1   **Detoxification of host plant phenolic aglycones by the spruce budworm**

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7

8   **Abstract**

9   This study examines the post-ingestive fate of two host-plant derived small-molecule phenolics
10   (the acetophenones piceol and pungenol) that have previously been shown to be toxic to the
11   outbreaking forest pest, spruce budworm (*Choristoneura fumiferana*). We test first whether
12   these compounds are transformed during passage through the midgut, and second whether the
13   budworm upregulates activity of the detoxification enzyme glutathione-s-transferase (GST) in
14   response to feeding on these compounds. Insects were reared on either foliage or artificial diet to
15   the fourth instar, when they were transferred individually to one of two treatment diets, either
16   control or phenolic-laced, for approximately 10 days, after which midguts were dissected out and
17   used for Bradford soluble protein and GST enzyme activity analysis. Frass was collected and
18   subjected to HPLC-DAD-MS. HPLC showed that the acetophenones do not autoxidize under
19   midgut pH conditions, but that glucose- and glutathione- conjugates are present in the frass of
20   insects fed the phenolic-laced diet. GST enzyme activity increases in insects fed the phenolic-
21   laced diet, in both neutral pH and alkaline assays. These data show that the spruce budwom
22   exhibits counter-adaptations to plant phenolics similar to those seen in angiosperm feeders,
23   upregulating an important detoxifying enzyme (GST) and partially conjugating these
24   acetophenones prior to elimination, but that these counter-measures are not totally effective at
25   mitigating toxic effects of the ingested compounds in the context of our artifical-diet based
26   laboratory experiment.

27

28   **Introduction**

29   In the chemical arms race between plants and herbivores, insects exhibit multiple mechanisms

30   for dealing with plant secondary metabolites in their diet, ranging from avoidance, rapid egestion,

31   enzymatic detoxification via degradation or conjugation, or even sequestration for use in defense

32   [1]. Insect counter-defenses are often phenotypically plastic, especially in generalist feeders, and

33   induced by plant compounds in the diet. These counter-adaptations to plant defenses can also

34   occur at different time points: before ingestion, in the digestive tract prior to absorption or within

35   cells afterwards [2,3].

36   The midgut is the main site of digestion in insects and a major interface for detoxification

37   of plant allelochemicals [4]. The midgut is lined by a peritrophic membrane, a sheath of chitin

38   microfibrils crosslinked by proteins around the midgut lumen that prevents large molecules from

39   entering midgut cells, and thus constitutes a first line of defense against absorption of

40   allelochemicals [5]. In many Lepidopteran species, the midgut lumen enclosed by the

41   peritrophic membrane is highly alkaline, which in itself can be considered another counter-

42   adaptation to plant defenses, since high pH decreases the protein binding capacity of tannins,

43   improves the extraction of protein from leaf tissue and contributes to inactivating plant defensive

44   enzymes [6,7].

45   Phenolics are one of the most important classes of plant secondary metabolites. Their

46   detrimental effects have been linked to oxidation of biologically important molecules such as

47   proteins and DNA. Large-molecule phenolics such as tannins can be either autoxidized or

48   oxidized by plant enzymes in the midgut lumen, generating low molecular weight ROS that cross

49   the peritrophic membrane to enter cells [8,9]. However, smaller phenolic compounds could cross

50   the peritrophic membrane themselves and cause lesions and oxidative stress in cells directly [5].

51   Oxidative stress in midgut tissues, caused by either of the above mechanisms, is associated with

52   lower caterpillar performance [8].

53   One approach to understanding insect counter-adaptations to phenolic compounds

54   involves following the fate of phenolics in Lepidopteran midguts by assaying the original

55   compounds and their metabolites in the insects' frass. These studies suggest that the outcome

56   varies greatly depending on the compounds' structure: some move through the digestive tract of

57   the caterpillar intact, while others are modified by enzymes and eliminated in a less toxic form

58   [10]. Enzymatic detoxification depends on the compound and insect species, and can involve

59   glycosylation, glutathionation, sulfation or deacylation [10-13]. A broad range of detoxification

60   enzymes have been recorded from insect midguts [14]: many are located in the cytoplasm of

61   midgut cells where they act to prevent damage to biological molecules and hasten excretion of

62   toxic compounds, but some are secreted into the midgut lumen where they act on plant toxins

63   before they enter cells [3]. Lepidopteran digestive enzymes are known to be adapted to function

64   in alkaline conditions [15], and the same is likely to apply to secreted detoxification enzymes

65   [14].

66   Most work on phenolic mechanisms of toxicity and insect counter-adaptations has been

67   with angiosperm feeders [8]. However, phenolic compounds are known to play a key role in

68   conifer defense against herbivores [16], and the composition of conifer phenolics differs from

69   those found in angiosperms [9].

70   Two phenolic compounds have been suggested to play an important role in the defense of

71   coniferous trees against defoliation by the spruce budworm, *Choristoneura fumiferana* Clemens

72   (Lepidoptera: Tortricidae) [17,18], the most serious insect pest of coniferous forests of eastern

73   North America [19]. Two sets of acetophenones have been identified from white spruce (*Picea*

74   *glauca*) trees resistant to budworm attack which suffered only light defoliation when other trees

75   around them were heavily damaged [20]: piceol and pungenol (aglycones) and picein and

76   pungenin (their glycosides). The aglycones increase mortality and slow growth in bioassays, but

77   the glycosylated forms appear to have no effect on the budworm [18].

78   These findings have turned a spotlight onto the role of acetophenones in conifer defense

79   against folivores and in particular, against the spruce budworm. Since then, these acetophenones

80   have been shown to be broadly distributed across coniferous trees: ten of 12 surveyed Pinaceae

81   species accumulated at least one of the two glycosylated acetophenones in the foliage, of which 4

82   species accumulated both a glycoside and the corresponding aglycon. In general, the glycosides

83   were found alone or at higher concentrations than the aglycones [21]. Genetic analysis of white

84   spruce trees showed that a glucosyl hydrolase gene, *PgBgluc-1*, was constitutively highly

85   expressed in resistant trees, catalyzing formation of the aglycones from the glycosylated

86   compounds [22]. Levels of both the gene transcripts and the aglycones are highly heritable and

87   are thought to be maintained via selection pressures imposed by spruce budworm herbivory [17].

