# THE FECAL METABOLOME OF IRRITABLE BOWEL SYNDROME

# FECAL METABOLOMIC ANALYSIS OF IRRITABLE BOWEL SYNDROME USING MULTISEGMENT INJECTION-CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

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## Abstract

Irritable bowel syndrome (IBS) is increasingly common in Canada, effecting upwards of 18% of the population. The cause of functional gut disorders is not well understood, and new tools are urgently needed to help understand these complex chronic diseases and more accurately diagnose patients. Comprehensive metabolite profiling is a promising strategy to derive new insights into microbiome activity, but a clear set of guidelines for the handling and storage of human fecal specimens has yet to be thoroughly developed. The objective of this thesis is to create standardized sample handling procedures to enable reliable untargeted metabolite analysis of stool samples from IBS patients for biomarker discovery and differential diagnosis. Our results indicated that lyophilization prior to sample extraction not only increased the extraction efficiency on average by 28.5% compared to crude extraction, but also provided good long-term stability with less then 50% of metabolites showing altered responses after long-term storage while frozen up to 21 weeks. Additionally, lyophilization increased study repeatability, by simplifying the weighing and extraction process, reducing variability due to inconsistent stool water content as metabolite concentrations can be normalized to dried weight. This approach was subsequently applied in a pilot metabolomics study involving a cohort of IBS patients (n = 60) and healthy non-IBS controls (n = 20), where lyophilized stool extracts were analyzed by multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) with stringent quality control. The study included a differential stool metabolome analysis of diarrhoea and constipation predominate IBS subgroups (IBS-D; IBS-C), while also classifying IBS patients during contrasting periods of active or dormant symptoms based on their self-reported symptom severity and Bristol stool scale scores. Untargeted and targeted metabolite profiling of stool extracts by MSI-CE-MS under full-scam data acquisition in positive and negative ion modes revealed several promising biomarkers unique to

IBS subtypes and symptomology, while also identifying novel metabolic signatures underlying IBS pathophysiology. Stool metabolomic studies aim to better decipher the underlying mechanisms of debilitating digestive disorders having complex aetiologies, which may also improve diagnostic testing and therapeutic treatments optimal for individual patients.

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## List of Abbreviations and Symbols

| | |
|---|---|
| $[M+H]^+$ | Protonated molecular ion |
| $[M-H]^-$ | Deprotonated molecular ion |
| $^1$H-NMR | Proton nuclear magnetic resonance |
| AA | Amino acid |
| ACN | Acetonitrile |
| ADH | Alcohol dehydrogenase |
| ANCOVA | One way analysis of covariance |
| ANOVA | Analysis of variance |
| AUC | Area under the curve |
| BA | Bile acid |
| B-D | Bligh-Dyer (extraction) |
| BGE | Background electrolyte |
| BMI | Body mass index |
| BSFS/BSS | Bristol stool form scale/Bristol stool scale |
| CA | Cholic acid |
| CD | Crohn's disease |
| CDCA | Chenodeoxycholic acid |
| CE | Capillary electrophoresis |
| CE-MS | Capillary electrophoresis-mass spectrometry |
| CF | Cystic fibrosis |
| cGMP | Cyclic guanosine monophosphate |
| $CHCl_3$ | Chloroform |
| CI | Chemical ionization |
| CNS | Central nervous system |
| CV | Coefficient of variation |
| Da | Dalton/Atomic mass unit |
| DI | Direct infusion |
| EI | Electron ionization |

| | |
|---|---|
| EIE | Extracted ion electropherogram |
| ESI | Electrospray ionization |
| EOF | Electroosmotic flow |
| ESI-MS | Electrospray ionization coupled to mass spectrometry |
| EtOH | Ethanol |
| FA | Fatty acid |
| FODMAP | Fermentable oligosaccharides, disaccharides, monosaccharides and polyols |
| FT | Freeze-thaw |
| GBA | Gut-brain axis |
| GC | Gas chromatography |
| GCDCA | Glycochenodeoxycholic acid |
| GI | Gastrointestinal |
| HADS | Hospital Anxiety and Depression Scoring |
| HC | Healthy control |
| HCA | Hierarchical cluster analysis |
| HiREB# | Hamilton research ethics board number |
| HILIC | Hydrophilic interaction chromatography |
| HMBD | Human metabolomics database |
| HPLC | High performance liquid chromatography |
| IBS | Irritable Bowel Syndrome |
| IBS-C | Constipation predominant irritable bowel syndrome |
| IBS-D | Diarrhea predominant irritable bowel syndrome |
| IBS-M | Mixed irritable bowel syndrome subtype |
| IBS-U | Unclassified irritable bowel syndrome subtype |
| IBD | Inflammatory bowel disease |
| IS | Internal Standard |
| KDCA | Ketodeoxycholic acid |
| LC | Liquid chromatography |
| LC-MS | Liquid chromatography-mass spectrometry |
| MeOH | Methanol |

| | |
|---|---|
| MS | Mass spectrometry |
| MSI-CE-MS | Multisegment injection-capillary electrophoresis- mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| m/z | Mass/charge ratio |
| NAG | *N*-Acetyl glutamate |
| NAGS | *N*-Acetyl glutamate synthase |
| NBS | Newborn screening |
| NMR | Nuclear magnetic resonance |
| PCA | Principal component analysis |
| PI | Post infection |
| PLS-DA | Partial least-squared discriminant analysis |
| QA | Quality assurance |
| QC | Quality control |
| RMT | Relative migration time |
| RPA | Relative peak area |
| ROC | Receiver operations curve |
| RS | Recovery standard |
| SCFA | Short-chain fatty acid |
| SIBO | Small intestinal bacterial overgrowth |
| TOF | Time-of-flight |
| UC | Ulcerative colitis |
| VOC | Volatile organic compound |
| VIP | Variable importance projection |

# Chapter I: Introduction

# Fecal Sample Handling and Fecal Metabolome Characterization for New Insights into Irritable Bowel Syndrome

# Chapter I: Introduction – Fecal Sample Handling and Fecal Metabolome Characterization for New Insights into Irritable Bowel Syndrome

## 1.1 Introduction to Metabolomics

### 1.1.1 Overview of metabolomics

The concept of metabolomics extends back to over 3000 years ago when the importance of bodily fluids for disease recognition was discovered, as changes to the colour or taste of urine could be used as an indicator of disease status (van der Greef et al., 2013). This phenomenon is related to what we currently understand as aberrant changes in metabolism, such as elevated excretion of glucose in type 2 diabetes. Metabolomics refers to the comprehensive analysis of low molecular weight (< 1,500 Da) compounds in a specific biospecimen (e.g., cell, tissue, biofluid) within an organism (Wishart et al., 2013). Metabolites are intermediates and by-products of cellular metabolism that reflect cellular enzymatic activity and gene expression. In the human metabolome, metabolites can be either endogenous to the host and/or the gut microbiota (i.e., as a human-microbe superorganism), as well as a plethora of chemicals from environmental exposures, including diet, drugs, and pollutants. The ability of the metabolome to characterize the deleterious effects of gene expression and chemical exposures that are closely linked to phenotype (**Figure 1.1**), makes it an important clinical tool for biomarker identification relevant to new insights into disease pathophysiology (Johnson et al., 2016). Integration of metabolomics into clinical practice can improve disease screening, diagnosis, prognosis, and treatment responses to therapies on an individual level, which is important for early detection and disease prevention, while avoiding invasive and costly sampling procedures (e.g., colonoscopies or tissue biopsies) and symptomatic based screening tools prone to misinterpretation. For instance, universal newborn screening (NBS) programs represent one of the most successful public health policies for pre-symptomatic detection

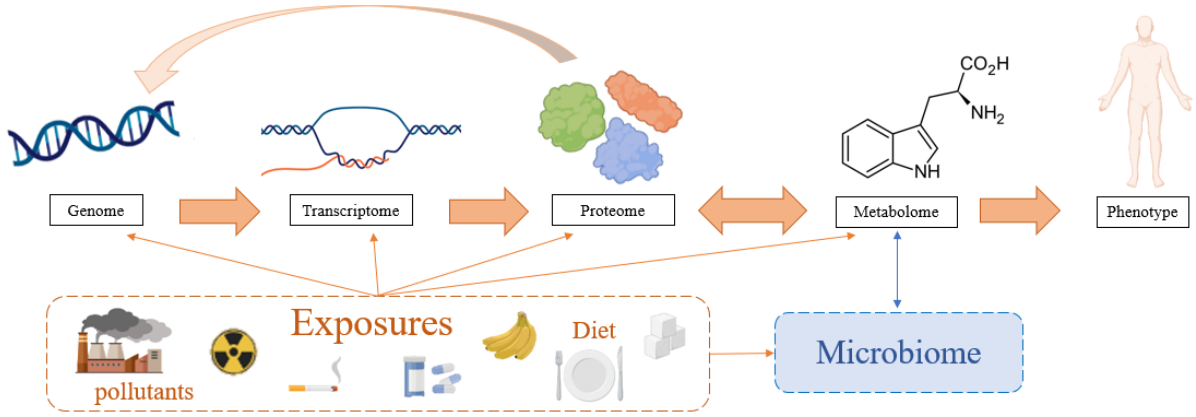


**Figure 1.1** The various different "-omics" approaches used in clinical research highlighting the close association of metabolomics to phenotype and the contribution of the microbiome and lifelong exposures

of rare,yet potentially life-threatening genetic diseases in the population, such as cystic fibrosis (CF) (Dibattista et al., 2018). Early treatment intervention as a result of NBS has shown to improve long-term clinical outcomes in affected children as compared to conventional and later stage symptomatic diagnosis (Sims et al., 2007). The most common biological specimens used for metabolomics are blood and urine due to their convenience for sampling, but increasingly so, many other biological samples, including saliva, tissue and stool have also been studied (Gowda et al., 2008). Each specimen type provides complementary biochemical information related to aberrant metabolism in specific human diseases, with fecal specimens providing unique insights into impaired nutrient absorption, colonic inflammation, and gut (microbial) dysbiosis (Vijayvargiya et al., 2013; Weir et al., 2013). The interaction of metabolites between the host and the gut microbiota are modulated to stay in a state of homeostasis, however abrupt changes in lifestyle and exposures, such as dietary habits, psychosocial stress, and drug usage (i.e., antibiotics) can disrupt this balance and contribute to a variety of disabling and chronic bowel disorders (Chen et al., 2019). As a result, It is essential to include microbial metabolism and its associated metabolites

when exploring the pathophysiology of complex gastrointestinal (GI) disorders of unknown or complex aetiologies. For example, irritable bowel syndrome (IBS) is one example of a multi-factorial GI disorder in which current diagnosis is reliant on patient reports of symptoms, as well as bowel habits and stool consistency (e.g., constipated, diarrhea) (L. Xiao et al., 2021). Yet, self reports do not provide as unambiguous diagnosis of IBS given similar overlap of symptoms in other chronic bowel disorders. For this reason, IBS could benefit from a quantifiable metabolic signature that differentiates from healthy and related bowel diseases (e.g., Crohn's disease or ulcerative colitis) while also offering clinicians a means to track treatment response to therapy. Furthermore, differential diagnosis between the two main subtypes of IBS, namely IBS-diarrhea (IBS-D) predominant and IBS-constipation (IBS-C) predominant may be achieved based on metabolic phenotype changes measured in stool extracts (L. Xiao et al., 2021). However, successful biomarker translation into clinical practice requires validated methods to process highly heterogenous human stool specimens together with high resolution instrumental techniques and robust data workflows to characterize novel metabolites associated with IBS disease progression.

### 1.1.2 Major analytical platforms for metabolomics

A major challenge facing metabolomics lies in the immense chemical diversity in physicochemical and stereochemical properties of metabolites present in an organism. Moreover, a large fraction of the human metabolome corresponds to unknown compounds that lack purified chemical standards and mass spectral libraries making comprehensive analyses under a single analytical platform extremely challenging (Dunn & Ellis, 2005). Also, the dynamic range of metabolite concentrations vary over nine orders of magnitude (from pmol to mmol) which requires analytical methods with exquisite sensitivity and low detection limits. To date, nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (MS) remain as the most commonly used

analytical platforms in metabolomics (Wang et al., 2010). As one of the first methods introduced for metabolomics, proton ($^1$H)-NMR is still highly popular due to its excellent reproducibility and long-term stability, including its ability to confidently identify and directly quantify metabolites with minimal sample processing (Wang et al., 2010). Nuclear magnetic resonance uses a magnetic field and electromagnetic radiation to produce a spectrum of peaks (i.e., chemically distinct proton signals) based on their chemical shift for a metabolite, that also allows for its unambiguous structural elucidation. Although NMR is non-destructive and robust (Wang et al., 2010), it is limited by large sample volume requirements (> 200 mL), high infrastructure costs, and suffers from poor concentration sensitivity which limits overall metabolome coverage (Emwas, 2015). In contrast, high resolution mass spectrometry (MS) offers greater sensitivity, lower sample volume requirements and better selectivity than one-dimensional $^1$H-NMR especially when coupled to high efficiency separation techniques for a more comprehensive coverage of the human metabolome. Depending on the exact separation platform and metabolite class to be analyzed, most MS-based metabolomic studies use electron ionization (EI), chemical ionization (CI) or electrospray ionization (ESI) as an ion source for generation of gas-phase ions. These ions are subsequently accelerated and transmitted through a mass analyzer, such as a time-of-flight (ToF) MS instrument, where they are resolved and detected based on their mass-to-charge (*m/z*) ratio reflecting differences in their characteristic drift times in a field-free vacuum (Ho et al., 2003). Depending on the volatility, polarity, and chemical stability of the compounds a suitable separation technique can be selected, mainly either gas chromatography (GC) which is best suits for non-polar, volatile compounds or liquid chromatography (LC) which can resolve complex mixtures of metabolites having a much wider range of polarities (J. F. Xiao et al., 2012). Both chromatographic separation techniques function by resolving metabolites and their isomers based on their

characteristic retention times in a column containing a specific stationary phase prior to their ionization and subsequent MS analysis. GC-MS offers high efficiency separations for thermally stable and low molecular weight compounds that are non-polar, but often requires complicated sample work-up procedures, such as pre-column chemical derivatization for the analysis of polar metabolites (Kornilova et al., 2013). These requirements for GC-MS reduce the overall coverage capabilities and limits its usefulness as an analytical platform for comprehensive metabolomic studies. In contrast, reversed-phase LC coupled to electrospray ionization (ESI)-MS is the most widely implemented analytical platform in metabolomics due to its reproducible retention times and versatility with interchangeable columns and tuneable elution systems, which can facilitate the resolution of a wide range of metabolites and lipids in complex biological samples (J. F. Xiao et al., 2012). Although LC offers several different column types, elution conditions and separation mechanisms (i.e., ion-exchange, size exclusion etc.), a combination of reversed-phase and hydrophilic interaction chromatography (HILIC) are widely used in ESI-MS as complementary chromatographic separation modes to expand metabolome coverage (Naser et al., 2018). Alternatively, capillary electrophoresis coupled to mass spectroscopy (CE-MS) offers a high efficiency separation technique that separates ions based on differences in their effective charge and hydrodynamic radius as reflected by their characteristic electrophoretic mobility. CE-MS often uses a coaxial sheath liquid interface which is applied to the outside of the capillary and provides a make-up flow, stabilizing spray formation together with a gas nebulizer. This interface for stable spray formation is also needed to decouple the electrical circuit applied for CE separation from ESI-MS. Unlike LC-MS, CE relies on an isocratic buffer system for electrophoretic separation, whereas a sheath liquid also provides a constant composition for solute ionization that can be independently optimized to enhance sensitivity. In gradient elution separations as used widely in

LC-MS analyses in metabolomics, solute ionization is dependent on the solvent composition and varies over the course of the separation (Mischak et al., 2009). Overall, CE-MS is ideal for the rapid separation of complex mixtures of polar/ionic metabolites from highly saline and volume-restricted biospecimens due to the low sample volume requirements needed for analysis (Gahoual et al., 2018). Additionally, CE-MS offers a cost-effective and versatile platform for analysis of the ionic metabolome with minimal sample workup and solvent consumption (Beretov et al., 2014). Although narrow fused-silica capillaries used in CE-MS allow for high efficiency separations due to their effective dissipation of Joule heating when using high applied electric field strengths, this limits sample loading capacity resulting in poor concentration sensitivity as compared to LC-MS (Mischak et al., 2009). This problem is enhanced by post-capillary dilution effects when using a coaxial sheath liquid to support solute ionization in ESI-MS. To combat this issue, sheathless and low sheath flow coupling interfaces have been developed (Moini, 2007) as depicted in **Figure 1.2** where an electrode is positioned at the end of the capillary to act as the electrical contact and the flow rate is dependent only on CE flow rate (~ 10 nL/min), with a glass emitter directing the spray directly to the inlet of the MS (Gahoual et al., 2018) with improved concentration sensitivity and lower detection limits (Gaspar et al., 2008). The reduced sheath liquid enhances sensitivity and creates improved desolvation but may result in poor spray stability making it less suitable for large-scale metabolomic studies. Another draw back to CE-MS is the poor reproducibility of apparent migration times for ions as compared to solute retention times in reversed-phase LC-MS, which are largely due to variations in the electroosmotic flow (EOF) as a result of changes in capillary surface (i.e., zeta potential), temperature or buffer composition (Kehl et al., 2022). However, the electrophoretic mobility of an ion is a fundamental physicochemical property in CE that can be

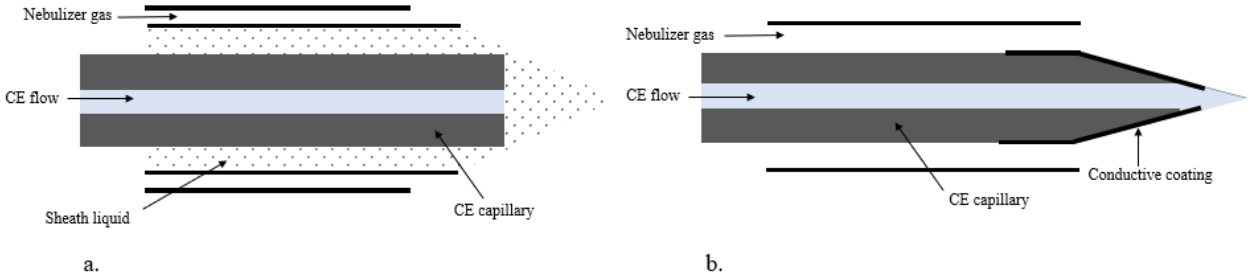


**Figure 1.2** Schematic of two common CE-MS interfaces for ESI in metabolomic studies, including (a) a coaxial sheath liquid interface and (b) a sheathless interface.

measured with better precision when using a neutral marker for the EOF to facilitate compound identification. Alternatively, an internal standard can be used to adjust for migration time variations due to changes in EOF between runs when reporting the relative migration time (RMT) of an ion (Nowak et al., 2017). Since CE-MS is amenable to multiplexed separations to improve sample throughput, large-scale metabolomic studies are feasible as highlighted by characterization of the maternal serum metabolome from a cohort of over a thousand pregnant women (Shanmuganathan et al., 2021).

### 1.1.3 Metabolomics workflow

Metabolomics is the comprehensive analysis of detectable metabolites in a specific biospecimen, which often uses both a targeted (i.e., hypothesis testing) and/or nontargeted (i.e., hypothesis generating) approach to identify and quantify compounds of clinical or biological significance. Metabolomics studies can provide novel insights into biomarkers of disease for improvements to diagnosis, treatment progression, or personalized treatment plans and medication. Overall, each step of the metabolomics workflow (**Figure 1.3**) is critical to minimize false discoveries, including pre-analytical study design, sample selection and sample handling, to analytical instrumentation selection and optimization, as well as post-analytical data

preprocessing, statistical analysis, and biochemical interpretation following unknown identification. Quality control (QC) and quality assurance (QA) are key elements in a robust metabolomics study in order to ensure that each step of the process is reproducible and bias-free while implementing standard operating protocols and preventative maintenance strategies.

The pre-analytical steps encompass a well thought out study design that will minimize bias and inadequate study power based on a clear description of cohort selection and exclusion criteria (i.e., consort diagram), clinical trial pre-registration (i.e., defined study objectives and clinical outcomes) and a standardized protocol (e.g., sample collection, handling, and storage) (Chidambaram & Josephson, 2019). It is important that the study design and objectives be put into place before initiation of cohort recruitment, sample collection or data acquisition to reduced bias during hypothesis formulation. Small pilot studies come with many limitations, including lack of study power and generalizability that cannot be independently validated, while large-scale metabolomic studies performed over extended periods of time can suffer from instrumental drift and signal fluctuation, so method validation and QC checks are invaluable to correct for instrumental bias. This can be achieved by inclusion of a pooled QC sample that is repeatedly analyzed over the duration of the study for assessment of intermediate precision, allowing for increased confidence in reported findings in discovery-based metabolomics research.

During sample collection and sample preparation, it is important to follow standardized protocols specific to biospecimen type as the introduction of unintentional freeze-thaw cycling may introduce unwanted variation and result in metabolite degradation, while improper extraction methods may lower metabolite extraction efficiency and selectivity. For example, aqueous solutions may not be able to extract as diverse classes of metabolites as a mixed polarity solvent

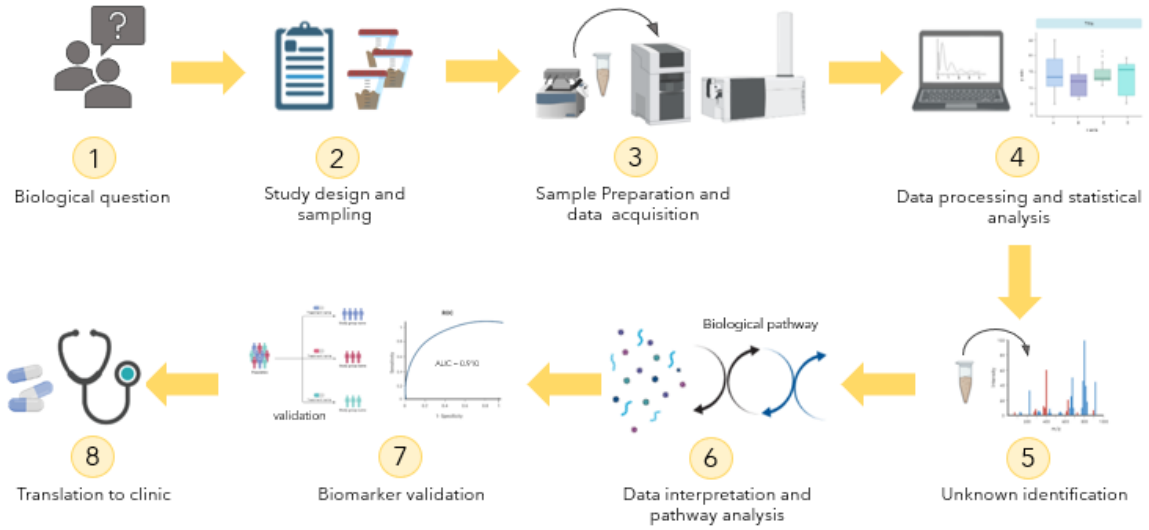


**Figure 1.3** An overview of the metabolomics data workflow using CE-MS for clinical biomarker discovery.

(i.e., methanol and water) as required to better solubilize lipophilic compounds (Ser et al., 2015). During data processing in metabolomics, raw data must go through various transformations which includes batch correction, normalization to an internal standard (IS), and corrections to dried specimen mass as done for lyophilized fecal samples. Normalization of the data is important as between runs as there can be long-term signal drift in detection and between-sample variations in injection volumes introduced on-column. In this case, an IS at a fixed concentration, is added to every sample that corrects for variations in sample injection or ionization conditions, where the integrated peak area of each metabolite response is normalized to the IS (van der Kloet et al., 2009). A recovery standard (RS) is also commonly added to samples prior to sample processing to assess extraction recovery while also correcting for variations in extraction efficiency between samples (Lippa et al., 2022). In terms of sample normalization, lyophilization of fecal samples is often performed to account for differences in water content between samples, as increased water

content may contribute to lower apparent metabolite concentrations upon extraction (Lemay et al., 2021). Freeze drying heterogenous stool samples for metabolomic analyses also facilitates sample handling, enables reproducible weighing, may improve long-term metabolite stability, and enhances their extraction efficiency, which is especially important when analyzing clinical samples from diarrhea-predominate IBS patients.

After data collection and quality assessment, the compilation of a curated metabolome data matrix often requires further processing to account for missing values, which are a result of low or undetectable metabolite levels that are due natural abundance variability (Do et al., 2018). To reduce data distortion, appropriate methods must be used to replace missing values with a non-zero numeric that most accurately represents the replaced value. A complete metabolomics data matrix will often undergo auto scaling and generalized *log* transformation prior to statistical analysis to improve data normality and reduce the impact of metabolite concentration differences. Furthermore, multivariate data analysis is often used in metabolomics to reduce data dimensionality, identify potential outliers, and reveal data trends (i.e., similarities), such as unsupervised methods based on principal component analysis (PCA) and hierarchical cluster analysis (HCA). Top-ranked metabolites as putative disease biomarkers can be ranked-ordered (e.g., disease versus healthy) when using supervised multivariate data analysis, such as partial least-squared discriminant analysis (PLS-DA) in conjunction with variable importance projection (VIP) score (Farrés et al., 2015; Sorochan Armstrong et al., 2022). These biomarker candidates can then be subsequently verified using univariate statistical analysis methods with appropriate adjustments (e.g., confounding variables), such as student's t-test and one-way analysis of covariance (ANCOVA) depending on study design and data normality. Otherwise, non-parametric statistical methods for analysis (Bartel et al., 2013) such as the Mann-Whitney U test or the

Kruskal-Wallis test may be needed for skewed data that is not normalized after data transformation. Data visualization using box plots, heatmaps and other graphical approaches act complementary to the descriptive statistics, presenting data trends visually which is important for identifying trends and correlations in larger data sets. Lastly, biochemical interpretation relies on the ability to identify metabolites with high confidence given the large number of potential isomers and isobaric compounds that exist for a candidate ion. top-ranking features undergo MS/MS spectral matching and authentic standard spiking (e.g., co-elution) for reliable metabolite identification.

