through the first hour, and the decrease over the first thirty

minutes was only 0.04 log CFU/mL or 0.39 %, suggesting that at least 99 % of the cells remained viable after thirty minutes of incubation in the acidified buffer.

Effect of Acetic Acid on Cell Survival

Cells exposed to acetic acid at a constant pH exhibited differences in inactivation kinetics. For cells grown in complex medium, this accounted for a difference of 2.88 log CFU/mL between acid challenge conditions, compared to a difference of 0.69 log CFU/mL for cells grown in minimal medium. In addition to the stress caused by low pH (an accumulation of hydrogen ions), the cells in 40 mM acetic acid were presumably under additional stress from the buildup of acetate anion inside the cytoplasm (Russell, 1992; Diez-Gonzalez *et al.*, 1997). The Henderson-Hasselbalch equation shows that based on the pK_a of acetic acid (4.75) and the pH of the solution (3.2), 97.2 % of acetic acid would be in its undissociated form and could diffuse across the cell membrane and dissociate inside the cytoplasm (Henderson, 1908; Hasselbalch, 1917). The intracellular accumulation of excess acetate anion may account for the decreased survival of cells in acetic acid relative to cells at the same pH (Russell, 1992; Diez-Gonzalez *et al.*, 1997).

Effect of Growth Medium on Cell Survival

The medium in which *E. coli* O157:H7 was grown had a noticeable effect on its survival in acidic environments. Cells that had been grown in complex medium grew to similar cell numbers in a lesser amount of time than did cells grown in minimal medium, by a difference of 0.38 log CFU/mL at the end of the incubation period. In pH 3.2 MG buffer in the absence of acetic acid, cells grown in complex medium decreased by 0.02 log CFU/mL, and cells grown in minimal medium decreased by 0.96 log CFU/mL. Conversely, in the presence of 40 mM acetic acid, the cells grown in minimal medium survived longer than those grown in complex medium by 2.60 log CFU/mL ($p = 0.009$). This finding is in agreement with work done by others who found better survival of *E. coli* O157:H7

strains which had been grown in minimal medium in response to osmotic or acid stress (McQuestin *et al.*, 2006; Bhagwat and Bhagwat, 2004). In developing this minimal medium, it was observed via noticeably increased turbidity that the addition of thiamine helped the cells grow to a higher CFU/mL than in unsupplemented growth medium. McQuestin *et al.* asserted that the ideal medium to provide protection from stress depends on the nature and intensity of the stress provided, due to the different mechanisms of action of each stressor (2006). For example, in this acid stress study, an ideal medium would provide amino acids such as glutamate, arginine, and/or lysine, for their use in amino acid decarboxylase mechanisms that help maintain pH homeostasis. Additionally, Roe *et al.* determined that the presence of methionine in the medium in which the cells were suspended provided protection against acetate stress (2002).

2.4 Conclusions

The purpose of this experiment was to establish a set of conditions and a time frame for which the survival of 90 % or greater of a population of *Escherichia coli* O157:H7 cells could be guaranteed. For the conditions of pH 3.2 buffer and pH 3.2 buffer with 40 mM acetic acid, cells grown in minimal medium can be expected to survive at greater than 99 % for at least an hour. This study also served to highlight the similarities in acid resistance between cells grown in complex and minimal growth media. For all growth media and conditions tested, the survival of greater than 90 % of the initial population of cells can be expected for at least a thirty minute time period following inoculation into the acid challenge buffer. Thus, it is proposed that when these acid-challenged cells are lysed and their metabolites extracted for analysis, the metabolites detected are those of live cells.

2.5 Acknowledgements

The author thanks Frederick Breidt, Jr. and Roger F. McFeeters for helpful discussions of this work and Sandra Parker for excellent administrative assistance.

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2.6 References

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Table 2.1 Survival of *Escherichia coli* O157:H7 during acid challenge.

Growth	Acid Challenge	$\log_{10}$ CFU/mL			$\log_{10}$ Change		p Value	
Medium	Treatment	Initial	30 min	60 min	30 min	60 min	30 min	60 min
LBG	pH 3.2	9.27 ± 0.015	N/A	9.32 ± 0.035	N/A	-0.045 ± 0.037	N/A	0.09
	pH 3.2 + 40 mM acetic	9.59 ± 0.090	9.44 ± 0.10	9.43 ± 0.090	0.15 ± 0.18	0.17 ± 0.13	0.14	0.08
M9-GT	pH 3.2	9.83 ± 0.047	9.82 ± 0.015	9.28 ± 0.073	0.010 ± 0.062	0.55 ± 0.062	0.40	0.00
	pH 3.2 + 40 mM acetic	9.8 ± 0.14	9.76 ± 0.10	9.55 ± 0.026	0.039 ± 0.20	0.25 ± 0.13	0.39	0.04

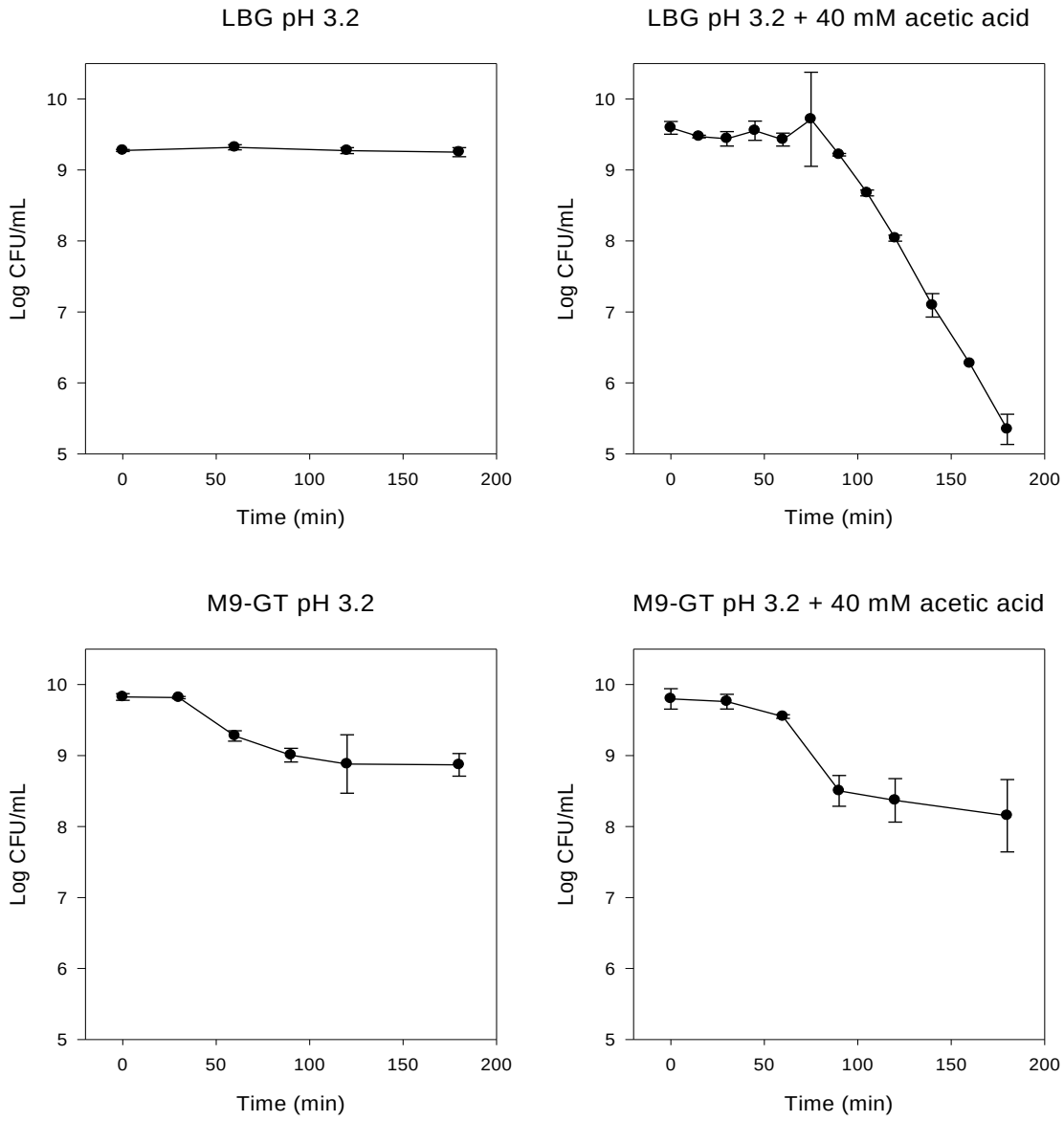

Figure 2.1 Survival under acid stress of *Escherichia coli* O157:H7 strain B241 grown in complex or minimal medium. Each point represents the average of three biological replicates, with the standard deviation shown by the error bars.

CHAPTER THREE

Methods Development: Calibration of Cell Quantity, Analysis of Derivatized Standard Compounds, and Measurement of Quenching Temperature for Metabolite Analysis by GCxGC-ToF-MS

Abstract

A microbial metabolomics method has several key requirements: an adequate amount of cells to allow detection of internal metabolites; confirmation of metabolite identifications with pure standard compounds; and validation that the conditions to quench metabolism are reached as quickly and repeatably as possible. The objectives of this study were (1) to determine the number of cells required for detection of cellular metabolites by GCxGC-ToFMS, (2) assemble a retention index database for chemically derivatized standard compounds, and (3) select an appropriate quenching procedure.

For determining the number of cells necessary to generate the optimal chromatogram, a relationship was established between cell dry weights and the visual appearance of the analogous chromatogram of derivatized cell extracts. Pure standard chemicals were derivatized using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS) in order to determine the retention times of these compounds for compilation of a database of standard compounds. Seven protocols for metabolic quenching were tested based on the most common recommendations made in the scientific literature, and the time and temperature data for these trials were recorded.

Based on observation of chromatographic peaks, the ideal number of cells filtered, was $4.16*10^9$ CFU, approximately 3 mg dry weight of cells. The standard compounds were located on the

chromatogram and the retention index and other spectral information for these were added to the author's database for future identification of metabolites. The best of the seven quenching methods was defined as the one which reached -40 °C most rapidly, with the filter added to a petri plate containing 60 % methanol in water solution, pre-cooled on a slab of dry ice (-78 °C) in an insulated foam cooler. The temperature reached -40 °C in 9.33 ± 2.50 seconds, with a quenching rate of -57.33 ± 11.984 °C/sec and reaching a final temperature of -76.60 ± 2.27 °C.

The results of these experiments indicate that the metabolomic study designed to observe acid stress-induced changes in *Escherichia coli* O157:H7 would be best carried out using $4.16*10^9$ CFU (3 mg dry weight) cells, with a database of pure standards for comparison, using a quenching method that employs -80 °C 60 % methanol under dry ice-cooled conditions.

3.1 Introduction

There are several considerations that go into the design of a microbial metabolomics study. Since each study is unique, there are some parameters that should be measured and tailored to the specific experiment. These include the amount of cells to be sampled and analyzed, the strength of the signal on the chromatogram, the standard compounds with which the tentative identifications of metabolites will be confirmed, and the conditions required to halt cellular metabolism (a process known as "quenching"). Knowing what these parameters are and the correct setting for each of them helps to develop an accurate and repeatable metabolomic analysis.

The dry weight of a cell is a measure of the mass of the cell that can be used to standardize cell numbers, in the same way that optical density or total protein concentration values could be used. Whereas the concentration of cells suspended in a growth medium or buffer can vary, the mass of a cell is constant. At present, there is no defined value in the microbial metabolomics literature characterizing the "ideal" amount of cells one must extract and derivatize in order to obtain the "best" possible chromatogram via two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS). The amount of *Escherichia coli* O157:H7 cells which equated to 2 mg of dry weight was hypothesized to generate the optimal signal intensity (McFeeters, unpublished data).

Additionally, a two-dimensional chromatogram generated using this technique should make use of the full range of detection of the instrument. The detection limit of an analytical technique such as GCxGC-ToF-MS varies based on the instrument and the analyte being detected, but can be as low as 0.2 pg/µL (LECO, 2011). Peaks should give areas above the baseline of detection so they are not confused with spectral noise. Additionally, peak areas should not be so large as to

oversaturate the detector and overload the chromatogram. Overloaded peaks generally have a long tail and are harder to both distinguish from neighboring peaks and quantify.

Another important component is the ability to identify analytes as they come off the column. In gas chromatography, this is commonly achieved through mass spectroscopy. However, the retention time of each analyte also is a component of the method used to confirm the identity of the analyte. It is useful to have a database of standard compounds whose retention times are known in order to aid in this identification. Retention times can vary slightly based on the specific GC instrument, so it is important to know where each compound elutes on that particular system. To compensate for that variation, retention indices are calculated for each compound using the equation developed by Kováts (1958).

Living cells are constantly undergoing metabolism. Metabolite pools can change in fractions of a second, based on signals the cell receives from its environment (Wittman *et al.*, 2004). Therefore, it is important to ensure that when sampling cell metabolites, those biological reactions are halted. Failure to do so could induce a stress response in the cells, altering their metabolite pools and resulting in unwanted bias in the analysis. In this research especially, the subject of study is the response to acid stress, not acid stress and starvation stress, which could induce cross-protection in a bacterium like *E. coli*.

In the field of microbial metabolomics, the act of halting metabolism is known as quenching. Although the scientific community has an agreed-upon definition for the term, to date there are no widely agreed-upon time and temperature parameters that define this state. In the literature, reports of how quenching was achieved vary widely. Mashego *et al.* reported that quenching can be achieved by one of two means: extreme temperature or extreme pH (2007). Many scientists favor the use of cold temperatures, though a wide range of values are presented for use. A summary of

the methods reported in the literature is presented in Table 3.1. The general consensus seems to be that quenching is best done at cold temperatures (between -20 and -80 °C), and that the cells should be kept cold through the lysis step. However, there is a larger range of temperatures for extraction of intracellular metabolites (from -80 to 70 °C). The higher temperatures are designed to inactivate enzymes and speed the rate of extraction of chemicals (Bennett *et al*. 2008); however, the lower temperatures are favored in extractions where there is a risk of metabolite leakage prior to lysing the cell membrane (Bolten *et al*. 2007).

In contrast to the amount of advice on how to quench cellular metabolism is the dearth of data confirming that the reported quenching conditions were reached. To the author's knowledge, no paper has been published which presents evidence to this end. Time and temperature data are essential in order to make the claim that metabolism was instantaneously halted.

The purpose of this work was to determine the conditions necessary to carry out a metabolomics study of *E. coli* O157:H7 under acid stress. The first objective was to determine the number of cells required for non-targeted metabolite profiling by GCxGC-ToF-MS. The second objective was to determine the retention characteristics and mass spectra of known standard compounds. The third objective of this experiment was to gather data on the quenching temperatures and quenching rates for various methods of quenching proposed for microbial metabolomics.

3.2 Materials and Methods

Chemicals

Sodium chloride, D-glucose, ribitol, xylitol, glucose-6-phosphate sodium salt, nicotinic acid, citrulline, magnesium sulfate, sodium phosphate monobasic, potassium phosphate dibasic, and

methoxyamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, phenyl glucoside, pyruvic acid, and phosphoric acid were purchased from Aldrich (Milwaukee, WI, USA). Uridine, putrescine, and guanosine were purchased from Acros Organics (Fair Lawn, NJ, USA). D4-alanine, ^{13}C-proline, D8-valine, and ^{13}C-salicylic acid were purchased from Icon Isotopes (Summit, NJ, USA). Hydrochloric acid, methanol, Luria-Bertani agar, and fumaric acid were purchased from Fisher (Fair Lawn, NJ, USA). L-glutamic acid was purchased from MP Biomedicals (Solon, OH, USA). Sylilation grade pyridine and BSTFA were purchased from Thermo Scientific (Rockford, IL, USA). Alkanes C8-C20, alkanes C21-C40, and ammonium chloride were purchased from Fluka (St. Louis, MO, USA). Fatty acid methyl ester reference standard GLC-409 was purchased from Nu-Chek Prep (Elysian, MN, USA). Liquid nitrogen was purchased from Airgas National Welders (Charlotte, NC, USA). Dry ice was purchased from Harris Teeter (Raleigh, NC, USA).

Media Preparation for Cell Dry Weight Optimization

M9-GT media was prepared according to recipe for M9 media (Sambrook *et al.*, 1989), but supplemented to 1 % total glucose and 0.005 % thiamine in media. Cells did not grow well beyond one overnight without the addition of a small amount of unlabeled thiamine to the medium (unpublished data).

Cell Culture for Cell Dry Weight Optimization

Escherichia coli O157:H7 strain B241 (28RC1), a bovine isolate obtained from the USDA-ARS Food Science Research Unit culture collection (Raleigh, NC), was chosen for its acid resistance (Oh *et al.* 2009). Cells were inoculated into M9-GT minimal media and incubated aerobically at 37 °C statically for 18 hours. Cells were harvested by centrifugation for 10 minutes at 5000g and resuspended in sterile 0.85 % saline at one hundred-fold concentration. Cells were plated on Luria-Bertani (LB) agar to obtain cell counts in CFU/mL. Two-fold, five-fold, and ten-fold dilutions of the

concentrated cells were prepared in sterile 0.85 % saline. An equal volume (250 µL) of each dilution, as well as the one hundred-fold stock, was vacuum-filtered onto a pre-tared 0.45 micron 25mm diameter polyethersulfone membrane filter (Sterlitech Corporation, Kent, WA, USA), which was placed into a pre-tared microfuge tube. Microcentifuge tubes were heated in a Thermomixer R (Eppendorf, Hamburg, Germany) for 10 minutes at 90 °C to inactivate pathogenic *E. coli*, then dried in a SpeedVac SVC 100 (Savant Instruments, Hicksville, NY, USA). Dried tubes were weighed on an analytical balance to determine the dry mass of cells on each filter. Dry weight was plotted versus cell concentration (CFU) in order to calculate which dilution most nearly approximated 2 mg dry weight of cells.

A second batch of *Escherichia coli* O157:H7 B241 cells were inoculated into M9-GT in triplicate and incubated at 37 °C statically for 18 hours. An aliquot of each replicate was transferred into fresh M9-GT and incubated at 37 °C statically for 18 hours. Cells were harvested by centrifugation for 10 minutes at 5000g and resuspended in an equal sterile 0.85 % saline. The process was repeated once, then centrifuged the same way again and resuspended in sterile 0.85 % saline at one hundred-fold concentration. Cells were diluted ten-fold into pH 7 MG buffer and plated on Luria-Bertani agar to obtain cell counts in CFU/mL. Aliquots of 0.25 mL, 1 mL, 2 mL, and 4 mL of the cell suspension were vacuum-filtered onto a 0.45 micron 25mm diameter polyethersulfone membrane filter (Sterlitech). Immediately, filters were placed in individual 35 mm petri dishes containing 0.75 mL 60 % methanol kept cooled on dry ice for quenching of metabolism at -80 °C. Petri dishes were kept chilled on dry ice until transfer of the liquid and filter into microfuge tubes. Additional aliquots of 0.25 mL, 1 mL, 2 mL, and 4 mL of the cell suspension were vacuum-filtered onto pre-tared a 0.45 micron 25mm diameter polyethersulfone membrane filters (Sterlitech) and placed into pre-tared microfuge tubes for dry weight measurement by the previously described

protocol. Filter blanks were prepared by vacuum-filtering 1 mL and 2 mL of sterile 0.85 % saline diluted ten-fold into pH 7 MG buffer.

Extraction and Derivatization of Intracellular Metabolites

Microcentrifuge tubes containing the filters with cells were retrieved from the -80 °C dry ice cooler. Tubes were mixed for 25 min at 70 °C and 300 rpm to inactivate enzymes. An aliquot of each cooled tube was transferred into GC vials. Internal standard solution containing 0.2 mM ^{13}C-salicylic acid and 0.2 mM D8-valine was added to each and vortexed. Samples were dried in a SpeedVac SVC 100 (Savant Instruments) on medium heat to complete dryness, then loaded into a CTC Combi PAL autosampler (LEAP Technologies, Carrboro, NC, USA), where the machine performed the following chemical reactions. A methoximation solution containing 20 mg/mL methoxyamine HCl in pyridine was added to each vial and mixed for 90 min at 70 °C and 300 rpm. An equal volume of BSTFA was added to each vial and mixed for 30 min at 70 °C and 300 rpm. Alkanes C8-C20 and C21-C40 were added to all samples for standardization of retention indices before injection onto the GCxGC-ToF-MS. Derivatization blanks were also prepared, containing empty vials to which the autosampler added the reagents. Sample order was randomized and proceeded one reaction at a time, such that there was no waiting period between each derivatization reaction and the subsequent injection onto the GCxGC-ToF-MS.

GCxGC-ToF-MS Method for Detection of Intracellular Metabolites

Qualitative analysis used two-dimensional GC with time-of-flight mass spectrometry according to Johanningsmeier (2011). Briefly, GCxGC-ToF-MS analysis was carried out using an Agilent 6890 (Agilent Technologies, Santa Clara, CA, USA) equipped with a 28.45 m BPX50 column (SGE Analytical Science, Austin, TX, USA) in the first dimension and a 1.0 m BPX5 column (SGE Analytical Science) in the second dimension. The temperature of the injection port was 260 °C. The

oven temperature program was 85 °C for four minutes, then from 85 to 245 °C at 5 °C/min then heating at 15 °C/min to 305 °C, and finally holding at 305 °C for 15 minutes. Helium was used as the carrier gas at a flow rate of 1.00 mL/min. The temperatures of the transfer line and the ion source were 280 and 250 °C, respectively. The detector was a Pegasus 4 time-of-flight detector (LECO Corporation, St. Joseph, MI, USA). Mass spectra were acquired over the mass range of 35-800 amu at an ionization energy of -70 Volts. Identification of individual components was done using the LECO-Fiehn Rtx5 library. The alkanes identified in a representative chromatogram were used to create a retention index method based on the retention times of alkanes C8-C40. ChromaTOF® (LECO Corporation) was used to compute the baseline, find peaks above the baseline, identify all peaks found, compute area and height of peaks, and compute retention index. The baseline offset was set to 0.8 on a scale of (0.5-3.0). Three data points were averaged for smoothing. The first dimension expected peak width was eleven seconds; in the second dimension, the expected peak width was 0.08 seconds. The match required to combine in the second dimension was 600. The minimum signal to noise ratio was set to 6. Common masses in derivatized products included 73, 147, and 217. The library search was performed forwards in normal mode, searching masses 85 to 800 and returning the ten best hits. The minimum and maximum molecular weights allowed were 40 and 1000. The max threshold (relative abundance of base ion) was set to 0 on a scale of (0-998). The minimum similarity match before name is assigned was set to 800 on a scale of (0-999). The library searched was the LECO-Fiehn Rtx5 library, and the mass used for area and height calculation was the unique mass.

Data Analysis for Cell Dry Weight Optimization

Cell dry weights were plotted versus the number of cells in CFU, and a linear regression equation was generated to calculate the amount of cells that corresponded to 2 mg dry weight. The

chromatograms were visually analyzed for signs of peak oversaturation caused by too strong a signal from the TMS-derivatized cell extracts, or too few peaks signifying not a large enough amount of cell extracts. Chromatograms deemed "ideal" had strong signals and visibly differed from filter blanks and derivatization blanks in the number of peaks detected. The dry weight measurements were obtained to correlate with cell counts in CFU/mL to calculate the mass of cells on each filter and the amount of cells that represented.