88   As their name implies, spruce budworm are early-spring feeders, attacking buds as they

89   begin to elongate. In white spruce, the aglycones begin to be expressed in current-year foliage

90   near the end of shoot elongation, when the budworm reach the final instars [17]. Budworm

91   feeding on white spruce are therefore exposed to these compounds in the final larval instars, as

92   well as early in the season when insects emerging from diapause feed on previous-year foliage

93   before buds become available. The final-instar spruce budworm midgut has a highly alkaline pH

94   (10.5 ± 0.12 [23]) and has been shown to express at least one major detoxification enzyme,

95   glutathione-s-transferase (GST) in response to feeding on foliage [24]. The present study aims to

96   determine the fate of piceol and pungenol after ingestion by the budworm, testing first whether

97   these compounds are transformed during passage through the midgut or eliminated unchanged,

98   and second whether the budworm upregulates GST activity in response to feeding on these

99   compounds.

100   **Materials and Methods**

101   *Experimental design*

102   Spruce budworm insects were obtained at the second instar larval diapausing stage from the

103   Great Lakes Forest Research Centre, (Canadian Forest Service, Sault Ste. Marie, ON, Canada).

104   The larvae were placed in groups of 10 in Solo cups (2 cm diameter, 4 cm long) and reared in a

105   laboratory incubator on pre-treatment diet (either white spruce foliage or artificial diet) at 23°C,

106   50% relative humidity. The two pre-treatment diets (foliage or artificial diet) were used to

107   control for possible down-regulation of detoxification enzymes when feeding on artificial diet

108   rather than foliage [24]. The experiment was run 3 times (once on foliage in 2016, twice on

109   artificial diet (2016 & 2017)).

110   At the moult to the fourth instar, larvae were placed individually in new cups containing

111   the treatment diet (either control or phenolic-laced) until one week after the moult to the sixth

112   instar when they were removed for immediate use in the experiment (N=48 larvae per treatment

113   per experimental run).

114   The experiment began by weighing the caterpillars, then dissecting them to remove

115   midguts for immediate biochemical analyses, specifically Bradford soluble proteins and

116   glutathione-*S*-transferase activity. Frass from the treatment cups was collected and frozen at -

117   80°C until HPLC analysis.

118   *Insect diets*

119   In the foliage pre-treatment, insects were reared until fourth instar on fresh current-year white
120   spruce foliage collected at Morgan Arboretum (45°53'N, 72°92'W): twigs were placed in water
121   picks and changed every three days to ensure freshness. In the artificial diet pre-treatment, initial
122   rearing was done on modified McMorran Grisdale artificial diet [25] prepared in the laboratory
123   as per the recipe provided by Insect Production Services, Canadian Forest Service (Sault Ste.
124   Marie, ON, Canada).

125   Piceol (4'-hydroxy-acetophenone) and pungenol (3', 4'-dihydroxy-acetophenone) were
126   purchased from Sigma-Aldrich (Oakville, ON, Canada). To prepare the phenolic-laced diets,
127   0.966 g each of the two compounds (to achieve the upper range of physiological concentrations
128   [18]) was dissolved in 10 ml of methanol and added to the artificial diet at the same time as the
129   vitamin mixture.

130   *HPLC-DAD-MS analysis*
131   High Performance Liquid Chromatography-Diode Array Detector-Mass Spectroscopy (HPLC-
132   DAD-MS) was used for chromatographic separation and identification of phenolics in the
133   budworm frass.

134   Since it was suspected that the alkaline environment of the caterpillar midgut may lead to
135   oxidative reactions [26], a mix of piceol and pungenol (1 mg/ml each) was incubated at either
136   neutral (pH 7.2) or alkaline (pH 9.5) conditions for 24 hours and subjected to HPLC-DAD-MS.

137   Phenolic compounds were extracted from the frass of spruce budworm pre-treated on
138   artificial diet (2017 run) following [22]. Frass from 30 individual caterpillars from each
139   treatment diet was pooled and dried in an oven for 24 hrs and ground to powdered form using
140   liquid nitrogen, then stored in 2 ml Eppendorf tubes at -80°C prior to analysis. 50–100 mg of fine
141   dried frass powder was extracted using 1 ml of 70% HPLC grade methanol. Benzoic acid (1
142   mg/ml) was used as an internal standard with 150 µl of benzoic acid added to 350 µl of the liquid
143   sample. 70% methanol (600 µl) was added to the frass powder and incubated at 4°C on a shaker.
144   After 6, 24 and 48 hours of incubation, the samples were centrifuged at 13 000 g for 10 mins.
145   The supernatants were pooled and kept at -80°C. A fresh 600 µl of aqueous methanol was added
146   to each sample, and after incubation, centrifugation was repeated. Extracts obtained after 6, 24 or
147   48 hours were pooled as a single extract for HPLC-DAD-MS analyses. Extraction and analysis
148   was replicated three times.

149   Phenolics were separated by HPLC-DAD-MS using a Spursil C18 3 µm column (150 x
150   2.1 mm) maintained at 25°C. The mobile phase consisted of (A) 0.1% formic acid and (B) 0.1%
151   formic acid in acetonitrile (ACN). The separation gradient was as follows: 0-12 min, 3-45% B;
152   12-13 min, 45-95% B; 13-15 min, 95% B and 15-18 min, 95-98% B. The column flow rate was
153   250 µl per min. The detection wavelength was at 280 nm. For mass spectrometry, a micromass
154   quantitative-time-of-flight (q-Tof) spectrometer (Ultima ^{TM} API instrument) with electrospray
155   ionization (ESI) in the positive mode was used for detection and identification of conjugated
156   forms of the phenolic compounds. The following parameters were used: scanning range between
157   *m/z* 200-500, 3.5 K volt with a scan time of 1 second, drying gas flow 6 mL/min, nebulizer
158   pressure 60 psi, dry gas temperature 300 °C, vaporizer temperature 250 °C. The instrument was
159   programmed to detect compounds with a molecular mass between 50 to 900 Da.