Several strategies have been developed for the structural annotation of unidentified metabolites of significance, where a combination of metabolite characteristics such as accurate mass (m/z), retention time, and most probable molecular formula can be used in conjunction with collision-induced dissociation coupled to tandem mass spectrometry (MS/MS) spectral matching. Annotation of MS/MS spectra based on the relative intensities of specific product ions and neutral losses can be matched to known compounds in spectral libraries that were acquired under similar experimental conditions (e.g., collisional voltage). However, many unknown compounds of clinical significance may not be matched to existing spectral databases as they remain incomplete, whereas MS/MS spectra may not be feasible to acquire for certain low abundance metabolites as precursor ions. **Table 1.1** summarizes the different levels of confidence recommended for metabolite identification in metabolomics research (Y. Li et al., 2020; Schrimpe-Rutledge et al., 2016). There is greater insistence for creating an open-access public repository of metabolomics data contributed by researchers to improve reproducibility and transparency in scientific research,

**Table 1.1** Confidence levels in metabolite identification adapted from (Schrimpe-Rutledge et al., 2016; Sumner et al., 2007)

| Confidence Level | Requirements |
|---|---|
| 1 | Structure has been confirmed using an authentic reference standard based on a minimum of two orthogonal features such as retention time and matching spectra (MS/MS) |
| 2 | Putative identification with a probable structure through physiochemical characteristics and matching reference spectra from a spectral library |
| 3 | Tentative identification based on spectral elucidation from MS/MS data and similarities to known metabolite classes |
| 4 | Tentative molecular formula and reliably quantifiable with one or more potential structures |
| 5 | Unique feature assigned an exact mass with mass error (± ppm) |

including depositing raw MS or MS/MS spectral data, study design description, sample preparation detail sand data acquisition parameters on specific instrumental platforms such as the metabolomeXchange initiative (www.MetabolomeXchange.org/; accessed 2022-06-29)

After compilation of a filtered metabolite data matrix comprising known and unknown yet fully annotated metabolites measured with adequate frequency (> 75%) and technical precision (CV < 30%), biological interpretation is an important next step in understanding the role of aberrant metabolism to disease pathophysiology. Metabolomic studies often produce a wide range of biomarkers that statistically differ in expression between two or more sub-groups of patients and controls, however the link between altered metabolite concentrations and biochemical pathways are not always evident. Putatively identified molecular features pose an even greater challenge for biological interpretation, since the exact chemical structure and likely biological function of an unknown metabolite is still unclear (e.g., substrate or product of a specific enzyme, dietary exposure from the intake of specific foods). To address this issue, correlated metabolite networking is one way to simplify larger, more complex data matrices by relating metabolites

based on statistical and chemical/biochemical similarities, where clustered metabolites are often co-regulated (Amara et al., 2022). Biomarkers that possess clinical significance and are associated with clinical outcomes (e.g., specific clinical events, hospitalization, or all-cause mortality) or an independent biochemical measure of disease severity (e.g., elevated fecal calprotectin in Crohn's disease) must be validated prior to implementation into clinical practice. Validation of lead biomarkers follows a series of steps as outlined by Hunter et al., 2010 that relate to its performance characteristics in a defined clinical context (e.g., screening, diagnosis etc.), such as its sensitivity, specificity, and overall predictive accuracy. This is often assessed using a receiver-operating characteristic (ROC) curve to evaluate the binary discrimination of a single biomarker, biomarker ratio or biomarker panel of metabolites (disease versus healthy control). After initial requirements are satisfied, a bioanalytical assessment is performed to gauge marker viability for routine use, which would require good reproducibility, high accuracy and precision along with high frequency of detection (Hunter et al., 2010). Finally, the probability of false-positives and false-negatives must be taken into account along with the positive predictive value of a biomarker through subsequent clinical trials in an independent cohort of patients.

### 1.1.4 Fecal metabolomics and sample handling protocols

Recently, there has been growing interest in coupling metabolomics with microbiome studies as it can provide functional insights into complex microbiota-host interactions as modified by environmental exposures. As stool is directly correlated to host colon function as well as the gut microbiome activity, multi-omic studies can provide information on the role of genetics and diet on the health of an individual (Mandal et al., 2020). Previous methods for assessing gut health typically require mucosal biopsy sampling of patients, which is highly invasive and can cause microbiome disturbances, leading to adverse reactions (Huse et al., 2014). Although the use of

fecal samples has the advantage of being non-invasive, it also faces several challenges in relation to the collection, preparation, and storage since it is a highly heterogenous biospecimen composed of host cells, unabsorbed nutrients and colon derived microbes. Due to fecal sample analysis being a relatively new field of research, a consensus on optimal sample handling and treatment has yet to be established (Mandal et al., 2020).

One of the concerns with analysis of feces is that even variations in sample collection technique and the time lapse between collection and long-term storage prior to analysis can introduce substantial variation. The time a sample is stored at ambient temperatures before being refrigerated or frozen can cause metabolite concentration changes due to the fermentative activity of bacteria present in stool samples (Gholib et al., 2018). In order to preserve sample integrity, a variety of techniques have been employed, including the addition of stabilizing solutions, immediate freezing of the sample, and freeze-drying/lyophilization (Liang et al., 2020; Nam et al., 2022). It is also good practice to aliquot and freeze samples as soon as possible after collection to avoid the need for multiple freeze-thaw cycles prior to analysis (Mandal et al., 2020). Other concerns with using fecal specimens for analysis and quantification of metabolites is the large variability between samples. Common practice in stool sample analysis currently involves using fresh wet fecal samples for metabolite extraction and recovery. There is concern with this method as stool is highly heterogeneous, with a water content ranging from 70-80% water in healthy individuals (Bothe et al., 2017). This range also does not include the further variations in water content for stool samples collected from patients with diseases directly affecting the gut's ability to absorb water, such as IBS-D. To accommodate for this, lyophilization as a pre-treatment step has been proposed to correct for variations in hydration status of stool. One of the first articles to apply freeze drying of fecal samples in their sample processing procedures was written by Fischer

et al. (1998) for the purpose of quantifying elastase-1 in patients with pancreatic exocrine insufficiency, with a stool water content above 85%. In this study it was shown that not only was the content of elastase able to be evaluated after lyophilization and reconstitution, but that the levels of elastase-1 were substantially elevated in the lyophilized samples as compared to the unprocessed samples (B. Fischer, 2009).

The freeze-drying process has been used as a preservation technique since ancient Egypt as a means of preserving the bodies of the deceased in hopes of obtaining immortality. The use of freeze drying has since evolved and was first introduced in a scientific setting back in 1890 by Richard Altmann for use on biological tissues (Simpson, 1941). The process of freeze drying involves the removal of water without the use of heat so as to keep the sample intact and reduce degradation and denaturation as would be seen in typical heated drying processes. Accordingly, the sample is first frozen, causing the formation of ice crystals within the product. After freezing, a vacuum is applied in order to reduce the pressure in the sample container until sublimation can occur and the water in the sample becomes vaporized (C. Jos, 2009). This process is thought to also induce cell lysis at the time of rehydration, releasing the internal constituents of biological cells contained within stool (Didovyk et al., 2017). The inner constituents of cells contain a variety of lipids, proteins and nucleic acids which hold importance when establishing a comprehensive fecal metabolite profile derived from the host, food/fiber and microbes (Kumar et al., 2016).

Lyophilization of fecal samples has brought about conflicting opinions with regards to the benefits of its use and has not been well studied to date. This process can be applied to samples before extraction for ease of handling or immediately following collection for better sample preservation and reduction of bacterial growth (Smith et al., 2020). By freeze drying samples prior to extraction, water can be removed allowing for quantitative data to be obtained, and clinical

reference values for key biomarkers can be established. Another key advantage of lyophilization is the ability to easily homogenize fecal samples while in a dry powdered form; this is important for producing reproducible results and for obtaining an accurate metabolite profile. Although many advantages are associated with lyophilization of stool specimens, the potential loss of volatile organic compounds (VOC) has been reported in some studies (Moosmang et al., 2019; Smith et al., 2020). This postulation should be tested further as other articles have also reported no loss of volatile SCFA after testing various solvents and extraction conditions for fresh, frozen and freeze-dried stool samples. For instance, Cheng et al. observed the greatest number of molecular features when using freeze dried stool samples extracted with methanol (MeOH) when performing metabolomic analyses (Cheng et al., 2020).

Comprehensive analysis of stool sample extraction methods is summarized in **Table 1.2** along with the principal metabolite classes extracted with each method. Overall, a review of the literature highlights a lack of consensus with regards to an optimal stool extraction method for comprehensive stool metabolome coverage since specific metabolite classes will be optimally

**Table 1.2** Review of recent literature on metabolite stool extraction techniques

| Article | Extraction method[e] | | Extracted metabolite classes |
|---|---|---|---|
| | Solvent system | Sample Information | |
| De Zawadzki et al. (2022) | Methanol Extraction:<br>400 *ul* 100% methanol | 3 stage homogenization. Stool was stored frozen, and 30 mg used for extraction | Bile acids, amino acids, free fatty acids, carbohydrates and TCA cycle metabolite |
| Hosseinkhani et al. (2021) | Methanol Water Extraction:<br>200 *ul* of *MeOH: H2O, 1:1*<br>Methanol Extraction:<br>200 *ul* of *MeOH*<br>Ethanol Extraction:<br>200 *ul* of *EtOH*<br>Water Extraction:<br>200 *ul* of *H2O*<br><br>MTBE Extraction:<br>400 *ul* MTBS:MeOH: *H2O, 3.6:2.8:3.5*<br>Chloroform Extraction:<br>400 *ul* Chloroform:MeOH: *H2O, 3.6:2.8:3.5* | Fresh samples stirred/aliquoted and stored at -80℃.<br>Used 0.5gfor extraction.<br>Extractions carried out in acidic, neutral and basic pH environments. (0.1% formic acid, ammonia hydroxide, or nothing) | Bile acids greater in MTBE and MeOH:water extracts. Polar metabolites. MTBE greatest efficiency (11 metabolite classes, including lipids) |

| | | | |
|---|---|---|---|
| Ruben et al. (2020) | Acetonitrile extraction:<br>*400 ul of ACN: $H_2O$ (1:3)*<br><br>Methanol extraction:<br>*200ng/ml tryptamine-d4, 500ng/ml L-tryptophan-d3, 100ng.ml 3-methylindol-d3, 200ng/ml indole-3-acetic acid-d5 in 80% MeOH* | Samples stored at -80℃ 100 mg sample extraction<br>50mg sample for extraction | ACN extraction:<br>metabolome profiling SCFA, bile acids, amino acids, nucleobases<br>MeOH Extraction:<br>Tryptophan pathway metabolites |
| Lemay et al. (2020) | Modified Bligh Dyer extraction:<br>*1.424 ul MeOH: H2O: $CHCl_3$, 4:3.6:4*<br>*2. 128 ul $CHCl_3$: H2O, 1:1* | Stored at 4C until storage at -80℃.<br>Samples were lyophilized.<br>~ 15mg used for extraction | SCFAs and electrolytes |
| Moosmang et al. (2019) | Acetonitrile/water extraction:<br>*500ul* ACN: *$H_2O$ (1:3) x 3*<br>Water extraction:<br>*500ul 100% water x 3*<br>Methanol extraction:<br>*500ul 100% MeOH x 3*<br>Methanol-water extraction: [c]<br>*500ul MeOH: $H_2O$ (4:1) x 3*<br>Chloroform-methanol-water extraction*:*<br>*500ul CHCl3: MeOH: $H_2O$ (3:1:2) x 3* | Stored at -80℃ until processing.<br>Extraction of 300mg of wet stool (fecal water) or 30mg lyophilized stool samples | untargeted comprehensive metabolite coverage (amino acids, sugars, lipids, SCFA, vitamins, bile acids, etc..) |
| Eun Song et al. (2019) | Water extraction:<br>*380ul $H_2O$ homogenized* | Stored at -80℃.<br>20-25 mg mouse feces for extraction | SCFAs |
| Wang et al. (2019) | Acetonitrile extraction:<br>*20 ml of 30% ACN in $H_2O$* | 2000mg wet stool sample homogenized | SCFAs |
| Yang et al. (2019) | Methanol extraction [d]<br>*600 ul 80% MeOH*<br>*Or 100% MeOH*<br>Acetonitrile extraction:<br>*600 ul 100% ACN*<br>Acetonitrile/chloroform extraction:<br>*600 ul ACN: $CHCl_3$, 3:1*<br>Methanol-chloroform extraction:<br>*600 ul MeOH: ACN, 3:1*<br>Isopropanol-acetonitrile-water extraction*:*<br>*600 ul APN: ACN: H2O, 3:2:2* | Stored at -80℃<br>10 mg samples for extractions.<br>After extraction, fecal extracts were dried for 3 h. | untargeted comprehensive metabolite coverage (amino acids, sugars, lipids, SCFA, lipids, nicotinamides, vitamins, bile acids, etc..) |
| Gholib et al. (2018) | MeOH- Water Extraction: [b]<br>*MeOH: $H_2O$, 80:20*<br>Ethanol-Water extraction:<br>*EtOH: $H_2O$, 80:20*<br>Alcohol-Water Extractions:<br>*Alco: $H_2O$, 80:20* | Feces homogenized and stored – 20℃<br>Samples underwent lyophilization, oven drying or no pre-treatment<br>50mg extraction sample | Hormone metabolites (estrogen, glucocorticoid) |
| Cesbron et al. (2017) | Methanol extraction<br>MeOH (150 ul) + $CHCl_3$ (380 ul) + $H_2O$ (120 ul)<br><br>Methanol extraction<br>855 ul MeOH | 150 mg or 1000 mg of lyophilized stool<br>Sample dilution with water 1:1 (50mg feces/ml, 1:3, or 1:10)<br><br>285 mg fresh stool | untargeted comprehensive metabolite coverage (amino acids, sugars, lipids, SCFA, vitamins, bile acids, etc..) |
| Dobrowolska-Iwanek et al. (2016) | Perchloric acid extraction:<br>*10 ml perchloric acid (0.15M) filtered with 0.45um pore size cellulose esters membrane filter (MCE) x 3* | Samples dried at 40℃ for 48 h.<br>1000mg used for extractions | SCFAs |
| Brown et al. (2016) | Methanol water extraction:<br>*Methanol extraction 5x methanol (5:1 methanol/water) extraction* | -80℃ Storage till analysis.<br>Sample extracts were dried under vacuum for a minimum of 18 h before derivatization for GC-MS analysis | untargeted comprehensive metabolite coverage (amino acids, sugars, lipids, SCFA, vitamins, bile acids, etc..) |

| | | | |
|---|---|---|---|
| Phua et al. (2013) | Methanol -water extraction: [d]<br>*MeOH: $H_2O$, 4:1*<br>*MeOH: $H_2O$, 2:1*<br>*Chloroform-methanol-water extraction:*<br>*$CHCl_3$: MeOH: $H_2O$, 2:5:2*<br>*Acetonitrile-water extraction:*<br>*ACN: $H_2O$, 4: 1* | Lyophilized samples stored at -80˚C | Overall metabolic coverage (amino acids, sugars, lipids, SCFA, vitamins, bile acids, etc..) |
| Zheng et al. (2013) [18] | NaOH extraction:<br>*@ 4˚C 1 ml of 0.005M aqueous NaOH with IS 5 ug/ml caproic acid-d3* | 50-150mg wet stool sample | SCFAs and branched-chain amino acids (BCAAs) |
| Humbert et al. (2012) | NaOH extraction: [a]<br>*2ml NaOH at 60˚C for 1 hr then + 4ml $H_2O$*<br>Acetonitrile or Isopropanol extraction:<br>*2ml $H_2O$ homogenized then + ACN or IPA (80% v/v)*<br>*Incubate at room temp. for 20 min.*<br>Water extraction:<br>*10ml $H_2O$ homogenized* | Samples were homogenized and stored at -80˚C. Lyophilized and 100mg used for extraction | Bile acids |
| Spiegeleer et al. (2020) | Methanol water extraction:<br>*MeOH: $H_2O$, 4:1*<br>Methanol/ MTB/ Water extraction:<br>*With 0.01% Butylated hydroxytoluene and 2.5% trichloroacetic acid* | Samples were lyophilized (200 mg aliquots) and filtered with polyamide filter after extraction | Polar metabolites<br><br>Lipidome metabolites |
| Hsu et al. (2019) | Organic Solvent Extractions:<br>*Tested MeOH, ACN, $CHCl_3$, EtOH, Hexane extractions*<br><br>Liquid-Liquid Extraction:<br>*Butanol* | Samples were either Lyophilized, crude or wet extracts. Stored at 4˚C, -20˚C or -80˚C for varied periods of time | SCFAs |
| Erben et al., (2021) | Phosphate-buffered saline:<br>150µL PBS<br>Isopropanol<br>200µL isopropanol<br>Methanol/Acetonitrile/water:<br>200µL MeoH:ACN:$H_2O$, 2:2:1<br>Methanol/Acetonitrile:<br>MeOH:ACN, 1:1<br>Ethanol/Phosphate Buffer extraction:<br>150µL 85% EtOH:15% 20mM phosphate buffer followed by 150µL 20% EtOH:800% 20mM phosphate buffer<br><br>Ethanol extraction:<br>*200µL of 75% EtOH followed by 500µL MTBE +125µL $H_2O$*<br>Methanol/ MTBE extraction:<br>*225µL 100% MeOH followed by 750µL MTBE + 188µL $H_2O$ with 0.1% ammonium acetate + 2000µL 100% MeOH*<br>Methanol extraction:<br>*150µL 100% MeOH followed by 150µL 20% MeOH* | 50 mg frozen stool | Amino acids/polar metabolites, certain lipids<br><br>Triglycerides, acylcarnitines (organic solvent methods) |

[a] *NaOH extraction method showed greatest metabolite recovery with higher abundance of bile acids*

[b] *80% MeOH extraction and Lyophilization methods showed greatest extraction efficiency*

[c] *Based on dried residual weights the methanol-water extraction was the most efficient*

[d] *80% MeOH extraction saw the greatest metabolite coverage*

[e] *Extraction techniques do not include all extraction steps, i.e. vortexing and centrifugation*

extracted under different solvent systems (Moosmang et al., 2019). Organic solvents such as acetonitrile (ACN), ethanol (EtOH) or chloroform ($CHCl_3$) are useful for extracting lipids but tend

to have lower extraction efficiency for hydrophilic/ionic metabolites. In contrast, solvent systems containing water will be more capable of extracting hydrophilic metabolites. As shown in **Table 1.2**, a commonly reported extraction solvent for stool metabolite analysis consists of a combination of water and methanol (~80% MeOH), but excessively high organic solvent content can lead to poor results during analysis due to incompatibility with various analytical instrumentation techniques (Moosmang et al., 2019).

## 1.2 Introduction to Irritable Bowel Syndrome

### 1.2.1 Overview and prevalence of IBS

In Canada, the United States and the United Kingdom more then 1 in 4 adults are living with some kind of functional bowel disorder (Palsson et al., 2020). The prevalence of bowel related diseases is exceptionally elevated in Canada compared to the rest of the world with an estimated 18% of the population diagnosed with IBS, whereas globally this figure is closer to 11% (Lovell & Ford, 2012). Reviewing province specific prevalence data, a distinct trend moving from east to west reveals elevated IBS occurrence in east cost provinces, with the lowest noted prevalence found in Quebec (Fedorak et al., 2012). Bowel diseases are serious medical conditions which cause patients to endure a wide range of symptoms that have a major impact on everyday life. For diseases like IBS and IBD, treatment is usually symptom and patient specific. Irritable bowel syndrome can be divided into two main sub-groups depending on bowel patterns and Bristol stool scores. Patients that frequently experience loose and watery stools are classified as IBS-D where those who have difficulty passing stool accompanied by hardened stool forms are classified as IBS-C. Additionally, an IBS patient may experience bouts of chronic diarrhea that switch to extended periods of constipation, in which a patient may be classified as mixed IBS (IBS-M) (Canavan et al., 2014). Although altered bowel habits are key to IBS, many other undesirable

symptoms such as abdominal pain, and cramps may also be present (Irritable Bowel Syndrome: Overview - InformedHealth.Org - NCBI Bookshelf, 2019). Distribution of IBS among different age groups and sex, shows that occurrence is significantly higher in younger age groups with a 25% reduction in prevalence over the age of 50 while also presenting 1.5-3-fold higher in woman opposed to men (Canavan et al., 2014).

With recent advances in IBS research, its multi-factorial nature has become apparent, with etiology being linked to alterations in both the immune system and gut microbiota (Raskov et al., 2016). IBS has also been strongly associated with psychological stress showing comorbidity with both anxiety and depression. However, it remains uncertain whether there is a causal relationship between IBS and mental/mood disorders, or if altered bowel habits and associated symptoms causing a reduced quality of life is the major contributing factor (Mudyanadzo et al., 2018). Interactions between the nervous system and gut microbiota is a symbiotic bidirectional relationship denoted as the Gut-Brain Axis (GBA) (Raskov et al., 2016). Current research supports the link between the GBA and IBS, with dysbiosis in the brain or the gut inducing a cyclic chain of events where disturbances to gut microbiota can alter chemical neurohumoral signaling to the brain, which can cause central nervous system (CNS) abnormalities. This can activate an immunological response, perpetuating negative gastrointestinal responses via sympathetic and parasympathetic efferent pathways (Mudyanadzo et al., 2018). The ability of the gut to alter neurological signaling and chemical balance in the brain supports the idea that IBS can have a causal link to the pathogenesis of depression, although IBS pathophysiology overall is still not well understood.

### 1.2.2 Current practices: Diagnosis of IBS

Available diagnostic tools for IBS are limited, with diagnosis usually occurring over a span of years, starting with testing to rule out other potential diseases (Canavan et al., 2014; Moayyedi et al., 2017). Current practice for the diagnosis of IBS follows the Rome IV criteria which has been developed by The Rome Foundation and is periodically updated as our understanding of IBS evolves. With the Rome IV criteria, patients are required to track recurrent abdominal pain and its association with changes to stool frequency or form. A patient must experience recurrent abdominal pain for a minimum of one day a week for three continuous months to meet the current criteria (Lacy & Patel, 2017). The specific criteria of the IBS Rome IV diagnostic are outlined in **Table 1.3**. Classification of IBS subtype is based on the Bristol stool form scale (BSFS) consisting of 7 different stool types ranging from 1 to 7 where 1 designates a solid stool that is hard and lumpy, and 7 is designated for a watery stool containing no solid form and is completely liquid. IBS classification associates IBS-C and IBS-D with having >25% of bowel movements corresponding to stool forms 1 or 2 and 6 or 7 respectively (Lewis & Heaton, 1997). However, there is an urgent need for a more definitive and objective diagnostic biomarker of IBS and through the use of metabolomics this can hopefully be achieved. It is important to understand the potential of stool metabolomic diagnostic approaches for chronic diseases such as depression, diabetes, and cancer of which also have strong ties to the gut microbiome (W.-Z. Li et al., 2020; Mudyanadzo et al., 2018; Vivarelli et al., 2019).

Similar to IBS, the incidence of inflammatory bowel disease (IBD) is also on the rise (Alatab et al., 2020a; Peña-Sánchez et al., 2022). Inflammatory bowel disease is also divided into two subclasses depending on the location of inflammation within the gastrointestinal (GI) tract. Inflammatory bowel disease also does not currently have a definitive test for diagnosis and relies

**Table 1.3** Rome IV criteria for IBS diagnosis adapted from (www.theromefoundation.org; accessed 06-30-2022)

| **Recurrent abdominal pain ≥1 day per week for the last 3 months, associated with 2+ of the following criteria:** |
|---|
| ▪ Related to defecation |
| ▪ Associated with a change in frequency of stool |
| ▪ Associated with a change in form or appearance of stool |

**Criteria fulfilled for the last three months, with symptom onset for at least 6 months before diagnosis*

on a combination of patient history and confirmation using invasive colonoscopy and tissue biopsy (Nikolaus & Schreiber, 2007). Metabolomic analysis has led to the identification two key biomarkers of acute inflammation in the body showing increased levels of C-reactive protein and calprotectin in serum and fecal samples respectively (Kyle et al., 2021). These biomarkers may be beneficial for IBD diagnostics but still do not provide clinicians with the ability to differentiate between the subtypes of IBD. Crohn's disease (CD) and Ulcerative Colitis (UC) are the two sub-types of IBD and are both chronic inflammatory disorders of the GI tract. Ulcerative Colitis patients tend to have continuous inflammation that is confined to the mucosa and submucosa of the colon, whereas CD is characterized by transmural inflammation that extends throughout the GI tract (Qin, 2013). The pathophysiology of IBD is not fully understood, although it is believed to be some form of immune response which is prevalent in individuals with a genetic predisposition. Distinguishing between the two diseases is rather difficult and requires invasive testing for diagnosis (Alatab et al., 2020). Yamamoto et al. recently demonstrated that a panel of urinary biomarkers, such as indoxyl sulfate, sialic acid and phenylacetylglutamine, show potential for differentiation between two the major pediatric IBD sub-types (Yamamoto et al., 2021). These

results offer a glimpse at the translational potential of metabolite biomarkers, which may assist clinicians to more accurately and rapidly diagnose chronic bowel disorders, including IBS.

### 1.2.3 Current practices: IBS treatment options

Despite advancements in IBS research, underlying mechanisms of pathophysiology are not well understood and current potential biomarkers for diagnosis are limited and poorly validated. Currently, most treatment approaches focus on symptom reduction through use of pharmaceutical agents and dietary modifications, rather then treatment of underlying root cause(s). Alternative medicine treatments for IBS focus on treating underlying issues with use of essential oils and probiotic regimens, although scientific evidence backing up these methods are underpowered. For patients with more mild symptoms, over the counter anti-diarrheal medicine or laxatives can be used in conjunction with anti-depressants to reduce altered bowel habits and relieve psychological symptoms. For patients with more severe IBS, both lubiprostone and linaclotide have been approved for use by the Food and Drug Administration (FDA) for indication with IBS-C (Wall et al., 2014). In Canada there are currently only two pharmacological treatments, eluxadoline and rifaximin that have been approved for indication with IBS-D (Andrews & Bradette, 2020). Recent research efforts have helped to expand the available treatment options, although several medications are associated with negative side effects and the specificity of medications for either IBS-C or IBS-D poses a problem for a subset of IBS patients that experience mixed symptoms. One of the key medications for treatment of IBS-C, lubiprostone acts as an activator of the chloride-channel which promotes intestinal secretion and increased motility and clinical trials showed a 17.9% improvement to efficacy endpoint. Although no serious adverse reactions are typically noted with use of lubiprostone, there was a slight elevation in moderate adverse events including diarrhoea, abdominal distension and nausea (Drossman et al., 2009). Linaclotide is an

emerging IBS-C treatment that acts through guanylate cyclase receptor stimulation, causing elevation to guanylin and uroguanylin secretion. These compounds are secreted into the intestinal lumen and signal for a combined release of electrolytes and fluid into the bowels, helping to relieve constipation related symptoms (Bryant et al., 2010). With no known serious side effects and low (5.7%) incidence of minor side effects, linaclotide could offer a viable treatment option for severe IBS-C but suffers from high costs limiting its accessibility in the general population (Wall et al., 2014). Rifaximin is an example of an available treatment for IBS-D, which was suggest on principle that IBS-D may actually be small intestinal bacterial overgrowth (SIBO) or share a close relation to SIBO, but also only provides symptom relief, although improvements have been reported to last up to 10 weeks (Pimentel et al., 2011). Although more promising medication for IBS patients are increasingly becoming available, they work by treating the symptoms of IBS, and show little indication in curing the associated underlying conditions.

