Optimization And Validation Of The Extraction, Purification And Analysis Of Galacto-oligosaccharides (GOS) In Beans

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ABSTRACT

OPTIMIZATION AND VALIDATION OF THE EXTRACTION, PURIFICATION AND ANALYSIS OF GALACTOOLIGOSACCHARIDES (GOS) IN BEANS

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Pulses are rich in protein, dietary fiber, minerals and vitamins and their consumption promotes beneficial metabolic responses. However, postprandial gastrointestinal discomfort decreases the overall interest for pulses. Side effects are mostly attributed to Galacto-oligosaccharides (GOS), a group of indigestible carbohydrates formed by galactose monomers linked to a sucrose molecule by α (1→6) glycosidic linkage. Reducing these oligosaccharides is of interest to enhance pulse consumption; nevertheless, a reliable and reproducible extraction and analysis method needs to be developed. In this work, we optimized and validated the extraction and purification of GOS in red kidney bean (*Phaseolus vulgaris*), and the subsequent quantification through high-performance anion-exchange chromatography (HPAEC-PAD) and gas chromatography/mass spectrometry (GC/MS). With an optimized derivatization (to make them volatile) procedure, the GC/MS was more reproducible and reliable to identify and quantify the GOS content in 6 native bean kernels and canned whole beans exposed to different processing conditions.

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Table of *Contents*

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

α – Alpha

β – Beta

^{o}C – Degrees celsius

CH_3COONa – Sodium acetate

CVD – Cardiovascular disease

D-RSV - Raffinose, stachyose, verbascose standard solution for HPAEC-PAD analysis

EI – Electron impact

eV – Electronvolt (unit)

FODMAPs - Fermentable oligo-, di and monosaccharides and polyols

g – Grams

G-RSV - Raffinose, stachyose, verbascose standard solution for GC-MS analysis

GC-MS – Gas chromatography coupled with a mass spectrometry

GC-TMSi – Gas chromatography with N-trimethylsilylimidazole

GOS – Galacto-oligosaccharides

HPLC with ESLD – High-performance liquid chromatography with evaporative light scattering detector

HPLC-HR ESIMS – High-performance liquid chromatography- high resolution electrospray ionization mass spectrometry

HPLC-HRMS – High-performance liquid chromatography-high resolution mass spectrometry

HPAEC-PAD – High-performance anion exchange chromatography with pulsed amperometric detection

HPLC-RI – High-performance liquid chromatography with refractive index detection

IBS – Irritable bowel syndrome

IS – Internal standard

kg – Kilograms

LOD – Limit of detection

LOQ – Limit of quantification

min – Minutes

mg – Milligrams

µL – Microlitre

NH_2 – Azanide

ppm – Parts per million

R - Raffinose

R^2 – Coefficient of determination

RBF – Round bottom flask

RFO – Raffinose family oligosaccharides

RSV – Raffinose, stachyose, verbascose standard solution

s – Seconds

S - Stachyose

SCFA – Short chain fatty acid

SPE – Solid phase extraction

TFA – Trifluoroacetic acid

TMS – Hexamethyldisilizane

USD – United states dollar

V – Verbascose

LIST OF APPENDICES

1 Introduction

1.1 Pulses

Pulses are dry seeds that are harvested from leguminous plants, belonging to the plant family *Leguminosae* (Tiwari, Gowen, & McKenna, 2011). For thousands of years, it has been categorized as one of the important food categories for humans which makes it the second most consumed food aside from cereal grains (Dilis & Trichopoulou, 2009; Tiwari et al., 2011). Examples of pulses includes but are not limited to dry beans (i.e. kidney beans, dry broad bean, dry peas, chickpeas, dry cowpeas, pigeon peas, lentils, vetch, lupins) (Tiwari et al., 2011). The common bean, *Phaseolus vulgaris*, also known as kidney bean, is the pulse of interest in this research project. In general, pulses are rich in minerals and vitamins, a rich source of proteins, dietary fibers, and carbohydrates which have a low glycemic index (Tiwari et al., 2011; Vidal-Valverde et al., 2002). Pulses are one of the commodities which contributes to promote economic growth in countries such as Canada. The largest global producer and consumer of this commodity is India who is known to contribute 25-28% of the world's pulse production (Tiwari & Singh, 2012). Canada is also one of the leading producers and exporter of pulses who supplies to 150 different countries (Statistics Canada, 2018). In 2011, a significant economic growth occurred which resulted in a 1.5 billion Canadian dollars of cash receipts (Statistics Canada, 2018). Though pulses are nutritious and are very beneficial to overall health, the North American demographic consumes the least amount; 3.5 kg/capita per year, compared to South Asia and Latin America (10-40 kg/capita per year) (Mudryj et al., 2012). In fact, increased consumption of pulses by 100 g/day per person would decrease incidence of type 2 diabetes and cardiovascular diseases

(CVD). This would save approximately 315 million Canadian dollars on healthcare per annum (Abdullah, Marinangeli, Jones, & Carlberg, 2017).

1.2 Pulses Composition: Nutritional components

With its high nutritional value, especially with its rich dietary fiber content, pulses have the potential to reduce risk of diet related diseases such as cardiovascular disease, obesity, type 2 diabetes, intestinal cancer and serum cholesterol (Njoumi, Joseph, Rochette, Bellagha, & Mouquet-Rivier, 2019). In addition, it could also be used as an aid to treat constipation and promote growth of beneficial microorganisms in the large intestine (Njoumi et al., 2019). One attractive trait of pulses is its high fiber, low lipid content, relatively high protein content as well as biologically active compounds (Campos-Vega et al., 2009; Dilis & Trichopoulou, 2008). For example, the common bean, *Phaseolus vulgaris* contains 20-25% protein, and 50-60% carbohydrates (Campos-Vega et al., 2009).

1.2.1 Antinutrients

Pulses are relatively high in proteins; however, it is inferior to animal protein due to its lack of sulphuric amino acids which is important in maintaining and preserving integrity of cellular system through influencing cellular detox state and cellular capacity to detoxify toxins, reactive oxygen species and free radicals (Townsend, Tew, & Tapiero, 2004; Dilis & Trichopoulou, 2009). Furthermore, pulses contain high amounts of complex carbohydrates (e.g. starch and dietary fibre), B vitamins including thiamin, riboflavin, niacin, folacin, as well as inorganic compounds such as iron, magnesium, zinc and phosphorous (Dilis & Trichopoulou, 2008). Nevertheless, vitamins and minerals as well as other non-nutrient compounds leach out when soaked and cooked (Dilis & Trichopoulou, 2009). With that said, the human body could utilize only 32% to 78% of protein

that is ingested as proteolysis of the seed is limited and the presence of antinutritional compounds reduces protein bioavailability (Dilis & Trichopoulou, 2009; Burbano et al., 1999).

These antinutritional compounds include enzymes or protease inhibitors (trypsin, chymotrypsin, α-amylase), phytic acid, tannins, lectins, oxalates, phenolic compounds, saponins, and oligosaccharides (Dilis & Trichopoulou, 2009; Sathe & Deshpande, 2003). Protease inhibitors or enzyme inhibitors reduce the absorption of other proteins present in pulses; however, they are denatured during cooking thus their inhibitory effects are reduced (Dilis & Trichopoulou, 2009). Lectins are glycoproteins which function to agglutinate red blood cells, allowing it to bind to specific receptor sites of epithelial cells of the small intestine. This decreases the ability to absorb sugars, amino acids, vitamin B12 and lipids (Dilis & Trichopoulou, 2009). Furthermore, lectins decrease digestive enzyme activity or damage intestinal lining which allows bacteria to penetrate into the blood stream (Thompson, 1993). Phytic acid also known as inositol hexaphosphate is crucial for regulating the different functions of human cells (Dilis & Trichopoulou, 2009). It contributes to the anticarcinogenic property of dietary fibre by creating complexes with iron (Fe) which prevents lipid peroxidation and hydroxyl radical generation (Fenton reaction) (Graf, Empson & Eaton, 1987). In addition, phytic acid can bind to zinc (Zn) and iron (Fe) which converts it to phytate, thus, decreasing bioavailability of the minerals (Dilis & Trichopoulou, 2009). Saponin is a glucoside belonging to the steroid or triterpenoid group. It is an amphiphilic molecule that pose both beneficial and detrimental effects to the human body (Dilis & Trichopoulou, 2009). Saponins are able to bind to bile acids and cholesterol, thus decreasing blood cholesterol levels through faecal excretion (Dilis & Trichopoulou, 2009; Thompson, 1993; Sidhu & Oakenfull, 1986). However, saponins may cause lysis of red blood cells and intestinal mucosa cells which

reduces nutrient absorption through enzyme inactivation or binding (Thompson, 1993). Phenolics most commonly are flavonoids, are widely distributed in plants as it serves as a protection mechanism from viruses, fungus, or chemical attacks (Champ, 2002). Phenolics are well known for their antioxidant activity which decreases ageing rate and development of degenerative diseases, however, they are also responsible for reduced bioavailability of iron (Fe) (Harman, 1956; Sandberg, 2002). The last main antinutrient in pulses are the oligosaccharides. The main oligosaccharides in pulses are the alpha-galactooligosaccharides (GOS) (i.e. raffinose, stachyose, and verbascose) however, there are other minor ones which are ciceritol and ajugose (Dilis & Trichopoulou, 2009; Kannan, Sharma, Gangola002C & Chibbar, 2018).

1.2.1.1 FODMAPs

GOS belongs to a group called fermentable oligo-, di and monosacharides and polyols (FODMAP) which includes a group of short chain carbohydrates that cannot be digested by the human body and therefore, cannot produce energy (Gibson & Shepherd, 2005; Halmos, Power, Shepherd, Gibson, Muir, 2014). There are five main FODMAP groups i) polyols, ii) fructans, iii) fructose, iv) lactose and v) galactooligosaccharides (Gibson & Shepherd, 2005), which will be the FODMAP of interest in this thesis project. galacto-oligosaccharides (GOS), also known as alpha-galactosides or Raffinose family of sugars (RFO), consist of galactosyl residues linked by α-1,6 to the glucose moiety of sucrose and are present abundantly in storage organs of plants such as tubers and seeds (Njoumi et al., 2019; Jones, DuPont, Ambrose, Frias, & Hedley, 1999). In nature, GOS accumulates during seed development; however, raffinose and stachyose will start to be produced during seed maturation which functions as a reserve for carbon to aid in germination (Frias, Diaz-Pollan, Hedley, & Vidal-Valverde, 1996; Lowell & Kuo, 1989; Dey, 1990). Raffinose and

stachyose concentration is most abundant in the hypocotyl of *Phaseolus coccineus* (Kosson, 1998). In order to understand the hypocotyl, one must have knowledge in the basics of bean seed structure. Bean seeds consist of an embryo – a baby plant where food and nutrients are stored for germination (Shakuntala, 2007). The embryo has different structures consisting of the radicle which would grow into the root, and plumule which forms the shoot of the plant (Shakuntala, 2007). Seeds can be classified as monocots; one cotyledon, and dicots which means two cotyledons, and this is the structure of bean seeds (Shakuntala, 2007). The stem of the plant is classified into 2 parts; epicotyl and hypocotyl (Shakuntala, 2007). Epicotyl is the upper section of the stem above the cotyledon, and hypocotyl is the lower part of the cotyledon (Shakuntala, 2007). The morphology of a dicot bean and the location of epicotyl and hypocotyl of a stem can be seen in **Figures 1** and **2** respectively**.** The hypocotyl plays an important role in a growing red kidney bean as it holds most of all the glycosidases (enzymes) which functions to degrade wall and storage carbohydrates, to mobilize food into the embryo (Nevins, 1970). As germination continues, raffinose and stachyose would decrease however, fructose would gradually increase during the process (Silva & Luh, 1979). Thus, germination is known as one of the methods to decrease GOS content in beans (Petrova, Marinova, & Tchorbanov, 2010).

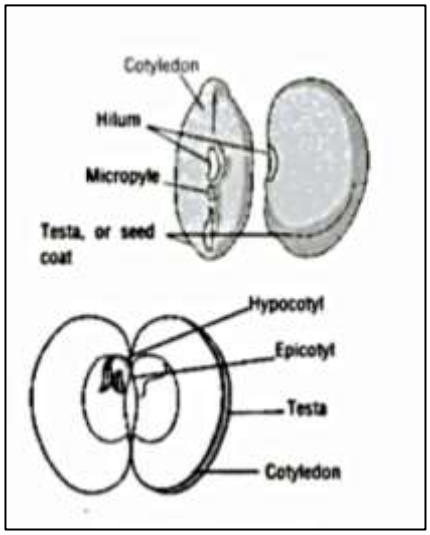

Figure 1. Kidney bean anatomy, showing the location of the hypocotyl. Obtained from Shakuntala (2007).

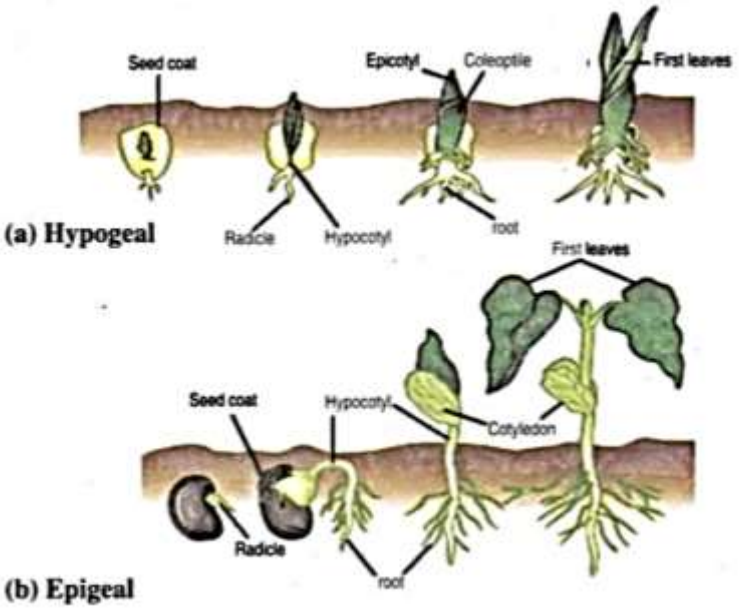

Figure 2. Growth stages of bean seed showing the location of hypocotyl and epicotyl. Obtained from Shakuntala (2007).

1.2.1.2 GOS Structure

The structure of GOS comprises of 1 to 3 galactose units linked to a sucrose molecule via an α-1-6 glycosidic bond attached to C-6 of the glucose in the sucrose moiety (Njoumi et al., 2019). This can be seen in **Figures 3** and **4**.

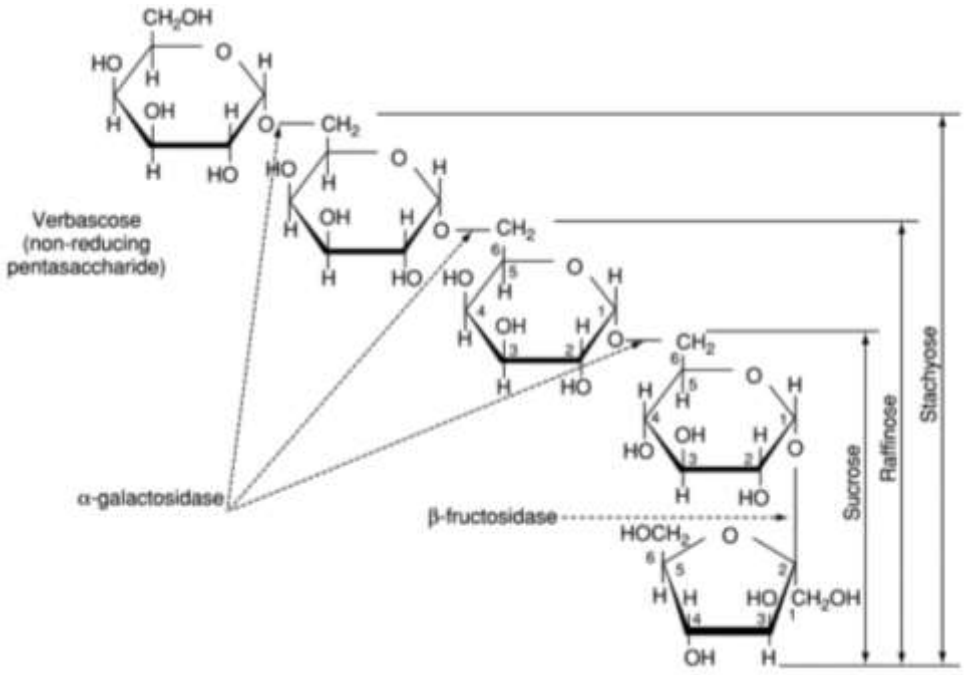

Figure 3. The structural relationship between sucrose and the three-common GOS (raffinose, stachyose and verbascose). Obtained from Tester & Karkalas, (2003).

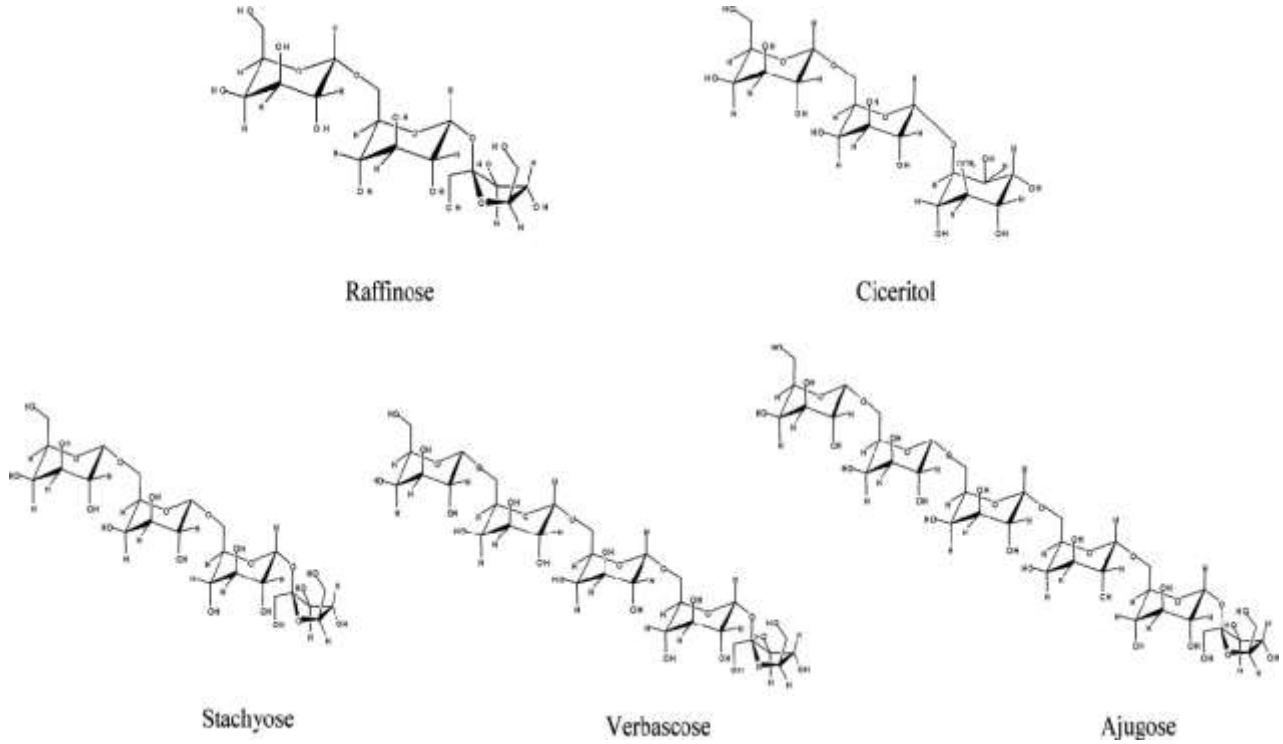

Figure 4. Structure of various galacto-oligosaccharides present in beans. Obtained from Martínez-Villaluenga, Frias, & Vidal-Valverde, (2008).