160   *Biochemical analyses*
161   In each of the three runs of the experiment, sixth instar caterpillars were dissected to remove the
162   midguts. Four midguts were pooled for each sample. Midguts were rinsed in saline dissection
163   buffer and placed four together in an Eppendorf tube that contained 600 µl of sterile dissection
164   buffer and 600 µl of 2X protease inhibitor cocktail. The midgut samples were homogenized and
165   the homogenates centrifuged at 13 000 rpm at 4°C for 5 minutes. The supernatants were
166   transferred to new, sterile Eppendorf tubes. 5 µl aliquots of gut homogenate were used for the
167   soluble protein assay and 10 µl aliquots of the homogenate was used for the assay measuring
168   GST activity on the same day as dissection. Enzyme assays were conducted at both neutral and
169   alkaline pH, to mimic conditions inside cells and in the midgut lumen [23].

170   *Soluble protein assay*
171   The soluble protein concentration of each midgut sample was determined by the use of the
172   modified Bradford assay (Bradford, 1976; Zor & Ernst, 2010). The buffer used was 0.1M
173   phosphate buffer, pH 7.2. The Bradford reagent (Bio-Rad) was diluted in distilled water as per
174   manufacturer's instructions. The linear concentration range was between 0.1-1.4 mg/ml. Bovine
175   serum albumin (BSA) was used to make a standard curve. All samples and standards were
176   analyzed in triplicate and a negative control was included. The absorbance was measured at both
177   590 nm and 450 nm using an Infinite PRO 200 spectrophotometer (Tecan). The soluble protein

178   concentration of the unknown samples was determined by comparing the $A_{590/450}$ values against

179   the standard curve.

Glutathione-S-transferase enzyme assay

181   Glutathione-*S*-transferase (GST) catalyzes the addition of reduced glutathione (GSH) to the

182   substrate, in this case 1- chloro 2, 4 dinitrobenzene (CDNB). The product of the reaction formed

183   is a yellow colored product that can be monitored at 340 nm. Each 96-well plate included a

184   positive control (GSH, buffer, GST enzyme and CDNB), a negative control (GSH, CDNB and

185   buffer) and experimental samples (GSH, CDNB, gut homogenate and buffer), all of which were

186   assayed in triplicate. The assay was conducted at both neutral pH, 100 mM potassium

187   bicarbonate buffer (pH 7.2) as well as at alkaline pH, 100 mM sodium bicarbonate buffer (pH

188   9.2). Each reaction mixture contained the following: 25 µl of 10 mM GSH, 25 µl 10 mM CDNB

189   dissolved in 0.1% v/v in 95% ethanol, 10 µl of 0.1 U/ml GST enzyme, 10 µl of gut homogenate,

190   and was transferred into a ultraviolet microplate well that contained appropriate buffer up to a

191   total volume of 250 µL. Enzyme activity was determined by monitoring changes in absorbance

192   at 340 nm measured every 15 seconds for 2 minutes under the spectrophotometric kinetic mode,

193   at a constant temperature of 25°C.

Statistical analysis

195   Caterpillar mass at the beginning of the experiment was analyzed by two-factor Anova, in order

196   to evaluate the effects of the acetophenones on growth, as a proxy for performance. Soluble

197   protein and GST activity were compared between the phenolic-laced and control diets with

198   separate t-tests for each run of the experiment: these data could not be compared directly

199   between different runs of the experiment as assays were conducted separately.

Results

Caterpillar mass

202   Two-way analysis of variance showed that growth of budworm was lower on phenolic than on

203   control diet ($F_{1, 269} = 7.62$, $p = 0.006$). Growth also differed between the three runs of the

204   experiment ($F_{2, 269} = 111.9$, $p < 0.001$), being lower on the artificial diet than on the foliage pre-

205   treatment, see Figure 1. The interaction term was not significant ($F_{2, 269} = 1.44$, $p = 0.23$).

HPLC-DAD-MS of incubated phenolic solutions

207   Peaks corresponding to piceol and pungenol standards were observed in the mixed solutions
208   incubated at neutral and alkaline pH. Several other peaks were also observed after incubation in
209   the alkaline solution (Figure 2.) One of these peaks was also observed after incubation at neutral
210   pH at a similar level, but the other two were at much lower concentrations. The molecular
211   masses (m/z 285, 287 and 303) of these compounds suggest that they may be dimers of piceol
212   (m/z 136.45) and pungenol (m/z 152.15).

213   *HPLC-DAD-MS of budworm frass*
214   In the frass from caterpillars fed on phenolic diet, peaks were detected corresponding to piceol
215   (m/z 136.45) and pungenol (m/z 152.15) and to one of the putative dimers observed in the
216   alkaline incubation (m/z 287) (Figure 3). Four additional peaks were also observed, and
217   tentatively identified as the phenolic glycosides picein (m/z 299.09) and pungenin (m/z 315.11),
218   and as the glutathionylated conjugates of piceol (m/z 425.28) and pungenol (m/z 441.23). These
219   m/z represent the loss of water plus addition of glutathione to the phenolic and the elimination of
220   a proton. As expected, frass from caterpillars fed on control diet did not contain any of these
221   compounds.

222   *Midgut soluble protein levels*
223   The midgut soluble protein levels of the spruce budworm caterpillars fed on control diet were not
224   significantly different from those of budworm fed on phenolic diet in any of the three
225   experimental trials: foliage to artificial diet: t_{22} = -1.52, p = 0.14; artificial to artificial diet: t_{22} = -
226   1.59, p = 0.14 (2016); t_{22} = -0.622, p = 0.54 (2017).

