Partial Characterization of Yeast Hydrolysates for insights on Chemically Defined Media for Sf-9 Insect Cells

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Yeast extract, or other varieties of yeast hydrolysate, have been used in combination with a lipid supplement to completely eliminate the need for fetal bovine serum during *in vitro* cultivations of insect cells. This has made the process cheaper and much more attractive for large industrial uses due to the higher availability of yeast extract over serum. While yeast extract represents a vital step forward in culture media, the gold standard of a chemically defined media with the same growth parameters has not been achieved. To better understand the hydrolysate composition, it was subjected to trace mineral analysis via ICP-OES while H-NMR was used to quantify the detectable organic compounds before, during, and after acid hydrolysis.

Trace mineral analysis showed that yeast extract was the sole provider of silicon, chromium, nickel and vanadium, as well as a majority provider of zinc, manganese, copper, cobalt and iron. While silicon has no known function in metabolism, the other elements are of considerable interest complicated by their contributions to detrimental free radical reactions. Yeast extract also provides antioxidants to help combat some of these reactions while possibly assisting in the trace metal dissolution due to the presence of several organic molecules which function as chelators.

Through the hydrolysis studies, the yeast extract was shown to be composed of peptides heavily consisting of glycine, lysine and proline. Yeast extract also contains a significant amount of nucleic material, mostly from RNA due to the presence of uridine. Further, several B-vitamins were quantified, including significant amounts of B3 and myo-inositol, each more than 10x their current respective defined supplementation.

Metabolic studies of *Spodoptera frugiperda* Sf-9 cell cultures in media containing hydrolysates helped to illuminate which compounds are active metabolites over the initial growth phase as well as determining any compounds which become completely exhausted. However, consistent cell growth after the commercial media was diluted sufficiently over 3 passages was only obtained with one of the yeast extract lots studied, possibly highlighting deeper concerns with the manufacturing process overall.

Along with a thorough literature review, all of this data was combined into a chemically defined supplement to replace the yeast extract. No significant growth was obtained once the commercial media had been diluted from the system over several passages. This suggests that only a small amount of the crucial growth promoting compound is required, but more rigorous investigations (e.g. GC-MS, fractionation analysis) are still required to pinpoint its identity.

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List of Abbreviations

(H) [C] NMR	(Hydrogen) [Carbon] Nuclear Magnetic Resonance Spectroscopy
a-GPC	alpha-glycerophosphocholine
AKG	alpha-ketoglutarate
Ala	Alanine
ALIM	Aucoin Lab Insect-cell Media
AN/TN	Amino Nitrogen to Total Nitrogen ratio
ANOVA	Analysis of Variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Asx	Combined Asp and Asn
ATP	Adenosine triphosphate
ATPase	enzyme using ATP for energy
BD	BD Biosciences
BEVS	Baculovirus Expression Vector System
CDM	Chemically Defined Media
CoV	Coefficient of Variation
Cys	Cystine
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
FBS	Fetal Bovine Serum
FL	ThermoFisher
For	Formate
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
Glx	Combined Glu, Gln, Pglu
Gly	Glycine
His	Histidine
ICP OES	Inductively Coupled Plasma Optical Emission Spectroscopy
Ile	Isoleucine
ITS	Insulin, Transferrin, Selenium (Media Supplement)
LDL	Lower Detection Limit
Leu	Leucine
Lev	Levulinate
Lys	Lysine
Met	Methionine
ODE	Ordinary Differential Equations
Osm	Osmoles (measure of osmolality)
PCA	Principal Component Analysis
Pglu	Pyroglutamate
Phe	Phenylalanine
Pro	Proline
RPM	Revolutions per minute
Ser	Serine
Sf-9	Spodoptera frugiperda clone line 9
TCA	Tricarboxylic Acid (cycle)
Thr	Threonine
Try	Tryptophan
Tyr	Tyrosine
Val	Valine
w%	Weight percent

Highlights

- Hydrolysates, such as yeast extract, are known to be highly variable in composition. While the relative amounts of free amino acids and most other compounds were similar in a yeast extract lot analysis, some instances of extreme variability were noted, including a doubling of trehalose content and different detected minerals, which could explain their significantly different effects on cell growth.
- As expected, yeast hydrolysates of different origins have different compositions. This highlights the need for either strict industry process control parameters or the much more achievable goal of a completely defined media to completely eliminate the variable hydrolysate supplementation.
- From various analytical methods, several trace metals, vitamins and other possibly biologically active molecules were identified as solely being provided by the yeast extract. A literature review identified several others highly likely to be present or other more readily available components with similar effects.
- This data was combined into a fully chemically defined media supplement to replace the yeast extract but no significant growth was obtained, suggesting more rigorous investigation methods (eg. GC-MS) are still required.

1.0 Introduction

During the early days of *in vitro* cell culture, significant growth for many cell lines was only obtained through the use of a mammalian blood serum (normally fetal bovine). It provides functional proteins, amino acids, lipids, sugars, salts and trace metals for various metabolic purposes, as well as having several general stabilizing aspects allowing the cells to better grow *in vitro*[1]. However, it is known to be highly variable between lots, prone to high cost and low availability, as well as potentially containing contaminants or other unwanted components which could pose a problem immediately or in later downstream purification processes[1, 2]. This was a major driving force behind producing chemically defined media for a variety of cell lines with immediate therapeutic potential. It was not until recent advances in developing baculovirus expression vector systems (BEVS) as a biomanufacturing platform for a variety of therapeutics in insect cell lines[97] that there has been a need for a high performing chemically defined medium (CDM). There are currently nine approved applications for using BEVS to create these therapeutics, most commonly in Lepidopteran cell lines[98], as detailed in Table 1.1 (modified from Felberbaum[99]). Although the current production systems do not use FBS, they use other complex additives, meaning there is still some inherent variability between different lots of media. Improving the media to ensure only the required components are present will allow for the best understanding of the exact metabolic processes involved, will have highly reproducible results, and should be easier to seek regulatory approval[2].

Table 1.1: Current approved uses of BEVS. VLP: Virus like particle

Vaccine Name	Manufacturer	Treatment	Product Form
Porcine			
Porcilis™ Pesti	MSD Animal Health	Classical Swine Fever	Subunit
BAYOVAC CSF E2™ / Advasure™	Bayer AG / Pfizer Animal Health		
Circumvent™ PCV	Merck Animal Health	Porcine Circovirus type 2	VLP
Ingelvac CircoFLEX™	Boehringer Ingelheim Vetmedica		
Porcilis™ PCV	MSD Animal Health		
Human			
Cervarix™	GlaxoSmithKline	Human Papillomavirus	VLP
Flublok™	Protein Sciences Corporation	Influenza	subunit
Provenge™	Dendreon	Prostate Cancer	immunotherapy
Glybera™	uniQure	Lipoprotein lipase deficiency	rAAV-based gene therapy

Since the development of IPL-41 by Weiss et al.[3], industrial scale serum-free culturing has been shown possible by supplementing yeast extract and a lipid emulsion to a chemically defined basal medium consisting of amino acids, sugars, salts, vitamins and trace metals. Yeast extract, also referred to as yeastolate or yeast hydrolysate, is the water soluble portion of autolyzed yeast biomass (normally *Saccharomyces cerevisiae* [i.e. Brewer's yeast] due to its abundance but any other species could be used) primarily containing amino acids and peptides, but also a source of B-vitamins, sugars, salts, and trace metals. The hydrolysate contains most of the components provided by serum, while the lipid solution provides others, but a majority of the larger compounds in the serum can be excluded without detrimental effects (as attested from the performance of the commercial media). Although most serum-free media use yeast extract, other hydrolysates (e.g. meat, egg, milk, soy[4-8]) have also shown positive effects in culture, but the results are normally dependent on the cell line and the optimum type and level of useful hydrolysates must undergo extensive investigation[1]. Even once suitable hydrolysate levels are found, screening methods are normally required to ensure that the hydrolysate lots pass some base criteria before being deemed appropriate for full scale use[9, 10]. Therefore, while hydrolysates represent an important step forward in insect cell culture research, only very recently has there been a reported CDM for Sf9 insect cells (i.e. SpodOmics™, Cell Culture Technologies) but it still does not perform as well as those containing hydrolysates (e.g. Sf-900 III™, Gibco), meaning improvements can still be made to completely eliminate any potential for batch-to-batch variability.

By analyzing yeast hydrolysate, insights on the specific quantities of certain components could help to open up new windows of investigation to create this elusive CDM.

1.1 Hypothesis

While a CDM is available for insect cells, it still does not perform as well as those containing hydrolysate supplements. It is hypothesized that a thorough characterization of yeast extract by H-NMR spectroscopy and trace metal analysis will provide the required information to derive a chemically defined replacement for yeast extract and other hydrolysates.

1.2 Objectives

To most efficiently analyze the yeast extract, trace metal analysis and hydrolysis techniques will be combined with targeted H-NMR spectroscopy to ensure the largest amount of compounds are quantified.

Acid hydrolysis has long been a favoured technique to determine amino acid sequence of peptide chains[14]. The extreme conditions allow for the complete hydrolysis of peptide bonds but has several side effects: the complete destruction of tryptophan (Try) and cystine (Cys); the slight destructions of serine (Ser) and methionine (Met); the conversions of glutamine (Gln), and asparagine (Asn) to glutamate (Glu), and aspartate (Asp), respectively; and the conversion of sugars to levulinic acid, formic acid, and several by-products[31]. Since about half of the nitrogen in the yeast extract is in peptide form[16], and it contains a variety of other organic bonds (e.g. phosphate, ester, or glycosidic), this will allow for a significant amount of new material to be quantified.

H-NMR spectroscopy allows a wide variety of molecules to be detected in a single spectrum, which can then be easily interpreted through available software packages. While a wide range of components are detected at once, there are still some components that could be in yeast extract that are undetectable to some scan parameters because they have an inappropriate hydrogen ion (e.g. guanidine and uric acid). Although this methodology will be unable to account for all the contributions in one hydrolysate, it provides extensive information which would otherwise require significantly more resources. However, as this is a study on H-NMR spectroscopy and its applicability to complex organic solutions it is accepted that some components may not be identified. The success of using NMR in this manner can be concluded based on the percentage of the original material that is quantified.

Yeast extracts are known to be heavily variable in trace metal composition[22, 23], which can be involved in free radical reactions that harm other biological molecules[27]. A chemically defined medium will require carefully controlled levels of these compounds to be as effective as possible. Inductively-coupled plasma optical emission spectroscopy (ICP-OES) will be used to measure the quantity of twenty different cations present in yeast extract, as well as the overall level in a variety of media.

All of this information will be utilized to compare the growth of *Spodoptera frugiperda* Sf-9 clone cells in commercial media with hydrolysates to an in-house media with and without hydrolysates. Growth profiles will be maintained for each culture as they are adapted to the new media, and cultures exhibiting consistency in reaching at least $6x10^6$ cells/mL in less than 10 days were subjected to metabolic analysis.

1.3 Anticipated Contributions and Impact of Analysis

This thesis will hopefully provide several insights on various methodologies and phenomena surrounding cell culture growth, roughly corresponding to the organization of the report.

- Thorough statistical analysis using H-NMR spectra to quantify the semi-defined product of yeast extract and to test the variability of the analytical process as it compares to the variability between different lots and suppliers.
- Novel acid hydrolysis methodology and modelling of yeast extract to determine if this provided new areas of interest when creating a chemically defined medium for insect cells.
- Understanding and comparing the metabolism of a cell line due to different complex additives or a chemically defined replacement, including any enzymatic activity of the culture supernatant.

2.0 Insect Cell Media and Metabolism

2.1 Historical Evolution of Insect Cell Lines and Media

The basis of all media is the same: an aqueous mixture of carbohydrates, amino acids and salts with a suitable buffer. However, the quantities of these components is heavily dependent on the family, species and even specific cell lines[1].

Although hundreds of cell lines from various species and orders have been isolated[100], the Lepidopteran species *Spodoptera frugiperda* produced two of the most popular industrial cell lines: Sf-21 and Sf-9 clones[99]. Sf-21 cells were established from isolated ovarian cells by Vaughn et al.[101] This cell line was then developed into Sf-9 cells by Smith and his group[99, 102]. These cell lines have become popular since they are highly susceptible to infection by *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) or other baculoviruses, creating a rigorous biomanufacturing platform called insect cell BEVS (IC-BEVS). With the complete DNA sequence and individual gene functions of the AcMNPV highly studied[103], a number of different promoters have been analyzed to assess their performance for protein production. Usually, the gene for the production of polyhedrin (proteins used to protect the virus in the natural environment and are therefore unnecessary in cell culture) is replaced with the protein of interest due to it being controlled by a very strong promotor and thus results in high protein yields[99]. The infection cycle will generally start expressing this product 18 hours post infection (h.p.i), but it is overexpressed 48 to 120 h.p.i.[104], after which the cytopathic effects from infection result in cell lysis and the release of the product into the supernatant. While there are many benefits for IC-BEVS, the two most important are: their inherently safe nature since they are unable to infect native mammalian cells[105] (and the modified baculovirus is unlikely to survive in nature without the polyhedrin); and they are able to express genes from eukaryotic, prokaryotic and even viral organisms[106, 107].

Initial media for Lepidopteran cells came from analyzing hemolymph. Grace's[35], TNM-FH[36], and TC-100[37] were all initial attempts at media for *in vitro* insect cell culture and contain carbohydrates, amino acids, organic acids, and vitamin mixtures supplemented with 5-10% fetal bovine serum (FBS). FBS is a complex, protein-containing supplement providing detoxifying effects, carrier molecules, and protection from shear stress[1]. However, FBS in non-ideal for several reasons: high cost and low availability; batch-to batch variability; and possible contaminants or unwanted compounds[1]. Further, since the FBS is protein-rich, this adds complications when the trying to prepare a purified protein product made through recombinant protein production (i.e. downstream processing). Serum heavily stimulates growth in media,

but due to the issues surrounding its use many groups attempted to replace it with less variable and more defined supplements.

Several possible serum substitutes have been investigated, including peptones (hydrolyzed animal tissues), egg yolk emulsions, and lactalbumin (milk proteins) [4-6, 79]. However the greatest effect is achieved with yeast extract or yeastolate, products of autolyzed yeast biomass[1, 79]. Supplemented in conjunction with a lipid emulsion, yeast extract is able to provide excellent growth for Lepidopteran lines[1, 79]. Yeastolate is known to be a heavy provider of vitamins, trace metals, and bioactive peptides known to stimulate either cell growth or viral protein production[15]. Yeast extract is also beneficial as it is a non-animal derived source, increasing the safety and reproducibility of the product (although there is still some batch-to-batch variability in yeast, it can be minimized through ultrafiltration[79]). Nowadays, several serum-free commercial media are available but due to their proprietary nature, the exact formulations are unknown (and thus could be animal-derived if not otherwise acknowledged), but in any case, contain some sort of hydrolysate. Commercial media is generally restricted to a specific cell line or a narrow range of similar lines when claiming growth parameters. This means that while cells could stay viable in a non-specific media, their growth will most likely be heavily affected away from optimum values.

2.2 Culturing Parameters

Culture parameters, such as temperature, pH, osmolality, dissolved gases, and shear stresses, heavily influence the growth of cells. Insect cells obtain a maximum growth rate and cell density at a temperature of 27 ^{0}C[38], but are tolerant between 25 and 30 ^{0}C. Higher temperatures can reduce overall protein yield in Sf-9 cells[39]. A medium pH between 6.0 and 6.8 is acceptable, requiring only a phosphate buffer (as opposed to a pH of 7.4 and the CO_2/bicarbonate system required for mammalian systems). Sf-9 cells prefer a medium between 6.2 and 6.4[40]. Insect cells can also tolerate media with an osmolality anywhere from 250-450 mOsm/kg, but is generally kept between 320 and 385 mOsm/kg[41]. Dissolved oxygen is an important factor during both growth and infection stages, with oxygen uptake rates increasing during infection[42]. However, more dissolved oxygen results in increased CO_2, which is known to inhibit growth and protein production beyond 24 mM, possibly due to intracellular pH changes[43-45]. Suspension cultures require some sort of agitation to maintain their suspension nature as well as to ensure proper oxygen transfer rates but cannot withstand the force generated by a classic stirred tank or bubble column[46], so careful engineering is required to keep these forces to a minimum for large scale designs. For small scale shake flasks, rotation agitation between 100-150 rpm with a culture volume 25-30% the total flask volume

is preferred[47-48]. A non-ionic polyol surfactant (such as Pluronic® F-68[Gibco©]) is added to help control shear forces as well as minimize any surface adhesion.

2.3 Media Component Groups

Altogether, insect cell media can contain 75-100 individual components, making the development of an optimal medium a tedious process. Compounds can be grouped into the following major categories:

- **Bulk Ions** (or **Osmolites**) [Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}]
    - While not explicit metabolites, Na^+ and K^+ are directly coupled to ATPase pumps, which provide secondary potential to transport larger molecules into the cell, while Ca^{2+} and Mg^{2+} are essential components of many enzymes.
- **Sugars and Organic Acids**
    - While cultures can grow solely in the presence of glucose, some disaccharides can be externally degraded in the supernatant by excreted amylases. This could allow for lower levels of glucose while lowering the accumulation of by-products that occur under glucose excess. While some organic acids are used as carbon sources, this is heavily dependent on the media and the other metabolites it provides[50].
- **Amino Acids**
    - The amino acids have many interacting effects[50]. As such, understanding optimal supplementation requires detailed experimental design. It is also highly likely that this will be heavily affected by the protein production phase (i.e. there should be more than enough amino acids for both stages of the culture).
- **Lipids** [fatty acids, sterols]
    - Being the main constituents of cell walls, these compounds should have a heavy influence on growth rate. Since only a maximum of 20% of glucose is converted into lipids[52, 53], lipid supplementation would allow the cell to have an initial pool of these metabolites without having to wait for the full conversion process from glucose.
- **Vitamins and Trace Metals**
    - These compounds are important co-factors for several enzymes and other metabolic processes. Although only present in small amounts, these compounds are crucial for growth but are probably heavily involved in determining oxidative stress (the term for the collective level of oxygen or other radicals) present in the media[27].

- **Antioxidants** [e.g. ascorbate, glutathione, Vitamin E]
    - Due to the dissolved oxygen and aerobic conditions, the media is an actively oxidative environment, able to damage macromolecules through perpetuating free radical reactions[27]. Further, cultures can become much more oxidative during viral infection stages[81]. Therefore supplementation of antioxidants help to eliminate some of these radicals and create a more conducive environment for growth.
- **Micronutrients**
    - These are classified as biologically active compounds wherein their supplementation has not ever, or fully been, investigated in insect cell culture. These compounds include polyamines (e.g. cadaverine), phospholipid head groups (e.g. ethanolamine), and other lipid related compounds (e.g. carnitine).

2.4 Insect Metabolism in Suspension Culture

Understanding the metabolism of cells is important to ensure sufficient quantities of substrates are available in the media to sustain prolonged growth.

2.4.1 Carbohydrates

Glucose is the preferred carbon-source for insect cells and is consumed at a much higher rate than in mammalian cells[21]. Other carbon sources (e.g. fructose and lactate), can be utilized after glucose has been depleted from the media[50, 51]. Supernatant from Sf-9 cultures actively digests maltose and trehalose into glucose, suggesting the presence of secreted amylases (data forthcoming). Insect cells regulate their fluxes such that 80% of the pyruvate formed from glycolysis eventually feeds into the TCA cycle, leaving the remainder for lipid and alanine production[52, 53]. While glucose is required for growth, cells are able to remain viable in the media if sufficient amino acids are available but excess glucose can lead to the accumulation of lactate and alanine[50, 54], reducing the efficiency with which other active metabolites are produced. While TCA intermediates can be used as a carbon source, they are not consumed during the growth phase[6], and their absence does not affect growth[37].

2.4.2 Amino Acids

Gln is the main amino acid used as an energy source for Sf lines[54] possibly through conversion into α-ketoglutarate (AKG) through Glu, but while Sf-9 cells possess a Gln synthase[54] (to create Gln from Glu), its absence from media significantly affects growth, suggesting this pathway is not very efficient[55, 56]. Other

energy sources for Sf-9 cells are arginine (Arg), Asn, Asp, Met, and Ser[49]. The majority of amino acids are not synthesized by the cell[57], necessitating their supplementation. Isoleucine (Ile), lysine (Lys), histidine (His), leucine (Leu), Met, and threonine (Thr) are all crucial for Sf-9 growth and delaying cell death[55]. The amino acids Arg, Asn, Asp, Gln, Glu, glycine (Gly), and Ser are required to sustain high growth of Sf-9 cells[58] as well as being necessary during infection phases to obtain high protein production[31].

2.4.3 By-products of metabolism

The most significant by-product that accumulates over the course of an Sf-9 culture is alanine (Ala). When Asx is converted to oxaloacetate, or Glx is converted to AKG, pyruvate acts as an ammonium sink to produce Ala[56]. While ammonium toxicity is not fully studied in insect cells[99], its effect is probably similar to that of mammalian cells: the acidification of the cytoplasm and mitochondria coupled with competition of ammonium and K^+ through ATPase transport pumps[60, 61]. Insect cultures are generally more tolerant than mammalian cultures to ammonium content[50, 61], and can tolerate up to 100mM of Ala[28].

2.4.4 Lipids

It is known that insect cells have an incomplete lipid metabolism, leaving them unable to synthesize, desaturate and elongate fatty acids as well as being unable to anabolize cholesterol[57]. Supplementation of long chain, unsaturated fatty acids (i.e. oleic, linoleic, and linolenic acids) improve growth of insect cultures significantly, and these fatty acids are used directly to create more cellular membranes[62].

2.5 Yeast Hydrolysates

2.5.1 Production of Hydrolysates

There are two main methods for the production of hydrolysates. Either biomass can be exposed to enzymatic hydrolysis (through the initiation of autolysis or the addition of external proteases[11-14, 16]), or through acidic or alkaline hydrolysis[12, 14]. The latter process occurs in extremely alkaline or acidic conditions and at relatively high temperatures (e.g. HCl digestion occurs at 6M acid and 120^0C). These extreme conditions completely hydrolyze all organic bonds at various rates but results in a product of relatively high salt content. Enzymatic digestion, on the other hand, occurs at relatively moderate conditions (autolysis at about 60^0C[11] or proteases at 37^0C[13, 14]) allowing the final overall salt concentration to remain low. Further, these more moderate conditions also preserve several components lost through acid digestion (Try, Asn, Gln, Cys, disulfide bonds, etc.) and do not result in complete hydrolysis, meaning

the final product is a mix of free amino acids and relatively short oligopeptide chains. Experiments have shown the most positive results are obtained using the fraction of yeast extract less than 3kD in size[15, 79], indicating that the short chain oligopeptides and free components are the most important.

To produce yeast hydrolysate through autolysis, a high density culture of yeast cells is exposed to either a heat or osmotic shock to kill the cells without inactivating the autolytic enzymes. The autolysis is then allowed to continue for up to 60 hours. The slurry is then filtered and centrifuged to remove insoluble and lipid components. The final product can then either be concentrated into a slurry or spray-dried into a powder[16, 17]. From this simplified process, it becomes clear why hydrolysates can vary so widely. Not only will the specific strain and their growth conditions provide some inherent differences, but the length and method of hydrolysis as well as any downstream processing will be important[18-20]. However, even with all of these potential differences, differing lots produced years apart can have similar effects on a cell culture. This means the most important compounds are not affected, again indicating that the most necessary compounds are probably small individual components as opposed to a long specific oligopeptide sequence (the experiments by Chou et al.[15] show that growth is detrimentally affected when compounds <1kD are excluded). One study[80] identified four unique peptide strands (heavily composed of Pro and Lys) from yeast specific protein sequences in 8 different lots of yeast extract, attesting to the continuity of the product over time.

2.5.2 Composition of yeast extract

As initially stated, yeast extract is composed of amino acids, short polypeptides, carbohydrates, salts, trace metals, and vitamins. As most of these components are easily measureable with relatively simple tests (e.g. liquid chromatography (LC) for amino acids and carbohydrates, inductively couple plasma optical emission spectroscopy (ICP EOS) for trace metals and salts, the Kjeldahl method for total nitrogen), crude compositions are readily available. However, there are very few comprehensive studies of yeast extract available and several techniques, including surface-enhanced Ramen scattering[93] and fractionation followed by high performance LC[87, 92] have been useful to screen lots or analyze for a specific chemical, as opposed to complete characterization. This is further complicated by the high variability between lots and suppliers of yeast extract which makes extrapolating components and even concentrations between lots highly risky[22, 87, 88, 90]. Therefore, while other papers have been cited appropriately, they would be hard to directly compare to any analysis performed in this work. However, BD Biosciences supplies technical results for a variety of their yeast extract products, including the one used in this study (results are for a

different lot but the same product), and provides the best point of comparison to check this study's result. Table 2.1 provides a crude breakdown of the yeast extract lot, as analyzed by BD Biosciences.

Table 2.1: Crude composition of yeast extract[16]

Total Nitrogen (w%)	Amino Nitrogen (w%)	Degree of Hydrolysis (AN/TN)	Total Carbohydrate (w%)	Ash (w%)	Loss on Drying (w%)
10.7	6.0	0.56	14.3	11.7	2.2

Although this crude breakdown does not help to identify the actual beneficial components, it does provide the first hints into the overall content of yeast extract. Firstly, the degree of hydrolysis shows that over half of the nitrogen is free amino acids; the other nitrogen would be mostly in the oligopeptides, but also in any vitamins and nucleic material. The total carbohydrates (based on a sulfuric acid-phenol colorimetric assay) represent the common sugars (glucose and fructose) and their polymers as well as any (deoxy)ribose due to nucleic material. Ash can be taken as a general measurement of the total inorganic ions present (sodium and potassium account for most of the weight).

Further refinement on the composition can be achieved with more rigorous analysis. For the BD yeast extract summarized in Table 2.2, this included: 20 hour acid digestion for total amino acid levels; ion chromatography for chloride, sulphate and phosphate anions; ICP OES for elemental cations. Table 2.3 compares the levels in the IPL-41 chemically defined basal media to supplementation at 4 g/L.

Table 2.2: More detailed analysis of yeast extract components (modified from BD Biosciences[16]) before and after acid hydrolysis**.** Components with an asterisk are completely or partially destroyed, as noted by BD.

Amino acid	Free (w%)	Total (w%)	Amino acid	Free (w%)	Total (w%)	Minerals	µg/g
Alanine	4.6	4.6	Leucine	3.5	4.9	Calcium	228
Arginine	1.7	2.4	Lysine	2.3	4.2	Iron	73.7
Asparagine*	1.2	--	Methionine*	0.8	0.8	Magnesium	250
Aspartic Acid	1.8	4.8	Phenylalanine	2.3	3.3		
Cystine*	0.2	--	Proline	0.9	1.8	**Other Ions**	**(w%)**
Glutamic Acid	6.6	8.7	Serine*	1.5	1.4	Potassium	5.09
Glutamine*	0.3	--	Threonine	1.3	1.4	Sodium	0.82
Glycine	1.3	2.7	Tryptophan*	0.6	--	Chloride	0.3
Histidine	0.5	1.1	Tyrosine	0.8	0.9	Sulfate	0.49
Isoleucine	2.1	3.6	Valine	2.4	3.7	Phosphate	2.63
			Total	**36.7**	**50.3**	**Total**	**9.33**

Table 2.3: Comparison of concentration from yeast extract and chemically supplied in IPL-41 basal medium. Iron is highlighted as it is the only compound supplied more by the yeast extract than the basal medium

	g/L			g/L			mg/L	
Amino Acid	**BD Yeast at 4g/L**	**IPL-41 Basal Medium**	**Amino Acid**	**BD Yeast at 4g/L**	**IPL-41 Basal Medium**	**Other**	**BD Yeast at 4g/L**	**IPL-41 Basal Medium**
Alanine	0.18	0.00	Leucine	0.20	0.25	Calcium	0.9	181
Arginine	0.10	0.66	Lysine	0.17	0.56	Iron	0.29	0.11
Asparagine*	0.05	1.30	Methionine*	0.03	1.00	Magnesium	1.0	185
Aspartic Acid	0.19	1.30	Phenylalanine	0.13	1.00	Potassium	204	629
Cystine*	0.01	0.10	Proline	0.07	0.50	Sodium	33	1361
Glutamic Acid	0.35	1.50	Serine*	0.06	0.40	Chloride	12	3454
Glutamine*	0.01	1.00	Threonine	0.06	0.20	Sulfate	20	732
Glycine	0.11	0.20	Tryptophan*	0.02	0.10	Phosphate	105	799
Histidine	0.04	0.20	Tyrosine	0.04	0.25			
Isoleucine	0.14	0.75	Valine	0.15	0.50			

From Table 2.3, the levels of most basal compounds are not significantly affected. However, when looking at the iron levels, IPL-41 supplements 110 µg/L (as $FeSO_4 \cdot 7H_2O$) and the yeast extract could provide 295 µg/L. From this preliminary analysis, media without yeast extract could only have 25% of the iron that would normally be present. Due to the importance of iron for a variety of metabolic processes, removing the yeast extract without further supplementation should have serious effects. Further, other reports[22, 23, 91] show a wider range of detected elements not added to the basal media, as well as significant variation between lots in some cases. Trace elements can have significant effects on cell culture both in metabolic roles[24-26] and as general toxins which lead to the formation of peroxide molecules that initiate free radical reactions[27]. All of these factors necessitate the need to carefully control the quantities of these components in the media and to exclude those without a metabolic role.

Yeast extract is also a known provider of nucleic acids and related nucleic material in both free nucleoside[28, 29] (most likely as ribonucleotides[32]) and phosphorylated forms[22, 28, 29]. These nucleosides have sizes between 3 and 25 bases, while accounting for about 1% of the total weight of the yeast extract[80], but could be as high as 10%[89]. Riboflavin (B2) has also been proven to be in yeast extract[30], but a variety of B-vitamins, including niacin or nicotinate (B3), biotin (B7), pantothenic acid (B5), thiamine (B1), choline, pyridoxine, and folic acid could be present as they are detected in the Baker's yeast strain[33, 34, 86].

2.5.3 Role of Yeast Hydrolysates

Due to the complex nature of yeast extract, it provides compounds from all of the presented categories of culture media. Therefore, the explicit benefit of the yeast extract to cell culture must be from components not already supplied. These are likely to be some trace metals, antioxidant chemicals, and possibly some minor, but important, bioactive chemicals (e.g. polyamines). Some hints at the necessary components can be deduced from those cell lines that once required hydrolysates, but have since been found to grow in media with completely chemically defined elements.

Wilkie et al[68] report a CDM for lepidopteran cells. While it included several additions over the normal CDM basal media, many groups report it was unable to sustain any high growth of cultures without adding additional compounds[58, 66]. Mitsuhashi developed a CDM for a *Sarcophaga peregrine* cell line (NIH-SaPe-4)[66, 67]. Compared to IPL-41, several additional compounds including nucleic, lipid, and other possibly biologically active components, including polyamines, are found.

An extensive review on the development of CDM for mammalian cells was compiled by van der Valk[63]. He shows that most media need an additive known as ITS, which provides insulin, transferrin, and selenium. Insulin and transferrin aid in glucose and iron membrane transport, respectively, whereas selenium is a necessary component for several antioxidant enzymes. Glucose already has higher uptake rates in insect cells than mammalian cells[49], so insulin is probably unnecessary for insect cells. While *in vivo* cultures of insects do use transferrin to regulate iron levels[64], some insect cell lines have shown its (and insulin's) supplementation does not eliminate the dependency on complex additives[65]. The addition of selenium presents the most interest due to the oxidative nature of *in vitro* cultures[27]. Since it is not added to a variety of basal medium, yeast extract could be the only source of this element (about 4 µg/g dry cell weight of yeast cultures[84] and mostly as selomethionine[85]).

With the knowledge gained through metabolic studies and drawing parallel conclusions to work performed in other eukaryotic systems, a more complete understanding of the requirements for a defined media will be obtained. Outside of carbohydrate and amino acids studies, other known requirements of media have been largely ignored, especially those which clearly show promise (i.e. lipids and antioxidants).

3.0 Selected Methodologies

Due to the variety of components expected to be detected in yeast extract (as well as in cell culture metabolism overall), identifying every component is possible through the use of multiple quantitative systems and extensive analysis for qualifying any unknown compounds. However, H-NMR spectroscopy coupled with an extensive software database (Section 3.1) allows for quick quantification for a large variety of compounds while providing insights on any unqualified compounds from residual spectral peaks. However, since yeast extract is known to contain a variety of organic bonds, these may need to be hydrolyzed before they can be detected by H-NMR. Acid digestion has long been a favoured practice to hydrolyze organic bonds, but due to the intense nature of the reaction, several considerations must be made (Section 3.2).

3.1 NMR spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an established technique to quantify a large selection of components found in a variety of biological fluids[69]. Briefly, a liquid sample is subjected to a strong magnetic field which magnetically aligns all components in the sample. Relaxing this magnetic field causes the molecules to re-align themselves to their biologically native state. The machine is then able to interpret this re-alignment and produce an appropriate spectrum to qualify or quantify components of interest.

Historically, carbon-13 NMR (^{13}C-NMR) was the established method to quantify components for use in intracellular flux analysis. However, it is relatively expensive due to the necessity of ^{13}C-labelled components as well as being heavily influenced from the complex interpretation of the spectra. Conversely, the use of proton NMR (or H-NMR) is a much simpler process in terms of sample preparation and data acquisition. Although it was initially used in order to determine the purity of pharmaceuticals, it has recently shown to be useful for the quantification of a wide range of metabolites in a variety of biological fluids, including media monitoring during cell culture and recombinant protein production[75].

The fundamental underlying principle of any NMR is that the intensity of the signal is linearly proportional to the number of nuclei responsible for that signal. These two parameters (signal intensity and number of nuclei present) can be equated by developing a constant specific to the spectroscopy unit dependent on many properties of the actual scan (Bharti and Roy[69] compiled an excellent review of all the specifics related to H-NMR and is recommended for those who would like a more in-depth discussion of

all parameters specific to the scan). These spectroscopy parameters can be accounted for by calibrating a known standard. This can include an internal calibration (a known quantity of a compound is added directly to the sample), external calibration (a known quantity of a component is scanned in a co-axial tube), or electronic calibration (a synthetic signal generated by the NMR). Metabolite concentrations can then be determined by comparing this standard and its intensity to that of the other compounds. This means that any error when generating the standard peak will be present in all subsequent quantities. One of the largest sources of error comes from improper phase and baseline corrections, a mostly user subjective process[70-72].

As implied, H-NMR measures protons, and therefore different functional groups possessing a hydrogen atom are able to be qualified through NMR since they will result in different signal patterns (i.e. a methyl group [-CH_3] will have a much different spin than an ethylene group [-CH=CH-]). These signals are also influenced by neighbouring functional groups and how they interact with protons (i.e. nearby, heavily polar functional groups can distort ideal readings). When performing aqueous H-NMR scans, the main pre-processing of the required signal is to suppress the water peak. If this is not adequately performed, the water signal would completely dwarf any other peaks, making acquisition useless. Due to this suppression, any hydrogens which are able to interchange with water are masked (i.e. uric acid). Further, compounds possessing peaks near water also suffer from inadequate water suppression issues.

As alluded to, proper preprocessing and calibration is required before the NMR spectra can produce any meaningful results. Three of these most important steps are: matching and tuning; water suppression; and shimming[69]. Matching and tuning are important to ensure the 90^0 pulse (i.e. the intense magnetic pulse which aligns all spins before the acquisition) is as effective as possible at exciting the molecular spins. Shimming helps to create a homogenous magnetic field to ensure the peaks are as ideal as possible. Water suppression helps to eliminate the influence of the water peak and increase the sensitivity to minor components. These steps are initially verified by visually inspecting the magnitude of the water peak and the shape of the internal standard.

The sensitivity of an NMR scan depends on a variety of factors, some of which are inherent issues of the NMR process. Since the NMR scan is dependent on the number of protons present, if a compound is at too low of a concentration, peaks can be indistinguishable from the baseline. Further, for those compounds present at a significantly high concentration, their peaks can completely dominate the spectrum and potentially convolute the appearance of the less concentrated components. This same issue can also be encountered if the water suppression is not performed accurately: a large water spike will

distort the spectrum's baseline. To complicate the issue more, the large variety of compounds present in yeast extract can create stearic interference, hindering the effectiveness of the magnetic field and subsequent acquisition. Therefore, while performing scans with higher concentrations could enable some peaks to be seen or become clearer, it could also create issues reading those compounds already present at detectable levels. To overcome this, differing concentrations of the yeast extract can be read to determine the effect the overall ionic strength has on the individual readings. Overall, quantification via H-NMR is expected to have about a 5-10% variance[75].

If the preprocessing is successful, the next step is analyzing and interpreting the resulting spectrum. Normalization to the area of the standard component provides the basis of quantification, which can then be converted into a molar concentration depending on the number of hydrogens represented by a specific peak. From here, there are two major methods of performing area analysis: binning or targeted-profiling. In binning, the spectrum is broken up into several individual sections and the total area in each bin is calculated and normalized. Since each peak location typically represents compounds with similar components, binning allows for an indirect analysis of component groupings. However, since many compounds have more than one readable hydrogen group, there will be multiple resulting peaks, meaning binning tends to increase the number of variables during analysis. Targeted profiling requires fitting a compound's known peaks to those found in the spectrum. This is easily done with software such as Chenomx© NMR Suite©. Chenomx© contains a large library of idealized components peaks, allowing for specific components to be analyzed and quantified, enabling more precise results to be obtained from analysis. However, since the library peaks are ideal (normally acquired in deionized water at various pH levels to ensure maximum applicability), complex mixtures and extreme pH variations cause the resulting peaks to be less ideal than expected, possibly introducing some error and uncertainty. A comparison of binning and targeted profiling is shown in Figure 3.1. Here, both binning and targeted profiling can produce four data points of analysis (although all six bins would be analyzed in practice). Targeted analysis provides evidence that levulinate, succinate, glutamate, and valine are all present in the sample. Binning convolutes many compounds (bin 2 contains three separate components as this spectral location seems to represent $–CH_2–$ between carbonyl [C=O]) and still requires compounds to be identified. While binning and targeting could produce the same amount of data points, this spectrum portion is less convoluted than typical cell culture spectrum. For example, in the work of Weljie's[70] group, 11 compounds required 45 custom bin sizes, over 4 times the amount of data obtained from targeted profiling. Since most of these bins contain redundant information (i.e. multiple bins are for the same compound), this vastly overcomplicates the spectral analysis.