These carbohydrates require the α-galactosidase enzyme activity to hydrolyze the α-1-6 bonds; however, this enzyme is absent from the human intestinal lining (Carlsson, Sandberg, & Karlsson, 1992; Njoumi et al., 2019) and consequently, these oligosaccharides can reach the colon intact. As a result, GOS are fermented by microorganisms in the human colon which leads to the production of short chain fatty acids (SCFA), carbon dioxide, methane and hydrogen gas (Njoumi et al., 2019). Due to this, most healthy individuals would experience increased flatulence production in the intestinal tract when consuming pulses resulting in the appearance of negative symptoms including abdominal discomfort, cramps pain, and diarrhea (Njoumi et al., 2019; Martinez-Villaluenga et al., 2008). Nevertheless, the resistance of these compounds in the initial

part of the gastrointestinal tract allows them to get into the large intestine intact and be fermented by the colon microbiota, encouraging the growth of *Lactobacilli* and *Bifidobacteria* and the decrease of enterobacteria growth in the microflora (Njoumi et al., 2019; Martínez-Villaluenga, Frías, & Vidal-Valverde, 2005). Since GOS is indigestible, it is classified as a prebiotic (Njoumi et al., 2019; Martínez-Villaluenga et al., 2005). Prebiotics are defined as non-digestible food ingredients that are beneficial to the host by stimulating the growth and activity of one, or a limited number of a selected bacteria in the colon (Martinez-Villaluenga et al., 2005). Thus, GOS has been used as a nutritional therapeutic agent to relieve constipation with a recommended serving size of 12 grams per day (Niittynen et al., 2007). As constipation is more prevalent with age and reduces the quality of life, incorporating adequate intake of dietary fiber and water are advised to soften the stool and increase bowel movement (Niittynen et al., 2007; Higgins et al., 2004).

1.2.1.3 GOS and Physiological implications: Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a gastrointestinal or bowel disorder which is caused by multiple factors causing symptoms such as abdominal pain, bloating; occurred in 60% of IBS patients, and altered bowel movements (De Giorgio, Volta, & Gibson, 2016; Staudacher, Irving, Lomer, & Whelan, 2014; Staudacher, & Whelan, 2017). Factors which leads to IBS varies ranges from an imbalance or changes in the gut microbiome, altered gut-brain axis with dysmotility and hypersensitivity, activated immune system, genetic factors, psychological factors and leaky gut barrier functions (De Giorgio et al., 2016; Chey & Menees, 2018). This syndrome affects 11-12% of the general population in America, Europe, Asia Pacific and Africa (Chey & Menees 2018; Staudacher et al., 2014). It has negative implications towards a person's productivity, social

functionality and overall quality of life (Chey & Menees, 2018; Staudacher et al., 2014). In fact, it has costed over $30 billion USD in the United States health care sector (Chey & Menees, 2018).

The relationship of FODMAPs and IBS has been a topic of interest since the 1900's until this day, where individuals are more concerned with lifestyle and diet choices compared to the past. It has been demonstrated that food items containing FODMAPs, includes wheat containing products (e.g. gluten), legumes and vegetables (GOS), proteins from yeasts and soy, dairy (lactose), certain fruits (e.g. fructose), and fructo-oligosaccharides (fructans) in asparagus, artichoke, garlic, trigger IBS symptoms (Biesiekierski et al., 2011; Gibson & Shepherd, 2005; Staudacher & Whelan, 2017; Lovegrove et al., 2017). The mechanism by which various food and its components, trigger IBS symptoms is illustrated in **Figure 5**. When an IBS patient ingests food containing FODMAPs, there are several events that could happen; activation of mast cells, chemosensory activation through the activity of bioactive molecules, mechanoreceptor activation through luminal distension (De Giorgio et al., 2016; Staudaccher et al., 2014; Sarna, 2010). All of which have a role to play in triggering bloating, abdominal pain and altered bowel habits (diarrhea and constipation) (De Giorgio et al., 2016; Staudaccher et al., 2014; Sarna, 2010).

The mechanisms of activation would be further explained. Mast cell activation occurs when food components (i.e. proteins pass through a leaky epithelial barrier) (Krystel-Whittemore, Dileepan & Wood, 2015; da Silva, Jamur & Oliver, 2014; Galli & Tsai, 2010; Jamur, et al., 2005; Metcalfe & Boyce, 2006). Mast cells are immune cells that are present in mucosal and epithelial tissues throughout the body, more specifically they are located in the gastrointestinal tract, skin and respiratory epithelium (Krystel-Whittemore, Dileepan & Wood, 2015; da Silva, Jamur & Oliver, 2014; Galli & Tsai, 2010; Jamur, et al., 2005; Metcalfe & Boyce, 2006). IBS patients are

known to have a higher amount of mast cells present in colonic and ileal mucosa which is one of the hypotheses that explains the relationship between mast cells and disturbed function in the colon (Chadwick, Chen, Paulus, Bethwaite & Wilson, 2002; O'Sullivan et al., 2000; Weston, Biddle, Bhatia & Miner, 1993). The activation of mast cells in the colon, increased excitement of enteric and primary afferent neurons (Reed et al., 2003; Gao et al., 2002; Nozdrachev et al., 1999) which leads to increased sensitivity to pain (visceral hypersensitivity) and disturbed gut motor function (Bueno, Fioramonti, Delvaux & Frexinos 1997; Castex Fioramonti, Fargeas, More & Bueno, 1994).

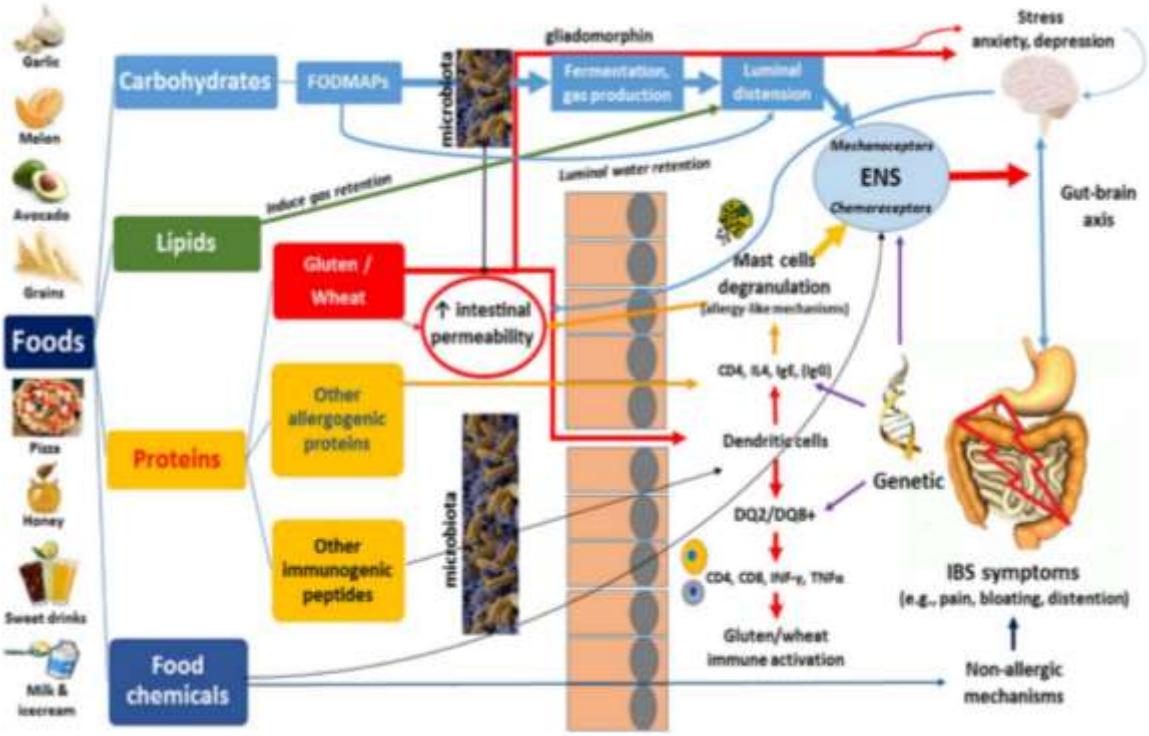

Figure 5. Schematic diagram of the mechanism which various foods containing carbohydrates, lipids, protein and other bioactives could trigger symptoms in IBS patients. Obtained from De Giorgio et al. (2016).

Furthermore, the physiological state of an IBS patient is also an important aspect to reduce symptoms. Clinical studies conducted in rats, under chronic stress, and not acute stress, triggers the symptoms of IBS (Bennet et al., 1998; Whitehead et al., 1992). Stress targets motor function and visceral hypersensitivity to aid in motoring stool and movement in the colon (Sarna, 2010). All of which are impaired in IBS patients (Sarna, 2010).

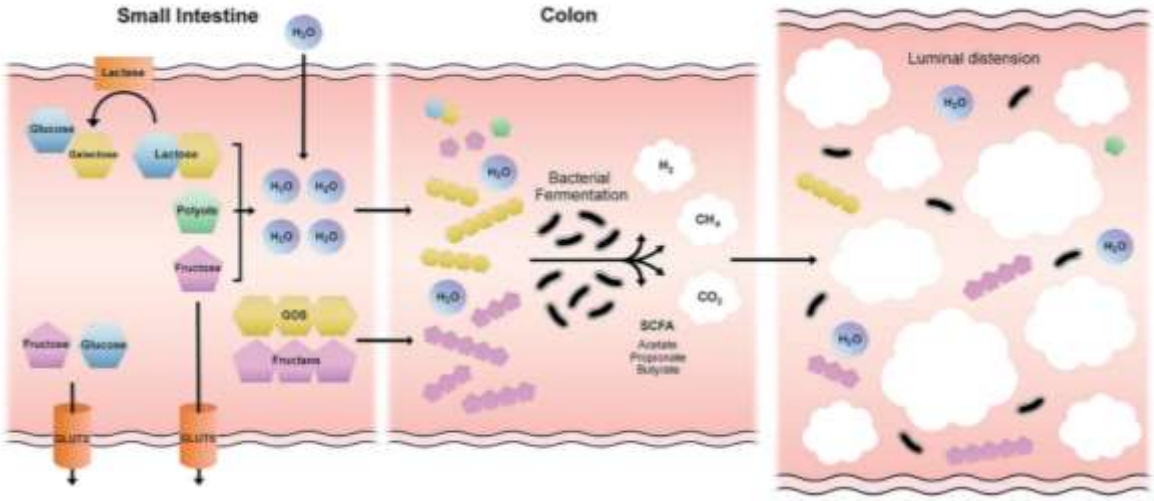

Figure 6. A Schematic diagram of certain FODMAPs ie. fructose, lactose and polyols fermented by the gut microorganisms in the colon, leading to lumen distension. Obtained from Staudacher & Whelan, (2017).

Luminal distension, illustrated in **Figure 6**, occurs when non-digested or non-absorbed short chain carbohydrates are available for fermentation in the colon, leading to an accumulation of gas such as hydrogen and methane (De Giorgio et al., 2016). Therefore, IBS symptoms would emerge for an individual who has visceral hypersensitivity (De Giorgio et al., 2016; Staudacher & Whelan, 2017). Visceral hypersensitivity is a term used to describe an intensified sensitivity towards pain (Pusceddu & Gareau, 2018). According to Distrutti, Monaldi, Ricci & Fiorucci

(2016) and Dupont (2014), the gut microbiome is correlated to visceral pain disorders. In fact, studies have shown that viral, parasitic and bacterial infections in the colon triggers IBS symptoms (Pusceddu & Gareau, 2018). A meta-analysis of 45 studies which included 21,421 people with intestinal inflammation (entiritis) showed that in a span of 12 months, the development of IBS increased more than 10% after the infection (Pusceddu & Gareau, 2018). This shows that a balanced population of the gut microbiome is very important in enteritis (Pusceddu & Gareau, 2018). In fact, this statement was based on the research review of Distrutti et al. (2016) who mentioned that a disturbed intestinal ecology leads to the development of IBS symptoms, thus manipulating the gut microbiome (i.e. expanding beneficial bacterial species such as *Lactobacilli* and *Bifidobacteria*) would result in a reduction in harmful microorganisms (i.e. *Clostridium, Escherichia coli, Salmonella Shigella* and *Pseudomonas*). This strategy would reduce the effect of IBS symptoms (Distrutti et al., 2016). With that said, it is essential to look further into the function and significance of the human gut microbiome.

The gut microbiome consists of 1000 species and 7000 strains of bacteria as a majority, and other microorganisms such as viruses, fungi, archaea, protozoa (Lloyd et al., 2016). The colon is heavily populated with these microorganisms, reaching a microbial concentration of $10^1 – 10^3$ cells per gram of fecal contents in the stomach, and up to $10^{11} – 10^{12}$ cells per gram of fecal contents in colon (Hyland & Cryan, 2016). This ecosystem has a symbiotic relationship with the host, which influences the organs, systems and functionality. The immune system is developed through the growth of the microbiota as a human grows and reaches adulthood (Gensollen, Iyer, Kasper, & Blumberg, 2016; Belkaid & Hand, 2014). Furthermore, the gut microbiome is also an important aspect in fermentation and gas production in the colon (Foley, Burgell, Barrett, & Gibson, 2014).

Altering the type of carbohydrates ingested, which preferably is low in FODMAPs, and the type of bacterial species would alter intestinal gas production (i.e. bloating sensations) (Foley et al., 2014). The treatment for IBS patients is exercising a low-FODMAP diet, excluding certain food items in their diet such as fat, alcohol, milk, and/or wheat as these would induce symptoms (Staudacher & Whelan, 2017). As there are many food restrictions for an individual who suffers from IBS, there will be an inadequacy of some important minerals such as calcium, or dietary fiber intake. Socializing would be difficult as social events and activities would include sharing of food (Staudacher & Whelan, 2017). Furthermore, other implications of practicing the low-FODMAP diet is the alteration of the gut microbiota. Most commonly it is the reduced abundance of *Bifidobacteria*. This effect is not yet known to have negative implications on the host, or whether it is long or short term. Nevertheless, the ingestion of *Bifidobacteria*, found in probiotic supplements, has shown to reduce symptoms of IBS (Venturi et al., 1999) and alleviate constipation (Kumemura et al., 1992; Kleessen, Sykura, Zunft, & Blaut, 1997) and prevent gastrointestinal infections through competitively excluding pathogens based on common binding sites of epithelial cells (Perdigon, Alvarez, Rachid, Aguero & Gobbato, 1995; Picard et al., 2005; Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2007). For example, *Clostridium perfringens* who is a toxin producer in the gut which causes diarrhea (Tanaka et al., 1983).

Overall, although there are some research studies surrounding IBS symptoms and dietary restrictions, it is still a complicated and multifactorial syndrome that requires further research as its main causes and cure are still undefined.

1.2.1.4 GOS Analysis

Research has been done to extract, purify and analyze oligosaccharides from a variety of legumes such as soybeans, chickpeas, lentils and mung beans.

1.2.1.4.1 Extraction and Purification

Extraction and purification of carbohydrates depends on the food that is being analyzed (Guo, Cui & Kang, 2014). Some aqueous food such as honey, syrups and fruit juice would require a simple and fast method to analyze its carbohydrate content (Guo, Cui & Kang, 2014). However, more elaborate foods such as beans, cereals, nuts, vegetables and breads would require a more extensive or elaborate extraction method, as the carbohydrates are chemically bound to the food matrix (Guo, Cui & Kang, 2014).

For an exhaustive extraction of carbohydrates, whole food samples (e.g. beans, nuts *etc.*) need to be ground into a flour and sifted, thus having the same particle size for even extraction (Pei, Zang & Xing, 2014; Gangola et al., 2014). If lipids are present in the sample, they need to be removed using non-polar solvents such as hexane or chloroform (Carlsson, Karlsson & Sandberg, 1992). This step enhances extraction yield and avoid contamination (Carlsson, Karlsson & Sandberg, 1992). Oligosaccharides and monosaccharides which have small molecular weight, could be extracted using 80% ethanol in the presence of heat (Gangola et al., 2014; Mosele et al., 2011; Dai et al., 2014; Sosulski, Elkowicz & Reichert, 1982). This initial heat extraction of carbohydrates would also extract minerals, vitamins, amino acids, organic acids and pigment into the ethanol supernatant (Brummer & Cui, 2005). These contaminants could be removed by adding clarifying agents (i.e. metal salts), into the extract or anion-exchange resin to remove charged unwanted molecules from the non-charged monosaccharides and oligosaccharides (Brummer &

Cui, 2005). The pellet which is left from the extraction would usually contain polysaccharides, cellulose, starch, pectin, protein and food gums (Guo, Cui & Kang, 2014). If any of these compounds are of interest, they could also be extracted using other solvents and physical purification techniques (Guo, Cui & Kang, 2014). Starch could be removed by adding α-amylase or amyloglucosidase (Guo, Cui & Kang, 2014). Water soluble polysaccharides could be removed using hot water and separated from the pellet using filtration or centrifugation. Insoluble polysaccharides are removed through mild base extraction (i.e 0.5 molar of potassium hydroxide (KOH) or sodium hydroxide (NaOH) solution) (Guo, Cui & Kang, 2014). Lastly, protein can be separated through physical (isoelectric precipitation), enzymatic (protease) and/or chemical methods (Staub, 1965; Guo, Cui & Kang, 2014).

1.2.1.4.2 Oligosaccharide analysis

The most common advanced analytical techniques used for the study of oligosaccharides are high-performance liquid chromatography (HPLC) techniques (i.e. high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-performance liquid chromatography coupled to a refractive index detector (HPLC-RI), and gas chromatography-mass spectrometry (GC-MS) (Gangola, Jaiswal, Khedikar, & Chibbar, 2014; Jones et al., 1999; Dai et al., 2014; Elkowicz & Sosulski, 1982). The GC-MS is usually the most preferred technique as it provides resolution power, robustness, sensitivity, and identification capabilities (Beveridge, Ford & Richards, 1977; Ruiz-Aceituno et al., 2017; Schweizer & Horman, 1981).

1.2.1.4.3 Current research to date

Based on recent studies, GOS content including raffinose, stachyose, and verbascose in legumes varies depending on internal and external factors such as origin, species, variety and farming conditions. Mung beans are a member of the legume family that is commonly eaten in Asian diets. In a recent study carried out by Carrero-Carralero et al. (2018), 6 different brands of mung beans originating from various origins were analyzed for GOS content. It was found that mung beans from Argentina had the lowest GOS content (68.2 mg/g of dry sample); whereas those from Spain with brand MBS2 had the highest levels of α-GOS (96.3 mg/g dry sample) (Carrero-Carralero et al., 2018). Out of the GOS, verbascose was the most abundant (50.4 – 74.03 mg/g dry sample), followed by stachyose (11.5 – 16.3 mg/g dry sample) (Carrero-Carralero et al., 2018). The concentration of raffinose was not reported. Results were obtained by an optimized microwave assisted extraction (MAE), and the GC-MS was used to characterize the bioactive carbohydrates (Carrero-Carralero et al., 2018). Prior to GC-MS analysis, mung bean extracts were volatilized using a two-step derivatization process; oximation (with 350µl, 2.5% hydroxylamine chloride in pyridine) and silylation (350µl hexamethyldisilizane (TMS) and 35µl trifluoracetic acid (TFA) (Carrero-Carralero et al., 2018). Carbohydrates of interest were identified by their linear retention indexes and mass spectra (Carrerro-Carralero et al., 2018). This derivatization method will be adopted and tailored for this thesis.