227   *Glutathione-S-transferase activity*
228   At alkaline pH, glutathione-*S*-transferase enzyme activity in the midgut of the budworm fed on
229   phenolic diet was significantly higher than in controls, in the foliage-to-artificial-diet experiment
230   (t_{22} = -4.03, p = 0.0006), as well as in the artificial-to-artificial-diet experiment: t_{22} = -3.20, p =
231   0.004 (2016) & t_{22} = -2.64, p = 0.017 (2017). See Figure 4**Error! Reference source not found.**.
232   At neutral pH, GST activity also increased on phenolic relative to control diet: foliage to
233   artificial diet: t_{22} = -2.11, p = 0.05; artificial to artificial diet: t_{22} = -4.28, p = 0.0007 (2016); t_{22} =
234   -3.53, p = 0.004 (2017). See Figure 4**Error! Reference source not found.**.
235

Discussion

Comparison of HPLC-DAD-MS chromatograms of acetophenones incubated at neutral and alkaline pH suggests that these compounds do not autoxidize under alkaline conditions. Similarly, previous work suggests that the phenolics of *Picea abies,* unlike those of many angiosperms, show only minor changes under alkaline incubation [26]. Interestingly, the chromatograms suggest that the acetophenones partially dimerize under high pH, but the biological significance of this effect is not clear.

HPLC-DAD-MS chromatograms of frass of insects fed acetophenone-laced diet showed, in addition to the original compounds and their dimers, novel peaks not observed in the alkaline-incubated solutions. Based on m/z, these compounds are putatively identified as glycosylated and glutathionated conjugates of the original piceol and pungenol. As expected, none of these compounds were detected in the frass of control insects. The novel peaks in the phenolic frass are therefore likely to be the products of enzymatic metabolism. Indeed, our results further show upregulation of glutathione-*S*-transferase in insects fed phenolic-laced artificial diet compared to controls.

Glycosylation and glutathionation of the acetophenones likely represent detoxification prior to elimination [10]. The acetophenone glycosides (picein and pungenin) are known to be less harmful to the budworm than are the aglycones [18]. Indeed, glycosides are often less toxic than their aglycones [2], and glycosylation is a common form of detoxification [3]. For instance, in *Epirrita autumnata,* phenolic glycosides were egested without metabolic modifications, but that phenolic aglycones were glycosylated prior to egestion{{23291 Salminen, J.P. 2004}}. The enzyme family likely responsible for glycosylation are the UDP-glycosyltransferases (UGTs) whose role in insect detoxification of plant compounds is as yet poorly understood [3]. UGTs have been demonstrated in several folivorous Lepidoptera [3,27,28], but not the spruce budworm; they have been demonstrated in white spruce and linked to glycosylation of the acetophenone aglycones for storage [29].

Glutathionation is better understood as a detoxification mechanism used by herbivorous insects, and detoxification of plant chemicals and/or insecticides via GST activitiy has been studied in numerous Lepidoptera [30], including the spruce budworm [24]. GSTs are a complex and widespread enzyme superfamily: multiple GSTs have been detected in all insects studied to-date, with variable specificities for a range of compounds. Generally insect GSTs catalyse the

267   conjugation of reduced glutathione (GSH) to electrophilic molecules; they thus generate
268   glutathione-S-conjugates that are more water soluble {{23689 Enayati, A.A. 2005}}, and can
269   prevent oxidative stress [31]. GSTs can be upregulated in response to plant allelochemicals in the
270   diet [30], and glutathione conjugates of plant metabolites have been detected in the frass of
271   several caterpillar species [12].

272   Previous work on the spruce budworm has shown higher levels of expression of
273   *Choristoneura fumiferana* GST mRNA and proteins in whole body extracts of sixth instar larvae
274   fed on balsam fir (*Abies balsamea*) foliage compared to caterpillars reared on artificial diet [24].
275   The authors suggested that GST could play an important role in detoxifying host secondary
276   metabolites, but could not identify the compounds responsible. Our results demonstrate that GST
277   is upregulated in the midgut in response to harmful aglycone acetophenones, and that these
278   compounds are partially glutathionated prior to elimination. Comparison of GST activity
279   between the two pre-treatment diets (foliage vs artificial diet) also suggests upregulation of the
280   enzyme prior to the beginning of the experiment in response to compounds in the white spruce
281   foliage [24].

282   Detoxification via GST activity has been recorded in the insect fat body, midgut and
283   haemolymph [30,32,33]. Most studies record intracellular GST activity as contributing to phase
284   II detoxification by binding lipophilic compounds so they are more easily removed from the cell
285   [32]. However, it remains unclear whether GST could also be secreted into the midgut lumen.
286   GST-catalysed conjugation neutralizes the electrophilic sites of lipophilic substrates by attaching
287   reduced GSH. GSTs have a high affinity towards GSH, which is generally present at high
288   concentrations intracellularly [12]. However, GSH has also been detected in caterpillar midguts,
289   and the ratio of reduced to oxidized GSH in the midgut lumen is used as an index of oxidative
290   stress from plant phenolics [34,35]. Schramm{{23662 Schramm, K. 2011}} detected GSH
291   conjugation to plant allelochemicals at high pH conditions representing those of caterpillar
292   midguts, but were unable to disentangle non-enzymatic from GST-catalyzed reactions. We
293   detected higher GST activity at alkaline than at neutral pH, suggesting that spruce budworm
294   GSTs could function in the midgut lumen. Previous studies suggest that GSH in the midgut
295   lumen could come, at least in part, from foliage, and spruce needles have been shown to contain
296   GSH [36]. Detoxification of plant compounds by enzymes in midgut tissues has been much
297   studied [3], but non-absorption of defensive chemicals due to conjugation in the midgut lumen

298   warrants further attention [14], since it would be a very powerful adaptive counter-measure to

299   neutralize toxins before they even contact damageable tissues.