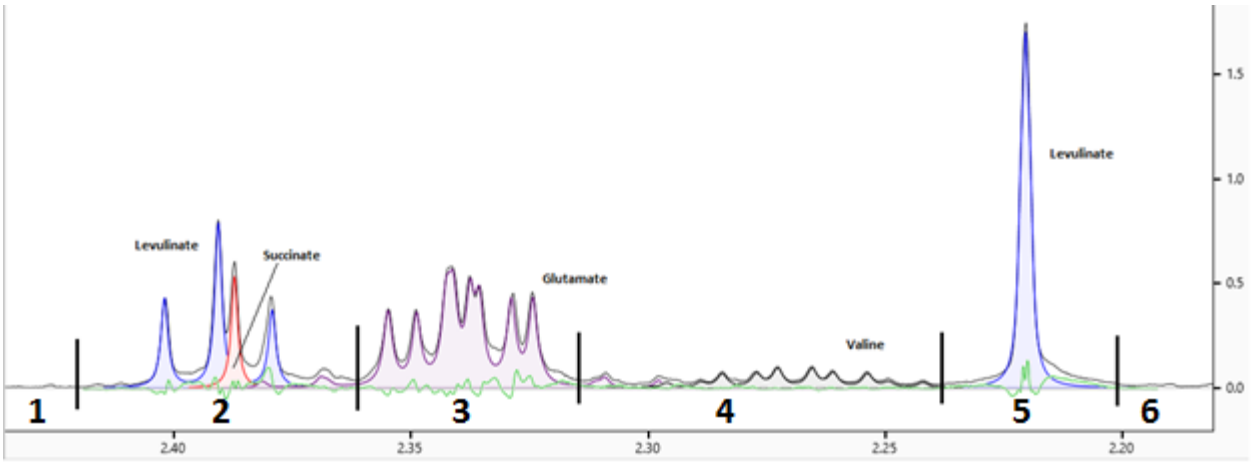

Figure 3.1: Comparison of binning and targeted-profiling techniques for a sample H-NMR spectrum**.** The green line represents the subtraction of the individual ideal peaks from the total spectral area, and here shows that there is very minimal residual leftover, indicating that the remaining area is most likely due to error when fitting an ideal profile in a less-than-ideal environment. Six possible bins of variable size are also shown for comparison.

With targeted profiling, it is important to remember that the confidence in identifying a compound results from the confidence in the presence of its peaks. Therefore if the peaks are heavily distorted or convoluted, or the compound is simple enough to only have one peak in H-NMR, then low confidence should be provided to that identification unless the compound is known to be present. This creates the paradox when trying to qualify unknown compounds in the spectra which only have one peak, or are present in heavily convoluted areas of the spectrum. For example, succinate and pyruvate are both simple organic acids used in the TCA cycle and therefore profiling both of them in the same solution could be possible. However, as shown in Figure 3.2, there is a slight spectral overlap of where these compounds are expected. Therefore, depending on the exact pH and ionic strength of the solution, it would not be impossible to have both peaks in this location. Further, as the peaks represent different quantities of measured hydrogens, the resulting quantification would be different depending on the peak assignment (Figure 3.2 has both components quantified at 0.5 mM). Therefore, to use H-NMR to quantify these compounds, various pH ranges and ionic strengths would have to be analyzed to see if this will separate the compounds far enough for one of them to move into their expected range (assuming the analyzed solutions are still in an appropriate state for quantification via NMR). Apart from the amino acids, glucose, and trehalose, which have been repeatedly quantified in yeast extract, other identified components must be based on how confident the profiling is based on the spectrum (i.e. any new compound should have at least two unconvoluted peaks to be completely confident in its identification). Those not meeting this requirement, or compounds whose presence is of questionable validity, should be considered below the detection limit.

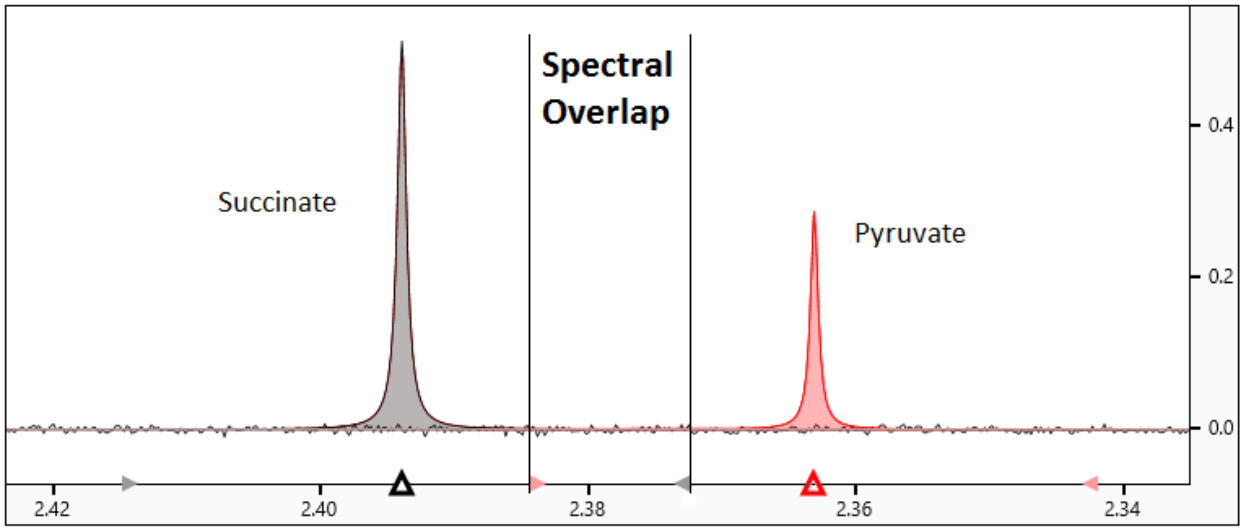

Figure 3.2: Comparison of expected H-NMR peak locations for succinate (black) and pyruvate (red). Both compounds are profiled at 0.5 mM to highlight the difference in quantification if not properly assigned

3.2 Acid Digestion

Acid hydrolysis of proteins has been in practice for decades since the extreme conditions allow for the complete hydrolysis of peptide bonds[14]. However, the process results in the complete destruction of Try and Cys from oxidation; the slight oxidations of Ser and Met; and the conversion of Gln and Asn to Glu and Asp, respectively. Other compounds with amide functional groups can also be converted into their carboxylic counterpart (e.g. niacin is converted to nicotinate). Further, acid digestion completely destroys pentoses and hexoses through several acid hydrolysis pathways[31]. The most prevalent reaction scheme to occur is the dehydration of the hexoses to 5-hydroxymethylfurfural, followed by its hydrolysis to formic acid and levulinic acid. A schematic of the hexose reaction is shown in Figure 3.3. A number of side reactions create glucose reversion products as well as insoluble black precipitate known as humins which must be removed or they will interfere with the NMR scan.

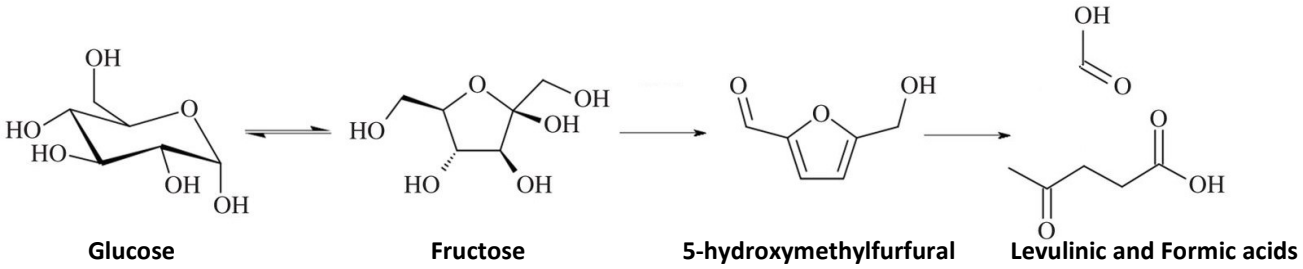

Figure 3.3: Acid hydrolysis mechanism for the conversion of glucose to levulinic acid

3.2.1 Kinetic Modelling

Due to the intense environment required for acid digestion, it is expected that some compounds will be degraded, meaning their final observed concentration could be an underestimation of the total initial content in the yeast extract. Therefore, observing how the solution evolves over time will provide the most comprehensive analysis of the products liberated from the yeast extract, in terms of both actual quantity and any compounds that may be liberated and destroyed within the span of the hydrolysis. Several reaction models are expected, as summarized in the following sections.

Amino Acids

Kinetic modelling of peptides is difficult to accurately model since compounds are liberated from their chains in a random order while many undergo a slight, constant degradation. While most reports will simply extrapolate to a zero time to overcome such issues, Robel and Crane[95] developed a novel (but rather simple) model to account for them directly. They assumed that the amino acids existed in one of three states (peptide, free, or destroyed) but that insignificant destruction of peptide bound amino acid occurs. Since the reaction is monitored by the amount of free amino acids, a simple material balance with a first order assumption on the free amino acids produces Equation 1.

$$r_F = hC - dF \qquad 1$$

Where *r* is the rate, *C* is the peptide concentration, *F* is the free amino acid concentration, *h* is the hydrolysis constant, and *d* is the destruction constant. Normalized amino acid amounts (i.e. g/g and not g/L) are normally used so that *h* and *d* represent a fractional conversion. In constant-volume batch reactor, the free amino acid concentration can be represented by Equation 2 where C_0 is the initial amount of amino acid contained in peptide form,

$$F = \frac{C_0 h}{h - d}\left(e^{-dt} - e^{-ht}\right) \qquad 2$$

As this model was just for a protein solution, Darragh and Moughan[94] extended (and validated) this model for more complex solutions which begin with an initial amount of free amino acids. They modified the model by adding a second destruction term for just the initial free amino acids, as shown in Equation 3 where B_0 represents the initial amount of free amino acid present

$$F = \frac{C_0 h}{h - d}\left(e^{-dt} - e^{-ht}\right) + B_0 e^{-dt} \qquad 3$$

As short chain oligopeptides are dominant in yeast extract, it is highly unlikely that hydrolysis times of over 150 hours is necessary to completely hydrolyze the sample as used in the previous reports[94-97] (12 hours was the chosen hydrolysis time; see Section 4.4 for complete experimental procedure). Since the destruction coefficients are generally 2 or 3 orders of magnitude less than the liberation ones (even for complex solutions[94]), any destruction over this experiment will be insignificant compared to the expected liberation (excluding the amino acids already mentioned). Since there is very little destruction, C_0 should be relatively close to the difference in the amino acid concentrations before and after acid digestion. The very little degradation may also mean that the destruction coefficient can be ignored if the observed reaction profiles do not warrant its inclusion (since NMR will vary by about 5-10%, the noticed decrease should be at least 15% to include the term). This would reduce the model to Equation 4.

$$F = C_o\left(1 - e^{-ht}\right) + B_0 \qquad 4$$

All of the above models assume closed batch kinetics. This is due to the standard practice of performing acid hydrolysis in specialized sealed vials meant to maintain a perfect vacuum batch reaction[14]. Due to economic and safety considerations, this report's hydrolysis is carried out under open reflux (i.e. the volume is not constant throughout the reaction; see Section 4.4 for complete experimental procedure). Using batch kinetics, we can relate the rate of the produced amino acid (N_P) to the reaction volume (V) and then estimate the influence of the volume change on a general rate of reaction (with k as the reaction constant and α as the reaction order), as shown in Equation 5.

$$\frac{dN_P}{dt} = r_P V \qquad \rightarrow \qquad \frac{\left(\frac{dN_P}{dt}\right)_1}{\left(\frac{dN_P}{dt}\right)_2} = \frac{k \left(\frac{N_P}{V_1}\right)^{\alpha} V_1}{k \left(\frac{N_P}{V_2}\right)^{\alpha} V_2} = \left(\frac{V_1}{V_2}\right)^{1-\alpha} \qquad 5$$

Since a first order assumption was shown to provide high correlations with the previous work cited, it will also be used here, meaning there would be no influence from the reaction volume on the overall rate of the reaction and the validated model[94] should be suitable.

Non-Amino Acid Compounds

Apart from glucose and its dehydration kinetics[31], there is very little other research into how the other components present in the yeast extract could possibly react.

Glucose and Dehydration products

Glucose and its expected products, levulinic and formic acids, will most likely have near first order reaction kinetics[31] (making them also unaffected by the open reflux system). While levulinic acid is stable throughout acidic hydrolysis[31], formate would be susceptible to degradation, modeled as a first order reaction. These will be the only components modelled by coupled ODEs, as presented in Equations 4-6, where C_i is the concentration, and *glc*, *lev*, and *for* refer to glucose, levulinate, and formate, respectively.

$$\frac{dC_{glc}}{dt} = d_{glc}C_{glc} \quad (6)$$

$$\frac{dC_{lev}}{dt} = h_{lev}C_{glc} \quad (7)$$

$$\frac{dC_{for}}{dt} = h_{for}C_{glc} - d_{d,for}C_{for} \quad (8)$$

This modelling choice should help to highlight some key aspects of the dehydration mechanism. Previous reports have suggested that very little of the intermediates accumulate during these reactions[31]. However, since there is severe material degradation into humins as well, using the concentration of glucose to drive all the reactions with independent reactions rates provides insights into the relative production rates without requiring the ability to measure the intermediates.

Other Components

For all the other components, visual inspection of their time course profiles provide the first insights into potential reaction mechanisms. Compounds showing an increasing trend and reaching their maximum concentration will be modelled similar to the amino acids, solving for the initial amount, and the hydrolysis and destruction coefficients. If a compound fails to reach a maximum value, a zero order assumption is used to model the initial leg of the true reaction. Compounds only showing a decrease will be modelled by a simple first order power law presented in Equation 9 (i.e. the same as the amino acids but with the hydrolysis constant set to 0):

$$F = B_0 e^{-dt} \quad (9)$$

Parameter estimation

All parameter estimation was performed using the MATLAB *lsqnonlin* and *ode45* built-in functions (where appropriate), along with a user generated script (provided in Appendix A).

4.0 Materials and Methods

4.1 Materials

Yeast extracts were purchased from ThermoFisher Scientific (Massachusetts, USA) and BD Biosciences (California, USA). Sf-900III media was purchased from ThermoFisher. All other components were purchased from Sigma-Aldrich (Missouri, USA) and were of the highest cell culture grade available. For the yeast extract products, three were ThermoFisher lots (Product: BP1422; FL1 [Lot: 163398], FL2 [Lot: 171921], and FL3 [Lot Unknown]) and one was a BD Biosciences lot (Product: 255772; BD [Lot: 6286813]). Sf-9 cells were purchased from ThermoFisher and cultures undergoing less than 50 passages were routinely maintained in the lab in Sf-900III medium.

4.2 H-NMR Spectroscopy

The method for H-NMR spectroscopy is the same as detailed in Sokolenko[71]. Samples were prepared by adding 70 µL of internal standard to 630 µL aqueous mixture for analysis. The standard used is 5 mM DSS (or 4,4-dimethyl-4-silapentane-1-sulfonic acid) dissolved in 99.9% D_2O with 0.2 w/v% sodium azide as an antimicrobial (supplied by Chenomx Inc., Edmonton, Canada). Sample solutions were vortexed and pipetted into 5 mm glass NMR tubes (NE-UL5-7, New Era Enterprises Inc., Vineland, NJ). Samples were scanned on a Bruker Avance 600 MHz spectrometer with triple resonance probe (TXI 600). Scans used the first increment of a 1D-NOESY pulse sequence with a 1 s pre-saturation pulse, 100 ms mixing time, and a 4 s acquisition. Spectra were analyzed using Chenomx© NMR Suite© 8.3 (Chenomx Inc., Edmonton, Canada). Baseline and phase corrections were first performed automatically by the software, then manually adjusted by a human operator, if necessary. The software is a targeted-profiling spectrum software containing a large database of idealized peaks for a variety of components, allowing for a human operator to manually fit convoluted peaks. Further, the software allows the creation of new compound profiles created by the user to expand their database for specific purposes. When analyzing samples from a time-course, the correction method developed by Sokolenko[71] was applied to the data in order to minimize the error associated with systematic sampling bias. On occasion, the spectra were profiled by a separate operator to determine profiling bias.

4.3 Statistical Analysis and Quantification in Hydrolysates

To determine whether the complex yeast extract solution would impart a higher degree of uncertainty in an NMR scan, three solutions of 10g/L were made by dissolving 0.5 g from one ThermoFisher lot (FL3) and adding 49.5mL of Milii-Q water and scanned in quadruplicate. Lot comparison was performed with four solutions each of the four yeast extract lots available subjected to duplicate scanning. The solutions and their spectra were statistically analyzed based on the resulting quantification ANOVA, as well as principal component analysis. Principal component analysis (PCA) is a multivariate statistical technique which models observations with inter-correlated variables as orthogonal, unrelated components to determine how alike the observations are or to be used as a model for future predictions[83]. All PCA was performed with R software using the RStudio interface and the ggplot2 and FactoMiner packages.

Compound identification and quantification was performed on both dilute (10g/L) and concentrated (>80g/L) solutions. This was to analyze how significantly the hydrolysate concentration contribute to the overall quantification of the spectra.

4.4 Acid Digestion Method

Acid hydrolysis of yeast extract was carried out according to classical procedures[14]. Yeast extract was digested in 6M HCl with 0.02 w% phenol. To see how the yeast extract responded during the reaction, a time course procedure under open reflux conditions was established. The high salt content of the native digestion solution upon neutralization would make analysis via NMR more difficult (i.e. all the ions interfere with the matching and shimming of the sample due to ionic strength) and thus the following protocol was established.

50mL of 12M HCl was added to the reactor, its mass was recorded, and was stirred on a magnetic hot plate set to 300^0C. Next, 40mL of a 100 g/L yeast extract solution and 10mL of a 0.2w% aqueous phenol solution were added to a 50mL Falcon™ tube and the total solution mass was recorded. The yeast extract solution was placed in a hot water bath (~70^0C) as the reactor was brought up to temperature. When the HCl began to boil (~50^0C), the yeast extract solution was added. The solution was maintained at a visual boil (~110^0C). These volumes were chosen in order to minimize the risk to the experimenter as well as the amount of material required for a digestion. The reaction was halted after a specified time by placing the reactor in an ice-water bath until its temperature equilibrated. An appropriate amount of 6M HCl was added to reacquire the initial starting mass of the digestion solution and a 1mL sample was withdrawn and added to 8mL of 150mM Na_2HPO_4 (pH 7) and 0.95mL of 6M NaOH. This step is crucial to neutralize

the sample and reduce the total amount of salt for NMR analysis. Additionally, the neutralization and dilution create ideal conditions to flocculate the humins and allow them to precipitate easier from the solution. The samples were centrifuged for 2 min at 2000 x g to pellet the humins as they interfere with the NMR analysis. In order to perform a time-course experiment without significantly affecting the total initial material in the reactor, batch reactions were performed for the length of the time point required. Since being performed under open reflux instead of in sealed containers, the volume of the solution was adjusted by adding about 10g of 6M HCl per hour to counteract the evaporation. The experiment was performed in triplicate.

4.5 ICP Analysis

The various yeast extracts were analyzed using ICP OES for 20 compounds: Mn, Fe, Ca, Cu, Zn, Na, Ni, Pb, Mg, K, Al, Ba, B, Cd, Cr, Co, Li, Sr, Si, and V. The samples were prepared by adding yeast extract to ultra-pure water and using an *aqua regia* digestion at 100^0C to remove the organic components, leaving the inorganic components for analysis. Spectra were analyzed on a Prodigy Higher Dispersion ICP (Teledyne Leemans Labs, New Hampshire, USA). The results were compared to the minimum detection limits of blank runs to eliminate results that may have been attributed to background noise. Stocks of *aqua regia* were made by mixing pure hydrochloric and nitric acids in a 4:1 volumetric ratio. When required, samples were diluted with ultra-pure water from a Simplicity® water purification system (Sigma-Aldrich).

4.6 Cell cultures

Cell cultures were performed the same as Sokolenko et. al[71]. Briefly, *Spodoptera frugiperda* (Sf9) cells were grown in shake flasks at 27 °C and 130 RPM using a fortified IPL-41 medium and commercial Sf-900III medium. The cells were routinely split to 0.6 x 10^6 cells/mL upon reaching a concentration of 2-3 x 10^6 cells/mL, with experiments carried out on cells that have undergone less than 50 passages. A 250 mL mother flask was seeded at 0.6 x 10^6 cells/mL with a working volume of 70 mL and grown up to 2-3 x 10^6 cells/mL. This flask was used to seed 125 mL flasks at 0.6 x 10^6 cells/mL at a working volume of 35 mL. Cells were counted and sampled for NMR every 24 h until reaching their maximum concentration. Cell counts were performed using 0.05% trypan blue in PBS, and counted on a hemocytometer. Trypan blue stains inactive cellular membranes, and thus provides a good indication of live or dead cell material during manual counting. The 1.5 mL samples of cell culture media were centrifuged for 10 min at 250 g, with the supernatant stored at -80 °C until analysis. Adapting the cells to new media was generally achieved by passaging the cells into 50% more new media when the cells reached at least 2 x 10^6 cells/mL and 85%

viability (i.e. the first passage was into media 50% Sf-900III and 50% new media, and all subsequent passages were in 100% new media).

For the various metabolic studies, one lot of basal media was used for FL3, while a second lot was used for FL1, FL2 and BD. This was due to FL3 being exhausted in the lab before the other yeast extracts had been obtained.

4.7 Media Preparation

Large batches of media were prepared by dissolving the appropriate amount of amino acids, salts, and sugars into approximately 700 mL of ultra-pure water (University of Waterloo E6 custom ultra-pure water filter, Durpro, Quebec, Canada) (R>17MΩ). Concentrates of lipids (Chemically Defined Lipid Solution 1, Sigma-Aldrich), Cys/Tyr, trace metals, and vitamins (all three solutions were made in house with chemicals purchased from Sigma) were then added and the solution was stirred for approximately 30 min without heating. The pH was adjusted to 6.2-6.4 with approximately 10mL of 2.5M NaOH and the solution volume was increased to 1 L using a volumetric flask. The solution was sterile filtered with a 0.2 µm PES vacuum filter into a sterile bottle. Media was stored at 4^0C and always less than 6 months old. The chemically defined basal medium was a fortified IPL-41 medium containing several improvements from various metabolic studies. This medium was designated the Aucoin Lab Insect-cell Medium, or ALIM. The complete formulation can be found in Appendix B.

5.0 H-NMR Analysis of Yeast Extract

These results are separated into two main sections. Section 5.1 will analyze the variability imparted by the H-NMR spectral quantification of a 10 g/L solution of yeast extract (specifically lot FL3) without acknowledging the appropriateness of the qualification of those peaks. Section 5.2 will analyze the variability between the lots, as well as providing a biological rational for each of the qualified compounds.

5.1 Statistical Analysis of Yeast Extract

In total, 50 compounds were identified in the 10g/L solutions and were used to determine the statistical variation between separate scans and solutions of yeast extract. These compounds were also compared to their levels found in more concentrated yeast extract solutions.

Figure 5.1a-d presents the boxplots for the compound concentrations and their variance between solutions. ANOVA highlighted only two statistically different compounds: glucose in solution C was different than A and B (which were not different from each other); and lactate was different between solutions A and B (neither were different from C). When the overall averages are examined (Table 5.1), glucose has a CoV of less than 5%, well within the expected error range; lactate is much larger at over 15% due to it being in a heavily convoluted location on the spectrum. Overall, compounds present at greater than 0.1 w% generally have a CoV of less than 10% and allow for accurate qualification in a 10g/L solution.

Due to the trace levels of some components, more concentrated yeast extract solutions were made to boost the signal intensity. Figure 5.2 displays the amount quantified in the more concentrated scan as compared to the dilute (1%) solution. On average, the higher concentrated solutions quantified about 90% of that determined from the 1% solutions. Only a few components did not consistently quantify within 25% of the detected amount in the 1% solutions: Arg, His, Lys, sarcosine, pantothenate, methanol and ornithine. This bias should mean that multiple concentrations of a complex solution should be analyzed in order to have a clearer quantification process. However, while this bias can be worrisome, the higher solution concentrations do reveal new compounds because of the increase in their absolute abundance and now allow for detection (if these peaks are in un-convoluted regions of the spectrum; as discussed in Section 5.2).

Table 5.1: Overall average weight percent of the quantified components: YX-FL3 – 10g/L. Italicized components are of low qualification confidence due to heavy peak convolution or near the apparent LDL. Yellow: CoV>5%; Red: CoV>10%. *a-glycerophosphocholine

Amino Acids	**W%**	**CoV**
Alanine	3.39%	3.25%
Arginine	1.72%	2.27%
Asparagine	1.71%	2.17%
Aspartate	2.20%	2.84%
Cystine	*0.06%*	*21.90%*
Glutamate	7.12%	3.07%
Glutamine	*0.37%*	*5.32%*
Glycine	1.48%	3.41%
Histidine	0.73%	2.94%
Isoleucine	2.64%	3.02%
Leucine	3.77%	2.60%
Lysine	1.91%	3.15%
Methionine	1.02%	2.27%
Phenylalanine	2.43%	2.58%
Proline	0.95%	2.81%
Pyroglutamate	1.52%	3.62%
Serine	2.18%	4.06%
Threonine	2.09%	2.40%
Tryptophan	0.70%	2.09%
Tyrosine	0.66%	3.12%
Valine	2.96%	3.63%
Total	**41.62%**	

Sugars	**W%**	**CoV**
Glucose	0.56%	3.65%
Trehalose	4.49%	2.34%
Total	**5.05%**	

Nucleic Material	**W%**	**CoV**
Adenine	*0.02%*	*10.18%*
Adenosine	1.35%	2.14%
Cytidine	*0.05%*	*15.21%*
Guanosine	*0.14%*	*3.02%*
Hypoxanthine	0.04%	6.76%
Inosine	*0.02%*	*16.13%*
Uracil	0.16%	3.43%
Uridine	0.35%	6.16%
Total	**2.13%**	

Vitamins	**W%**	**CoV**
Choline	0.03%	4.20%
Niacinamide	0.06%	6.20%
Nicotinate	0.05%	7.31%
Pantothenate	*0.12%*	*9.58%*
myo-Inositol	0.13%	14.08%
Total	**0.38%**	

Micronutrients	**W%**	**CoV**
4-Aminobutyrate	0.10%	3.36%
Betaine	1.22%	2.33%
Glycerol	0.08%	7.51%
Ornithine	0.18%	4.55%
Sarcosine	*0.01%*	*13.14%*
a-GPC*	0.30%	3.03%
Total	**1.88%**	

Organic Acids	**W%**	**CoV**
Acetate	0.41%	4.44%
Formate	0.03%	5.96%
Lactate	*0.06%*	*15.82%*
Succinate	0.60%	2.33%
Total	**1.10%**	

Alkylates	**W%**	**CoV**
2-Hydroxyisobutyrate	*0.01%*	*8.70%*
3-Hydroxyisovalerate	*0.06%*	*4.51%*
Butyrate	0.09%	6.23%
Isobutyrate	*0.16%*	*3.05%*
Total	**0.32%**	

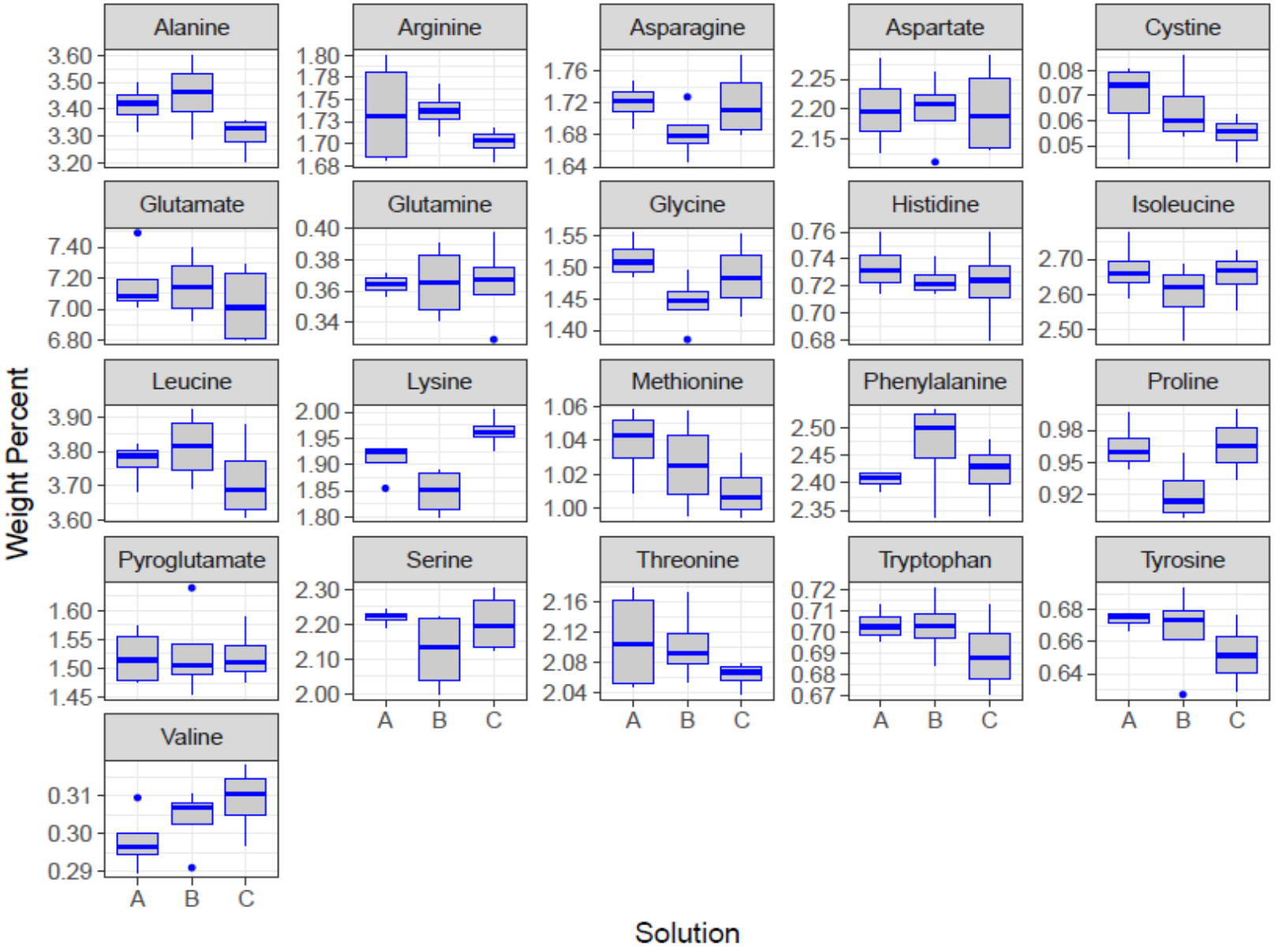

Figure 5.1a: Amino acid quantification variability in three different 10 g/L solutions of yeast extract FL3

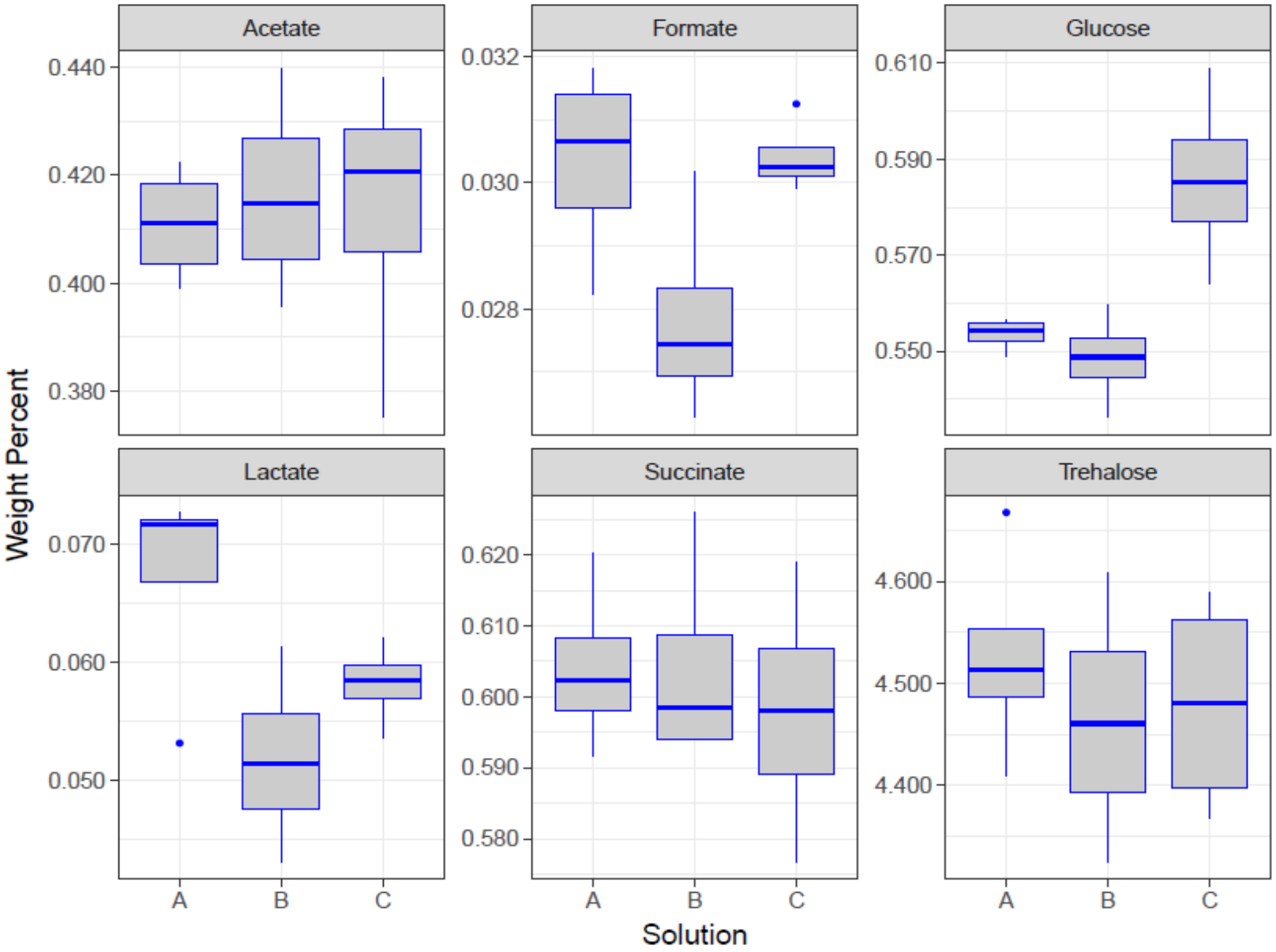

Figure 5.1b: Organic acids and sugars quantification variability in three different 10 g/L solutions of yeast extract FL3