Sample Preparation for Standard Compounds

Three concentrations of each standard (0.4 nmol, 3 nmol, and 20 nmol) were prepared from stock solutions. Internal standards (2 nmol ribitol, 2 nmol phenyl glucoside, 4 nmol D4-alanine, 6 nmol C^{13}-proline, and 4 nmol C^{13}-salicylic acid) and 12% NaCl solution (for ionic strength) were added to each sample. Samples were dried in a SpeedVac SVC 100 (Savant Instruments, Hicksville, NY, USA) on medium heat until completely dry by mass. They were derivatized by hand following the protocol described above, with the following deviations. After the addition of BSTFA, the tubes were immediately placed into ice and an alkane series was prepared containing the methoximation solution, BSTFA, the fatty acid methyl ester (FAME) reference standard GLC-409, alkanes C8-20, and alkanes C21-40. Derivatization blanks were also prepared containing the methoximation solution and BSTFA. FAME were added to each of the samples cooling in ice. All samples, blanks, and the alkane series were centrifuged under refrigeration for 7 min at 13000rpm. A sufficient volume for injection was transferred from each tube into GC vials. Sample run order was randomized.

Data Analysis for Standard Compounds

Data were analyzed in ChromaTOF® (LECO Corporation). A table of retention times, retention indices, and other pertinent information was compiled in Excel 2007 (Microsoft, Redmond, WA, USA). Chromatograms were reviewed visually in ChromaTOF® to locate the standard peaks and

to compare the mass spectra to the Fiehn library. A unique mass was chosen to distinguish the standard peaks from others in the chromatogram, and the chromatograms resulting from three concentrations of each standard compound were overlaid for comparison.

Sample Preparation for Quenching Validation

Seven conditions were tested in order to determine the conditions most likely to rapidly induce quenching of cell metabolism. All quenching treatments were replicated eight times, with the exception of the sixth trial, which was done in triplicate. All trials involved taping a thin-wire type T thermocouple (Omega Engineering, Inc., Stamford, CT, USA) to the center of each 25 mm polyethersulfone membrane filter (Sterlitech, Kent, WA, USA) with tape, leaving the tip of the thermocouple exposed. Temperatures were monitored using a TempScan model 1100 with 32 channels (Omega Engineering, Inc.) from one minute prior to chilling until one minute following the attainment of the minimum temperature. In the first trial, the filter was inserted into a microfuge tube, sealed snugly around the thermocouple, and dropped into an insulated dewar containing liquid nitrogen. In the second trial, microfuge tubes were pre-cooled to -80 °C for thirty minutes prior to inserting each filter, sealing, and dropping the combined unit into the liquid nitrogen dewar. In a third trial, a quenching solution of 60 % methanol in water was pre-cooled to -80 °C overnight. The quenching solution was then added, and the tubes were kept in a microfuge tube rack at room temperature. The fourth trial was similar to the third, with the exception that the tube was immediately sealed and dropped into the liquid nitrogen dewar instead of remaining at room temperature. In the fifth trial, 60 % methanol pre-cooled to -80 °C was added to a 35 mm petri plate into which the filter was placed. In the sixth trial, the petri plate was pre-cooled on a flat chunk of dry ice in an insulated foam container. The filter was placed in the petri plate, and chilled (-80 °C) 60 % methanol was pipetted directly onto the filter. In the seventh and final trial, the chilled (-80 °C) 60

% methanol was added to the petri plate, pre-cooled on the flat slab of dry ice in the insulated foam cooler, before the filter was added.

Data Collection and Analysis for Quenching Validation

Data were collected using TempView software version 4.1 (IOtech, Inc., Cleveland, OH, USA). Excel 2007 (Microsoft, Redmond, WA, USA) was used to compile and tabulate data.

3.3 Results and Discussion

Cell Dry Weight Optimization

This experiment mathematically confirmed a strong positive correlation between the quantity of cells and their dry mass, with an R^2 value of 0.9991 (Figure 3.1). This suggests the experiment was performed accurately and that the results of this experiment are repeatable. The dry mass of one cell of *E. coli* calculated using this method was found to be $5.14 * 10^{-12}$ g/CFU, which fell within the 95 % confidence range of a previously calculated figure obtained using the dry mass of *E. coli* K-12 cells ($6.06 * 10^{-12} \pm 1.82 * 10^{-12}$ g/CFU, unpublished data), but is an order of magnitude greater compared to previously published values for the dry weight of *E. coli* cells obtained using transmission electron microscopy and densitometric image analysis (Loferer-Krößbacher *et al.*, 1998). This could be an indication of incomplete dryness of the cells which were collected for that portion of the analysis.

Visual analysis of the chromatograms obtained in the second portion of this work showed that the signal intensity (represented by "warmer," red-orange colors in the z-axis on the contour plot) increased as the number of cells extracted, and thereby the concentration of metabolites derivatized, increased. Figure 3.2a shows a typical chromatogram where $2.88*10^8$ CFU (0.38 mg dry weight of cells) was collected for extraction and derivatization; there is a clear signal demonstrated

by the multitude of peaks detected on the chromatogram. However, as shown in Figures 3.2b and 3.2c, the collection of larger volumes of cell suspension resulted in a greater and greater signal detected by the GCxGC-ToF-MS, and thus more peaks with larger peak areas were detected. Figure 3.2c is an excellent visual example of why it is not always best to simply inject the highest possible concentration of samples, as it showed peaks becoming overloaded and exhibiting "tailing," which does not make for an ideal chromatogram. While it was true that all three chromatograms showed some degree of tailing peaks, it was most pronounced in the last figure representing eight times more cells than the first (by dry weight), with virtually all peaks having this issue. Figure 3.2b represents the best compromise between small and large sample sizes for the detection limit of the instrument as well as the quality and interpretation ease of the resulting chromatographic data.

The amount of cells collected to generate the chromatogram in Figure 3.2b corresponded to approximately $4.16*10^9$ CFU (3 mg dry weight of cells) based on the data in Figure 3.1. This mass was slightly larger than the value predicted by McFeeters, but gave a good relationship between the chromatographic detection and resolution and the amount of filtered cell suspension required. Additionally, when taking into account the practical limitations such as the time required to filter the cell suspension, this dry weight value represented the upper limit for how many cells could reasonably be filtered in thirty seconds or less.

Standard Compounds

Analysis of pure standard compounds allowed for the development of a table containing information on retention times, retention indices, quantifying masses, and other pertinent mass spectral data to serve as a reference for future chromatographic-mass spectral work (Table 3.2). These standards may lead to the identification of these compounds in other sample matrices. They also may aid in identifying ^{13}C-containing peaks that have the same retention time as their ^{12}C

counterparts, which would be misidentified by the library search due to the heavier masses in the spectrum.

Due to the chemistry of the oximation and silylation reactions, multiple derivatization products can result from a single starting compound (Little, 1999). The mass spectral library identifies each of these separately as compound 1, compound 2, and so on, as necessary. These derivatization products were not detected in every chromatogram for a few reasons. First, some derivatization reactions only resulted in a single product. Reactions that result in more than one product can do so because the stereochemistry of the reaction results in isomeric products, or because the reaction was incomplete and not all of the possible sites on the molecule were replaced with TMS groups (Little, 1999). Another reason is that for thermodynamic reasons, the reaction may favor the formation of one derivatization product over the other(s) (Little, 1999).

Fifteen compounds in total were derivatized and injected onto the gas chromatograph. However, four compounds were unable to be detected by the mass spectrometer. Two of these (butyric acid and ethanol) were volatile compounds and were lost during sample preparation.

Quenching Validation

Research into the scientific literature, following preliminary experiments, raised questions as to whether quenching was occurring, and whether or not it satisfied the commonly held definition of quenching. The existing experimental protocol called for the cell suspension to be filtered, and the filter to be placed in a microfuge tube and dropped into liquid nitrogen in a total time of under thirty seconds. Bennett and coworkers (2008) detailed a protocol in which -80 °C quenching solution was kept in a pre-cooled petri dish, and the filter containing the cells was added and kept at -80 °C on dry ice for at least fifteen minutes. However, those scientists grew their cells on the filter, whereas in this experiment cells were filtered out of the suspension medium.

This study tested seven different protocols for quenching in order to determine which method reached -40 °C most rapidly and to measure the rate at which cooling occurred. The first trial in this work tested the Bennett *et al.* (2008) protocol using a no-cell control, and found that it took nearly eleven seconds to reach -40 °C on the surface of the filter, the temperature at which many authors consider quenching to occur (Table 3.3). This discovery disproved the assumption that cooling would occur instantaneously, translating into rapid quenching of metabolism. It was hypothesized that the microcentrifuge tube was insulating the transfer of heat from the filter to the liquid nitrogen and delaying the onset of quenching. To test this, a second trial modified the existing protocol by pre-cooling the microcentrifuge tubes to -80 °C. However, Table 3.3 shows that this alteration resulted in the tubes reaching -40 °C nearly three seconds longer after the initiation of cooling. This could be due to the length of time required to seal the cap and drop the tube into the dewar: A method with less handling time might be better suited for this purpose. These trials disproved the notion that cooling of the sample occurs instantaneously in liquid nitrogen by this method, most likely due to the lack of direct exposure between the cells and the liquid nitrogen.

A different approach was considered next: perhaps the addition of a chemical-based quenching agent would prove successful. The existing protocol called for a solution of 60 % methanol in water in order to lyse the cell membrane. With the membrane lysed, the cell is no longer alive, and its metabolic activity stops. A third trial was developed using that principle, in which the lysing solution was pre-cooled to -80 °C. This would serve the dual purpose of simultaneously lysing the cell membrane and cooling the intracellular metabolites, ensuring metabolism was completely halted. However, the desired quenching temperature of -40 °C was never reached (Table 3.3). In the fourth trial, with the addition of liquid nitrogen, the temperature reached -40 °C in nearly fifteen seconds after the onset of cooling, the slowest time yet. However, it

was later realized that to pipet the methanol into the tube, seal it, and physically place the tube in the dewar of liquid nitrogen could require approximately four or more seconds to complete, a factor which is not accounted for in the calculated time.

Since it was still thought that the insulating properties of the microfuge tube could be having an adverse effect on cooling, the quenching environment was changed to a petri plate just larger than the diameter of the filter. Here, the chilled methanol solution could have the largest surface area to exchange heat with the filter. This fifth trial yielded promising results, as reflected by the rapid drop to a temperature of -20 °C; however, the final temperature never reached the goal of -40 °C (Table 3.3). In the sixth trial, dry ice was used to pre-chill the petri plates inside an insulated foam cooler. This resulted in the temperature dropping to -40 °C in a matter of 17 seconds, and settling at a minimum temperature of around -54 °C (Table 3.3). However, the effect of the approximately four-second-long pipetting delay was also not considered in either trial, so it is possible that quenching occurred even more rapidly than what was recorded by the thermocouples. To correct for this delay, a seventh trial was performed in which the chilled (-78 °C) 60 % methanol was already in the petri dish before the no-cell control filter with the thermocouple was added; this was intended to observe the temperature change while correcting for the pipetting delay. In this trial, the 60 % methanol was added to the petri plate before the no-cell control filter, so that the entire experiment was maintained at the equilibrium temperature of the system (approximately -77 °C). Table 3.3 shows that the temperature reached the desired -40 °C in just over nine seconds, the fastest trial yet. The cooling curve for this trial was plotted against the previous six in Figure 3.3.

Also important to these experiments was the rate at which quenching occurred. Consistent with the findings for quenching times, the sixth trial had the highest quenching rate, dropping an average of about 57 °C/sec (Table 3.3). Samples exposed to dry ice through the chilled petri plates

cooled faster than samples indirectly exposed to liquid nitrogen despite the relatively higher temperature of the medium. One explanation for this observation is that heat transfer occurred more readily through the plastic petri plates than through the plastic microfuge tubes due to the difference in surface area between the two receptacles. The importance of validating one's presumed quenching method, as demonstrated above, is illustrated in works such as Bennett *et al*. (2008).

3.4 Conclusions

From these data, it can be concluded that 2 mg of dry weight of cells can be collected accurately and repeatably, and that using a slightly larger quantity of cells (3 mg dry weight) generates a chromatogram that can be visually distinguished from the chromatogram of a blank and exhibits signs of being neither at or below the detection limit of the instrument nor having overloaded it. The majority of chemicals that were derivatized and run as standards provided valuable chromatographic and mass spectral data. The collection of these data allowed for the creation of a table which will be used as a reference for confirming tentative peak identifications. The retention index information in particular will prove useful to this goal. Quenching carried out in a pre-cooled petri plate on dry ice containing -78 °C lysis solution exhibited the fastest overall cooling time and the highest cooling rate of all quenching conditions tested. In addition, the protocol for quenching in this manner is very easily adaptable to the current methods developed for acid-challenging and harvesting cells for trimethylsilyl derivatization and subsequent analysis by two-dimensional gas chromatography-time-of-flight mass spectrometry.

3.5 Acknowledgements

The author thanks Roger F. McFeeters and Suzanne D. Johanningsmeier for helpful discussions of this work, D. Seth Fornea for analytical expertise and instrument maintenance, Josip Simunovic for his temperature-recording equipment and training in its use, and Sandra Parker for excellent administrative assistance.

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Table 3.1 Summary of quenching, lysis, extraction, and storage protocols found in the metabolomics literature.

Protocol Type	Temperature of Protocol (° C)	Notes	References
Quenching	-20		Bolten *et al.*, 2007; Wittman *et al.*, 2004
Quenching	-27	Cold methanol	Carnicer *et al.*, 2012
Quenching	-40 to -50		Castrillo *et al.* 2003; Koek *et al.*, 2006;
Quenching	Unspecified	Liquid nitrogen	Fiehn *et al.*, 2008; Jozefczuk *et al.*, 2010; Rudell *et al.*, 2008; Zhao *et al.*, 2011
Quenching, Lysis, and Extraction	-20 to -80	Temperature depends on solution used	Bennett *et al.*, 2008
Quenching, Lysis, and Extraction	-40 to -45	Cold lysing agent	Maharjan and Ferenci, 2003; van den Berg *et al.*, 2006; Villas-Boas *et al.*, 2005; Yang *et al.*, 2010
Lysis	4		Börner *et al.*, 2007
Lysis and Extraction	-80	Cold lysing agent	Villas-Boas *et al.*, 2005; Yang *et al.*, 2010; Zhao *et al.*, 2011
Extraction	70		Börner *et al.*, 2007
Extraction	4		Jozefczuk *et al.*, 2010
Extraction	-45		Koek *et al.*, 2006
Storage	-80		Fiehn *et al.*, 2008
Storage	-20		Castrillo *et al.* 2003; Villas-Boas *et al.*, 2005, Wittman *et al.*, 2004; Yang *et al.*, 2010
Storage	4	Refrigeration	Shimazu *et al.*, 2004; Szeto *et al.*, 2011

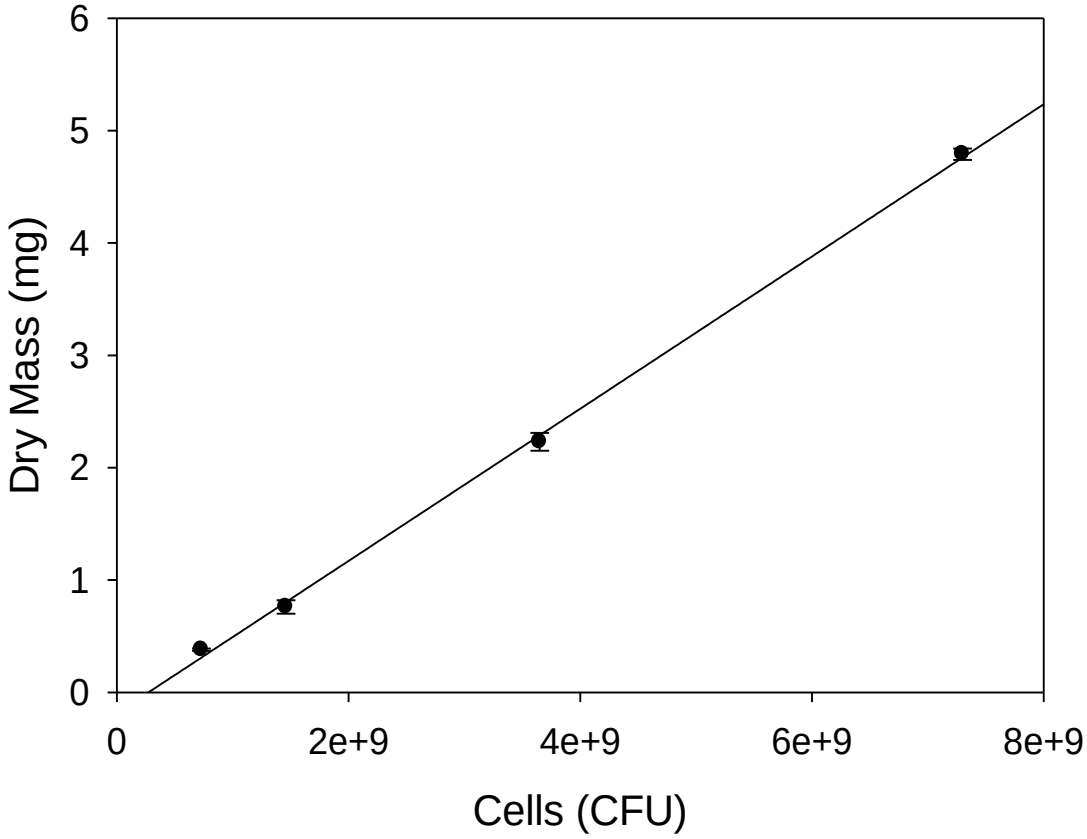

Figure 3.1 Dry mass versus number of cells. The trend line shown is linear with the equation y = $6.7738*10^{-10}$ x – 0.1852 and an R^2 value of 0.9991.

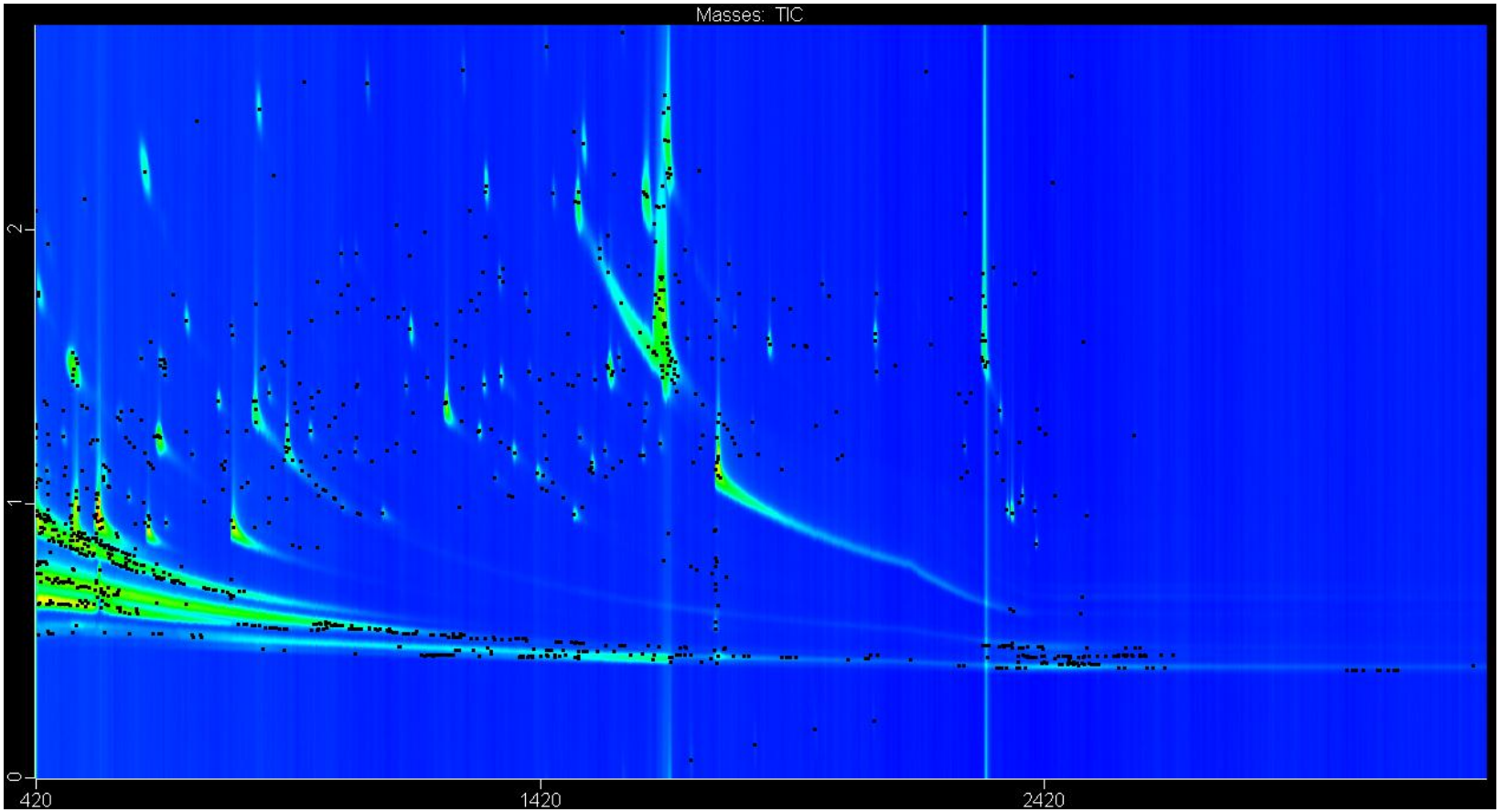

Figure 3.2a Total ion current chromatogram of TMS-derivatized metabolites extracted from 0.38 mg dry weight of *Escherichia coli* O157:H7 cells. The chromatogram above was obtained from 0.25 mL filtrate of 10x concentrated cell extracts. The x axis represents the first dimension retention time (s) and the y axis represents the second dimension retention time (s). The color intensity in the z axis represents the height of the peak detected.

