How spruce budworm *Choristoneura fumiferana* detoxify host plant toxins?

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ABSTRACT

How spruce budworm *Choristoneura fumiferana* detoxify host plant toxins?

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The spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), is one of the destructive insect species of the boreal forest in eastern North America. Recent studies have discovered two sets of phenolic compounds that appear to play an important role in the resistance of coniferous trees to the spruce budworm. The phenolic glycosides, picein and pungenin are present in most of the susceptible white spruce trees, but their aglycone forms, piceol and pungenol are found only in white spruce trees resistant to the spruce budworm. These compounds have been shown to retard development time, reduce budworm survival and pupal mass. This research focused on monitoring the fate of these phenolic aglycones (acetophenones) after ingestion by the budworm and aimed at determining how the compounds were detoxified. High performance liquid chromatography-mass spectrometry identified glycosylated and glutathionylated-metabolite of piceol and pungenol in the frass of the caterpillars. Midgut enzyme assays were conducted at neutral and alkaline pH to measure the activity of detoxification enzyme glutathione-*S*-transferase.

In this study, spruce budworm larvae were reared on either artificial diet only (control diet) or artificial diet containing combined acetophenones (piceol and pungenol). Our results suggest that the insects upregulated production of the detoxifying enzyme, glutathione-*S*-transferase, in response to feeding on diet containing acetophenones. The acetophenones were thus detoxified by conversion to glycosylated and glutathionylated form in the gut.

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1.0 Introduction

1.1 Spruce budworm

The spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), is one of the most serious insect pests in forests of eastern North America (Blais, 1983; Sanders, 1991). The insects feed primarily on three spruce tree species, *Picea glauca*, *Picea mariana*, and *Picea rubens* and balsam fir, *Abies balsamea*, which is the most vulnerable host species to *C. fumiferana* (Maclean, 1980). Spruce budworm adult moths emerge in July and lay egg masses each containing 10-150 eggs on host tree needles. The first instar builds a hibernaculum in bark cracks or in old conifers, moults to second instar and overwinters until early spring. Second instars emerge from diapause 2-3 weeks prior to vegetative budbreak and mine old foliage. Spruce budworm damage can begin even before buds have flushed out.

At budbreak, larvae feed on current year foliage and undergo four additional larval stages before turning into pupae in early July (Fig 1). Balsam fir trees die from severe defoliation after 3-4 years, where as white and red spruce trees die from severe defoliation after 4-5 years. Late instar larvae are voracious feeders, chewing off needles at their bases. In heavy infestations, old foliage is also eaten. Tree growth loss, tree deformity, and mortality follow several years of heavy infestation (Blais, 1983). The spruce budworm outbreak of 1950-1993 covered an area of 850,000 km^2 in Canada, and killed 45-58% of tree hosts in highly affected areas and decreased wood yields by 300-6800 m^3 km^{-2} (Gray and MacKinnon, 2006). A more recent outbreak began in 2006 along the north shore of St. Lawrence river, affecting a spruce tree population of about 3,000 hectares. Over 3.2 million hectares of forest in Quebec alone has suffered moderate to heavy defoliation by the spruce budworm in 2013. A 2016 spruce budworm infestation resulted in defoliation of 7.2 million hectares of forest.

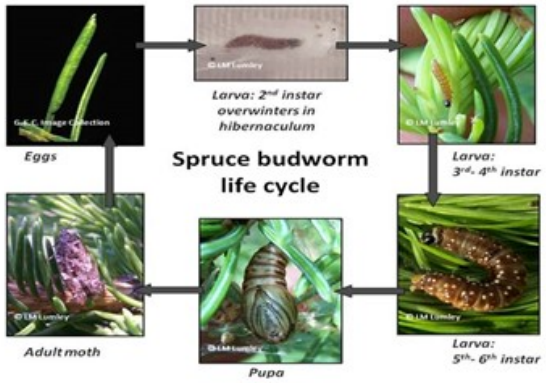


Fig 1. Spruce budworm life cycle. Image source: Michel Cusson.

Host resistance against spruce budworm has been associated with growth phenology and the chemical nature of foliage (Clancy, 2002; Daoust *et al.,* 2010; Delvas *et al*., 2011). Two sets of phenolic compounds appear to play an important role in the resistance of white spruce trees to spruce budworm defoliation. These compounds are acetophenones, organic compounds that consist of a benzene and ketone structure. They were identified from trees resistant to budworm attack which suffered only light defoliation when other trees around them were heavily damaged (Daoust *et al*., 2010). Piceol and pungenol compounds have been shown to increase mortality and slow growth in bioassays, but the glycosylated forms, picein and pungenin appear to have no effect on the budworm (Delvas *et al*., 2011). Both susceptible and resistant trees contain the glycosylated compounds, picein and pungenin, but only resistant trees contain the acetophenones piceol and pungenol (Delvas *et al.,* 2011). A glucosyl hydrolase gene, *PgBgluc-1*, was highly expressed in resistant trees, catalyzing formation of the acetophenones from the glycosylated compounds (Mageroy *et al*., 2014). The present study aims to determine how piceol and pungenol are detoxified in the midgut of the spruce budworm asking whether the budworm has counter-measures to protect it from these toxic compounds. Specifically, this study first tests whether these compounds are egested unchanged or in modified form, and second whether the budworm upregulates detoxification enzymes in response to feeding on these compounds. The biochemical transformation of plant toxins by insects is one of the major schemes that herbivorous insects have evolved in their arms race with plants (Berenbaum, 2002).

1.2 Insect metabolism of host plant compounds

Insect herbivores exhibit multiple mechanisms for dealing with plant secondary metabolites in their diet. Examples of these mechanisms include deactivation of host plant toxins, metabolism, excretion, sequestration, detoxification, and target-site resistance of the toxins (Despres *et al*., 2007).

Metabolic resistance often results in the production of detoxifying enzymes that metabolize host plant toxins or dietary host toxins (Meyran *et al*., 2002). Insects counter- defense against host toxins may be activated by specific genes encoding enzymes, generating enzyme-catalyzed reactions that modify toxins. For example, the fifth instar spruce budworm larvae induce the expression of glutathione-*S*-transferase (GST) in response to several insecticides (Feng *et al*.,

2001). GST catalyzes the conjugation of glutathione to toxic electrophilic compounds enhancing their water solubility and elimination by the insect (Enayati *et al*., 2005).

Another enzyme responsible for detoxification-mediated activity is the UDP-glycosyltransferases (UGTs). UGTs catalyze the conjugation of xenobiotics with glucose making these compounds water-soluble for excretion in the insect. For example, the Lepidopteran species *H. armigera*, *H. zea*, and *H. assulta* is resistant to capsaicin active compounds found in chilli peppers. The capsaicin produces a burning sensational taste against mammals and also serves as anti-feedant against insects. The three *Helicoverpa* species metabolized the capsaicin bioactive compound in chilli peppers when they fed on them via glycosylation through their UGT detoxification system (Ahn *et al*., 2011).

The above-mentioned GSTs and UGTs are generalist enzymes that confer resistance to multiple toxins. In other cases, induction of specialized detoxifying enzymes can be an initial step toward enabling an herbivore to specialize on a particular host plant (Le Goff *et al*., 2006). Specialized detoxification enzymes against plant host toxins have been found in specialized insects for example the parsnip webworm (*Depresaria pastinacella*). The parsnip webworm feeds on furanocoumarin containing plants and principally relies on cytochrome P450 detoxification enzyme against the host toxins contained in these plants. It has been identified that the specialized enzyme encoded gene, CYP6B in the insect produces biochemical resistant mechanism to metabolize high levels of furanocoumarin toxins in its diet (Mao *et al*., 2006).

After metabolism, a large proportion of the plant chemical compounds can be egested in a modified, less toxic form. Other compounds also move through the digestive tract of the insect intact without any metabolic modification and therefore egested in the frass in the same form as they were ingested by the insect.

Another way of dealing with host plant toxins by the insects is to decrease production of defensive compounds in plants. A study (Musser *et al*., 2002) showed that saliva of the caterpillar species, *Helicoverpa zea,* contains an enzyme, glucose oxidase that decreases the level of nicotine in the leaves of *Nicotiana tabacum* when the insect feeds on these leaves, so that this plant becomes less toxic to the herbivore.

Finally, plant toxic compounds can also be sequestered in other parts of the insect's body like the wings and later re-used for purposes of defence and protection against predators or disesase causing organisms (Willinger and Dobler, 2001). Insects also ensure sequestered toxic compounds

are transported and stored selectively to avoid breakdown of its physiological activities (Kuhn *et al*., 2004).

1.3 Phenolic compounds

Phenolics are an important group of plant specialized compounds with high structural diversity (Harbone, 1984). They form one of the major classes of carbon-based specialized compounds in conifers, and play several important roles in trees (Bravo,1998; Wink, 2003). They are frequently involved in plant defence against herbivores and pathogens (Abou-Zaid *et al*., 2000).

A phenolic compound is a compound that has a six carbon aromatic ring with one or several hydroxyl groups (Quideau *et al*., 2011). Plants produce many phenolic compounds during their growth and development (Johnson and Felton, 2001) and this may range from simple to complex compounds. Phenolics can be further grouped, for example as tannins, phenolic acids and flavonoids (Rehman *et al*., 2012). Phenolics also provide some defense and protection against plant herbivory as they may act as toxic substances, retarding growth and development in insects (Close and McArthur; 2002; Delvas *et al*., 2011).

Herbivorous insects may find plant phenolic compounds toxic or anti-digestive. Phenolic compounds have been shown to have both positive and negative effects on the growth and feeding behaviour of larvae (Johnson and Felton, 2001; Ikonen *et al*., 2001). The effects of phenolics depend upon their biological activities in a particular biochemical environment (Bi *et al*., 1997; Johnson and Felton, 2001). The biochemical mode of action of phenolic compounds in herbivorous insects depends on the gut pH and the presence of detoxification enzymes. For example, the midgut of the spruce budworm larvae, like that of most caterpillars, has an alkaline pH (10.5 ± 0.12, Gringorten *et al*., 1993) which can lead to oxidation of phenolics, causing oxidative damage to the insect (Barbehenn *et al*., 2006a).

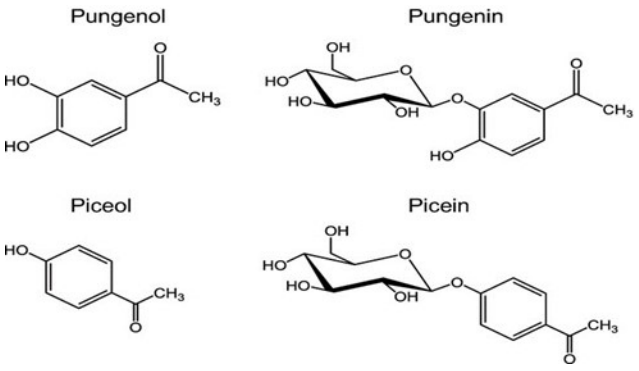


Fig 2. The structures of acetophenones, pungenol and piceol, and their respective glycosides, pungenin and picein in white spruce trees (Delvas *et al*., 2011).

One way to examine metabolism of phenolics by herbivores that ingest them is to assay the original compounds and their metabolites in the insect's frass. Depending on the compound and insect species, phenolics can be glycosylated, glutathionylated, sulfated, deacylated or deglycosylated in insect guts (Ferreres *et al*., 2008; Schramm *et al*., 2011; Salminen *et al*., 2004).

In the moth *Acentria ephemerella*, a major dietary phenolic ellagitannin was not detected in the larval frass possibly suggesting that this compound had been degraded in larval metabolism (Gross *et al*., 2008). Similarly, (Ruuhola *et al*., 2001) studied the degradation rates of flavonoids in lepidopteran larvae by analyzing the frass of the *Salix*-feeding *Operopthera brumata.* They found that generally more than 60% of the total flavonoids (including flavones and flavonols) had been degraded by larval metabolism. Chemical modifications of phenolic compounds were also detected in the frass of several Lepidopteran species (Vihakas *et al*., 2015). These modified phenolics included kaempferol and quercetin sulphates, and similar types of compunds were earlier detected in the frass of the Lepidopteran *Pieris brassicae* via metabolism through deglycosylation, deacylation and sulfating processes (Ferreres *et al*., 2008).

The present study used high performance liquid chromatography-mass spectrometry (HPLC-MS) to test whether piceol and pungenol incorporated in artificial diet or their metabolites are recovered in spruce budworm frass. If the original compounds are recovered from the frass, then this suggests that the phenolic compounds passed through the larval midgut intact without any biochemical modification. If compounds are missing or absent in the spruce budworm frass, then this may suggest a plausible form of metabolic modification of the compounds in the larval midgut and subsequent release of its metabolic-byproduct in frass.

1.4 Spruce budworm gut structure

The digestive tract of insects is broadly divided into three sections, namely: foregut, midgut and hingut (Terra *et al*., 1996). Leaf chewing insects, like the spruce budworm, use their mouthparts (e.g. mandibles) for cutting and grinding the tissues of their host plant (Smith, 1985). Food first enters the foregut. Lepidopteran larval foreguts are reported to range from slightly acidic to neutral (Appel and Maines, 1995; Barbehenn and Martin, 1994), but the conditions may be alkaline in some species (Appel and Martin, 1990).

Food from the foregut moves into the midgut. The midguts of different species of Lepidopteran larvae are highly alkaline (Barenbaum, 1980; Dow, 1984), which would favour oxidation reactions (Appel, 1993). Digestive enzymes, like amylases of Lepidopteran species, are adapted evolutionarily to function in alkaline midgut (Pytelkova *et al.*, 2009). In the midgut, most of the food substances are processed by these larval digestive enzymes and absorbed. Several studies of plant-insect interaction have shown the midgut tissue as the major interphase for a host of detoxification enzymes (Hakim *et al*., 2010; Rajarapu *et al*., 2011). Midguts contain detoxification enzymes to process plant specialized metabolites, such as glutathione-*S*-transferase and cytochrome P450s enzymes. GSTs and P450s aid by conjugating a moiety to these compounds in the midgut to detoxify them. In the hindgut, the waste metabolic products are emptied from the Malpighian tubules and dumped with the faeces as frass.

1.5 Detoxification enzymes

Detoxification enzymes found in the caterpillar midgut typically include three main super-families: the cytochrome P450 monooxygenases (P450s), the glutathione-*S*-transferases (GSTs), and the carboxylesterases (COEs), (Despres *et al*., 2007). Detoxification provides a critical line of defense through metabolism against xenobiotics such as plant allelochemicals or insecticides (Terriere, 1984). Detoxification happens in two phases- phase I and phase II. Phase I enzymes include P450s, and phase II enzymes include GSTs, COEs. Phase I enzymes occur through processes such as oxidation, reduction and hydrolysis. Mostly, oxidative reaction is seen in CP450 family of enzymes in phase I. The phase I reaction proceeds by introducing functional groups such as hydroxyl to produce more polar metabolites to be readily excreted. However, some products of the phase I are not eliminated, so they enter the next enzymatic phase II.

At phase II, the rest of the metabolites, combine with functional groups such as glutathione (GSH), sulphates, glucuronic acids to form more polar conjugates of the metabolites that can be readily egested in frass of insects. The two phases occur sequentially: phase I prepares a functional group enabling the conjugation with a polar compound in phase II. All these enzymes play a key role in insect-plant interactions. In this study, GSTs and β-glucosidase enzyme activities were measured in the midguts of the spruce budworm larvae.

1.6 Glutathione-*S*-transferases (GSTs)

Detoxification by GST is an important mechanism in insects as well as mammals. These enzymes belong to phase II in the detoxification pathway (Yu, 1992). GSTs are involved in the detoxification of various xenobiotics and induced by plant allelochemicals (Yu, 1992; Wadleigh and Yu, 1988). Generally in insects, GSTs catalyse the conjugation of reduced glutathione (GSH) to electrophilic molecules and thus generating glutathione-*S*-conjugates that are more water soluble and, thus, excretable metabolites (Enayati *et al*., 2005). The general reaction performed by GSTs is as follows:

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG + GS(\text{reduced glutathione})$$

GST mediated metabolism is often induced by the ingestion of plant allelochemicals and other toxic compounds. GSTs are thought to utilize over 3,000 compounds as their substrates (Jakoby and Habig, 1981). The induction of GST was first observed in houseflies exposed to phenobarbital (Ottea and Plapp, 1981).

The primary detoxification role of GSTs on plant chemicals has been studied in numerous Lepidopteran species and insects feeding on xenobiotics, crops and deciduous trees (Yu, 1996). The class I GST gene (i.e. D*mGST*D1) from *Drosophilia melanogaster* was induced to lower DDTase activity and this gene was expressed to produce GST enzymes to metabolize ingested DDT in the insect (Yu, 1996). Similar GST inductions were observed in *Musca persicae* when the insect was fed with Brassicaceae plants containing toxic isothiocyanates and glucosinolates. (Yu, 1996). Studies on fall armyworm, *S. frugiperda* (Lepidoptera: Noctuidae) feeding on cowpea, mustard and turnip demonstrated an induction of GSTs in response to host allelochemicals in their diets (Yu, 1982). The presence of insecticides and plant specialized compounds has been shown to induce the production of GSTs in Lepidopteran species (Feng *et al*., 2001; Sintim *et al*., 2012;

Sonoda and Tsumuki, 2005; Ugale *et al*., 2011b; Yamamoto *et al*., 2008; Zhang *et al*., 2011b). Several works of GST from these authors indicate that most herbivorous insects can selectively express GST enzymes for detoxification of allelochemicals in their diets and host plants. This study hypothesizes that GST will be upregulated in the presence of piceol and pungenol in the caterpillar's diet (Feng *et al*., 2001).

