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Differential detoxification strategies in closely related herbivore species across a sharp ecotone

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ABSTRACT

Herbivores face a continual challenge of balancing their nutritional needs with the toxicity that they encounter in their diets. Plants produce toxic phytochemicals to ward off herbivores, while herbivores have evolved methods of detoxifying these chemicals. One such method is the use of cytochrome P450 enzymes (CYPs), many of which are found in the liver. While there has been much study of CYPs in model systems, there has been little research on CYPs in wild systems.

The woodrat species *Neotoma lepida* and *N. bryanti* live across a sharp ecotone in which they encounter plants with vastly different chemistry. *Neotoma lepida*, more of a specialist, prefers the cyanogenic glycoside-containing *Prunus fasciculata*, while *N. bryanti*, more of a generalist, has a more varied diet including a large proportion of the anthraquinone-containing *Frangula californica*. We investigated woodrat CYP activity to answer the following questions: 1) How to CYPs detoxify specialized diets in *N. lepida* and *N. bryanti*? 2) How does CYP detoxification ability limit diet switching in *N. lepida* and *N. bryanti*? To answer these questions, we developed an *in vitro* assay to isolate liver CYPs and test their activity on plant extracts as well as individual compounds, using LCMS-TOF to compare post-assay chemistry.

We found that *N. bryanti* and *N. lepida* do use CYPs for detoxification, on both known and unknown plant secondary metabolites. Additionally, we found that while both woodrat species were able to modify plant toxins in the unfamiliar diet, it was not to the same ability for both species on plant compounds, indicating species and prior diet exposure are both important factors in detoxification chemistry. This lowered detoxification ability for the unfamiliar diets limits their potential to expand their range

across this sharp ecotone as well as lowering the likelihood of hybridization with the other *Neotoma* species nearby, as they would be less likely to spend time on the opposite side of this ecotone, thereby reinforcing the species boundary.

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INTRODUCTION

Plants produce toxic phytochemicals as a defense against herbivory. Consequently, herbivores must find a balance between their nutritional needs and the energetic cost of tolerance or detoxification of these plant secondary metabolites (PSMs) (Mangione et al., 2004; Sorensen, Heward, et al., 2005). This detoxification ability limits both the plant species and the quantity of plant that an animal can easily consume (Freeland & Janzen, 1974). Animals utilize various strategies to cope with these plant secondary metabolites (PSMs), including behavioral avoidance, adapting a tolerance to the negative effects of PSMs, and detoxification either by enzymatic processes or with the assistance of the gut microbiome.

In mammalian herbivores, hepatic cytochrome P450 enzymes (CYPs) serve as Phase I detoxification enzymes. They transform xenobiotic compounds into more hydrophilic forms, often through the addition of an oxygen atom, which facilitates the organisms ability to excrete these modified toxins (Werck-Reichhart & Feyereisen, 2000). While liver CYPs have been studied extensively in model systems, especially humans, there has been far less research on CYP activity in non-model systems; additionally, studies of CYPs in wild mammalian systems tend to focus on gene expression, rather than CYP activity itself (Guengerich, 2008; Malenke et al., 2012).

Herbivores tend to fall on a spectrum somewhere between diet specialists, who only consume specific plant species, and generalists, who consume a wide variety of plant species. While most mammals would be classified as dietary generalists, there are some that do specialize on toxic plants. Diet specialists have often evolved to handle the specific PSMs of their host plant, thus they are able to tolerate or quickly detoxify these

compounds. Mammalian herbivore specialists have been found to have decreased absorption rates of plant toxins compared to generalists (Sorensen et al., 2004; Sorensen & Dearing, 2003). Additionally, these specialists often have more copies of CYP genes than generalists, which indicates more different variants and greater Phase I detoxification ability (Kitanovic et al., 2018). However, mammalian specialists have been found to be more negatively impacted when exposed to novel plant toxins than generalists (Sorensen, McLister, et al., 2005); mammalian generalists have less difficulty incorporating a new host plant into their diet. This ability to switch diets, or lack thereof, has wide-ranging ecological implications, influencing species range as well as interspecific gene flow and hybridization, especially across ecotones.