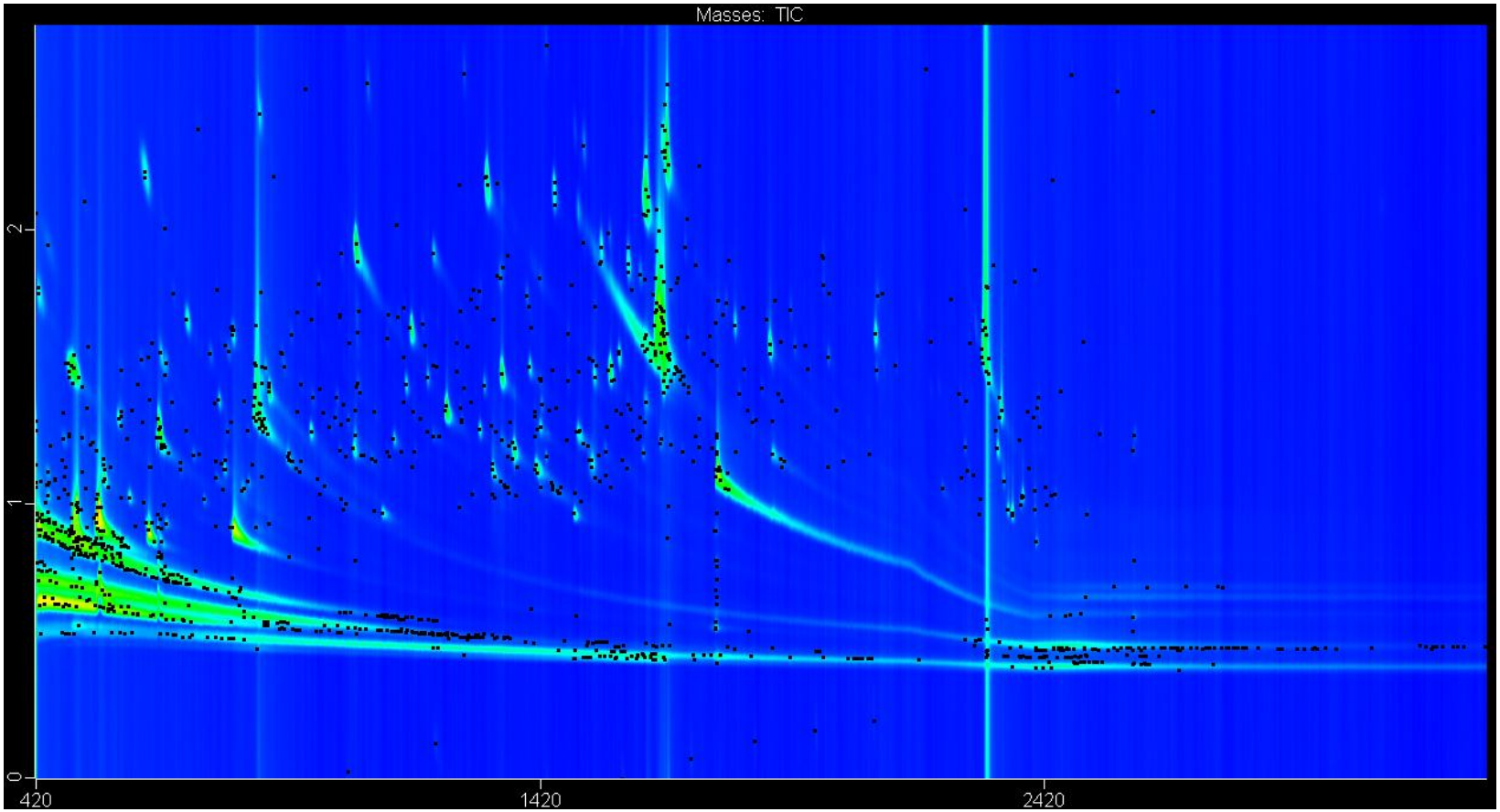

Figure 3.2b Total ion current chromatogram of TMS-derivatized metabolites extracted from 1.89 mg dry weight of *Escherichia coli* O157:H7 cells. The chromatogram above was obtained from 2 mL filtrate of 10x concentrated cell extracts. The x axis represents the first dimension retention time (s) and the y axis represents the second dimension retention time (s). The color intensity in the z axis represents the height of the peak detected.

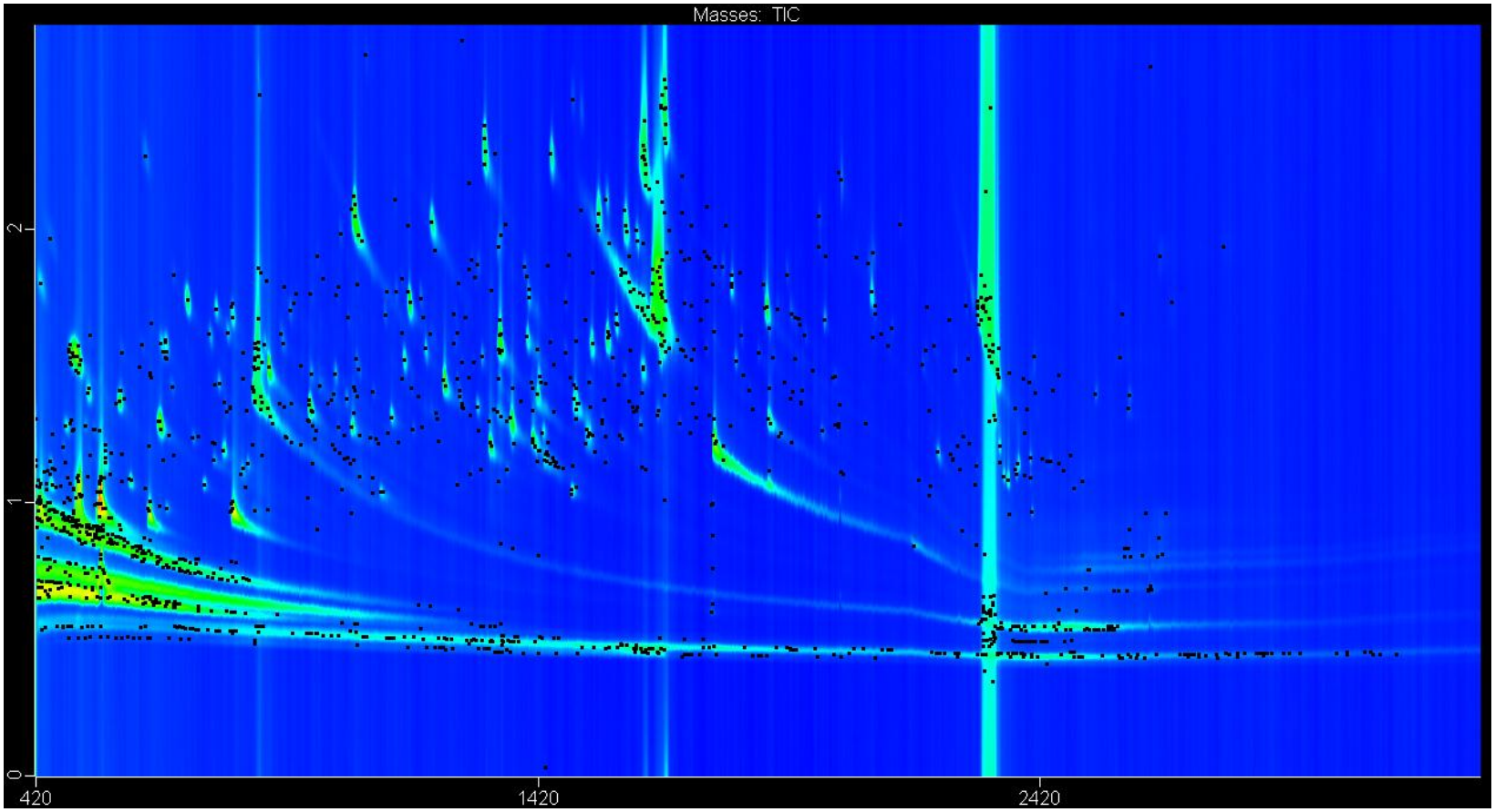

Figure 3.2c Total ion current chromatogram of TMS-derivatized metabolites extracted from 7.70 mg dry weight of *Escherichia coli* O157:H7 cells. The chromatogram above was obtained from 4 mL filtrate of 10x concentrated cell extracts. The x axis represents the first dimension retention time (s) and the y axis represents the second dimension retention time (s). The color intensity in the z axis represents the height of the peak detected.

Table 3.2 Chromatographic data for known standards derivatized and analyzed by GCxGC-ToF-MS.

Peak #	Compound	Fiehn Library Name	Derivative Name	Amount in Tube (nmoles)	Compound CAS #	Retention Index	1st Dimension Time (s)	2nd Dimension Time (s)	Quant Masses	Area	Similarity	Reverse	Probability
425	Phosphoric Acid	phosphate	Phosphoric acid, tris(trimethylsilyl) ester	0.4	7664-38-2	1334.9	887.5	1.465	299	225066	918	918	9817
443	Phosphoric Acid	phosphate	Phosphoric acid, tris(trimethylsilyl) ester	3	7664-38-2	1333.1	884.75	1.47	299	618501	937	938	9819
492	Phosphoric Acid	phosphate	Phosphoric acid, tris(trimethylsilyl) ester	20	7664-38-2	1333.1	884.75	1.5	299	2310547	947	947	9757
452	Nicotinic Acid	nicotinic acid	3-Pyridinecarboxylic acid, trimethylsilyl ester	3	59-67-6	1466.9	1091	1.255	180	568317	899	908	9760
720	Nicotinic Acid	nicotinic acid	3-Pyridinecarboxylic acid, trimethylsilyl ester	20	59-67-6	1458	1071.75	1.275	180	3717824	926	926	9828
741	Nicotinic Acid	nicotinic acid	3-Pyridinecarboxylic acid, trimethylsilyl ester	100	59-67-6	1458	1071.75	1.29	180	1232527	915	915	9780
527	L-glutamic acid	Unknown 506	--	3	56-86-0	1659.2	1366	1.655	246	32526	640	828	9630
495	L-glutamic acid	glutamic acid	L-Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester	20	56-86-0	1659.2	1366	1.66	246	45632	909	920	9813
317	Pyruvic Acid	Pyruvic acid	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	0.4	127-17-3	1179.7	645.5	1.135	174	9240.4	689	873	9489
361	Pyruvic Acid	Pyruvic acid	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	3	127-17-3	1183.2	651	1.13	174	55938	924	932	9639
318	Pyruvic Acid	Pyruvic acid	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	20	127-17-3	1181.4	648.25	1.135	174	295223	944	945	9587
444	Fumaric Acid	Analyte 532	--	0.4	110-17-8	1404.6	994.75	1.57	45	79337	693	789	7645
479	Fumaric Acid	fumaric acid	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	3	110-17-8	1404.6	994.75	1.57	245	267901	930	937	9754
415	Fumaric Acid	fumaric acid	2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	20	110-17-8	1404.6	994.75	1.595	245	496567	932	933	9740
968	Uridine	uridine 1	Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	3	58-96-8	2690.5	2372.5	1.07	169	551636	821	841	3899
808	Uridine	uridine 2	2',3'-Bis-(trimethylsiloxy)-uridine	20	58-96-8	2592.1	2325.75	1.17	245	25958	914	919	9746
816	Uridine	uridine 1	Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	20	58-96-8	2637.8	2347.75	1.18	169	430071	889	895	8072

Table 3.2 Continued

Peak #	Compound	Fiehn Library Name	Derivative Name	Amount in Tube (nmoles)	Compound CAS #	Retention Index	1st Dimension Time (s)	2nd Dimension Time (s)	Quant Masses	Area	Similarity	Reverse	Probability
834	Xylitol	xylitol	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	0.4	87-99-0	1607.9	1289	2.435	189	53367	898	907	2212
765	Xylitol	xylitol	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	3	87-99-0	1607.9	1289	2.445	189	12582	880	894	2243
643	Xylitol	xylitol	Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	20	87-99-0	1607.8	1289	2.315	189	3034.8	847	855	2462
1136	Glucose-6-Phosphate Sodium Salt	glucose-6-phosphate 1	6-O-(Bis[(trimethylsilyl)oxy]phosphoryl)-1,2,3,4-tetrakis-O-(trimethylsilyl)hexopyranose	3	56-73-5	2286.1	2097.5	1.945	103	15283	866	866	8953
900	Glucose-6-Phosphate Sodium Salt	glucose-6-phosphate 1	6-O-(Bis[(trimethylsilyl)oxy]phosphoryl)-1,2,3,4-tetrakis-O-(trimethylsilyl)hexopyranose	20	56-73-5	2288.8	2100.25	2.025	103	144519	876	876	9191
918	Glucose-6-Phosphate Sodium Salt	glucose-6-phosphate 2	6-O-(Bis[(trimethylsilyl)oxy]phosphoryl)-1,2,3,4-tetrakis-O-(trimethylsilyl)hexopyranose	20	56-73-5	2304.9	2116.75	1.875	357	34031	808	820	6719
796	Putrescine	putrescine 2	1,4-Butanediamine, N,N,N'-tris(trimethylsilyl)-	0.4	110-60-1	1638.6	1330.25	2.46	174	331261	923	9502	9502
765	Putrescine	putrescine 2	1,4-Butanediamine, N,N,N'-tris(trimethylsilyl)-	3	110-60-1	1638.6	1330.25	2.475	174	2366912	951	952	9695
814	Putrescine	putrescine 1	1,4-Butanediamine, N,N,N',N'-tetrakis(trimethylsilyl)-	3	110-60-1	1861.6	1619	2.02	174	13899	843	886	8377
734	Putrescine	putrescine 2	1,4-Butanediamine, N,N,N'-tris(trimethylsilyl)-	20	110-60-1	1634.5	1324.75	2.495	174	6019624	944	945	9658
773	Putrescine	putrescine 1	1,4-Butanediamine, N,N,N',N'-tetrakis(trimethylsilyl)-	20	110-60-1	1861.6	1619	2.015	174	238304	920	920	8929

Table 3.2 Continued

Peak #	Compound	Fiehn Library Name	Derivative Name	Amount in Tube (nmoles)	Compound CAS #	Retention Index	1st Dimension Time (s)	2nd Dimension Time (s)	Quant Masses	Area	Similarity	Reverse	Probability
798	Citrulline	citrulline 1	Citrulline, tri(trimethylsilyl)-	40	372-75-8	1881.7	1643.75	1.66	157	20122	686	852	9589
834	Citrulline	citrulline 1	Citrulline, tri(trimethylsilyl)-	200	372-75-8	1881.8	1643.75	1.67	157	660986	921	922	9744
836	Citrulline	citrulline 2	Citrulline, tetra(trimethylsilyl)-	200	372-75-8	1892.4	1657.5	1.41	70	386510	933	939	9491
1167	Citrulline	citrulline 1	Citrulline, tri(trimethylsilyl)-	1000	372-75-8	1881.8	1643.75	1.77	157	73675	889	889	9737
1172	Citrulline	citrulline 2	Citrulline, tetra(trimethylsilyl)-	1000	372-75-8	1890.3	1654.75	1.43	70	1950126	941	941	9416
933	Guanosine	guanosine	9H-Purin-2-amine, N-(trimethylsilyl)-6-[(trimethylsilyl)oxy]-9-[2,3,5-tris-O-(trimethylsilyl)-β-D-ribofuranosyl]-	3	118-00-3	2908.7	2466	1.315	103	97125	879	904	9803
942	Guanosine	guanosine	9H-Purin-2-amine, N-(trimethylsilyl)-6-[(trimethylsilyl)oxy]-9-[2,3,5-tris-O-(trimethylsilyl)-β-D-ribofuranosyl]-	20	118-00-3	2908.7	2466	1.32	245	339186	774	797	9844

* 1 or 2 designates multiple TMS-derivatized forms of the same initial standard compound.

Table 3.3 Cooling times and rates for experimental quenching protocols.

Trial	Description	Time to -20 °C (sec)	Time to -40 °C (sec)	Min T (°C)	Cooling Rate (° C/sec)
1	Cells on filter in microfuge tube, immersed in liquid nitrogen	8.35 ± 2.96	10.97 ± 4.47	-194.55 ± 4.01	-1.82 ± 0.656
2	Cells on filter in pre-chilled (-80 °C) microfuge tube, immersed in LN_2	11.83 ± 2.57	13.9 ± 2.77	-194.85 ± 3.4	-2.15 ± 0.441
3	Cells on filter in microfuge tube, -80 °C 60 % MeOH added to tube	N / A	N / A	-13.99 ± 1.87	-24.38 ± 8.22
4	Cells on filter in microfuge tube, -80 °C 60 % MeOH added to tube, tube immersed in LN_2	9.68 ± 1.61	14.93 ± 3.12	-149.84 ± 21.59	-31.78 ± 8.96
5	Cells on filter placed into 35 mm petri plate, followed by addition of -80 °C 60 % MeOH	1.28 ± 0.28	N / A	-30.63 ± 3.45	-9.33 ± 6.17
6	Cells on filter placed into 35 mm petri plate on dry ice slab in foam cooler, followed by addition of -80 °C 60 % MeOH	7.08 ± 3.17	17.33 ± 9.19	-53.94 ± 5.58	-10.31 ± 3.68
7	Cells on filter placed into 35 mm petri plate on dry ice slab in foam cooler, -80 °C 60 % MeOH already in plate	5.13 ± 1.47	9.33 ± 2.50	-76.60 ± 2.27	-57.33 ± 12.0

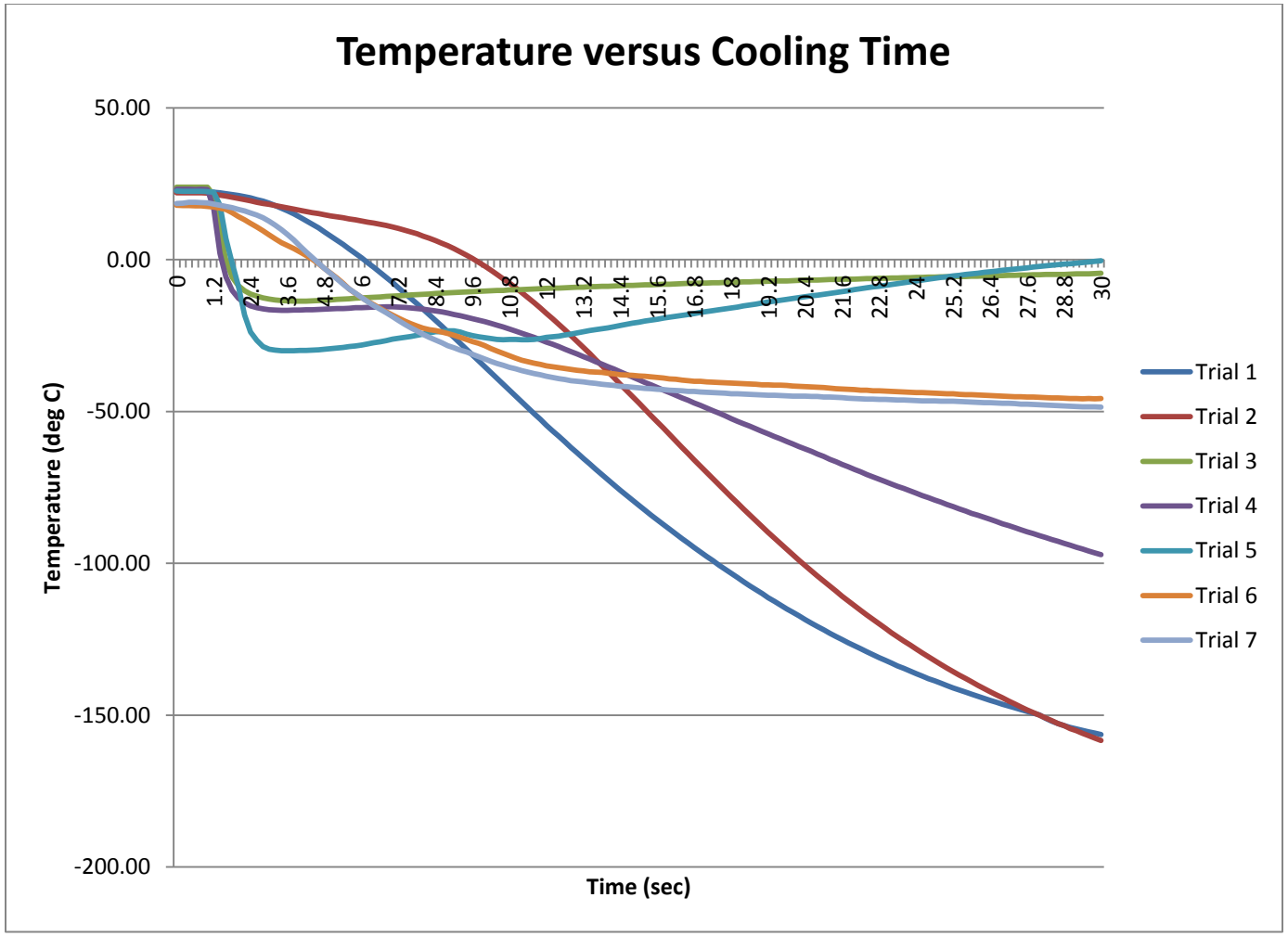

Figure 3.3 Graph of cooling rate for each trial, as recorded by thermocouples attached to no-cell control filters.

CHAPTER FOUR

Detection of Changes in the *Escherichia coli* O157:H7 Metabolome under Acid pH and Organic Acid Stress using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry

Abstract

Escherichia coli O157:H7 is a pathogen noted for its ability to survive in acid pH foods by resisting acidification of the cytoplasm. A study was undertaken to elucidate the changes in the intracellular metabolite pool during acid stress for the purpose of using the knowledge to improve the safety of acid and acidified food products. Two dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS) was used to detect TMS-derivatized metabolites in the extracts of cells exposed to neutral pH (7), acid pH (3.2), or acid pH in the presence of organic acid (40 mM acetic acid at pH 3.2). Analysis of variance (ANOVA) and hierarchical clustering analysis selected 90 out of a total of 2488 detected peaks whose areas changed significantly ($p < 0.001$) in response to acid stress. The tentatively identified peaks included metabolites involved in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, the pentose phosphate pathway, the urea cycle and amino acid synthesis and breakdown. This study uncovered evidence that glutamic acid was decreased and γ-aminobutyric acid (GABA) was increased at acid pH (with and without organic acid), which supports the prevailing hypothesis that O157:H7 uses the glutamate-dependent acid resistance mechanism to counter decreases in cytoplasmic pH. Two clusters of metabolites were uncovered whose concentrations significantly increased or decreased at acid pH only when acetic acid was present, suggesting that intracellular acetate triggered a separate stress response to the anion buildup independent of the pH effect. Additionally, the detection of metabolites involved in pathways producing ATP energy also strengthened the concept that acid-stressed cells use ATP to

pump out protons from the cytoplasm in the opposite direction of the proton motive force. This work could impact both metabolomics and food safety circles by aiding in the understanding of acid stress responses and the application of that knowledge to make food products less hospitable to the survival of this acid-resistant pathogen.

4.1 Introduction

Escherichia coli O157:H7 is a pathogen whose resistance to acid poses a food safety problem. *E. coli*, including the O157 serotype, has four well-documented acid response mechanisms, as described by other researchers (Lin *et al.*, 1995; Hersh *et al.*, 1996; Richard and Foster, 2004). The first ("AR1") is a glucose-repressed oxidative system that is induced in stationary phase cells (Castanie-Cornet *et al.*, 1999). The second ("AR2") uses the enzyme glutamate decarboxylase (GAD) to convert glutamate into γ-aminobutyrate (GABA), which consumes a proton and helps regulate intracellular pH (Hersh *et al.*, 1996; De Biase *et al.*, 1999; Shin *et al.*, 2001; Ma *et al.*, 2003). The third acid resistance system ("AR3") is similar to the second, involving the decarboxylation of arginine by arginine decarboxylase, having the same net effect on pH homeostasis in the cytoplasm (Lin *et al.*, 1995; Lin *et al.*, 1996). The fourth acid resistance system ("AR4") involves the decarboxylation of lysine similar to the other amino acid decarboxylases, with a similar final result (Diez-Gonzalez *et al.*, 2004). In stationary phase *E. coli* O157:H7, the glutamate-dependent system was the most active, and the arginine- and lysine-dependent systems were active to a lesser degree (Diez-Gonzalez *et al.*, 2004).