1.7 βeta-glucosidase enzyme

Another enzyme studied in this research was β-glucosidase. This enzyme cleaves the glycosidic bonds of phenolic glycosides to release aglycones (Ferreira *et al*., 1997; Lindroth, 1988). In some insect species, they also aid in digesting cellulose (Tokuda *et al*., 2009). For example, when generalist gypsy moth and forest tent caterpillars, were fed on a diet that contained a high concentration of the salicinoid salicortin, β-glucosidase levels in their midguts were reduced to avoid the formation of toxic aglycones (Hemming and Lindroth, 2000). This study predicts that β-glucosidase will be similarly downregulated in the presence of piceol and pungenol.

1.8 Objectives

The research focus was to determine the fate of the phenolic compounds after ingestion by the spruce budworm and, hence, to uncover their mode of detoxification. The study was aimed at resolving the following questions:

Does the spruce budworm modify the phenolic compounds during their passage in the midgut?

Does the spruce budworm produce detoxifying enzymes in response to feeding on the artificial diet containing the acetophenones?

In this study, spruce budworm larvae were fed on either control or phenolic-laced artificial diet from the fourth instar onward. Soluble proteins, as well as β-glucosidase and GST enzyme activity in midguts were measured in the midguts of sixth and final instar caterpillars. Enzyme activities were measured at both neutral and physiological (ie highly alkaline) pH. Two variations of the experiment were conducted: in the first, larvae were pre-treated on white spruce foliage and switched to artificial diet at the fourth instar. In the next experiment, spruce budworm larvae were pre-treated on control diet prior to the switch to control or phenolic-laced diet. These two versions

of the experiment were conducted to control for a potential effect of prior diet on midgut physiology.

2.0 Methods

2.1 Experimental design

Spruce budworm insects were obtained at the second instar larval diapausing stage from the Great Lakes Forest Research Centre, (Canadian Forest Service, Sault Ste. Marie, ON, Canada). They were delivered and maintained in cheese cloth at -4°C until their emergence from diapause. The larvae were reared in a laboratory incubator on pre-treatment diet (foliage in experiment 1; initial sample size, N = 200), (artificial diet in experiment 2; initial sample size, N = 200) at 23°C, 50% relative humidity. Larvae were placed in groups of 10 in Solo cups (2 cm diameter, 4 cm long).

At moult to the fourth instar, larvae were placed individually in new cups containing the treatment diet (either control or phenolic-laced) until one week after the moult to the sixth instar when they were removed for use in the experiment.

The experiment began by weighing the caterpillars, then dissecting them to remove midguts for biochemical analyses: Bradford soluble proteins, glutathione-*S*-transferase and β-glucosidase enzyme analyses. Frass from the treatment cups was collected and frozen at -80°C until HPLC analysis.

2.2 Pre-treatment diets

In experiment 1, insects were reared until fourth instar on current-year white spruce foliage collected at Morgan Arboretum (45˚53'N, 72˚92'W) on May 24 and June 17, 2016.

In experiment 2, initial rearing was done on modified McMorran Grisdale artificial diet (Grisdale, 1973) prepared in the laboratory as per the recipe provided by the Insect Production Services, Canadian Forest Service (Sault Ste. Marie, ON, Canada). Ingredients for 1 L diet, included 220 ml distilled water, 17.36 g agar, 35 g casein, 35 g sugar, 4M KOH, 5 g alphacel, 10 g Wesson's salt, 30.69 g toasted wheat germ, 1 g choline chloride, 4 g ascorbic acid, 1.5 g methyl paraben, 2.1 g aureomycin, 5 g raw linseed oil and 10 g vitamin solution. The ingredients above were mixed in a blender leaving out the vitamin solution. 620 ml distilled microwaved water and agar were placed evenly into two separate microwavable containers and heated for 10 mins, stirred, and heated for another 10 minutes until the temperature reached 85°C. Half of the heated agar solution was added to the blender and mixed. The second half of the agar solution was added to the blender and mixed

for 2 minutes. Ingredients were mixed together for about 1 minute until temperature dropped to 55°C. At 55°C, the vitamin solution was added and, finally, 10 ml of methanol solution containing the individual phenolic compounds was added and poured into the diet to form a mixture. When the diet was ready, it was poured into small plastic cups. The diets in the cups were allowed to dry for 30 minutes after pouring. Artificial diets were stored at -20 °C prior to use. Experiment 2 was replicated twice (once in 2016 and once in 2017).

2.3 Preparation of phenolic compounds

Piceol (4'-hydroxy-acetophenone) and pungenol (3',4'-dihydroxy-acetophenone) were purchased from Sigma-Aldrich (Oakville, ON, Canada). For both compounds, 0.966 g was dissolved in 10 ml of methanol. Piceol and pungenol compounds were then added to the artificial diet at a physiological concentration comparable to current year shoots found in natural foliage (Delvas *et al.,* 2011) to obtain the phenolic diets: 10 ml of methanol solution containing the individual phenolic compounds was added to the artificial diet at the same time as the vitamin mixture.

2.4 HPLC-DAD-MS analysis

The chromatographic separation and quantification of phenolics in the budworm frass were obtained using a LC-DAD-MS system. Specifically, we test whether the acetophenones or modified forms are present in the frass of insects fed the phenolic-laced diet.

2.4.1 HPLC-DAD-MS approach

Detection techniques for HPLC methods are various but diode array detection (DAD) is currently the most widely available and commonly used technique for routine qualitative and quantitative analysis of phenolic compounds (Merken and Beecher, 2000; He, 2000). These two instruments are coupled in line, so that the eluent flow from LC first passes through an UV-vis detector, after which the eluent is directed to MS detector (LC-DAD-MS). Phenolic compounds were identified on the basis of their ultraviolet absorption spectra, mass spectra, and retention times (Ossipov *et al*., 1995, 1996; Salminen *et al*., 1999, 2001; Valkama *et al*., 2003).

The diode array detector simultaneously measures a range of wavelengths (e.g., 200-500 nm), which enables the measurement of ultraviolet-vis spectra of phenolic compounds. Phenolic metabolites can be detected at one or more wavelengths, based on their absorbance spectra and are separated based on retention times (Santos-Buelga *et al*., 2003).

After separation of the phenolic compounds by HPLC, the mass spectrometer was used in the positive ionization mode. Depending on conditions, phenolic compounds such as monomeric flavan-3-ols and dimeric and trimeric proanthocyanidins, are protonated to positive ions (Lin *et al*., 2000) and deprotonated to negative ions (Poon, 1998; Friedrich *et al*., 2000; Hammerstone *et al*., 1999). Flavonol glycosides show responses in both positive and negative ion modes (Hakkinen and Auriola, 1998; Andlauer *et al*., 1999). Phenolics in their positive ionization mode can sometimes give more structural and fragmentation information (Cuyckens and Claeys, 2004). For mass spectrometry, a micromass Q-Tof Ultima ™ API instrument with electrospray ionization (ESI) in positive mode was used for detection and identification of conjugated forms of the phenolic compounds with scanning range between *m/z* 200-500, 3.5 K volt with a scan time of 1 second, drying gas flow 6 mL/min, nebulizer pressure 60 psi, dry gas temperature 300 °C, vaporizer temperature 250 °C. The instrument was programmed to detect the molecular mass of compounds between 50 to 900 Da.

2.4.2 **Standards**

The commercial standards, piceol (4'-hydroxy-acetophenone) and pungenol (3',4'-dihydroxy-acetophenone) were purchased from Sigma-Aldrich (Oakville, ON, Canada). These standards, 2 mg were dissolved in 1 ml of 70% methanol.

Phenolics were separated through Spursil C18 3 µm column (150 * 2.1 mm). The mobile phases consisted of (A) 0.1% formic acid and (B) (0.1% formic acid in acetonitrile (ACN). The mobile phase gradient was as follows: 0-12 min, 3-45% B; 12-13 min, 45-95% B; 13-15 min, 95% B and 15-18 min, 95-98% B. The column flow rate was 250 µl per min. The detection wavelength was at 280 nm for phenolic frass and 275 nm for the standards. The column temperature was 25 °C. 10 µl of extract was injected into the column.The experiment was repeated three times.

2.4.3 **Preparation of frass samples**

A method based on (Mageroy *et al*., 2014) was used for the extraction of phenolic compounds from the frass of the spruce budworm from Experiment 2 (2017). Frass from 30 individual caterpillars from each treatment diet was pooled and dried in an oven for 24 hrs and grinded to powdered form using liquid nitrogen, then stored in 2 ml Eppendorf tubes at -80°C prior to analysis. 50–100 mg of fine dried powder of frass was extracted using 1 ml of 70% HPLC grade methanol. Benzoic acid (1 mg/ml) was used as an internal standard with 150 µl of benzoic acid added to 350 µl of the liquid sample. 70% methanol (600 µl) was added to the frass powder and incubated at 4°C on a shaker. After 6, 24 and 48 hours of incubation, the samples were centrifuged at 13 000 g for 10 mins. The supernatants were pooled and kept at -80°C. A fresh 600 µl of aqueous methanol was added to each sample, and after incubation, centrifugation was repeated. Extracts obtained after 6, 24 or 48 hours were pooled as a single extract for HPLC-DAD-MS analyses. Extraction and analysis was replicated twice.

2.4.4 **pH effects on acetophenones**

Piceol and pungenol were incubated together at a neutral pH 7.2 and at an alkaline pH 9.2 for 24 hours and analyzed by LC-DAD-MS to test whether pH modifies the structure of these compounds. The concentration of the piceol and pungenol in the neutral buffer (potassium hydrogen phosphate) and alkaline buffer (sodium bicarbonate) solutions were 1 mg/ml for each compound. This experiment was replicated twice.

2.5 Enzyme analysis

2.5.1 Midgut sample preparation

Sixth instar caterpillars were dissected to remove the midguts and four midguts were pooled for each sample. Midguts were rinsed in saline dissection buffer and placed four together in a prelabelled Eppendorf tube samples that contained 600 µl of sterile dissection buffer and 600 µl of protease inhibitor cocktail. The midgut samples were homogenized in each Eppendorf tube sample, and the homogenates centrifuged at 13 000 rpm at 4°C for 5 minutes. The supernatants were transferred to a new, labelled Eppendorf tubes. 5 µl aliquots of gut homogenate were used

for the Bradford protein assay and 10 µl of the homogenate was used for the enzyme assays. For each of the three biochemical assays, the design of the microplate included a positive control, negative control, gut samples, each done in triplicate. Assays were conducted at both alkaline and neutral pH, 9.2 and 7.2 respectively. The β-glucosidase enzyme assay was run at a static read and the glutathione-*S*-transferase enzyme assay was run at a kinetic read.

2.5.2 **Bradford protein assay**

The soluble protein concentration of each midgut sample was determined by the use of the Bradford reagent (Bradford, 1976). The buffer used was 0.1M phosphate buffer, pH 7.2. The linear concentration range was 0.1-1.4 mg/ml of protein using bovine serum albumin (BSA) to make a standard curve of known concentrations. The Bradford reagent (Bio-Rad) was diluted in distilled water in a 2.5 fold dilution factor; 10 ml of the Bradford reagent was added to 15 ml of distilled water in a 45 ml centrifuge tube. The total volume in all the wells was 255 µl.

The absorbance was measured at both 590 nm and 450 nm using the Tecan spectrophotometer. The absorbances of the samples were recorded before the 60 minute time limit. A calibration curve was prepared by finding the ratio net absorbance values at 590 nm and 450 nm versus the protein concentration of each standard. The soluble protein concentration of the unknown samples was determined by the $A_{590/450}$ values against the standard curve.

2.5.3 **β-glucosidase enzyme assay**

In this assay, the β-glucosidase enzyme reacts with the substrate 4-methylumbelliferyl β-D-glucopyranoside to produce a violet colored complex in a black well microplate system. The product formed was 4-methylumbelliferone with an absorbance at 450 nm. The standard curve was prepared by using the reagent 4-methylumbelliferone. A stock of 5 U/ml was serially diluted to create six concentrations from a highest point of 5 U/ml to a lowest point of 0.7 U/ml. The samples were diluted in a ratio of 1:1 with buffer to determine the optimal amount of sample for the assay. After the assay was set up, the black plate was incubated for 30 minutes at 35°C. The reaction was visualized and stopped after 30 minutes by adding a stopping buffer of 50 µl of 5 mM NaOH to all the wells. The black plate was inserted into the spectrophotometer and absorbance

values recorded at 450 nm. The β-glucosidase activity in U/ml was corrected according to the soluble protein levels (U/mg soluble proteins).

2.5.4 **Glutathione-*S*-transferase enzyme assay**

This enzyme catalyzes the addition of glutathione to the substrate, 1- chloro 2, 4 dinitrobenzene (CDNB), that can be seen at 340 nm with the use of the spectrophotometer. One unit of the GST enzyme conjugates 10 nMol of CDNB with reduced glutathione per minute at 25°C. The product of the reaction formed a yellow colored product.

25 µl of 10 mM reduced glutathione (GSH), 25 µl of 10 mM 1-chloro, 2,4 dinitrobenzene (CDNB) dissolved in (0.1% v/v in 95% ethanol), 0.1 U/ml glutathione-*S*-transferase (GST) enzyme and 10 µl of gut homogenate were transferred into a clear ultraviolet microplate well at neutral or alkaline pH. Enzyme activity was determined in U/ml by monitoring changes in absorbance at 340 nm, measured every 15 seconds for 2 minutes under the spectrophotometric kinetic mode, at a constant temperature of 25°C. The GST enzyme activity in U/ml was then corrected for its soluble protein level (U/mg soluble proteins).

2.5.5 **Statistics**

Student's t-tests were used to compare caterpillar mass, total soluble protein, β-glucosidae activity and GST activity between insects fed control and phenolic-laced diets. Analyses were done using SPSS version 21.

3.0 Results

3.1 Caterpillar mass

In all three experiments, growth of the spruce budworm larvae reared on phenolic diet was lower compared to control diet but not significant (Fig 3). Experiment 1 (foliage to artificial diet, 2016): P = 0.240, d.f. = 22, t stat = 1.5321; experiment 2 (artificial diet to artifical diet) : P = 0.102, d.f. = 22, t stat = 1.3213 (2016), P = 0.105, d.f. = 22, t stat = 1.3013 (2017).

3.3 HPLC-DAD-MS

3.3.1 Identification of standards

The chromatographic analyses of piceol and pungenol standards produced sharp peaks at different retention times which were 7.980 mins and 10.369 mins (Fig 4).

3.3.2 HPLC-DAD detection of phenolics in budworm frass

Peak assignments of phenolic compounds in the chromatograms were based on the comparison of their spectral characteristics with their retention times to the internal standards, piceol and pungenol. Frass from caterpillars fed on control diet did not contain any phenolic compounds (Fig 8). In the frass from caterpillars fed on phenolic diet, piceol and pungenol were not detected (Fig 9), but other peaks were observed.

3.3.3 Putative identification of compounds by HPLC-MS

Four phenolic metabolites were detected in the frass of the caterpillars fed on phenolic diet; these were identified as glycosylated and glutathionylated-S-conjugated forms of piceol and pungenol (Fig 10B), (Fig 11B), (Fig 12B) and (Fig 13B).

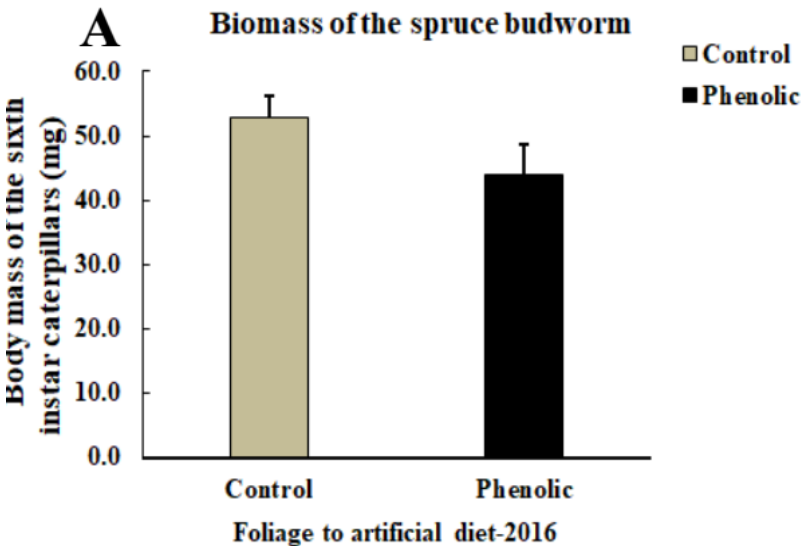
3.3.4 HPLC-DAD-MS of phenolic compounds incubated at pH 7 and pH 9

The incubation of piceol and pungenol compounds in alkaline and neutral conditions produced different coloured products: neutral solutions remained clear, but at high pH the solution turned dark red. The colour change suggests possible transformation of these compounds under pH conditions similar to those in the budworm midguts (Fig 5). HPLC-DAD-MS analysis of the

mixtures detected novel compounds, detected at low concentrations at pH 7 and at high concentrations at pH 9 (Fig 6 and 7). The molecular masses of the compounds detected suggest the formation of dimers. These compounds were not detected in the frass samples.