Besides pharmacological options, diet intervention and other natural approaches have been suggested for IBS treatment. A correlation between symptom flair-ups and diets high in processed foods, dairy, grains, and protein provides reasonable support to the treatment of IBS via specific dietary restrictions (Capili et al., 2016; Cozma-Petrut et al., 2017). Unbiased dietary studies with adequate controls are limited due to the technical feasibility of creating a true placebo that patients will not be able to identify, as personal beliefs or expectations can impart an apparent placebo effect (Werlang et al., 2019). Recent literature suggests diets low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) along with the exclusion of glucose, can provide relief of IBS symptoms, from which one study showed an improvement for approximately 75% of patients (Lacy, 2015). However, this study is limited by loose exclusion criteria and non-standardized diets and questionnaires, so the presented data should not be used to rule out other,

more thoroughly investigated treatment options. Alternative therapeutic options focus on root cause treatments, commonly through use of probiotics containing *Lactobacillus, Bifidobacterium,* and *Propionibacterium* species. Probiotic therapy has high compatibility to multifactorial GI disorders like IBS, as treatments can be customized to patient specific disease factors (Singh & Natraj, 2021). So far, probiotic studies have shown reasonable effectiveness for treatment of IBS, through suppression of pathogenic bacteria and the secretion of important short chain fatty acids (SCFAs) that in combination help heal intestinal mucosa, improve barrier function, have a preventative effect on epithelial cell apoptosis (Aragon et al., 2010). More work into understanding fundamental disease mechanisms is needed to formulate better treatments and treatment plans that can effectively cure IBS and reduced its overall prevalence globally.

## 1.3 Current Progress Towards the Use of Fecal Metabolomics for IBS

### 1.3.1 Metabolite discoveries for diagnosis of IBS

Mechanisms of disease pathophysiology and established risk factors for IBS have shaped the development of diagnostic practices. One of the highest risk factors for development of IBS is previous infection of the small intestine, with previously infected individuals having a 4.2-fold higher risk of IBS. Study specifics show parasitic, or protozoa infections lead to a 41.9% development of post-infection IBS compared to the 13.8% that developed IBS after bacterial infection, indicating increased disruption of gut microbiota by parasitic organisms (Klem et al., 2017). With microbiota dysbiosis having a substantial contribution to IBS, a lot of focus for IBS diagnosis has shifted to by-products of microbial metabolism. A recent review of current biomarkers of IBS highlights the use of SCFAs, a product of microbial fermentation, as a sensitive and specific marker of IBS, with propionic acid and butyric acid having the best recorded

specificity and sensitivity (Kim et al., 2017). Stress response is also an important contributor to IBS symptomology, and studies have shown altered steroid hormone biosynthesis pathways as well as histidine metabolism in IBS patients is indication of increases stress and inflammation in the body, with histidine and cortisone being good markers of IBS (Yu et al., 2019). It has also been suggested that endocrine cells in the gut may play a role in abdominal pain as a consequence of increased gas production and intestinal distention when digesting FODMAPs (El-Salhy et al., 2014). In accordance with these findings, many other studies have found granins, chromogranins and secretogranis, which may be representative of enteric neuroendocrine system activation, as novel markers of IBS. The increased fecal levels of granins is opposite to the reduced levels of granins reported in the mucosa of IBS patients when compared to healthy controls (Nakov et al., 2022). Interestingly, a study was conducted on assessment of volatile organic metabolites and was able to not only distinguish IBS-D patients from healthy controls, but also from current IBD patients with high specificity (82%) and sensitivity (80%) highlighting the potential for differentiation of IBS from other GI related diseases, which is largely unexplored to date (Ahmed et al., 2013).

### 1.3.2 Differential diagnosis of IBS subtypes: IBS-D and IBS-C

In comparison to the quantity of studies on the differential diagnosis of IBS patients from healthy controls, surprisingly few studies focus on differentiating between IBS subtypes, primarily IBS-D and IBS-C. Recently, a study examining gene and serological expression reported a panel containing 34 biomarkers that not only differentiated between IBS and healthy non-IBS controls, but also could differentiate between IBS-C and IBS-D. Of this large panel, 4 key biomarkers, histamine, Neutrophil Gelatinase-associated lipocalin (NGAL), Molecules Interacting with CasL Like protein 1 (MICALL-1) and Ring Finger protein 26 (RNF26) had a large apparent impact on

the overall diagnostic capabilities. This biomarker panel showed a correlation to pathways and processes with substantial impairment in patients with IBS. For example, NGAL which plays a role in mucosal regeneration, while MICAL-like 1 is a cytoskeletal regulator involved in tight junction activity and assembly. Being able to differentiate between IBS subgroups suggests that some aspects of their mechanisms of disease may be distinct (Jones et al., 2014). Even now studies struggle to validate robust biomarkers to differentiate between subtypes, but with further research and by expanding nontargeted metabolite discovery-based studies, formulating a concrete panel of differential metabolites may be feasible.

### 1.3.3 Metabolomics for monitoring and treatment of IBS

Although efforts have been made to explore the metabolic mechanisms of IBS with hope of finding quality markers characteristic of the disorder, there is a gap in available studies looking at IBS metabolite profiles long term. Longitudinal studies offer various benefits, giving insights into how the disease changes over time, especially with fluctuations in active and dormant disease states. Long term metabolic tracking can also act as an indicator of treatment progression and how certain treatments effect long term remission. By studying IBS patients at various time points and assessing biochemical changes associated with increased symptom severity, we may be able to gain a better understanding of symptom perturbations and provide better treatment options. Looking at periods of symptomatic and asymptomatic IBS, in conjunction with the days or weeks prior to symptom flair ups, it may become apparent that certain activities or metabolic changes may be responsible for immediate as well as delayed symptom responses. Whereas current research often will only look at IBS at a singular point in time or at immediate indication of worsening symptoms. One longitudinal study conducted by Mars et al. (Mars et al., 2020) integrated metabolomics with microbiome data, epigenomics and transcriptomics, in a long-term

study extended over 7 sampling points. In this study it was discovered that there was a higher abundance of over 20 lactobacillus species in severe IBS-D when compared to times of mild to moderate IBS-D. This data, among other findings, such as elevated alcohol dehydrogenase (ADH) in severe IBS-C and IBS-D are findings unique to longitudinal studies, highlighting their importance in the future of IBS research .

### 1.3.4 Challenges and deficiencies in current research

Many recently published studies pertaining to IBS only look at metabolic and microbial differences between health controls and IBS, with results that are static, often looking at patient profiles for a singular point in time. Some longitudinal studies (Mars et al., 2020a) have been completed and provide novel insights into mechanisms of IBS as discussed earlier, but studies such as this one lack exploratory findings and focus on previously discovered metabolites. Some studies are more exploratory in nature (Han et al., 2021; Stenlund et al., 2021) but results focused on only well-known metabolites, so it is unclear if untargeted analysis produced any novel markers. Other studies extend over long periods of time, but only compare baseline assessments to a single post treatment time point, as outlined in this study (Jacobs et al., 2021) looking at cognitive behavioral therapy effects on IBS and metabolites associated with higher treatment response. As stated above, longitudinal studies offer many benefits with regards to enhancing mechanisms of disease and treatment monitoring. Disorders such as IBS are dynamic in nature, and what may have initially acted as the trigger may be different from what is currently perpetuating the condition. Additionally, longer studies will help to better evaluate chemical changes occurring in the body during altered symptom states which may also offer valuable insights into pathophysiology.

As we begin to better understand the importance of the gut microbiome in relation to health and disease, the fecal metabolome has gained substantial popularity. The fecal metabolome and ultimately fecal samples act as a functional read out of the gut, including the gut microbiome and all associated metabolites of microbial metabolism (Zierer et al., 2018). Human fecal samples have an abundance of information due to their complexity and provide researchers with a new outlet for gaining novel insights to the various GI disorders present today. However, as a relatively new field it lacks the robust sample handling standardization of more commonly studied biospecimen, leading to contradicting results from different studies. The following two studies examined SCFA levels in IBS patients, but came to opposite conclusions with contradicting results (Fukui et al., 2020; Mars et al., 2020); propionate levels were shown to be highest in the IBS-C subgroup compared to IBS-D and HCs by Fukui et al., but conversely Mars et al. reported that propionate was lowest in the IBS-C group for the same comparison. Stool is very heterogeneous and the way the samples have been prepared or extracted can have impact on the types and quantities of extracted metabolites. Standardization of fecal sample handling will help reduce variability across different studies and lead to more reproducible results. For complex disorders like IBS, often symptoms arise due to elevated stress, altered mental state and dysbiosis of the gut microbiome which can arise on its own or in part due to the previous two factors. IBS is not a disease or heavily reliant on genetics for pathophysiology and so natural remedies for relief of stress or anxiety (Abrahão et al., 2019), as well as dietary modifications/probiotics have the potential to be an effective treatment for IBS. There is a need for more research into treatments of underlying mechanisms of IBS, rather then drugs meant only for symptom relief. Some work has been done, but most studies lack statistical power and have major flaws such as lack of rigorous study design and improper study implementation (Nahin & Straus, 2001). In this respect, metabolomic studies

have greatly improved our understanding of IBS, as well as the complex dynamics of host-microbiota interactions for a range of GI disorders.

## 1.4 Thesis Objectives and Motivation: Analysis of the Fecal Metabolome and Applications to Irritable Bowel Syndrome

### 1.4.1 Overview

IBS is one of the most prevalent gastrointestinal disorders worldwide with the greatest prevalence in western countries, with a higher occurrence in younger females. Worldwide incidence of IBS has seen a recent incline, with no known cure available to date. The underlying mechanisms of pathophysiology remain inadequately understood preventing effective diagnostics and treatment plans for sufferers of this chronic condition. Recently, stool has emerged as a viable and non-invasive biospecimen for directly analysing the endpoint of host metabolism, as well as the functional readout of the gut and associated microbial metabolism. The ability to compare metabolites generated by bacterial and host metabolism, offers crucial information on the host-bacterial interplay and the extent of the GBA. By exploring the stool metabolome, we may be able to uncover novel insights into IBS pathophysiology, along with many other functional gut disorders that may also benefit from discovery-based stool metabolomic studies. As a new addition to metabolomics research, stool metabolomics is still in need of standardized sample handling protocols. Its heterogeneous nature creates a highly variable biospecimen that is in desperate need of standardized sample handling protocols to improve reproducibility required for use in a clinical setting. To further the field of stool metabolomics various sample workup procedures were investigated, with focus on developing a simple but effective solvent extraction system for analyzing the stool metabolome, along with ways to improve the ease of use, stability, and metabolite abundance (coverage and quantity) when using lyophilization. Through literature

recommendations and evaluation of our study findings, a reliable and effective stool preparation technique was developed and used to conduct a pilot study on a cohort of IBS patient samples using multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) as a high throughput platform for characterizing the ionic metabolome (Yamamoto et al., 2016). The longitudinal nature of the study provided a unique set of samples allowing for potential identification of stool metabolic signatures associated with symptomatic and asymptomatic time periods for each IBS patient.

### 1.4.2 Fecal extraction method optimization

Fecal samples contain an abundance of information (Jain et al., 2019), but in order to access this information an effective extraction protocol must be used. As summarized in **Table 1.2**, an optimal extraction condition for comprehensive fecal metabolome coverage uses a mixed solvent system of methanol and water in a 4:1 ratio (MeOH:$H_2O$, 4:1). This solvent system allows for extraction of polar metabolites simultaneously with polar hydrophobic metabolites due to the combined aqueous-organic nature of the extraction solvent. As our samples will be analysed using MSI-CE-MS, we have altered the extraction solvent for compatibility with our instrument choice. CE requires the use of two aqueous background electrolyte (BGE) systems for separation of ionic metabolites under acidic and alkaline conditions when using positive and negative ion mode detection, respectively. As the BGE interacts with the sample solution, the sample solution will require equilibration with the BGE; if the two are not compatible this process may take longer and result in poor peak shapes. The commonly used extraction solvent mixture of MeOH and water has high organic content, making it incompatible with the BGE system used for the CE separation, so the ratio of methanol to water was adjusted. Within our lab, two key stool extraction methods are employed for metabolomic analysis using MSI-CE-MS. The modified Bligh Dyer extraction

was optimized by former PhD student, M. Yamamoto from the Britz-McKibbin laboratory. The extraction called for lyophilized stool (~15 mg) to be to be mixed with a solvent solution ($V_T$ = 424 µL) of chilled methanol, deionized water and chloroform in a 4:3.6:4 ratio. The combined solution is vortexed and centrifuged before the aqueous layer is extracted and a second extraction step is conducted using a methanol water mixture (1:1, $V_T$ = 128 µL). After further vortexing and centrifugation the aqueous layer is once again extracted and combined with the aqueous layer from the first extraction step. The other extraction protocol although similar is less complex and requires fewer extraction steps and solvent solution preparation. The methanol-water stool extraction calls for 5 mg of lyophilized stool to be combined with 100 µL of a 30:70 MeOH:$H_2O$ solution followed by vortexing and centrifugation. The supernatant is then extracted, and the same procedure is followed for the second extraction step (Lemay et al., 2021). Although the modified Bligh Dyer extraction was more commonly used prior to this study, this extraction comparison study will help to determine the most suitable extraction protocol for future studies. The capability to detect a wide range of metabolites is paramount for studies where accurate metabolite profiles are needed, but it is also very important to have extractions that are simple and highly reproducible, especially when dealing with large scale projects that are time consuming and prone to variability (Moosmang et al., 2019).

### 1.4.3 Wet extraction versus lyophilization for sample preparation optimization

The handling of fecal samples has yet to be standardize and there is currently no consensus concerning the use of lyophilization as a pre-treatment step for stool sample preparation; we hope to further explore the use of lyophilization as it can offer several additional benefits. It has been reported in a limited number of studies that lyophilization may actually increase metabolite recovery due to the potential of the process to lyse bacterial cells, thus releasing the intracellular

components (Didovyk et al., 2017b). As a very heterogeneous medium, stool has highly variable water content and metabolite/bacterial composition depending on sampling location. Lyophilization would allow for the generation of reproducible and clinically relevant metabolite quantifications through normalization of metabolite abundances to dried fecal mass and by creating a fine powder that can be more readily homogenized. There are some concerns with regards to loss of more volatile stool metabolites, such as SCFAs so to solve this, we tested lyophilized samples against fresh wet stool samples for metabolite recovery and the retention of volatile organic compounds. In conjunction with this information, the overall stability was also assessed for fecal samples in either form, to determine the best means of preserving stool samples long-term for biobanking purposes.

### 1.4.4 Storage and stability of fecal samples

In order to increase confidence in fecal metabolomic studies, it is important to assess the stability of samples based on their storage state. There is a need for more longitudinal studies, but in order to produce reliable results, samples must be stable over time without metabolite degradation during the duration of the study. Stool samples are stored in various states (i.e., crude, wet extract, lyophilized) at various temperatures (-20 °C or -80 °C) and have undergone repeat freeze and thaw cycling according to the study design. To ensure the stability of our samples and assess the potential benefit of lyophilization for reduced microbial activity and increased long term stability, a stool stability study was conducted. The three main stool states were systematically compared after 1, 2 or 3 freeze-thaw (FT) cycles and storage at -80 °C for 48 h, 1 week, 8 weeks, and 21 weeks. An overview of the literature for stool sample collection, preparation, and storage methods, including long term stability studies is summarized in **Table 1.3**. Regardless of stool state, all samples underwent a MeOH: $H_2O$ extraction as detailed in section 2.3.5.

### 1.4.5 Fecal biomarkers for differential diagnosis of IBS

The growing number of fecal metabolomic studies for IBS has led to the discovery of promising biomarkers associated with IBS pathophysiology, but further studies are needed to rigorously validate a reliable and robust diagnostic or prognostic metabolite panel. By taking advantage of a prospective study design involving repeat stool specimens collected for a cohort

**Table 1.4** Stool sample collection, preparation and storage/stability studies in recent literature for stool metabolomics

| | Sample Workup Information | | | |
|---|---|---|---|---|
| Article | Extraction Solvent System | Sample Preparation | Storage Conditions | Key Findings/Conclusions |
| Mandal et al. (2020) | N/A[a] | 1. Homogenized in water + Lyophilization<br><br>2. Homogenized in water<br><br>3. Homogenized in water | 1. 4˚C<br>2. -80˚<br>3. 4˚C | Different preservation techniques introduce varying metabolite biases<br><br>Lyophilized samples as well as samples stored at – 80 °C are the most suitable for RM development |
| Gratton et al. (2016) | Left as crude feces, or fecal water extraction performed | 1. Manual Homogenization<br><br>2. Manual Homogenization + addition of water and vortexing<br>One/two Freeze thaw cycles (-80˚C 24 hrs + thaw at RT for 2 hrs) | 1. a. *RT for 0, 1, 5, 10 & 24 hrs*<br>b. *4˚C for 1, 5, 10, 24 and 24+ RT 5 hrs*<br>c. *-20˚C for 1, 5, 10, 24 and 24 + RT 5 hrs*<br><br>2. a. *RT for 0, 1, 5, 10 & 24 hrs*<br>b. *4˚C for 1, 5, 10, and 24 hrs*<br>c. *-20˚C for 1, 5, 10 and 24 hrs*<br>All samples stored at -80˚C until analysis | Sample collection and extraction should be performed within 1 h of stool collection.<br><br>Samples should stay refrigerated to prevent the metabolic changes that occur during thawing<br><br>kept on ice throughout the extraction process. The sample should be homogenized immediately post collection<br><br>More than one freeze−thaw cycle of fecal water is not recommended; hence, aliquoting fecal water prior to freezing is essential (FT increases some AA)<br><br>significant metabolic changes occurred after 5-h storage at room temperature and after 24 h at 4 °C. (SCFA/TCA met. Increased + others decreased over storage time) |
| Liang et al. (2020) | N/A[a] | 1.Head, body and tail slices taken and analyzed separately<br><br>2.Seprate sample homogenized sample to evaluate storage and thawing conditions | 2. Storage in RNALater and gradual vs fast thawing | 22 of 176 identified metabolites varied significantly across different sampling regions.<br><br>Sample storage in RNALater showed a significant level of variation in both microbiome and metabolome profiles and is not recommended<br><br>There were no significant differences between thawing methods |
| Spiegeleer et al. (2020) | Methanol water extraction<br><br>Methanol/ MTB/ Water extraction with 0.01% Butylated hydroxytoluene and | 1. Lyophilized in 200mg aliquots | 1.-80 *˚C with 0, 1, 2 FT cycles and long-term storage at -80 ˚C and -20 ˚C under both Aerobic and Anaerobic conditions*<br>Storage time up to 25 weeks | recommendations are that intact stool samples should be divided into<br>aliquots, lyophilised and stored at 80 ˚C for a period no longer than 18 weeks, and avoiding any freeze thawing |

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|---|---|---|---|---|
| | 2.5% trichloroacetic acid | | | |
| Hsu et al. (2019) | Organic Solvent Extractions: *Tested MeOH, ACN, $CHCl_3$, EtOH, Hexane extractions*<br><br>Liquid-Liquid Extraction: *Butanol* | Samples were either Lyophilized, crude or wet extracts. | Stored at 4˚C, -20˚C for 7 or 30 days + Crude stored at RT for 1, 6 and 24 hrs<br><br>Stored at -80˚C until analysis | Lyophilization minimized biases due to water content<br><br>Lyophilization provided better stability for SCFAs |
| Ueyama et al. (2020) | Liquid-Liquid Extraction: Hexane | Freeze-Dried Samples: frozen for 24 hrs at -80˚ prior to lyophilization<br><br>Crude feces frozen at -80˚C | Stored at -80˚C and RT<br><br>Crude Stored at -80˚C ( 30, 60 days) and 4˚C (1, 2 days) | Freeze drying reduced variability<br><br>Decreased SCFA concentrations with storage at 4˚C for 24 hours<br><br>Freeze drying gives stability to samples even being stored at room temperature for 3 days |
| Newland et al. (2021) | N/A* | Fresh crude stool | Used in 10 minutes, -4 ˚C for 60 mins, anaerobic chamber @ 4˚C 60 mins, -20˚C 60 mins, -80˚C 2 weeks | Freezing at -80 had significant effects on microbial numbers<br><br>Storage at 4˚C had minimal effects on metabolic profile and good for short term storage until analysis |

[a] *Information was not available or provided*

of IBS patients, we were able to identify biomarkers representative of a patient's symptom state and track the ways in which a patient's stool metabolic profile is linked to clinical outcomes over time. We are primarily focused on distinguishing between asymptomatic and symptomatic states of IBS-C and IBS-D patients, and comparing the metabolic phenotypes of stool that distinguish between IBS subtypes and healthy controls when using an exploratory and untargeted metabolomics approach to biomarker discovery with MSI-CE-MS. Most IBS stool metabolomics studies reported to date have been targeted in nature with limited overall metabolome coverage, removing the possibility for new discoveries. There has been one longitudinal IBS study which analyzed primarily gut microbial activities via genome sequencing, but only measured a targeted set of fecal metabolites collected during active and dormant symptom timepoints (Mars et al.,

2020). To the best of our knowledge, no untargeted longitudinal stool metabolomics study looking at the different symptom states of IBS patients has been reported, which opens up the opportunity for new biomarkers indicative or predictive of symptom severity.

## 1.5 References

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# Chapter II:

# The Development of Standardized Sample Handling Protocols for Fecal Metabolomic Studies of Irritable Bowel Syndrome

# Chapter II: The Development of Standardized Sample Handling Protocols for Fecal Metabolomic Studies of Irritable Bowel Syndrome

## 2.1 Introduction

Irritable bowel syndrome (IBS) is a major functional gastrointestinal (GI) disorder characterized by reoccurring bloating, abdominal pain, and stool irregularities (Enck et al., 2016). Canada has one of the highest rates of IBS as compared to an estimated pooled global prevalence of 11.2% depending on exact criteria used for diagnosis (Lovell & Ford, 2012). Irritable bowel syndrome can often be a lifelong affliction that is most common in women and young adults causing a serious burden to their quality of life and workplace productivity, while contributing to greater healthcare expenditures (Sperber et al., 2021) with frequent psychiatric comorbidities, including anxiety disorders and depression (Hu et al., 2021; Mudyanadzo et al., 2018). There is currently no cure for IBS as current pharmacological treatments (i.e., rifaximin, guanylate cyclase) are aimed at attenuating symptoms while not addressing the complex underlying factors implicated in IBS etiology, including diet, lifestyle, trauma history and/or environmental exposures that modulate the human gut microbiome. Irritable bowel syndrome diagnosis has evolved based on a consensus of predominate symptoms reflecting the frequency of abdominal pain, duration of altered stool habits, along with reported stool consistency based on the Bristol stool form scale (Lacy & Patel, 2017). For example, the Rome IV criteria is used to diagnose and classify IBS patients into 4 major sub-types to better guide patient treatment, including IBS-predominant constipation (IBS-C), IBS-predominate diarrhea (IBS-D), IBS-predominate mixed bowel habits (IBS-M), and IBS-unclassified (IBS-U). Furthermore, symptom prevalence is highly variable ranging in severity and frequency, with definitive diagnosis often taking well over 6 months, with the average diagnosis taking up to 4 years after excluding other GI diseases and disorders (*IBS*

*Global Impact Report 2018 - Gastrointestinal Society*, n.d.; Jeffery, Das, O'Herlihy, et al., 2020; Lacy & Patel, 2017). There is a demonstrated need for validated biological markers for accelerated diagnosis and classification of IBS and its associated subtypes. Moreover, disease pathophysiology remains poorly understood, but is generally attributed to a dysfunction of the microbiome-gut-brain axis (Kennedy et al., 2014). There is a high frequency of postinfectious (PI) IBS, with a reported incidence as high as 32%, leading researchers to believe the gut may play an essential role in not only etiology, but as a potential inception point for IBS (Thabane & Marshall, 2009). Increased focus on the role of gut health in the pathophysiology of IBS has expanded research on the human stool metabolome.

To date, most stool metabolome analyses have relied on cross-sectional study designs for differentiation of IBS patients from healthy controls, that cannot properly capture the dynamic nature of the disorder, disregarding changes in individual symptom severity (Aggio et al., 2017; ena et al., 2011; Han et al., 2021; Jeffery, Das, O'herlihy, et al., 2020; Liu et al., 2020). Recently, a longitudinal multi-omics study revealed stool metabolic signatures associated with symptomatic illness and microbial composition changes among IBS specific sub-sets of patients, including SCFAs, hypoxanthine, tryptamine and bile acids (Mars et al., 2020). Importantly, fecal cholic acid and chenodeoxycholic acid were identified in both IBS-D and IBS-C patients undergoing a transient flare up in symptoms relative to baseline that coincided with lower microbiome diversity on a species level (Mars et al., 2020). Nuclear magnetic resonance (NMR) and mass spectrometry (MS)-based metabolomic studies in stool and other complementary biospecimens, such as urine (Keshteli et al., 2019) and serum (Noorbakhsh et al., 2019) have also been reported to evaluate treatment responses of IBS patients following a low fermentable oligo-di-monosaccharides and polyols (FODMAP) diet and probiotics (Bennet et al., 2020). In contrast, few studies have

identified differentiating fecal metabolites as potential biomarker candidates for IBS subtype classification (Dior et al., 2016; Wong et al., 2012), and so a more extensive panel of metabolites from stool extracts is still needed and will require independent validation in a larger prospective cohort. For instance, differential metabolites for IBS subtyping, such as the short-chain fatty acids (SCFAs), have shown contradictory results between studies, likely due to the lack of standardized protocols used for stool sample preparation prior to metabolomic analyses (Ahluwalia et al., 2021).

Although fecal metagenomics sequencing is widely used to characterize differences in gut microbial composition and their abundances in IBS patients (Phan et al., 2021), fecal metabolomics is a rapidly expanding area that offers a functional assessment of microbial activity and host-microbial interactions for new insights into disease mechanisms (Zierer et al., 2018). However, there are several analytical challenges when performing nontargeted stool metabolite profiling that can contribute to bias and poor precision without rigorous standardization, including sample collection, storage, preparation, and analysis (Karu et al., 2018; Mandal et al., 2020). For instance, lyophilization of heterogeneous fecal specimens can adjust for variations in hydration and stool form while also allowing for normalization of metabolite concentrations to dried weight for more reliable quantitative comparisons (Lemay et al., 2021a). Additionally, differences in extraction protocols will produce variable results based on its ability to effectively extract various metabolite classes (Table 1.2) (Erben et al., 2021; Yang et al., 2019) .

To supplement stool handling optimization research and to expand upon the characterization of IBS through stool metabolomics, herein we report a comprehensive stool metabolomic study on a cohort of age and sex-matched IBS sub-types of patients exhibiting self-reported symptomatic and asymptomatic periods of bowel dysfunction and psychiatric distress. Also, a lyophilization and stability study was performed on human stool specimens to compare the

effects of sample processing and storage conditions for more robust metabolomic analyses when using higher throughput methods based on multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) (Saoi, Li, et al., 2019; Shanmuganathan, Kroezen, et al., 2021; Yamamoto et al., 2021).

## 2.2 Experimental

### 2.2.1 Chemicals and reagents

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. All buffers, stock solutions for calibrants and sheath liquid were prepared with ultra grade LC-MS grade water obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada) with the exception of the extraction solvent solution stock for the lyophilization study, which was prepared with deionized (DI) water using a Thermo Scientific Barnstead EasyPure II LF ultrapure water system. Standards and extraction solvent solutions (30:70, methanol: deionized water) were stored at 4 °C, whereas background electrolytes (BGE) and sheath liquid solutions used for CE-MS were stored at room temperature.