There are several approaches to studying the effects of acid pH and organic acid stress on cells of *Escherichia coli* O157:H7. Some of the more recently popularized methodologies for this purpose are the "omics", which apply a systems biology approach to the study of transcription (transcriptomics), protein expression (proteomics), and metabolite pools (metabolomics). Researchers have had some success in both transcriptomic (Allen *et al.*, 2008; Arnold *et al.*, 2001; King *et al.*, 2010; Riordan *et al.*, 2010) and proteomic (Fagerquist *et al.*, 2010; Huang *et al.*, 2007) studies, as well as the combination of the two (Kocharunchitt *et al.*, 2012) in uncovering new information about the acid resistance capabilities of O157:H7. However, metabolomic studies on the

response of *E. coli* O157:H7 to acid stress have not been published in the literature. Many studies on other types of cells have proven the validity and feasibility of this technique (Almstetter *et al.*, 2009; Bennett *et al.*, 2009; Cevallos-Cevallos *et al.*, 2011; Koek *et al.*, 2006; Mashego *et al.*, 2006; Park *et al.*, 2005; Rabinowitz, 2007), yet it has not to the author's knowledge yet been applied to answer the question of what happens to the metabolite pool of *E. coli* O157:H7 as it undergoes acid stress.

The literature describes several metabolomic analyses of *Escherichia coli* K-12, and a few proteomic or transcriptomic analyses of O157:H7. In *E. coli* K-12, King and coworkers found transcriptomic evidence for the cellular response to inorganic acid (hydrochloric) and organic acids (acetic and lactic); the *gadE* gene responsible for glutamic acid decarboxylase was downregulated in K-12 and upregulated in O157:H7 for all acidulants (King *et al.*, 2010). Several other studies examined non-O157 strains of *E. coli*. Almstetter and others were able to differentiate two non-O157 strains from each other by 48 statistically significant peaks using GCxGC-ToF-MS (Almstetter *et al.*, 2009). Of these 48 peaks, those identified were tricarboxylic acid cycle intermediates, glycolysis intermediates, amino acids, sugars, and several other compounds (Almstetter *et al.*, 2009). Jozefczuk and coworkers analyzed a non-O157 strain for its stress responses to cold, heat, oxidation, and glucose-lactose shift, and found numerous metabolite patterns changing in response to each condition, including many of the same classes of compounds found by Almstetter and coworkers (Jozefczuk *et al.*, 2010). Studies have also been undertaken on O157:H7; Fagerquist and others took a proteomic approach to identify the acid stress protein HdeA in *E. coli* O157:H7 using matrix-assisted laser desorption ionization coupled to tandem time-of-flight mass spectrometry (MALDI-ToF-ToF) (2010). They identified several protein biomarkers, such as acid stress chaperone proteins, that differentiated pathogenic O157:H7 from nonpathogenic *E. coli* strains, but not from genetically similar non-O157 pathogenic strain O55:H7 (Fagerquist *et al.* 2010). More recently, King and

coworkers did not observe upregulation of any acid resistance mechanisms in O157:H7 except for the gene encoding the lysine-dependent system (*cadB*), in response to hydrochloric acid as the acidulant (2010). With acetic acid as the acidulant, they observed upregulation of three genes (*yojI*, a multidrug transporter membrane component and ATP-binding component; *yfdX*, which codes for a hypothetical protein; and *yqgA*, which encodes a predicted inner membrane transport protein) and three intergenic regions (King *et al.*, 2010). Though many metabolomic studies have been undertaken on *Escherichia coli*, both O157 and non-O157 strains, under various conditions, both stressed and unstressed, there is a lack of information on metabolomic changes in O157:H7 resulting specifically from acid pH and organic acid stress. Elucidating these changes will lead to a better understanding of the acid stress response of this pathogen. This, in turn, could be used to develop novel control strategies based upon the uncovered metabolites and ultimately result in safer acid and acidified food products. The objective of this study was to determine the changes in the intracellular metabolite profiles of *E. coli* O157:H7 under acid pH and acetic acid stress.

4.2 Materials and Methods

Chemicals

D-glucose, sodium chloride, methoxyamine hydrochloride, magnesium sulfate, sodium phosphate monobasic, morpholine-4-ethanesulfonic acid hydrate (MES), and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide was purchased from Aldrich (Milwaukee, WI, USA). Hydrochloric acid and methanol were purchased from Fisher (Fair Lawn, NJ, USA). ^{13}C-salicylic acid and D8-valine were purchased from Icon Isotopes (Summit, NJ, USA). Pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Thermo

Scientific (Rockford, IL, USA). Alkanes C8-C20, alkanes C21-C40, and ammonium chloride were purchased from Fluka (St. Louis, MO, USA).

Media Preparation

M9-GT media was prepared according to recipe for M9 media (Sambrook *et al.*, 1989), but supplemented to 1 % total glucose and 0.005 % thiamine. Cells did not grow well beyond one overnight without the addition of a small amount of unlabeled thiamine to the medium.

Buffer ("MG") was made containing 20 mM MES and 20 mM gluconic acid as noninhibitory buffers (Good *et al.*, 1966; Breidt *et al.*, 2004), and 0.85 % sodium chloride, and adjusted to a final pH of 3.2 with hydrochloric acid or sodium hydroxide as necessary. An identical buffer was made which also contained 40 mM protonated acetic acid and was adjusted to pH 3.2 with hydrochloric acid or sodium hydroxide as necessary. A third buffer was made containing 20 mM MES, 20 mM gluconic acid, and 0.85 % sodium chloride, which was adjusted to a final pH of 7.0 with hydrochloric acid or sodium hydroxide as necessary.

Cell Preparation and Treatment

Escherichia coli O157:H7 strain B241 (28RC1), a bovine isolate obtained from the USDA-ARS Food Science Research Unit culture collection (Raleigh, NC), was chosen for its acid resistance (Oh *et al.* 2009). An LB agar plate was streaked from the frozen stock and incubated overnight at 37 °C; then, three separate colonies were picked and inoculated into 10 mL M9-GT in triplicate, and incubated at 37 °C statically for 18 hours. A 250 µL aliquot of each replicate was transferred into 25 mL of fresh M9-GT and incubated at 37 °C statically for 18 hours. Cells were harvested by centrifugation for 10 minutes at 5000 x g and resuspended in an equal amount of sterile 0.85 % saline. The process was repeated once, and cells were resuspended in sterile 0.85 % saline at one hundred-fold concentration (approximately $5 * 10^{10}$ CFU/mL). Cells were tenfold diluted to

approximately 5 * 10^9 CFU/mL in pH 3.2 MG buffer with no acetic acid (final pH 3.19) or 40 mM total acetic acid (final pH 3.19) or in pH 7 MG buffer (final pH 6.99). The buffers were then incubated at 30 °C for thirty minutes. A 2 mL aliquot of the cell suspension in buffer was vacuum-filtered such that the cells were collected onto a 0.45 micron 25mm diameter polyethersulfone membrane filter (Sterlitech Corporation, Kent, WA, USA). Immediately after filtration, filters were placed in individual 35 mm petri dishes containing 0.75 mL 60 % methanol kept cooled on top of a flat block of dry ice for quenching of metabolism at -80 °C. Filter blanks (no-cell controls) were prepared by incubating sterile 0.85 % saline diluted ten-fold into the appropriate buffer for thirty minutes at 30 °C, and then filtering a 2 mL aliquot and quenching as described. Petri dishes containing the cell extracts were kept chilled on dry ice until transfer of the liquid and filter into microfuge tubes. The experimental design was repeated with seven biological replicates.

Extraction and Derivatization of Cellular Metabolites

Microfuge tubes containing the filters and cell extracts were retrieved from the -80 °C dry ice cooler. Tubes were mixed for 25 min at 70 °C and 300 rpm in a Thermomixer R (Eppendorf, Hamburg, Germany) to inactivate enzymes. A 700 µL aliquot of each cell extract was transferred into GC vials (product number 11-1500-S, MicroLiter Analytical Supplies, Suwanee, GA, USA). Internal standard solution containing 0.2 mM ^{13}C-salicylic acid and 0.2 mM D8-valine was added to each and vortexed. Samples were dried in a SpeedVac SVC 100 (Savant Instruments, Hickville, NY, USA) on medium heat to complete dryness, then loaded into a CTC Combi PAL autosampler (LEAP Technologies, Carrboro, NC, USA), for automated chemical derivatization and injection. A methoximation solution containing 20 mg/mL methoxyamine hydrochloride in pyridine was added to each vial and mixed for 90 min at 70 °C. An equal volume of BSTFA was added to each vial and mixed for 30 min at 70 °C. Alkanes C8-C40 were added to all samples before injection into the

GCxGC-ToF-MS for calculation of retention indices. Seven derivatization blanks were also prepared, containing empty vials to which the autosampler added the reagents. Sample order was randomized and staggered one reaction at a time, such that there was no waiting period between each derivatization reaction and the subsequent injection into the GCxGC-ToF-MS.

GCxGC-ToF-MS Method

Qualitative analyses used two-dimensional GC with time-of-flight mass spectrometry. GCxGC-ToF-MS analysis was carried out according to the method developed by Johanningsmeier (2011). Briefly, an Agilent 6890 (Agilent Technologies, Santa Clara, CA, USA) equipped with a 28.45 m BPX50 column (SGE Analytical Science, Austin, TX, USA) in the first dimension and a 1.0 m BPX5 column (SGE Analytical Science) in the second dimension was used for separation of TMS-derivatized metabolites. The temperature of the injection port was 260 °C. The oven temperature program was 85 °C for four minutes, then from 85 to 245 °C at 5 °C/min then heating at 15 °C/min to 305 °C, and finally holding at 305 °C for 15 minutes. Helium was used as the carrier gas at a flow rate of 1.00 mL/min. The temperatures of the transfer line and the ion source were 280 and 250 °C, respectively. The detector was a Pegasus 4 time-of-flight detector (LECO Corporation, St. Joseph, MI, USA). Mass spectra were acquired over the mass range of 35-800 amu at an ionization energy of -70 Volts.

Data Analysis

Identification of individual components was done using the LECO-Fiehn Rtx5 (Kind *et al.*, 2009), Max_Planck_TOFMS (Wagner *et al.*, 2003), and the 2011 version of the NIST mainlib (National Institute of Standards and Technology Gaithersburg, MD, USA) libraries. The alkane standards identified in a representative chromatogram were used to create a retention index method based on the retention times of alkanes C8-C40. ChromaTOF® (LECO) software was used to determine the

baseline, find peaks, search the library, calculate heights and areas, and calculate retention indices. Data were analyzed in ChromaTOF® using the Statistical Compare feature to align peak names and numbers of analytes. Data were exported and statistically analyzed in JMP Genomics version 5.0 (SAS Institute, Cary, NC, USA) using a basic expression workflow that treated the differences in peak areas as analogous to the differences in gene expression that one might observe in a microarray experiment.

4.3 Results and Discussion

In order to form a solid base of knowledge from which to draw conclusions, extensive chemical confirmation was undertaken. Five peaks were chosen as landmarks for analysis in further depth: succinic acid, 4-aminobutanoic acid, L-aspartic acid, glutamic acid, and N-acetylglutamic acid. The peaks were selected based on confidence in the tentative identity of the peak and its connection to published acid resistance mechanisms in *Escherichia coli* O157:H7. The first peak selected as a landmark, succinic acid, is a tricarboxylic acid cycle (TCA) intermediate whose conversion from succinate to fumarate generates the equivalent of 1.5 ATP for the cell. The second chosen peak, 4-aminobutanoic acid, also called γ-aminobutyric acid (GABA), is the end product of the decarboxylation of glutamate by glutamate decarboxylase (GAD), which is documented as an acid resistance mechanism in *E. coli* O157:H7 due to the resulting consumption of a proton inside the cytoplasm and extracellular alkalinization following export of GABA (Diez-Gonzalez *et al.*, 2004; Lin *et al.*, 1996). GABA can also be converted to succinic acid by transamination and oxidation, and subsequently fed back into the TCA cycle, or removed from the cell via the antiporter encoded by *gadC* (Castanie-Cornet *et al.*, 1999). The third selected landmark, L-aspartic acid, is one of the proteinogenic amino acids that can be used to synthesize lysine, whose decarboxylation into

cadaverine is said to be partially responsible for acid resistance in *E. coli* O157:H7 (Diez-Gonzalez *et al.*, 2004). In the urea cycle, aspartate can be converted to arginosuccinate and then to fumarate, which is a TCA cycle intermediate. Additionally, aspartate can be converted to oxaloacetate, another TCA cycle intermediate, which can be converted to pyruvate and thus have an effect on the rate of gluconeogenesis in the cell. Pyruvate, an α-keto acid, is also capable of reacting with toxic oxygen species such as peroxides in an antioxidant capacity, which may help stave off cell death. The other half of the GAD acid response mechanism is glutamic acid, an amino acid which together with TCA cycle intermediate oxaloacetate can be reversibly converted to aspartate. It can also be synthesized reversibly from the reaction of alanine and TCA intermediate α-ketoglutarate, forming not only glutamate but also pyruvate, whose effect on cell metabolism has previously been described. The fifth chosen landmark peak is N-acetylglutamic acid. In the urea cycle, it activates the enzyme CPS-1 that catalyzes the first reaction in the cycle. Its synthesis in the cell is stimulated by the presence of glutamate and arginine, whose involvement in the acid resistance mechanisms of *E. coli* O157:H7 is proposed by several authors (Diez-Gonzalez *et al.*, 2004; Foster and Moreno, 1999).

The retention index (RI) reproducibility, library similarity, and mass spectra for landmark peaks were analyzed in depth for confirmation of their identities. The data indicate that the RIs were highly reproducible, ranging from 0.03 % RSD for succinic acid to 0.06 % RSD for N-acetylglutamate. The similarity score of each landmark peak was plotted in Figure 4.2 as a measure of how well each obtained mass spectrum matched the reference spectrum given in the library. The box-and-whisker plot shows the average similarity score, as well as the high and low scores, for each peak, with a user-specified score of 800 (out of a maximum 1000) required in order to assign a name to the peak. The library matching gave a higher similarity value for the identities of succinic acid, GABA, and L-aspartic acid than for glutamic acid and N-acetylglutamic acid. However the comparison of the

retention index for the peak identified as glutamic acid to a pure standard compound indicated that the tentative identity was a match (data shown in Chapter 3, Table 3.2). The mass spectra of the landmark peaks (Figure 4.3) were also analyzed in detail for confirmation that the peak in question was properly identified by the mass spectral library.

Also of interest in this analysis was the reproducibility of peaks in this experiment. The peak area percent relative standard deviation (%RSD) was sorted by treatment and plotted in Figure 4.4. The histogram in Figure 4.4a shows a Gaussian distribution of %RSD values for the peaks in the pH 7 samples, with some positive skew indicating a large number of peaks with relatively higher variability. Figure 4.4b shows less positive skew of the distribution of %RSDs for the pH 3.2 samples, the variability seemingly increasing in response to the addition of the acid pH variable. Figure 4.4c shows positive skew as well with the additional variable of the presence of acetic acid. These histograms demonstrate that the majority of peaks fall between 20 % and 70 %, indicating good reproducibility. The analytical (instrumental) variability estimated by the %RSD of the internal standard ^{13}C-salicylic acid was 15.4 %. Despite the variability, this work was able to detect differences in a number of important metabolites.

The Statistical Compare function in ChromaTOF® aligned a total of 2488 peaks across the 37 samples, no-cell controls, and derivatization blanks. All peaks were imported into JMP Genomics, where analysis of variance (ANOVA) identified 90 peaks which changed significantly in response to acid stress ($p < 0.001$). The hierarchical clustering analysis (Figure 4.1) showed that the peaks grouped into five clusters based on the trends in peak areas. The first cluster encompassed analytes which had higher peak areas in the pH 7 samples, but lower in both pH 3.2 and pH 3.2 with 40 mM acetic acid (Table 4.1). These metabolite peaks can be described as compounds that decreased due to acid pH stress, regardless of the presence of organic acid. The second cluster was composed of

analytes whose peak areas were highest in the pH 3.2 samples, and lowest at pH 7 and pH 3.2 with 40 mM acetic acid (Table 4.2). These compounds may represent biochemicals which increased in concentration in response to acid pH stress alone, but were ameliorated by the presence of acetate. However, manual confirmation of these peaks is warranted. The third cluster included analytes that had the smallest peak areas in the samples at pH 3.2 with 40 mM acetic acid (Table 4.3). This represents an interesting trend in which the metabolites decreased in response to the organic acid stress presented by the acetic acid, in an effect that is clearly separate from the stress introduced by acid pH alone. In the fourth cluster, there were 9 peaks whose areas were increased in the samples at pH 3.2 with and without the added acetic acid (Table 4.4). This trend represents the complementary set of analytes to those in the first cluster, in which these peaks are increased due to acid pH stress on the *E. coli* cells. The fifth cluster represented peaks whose areas were larger in the pH 3.2 samples with 40 mM acetic acid present (Table 4.5). These are complementary to the peaks in the third cluster, suggesting that these compounds are increasing in response to the organic acid stress and are not triggered solely by the increased concentration of hydrogen ions present in acid pH environments.

Out of the 90 statistically significant peaks, 37 were identified by name by the mass spectral library. An overview of their potential significance is presented in Table 4.6. Most of these peaks have close metabolic ties to the tricarboxylic acid cycle, the urea cycle, glycolysis/gluconeogenesis, and amino acid metabolism. Glycolysis, gluconeogenesis, and the TCA cycle are ways by which the cell generates energy in the form of ATP. Perhaps the cells, under acid stress, produce ATP in order to power the expulsion of excess protons from the cytoplasm, re-equilibrating the internal pH and restoring homeostasis (Booth, 1985). Amino acid metabolism is a precursor to protein synthesis, which acid-stressed cells could be using for signaling purposes. The urea cycle is a means for cells to

remove ammonia by converting it to less-toxic urea. Compounds detected in this analysis with ties to the urea cycle are those which are used in the latter steps that take place in the cytoplasm.

Specifically, at pH 7, representing an unstressed condition for the cells of *E. coli* O157:H7, several amino acids such as glutamate and TCA cycle intermediates were present at significantly higher levels than in acid pH conditions. Peaks that were significantly increased at acid pH included GABA. These data support the idea that the glutamate-dependent acid response system is not a significant occurrence at pH 7, and that the converse is true at pH 3.2 with and without the additional stress presented by acetic acid. At pH 3.2 with organic acid stress, there are increased concentrations of several lactones with ties to the pentose phosphate pathway (PPP) and ascorbate metabolism, and decreased amounts of various amino acids (glycine, alanine, isoleucine, proline, threonine, and tyrosine), TCA cycle intermediates, and GABA precursors. Synthesizing these metabolomic clues suggests that the cells may have converted those compounds to other metabolic products for their ATP equivalency, in order to have that energy available to pump out excess protons from the cytoplasm. The reason these metabolic changes are occurring in response to acetic acid is yet unknown.

The findings in this study can be related to the results of other work that has been done with a proteomic or transcriptomic approach, rather than one oriented towards the detection of small, intracellular metabolites. Fagerquist and coworkers were able to identify the acid stress protein HdeA in *E. coli* O157:H7 using matrix-assisted laser desorption ionization coupled to tandem time-of-flight mass spectrometry (MALDI-ToF-ToF) (2010). The HdeA protein, one of the most abundant in the periplasm, is one regulated by the RpoS sigma factor, expressed from the hdeAB operon of the genetic sequence (Booth *et al.* 2002). It is responsible for preventing acid-induced aggregation of proteins in the cell, which would greatly reduce the functionality of proteins

necessary for survival (Booth *et al.*, 2002). Huang and others also used MALDI-ToF-ToF to identify proteins isolated from a Taiwanese outbreak strain of O157:H7 (2007). They detected the proteins glutamate decarboxylase α and glutamate decarboxylase β, the two isozymes responsible for the conversion of glutamate to GABA in the glutamate-dependent acid response mechanism. Both glutamate and GABA were detected in this analysis, in agreement with the findings of Huang and coworkers. Arnold and others employed a transcriptomic approach to show that a number of genes were upregulated following exposure to acetate, including *gadA*, *gadB*, and *gadC*, the genes encoding the two glutamate decarboxylase isozymes involved in the acid resistance of *E. coli* O157:H7, and the amino acid antiporter, respectively (2001). This suggests that the glutamate-dependent acid resistance mechanism is active in the pH 3.2 samples as well as those at pH 3.2 with 40 mM acetic acid present. More recently, King and coworkers did not see any upregulation of any acid resistance mechanisms in O157:H7 except for the gene encoding the lysine-dependent system (*cadB*), in response to hydrochloric acid as the acidulant (2010). With acetic acid as the acidulant, they observed upregulation of three genes (*yojI*, a multidrug transporter membrane component and ATP-binding component; *yfdX*, which codes for a hypothetical protein; and *yqgA*, which encodes a predicted inner membrane transport protein) and three intergenic regions, and the fold-increase of these were similar to those observed in nonpathogenic *E. coli* K-12 (King 2010). As the present work did not detect any tentatively named metabolites corresponding to the genes mentioned by King and others, it is difficult to draw comparisons between the two studies. Riordan *et al.* studied acid resistance and virulence in *E. coli* O157:H7 Sakai and found that in RpoN mutants, stress resistance genes such as *gadA*, *gadB*, *gadC*, *gadE* (the acid-responsive regulator gene), and *gadX* (encoding the acid sensitivity protein) were expressed three to five or more times higher during exponential phase than in wild-type Sakai (2010). However, when the cells reached stationary phase, expression of

these genes in the mutant and wild-type O157 strains were not significantly different (Riordan 2010). The cell cultures in the present experiment had been grown to stationary phase and presumably possessed unaltered RpoN, so they can only fairly be compared to the findings for wild-type O157:H7 Sakai at stationary phase. However the Riordan study did show that *gadE* is necessary for the glutamate-dependent acid resistance system to work during exponential phase (2010).

4.4 Conclusions

The findings of this experiment support the current hypotheses for acid resistance in *E. coli* O157:H7 and suggest some other pathways that may be activated in response to the acid pH stress and organic acid stress presented at pH 3.2 in the presence of acetic acid. This work presented metabolomic evidence that the glutamate-dependent acid resistance mechanism was active in both the presence and absence of acetic acid. The results of this experiment also shed light on several other metabolites involved in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, the pentose phosphate pathway, the urea cycle and amino acid synthesis and breakdown, whose concentrations increased or decreased in response to the conditions to which *E. coli* O157:H7 were exposed, warranting further research into the significance of these biochemical changes. The GCxGC-ToF-MS metabolomics approach allowed the direct observation of molecular-level changes in *E. coli* O157:H7 which provided insight into the acid stress response unattainable by any other presently used analytical technique. Future work in this area should be directed toward delving deeper into the changes induced in the intracellular metabolite pool by these conditions, as well as investigating the effect of other organic acids commonly encountered in food environments. Clues from this study and others, as well as future work in this area, may highlight new or improved ways to prevent *E. coli* O157:H7 from surviving in food products and causing potentially deadly foodborne outbreaks.

4.5 Acknowledgements

The author thanks Frederick Breidt, Jr., Suzanne D. Johanningsmeier, Sophia Kathariou, and David C. Muddiman for helpful discussions of this work, D. Seth Fornea for analytical expertise and instrument maintenance, and Sandra Parker for excellent administrative assistance.