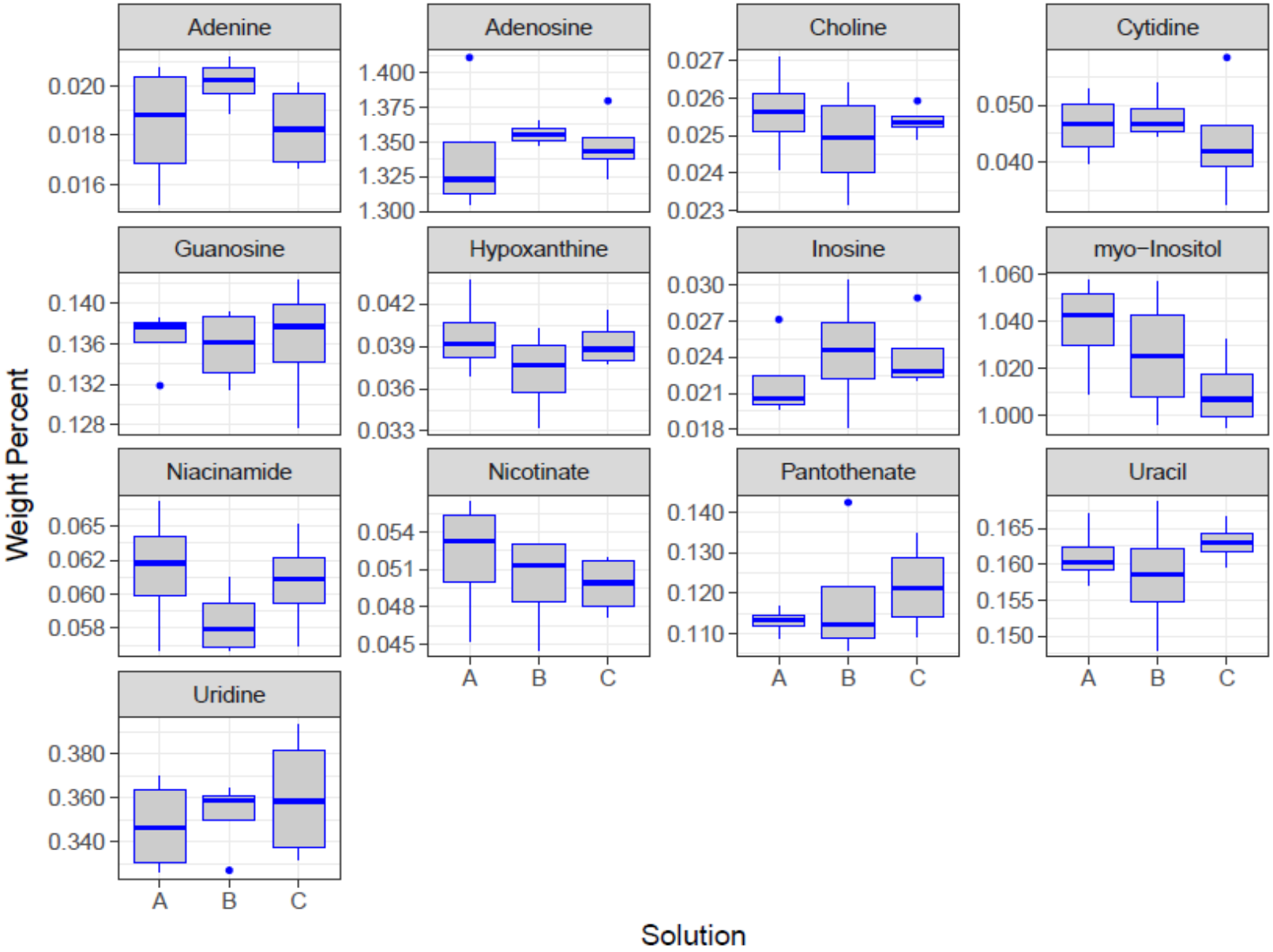

Figure 5.1c: Nucleic material and vitamins quantification variability in three different 10 g/L solutions of yeast extract FL3

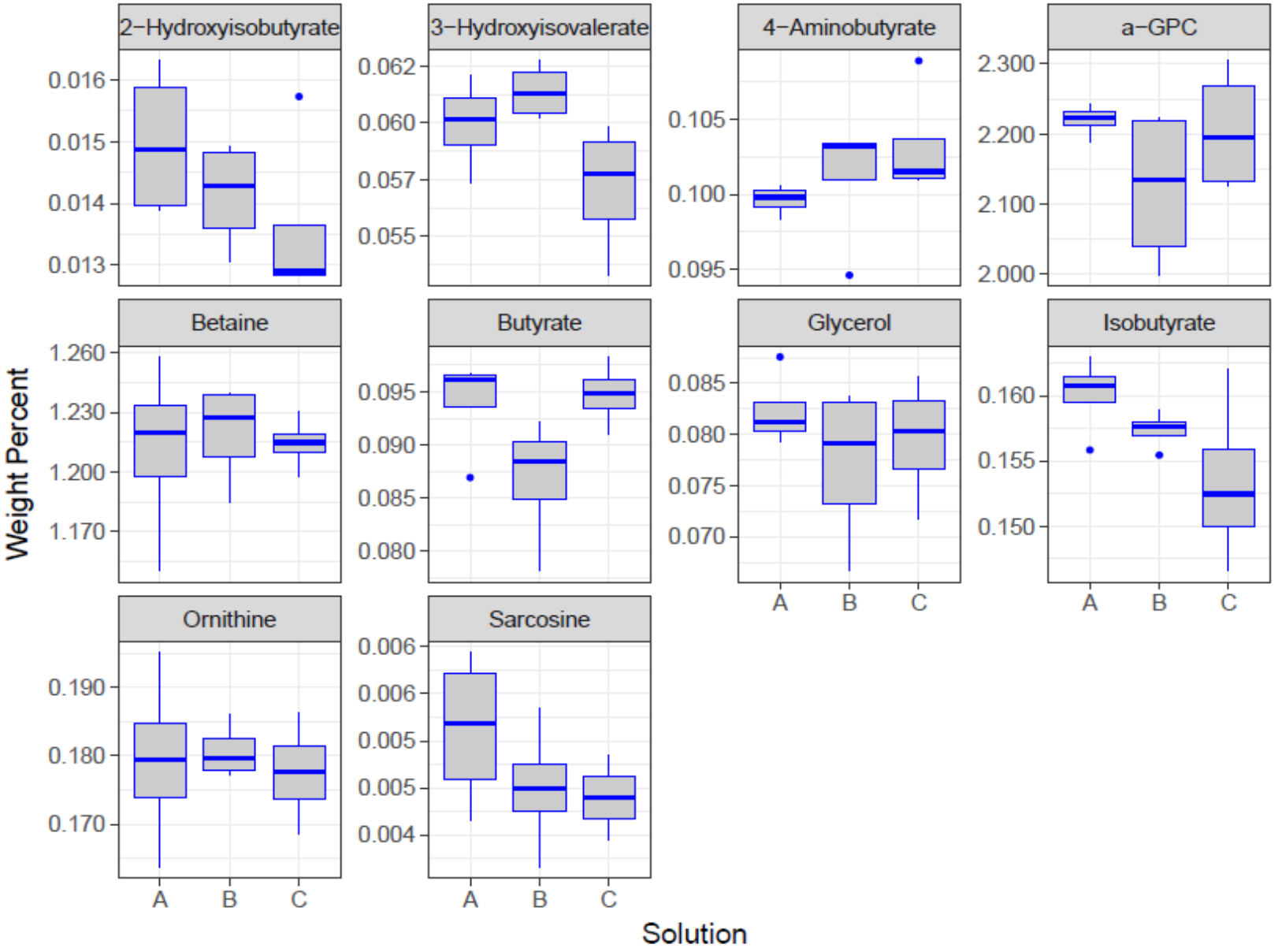

Figure 5.1d: Micronutrients and alkylates quantification variability in three different 10 g/L solutions of yeast extract FL3. a-GPC: a-glycerophosphocholine

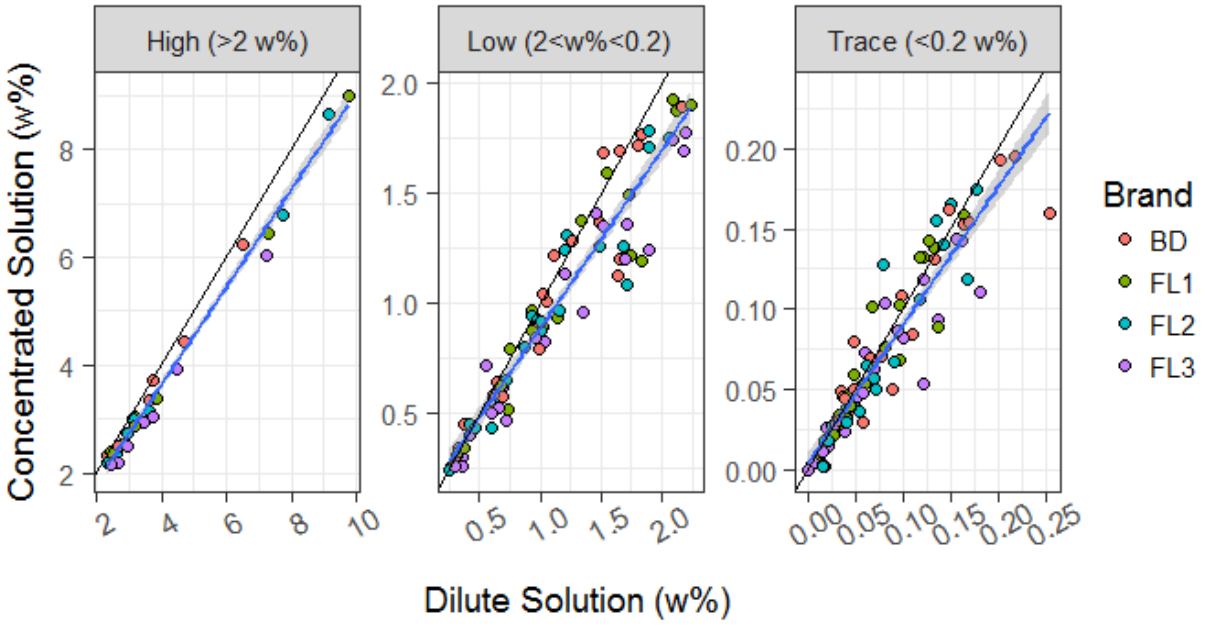

Figure 5.2: Quantification between dilute (10 g/L) solutions and more concentrated solutions (80 g/L for BD and FL3; 100g/L for FL1 and FL2. Black line: y = x; blue line: trendline with 95% confidence limits

5.2 Compound Identification and Quantification

In total, 60 compounds were qualified in the yeast extract, 42 of them with high confidence, as detailed in Table 5.2.

Table 5.2: Identified components in all yeast extract lots. Italicized components are of low qualification confidence due to heavy peak convolution or near the apparent lower detection limit (LDL)

Amino Acids		**Sugars**	**Nucleic Material**	**Organic Acids**
Alanine	Lysine	Glucose	*Adenine*	Acetate
Arginine	Methionine	Trehalose	Adenosine	Citrate
Asparagine	Phenylalanine		*Cytidine*	Formate
Aspartate	Proline	**Micronutrients**	*Guanosine*	*Fumarate*
Cystine	Pyroglutamate	4-Aminobutyrate	Hypoxanthine	*Lactate*
Glutamate	Serine	a-GPC	*Inosine*	Succinate
Glutamine	Threonine	Betaine	Uracil	*trans-Aconitate*
Glycine	Tryptophan	*Ethanol*	Uridine	
Histidine	Tyrosine	Glycerol	**Vitamins**	**Alkylates**
Isoleucine	Valine	*Methanol*	*4-Pyridoxate*	*2-Hydroxyisobutyrate*
Leucine		Ornithine	Choline	*3-Hydroxybutyrate*
		Sarcosine	myo-Inositol	*3-Hydroxyisovalerate*
		Trigonelline	Niacinamide	Butyrate
			Nicotinate	*Isobutyrate*
			Pantothenate	*Isovalerate*

A wide cross section of a variety of biological classes were obtained: amino acids, sugars, nucleic material, vitamins, organic acids, and other active biological molecules. Figure 5.3 presents the lot-to-lot variability of the yeast extracts, broken into rough quantity bins depending on the level quantified. Overall contribution based on these identified biological groupings are shown in Table 5.3

Table 5.3: Overall contributions of the biological groups for each yeast extract lot (w%).

	BD	FL1	FL2	FL3
Alkylates	0.24	0.28	0.29	0.37
Amino Acids	38.86	41.66	39.83	41.68
Micronutrients	3.52	1.65	2.10	1.88
Nucleic Material	1.60	1.64	1.71	2.15
Organic Acids	1.19	1.19	1.31	1.13
Sugars	5.94	10.48	10.13	5.04
Vitamins	0.63	0.38	0.39	0.41
Total	**51.98**	**57.29**	**55.77**	**52.68**

5.2.1 Amino Acids and Sugars

All twenty standard amino acids were found, along with pyroglutamate. Pyroglutamate is produced from phosphate reacting with glutamine and is most likely formed during the production of the yeast extract. These compounds were expected to be present, and results are similar to the values previously cited[16]. Only cystine and glutamine quantification are uncertain due to being near the LDL. Glucose and trehalose were the main sugars quantified but there were drastic differences in the trehalose content between the four lots, which could be an indication of altered biomass growth or downstream processing of the yeast extract.

5.2.2 Organic Acids

A few TCA cycle intermediates were identified; of them, citrate was the least confidently quantified. Even though fumarate and trans-aconitate were only detected at the higher concentrations, the observed peaks are not convoluted with any others, lending them relatively higher confidence. Formate and acetate, although not a part of the TCA, are integral organic acids in a variety of reactions. Both compounds have a single peak, but still allow relative confidence in their quantification (acetate is slightly convoluted but not to a concerning degree). Lactate is present as by-product of the TCA or produced by lactic-acid bacteria contamination of the hydrolysate lot[82]. The lactate readings are highly variable, and its peaks are normally heavily convoluted in all spectra, imparting very little confidence on its quantification.

5.2.3 Vitamins

Several B-Vitamins were identified, in agreement with other reports[33, 34]. Vitamin B3 (as nicotinate and niacinamide) was confidently identified, whereas vitamins B5 (pantothenate) and B6 (as 4-pyridoxate) were tentatively quantified due to the convolution of their peaks. Further, myo-inositol and choline, which have historically been considered B vitamins were also identified. Overall, the identified vitamins account for about 0.5 w% of the yeast extract, or about 20 mg. As a contrast, these vitamins are only added to IPL-41 at a total mass of 1mg. With the many functions performed by the vitamins, an over 20x reduction in the overall vitamin levels should have a detrimental impact on the growth of a culture when grown without yeast extract.

5.2.4 Nucleic Material

A significant amount of nucleic material was also quantified in agreement with previous reports[76, 77]. In this study, adenosine, uridine and uracil were confidently profiled, whereas all the others have considerable uncertainty. Further, there were several areas of the spectrum around identified nucleic material with clear peaks but could not be identified. From their location and shape, they could be indicative of more material containing nucleic components (more evidence of this can be seen in the time-course study in Section 5.1.3). Since ribose and nucleotides are not supplied in the basal medium, this material would allow the cell to have an active pool of nuclear metabolites immediately available (possibly allowing for a quicker adaptation and faster growth as cellular resources do not have to be diverted to create these compounds) or as they could be acting in some signaling capacity informing the cell of the quality of its surroundings.

5.2.5 Other Biological Components

Betaine, choline, and sarcosine are related compounds active in a variety of ways, notably in glycine metabolism and as a head group in phospholipids. Betaine and choline were profiled with high accuracy whereas sarcosine only has one unconvoluted peak. Ornithine is a non-standard amino acid and an intermediate product when arginine is converted into putrescine (a polyamine). Since several reported chemically defined media require the addition of a polyamine[66, 67], the ornithine could be acting in this capacity. Glycerol is essential to the production of phospholipids required for the various membranes of the cells. While it could potentially be added to media formulation, the separate lipid supplementation has been shown to provide adequate lipid metabolites. There is also significantly more choline and a-

glycerophosphocholine (a-GPC) in the BD lot as compared to the FL lots. Since a-GPC is a major storage molecule for choline in yeast cells, the significantly higher levels could indicated that the BD growth medium contains significantly more choline than the medium used in the FL lots.

5.2.6 Alkylates

The final group of identified compounds, designated the alkylates, are highly suspect except for butyrate. The other compounds are present in very low quantities, or only have one unconvoluted peak. While 3-hydroxyisobutyrate and 2-hydroxyisovalerate are known to be active in a couple of yeast metabolic pathways, the others do not have any specific role. More likely, these components are place holders in the analysis for other methyl or ethyl groups on otherwise unidentified compounds. Therefore, while they are profiled in subsequent spectra, they are not expected to be required in a chemically defined medium.

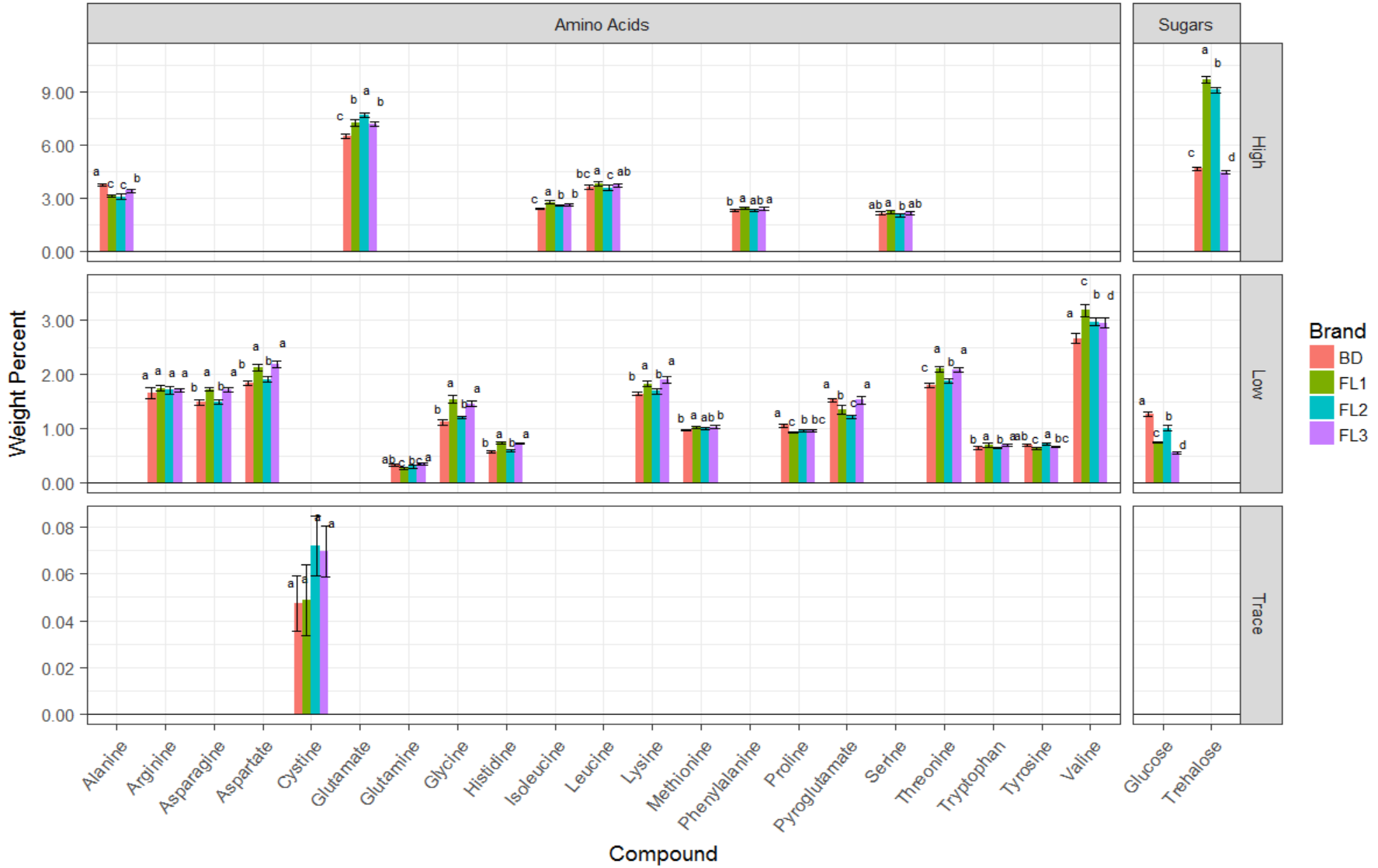

Figure 5.3a: Lot variation of amino acids and sugars. Components levels are based on 10g/L solution concentrations: "High": >2%; "Low": 2%< x<0.1%; "Trace" : <0.1%. Bars with the same letters are not significantly different.

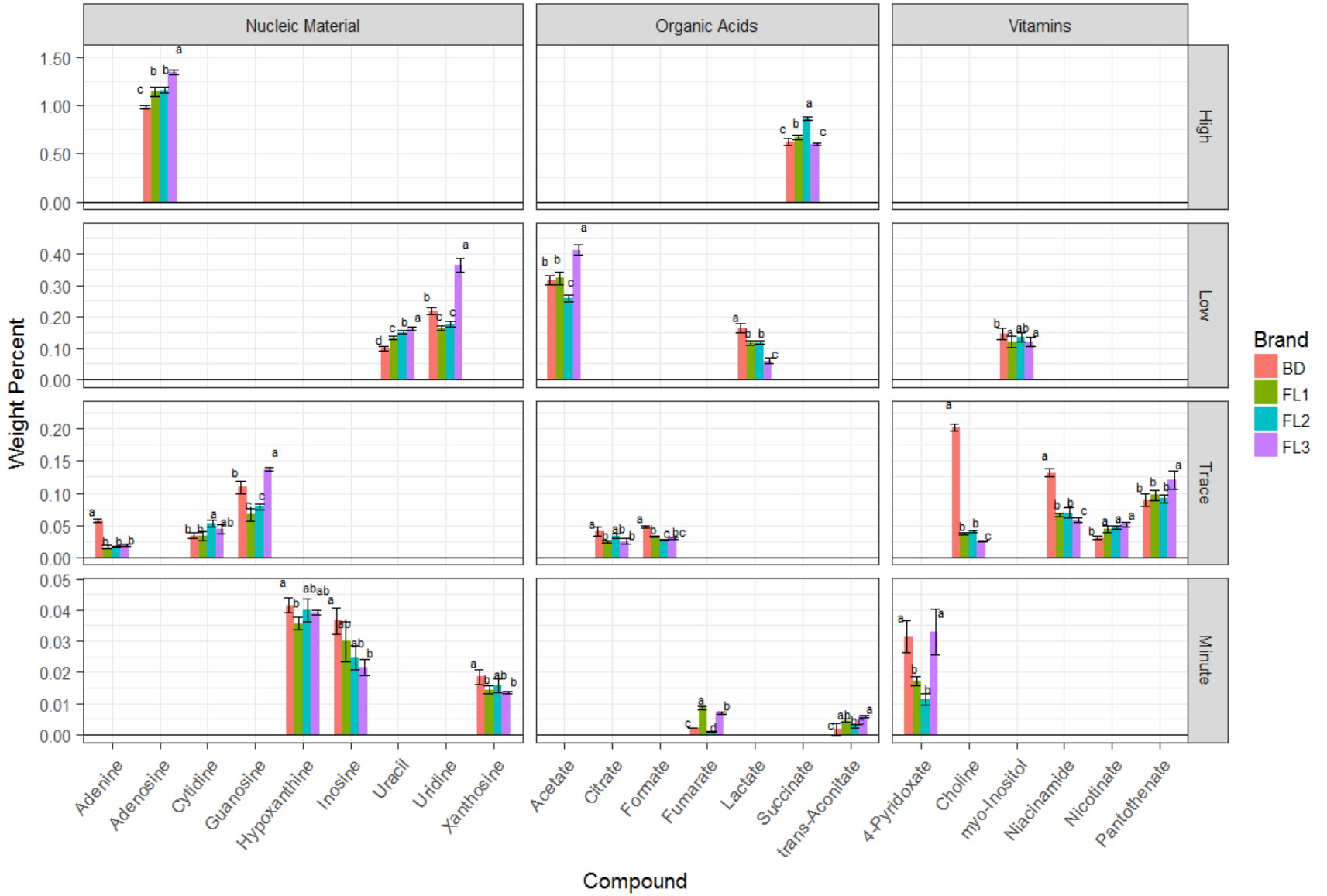

Figure 5.3b: Lot variation of nucleic material, organic acids and vitamins. Components levels are based on 10g/L solution concentrations: “High”: >1%; “Low”: 1%< x<0.1%; “Trace”: 0.15%< x<0.05%; “Minute” : <0.05%. Bars with the same letters are not significantly different.

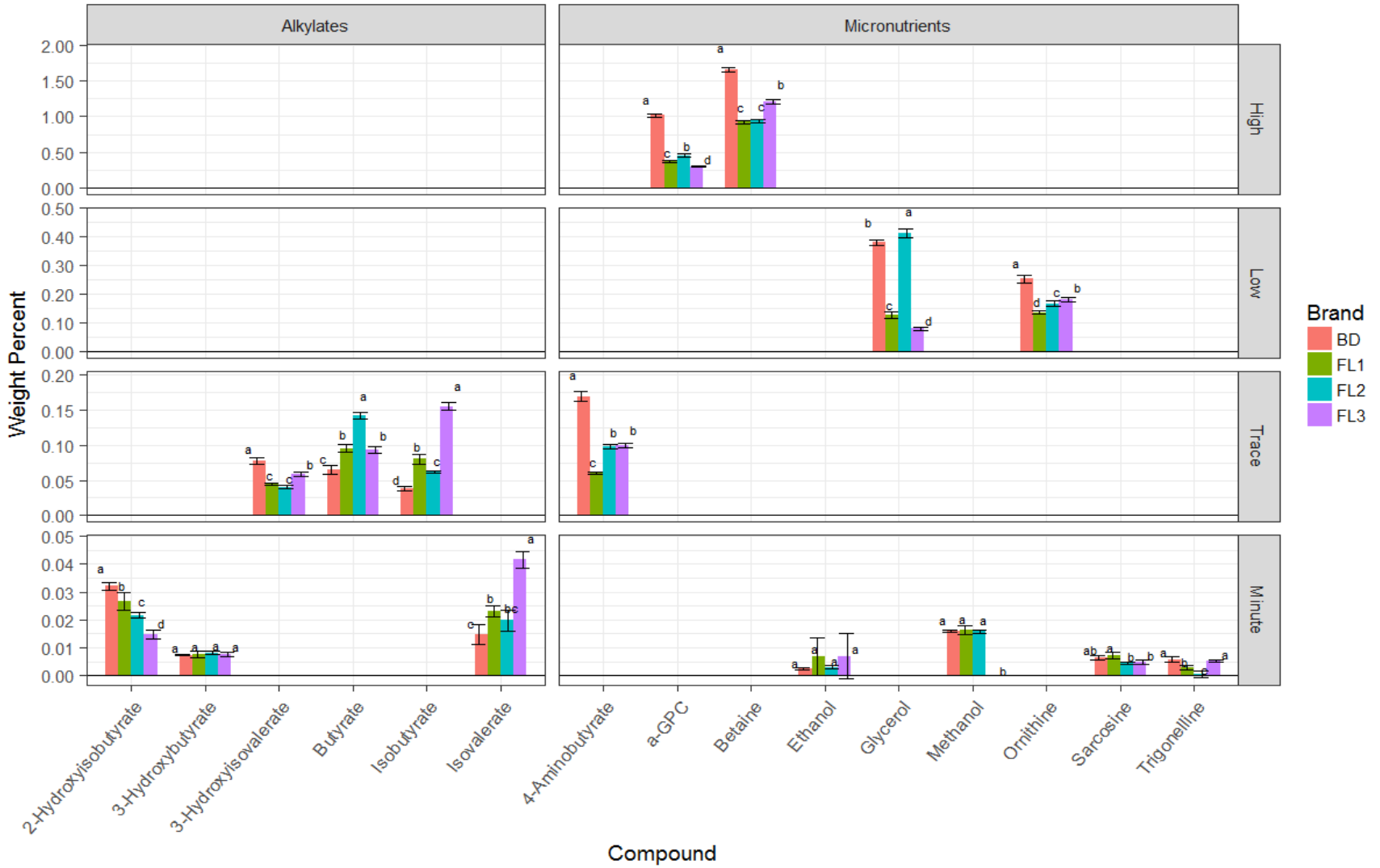

Figure 5.3c: Lot variation of micronutrients and alkylates. Components levels are based on 10g/L solution concentrations: “High”: >1%; “Low”: 1%<x<0.1%; “Trace”: 0.15%<x<0.05%; “Minute”: <0.05%. Bars with the same letters are not significantly different.

5.3 Principal Component Analysis

As stated in Section 4.3, PCA can be used to determine how alike a group of observations are[83]. By using it on the quantified components in yeast extract, it should be able to distinguish the major differences from various scans of the same solution, as well as how different the various lots of yeast extract are.

5.3.1 Analysis of Scanning Variation

Eight principal components accounted for 89% of the variance. As the solutions were all of the same lot of yeast extract, a high number of components indicates a very similar dataspace and the model is overcompensating to fit the data due to random error[83]. Figures 5.4 - 5.6 represent the compound loadings and Figures 5.7 – 5.9 represent the solution scores for the first three components only (49.0% of the total variance). Table 5.4 documents the most variable compounds, while Table 5.5 documents the most variable scans for each of the first three components.

Table 5.4: Top five variable variance contributions for the first three principal components, single lot analysis

Component					
1	Butyrate (8.6%)	Leucine (7.9%)	Glycerol (6.3%)	Phenylalanine (6.3%)	*Lactate* (5.1%)
2	Trehalose (9.0%)	Uracil (7.5%)	Pyroglutamate (6.8%)	Methionine (6.2%)	Proline (5.4%)
3	Glutamate (10%)	*Cystine* (8.6%)	4-Aminobutyrate (7.4%)	Niacinamide (6.2%)	Nicotinate (5.8%)

Table 5.5: Top five individual variance contributions for the first three principal components, single lot analysis. ID format: Solution-Scan

Component				
1	B4 (52.0%)	B1 (15.4%)	C1 (11.4%)	C2 (10.3%)
2	A1 (35.6%)	B4 (16.6%)	C3 (14.4%)	B1 (12.0%)
3	C2 (27.9%)	A1 (13.9%)	B2 (13.3%)	A2 (13.3%)

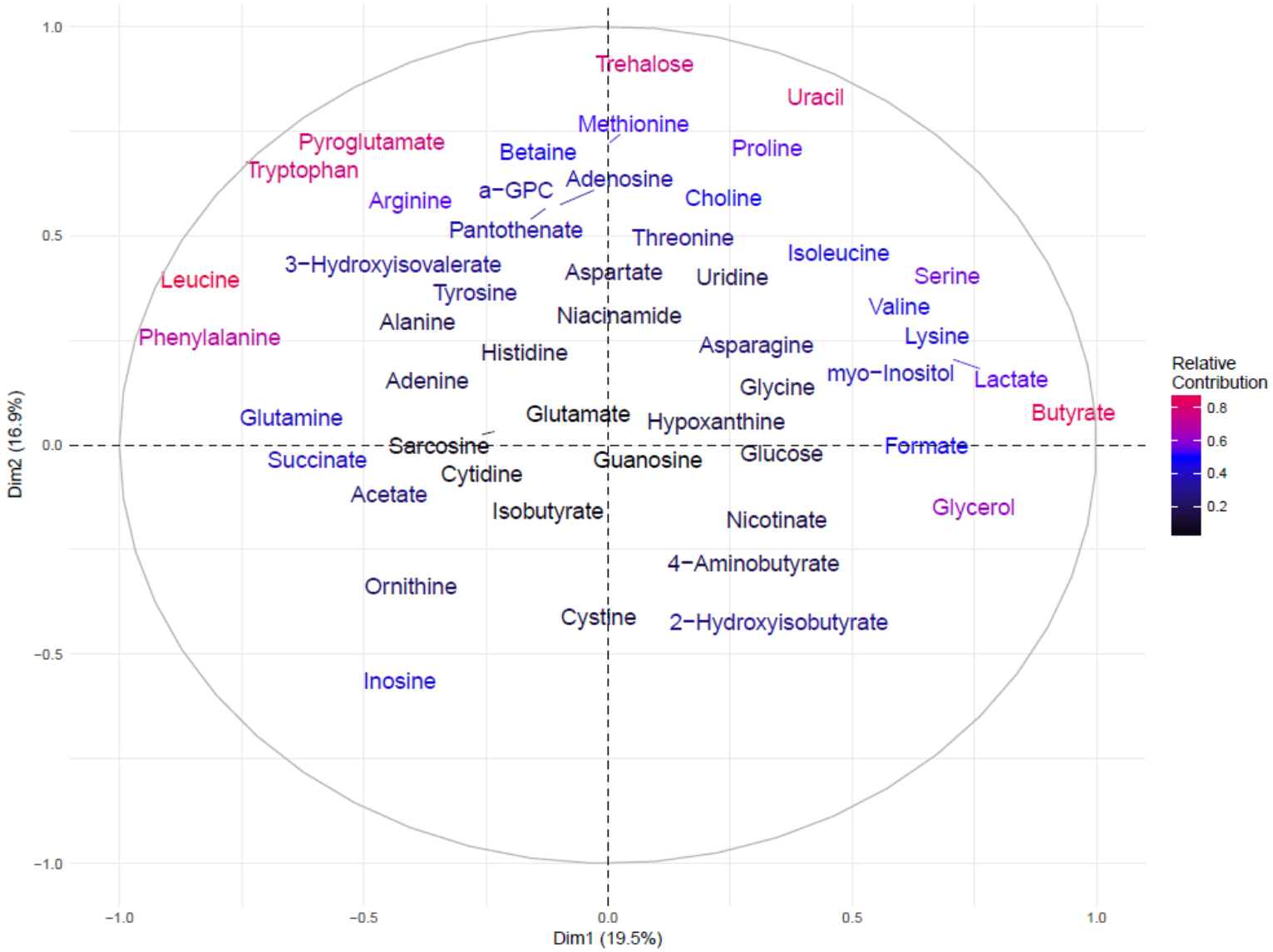

Figure 5.4: First and second principal component loadings for the scanning variance analysis

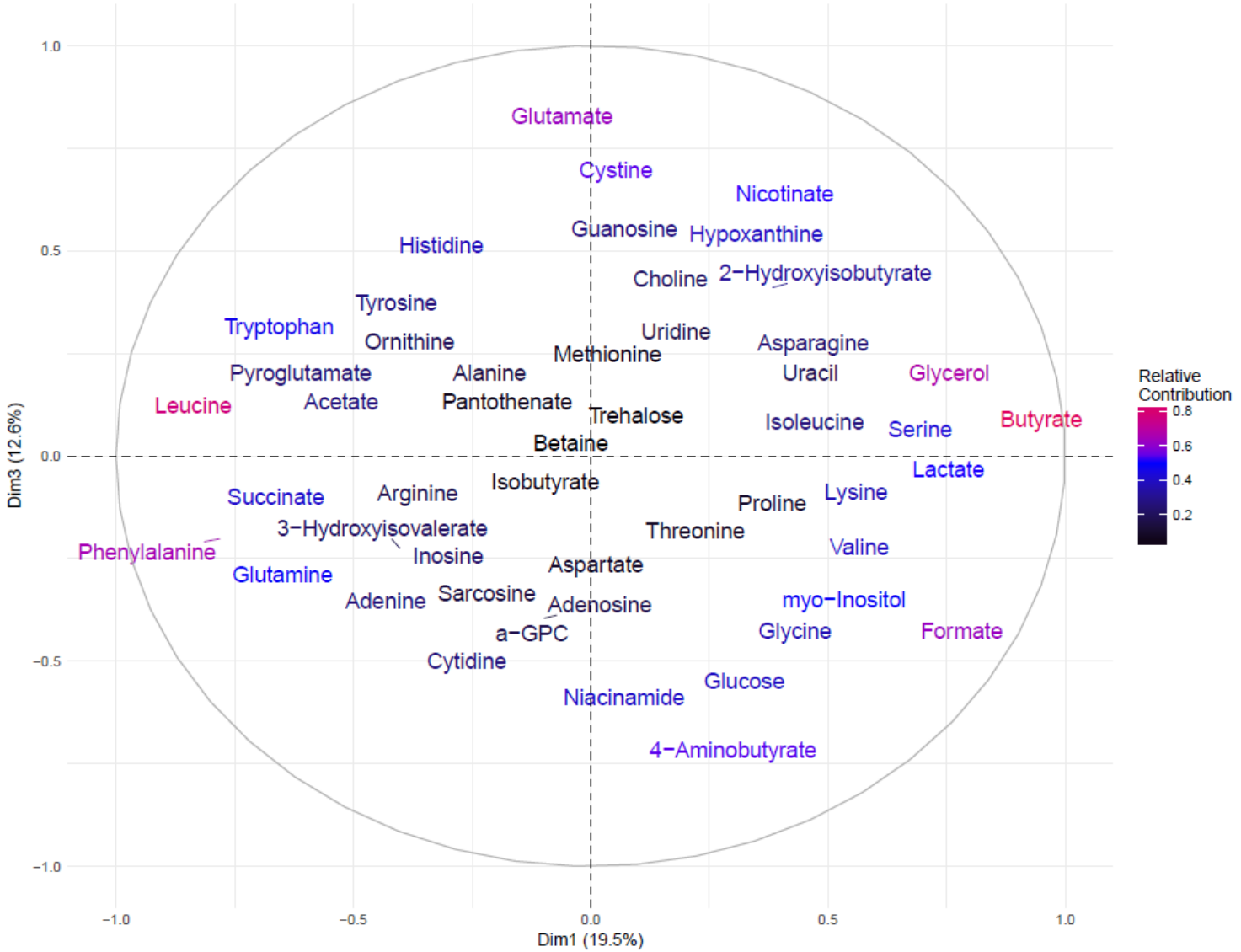

Figure 5.5: First and third principal component loadings for the scanning variance analysis