In this study we compare two closely related mammalian herbivores, *Neotoma lepida* and *N. bryanti*, to understand the role of detoxification in maintaining species diversity. These woodrat species have large ranges that meet in a hybrid zone with a sharp ecotone; while they can hybridize with each other, the hybrids often have lower survival, and will only produce off spring with members of one of the parental species (Matocq et al., 2020; Shurtliff et al., 2014). *Neotoma lepida* and *N. bryanti* differ in diets that contain toxic plant secondary compounds. *Neotoma lepida* is locally specializes on *Prunus fasciculata*, of the family *Rosaceae*, which contains cyanogenic glycosides; *N. bryanti* is a facultative generalist, consuming a more varied diet that includes the anthraquinone-containing *Frangula californica*, of the *Rhamnaceae* family, which they consume more of than any other single plant (Nielsen & Matocq, 2021). While both anthraquinones and cyanogenic glycosides are toxic, they are structurally dissimilar and would require different detoxification methods. Additionally, their diets are based not

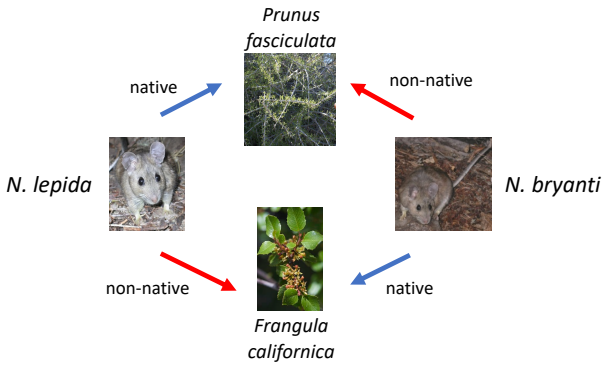
only on availability, with each plant occupying one side of the sharp ecotone at our study site, but also on preference. Previous work has shown that there is asymmetry in the ability of these woodrats to switch diets; *N. bryanti* can consume *P. fasciculata*, while *N. lepida* struggles to manage on a diet of *F. californica* (Nielsen, 2022). When exposed to the opposite diet, *N. lepida* showed shifts in gene expression in the liver and caeca, as well as changes in the composition of the gut microbiome. All these factors affect the ability of *N. bryanti* and *N. lepida* to not only expand their range, as their preferred plants tend to only occur in their current range, but also hybridize with each other.

We investigated woodrat CYP activity to answer the following questions: 1) What is the role of CYPs in detoxifying specialized diets in *N. lepida* and *N. bryanti*? 2) How does CYP detoxification ability limit *N. lepida* and *N. bryanti* from consuming each other's dominant host species? To answer these questions, we developed an assay to isolate liver CYPs and test their activity on plant extracts and individual compounds, using LCMS-TOF to compare post-assay chemistry. This will improve our understanding of the maintenance of the species boundary at this hybrid zone, as well as give us further insight into detoxification strategies in non-model systems.