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Table 4.1 Metabolite peaks that decreased in response to acid stress ($p < 0.001$)

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Analyte 375	--	--	--	--	--	942	534.4	1.21	173
Analyte 925	--	--	--	--	--	1266	777.8	1.14	82
Analyte 1009	--	--	--	--	--	1320	840.4	0.99	55
2-Ketoisocaproic acid	816-66-0	MS	807	824	8087	1327	851.3	1.13	82
Succinic acid	110-15-6	MS, ST	834	916	6017	1401	967.1	1.25	75
Analyte 1250	--	--	--	--	--	1417	992.0	1.70	103
Analyte 1315	--	--	--	--	--	1454	1049.8	0.56	74
L-Aspartic acid	56-84-8	MS	788	831	8473	1554	1197.8	1.43	232
Analyte 1547	--	--	--	--	--	1600	1266.0	1.28	84
Analyte 1568	--	--	--	--	--	1618	1290.7	1.04	89
Analyte 1599	--	--	--	--	--	1644	1325.1	1.17	116
glutamic acid	56-86-0	MS, ST	688	879	9575	1658	1343.4	1.44	246
N-Acetylglutamic acid	1188-37-0	MS	761	888	9553	1658	1344.6	1.21	84
Analyte 1628	--	--	--	--	--	1668	1358.1	1.02	89
Analyte 9999	--	--	--	--	--	1675	1366.7	1.39	246
oxoproline	98-79-3	MS	668	897	7919	1679	1372.6	1.17	156
Analyte 1687	--	--	--	--	--	1724	1431.8	1.15	55
Analyte 1713	--	--	--	--	--	1747	1461.6	1.04	73
Analyte 1831	--	--	--	--	--	1823	1558.5	1.04	116
Analyte 1847	--	--	--	--	--	1828	1565.1	1.04	56
citric acid	5949-29-1	MS, ST	667	861	8859	1838	1576.8	1.51	273
gluconic acid 1	526-95-4	MS, ST	808	929	5817	1878	1625.3	2.08	103
Analyte 1982	--	--	--	--	--	1909	1664.1	1.74	156

Table 4.1 Continued

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Analyte 2082	--	--	--	--	--	1973	1740.0	1.44	82
Analyte 2227	--	--	--	--	--	2111	1664.1	1.74	156
Analyte 2258	--	--	--	--	--	2164	1740.0	1.44	82
Analyte 2512	--	--	--	--	--	2628	2332.9	1.34	75

Table 4.2 Metabolite peaks that increased in the pH 3.2 stressed *E. coli* O157:H7 cells ($p < 0.001$)

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Threonic acid	3909-12-4	MS	856	913	4169	1512	1137.8	1.65	147
Analyte 1530	--	--	--	--	--	1587	1246.3	1.51	103
Analyte 1578	--	--	--	--	--	1626	1300.0	1.48	112
Analyte 1601	--	--	--	--	--	1645	1326.2	1.65	147
Analyte 1704	--	--	--	--	--	1737	1448.4	1.41	159
Analyte 1869	--	--	--	--	--	1853	1594.3	1.84	103

Table 4.3 Metabolite peaks that decreased in pH 3.2 acid-stressed *E. coli* cells only in the presence of 40 mM acetic acid ($p < 0.001$)

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Glycine	56-40-6	MS	822	873	9413	814	448.2	1.02	118
L-Alanine	56-41-7	MS	882	896	7323	929	521.9	1.34	116
L-Alanine	56-41-7	MS	786	854	5941	949	541.9	1.31	116
2-hydroxypentanoic acid	109-52-4	MS	802	882	4015	1031	608.9	1.41	145
Analyte 795	--	--	--	--	--	1169	694.3	1.34	174
2-hydroxy-4-methylpentanoic acid	646-07-1	MS	756	808	4389	1215	729.3	1.43	103
4-hydroxybutanoic acid	107-92-6	MS	791	863	5667	1266	777.5	1.34	147
L-Isoleucine	73-32-5	MS, ST	800	862	5946	1295	803.9	1.53	158
2-pyrrolidinone	616-45-5	MS	815	837	9025	1315	832.4	1.05	142
Analyte 1051	--	--	--	--	--	1338	864.0	1.74	117
L-Proline	147-85-3	MS	801	885	9651	1338	868.5	1.42	142
Analyte 9998	--	--	--	--	--	1348	884.4	1.38	102
L-threonine	72-19-5	MS	810	838	8767	1375	926.0	1.59	57
Analyte 1150	--	--	--	--	--	1378	932.9	0.55	170
L-Malic acid	97-67-6	MS	616	877	6786	1523	1154.1	1.41	55
Analyte 9997	--	--	--	--	--	1529	1161.4	1.62	174
Analyte 1453	--	--	--	--	--	1530	1162.5	1.87	103
Analyte 1528	--	--	--	--	--	1583	1241.1	0.52	79
2-Hydroxyglutaric acid	2889-31-8	MS	818	908	9558	1619	1291.8	1.39	129
β-Phenyllactic acid	501-97-3	MS	810	842	6910	1681	1374.3	1.31	193
Proline	147-85-3	MS	783	908	9554	1741	1454.0	1.17	142
Analyte 2065	--	--	--	--	--	1962	1726.4	1.14	71
β-(4-hydroxyphenyl)lactic acid	501-97-3	MS	777	874	7679	1966	1731.8	1.42	179

Table 4.3 Continued

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Analyte 2101	--	--	--	--	--	1993	1763.5	1.18	173
L-Tyrosine	60-18-4	MS	783	872	7189	1999	1770.0	1.46	218
Analyte 2179	--	--	--	--	--	2064	1844.5	1.14	158
Analyte 2619	--	--	--	--	--	2796	2408.3	1.07	258

Table 4.4 Metabolite peaks that increased due to acid stress ($p < 0.001$)

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Analyte 1206	--	--	--	--	--	1400	966.2	1.25	247
4-aminobutanoic acid	56-12-2	MS, ST	836	852	8415	1532	1165.8	1.60	174
14-acetoxy-3,6,9,12-tetraoxatetradecan-1-oate	N/A	MS	663	764	1567	1725	1432.6	1.16	59
Analyte 1767	--	--	--	--	--	1777	1498.7	2.09	70
mannose 2	3458-28-4	MS	748	909	2748	1823	1557.0	1.95	160
Analyte 1881	--	--	--	--	--	1866	1610.8	1.39	147
Analyte 2066	--	--	--	--	--	1962	1726.3	1.56	73
Analyte 2492	--	--	--	--	--	2597	2317.6	1.08	73

Table 4.5 Metabolite peaks that increased in pH 3.2 acid-stressed *E. coli* cells only in the presence of 40 mM acetic acid ($p < 0.001$)

Metabolite ID	CAS Registry #	Method of Identification	Similarity	Reverse	Probability	RI	t_{r1} (s)	t_{r2} (s)	Mass
Analyte 754	--	--	--	--	--	1150	681.3	1.52	152
Analyte 1848	--	--	--	--	--	1831	1567.1	2.15	73
Tetradecanoic acid	544-63-8	MS	817	841	7920	1896	1648.7	1.52	132
L-Gluconic acid lactone	133-42-6	MS	817	848	2560	1902	1655.5	1.72	148
Gluconic lactone 2	90-80-2	MS	802	826	3995	1904	1660.8	1.85	111
Analyte 1994	--	--	--	--	--	1912	1667.7	1.11	192
Analyte 2043	--	--	--	--	--	1941	1701.5	1.70	73
Analyte 2050	--	--	--	--	--	1950	1712.6	1.58	205
Analyte 2090	--	--	--	--	--	1983	1751.0	1.63	43
D(-)-Galactono-1,4-lactone	2782-07-2	MS	746	825	1454	1988	1756.7	1.58	217
Analyte 2132	--	--	--	--	--	2009	1781.3	1.78	43
Analyte 2163	--	--	--	--	--	2036	1812.4	1.75	73
Analyte 2164	--	--	--	--	--	2042	1820.1	0.44	75
Analyte 2171	--	--	--	--	--	2048	1825.3	1.36	43
Analyte 2180	--	--	--	--	--	2059	1838.7	1.31	43
Analyte 2181	--	--	--	--	--	2060	1839.0	1.69	73
Analyte 2256	--	--	--	--	--	2158	1946.4	1.55	73
Analyte 2326	--	--	--	--	--	2265	2058.8	2.08	73
Analyte 2582	--	--	--	--	--	2738	2384.4	1.00	73
EITTMS_N12C_ATHL_3452.3_1135EC24_G	N/A	MS	786	857	968	3177	2589.8	2.55	73

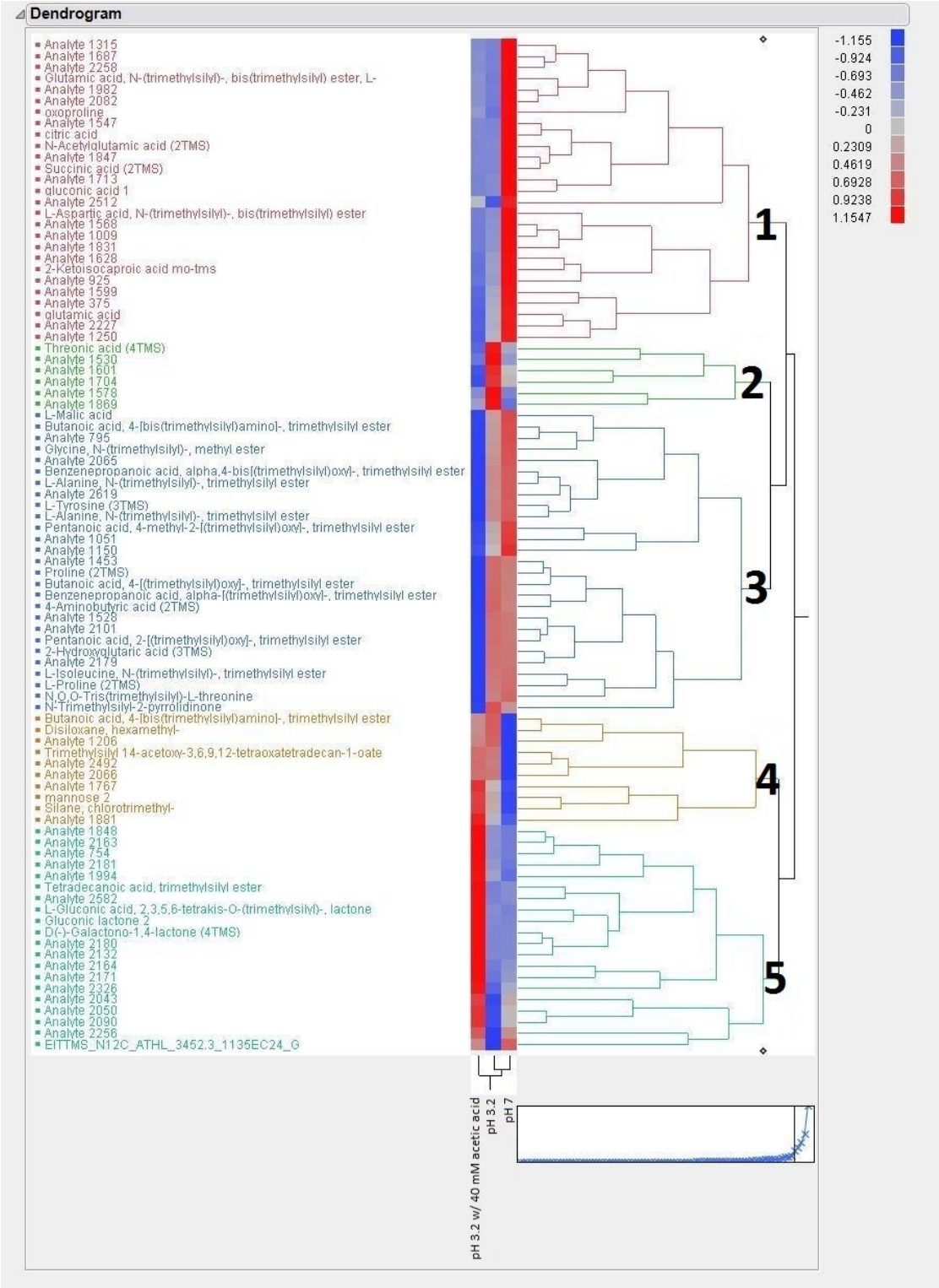


Figure 4.1 Hierarchical clustering analysis of peaks which were significantly different among acid stress treatments ($p < 0.001$)

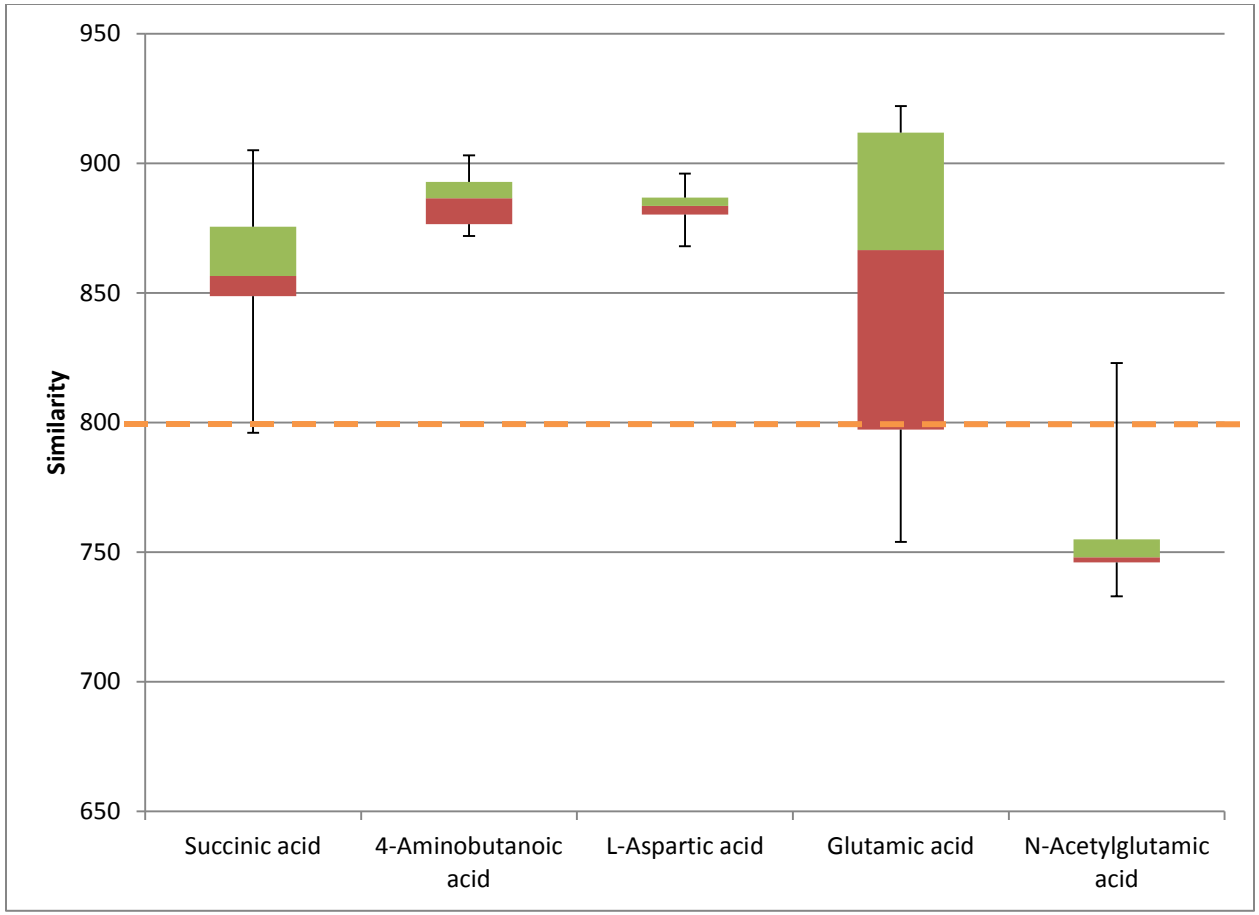
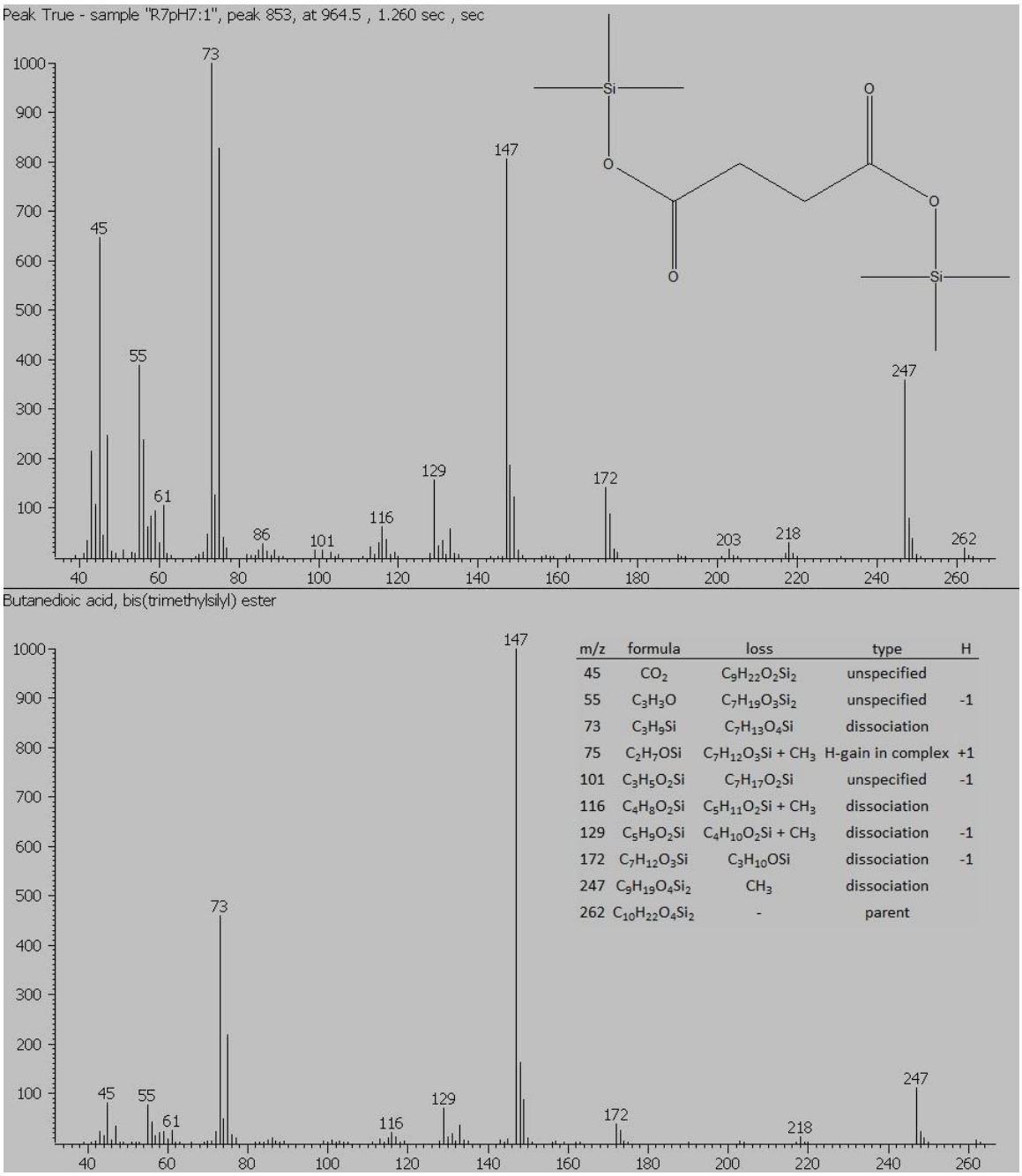


Figure 4.2 Average library similarity of all aligned peaks for landmark peaks. A similarity value of 800 is required before assigning a name to the peak. The lighter green color indicates the upper quartile, and the darker red color indicates the lower quartile.



m/z	formula	loss	type	H
45	CO_2	$C_9H_{22}O_2Si_2$	unspecified	
55	C_3H_3O	$C_7H_{19}O_3Si_2$	unspecified	-1
73	C_3H_9Si	$C_7H_{13}O_4Si$	dissociation	
75	C_2H_7OSi	$C_7H_{12}O_3Si + CH_3$	H-gain in complex	+1
101	$C_3H_5O_2Si$	$C_7H_{17}O_2Si$	unspecified	-1
116	$C_4H_8O_2Si$	$C_5H_{11}O_2Si + CH_3$	dissociation	
129	$C_5H_9O_2Si$	$C_4H_{10}O_2Si + CH_3$	dissociation	-1
172	$C_7H_{12}O_3Si$	$C_3H_{10}OSi$	dissociation	-1
247	$C_9H_{19}O_4Si_2$	CH_3	dissociation	
262	$C_{10}H_{22}O_4Si_2$	-	parent	

Figure 4.3a Mass spectrum of succinic acid (2TMS)

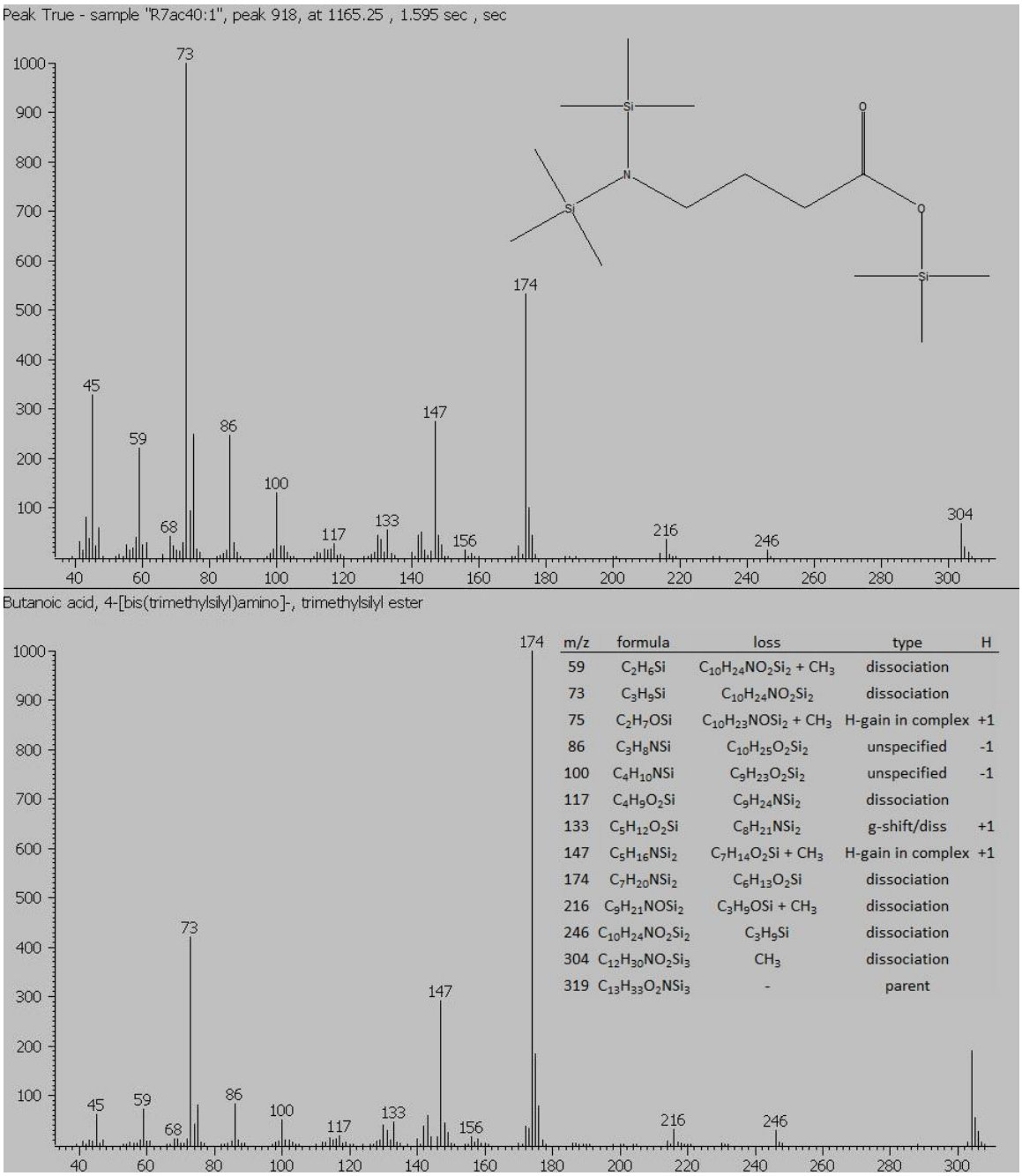


Figure 4.3b Mass spectrum of 4-aminobutanoic acid (3TMS)