There is more research surrounding the analysis of galactooligosaccharides in peas, beans and lentils (e.g. chickpeas, kidney beans, navy beans, marama beans, soybeans). A variety of chromatography techniques have been used to analyze oligosaccharides profiles in these pulses; which includes HPAEC-PAD, GC-MS, HPLC-RI, GC-TMSi (Gangola et al., 2014; Carrero-

Carralero et al., 2018; Jones et al., 1999, Dai et al., 2017; Njoumi et al., 2019; Mosele et al., 2011; Da Silva Fialho et al., 2006; Xiaoli et al., 2008; Quemener & Brillouet, 1983; Frias, Hedley, Price, Fenwick, & Vidal-Valverde, 1994). A study conducted by Gangola et al. (2014), found that the most abundant α-galactoligosaccharide present in chickpea meal extracts was stachyose (1.88-2.83 mmol/100g chickpea meal), followed by raffinose (1.22-1.87 mmol/100g chickpea meal), and verbascose (0.06-0.14 mmol/100g chickpea meal). These results were obtained through the HPAEC-PAD, gradient mode with CarboPac PA 100 column. Another study conducted by Xiaoli et al. (2008), found that chickpea seeds contains 50% ciceritol which is another class of GOS, and 35% of stachyose. Here, Xiaoli et al. (2008), acquired the high-performance liquid chromatography with refraction index detector (HPLC-RID) for GOS analysis. Despite the chromatography instrument utilized, both studies showed the same trend for the abundancy for α-galactoligosccharide content in chickpeas: stachyose > raffinose > verbascose (Gangola et al., 2014; Xiaoli et al., 2008). However, Xiaoli et al. (2008) mentioned that verbascose could only be detected in seven chickpeas cultivars, as verbascose is dependent on genotype of the pea, whereas raffinose and stachyose levels are dependent on environmental factors (Trugo, Almeida & Gross, 1988). Furthermore, it must be highlighted that among all the studies reviewed, Gangola et al., 2014 were the only authors validating the chromatographic method employed, the linearity of calibration curves (R^2), limit of detection (LOD), limit of quantification (LOQ), recovery percentage, repeatability, and intermediate precision were calculated (Gangola et al., 2014).

Furthermore, Da Silva Fialho et al. (2006) analyzed α-GOS content in various cultivars of common beans (*Phaseolus vulgaris*) by means of the HPLC-RID with LC-NH2 Supelcosil column (5μm, 250 x 4.6 mm). It was concluded that stachyose was the major GOS in *Phaseolus vulgaris*

(Da Silva Fialho et al., 2006; Burbano et al., 1999). This finding is coherent with Queiroz, Oliveira & Helbig (2002) who also found that stachyose is most abundant in raw *Phaseolus vulgaris* which also used HPLC-RID with NH_2 column (12.5 x 4mm). Nevertheless, it is unclear whether the extraction method used was validated as Queiroz, Oliveira & Helbig (2002) adopted the extraction method from Vidal-Valverde et al. (1993). Thus, more studies are required to validate extraction methods for the bean matrix with low limits of detection.

Although stachyose is the dominant GOS present in chickpeas and common beans (*Phaseolus vulgaris*), as discussed in the four studies presented above, it is not always be true for all legumes. **Table 1** provides a summary of recent findings of GOS content present in mainly consumed pulses.

Table 1. A summary of common oligosaccharide contents (raffinose, stachyose, verbascose, ciceritol, ajugose) found in pulses.

	Galactooligosaccharide							
Pulse Type	**Raffinose**	**Stachyose**	**Verbascose**	**Ciceritol**	**Ajugose**	**Units**	**Analysis method**	**Reference**
Chickpeas (*Cicer arietinum*)	0.320 ± 0.030	1.770 ± 0.060	-	2.76 ± 0.100	-	% Dry basis	HPLC-RID	(Aguilera et al., 2009)
Lentils (*Lens culinaris*)	0.10 - 0.53	1.10 - 2.97	ND - 0.97	0.24 - 1.01	ND	% Dry basis	HPAC-PAD	(Frias et al., 1994; Vidal-Valverde et al., 1993)
Green Peas (*Pisum sativum*)	0.83 - 1.12	2.48 - 3.69	1.07 - 2.22	-	-	% Dry basis	HPAEC-PAC	(El-Adawy et al., 2003; N.Wang, Hatcher & Gawalko, 2008)
Cowpeas (*Vigna unguiculata*)	0.780 ± 0.060	3.530 ± 0.100	Not tested	Not tested	not tested	% Dry basis	Thin Layer Chromatography	(Egonunlety & Aworth, 2003)
Pigeon peas (*Cojanus cajan*)	0.520 – 1.220	0.720 – 1.820	3.600 – 7.600	-		% Dry basis	Solvent system (n-propanol, ethylacetate, and water 6:1:3) on cellulose thin-layer chromatogram	(Devindra et al., 2011; Mulimani & Devindra, 1998)
Common Bean (*Phaseolus vulgaris*)	0.130 - 0.71	3.040 - 3.77	0.023 - 1.51	-	-	% Dry basis	Thin Layer chromatography	(Silva & Braga, 1982)
Common Bean (Pink mottled cream) (*Phaseolus vulgaris*)	0.160 ± 0.020	2.530 ± 0.010	ND	ND	ND	% Dry basis	HPLC-RID	(Aguilera et al., 2009)
Common Bean (White) (*Phaseolus vulgaris*)	0.19 ± 0.080	2.480 ± 0.100	ND	ND	ND	% Dry basis	HPLC-RID	(Aguilera et al., 2009)
Mung Bean (*Vigna radiata*)	0.300 - 0.430	0.630 - 1.520	2.400	-	-	% Dry basis	HPLC-HR ESIMS	(El-Adawy et al., 2003; Fan, Zang & Xing 2015; SU & Chang, 1995)

(-): sugar not present; ND: sugar not detected; not tested: means not tested

Table 2. The continuation of Table 1.

	Galactooligosaccharide							
Pulse Type	**Raffinose**	**Stachyose**	**Verbascose**	**Ciceritol**	**Ajugose**	**Units**	**Analysis method**	**Reference**
Soybeans (*Glycine max*)	0.984 ± 0.008	1.646 ± 0.010	ND	-	-	% Dry basis	Ascending chromatography with mixture of n-propanol, ethyl acetate, and water (6:1:3 v/v)	(Singh & Kayastha, 2013)
Black gram (*Vigna mungo*)	0.36 ±0.000	0.420 ± 0.010	3.160 ± 0.020	-	-	% Dry basis	HPLC-RID	(Gingowda, Prashnath & Mulimani, 2005)
Adzuki (*Vigna angularis*)	2.00 ± 0.500	44.40 ± 4.700	2.400 ± 0.500	not tested	not tested	mmog/kg	HPLC-HRMS	(Pei, Zang & Xing, 2014)
Broad Bean (*Vigna faba*)	48.00 ± 1.400	10.10 ± 1.400	22.80 ± 4.00	not tested	Not tested	g/kg	HPLC-HRMS	(Pei, Zang & Xing, 2014)
Black Turtle (*Phaseolus vulgaris*)	3.77	11.95	ND	not tested	not tested	mg/g	HPLC-RID	(Phillips & Abbey, 1989)
Dark Red Kidney (*Phaseolus vulgaris*)	3.18	16.03	ND	not tested	not tested	mg/g	HPLC-RID	(Phillips & Abbey, 1989)
Pinto (*Phaseolus vulgaris*)	2.48	10.17	ND	not tested	not tested	mg/g	HPLC-RID	(Phillips & Abbey, 1989)
Romano (*Phaseolus vulgaris*)	0.48	0.52	ND	not tested	not tested	g/100g	HPLC with ESLD	(Biesiekierski et al., 2011)
White Navy (*Phaseolus vulgaris*)	4.52	11.9	ND	not tested	not tested	mg/g	HPLC-RID	(Phillips & Abbey, 1989)
White Kidney (*Phaseolus vulgaris*)	4.60 ± 0.40	44.20 ± 1.00	0.60 ± 0.20	not tested	not tested	g/kg	HPLC-HRMS	(Pei, Zang & Xing, 2014)

(-): sugar not present; ND: sugar not detected; not tested: means not tested

Based on the information gathered above, there is a clear balance in the consumption of pulses (i.e beans). Whether we would like to enhance GOS consumption for its prebiotic effects, or we would like to limit its presence in our food due to undesirable side effects such as increased gas production, it is necessary to have an accurate and reliable method in place to quantify GOS from our food. Very few studies have optimized and validated an efficient method to quantify GOS from beans, specifically. Therefore, this thesis project focuses on developing an optimized and validated extraction and chromatography method to achieve an efficient, reliable and accurate GOS quantification from bean matrix. Along with the quantification of GOS in beans, future studies should explore in finding an efficient and cost-effective technique to reduce it while preserving the bean matrix as this is important to expand product variety sold in the food industry. With an optimized and validated method for GOS extraction from beans, individuals especially IBS patients, could obtain nutritional benefits of beans, without compromising their overall quality of life.

1.3 Hypothesis

Three hypotheses are proposed for this thesis:

1) Gas chromatography coupled to a mass spectrometry (GC-MS) is a more reliable technique to study galacto-oligosaccharides (GOS) in beans in comparison with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)
2) The GOS content including raffinose, stachyose and verbascose significantly varies depending on the bean variety
3) Canned beans that are exposed to high treatment conditions have a lower GOS content

1.4 Research Objectives

The following are the main objectives for this thesis:

1) Optimize and validate an analytical and chromatographic method for the extraction and analysis of GOS from beans in an efficient and reliable manner
2) Compare two chromatography methods (HPAEC-PAD and GC/MS) to quantify GOS content in beans
3) Demonstrate the application of the optimized methodology to characterize and differentiate the GOS content in 6 bean varieties, and 4 canned bean samples exposed to low and high treatment conditions

2 Materials and Methods

2.1 Samples

2.1.1 Whole bean powder preparation

Six (6) kidney bean (*Phaseolus vulgaris*) varieties including Black turtle, Dark red kidney, Pinto, Romano, White navy, and Yellow eye bean were purchased in a local supermarket (Guelph, ON, Canada). To prepare whole bean flour, the raw beans were frozen with liquid nitrogen and subsequently ground with a grinder (Black and Decker, Mississauga, ON, Canada) for a minimum of two minutes. To obtain uniform particle size, the flour was sieved through a 250μm sieve (Retsch, Newton, USA). The bean flour samples were stored at –20°C until further GOS extraction and analysis.

2.1.2 Preparing Canned Bean flour and Brine flour

Canned red kidney beans exposed to low and high treatment conditions were donated from a multinational European company. Duplicates of all the treatments were obtained. Canned beans were separated from the brine using a regular kitchen standard sieve. Canned beans from the same treatment were mixed before mashing in a pestle and mortar. After a paste was obtained, a thin layer of bean paste was laid out in a plastic container, stored at -80 ^{o}C for at least 4 hours and freeze dried (Labconco, Kansas City, MO, USA) for 24 hours. For the brine, the two replicates from each treatment were combined and mixed. A portion of it was placed in a plastic container and stored at -80 ^{o}C for at least four hours before freeze drying for 24 hours. The freeze dryer was set to a pressure of 0.003 (mbar), with temperature of – 104 to 105 ^{o}C. All samples were stored at -80 ^{o}C until further extraction.

2.1.3 Moisture content measurement

Moisture content of the six (6) bean varieties, four (4) canned beans and their brines were measured with a moisture balance (Sartorius Canada Inc., Oakville, Ontario, Canada). One (1) gram of sifted bean flour was used and the moisture percentage for each sample obtained. Measurements were performed in duplicate.

2.2 GOS Extraction Method

The GOS were extracted as follows: 200 milligrams (0.2 g) of bean flour were weighed and mixed with 3mL of 70% ethanol solution. The mixture was subsequently homogenized with a vortex (Thermo Fisher Scientific, Mississauga) for 10 seconds, and sonicated for 2 hours at 80 ^{o}C (Ultrasonic Cleaner Digital Pro, Flexzion, Los Angeles, CA, USA). After cooling down the samples at room temperature, they were centrifuged for 10 min at 3500 rpm and the supernatant collected. To ensure a complete extraction of the GOS, another 3 ml of 70% ethanol solution was added to the pellets and the samples were vigorously shaken for 30 minutes using a multi-wrist shaker (Lab-Line, Markham, ON, Canada). Samples were then centrifuged under the same conditions described above and both were supernatants combined. A final 15 minutes centrifugation cycle with the combined supernatants was performed to ensure no remaining particles were present in the mixture.

2.2.1 Extracts Clean up: Carrez procedure and Solid Phase Extraction (SPE)

The Carrez reagent is used to selectively precipitate colloidal interferences such as fats, proteins, and redox compounds (Sigma Aldrich, 2020; Culhaoglu, Zheng, Mechin, & Baumberger, 2011). It consists of two reagents: (1) Carrez I, a zinc acetate solution to precipitate polysaccharides, and (2) Carrez II, a potassium ferrocyanide solution which precipitates excess

zinc acetate (Culhaoglu et al., 2011). To test the effectiveness of the Carrez procedure, 250µl Carrez I diluted 1:100, and 250µl Carrez II diluted 1:100 were added to the total supernatant collected from both heat extraction and wrist shaking. The mixture was then wrist shaken for 5 minutes at room temperature, maximum speed, and then centrifuged at 3500rpm for 15 minutes. The supernatant was collected and dried using a Nitrogen evaporator. 2mL of 70% ethanol solution was added to reconstitute the dried sample.

To test if the solid phase extraction (SPE) process could improve the extraction, remove possible interferences, and concentrate the GOS, solid phase extraction (SPE) was performed in red kidney beans extracts as follows: the solvent of the sample extracted was evaporated until complete dryness under a gentle stream of nitrogen using the N-EVAP 112 nitrogen evaporator (Organomation, Fisher Scientific Mississauga, ON, Canada) and the remained dry pellet reconstituted in 3 mL of Milli-Q water. For the SPE, OASIS HLB 1cc (30mg) cartridges with a polymeric reversed-phase sorbent were employed. SPE was performed as follows:

a. Cartridges were activated using 5 mL of 100% methanol.
b. Aqueous samples were loaded by adding 1 mL, 3 times.
c. Soluble interferences were eluted by adding 1mL of NaH_2PO_4 0.05 M (pH 3).
d. The aqueous samples containing the GOS were collected from the cartridge by adding 3 mL of 70% ethanol solution.

For the HPAEC-PAD analysis, samples collected after SPE were dried and reconstituted in 2 mL of Milli-Q water under sonication for 2 minutes and immediately analyzed. For the GC/MS analysis, samples collected were kept in 70% ethanol and immediately derivatized and analyzed.

2.3 Chromatographic Analysis of GOS

2.3.1 High-performance anion exchange chromatography – pulsed amperometric detection (HPAEC-PAD) materials, reagents and standard solution preparation

Several solutions were prepared to analyze GOS by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-300, Fisher Scientific, Mississauga, ON, Canada). NaOH solution concentrations from 1M NaOH solution (Fisher Scientific, Mississauga, ON, Canada) were prepared to test which one was the best mobile phase in the analysis of GOS by HPAEC-PAD. NaOH solution was sonicated for 5 to 10 minutes and then bubbled with helium for approximately 15 minutes to minimize bi-carbonation formation in the mobile phase. A standard stock solution (D-RSV) of raffinose (500ppm), stachyose (250ppm), and verbascose (25ppm) was prepared in Milli-Q water. A 1 Litre, 500mM stock solution of sodium acetate (CH_3COONa) (Sigma Aldrich, Oakville, Ontario, Canada) was prepared by dissolving 41.017 grams of CH_3COONa in 600mL of Milli-Q water with a sonicator, then the solution is made up to 1 Litre. The solution was filtered through a Millipore filter apparatus (Millipore Canada, Etobicoke, Ontario, Canada) to remove traces of salt from the sodium acetate solution. A calibration curve was developed with a concentration ranging from 500 to 10 ppm in the case of raffinose, 250 to 5 ppm in stachyose and from 25 to 2.5 ppm in the case of verbascose.

2.3.2 HPAEC-PAD chromatographic conditions

Extracted GOS mixtures were filtered through a 0.22 μm filter (Fisher Scientific, Mississauga, ON, Canada) to ensure no particles remained in the solution before the chromatographic analysis. A Dionex ICS 3000 high-performance anion exchange chromatography-pulsed amperometric detector (Fischer Scientific, Mississauga, ON, Canada) was used. Column separation effectiveness was evaluated on Dionex CarboPac PA-100 (4x250mm)

and CarboPac PA-100 (4x250mm) columns (Thermo Scientific, Mississauga). In both cases, a Dionex Guard (4x50 mm) precolumn with the same stationary phase as the column used. The column and precolumn were at room temperature during the analysis. A constant flow rate of 1.0 mL/min and an injection volume of 25μL were employed. The system used an AgCl reference electrode. Different waveform potentials were tested and will be described in the results and discussion section. Both pumps and the detector were purged before the first injection. The whole system assembly was controlled by Chromeleon 7.2 SR4 Software (Thermo Scientific, Dionex, Chromeleon, Mississauga, ON, Canada)**.**

2.3.3 GOS analysis by Gas Chromatography/ Mass Spectrometry (GC/MS)

2.3.4 Gas Chromatography/ Mass Spectrometry (GC/MS) standard solution and derivatization materials preparation

Several solutions were prepared to analyze GOS by gas chromatography/ mass spectrometry (Agilent Technologies, Mississauga, ON, Canada). As an internal standard (IS), a stock solution of 125 ppm phenyl-β-D-glucoside (Sigma Aldrich, Oakville, ON, Canada) was prepared in a volumetric flask by dissolving 1.25 mg of phenyl-β-glucoside in 10mL of 100% methanol. In addition, a standard stock solution, named G-RSV, of IS (2000 ppm), raffinose (2000ppm), stachyose (1000 ppm), and verbascose (3000 ppm) was prepared in 100% methanol. A matrix-matched calibration curve was prepared by spiking the GOS extracted from red kidney bean (blank) with 1 ml of G-RSV. A non-spiked blank was also analyzed. Seven points were included in the calibration curves and the non-spiked blank sample was employed to subtract the blank signal. For the IS and raffinose with the calibration curve ranged from 2000 to 100 ppm, for stachyose from 1000 to 50 ppm, and for verbascose from 3000 ppm to 200 ppm. For the oximation and silylation of the galacto-oligosacccharides, a 2.5% hydroxylamine in pyridine solution was

prepared in a 250mL volumetric flask by dissolving 6.25g of hydroxylamine chloride (Sigma Aldrich, Oakville, ON, Canada) in 250mL of pyridine (Fisher Scientific, Mississauga, ON, Canada). Tetramethylsilane (TMS) and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (Oakville, ON, Canada).

2.3.5 Derivatization of GOS: Oximation and Silylation

GOS extraction mixture solvent (sections 2.2 and 2.2.1) was evaporated with a N-EVAP 112 Nitrogen Evaporator (Organomation, Fisher Scientific, Mississauga, ON, Canada). 1mL of IS (phenyl-β-glucoside, 125ppm) was added and dried. To ensure the complete water removal from the sample, 3mL of acetone (Fisher Scientific, Mississauga, ON, Canada) were added and evaporated. For the oximation process, 500µL of 2.5% hydroxylamine chloride solution were added, vortexed to fully reconstitute the dried sample and incubated at 75ºC for 30 minutes in a Thermomixer F2.0 (Eppendorf, Mississauga, ON, Canada). After cooling down the sample at room temperature, silylation was initiated by adding 1 mL of tetramethylsilane (TMS) and 100µL of trifluoroacetic acid (TFA). The mixture was incubated at 45ºC for 1 hour. After incubation, samples were centrifuged with a Legend Micro 21R microcentrifuge (Thermo Scientific, Mississauga, ON, Canada) for 15 minutes at 20g. To avoid moisture in the vial, the headspace of the vial was flushed with a gentle stream of nitrogen for 60 seconds and stored in a -20 ºC freezer until further analysis by GC/MS.