300   Low molecular weight phenolics from angiosperms have been shown to cause oxidative

301   stress in caterpillar midgut tissues but it is not always clear whether the damage is caused by

302   ROS created by oxidation of the phenolics in the midgut lumen or directly by the phenolics

303   themselves after their absorption in midgut tissues {{72 Barbehenn,R V. 2011}}. We show no

304   evidence of oxidation of piceol and pungenol under high pH conditions, consistent with previous

305   work on *Picea* phenolics. If small compounds like acetophenones can cross the peritrophic

306   membrane and directly damage tissues, secreted GSTs could limit absorption of these

307   compounds by making them larger and more hydrophilic and less likely to be absorbed, and

308   cytosolic GSTs could limit damage caused by phenolics that have crossed the peritrophic

309   membrane and been absorbed. We show GST activity at both neutral and alkaline pH, but our

310   results cannot resolve whether the enzymes responsible are cytosolic or secreted. In general, few

311   studies pinpoint the exact location of enzyme activity [14], leaving it a question open to further

312   investigation.

313   Finally, our results showed that the acetophenones are only partially conjugated and that

314   they still negatively affect budworm performance, at least in the context of an artificial diet-

315   based laboratory assay. Indeed, addition of piceol and pungenol to artificial diet reduced growth

316   of spruce budworm larvae in comparison to caterpillars fed control artificial diet. Similarly,

317   Schramm {{23662 Schramm, K. 2011}} concluded that GST-conjugation of ingested plant

318   compounds occurs, but only partially, and is of limited importance relative to other

319   detoxification mechanisms, including rapid elimination. One possible explanation is that this

320   conjugation is costly, as GSH contains 3 Nitrogen atoms, a nutrient often limiting in herbivores,

321   including the spruce budworm [37]. The sulfur amino acids required for GSH synthesis are

322   particularly low in foliage: for instance, close to 20% of sulfur amino acids ingested by two

323   angiosperm-feeding caterpillars are directed to GSH production [38]. This would support a well-

324   known relationship between protein nutrition and allelochemicals, whereby insects deficient in

325   dietary N are often more sensitive to plant defensive compounds [39].

326   In general, organisms with an evolutionary history of consuming phenolics are less

327   sensitive to their deleterious effects, via physical and biochemical defenses in the midgut

328   including surfactants, high pH, low redox potential, low oxygen levels, antioxidants, antioxidant

329 enzymes, and detoxification enzymes [8]. We show that the spruce budworm exhibits counter-
330 adaptations to plant phenolics similar to those seen in angiosperm feeders, upregulating an
331 important detoxifying enzyme (GST) and partially conjugating these acetophenones prior to
332 elimination, but that these counter-measures are not totally effective at neutralizing effects of the
333 ingested compounds in the context of our artificial-diet based laboratory experiment. As a
334 further twist in the co-evolutionary arms race between plants and their insect herbivores, conifers
335 have been shown to produce flavonoids that act as GST inhibitors in vitro and increase
336 insecticide mortality; these could thus represent counter-counter-adaptations against insect
337 detoxifying enzymes that synergize the plants` defensive phenolics [40].

Acknowledgements

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454

Figure captions:

456

457   *Figure 1: Body mass of sixth instar budworm caterpillars (mean±SE, N=50) fed on either control- or phenolic-laced artificial*
458   *diet in the three experimental runs (pretreatment on foliage (2016) or on artificial diet (2016 & 2017)).*

459 *Figure 2: HPLC-DAD chromatograms of the piceol/pungenol mixture incubated at A. neutral and B. alkaline pH. Numbers*
460 *represent m/z of identified peaks: 137 = piceol, 153 = pungenol, 285, 287 and 303 = putative dimers.*

461 *Figure 3: HPLC-DAD chromatograms of frass from caterpillars fed A. control or B. phenolic-laced artificial diet. Numbers*
462 *indicate m/z of identified peaks: 137 = piceol, 153 = pungenol, 287 = putative dimer, 299 = tentatively identified as picein, 315*
463 *= tentatively identified as pungenin, 425 = tentatively identified as a GSH-piceol conjugate, 441 = tentatively identified as a*
464 *GSH-pungenol conjugate.*

465 *Figure 4: Glutathione-S-transferase enzyme activity of spruce budwormthe midgut tissue of the spruce budworm at neutral and*
466 *alkaline pH, represented per mg soluble protein (mean ± SE, N=12).*