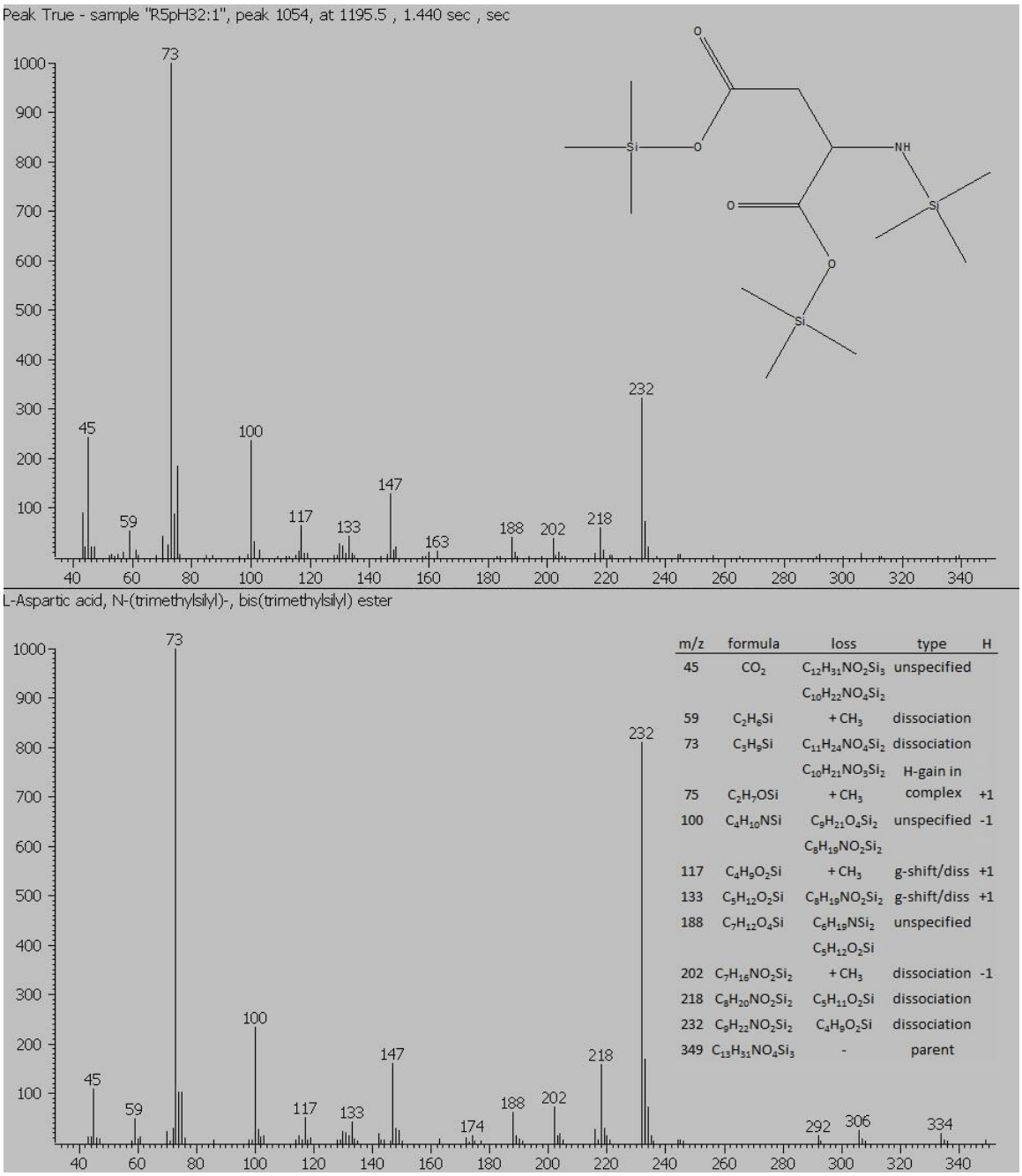


Figure 4.3c Mass spectrum of L-aspartic acid (3TMS)

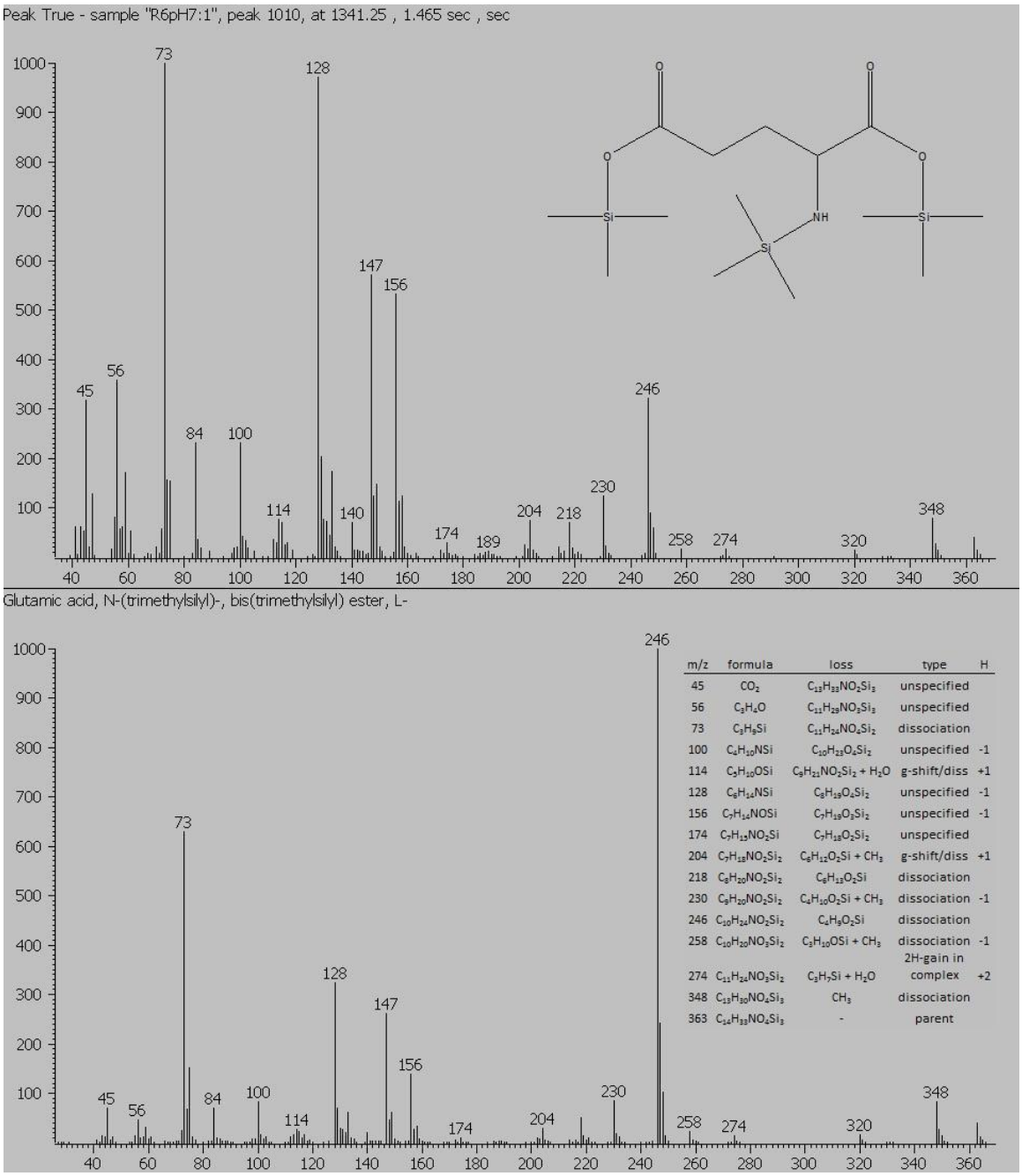
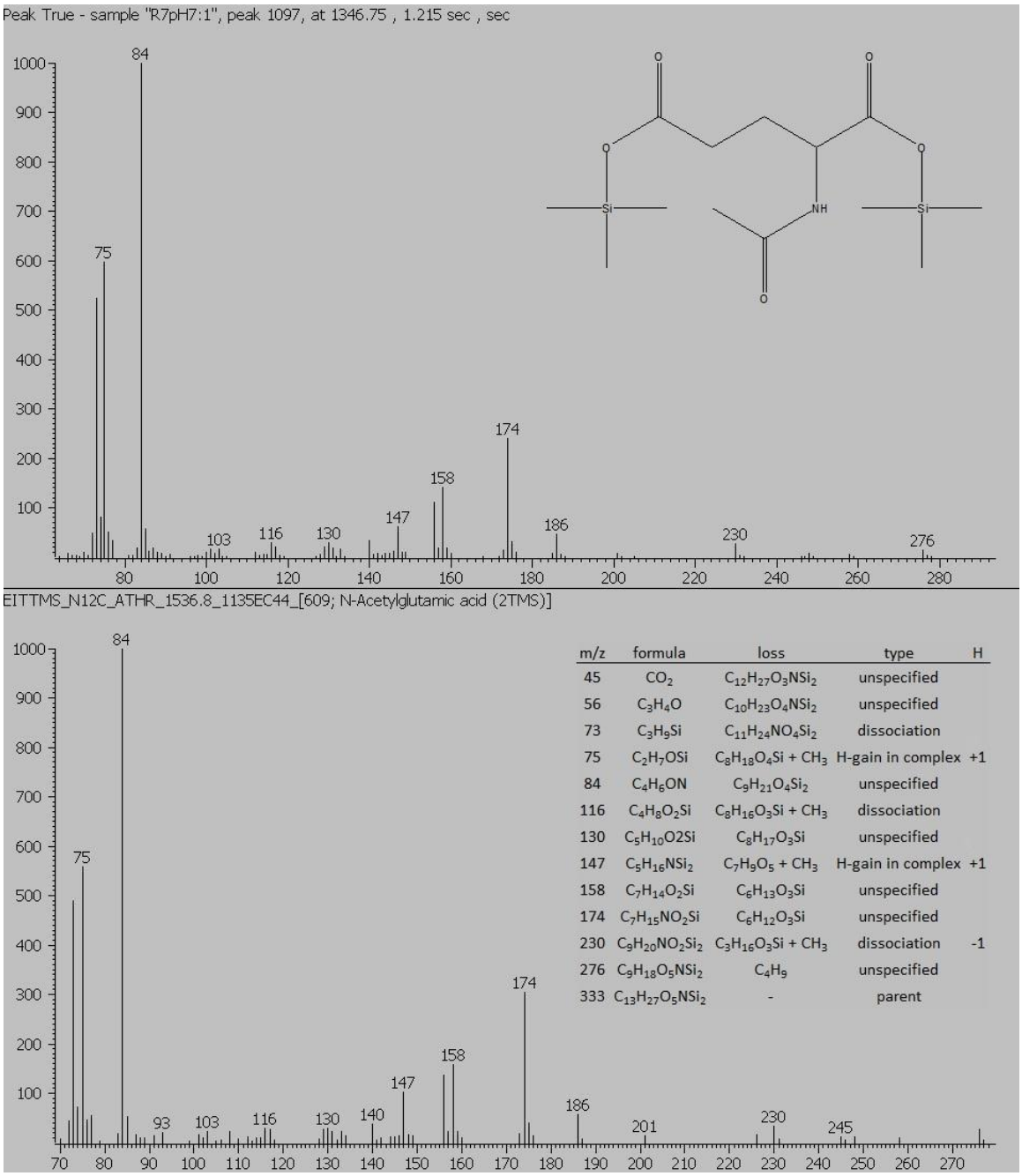


Figure 4.3d Mass spectrum of glutamic acid (3TMS)



m/z	formula	loss	type	H
45	CO_2	$C_{12}H_{27}O_3NSi_2$	unspecified	
56	C_3H_4O	$C_{10}H_{23}O_4NSi_2$	unspecified	
73	C_3H_9Si	$C_{11}H_{24}NO_4Si_2$	dissociation	
75	C_2H_7OSi	$C_8H_{18}O_4Si + CH_3$	H-gain in complex	+1
84	C_4H_6ON	$C_9H_{21}O_4Si_2$	unspecified	
116	$C_4H_8O_2Si$	$C_8H_{16}O_3Si + CH_3$	dissociation	
130	$C_5H_{10}O2Si$	$C_8H_{17}O_3Si$	unspecified	
147	$C_5H_{16}NSi_2$	$C_7H_9O_5 + CH_3$	H-gain in complex	+1
158	$C_7H_{14}O_2Si$	$C_6H_{13}O_3Si$	unspecified	
174	$C_7H_{15}NO_2Si$	$C_6H_{12}O_3Si$	unspecified	
230	$C_9H_{20}NO_2Si_2$	$C_3H_{16}O_3Si + CH_3$	dissociation	-1
276	$C_9H_{18}O_5NSi_2$	C_4H_9	unspecified	
333	$C_{13}H_{27}O_5NSi_2$	-	parent	

Figure 4.3e Mass spectrum of N-acetylglutamic acid (2TMS)

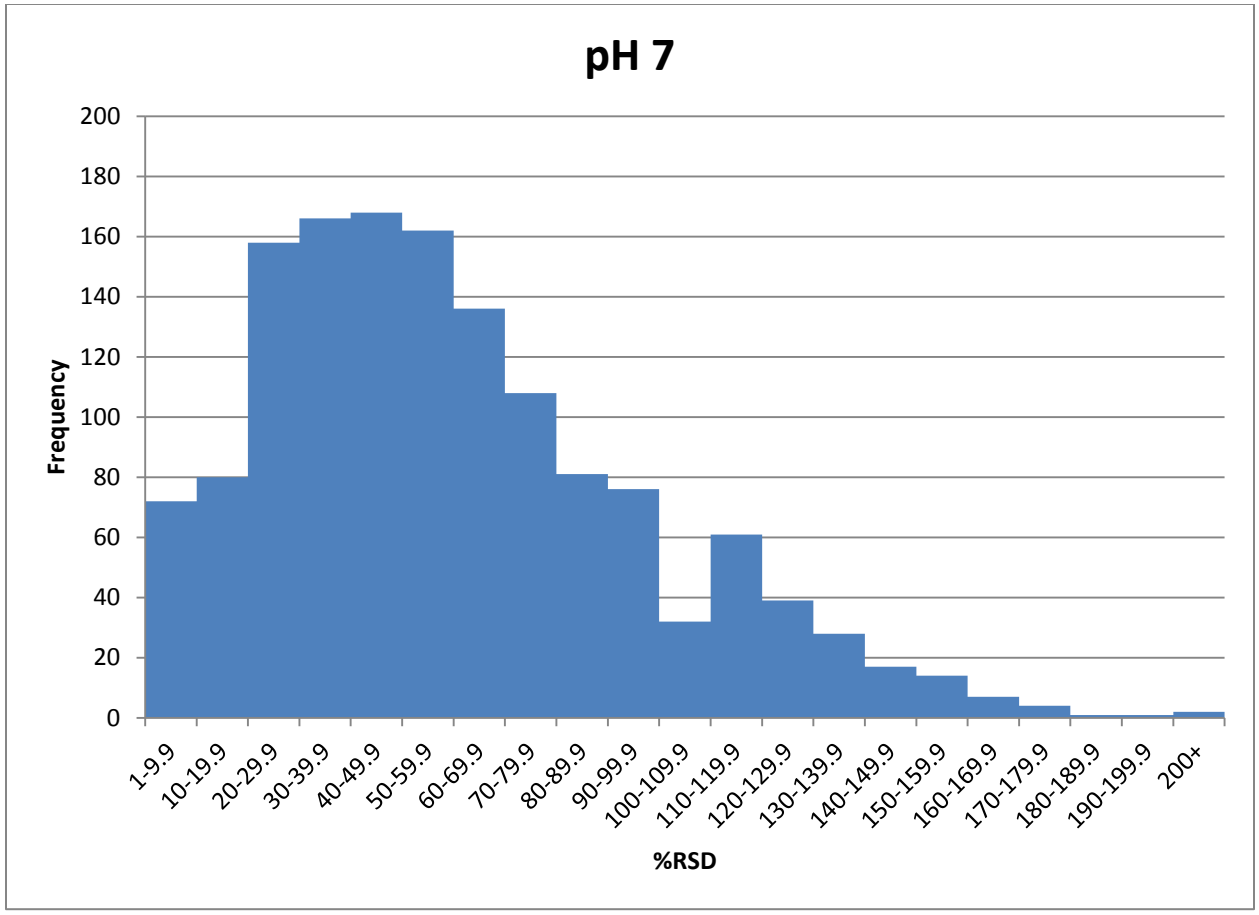


Figure 4.4a Histogram showing %RSD of peak areas sorted by pH 7 treatment.

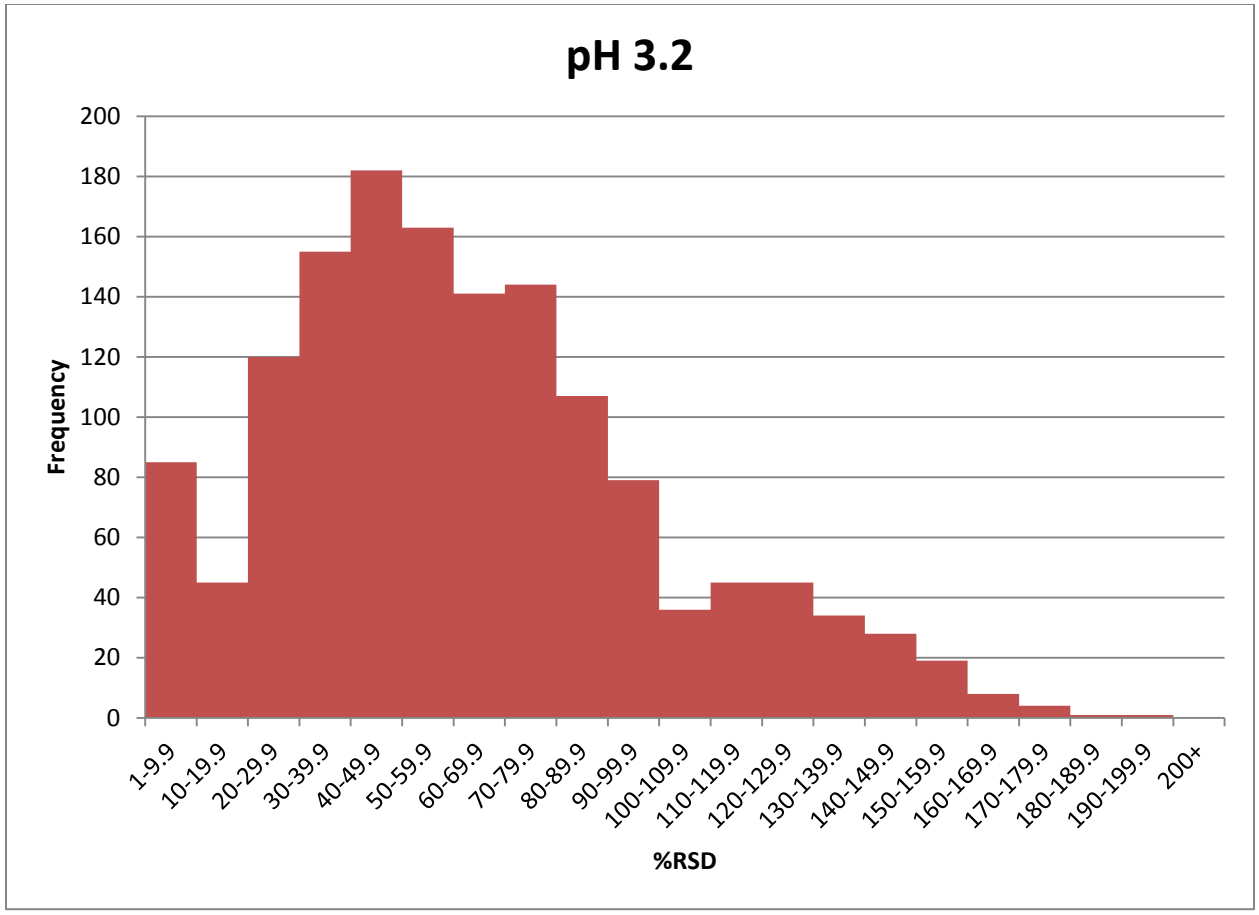


Figure 4.4b Histogram showing %RSD of peak areas sorted by pH 3.2 treatment.