### 2.2.2 Sample collection and study design: Lyophilization versus wet extraction

A fresh whole stool sample was initially collected at McMaster University from a healthy 24-year-old female donor using a sterilized collection pan. After collection, the sample was promptly (< 5 minutes) placed on ice for preservation. Using a scalpel, slices were taken from the head, body and tail of the intact stool sample and combined in a sterile collection container. This sampling approach ensures that our sample is representative of all the metabolically distinct regions of the fecal sample (Liang et al., 2020). Combined slices were then manually homogenized with a wooden depressor and the addition of 4.0 mL of HPLC grade water as a soft homogenization

technique to prevent potential cell lysis. The homogenized fecal paste was then weighed out (~15mg) accurately on an electronic balance into 1.5 mL microcentrifuge tubes. For half of the samples (n = 6) a solvent extraction (**Figure S2.1**) was immediately performed by adding 300µL of a methanol:water (30:70) solution containing 20 µM of 3-chlorotyrosine (Cl-Tyr), 4-fluorophenylalanine (F-Phe), γ-aminobutyric acid-d6 (GABA-D6), choline chloride (choline-D9) and naphthalene monosulfonic acid (NMS) as internal standards. The mixture was vortexed for 10 minutes, followed by centrifugation for 10 min at 14,000 *g*. The supernatant (270 µL) was then transferred to a new microcentrifuge tube, and the extraction process was repeated a second time on the same stool specimen to maximize metabolite recovery. The two supernatant extracts were combined and then frozen at –80 °C until analysis. The other half of the samples were frozen at –80 °C immediately after being weighed out. After 24 h, these frozen stool samples were removed from the freezer and a double layer of Kimwipe tissue was secured over the top of the microcentrifuge tubes with rubber bands in place of the centrifuge caps. Samples were then placed in a frozen lyophilization capsule and lyophilized (Labconco FreeZone Freeze Dry System; MO, USA) from frozen over a span of 24 h. Following lyophilization, samples were re-weighed (dry weight) to obtain water content estimates (~75% water) based on weight reduction attributed to water loss and were extracted following the same protocol as detailed above and remained on ice until analysis with MSI-CE-MS. The frozen extracts from the non-lyophilized samples were thawed slowly on ice, and once thawed the samples from both sample sets were vortexed (1 min) and centrifuged (3 min). Small aliquots from each individual sample extract were pooled together to make a quality control (QC) sample to monitor instrumental variability during stool metabolome data acquisition. As the samples were all derived from the same homogenized stool specimen, the six replicates in each group allow for adequate evaluation of the reproducibility of reported results.

Additionally, by sampling from a singular homogenized sample, bias was reduced when comparing the metabolome extraction efficiency and overall stool metabolome coverage between treatment groups.

### 2.2.3 Sample collection and study design: Longitudinal stability study

A fresh whole stool sample was collected and processed as describe in section 2.2.2, but due to the difference in sample water content the sample was homogenized with slightly less water (3 mL) to achieve a homogenized fecal paste. The homogenized sample was then weighed out (~20 mg) accurately on an electronic balance into 1.5 mL centrifuge tubes. A total of 135 samples (plus 3 QC designated samples) were weighed out and divided into three main treatment groups (crude, lyophilized and extracts) as detailed in the study design (**Figure 2.1)**. Each group was assigned 36 samples, for the crude treatment group the samples were immediately stored in the freezer at -80 °C until analysis. The lyophilization treatment group was frozen at –80 °C for 24 h prior to subsequent lyophilization at –80 °C for 24 h. For the wet extract treatment group, a methanol and water extraction (**Figure S2.1**) was immediately performed. The extraction was performed as described in section 2.3.2 with an adjustment to the amount of extraction solvent (400 µL) added to the sample to account for the increase in sample size from 15 mg to 20 mg (100 µL per 5 mg) for this study. After the two supernatant fractions were combined, and the extracts were then stored at –80 °C until analysis. After initial processing, samples underwent repeat freeze-thaw (FT) cycling according to study design (**Figure 2.1**), with 1 hour freezing at –80 °C and 1 hour thawing on ice. The 1 FT cycle samples remained in the freezer and were only thawed at time of analysis. At each of the designated timepoints, select samples were removed from the freezer

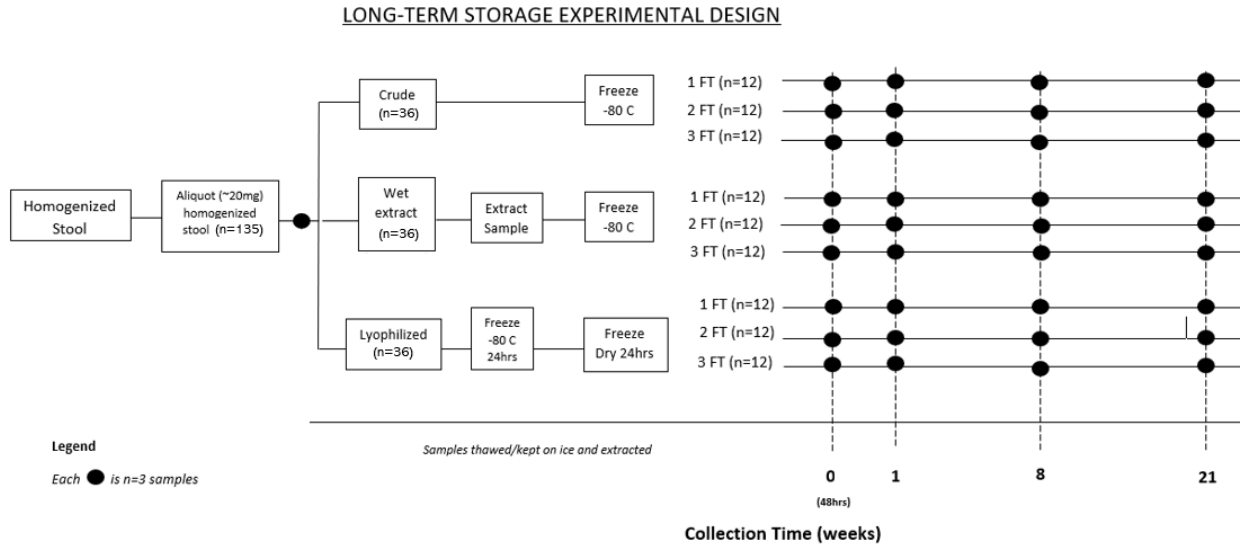


**Figure 2.1** Study design for long-term storage study comparing storage of three main fecal types (crude, wet extracts, lyophilized) with nontargeted metabolite profiling by MSI-CE-MS performed at select time periods over 21 weeks following up to 3 repeat freeze-thaw cycles.

and thawed slowly on ice. After thawing, the crude and lyophilized treatment groups were extracted following the same procedure the wet extract group underwent prior to storage. QCs were prepared using a subset (n = 3) of 20 mg of lyophilized stool samples that were extracted, pooled, and diluted 5-fold before aliquoting into sample vials. The QC samples were included in every run for each time point to monitor between run instrumental variation.

### 2.2.4 Cohort and pilot study sample selection: Longitudinal IBS patient study

Samples were selected from a cohort of IBS patients and healthy controls for the Role of Microbiota in Irritable Bowel Syndrome study which was approved by the Hamilton Research Ethics Board (HiREB# 11-445) with participants providing informed consent. The original study had 28 patients enrolled (20 IBS-D, 8 IBS-C) with 10 healthy controls (non-IBS) who provided stool samples and survey results each week for up to 25 weeks. The study recruited males and females from 18 and 60 years old who had a positive IBS diagnosis based on Rome III criteria.

Patients were excluded if they had concurrent systemic disease, organic GI pathology (except for benign polyps), diverticulosis, hemorrhoids, are pregnant or breastfeeding, and if they were receiving antibiotic therapy within the last three months. Patients were also excluded if they had severe depression or anxiety based on the Hospital Anxiety and Depression Scoring (HADS ≥15) or had a history of alcohol or drug abuse. In our pilot study we had 25 participants, which included 10 IBS-D group patients, 5 IBS-C group patients, and the 10 healthy control patients that were age, sex and BMI matched. From the 25 weeks of collected samples, 4 samples were selected from each IBS patient at timepoints during heightened (n=2) or reduced (n=2) symptomology based on overall symptom scores from survey results, which included self-reported abdominal pain, stool form and psychological distress. Symptomatic and asymptomatic samples were selected from both extremes of the symptom scoring to maximize contrast between IBS subgroups. Overall, a subset of 80 samples out of the 914 samples available were used for our metabolomics study.

### 2.2.5 Fecal sample handling and pre-treatment: IBS study

Stool samples were collected at home by participants recruited locally from the Hamilton (ON, Canada) region on a weekly basis within 24 h of completing both symptom and Hospital Anxiety and Depression Scoring (HADS) questionnaires. Patients were provided a collection kit, and a couple grams of sample were placed in a sterile 50 mL vial with the lid only lightly closed. Sample vials were placed in an air-tight sachet with an oxoid anaerobic packet and sealed. The samples were transferred on a cooling pad to McMaster University within six hours of collection. In the lab, samples were transferred to 2 mL cryovials and snap frozen with liquid nitrogen. Samples were stored at –80 °C until they were transferred to us for lyophilization and metabolomic analysis. When aliquoting samples for the metabolomic analysis, punches of the frozen sample were taken to avoid any freeze thaw cycling. Frozen samples had Kimwipes secured over the vial

openings and were lyophilized from frozen for 24 hours at room temperature. The lyophilized samples were homogenized by vortexing at 3000 *g* for 10 min, and then accurately weighed (~15 mg) on an electronic balance into 1.5 mL microcentrifuge tubes. Pooled subgroup samples, including repeat stool samples collected from the same individual during asymptomatic (A) and symptomatic (S) periods of time (i.e., IBS-D_A, IBS-D_S, IBS-C_A, IBS-C_S) and healthy controls (HC) were also prepared, by combining small aliquots of lyophilized stool from each sample within the respective subtypes for a subgroup analysis. Additionally, a pooled QC was also prepared by transferring some stool from each of the pooled subgroup samples into a clean vial. IBS Subgroup and pooled QC stool vials were homogenized (vortexed) and weighed out into 15 mg samples. All samples were extracted as detailed previously, but 2 mM of a stable-isotope analog of glucose ($6C_{13}$-Glu) was added to the extraction solvent, and the internal standards (IS) had their concentrations doubled to 40 µM. Just prior to analysis, all stool extracts were sedimented (8 min at 14,000 *g*) to ensure there was no residual particulates suspended in the extracts.

### 2.2.6 Data workflow and stool metabolomic characterization by MSI-CE-MS

All MSI-CE-MS metabolomic analyses were performed on an Agilent G7100 capillary electrophoresis system (Agilent Technologies Inc., Mississauga, ON, Canada) with a coaxial sheath liquid Jetstream (Dual AJS) electrospray ionization source in combination with an Agilent 6200 high resolution time-of-flight mass spectrometer (TOF-MS). The separation was performed with an applied voltage of 30 kV at 25 °C using two different BGE conditions on an open-tubular uncoated fused-silica capillary (Polymicro Technologies Inc. Phoenix, AZ, USA) with a total length of 120cm and an inner diameter of 50 µm. The BGE consisted of 1.0 M formic acid with 15% vol acetonitrile (pH 1.8) under positive ion mode and 50 mM ammonium bicarbonate (pH 8.5) under negative ion mode. The sheath liquid composition used under positive and negative ion

mode in MSI-CE-MS consisted of 60% vol methanol, whereas positive ion mode detection also included 0.1% vol formic acid. Prior to sample acquisition, the capillary was flushed at 950 mbar with methanol (15 min), 1.0 M sodium hydroxide (15 min), water (15 min) and BGE (30 min). A 13-plug serial injection format in MSI-CE-MS was used during sample injection to increase throughput as described elsewhere (Saoi, Li, et al., 2019; Shanmuganathan, Sarfaraz, et al., 2021). Individual samples and a single QC were analyzed in a randomized sequence for each run with the exception of the lyophilization versus wet extract analysis in which samples were run in triplicate to act as their own quality control and a single blank was injected instead. All stool extracts were injected hydrodynamically at 50 mbar for 5 s, followed by an electrokinetic injection of the BGE (30 kV for 75 s) that was repeated with each subsequent stool extract sample. This approach enables for improved zonal separation of stool metabolites without loss in resolution and effective capillary length when relying on serial sample and BGE hydrodynamic injections alone (Kuehnbaum et al., 2013). Furthermore, to ensure correct peak assignments from different samples, a temporal signal pattern was introduced for the lyophilization study and for untargeted analysis of the pooled subgroup samples for the IBS study.

Raw data acquired under full-scan acquisition (*m/z* 50 to 800) and was processed using Mass Hunter Workstation Software (Qualitative Analysis, version B.8.00, Agilent Technologies, 2016). Molecular features were extracted based on accurate mass (*m/z*) corresponding to a protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ion using an internal curated target stool metabolite list and the tentatively assigned metabolites identified during untargeted analysis as detailed elsewhere. All ions were extracted using the Molecular Feature Extractor in profile mode, with an allowed 10 ppm mass window and smoothed using the Savitzky-Golay quadratic/cubic function (7-point smoothing) prior to peak integration of each ion relative to an internal standard

migrating from the same injection position. Peak areas and migration times for all features and internal standards were exported to Microsoft Excel and normalized to internal standard Cl-Tyr or NMS to obtain relative peak area (RPA) and relative migration time (RMT) for positive and negative mode detection respectively. Relative peak area values were normalized to dry weight where applicable (e.g., solvent extraction comparison, IBS pilot study) to account for differences in stool water content across samples. For the studies comparing metabolite recoveries where not all samples underwent lyophilization pre-treatment, such as the lyophilization versus wet extraction and the longitudinal stability study, samples were accurately weighed out before lyophilization, and RPAs were normalized to wet weight. Final data matrices were generated after the removal of metabolites with poor technical precision (CV > 40%) and low frequency of detection (< 75%) with the exception of IBS subgroup specific features that were only/mainly detected in one IBS subgroup which has a frequency of detection < 75%.

### 2.2.7 Untargeted data analysis for unknown metabolite identification

Temporal signal patterning was integrated into certain sample runs using a serial 13-plug sample injection for nontargeted metabolite profiling for the lyophilization versus wet extraction study as well as the IBS pilot study. For the IBS pilot study, a pooled subgroup analysis with a 1:2:1:1:1:1:blank:2:1:1:1:1:2 dilution pattern was used, and allowed not only for easy authentic peak identification, but also simple subgroup assignments based on peak position within the run. Raw data from select runs were converted to mzXML format and analysed using MZmine 2 for feature generation (**Supplemental Figure S2.3**). The initial feature list (i.e., IBS pilot study: 66,108 positive mode, 21,112 negative mode) was detected in MZmine 2 (Pluskal et al., 2010) using MSI-CE-MS with temporal signal pattern recognition based on a multi-tiered data workflow as described elsewhere for rigorous metabolite or lipid credentialization (Ly et al., 2022). A

screened list of reliable and likely authentic stool metabolites (IBS pilot study: 401 positive mode, 300 negative mode) was compiled and their accurate masses (*m/z*) were then extracted in Mass Hunter Workstation Software (Qualitative Analysis, version B.8.00, Agilent Technologies, 2016) as a way to remove redundant and non-informative signals, including background ions, artifactual signals, and ions measured inconsistently. A second stage of filtering was also performed in Mass Hunter to remove in-source fragments, adduct ions or isotope contributions, which were rigorously excluded if they co-migrated with a molecular ion, or had a detectable signal in blank extract. This nontargeted approach identified additional authentic yet unknown ions in IBS patients (n = 66) that expanded our initial targeted panel of stool metabolites. These unknown ions discovered in our stool extracts were further characterized based on their charge state and most likely molecular formula generated from Mass Hunter Molecular Formula Generator with putative structural identification using online metabolomic databases, such as the Human Metabolome Database (HMBD) (http://hmbd.ca). From our lyophilization versus wet extraction study samples 32 cationic and 12 anionic unknowns were discovered and added to the final data matrix. Alternatively, in our pooled stool samples from the IBS pilot study 50 cationic and 16 anionic unknowns tentatively assigned features were included in our final data matrix with several unknown metabolites being common between the data sets.

### 2.2.8 Statistical analysis

Final data matrices were converted to .cvs files and uploaded to Metaboanalyst 5.0 for univariate and multivariate analysis (http://metaboanalyst.ca) including principal component analysis (PCA), receiver operating characteristic (ROC) curves, orthogonal partial least squares discriminant analysis (OPLS-DA) and correlation analysis. For data matrix completeness, missing

values resulting from low or undetectable signals were replaced with half of the lowest detected value within a specific stool group or IBS subgrouping (i.e., IBS-C, IBS-D, lyophilized, crude, etc.). The Statistical Package for the Social Sciences (IBM SPSS Software version 22) was used to perform the analysis of variance (1-way ANOVA), Mann-Whitney U-test, normality test (Shapiro-Wilk), Independent Samples student t-Test and Kruskal-Wallis test. Normality assumptions were valid and parametric statistical tests were used for the majority of the studies after *log* transformation. In contrast, for the IBS pilot study, most metabolites from the stool extracts (72.3%) failed the Shapiro-Wilk normality test ($p < 0.05$) after *log* transformation, thus requiring non-parametric univariate and multivariate testing on untransformed data.

The QC sample included in each run was used to obtain coefficient of variation (CV) values. Coefficient of variation (CV) values could be calculated by averaging the relative peak areas (RPAs) for each metabolite in the QC sample from each run and dividing by the standard deviation. For metabolomic analyses that did not include a QC sample in each run, samples were run in triplicate and each sample acted as its own QC from which CV values could be calculated. Additionally, control charts displaying the RPAs of internal standards were generated for both positive and negative ion modes to assess instrumental fluctuations across runs and study timepoints for studies where runs took place over several days. Control charts with low between batch variation (CV< 20%) were accepted, but an inter-batch QC-based correction was performed in R (Shanmuganathan, Kroezen, et al., 2021) for the longitudinal stability study which had substantial between batch variation due to sample analyses being run up to 21 weeks apart. The inter-batch QC correction resulted in substantial improvements to technical variability from a median CV of 33% to 6.9% (**Supplemental Figure S2.4**).

## 2.3 Results

### 2.3.1 Extraction of lyophilized stool metabolites by MSI-CE-MS

In order to establish a robust yet simple extraction protocol for our stool metabolomic studies, we reviewed the recovery and reproducibility of extraction protocols used in literature (**Table 1.2**) and performed a modest two-way extraction protocol comparison study using a subset of lyophilized pooled stool samples ($n$ = 6), comparing a single-phase methanol-water extraction (Erben et al., 2021b; Yang et al., 2019) and an internal biphasic modified Bligh-Dyer (B-D) extraction (Lemay et al., 2021b; Saoi, Percival, et al., 2019) which uses a combination of methanol, water and chloroform. The samples were weighed out in duplicate and extracted by both protocols for direct comparison. Samples were analysed using MSI-CE-MS (**Figure 2.2**) which is a high throughput platform for metabolomics, which allowed for a 13-plug serial injection of six samples for each extraction group, separated by a blank extract and analysed in a single run. The absence of a peak in the blank position ensures there was no sample contamination or carry-over. Metabolite responses were normalized to an internal standard (Cl-Tyr) and the dried stool weight (15 mg modified B-D, 5 mg MeOH/water) for each sample. Although both methods were able to extract a broad range of metabolite classes (i.e., SCFAs, amino acids, bile acids, sugars, etc.) the methanol-water solvent mixture had better extraction efficiency as represented by the greater overall metabolite abundance (mean difference of 66.1%) based on Bland Altman % difference plot of a targeted list of stool metabolites (n = 55).

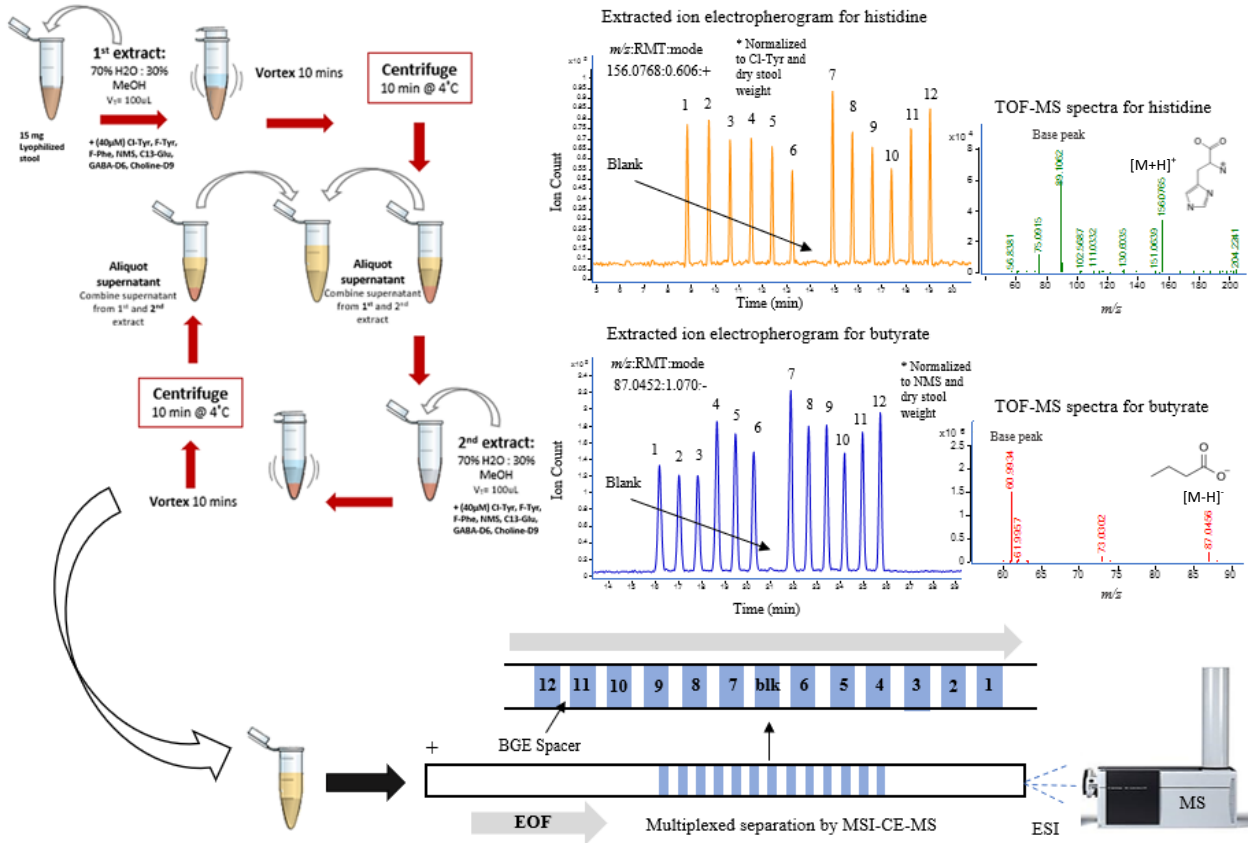


**Figure 2.2** Lyophilized stool extraction protocol and high-throughput metabolite profiling platform by MSI-CE-MS using a 13 serial sample injection with representative extracted ion chromatograms for histidine (+ mode) and butyrate (- mode).

### 2.3.2 Lyophilization versus wet extraction to ensure high stool metabolite recovery

After removal of low precision (CV > 40%) and infrequently detected (< 75%) metabolites in MSI-CE-MS, there was a total of 102 stool metabolites identified in the lyophilized group, and 96 metabolites identified in the wet extract group, with no metabolites being uniquely present in the non-lyophilized wet extract group. Additionally, metabolites detected exclusively in the lyophilized extracts were all unknowns identified during untargeted analysis. After normalization of stool RPAs to dried weight and internal standards, our data showed great technical precision (CV = 10.3%) and reduced biological variation for lyophilized samples with a CV of 7.4% compared to the non lyophilized samples which had a CV of 10.0%. Furthermore, looking at the

quantitative recoveries as per the method comparison with Bland-Altman regression, there was an overall mean bias (28.5%) for the lyophilized treatment group, with several amino acids (i.e., citrulline, alanine, methionine, lysine, etc.) and tentatively identified unknowns showing elevated abundances in the lyophilized sample group (**Supplemental Figure S2.5**).

Although lyophilization allowed for more consistent comparison of stool metabolite levels independent of hydration and stool form (Hsu et al., 2019; Lemay et al., 2021), we next examined whether there was any loss in volatile low molecular weight classes of metabolites, such as SCFAs following lyophilization as compared to wet stool extractions without lyophilization. Comparison of SCFAs between lyophilized and non-lyophilized treatment groups was performed on *log* transformed data using students t-test, and the abundances for both treatment arms were highly comparable ($p > 0.05$) as shown in **Figure 2.3**.

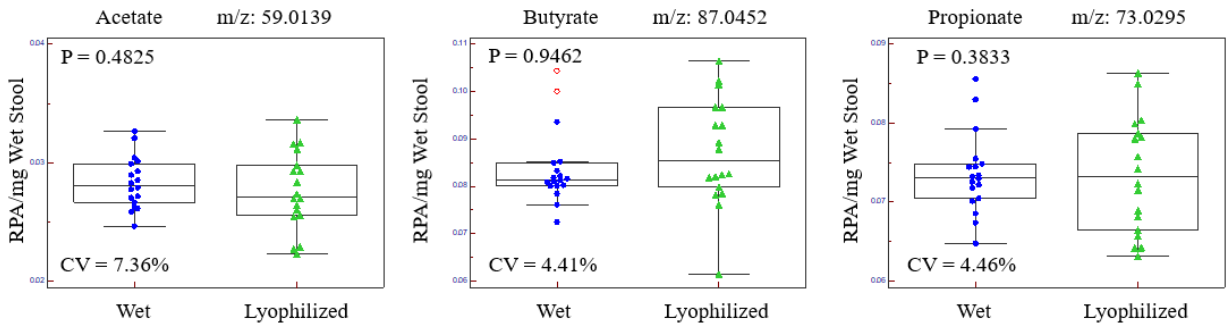


**Figure 2.3** Box plots showing the abundance of key VOCs such as , acetate, butyrate, and propionate with (lyophilized) and without (wet) lyophilization pre-treatment with RPAs normalized to wet weight of the stool after normalization to Cl-Tyr IS.

### 2.3.3 Comparison of stool storage form for optimal long-term chemical stability

Next, a systematic study of different sample preparation methods, storage durations and repeat freeze-thaw cycling was performed on stool samples in order to identify potential sources of variation and bias that may alter metabolite recovery. As all samples were derived from the

same homogenized stool sample, differences due to biological variation between participants were minimized, while additionally using the same optimized extraction protocol based on methanol:water (30:70) as previously described.

We first performed a comparison of three stool preparation methods for analysis of representative metabolite classes relevant to host and microbial interactions (*e.g.*, SCFAs, bile acids, biogenic amines etc.) that were re-analyzed over a period of 21 weeks while frozen at – 80 °C. A total of 52 stool metabolites (38 cations, 14 anions) were reliably (CV < 40%) detected in all treatment groups with an overall median technical CV of 6.9% after QC-based batch correction. Crude samples analysed at 48 h acted as the control for the samples stored in both crude and wet extract forms as it is most similar to a fresh minimally processed stool sample and will best represent the original crude stool composition. As lyophilization alters metabolite profiles, likely due to changes in extraction efficiency and cell lysis as reported in literature and as displayed by our own comparative study previously detailed, the freeze dried stool extracts analyzed at 48 h will act as the control for the samples the underwent lyophilization prior to storage (Moosmang et al., 2019).