467

468

Figure 1
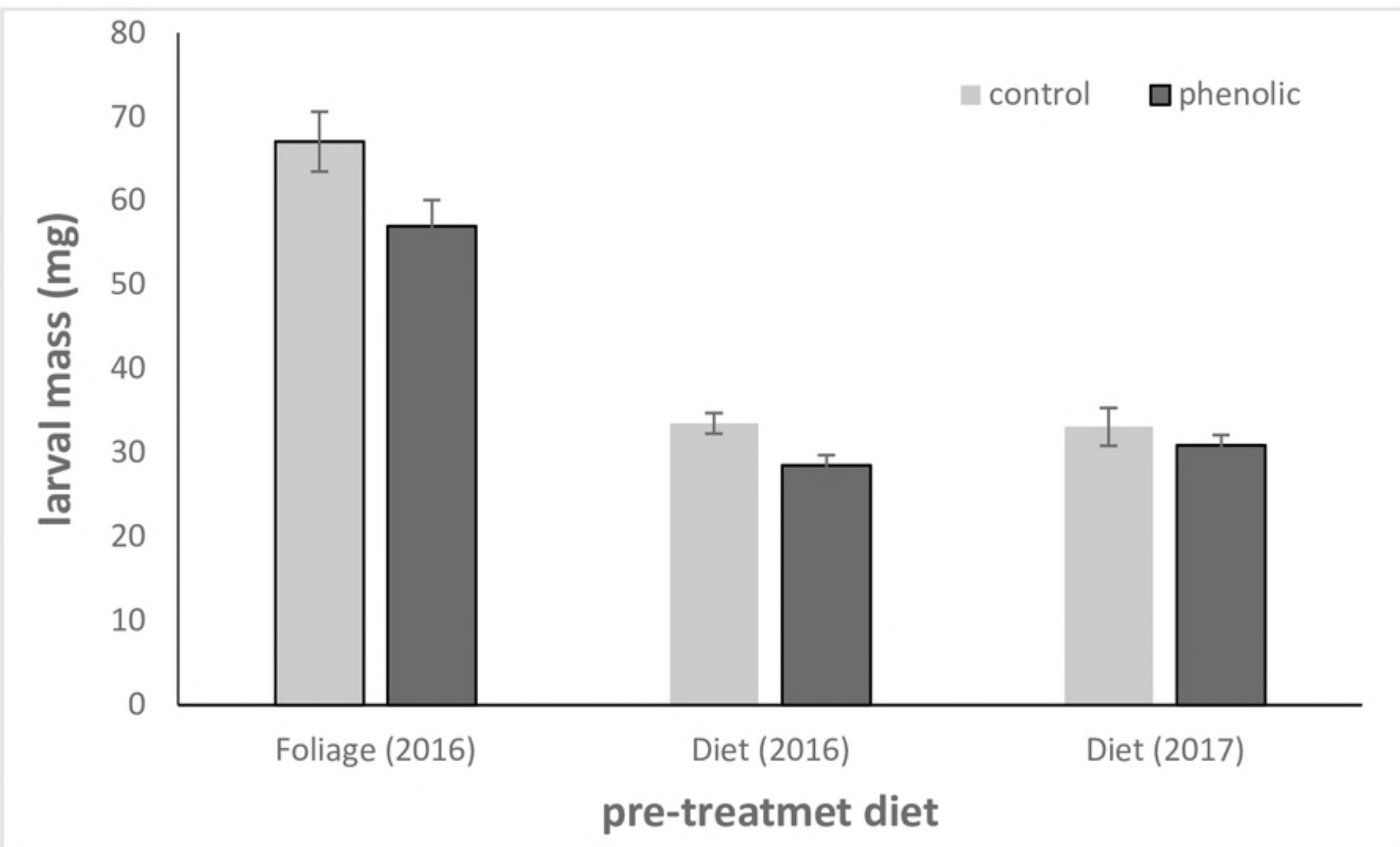