3.3.5 Figures

Experiment 1



Experiment 2

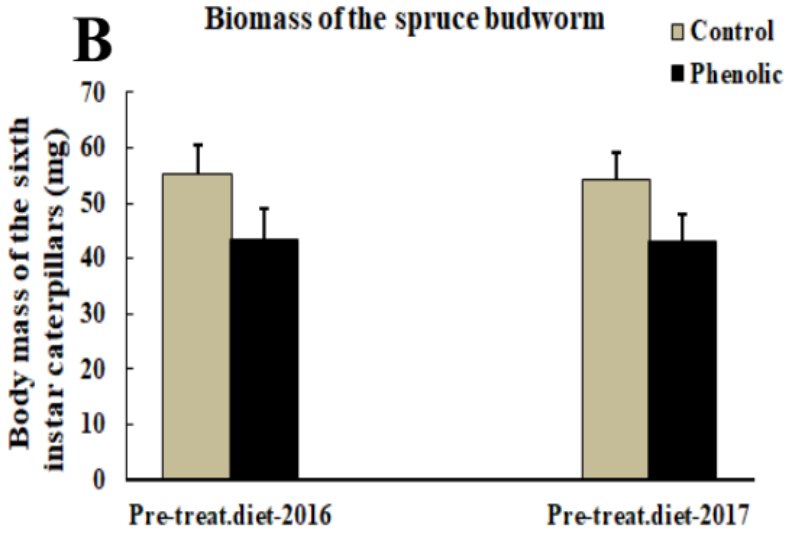


Fig 3. Body mass of sixth instar budworm caterpillars (mean±SE), fed on **A)** Foliage to artificial diet or **B)** Artificial diet to artificial diet.

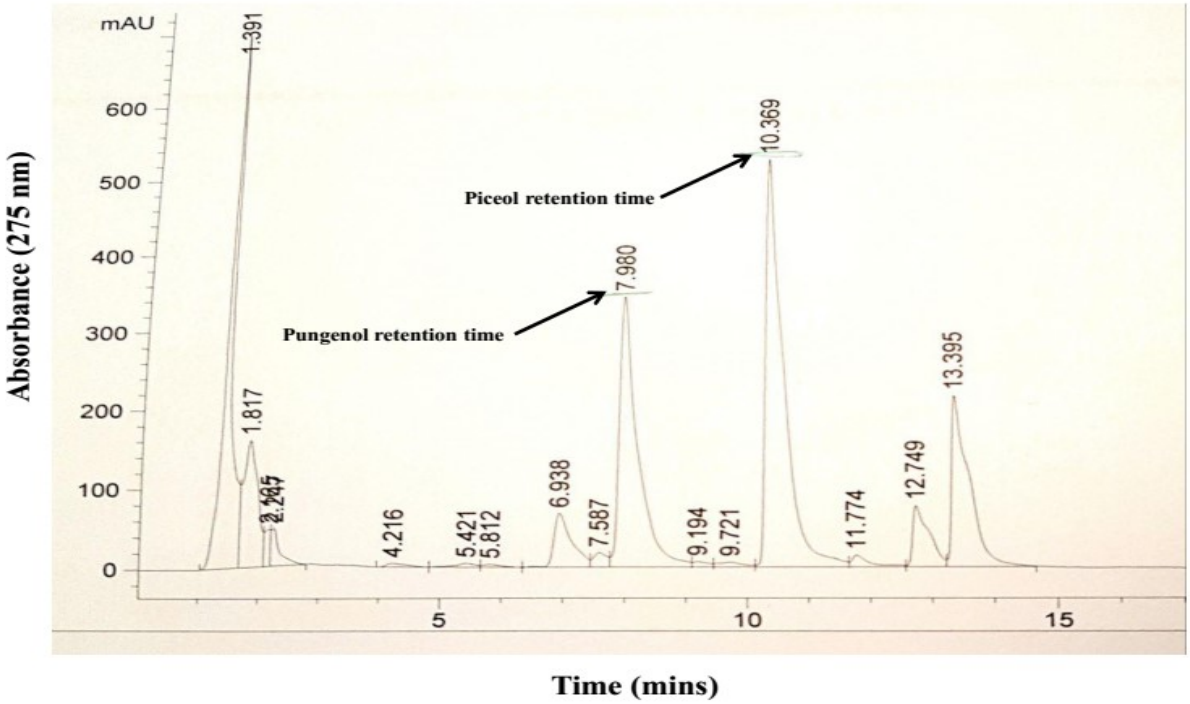


Fig 4. HPLC-DAD of piceol and pungenol standards at a detection wavelength of 275 nm. The retention times for pungenol and piceol were 7.98 mins and 10.37 mins, respectively.

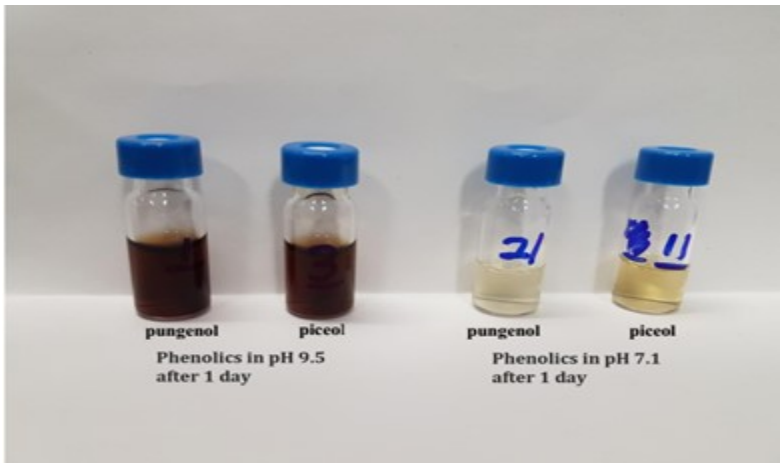


Fig 5**.** Incubation of phenolic samples in neutral and alkaline solutions. The first two solutions represent piceol and pungenol compounds after incubation at an alkaline pH 9.5 for 24 hours and the second set of solutions represent piceol and pungenol compounds after incubation at a neutral pH 7.1 for 24 hours.

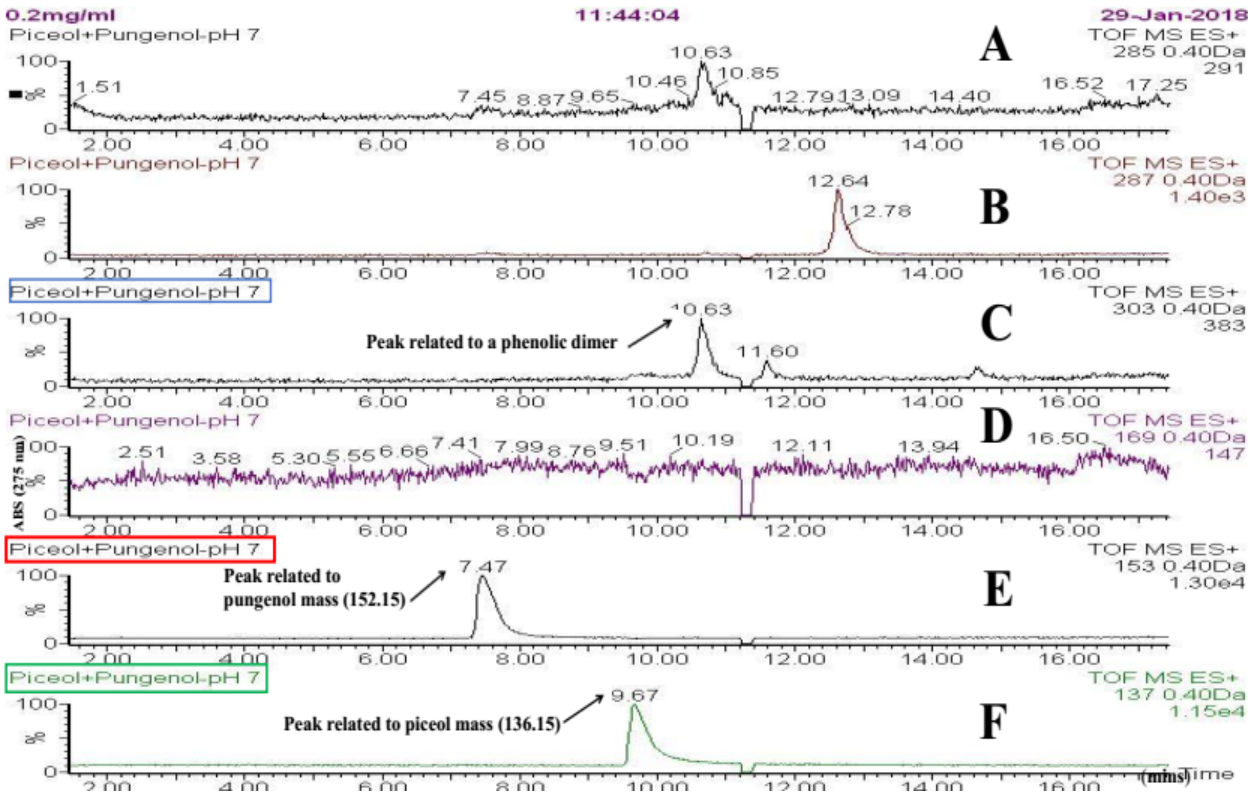


Fig 6. HPLC-MS chromatograms of piceol and pungenol compounds incubated at pH 7.2, at a detection wavelength of 275 nm, measured at m/z **A)** 285 **B**) 287 **C)** 303 **D)** 169 **E)** 153 **F)** 137. The peak at m/z 285 with retention time 10.63 mins is likely to represent a phenolic dimer by the combination of two piceol compounds. The peaks at m/z 287 with retention time 12.64 mins and at m/z 303 with retention time 10.63 min are all suggested to be a phenolic dimer. The m/z at 169 with the various retention times may represent the oxidised form of the pungenol compound. The retention times for pungenol (m/z 153) and piceol (m/z 137) compounds were seen at 7.47 min and 9.67 min respectively.

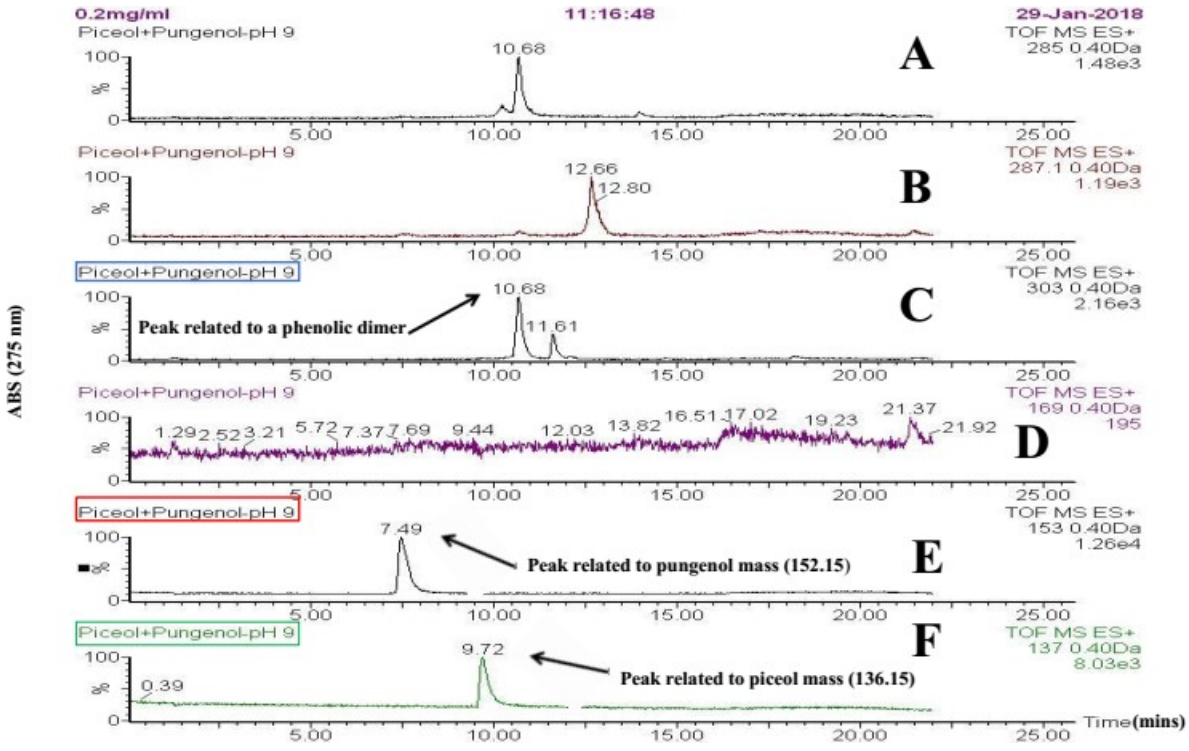


Fig 7. HPLC-MS chromatograms of piceol and pungenol compounds incubated at pH 9.5, at a detection wavelength of 275 nm, measured at m/z **A)** 285 **B)** 287 **C)** 303 **D)** 169 **E)** 153 **F)** 137. See fig. 6 for explanation of peaks.

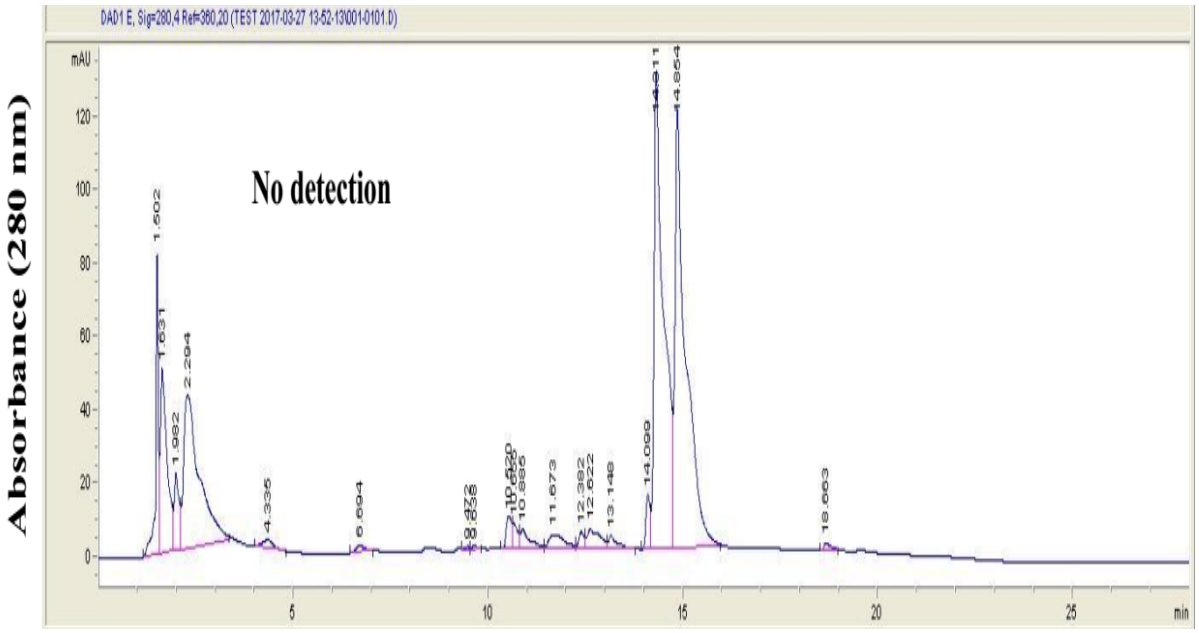


Fig 8. HPLC-DAD chromatogram of frass from caterpillars fed on artificial diet alone (control) recorded at 280 nm. As expected, the phenolic compounds pungenol and piceol, were not detected at their retention times, 7.980 mins and 10.369 mins respectively.