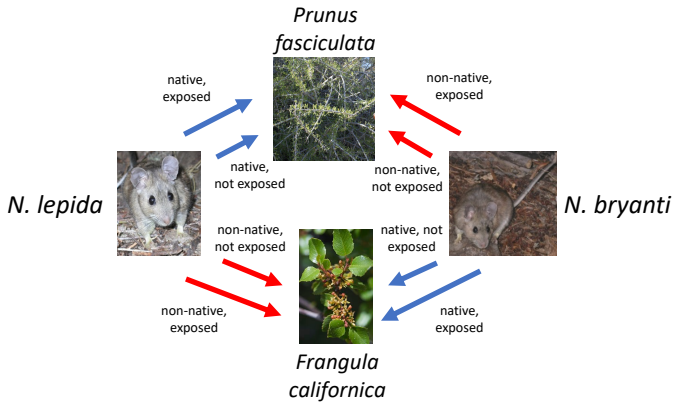
MATERIALS AND METHODS

Experimental Overview

Woodrats of both species were collected from our study site at this hybrid zone. These woodrats then went through feeding trials on plant secondary compounds from either their native or non-native diet, creating four different diet treatment groups. After the feeding trials, liver tissue was collected and CYP enzymes were isolated by creating microsomes. These microsomes were then exposed to single known plant secondary compounds, as well as a mixture of these compounds from plant extracts, in the presence of NADPH. All woodrat diet treatment groups were exposed to all single compounds and plant extract mixtures, to observe the effects of prior dietary exposure on detoxification chemistry for native and non-native woodrats. Changes in chemistry over the course of these assays were observed using liquid chromatography-mass spectrometry.



Schematic A: Visual depiction of woodrat feeding trials.



Schematic B: Visual depiction of sample for CYP assays; prior exposure refers to which diet the woodrat was fed during the earlier feeding trials.

Study system and collection

Our study site is located at the hybrid zone of *N. bryanti* and *N. lepida* at the southern end of the Sierra Nevada mountains in Kern County, CA, (35°25′45 N, 118°15′2 W) where the xeric "flats" habitat meets the mesic "hill" habitat at a sharp ecotone. We collected adult *N. lepida* and *N. bryanti* woodrats from either side of the hybrid zone in locations where *P. fasciculata* and *F. californica* were present. Woodrats were captured in tomahawk live traps using peanut butter and oats as bait. Body measurements and sex were recorded before the woodrats were transported back to the university in shoebox cages with ad libitum food and water.

Feeding trials

Upon arrival to the lab, the woodrats were kept in a 15:9 light:dark cycle at 23°C and given water and high-fiber rabbit chow ad libitum; the rabbit chow was in a powdered form so as to prevent caching. All animal care procedures were approved by the UNR Institutional Animal Care and Use Committee and the California Department of Fish and Wildlife, and consistent with the guidelines of the American Society of Mammologists (Sikes et al., 2016).

Each woodrat was fed a diet of rabbit chow mixed with a plant extract from either their native diet, to control for any differences in diet that may have occurred in the wild prior to the experiment, or their non-native diet, to observe how adjustments in gene expression due to prior exposure might affect detoxification chemistry. We prepared extracts for both *P. fasciculata* and *F. californica* by crushing dried leaf material in a mortar and pestle material under liquid nitrogen, then transferring to a large flask and

adding 5 mL of methanol per gram of dried leaf material and let stir overnight. The mixture was then filtered using a Buchner funnel to remove the leaf material, and the liquid was dried using a Genevac EZ-2 (SP Scientific) followed by an overnight high vacuum to remove any remaining solvent. The dosage was determined by dividing the mass of the dried extract by the mass of the starting plant material, and the maximum dosage (100%) was the maximum amount of extract that the animal would be exposed to in the wild if it only consumed that plant. The dried extract was then thoroughly mixed with the rabbit chow. Each dose was maintained for two days (except the maximum dose, which was maintained for three days) to ensure liver enzyme induction; the doses started at 0% extract, followed by 1%, 5%, 10%, and then increased by 10% until 100%. The maximum tolerable dosage was determined by the percentage of extract just prior to a significant decrease in food intake, a significant increase in water intake, or a 10% decrease in weight.

The woodrats were euthanized with isoflurane and cervical dislocation as a secondary method of euthanasia. Liver tissue was rapidly harvested and rinsed with sterile saline, minced on an ice-cold tray, and placed in a cryovial in liquid nitrogen before being moved to a -80°C freezer for storage. All tissues were handled with sterile tools that were changed across individuals and tissue types.