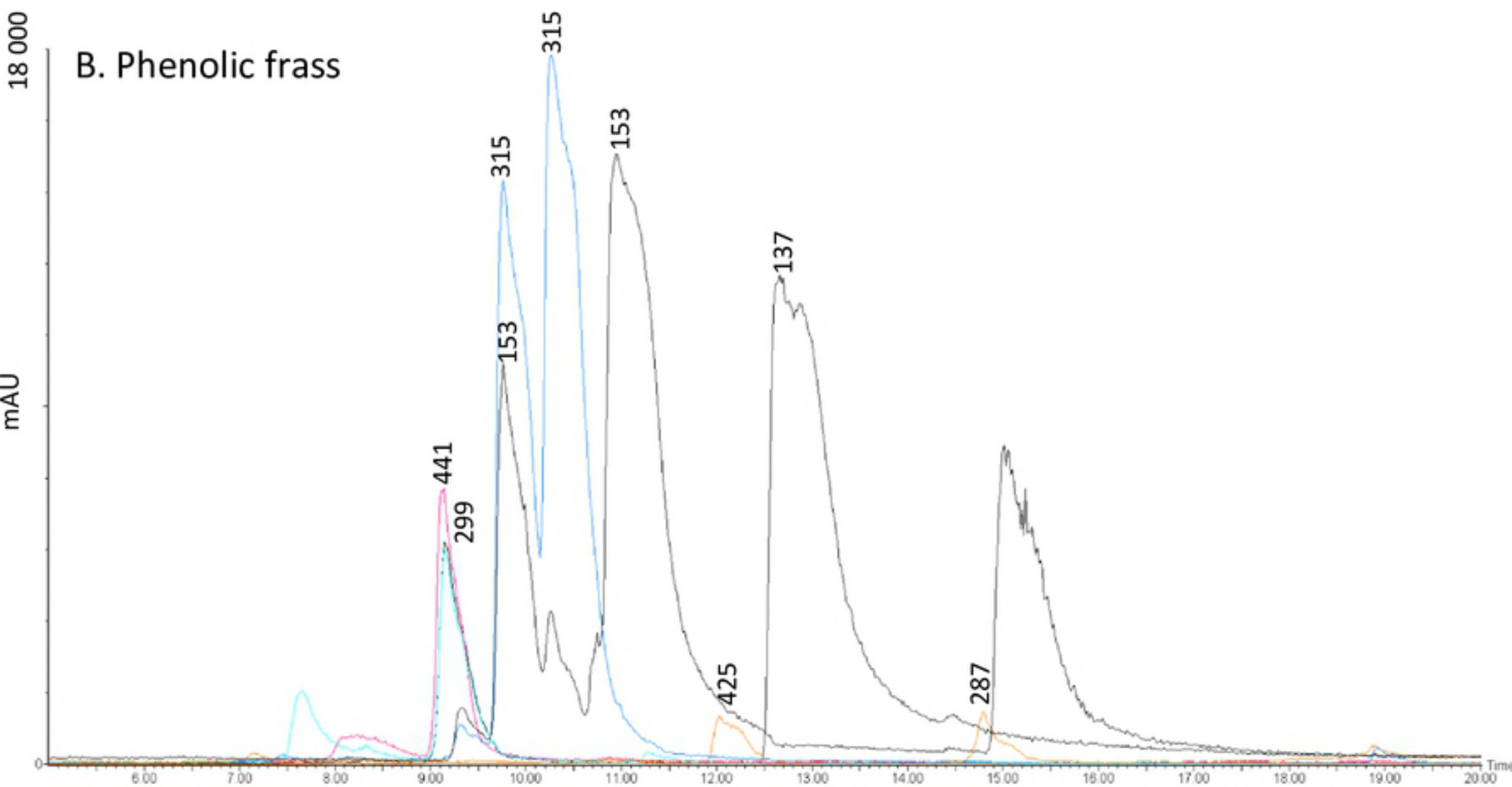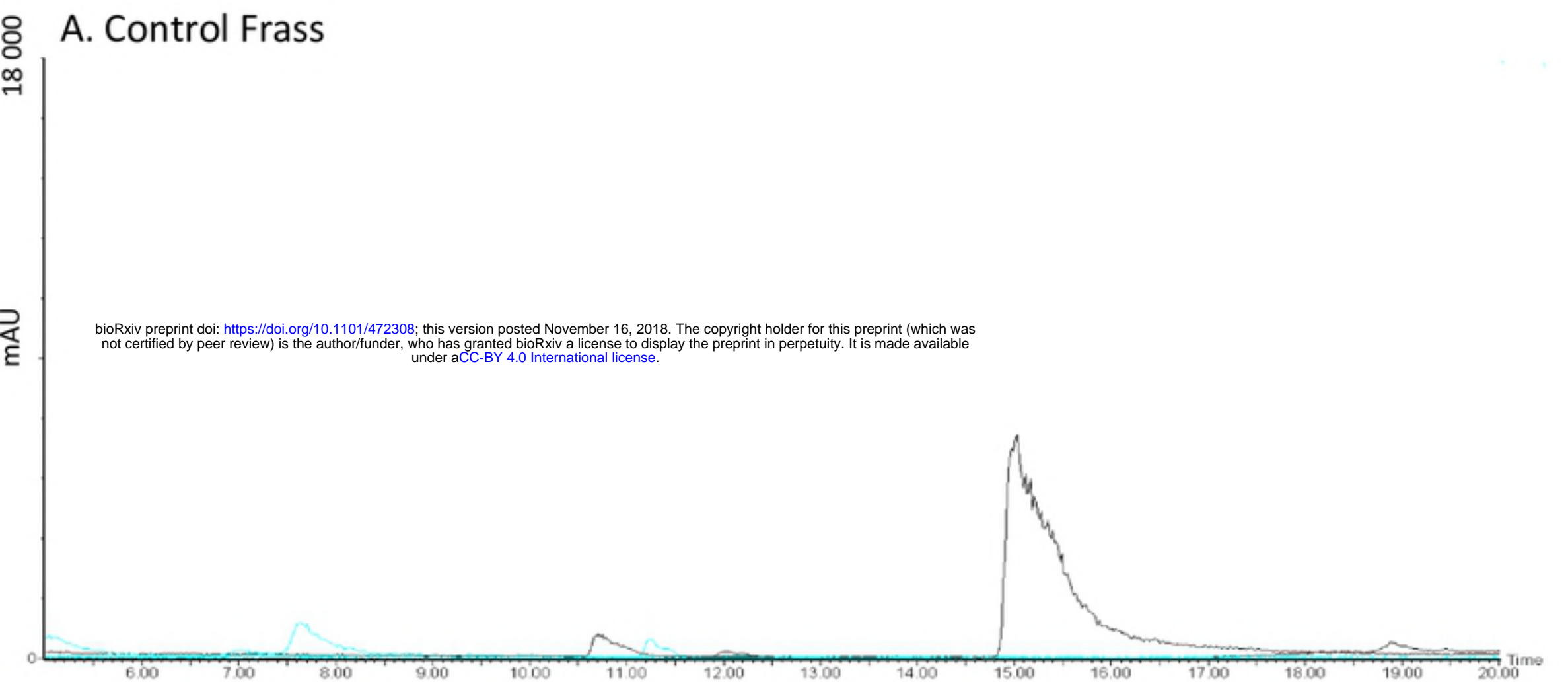