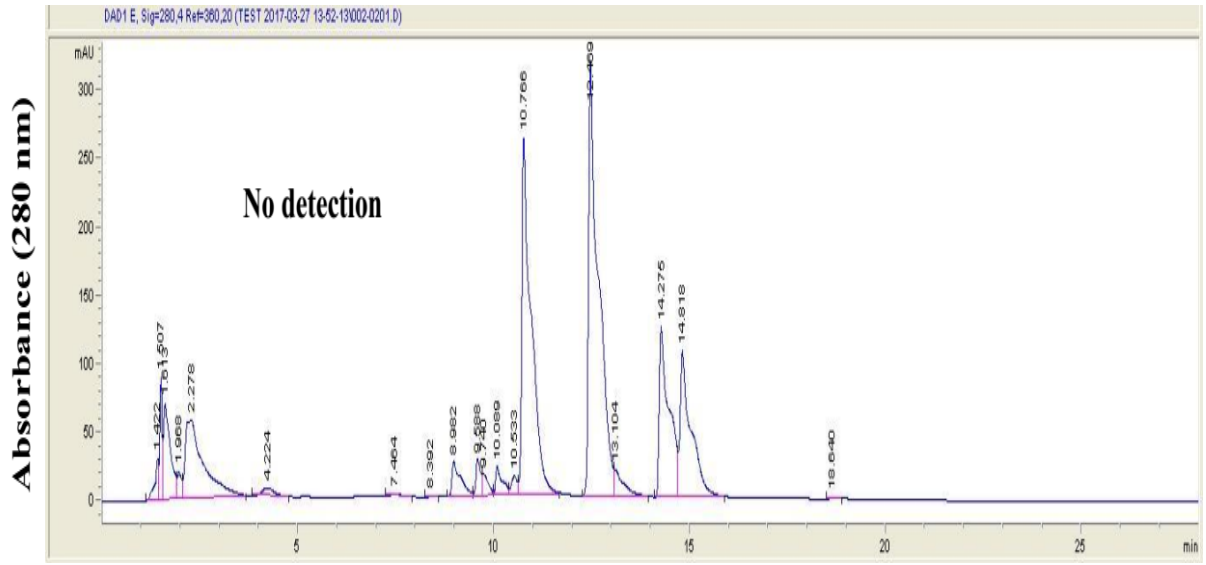


Fig 9**.** HPLC-DAD chromatogram of frass from caterpillars fed on artificial diet containing phenolic compounds (phenolic) recorded at 280 nm**.** The phenolic compounds, pungenol and piceol, were not detected at their retention times 7.980 mins or 10.369 mins respectively.

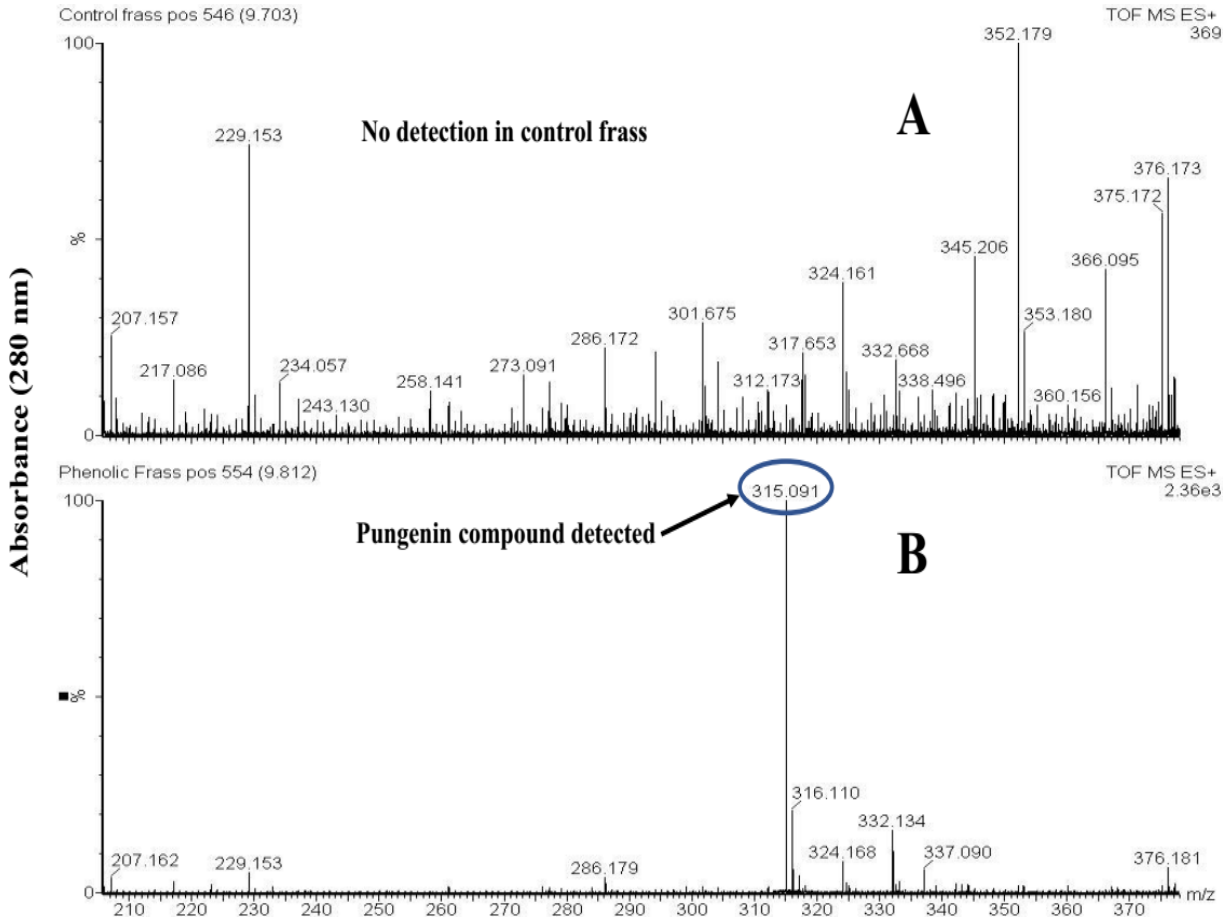


Fig 10. HPLC-MS chromatograms of frass of spruce budworm caterpillars fed on either **A)** Control diet or **B)** Phenolic diet. Peak detected in the frass of the spruce budworm caterpillars fed on the phenolic-spiked diet approximate to the molecular mass of pungenin (315.11) with retention time 9.812 mins. Pungenin was not detected in the frass of the spruce budworm caterpillars fed on control diet.

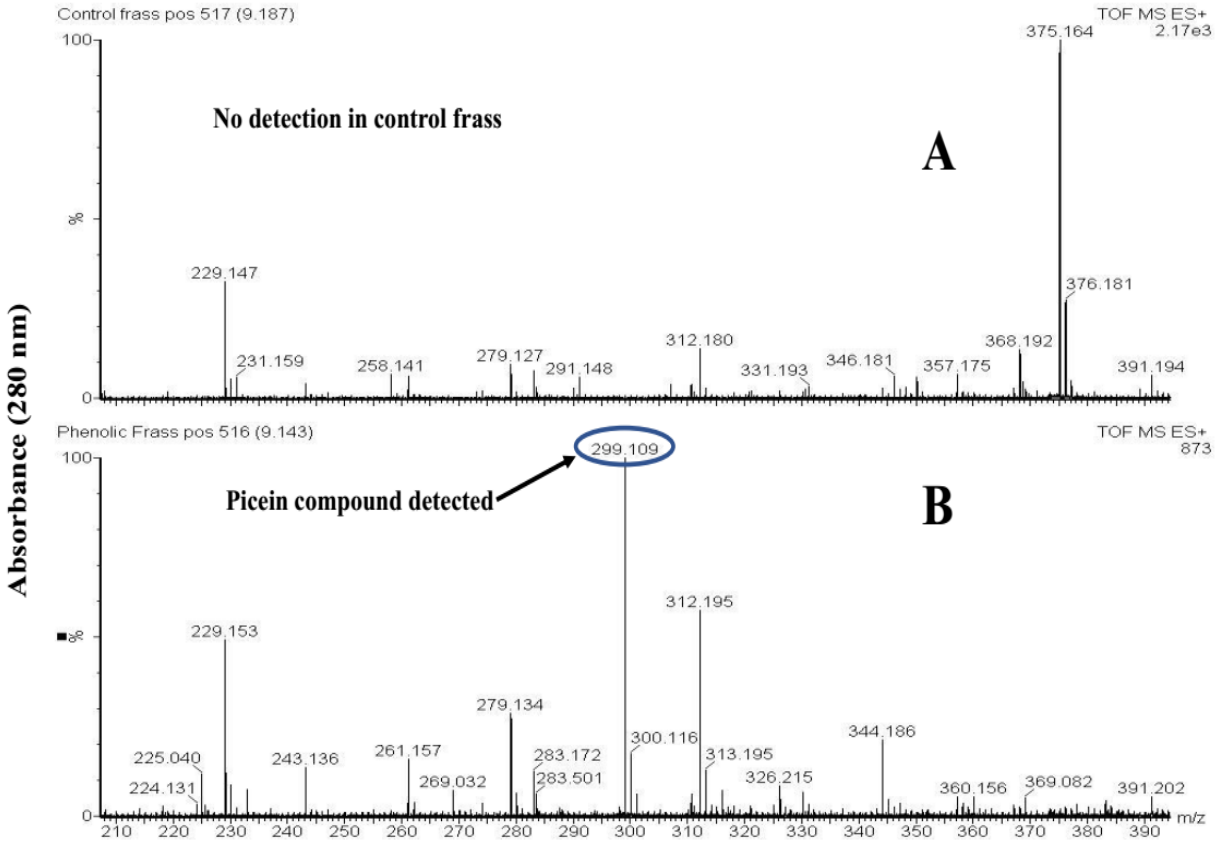


Fig 11**.** HPLC-MS chromatograms of frass of spruce budworm caterpillars fed on either **A)** Control or **B)** Phenolic diet. Peak detected in the frass of the spruce budworm caterpillars fed on the phenolic-spiked diet approximate to the molecular mass of picein (299.11) with retention time 9.143 mins. Picein was not detected in the frass of the spruce budworm caterpillars fed on control diet.

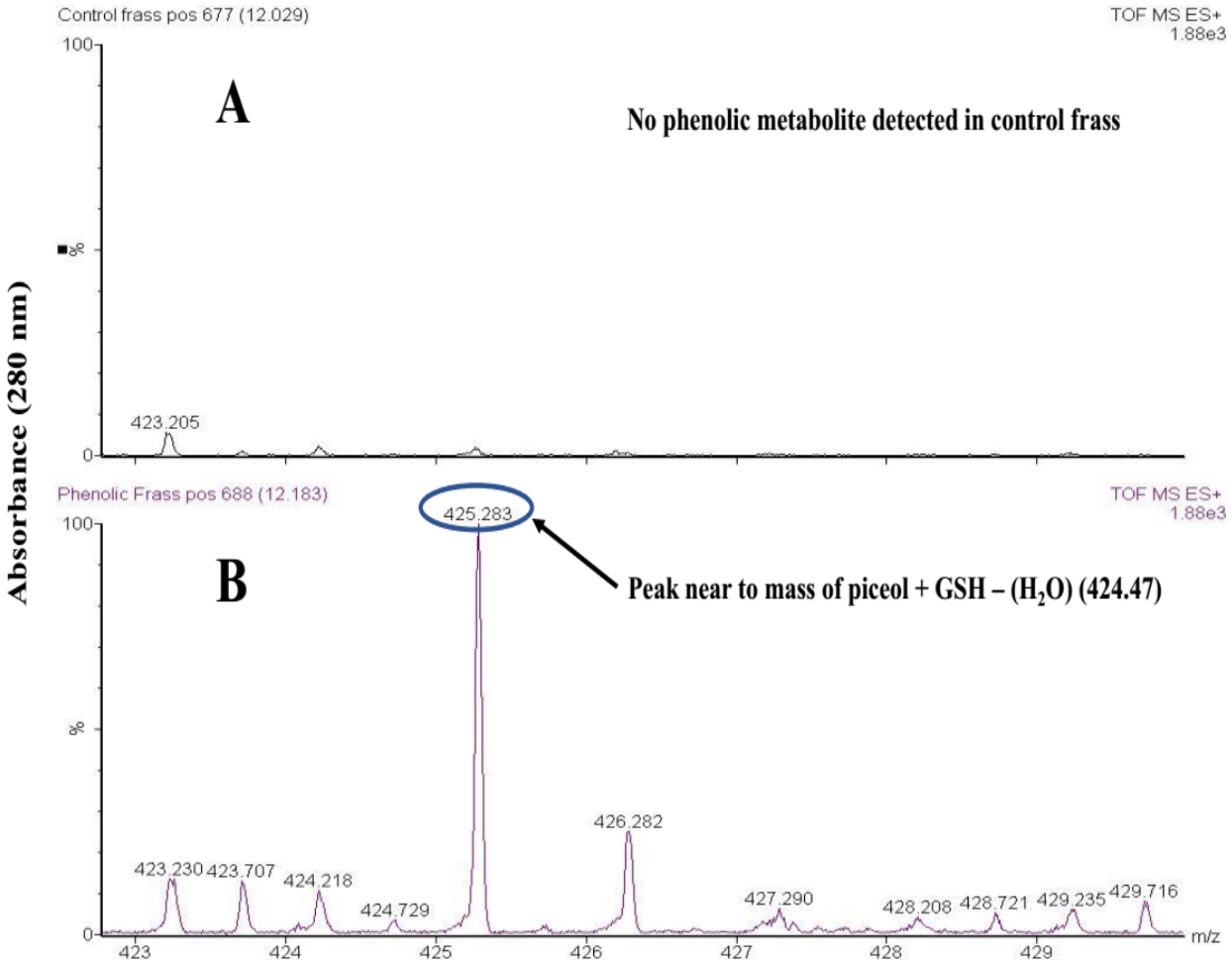


Fig 12. HPLC-MS chromatograms of frass of spruce budworm caterpillars fed on either **A)** Control diet or **B)** Phenolic diet. A putative glutathionylated conjugate of piceol was detected in the frass of the spruce budworm caterpillar fed on phenolic-spiked diet. The m/z of piceol is 137. The compound at m/z 425.28 **(**retention time 12.183 min) could represent loss of water (H_2O) plus addition of glutathione to piceol, and elimination of a proton. This compound was not detected in frass of the spruce budworm caterpillar fed on control diet.

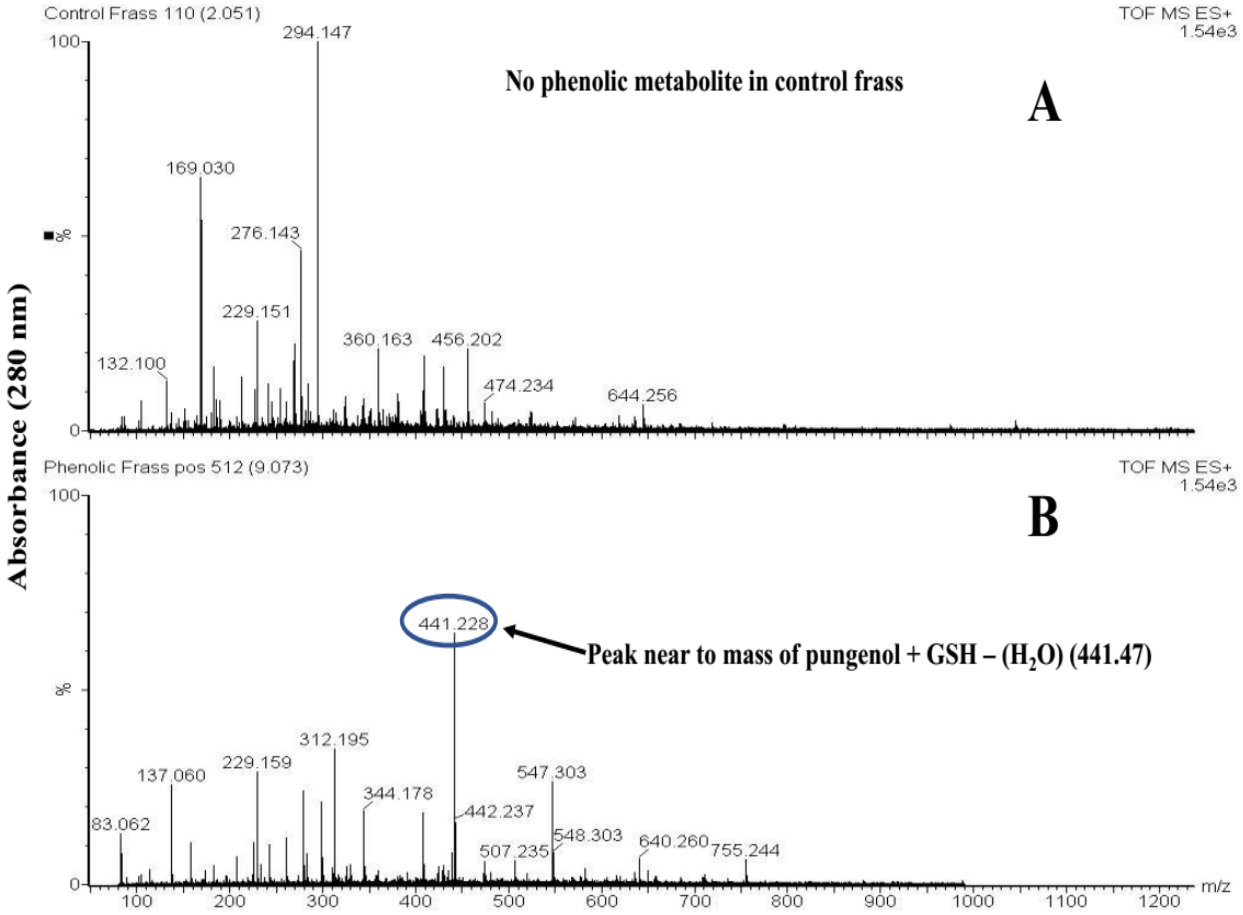


Fig 13. HPLC-MS chromatograms of frass of spruce budworm caterpillars fed on either **A)** Control diet or **B)** Phenolic diet. A putative pungenol glutathione-S conjugate was detected in the frass of the spruce budworm caterpillar fed on phenolic diet. This was identified as putatively glutathionylated conjugate of pungenol. The m/z of pungenol is 153. The compound with m/z 441.23 **(**retention time 9.073 min) could represent the loss of water (H_2O) plus addition of glutathione to pungenol, and elimination of a proton. This compound peak was not detected in frass of the spruce budworm caterpillars fed on control diet.