ANOVA comparison of stool metabolite profiles for the 48 h controls (crude and lyophilized controls) as compared to the metabolite profiles after 21 weeks of storage for samples that had undergone one FT cycle, showed good chemical stability for crude and lyophilized samples. This was reflected by only 40% of crude and 46% of lyophilized metabolites being significantly altered after 21 weeks of storage, compared to the wet extract samples, where 62% of metabolites were significantly altered over the same duration. Comparison of the chemical stability for all three stool forms (crude, lyophilized, wet extract) up to 21 weeks of storage is shown in **Figure 2.4** for several metabolites from biochemically relevant metabolite classes

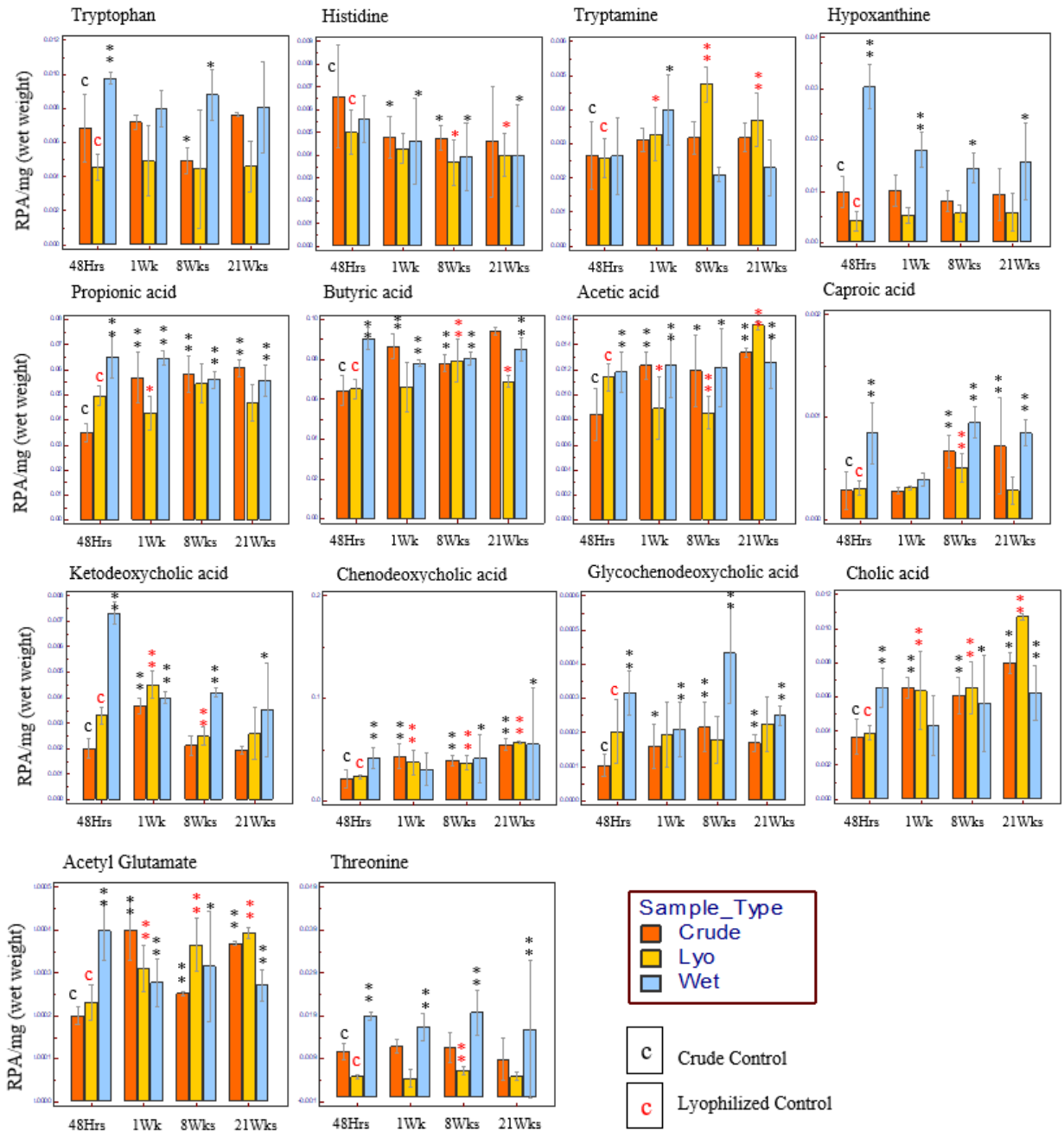


**Figure 2.4** Bar graph plots representing the chemical stability of select key stool metabolites after long-term storage up to 21 weeks at -80˚C. the "c" represents the control (crude untreated stool at 48-hour baseline or lyophilized stool at 48-hour baseline). Samples depicted in these plots have undergone one FT cycle. Significant differences between the control and each condition indicated by * ($p < 0.05$) or ** ($p < 0.01$) after students t-test analysis.

associated with microbiome activity. For example, this analysis showed that some bile acids (e.g., lithocholic acid, ketodeoxycholic acid and sulfolithocholic acid) were substantially reduced ($p <$

0.05) across all storage forms, whereas some amino acids (e.g., histidine, glutamine, serine) were reduced in the wet and crude storage forms after just one week as depicted in **Figure 2.5**.

Freeze thaw (FT) cycling by thawing samples on ice for one hour and freezing at -80 ˚C for an hour up to three times before storage, had negligible effects ($p > 0.05$) on sample stability for crude and lyophilized samples after multivariate analysis of the effects of FT cycling and storage duration on stool composition, with a Wilks' Lambda significance value of 0.296 and 0.300 for the effect of FT cycling on crude and lyophilized samples respectively. Wet extract samples did show marginally significant differences to metabolite profiles with respect to the number of FT cycles with a Wilks' Lambda significance of 0.038, indicating differences to the stool composition based on FT cycles regardless of storage duration. The effects of FT cycling are shown in **Figure 2.6** for a set of metabolites extracted from the lyophilized samples. The effects of FT cycling on crude samples after storage up to 21 weeks can be seen in **Supplemental Figure S2.7**. Compared to FT cycling, storage duration had a much larger effect on the composition of stool samples, with Wilks' Lambda significance values below 0.05 for all stool forms regardless of the number of FT cycles.

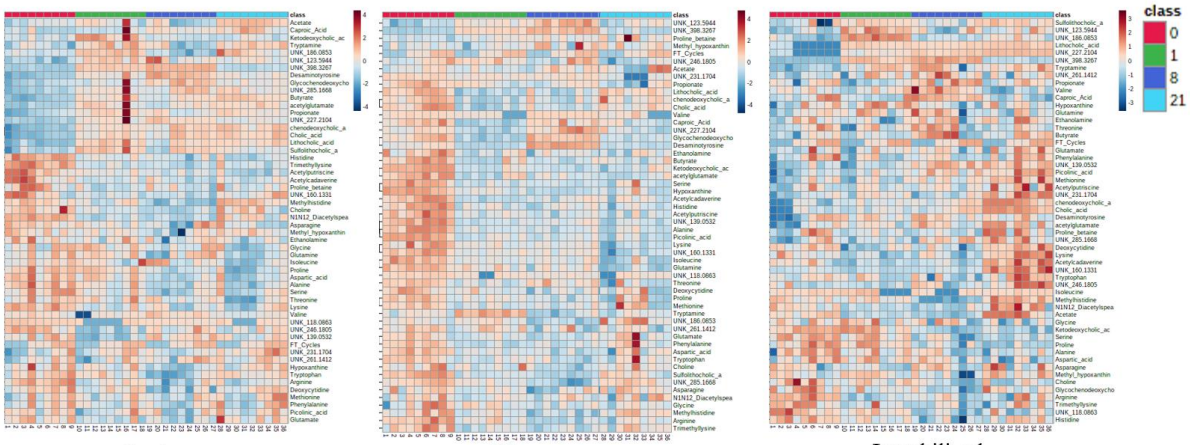


**Figure 2.5** 2D heatmap reflecting changes in stool metabolome abundances for 52 metabolites over 1, 8, and 21 weeks of storage at –80 °C from baseline (48 h) for samples stored in crude, wet extract and lyophilized forms.

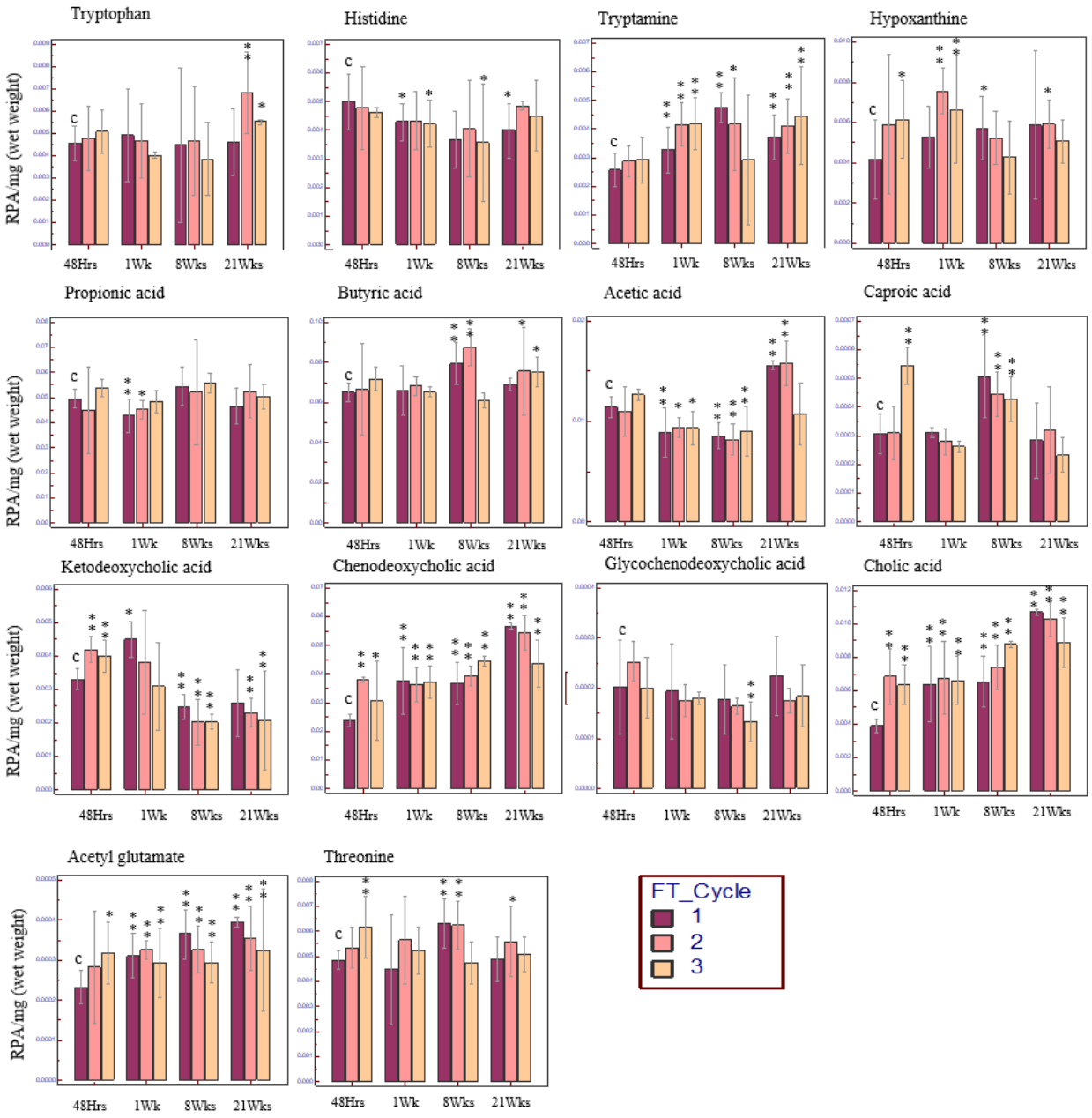


**Figure 2.6** Bar graph plots representing the chemical stability of select stool metabolites extracted from stool and analysed using MSI-CE-MS after lyophilization and 1, 2, or 3 Freeze-thaw cycles after storage at -80˚C for a duration of up to 21 weeks.

### 2.3.4 Differentiating stool metabolites associated with IBS from non-IBS

During sample selection IBS subgroups were age, sex and BMI matched as summarized in **Table 2.1,** to avoid the need for covariable adjustments during statistical analysis. A total of 136

metabolites were initially included in our data matrix and displayed adequate precision (CV < 40%) and detection frequency (> 75%). When the frequency of detection cut-off of 75% was removed in order to identify biomarkers that may be subgroup specific, unknown *m/z* 264.1966 and unknown *m/z* 344.1041 were reintroduced to the study data matrix. IBS samples (n = 60) from all IBS subtypes and symptom states were collectively compared against the non-IBS samples (n = 20). Fecal metabolomes for IBS patients and non-IBS were compared using Mann-Whitney U-test and results for top ranking biomarker candidates ($p < 0.05$) are summarized in **Table 2.2**. Additionally, we compared non-IBS samples to symptomatic IBS with a Man-Whitney U-test, as well as to IBS-C and IBS-D samples separately using a Kruskal Wallis test to identify any additional representative markers of IBS that may be subgroups specific and not apparent due

**Table 2.1** Summary of IBS pilot study cohort patients with clinical measurements for BMI, anxiety and depression as per Hospital Anxiety and Depression Score (HADS), inflammatory markers defensin and calprotectin, along with average Bristol Stool Scores (BSS) and symptom severity as per the Birmingham symptom severity scale for the 25 weeks of sample collection with associated standard errors.

| Parameter | Healthy Controls (samples; n = 20) (patient; n = 10) | IBS-D (samples; n = 40) (patients; n = 10) | | IBS-C (samples; n = 20) (patients; n = 5) | | Total (samples; n = 80) (patients; n =25) |
|---|---|---|---|---|---|---|
| | | Asymptomatic (samples; n = 20) | Symptomatic (samples; n = 20) | Asymptomatic (samples; n = 10) | Symptomatic (samples; n = 10) | |
| **Age – years, mean ± SD** | 39 ± 13 | 47 ± 15 | | 51 ± 8 | | 44 ± 14 |
| **BMI – kg/m$^2$, mean ± SD** | 23.3 ± 3.1 | 29.2 ± 6.2 | | 26.4 ± 2.6 | | 26.3 ± 5.1 |
| **BMI – no./total no. (%)** | | | | | | |
| <18.5 kg/m$^2$ | 0/10 (0%) | 0/10 (0%) | | 0/5 (0%) | | 0/25 (0%) |
| 18.5 – 24.9 kg/m$^2$ | 6/10 (60%) | 3/10 (30%) | | 2/5 (40%) | | 11/25 (44%) |
| >25.0 kg/m$^2$ | 3/10 (30%) | 6/10 (60%) | | 3/5 (60%) | | 12/25 ( 48%) |
| Not reported | 1/10 (10%) | 1/10 (10%) | | 0/5 (0%) | | 2/25 (8%) |
| **Sex – no./total no. (%)** | | | | | | |
| Female | 5/10 (50%) | 4/10 (40%) | | 4/5 (80%) | | 13/25 (52%) |
| Male | 5/10 (50%) | 6/10 (60%) | | 1/5 (20%) | | 12/25 (48%) |
| Anxiety | 2.4 ± 3.0 | 2.7 ± 3.0 | 3.5 ± 3.4 | 7.9 ± 2.2 | 6.9 ± 3.2 | 3.7 ± 3.3 |
| Depression | 1.8 ± 1.8 | 2.8 ± 2.3 | 4.4 ± 2.9 | 7.4 ± 3.8 | 6.9 ± 3.7 | 3.8 ± 3.6 |
| Bristol | 3.8 ± 0.6 | 3.9 ± 0.7 | 5.1 ± 1.9 | 4.1 ± 0.7 | 3.3 ± 2.6 | 4.1 ± 1.6 |
| Severity | 0.3 ± 0.6 | 6.1 ± 5.3 | 17.9 ± 6.0 | 7.9 ± 6.9 | 25.0 ± 11.6 | 10.2 ± 10.5 |
| Calprotectin | 37.8 ± 38.3 | 57.9 ± 58.2 | 47.0 ± 60.4 | 13.0 ± 11.2 | 48.3 ± 43.6 | 47.1 ± 56.8 |
| Defensin | 24.9 ± 22.5 | 34.6 ± 33.7 | 60.0 ± 68.7 | 55.4 ± 103.5 | 49.2 ± 84.4 | 42.8 ± 65.5 |

**Table 2.2** Top ranking stool metabolite candidates identified by MSI-CE-MS for differentiating IBS (n = 60) and non-IBS controls (n = 20) from extracted lyophilized stool samples normalized to dried mass

| ***m/z*:RMT:mode** | **Chemical ID[1] (mass error)** | ***p*-value[2]** | **Median FC[3]** | **Effect size[4]** |
|---|---|---|---|---|
| 407.2803:0.668:n | Cholic acid | $5.96 \times 10^{-4}$ | 1.83 | 0.38 |
| 159.1496:0.771:p | $C_8H_{18}N_2O$ (2.6 ppm) | $6.19 \times 10^{-4}$ | 5.50 | 0.38 |
| 156.0768:0.596:p | Histidine | $7.61 \times 10^{-4}$ | 2.30 | 0.38 |
| 139.0493:0.706:p | $C_4H_4N_5O$ (3.2 ppm) | $2.51 \times 10^{-3}$ | 0.63 | 0.34 |
| 155.0092:1.194:n | Orotic acid | $3.21 \times 10^{-3}$ | 0.63 | 0.33 |
| 227.1990:0.745:n | $C_{10}H_{24}N_6$ (0.1 ppm) | $5.25 \times 10^{-3}$ | 1.19 | 0.31 |
| 115.0821:0.945:n | Caproic acid | 0.0109 | 0.38 | 0.28 |
| 161.1073:0.790:p | Tryptamine | 0.0132 | 1.52 | 0.28 |
| 137.5999:0.590:p | $C_{11}H_{25}N_6S$ (3.3 ppm) | 0.0146 | 2.44 | 0.27 |
| 232.1411:0.533:p | $C_{10}H_{19}N_2O_4$ (2.9 ppm) | 0.0152 | 1.89 | 0.27 |
| 142.5919:0.444:p | Unknown* | 0.0174 | 1.52 | 0.27 |
| 188.0558:1.455:n | *N*-Acetyl glutamate | 0.0208 | 0.67 | 0.26 |
| 73.0295:1.188:n | Propionic acid | 0.0255 | 1.74 | 0.25 |
| 116.5755:0.533:p | $C_{13}H_{17}N_3O$ (3.0 ppm) | 0.0293 | 2.36 | 0.24 |
| 186.0860:0.938:p | $C_5H_9N_6O_2$ (0.1 ppm) | 0.0302 | 0.52 | 0.24 |
| 405.2646:0.674:n | Ketodeoxycholic acid | 0.0362 | 1.34 | 0.23 |
| 391.2865:0.678:n | Chenodeoxycholic acid | 0.0443 | 1.66 | 0.22 |

[1]*Unknown stool metabolites were characterized by their accurate mass, relative migration time. and their most likely molecular formula, whereas other metabolites were identified by spiking with authentic chemical standard.* *No suitable molecular formula*
[2] *Differentiating stool metabolites were classified when using a Mann-Whitney U-test ($p < 0.05$).*
[3]*Median fold change calculated as a ratio of ion response for stool metabolites normalized to dry weight as IBS/HC*
[4]*Effect size calculated as Mann-Whitney U-test test statistic Z/sq rt of sample size N.*

to data skewing that may occur when subgroups are analysed together. There were clear differences between IBS patients and healthy controls when comparing the levels of certain bile acids and short chain fatty acids. IBS patients showed significantly ($p < 0.05$) higher levels of bile acids such as cholic acid (CA), ketodeoxycholic acid (KDCA) and chenodeoxycholic acid (CDCA). In contrast, fecal SCFAs showed opposing trends, with propionic acid being more abundant, whereas caproic acid was significantly reduced in the IBS stool extracts as compared to controls. Other stool derived metabolites, such as orotic acid and *N*-acetyl glutamate were also notably reduced in patients with IBS and displayed a median fold change of 0.63 and 0.67

respectively. Several unknown metabolites with indeterminate chemical structures after searching in public databases were nonetheless detected consistently in most lyophilized stool specimens (> 75%) require structural elucidation by MS/MS with collision-induced dissociation. When the non-IBS participants were compared to IBS-D and IBS-C sub-groups separately to remove potential confounding from differences in underlying disease phenotype, only guanosine was newly identified as being a significant ($p$ = 0.0172) marker for differentiating between healthy controls and IBS-D as depicted in **Figure 2.7**. This was initially unclear due to the reduction of guanosine in the IBS-C group, making it appear as though guanosine levels were comparable between IBS and non-IBS patients. When the diagnostic capabilities of the key metabolites were assessed using receiver operating characteristic (ROC) curves, cholic acid was found to be the most significant singular feature with and AUC = 0.758 and a $p$ = 0.0020, while a ratio of cationic unknown ions, *m/z* 159.1496 and *m/z* 139.0493 was the most definitive ratiometric marker with and AUC = 0.848 and a $p < 0.001$. Based on a Spearman rank correlation analysis, the unknown cation, *m/z* 159.1496 was moderately correlated with SCFAs propionate ($r$ = 0.40) and butyrate ($r$ = 0.36). Additionally, this unknown ion also displayed the strongest positive correlation to symptom severity ($r$ = 0.41) with respect to all top ranked metabolites, whereas unknown ion *m/z* 139.0493 only showed modest correlation ($r$ = 0.37) to *N*-acetyl glutamate with no strong association to any psychological or symptom based clinical measurements. When examining combination of stool metabolite ratios using only known compounds, the ratio of histidine/orotic acid, also showed good predictive accuracy to differentiate IBS patients from non-IBS controls with an AUC = 0.802 and $p < 0.001$ as shown in **Figure 2.7.**

Unknown IBS stool metabolites characterized by non-targeted MSI-CE-MS approaches and authenticated by our multi-tiered exclusion criteria and temporal signal pattern recognition

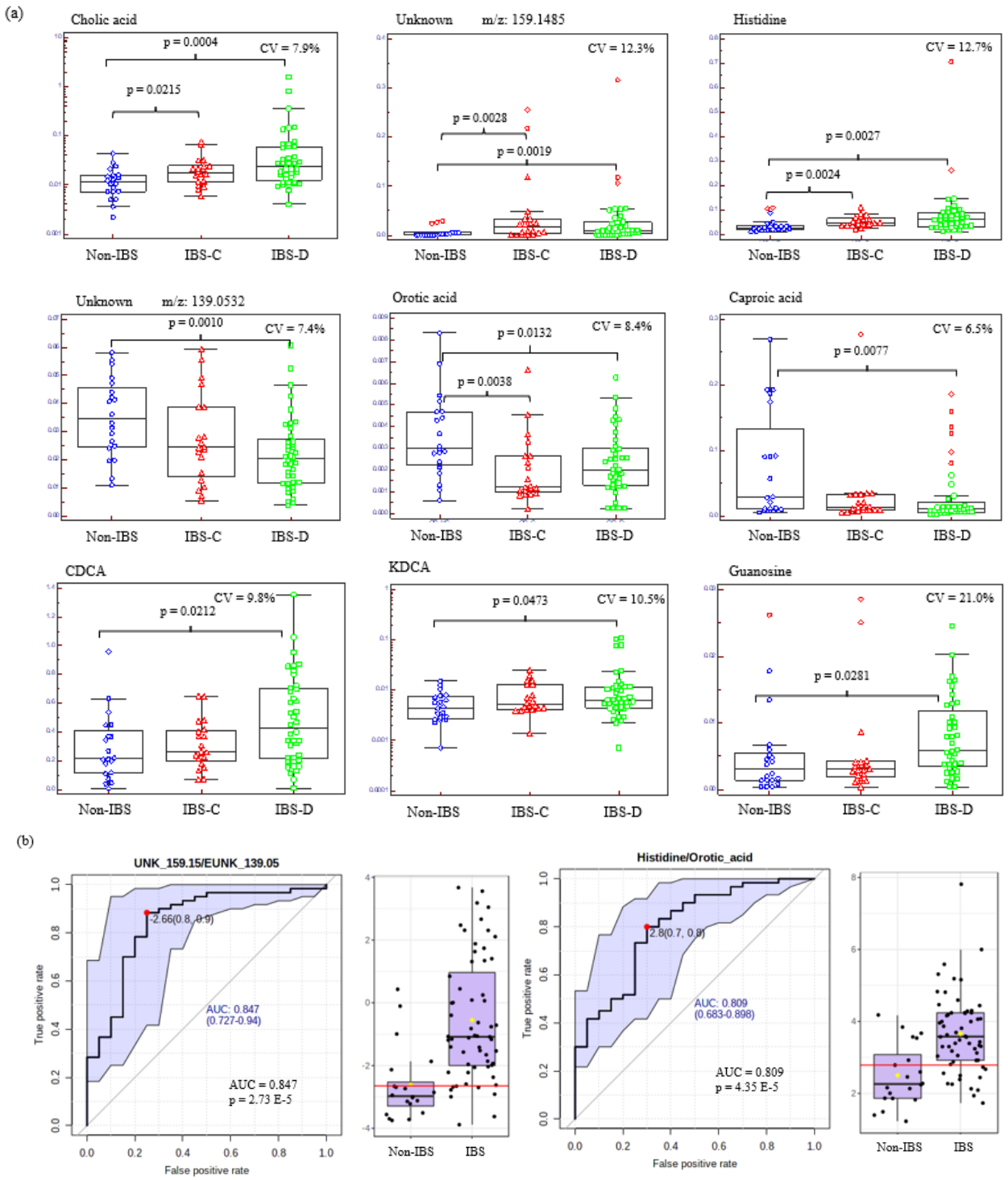


**Figure 2.7** (a) Box-whisker plots of stool metabolites differentially excreted in IBS patient subgroups as compared to non-IBS controls when using Mann-Whitney U-test ($p < 0.05$). (b) Receiver-operating characteristic (ROC) curves for the top-ranking ratiometric (known/unknown) stool metabolites associated with IBS with a 95% confidence interval.

that showed significant between group differentiation were assigned most likely chemical formulas based on accurate mass (*m/z*) are shown in **Table 2.2**. Representative extracted ion electropherograms (EIE) and high-resolution time-of-flight (TOF) MS spectra for key unknowns are depicted in **Supplemental Figure S2.8**. Most probable chemical formulas were searched in the HMBD database to formulate a list of potential metabolite candidates, as was done with the unknown, singly charged $[M+H]^+$ as *m/z* 159.1496 ($C_8H_{18}N_2O$) which matched with several plausible compounds based on chemical formulas and likely electrophoretic mobility, such as *N*-nitrosodibutylamine, monopropionylcadaverine or *N*-acetyl-1,6-diaminohexane. Lead candidate metabolites having likely chemical structure identities consistent with a targeted stool metabolite in-house database, were further validated by spiking a pooled stool sample with authentic

standards to confirm their co-migration.