2.3.6 GC/MS Instrumentation Conditions

GC/MS analysis of the derivatized GOS was done using a 7890A gas chromatography coupled to a 5975C inert XL EI MSD with Triple-Axis Detector Mass Spectrometer (Agilent Technologies, Mississauga, ON, Canada). The silylated GOS were separated in a HT-5 (5% Phenyl

Polycarborane-siloxane) column (12m x 0.22mm I.D., 0.1 µm film thickness) (SGE, Ringwood, Australia). Helium was utilized at constant flow rate of 1mL/min as a carried gas. The temperature gradient used was as follows: oven temperature was initially set at 155°C (10 min hold), increased to 200 °C at 5 °C/min (15 min hold), to 270 °C (1min hold), to 270 °C at 15 °C /min (1min hold), to 290 °C at 15 °C /min (1min hold), to 300 °C at 15 °C /min (15min hold) and to 350 °C (15min hold). Injection volume was 3 µL with a solvent delay of 10 min. Total run time was 76.0 min. Mass spectra were recorded at an ionisation energy of 70 eV, with data acquisition conducted in the scan mode from 20 to 450 m/z range. The injector was set at 320 °C working in a splitless mode for the analysis of verbascose and in split 1:100 mode for raffinose and stachyose analysis. The detector and ionization source were at 320 and 230 °C respectively. The system was operated with a ChemStation Software version E. 02. 02 (Agilent Technologies, Mississauga, Ontario, Canada). Identification of the GOS was based on the comparison of the retention time and target ion of the compound with the individual raffinose, stachyose and verbascose standards mass spectra. At least 2 replicates were employed per bean variety and canned beans served as the experimental treatment.

2.4 Validation

Method validation is required to ensure that the developed and optimized method is robust and reliable to analyze the biological matrix of interest (Rogatsky & Stein, 2005). In this work, whole red kidney beans were used to obtain information on the strength of the matrix effect, the sensitivity of the chromatography conditions and the reliability of the HPAEC-PAD and the GC/MS techniques to study GOS. The validation method was carried out according to AOAC

guidelines (2002) by establishing the linearity (intraday and interday repeatability), percentage recovery, and matrix effect.

2.4.1 Linearity

Calibration curves were developed at nine and seven different concentrations for HPAEC-PAD and GC/MS analysis respectively. The calibration curve of each standard was established by plotting the area under the peak of the standard in the chromatogram and the known concentrations. The linear regression and the coefficient of determination (R^2) value were calculated. The plots of the calibration curve, together with linear regression and the R^2 values are shown in **Appendix 1**. For the GC/MS analysis, two types of calibration curves were created: high concentration raffinose (2000 to 1200 ppm), stachyose (1000 to 150ppm), verbascose (3000 to 1000ppm) and low concentration of raffinose (400 to 100 ppm), Stachyose (200 to 50ppm), verbascose (1000 to 250ppm) (see **Appendix 2** and **3**). This was performed because the linear relationship between concentration of GOS standards and area was lost at a certain point. For the HPAEC-PAD analysis only one calibration curve was created.

2.4.2 Precision: Inter- and Intraday repeatability

For the intra-day repeatability, GOS extracted from Red kidney beans were injected in duplicate. For the inter-day repeatability, GOS extracted from Red kidney beans were injected in duplicate on three alternate days. The RSD (%) of raffinose, stachyose and verbascose for inter- and intraday repeatability was calculated.

2.4.3 Accuracy: Quantification of GOS

The percentage recovery (%R) and matrix effect (%ME) were calculated. To this aim, blank, matrix effect and spiked red kidney beans samples were prepared as follows: *Blank bean samples* (control samples) were extracted and injected in the HPAEC-PAD and GC/MS as outlined in their respective methods (sections 2.2). *Spiked bean samples* were spiked with 200µL of D-RSV (HPAEC-PAD) or 200µL of G-RSV (GC-MS) into the dry bean flour. The wet sample was dried using a gentle stream of nitrogen at 55 – 60 ^{o}C for approximately 45 minutes, until a completely dry powder was obtained. To prepare the *Matrix Effect samples* for HPAEC-PAD analysis, 200µL of D-RSV were added to the 3mL of extracted GOS mixture collected from the SPE (section 2.2.1). This solution was dried using a N-EVAP nitrogen evaporator (Organomation, Fisher Scientific, Mississauga, ON, Canada). Then 1.8mL of Milli-Q water was added to reconstitute the dried sample, making a total volume of 2mL. To prepare Matrix Effect samples for GC/MS analysis, 200µL of G-RSV were added to 3mL of extracted GOS mixture collected after extraction (section 2.2) and after SPE cleaned up (section 2.2.1). Matrix effect samples were dried using the N-EVAP Nitrogen evaporator and 3mL of acetone added and evaporated to remove possible remaining moisture from the powder. Samples analyzed by GC/MS were oximated and silylated as described in section 2.4.2. The percentage recovery (%R) and the matrix effect (%ME) of raffinose, stachyose and verbascose were calculated using the following equations:

$$\%\,R = \frac{Area\ of GOS\ peak\ in\ Spiked\ sample}{Area\ of\ GOS\ peak\ in\ Matrix\ Effect\ sample} \times 100$$

$$\%ME = \frac{Area\ of\ GOS\ peak\ in\ Matrix\ Effect - Area\ of\ GOS\ peak\ in\ Blank}{Area\ of\ GOS\ peak\ in\ solution} \times 100$$

To quantify GOS expressed as mg GOS per 100 g of dry bean, the calibration curves for raffinose, stachyose and verbascose, the matrix effect and the moisture content were considered:

$$\frac{mg}{100g}GOS = \frac{Area\ Sample - Intercept\ \times 0.0016L}{Slope\ \times Weight\ sample\ (dry\ basis, g)} \times 100$$

2.4.4 Statistical analysis

Statistical analysis was conducted using XLSTAT software to analyze variations of the GOS content in six (6) bean varieties, four (4) canned beans and four (4) brines. Two biological replications of each sample were analyzed in duplicate. One-way analysis of variance (ANOVA) was used to determine if there were significant differences between the samples. Where GOS content was significant, the means were compared with Fisher's Least Significant Difference (LSD), $\alpha = 0.05$. Statistical analysis and figures were done using XLSTAT Premium version.

3 Results and Discussion

3.1 Optimization of the chromatographic conditions

The HPAEC-PAD and GC/MS method optimization was performed with the individual retention times of raffinose (R), stachyose (S), and verbascose (V) standards. A stock solution of GOS standards (RSV) was also prepared in 100 % MiliQ water (D-RSV) and 100 % methanol (G-RSV) for HPAEC-PAD and GC/MS analysis, respectively. The standards retention times and the areas of the peaks detected were used to select the optimum chromatographic conditions for GOS analysis.

3.1.1 HPAEC-PAD

3.1.1.1 Optimization of the mobile phase and chromatographic column

Sodium hydroxide (NaOH) and sodium acetate (CH_3COONa) in isocratic and gradient modes as mobile phases, and two different chromatographic columns (CarboPac-PA1 and CarboPac-PA100) were evaluated to separate and analyse GOS by HPAEC-PAD. To test the mobile phases and columns efficiency, D-RSV stock solution was used to identify the standard peak retention times and the peak area produced. Eleven (11) different gradients of 145mM of 100% NaOH as eluent A and 12.5mM, 25mM and 75mM sodium acetate (CH_3COONa) as eluent B were evaluated with the CarboPac-PA1 chromatographic column. Eluent A was tested with a binary isocratic condition ranging from 85 – 99%. From these tests, it was found that Eluent A: 145mM NaOH and Eluent B: 145mM NaOH + 75mM CH_3COONa, with the gradient given in **Table 3**, obtained the best chromatographic results of GOS. This is, the retention times of raffinose, stachyose, and verbascose were 7.54, 8.08, 9.64 min respectively; however, the resolution of raffinose and stachyose was not efficient as the two peaks coeluted (**Figure 7**). Due to this, CarboPac-PA100 column was tested with twelve (12) different concentrations including 0.25, 1, 10, 15, 37.5, 75, 100, 145, 175, 200, 250, 500mM of 100% NaOH as this column has been described to be very efficient in the separation of oligosaccharides (Sancho, Souza, de Lima & Pastore 2017; Frias et al., 1994; Frias et al., 1996). Results demonstrated that the 10mM 100% NaOH mobile phase with a CarboPac-PA100 column resulted in the most efficient separation of raffinose, stachyose and verbascose peaks at 2.18, 2.35 and 2.87 minutes respectively.

Table 3. Gradient condition with Eluent A: 145mM NaoH and Eluent B: 145mM NaOH + 75mM CH_3COONa in HPAEC-PAD.

Time (min)	% A	% B
-5	99.5	0.5
0	99.5	0.5
0.5	100	0
20	100	0

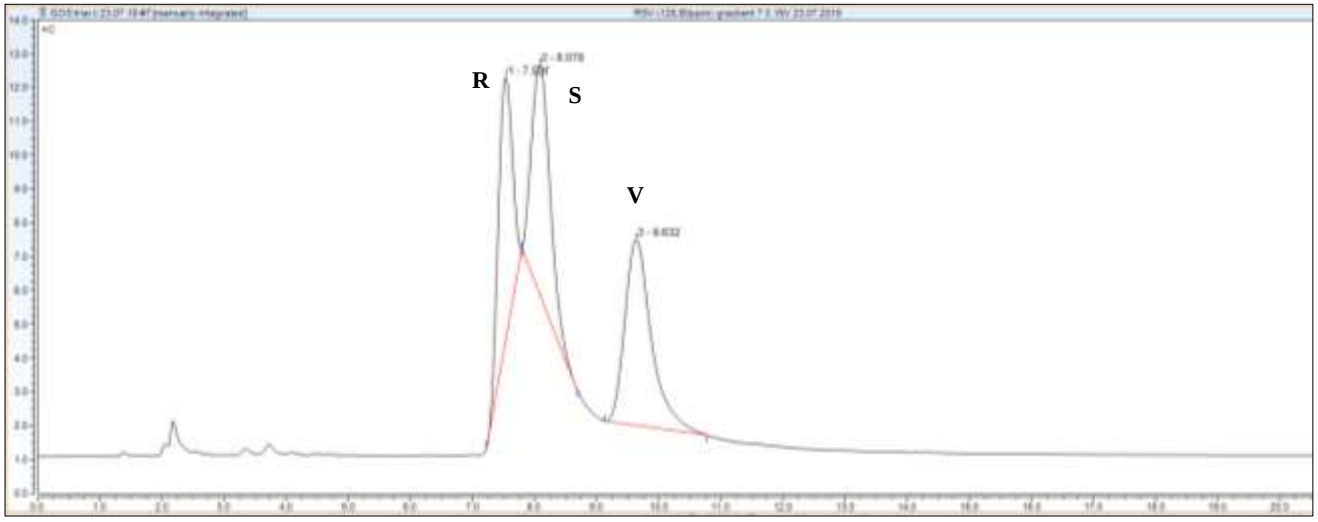

Figure 7. Chromatogram showing the co-elution of (R) raffinose (125ppm), (S) stachyose (125ppm) and (V) verbascose (50ppm) with Eluent A: 145mM NaOH and Eluent B: 145mM NaOH + 75mM CH3COONa, in gradient mode (Table 3) obtained by HPAEC-PAD.

3.1.1.2 Optimization of Waveform potentials

The optimization of the pulse amperometric detector (PAD) was conducted by optimizing the current waveform potentials. PAD is based on the application of triple-step potential waveforms (E1, E2 and E3) to integrate amperometric detection with alternated anodic and cathodic polarizations to clean and reactivate electrode surfaces (Lacourse et al, 1991) (**Figure 8**).

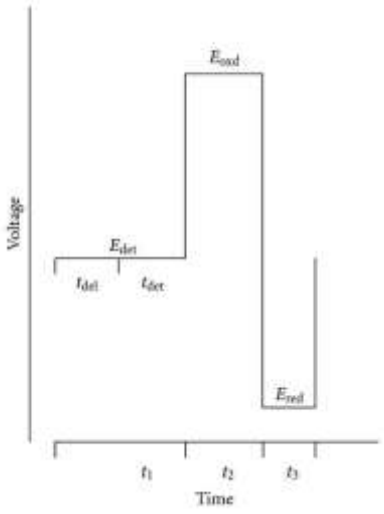

Figure 8. A diagram of a triple-pulsed potential waveform PAD. Edet : referred as E1, is the constant potential employed during t1; Eoxd : referred as E2, is the positive oxidation working potential during t2; Eoxd : referred as E3, is the negative reducing working potential during t_3.

The waveform detector potential E1 was tested from +0.05V to +0.20V for 300msec. The E2 (oxidation) and E3 (reduction) waveform potentials were maintained at +0.60V (120msec) and -0.60V (300msec) respectively, in accordance with the methodology of other authors (Frias et al., 1994). The highest area of the D-RSV standard mix generated from all the waveform potential tests was obtained with E1: 0.15V. Thus, the optimal waveform potential in the GOS standard mix analysis was E1: +0.15V (300msec), E2: +0.60 (120msec), E3: -0.60V (300msec). However, this waveform potential conditions produced great signal for standards but low signals for bean samples. Hence, the Gold AAA waveform potential with the parameters shown in **Table 4** was also tested, resulting in a significant increase in signal of raffinose, stachyose, and verbascose in bean samples (**Figure 9**). This waveform was successfully selected for GOS analysis on beans.

Table 4. Gold Standard AAA PAD waveform potentials

No.	Time (s)	Voltage (V)	Gain Region	Ramp	Integration
1	0	-0.2	Off	ramp	Off
2	0.04	-0.2	Off	ramp	Off
3	0.05	0	Off	ramp	Off
4	0.21	0	On	ramp	On
5	0.22	0.22	On	ramp	On
6	0.46	0.22	On	ramp	On
7	0.47	0	On	ramp	On
8	0.56	0	Off	ramp	Off
9	0.57	-2	Off	ramp	Off
10	0.58	-2	Off	ramp	Off
11	0.59	0.6	Off	ramp	Off
12	0.6	-0.2	Off	ramp	Off

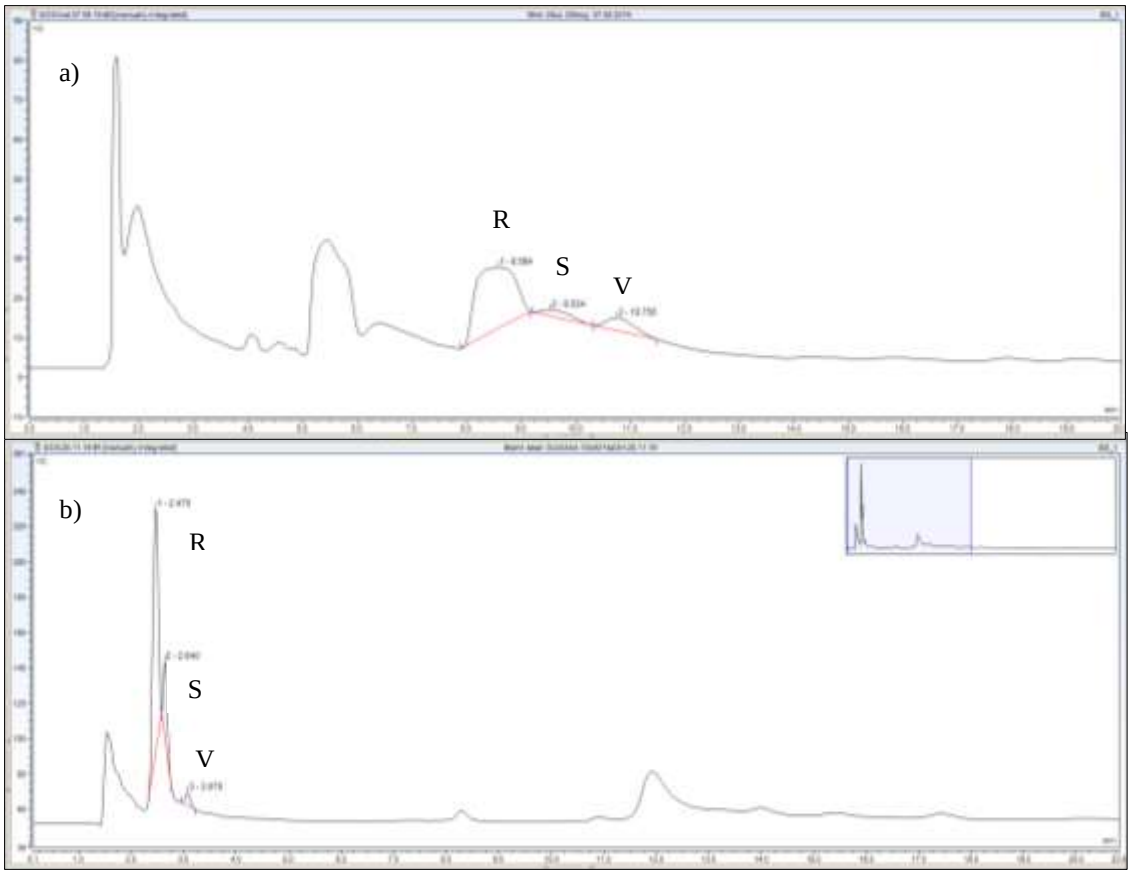

Figure 9. Comparison of the retention times and peak resolution of raffinose (R), stachyose (S), verbascose (V) extracted from red kidney beans and studied by HPAEC-PAD using (a) E1: 0.15V and (b) Gold AAA potential.

3.1.2 GC/MS

3.1.2.1 Optimization of the oximation and silylation procedure

Oligosaccharides are non-volatile polar molecules that contain hydroxyl groups. To analyze them by GC/MS, a derivatization process is needed to convert them in volatile compounds (Tea & Tcherkez, 2017). Derivatization is a two-step process: (1) oximation and (2) silylation. *Oximation* is the condensation reaction between ketones and aldehydes with hydroxylamine (NH_2OH) in pyridine to produce oximes or ketoximes (see **Figure 10**).

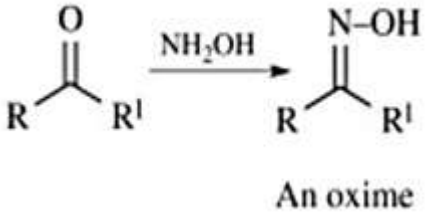

Figure 10. Ketone reacting with hydroxylamine (NH2OH) to produce an oxime. (Smith, 2017).

This reaction blocks the anomeric carbons such that reducing sugars are unable to return into their cyclic structure (Smith, 2017; Ruiz-Matute, Hernandez-Hernandez, Rodriguez-Sanchez, Sanz & Martinez-Castro, 2011). This fact is important when analyzing oligosaccharides by GC/MS because it avoids the appearance of multiple peaks in the chromatogram due to sugar isomerization (Ruiz-Matute et al., 2011). GOS compounds such as raffinose, stachyose and verbascose are non-reducing sugars; however, up to 0.82% of total soluble sugars are reducing sugars in beans (Ruiz-Matute et al., 2011; Rehman, Salariya, & Zafar, 2001); hence, this reaction is important to obtain clear chromatograms of bean oligosaccharides.

The second step in the derivation process is the *silylation*, a reaction that converts non-volatile oligosaccharides to volatile molecules by replacing -OH groups of oximes with tri-methyl silyl groups (TMS) (see **Figure 11**) (Ruiz-Matute et al., 2011; Tea & Tcherkez, 2017). The combination of silylation and oximation reactions generates a molecule named trimethylsilyl oximes (TMSO).