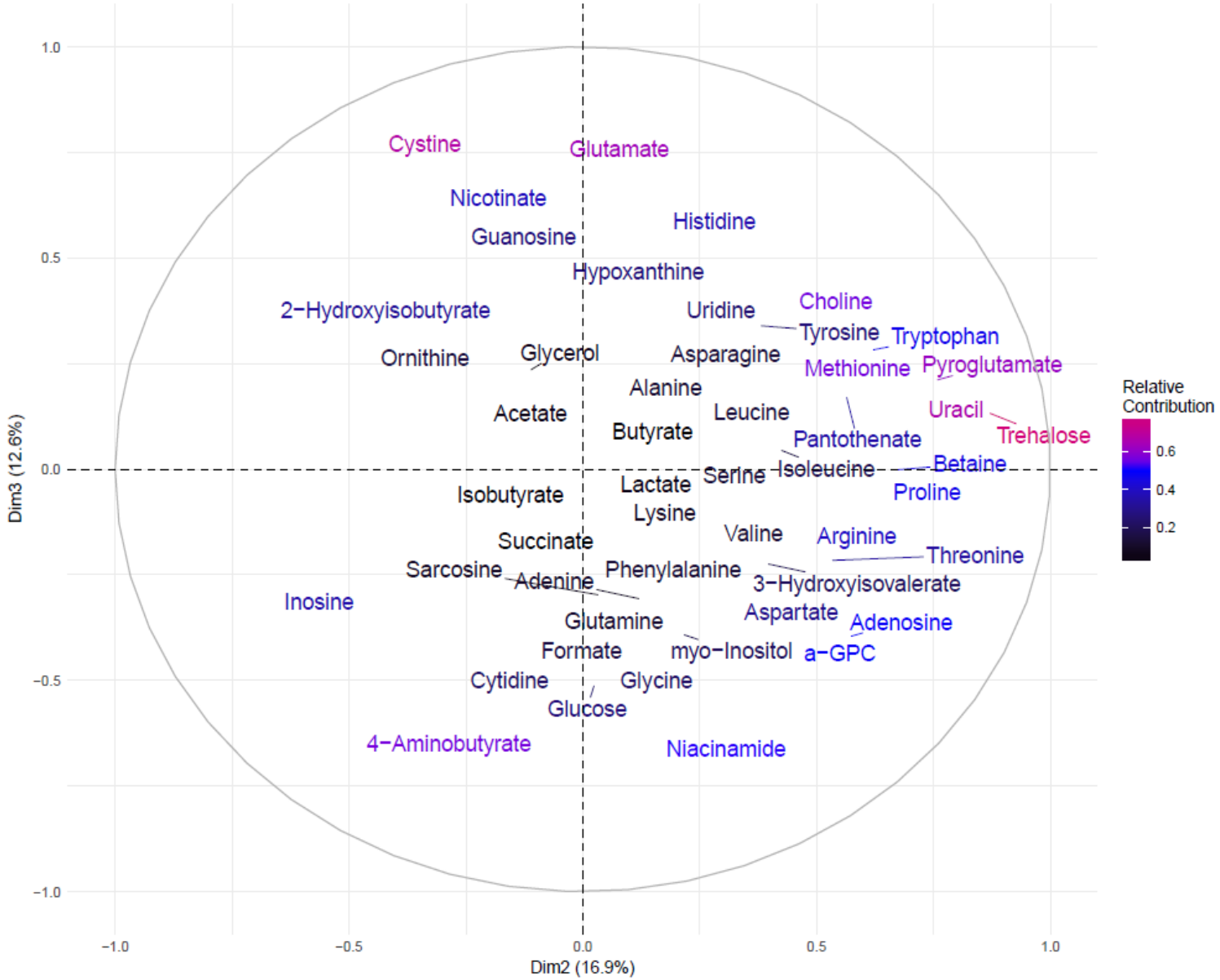

Figure 5.6: Second and third principal component loadings for the scanning variance analysis

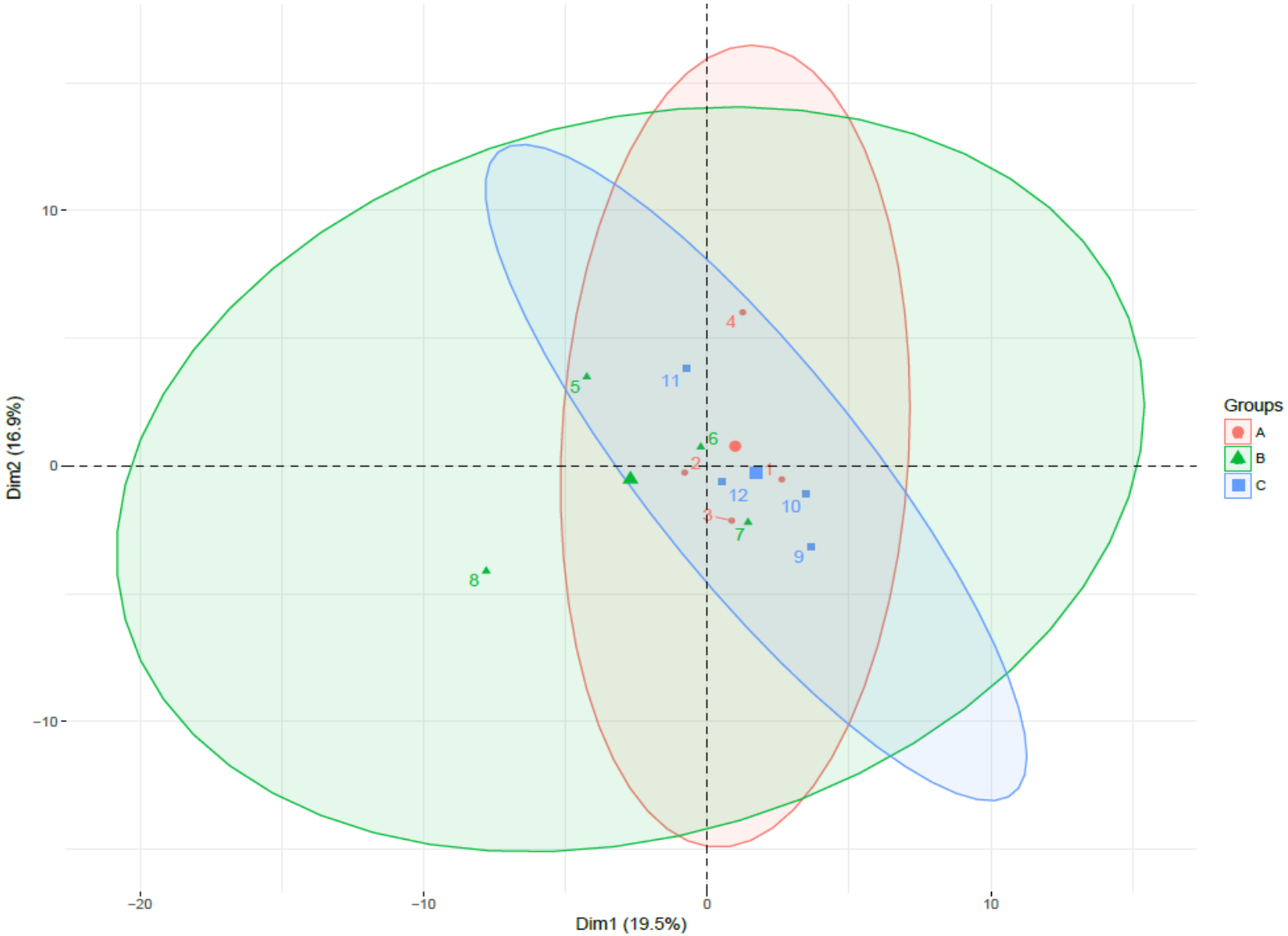

Figure 5.7: First and second principal component scores for the scanning variance analysis. Scans: 1-4: solution A; 5-8: solution B; 9-12: solution C. Ellipses represent the 95% confidence interval for the groups.

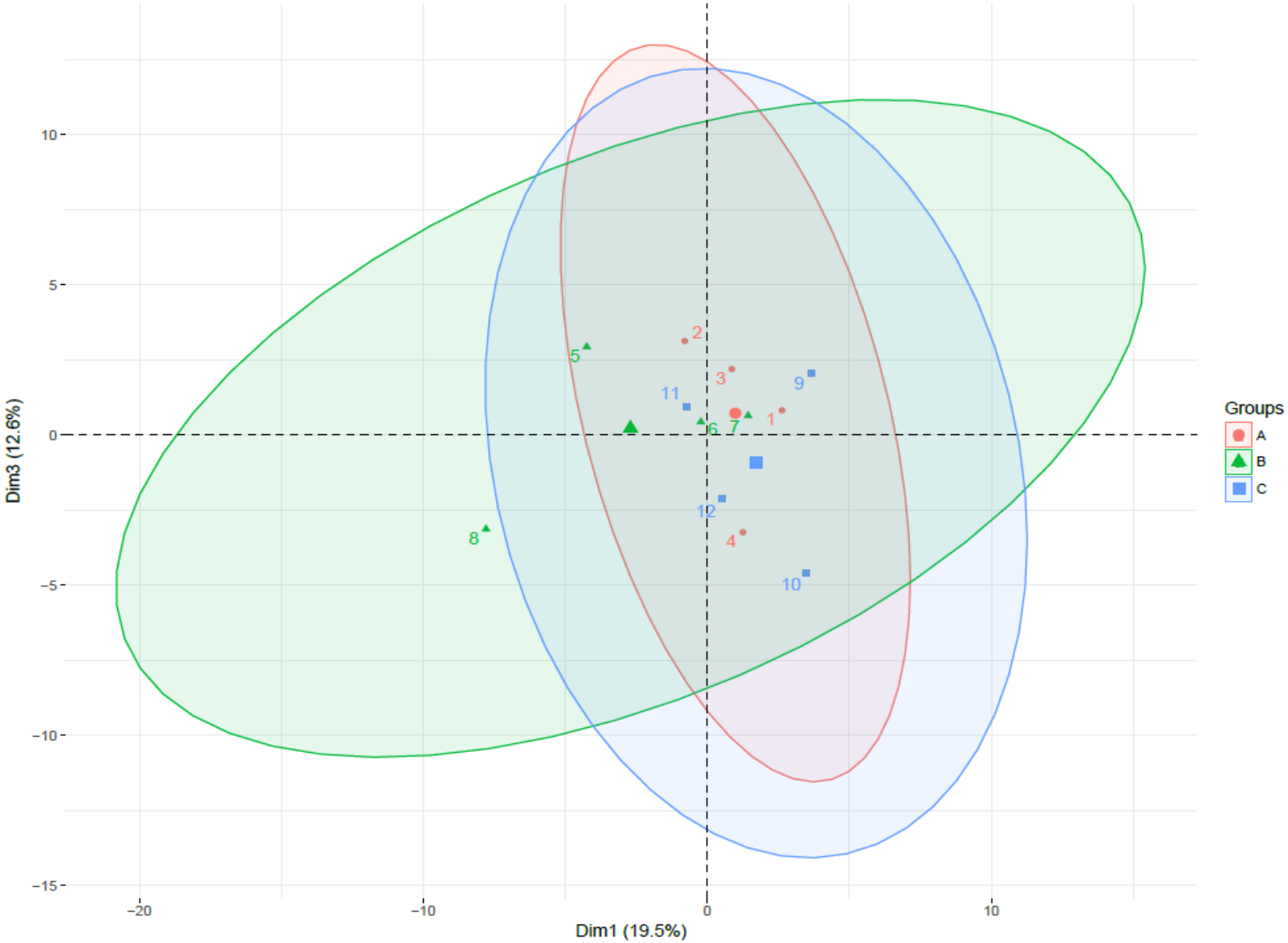

Figure 5.8: First and third principal component scores for the scanning variance analysis. Scans: 1-4: solution A; 5-8: solution B; 9-12: solution C. Ellipses represent the 95% confidence interval for the groups.

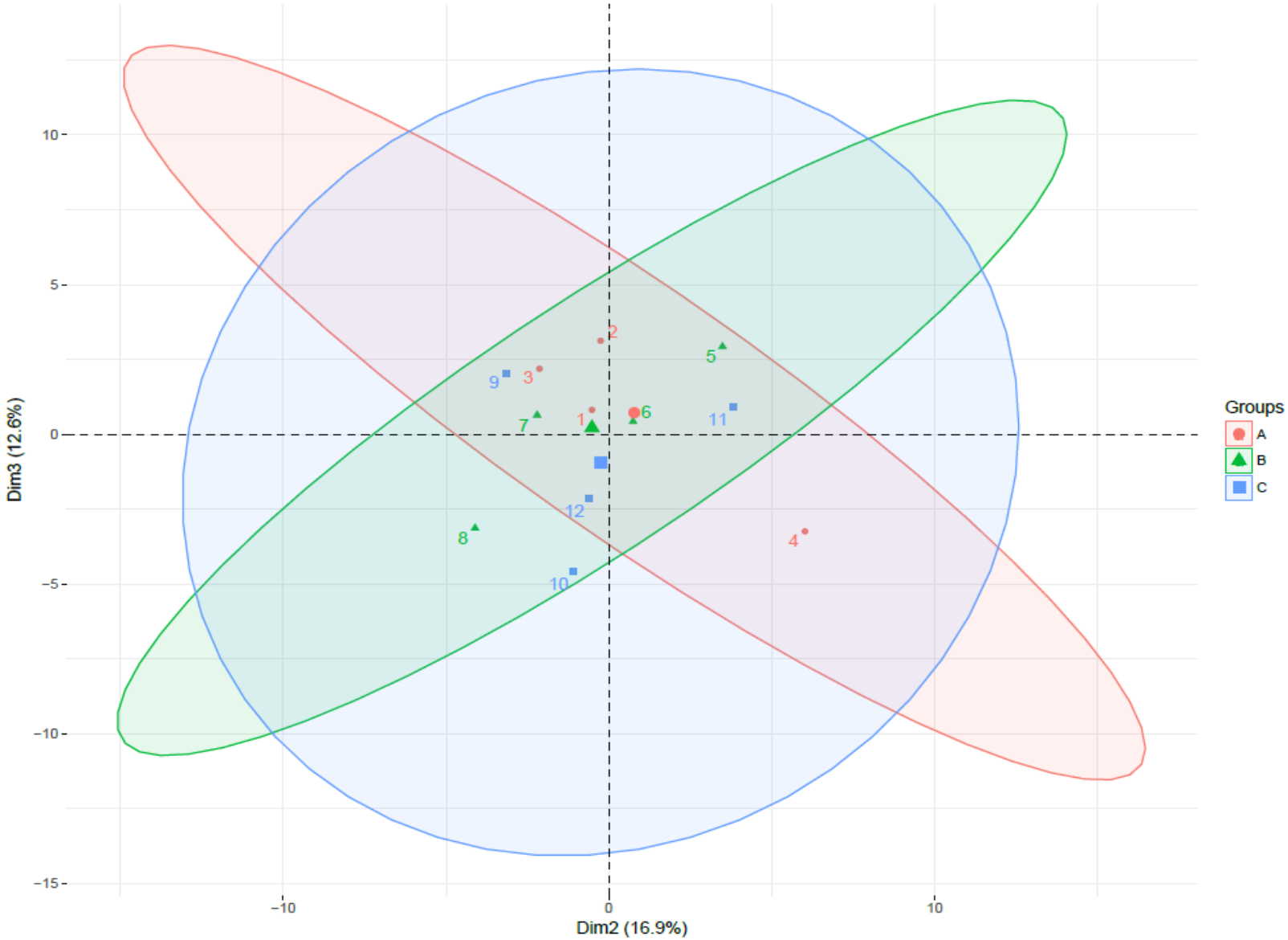

Figure 5.9: Second and third principal component scores for the scanning variance analysis. Scans: 1-4: solution A; 5-8: solution B; 9-12: solution C. Ellipses represent the 95% confidence interval for the groups.

As expected, PCA for the FL3 solutions show heavy clustering across the first three components; solution 8 (Sample B4) is the most significant outlier (as it accounts for 52% and 16% of the variation in the 1st and 2nd components respectively [Table 4.5]). During processing, Sample B4 had a worse shim on the spectrum than the other samples, meaning there would be more uncertainty in its quantification (overall, its quantification error was in the typical ranges expected for each compound when compared to the overall average of the three solutions). This shows that when performing PCA on spectra, preprocessing is crucial in order to eliminate as much variability as possible. However, it can still be seen that the loadings are relatively evenly dispersed across the first three components and the average scores for all the three solutions are within the 95% confidence ellipses for each other, indicating an insignificant difference between the solutions overall. Further, all of the most variable compounds (contribution >0.6 in Figure 5.4) have at least one or two unconvoluted peaks, removing this as a potential error source.

5.3.2 Lot-to-Lot Variation

The lot-to-lot variation was determined by incorporating both the lower and higher concentrations of yeast extract scans. Here, 6 components account for over 90% of the variation, indicating a more diverse dataspace than the single lot analysis[83]. Figures 5.10 - 5.12 represent the compound loadings and Figures 5.13 – 5.15 represent the solution scores for the first three components (77.3% of the total variance). Table 5.6 documents the most variable compounds, while Table 5.7 documents the most variable scans for each of the first three components.

Table 5.6: Top five variable variance contributions for the first three principal components, lot comparison

Component					
1	Adenosine (4.5%)	Threonine (4.2%)	Aspartate (4.1%)	Glucose (3.9%)	Asparagine (3.6%)
2	Alanine (6.3%)	*3-Hydroxyisovalerate* (6.2%)	Ornithine (5.9%)	4-Aminobutyrate (5.0%)	Betaine (4.9%)
3	Succinate (11.4%)	Trehalose (8.8%)	Acetate (6.8%)	Methanol (6.1%)	Glutamate (5.7%)

Table 5.7: Top four individual variance contributions for the first three principal components, lot comparison. Sample ID format: Brand-Concentration(g/L)-Scan. Asterisk indicates the spectrum was profiled by the second operator

Component				
1	BD-80-4 (6.1%)	BD-80-3 (6.1%)	BD-80-3* (6.1%)	BD-80-2 (5.0%)
2	BD-10-1 (7.2%)	BD-10-3 (6.7%)	BD-10-2 (6.1%)	FL1-100-1 (5.9%)
3	FL2-10-2 (9.1%)	FL2-10-1 (8.8%)	FL3-100-2 (8.1%)	FL2-10-3 (7.4%)

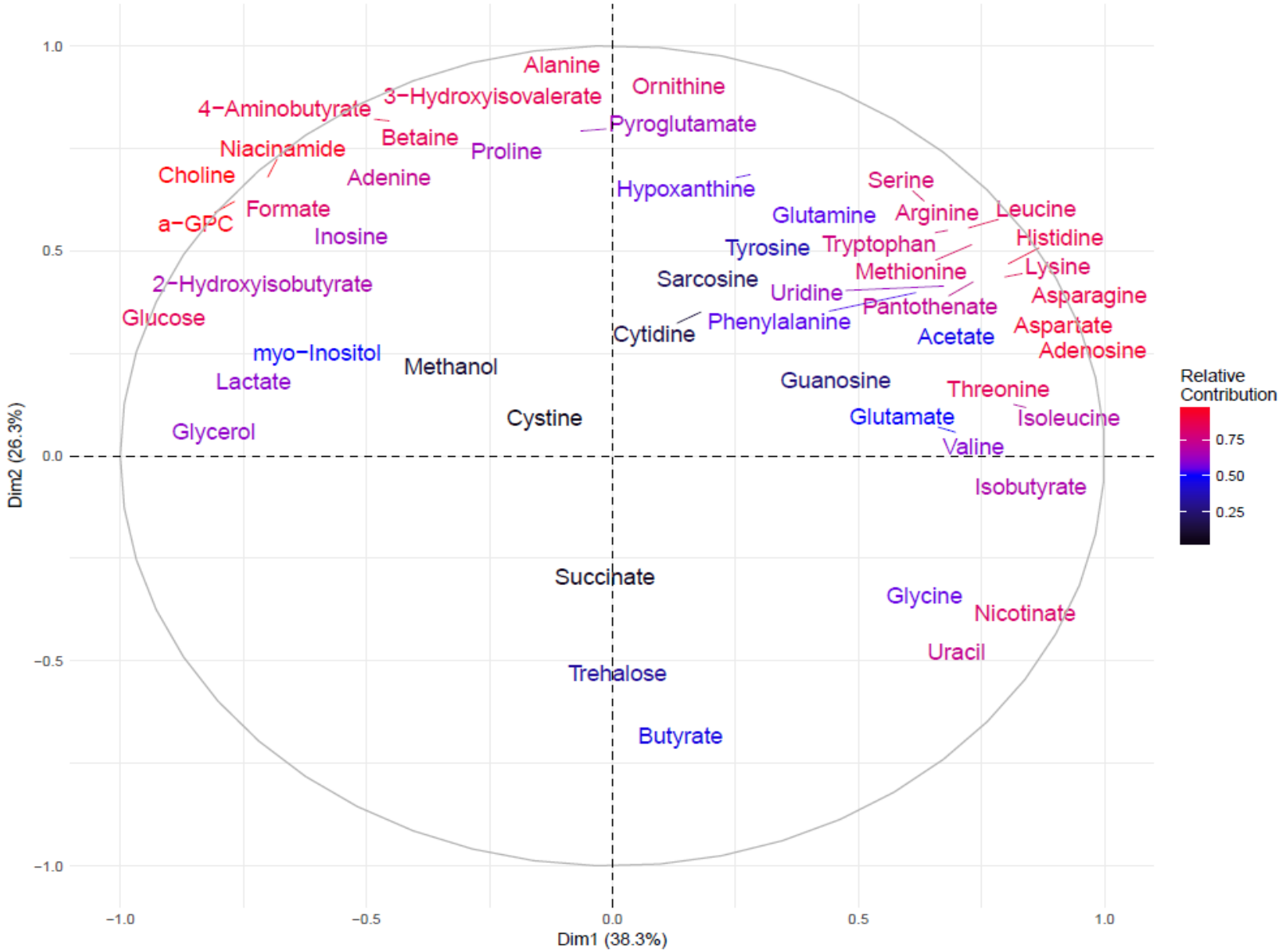

Figure 5.10: First and second principal component loadings for the lot comparison analysis

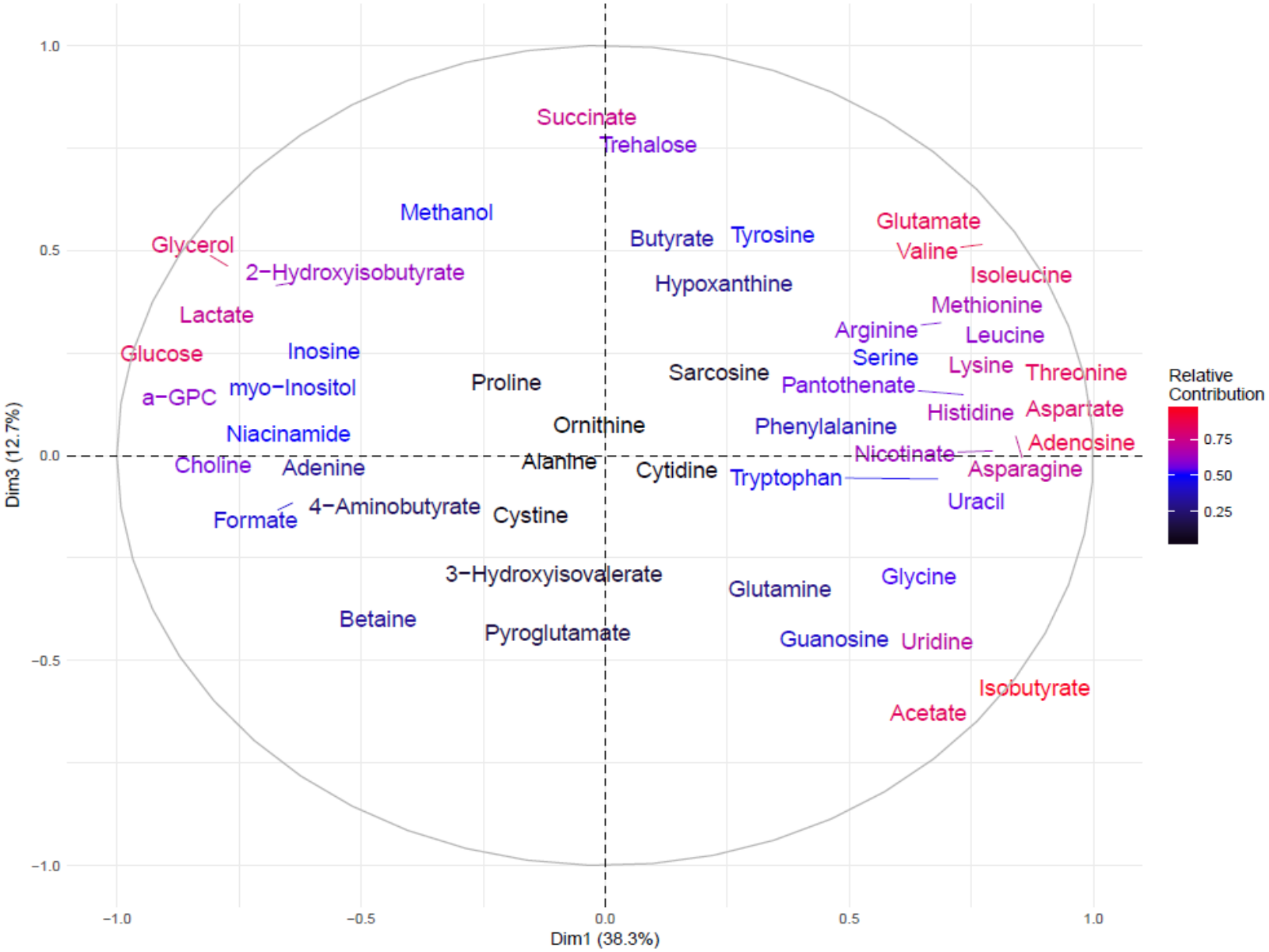

Figure 5.11: First and third principal component loadings for the lot comparison analysis

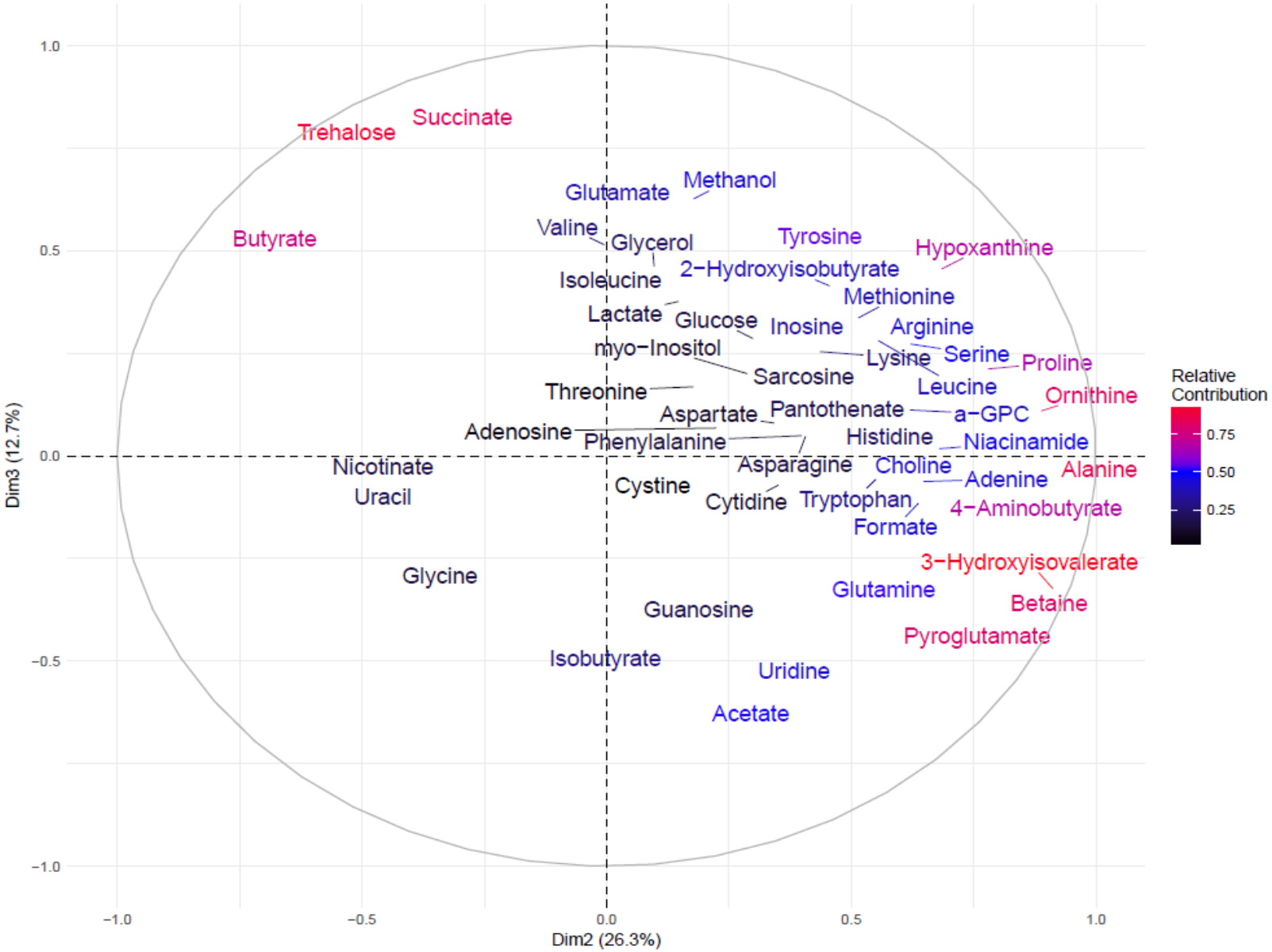

Figure 5.12: Second and third principal component loadings for the lot comparison analysis

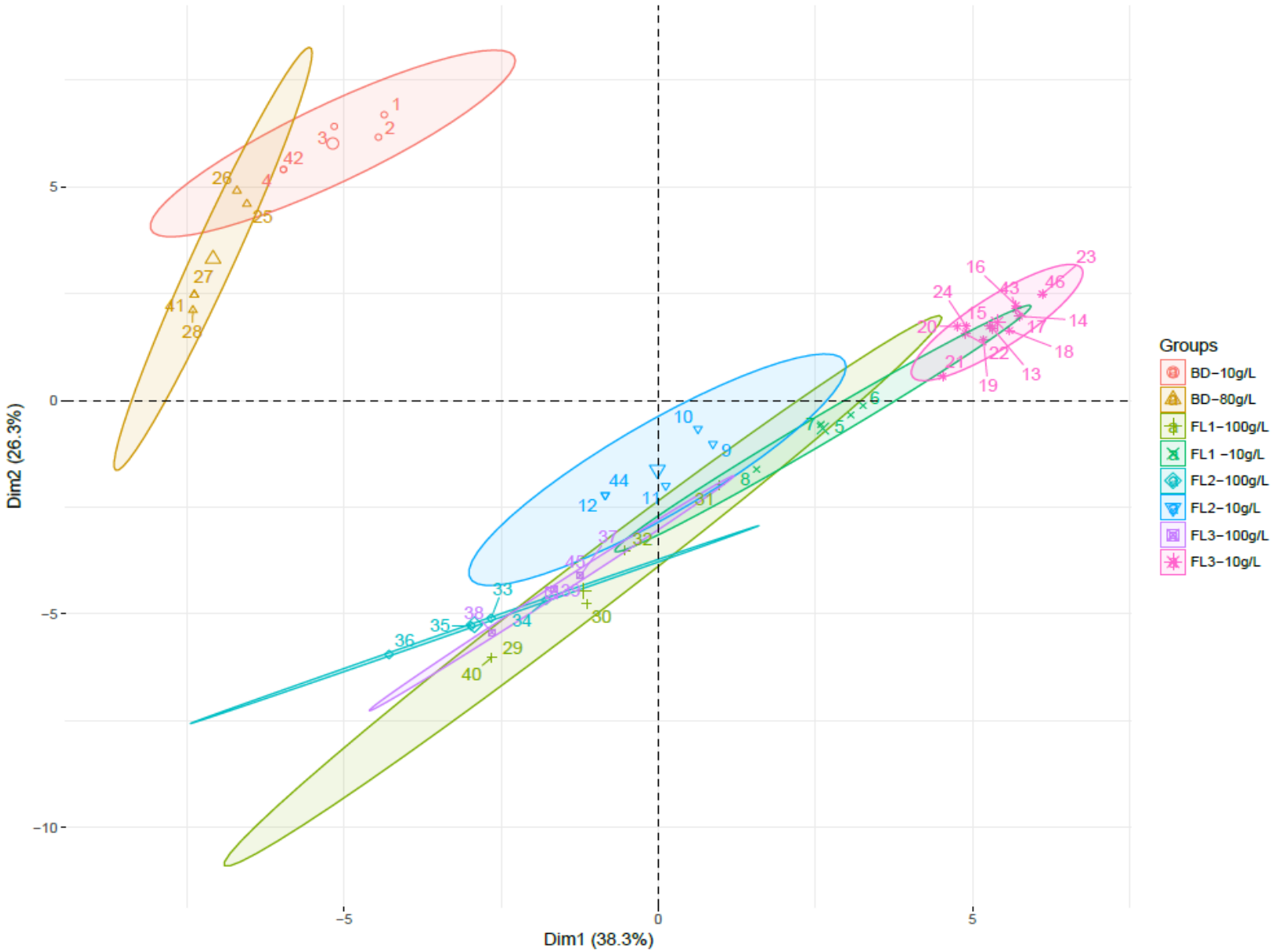

Figure 5.13: First and second principal component scores for the lot comparison analysis. Ellipses represent the 95% confidence interval for the groups.

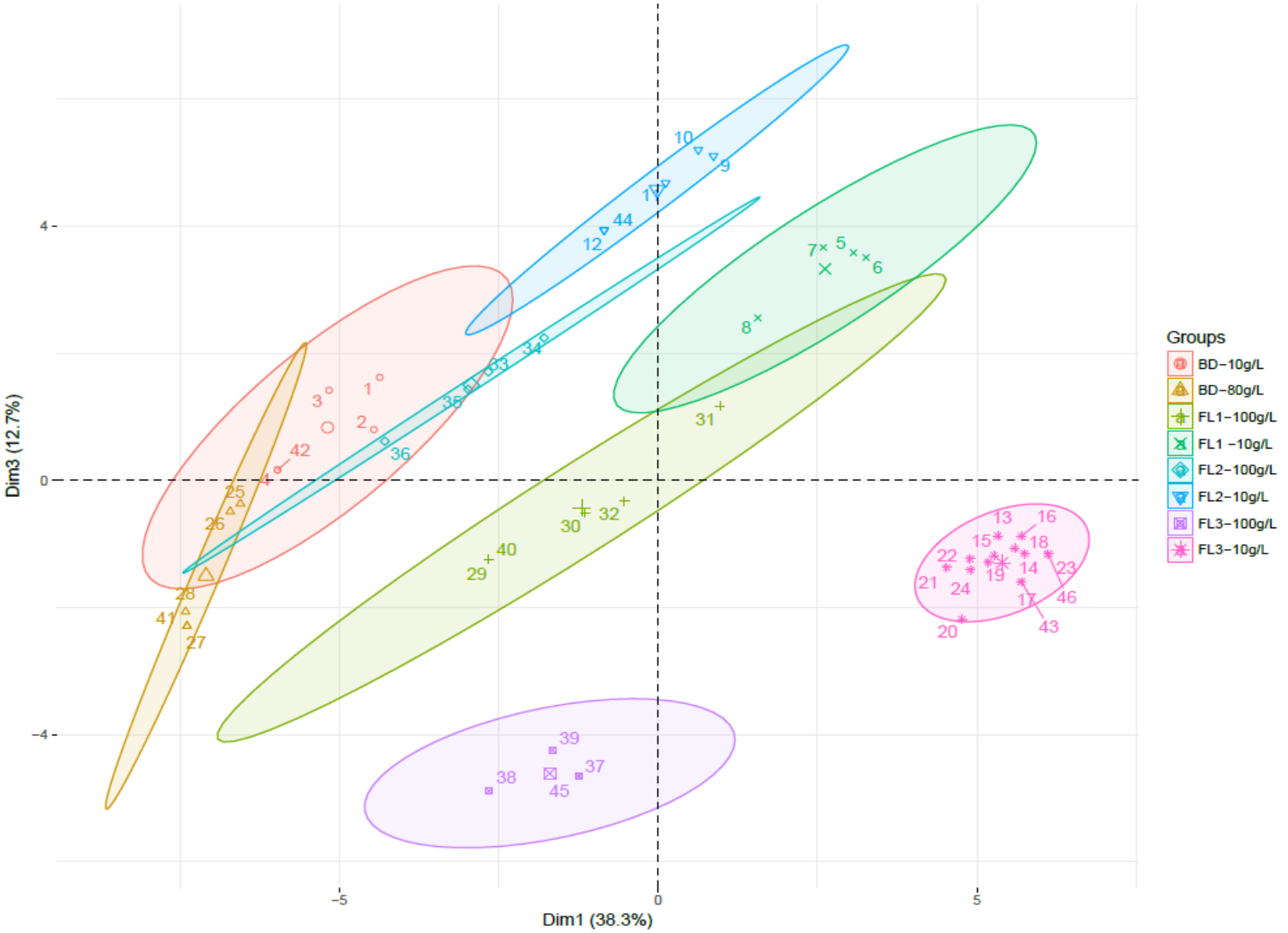

Figure 5.14: First and third principal component scores for the lot comparison analysis. Ellipses represent the 95% confidence interval for the groups.