3.4 Enzyme analysis

3.4.1 Midgut soluble protein levels

The midgut soluble protein levels of the spruce budworm caterpillars fed on control diet were not significantly different from those of budworm fed on phenolic diet in any of the three experimental trials: Experiment 1 (foliage to artificial diet): P = 0.143, d.f. = 22, t stat = -1.5196, Fig 14A; Experiment 2 (artificial to artificial diet): P = 0.143, d.f. = 22, t stat = -1.5866 (2016), (P = 0.541, d.f. = 20, t stat = -0.6224 (2017), Fig 14B.

3.4.2 β-glucosidase enzyme activity

There was no significant difference in β-glucosidase enzyme activity per mg soluble protein between control caterpillars and those fed on artificial diet containing the phenolic compounds in any of the three experimental trials, at either neutral or alkaline pH: Experiment 1 (foliage to artificial diet, Fig 15) : P = 0.4967, d.f. = 22, t stat = 0.6910 (neutral, Fig 15A), P = 0.402, d.f. = 22, t stat = -0.8544 (alkaline, Fig 15B); Experiment 2 (artificial to artificial diet, Fig 16): P = 0.4902, d.f. = 22, t stat = 0.7016 (2016 neutral), P = 0.8199, d.f. = 20, t stat = 0.2305 (2017, neutral), P = 0.101, d.f. = 22, t stat = -1.8997 (2016 alkaline), P = 0.07, d.f. = 20, t stat = 1.9442 (2017 alkaline).

3.4.3 Glutathione-*S*-transferase enzyme activity

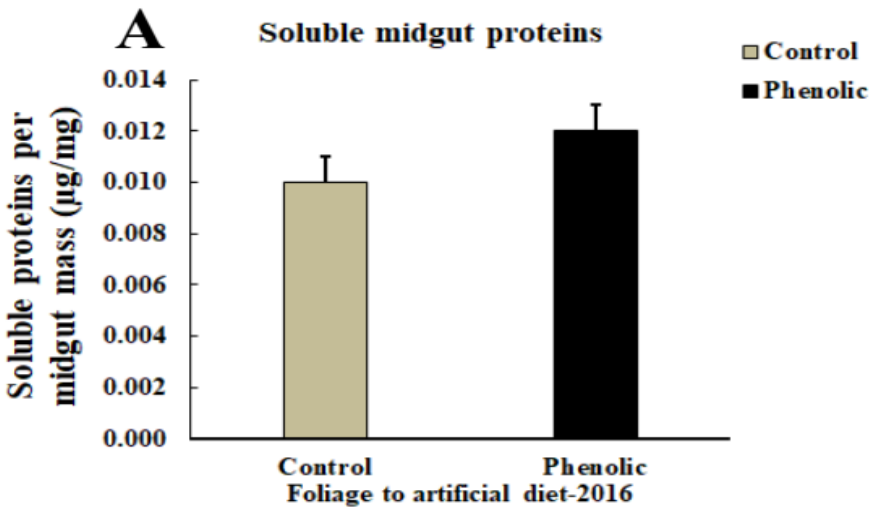
Results from neutral pH assays from 2016 (both Experiment 1 and Experiment 2) are not presented due to difficulties optimizing the assay.

In Experiment 1 (foliage to artificial diet) at alkaline pH, glutathione-*S*-transferase enzyme activity in the midgut of the spruce budworm larvae fed on phenolic diet was significantly higher than that in the midgut of the spruce budworm larvae fed on control diet (P = 0.0006, d.f. = 22, t stat = -4.0338, Fig 17).

Similarly, in Experiment 2 (artificial to artificial diet), glutathione-*S*-transferase enzyme activity was significantly higher in in the midgut of the spruce budworm larvae fed on artificial diet with phenolics than in controls, at neutral pH (P = 0.007, d.f. = 18, t stat = -3.071 (2017), Fig 18A). and at alkaline pH (P = 0.004, d.f. = 22, t stat = -3.2047 (2016) & P = 0.017, d.f. = 18, t stat = -2.6419 (2017), Fig 18B).

3.4.4 Figures

Experiment 1



Experiment 2

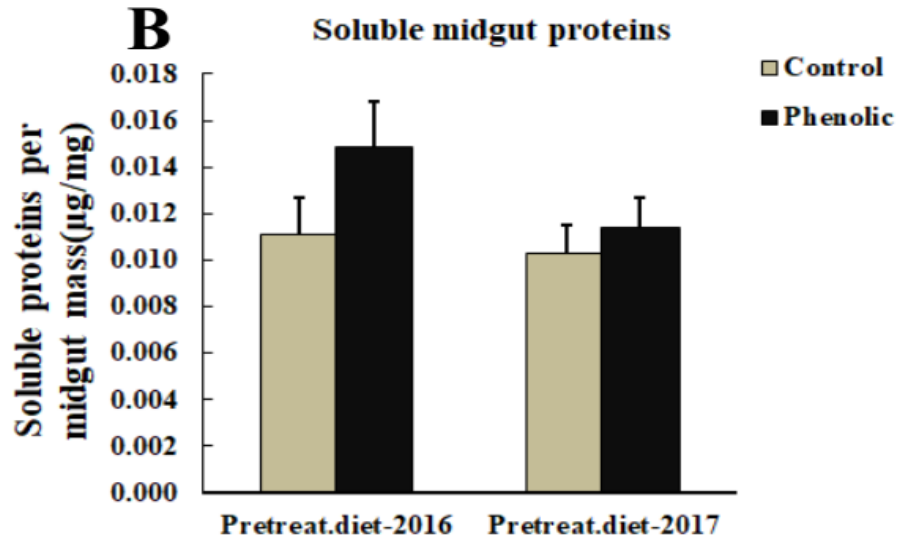


Fig 14. Soluble midgut proteins from sixth instar spruce budworm fed on **A)** Foliage to artificial diet or **B)** Artificial diet to artificial diet. Soluble protein levels are measured by modified Bradford method (Bradford, 1976) and expressed as soluble protein (µg soluble proteins per midgut mass (µg/mg), (mean±SE, N = 12).

Experiment 1

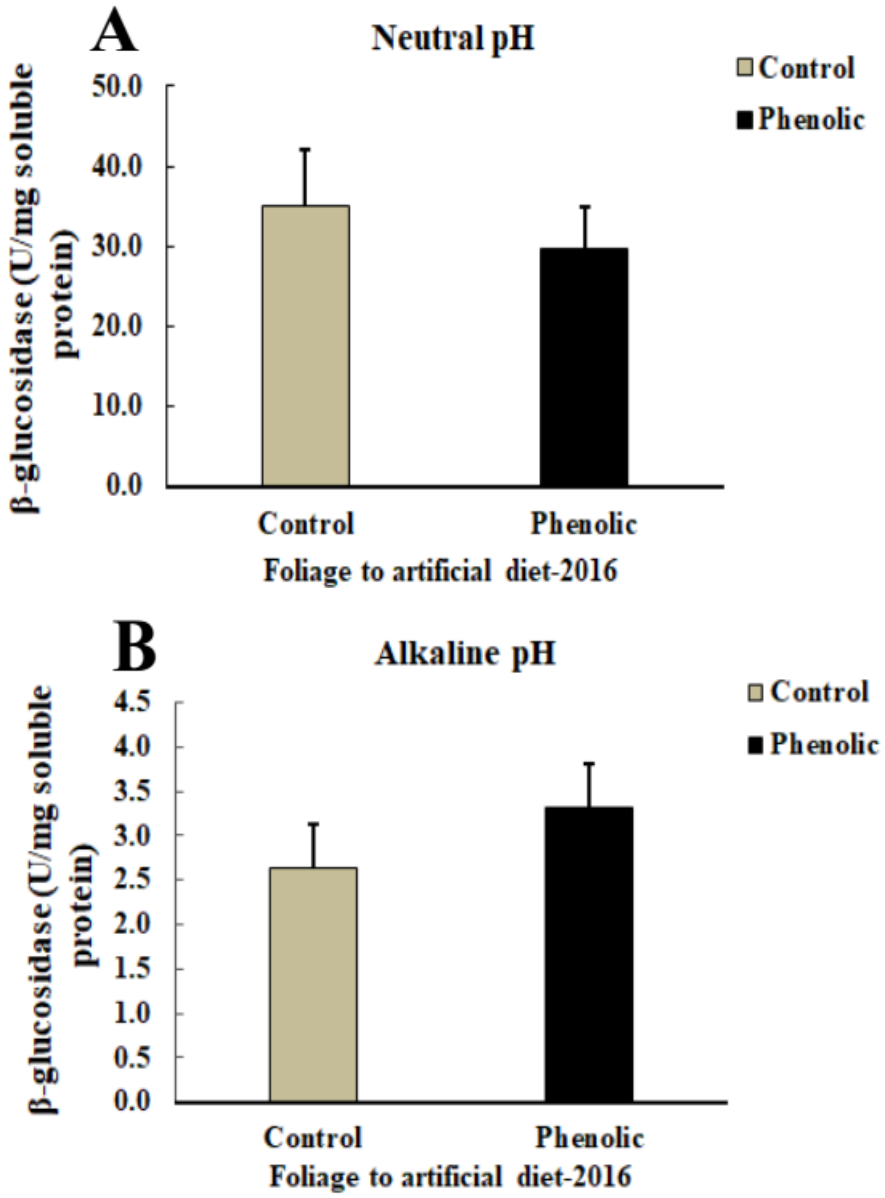


Fig 15. βeta-glucosidase activity of the midgut tissue of the spruce budworm on foliage to artificial diet at **A)** pH 7.2 or **B)** pH 9.2. The activity is represented as β-glucosidase enzyme activity per mg soluble protein of the midguts (mean±SE, N =12).

Experiment 2

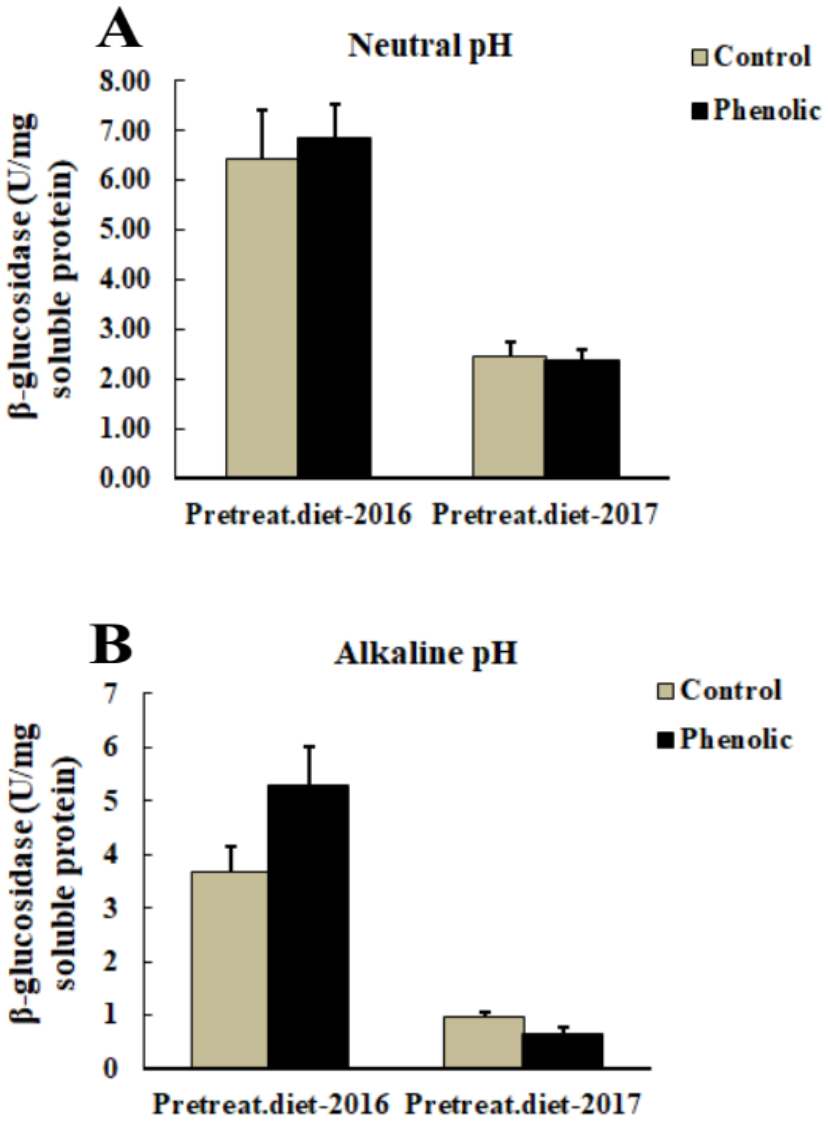


Fig 16. βeta-glucosidase activity of the midgut tissue of the spruce budworm on artificial diet to artificial diet at **A)** pH 7.2 or **B)** pH 9.2. The activity is represented as β-glucosidase enzyme activity per mg soluble protein (mean±SE, N =12).

Experiment 1

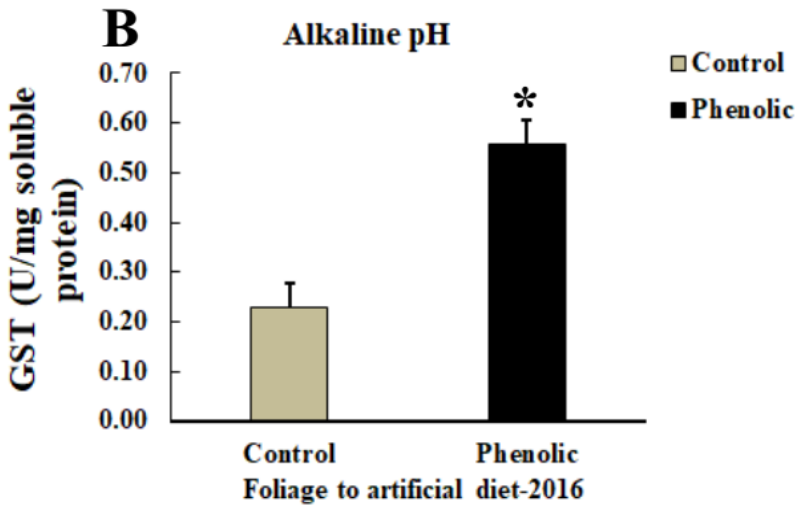


Fig 17**.** Glutathione-*S*-transferase enzyme activity of the midgut tissue of the spruce budworm on foliage to artificial diet at pH 9.2. The activity is represented as glutathione-*S*-transferase enzyme activity per mg soluble protein (mean±SE, N=12). Asterisks **(*)** indicates significant difference ($P < 0.05$).