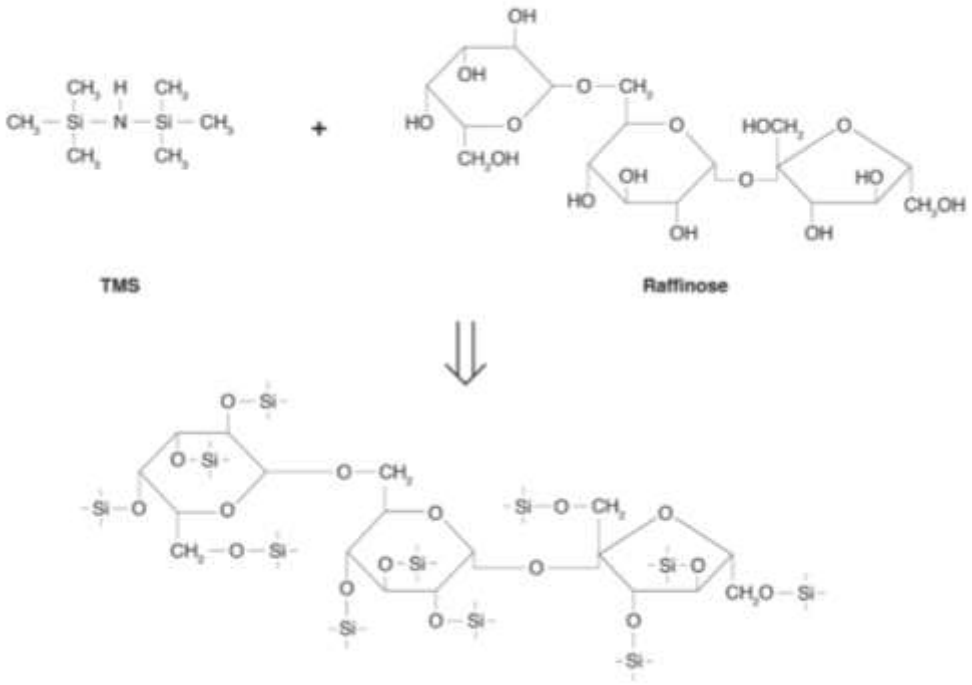

Figure 11. The condensation reaction of tri-methyl silyl groups (TMSO) displacing hydroxyl groups (OH) of raffinose, creating an unstable TMS-raffinose molecule.

The optimization of oximation and silylation procedures were conducted following the method described by Ruiz-Aceituno et al. (2017). Hydroxylamine, hexamethyldisilizane (TMS) and trifluoracetic acid (TFA) volumes as well as incubation temperature and time were optimized to achieve a complete silylation of the alpha-galactooligosaccharides. To do this, retention time and peak areas of raffinose, stachyose, and verbascose standards were considered.

The Oximation process was tested by dissolving 8mg of raffinose and stachyose and 2mg of verbascose standard in 500μL of 2.5% hydroxylamine in pyridine and incubating the solution at 75 ºC for 30 minutes. (Ruiz-Aceituno et al., 2017; Ruiz-Matute et al., 2011). For the *silylation process*, different temperatures (45, 60, 100 ºC) and incubation times (30 and 60 min) were studied to identify the optimum silylation conditions. In all these tests, 500 μL TMS and 50 μL of TFA (10:1; v/v) were used. The incubation temperature at 45 ºC for 60 min showed a chromatogram with symmetric and sharp peaks of raffinose, stachyose and verbascose at the retention times, with the target ion (the most abundant ion in the mass spectrum) and the areas shown in **Table 5**.

Table 5. Molecular weight, boiling point, retention time, target ion, and area counts obtained from the peak integration of the target ions from the chromatographic analysis of partially silylated raffinose, stachyose and verbascose standards.

Standard	Molecular weight	Boiling point (ºC)	Retention time (min)	Target Ion	Area counts
Verbascose	828.72	1185	34.00	252	921,243
Stachyose	666.58	1044	36.00	277	8,573
Raffinose	504.42	884	41.65	361	8,527

As it can be observed, the order of elution of these oligosaccharides was verbascose, stachyose and raffinose; this is, the highest molecule with the highest number of hydroxyl groups eluted first. However, considering that the hydroxyl groups have been silylated through the derivatization process and the molecules are converted to non-polar compounds, the order of elution should be based on the molecular weight and the boiling point of the molecules (the smallest compounds with lowest boiling points eluting first) due to the affinity of non-polar compounds (the complete silylated GOS) to non-polar capillary column (HT5 column) (Ruiz-Aceituno et al., 2017). Thus, it was hypothesized that this elution (retention times) and target ions of GOS were a result of a *partially silylated* GOS which contained non-silylated hydroxyl groups and that a complete

silylation needed to be achieved in order to properly analyzed GOS in beans. Moreover, due to poor peak reproducibility of the standards, evaporation of the ethanol solvent by a gentle stream of nitrogen steps followed by the addition and evaporation of acetone was also included in the procedure. The latter step aims to remove excess moisture which enhances the stability of TMS-derivatives as they are highly sensitive to moisture that are naturally present in the air (Gutnikov & Scott, 2000; Dasgupta, 2009).

To achieve a *complete silylation* of GOS, TMS and TFA volumes were increased to 1000μL and 100μL respectively to completely displace hydroxyl groups on sugars with silylmethyl groups (TMS). The incubation temperature and time remained unchanged (45 ^{o}C for 60 min). After the addition of TMS and TFA to the oximated sample, an opaque white precipitate formed by the reaction of hydroxylamine with excess TMS was formed indicating that there was sufficient reagent to produce the reaction. The chromatographic analysis of the complete derivatized GOS showed three symmetric and sharp peaks at 32.00, 40.18 and 73.65 min corresponding to the internal standard (IS), raffinose (R) and stachyose (S), respectively. These retention times were obtained with the initial temperature gradient given in **Table 6**. However, with the complete silylation of GOS, verbascose (V) did not elute and an optimization of the chromatographic temperature gradient was necessary.

3.1.2.2 Optimization of the temperature gradient

The initial temperature gradient conditions were adapted following Fuzfai, Katona, Kovacs & Molnar-Perl (2004) with slight modifications (see **Table 6**). However, as above commented, verbascose has a high boiling point (1185 ^{o}C) and high molecular weight (828.72 g/mol) and could

not be eluted under these conditions; thus, an optimization of the gradient with higher temperatures was needed.

Table 6. Initial temperature gradient in the GC/MS method for the analysis of GOS.

ºC/min	Temperature (ºC)	Time (min)
	60	2
20	120	3
6	155	16
13	250	35
50	325	45

Table 7 shows the ramp temperature developed. A higher temperature (155 ºC) was set as initial point with a hold time of 10 min. Then, the temperature was increased to 200 ºC with a hold time of 15 min., an increase until 300 ºC was set at 15 ºC/min with a hold time of 15 min. and the final temperature was achieved at 350 ºC and it was held there for 15 min to ensure the complete elution of the target compounds. Total run time was 76.0 min.

Table 7. Final temperature gradient in the GC/MS method for the analysis of GOS.

ºC/min	Temperature (ºC)	Time (min)
	155	10
5	200	15
15	270	1
15	290	1
15	300	15
15	350	15

This temperature ramp was tested with a stock solution of 125 ppm internal standard, 25ppm raffinose, 125ppm stachyose, and 500ppm verbascose. As observed in **Figure 12a** and **b** in which an overlapped chromatogram of the standards run with the temperature gradient of Table 6 and 7 respectively is shown, and the retention times, target ions and peak areas of the target ions of the

standards run with the temperature gradient of Table 7 are given. The temperature gradient in Table 7 was found to be efficient in the analysis of GOS standards and thus, the GC/MS method was optimized for the analysis of GOS in beans.

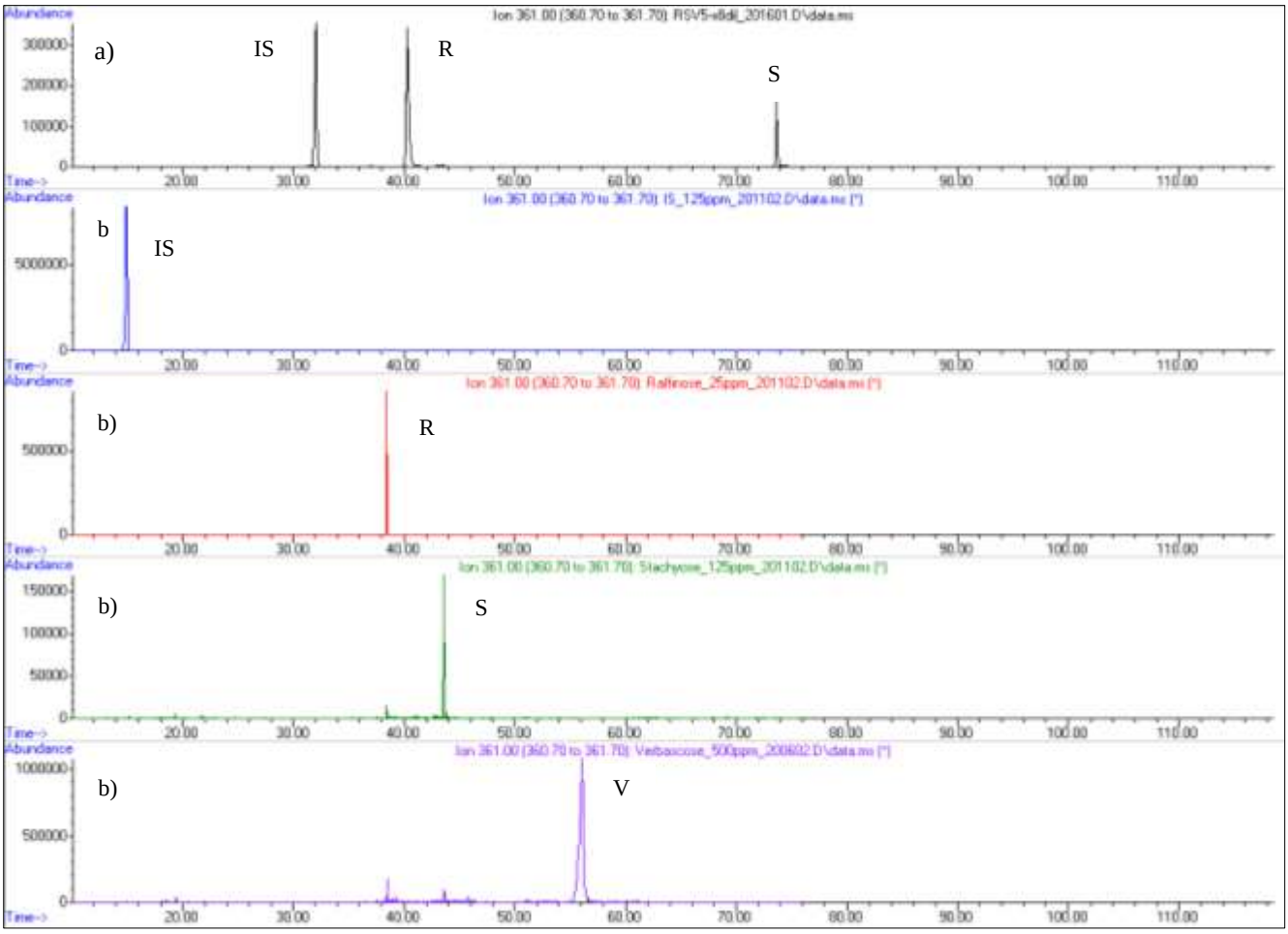

Figure 12. Retention time of internal standard (IS), raffinose (R), stachyose (S), and verbascose (V) with the **a)** initial temperature gradient shown in Table 6, and **b)** with final temperature gradient shown in Table 7.

Table 8. Retention times, target ions and area counts obtained from the peak integration of the target ions obtained from the analysis of silylated raffinose, stachyose and verbascose solution by GC/MS with the temperature gradient shown in Table 7.

Sample	Retention time (min)	Target Ion	Area counts
Raffinose	38.42	361.2	1,921,611
Stachyose	43.57	361.2	855,988
Verbascose	56.08	361.2	27,267,903

3.1.3 Development and Optimization of GOS extraction procedure from beans

As above commented, the GOS extraction optimization method was performed with whole red kidney beans, as they are common beans. A revision on the literature available showed that there is a wide range of different parameters used for the GOS extraction. This is, from 0.1 mg to 1.0 g of sample, heating temperature varies from 50 to 90 ^{o}C, the extraction time from 20 min to 120 min, ethanol concentration and volume from 50 to 85% and 3 to 10 mL respectively (Gangola et al., 2014; Carrerro-Carralero et al., 2018; Bainy, Tosh, Corredig, Poysa, & Woodrow, 2008; Dai et al., 2014; Njoumi et al., 2019; Mosele et al., 2011). To ensure an efficient and optimized extraction of GOS from beans, several extraction conditions were tested, including the amount of sample, solvent concentration, extraction temperature and time, as well as the use of solid phased extraction (SPE) procedure to remove sample interferences. A total of eight (8) experiments were carried out, with test 1 as the control (**Table 9**). Wrist shaking for 15 and 30 minutes was also added to tests 1, 3, 6, and 8 to observe whether an increase in GOS extracted was obtained. The supernatant collected from heat treatment and wrist shaking were individually analysed by HPAEC-PAD to test the extraction efficiency.

Table 9. Summary of the GOS extraction conditions in red kidney beans including sample weight expressed in mg, ethanol percentage (%), temperature of extraction (ºC), and heating method, together with the retention time (RT) and peak area counts of raffinose, stachyose, verbascose obtained by HPAEC-PAD.

	GOS Extraction conditions				Raffinose		Stachyose		Verbascose	
Test	**Weight (mg)**	**Ethanol (%)**	**Temp. (ºC)**	**Heating method**	**RT (min)**	**Area**	**RT (min)**	**Area**	**RT (min)**	**Area**
1*	100	80	90	Plate	11.162	5.1469	11.791	0.3979	14.196	0.5534
2	200**	80	90	Plate	11.039	7.5923	11.532	0.2854	14.073	0.8986
3	100	80	90	Plate	11.063	4.1466	11.335	0.5886	14.048	0.9535
4	100	50	90	Plate	11.026	5.2624	11.384	0.2957	13.912	0.857
5	100	60	90	Plate	10.977	5.5315	11.618	0.4281	13.875	0.8363
6	100	70**	90	Plate	10.927	5.4129	11.322	0.392	13.826	0.8248
7	100	80	75 - 80	Sonication	10.866	6.1846	11.31	0.3369	13.789	0.8616
8	100	50	75 - 80	Sonication**	10.792	5.6938	11.162	0.2799	13.653	0.9661

1* Control experiment; ** Parameters chosen for optimized method of extraction; Parameters chosen for optimized method were compared to areas of raffinose, stachyose and verbascose of the control (1*)

As it can be observed in **Table 9**, the experimental conditions which led to an increment in peak area of raffinose, stachyose and verbascose, marked in orange colour and **, compared to the control (test 1) were chosen and combined to be the proposed optimized method of extraction of GOS from beans. In addition, wrist shaking increased GOS extraction by 1.7 – 2.5 and 1.4 times of raffinose and verbascose respectively, as observed in the areas summarized in **Table 10**.

Table 10. Retention time and peak area of raffinose, stachyose and verbascose from supernatant obtained after wrist shaking of bean pellet with its corresponding ethanol concentration (refer to Table 9).

		Raffinose		Stachyose		Verbascose	
Test	Wrist Shaking (min)	RT (min)	Area	RT (min)	Area	RT (min)	Area
1	15	11.150	3.871	nd	nd	13.727	0.209
3	30**	11.162	7.968	nd	nd	13.641	0.349
6	15	11.125	6.145	nd	nd	13.653	0.275
8	15	11.051	5.987	nd	nd	13.579	0.280

**Parameters chosen for optimized method of extraction; nd: not detected

Thirty (30) minutes wrist shaking of bean samples (test 3) was shown to be the most effective in enhancing raffinose and verbascose extraction from the bean pellet. It must be highlighted that stachyose was undetected in wrist shaking supernatants which indicated that heat treatment extraction was sufficient to remove stachyose from the bean matrix. As a summary, the optimized extraction method conditions were 200 mg of bean powder extracted with 70% ethanol solution at 80 ^{o}C sonication bath for 120 min., followed by a second extraction with wrist shaking for 30 minutes at maximum speed at room temperature.

3.1.3.1 Purifying GOS extracts: evaluation of the efficiency of Carrez and SPE procedures

The HPAEC-PAD chromatograms obtained from the GOS extracted from beans showed a large peak in front of raffinose, stachyose and verbascose evidencing a co-extraction of other compounds or interferences. In an attempt to reduce interferences in the samples, two different methods, *Carrez Reagent to precipitate proteins* and *Solid Phase Extraction (SPE)*, were evaluated as an extra step in the extraction process. The Carrez reagent is used to selectively precipitate colloidal interferences such as fats, proteins, and redox compounds (Sigma Aldrich 2020; Culhaoglu, Zheng, Mechin & Baumberger, 2011). It consists of two reagents: (1) Carrez I, a zinc acetate solution to precipitate polysaccharides, and (2) Carrez II, a potassium ferrocyanide solution which precipitates excess zinc acetate (Culhaoglu et al., 2011). In the case of SPE, the procedure consists of 4 steps: (1) condition the column with 5mL methanol, (2) load 3mL sample, (3) elute with 1mL monosodium phosphate (NaH_2PO_4) buffer to remove soluble interferences, (4) wash

with 3mL 70% ethanol to collect analyte of interest. This is described in section 2.2.1 of the materials and methods and illustrated in **Figure 13**.

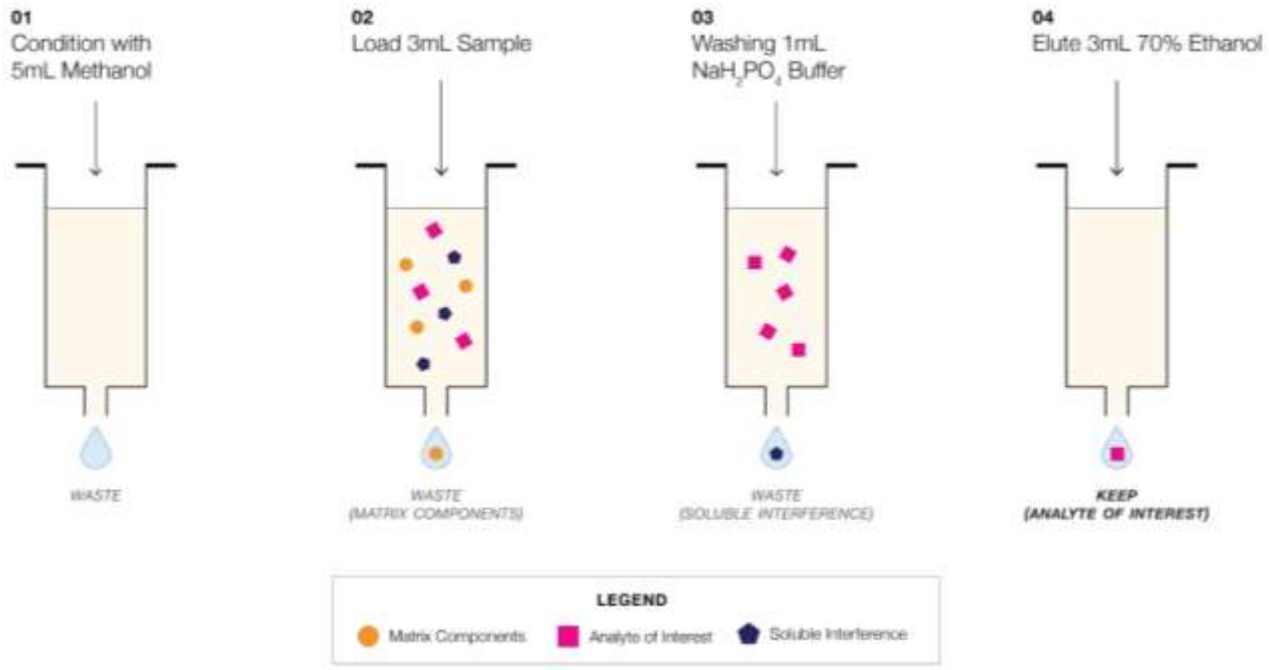

Figure 13. Illustration of Solid Phase Extraction (SPE) procedure. Adopted from Weinberger (2000).

As can be observed in the HPAEC-PAD chromatograms given in **Figure 14**, the Carrez reagents did not reduce the interference (I) present at early retention times of the chromatogram; however, SPE significantly decreased the signal of the interference in red bean kidney extract. To confirm the efficiency of SPE in reducing interferences while keeping the compounds of interest, extracts were also analyzed by GC/MS. Derivatized GOS, after oximation and silylation, analyzed by GC/MS showed a significant decrease in the areas of the raffinose, stachyose and verbascose standards (see **Figure 15**). Due to this, Carrez reagent and SPE were not utilized in the procedure of GOS extraction.