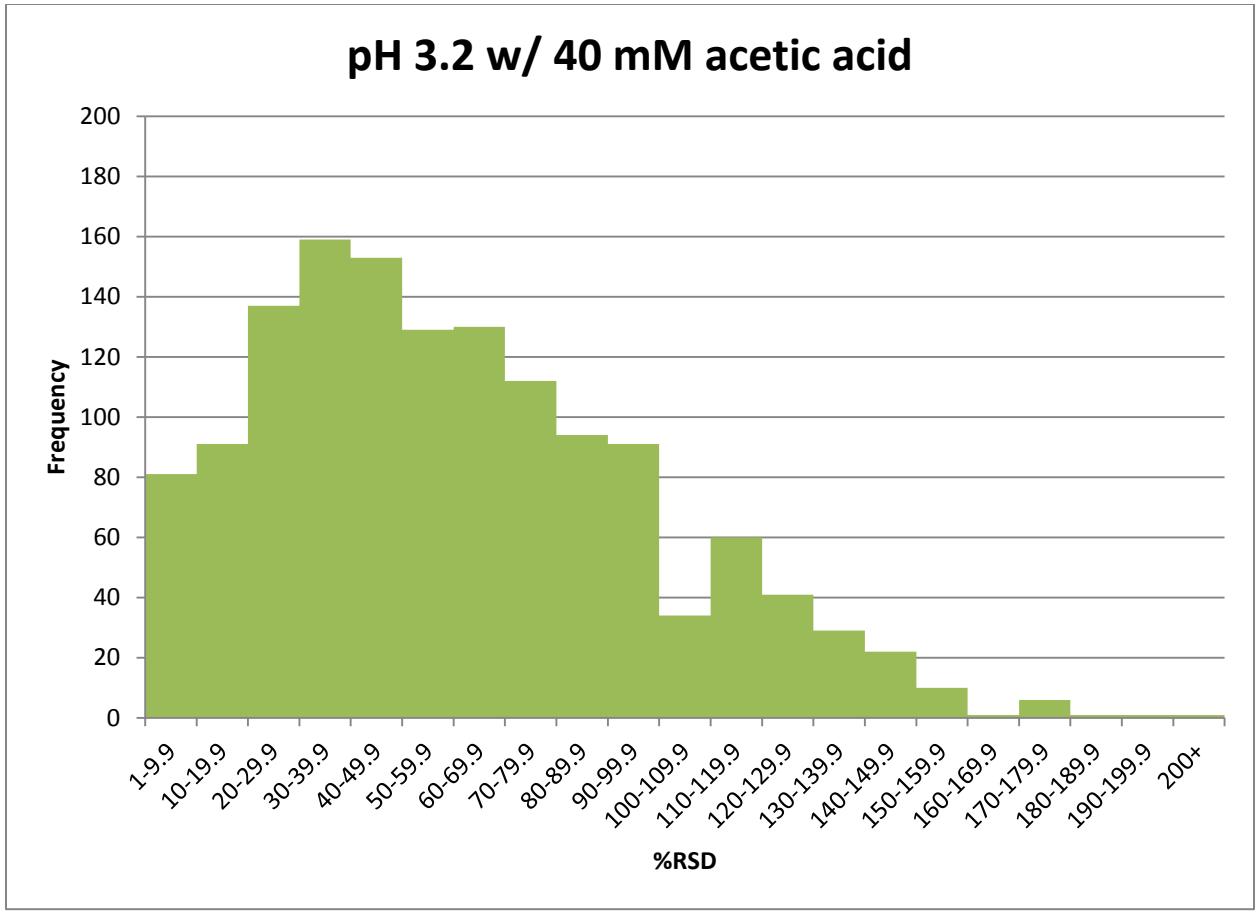


Figure 4.4c Histogram showing %RSD of peak areas sorted by pH 3.2 treatment with 40 mM acetic acid.

Table 4.6 Summary of biological significance of tentatively identified metabolites that changed due to acid stress.

Cluster #	Metabolite ID	pH 7 Peak Area	pH 3.2 Peak Area	Acetic Acid Peak Area	Common Names	Biochemical Significance
1	2-Ketoisocaproic acid	10988 ± 2987	24401 ± 0	N / A	α-ketoisocaproic acid	intermediate in leucine metabolism
1	Succinic acid	1557060 ± 315610	N / A	N / A	succinate	TCA cycle intermediate
1	L-Aspartic acid	114587 ± 28778	13596 ± 3903	10541 ± 1239	aspartate	proteinogenic amino acid; converted to oxaloacetate (TCA cycle) or arginosuccinate (urea cycle)
1	glutamic acid (confirmed)	490277 ± 358958	40866 ± 10959	10942 ± 1922	glutamate	proteinogenic amino acid; GAD acid resistance mechanism; converted to α-ketoglutarate (TCA cycle) or ornithine (urea cycle)
1	N-Acetylglutamic acid	265212 ± 333975	N / A	N / A	N-acetylglutamate	activates urea cycle enzyme CPS-1; converted to ornithine (urea cycle)
1	oxoproline	3262593 ± 639954	N / A	354506 ± 0	pyroglutamic acid, pidolic acid	lactam form of glutamic acid
1	citric acid	441512 ± 287848	54006 ± 68837	50846 ± 45659	citrate	TCA cycle intermediate
1	gluconic acid 1	897595 ± 383941	N / A	N / A	gluconic acid	non-inhibitory buffer; pentose phosphate pathway
2	Threonic acid	43398 ± 7128	62742 ± 8985	28521 ± 5361	threonic acid	sugar acid; derivative of ascorbic acid
3	Glycine	27266 ± 3097	17958 ± 4739	7012 ± 2950	glycine	proteinogenic amino acid; degraded to pyruvate
3	L-Alanine	3173043 ± 772754	2260666 ± 1027538	497468 ± 140812	alanine	proteinogenic amino acid; synthesized from pyruvate (TCA cycle, gluconeogenesis)
3	L-Alanine	26964 ± 10213	21458 ± 12023	N / A	alanine	proteinogenic amino acid; synthesized from pyruvate (TCA cycle, gluconeogenesis)
3	2-hydroxypentanoic acid	27459 ± 10508	30493 ± 14605	N / A	valeric acid	structurally similar to GABA
3	2-hydroxy-4-methylpentanoic acid	78983 ± 14645	28982 ± 22943	N / A	isocaproic acid	leucine, isoleucine, and valine biosynthesis

Table 4.6 Continued

Cluster #	Metabolite ID	pH 7 Peak Area	pH 3.2 Peak Area	Acetic Acid Peak Area	Common Names	Biochemical Significance
3	4-hydroxybutanoic acid	21339 ± 6767	29997 ± 13544	12388 ± 3145	γ-hydroxybutyrate (GHB)	converted to GABA or succinic acid
3	L-Isoleucine	148566 ± 75129	139450 ± 107686	148566 ± 75129	isoleucine	proteinogenic amino acid; synthesized from pyruvate (TCA cycle, gluconeogenesis)
3	2-pyrrolidinone	1055052 ± 427494	1362698 ± 672189	240440 ± 126252	2-pyrrolidinone	lactam
3	L-Proline	1042502 ± 306259	926219 ± 585063	76502 ± 63813	proline	proteinogenic amino acid; synthesized from glutamate
3	L-threonine	101735 ± 22526	73370 ± 22348	10985 ± 5222	threonine	proteinogenic amino acid; synthesized from aspartic acid; converted to pyruvate
3	L-Malic acid	239430 ± 84128	42598 ± 19134	11645 ± 10261	malate	TCA cycle intermediate
3	2-Hydroxyglutaric acid	34703 ± 13568	41626 ± 46266	N / A	α-hydroxyglutarate	converted to α-ketoglutarate (TCA cycle intermediate)
3	β-Phenyllactic acid	14342 ± 3464	26557 ± 11550	N / A	β-phenyllactic acid	structurally similar to tyrosine
3	Proline	206483 ± 106751	169574 ± 67229	16868 ± 2569	proline	proteinogenic amino acid; synthesized from glutamate
3	β-(4-hydroxyphenyl)lactic acid	34617 ± 6887	27357 ± 11849	N / A	β-(4-hydroxyphenyl)lactic acid	structurally similar to tyrosine
3	L-Tyrosine	33927 ± 15359	22825 ± 8616	N / A	tyrosine	proteinogenic amino acid; degraded to fumarate (TCA cycle intermediate)

Table 4.6 Continued

Cluster #	Metabolite ID	pH 7 Peak Area	pH 3.2 Peak Area	Acetic Acid Peak Area	Common Names	Biochemical Significance
4	4-aminobutanoic acid	1232330 ± 471367	1211234 ± 638935	229224 ± 79837	GABA	synthesized from glutamate; GAD acid resistance mechanism; converted to succinate (TCA cycle) or ornithine (urea cycle)
4	14-acetoxy-3,6,9,12-tetraoxatetradecan-1-oate	N / A	60598 ± 34763	77479 ± 15952	N/A	unknown
4	mannose 2	27780 ± 3314	170400 ± 58896	438593 ± 79940	mannose	C2 epimer of glucose (glycolysis); sugar metabolism
5	Tetradecanoic acid	N / A	N / A	185943 ± 108264	myristic acid	cell membrane component
5	L-Gluconic acid lactone	N / A	N / A	154734 ± 45930	gluconic acid δ-lactone	non-inhibitory buffer; pentose phosphate pathway
5	Gluconic lactone 2	N / A	N / A	14565 ± 8292	gluconic acid δ-lactone	non-inhibitory buffer; pentose phosphate pathway
5	D(-)-Galactono-1,4-lactone	14067 ± 2375	12050 ± 2067	78877 ± 16681	galactonic acid γ-lactone	non-inhibitory buffer; ascorbate metabolism

APPENDIX

APPENDIX ONE

Development of a Global Internal Standard for Microbial Metabolomics

using Uniformly ^{13}C-Labeled D-glucose

Abstract

A protocol for the use of ^{13}C-labeled extracts of *Escherichia coli* O157:H7 as a global internal standard was developed for the purpose of using this internal standard to help quantify the error present in the biological, technical, and analytical aspects of metabolomics experiments. *E. coli* was grown in minimal medium for one or two periods of overnight growth lasting between 15 and 18 hours. The growth medium contained either ^{12}C D-glucose or ^{13}C D-glucose as the carbon source. Cells were then collected and lysed for two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToF-MS) analysis of the ^{12}C and ^{13}C labeled cell extracts, as well as an equal mixture of ^{12}C and ^{13}C extracts. The results demonstrated that ^{13}C labeling of *E. coli* metabolites was taking place to different degrees varying from 77 % to 98 %. These differences were dependent on the identity of the metabolite, the number of carbon atoms present in the metabolite for labeling, and the duration of incubation in the isotope-rich growth medium. However, labeling was not uniform enough or complete enough to justify the use of this methodology for its intended purpose as a global internal standard for microbial metabolomics.

A1.1 Introduction

The field of metabolomics employs a systems biology approach to identifying the biochemical fingerprint left behind by the reactions of metabolism. This fingerprint provides clues to the state of the organism. The metabolite pools of living things as large as humans or plants and as small as single-celled organisms like bacteria and yeasts have been studied (Adahchour *et al.*, 2006; Barsch *et al.*, 2003; Castrillo *et al.*, 2008; Coucheney *et al.*, 2008; Fiehn *et al.*, 2000; Jozefczuk *et al.*, 2010; Koek *et al.*, 2006; Lisec *et al.*, 2006; Maharjan and Ferenci, 2003; Park *et al.*, 2005; Pasikanti *et al.*, 2008; Plassmeier *et al.*, 2007; Roessner *et al.*, 2000; Rudell *et al.*, 2008; Szeto *et al.*, 2011; Villas-Bôas *et al.*, 2005). A key aspect of this approach is that it must be non-targeted in nature, such that the technique used to isolate, identify, or quantify the metabolites does not bias their detection.

There are several commonly-used techniques that are generally accepted as platforms for non-targeted metabolite analysis. These include nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and various adaptations of mass spectroscopy including tandem techniques. The value of each of these platforms has been demonstrated in a wide variety of metabolomics studies (Adahchour *et al.*, 2006; Barsch *et al.*, 2003; Castrillo *et al.*, 2008; Coucheney *et al.*, 2008; Fiehn *et al.*, 2000; Koek *et al.*, 2006; Lee *et al.*, 2010; Lisec *et al.*, 2006; Mashego *et al.*, 2007; Pasikanti *et al.*, 2008; Plassmeier *et al.*, 2007; Rabinowitz and Kimball, 2007; Roessner *et al.*, 2000; Rudell *et al.*, 2008; Smilde *et al.*, 2009; Szeto *et al.*, 2011; Villas-Bôas *et al.*, 2005, Williamson and Bartlett, 2007; Yang *et al.*, 2010). One analytical technique with notable contributions to the field is two-dimensional gas chromatography-time-of-flight mass spectrometry, or GCxGC-ToF-MS (Adahchour *et al.*, 2006). This two-dimensional approach offers much higher resolution of analytes, which, when coupled with a highly sensitive detector such as time-of-flight, allows for the analysis of complex, dilute metabolite

pools which may be more difficult or even impossible by less capable instrumentation (Adahchour *et al.*, 2006).

In metabolomics, variability can come from biological variation among organisms, the sample preparation steps, or the analytical method used. In order to minimize the variability, it is useful to have some measurement by which to gauge the current magnitude of the experimental error, and the amount by which it improves when modifications are made to the experimental protocol.

Naturally-existing isotopes can be used to label molecules for many purposes, such as following chemicals through a metabolic pathway or distinguishing molecular fragments in a mass spectrum. Carbon-13 is one such naturally occurring isotope; however, its prevalence is much lower than the more abundant carbon-12. Therefore, labeling of intracellular metabolites with carbon-13 atoms typically begins with a uniformly ^{13}C labeled substance that is provided to the cells as the sole carbon source. The cells metabolize the labeled molecules, and any metabolites downstream from that energy source thus incorporate the heavier labeled atoms into their structure. The labeled metabolites will be located on the two-dimensional gas chromatogram at the same first and second dimension retention times as the unlabeled counterparts, but a closer look at the mass spectrum for that chromatographic peak will have the same mass fragmentation pattern, shifted in m/z by the number of carbon atoms which were successfully labeled with the ^{13}C isotope.

The purpose of this experiment was to determine the labeling efficiency of the internal metabolites of *E. coli* O157:H7 grown on uniformly labeled ^{13}C-glucose. This was done in order to evaluate its utility as a quantifier, so that the various sources of error present in the current experimental protocol could be identified.

A1.2 Materials and Methods

Chemicals

D-glucose, sodium chloride, ribitol, methoxyamine hydrochloride, magnesium sulfate, sodium phosphate monobasic, and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide and phenyl glucoside were purchased from Aldrich (Milwaukee, WI, USA). Hydrochloric acid, methanol, and Luria-Bertani agar were purchased from Fisher (Fair Lawn, NJ, USA). D4-alanine, ^{13}C-proline, and ^{13}C-salicylic acid were purchased from Icon Isotopes (Summit, NJ, USA). Pyridine and BSTFA were purchased from Thermo Scientific (Rockford, IL, USA). Alkanes C8-C20, alkanes C21-C40, and ammonium chloride were purchased from Fluka (St. Louis, MO, USA). U-^{13}C-D-glucose was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Fatty acid methyl ester reference standard GLC-409 was purchased from Nu-Chek Prep (Elysian, MN, USA). Liquid nitrogen was purchased from Airgas National Welders (Charlotte, NC, USA).

Media Preparation

M9-GT media was prepared according to recipe for M9 media (Sambrook *et al.*, 1989), but supplemented to 1 % total glucose and 0.005 % thiamine in media. One volume of media was prepared with carbon-12 glucose and another volume of media with carbon-13 glucose. Cells did not grow well beyond one overnight without the addition of a small amount of unlabeled thiamine to the medium (unpublished data).

Cell Culture

Escherichia coli O157:H7 strain B241 (28RC1), a bovine isolate obtained from the USDA-ARS Food Science Research Unit culture collection, was chosen for its acid resistance (Oh *et al.* 2009). A Luria-Bertani (LB) agar plate was streaked from the freezer stock and incubated overnight at 37 °C.

Unique colonies were picked off the plate and the cells were inoculated into M9-^{12}GT in triplicate and incubated at 37 °C statically for 15 hours. An aliquot of each replicate was transferred into both fresh M9-^{12}GT and also M9-^{13}GT and incubated at 37 °C statically for 15 hours. A small volume of each portion of cells was collected on a sterile 0.45 micron 25mm diameter polyethersulfone membrane filter (Sterlitech Corporation, Kent, WA, USA) and rinsed twice with 2.6 % NaCl (Bolten *et al.*, 2007). The filter was then aseptically transferred to a microfuge tube and dropped in liquid nitrogen to quench metabolism.

Each replicate of ^{12}C-grown cells was mixed with an equal number of ^{13}C-grown cells based on optical density measurements taken at 600 nm. A small volume of each portion of cell mixture was collected on a sterile 0.45 micron 25mm diameter polyethersulfone membrane filter and rinsed twice with 2.6 % NaCl (Bolten *et al.*, 2007). The filter was then aseptically transferred to a microfuge tube and dropped in liquid nitrogen to quench metabolism.

E. coli O157:H7 cells were also inoculated into M9-^{13}GT in triplicate and incubated at 37 °C statically for 18 hours. Cells were centrifuged and resuspended in sterile saline at ten-fold concentration. A small volume of each portion of cell mixture was collected on a sterile 0.45 micron 25 mm diameter polyethersulfone membrane filter and rinsed twice with 2.6 % NaCl (Bolten *et al.*, 2007). The filter was then aseptically transferred to a microfuge tube and dropped in liquid nitrogen to quench metabolism. A small aliquot of each of the concentrated cell cultures was transferred into fresh M9-^{13}GT media and incubated at 37 °C statically for 18 hours. After growth, cells were centrifuged and resuspended in sterile saline at ten-fold concentration. A small volume of each portion of cell mixture was collected on a sterile 0.45 micron 25mm diameter polyethersulfone membrane filter and rinsed twice with 2.6 % NaCl (Bolten *et al.*, 2007). The filter was then aseptically transferred to a microfuge tube and dropped in liquid nitrogen to quench metabolism.

Cells were plated on LB agar to obtain cell counts in CFU/mL and the optical density was recorded at 600 nm at each stage.

Sample Preparation

Microfuge tubes containing the filters with cells were retrieved from the -80 °C freezer. A solution of 60 % methanol chilled to -20 °C was added to cover the frozen filter in each tube. Tubes were mixed for 25 min at 70 °C and 300 rpm. An aliquot of each cooled tube was transferred into new microfuge tubes. Internal standard solution was added to each and vortexed. Samples were dried in a SpeedVac SVC 100 (Savant Instruments, Hicksville, NY, USA) on medium heat for 3 hours. A methoximation solution containing 20 mg/mL methoxyamine HCl in pyridine was added to each tube and mixed for 90 min at 70 °C and 300 rpm. An equal volume of BSTFA was added to each tube and mixed for 30 min at 70 °C and 300 rpm. The tubes were immediately placed into ice and an alkane series was prepared containing the methoximation solution, BSTFA, the fatty acid methyl ester (FAME) reference standard GLC-409, alkanes C8-20, and alkanes C21-40. Derivatization blanks were also prepared containing the methoximation solution and BSTFA. FAME was also added to each of the samples cooling in ice. All samples, blanks, and the alkane series were centrifuged under refrigeration for 8 min at 13000rpm. A sufficient volume for injection was transferred from each tube into GC vials. Sample order was randomized and a hexane blank was injected after every ^{12}C or ^{13}C injection to test for the possibility of carryover.

GCxGC-ToF-MS Method

Qualitative analyses used two-dimensional GC with time-of-flight mass spectrometry. GCxGC-ToF-MS analysis was carried out according to Johanningsmeier (2011) using an Agilent 6890 (Agilent Technologies, Santa Clara, CA, USA) equipped with a 28.45 m BPX50 column (SGE Analytical Science, Austin, TX, USA) in the first dimension and a 1.0 m BPX5 column (SGE Analytical Science) in

the second dimension. The temperature of the injection port was 260 °C. The oven temperature program was 85 °C for four minutes, then from 85 to 245 °C at 5 °C/min then heating at 15 °C/min to 305 °C, and finally holding at 305 °C for 15 minutes. Helium was used as the carrier gas at a flow rate of 1.00 mL/min. The temperatures of the transfer line and the ion source were 280 and 250 °C, respectively. The detector was a Pegasus 4 time-of-flight detector (LECO Corporation, St. Joseph, MI, USA). Mass spectra were acquired over the mass range of 35-800 amu at an ionization energy of -70 Volts. Identification of individual components was done using the LECO-Fiehn Rtx5 library. The alkane series was used to create a retention index method based on the retention times of alkanes C8-C40. ChromaTOF® (LECO Corporation) was used to determine the baseline, find peaks, search the library, calculate heights and areas, and calculate retention indices.

Data Analysis

Chromatograms of the first set of ^{12}C cells were analyzed for identification of standard compounds based upon retention index and mass-to-charge ratio. The ^{13}C cell chromatograms were also analyzed for peaks with mass-to-charge ratios shifted by the number of carbons in the standard molecule. Labeling efficiency was calculated by dividing the ^{13}C abundance by the total ^{12}C and ^{13}C abundance. The chromatograms of mixed ^{12}C and ^{13}C cells were also analyzed for the presence of a doublet of masses of equal intensity at the expected mass-to-charge ratio based on the number of carbons in the molecule. Labeling efficiency was calculated for all of the tentatively identified standard compounds.

The second set of chromatograms was analyzed for peaks with mass-to-charge ratio shifted by the number of carbons in the standard molecules. Average labeling efficiencies and standard deviations were calculated for the standard compounds which were tentatively identified. Labeling efficiency was graphed versus the number of overnights.

A1.3 Results and Discussion

Isotopic labeling of metabolites has been accomplished before with some success. A study by Bennett *et al.* demonstrated the utility of carbon-13 labeled molecules as a means to quantify the concentrations of intracellular metabolites in metabolomics studies (2008). The protocol employed therein used different equipment and apparati, as well as a different method, to grow, harvest, and analyze the cells. For example, the authors of that paper grew their cells on a filter on top of the isotope-containing agar rather than growth in isotope-containing broth culture followed by rapid filtration. Additionally, a different extraction solution was utilized and the metabolite extracts were analyzed with HPLC-MS/MS rather than GCxGC-ToF-MS. Furthermore, the lysing and quenching procedures also differed in terms of the chemicals, temperatures, and times involved. Rather than adopting an already published method, the author decided to develop a different one more suited to its specific application. Using the Bennett paper as a guideline, this work presents modifications to their methodology which allowed the authors to perform an experiment under similar conditions and obtain comparable results.

This experiment demonstrated that the internal metabolites of *E. coli* O157:H7 can successfully be labeled with carbon-13 isotopes using this method. The chromatograms obtained showed that analytes eluted at the same retention indices and retention times whether or not they contained carbon-13 atoms (see Figures A1.1a and A1.1b for an example). The analytes were distinguishable not through their retention on the column, but by the shift in mass-to-charge ratio plotted on the corresponding mass spectra. Figure A1.2 shows that samples comprised of an equal mixture of ^{12}C- and ^{13}C-containing analytes exhibited “doublets” of roughly equal intensity at the m/z ratios expected for labeled and unlabeled pyruvate.

Labeling varied among individual metabolites. Table A1.1 shows that intracellular citric acid and pyruvic acid were labeled with 97.1 % and 97.8 % efficiency, respectively, in the first overnight. However, citric acid did not label as well in the second overnight, whereas pyruvic acid became more efficiently labeled during that time period, achieving 87.1 % and 98.4 % labeling, respectively. This can be visually observed in the mass spectrum presented in Figure A1.3. The majority of fumaric acid and glutamic acid molecules became labeled, but not to a percentage high enough for use as an internal standard. Fumaric acid labeling improved slightly after an additional overnight incubation in fresh ^{13}C media, but the labeling of glutamic acid decreased markedly in the same period. Overall, labeling efficiency did not increase when cells were transferred into fresh ^{13}C-rich media for a second period of overnight growth.

Furthermore, carbon-13 labeling in the second experiment was incomplete. In a molecule of pyruvic acid, containing three carbon atoms, the mass spectrum showed that carbon-13 replaced carbon-12 atoms at all three sites. However, for citric acid and glutamic acid, carbon-13 incorporation only occurred at some of the possible locations, as indicated by the difference in mass-to-charge ratios on the mass spectrum for those analytes (Figure A1.4).

A1.4 Conclusions

This experiment demonstrated that the internal metabolites of *Escherichia coli* O157:H7 can be labeled with ^{13}C-glucose using this protocol. It also confirmed that carbon-13 metabolites were distinguishable from carbon-12 metabolites in time-of-flight mass spectra. The application of this technique to the existing microbial metabolomics protocol would ideally aid in uncovering the source and magnitude of the variability and the extent upon which it affects the precision of the experiment. However, despite efforts to adapt and improve the technique, the labeling efficiency

was not high enough or uniform enough for use as a global internal standard. Future research in this area should continue to develop methods for labeling analytes more efficiently and more completely. Perhaps the use of U-^{13}C-thiamine and additional or longer overnight growth periods would improve labeling efficiency of *E. coli* metabolites. Finally, more TMS-derivatized standard compounds should be run so that labeling efficiency calculations can be based upon a larger quantity and variety of identified analytes.