Experiment 2

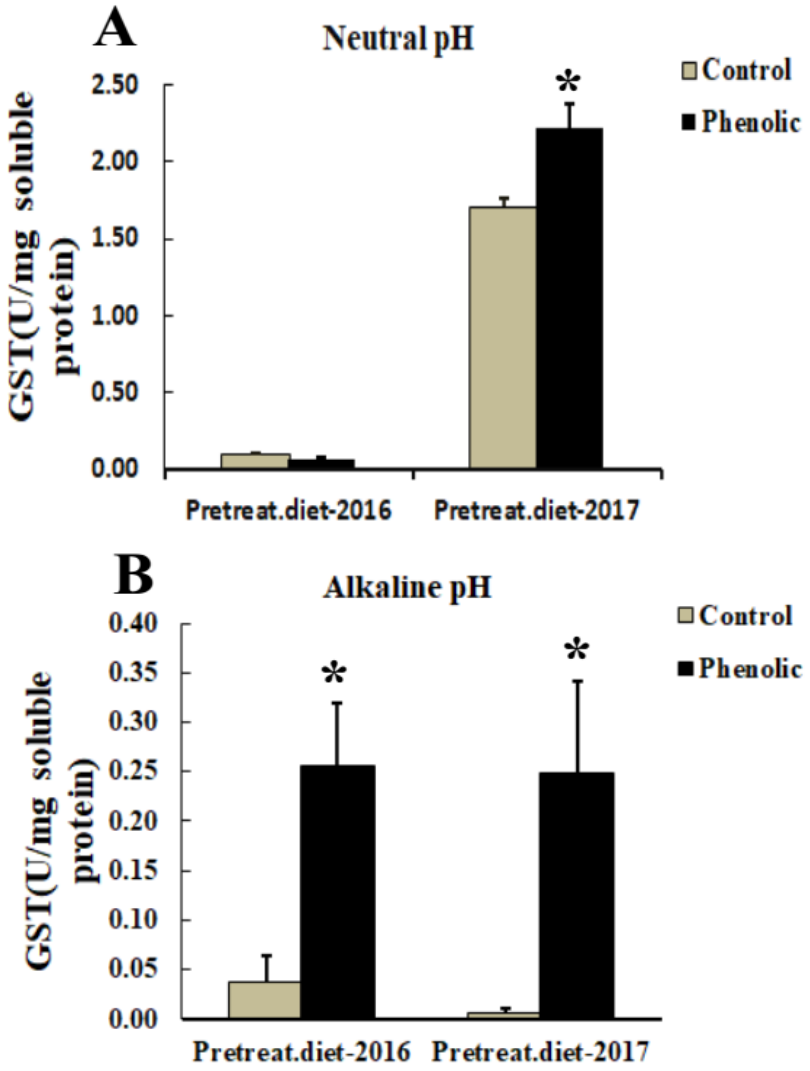


Fig 18. Glutathione-*S*-transferase enzyme activity per mg soluble protein (mean±SE, N=12) of the midgut tissue of the spruce budworm in Experiment 2 (artificial diet to artificial diet) at **A)** pH 7.2 or **B)** pH 9.2. Asterisks **(*)** indicate significant difference ($P < 0.05$).

4.0 Discussion

4.1 Fate of the acetophenones in the spruce budworm midgut

The addition of the phenolic compounds, piceol and pungenol to the spruce budworm diet reduced the body mass of the spruce budworm in comparison to the spruce buworm fed artificial diet only. The combined effect of piceol and pungenol in caterpillar diet affected the growth of the spruce budworm relative to the spruce budworm fed control diet without phenolics, though ingested phenolics were potentially detoxified by larval digestive enzymes.

The HPLC-DAD chromatograms of the frass of the spruce budworm caterpillars fed on control diet did not contain the acetophenones (Fig 8). This was expected as no phenolic compounds were added to the control diet. However, the acetophenones (Fig 9) were not detected in the frass of the spruce budworm caterpillar fed on phenolic diet either. Other compounds were present in this frass and absent from the control frass, but their retention times (Fig 9) did not match the retention times of the standard solutions of piceol and pungenol (Fig 4). This suggests that these compounds may be products of metabolism of the original acetophenones.

The midgut pH of the spruce budworm is strongly basic like in most Lepidopteran species (Martin and Martin, 1983; Appel, 1993). The massive colour changes in the piceol and pungenol mixture incubated at pH 9.2 compared to pH 7.2 suggests the acetophenones were transformed and dimerized under alkaline conditions (Fig 5). The HPLC-MS analysis identifies the molecular masses of the pungenol and piceol compounds at m/z 153 and 137, respectively, at both pH 7 and pH 9. The HPLC-MS also detected a putative phenolic dimer at m/z 303 at a higher intensity at alkaline than neutral pH, which could underlie the observed colour change seen at alkaline pH (Fig 6 & Fig 7). Previous research also shows colour changes in phenolics incubated at neutral or alkaline pH depending on the chemical properties of these phenolics (Vihakas *et al*., 2015). The accumulation of colorful products after incubation at alkaline pH could be thought to represent the biochemical transformation these phenolics undergo at the highly alkaline midgut environment of the Lepidopteran larvae (Vihakas *et al*., 2015).

However, these dimers were not detected in either control or phenolic frass. Thus, although some chemical changes do occur to these compounds at midgut physiological pH, high pH alone does not explain the new peaks observed in the phenolic-fed budworm frass. The novel peaks in the phenolic frass are, therefore, likely to be the products of enzymes, and may represent glycosylated and glutathionylated metabolites of the ingested acetophenones. This likely represents a form of detoxification prior to egestion, as the glycosides are known to be less harmful to the budworm than are the aglycones (Delvas *et al*., 2011).

Biochemical analysis of caterpillar midguts showed upregulation of glutathione-*S*-transferase in insects fed phenolic-laced artificial diet compared to controls. This result was consistent between insects pre-treated on foliage and on artificial diet. Enzyme assays were conducted at both neutral and alkaline pH but the result at alkaline pH is more representative of true midgut conditions. Our results demonstrate the feasibility of running enzyme assays under midgut physiological conditions. The midgut enzymes at the alkaline pH were generally seen to be higher than at neutral pH, reflecting the fact that these enzymes have evolved to operate at high pH. Therefore, conducting the biochemical assay at pH 9.2 provides more biologically relevant information about metabolic processes in the midgut of spruce budworm larvae.

4.2 Metabolism of phenolics by insects

Previous work on the fate of phenolic compounds in insect midguts show that the outcome depends both on the individual compound and the conditions in the gut lumen pH (Appel, 1993). Phenolic compounds may be egested unchanged or transformed by metabolism. The alkaline conditions of the midgut lumen in most Lepidopteran species will oxidise phenolic compounds (Barbenhen *et al*., 2006a; Moilanen and Salminen, 2008).

A study by (Salminen *et al*., 2004) highlights the fact that individual phenolics face different fates in the digestive tract of the Lepidopteran herbivore, *Epirrita autumnata* in which chlorogenic and p-coumaroylquinic phenolic acids were isomerised from the gut, flavonoid glycosides were egested without visible metabolic modifications, and flavonoid aglycones were partially detoxified into acacetin-7-O-glucoside and kaempferide-3-O-glucoside via glycosylation. This study shows

that piceol and pungenol are detoxified by glutathionylation and glycosylation and subsequently egested.

Some phenolic compounds, like quercetin and catechin, upregulated antioxidant activities in the midgut lumen of lepidopteran larvae (Johnson and Felton, 2001; Johnson, 2005). The authors observed that the Lepidopteran species tobacco budworm, *Heliothis virescens* after being fed phenolic compounds enhanced its midgut antioxidant properties to act as physiological barrier against reactive oxygen species in the midgut lumen. The antioxidant activity of the midgut lumen produced biochemical mechanisms to suppress prooxidant activities that could possibly lead to oxidative stress. Examples of these mechanisms include production of antioxidants such as glutathione, ascorbate, uric acid (Summers and Felton, 1994; Barbehenn *et al*., 2001) or enzymes such as catalase (Felton and Duffrey, 1991), or glucose oxidase (Johnson and Barbehenn, 2000). The present study shows how the anti-oxidant glutathione is conjugated to phenolics in the spruce budworm midgut, presumably decreasing their oxidative capacity and hence their toxicity.

4.3 Glutathionylation

Detoxification is one of the important mechanisms in insects to deal with plant allelochemicals (Terriere, 1984). Detoxification defenses are essential to enable herbivorous insects to overcome the chemical defenses of their host plants (Ahmad, 1992; Felton and Summers, 1995). Glutathione (GSH) chemically reduces a variety of electrophilic compounds, typically by glutathione-*S*-transferase enzyme-catalysed reactions. As a detoxification compound, GSH forms covalent adducts with reactive toxins and other quinones, which are excreted (Gant *et al*., 1988; Hayes and McLellan, 1999; Masella *et al*., 2005).

(Schramm *et al*., 2011) detected glutathione conjugates of glucosinolates-derived isothiocyanates in the frass of lepidopteran species, such as *S. exigua*, *H. armigera*, *T. ni*, and *M. brassicae* and *S. littoralis* larvae after feeding on glucosinolate-containing plants. When the Lepidopteran herbivores were fed Brassicaceae plants containing the toxic isothiocyanates, they were able to metabolize a substantive portion of the ingested toxins by conjugation with GSH and these GSH conjugated form of isothiocyanates were detected in the faeces of *S. exigua*, *H. armigera*, *T. ni*, and *M. brassicae* and *S. littoralis* larvae as GSH-cysteinylglycine and GSH-cysteine. The

detection of GSH conjugates of isothiocyanates in the larval faeces may suggest detoxification of the toxins induced by glutathione-*S*-transferase enzyme catalyzed reaction.

Higher levels of expression of *Choristoneura fumiferana* GST mRNA and proteins were induced in sixth instar larvae when they were fed on balsam fir foliage, compared to budworm larvae that fed on artificial diet only (Feng *et al*., 2001). The induction of the *Cf*GST enzymes played a key role in the detoxification of the toxic compounds in the balsam fir leaves. The present study shows that GST also plays a role in detoxifying piceol and pungenol.

4.4 Glycosylation

Detoxification of chemical compounds by glycosylation can involve the activation of enzymes such as glycosidases found in insects that catalyse the reaction of glycosidic bonds between two carbohydrates or between a carbohydrate and an aglycone moiety. (Salminen *et al*., 2004) observed the chemical transformation of the flavonoid aglycones acacetin and kaempferide fed to the fifth instar *Epirrita autumnata* larvae. They found their corresponding glycosides, acacetin-7-*O*-glucoside and kaempferide-3-*O*-glucoside in the larval frass after detoxification via glycosylation. The presence of picein and pungenin in the present study suggests that the spruce budworm also glycosylates plant toxic compounds prior to egestion as a detoxification mechanism. Enzymes that could be responsible have not previously been studied in this species.

5.0 CONCLUSION

HPLC-DAD-MS showed the presence of putative glutathionylated and glycosylated phenolic metabolites in the frass of the *C. fumiferana* larvae fed on artificial spiked phenolic diet. Biochemical analyses of the midguts showed glutathione-*S*-transferase enzyme activity was more highly expressed in the midguts of the *C. fumiferana* larvae fed on artificial diet containing the phenolics than on control artificial diet. Together these results suggest that spruce budworm have counter-defenses to these compounds and can detoxify them by glutathionylation and glycosylation prior to egestion. The conclusion is supported by the larval mass data which shows no significant difference in growth between larvae fed the control and phenolic artificial diets.

6.0 FUTURE RESEARCH DIRECTION

Genomic approach using transcriptomic profiling of resistant and susceptible white spruce trees, together with treatment diets could be a future work to be considered. Transcriptomic screening could be done to analyze and characterize the gene and its corresponding enzymes responsible for the differences in aglycone levels in resistant and non-resistant white spruce trees and treatment diets.

Redox activity of the spruce budworm midgut could be measured to determine whether the phenolic compounds are oxidized to produce reactive oxygen species.

7.0 REFERENCES

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Appendix A

Experiment 1: Mean body mass (mg) of spruce budworm larvae reared on control and phenolic diet

Foliage to diet-2016	
Control	Phenolic
52.81	43.84

Standard error of the mean body mass

0.07	0.05

Experiment 2: Mean body mass (mg) of spruce budworm larvae reared on control and phenolic diet

	Pre-treat.diet-2016	Pre-treat.diet-2017
Control	55.25	54.34
Phenolic	43.38	43.26

Standard error of the mean body mass

Control	5.12	4.79
Phenolic	5.68	4.89

Experiment 1 (2016) - Soluble midgut proteins

Samples	Control (ug/mg)	Phenolic (ug/mg)
1	0.012361	0.028023
2	0.017667	0.027562
3	0.021729	0.009027
4	0.013895	0.012737
5	0.008014	0.010133
6	0.012687	0.012389
7	0.012881	0.013403
8	0.013784	0.010574
9	0.008390	0.009076
10	0.003580	0.018786
11	0.004462	0.014678
12	0.003668	0.012405

Experiment 2 (2016): Soluble midgut proteins

Samples	Control (ug/mg)	Phenolic (ug/mg)
1	0.012361	0.028023
2	0.017667	0.027562
3	0.021729	0.009027
4	0.013895	0.012737
5	0.008014	0.010133
6	0.012687	0.012389
7	0.012881	0.013403
8	0.013784	0.010574
9	0.008390	0.009076
10	0.003580	0.018786
11	0.004462	0.014678
12	0.003668	0.012405

Experiment 2 (2017): Soluble midgut proteins

Samples	Control (ug/mg)	Phenolic (ug/mg)
1	0.017431	0.017749
2	0.013189	0.007805
3	0.010038	0.014467
5	0.017443	0.014665
6	0.005115	0.005879
7	0.009586	0.018735
8	0.005998	0.007279
9	0.008061	0.010743
10	0.009018	0.009062
11	0.008663	0.010345
12	0.008710	0.008844

Experiment 1 (2016) - Bradford protein assay table

					590nm							
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.671	0.6732	0.6746	0.4999	0.4856	0.4801	0.3309	0.4026	0.5019	0.4575	0.4606	0.4657
B	0.6686	0.6403	0.67	0.4783	0.514	0.4756	0.3672	0.4247	0.4884	0.472	0.4579	0.4932
C	0.4957	0.5156	0.5946	0.4617	0.4575	0.4724	0.5494	0.5279	0.5192	0.489	0.4744	0.4271
D	0.4172	0.4188	0.425	0.4688	0.4579	0.4749	0.6112	0.5837	0.6011	0.6185	0.4991	0.5625
E	0.3761	0.3606	0.3544	0.5188	0.5297	0.5202	0.4639	0.452	0.4521	0.5913	0.5647	0.5611
F	0.2865	0.2656	0.2646	0.5177	0.4867	0.4986	0.6028	0.5553	0.597	0.54	0.5139	0.4957
G	0.4866	0.4763	0.4668	0.4747	0.4723	0.4509	0.4475	0.456	0.4684	0.2532	0.2302	0.2196
H	0.5081	0.4893	0.4949	0.3383	0.3972	0.4209	0.5569	0.5427	0.5083	0.0378	0.0382	0.0378
					450nm							
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0864	0.085	0.087	0.1203	0.1173	0.1154	0.18	0.1503	0.1147	0.1238	0.1179	0.1186
B	0.0906	0.0972	0.0914	0.1321	0.1192	0.1276	0.1941	0.1435	0.1265	0.1206	0.1201	0.1148
C	0.1234	0.1188	0.1035	0.1358	0.1394	0.127	0.1079	0.1066	0.1128	0.1168	0.1214	0.1265
D	0.1521	0.1534	0.147	0.1287	0.1371	0.1295	0.1004	0.1022	0.1019	0.0957	0.1073	0.1034
E	0.1675	0.1673	0.17	0.1204	0.1123	0.1133	0.1266	0.121	0.1309	0.0984	0.0987	0.1022
F	0.1991	0.2052	0.2096	0.1118	0.1227	0.1213	0.1008	0.1072	0.1034	0.1079	0.1127	0.1084
G	0.1218	0.1217	0.129	0.123	0.1223	0.127	0.1339	0.1208	0.1195	0.2094	0.2055	0.2004
H	0.1114	0.1203	0.1165	0.1899	0.157	0.1488	0.1089	0.1075	0.1128	0.0356	0.0358	0.0354

CONTROLS (590/450)	
G(10-12)	NEUTRAL BUFFER CONTROL
H(10-12)	WATER CONTROL
A-F(1-3)	STANDARD CURVES
	CONTROL DIET SAMPLES
	PHENOLIC DIET SAMPLES

Experiment 1 (2016) - βeta-glucosidase assay table (pH 7)