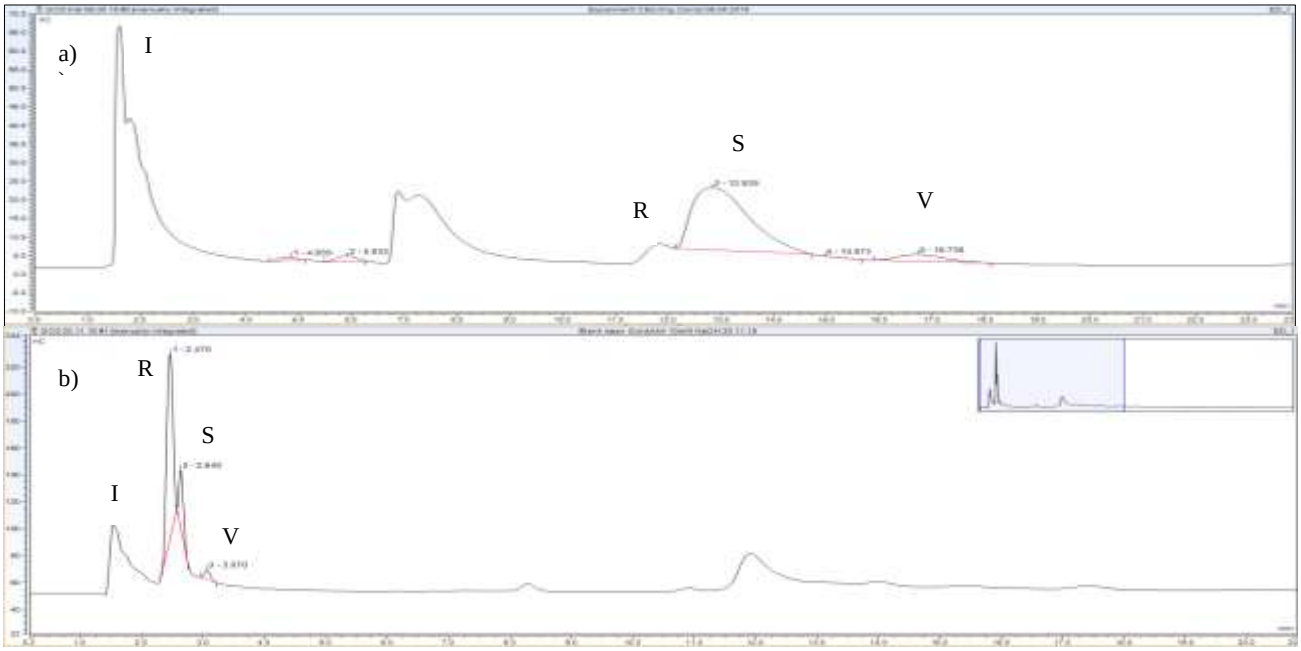

Figure 14. A comparison of the two methods of interference (I) removal using **a)** 250µL Carrez I (diluted 1:100) & 250µL Carrez II (diluted 1:100) reagents, and **b)** solid phase extraction (SPE), analyzed with HPAEC-PAD.

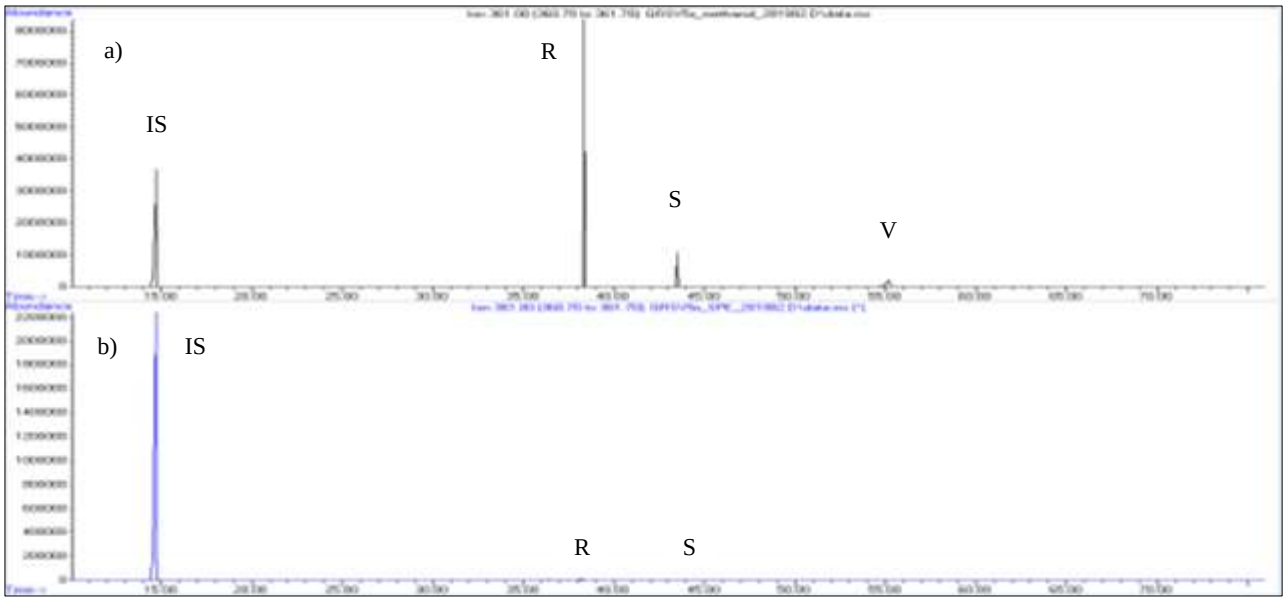

Figure 15. GC/MS chromatograms of internal standard (IS, 2000ppm) raffinose (R, 2000ppm), stachyose (S,1000ppm), verbascose (V, 100ppm) **a)** before SPE and **b)** after SPE procedure.

3.1.3.2 Validation of HPAEC-PAD for GOS analysis on beans

After selecting the appropriate mobile phase, column and waveform potential with the GOS standards (*section 3.1.1*), the HPAEC-PAD chromatographic method was validated with the GOS extracted from beans with the optimized method. **Figure 16** shows the chromatogram of the GOS extracted in beans and analyzed by HPAEC-PAD. As it can be observed, there are 3 main peaks belonging to raffinose (R), stachyose (S) and verbascose (V), respectively.

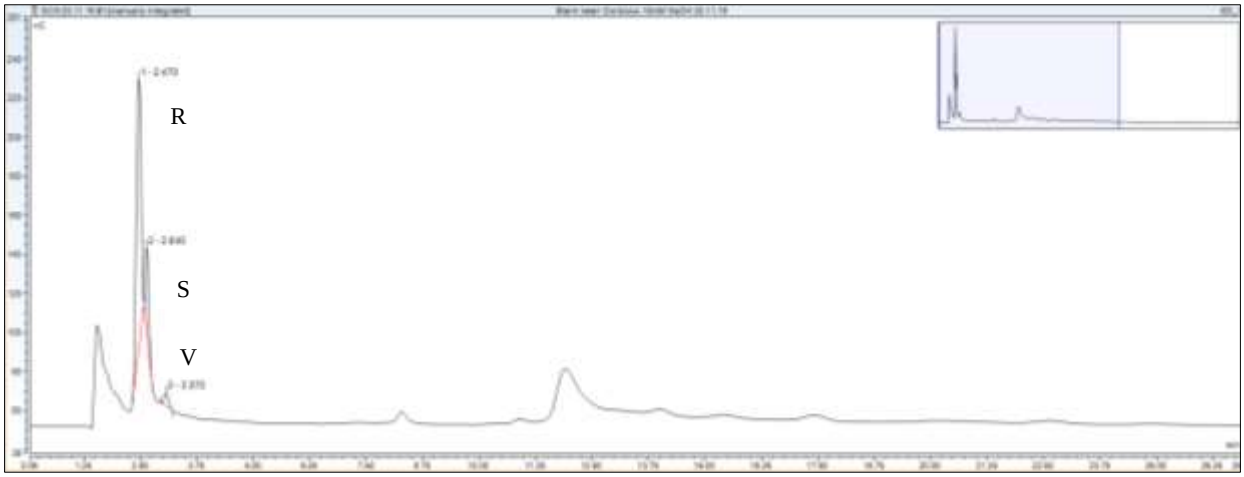

Figure 16. HPAEC-PAD chromatogram showing raffinose (R), stachyose (S) and verbascose (V) from red kidney bean extract, obtained with 10mM NaOH mobile phase, and Gold AAA waveform potential.

A 9-points calibration curve was made for each of the GOS standards as it can be seen in **Appendix 1**. As shown in **Table 11**, the D-RSV (Dionex-Raffinose-stachyose and verbascose) calibration curves were found to be linear with coefficients of determination (R^2) higher than 0.99 in the specific ranges. The percentage recovery (%R) was calculated by comparing the area of each peak in the spiked and the matrix effect samples ranging from 85.81% to 88.15%. As a guide, a percentage recovery within 80-120% is considered to be accurate (AOAC guidelines, 2002). Due to this, the method can be considered accurate. For the method precision, intraday repeatability

ranged from 1.81% for stachyose to 12.58% for verbascose. Following the AOAC guidelines (2002), maximum RSD of 15% is accepted for the repeatability. However, the interday repeatability which was performed by injecting control samples (or blanks) on three alternate days showed a huge variation on the peak areas (up to 400%). Hence, it can be concluded that the quantitative method is not sufficiently precise when running the extracted GOS samples on different days and therefore, it is convenient to add a known concentration of internal standard if samples are not immediately analyzed.

Table 11. Summary of GOS detected by HPAEC-PAD, their retention time (RT), range of concentrations in the calibration curve in ppm, coefficient of efficiency of standard curve (R^2), percentage recovery (%R), and inter- and intraday repeatability of GOS in beans

Compound	RT (min)	Range (ppm)	R^2	%R	%RSD intraday	% RSD interday
Raffinose	3.160	500-10	0.9954	88.15	10.36	-
Stachyose	3.310	250-5	0.9943	87.86	1.81	-
Verbascose	3.560	25-0.5	0.9928	85.81	12.58	-

It must also be highlighted that retention times of the peaks significantly varied during the chromatographic analysis of GOS. This is, the peaks obtained from the runs of the same standard stock solution in two different days had a shift of 0.78, 0.82 and 0.94 min for raffinose, stachyose and verbascose respectively. Considering that GOS identification with HPAEC-PAD relies on the comparison of peak retention times of the standard compounds, with ones obtained in the unknown sample, it was necessary to optimize and validate the analysis of GOS by gas chromatography/ mass spectrometry (GC/MS). The latter is known to be a more reliable and precise chromatographic technique than the HPAEC-PAD.

3.1.3.3 Validation of the GC/MS method for GOS analysis on beans

After selecting the optimized derivatization conditions and temperature gradient of the GC/MS (*section 3.1.2*), GOS from beans were analyzed. An example of a GC/MS GOS chromatogram profile from red kidney bean can be seen in **Figure 17**. It is shown that there are three (3) main peaks belonging to raffinose (R), stachyose(S) and verbascose (V), respectively.

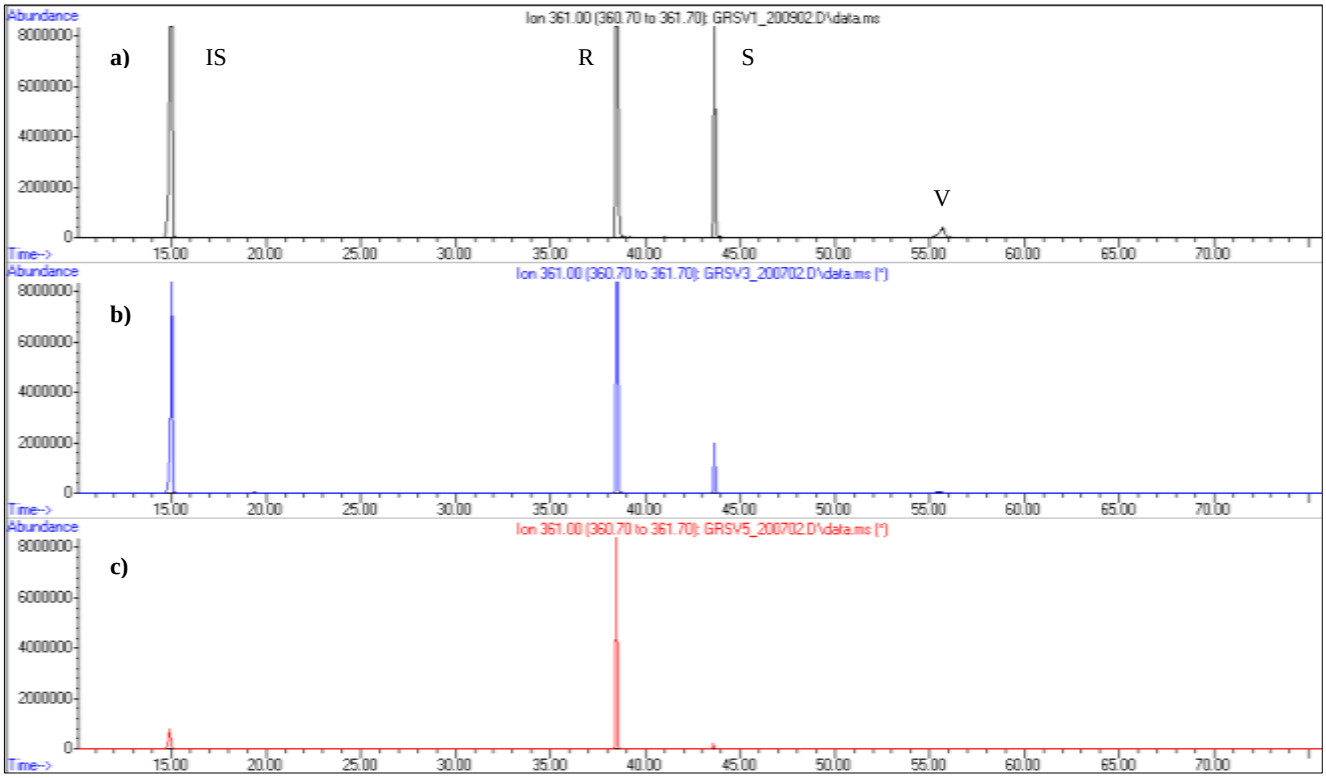

Figure 17. GC/MS chromatogram of GOS beans spiked with **a)** IS (125ppm, RT 14.90 min), raffinose (R, 125 ppm, RT 38.42 min), stachyose (S, 125ppm, RT 43.57 min) and verbascose (V, 500ppm, 56.08min), **b)** IS (75ppm, RT 14.90 min), raffinose (R, 75 ppm, RT 38.42 min), stachyose (S, 75ppm, RT 43.57 min) and verbascose (V, 300ppm, 56.08min) and **c)** IS (25ppm, RT 14.90 min), raffinose (R, 25 ppm, RT 38.42 min), stachyose (S, 25ppm, RT 43.57 min) and verbascose (V, 100ppm, 56.08min)

It was observed that the *response factor*, which is the sensitivity of the detector towards the substance, of raffinose, stachyose and verbascose was significantly different, verbascose having the lowest response factor in comparison with raffinose and stachyose. A high response factor means that a high signal could be obtained with a low concentration of the substance, and vice versa for low response factors (Ettre, 1993). The response factor can be calculated as shown below:

$$Response\ factor = \frac{Peak\ Area_{compound\ A}}{Concentration_{compound\ A}}$$

Thus, a much higher concentration of verbascose was required to be detected by the MS. Response factors for raffinose, stachyose and verbascose are shown in **Table 12**.

Table 12. Response factors of raffinose, stachyose, and verbascose obtained from GC/MS

Compound	Peak area	Concentration (ppm)	Response Factor
Internal Standard	107,145,640	125	857,165
Raffinose	97,963,233	125	783,705
Stachyose	51,405,391	125	411,243
Verbascose	8,173,528	500	16,347

As observed in **Figure 17**, a saturated peak was obtained for raffinose at 125ppm whereas a small peak was seen for 500ppm of verbascose. The low response factor for verbascose is contributed to its large molecular weight which is caused by the assumption of discrimination of the injector towards higher molecular weight compounds or derivatives (Karoutis, Tyler, & Slater, 1992). Due to the different response factors of raffinose, stachyose and verbascose, two different chromatographic injections were performed for each sample: (1) in splitless mode for the analysis

of verbascose, and (2) in split 1:100 mode for the analysis of raffinose and stachyose. In this latter, a valve in the GC/MS injector controls the amount of sample entering in the column. In this case, for every hundred parts only one part enters the column and the remaining part is vented out of the system.

As performed with the HPAEC-PAD, the percentage recovery and matrix effect parameters were calculated. The *percentage recovery (%R)* obtained by comparing the area of each peak in the spiked and the matrix effect samples ranged from 92 to 112% (see **Table 13**). According to AOAC guidelines (2002) a percentage recovery ranging from 80 – 120% is classified to be accurate. The intraday repeatability for raffinose, stachyose and verbascose were 6.88, 3.27 and 10.90% respectively. Nevertheless, the interday repeatability of raffinose and stachyose showed high variability of peak areas at different days, up to 77% which falls out of the maximum standard for reproducibility based on AOAC guidelines (2000). Thus, derivatized bean extracts changed over time and it should be injected on the same day for high reliable results, or it should be stored in the a -20^{o}C freezer if it is to be injected on the next day.

Table 13. Summary of the GOS kidney bean detected by GC/MS, their retention time (RT), range of concentrations in the calibration curve in ppm, coefficient of efficiency of standard curve (R^2), percentage recovery (%R), matrix effect (%ME) and inter- and intraday repeatability.

Compound	RT (min)	Range (ppm)	R^2 (High)	R^2 (Low)	%R	%ME	%RSD intraday	% RSD interday
IS	14.38	2000-100	0.999	0.992	100.40	137.85	12.05	nd
Raffinose	38.42	2000 - 200	0.985	0.995	103.93	5088.30	6.88	50.10
Stachyose	43.57	1000 - 50	0.983	0.915	112.00	1003.23	3.27	77.76
Verbascose	56.08	3000 - 250	0.986	0.999	94.92	430.46	10.90	nd

nd: not determined

A matrix-enhancement effect was obtained in GC/MS for GOS extracted from red kidney beans. Matrix effect is often caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds in the same matrix, affecting the performance of the chromatographic method (Panuwet et al., 2016). In this case, an enhancement of the signal of GOS extracted from beans was observed in comparison to that obtained with the GOS standards in solution (methanol). The matrix effect percentage for raffinose, stachyose and verbascose was 5088.30%, 1003.23% and 430.46% respectively. In a bean matrix extract, this phenomenon could be induced when matrix compounds adhere to active sites of the GC which increases the rate of analytes (GOS) transferred to the detector (Kwon, Lehotay, & Geis-Asteggiante, 2012). Whereas, in a standard solution without matrix, analytes (GOS) would adhere to the active sites in the GC which decreases the amount of analytes that is detected by the detector (Kwon et al., 2012). This can be seen schematically in **Figure 18**.