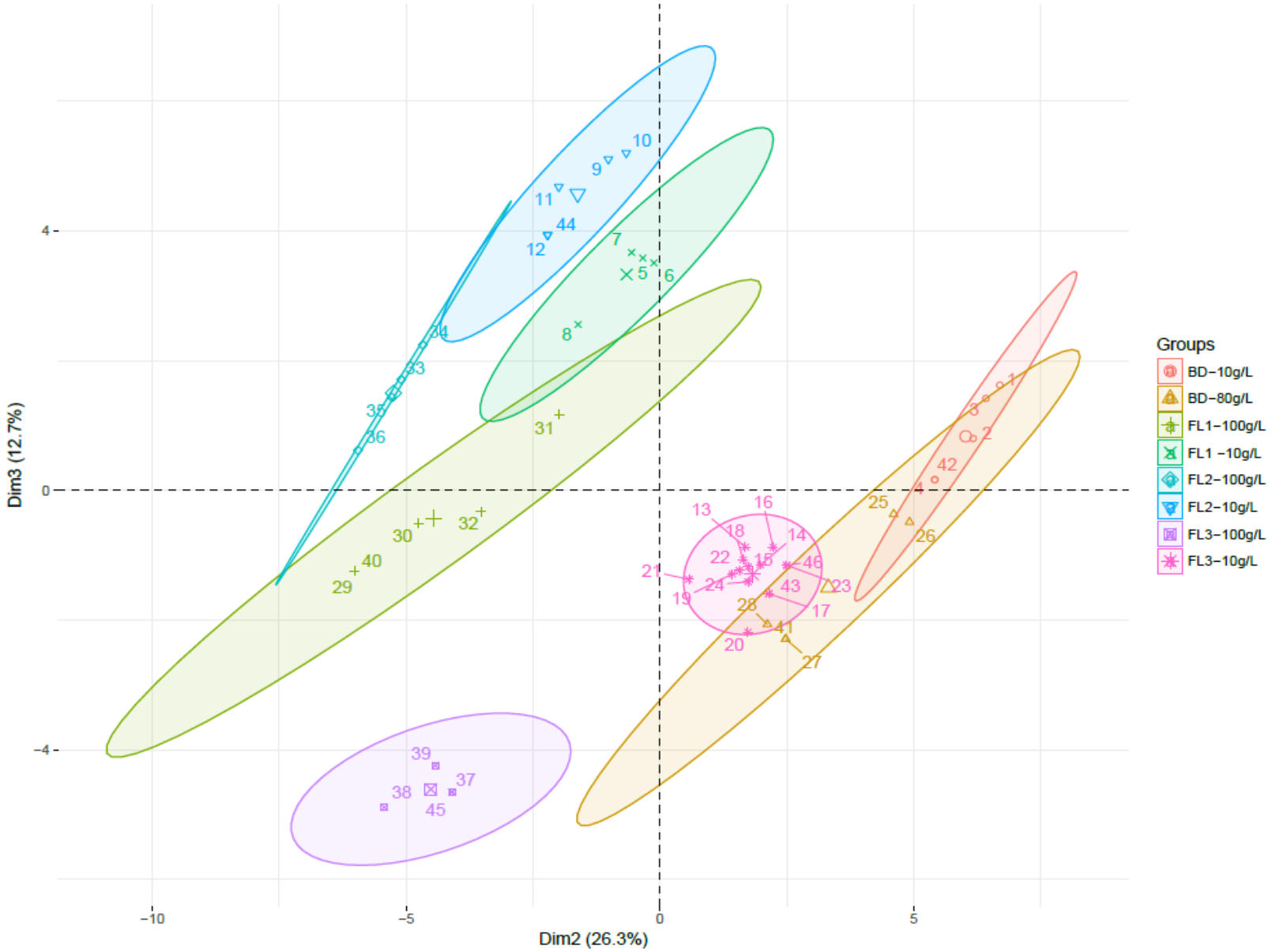

Figure 5.15: Second and third principal component scores for the lot comparison analysis. Ellipses represent the 95% confidence interval for the groups.

There is a clear separation between the BD and FL yeast extracts between the 1st and 2nd components as well as a possible separation between the 2st and 3rd components. The separation in the 1st and 2nd components is a mainly a result the vitamin differences (niacinamide, a-GPC, choline, myo-inositol), but accounts for the other most variable components between the yeast extracts (glucose, betaine, adenine, etc.). Although all of the FL seem to be more similar to each other than to the BD lot, their wide spread across the 1st and 2nd components is most likely due to the drastically different levels of trehalose in FL1 and FL2 as compared to FL3. The separation in the 2nd and 3rd components could be also be due to the significantly different trehalose levels between all the lots tested. Most of the overlap between Table 4.4 and Table 4.6 is due to those compounds having significantly different levels between lots, and is not an indication of any interactions between NMR and PCA.

In all cases, the higher concentration scans had lower scores for that yeast extract. Since a minority of compounds have loadings in the third quadrant, the shift towards this side indicates the analysis determining a lack of the compounds in the first quadrant, as was previously established when comparing the relative concentrations in Section 5.1. While the sample concentration influenced the PCA, the operator who profiled the spectrum does not. In Figures 5.13 – 5.15, scan numbers 40-47 were spectra quantified by a separate operator, and yet they overlay nearly exactly with those performed by this author. However, this does not mean the operators' results were identical; some of the more minor components have a variety of potential peak options in the spectra and thus their location must be standardized between operators for more accurate quantification. Since the majority of the peaks were identically assigned by both operators, PCA was unable to distinguish the minute differences between the scans, even though it could detect the minor variations in the multiple yeast extracts profiled by a single operator. This shows the strength of using PCA for lot screening as the operator does not impart enough additional variability in the scans to create a significantly different result.

6.0 ICP Analysis of Yeast Extract

In order to determine the variability in the ICP reading due to a complex solution, individual concentrated solutions of Co, Fe, Mn, Cu, and Zn salts were created and then combined into one solution at a 100-fold dilution. All six solutions were subjected to ICP analysis, with the results presented in Figure 6.1. As can be seen, the supplied elements behaved accordingly upon dissolution (Figure 6.1a, "High") but could be detected in the other defined solutions, along with several other contaminants not present in any of the individual concentrates at the required level seen in the combined solution (i.e. the levels in 6.2b "Low" are all pure solutions but do not match 6.2b "High" detections). This tends to suggest that these are not true readings in complex solutions and throw doubt into whether these compounds were detected in yeast extract. Considering the vast number of non-supplied chemicals detected, more serious investigation is required to determine where this is from and if it would in any way be affecting the detection in the yeast extract. The most probable explanation for these readings are the contaminants are present in any of the waters used to make the yeast extract solution or during the ICP *aqua regia* hydrolysis.

From the analysis of the 10 g/L solutions, 13 elements were detected, however not every element was present in every lot, as shown in Figure 6.2.

The bulk ions (Na, K, Ca, and Mg) represent very little contribution when compared to the levels already supplied in IPL-41 (i.e. 40%, 16%, 5% and 5% respectively). The yeast extract is a majority supplier of Co, Fe, Mn, Cu and Zn, and would be the only supplier of Cr, Ni, Si, and V. Compared to the IPL supplement, the yeast extract provides significantly more of these trace metals (Mo is the only supplied element which cannot be analyzed). Multivalent trace ions (e.g. Fe, Cu, and Mn) are responsible for producing free oxygen radicals in media. These radicals cause harm to major biological macromolecules and therefore their levels need to be carefully controlled for optimal growth. However, these three all have known biological roles, necessitating their supplementation. Except V, none of the elements detected in the yeast extract but not already supplied are suspected of having biological roles for Sf-9 cells and could be causing more harm than good in the media. The variability in both the quantity and presence of a variety of ions can have a wide range of unexpected effects during cell growth and demonstrates a need for fine supplementation control.

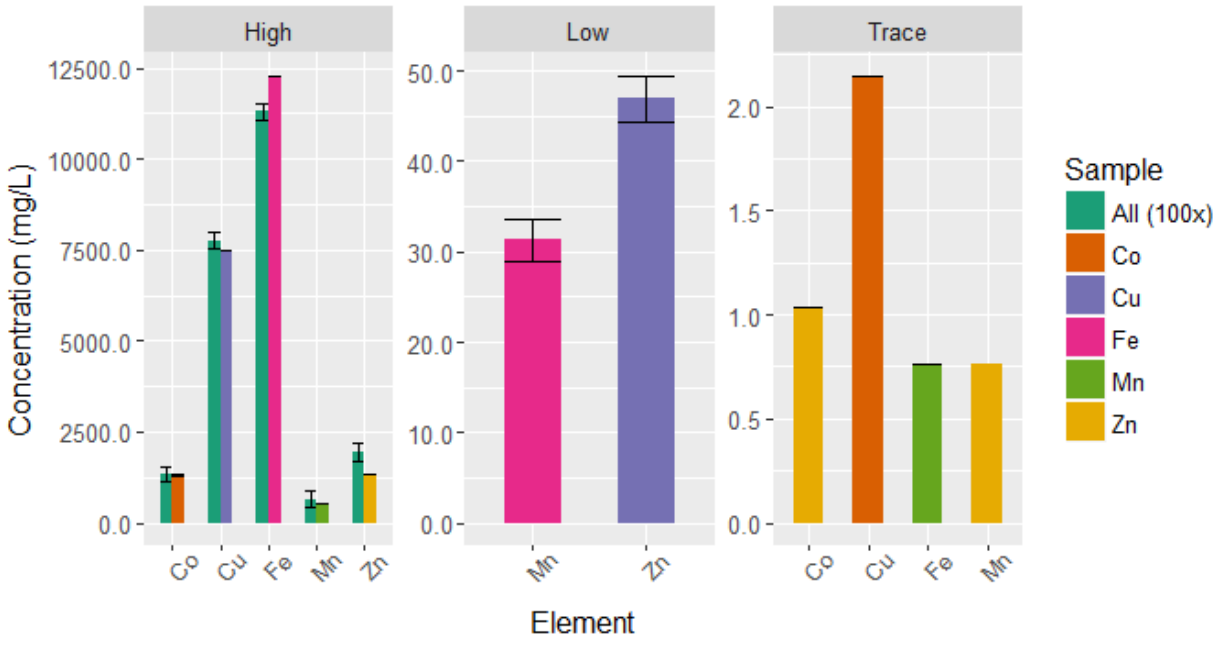

Figure 6.1a: Quantification of supplied metals as individual concentrated supplements and their detected level as a combined solution. "ALL (100x)" refers to the detected amount in the 100-fold diluted combined solution, but is plotted at 100x the detected amount for comparison to the concentrated samples. Error bars are neglected when they exceeded the magnitude of the average value.

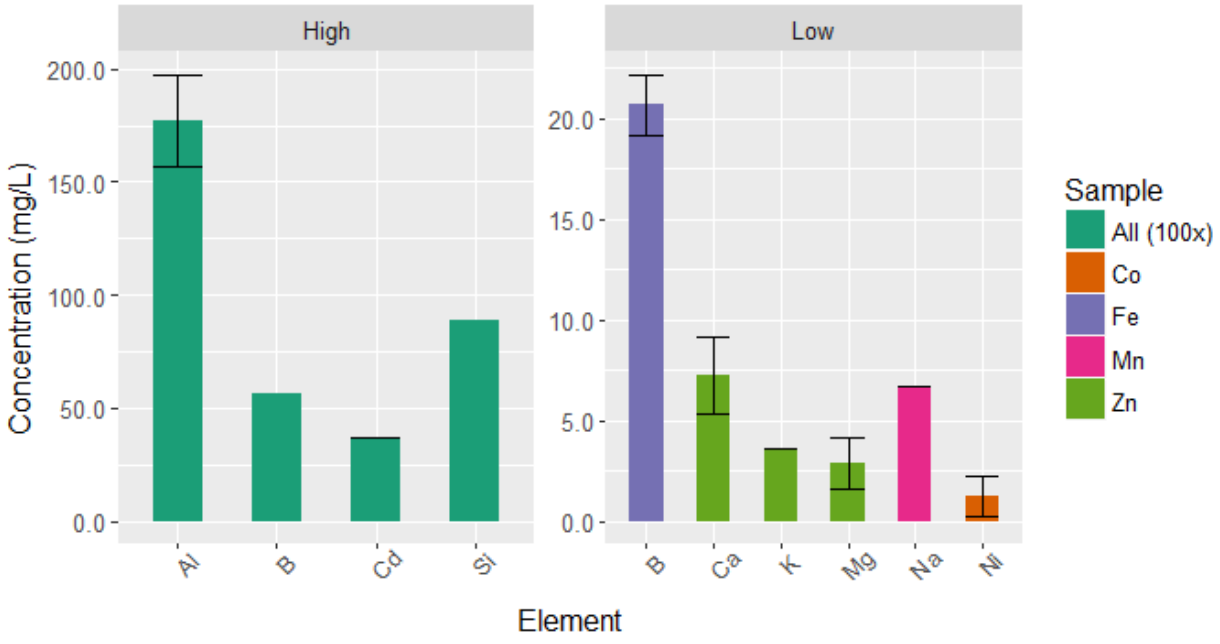

Figure 6.1b: Quantification of contaminants in concentrated supplements and their detected level in the combined solution. Error bars are neglected when they exceeded the magnitude of the average value.

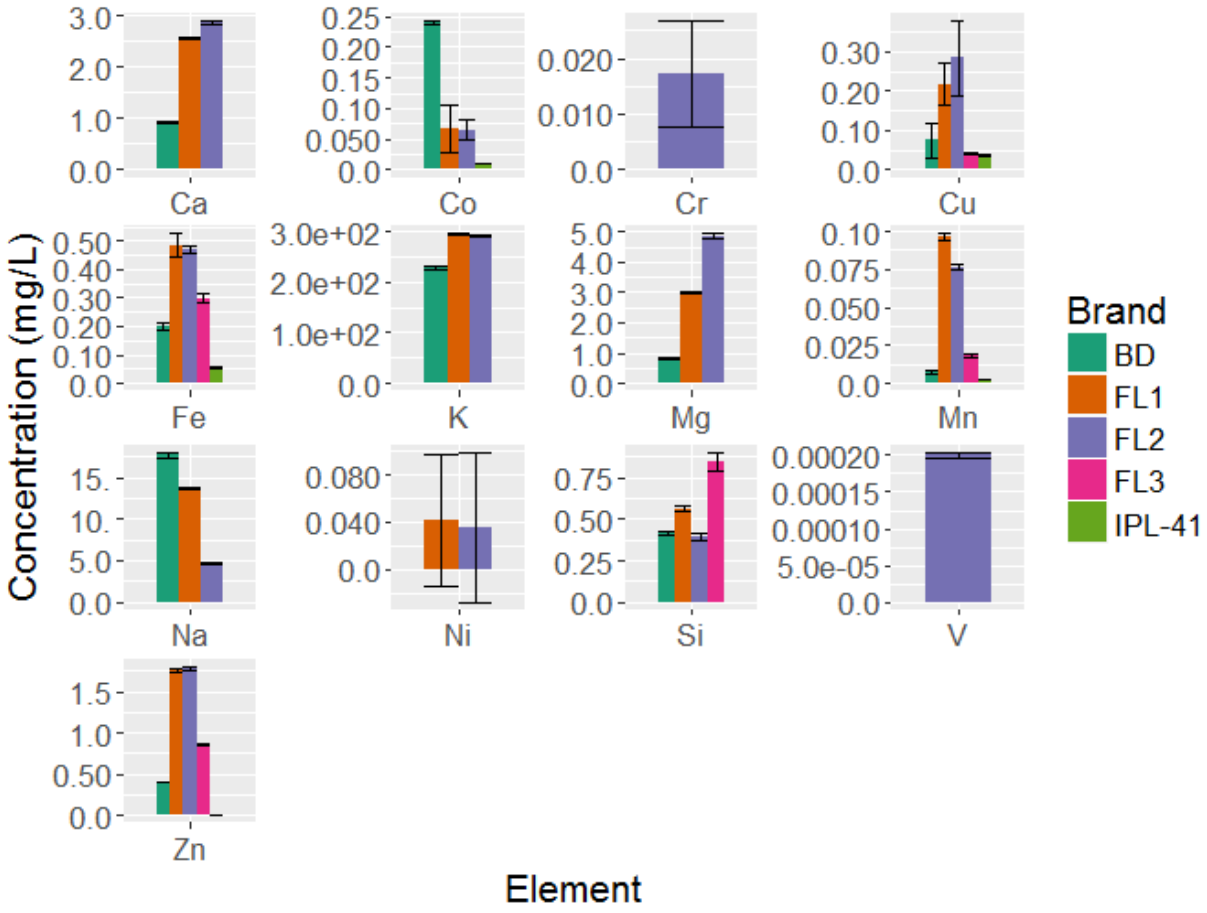

Figure 6.2: Detected metal ions along with the IPL-41 trace supplement levels. The IPL-41 trace metal supplement levels are shown for comparison

7.0 Acid Digestion of Yeast Extract

As stated in Section 3.2, yeast extract is composed of many different organic bonds and thus acid hydrolysis was chosen to digest the base yeast extract. A full time course was studied to ensure the most accurate representation of the overall process was achieved. Some components degraded immediately whereas others were essentially stable over the analysis. An appropriate kinetic model was chosen based on the observed data. Over the duration of the acid hydrolysis, 53 compounds were monitored.

7.1. Amino Acids

Amino acids with modelled kinetics are shown in Figure 7.1a, whereas those without modelled kinetics are in Figure 7.1b. Table 7.1 presents the estimated initial amount (C_0) to show dominant amino acids, and Table 7.2 presents the determined hydrolysis constants.

Table 7.1: Initial estimated amount and its confidence interval for each amino acid with modelled kinetics. Asp and Glu were modeled using the total initial amount of Asx and Glx, respectively.

	C_o (w%)	C_o CI%		C_o (w%)	C_o CI%
Aspartate	3.11	2.8	**Alanine**	0.95	7.9
Glutamate	3.06	7.0	**Isoleucine**	0.93	14.7
Lysine	2.68	6.8	**Arginine**	0.91	8.1
Glycine	1.59	5.5	**Leucine**	0.60	8.1
Proline	1.56	3.5	**Histidine**	0.58	7.9
Threonine	1.46	7.3	**Tyrosine**	0.52	6.7
Serine	1.32	10.3	**Phenylalanine**	0.46	12.8
Valine	1.13	8.5	**Methionine**	0.15	52.3

Table 7.2: Estimated hydrolysis rate and its confidence interval for each amino acid with modelled kinetics. Asp and Glu were modeled using the total initial amount of Asx and Glx, respectively

	h (min^{-1})	h CI%		h (min^{-1})	h CI%
Alanine	0.0325	42.4	**Tyrosine**	0.0164	24.0
Glycine	0.0294	27.8	**Threonine**	0.0151	25.0
Aspartate	0.0212	11.4	**Histidine**	0.0144	26.6
Serine	0.0210	42.1	**Lysine**	0.0142	22.6
Phenylalanine	0.0204	51.5	**Proline**	0.0137	11.4
Arginine	0.0203	32.4	**Methionine**	0.0099	148
Leucine	0.0201	32.4	**Valine**	0.0097	23.9
Glutamate	0.0192	27.3	**Isoleucine**	0.0091	40.2

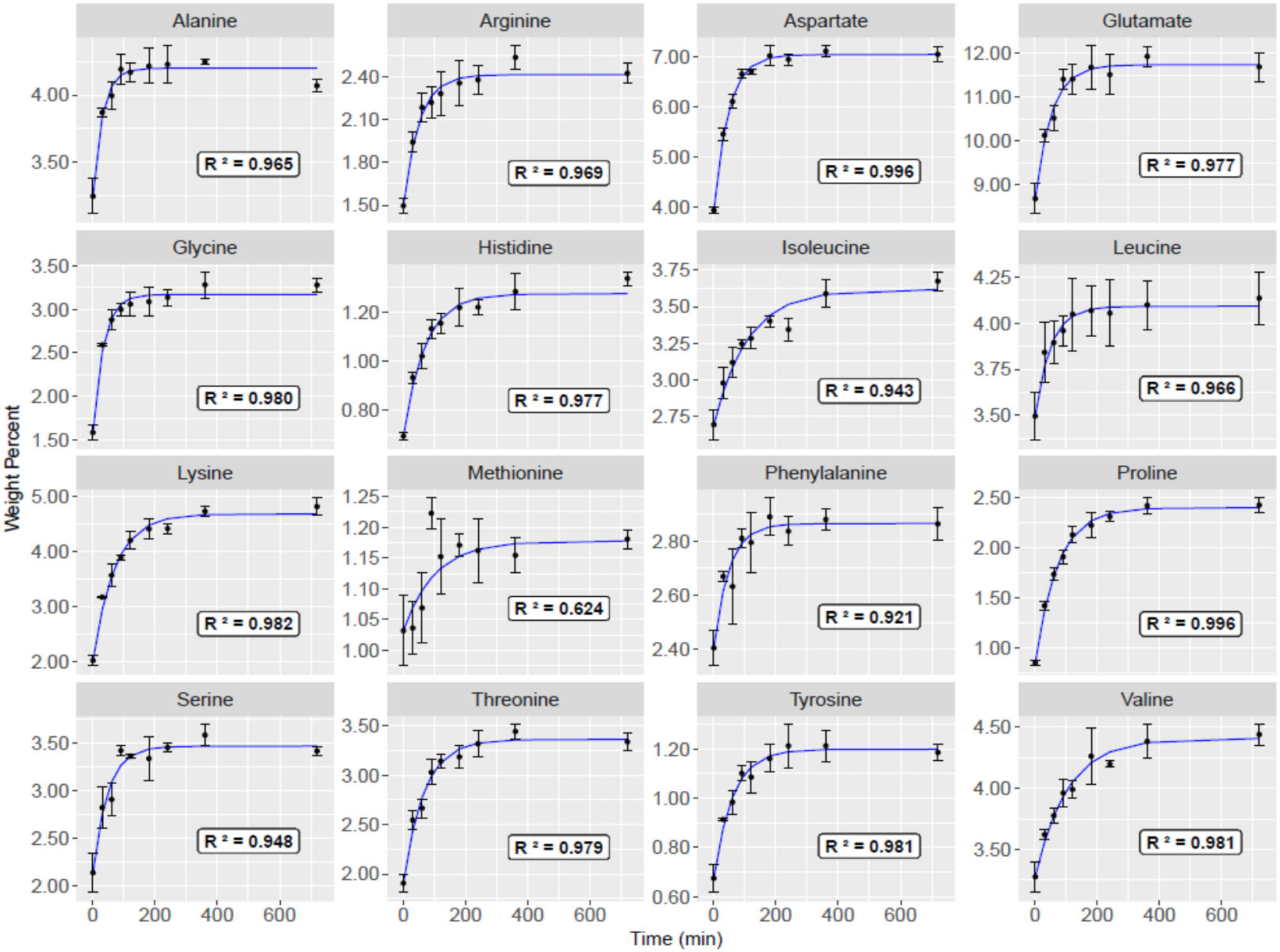

Figure 7.1a: Amino acids with discernable reaction kinetics. Asp and Glu were modeled using the total initial amount of Asx and Glx, respectively.

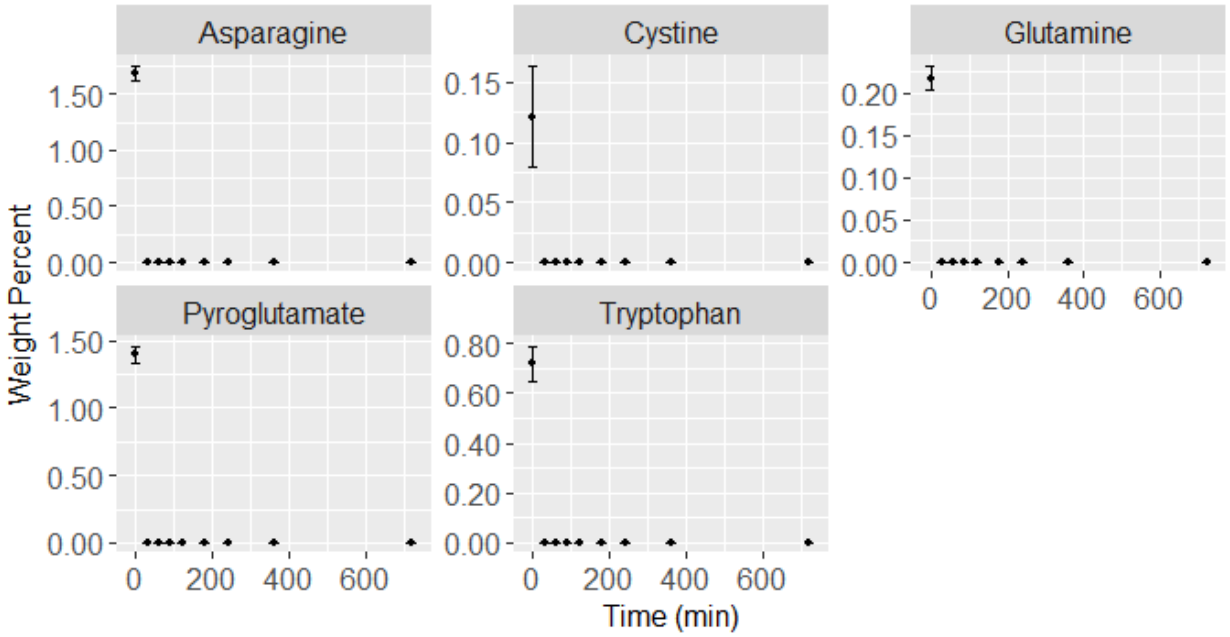

Figure 7.1b: Amino acids experiencing instant degradation.

As can be seen, most amino acids do not significantly change after 300 min. Since 24 hours is recommended for the complete hydrolysis of full length proteins[14], the shortened amount of time is consistent with having a majority of short oligopeptides present. The hydrolysis constants show a similar pattern to that observed by Darragh and Moughan[94], with Asp and Ala having the highest rates and Ile and Val having the lowest.

When analyzing the overall change in amino acid levels, it helps to not only look at how its overall contribution changes, but how it compares to the total amount of free amino acids detected, as reported in Table 7.2. All the post digestion levels are similar to values previously reported for this yeast extract product[16] to be considered evidence of minor batch-to-batch variation; the degree of hydrolysis (i.e. the initial free amino acids to the total free amino acids) is also very similar at 0.66. However, even some components with a modest overall increase and are present at the average rate in the peptides (e.g. Ser, Val) do not have as significant of a change when compared to the total amino acids. Noting their increases in this regard helps to show that Gly, Lys and Pro are more heavily contained in oligopeptides than in the initial free amino acids than components like Leu and Phe. Only a limited number of proteases are able to hydrolyze Pro linkages[77], and therefore its heavy presence in the oligopeptide fractions is expected; the increases of Lys and Pro coupled with the high presence of Glx and Asx could be related to previous reports that the most bio-active fraction of yeastolate contains a higher proportion of positively-charged side chains[15].

Table 7.2: The change in the amino acids, normalized to the overall initial yeast extract and the final free amino acid levels. Methionine experiences severe degradation and is most likely present in higher amounts overall. Asp and Glu represent Asx and Glx respectively. Highlighted compounds undergo the most significant changes. $B_o'_{,i} = B_{o,i} / B_{o,\,total}$ (same for C_o' and T_o'); %change compares the increase (C_o) and total (T_o) (i.e. 1 - B_o / T_o)

	Overall (w%)				Amino acid (aa%)			
	B_o	C_o	T_o	%Change	B_o'	C_o'	T_o'	%Change
Alanine	3.25	0.95	4.20	22.6	8.10	4.52	6.87	-15.2
Arginine	1.50	0.91	2.41	37.9	3.73	4.34	3.94	5.7
Aspartate	3.94	3.11	7.05	44.1	9.81	14.8	11.5	17.5
Glutamate	8.68	3.06	11.74	26.1	21.6	14.6	19.2	-11.2
Glycine	1.58	1.59	3.17	50.0	3.95	7.55	5.19	31.4
Histidine	0.69	0.58	1.27	45.5	1.73	2.76	2.08	20.5
Isoleucine	2.69	0.93	3.62	25.7	6.71	4.42	5.92	-11.7
Leucine	3.50	0.60	4.09	14.6	8.71	2.84	6.70	-23.1
Lysine	2.01	2.68	4.69	57.1	5.02	12.75	7.67	52.9
Methionine	1.03	0.15	1.18	12.4	2.57	0.70	1.93	-25.1
Phenylalanine	2.41	0.46	2.87	16.0	6.00	2.18	4.69	-21.8
Proline	0.85	1.56	2.41	64.7	2.12	7.41	3.94	86.0
Serine	2.14	1.32	3.47	38.2	5.34	6.30	5.67	6.2
Threonine	1.91	1.46	3.36	43.3	4.75	6.93	5.50	15.8
Tyrosine	0.67	0.52	1.20	43.7	1.68	2.50	1.96	16.7
Valine	3.28	1.13	4.41	25.6	8.16	5.38	7.21	-11.7
Total	40.1	21.0	61.1	34.4				

7.2 Sugars and Conversion Products

As expected, the acid digestion of yeast extract resulted in the formation of visible black particulate known as humins. Trehalose was converted instantly into glucose (its mass is included in the initial glucose amount during fitting), which was then slowly degraded into levulinate and formate. Their trends are presented in Figure 7.2 with their respective parameters in Table 7.3.

Table 7.3: Fitted glucose reaction kinetic parameters.

	h (min^{-1})	h CI%	d (min^{-1})	d CI%
Formate	0.00517	9.4	0.00245	24.8
	B_o (w%)	C_o CI%	h (min^{-1})	h CI%
Glucose	11.35	4.1	0.0181	7.7
Levulinate	--	--	0.0104	2.3

As expected, levulinate was stable after production whereas formate degrades. The estimated initial amount of glucose (11.35 w%) is very similar to the mass achieved from the measured initial amounts of glucose and trehalose combined (11.30 w% as glucose), indicating there is very little, if any, glucose contained in other forms before digestion. Overall, about 2.4 mM of glucose were reacted to obtain about 2.1 mM of levulinate, representing an almost 88% molar conversion and 60% mass yield.

While this study was performed at more dilute concentrations than those documented by Mukherjee et al.[78], it has a better conversion and yield than most other conditions. Further, this study also used a relatively lower amount of additive (i.e. 0.02% phenol) than others. Since phenol was shown to be degraded in this study, it would not have any impact during final purification of the levulinate. With the increased popularity in producing levulinic acid from biomass feedstock[31], it would be worthwhile to see if the increased conversion and yield hold for higher glucose concentrations under the phenol addition. Even though levulinate and formate are deemed to be formed at the same time when 5-HMF decomposes (Figure 3.2), levulinate has a hydrolysis constant almost twice that of formate, suggesting that the mechanism for this decomposition first releases levulinate before formate detection.

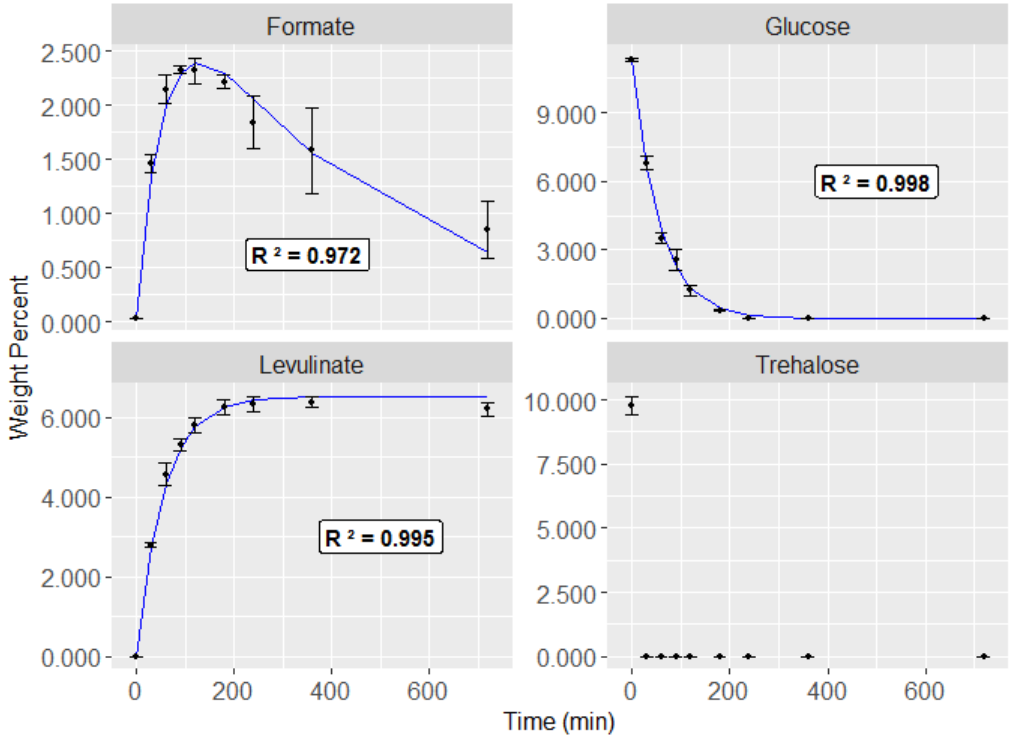

Figure 7.2: Kinetic trends for the sugars and their conversion products

7.3 Nucleic Material

Of the identified nucleic material in the undigested scan, acid digestion expectedly cleaved all the nucleotides from their sugars. However, while this happened instantaneously for the purines (adenosine, guanosine, and inosine), the pyrimidines (uridine and cytidine) seemed to be much more resilient to hydrolysis. All of the profiles and kinetic parameters can be found in Figure 7.3 and Table 7.4.

Table 7.4: Fitted nucleic material kinetic parameters (cytosine is a 0 order model). Hypoxathine was modelled with the initial amount determined from the more concentrated scans.

	C_o or B_o (w%)	C_o or B_o CI%	h or d (min^{-1})	h or d CI%
Adenine	1.30	6.2	0.0012	25.3
Uracil	0.99	25.7	0.0025	44.2
Cytidine	0.65	18.1	0.0024	48.9
Uridine	0.28	14.2	0.0035	32.2
Hypoxanthine	0.07	27.8	0.0083	72.9
Cytosine	--	--	0.0004	6.5

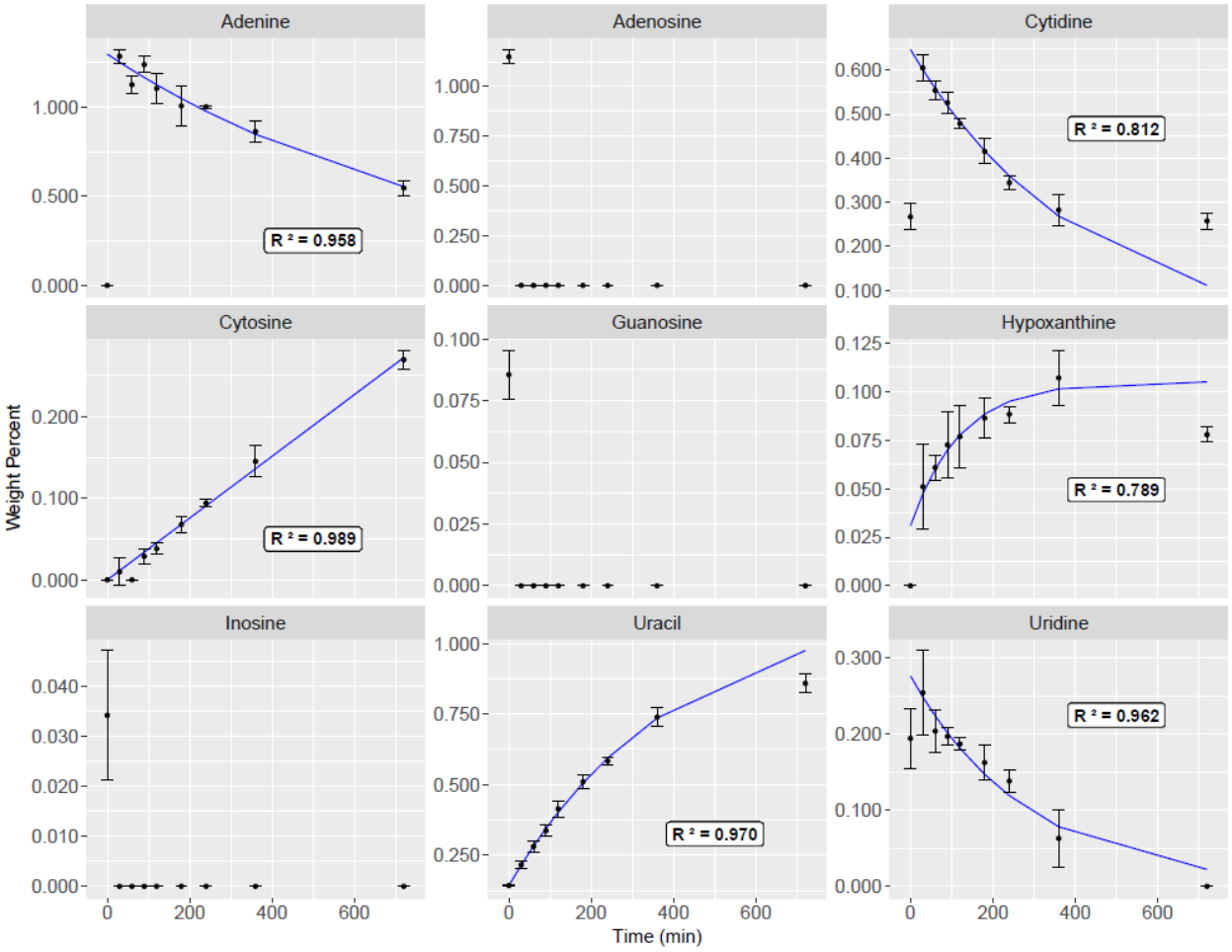

Figure 7.3: Kinetic trends and parameters for identified nucleic material (cytosine is a 0 order model). Hypoxanthine was modelled with the initial amount determined from the more concentrated scans.

The considerable increases in cytosine and uracil heavily suggest that they are main monomers of oligonucleoside fragments. No thymidine was detected throughout the study, which, along with the heavy presence of uridine, indicates that the nucleosides are mostly from RNA fragments and not DNA, in agreement with previous reports[28, 29, 32]. Further, as the uracil and cytosine levels do not reach a maximum,

this lends evidence that there are still fragments which have not been hydrolyzed even after 12 hours. While a first or zero order assumption fits most compounds well, cytidine is clearly present after 12 hours, even though it is an outlier for this assumption. This could be evidence cytidine is also being produced to some degree, again implying a significant amount of nucleic material. The most surprising find is that there is about twice as much adenine detected at 30 min (0.36 mM) than was expected based on the initial adenosine (0.16 mM). Since no other adenine containing components could be identified this could be due to residual poly-A tails left over from mRNA fragments.

7.4 Organic Acids, Vitamins, Micronutrients

Only acetate, fumarate, glycerol, and myo-inositol had any noticeable kinetics, as shown in Figure 7.6 and Table 7.5.

Table 7.5: Fitted kinetic parameters for other modelled compounds. Fumarate was modelled using the initial amount quantified from concentrated yeast extract spectra.