Figure 1

Figure 2

Figure 2

Figure 3

A. Control Frass

B. Phenolic frass
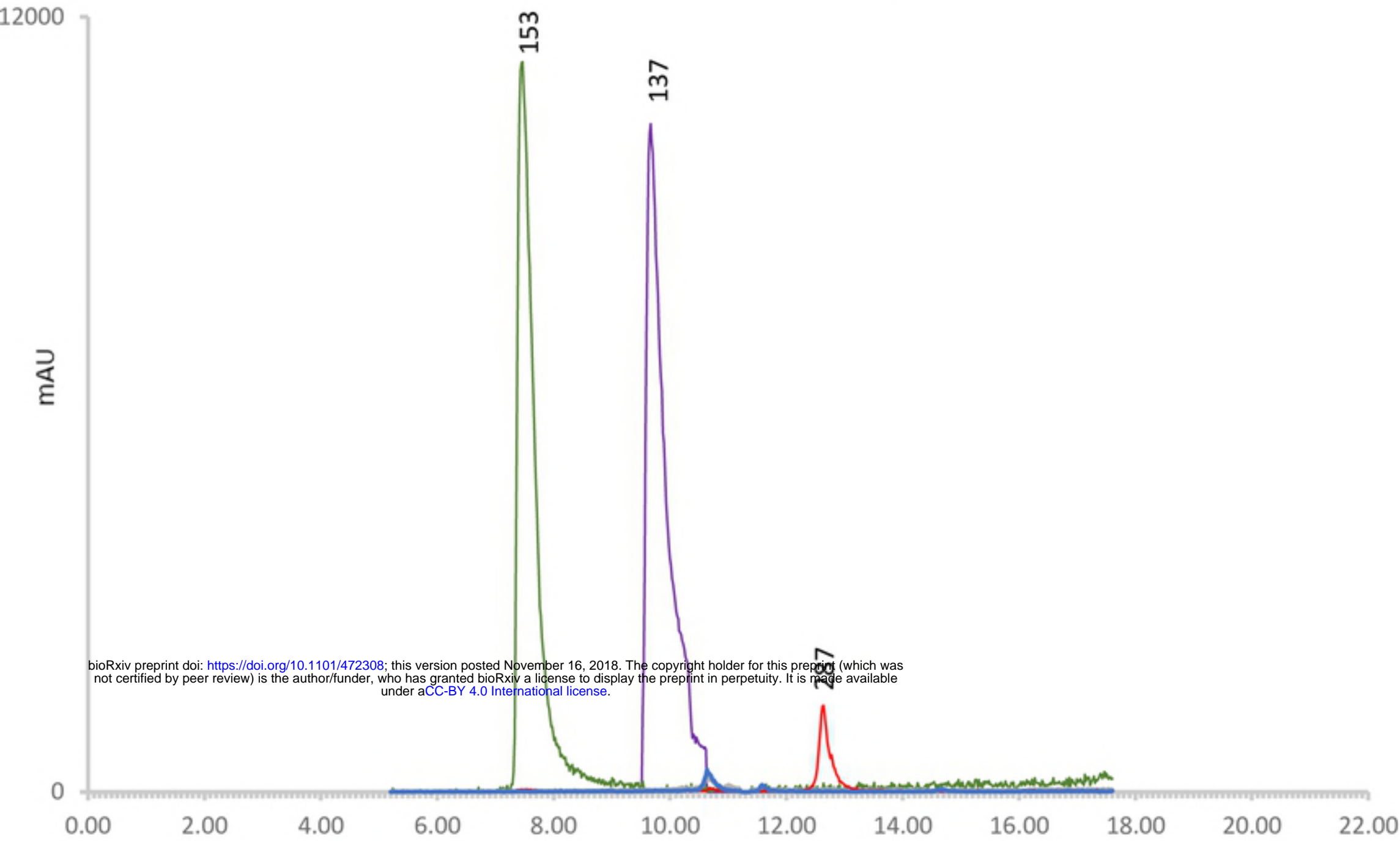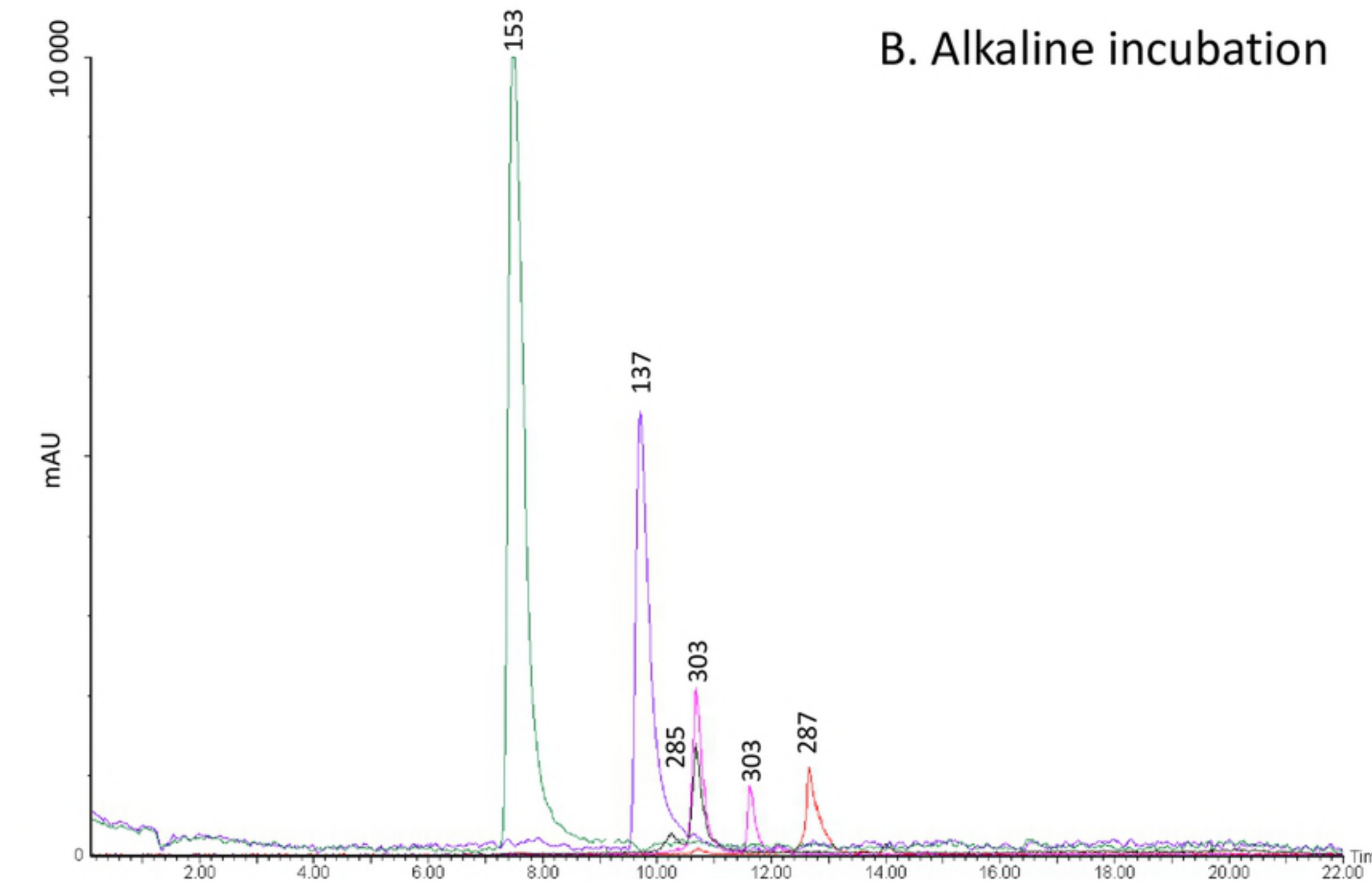
Figure 3

Figure 4

Figure 4