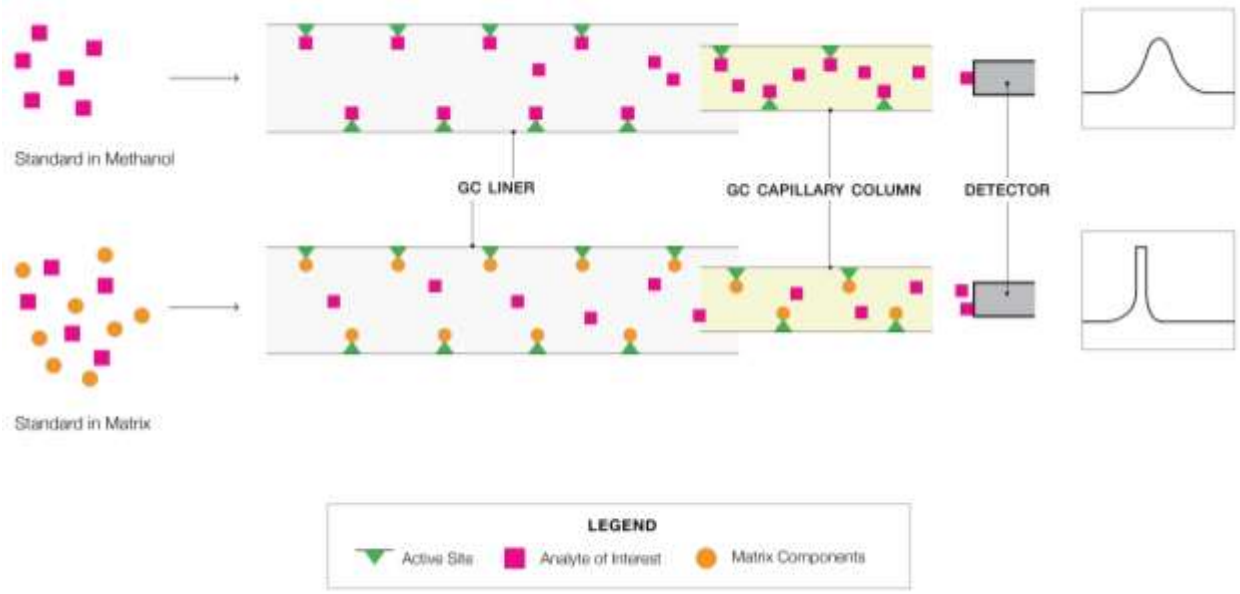

Figure 18. Illustration of matrix-effect enhancement of an analyte in GC/MS. Adopted from Rahman, El-Aty & Shim (2013).

To avoid the matrix enhancement effect in the GOS analysis in beans, a matrix-matched calibration curve was developed. To do this, GOS extracted from red kidney beans were spiked (spiked blank) with 1 mL of standard solution at different concentrations. A non-spiked red kidney bean (blank) was also extracted and diluted under the same conditions as the spiked blank. To calculate each standard area from the spiked blank, the area of each GOS peak from the blank was subtracted. The matrix-matched calibration curved could be found in **Appendix 2 and 3**. It is worth to mention that, due to a loss of linearity at a certain concentration of raffinose, stachyose, and verbascose, high and low concentration calibration curves were developed. The G-RSV calibration curves were found to be linear with coefficient of determination (R^2) of 0.98 and up to 0.99 for high and low concentrations of GOS respectively (see **Table 13**).

3.2 A case of study: application of GC/MS method to analyze GOS in beans

3.2.1 Extraction, detection and quantification of GOS content in 6 bean varieties

Six (6) bean varieties including Black turtle (*Phaseolus vulgaris*), Dark red kidney (*Phaseolus vulgaris*), Pinto (*Phaseolus vulgaris Pinto group*), Romano (*Phaseolus vulgaris*), White navy *(Phaseolus vulgaris*), Yellow eye (*Phaseolus vulgaris*) were analyzed and quantified for GOS content by GC/MS. GOS content expressed as mg/ 100 g of dry beans is given in **Table 14**.

Table 14. Raffinose, stachyose, verbascose and total GOS content (mg/100g dry bean) of six bean samples analyzed with GC/MS.

	Average GOS mg/100g of dry bean*			
Bean variety	**Raffinose**	**Stachyose**	**Verbascose**	**Total GOS**
Black turtle (*Phaseolus vulgaris*)	506.50 ± 166.29	506.50 ± 166.29	398.61± 180.54	1854.20 ± 58.41
Dark red kidney (*Phaseolus vulgaris*)	282.31 ± 13.73	1024.34 ± 55.66	245.10 ± 26.71	1590.44 ± 60.29
Pinto *(Phaseolus vulgaris)*	405.16 ± 23.01	834.47 ± 98.26	237.15 ± 5.97	1519.43 ± 68.26
Romano (*Phaseolus vulgaris)*	260.22 ± 4.17	316.05 ± 37.24	291.67 ± 20.12	897.34 ± 23.68
White navy (*Phaseolus vulgaris)*	380.47 ± 27.47	554.52 ± 75.27	240.56 ± 8.62	1213.48 ± 126.61
Yellow eye (*Phaseolus vulgaris)*	241.04 ± 6.97	923.54 ± 12.04	250.11 ± 6.23	1438.67 ± 7.42

*mean ± standard deviation

As it can be seen, stachyose was the most abundant GOS in all bean samples, followed by raffinose and verbascose which is present in lower amounts. These findings are in accordance with previous research by Martinez-Villaluenga et al. (2008); Fan, Zang & Xing (2015); Gangola et al. (2014); Burbano et al. (1999), who reported that stachyose is the most abundant GOS in pulses (i.e. *Phaseolus vulgaris,* chickpeas, soybeans, *etc*). Out of all the six beans, Black turtle (*Phaseolus vulgaris*) variety has the highest raffinose content (572. 62 mg/100g dry beans) and Yellow eye *(Phaseolus vulgaris)* the lowest (265.03 mg/100g dry beans). The one-way analysis of variance

(ANOVA) of the raffinose concentration in the six bean varieties showed a significantly higher content ($p<0.05$) of this oligosaccharide in Black turtle variety (see **Figure 19**).

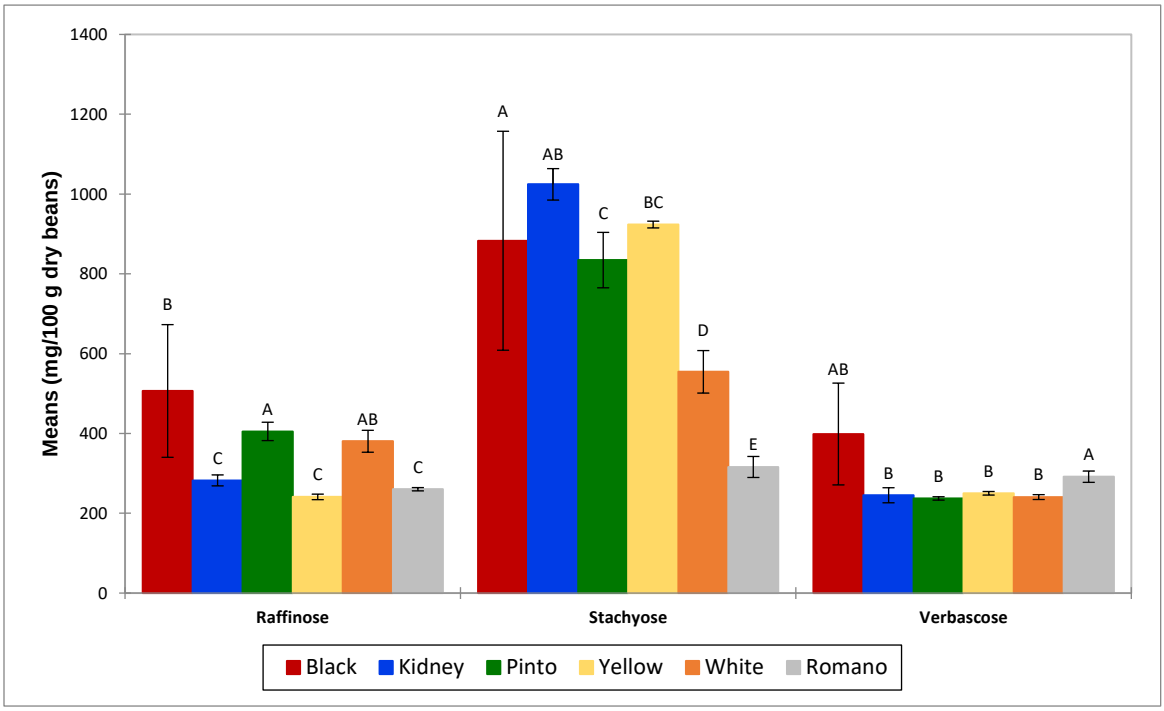

Figure 19. Raffinose, stachyose and verbascose content, expressed as mg/100g of dry beans, from 6 bean varieties. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, $n = 2$ per experimental replicate.

The Pinto and White varieties have an intermediate content of raffinose in comparison to Black and the rest of the bean varieties. Regarding stachyose, Dark red kidney (*Phaseolus vulgaris*) variety had significantly higher level (1024.34 mg/100g dry beans) of this GOS than the other varieties, followed by Yellow eye (*Phaseolus vulgaris)* (923.54 mg/100g dry beans) and Romano (*Phaseolus vulgaris)* which had the lowest (316.05 mg/100g dry beans). Verbascose content was not significantly different among the six bean varieties ($p=0.053$, $\alpha=0.05$). With Black

turtle (*Phaseolus vulgaris*) as the variety with the highest (398.61 mg/100g dry beans) and Pinto (*Phaseolus vulgaris)* as the lowest (237.15 mg/100g dry beans) level of this GOS.

The total GOS content was also calculated by summing the raffinose, stachyose and verbascose concentration in each bean and it can be seen in **Figure 20**. It was found that Black turtle (*Phaseolus vulgaris*) has the highest concentration ($p<0.05$) of total GOS followed by Dark red kidney (*Phaseolus vulgaris*) > Pinto (*Phaseolus vulgaris*) > Yellow eye *(Phaseolus vulgaris)* White navy (*Phaseolus vulgaris)* > Romano (*Phaseolus vulgaris).*

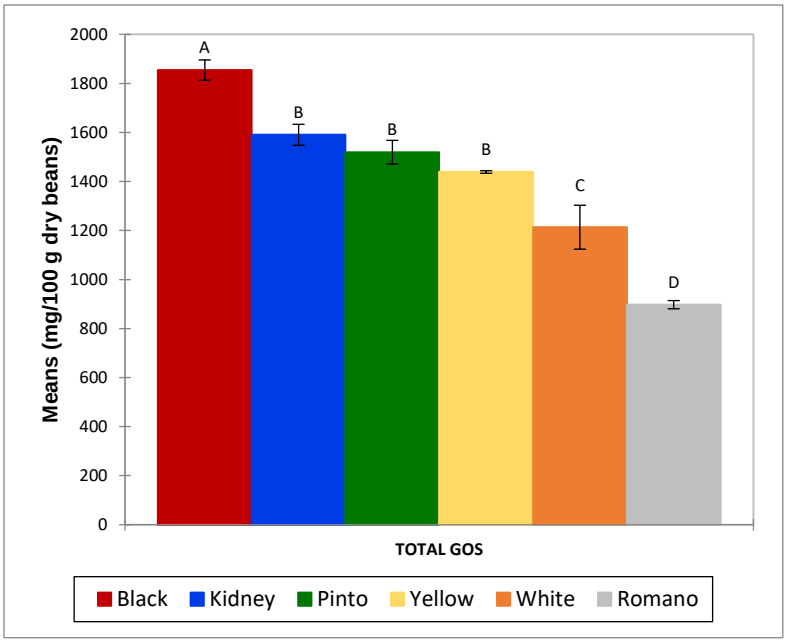

Figure 20. Total GOS content, expressed as mg/100g of dry beans, from 6 bean varieties. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, $n = 2$ per experimental replicate.

The GOS content obtained in this study is in accordance with results obtained in previous publications in which the raffinose, stachyose and verbascose content in *Phaseolus vulgaris* varied from 0.2 to 2.5%, 0.2 to 4.2%, and from 0.1 to 4.0% dry matter of total GOS, respectively (Reddy, Pierson, Sathe, & Salunkhe, 1984; Rackis, 1975; Salunkhe, Sathe & Desphande, 1989; Vidal-Valverde, Frias, & Valverde, 1993; Troszynska, Honke, Waszczuk, & Kozlowska, 1995). However, it must be highlighted that there are some discrepancies in the literature referring to GOS content (Phillips & Abbey, 1989), this could be due to the method of extraction and the detection sensitivity of the instrument used to analyze GOS.

The contrasting differences in total GOS content, and specifically on the stachyose content, between Romano (*Phaseolus vulgaris*) and Black turtle (*Phaseolus vulgaris*) could be due to the nature of the bean variety and/or to previous farming and climatic conditions to which they were exposed. GOS plays a physiological role in a plant seed namely tolerating frost, droughts and desiccation (Clegg et al., 1982; Obendorf et al., 1998). The synthesis and accumulation of GOS in leaves and seeds is a result of a protective mechanism to withstand cold climates and prevent the denaturation of membrane proteins (Martinez-Villaluenga et al., 2008). GOS protects the protoplasmic membrane of plant cells which are damaged by frost and drought through replacement of water and glass formation (Martinez-Villaluenga et al., 2008). For example, raffinose is more effective to stabilize chloroplast membranes from effects of stressors i.e. temperature and water than compared to sucrose and glucose (Santarius, 1973). There are two theories behind this statement: (1) the replacement of water from hydroxyl groups from GOS allows hydrophilic interactions between cellular membrane, and protein to take place and stabilizes the membrane of the seeds, and (2) the synthesis of GOS inhibits sucrose crystallization during

desiccation (Bryant & Wolfe, 1992; Vertucci & Farrant, 1995). The crystallization of sucrose is undesirable as its hydroxyl groups would not be readily available to bind to the membrane to aid in replacing water content in the membrane (Koster & Leopold, 1998; Sun & Leopold, 1993; Leopold et al., 1994). This helps to protect the membrane from the drying effects of desiccators.

Furthermore, the tolerance towards desiccators through GOS synthesis aids as a protective role for seeds (Martinez-Villaluenga et al., 2008). Monosaccharide content decreases as result of GOS synthesis which is undesirable for the plant as a low monosaccharide content would inhibit respiration (Vertucci & Farrant, 1995). For example, the use of desiccators and drying the bean seeds slowly, enhances raffinose and stachyose production in seeds than compared to when it is allowed to mature naturally (Martinez-Villaluenga et al., 2008). In addition, GOS plays a role in maturing bean seeds' ability to cope with temperature stressors. A maturation temperature of 18 ^{o}C increased stachyose and verbascose, compared to seeds matured at 25 ^{o}C (Gorecki et al., 1997). This shows the importance of temperature and possibility relative humidity conditions at which beans were matured and stored before consumption.

3.2.2 Analysis and quantification of GOS in 4 canned beans exposed to low and high treatment conditions

Side effects of bean consumption, such as gas production and bloating feeling are mostly attributed to the GOS content. Hence, reducing these oligosaccharides by exposing the beans to different processing methods is of great interest to enhance pulse consumption. Low (HRL1, L2HR) and high (E3L1, E7L1) treatment conditions were applied to dark red kidney beans with the aim to reduce GOS content. HRL1 and L2HR were exposed to milder treatment conditions, whereas E3L1 and E7L1 were subjected to high treatment conditions before canning. The raffinose, stachyose, verbascose and total GOS content on these beans and the corresponding

brines was analyzed by GC/MS. Several factors play a role in the reduction of GOS during processing treatments such as soaking (Vidal-Valverde, Frias, & Valverde 1992; Njoumi et al., 2019). Soaking induces the leaching of water-soluble molecules such as raffinose, stachyose, verbascose sucrose and fructose into the water (Njoumi et al., 2019). Endogenous enzymes (i.e. alpha-galactosidase) is activated during soaking which converts raffinose, stachyose and verbascose into lower molecular weight products in the seeds, allowing its diffusion into the water (Quieroz, Oliveira & Helbig, 2002; Vidal-Valverde, Frias & Verde, 1992; Njoumi et al., 2019). In fact, large amounts of galactose, glucose and fructose were present in the soaking water, with low amounts of GOS molecules. This indicates that hydrolysis of GOS occurred during this process (Njoumi et al., 2019). In addition, a maximum soaking time of 16 hours is advisable to reduce GOS whereas a small increase in GOS content occurred between 16 to 24 hours (Njoumi et al., 2019).

Concentration, expressed as mg of GOS per 100 g of dry beans or dry brine, of raffinose, stachyose, verbascose and total GOS in beans and their brines is summarized in **Table 15**.

Table 15. Raffinose, stachyose, verbascose and total GOS content in canned red kidney beans and their corresponding brines analyzed and quantified by GC/MS.

	GOS mg/100g of dry bean or dry brine			
Bean samples	**Raffinose**	**Stachyose**	**Verbascose**	**Total GOS**
L2HR Bean	275.52 ± 16.07	953.82 ± 44.01	271.83 ± 0.78	1511.18 ± 26.58
HRL1 Bean	256.91 ± 8.37	632.47 ± 76.82	262.10 ± 0.08	1159.13 ± 68.28
E3L1 Bean	225.27 ± 3.30	89.32 ± 0.63	244.30 ± 0.65	565.38 ± 4.68
E7L1 Bean	228.07 ± 4.68	89.88 ± 1.02	244.65 ± 0.94	569.84 ± 6.79
Brine samples	**Raffinose**	**Stachyose**	**Verbascose**	**Total GOS**
L2HR Brine	434.85 ± 33.42	2167.57 ± 239.73	294.03 ± 12.14	2923.11 ± 287.34
HRL1 Brine	385.83 ± 13.39	1937.36 ± 100.42	291.14 ± 0.69	2637.60 ± 115.30
E3L1 Brine	286.81 ± 9.25	214.62 ± 56.27	257.29 ± 3.47	775.77 ± 69.54
E7L1 Brine	252.29 ± 4.40	108.28 ± 5.22	239.30 ± 6.91	614.87 ± 2.97

As it can be observed, high treatment conditions (E3L1, E7L1) showed an effective removal of GOS from beans. **Figure 21** and **22** show that the raffinose, stachyose, verbascose and consequently total GOS content is significantly lower ($p<0.05$) in E3L1 and E7L1 samples compared to L2HR and HRL1, stachyose being up to 2.0 times lower. In addition, GOS content in L2HR and HRL1 beans is significantly different ($p<0.05$) for stachyose and verbascose whereas no differences were found in the raffinose level. Overall, the descending order of raffinose, stachyose, verbascose and total GOS content in canned beans is L2HR > HRL1 > E7L1 = E3L1.

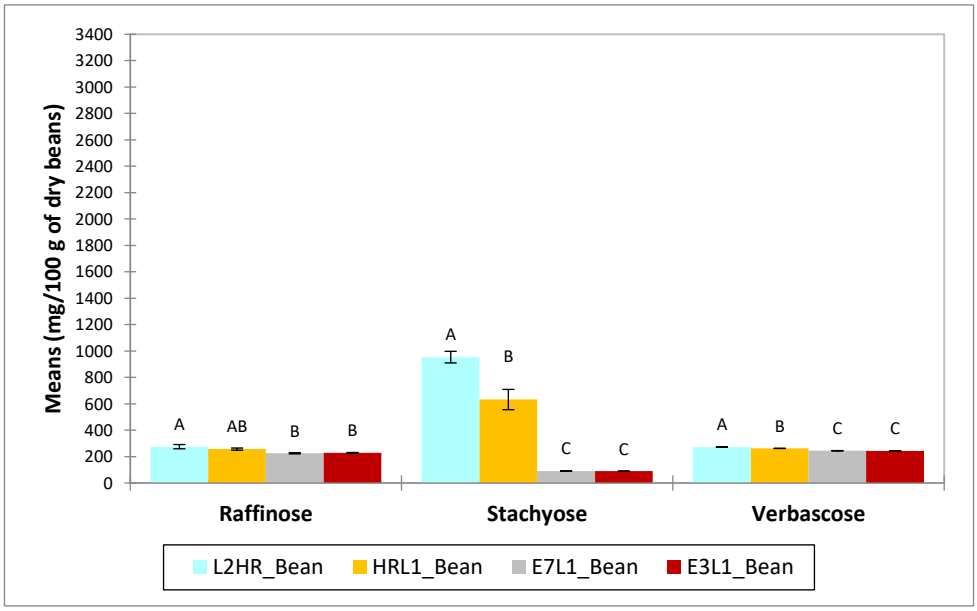

Figure 21. Raffinose, stachyose and verbascose content, expressed as mg/100g of dry beans in canned beans exposed to different treatments. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, $n = 2$ per experimental replicate

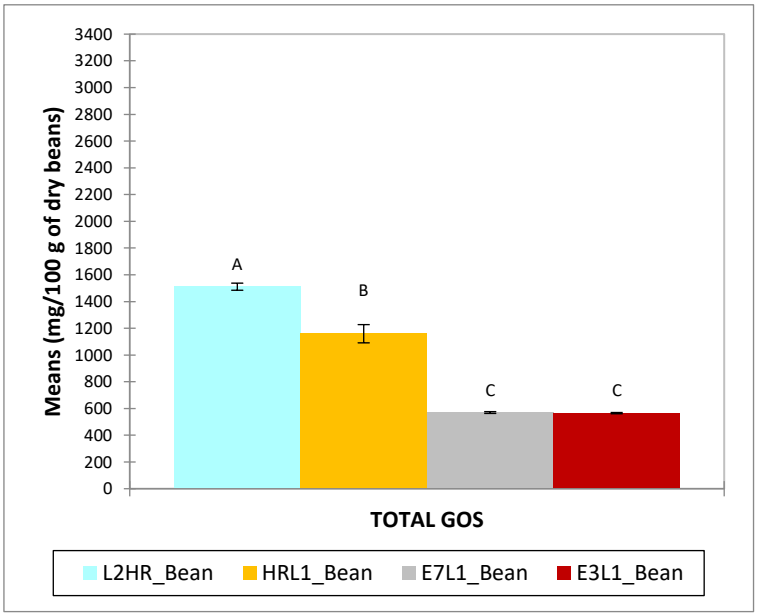

Figure 22. Total GOS content, expressed as mg/100g of dry beans in canned beans exposed to different treatments. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, n = 2 per experimental replicate.