	C_o or B_o (w%)	C_o or B_o CI%	h or d (min^{-1})	h or d CI%
Acetate	0.56	7.8	0.0018	25.0
Glycerol	0.061	18.8	0.0056	42.2
myo-Inositol	0.050	32.9	0.0093	90.3
Fumarate	0.017	46.3	0.0029	84.4

The initial increase of acetate could be due to the hydrolysis of acetyl ester bonds, although there were no significant quantities of any these compounds detected. Most of the glycerol and choline increase will come from the conversion of a-GPC. However, since choline experienced an immediate increase compared to the more gradual release of glycerol, this implies that the dephosphorylation of 1-phosphoglycerol is the rate limiting step, as shown in Figure 7.5. Of the initial 13 µmol a-GPC/g-yeast extract, only about 5 µmol glycerol/g-yeast extract was detected at the end of the hydrolysis (i.e. 38% total hydrolysis), meaning there is probably some residual 1-phosphoglycerol, or that glycerol itself decomposes significantly. Conversely, as much as 92% of the choline from a-GPC was quantified, suggesting there is no residual choline bonds. As myo-inositol is a common phospholipid head group, its increase could indicate there are residual phospholipids in the yeast extract. Phospholipids would also provide small amounts of glycerol and serine. It is unknown why fumarate would increase. The initial increase of nicotinate is from the conversion of niacinamide, but is still at low overall quantities, resulting in its more variable quantification. Although ornithine might also appear to have increased, the readings are too noisy to make any definitive claims. Lactate, sarcosine, and 4-aminobutyrate appear to have an

overall decreasing trend, but again the data is too noisy to make any definitive claims. Succinate, betaine, and citrate are stable even after 12 hours of acid digestion.

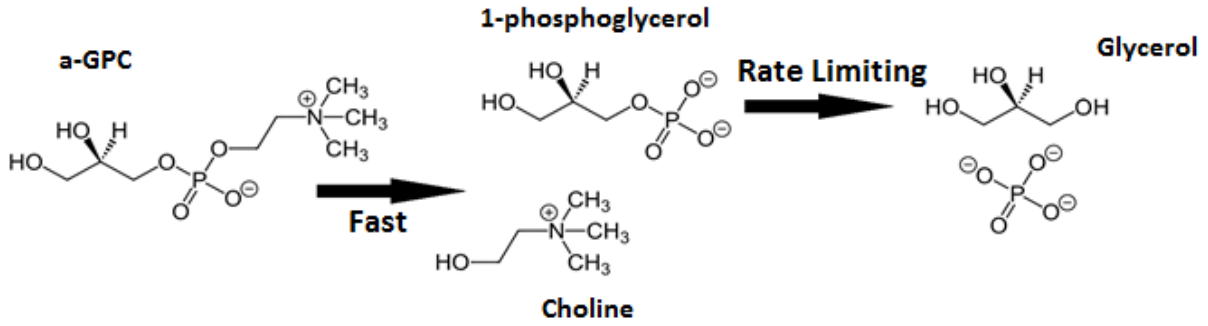

Figure 7.4: Proposed mechanism for the acid hydrolysis decomposition of a-GPC. Charges are assumed to be balanced by the abundance of ions in the overall solution.

7.5 Alkylates and Phenol

Figure 7.7 and Table 7.6 present the kinetic profiles for phenol and the alkylates.

Table 7.6: Fitted alkylate and phenol kinetic parameters

	C_o or B_o (w%)	C_o or B_o CI%	h or d (min^{-1})	h or d CI%
Butyrate	0.081	12.0	0.0029	33.7
Isobutyrate	0.058	27.1	0.0090	53.4
3-Hydroxybutyrate	0.056	13.4	0.0009	61.3
Isovalerate	0.030	18.4	0.0036	47.9
3-Hydroxyisovalerate	0.009	28.1	0.0033	52.7
Phenol	--	--	0.0262	18.1

As stated previously, only butyrate has any qualification confidence of the alkylates, and therefore the accuracy of the other compounds is debatable. Isobutyrate and isovalerate could not be accurately modelled, likely due to the only peaks used to quantify these compound becoming obscured heavily after 90 min. Phenol was completely degraded after 120 min in all cases. Although it appears 3-hydroxyvalerate increases over the hydrolysis, all of the values are still within + 10% of the mean, indicating it could be stable in the solution.

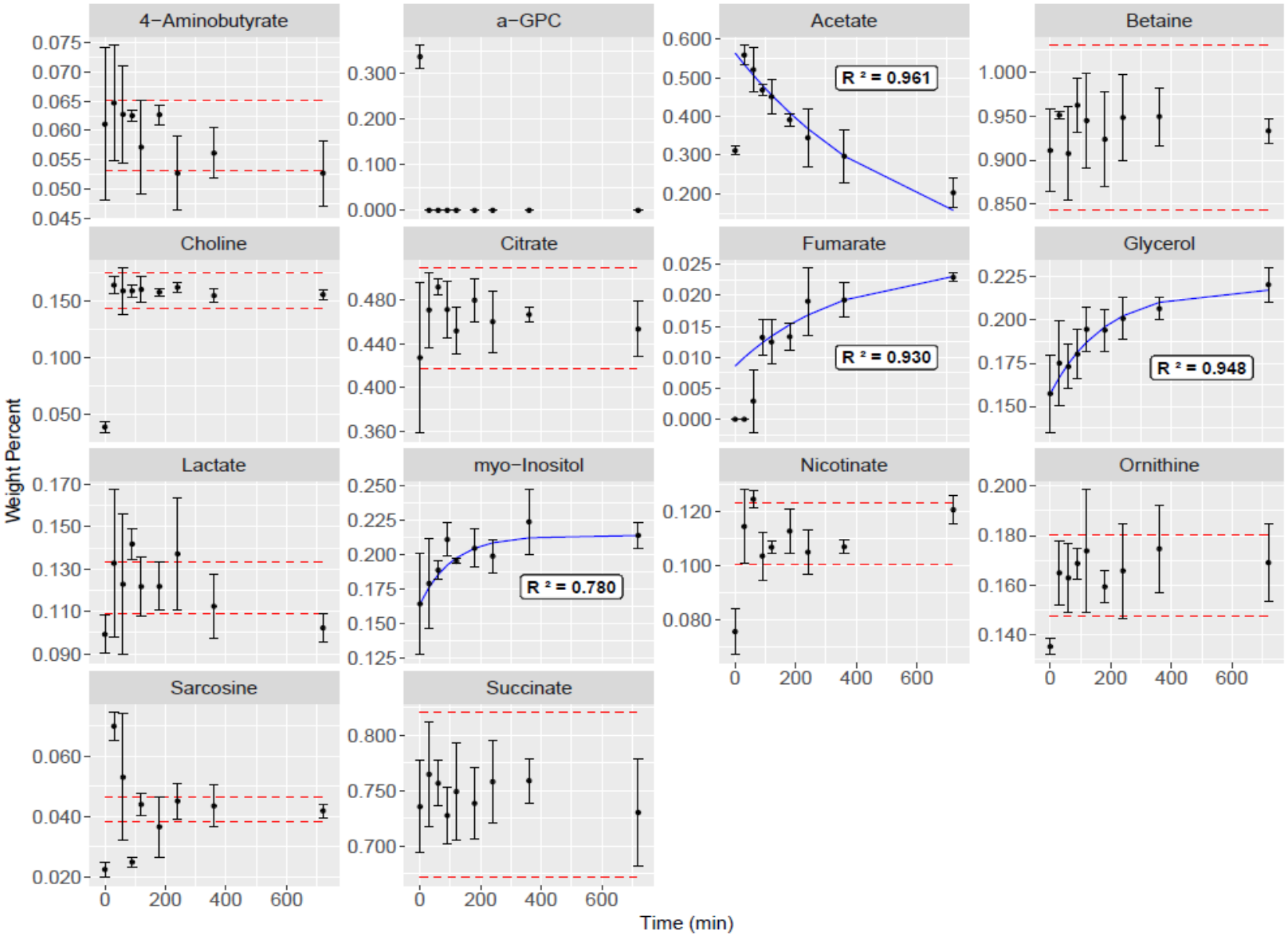

Figure 7.6: Kinetics of other compounds during acid hydrolysis of yeast extract. The red lines provide a ±10% boundary from the mean value of the scans to show stability of compounds with no modelled kinetics after any initial conversions.

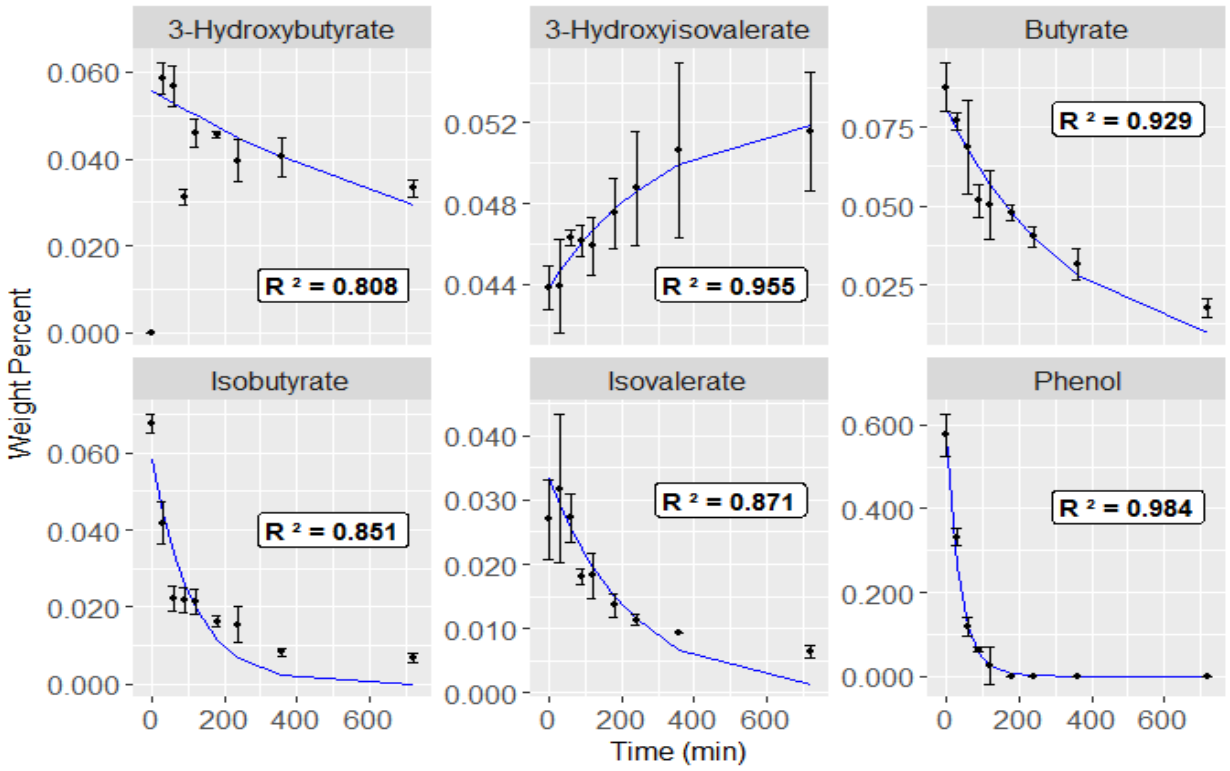

Figure 7.7: Kinetics of alkylates and phenol during acid hydrolysis of yeast extract. Phenol is normalized to the amount of yeast extract in order to keep units consistent (initial solution concentration is 0.02w%)

7.6 Final Acid Hydrolysis Composition

A hypothesized composition for FL3 is presented in Table 7.5. To account for the hydrolysis, the following was performed:

- The B_o and C_o terms for each amino acid and the other increasing compounds were summed;
- Compounds undergoing decomposition only were reported as their estimated B_o value;
- Compounds with no modelled kinetics were reported as the overall average amount;
- The a-GPC compounds are reported as their individual initial amounts;
- The estimated C_o for adenine, hypoxanthine, and uracil was converted to adenosine, inosine, or uridine, respectively, since these are the most likely precursors for these products; and
- Cytidine was reported as its B_o value. Cytosine was not used in the estimation since it had not reached a maximum value (but its final amount would translate into about 0.59w% cytidine)

Table 7.7: Hypothesized initial composition of FL3, weight percent. Glu and Asp account for Glx and Asx, respectively

Amino Acids	**w%**	**Sugars**	**w%**	**Organic Acids**	**w%**
Glutamate	11.74	Trehalose	9.78	Succinate	0.75
Aspartate	7.05	Glucose	1.01	Acetate	0.56
Lysine	4.69	**Total**	10.8	Citrate	0.46
Valine	4.41	**Nucleic Material**	**w%**	Lactate	0.12
Alanine	4.20	Adenosine	3.71	Formate	0.03
Leucine	4.09	Uridine	2.36	Fumarate	0.01
Isoleucine	3.62	Cytidine	0.65	**Total**	1.9
Serine	3.47	Inosine	0.18	**Micronutrients**	**w%**
Threonine	3.36	Uracil	0.14	Betaine	0.94
Glycine	3.17	Guanosine	0.09	a-GPC	0.34
Phenylalanine	2.87	Hypoxanthine	0.03	myo-Inositol	0.21
Arginine	2.41	Adenine	0.01	Ornithine	0.16
Proline	2.41	**Total**	7.2	Glycerol	0.16
Histidine	1.27	**Alkylates**	**w%**	Nicotinate	0.11
Tyrosine	1.20	Butyrate	0.08	4-Aminobutyrate	0.06
Methionine	1.18	Isobutyrate	0.07	Sarcosine	0.04
Total	61.1	3-Hydroxybutyrate	0.06	Choline	0.04
		3-Hydroxyisovalerate	0.05	**Total**	2.1
		Isovalerate	0.03		
		Total	0.3	**Grand total**	**83.4**

About 83.4% of the yeast extract can be accounted for through acid digestion and NMR analysis. Combining this with the ICP results (8.4 w%) brings the total accounted mass up to about 91.8 w% for this study. Assuming a similar inorganic anion composition (i.e. PO_4^{3-}, SO_4^{3-}, and Cl^-) as previously reported for this yeast extract product (3.4 w%[16]) allows for about 95 w% of the material to be determined. With very little mass unaccounted for, this indicates that only trace components, and those undetectable by the NMR are left unqualified. This proves that H-NMR spectroscopy is a highly useful tool for quantifying components in a complex solution with only minimal foreknowledge of possible constituents.

As expected, the yeast extract was mostly amino acids and sugars. Surprisingly, there was also a very significant amount of nucleic material detected, meaning the initial supplement pool for these compounds would be quite significant when using yeast extract for cell culture work. It could be very likely that these compounds could be necessary for ensuring the cells acclimatize properly to their media.

8.0 Metabolic Studies

Many groups have used the same defined basal media with different lots of various yeast extracts without seeing significant effects on overall growth. This suggests that the essential compounds for growth can be found in a large variety of products, independent of the various production parameters that have been employed and improved over the years. However, changes in media composition are known to influence cell growth and therefore a more complete metabolic analysis will help to illuminate how the growth is influenced by various lots of yeast extract.

8.1 Yeast extract containing medium

The metabolism of Sf-9 cells was studied as they grew in Sf-900III, one of the highest performing commercial media available, as well as the various in-house media. This is then compared to adapted cells in various yeast extracts to see how the metabolism has been influenced. As they are routinely maintained in this media, no adaptation period was necessary.

8.1.1 Commercial hydrolysate-containing media – Growth and Metabolite Trends

Figure 8.1 presents the growth rate for Sf-9 cells in Sf-900III media. The cells had an average growth rate of 0.0225 h^{-1} and reached an average maximum cell density of $13.5x10^6$ cells/mL. These will be the two main statistics used to judge how well the in-house media with various other yeast extracts perform. The trends for the 52 identified metabolites are shown in Figures 8.2 – 8.5, while Table 8.1 indicates the qualification confidence for each of them.

Table 8.1: Identified metabolites during Sf-900III cultures. Italicized components are of low qualification confidence due to heavy peak convolution or near the apparent LDL

Amino Acids		**Sugars**	**Biologicals**	**Organic Acids**
Alanine	Lysine	Fructose	4-Aminobutyrate	1,3-Dihydroxyacetone
Arginine	Methionine	Glucose	*a-GPC*	*2-Oxoglutarate*
Asparagine	Phenylalanine	Maltose	*Choline*	Citrate
Aspartate	Proline	*Mannose*	Nicotinate	Formate
Cystine	Pyroglutamate	Sucrose	Ornithine	Fumarate
Glutamate	Serine	Trehalose	Sarcosine	Lactate
Glutamine	Threonine			*Maleate*
Glycine	Tryptophan	**Nucleic Material**	**Others**	*Pyruvate*
Histidine	Tyrosine	*Guanosine*	*Acetone*	*Succinate*
Isoleucine	Valine	Hypoxanthine	Isobutyrate	
Leucine	b-Alanine	Inosine	Ethanol	
		Uracil	Methanol	
		Uridine		

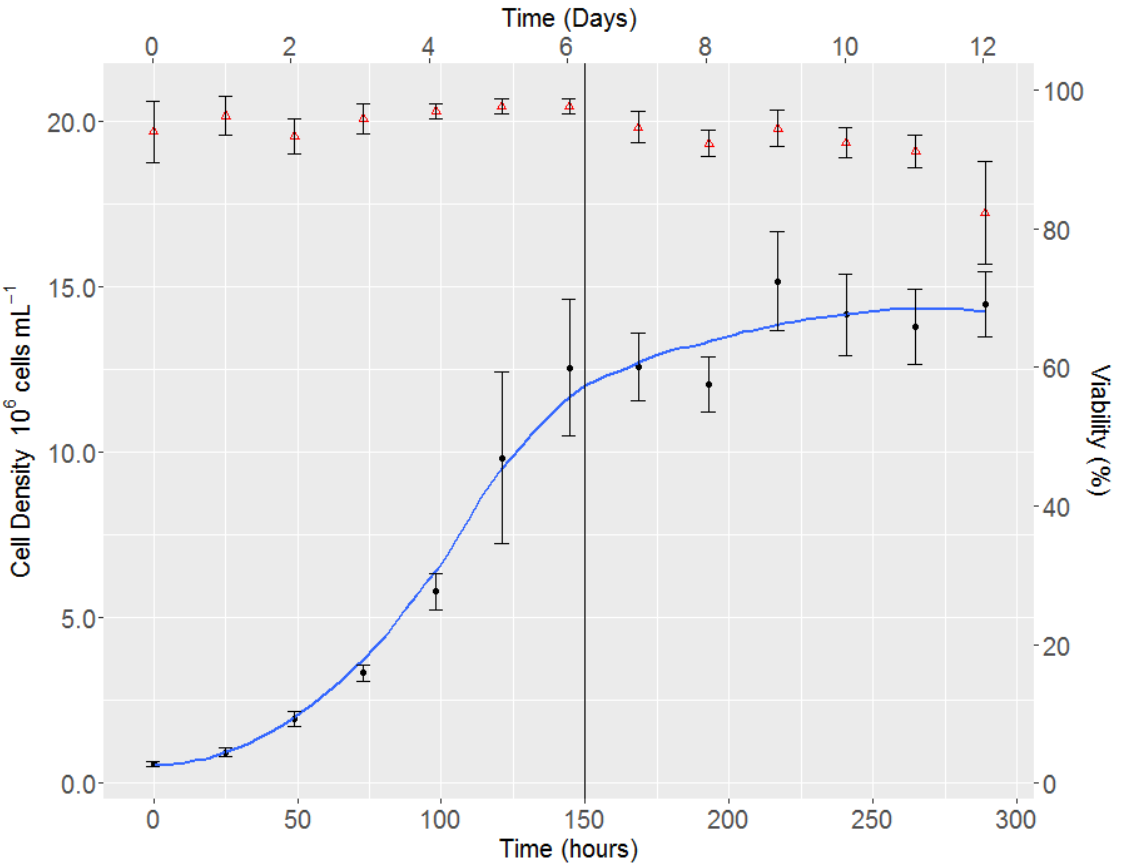

Figure 8.1: Growth of Sf-9 cells in Sf-900III media, average of three biological replicates. Vertical line represents the approximate end to exponential growth phase. Circle: cell density; triangle: viability.

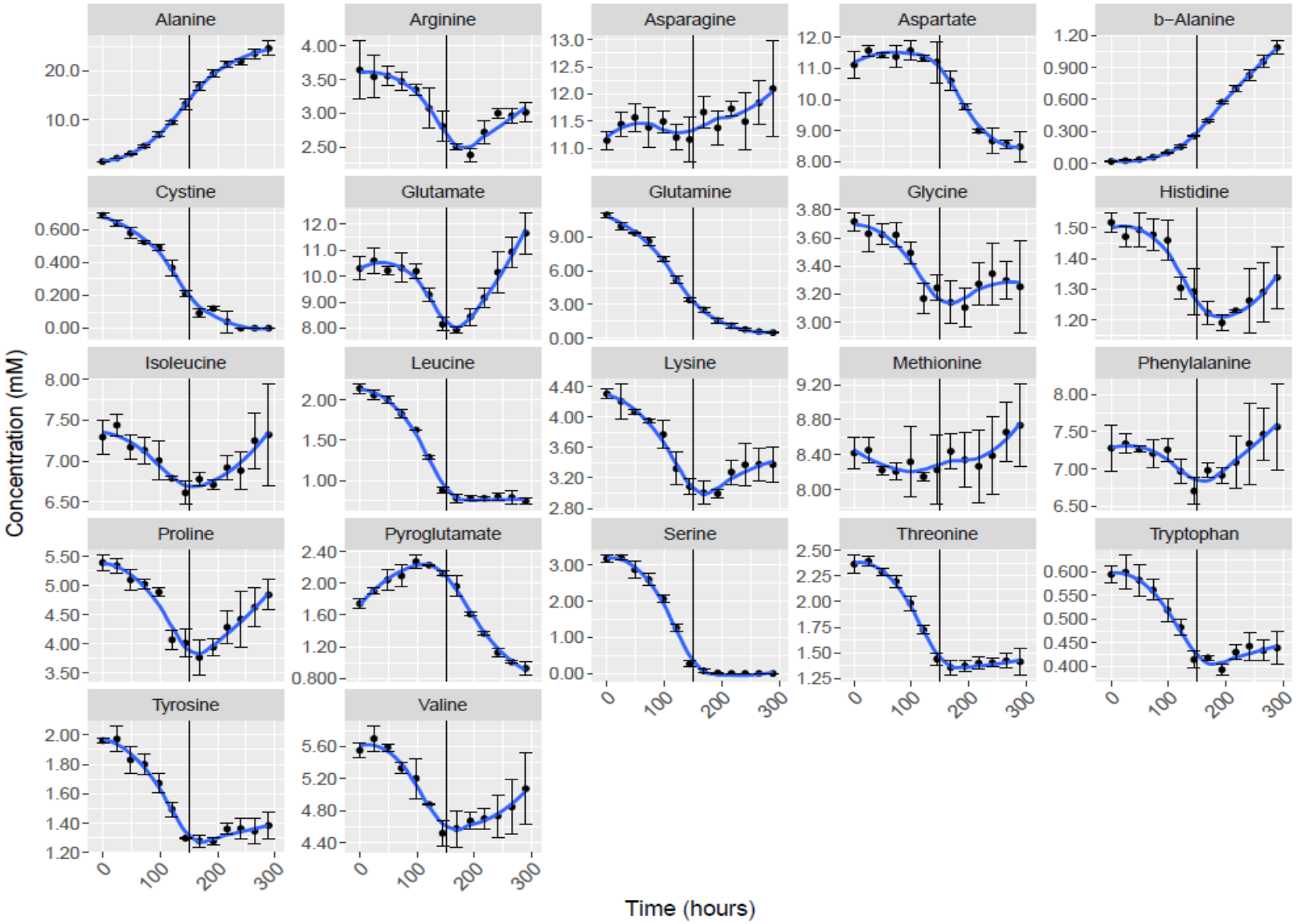

Figure 8.2: Trends of amino acids during metabolic studies in Sf-900II media, average of three biological replicates. Vertical line represents the approximate end to exponential growth phase.

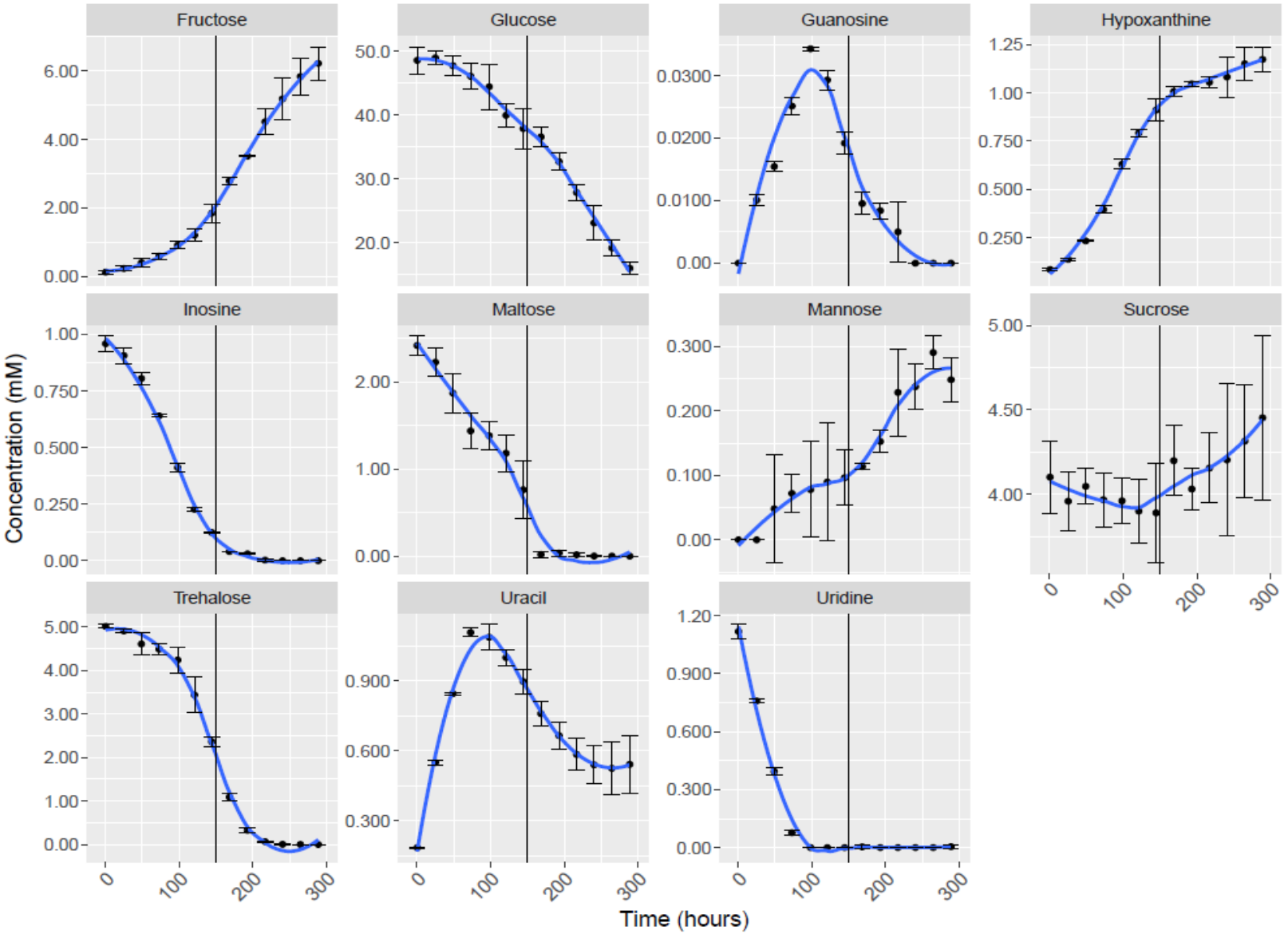

Figure 8.3: Trends of sugars and nucleic acids during a metabolic study in Sf-900II media, average of three biological replicates. Vertical line represents the approximate end to exponential growth phase.

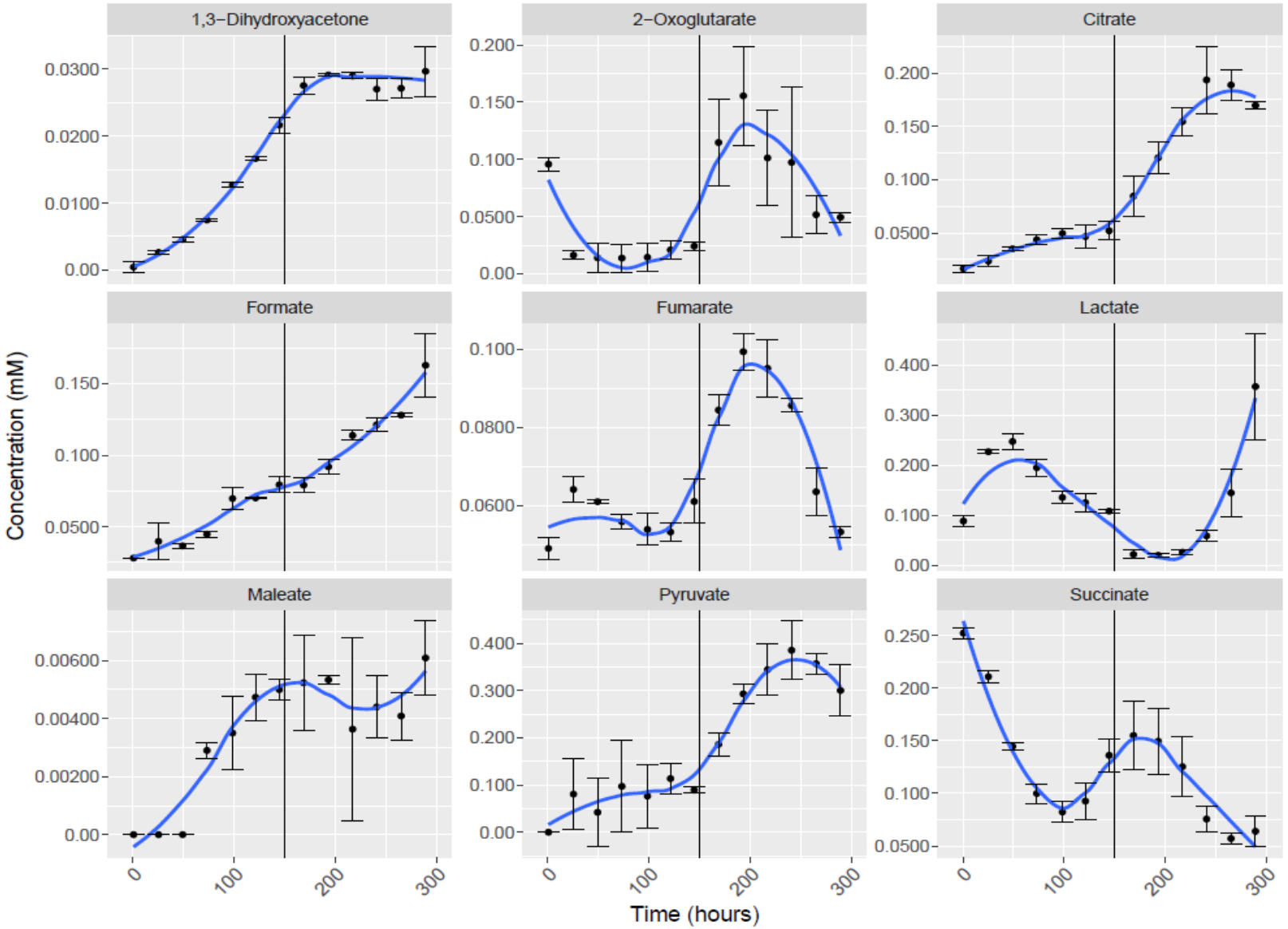

Figure 8.4: Trends of organic acids during a metabolic study in Sf-900II media, average of three biological replicates. Vertical line represents the approximate end to exponential growth phase.

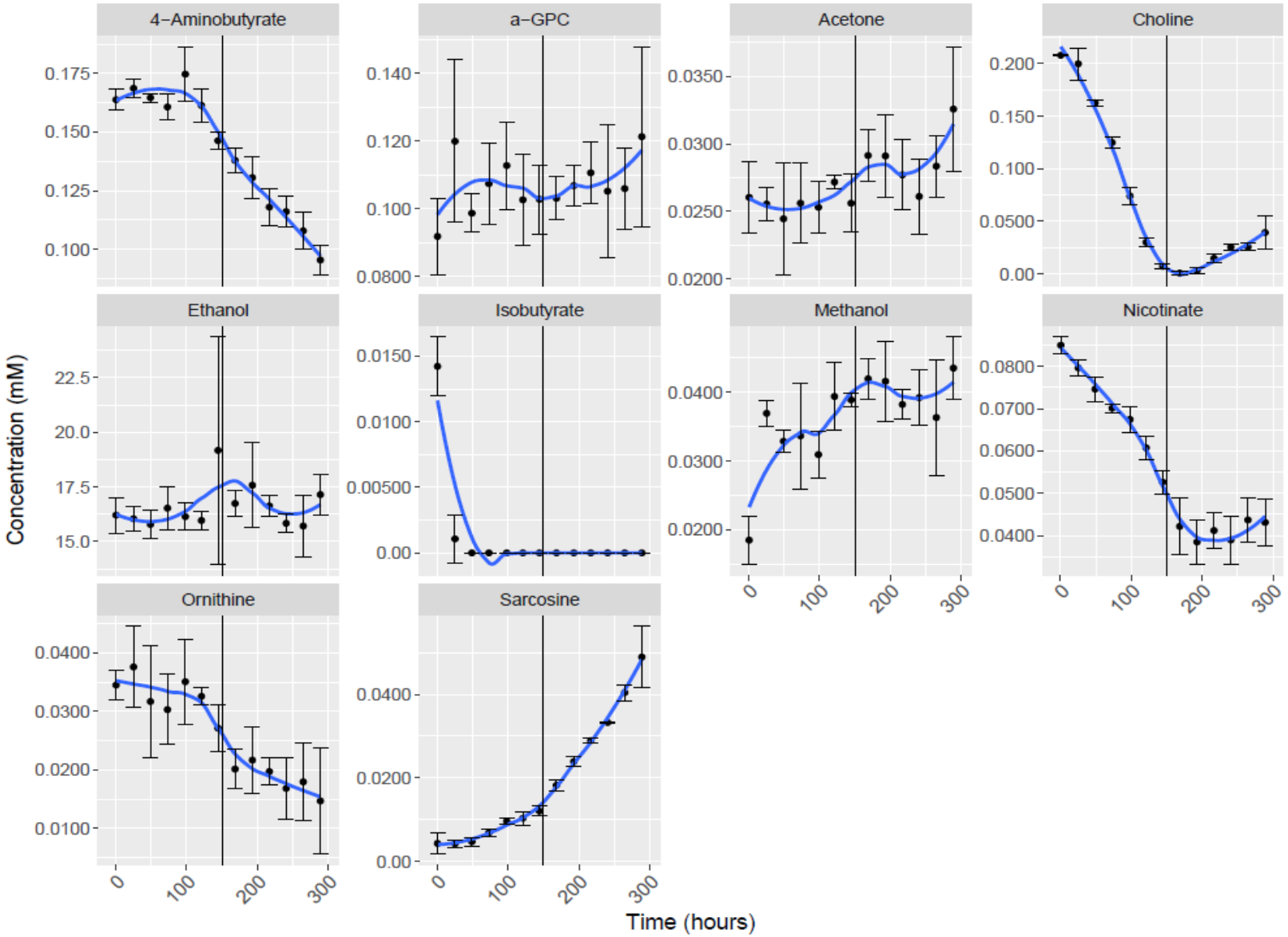

Figure 8.5: Trends of other biological and profiled compounds during a metabolic study in Sf-900II media, average of three biological replicates. Vertical line represents the approximate end to exponential growth phase.

These profiles are very similar to others found for Sf-9 cells in Sf-900II media[108, 109].

Amino acids

During exponential growth, the amino acids Ala, b-Ala[a], and pGlu increased whereas Asp, Asn and Phe stay relatively stable. The rest of the amino acids decreased, but only Cys and Ser are exhausted by the end of the culture. Once exponential growth was over, several amino acids began to accumulate in the media. No papers with an extensive metabolic analysis without media replenishment after the growth phase concluded could be found making it is hard to validate the trends.

Sugars

The Sf-9 cells consumed glucose and maltose and produced fructose and mannose. Sucrose is initially present in the media but appeared to be relatively stable over the culture, indicating it is not used at any point for energy production, contradicting other reports[110]. The significant amount of fructose produced only occurs when glucose is heavily supplied initially (i.e. cultures with lower initial levels of glucose, like the ALIM run in Section 8.1.2, do not produce this fructose). This suggests that the phosphofructokinase producing fructose-1,6-bisphosphate during glycolysis is not as active as those able to produce fructose and mannose (possibly mannose isomerase). Upregulating the phosphofructokinase genes would provide an interesting avenue for enhancing Sf-9 metabolism.

Nucleic material

Uridine was rapidly depleted from the media and resulted in a corresponding increase of the uracil, before the nucleotide was itself consumed. This indicates that the ribose is actively cleaved from the nucleoside, either by uridine ribohydrolase (which simply removes the ribose) or uridine:phosphate alpha-D-ribosyltransferase (which transfers the ribose to an inorganic phosphate). Similarly, inosine was depleted from the media and hypoxanthine was produced. However, as there is more final hypoxanthine than there was initial inosine, other mechanisms must be responsible for this discrepancy. At no point was xanthine detected though, indicating that the Sf-9 cells do not have an efficient xanthine dehydrogenase. It is hard to rationalize why guanosine would increase and decrease so rapidly but there was higher variability with this compound's spectral location than the other nucleosides and it could indicate that some improper qualification has occurred. However, no ribose was detected at any time. Therefore, while it is clear that

[a] Alpha-alanine is 2-aminopropanoic acid whereas beta-alanine is 3-aminopropanoic acid. These compounds have vastly different metabolic pathways and are not directly convertible. It is grouped here since it is still technically an amino acid.

the cells are metabolizing the ribose, it is unknown if the hydrolysis is occurring in the supernatant with a very quick consumption rate or inside the cell itself followed by nucleotide secretion.