### 2.3.5 Stool metabolites for differentiation of IBS-D from IBS-C

Comparison of stool metabolic markers of IBS-D and IBS-C was performed in the same way as the comparison between non-IBS controls and IBS patients in section 2.3.4. Univariate analysis results from the Mann-Whitney U-test are summarized in **Table 2.3**. Of the metabolites with verified identities, histamine ($p$ = 0.0352) and guanosine ($p$ = 0.0144) were the only metabolites found to be differentially abundant between the IBS-D and IBS-C subgroups (**Figure 2.7**). With inclusion of unknown metabolites characterized through non-targeted analysis, 13 other differential markers (Mann-Whitney U-test $p < 0.05$) were identified and are listed in **Table 2.3**. Due to subgroup specificity, unknown ions, *m/z* 264.1966 and *m/z* 344.1041 were reintroduced to the study data matrix as described previously. The frequency of detection for these two features were 20% and 25% in the IBS-C group respectively and were nearly exclusively detected in the IBS-C stool extracts. When ROC curves were used to evaluate the metabolites and metabolite

**Table 2.3** Top ranking stool metabolite candidates identified by MSI-CE-MS for differentiating between IBS-D (n = 40) and IBS-C (n = 20) from extracted lyophilized stool samples normalized to dried mass

| ***m/z*:RMT:mode** | **Chemical ID[1] (mass error)** | **p-value[2]** | **Median FC[3]** | **Effect size[4]** | **Frequency of Detection[5]** |
|---|---|---|---|---|---|
| 344.1041:0.705:p | $C_{14}H_{19}N_2O_6S$ (1.3 ppm) | $1.12 \times 10^{-4}$ | N/A* | 0.50 | 7.5% |
| 137.5999:0.590:p | $C_{11}H_{25}N_6S$ (3.3 ppm) | $4.14 \times 10^{-4}$ | 3.12 | 0.46 | 88% |
| 264.1966:0.900:p | $C_{16}H_{25}NO_2$ (3.0 ppm) | $1.07 \times 10^{-3}$ | NA* | 0.43 | 5% |
| 123.5964:0.577:p | $C_{14}H_{21}N_4$ (6.5 ppm) | $2.01 \times 10^{-3}$ | 2.64 | 0.40 | 98% |
| 142.5919:0.444:p | Unknown* | $4.53 \times 10^{-3}$ | 1.96 | 0.37 | 91% |
| 102.5722:0.517:p | $C_{11}H_{15}N_4$ (0.9 ppm) | $7.68 \times 10^{-3}$ | 1.68 | 0.34 | 98% |
| 116.5755:0.533:p | $C_{13}H_{17}N_3O$ (2.2 ppm) | 0.0132 | 3.29 | 0.32 | 90% |
| 138.5839:0.606:p | $C_9H_{21}N_7OS$ (1.5 ppm) | 0.0138 | 2.42 | 0.32 | 100% |
| 284.0989:1.152:p | Guanosine | 0.0144 | 1.94 | 0.32 | 93% |
| 285.1730:0.712:n | $C_{18}H_{24}NO_2$ (1.5 ppm) | 0.0305 | 1.53 | 0.28 | 85% |
| 152.5865:0.619:p | $C_{16}H_{21}N_3O_3$ (0.5 ppm)/ Valyl tryptophan | 0.0323 | 1.94 | 0.27 | 98% |
| 112.0875:0.302:p | Histamine | 0.0352 | 3.51 | 0.27 | 73%** |
| 202.1923:0.505:p | $C_{12}H_{25}O_2$ (2.1 ppm) | 0.0446 | 2.01 | 0.26 | 79% |
| 188.1758:0.484:p | $C_9H_{21}N_3O$ (0.3 ppm) | 0.0464 | 2.31 | 0.26 | 100% |
| 136.5919:0.566:p | $C_{16}H_{21}N_3O$ (2.9 ppm) | 0.0509 | 1.93 | 0.25 | 76% |

*[1] Unknown stool metabolites were characterized by their accurate mass, relative migration time. and their most likely molecular formula, whereas other metabolites were identified by spiking with authentic chemical standard. *no suitable formula could be assigned*

*[2] Differentiating stool metabolites were classified when using a Mann-Whitney U-test ($p < 0.05$).*

*[3] Median FC calculated as ratio of ion response for stool metabolite normalized to dried weight in IBSD/IBSC. *Due to low frequency of detection (<50%) in either subgroup the median values were the same for both groups and a representative median FC cannot be calculated*

*[4]Effect size calculated as Mann-Whitney U-test test statistic Z/sq rt of sample size N*

*[5]Metabolites with frequency of detection < 75% were kept if low detection frequency is due to subgroup specificity of the metabolite*

ratios of the known compounds, guanosine was the most significant with a satisfactory AUC = 0.695. When including unknown ions, *m/z* 137.5999 became the single most significant biomarker for differentiation between IBS sub-types with an AUC = 0.793. A combination of unknown ions, namely *m/z* 137.5999 and *m/z* 344.1041 also showed potential for discriminating between IBS subtypes (AUC = 0.874), but was limited by the low frequency of detection (7.5%) reducing its overall applicability.

### 2.3.6 Stool metabolic responses associated with IBS symptom flair ups

Sample data from IBS-C and IBS-D subgroups were combined and analyzed based on symptom state to assess markers indicative of elevated symptom severity. A Mann-Whitney U-test was performed and found that no metabolites were differentially abundant based on symptomology when IBS subgroups were pooled together. This was a result of subgroups having opposing trends for certain metabolites during symptom flare-ups. An example of this is shown when looking at glycochenodeoxycholic acid (GCDCA), which is reduced in IBS-D patients, but elevated in the IBS-C patients during symptom flair up as depicted in **Figure 2.8**. Calprotectin and defensin were notably elevated ($p < 0.05$) in the symptomatic IBS-C group. Whereas only defensin was elevated ($p < 0.05$) in the symptomatic IBS-D group as shown in **Figure 2.8**. We were able to identify 4 differentially excreted ($p < 0.05$) stool metabolites when comparing asymptomatic and

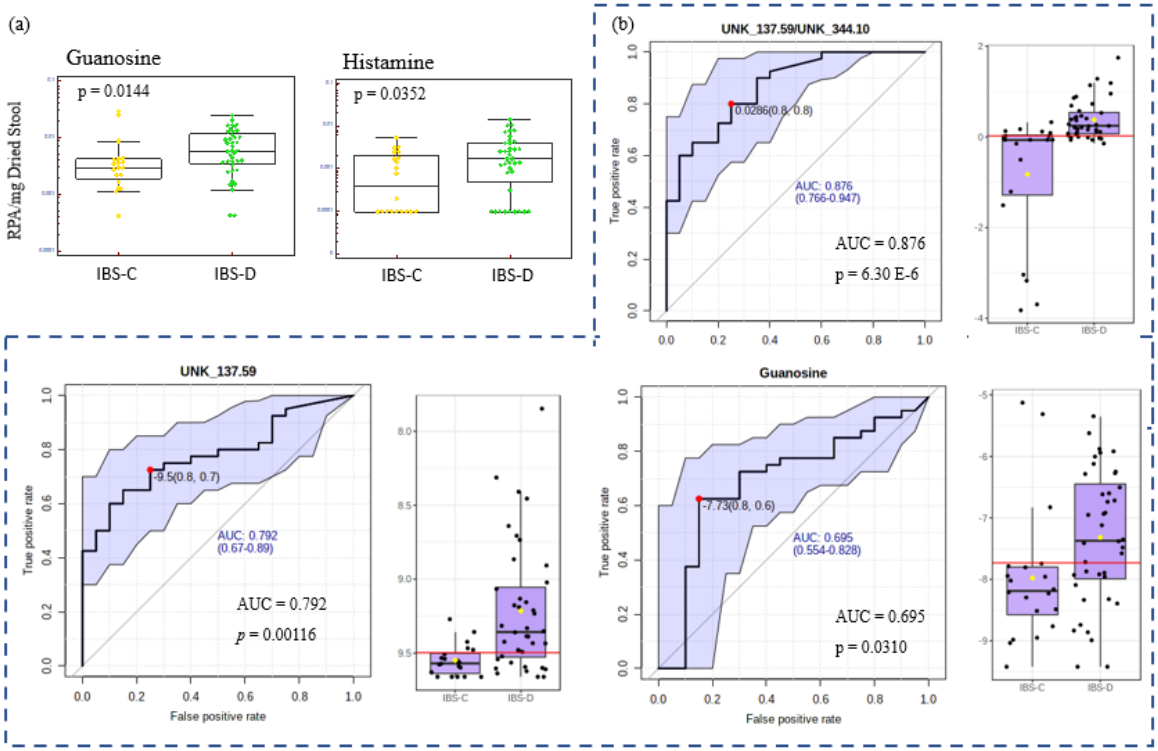


**Figure 2.8** (a) Box-whisker plots of stool metabolites differentially excreted by IBS-D and IBS-C patient subtypes when using Mann-Whitney U-test ($p < 0.05$). (b) receiver operating characteristic (ROC) curves for the top ranking singular and ratiometric (known/unknown) stool metabolites differentially excreted in IBS-D and IBS-C subtypes with a 95% confidence interval.

symptomatic IBS patient samples. Of these stool derived metabolites, two compounds (i.e., ornithine, $p$ = 0.0242 and GCDCA, $p$ = 0.0265) were associated with differentiating IBS-D symptom states and two other compounds (i.e., threonine, $p$ = 0.0413 and cystine, $p$ = 0.0021) were able to differentiate IBS-C symptom states (**Figure 2.9**). However, partial least squares discriminant analysis (PLS-DA) modeling showed moderate discrimination, likely in part due to the low sample numbers. Subsequent analysis of the full cohort of IBS samples available from the original study will likely increase the number of differentially abundant metabolites between symptom groups (Ruiz-Perez et al., 2020).

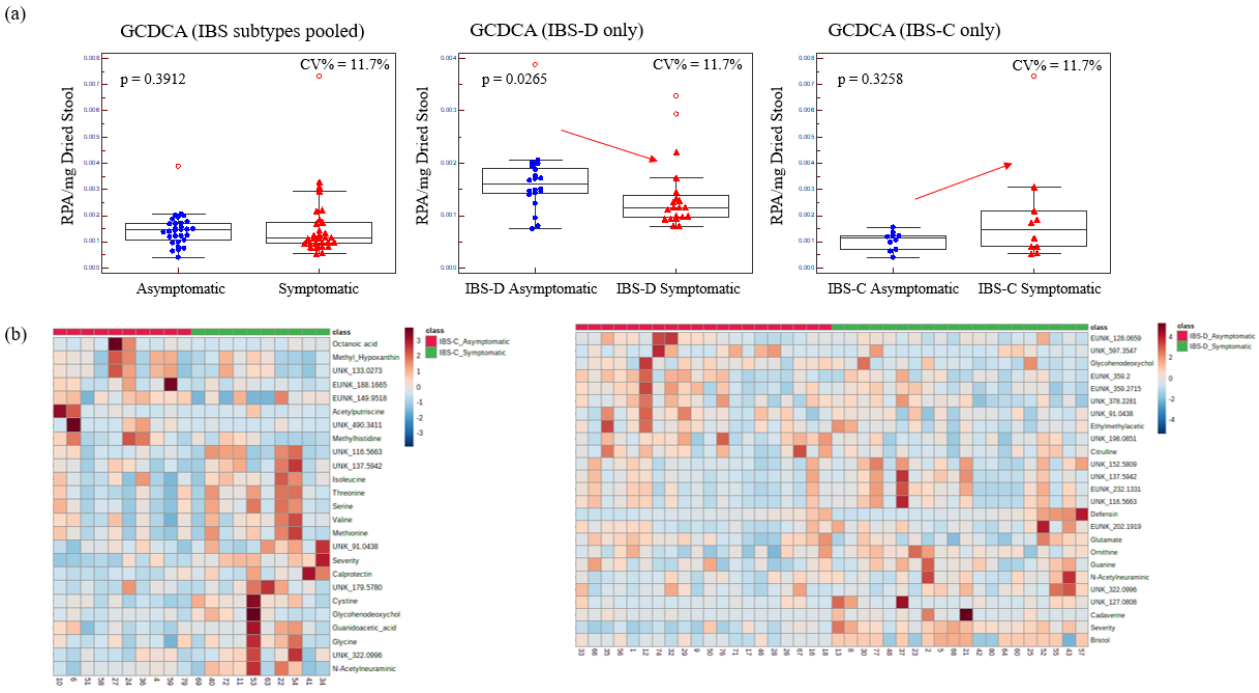


**Figure 2.9** (a) Box-whisker plots displaying changes in metabolite abundances for GCDCA based on symptom state and IBS subgroup. (b) Heatmaps for IBS-C (left) and IBS-D (right) metabolite abundances of the top-ranked markers for classifying patients by symptom state and highlighting differences in metabolic signatures for IBS subtypes during elevated symptom severity.

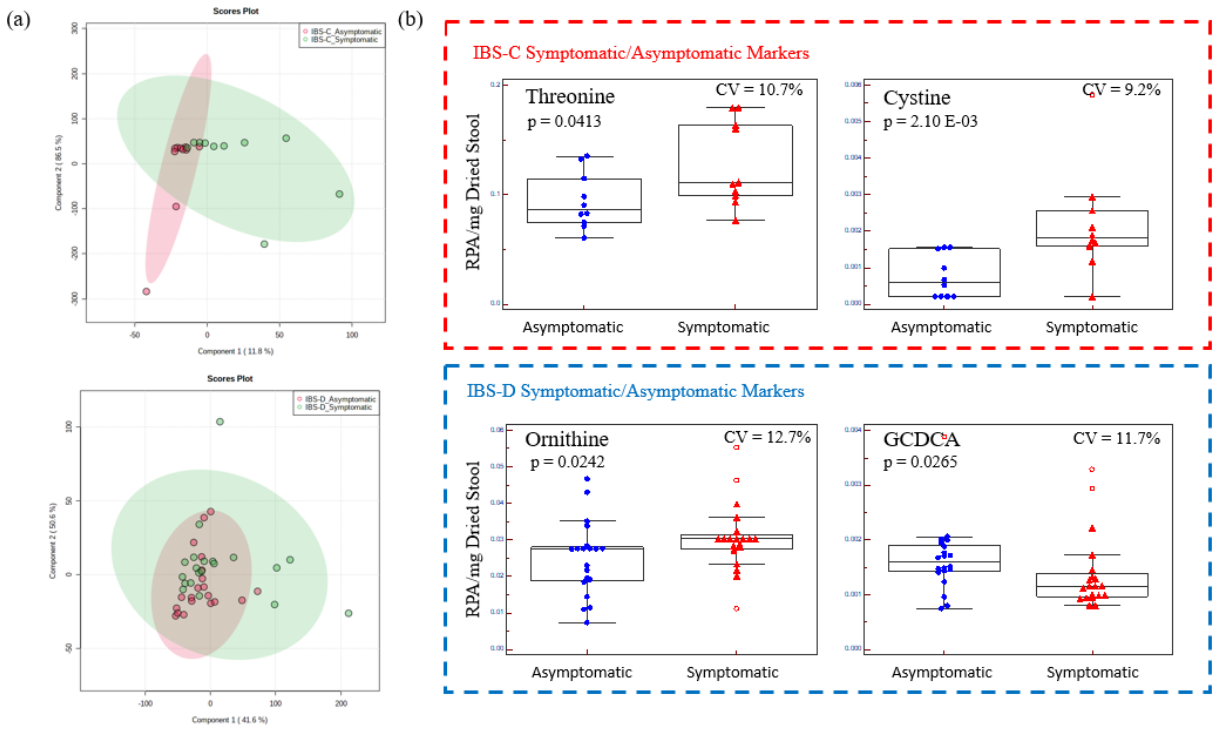


**Figure 2.10** (a) Partial least square discriminant analysis (PLS-DA) for IBS-C and IBS-D stool extracts modeling differences between symptom states. (b) Box-whisker plots for all symptomatically differential metabolites in IBS-C and IBS-D stool extracts.

## 2.4 Discussion

Stool metabolomics is an underdeveloped branch of human metabolomics research that holds abundant potential for metabolite profiling of gastrointestinal diseases. By expanding efforts to standardize all stages of stool sample processing, it can become an invaluable biospecimen for the diagnosis of chronic diseases of unknown or poorly understood aetiologies, including IBS. Stool metabolomics offers a complimentary platform for studies that use functional genomics to characterize gut microbial composition, and often rely on predictive modeling to measure microbial metabolic activity (Hsu et al., 2019; Zimmermann et al., 2021). Several studies in this thesis were performed to address deficiencies in literature pertaining to the number of comparative stool metabolomic studies focused on optimization of stool sample handling and storage protocols.

By performing these studies using the MSI-CE-MS platform were able to rapidly analyze stool samples through both targeted and untargeted approaches due to the uniqueness of the serial injection format and temporal signal pattern recognition, which can be easily integrated into our sample runs. From the first study comparing extraction protocols, we demonstrated that a methanol/water solvent system with repeat (combining two extracts) extractions is able to effectively extract a wide variety of metabolite classes with a 66.1% increase in overall metabolite abundance when compared to the modified B-D extraction. Current stool metabolomics studies often extract metabolites from the crude sample, but the variability in water content from fecal samples, especially from patients suffering from colonic water absorption issues, can limit the reproducibility of study outcomes (Hosseinkhani et al., 2021). It has also been suggested that the lyophilization process reduces or even removes VOCs from the sample, which are often key metabolic markers of disease and are important for metabolomic studies (Saric et al., 2008). To combat these issues, we assessed the use of lyophilization as a pre-treatment step in stool sample work-up and found that lyophilisation did not cause the loss of any metabolite classes, but instead increased the overall metabolite extraction abundance by 28.5% when compared to samples extracted from untreated crude stool samples using Bland Altman correlation analysis of 102 stool metabolites. By comparing the VOCs abundance directly with un lyophilized samples, we can validate the retention of VOCs during the lyophilization process. For instance, all fecal SCFAs that were detectable in non-lyophilized crude stool extracts, including acetate, butyrate, and propionate, were measurable without a reduction in their abundance following lyophilization despite their inherent volatility in free solution.

Upon inspection of the metabolite classes with increased abundance, several are known intracellular metabolites, including amino acids and glycolysis metabolites such as serine, alanine,

and citrulline, supporting the presumption that lyophilization induces cell lysis (Mielko et al., 2021; Tian et al., 2019). Based on our results which show a lower level of intracellular metabolites in the non-lyophilized group, it can be speculated that the solvent solution composition, which is only 30% methanol (70% water) as is required for instrumental compatibility, may not be adequate to disrupt the cell barrier for all bacterial types. In literature, pure methanol is commonly reported for induction of cell lysis and methods using methanol mixtures note additional steps for completing the cell lysis process (Sapcariu et al., 2014; Ser et al., 2015). The thawing of the frozen samples under vacuum during lyophilization is suspected to lyse the cell membrane and release intracellular components, reducing the need for addition cell lysis steps. The lack of oxygen and removal of water preserves the sample integrity during the process by limiting essential resources required for bacterial metabolism (Novak Babič et al., 2020). Additionally, lyophilization offers ease of handling, as powdered stool is easier to homogenize and weigh out more consistently, while offering the additional benefit of pre-concentrating the samples prior to extraction for increased detection of low abundance metabolites. Furthermore, stool extracts that underwent lyophilization prior to extraction showed lower overall biological variation (CV = 7.4%) when compared to non-lyophilized stool extracts (CV = 10.0%) despite coming from the same homogenized stool sample, signifying superior method reproducibility which could assist in harmonizing results across stool metabolomic studies. The results from these comparative studies provided the confidence needed to implement these methods on the IBS cohort samples and justified a more in-depth analysis of lyophilization potential in stool metabolomic research going forward. By limiting biological activity and reducing metabolite degradation, lyophilization prior to storage could provide researchers with a way to biobank stool samples for future analyses.

All stool forms (crude, wet, and lyophilized) showed adequate stability after storage at -80˚C for 21 weeks, but crude and lyophilized samples offered the most consistent metabolite profile when compared to metabolite abundances recorded at the 48-hour baseline measurements with only 40% and 46% of the metabolites (n = 52) showing significant alterations respectively. Stool samples stored as wet extracts for the study duration experienced significant alterations to metabolite abundances for 62% of the metabolites, indicating that without the addition of stabilizing solutions, extracted stool samples display a significantly different metabolite profile compared to crude samples. As shown in **Figure 2.6**, the low overall variability in metabolite concentrations across all weeks of the study for lyophilized samples demonstrated the potential for lyophilization to preserve samples long term after extended periods of storage. Of our main metabolites of interest based on our findings in the IBS pilot study that were also detectable in our stability study, cholic acid displayed the greatest difference (crude, $p < 0.001$) to baseline measurements across all stool forms. The majority of bile acids showed poor stability at 21 weeks of storage, with significantly altered metabolite concentrations ($p < 0.05$) for all stool forms, with the exception of certain bile acids from the samples that were lyophilized prior to storage and saw no significant changes ($p > 0.05$) to (KDCA and GCDCA levels even at 21 weeks. Other metabolite classes that showed significant alterations after long term storage included some of the SCFAs and amino acids which is consistent with reports in literature (de Spiegeleer et al., 2020; O'Sullivan et al., 2018). The exception was for propionate levels in lyophilized samples, which were consistent throughout the study.

The three-way comparison of metabolite stability depicted in **Figure 2.4** is representative of samples that have undergone one freeze thaw cycle. However, the addition of up to three FT cycles prior to storage had no apparent effect (Wilks Lambda $p > 0.3$) on metabolite concentrations

for crude or lyophilized samples. Freeze thaw cycling was performed by thawing samples (15mg) on ice for one hour, followed by freezing at -80˚C for one hour, repeated up to 3 times. This suggests that as long as freeze thaw cycles are short in duration and do not involve thawing/extended periods at room temperature, that the chemical stability of stool samples can be maintained for select stool storage forms. This information is important as it ensures that freeze thaw cycling that may be introduced during initial sample work-up will not compromise sample integrity for these sample types. Studies investigating the effects of freeze thaw cycling on stool, and especially how it effects different stool forms is very limited, and studies that have looked at the effects of FT cycling tend to have inconsistent FT cycling protocols (i.e., longer/shorts freeze and thawing times, thawing at room temperature vs thawing on ice, etc.) (de Spiegeleer et al., 2020; Gratton et al., 2016). It would be beneficial to also assess the effects of freeze thaw cycling that occurs after samples have already undergone storage for long periods of time, as longitudinal studies involving multiple collection timepoints may see accidental FT cycling introduced at intermediate phases of the study. Based on our results from our stability study and comparative study for extraction of stool metabolites from lyophilized and non-lyophilized samples, it is recommended to lyophilize samples prior to storage and/or analysis to preserve an accurate metabolite profile, while also extracting the greatest number and abundance of metabolites. Subsequently, as reported for tissue samples (Molnar et al., 2021) and as noted in our lyophilization pre-treatment comparison study, lyophilization appears to also reduce biological variation which could aid in the harmonization of stool metabolomic studies. General recommendation in literature are inconsistent, and most studies look at only one stool form, making it difficult to determine the best storage protocol due to differences in sample handling across studies. Stool samples obtained for our pilot IBS study were stored in crude form at –80 °C prior to metabolomic analysis, and

although lyophilization shows greater stability for certain metabolite classes such as the acids and SCFAs, crude samples demonstrated equivalent overall metabolite preservation. The results from these comparative studies provided the confidence needed to implement these methods on the IBS cohort samples and justified a more in-depth analysis of lyophilization potential in stool metabolomic research going forward.

The diagnosis of IBS is heavily reliant on patient symptom reporting and is lacking quantifiable markers that can produce a more definitive diagnosis (Saha, 2014). Efforts are being made to create a panel of diagnostic biomarkers, but there is still a sever deficiency in reliable and validated markers limiting the ability to introduce biomarker-based diagnosis strategies into clinical practice. Therefore, for our study we wanted to examine the stool metabolome of IBS patients, comparing their metabolic profiles to healthy controls. We found that bile acids (BA), including CA, KDCA and CDCA were consistently elevated (FC 1.34 - 1.83, $p < 0.05$), in stool from IBS patients. These primary and secondary bile acids play an important role in digestion and solubilization of fats, as well as acting as regulators for bile acid synthesis (Keitel et al., 2008; Camilleri & Vijayvargiya, 2020). The primary factors causing increased bile acid excretion in IBS patients is not well understood but could be related to elevated production of BAs or conversely limited reabsorption. An increase in the conversion of cholesterol to CA and CDCA may be responsible for the elevated levels seen in excreted stool and play a role in the increased colonic motility experienced by IBS-D patients (Zhao et al., 2019). Alternatively, issues with BA reabsorption can also explain the indicated elevations, although significantly elevated ($p = 0.0215$) CA levels seen in IBS-C patients, suggests that potential reabsorption issues may be multifactorial and not just due to increased bowel motility. Of the other significantly altered metabolites in IBS patients such as histidine, many of our findings are well matched to findings reported in literature

(Fukui et al., 2020; Mars et al., 2020b; Xiao et al., 2021). The elevation of histidine in the stool of IBS patients when compared to healthy controls may act as an indicator of increased inflammation and oxidative stress which matched reports of elevated urinary histidine levels in IBS patients (Yu et al., 2019). Not reported previously in literature to the best of our knowledge, was the discovery of reduced fecal abundances of orotic acid and N-acetyl glutamate (NAG) in association with IBS. N-acetyl glutamate ($p = 0.0209$) and orotic acid ($p = 0.0032$) levels were significantly reduced in the IBS extracts and share connecting metabolic pathways. Looking at ammonia breakdown and the urea cycle we can see how the two metabolites are closely associated with each other (**Figure 2.11**). The normal abundance of glutamate in IBS stool samples indicated that glutamate availability was not a contributing factor to the reduction of NAG, suggesting reduced levels or enzymatic activity of the *N*-acetyl glutamate synthase (NAGS) enzyme. NAG is a key metabolite in the detoxification of ammonia, which is produced by bacteria present in the gastrointestinal tract during the break down of proteins and amino acids. To further support the hypothesis that reduced NAG/NAGS is partially inhibiting ammonia conversion to carbamoyl phosphate we examined orotic acid levels, as it is a close by-product formed from carbamoyl phosphate metabolism. Orotic acid is also significantly reduced and highly correlated to NAG ($r > 0.60$) supporting a strong correlation to NAG reduction and reduced carbamoyl phosphate/carbamoyl phosphate by-products (**Supplemental Figure S2.9**). Other studies unrelated to IBS have also noted orotic acid levels being a good indicator of enzymatic CPS1 or NAGS deficiency when analysing urea cycle disorders (Mew et al., 2017). Additionally, we see reduction of other urea cycle metabolites such as ornithine, although not to the same degree, as many urea cycle metabolites are also exogenously sourced from our diets and do not solely rely on carbamoyl phosphate for synthesis. This connection between NAG and reduced detoxification and break down of ammonia may play a role

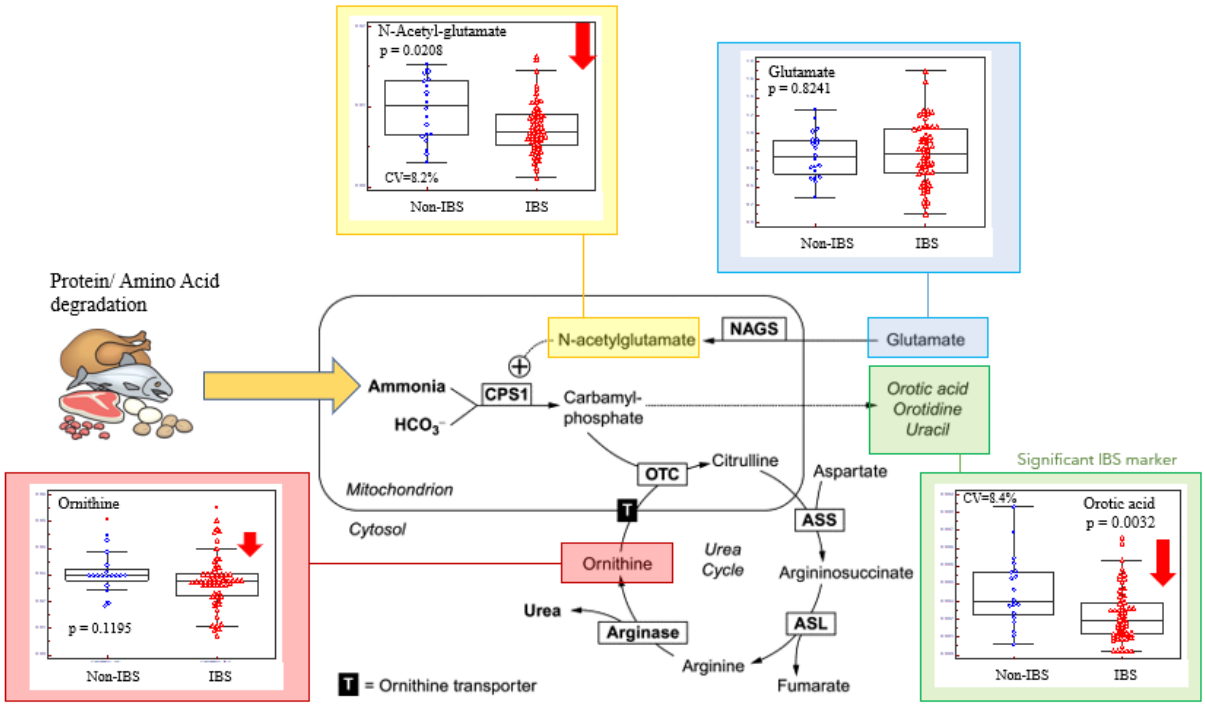


**Figure 2.11** Ammonia conversion to carbamoyl phosphate and the Urea Cycle and associated metabolites with representative box-whisker plots displaying metabolite level alterations in IBS patient samples.

in not only the pathophysiology of IBS, but also the associated symptoms. It is well understood that excess ammonia levels can lead to a wide range of undesirable conditions, with substantially elevated ammonia levels leading to hyperammonemia (Odigwe et al., 2015). Reduced urea cycle metabolites and excess ammonia levels can cause impaired colonic barrier function, stimulate the release of pro-inflammatory cytokines and cause general fatigue and headaches, which correlates with some current hypothesizes relating inflammatory cytokines with IBS pathophysiology and commonly reported symptoms of IBS (bin Abdulrahman et al., 2022; Carco et al., 2020; Gomborone et al., 1996).