Microsome Preparation

We prepared liver microsomes for each woodrat using a BioVision Microsome Isolation Kit. Approximately 0.1 g of liver tissue was measured and combined with 500 uL per gram of ice-cold homogenization buffer, then homogenized with a Dounce tissue

grinder. This homogenized tissue was vortexed for 30 seconds, chilled on ice for 1 minute, and then centrifuged at 10,000 x G for 15 minutes at 4°C. After centrifugation, the thin top layer was aspirated, and the supernatant was transferred to a new pre-chilled tube, then centrifuged for 20 minutes at 4°C at 20,000 x G; the supernatant was discarded and the pellet was first washed with 500 uL per gram tissue of homogenization buffer, then resuspended in the same volume of storage buffer. A BCA protein assay was used to determine total protein concentration of the microsomes. CYP presence was verified with a western blot using CYP11A1 rabbit antibodies with VDAC rabbit antibodies as a control (Figure S1).

Extract fraction preparation

To prepare the plant extracts for the CYP assay, we needed to make sure that the plant compounds would be similar to the compounds that liver CYPs would actually encounter, as they first encounter the digestive system before they are absorbed by the body. The plant extracts were dissolved in a 0.1g to 15 mL ratio in a 20 mM sodium acetate buffer with a pH of 5. One milligram of β-glucosidase was added per 15 mL, and the mixture was incubated and shaken for 24 hours and then dried using the Genevac; this was to mimic the activity of stomach enzymes and modify the extracts to be more similar to what chemicals would reach the liver. A solid-phase extraction was performed with a Sep-Pak C18 cartridge to separate each extract using five combinations of water and methanol, starting with 0% methanol, followed by 35%, 60%, 80%, and 100% methanol. Each fraction was dried using the Genevac, then redissolved in methanol at a concentration of 28 mg/mL to create a stock solution. The stock solutions were diluted

for use in the assay to a concentration of 0.55 ug/mL using a 0.01 mM phosphate buffered saline solution (PBS) with a pH of 7.4.

CYP Assay

CYP assays for all single compounds and extract fractions were performed on liver microsomes from both woodrat species and both diet treatment groups. For our single-compound assays, we used emodin, a known compound found in *F. californica*, and benzaldehyde, which while not found in *P. fasciculata* is known to be a product of prunasin and amygdalin (both known *Prunus* compounds) when they are modified before reaching the liver (Laham et al., 1988; Sendker et al., 2016). The 35% methanol fractions for both *P. fasciculata* and *F. californica* were chosen for use in the CYP assays (later referred to as *Prunus* and *Frangula* extracts) due to the higher peak richness encompassed compared to the other fractions (Figure S2), as well as the presence of emodin and emodin metabolites in the *Frangula* fraction.

A combination of 141 uL of PBS, 33 uL of freshly prepared 10 mM NADPH solution, and 9 uL of liver microsomes were put in a 1.5 mL microcentrifuge tube and kept on ice; the volumes of microsomes and PBS were adjusted based on the protein concentration of the microsomes, as these varied slightly between livers. To start the reaction, 150 uL of the plant extract fraction or individual compound was added to the tube, which was placed in an incubator at 37°C. At each time point (10, 20, 40, and 60 minutes), 66 uL was removed from the tube and combined with 33 uL of ice-cold methanol in a new tube and kept on ice; after 80 minutes we added 33 uL ice-cold methanol to the original tube for the final time point and kept on ice. All tubes were

centrifuged at 4°C for 10 minutes at 20,000 x G to separate the microsomes; the pellet was discarded while the supernatant was kept and stored at -80°C until LCMS-TOF analysis. All CYP assays were performed on microsomes from six woodrats per species, three of each species with previous *F. californica* diet exposure and three with prior *P. fasciculata* diet exposure, along with three lab rats as a control group. The resulting detoxification chemistry of the lab rats, however, was much different from that of the woodrats and they were excluded from most analyses (Figure S3).