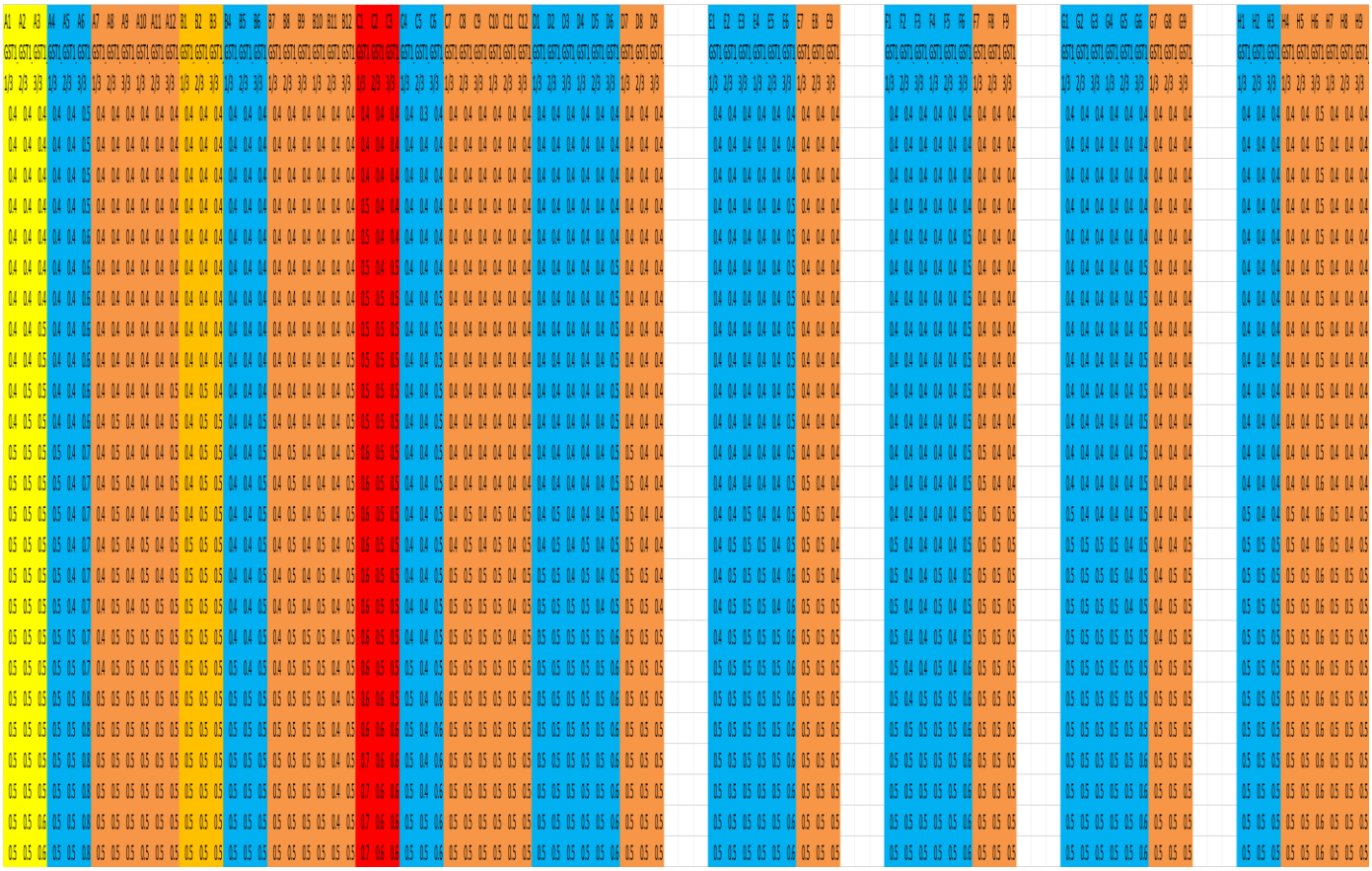
◇	1	2	3	4	5	6	7	8	9	10	11	12
A	6601	6236	6495	17742	18517	19406	35141	35099	34486	10764	10844	11645
B	2215	2127	2247	41847	43437	40531	7910	6077	5770	31855	31025	28990
C	2408	2318	2310	15886	18956	18691	26274	26646	24643	25369	26213	26368
D	1260	1277	1133	8718	9432	9218	17045	16046	15232	4582	5006	4888
E	1719	1423	453	46293	40629	39638	24280	25505	23936	10882	9551	10985
F	1012	1782	1476	20042	16308	16310	35576	34232	33754	31370	32143	32789
G	758	649	595	7199	6154	7113	10634	10560	10340	35666	38709	37231
H	931	867	660	15096	11275	13548	17934	17999	18016	15672	15374	16202
		A-F(1-3)	STANDS.			CONTROL DIET						
		G(1-3)	NEG CON			PHENOLIC DIET						
		H(1-3)	POS CON									

Experiment 1 (2016)- βeta-glucosidase assay table (pH 9)

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	40100	34548	32000	8631	8992	9138	6109	6143	6147	6891	5992	5705
B	9416	9463	9393	21162	17834	16635	3442	3128	3156	16475	16328	16483
C	4731	4630	4650	8340	7541	8234	15713	14991	16551	22080	20399	20644
D	1545	1440	1483	3312	3273	3296	11880	10453	10831	10269	9536	9521
E	1422	1275	1172	3590	3814	3689	11396	11350	10854	23295	20054	21821
F	609	605	1158	10375	7810	8767	14502	16281	17533	14785	18027	16796
G	621	526	499	3847	3971	3722	5377	5395	5691	21885	20945	20806
H	893	844	857	6889	7200	5686	12139	10241	12409	24144	24110	23770

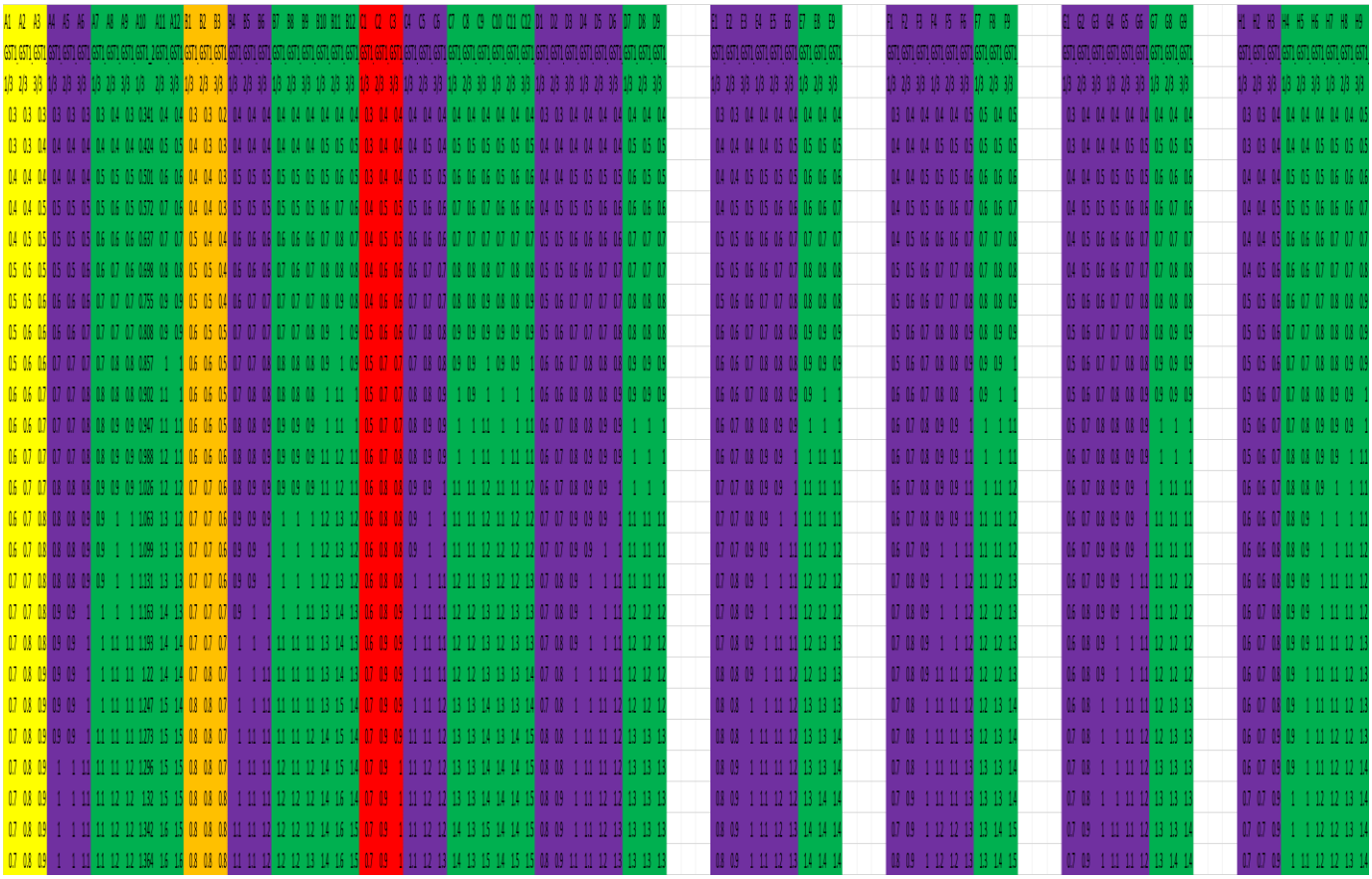
			A-F(1-3)		STANDARD CURVE			CONTROL DIET SAMPLES				
			G(1-3)		NEGATIVE CONTROL			TREATMENT DIET SAMPLES				
			H(1-3)		POSITIVE CONTROL							

Experiment 1 (2016): Glutathione-*S*-transferase assay table (pH 7)



A(1-3)		NEGATIVE CONTROL 1
B(1-3)		NEGATIVE CONTROL 2
C(1-3)		POSITIVE CONTROL
		CONTROL DIET SAMPLES
		TREATMENT DIET SAMPLES

Experiment 1 (2016)- Glutathione-S-transferase assay table (pH 9)



A(1-3)		NEGATIVE CONTROL 1
B(1-3)		NEGATIVE CONTROL 2
C(1-3)		POSITIVE CONTROL
		CONTROL DIET SAMPLES
		TREATMENT DIET SAMPLES

Experiment 2 (2016) - Bradford protein assay table

						590 nm						
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.671	0.6732	0.6746	0.4999	0.4856	0.4801	0.3309	0.4026	0.5019	0.4575	0.4606	0.4657
B	0.6686	0.6403	0.67	0.4783	0.514	0.4756	0.3672	0.4247	0.4884	0.472	0.4579	0.4932
C	0.4957	0.5156	0.5946	0.4617	0.4575	0.4724	0.5494	0.5279	0.5192	0.489	0.4744	0.4271
D	0.4172	0.4188	0.425	0.4688	0.4579	0.4749	0.6112	0.5837	0.6011	0.6185	0.4991	0.5625
E	0.3761	0.3606	0.3544	0.5188	0.5297	0.5202	0.4639	0.452	0.4521	0.5913	0.5647	0.5611
F	0.2865	0.2656	0.2646	0.5177	0.4867	0.4986	0.6028	0.5553	0.597	0.54	0.5139	0.4957
G	0.4866	0.4763	0.4668	0.4747	0.4723	0.4509	0.4475	0.456	0.4684	0.2532	0.2302	0.2196
H	0.5081	0.4893	0.4949	0.3383	0.3972	0.4209	0.5569	0.5427	0.5083	0.0378	0.0382	0.0378
						450 nm						
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0864	0.085	0.087	0.1203	0.1173	0.1154	0.18	0.1503	0.1147	0.1238	0.1179	0.1186
B	0.0906	0.0972	0.0914	0.1321	0.1192	0.1276	0.1941	0.1435	0.1265	0.1206	0.1201	0.1148
C	0.1234	0.1188	0.1035	0.1358	0.1394	0.127	0.1079	0.1066	0.1128	0.1168	0.1214	0.1265
D	0.1521	0.1534	0.147	0.1287	0.1371	0.1295	0.1004	0.1022	0.1019	0.0957	0.1073	0.1034
E	0.1675	0.1673	0.17	0.1204	0.1123	0.1133	0.1266	0.121	0.1309	0.0984	0.0987	0.1022
F	0.1991	0.2052	0.2096	0.1118	0.1227	0.1213	0.1008	0.1072	0.1034	0.1079	0.1127	0.1084
G	0.1218	0.1217	0.129	0.123	0.1223	0.127	0.1339	0.1208	0.1195	0.2094	0.2055	0.2004
H	0.1114	0.1203	0.1165	0.1899	0.157	0.1488	0.1089	0.1075	0.1128	0.0356	0.0358	0.0354

A-F (1-3)	STANDARD CURVES
	CONTROL DIET SAMPLES
	PHENOLIC DIET SAMPLES
G (10-12)	WATER CONTROL
H (10-12)	BUFFER CONTROL

Experiment 2 (2016) – βeta-glucosidase assay table (pH 7)

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	186	174	164	5984	6069	6054	11199	19157	19269	11869	12898	13536
B	451	423	399	2936	2858	2853	13569	20619	22206	12714	10081	13247
C	26430	25375	25656	26449	25588	25913	5938	8720	8936	17740	21843	19920
D	17357	16631	16986	7819	8335	7526	32381	32624	30715	30766	26196	25971
E	10926	10579	10501	32000	31695	30493	20572	20588	20009	11942	12312	11851
F	5097	5012	5007	9143	8764	9345	19829	19905	19133	16270	16917	16307
G	3767	3541	3520	11043	10711	11116	45637	43801	43385	17719	20258	18371
H	2122	2039	1992	8610	8678	9067	18025	18883	18416	11712	10223	13339

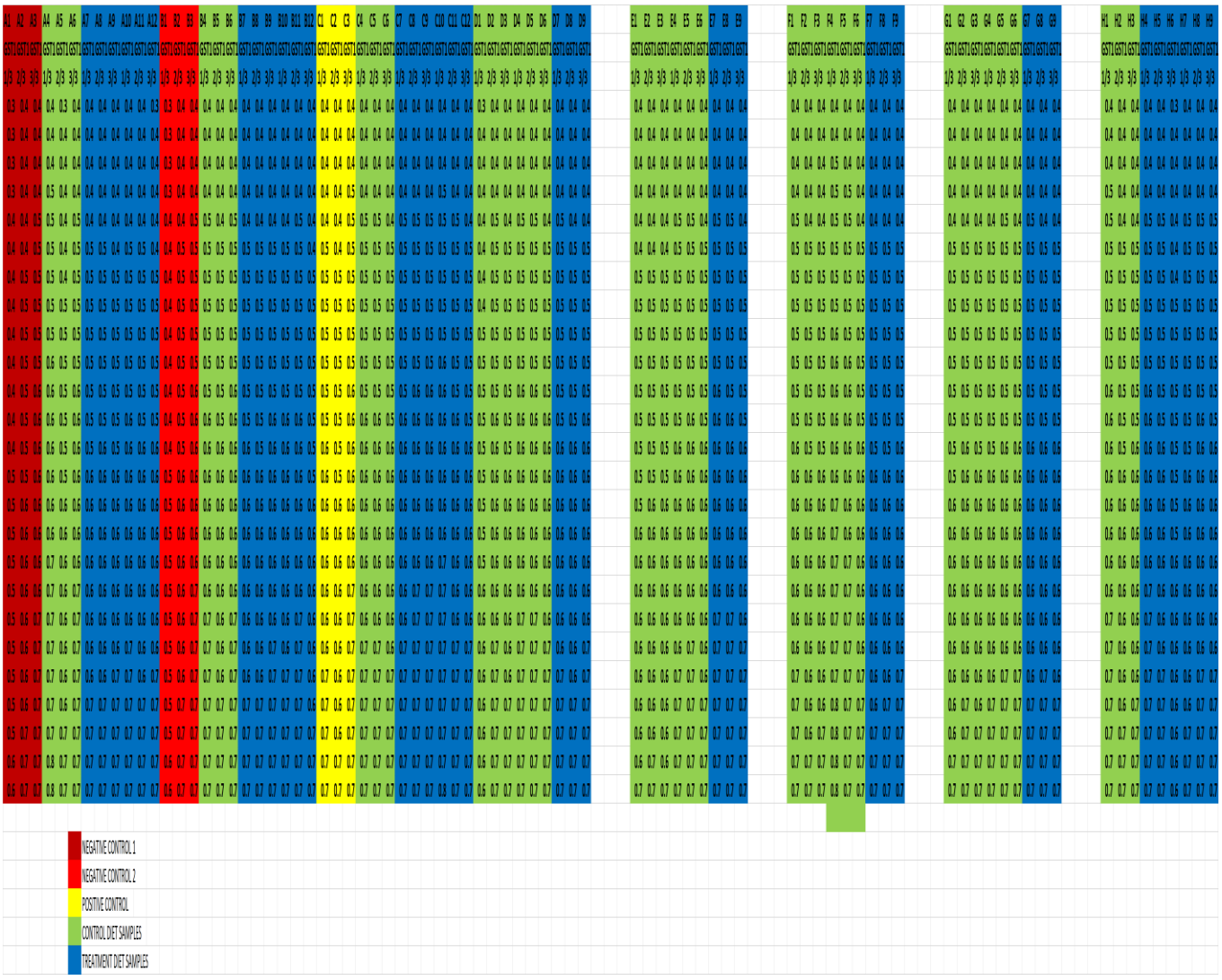
A(1-3)		NEGATIVE CONTROL
B(1-3)		POSITIVE CONTROL
C-H(1-3)		STANDARD CURVE
		CONTROL DIET SAMPLES

Experiment 2 (2016) – βeta-glucosidase assay table (pH 9)