Equally important to the initial IBS diagnosis is the ability to reliably differentiate between IBS-C and IBS-D subtypes. Current treatments for symptom relief are mostly IBS subtype specific, so access to objective biomarkers for classifying IBS subtypes, while also offering new insights into the distinct mechanisms of pathophysiology is needed (Wall et al., 2014). The large majority of stool derived metabolites differentiating IBS-D from IBS-C patients were identified during untargeted analysis and only have tentatively assigned molecular formulas based on their accurate mass (*m/z*) and will be further examined in the future to confidently assign the structural identities. Correlation analysis demonstrated that unknown ions *m/z* 227.1990 and *m/z* 159.1496 in addition to orotic acid, had the strongest correlation to symptom severity scores, with the unknown ions showing an increase in abundance in stool samples collected from IBS patients during high reported symptom severity, whereas fecal orotic acid has a negative correlation, with lower levels being associated with increased severity. Other unknown ions (*m/z* 137.5999, *m/z* 102.5722, *m/z* 123.5964, and *m/z* 116.5755) also showed significant correlation ($r > 0.45$) to propionate and butyrate, which may indicate some association to the metabolic pathways related to SCFAs. Alternatively, two metabolites with known chemical structures, namely histamine and guanosine were elevated in IBS-D patients. Histamine has been frequently reported in relation to IBS (Lee & Lee, 2016), however elevated guanosine has not been reported to date. Guanosine and cyclic guanosine monophosphate (cGMP) are connected by *de novo* synthesis pathways in the liver and elevated levels of guanosine may also be indicative of cGMP elevation as well. If there is a connection between these compounds, this may help to explain chronic diarrhoea in IBS-D patients, as diarrhoea has previously been linked to elevated levels of cGMP (Camilleri, 2012). Also notable is the introduction of the new drug linaclotide, which has been indicated for use with

IBS-C patients experiencing chronic constipation with cGMP being one of the main active ingredients used to reduce intestinal water absorption and relive constipation (Ahsan et al., 2017).

The pathophysiology of IBS is still not well understood due to its multifactorial nature which is influenced by both physical and psychological stressors. In order to increase our understanding of this complex disorder, our study also focused on more unexplored aspects of IBS, including alterations to metabolic signatures during active and dormant symptom states. Our comparison of changes to the stool metabolome phenotype with symptom flair-ups for IBS patients was unrevealing, but examining IBS-C and IBS-D patients separately revealed 4 stool metabolites of significance, with two metabolites associated with each subtype. This suggests that there are distinct metabolic signatures and mechanisms associated with increased symptom severity between the two IBS subgroups. In the IBS-D subgroup, ornithine and GCDCA showed altered abundances during periods of increased symptom severity. The bacterial metabolite, GCDCA was found in lower levels in stool extracts during symptom elevation and may be indicative of microbial dysbiosis in the gut. Gut composition alterations can have a substantial effect on the expression of certain transporters, especially organic anion transporting polypeptides such as OATP1B1 and OATP1B3 which have preferential uptake of glycine conjugated bile acids (Suga et al., 2017). If the glycine conjugated bile acid was more rapidly removed from the small intestine, it is reasonable that there would be lower levels in excreted stool. On the other hand, diets high in protein can worsen IBS-D symptoms, and ornithine elevation may simply be an indicator of elevated protein intake (Cozma-Petrut et al., 2017). Both threonine and cystine were found to be significantly ($p < 0.05$) elevated in IBS-C patients during symptom flair-ups. Threonine is an important component of mucin proteins which are responsible for maintaining mucus lining integrity to protect against pathogens. The reason for elevation of threonine in symptomatic IBS-

C is not apparent, but may reflect some form of inflammatory response (Rémond et al., 2009). Cystine on the other hand also presents questions as to what may be causing its elevation, as it uses the same intestinal amino acid transport as ornithine, which did not show any elevation in the symptomatic IBS-C group, decreasing the probability of reduced transporter activity. This suggests that the cause may be more so related to cystine's low solubility and the reduced stool water content during bouts of constipation (Carta, 1999).

Although our study size was modest and consequently did not allow for covariable adjustments, the sample cohort was carefully selected to reduce variability between subgroups. Due to the nature of the study, and the long storage time prior to analysis, although relative metabolite abundances can be used to identify key stool biomarkers, absolute metabolite concentrations would not be clinically relevant for diagnostic purposes and were not assessed. Although our study faces these limitations, our rigorous quality controls with QCs integrated into each sample run, along with the stability and lyophilization studies also presented, were able to ensure high data integrity and support the selection of sample handling protocols. With lyophilization sample pre-treatment we also had the added benefit of sample normalization to dry weight, reducing bias from stool water content inconsistencies. Overall, by using both targeted and untargeted approaches, and by exploring markers consistent with altered symptom states, we were able to uncover many novel biomarkers and have presented several new mechanisms of pathophysiology. Several stool derived biomarkers, such as tryptamine and histidine showed trends consistent with literature, while additional novel biomarkers of IBS, such as *N*-acetyl glutamate and orotic acid, or IBS-C/IBS-D differential marker guanosine were reported for the first time in our study. Finally, we explored the effects of symptom state on IBS metabolite profiles which has only been reported in only one recent study to date (Mars et al., 2020b). A similar study

should be performed on a larger cohort to independently validate our findings with greater study power. Once a panel of stool specific metabolites have been validated for their clinical utility in IBS differential diagnosis, integration with other promising biomarkers in literature, may prove to be a powerful non-invasive diagnostic tool for more definitive IBS diagnosis, as well as IBS-D and IBS-C differentiation.

## 2.5 References

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## 2.6 Supplemental Tables and Figures

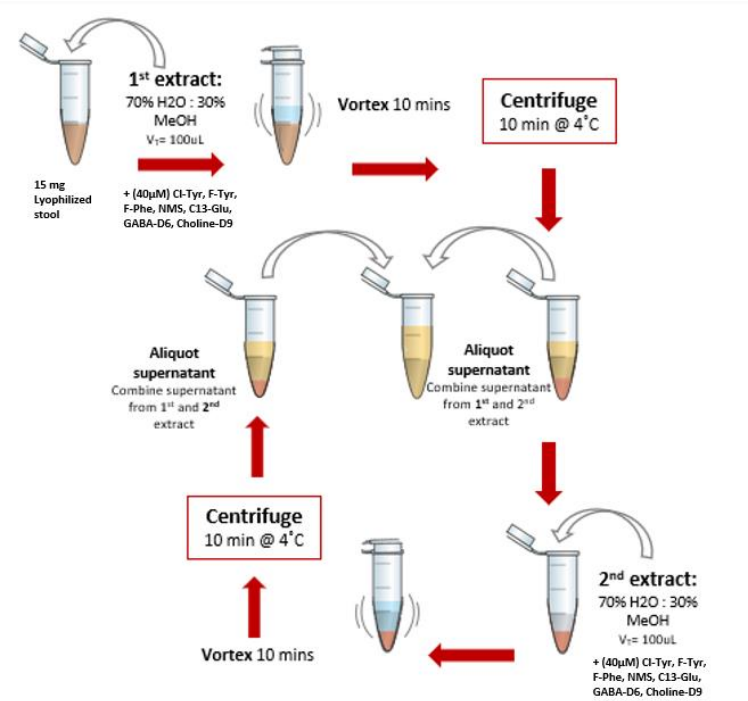



**Figure S2.1** Methanol and water extraction procedure and solution composition for processing of stool prior to metabolomic analyses.

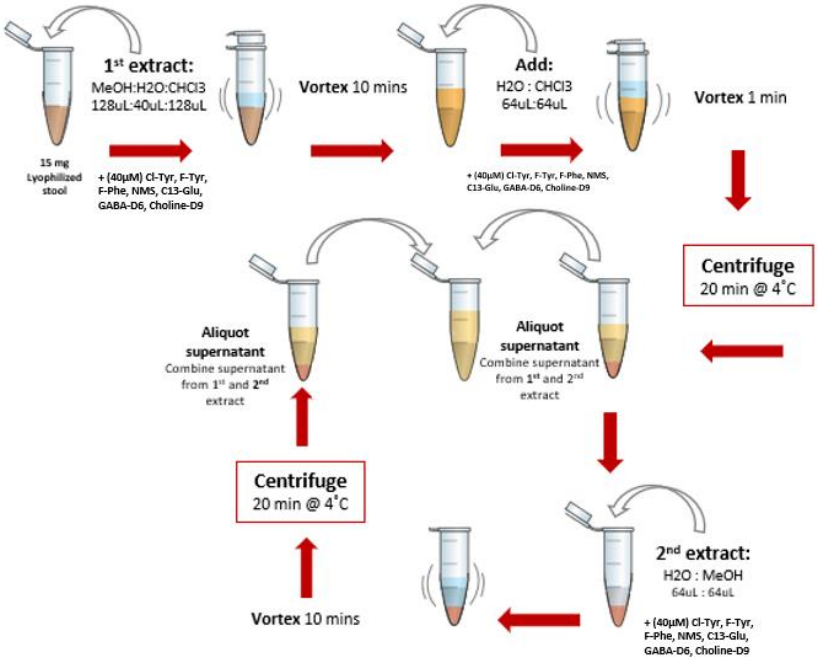



**Figure S2.2** Modified Bligh Dyer extraction procedure and solvent compositions for processing of stool prior to metabolomic analyses.

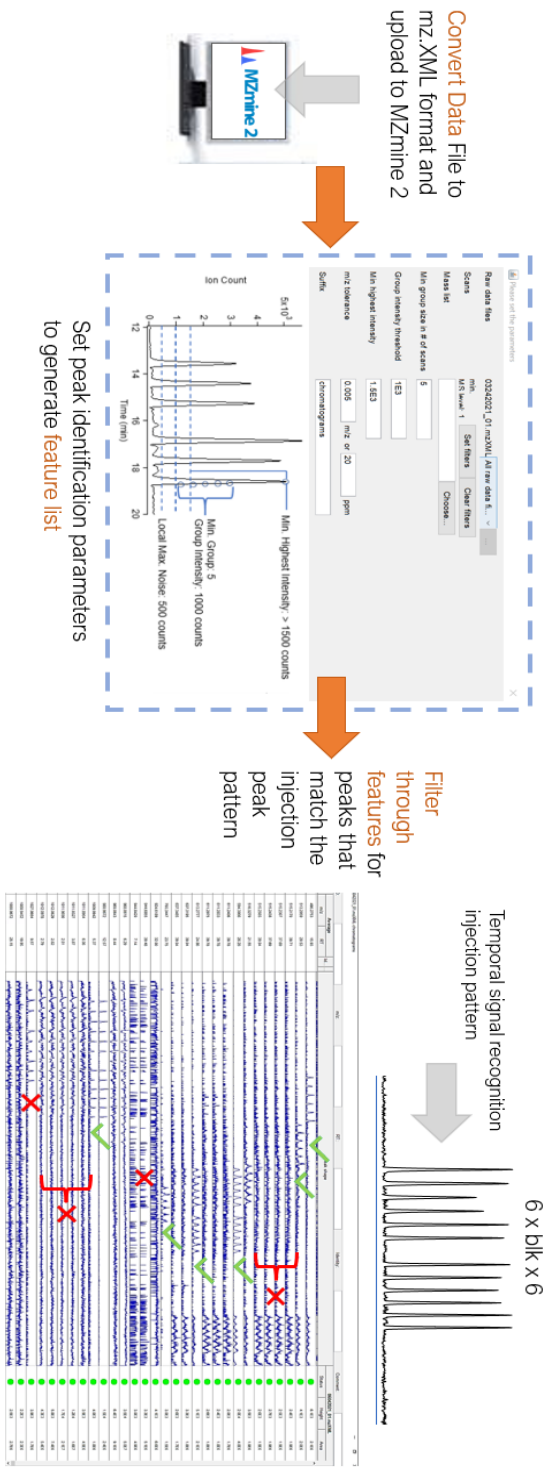



**Figure S2.3** Untargeted metabolomics workflow using MZmine 2, open-source mass-spectrometry data processing software for characterization of the stool metabolome when using dilution pattern recognition in MSI-CE-MS.

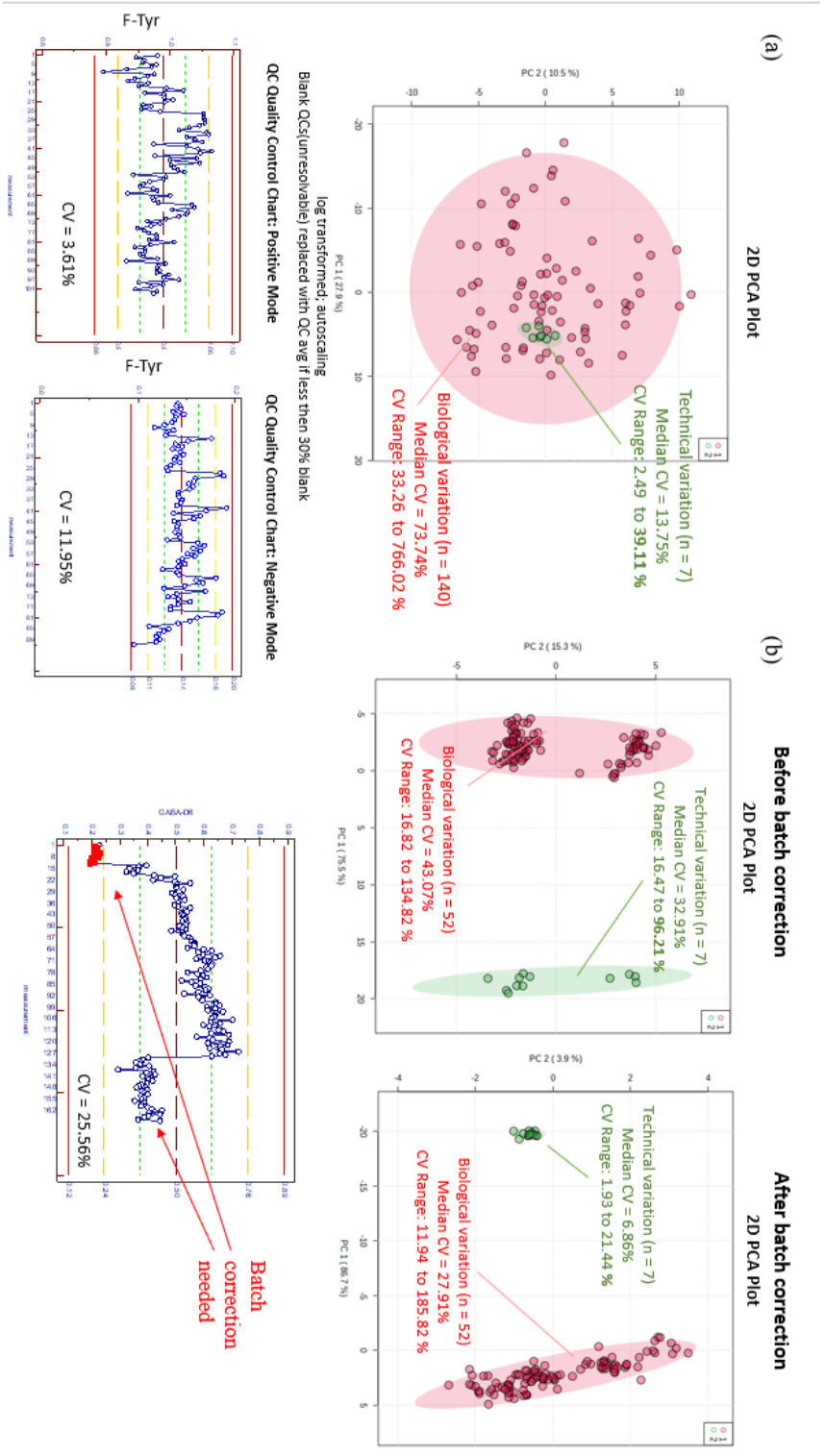



**Figure S2.4** Data quality checks based on PCA 2D scores plots with QCs, and control charts for recovery standards for (a) IBS metabolomics study that was acquired in a single batch of runs by MSI-CE-MS over 3 days of operation as compared to (b) long-term storage stability study quality control charts that was acquired intermittently over a 21-week period before and after batch correction

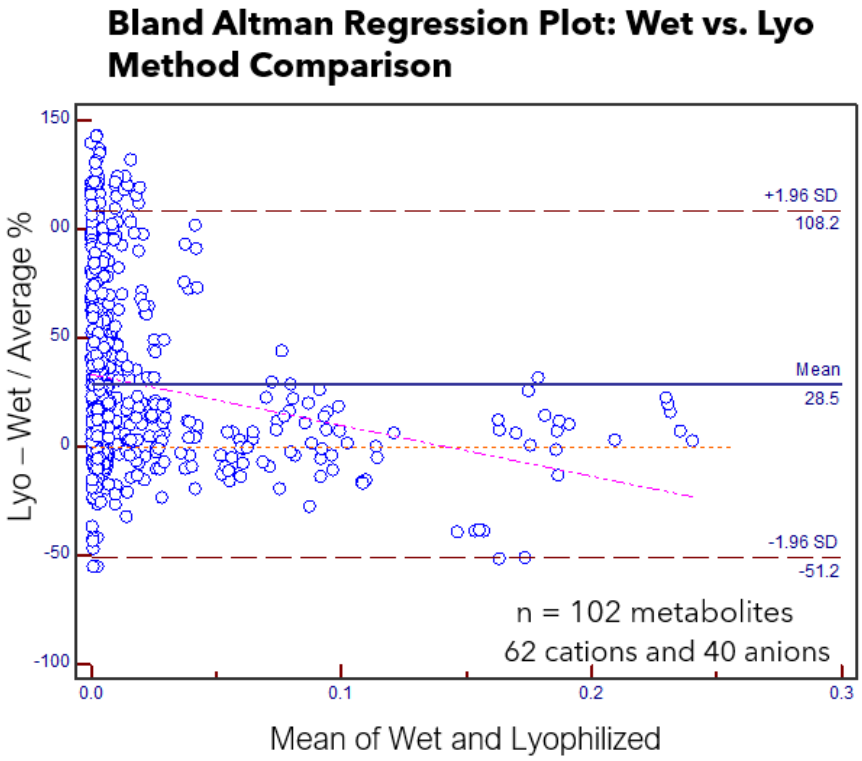



**Figure S2.5** Bland Altman regression plot (MedCalc statistical software) comparing the effects on metabolite abundance for stool samples with and without lyophilization pre-treatment