LCMS-TOF Analyses

Metabolomic (LC-MS) analyses of plant extracts, emodin, benzaldehyde and their CYP-transformed products were carried out on an Agilent 1290 Infinity II Ultrahigh performance liquid chromatograph (UPLC) equipped with a binary pump, multisampler, column compartment and diode array UV/Vis detector, coupled to an Agilent 6560 Ion-Mobility Quadrupole-Time-of-Flight (IM-QTOF) mass spectrometer via a Jet Stream electrospray ionization source (ESI-TOF; gas temperature: 300 °C, flow: 11 L/m; nebulizer pressure: 35 psig; sheath gas temp: 350 °C; sheath gas flow: 12 L/m; VCap: 3500 V; nozzle voltage: 500V; fragmentor: 350 V; skimmer: 65 V; octopole: 750 V). Samples were analyzed in low-mass (100-1700 m/z) TOF mode at a rate of 1 spectra/s (8131 transients/spectrum). Samples (10.00 µL) were co-injected with daidzein internal standard (1.00 µL, 10 µM in MeOH) and eluted in positive mode at 0.400 mL/min through a Phenomenex EVO C18 column (2.6 x 100 mm, 2.1 µ, 100 Å) at 40 °C run with a linear binary gradient comprised of buffers A (water containing 0.1 % formic acid) and B (99% aqueous acetonitrile containing 0.1 % formic acid) changing over 15 minutes: 0

min 1% B, ramp to 100% B at 10 min, hold at 100%B for 10-12 min, ramp to 1% B at 13 min, 13-15 min hold at 1% B. Samples were also run in negative mode at 0.400 mL/min through an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl column (2.1 x 150 mm, 1.8 µ, 100 Å) at 40 °C run with a linear binary gradient was comprised of buffers A (water containing 10 mM ammonium acetate) and B (99% aqueous acetonitrile) changing over 16 minutes: 0 min 1% B, ramp to 100% B at 10 min, hold at 100% B at 10-12 min, ramp to 1% B at 14 min, 14-16 min hold at 1% B. Peaks were extracted, retention time corrected, aligned and grouped using recursive small molecule analysis in Agilent Profinder v10.0 with a minimum height cut-off of 20,000 counts. These data were imported into Agilent Mass Profiler Professional v15.1 (MPP) where peak areas were normalized to daidzein internal standard. The positive mode entity list was used for statistical analyses. Representative replicates from experimental groups were analyzed in negative mode Q-TOF using iterative data-dependent acquisition (collision energy = 20, 40 V; precursor threshold = 10k counts). Putative annotations were based on high-resolution mass in positive and negative modes as well as MS/MS spectral confirmation from the METLIN mass spectral database in MassHunter Qualitative Analysis when possible.

To remove any chemical peaks from woodrat livers that may have remained in the sample after the assay, we removed any peaks found across both emodin and benzaldehyde assays; as the metabolites of these compounds are different from each other, thus any matching peaks across these samples would likely be due to liver contaminant that remained.

Statistical analyses

All analyses were performed in R version 4.3.0, MassHunter Qualitative Analysis 10.0, and MetaboAnalyst 5.0.

To quantify the changes in metabolites over time we calculated compound richness and diversity using hill numbers (add R function here, and package) for each assay at each different time point.

A partial least squares discriminant analysis (PLS-DA) was also performed, comparing CYP assay chemistry of each time point to investigate patterns of changing chemistry over time as well as influential peaks that separated each group.

A signed weighted gene coexpression network analysis (WGCNA) was performed on the extract fraction assays. This grouped the chemical peaks into modules based on peaks that covaried together. We then analyzed how the modules changed over time as well as the correlation between modules. This approach allowed us to identifying the peaks that decreased in concentration over time with peaks that increases over time, presumably as products of biotransformation.

RESULTS

We found that both *N. bryanti* and *N. lepida* cytochrome P450s metabolized not only the individual compounds in the assay, but also many different compounds from their respective plant extracts. There was far greater richness and chemical diversity in both the original *Frangula* extract fractions and subsequent CYP assays compared to those of *Prunus*.