A1.5 Acknowledgements

The author thanks Frederick Breidt, Jr., David C. Muddiman, and Suzanne D. Johanningsmeier for helpful discussions of this work, D. Seth Fornea for analytical expertise and instrument maintenance, and Sandra Parker for excellent administrative assistance.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.

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Table A1.1 Labeling efficiencies for selected analytes.

O/N	Metabolite	Average LE(%) by Abundance	Average LE(%) by Intensity	Expected RI	Observed RI	Expected RT_1 (s)	Observed RT_1 (s)	Expected RT_2 (s)	Observed RT_2 (s)
1	Citric Acid	97.1 ± 1.26	96.2 ± 1.29	1839.5	1840.5	1604.33	1599.75	1.393	1.743
1	Pyruvate	97.8 ± 0.0694	97.7 ± 0.0821	1181.4	1181.5	648.25	649.17	1.133	1.137
1	Fumaric Acid	82.6 ± 1.77	82.5 ± 1.81	1404.6	1403.5	994.75	992.92	1.589	1.572
1	Glutamic Acid	90.6 ± 4.61	89.2 ± 4.30	1659.2	1659.1	1366.00	1366.92	1.658	1.647
	Average	92.0 ± 1.93	91.4 ± 1.87						
2	Citric Acid	87.1 ± 5.43	86.4 ± 5.00	1839.5	1837.0	1604.33	1599.75	1.393	1.740
2	Pyruvate	98.4 ± 0.699	98.3 ± 0.666	1181.4	1179.4	648.25	651.00	1.133	1.135
2	Fumaric Acid	84.2 ± 0.251	84.2 ± 0.238	1404.6	1404.1	994.75	993.83	1.589	1.570
2	Glutamic Acid	77.3 ± 5.16	76.8 ± 4.43	1659.2	1653.2	1366.00	1366.00	1.658	1.655
	Average	86.7 ± 2.88	86.4 ± 2.58						

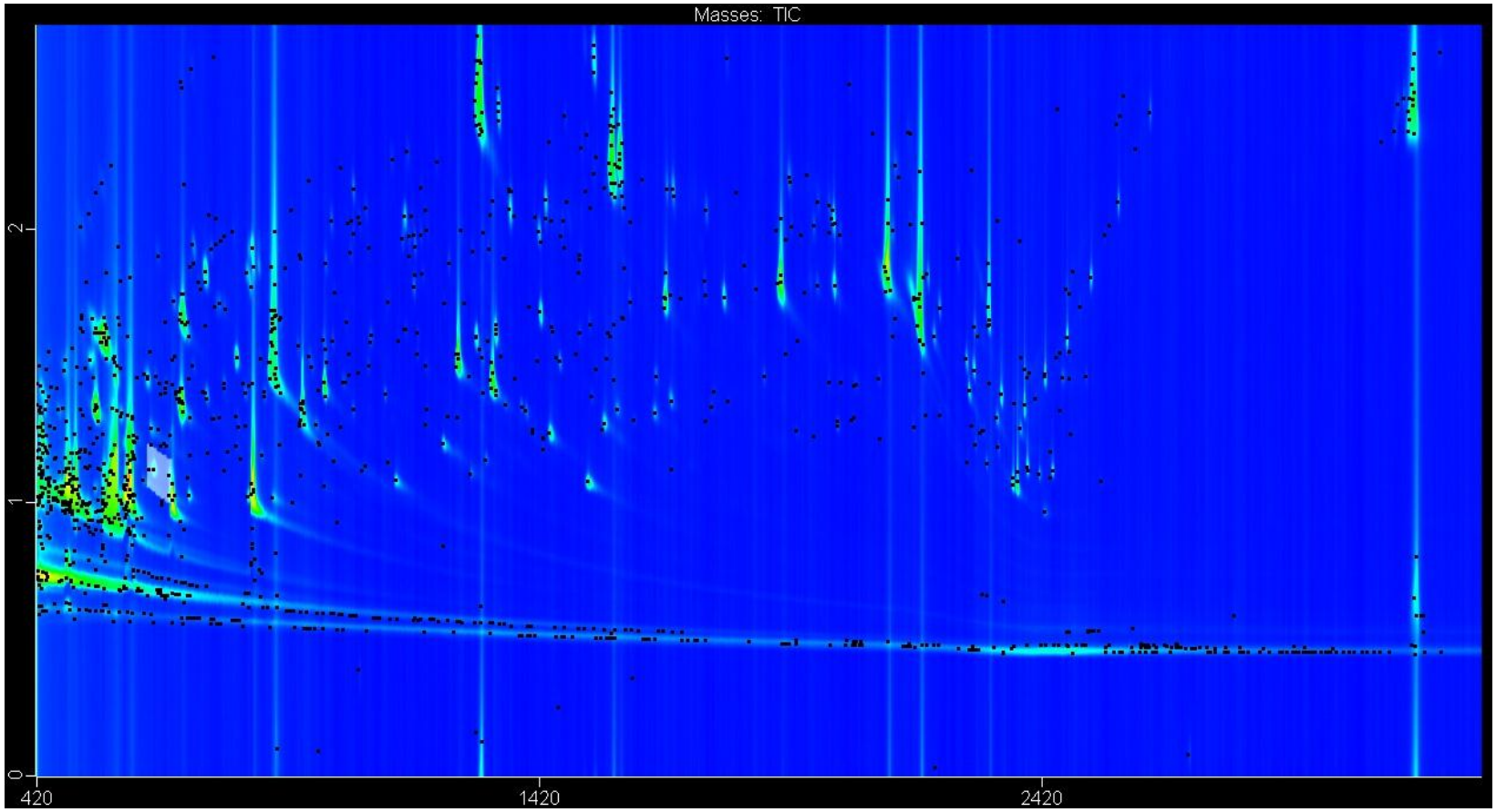

Figure A1.1a Chromatogram of ^{12}C-grown *Escherichia coli* O157:H7 extract. The x-axis represents first dimension retention time (s) and the y-axis represents second dimension retention time (s). The intensity of the color represents the height of the peak in the z-axis. The highlighted peak at (653.75s, 1.125 s) was tentatively identified by the mass spectral library as pyruvate.

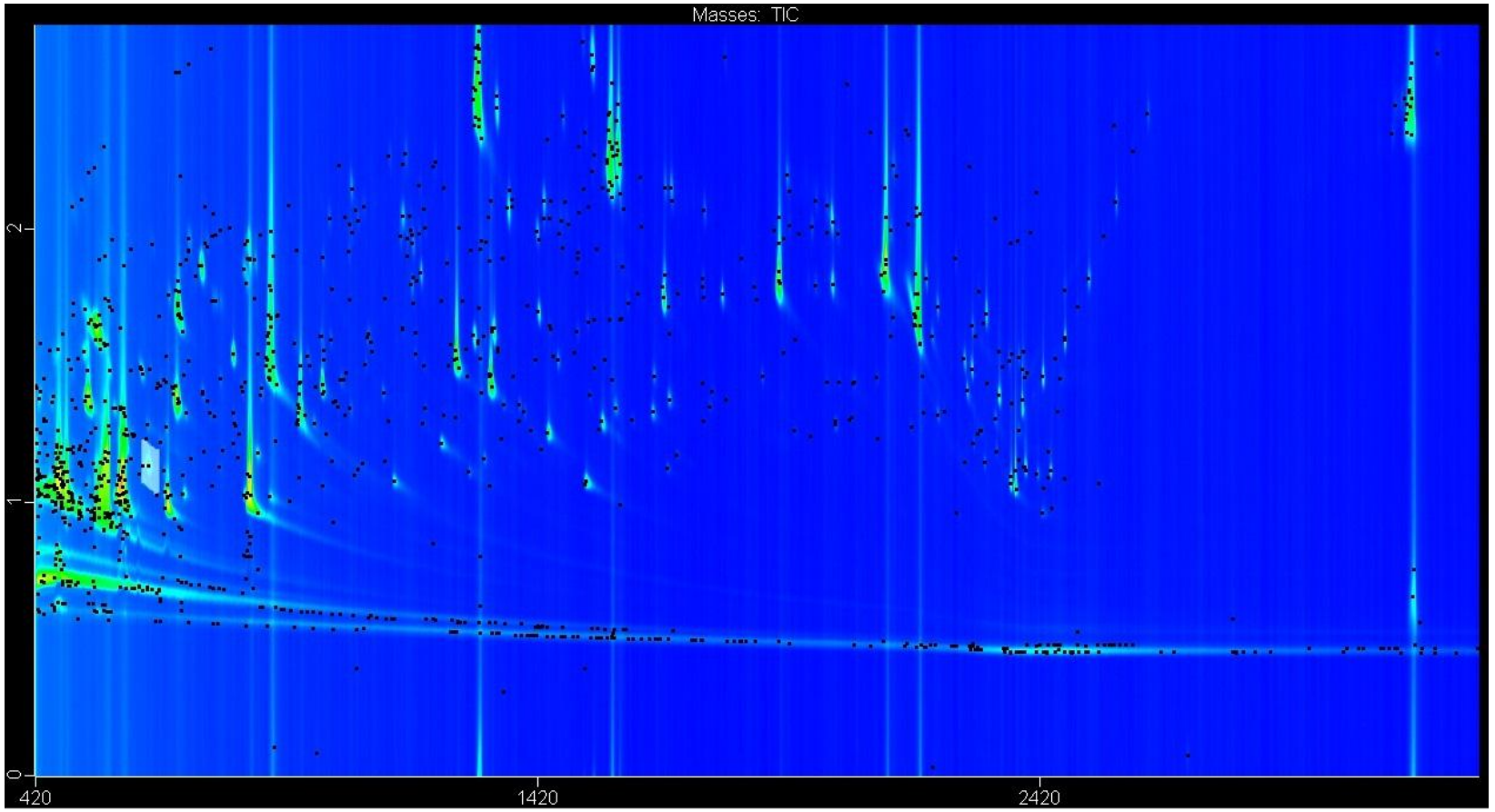

Figure A1.1b Chromatogram of ^{13}C-grown *Escherichia coli* O157:H7 extract. The x-axis represents first dimension retention time (s) and the y-axis represents second dimension retention time (s). The intensity of the color represents the height of the peak in the z-axis. The highlighted peak at (645.5 s, 1.140 s) was tentatively identified by comparable retention times to ^{12}C-pyruvate.

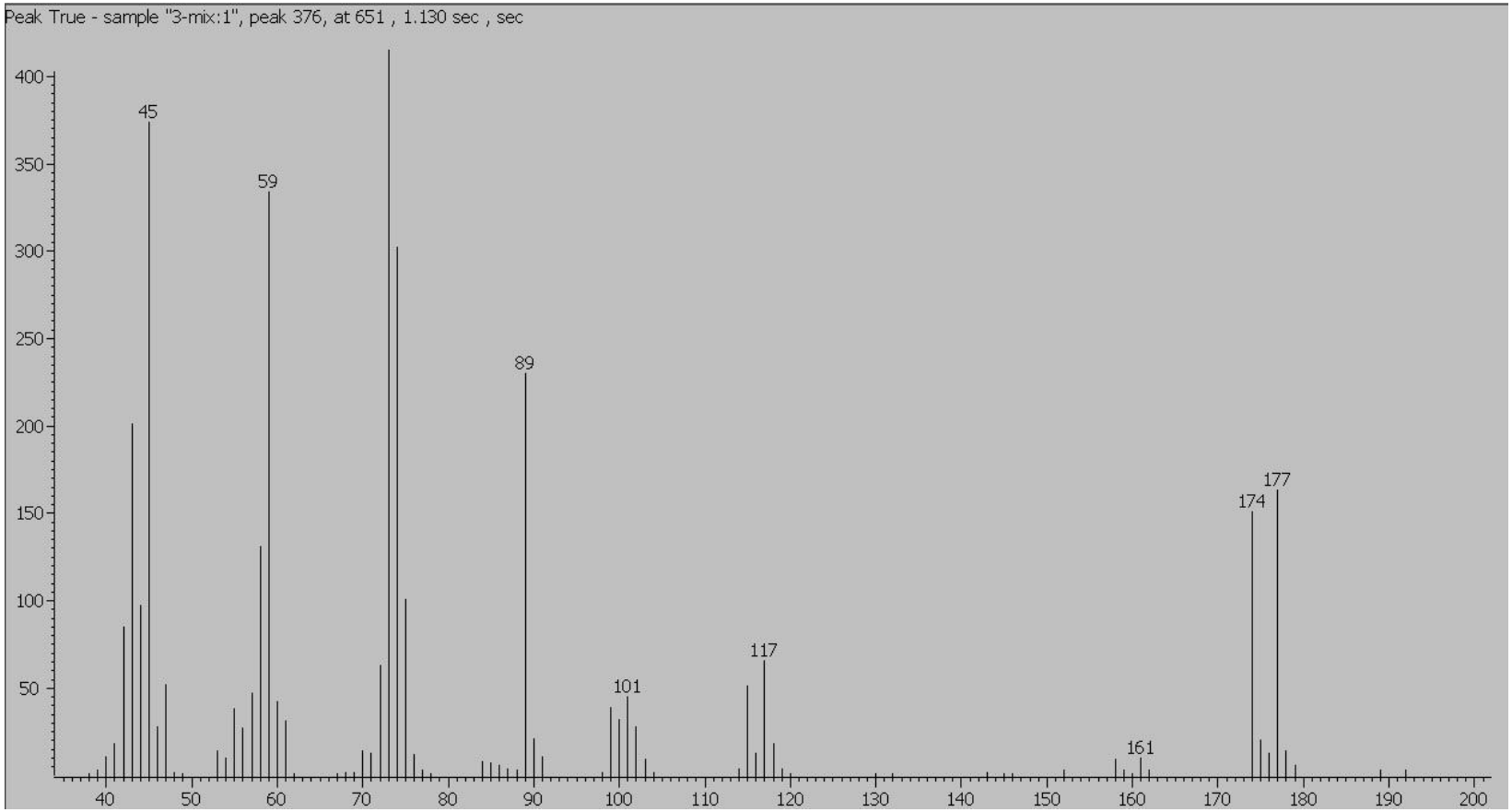

Figure A1.2 Mass spectrum of pyruvic acid peak in 1:1 mixture of ^{12}C- and ^{13}C-labeled extracts of *Escherichia coli* O157:H7. The "doublet" of peaks of roughly equal height at m/z 174 and 177 represent the quantifying masses of carbon-12 pyruvate and carbon-13 pyruvate.

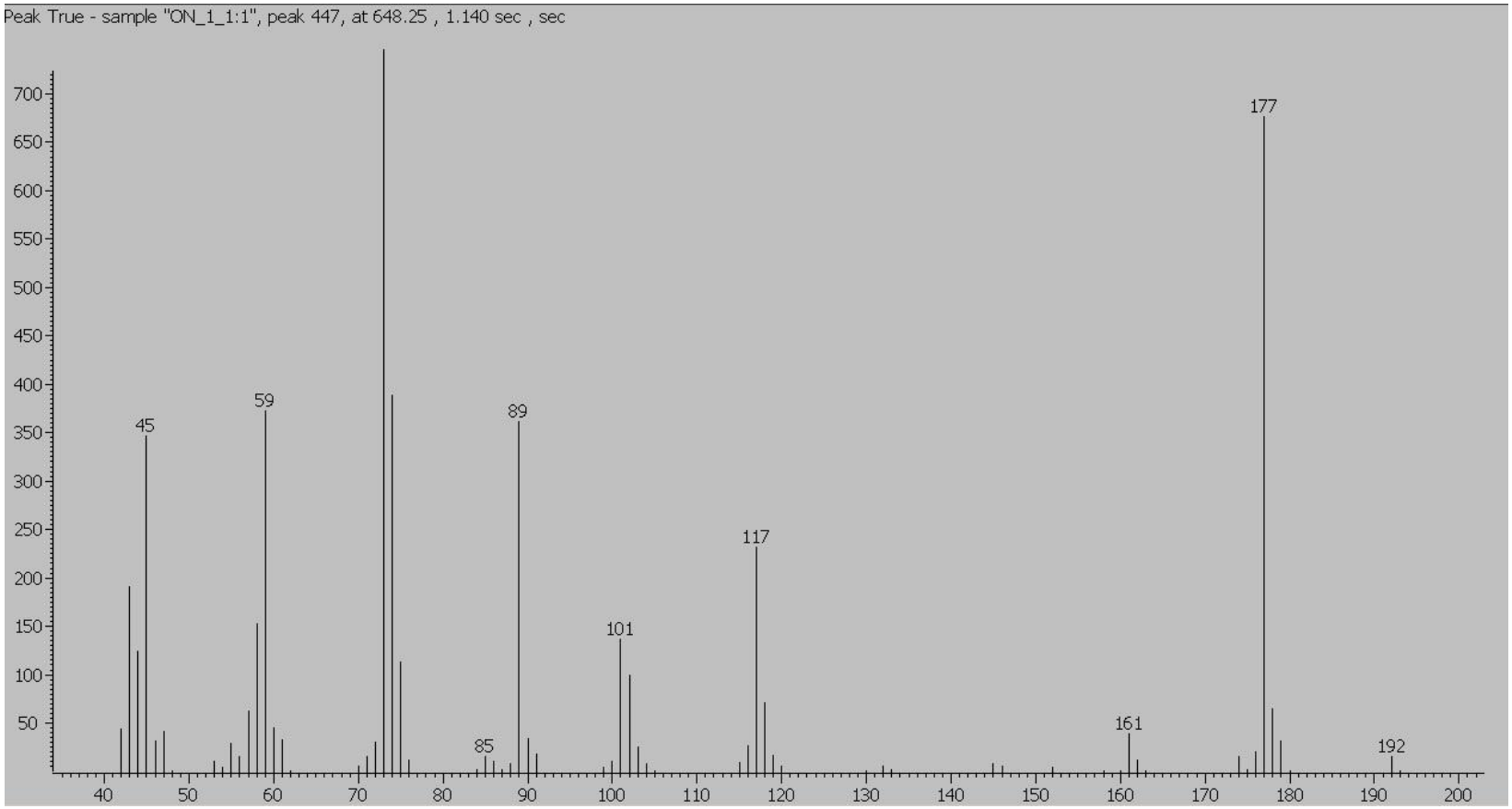

Figure A1.3 Mass spectrum of pyruvic acid peak in ^{13}C-labeled extract of *Escherichia coli* O157:H7. The peak at m/z 177 represents the quantifying mass of ^{13}C-labeled pyruvic acid, whereas the small peak remaining at m/z 174 represents the quantifying mass of unlabeled pyruvic acid that remains unlabeled.

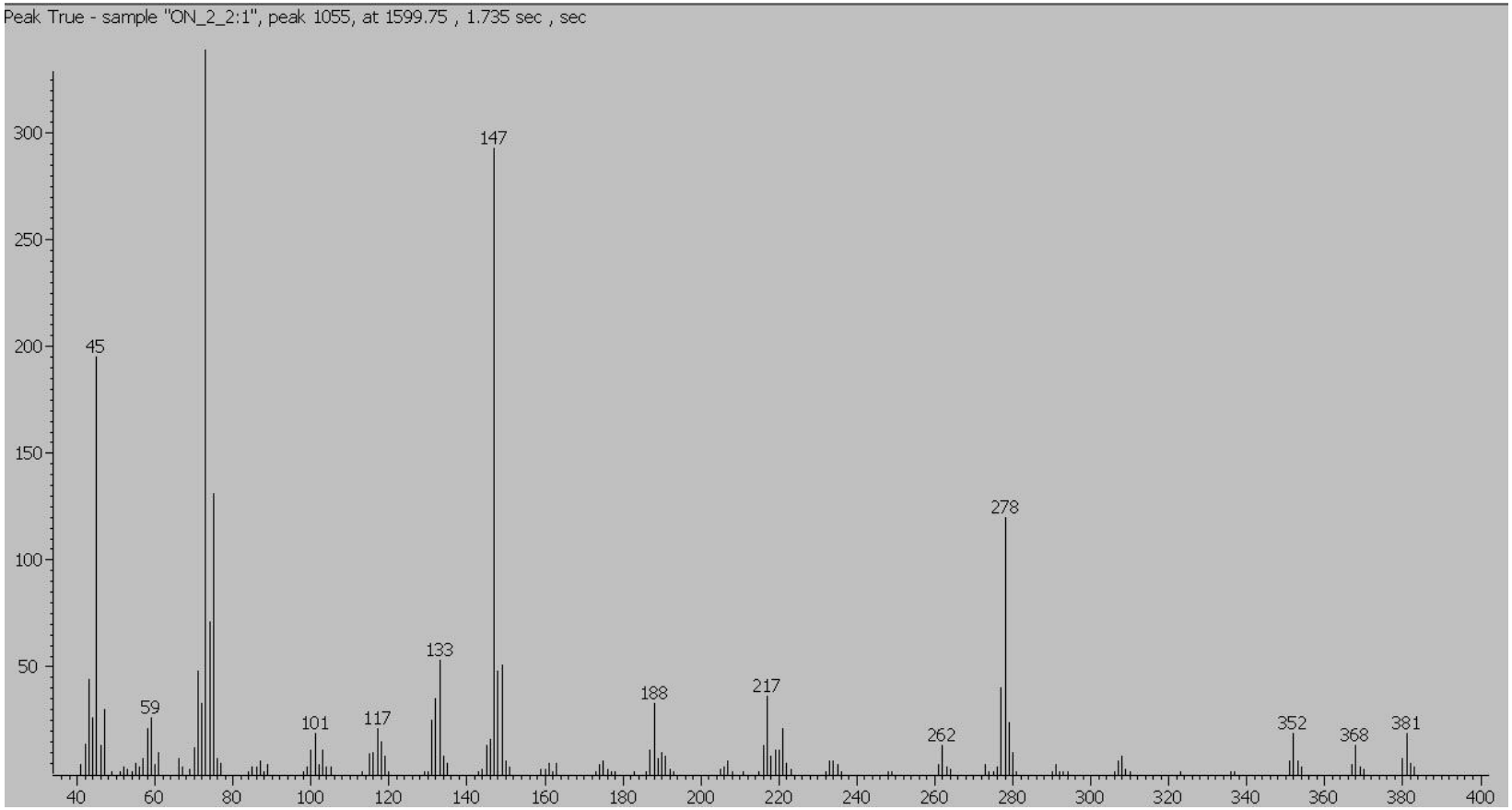

Figure A1.4 Mass spectrum of citric acid peak in ^{13}C-labeled extract of *Escherichia coli* O157:H7. The peak at m/z 278 represents the quantifying mass of incompletely ^{13}C-labeled citric acid, whereas the small peak remaining at m/z 273 represents the quantifying mass of unlabeled citric acid. The difference of 5 m/z units instead of an expected 6 m/z units indicates that carbon-13 labeling did not occur at every possible carbon atom in the molecule. For completely labeled citric acid, the peak at m/z 273 would be shifted to m/z 279, and each other peak in the mass spectrum would be shifted by the number of labeled carbon atoms in that mass fragment.