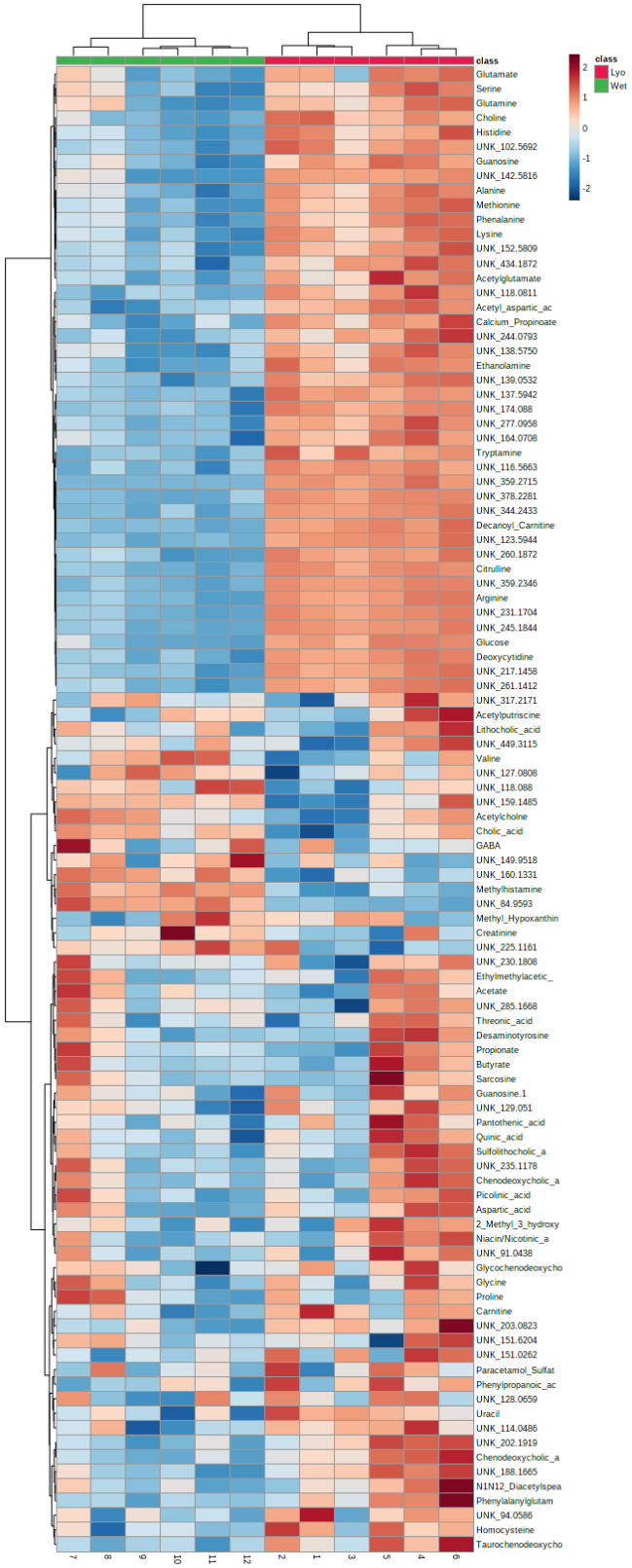



**Figure S2.6** Heatmap of stool metabolite abundances for the wet versus lyophilization pre-treatment comparison study.

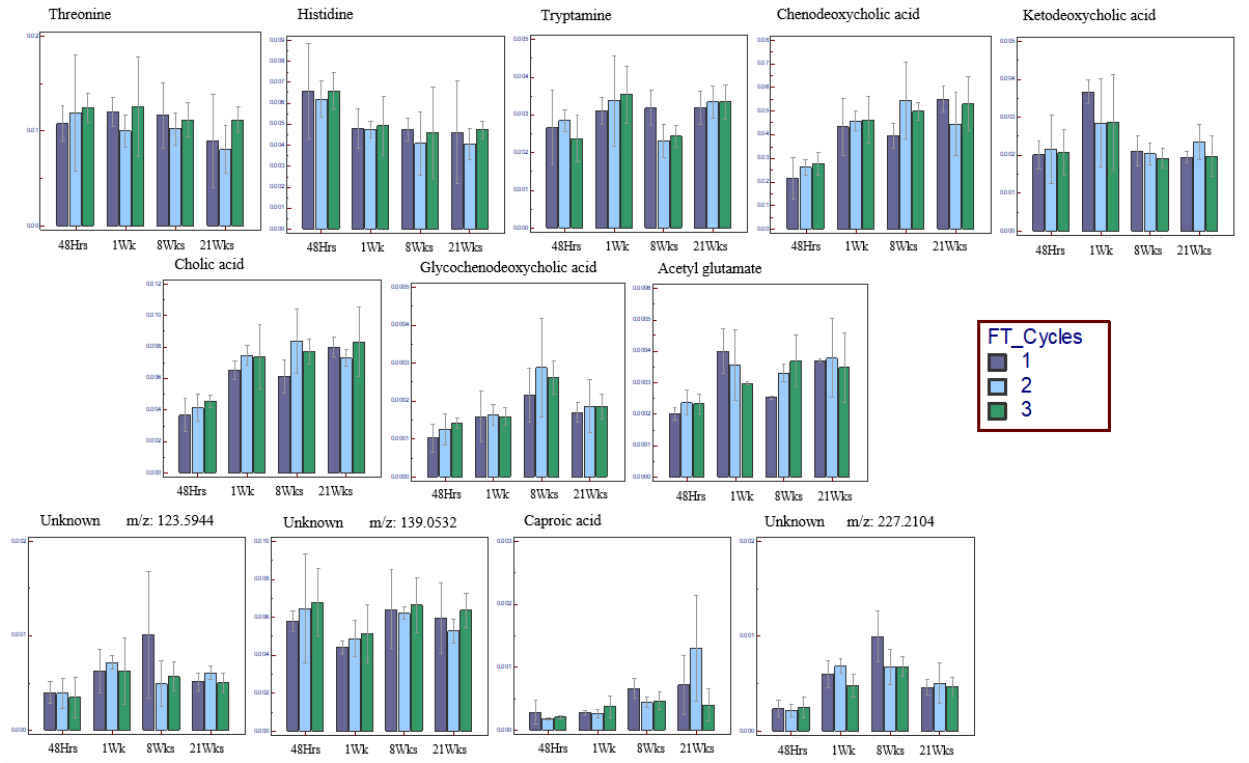
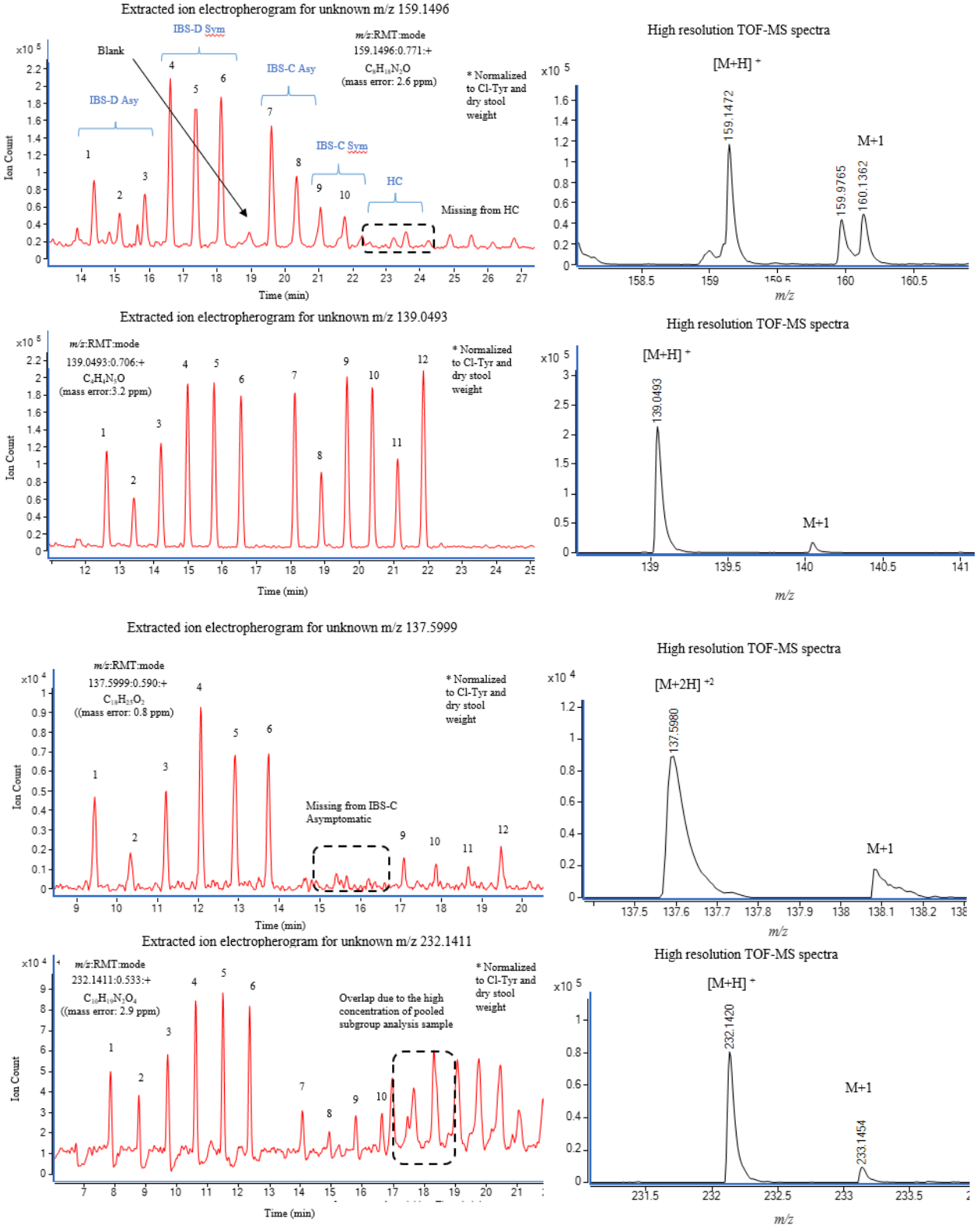



**Figure S2.7** Stability profiles for crude samples after 1, 2 or 3, freeze and thaw cycles, stored at -80˚C for up to 21 weeks


Extracted ion electropherogram for unknown m/z 159.1496
IBS-D Sym
IBS-D Asy
IBS-C Asy
IBS-C Sym
HC
Blank
m/z:RMT:mode
159.1496:0.771:+
C8H18N2O
(mass error: 2.6 ppm)
* Normalized to Cl-Tyr and dry stool weight
Missing from HC
Ion Count
Time (min)
High resolution TOF-MS spectra
[M+H] +
159.1472
M+1
159.9765
160.1362
m/z
Extracted ion electropherogram for unknown m/z 139.0493
m/z:RMT:mode
139.0493:0.706:+
C8H7N3O
(mass error:3.2 ppm)
* Normalized to Cl-Tyr and dry stool weight
Ion Count
Time (min)
High resolution TOF-MS spectra
[M+H] +
139.0493
M+1
m/z
Extracted ion electropherogram for unknown m/z 137.5999
m/z:RMT:mode
137.5999:0.590:+
C10H25O2
((mass error: 0.8 ppm)
* Normalized to Cl-Tyr and dry stool weight
Missing from IBS-C Asymptomatic
Ion Count
Time (min)
High resolution TOF-MS spectra
[M+2H] +2
137.5980
M+1
m/z
Extracted ion electropherogram for unknown m/z 232.1411
m/z:RMT:mode
232.1411:0.533:+
C10H21N3O4
((mass error: 2.9 ppm)
* Normalized to Cl-Tyr and dry stool weight
Overlap due to the high concentration of pooled subgroup analysis sample
Ion Count
Time (min)
High resolution TOF-MS spectra
[M+H] +
232.1420
M+1
233.1454
m/z

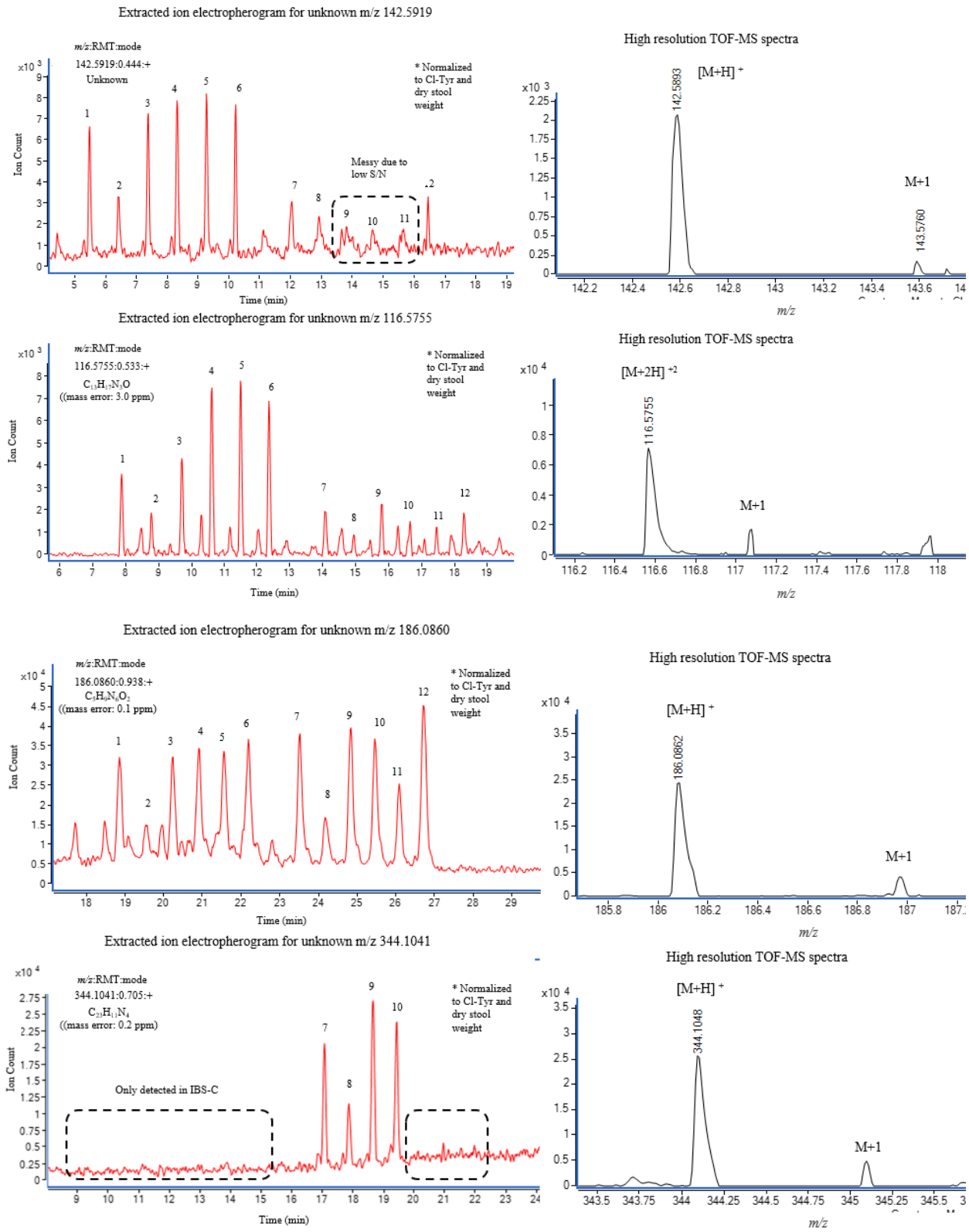



**Figure S2.8** Extracted ion electropherograms and high resolution TOF-MS spectra for key unknowns identified in the IBS Pilot Study.

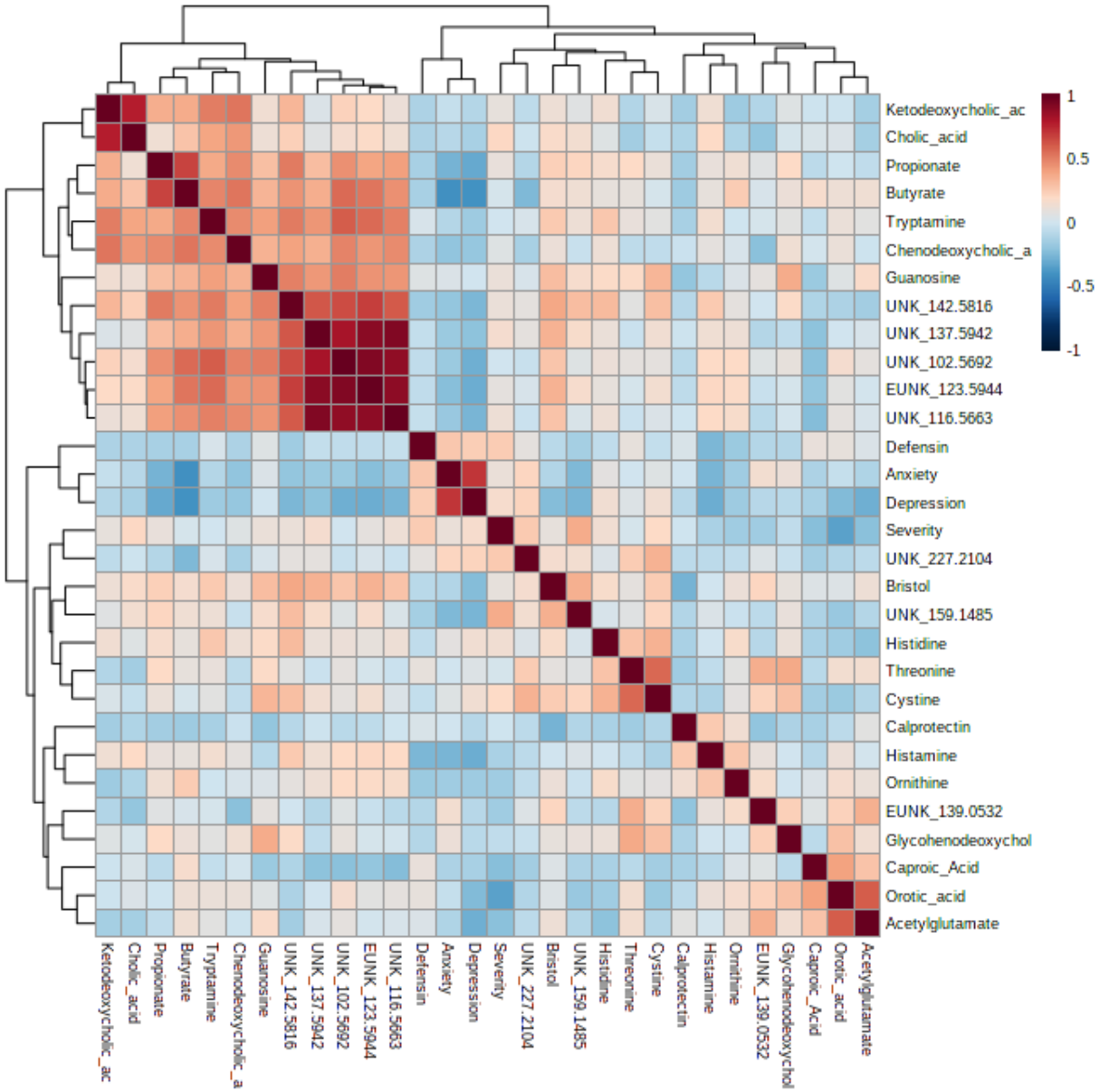



**Figure S2.9** Correlation heatmap (Spearman rank correlation) for top-ranked stool metabolites and clinical measurements from IBS patients and non-IBS controls.

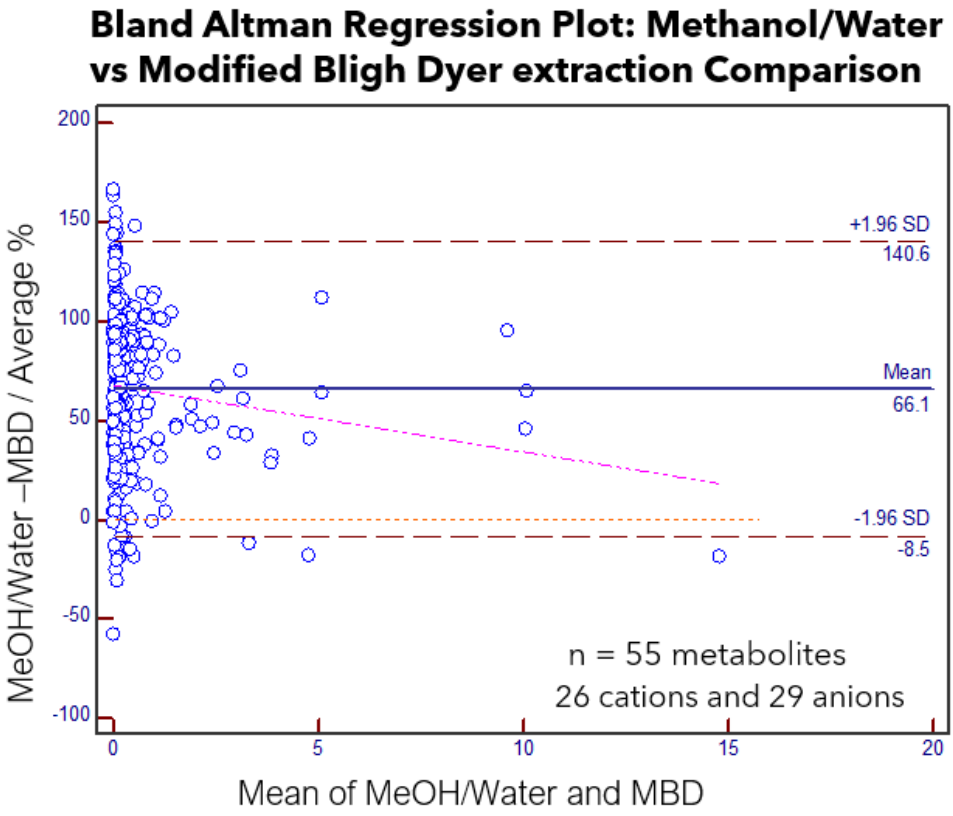



**Figure S2.10** Bland Altman regression plot (MedCalc statistical software) comparing the effects on metabolite abundance for stool samples after MeOH:water (30:70) extraction versus a modified Bligh Dyer extraction. (n = 55 metabolites) Each data point represents the difference in metabolite abundance for each sample extracted with both methods (n = 6 samples)

# Chapter III:

# Conclusion and Future Directions

# Chapter III: Conclusion and Future Directions

## 3.1 Conclusions and major thesis research contributions

The expanding field of metabolomics offers an exciting new approach for functional characterization of the human microbiome as required for new mechanistic insights into chronic diseases of complex etiology, such as IBS. In this thesis, we aimed to optimize a rigorous methodology for sample processing and data analysis when performing nontargeted analysis of metabolites from lyophilized stool extracts, which also improves long-term chemical stability. In *Chapter I* an overview of metabolomics and more specifically stool metabolomics was presented along with its applications to human health when using complementary instrumental platforms based on NMR and separation techniques coupled to high resolution MS. The specific platform used in this thesis, namely multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) was presented along with its major attributes that make it suitable for higher throughput analysis of polar/ionic metabolites from stool extracts with good data fidelity. In addition, this chapter introduced the prevalence and current practices associated with a growing global prevalence of IBS, highlighting on-going deficiencies in diagnostic testing and therapeutic treatments for a debilitating and highly variable chronic GI disorder that has been associated with perturbations in the human microbiome (i.e., dysbiosis). In this case, stool metabolomic studies may provide new insights into disease mechanisms for distinct IBS sub-types that can better inform clinical practices based on new advances in personalized medicine.

In *Chapter II* , complementary targeted and untargeted metabolomic data workflows were integrated when characterizing the stool metabolome by MSI-CE-MS, while also comparing the effects of sample workup procedure and storage conditions on overall metabolome coverage,

reproducibility and long-term stability in order to reduce pre-analytical sources of bias. This work not only was critical to develop a standardized stool handling protocol, but was also the first report to take an untargeted approach to identify clinically significant stool metabolites associated with IBS patient sub-groups (IBS-C vs IBS-D) during transient flare up periods reflecting GI dysfunction and psychological distress from self-reports (symptomatic vs asymptomatic/baseline). Our study confirmed that a repeat ice-cold methanol:water (30:70) solvent extraction procedure on lyophilized stool was optimal for maximizing the recovery of diverse classes of polar/ionic metabolites while normalizing their concentrations to total dried weight. This protocol was then applied to a study comparing lyophilized to crude extracted stool, where we were able to confirm the retention of volatile organic compounds, such as SCFAs (e.g., acetate, butyrate, propionate, and caproic acid) unexpectedly detected in both stool specimen types in equivalent amounts. However, the lyophilized stool samples exhibited greater overall abundance for common intracellular bacterial metabolites, that may indicate a greater extent of cell lysis (host/bacterial), and greater overall metabolome coverage than extraction of matching non-lyophilized stool specimens. Stool samples are highly heterogeneous and biologically active due their high content of bacterial mass, which can compromise sample stability if stored incorrectly. This chapter also presented a long-term (21 week) stability study, where the metabolite recovery from extraction of various stool forms after extended periods of storage at -80 °C were compared, including after repeat freeze-thaw cycles. Our work demonstrated acceptable long-term storage stability for crude and lyophilized stool forms, with low variability from start to end for the majority of extracted metabolites, showing a similar metabolome profile to their 48-h baseline measurement. The main focus of *Chapter II* was the characterization of the stool metabolome from a cohort of IBS participants in a cross-sectional prospective study as compared to non-IBS/healthy controls,

applying our recently optimized sample handling protocols for stool metabolome analyses when using MSI-CE-MS. This work identified several novel biomarkers associated with IBS, such as significantly reduced levels ($p < 0.05$) of orotic acid and *N*-acetyl glutamate in IBS patients as compared to age/sex-matched non-IBS controls. Lower elimination of these stool derived metabolites suggests lower *N*-acetyl glutamate synthase (NAGS) activity, reducing conversion of glutamate to *N*-acetyl glutamate, and ultimately impairment to the breakdown of ammonia to carbamoyl phosphate, which is required for orotic acid production. A follow-up study should be conducted to assess if these results can be replicated in a larger IBS patient cohort while analyzing circulating blood ammonia levels for further mechanistic validation. Also, differentiating stool biomarkers between IBS-D and IBS-C patients have been sparsely reported in the literature to date, but in our study, unexpectedly guanosine levels were specifically elevated in the IBS-D sub-group, which may suggest a link to elevated levels of secretory activator cGMP as it has been implicated with chronic diarrhea (Friebe et al., 2020). Concluding the study was the analysis of stool extracts from IBS-D and IBS-C patient sub-groups collected during symptomatic and asymptomatic timepoints to assess dynamic metabolic changes associated with active IBS flair-ups. Metabolic changes were specific to each IBS subtype, with altered profiles identified for ornithine and glycochenodeoxycholic acid in IBS-D patients, and threonine and cystine alterations in IBS-C patients, showing that subtypes may have distinct mechanisms causing elevated symptom severity coinciding with abdominal discomfort and psychological distress.

## 3.2 Non-aqueous MSI-CE-MS analysis: Fat absorption in IBS patients

IBS has been associated with altered bile acid levels, which are important emulsifiers that aid in the digestion and absorption of lipids (Staels & Fonseca, 2009). The method outlined in this thesis used an aqueous background electrolyte in MSI-CE-MS for the separation of ionic

metabolites from a largely polar extraction solvent system (70% water) that may limit overall lipidome coverage (e.g., bile acids, short-chain fatty acids). This prevented analysis of non-esterified fatty acids, fat-soluble vitamins and a diverse range of glycerophospholipids that may be relevant in IBS disease pathophysiology, such as lower intestinal fat absorption during active flare up periods. In this case, an analysis of the IBS stool extracts based on a Bligh-Dyer method (chloroform/methanol/water) or preferably using methyl-*tert*-butyl ether should be performed by multisegment injection-nonaqueous-capillary electrophoresis-mass spectrometry (MSI-NACE-MS), which allows for separation of non-polar yet ionic classes of lipids when using a non-aqueous background electrolyte system (Azab et al., 2019; Ly et al., 2022). Previous studies have reported the relevance of altered fatty acid profiles among IBS patients, such as elevated plasma arachidonic acid, whereas another study demonstrated a reduction in circulating arachidonic acid concentrations after long-term diet changes (Clarke et al., 2010; Orlando et al., 2020; Erben et al., 2021). Currently, available IBS stool metabolomic studies have primarily focused on short-chain fatty acids with few studies investigating metabolic changes upon symptomatic disease in IBS patients prospectively from a stable baseline (Mars et al., 2020). Overall, stool specimens are recommended to be lyophilized when performing lipid extraction since cell lysis enables greater recovery of host and bacterial cells metabolite, increasing lipid extraction yields (Gregory et al., 2013). Fatty acids, and of particular interest, medium-chain and long-chain fatty acids are increasingly associated to various GI disorders that are also relevant to explore in IBS pathophysiology (Mosińska et al., 2020). Future work using MSI-NACE-MS will evaluate various lipids derived from lyophilized stool extracts in IBS patients that may be able to better differentiate IBS subtypes and reflect dynamic changes to patient symptom states.

### 3.3 Lyophilization vs manual cell lysis comparison

As presented in *Chapter II* of this thesis, lyophilization of fecal samples produced greater overall metabolite recovery compared to non-lyophilized sample, showing particular elevations for intracellular metabolites (e.g., amino acids), suggesting that the lyophilization process may induce a greater extent of cell lysis. This theory has been supported in other recent publications, which have also presented similar findings with elevated metabolite levels following stool lyophilization (Nam et al., 2022; Shen et al., 2021). Although other studies have also suggested an association between lyophilization and cell lysis, this has yet to be confirmed in human stool samples. In order to determine the origin for the elevated metabolite levels from lyophilized stool extracts, a study should be conducted to rule out other possible mechanisms. Alternative more harsh homogenization techniques, such as bead blender homogenization, has been used as a mechanical lysis method for fecal samples (Vandeventer et al., 2011). Therefore, to test if changes in stool metabolome coverage after lyophilisation are entirely or in part a result of cell lysis during the lyophilization process, all samples will first be homogenized using a milder homogenization method, such as manual mixing of stool samples with a wooden depressor with a small addition of water. From this mixture a third of our total study sample size (n = 30) is proposed to be aliquoted and set aside as a control. The remaining samples will be blended using a bead blender to induce cell lysis, and the rest of the study samples will be aliquoted out, with half of these samples undergoing lyophilization. It is anticipated that no difference between the blender lysed and blender lysed with lyophilization in stool extracts would occur if the metabolite abundance changes were only due to cell lysis, but both sub-groups should show substantial differences from the non-lysed control group. Additionally, it has been postulated that the lyophilization process, by removing water, may allow for more efficient metabolite extraction as a fine powder that can be more easily weighed out with greater precision. A dry sample without water maybe be more

readily extractable as the solvent can make better contact with target molecules. Drying the sample will reduce dilution by stool water content giving more extraction power to the methanol component of the solvent mixture. This is especially true when extracting lipids using a purely organic extraction solvent, where high water content can reduce solvent interaction with lipids and reduce extraction efficiency due to some metabolites partitioning between the organic and water phase (Saini et al., 2021). This study will help us to better understand the effects of lyophilization on stool samples and will confirm if the differences in metabolite abundances between sample treatment groups is due to cell lysis or as result of other sample effects that occur when stool is dried to powder form. Knowing the effects of lyophilization is important before including this technique as a standardized protocol for preparing and storing stool specimens.

## 3.4 Overall Perspective

This thesis provides novel insights into IBS pathophysiology and the metabolic phenotypes associated with active and dormant symptom states from patients having contrasting disease sub-types using both targeted and untargeted stool metabolomic strategies for MSI-CE-MS with stringent quality controls. There are several important research contributions to the standardization of stool sample handling methods, assessing extraction protocols, sample pre-treatment steps and the long-term stability of various stool forms, helping to solidify our current understanding of best sample handling practices. Overall, standardized stool collection without delays to storage along with lyophilization, offers a more robust approach when conducting stool metabolomic studies with greater reproducibility and metabolome coverage while also affording great long-term stability. The information presented in this thesis will support the development of a stool metabolite panel for better differentiation of IBS from non-IBS/healthy controls, and help to classify patients based on their symptom status and IBS disease subtype for more rapid diagnoses

and personalized treatment interventions. The proposed future studies will expand IBS metabolite profiling by exploring stool lipidomics using MSI-NACE-MS in a larger cohort of IBS patients from different clinical sites, while also deepening our fundamental understanding of underlying disease mechanisms in IBS.

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