CYPs metabolizing native diets

Neotoma bryanti quickly metabolized emodin (Figure 1) into multiple products, including the compounds shown in Figure 2. The peak richness of the emodin assay increased at later time points (Figure 3). The overall peak richness and diversity of the *Frangula* fraction fluctuated over time, first decreasing and then later increasing towards the end of the assay (Figure 4). The overall chemical composition over time were similar during the first 40 minutes with the highest variability at 60 minutes (Figure 5). Additionally, known metabolites of emodin, identified by known masses with either one or two oxygen atoms added to the original molecule, changed over time (Figure 6). The WGCNA analysis identified modules with chemical peaks of interest (Figures 7 and 8). Modules of interest were composed of peaks that negatively correlated with other modules, or that had a strong positive or negative relationship with respect to time (selected peaks shown in Figure 9). The masses of the peaks of interest were compared to a database of known secondary metabolites of the genus *Frangula*; however, there are limited data for non-model systems resulting in no matches outside of the aforementioned emodin metabolites.

Neotoma lepida was able to metabolize benzaldehyde into benzoic acid, which generally increased in concentration throughout the assay (Figure 10). Additionally, the chemical richness and diversity increased throughout the assay (Figure 11), indicating that there were other products formed in the detoxification process. In contrast, the peak richness and diversity for the *Prunus* extract decreased over time (Figure 12). A discriminant analysis corroborates this, showing increasingly similar composition over time (Figure 13). In a WGCNA (Figures 14 and 15); we identified the black and green modules as containing compounds of interest as they were the most negatively correlated with each other modules (Figures 16 and 17). These peaks were compared against a database of known secondary metabolites of the genus *Prunus*, but there were no matches for these peaks of interest, as there is limited data available for non-model systems.

CYPs metabolizing non-native diets

We found that prior exposure to *F. californica* led *N. lepida* to detoxify emodin at a similar rate to *N. bryanti*, which was faster than *N. lepida* that had not been exposed previously (Figure 18). Additionally, known metabolites of emodin (Figure 19) were produced by both species of woodrat, regardless of prior diet exposure. Interestingly, the *N. lepida* without prior exposure to *F. californica* had much lower chemical diversity for the single compound emodin assay than their exposed counterparts as well as *N. bryanti*, despite having a similar peak richness (Figure 20).

Overall detoxification chemistry of *N. lepida* on the *Frangula* extract did not substantially differ between the diet treatments (Figure 21); it also varied less between individual assays than *N. bryanti* (Figure 22). Additionally, the peak richness and

diversity was lower for *N. lepida*, regardless of diet treatment, compared to the native *N. bryanti* (Figure 23). *Neotoma lepida* produced less emodin metabolites compared to *N. bryanti*, regardless of prior exposure to *F. californica*, and contrary to what was produced in the single compound emodin assay (Figure 24). Due to the differences between species and prior diet exposure, there were fewer modules with strong relationships with respect to time in the WGCNA analysis of the *Frangula* extract assays. Consequently, we used a two-way analysis of variance (ANOVA) on each module to compare differences in chemistry based on woodrat species and prior diet exposure to determine additional modules of interest. Peaks of interest were compared to a known database of PSMs from the genus *Frangula*, but as this is a non-model system there is little data available and so there were no matches aside from the known emodin metabolites.

We found that while *N. lepida* slowly modified benzaldehyde into benzoic acid regardless of prior diet treatment, but the most benzoic acid was produced by the specialist *N. lepida* on its native *Prunus* diet (Figure 25). Additionally, there was slightly higher peak diversity for *N. lepida* later on in the assay than *N. bryanti*, regardless of diet treatment (Figure 26).

Both *N. bryanti* and *N. lepida* varied chemically in the *Prunus a*ssays, but in different ways from each other (Figure 27). For *N. bryanti,* prior exposure to *P. fasciculata* resulted in higher variability in the response to *Prunus* extract than those without prior exposure (Figure 28). This is corroborated by the higher peak richness and Shannon's diversity in *N. bryanti* with prior exposure compared to their naïve counterparts (Figure 29). In a WGCNA on the *Prunus* extract fraction there were similarly few strong relationships with respect to time due to differences in species and

previous diet exposure. Thus, we used a two-way ANOVA to compare the effects of woodrat species and prior diet exposure on detoxification chemistry of peaks the modules to determine more modules of interest (selected peaks from modules of interest shown in Figures 30 and 31). The peaks of interest were compared to a known database of secondary metabolites of the genus *Prunus*, but there was little data to compare to in this genus, and no matches were found.