The corresponding canned beans brines were also analyzed. As observed in **Table 15**, the GOS content was remarkably high, most of the cases having higher GOS content than the beans. In accordance with results obtained in the beans, E7L1 and E3L1 brine samples contained the lowest amount of GOS in comparison to L2HR and HRL1 brines. This indicates that the processing parameters and conditions applied to E3L1 and E7L1 samples were effective in reducing the GOS from red kidney beans. The concentration differences and their significance in the raffinose, stachyose, verbascose and total GOS content of L2HR, HRL1, E7L1, and E3L1 brines are given in **Figures 23** and **24.**

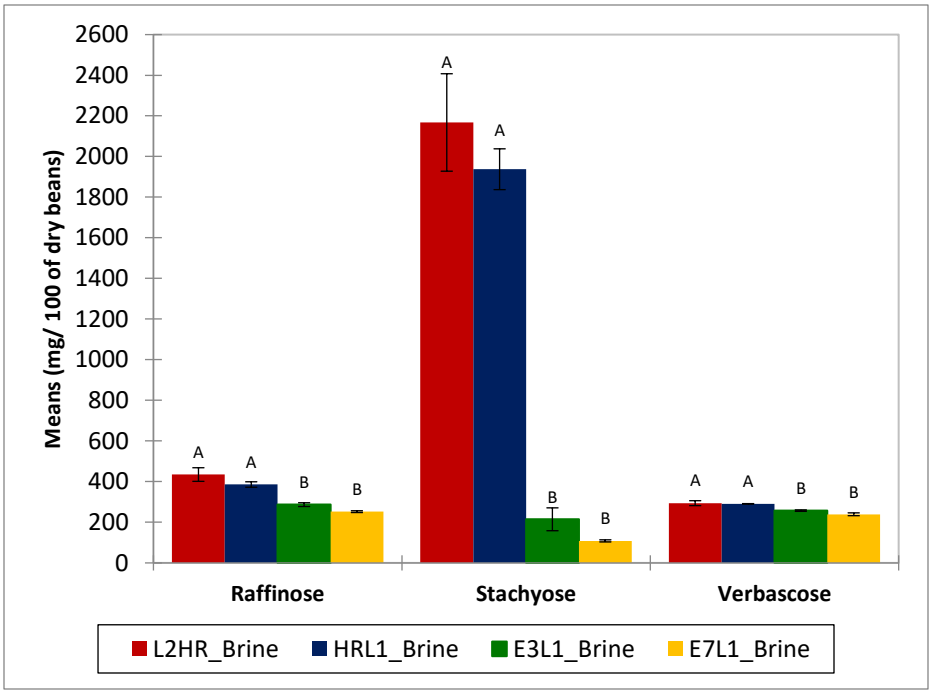

Figure 23. Raffinose, stachyose and verbascose content, expressed as mg/100g of dried brines from canned beans exposed to different treatments. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, n =2 per experimental replicate.

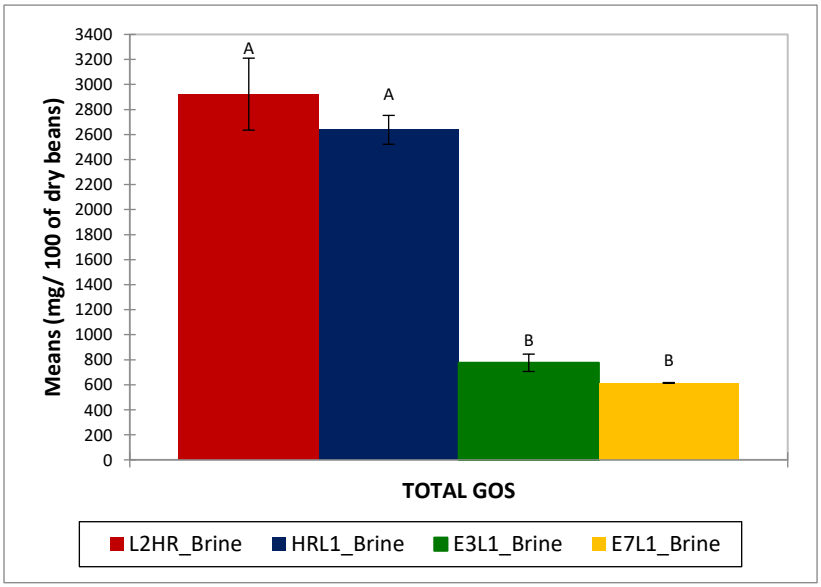

Figure 24. Total GOS content, expressed as mg/100g of dried brines from canned beans exposed to different treatments. Values in bar chart represent means ± standard errors. Means in the same row accompanied by different superscripts are significantly different at LSD $\alpha = 0.05$, n = 2 per experimental replicate.

4 Conclusion

The extraction of galacto-oligosaccahrides (GOS) in beans including raffinose, stachyose and verbascose and its subsequent analysis by gas chromatography/ mass spectrometry (GC/MS) was successfully optimized and validated. The comparison of the high-performance anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and GC/MS, showed that the latter (GC/MS) is a more reliable and reproducible instrument, to identify, detect and quantify GOS content in beans. The optimized and validated method was used to measure the GOS content in six bean varieties and canned beans exposed to different treatments in order to reduce GOS content. It was found that GOS is significantly higher in Black turtle beans, whereas Romano beans had the lowest. In addition, high treatment conditions of canned beans significantly reduced the level of GOS in beans. Moreover, results demonstrated that GOS can diffuse out from the beans to the brines during canning time. This thesis could serve as a foundation for pulse sectors to create methods to reduce GOS and implement the validated extraction and analysis method developed. This would expand the pulse industry as low GOS bean products could be marketed and produced which will aid in providing the nutritional benefits of beans without its undesirable side-effects, for IBS patients and the global population.

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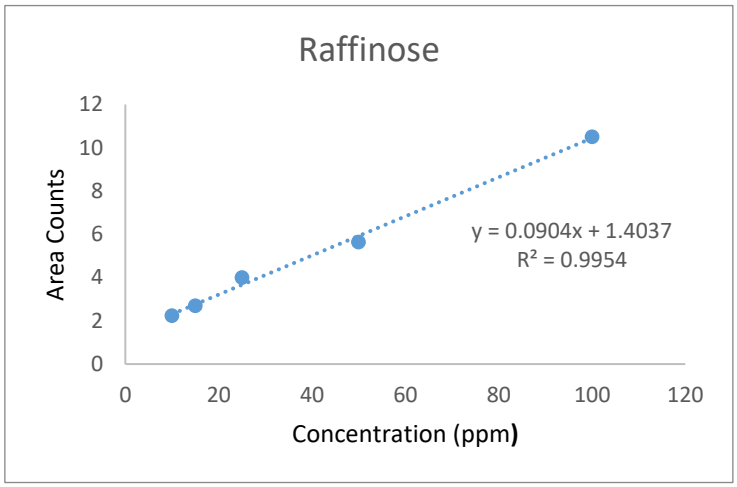
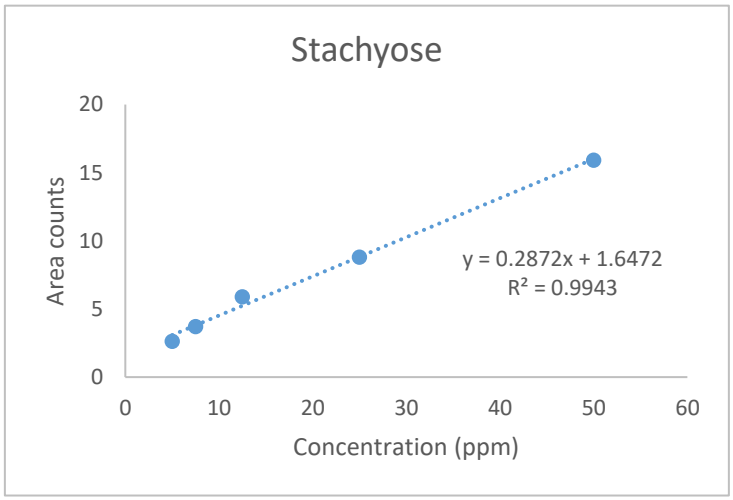
APPENDICES

Appendix 1. RSV Standard Solution Calibration Curve (HPAEC-PAD)

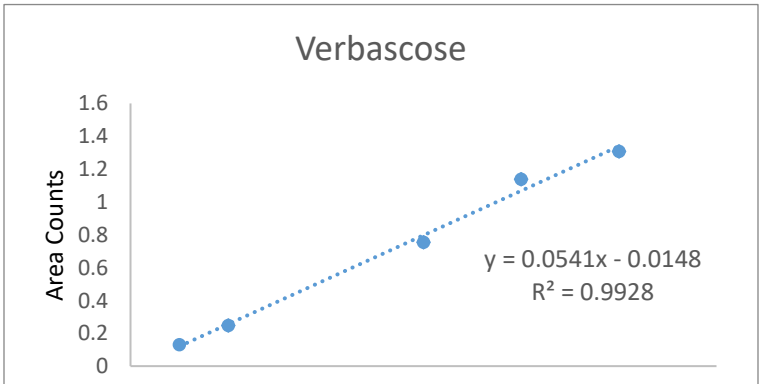
Raffinose	
Concentration (ppm)	Area
100	10.5153
50	5.6406
25	4.0004
15	2.7035
10	2.2367



Stachyose	
Concentration (ppm)	Area
50	15.910
25	8.809
12.5	5.892
7.5	3.707
5	2.635



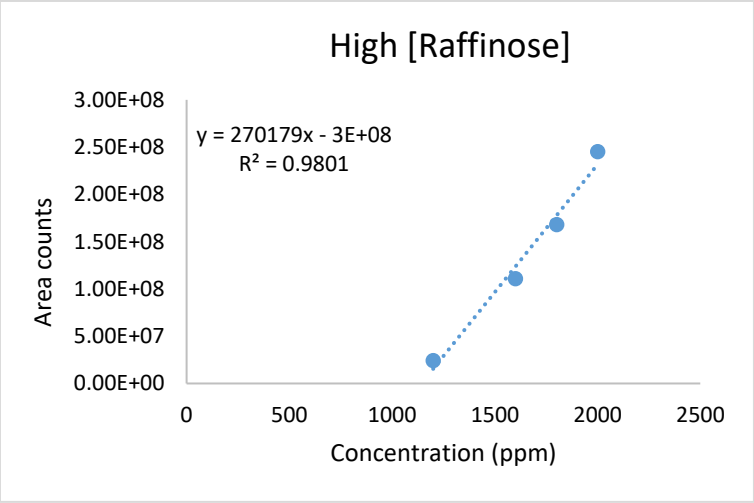
Verbascose

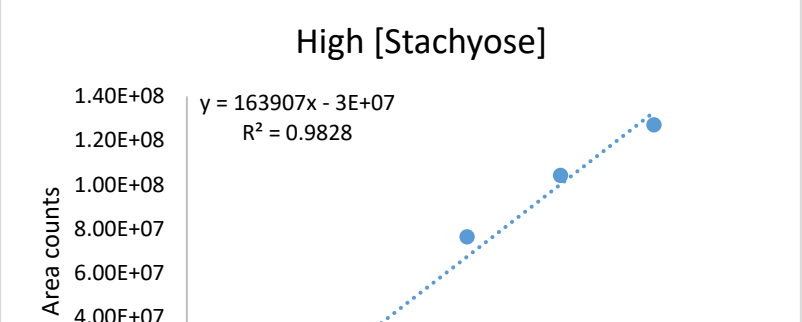


Concentration (ppm)	Area
25	1.309
20	1.138
15	0.754
5	0.248
2.5	0.131

Appendix 2. RSV Standard Calibration Curve High Concentration (GC-MS)

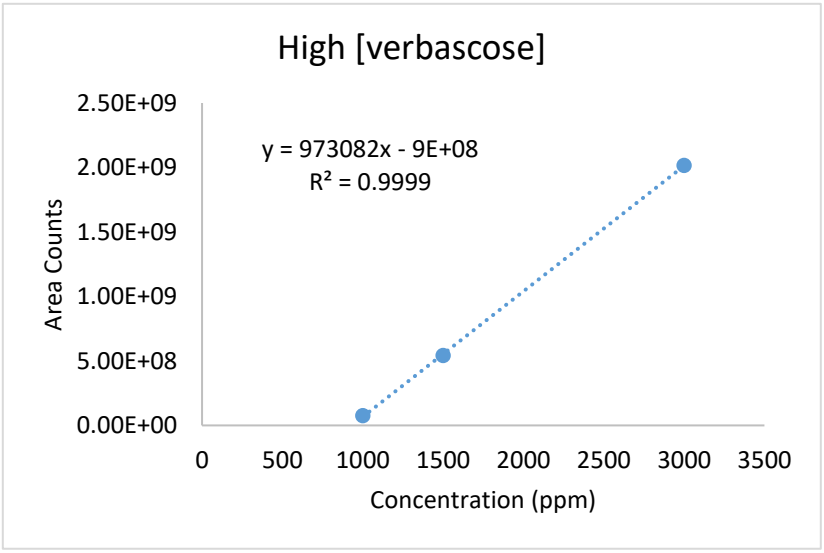
Raffinose	
Concentration (ppm)	Area
2000	245228077
1800	167860135
1600	110844085
1200	24231390

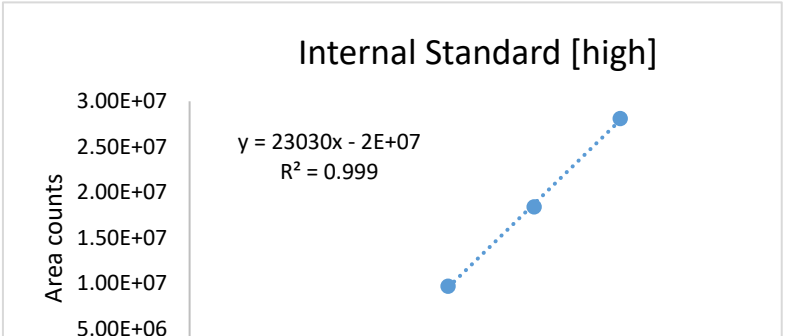




Stachyose	
Concentration (ppm)	Area
1000	127080125
800	104156692
600	76297617
400	26980553
200	1761184

Verbascose	
Concentration (ppm)	Area
3000	2015418475
1500	541719788
1000	74884300

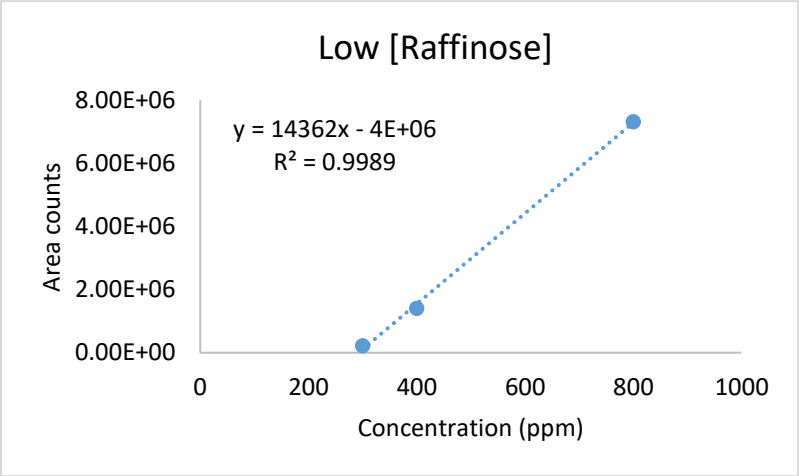


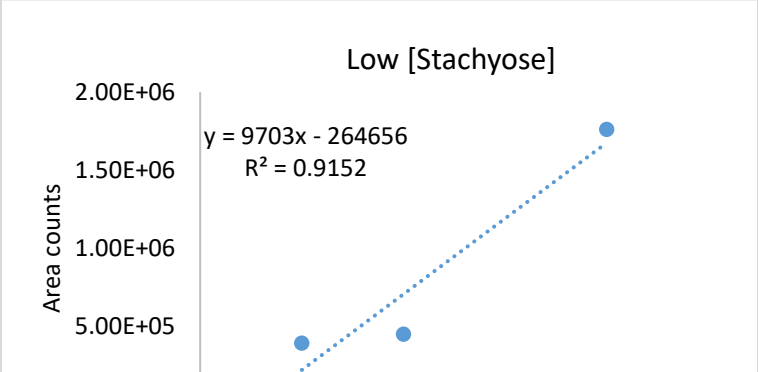


Internal Standard	
Concentration (ppm)	Area
2000	28109490
1600	18391189
1200	9685783

Appendix 3.RSV Standard Calibration Curve Low Concentration (GC-MS)

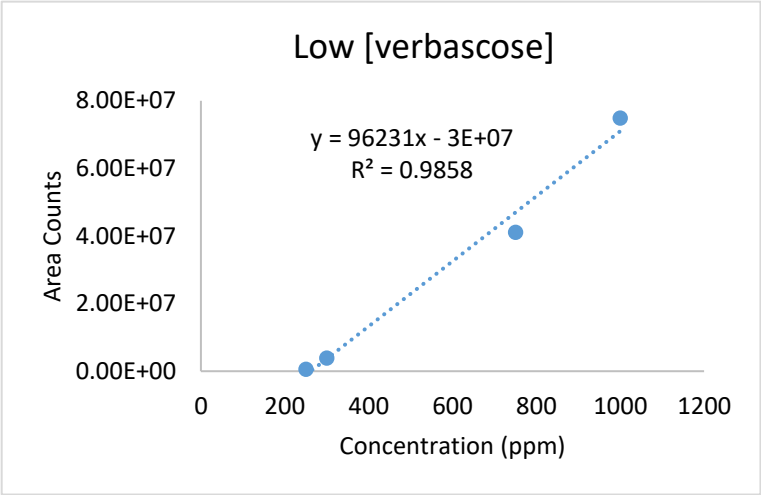
Raffinose	
Concentration (ppm)	Area
800	7319748
400	1408315
300	221857





Stachyose	
Concentration (ppm)	Area
200	1761184
100	449938
50	390967

Verbascose	
Concentration (ppm)	Area
1000	74884300
750	41087245
300	3895884
250	535512



Internal Standard	
Concentration (ppm)	Area
1200	9685783
800	5953874
400	2008053
200	525879
100	337181