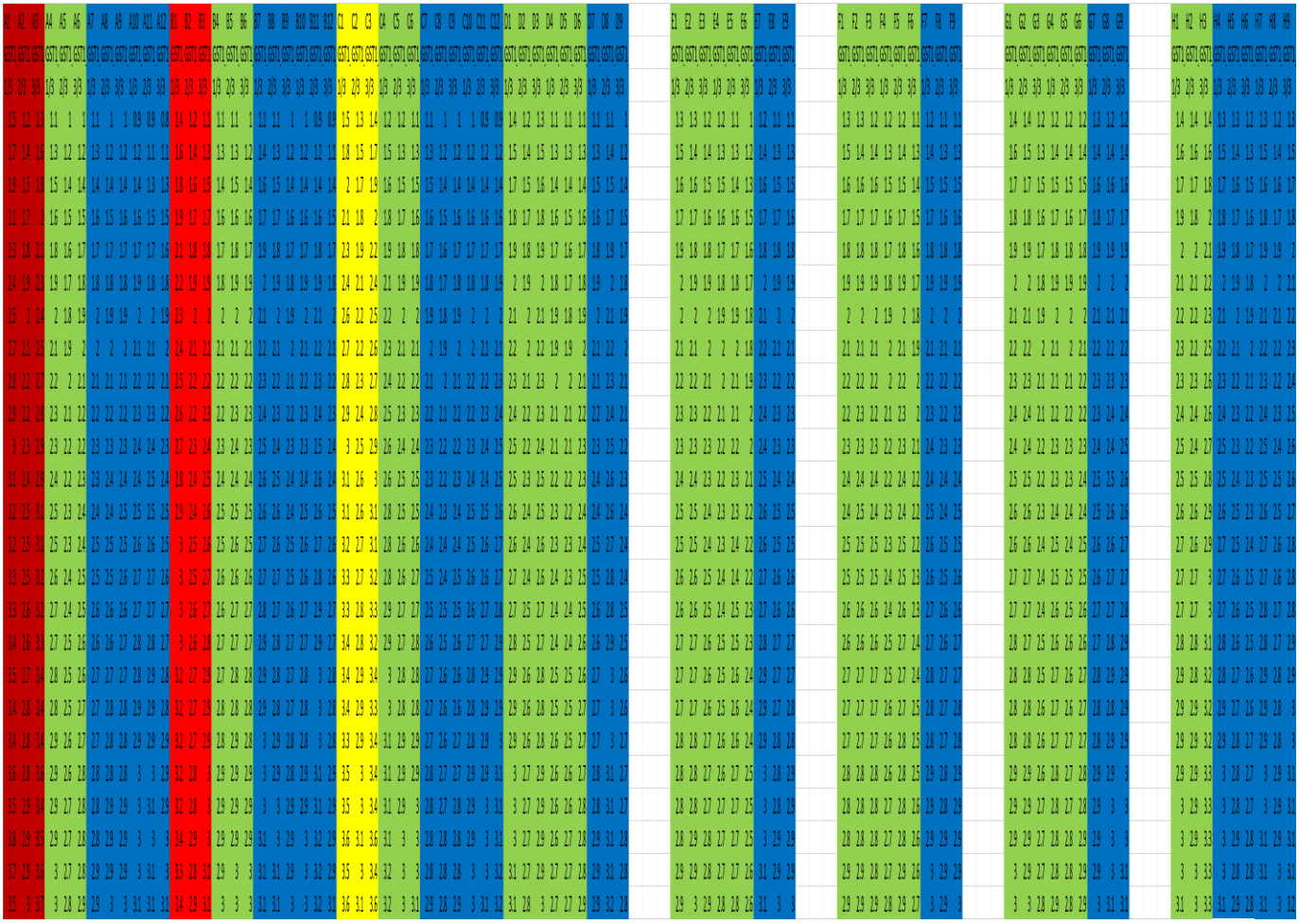
	1	2	3	4	5	6	7	8	9	10	11	12
A	151	129	119	13250	10720	12736	17140	16077	16901	14248	14308	14442
B	350	316	292	2679	2522	3041	26495	26637	23039	14356	13403	12949
C	33753	33494	32306	27339	25882	25939	8743	8481	2754	18258	18350	18702
D	25844	25009	25511	6897	7081	6408	26009	28913	26584	26860	26695	24243
E	17934	17846	17880	27367	27433	26479	17230	17732	16883	9397	9383	10027
F	9107	9144	8955	7932	8470	8834	15635	14926	15886	13732	13979	14278
G	6605	6354	6385	8407	9199	8980	39811	39947	39230	17555	18201	19094
H	3876	3783	3763	7569	9090	8391	17293	17598	16924	13024	13817	13978

A(1-3)	NEGATIVE CONTROL
B(1-3)	POSITIVE CONTROL
C-H(1-3)	STANDARD CURVE
	CONTROL DIET SAMPLES
	PHENOLIC DIET SAMPLES

Experiment 2 (2016): Glutathione-*S*-transferase assay table (pH 7)



Experiment 2 (2016)-Glutathione-S-transferase assay table (pH 9)



A(1-3)		NEGATIVE CONTROL 1
B(1-3)		NEGATIVE CONTROL 2
C(1-3)		POSITIVE CONTROL
		CONTROL DIET SAMPLES
		TREATMENT DIET SAMPLES

Appendix B

Experiment 2 (2017) – Bradford protein assay table

590nm

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.7961	0.7961	0.7962	0.6434	0.6864	0.6586	0.6681	0.6412	0.6626	0.5962	0.6059	0.6095
B	0.6786	0.6786	0.6787	0.5611	0.5549	0.5739	0.528	0.5441	0.5417	0.5099	0.5067	0.5225
C	0.6468	0.6407	0.619	0.6369	0.6311	0.6222	0.6877	0.6956	0.6776	0.6118	0.6019	0.6254
D	0.4787	0.4973	0.4936	0.5374	0.5722	0.5636	0.5599	0.5337	0.525	0.5701	0.578	0.5747
E	0.4051	0.4027	0.3976	0.6571	0.6751	0.6645	0.6129	0.6202	0.6184	0.6179	0.6277	0.6313
F	0.361	0.3697	0.3708	0.453	0.4819	0.4623	0.5148	0.5258	0.491	0.5433	0.5425	0.5532
G	0.0403	0.0392	0.041	0.6375	0.6725	0.6804	0.712	0.7195	0.7069	0.5956	0.5884	0.5736
H	0.053	0.0526	0.0536	0.5085	0.4994	0.5126	0.3848	0.3901	0.391	0.5542	0.5426	0.5212

450nm

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1015	0.1028	0.1105	0.1227	0.1197	0.1224	0.1272	0.1286	0.1272	0.1353	0.132	0.1327
B	0.1059	0.1058	0.1071	0.134	0.1393	0.1335	0.149	0.1449	0.1461	0.1571	0.1571	0.1563
C	0.1209	0.1223	0.128	0.1252	0.1269	0.1286	0.1237	0.1236	0.1278	0.132	0.1337	0.1325
D	0.1558	0.1585	0.1621	0.1391	0.1348	0.1384	0.1438	0.1483	0.1509	0.1424	0.1403	0.141
E	0.1811	0.1853	0.1907	0.1207	0.1226	0.122	0.1306	0.1286	0.1279	0.1293	0.1338	0.1274
F	0.1996	0.2	0.2013	0.1628	0.161	0.1667	0.1521	0.1469	0.1621	0.1435	0.1472	0.1465
G	0.0433	0.0434	0.0444	0.124	0.1218	0.122	0.1188	0.121	0.1191	0.132	0.1341	0.1383
H	0.057	0.0576	0.0588	0.1456	0.1548	0.1511	0.1966	0.1974	0.1957	0.1445	0.1456	0.1529

590/450

A-F(1-3)	STANDARD CURVE
G(1-3)	WATER CONTROL
H(1-3)	NEUTRAL BUFFER CONTROL
ARTIFICIAL DIET SAMPLES	
TREATMENT DIET SAMPLES	

Experiment 2 (2017) – βeta-glucosidase assay table (pH 7)

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	127	148	112	4881	5059	5098	2498	2308	2488	1180	1199	1331
B	260	275	311	1500	1482	1401	690	715	712	1494	1470	1554
C	43435	44394	37875	1992	2058	2007	3285	3331	3258	612	664	651
D	19648	18736	18785	846	849	855	881	960	921	910	944	1013
E	9837	9446	9711	2842	2650	2810	1548	1320	1349	1639	1655	1592
F	4401	4579	4465	1137	1450	1319	1089	1068	1129	466	491	507
G	3055	3010	2952	1305	1265	1221	1408	1437	1360	650	652	672
H	1731	1731	1659	691	688	629	1574	1531	1587	662	592	570

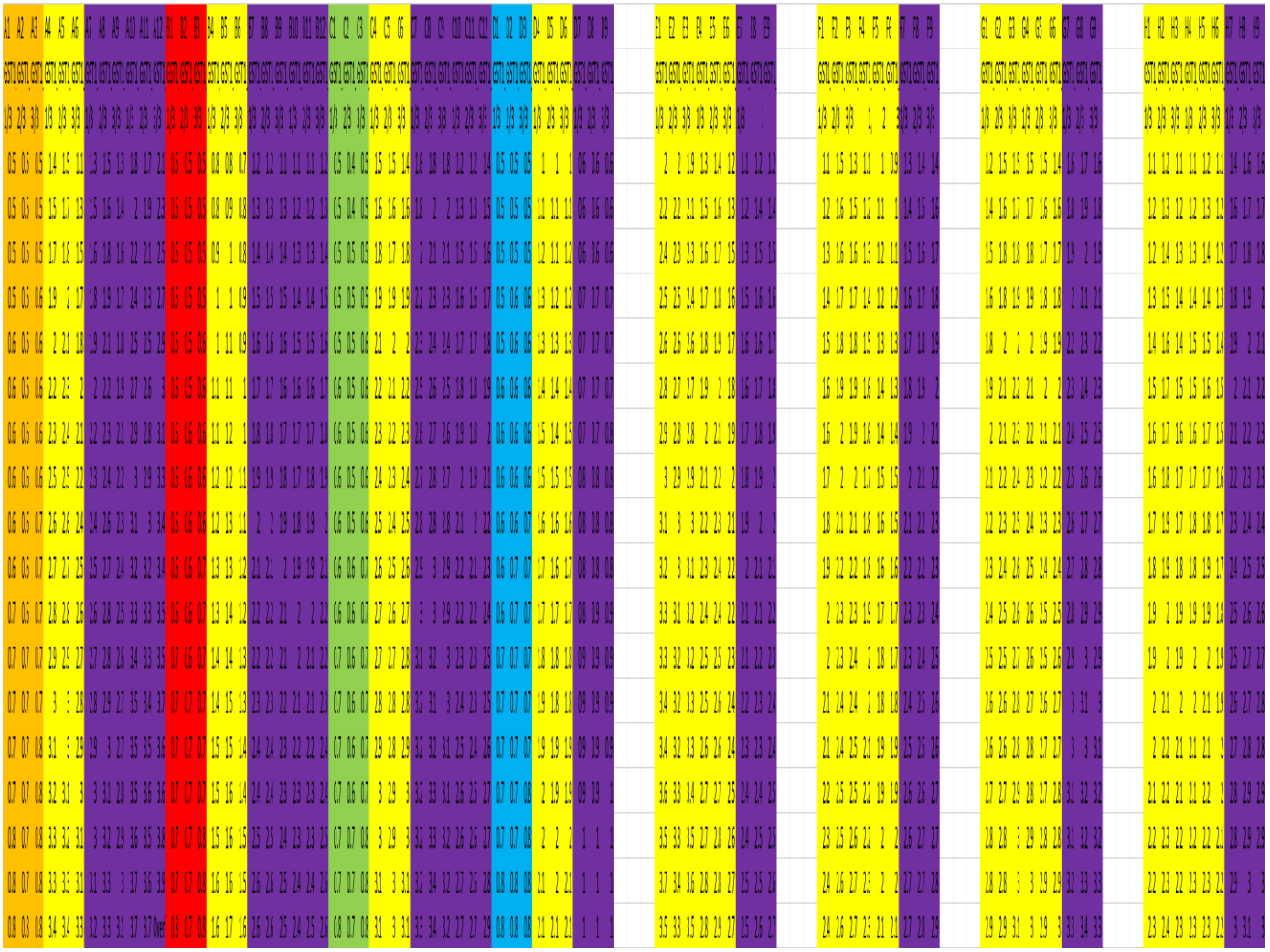
A(1-3)	NEGATIVE CONTROL
B(1-3)	POSITIVE CONTROL
C-H(1-3)	STANDARD CURVE
	ARTIFICIAL DIET SAMPLES
	TREATMENT DIET SAMPLES

Experiment 2 (2017) – βeta-glucosidase assay table (pH 9)

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	116	117	114	9283	9780	7798	5902	5799	5931	2143	2222	2250
B	215	221	271	2637	2922	2917	795	178	806	3852	3507	3502
C	45473	45872	44635	4895	4637	4647	7516	8031	7880	975	1006	999
D	38803	38527	38295	2059	2046	2036	1967	1981	1980	1922	1955	1974
E	27590	26225	26722	6582	6299	6338	1595	1630	1507	4357	4257	4262
F	15182	14271	14425	3051	3018	3010	1949	1602	2049	882	904	941
G	10476	10286	10305	2859	2963	2979	2845	2923	2832	1252	1237	1242
H	5901	5717	5612	1203	1234	1392	2839	2869	2836	1034	935	1002

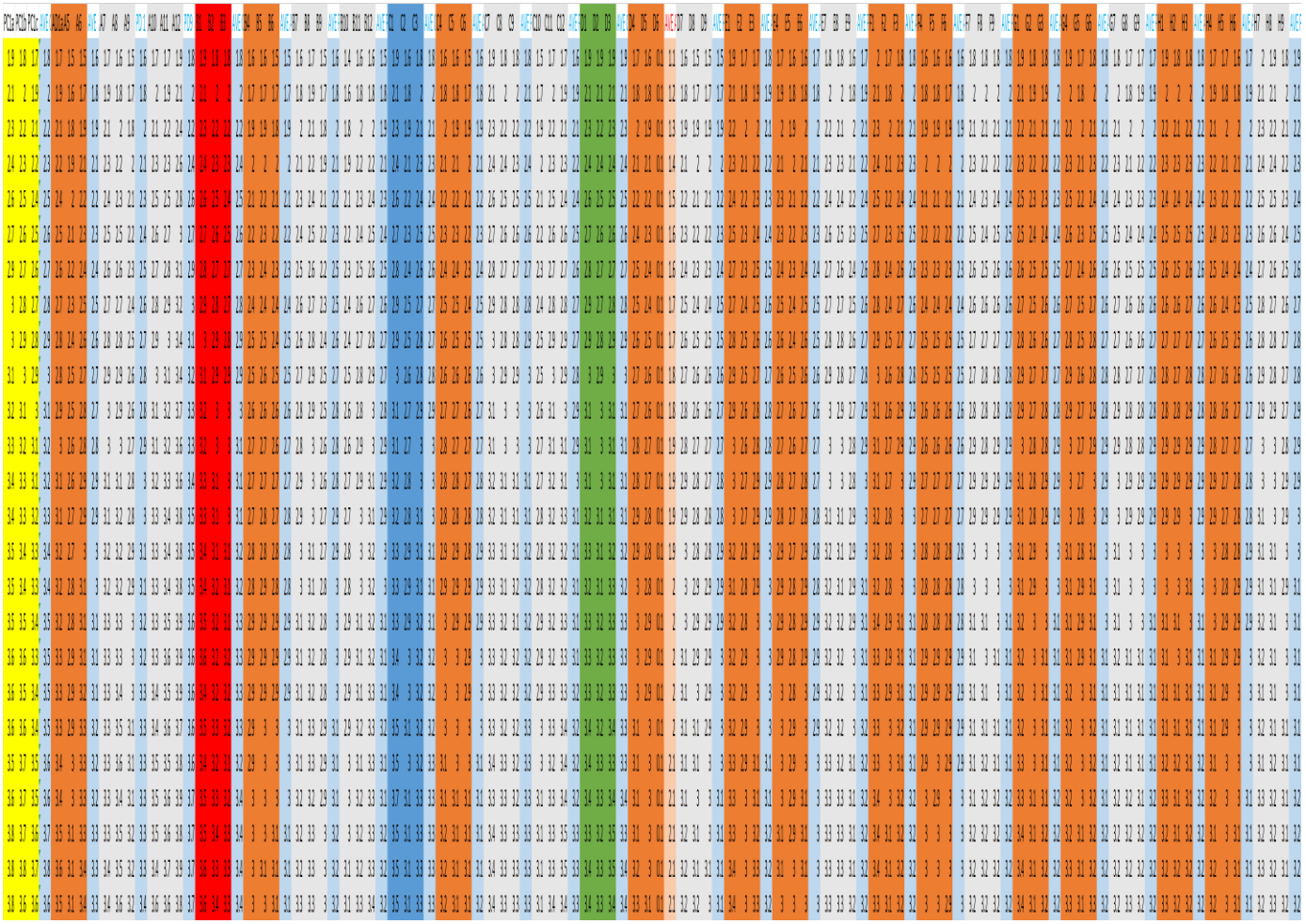
A(1-3)	NEGATIVE CONTROL
B(1-3)	POSITIVE CONTROL
C-H(1-3)	STANDARD CURVE
	ARTIFICIAL DIET SAMPLES
	TREATMENT DIET SAMPLES

Experiment 2 (2017)- Glutathione-S-transferase enzyme assay table (pH 7)



A(1-3)		POSITIVE CONTROL 1
B(1-3)		POSITIVE CONTROL 2
C(1-3)		NEGATIVE CONTROL 1
D(1-3)		NEGATIVE CONTROL 2
		ARTIFICIAL DIET SAMPLES
		TREATMENT DIET SAMPLES

Experiment 2 (2017)- Glutathione-S-transferase assay table (pH 9)



A(1-3)		POSITIVE CONTROL 1
B(1-3)		POSITIVE CONTROL 2
C(1-3)		NEGATIVE CONTROL 1
D(1-3)		NEGATIVE CONTROL 2
AD(1-12)		ARTIFICIAL DIET SAMPLES
PD(1-12)		TREATMENT SAMPLES