DISCUSSION

We found that that both *N. bryanti* and *N. lepida* detoxified single compounds of emodin and benzaldehyde easily as well as PSMs from their non-native diets. However, CYP assays revealed much higher variability in *N. bryanti* compared to *N. lepida*. Additionally, the prior diet exposure altered the results of CYP assays, especially for both woodrat species detoxifying *Prunus* compounds. In the wild, woodrats also utilize the gut microbiome in detoxification and may modify these compounds in one species but not another, which would also lead to differences in CYP detoxification chemistry. While we have identified a few peaks in the plant extracts and assays, there is still work to be done on this front to determine which peaks are being transformed into other peaks found later in the assays as well as the structures of these compounds.

CYP detoxification on native diets

In the single-compound CYP assay, emodin was quickly metabolized by *N. bryanti*. Similarly, previous studies in model systems have shown that emodin is often quickly metabolized in the liver specifically, usually fast enough to not spread elsewhere in the body (Liu et al., 2012). There are a number of known metabolites of emodin, three of which involve a simple monohydroxylation (Song et al., 2008). We were able to identify certain metabolites of emodin based on masses of monohydroxylated and dihydroxylated emodin, some of which are likely these known metabolites; however, there are many other peaks that are unidentified products of reactions throughout the course of the assay. Over the course of the *Frangula* extract fraction assay, there were interestingly more different potential metabolites of emodin, despite emodin itself being

eliminated much more quickly (likely due to its lower initial concentration, as it was in a mixture with many other PSMs). This is potentially due to synergistic effects leading to the production of different end products from the same original one.

Despite many peaks changing in concentration throughout the *Frangula* extract fraction assay, there were many compounds that stayed relatively stable the whole time. These compounds may be excreted quickly and not need detoxification, but they might also be detoxified elsewhere in the body, such as with the help of the gut microbiome. Several genera of gut microbes have been found to aid *N. bryanti* specifically in detoxification (Kohl et al., 2011). Gut microbiome in these woodrats is also greatly shaped by diet exposure; woodrats with previous exposure to a specific toxic diet have gut microbes that are better adapted to detoxify compounds from that specific diet (Nielsen et al., 2023). One intriguing path of study for the future would be to investigate gut microbiome detoxification strategies and compare to these; we would likely see different compounds being detoxified in the gut microbiome compared to those detoxified by liver CYPs.

Benzaldehyde was slowly transformed into benzoic acid by *N. lepida* in the single-compound CYP assay. This is a well-known detoxification reaction (Laham et al., 1988), and while benzaldehyde is not the same as the known *Prunus* compounds prunasin and amygdalin, the part of the structure that is modified has been found to follow this similar transformation (Sendker et al., 2016). We expect that if this assay was performed using prunasin or amygdalin instead of benzaldehyde, we would see this same type of transformation.

While many compounds changed greatly in concentration throughout the course of the *Prunus* extract fraction assay, there were many others that stayed relatively constant over time. These compounds are likely quickly excreted or detoxified elsewhere in the body; the gut microbiome, for example, is an important method for detoxification. In this system, in fact, the microbiome is greatly affected by the species of the host along with their diet (Nielsen et al., 2023). It would be unsurprising if some of these peaks that are unaffected by CYP activity were later modified by the gut microbiome. However, there is evidence of other *Neotoma* specialists absorbing lower amounts of toxins and quickly excreting them rather than detoxifying them (Sorensen et al., 2004); this may also be the case here with *N. lepida*.