Organic acids

Organic acid metabolism in Sf-9 cells was heavily dependent on the stage of the culture. Formate increased at a consistent rate regardless of the growth stage, whereas the increase of citrate was dependent on the stage of the culture, increasing much more rapidly once exponential growth had finished. 1,3-dihydroxyacetone (DHA) and maleate only increased during growth and stayed relatively stable during high density maintenance. Conversely, pyruvate, 2-oxoglutarate (i.e. AKG), and fumarate, were stable during the growth phase, but increased afterwards before being consumed again. Since maleate did not decrease after the growth phase, as noticed with fumarate, this suggests that the maleate cis-trans-isomerase is not very efficient in Sf-9 cells (maleate and fumarate are the common names for the cis and trans isomers of butenedioic acid, respectively). The profiles for succinate and lactate are highly unusual and might be metabolic in nature or they could be a result of the quantified peaks being highly variable in spectral location (succinate) and near the LDL (lactate).

Other biological and profiled components

Choline and nicotinate both decreased only during exponential growth, but whereas choline was fully depleted and then reappeared, nicotinate was stable after growth had finished. These are both explained by noting that these compounds are not energy related and are instead related more to the quantity of cells (i.e. choline in membranes and nicotinate as a vitamin). Although ornithine clearly decreased over the culture, it is hard to pinpoint if it is related to a specific growth stage due to the variable readings, unlike 4-aminobutyrate, which only decreased after growth had finished. Sarcosine can be created either through the methylation of glycine or the oxidation of choline to betaine and its demethylation into sarcosine. Of the other identified compounds, only isobutyrate was metabolized; all the other compounds were relatively stable over the culture.

8.1.2 ALIM Lot 1 with FL3 – Growth and Metabolite Trends

Figure 8.6a presents the adaptation of Sf9 cells in ALIM-4FL3 (i.e. ALIM media with 4g/L of FL3 yeast extract), while 8.6b focuses on the growth rate for the 8th passage (which was used for the metabolic study). The cells had an average growth rate of 0.0203 h^{-1} and a maximum cell density of 9.7 x 10^6 cells/mL, about 81% and 63% of the achievable statistics using Sf-900III, respectively. While their growth rates are

similar, the larger difference between the maximum cell densities and overall culture duration is due to glucose exhaustion after 7 days[49].

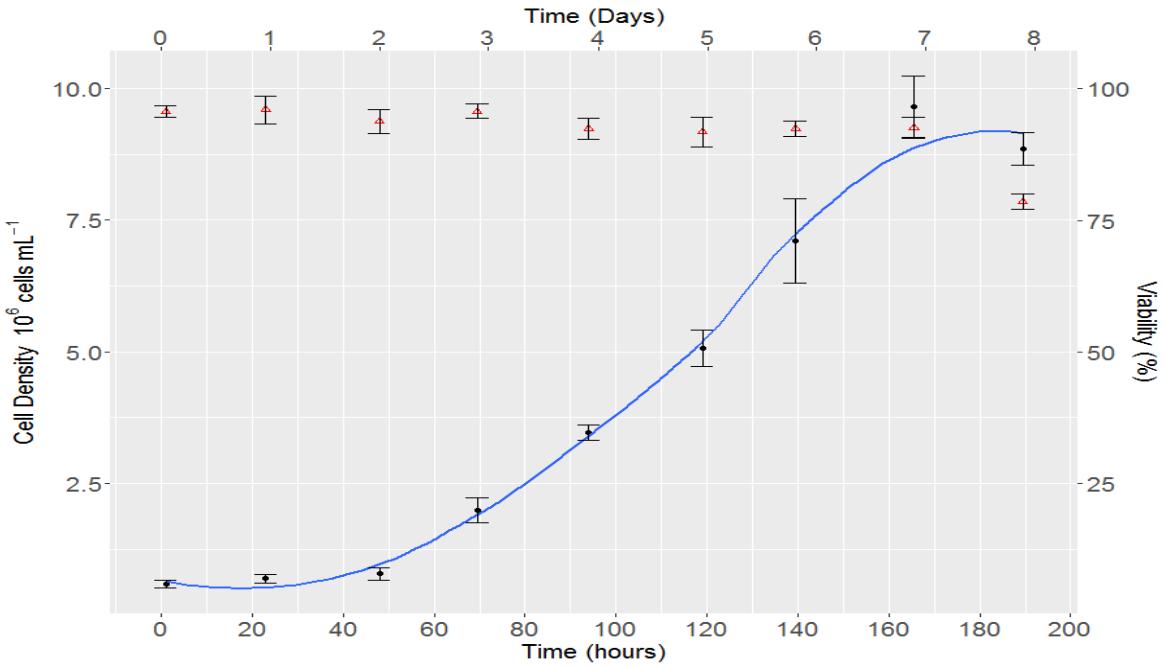

Figure 8.6b: Growth of Sf-9 cells in ALIM-4FL3 media after adaptation, average of four biological replicates. Circle: growth; triangle: viability.

In total, 52 compounds were profiled over the duration of the culture, 38 of which have reasonable certainty in their qualification (Table 8.2). Some compounds which had high confidence in their qualification for just the yeast extract scans became more convoluted due to the higher concentration of the amino acids and sugar (e.g. ornithine and acetate). The culture only lasted one day after exponential growth (i.e. the cultures were all dead on day 9). All metabolite trends can be found in Figures 8.7 – 8.10.

Table 8.2: Identified metabolites during ALIM cultures. Italicized components are of low qualification confidence due to heavy peak convolution or near the apparent LDL

Amino Acids		**Sugars**	**Biologicals**	**Organic Acids**
Alanine	Lysine	Glucose	*4-Aminobutyrate*	*Acetate*
Arginine	Methionine	Trehalose	Betaine	*Citrate*
Asparagine	Phenylalanine	.	Choline	Formate
Aspartate	Proline	**Nucleic Material**	*Glycerol*	Fumarate
Cystine	Pyroglutamate	Adenine	*Nicotinate*	Lactate
Glutamate	Serine	Adenosine	*O-Phosphocholine*	
Glutamine	Threonine	Cytidine	*Ornithine*	**Others**
Glycine	Tryptophan	Guanosine	*Sarcosine*	*3-Hydroxyisovalerate*
Histidine	Tyrosine	Hypoxanthine	Succinate	*Isobutyrate*
Isoleucine	Valine	Inosine	*a-GPC*	Ethanol
Leucine	*b-Alanine*	Uracil	1,3-Dihydroxyacetone	*Methanol*
		Uridine		

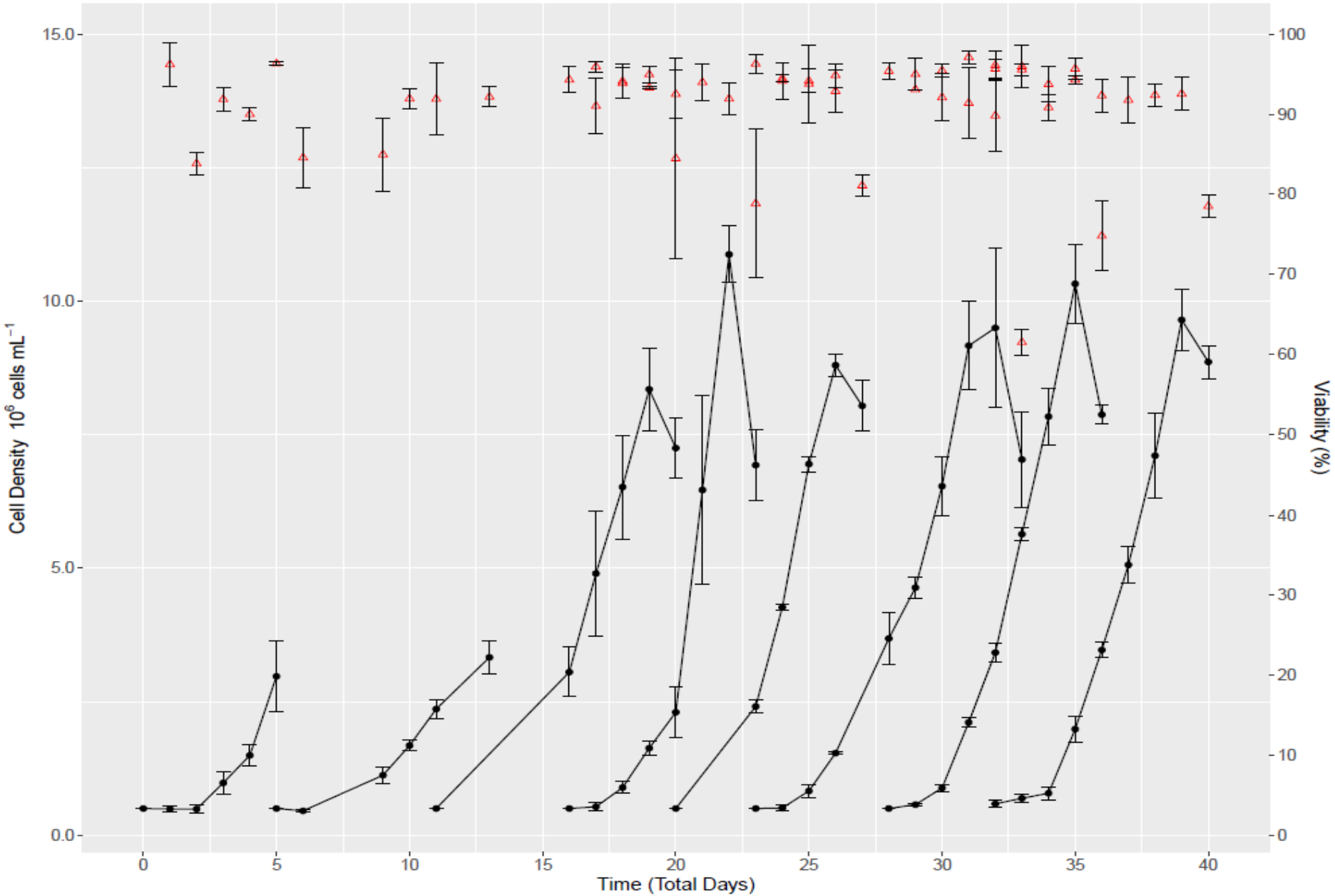

Figure 8.6a: Adaptation of Sf-9 cells in ALIM-FL3 media, average of two or four biological replicates. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 100% new media (passage 3 would be of negligible residual Sf-900III)

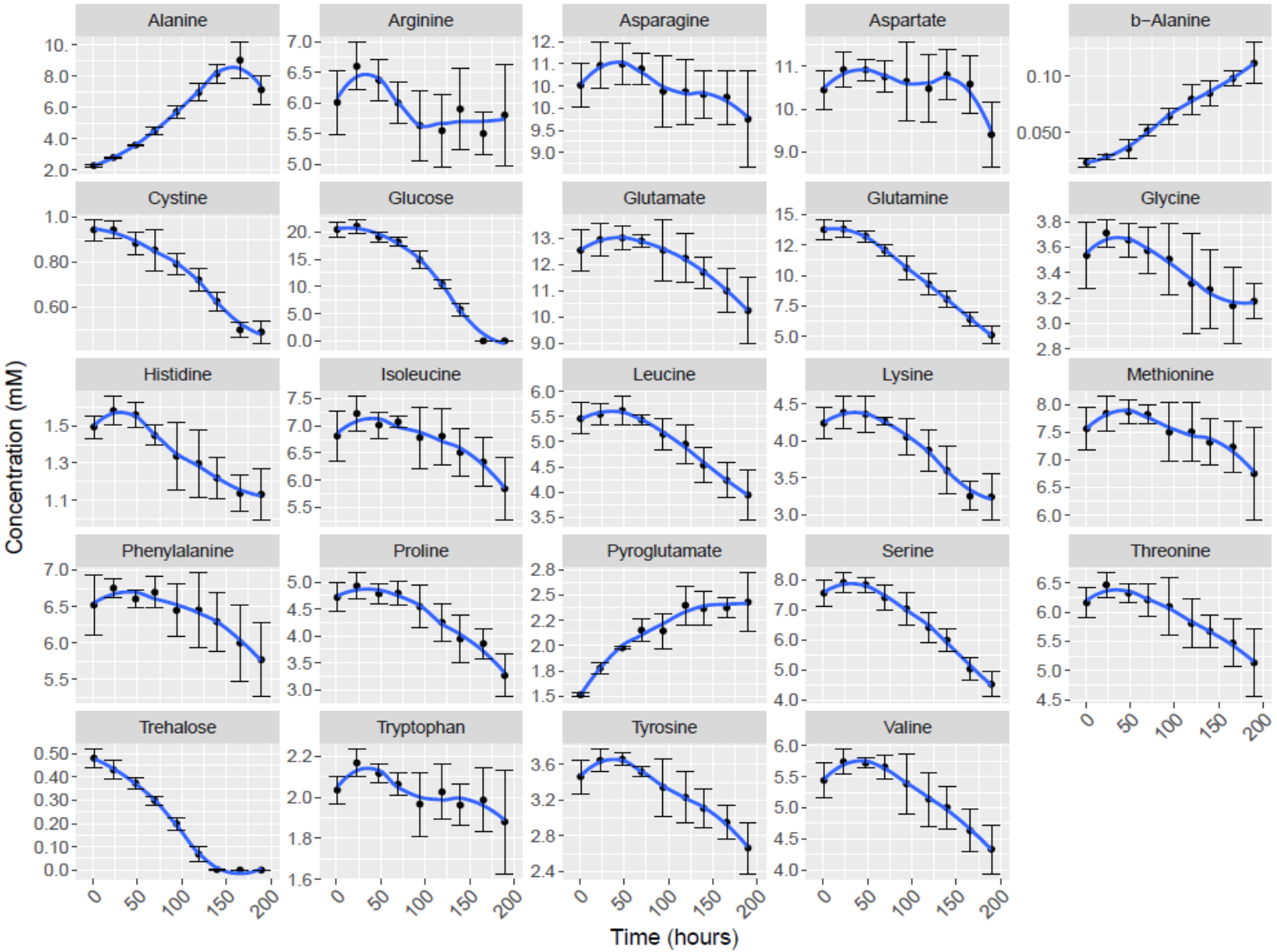

Figure 8.7: Trends of amino acids during metabolic studies in ALIM-FL3 media, average of four biological replicates.

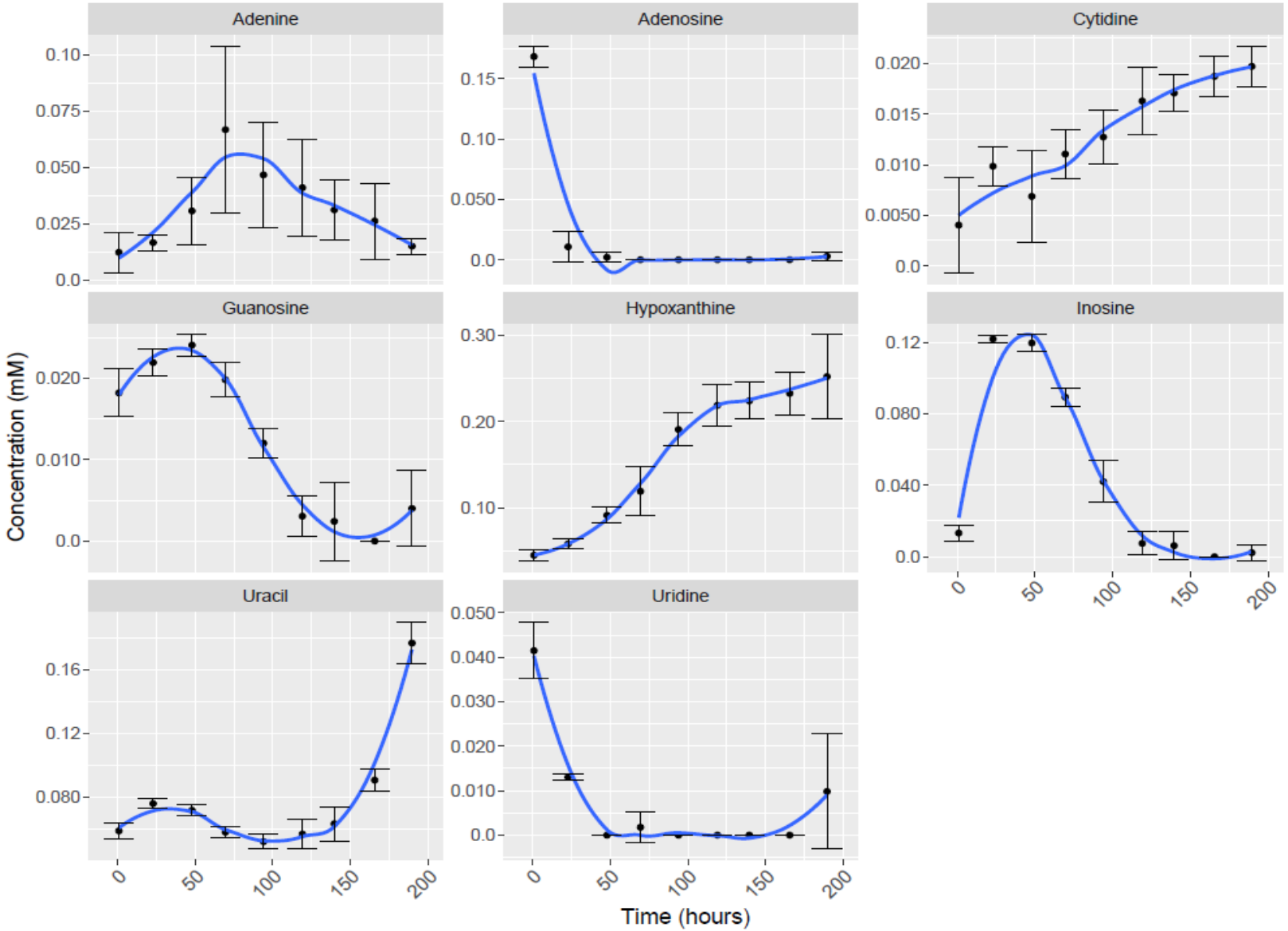

Figure 8.8: Trends of nucleic material during metabolic studies in ALIM-FL3 media, average of four biological replicates.

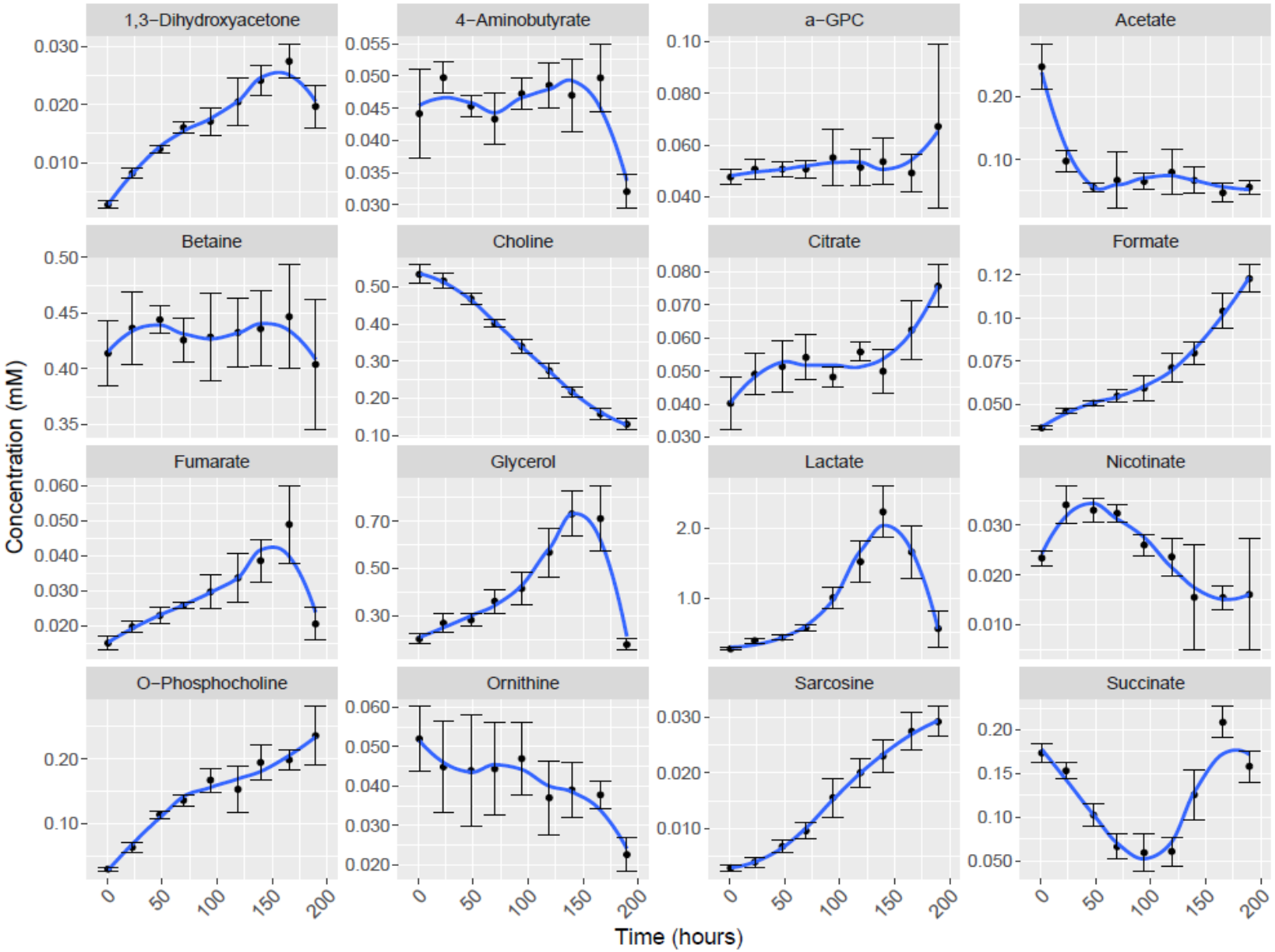

Figure 8.9: Trends of organic acids and other biological components during metabolic studies in ALIM-FL3 media, average of four biological replicates.

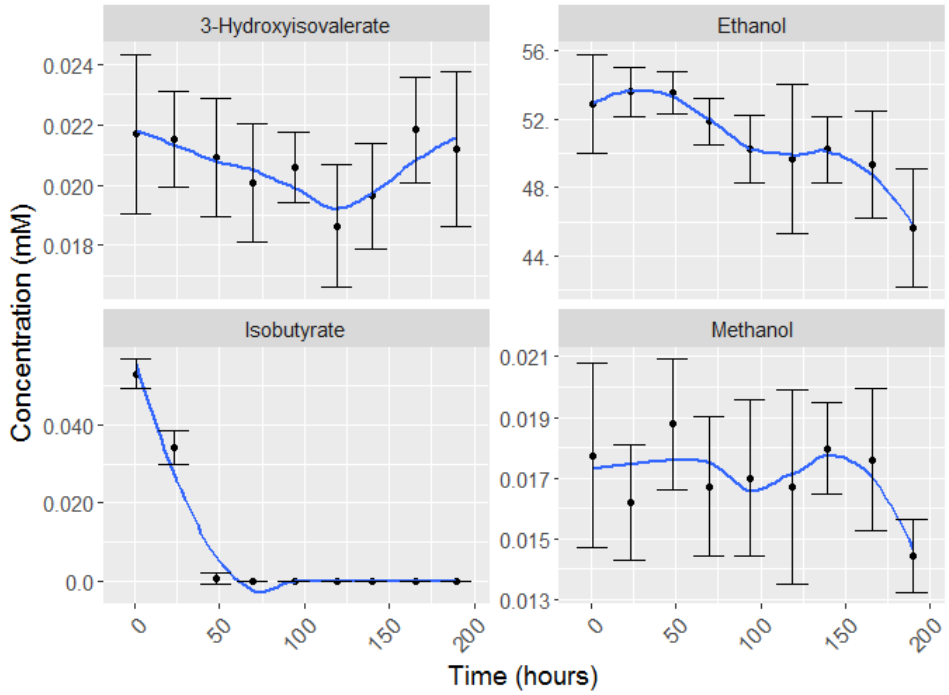

Figure 8.10: Trends of other profiled compounds during metabolic studies in ALIM-FL3 media, average of four biological replicates.

Amino Acids and Sugars

Only three amino acids show any significant increase during the culture: *n*- and β-alanine are metabolic by-products whereas pyroglutamate is a result of glutamine destruction. Of the remaining amino acids, only cystine and glutamine show close to a 50% reduction. Glutamine is a dominant carbon source for insect cells and so its reduction is expected. Cystine is the lowest supplied amino acid at less than 1 mM and this could be the reason why it has a more significant reduction compared to the other amino acids which are mostly supplied above 5mM. Overall, the trends closely mimic those in Sf-900III. Although no amino acids were completely exhausted, in most instances there was a more significant reduction after day seven, the time of the noticed glucose exhaustion. Therefore, with higher glucose supplementation, it could be possible to encounter glutamine exhaustion. However, excessive glucose increases the production of lactate, alanines and fructose, which are only consumed upon glucose exhaustion (as can be seen from the Sf-900III metabolite trends). The amino acid profiles compare reasonably well between the ALIM and Sf-900III cultures during exponential growth.

Nucleic Material

The cells seemed to metabolize the nucleosides and eventually secrete their respective nucleoside except for cytidine, which was continuously secreted over the duration of the culture with no cytosine being

detected at any point (guanine cannot be quantified by H-NMR so it is unknown if this is also increasing). Adenine is unique as it is secreted for the first couple of days before being metabolized. This could be due to the initial cells metabolizing the ribose from adenosine and having an excess of adenine, before the cell density is large enough to have a noticeable adenine metabolism. There was also a shift from having adenosine and little inosine in the media initially to very little adenosine and a lot of inosine after only 24 hours. All nucleosides increased on day 8, indicating that they are released from the cells during lysis. Most of the differences between these and the Sf-900III trends are due to the much lower initial supplied levels.

Organic Acids and Other Biological Compounds

A majority of these compounds show dramatic increases over the duration of the culture. Lactate is known to be produced by the cell in response to glucose metabolism; upon glucose exhaustion, the cell further metabolizes the lactate. Glycerol may be from glycolysis or fatty acid metabolism but either way, it also shows a heavy depletion after day seven meaning it could have been re-introduced into glycolysis as glycerol-3-phosphate after glucose exhaustion. The other TCA components are most likely present in the supernatant due to the cell producing a more than sufficient quantity for its needs and allowing a small amount to be excreted, but only fumarate and succinate show a noticeable decrease coinciding with glucose exhaustion. While betaine seems to be metabolized by the cell, its related compound choline is heavily used. The final combined amount of o-phosphocholine and sarcosine represents about half of the initial starting choline, suggesting that the choline is converted to these products and excreted, while the remainder is presumably used as a phospholipid head group. Succinate presents an interesting trend as it seems to be used over the initial growth of the culture but then it is excreted during the later stages.

Other Profiled Compounds

Four other compounds do not fit neatly into the above categories and thus have been separated. Ethanol is supplied in the lipid solution, whereas methanol could possibly be present as a contaminant (but more likely it is due to the spectral peak being improperly qualified). Although a decreasing trend can be fitted to ethanol, it only changes by about 6% over the course of the culture, well within the expected error for H-NMR, and therefore its trend could be a random outcome of the profiling. While 3-hydroxybutyrate seems to be stable over the culture, isobutyrate is completely metabolized.

Overall, the general trends obtained with ALIM-FL3 mimicked those observed with the Sf-900III media, confirming the hypothesis that the components necessary for growth are contained in a variety of hydrolysate lots and suppliers.

8.1.3 ALIM Lot 2 with FL1, FL2 and BD Yeast Extracts

As stated in Section 4.7, two different lots of ALIM were used due to not having the studied yeast extracts in the lab at the same time. While the media is completely chemically defined apart from the yeast extract, different lots of the defined lipid solution (purchased from SigmaAldrich) were used. Although it is hoped that the defined solution is made to the same standards every time, noticeable variability in the appearance of the solution was experienced by multiple users in the lab and it is unknown how this could affect lipid solubility and overall efficacy of the product. Therefore, these results are being presented with the major underlying assumption that the lipid solution was of similar quality and resulted in the same amount of overall lipid supplementation.

Figure 8.11 to 8.13 present the adaptation of Sf9 cells into ALIM with 4g/L of FL1, FL2 or BD yeast extracts, respectively.

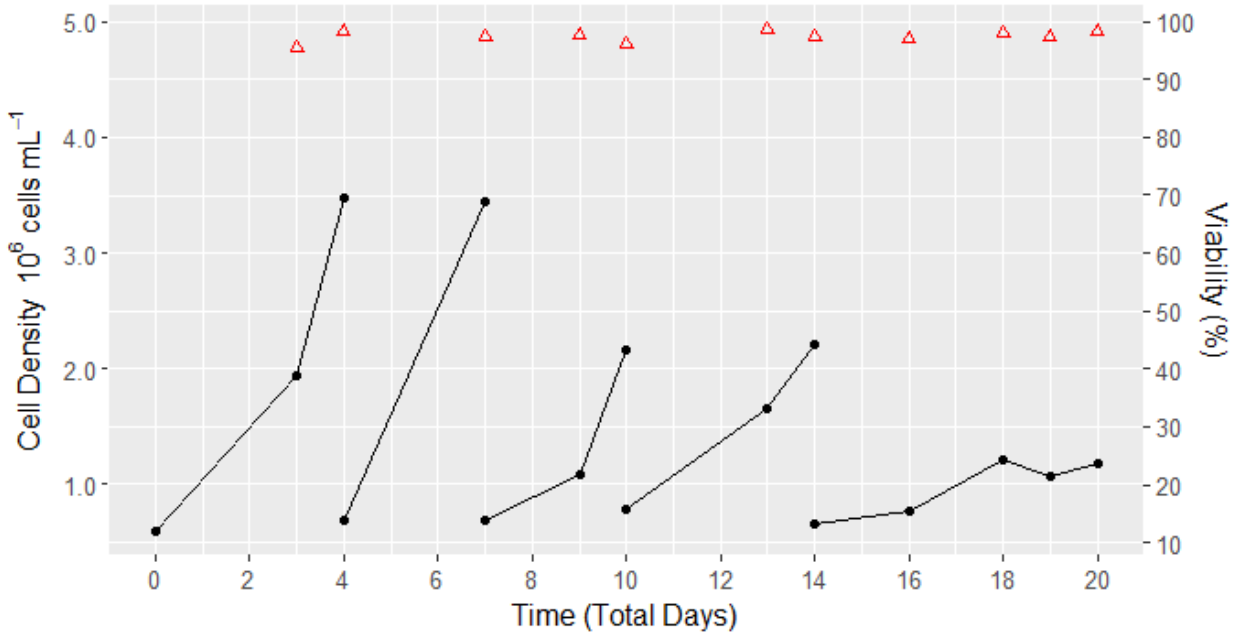

Figure 8.11: Adaptation of Sf-9 cells in ALIM-4FL1 media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% new media (passage 5 would be of negligible residual Sf-900III)

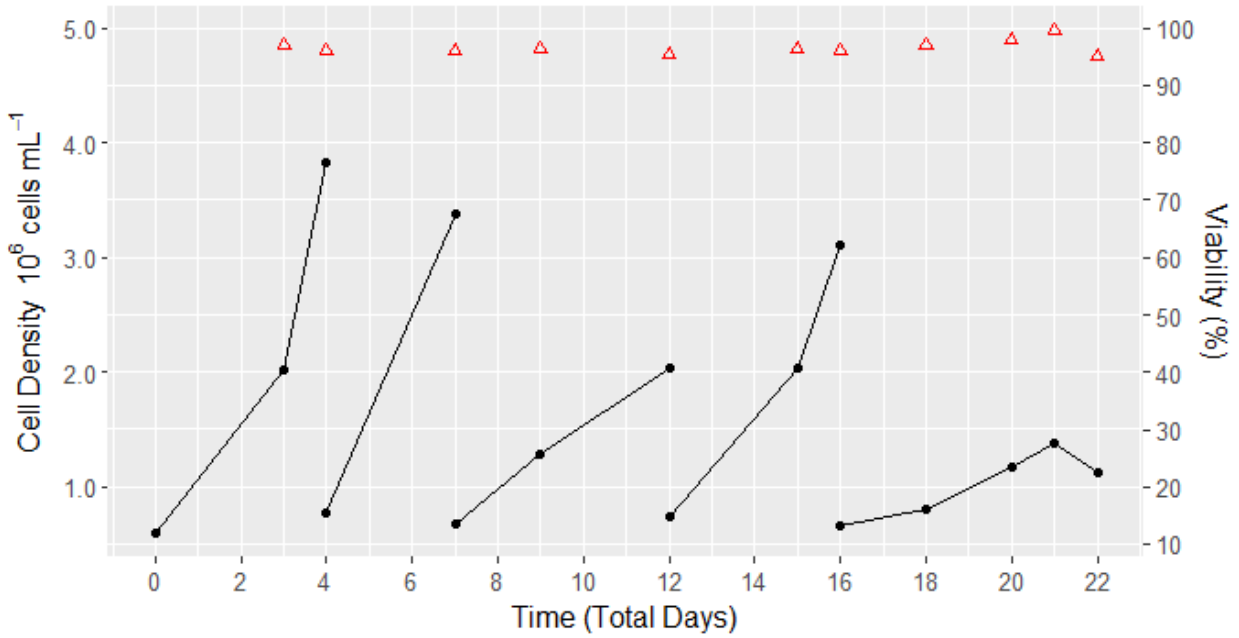

Figure 8.12: Adaptation of Sf-9 cells in ALIM-4FL2 media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% new media (passage 5 would be of negligible residual Sf-900III)

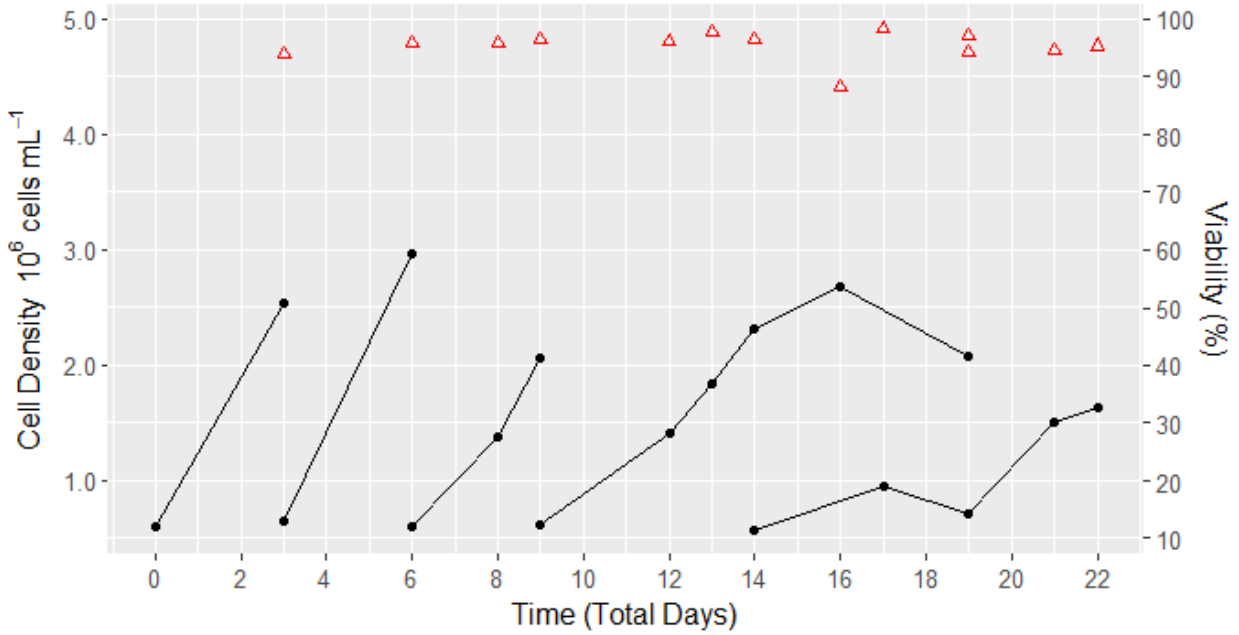

Figure 8.13: Adaptation of Sf-9 cells in ALIM-4BD media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% new media (passage 5 would be of negligible residual Sf-900III)

None of these yeast extracts were able to achieve growth above $2x10^6$ cells/mL after 4 or more passages. The major reason for this (again ignoring any potential issues from the lipid solution) would be due to the minor variations in composition affecting the overall supplementation levels. For example,

since FL2 and FL3 have a much higher weight percent of trehalose, other components would be affected negatively and show a small reduction in their contribution. While this may not be an issue for amino acids, sugars or ions more heavily provided by the defined basal medium, it could significantly affect trace components heavily provided by yeast extract. Therefore, 8g/L of yeast extract was tried in order to see if this would boost those trace components into active ranges, as documented in Figures 8.14 to 8.17.

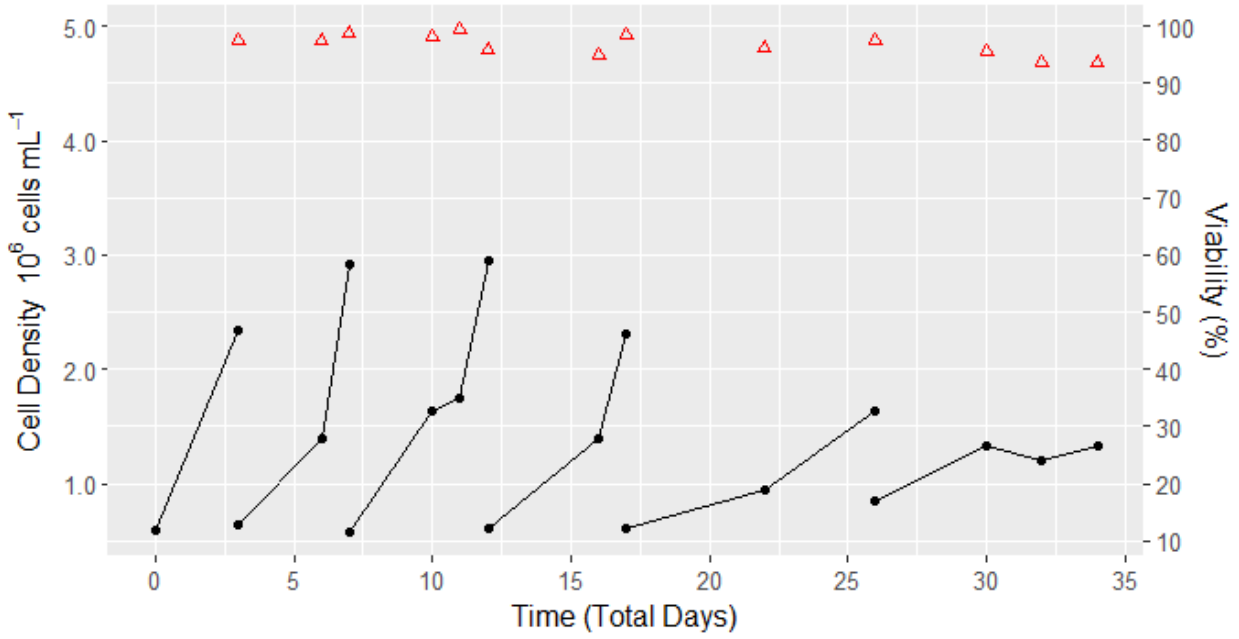

Figure 8.14: Adaptation of Sf-9 cells in ALIM-8FL1 media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% more new media (passage 5 would be of negligible residual Sf-900III)

Although there was no noticeable improvement using 8g/L of FL1, both FL2 and BD had a positive, albeit short-lived, effect. This would tend to suggest that there are some compounds in the yeast extract that need to be increased in order to improve the growth of Sf-9 cells. In all cases, the cells kept their high viability, even though there was no significant growth. Sf-9 cells are known to stay viable in media with a sufficient quantity of amino acids and sugars even if the compound required to initiate significant growth is absent[1]. ICP analysis on the media showed a remarkably high variation in the expected and actual trace metal supplementation, as shown in Figure 8.13. Of the trace metals supplied in the IPL supplement, only Cu was detected in the ALIM media and even this was at a much higher level than expected from the supplement. However, when the IPL trace metal concentrate was analyzed (Figure 8.14), all elements were detected but at about 20x lower than expected. Even though the IPL supplement is made as a 1000x concentrated solution, four elements require <0.030 g when making a 500mL batch, meaning there is a much higher variability in their measurements and this could be affecting final supplementation. Although

the yeast extract would provide more than the supplement for all of the detected elements, Mo cannot be detected. Therefore, if this compound is suffering from the same under-supplementation, and it is not supplied by the yeast extract, this could explain the severe differences in observed growth.

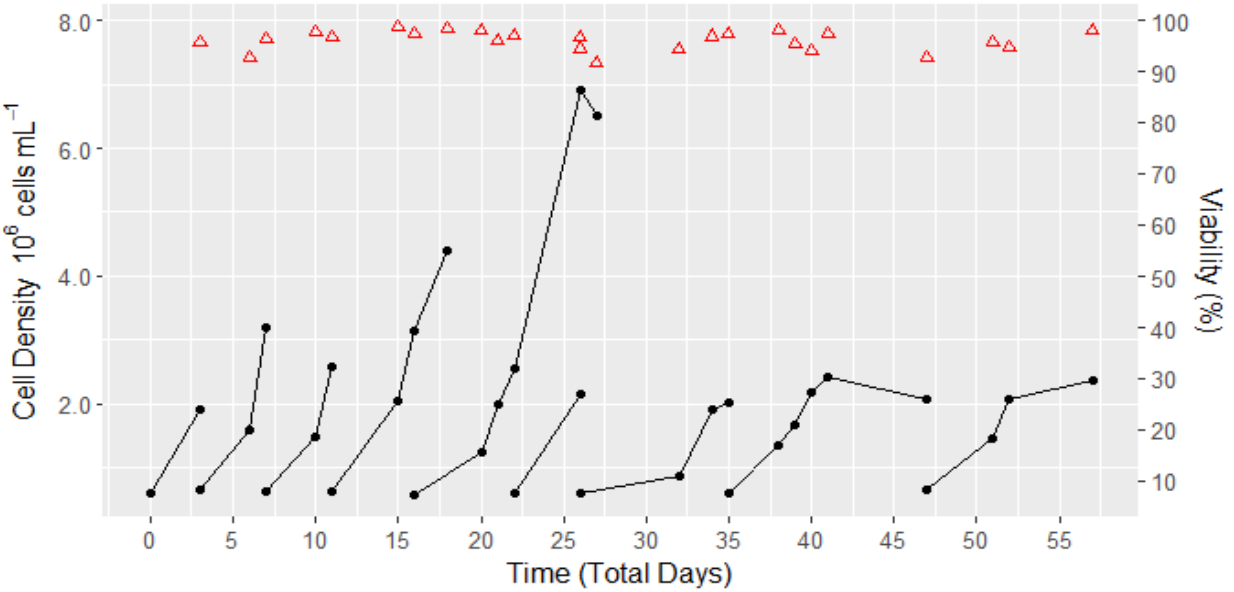

Figure 8.15: Adaptation of Sf-9 cells in ALIM-8FL2 media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% more new media (passage 5 would be of negligible residual Sf-900III)

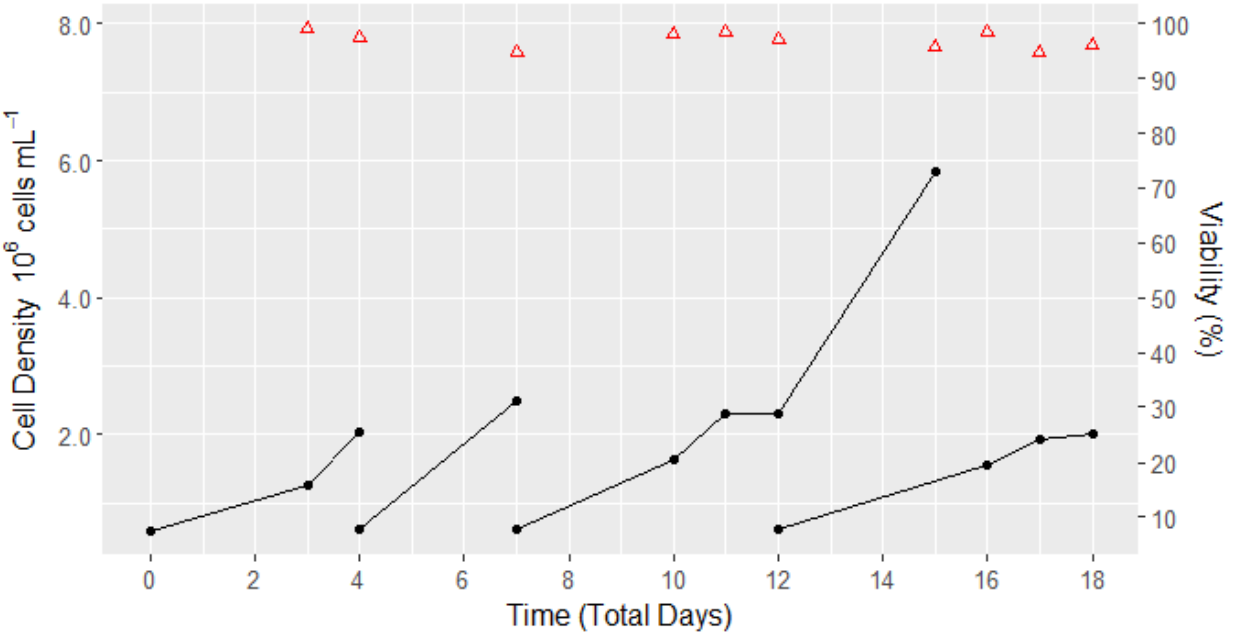

Figure 8.16: Adaptation of Sf-9 cells in ALIM-8BD media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 25% more new media (passage 5 would be of negligible residual Sf-900III)

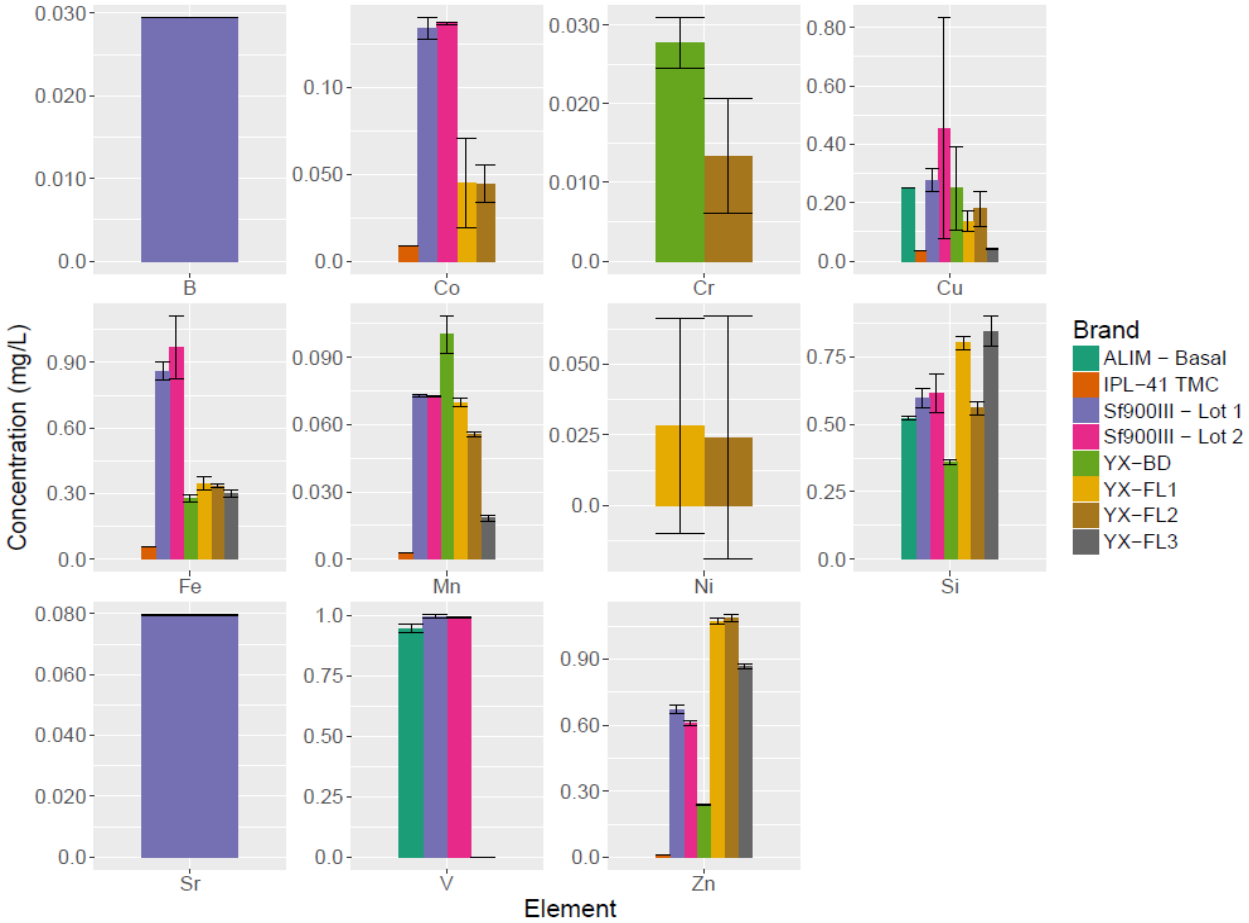

Figure 8.17: ICP results for each yeast extract, ALIM basal media, and two lots of Sf900III. TMC: Trace metal concentrate. Whereas the Sf-900III levels were consistent, ALIM did not have at least the amount expected from the supplement, but the yeast extract would more than cover for any deficiencies in it.

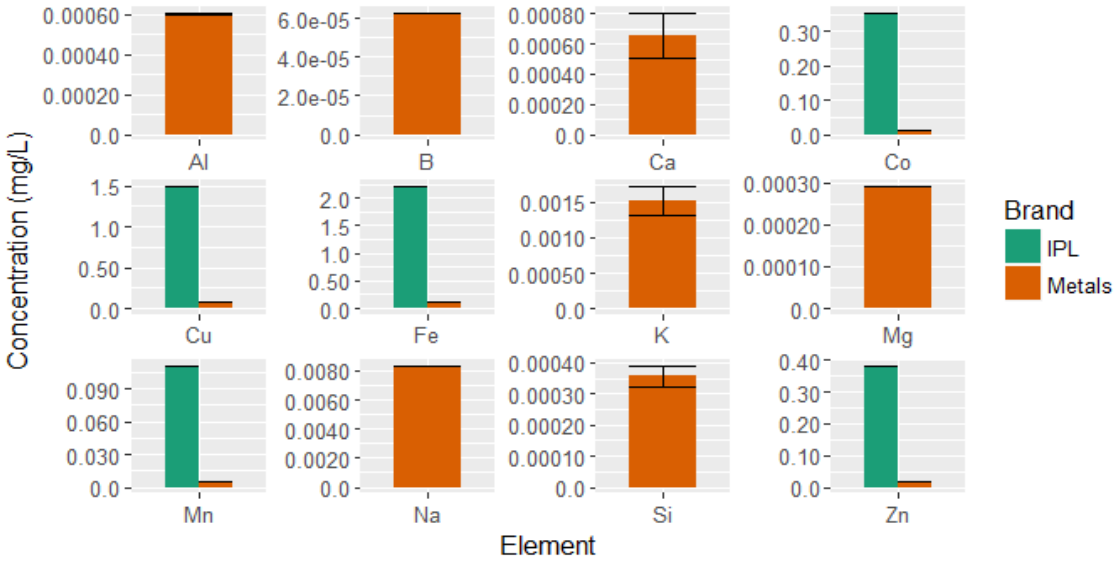

Figure 8.18: ICP results for trace metal concentrate (orange) as compared to the expected IPL trace metal supplement defined level (green). Significant differences in expected supplementation are obvious, as well as having a variety of contaminants (refer to Section 6 for related information).

8.2 Cell growth in yeast extract free medium

Based on the yeast extract and metabolic studies, the supplement in Table 8.3 was used as a CD replacement (when added to ALIM, the whole media is referred to as ALIM-CD).

Table 8.3: Supplement used as a replacement for yeast extract (final concentration when supplemented)

Trace Metal	**µM**	**%ALIM**
Co	0.65	309
Cu	1.5	110
Fe	3	152
Mn	1	994
Mo	0.05	154
Se	0.5	New
Zn	5	1704
Vitamin		
4-Aminobenzoic acid	10	429
Biotin	3	458
Cyanocobalamin	0.9	508
Folic acid	0.9	497
myo-inositol	20	901
Pantothenate	10	59567
Pyridoxone	5	257
Riboflavin	1	470
Thiamine	1.5	632
Vitamin B3	40	3078
Other		
Adenosine	150	New
Ascorbate	100	New
Ethanolamine	50	New
Ornithine	30	New
Putrescine	50	New
Succinate	250	New
Uridine	40	New

The Fe, Mn, and Zn levels were based off the ICP results and represent a 3, 10, and 17 fold increase over the IPL levels, respectively. Se at this level was not seen to be toxic to the cell culture; Mo is unable to be detected with this ICP analysis but is increased by about twice that in the IPL supplement (a similar increase as the other elements). The vitamins detected in yeast extract were supplemented at similar levels achieved from 4g/L; the other vitamins were increased about five-fold over the level in the IPL-41 supplement. The other components were based off the detected amount in yeast extract. Putrescine, ethanolamine and ascorbate were arbitrarily chosen, but significantly less than in other reported defined media for insect cells. Ascorbate is added to act as a general antioxidant since suspension cultures are a highly oxidative environment, even before the trace metals were significantly increased.

Figure 8.19 presents the adaptation of Sf-9 cells into ALIM-CD. As can be seen, the third passage didn't reach the required density needed to passage the cells and thus no further passages could be attempted. However, since passage 2 is about 10% Sf-900III and passage 3 is about 3%, this tends to suggest only a small amount of some compound is required to create any meaningful growth.

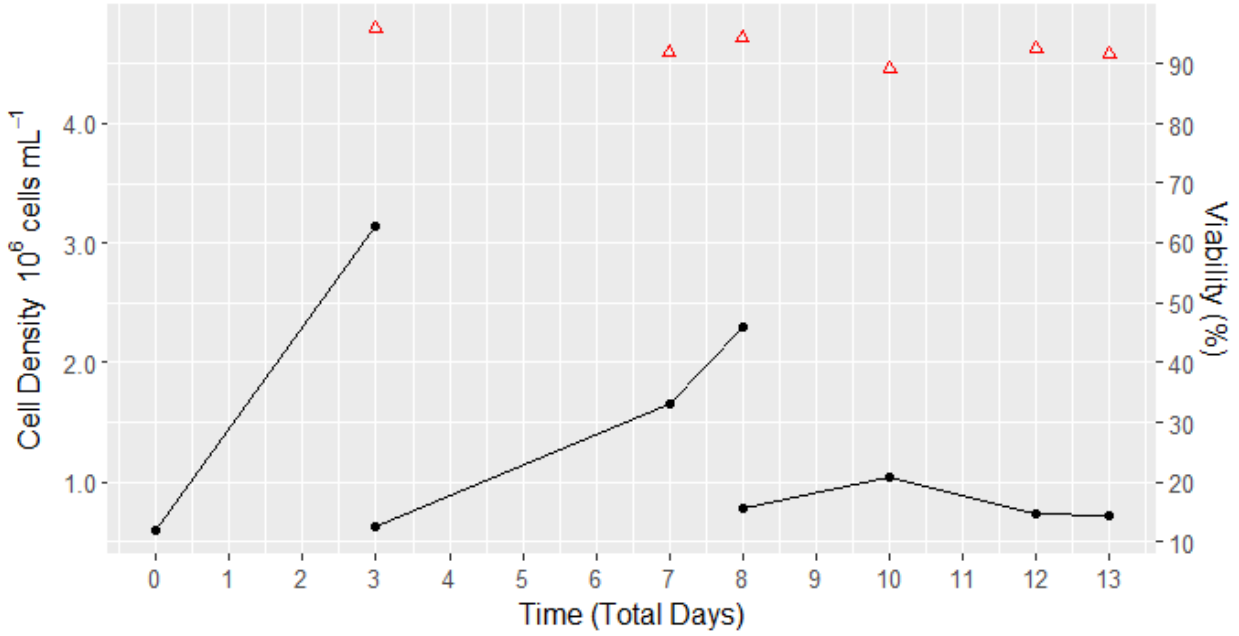

Figure 8.19: Adaptation of Sf-9 cells in ALIM-CD media. Circle: growth; triangle: viability. Connected points are of the same growth passage before being re-suspended into 50% more new media (passage 3 would be of negligible residual Sf-900III)

With all of the studied cell cultures having trouble adapting to their new medium, this could be a serious indicator that there are major problems with the lipid solution. As documented by Gilbert et al.[111], a lack of lipids had a serious effect on the growth of insect cell lines. While they determined the growth was mainly recovered when only Pluronic F-68 was added, it is hard to believe that the lipid solution used in this study (which is said to contain 100 mg/mL of it) would not be able to create this same effect at any point. More rigorous investigation is required in order to determine the exact effect and requirements of the lipid solution and if this is the cause of the lack of growth for all cultures.

9.0 Overall Conclusions

This work produced several important results, crucial to both the validation for the use of H-NMR spectroscopy in complex solution analysis and to the overall variability that exists in the commercial production of yeast extract, which would extend to any other hydrolysate production.

9.1 Analysis of Yeast Extracts by H-NMR and ICP

The complex nature of the yeast extract did not impart any additional variability when quantifying solution components, as verified through ANOVA and PCA. However, it was shown that PCA is highly sensitive to the NMR pre-processing steps and could identify likely spectra with this error.

While there was no additional variability in the quantification, there was a clear under bias imparted by increasing the overall hydrolysate concentration. Increasing the overall concentration did allow for the quantification of several compounds which were below the detection limit in more dilute samples. This highlights the need for several concentrations of hydrolysate to be analyzed during quantification and screening methods.

There was a high degree of lot and supplier variation. Some compounds (e.g. trehalose, choline products, and vitamins) showed a very significant variation between the studied lots. This highlights the need for stricter industry control parameters to minimize the variation between products. This variation, although seemingly minor based on this analysis, led to significantly different results when the product was used in an attempt to grow insect cells. The lot variation was also seen during the trace metal analysis, but these results were heavily influenced by the presence of several apparent contaminants, most of which are assumed to be a result of the water used to make the hydrolysate and *aqua regia* solutions.

Based on the acid digestion of the yeast extract, it was shown the product consisted of about 61% amino acids, 11% sugars, and 7% nucleic material. While the vitamins and trace metals only make up about 5% of the yeast extract, this still represents a significant increase in their current supplementation in media and highlights the need for a considerable increase when transitioning to a chemically defined medium.

9.2 Metabolic Analysis of Sf-9 Cells

The various yeast extracts produced significantly different results when cell cultures were attempted to be grown in supplemented medium. Only one lot of yeast extract was able to promote growth with the

in-lab media, but it closely mimicked growth obtained in commercial media. This reinforces the fact that the crucial component for growth can be found in a variety of products, but not in every lot of the same product. This again will require stricter industry control parameters to ensure that lots are more consistent with each other.

While a chemically defined supplement was devised based on the yeast extract analysis, it was unable to sustain any substation growth on its own. However, since growth was still obtained when the commercial media was diluted to about 5%, this shows that whatever the crucial component is, only very little of it is needed to initiate growth. The replacement contained several novel compounds without a significant amount of previous research, and therefore it is unknown if the supplementation used is sufficient.

10.0 Future Work and Recommendations

This analysis provides several interesting opportunities for further research.

10.1 Chemically Defined Medium Replacement

Most of the compounds used in this supplement have never been studied before and therefore it is unknown how close these compounds are to their individual, or any combined, toxicity limit. However, since the cells remained viable during the metabolic study, none of these compounds are currently at this threshold and thus increasing their concentrations may boost them into the necessary range. Since the vitamins and trace metals have already been increase substantially, the next step would be to see if increasing the novel components' concentration will have any benefits.

10.2 Lot and Supplier Variability of Yeast Extract

It would be beneficial to analyze several more lots and suppliers of yeast extract to have a more complete understanding of the possible variations that can occur between suppliers and their lots. This can be paired with more metabolic analysis to see which lots can induce growth in insect cells. These lots can then be subjected to more rigorous analysis techniques (e.g. GC-MS, fractionation), to see if any more similarities can be distinguished and allow for more insights into a defined supplement.

10.3 ICP Analysis

Due to the considerable amount of contaminants detected during the trace metal analysis, it is believed that the source of the water used for both the hydrolysate and *aqua regia* solutions are critical in order to eliminate all sources of error. Further, the current machine can be calibrated to detect selenium, a crucial element expected to play a significant metabolic role in insect cultures.

10.4 Bioactivity of Insect Culture Supernatant

To more fully understand any possible enzymatic activity which occurs in the supernatant separate from metabolic processes, disaccharides and yeast extract can be digested in supernatants of various ages to see which reactions occur. This could allow for a greater understanding of the metabolic needs of the insect cells, producing more avenues of investigation for the chemically defined supplement.

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Appendix A – Matlab Code – Curve Fitting

Coupled Glucose ODE Model

```
function ODE_nonlinfit

%grab data from relevant Excel Spreadsheet location
T = [0;30;60;90;120;180;240;360;720];
Yavg = xlsread('Triplicate','Biological Triplicate Others','B24:J24')';
Stdv = xlsread('Triplicate','Biological Triplicate Others','L24:T24')';
[~,tit]=xlsread('Triplicate','Biological Triplicate Others','A24');

%Data Plotting
figure(1)
errorbar(T,Yavg,Stdv,'ro','markersize',7)
hold on
xlim([0,750])
ylim([0.8*min(Yavg) 1.2*max(Yavg)])
set(gca,'ytick',[0.8*min(Yavg):(1.2*max(Yavg)-0.8*min(Yavg))/10:1.2*max(Yavg)])
set(gca,'yticklabel',num2str(get(gca,'ytick')','%0.2e'))
ylabel('Weight Fraction','FontSize', 14)
xlabel('Time [min]','FontSize', 14)
title(tit,'FontSize', 14)
hold on

%Parameter setting
alpha = 1
opts = statset('nlinfit');
opts.RobustWgtFun = 'bisquare';
B0=[0.01 0.0001]';

%ODE Parameter Solving
[beta,R,J,CovB,MSE,ErrorModelInfo] = nlinfit(T,Yavg,@odemodel,B0,opts);

%Optimum Parameter Solution
[~,Yest] = ode45(@(t,C)ode2(t,C,beta),T,[Yavg(1)]);
%Optimum Plotting
plot(T,Yest(:,1))

%R2 and CI calculations
avg = mean(Yavg).*ones(9,1);
SST = sum((Yavg-avg).^2);
SSE = sum(R.^2);
CI=nlparci(beta,R,'jacobian',J);
Beta_CI = [CI(:,1) beta CI(:,2)]
Beta_Range_Percent = (beta - CI(:,1))./beta
R2 = 1-SSE/SST

    function C = odemodel(B,t)
        function dC = ode(t,C)

           dC = zeros(2,1);                  %Coupled Glucose Kinetics
           dC(1) = -B(1)*(C(1))^alpha;
           dC(2) = B(1)*C(1)^alpha-B(2);
        end

        [t,C] = ode45(@ode,T,[Yavg(1)]);
        C=C2(:,2);
    end
```

```
    function dC=ode2(t,C,beta)

            dC = zeros(2,1);
            dC(1) = -beta(1)*C(1);
            dC(2) = beta(1)*C(1)-beta(2);
    end
  end
```

Other Component Modelling

```
function Acid_Digest_non_ODE_modelling_Aug1
clc
clear all
close all

% Grab data from relevant Excel Spreadsheet location
Yavg = xlsread('Triplicate-Reprofile July3-17 ML','Average', 'B33:J33')';%
[w%]
Stdv = xlsread('Triplicate-Reprofile July3-17 ML','Average', 'L33:T33')';%
[w%]
[~,tit]=xlsread('Triplicate-Reprofile July3-17 ML','Average','A33');
%compound name
T = [0;30;60;90;120;180;240;360;720];% [min] experimental times
%Yavg = [Yavg(1);Yavg(4:9)];
%Stdv= [Stdv(1);Stdv(4:9)];
Tspan = [0:5:720];% [min] modelled times

% Data Plotting - w% vs t, experimental points and error bars
figure(1)
errorbar(T,Yavg,Stdv,'ro','markersize',7)
hold on
xlim([0,750])
ylim([0.8*min(Yavg) 1.2*max(Yavg)])
set(gca,'ytick',[0.8*min(Yavg):(1.2*max(Yavg)-
0.8*min(Yavg))/10:1.2*max(Yavg)])
set(gca,'yticklabel',num2str(get(gca,'ytick')','%0.2e'))
ylabel('Weight Fraction','FontSize', 14)
xlabel('Time [min]','FontSize', 14)
title(tit,'FontSize', 14)
hold on

% Parameter setting
opts = statset('nlinfit');
opts.RobustWgtFun = 'bisquare';

% Initial Parameter estimates
%B0 = [max(Yavg)-Yavg(1),0.01]'; % Increasing kinetics [Co, h]
%B0 = [max(Yavg)-Yavg(1),0.01,0.0001]'; % Combined kinetics [Co, h, d]
B0 = [Yavg(1), 0.01]; % Decreasing kinetics [Bo, d]
%B0=0;

% Parameter Solving
```

```
[beta,R,J,CovB,MSE,ErrorModelInfo] =
nlinfit(T(1:9),Yavg(1:9),@model,B0,opts);

% Optimum Parameter Solution
%Yest = beta(1)*(1-exp(-beta(2)*Tspan)) + Yavg(1); % Increasing kinetics
%Yest = beta(1)*beta(2)/(beta(2)-beta(3))*(exp(-beta(3).*Tspan)-exp(-
beta(2).*Tspan)) + Yavg(1)*exp(-beta(3).*Tspan); % Combined kinetics
Yest=beta(1)*exp(-beta(2)*Tspan); % Decreasing kinetics
%Yest=Yavg(1)+beta(1)*Tspan;

% Optimum Plotting - add theoretical line
plot(Tspan,Yest)

csvwrite('ODE.csv',[Yest])

% R2 and CI calculations
avg = mean(Yavg(1:9)).*ones(9,1);
SST = sum((Yavg(1:9)-avg).^2);
SSE = sum(R.^2);
R2 = 1-SSE/SST

CI=nlparci(beta,R,'jacobian',J);
beta'
Beta_Range_Percent = (beta' - CI(:,1))./beta'

    function C = model(B,t)
        %C=B(1)*(1-exp(-B(2)*t)) + Yavg(1); % Increasing kinetics
        %C=B(1)*B(2)/(B(2)-B(3))*(exp(-B(3)*t)-exp(-B(2)*t)) + Yavg(1)*exp(-
B(3)*t); % Combined kinetics
        C=B(1)*exp(-B(2)*t); % Decreasing kinetics [Bo, d]
        %C=Yavg(1)+B(1)*t;
    end

  end
```

Appendix B – ALIM Media Formulation

Amino Acids	**g/L**	**Sugars**	**g/L**	**Trace Metals[2]**	**µg/L**
DL-Serine	0.74	Glucose	3.90	$(NH_4)Mo_7O_{24}\cdot 4H_2O$	40
Glycine	0.20			$CoCl_2\cdot 6H_2O$	50
L-Arginine	1.22	**Salts**		$CuCl2\cdot 2H_2O$	200
L-Asparagine	1.30	CaCl2	0.50	$FeSO4\cdot 7H_2O$	550
L-Aspartic acid	1.30	KCl	1.20	$MnSO_4\cdot H_2O$	17
L-Cystine[1]	0.20	$MgSO_4$ $7H_2O$	1.88	$ZnCl_2$	40
L-Glutamic acid	1.50	NaCl	2.72		
L-Glutamine	2.19	NaH_2PO_4	1.01	**Vitamins[2]**	
L-Histidine	0.20	NaHCO3	0.35	4-Aminobenzoic acid	320
L-Isoleucine	0.75			Biotin	160
L-Leucine	0.66		**mg/L**	Calcium pantothenate	8
L-Lysine HCl	0.70	Choline chloride	70	Cyanocobalamin	240
L-Methionine	1.00			Folic acid	80
L-Phenylalanine	1.00			myo-Inositol	400
L-Proline	0.50			Niacin	160
L-Threonine	0.60			Pyridoxine HCl	400
L-Tryptophan	0.41			Riboflavin	80
L-Tyrosine[1]	0.50			Thiamine	80
L-Valine	0.50				

Notes:

1: Made as a 75x solution in 1M HCl before adding 13.3mL to media
2: Made as a 1000x solution in DI before adding 1mL to media

Appendix C – Extended Figures and Tables

C1 Scanning Variance of FL3 Yeast Extract – Complete Results

Yellow – Cov > 5% Red – CoV > 10%

	A		B		C		Overall	
	Avg	CoV	Avg	CoV	Avg	CoV	Avg	CoV
2-Hydroxyisobutyrate	0.01%	8.18%	0.01%	6.24%	0.01%	10.50%	0.01%	8.70%
3-Hydroxyisovalerate	0.06%	3.35%	0.06%	1.97%	0.06%	5.12%	0.06%	4.51%
4-Aminobutyrate	0.10%	1.00%	0.10%	4.25%	0.10%	3.67%	0.10%	3.36%
Acetate	0.41%	2.59%	0.42%	4.59%	0.41%	6.57%	0.41%	4.44%
Adenine	0.02%	14.15%	0.02%	4.76%	0.02%	9.58%	0.02%	10.18%
Adenosine	1.34%	3.58%	1.36%	0.55%	1.35%	1.72%	1.35%	2.14%
Alanine	3.41%	2.23%	3.45%	3.84%	3.30%	2.15%	3.39%	3.25%
Arginine	1.74%	3.50%	1.74%	1.36%	1.70%	0.95%	1.72%	2.27%
Asparagine	1.72%	1.42%	1.68%	2.00%	1.72%	2.66%	1.71%	2.17%
Aspartate	2.20%	3.00%	2.20%	2.82%	2.20%	3.56%	2.20%	2.84%
Betaine	1.21%	3.73%	1.22%	2.12%	1.21%	1.10%	1.22%	2.33%
Butyrate	0.09%	5.02%	0.09%	7.04%	0.09%	3.16%	0.09%	6.23%
Choline	0.03%	4.83%	0.02%	5.73%	0.03%	1.68%	0.03%	4.20%
Cystine	0.07%	24.13%	0.07%	22.39%	0.05%	14.66%	0.06%	21.90%
Cytidine	0.05%	12.46%	0.05%	8.55%	0.04%	24.73%	0.05%	15.21%
Formate	0.03%	5.21%	0.03%	5.99%	0.03%	1.90%	0.03%	5.96%
Glucose	0.55%	0.60%	0.55%	1.76%	0.59%	3.17%	0.56%	3.65%
Glutamate	7.17%	3.05%	7.15%	2.97%	7.03%	3.67%	7.12%	3.07%
Glutamine	0.36%	1.71%	0.37%	6.47%	0.37%	7.65%	0.37%	5.32%
Glycerol	0.08%	4.43%	0.08%	10.32%	0.08%	7.59%	0.08%	7.51%
Glycine	1.51%	2.04%	1.44%	3.12%	1.49%	3.78%	1.48%	3.41%
Guanosine	0.14%	2.25%	0.14%	2.70%	0.14%	4.56%	0.14%	3.02%
Histidine	0.73%	2.58%	0.72%	1.67%	0.72%	4.49%	0.73%	2.94%
Hypoxanthine	0.04%	7.24%	0.04%	8.10%	0.04%	4.39%	0.04%	6.76%
Inosine	0.02%	15.75%	0.02%	20.72%	0.02%	13.33%	0.02%	16.13%
Isobutyrate	0.16%	1.89%	0.16%	0.90%	0.15%	4.26%	0.16%	3.05%
Isoleucine	2.67%	2.88%	2.60%	3.56%	2.66%	2.72%	2.64%	3.02%
Lactate	0.07%	14.01%	0.05%	14.79%	0.06%	5.97%	0.06%	15.82%
Leucine	3.77%	1.59%	3.81%	2.70%	3.72%	3.28%	3.77%	2.60%
Lysine	1.91%	1.87%	1.85%	2.44%	1.96%	1.61%	1.91%	3.15%
Methionine	1.04%	2.04%	1.03%	2.64%	1.01%	1.62%	1.02%	2.27%
Niacinamide	0.06%	7.37%	0.06%	4.28%	0.06%	6.10%	0.06%	6.20%
Nicotinate	0.05%	9.50%	0.05%	8.00%	0.05%	4.78%	0.05%	7.31%
Ornithine	0.18%	7.18%	0.18%	2.22%	0.18%	4.17%	0.18%	4.55%
Pantothenate	0.11%	2.84%	0.12%	14.02%	0.12%	9.41%	0.12%	9.58%
Phenylalanine	2.41%	0.63%	2.47%	3.61%	2.42%	2.43%	2.43%	2.58%
Proline	0.96%	1.97%	0.93%	2.44%	0.96%	2.42%	0.95%	2.81%
Pyroglutamate	1.52%	3.26%	1.53%	5.19%	1.52%	3.20%	1.52%	3.62%
Sarcosine	0.01%	14.56%	0.00%	13.77%	0.00%	7.82%	0.01%	13.14%
Serine	2.22%	1.03%	2.12%	5.37%	2.21%	4.05%	2.18%	4.06%
Succinate	0.60%	1.99%	0.60%	2.50%	0.60%	2.95%	0.60%	2.33%
Threonine	2.11%	3.25%	2.10%	2.38%	2.06%	0.86%	2.09%	2.40%
Trehalose	4.53%	2.35%	4.46%	2.71%	4.48%	2.42%	4.49%	2.34%

Tryptophan	0.70%	1.05%	0.70%	2.15%	0.69%	2.69%	0.70%	2.09%
Tyrosine	0.67%	0.73%	0.67%	4.27%	0.65%	3.11%	0.66%	3.12%
Uracil	0.16%	2.60%	0.16%	5.35%	0.16%	1.76%	0.16%	3.43%
Uridine	0.35%	6.19%	0.35%	4.83%	0.36%	8.16%	0.35%	6.16%
Valine	3.01%	3.28%	2.95%	5.52%	2.94%	1.49%	2.96%	3.63%
myo-Inositol	0.12%	15.62%	0.12%	16.92%	0.13%	9.43%	0.13%	14.08%
a-Glycerophosphocholine	0.30%	2.78%	0.30%	2.90%	0.31%	2.95%	0.30%	3.03%