There were fewer chemical peaks later in the assay, indicating that the original compounds (or intermediate metabolites of them) are completely eliminated from the mixture and are being detoxified in ways that result in similar end products. As a dietary specialist, *N. lepida* likely has adapted very efficient methods of detoxification for the PSMs that it encounters frequently in its host plant (Sorensen et al., 2004). While we were unable to identify any known compounds from the original extracts, we would expect to see that those original compounds would have multiple corresponding metabolites, like we saw for emodin in the single-compound assays.

CYP detoxification on non-native diets

Despite *N. lepida's* known distaste for *F. californica* (Nielsen & Matocq, 2021), it is evidently not due to an inability to detoxify emodin itself, as *N. lepida* on both diet treatments were able to detoxify most of the emodin they encountered; therefore, there

must be other compounds from *F. californica* that contribute to its avoidance by *N. lepida*. There are several known metabolites of emodin, most of which involve a mono- or dihydroxylation (Song et al., 2008); while unable to confirm exact structures, we identified some known metabolites of emodin based on their mass. However, there were differences in which of these known metabolites of emodin were produced by *N. lepida* based on prior diet exposure; the woodrats with prior exposure to *F. californica* showed more similarity in their emodin metabolites to *N. bryanti* than those not previously exposed. This is likely due to differences in gene expression, which we know occur in this system, especially with *N. lepida* exposed to *F. californica* (Nielsen, 2022).

While diet treatment was a notable factor determining detoxification of emodin as a single compound, species was a far more important factor when detoxifying emodin within the *Frangula* extract fraction as a whole. When examining the known metabolites of emodin within this fraction, *N. lepida* had far lower concentrations of all of these; this indicates that there is likely some antagonistic effect of other compounds within the mixture that influences emodin detoxification for *N. lepida*, preventing the production of the expected metabolites. Another possibility is that certain *F. californica* compounds may inhibit the CYPs that were able to modify emodin when encountered alone. This finding is especially notable and indicates the importance of studying detoxification of mixtures, rather than only single compounds of interest. Interestingly, previous research on other mammalian herbivores has shown that generalists tend to avoid more complex mixtures of plant toxins, while specialists on those plants have no preference (Nobler et al., 2019); however, this study was on a specialist consuming plant compounds from its

own native diet, which may help explain this difference. Nevertheless, it is still clear that chemical mixtures play an important role in diet preference and detoxification ability.

All *N. lepida* regardless of prior exposure to *F. californica* showed lower peak richness and chemical diversity than *N. bryanti* for the *Frangula* extract fraction assays, indicating that overall detoxification chemistry was more similar within species than between species, regardless of prior diet exposure. When examining various modules within the *Frangula* extract fraction, certain modules had higher concentrations in *N. bryanti* than in *N. lepida*, but other modules followed the opposite pattern. Despite the known differences in CYP gene expression of *N. lepida* when exposed to *F. californica* (Nielsen, 2022), prior exposure has little effect on much, but not all, of the detoxification chemistry of *N. lepida* when in this mixture. *N. lepida*, despite shifts in gene expression to accommodate a novel diet, still had trouble detoxifying these unfamiliar plant chemicals, suggesting that they may lack the genes to effectively detoxify some of these compounds. Additionally, prior research has shown that *N. lepida*, when forced to consume a diet with compounds from *F. californica*, cannot survive on as high of concentrations of those chemicals; these individuals were removed from feeding trials much earlier due to loss of body mass or increased water intake compared to *N. lepida* on its native *P. fasciculata* diet or *N. bryanti* fed either plant (Nielsen, 2022). This also follows the pattern of specialists being negatively impacted when introduced to novel plant toxins (Sorensen, McLister, et al., 2005).

Previous studies have shown changes in gene expression for *N. lepida* not only in our study system with exposure to *F. californica*, but also in other *N. lepida* populations. Different levels of CYP gene expression, as well as other biotransformation genes, were

found on populations of *N. lepida* feeding on creosote and juniper (Magnanou et al., 2013; Malenke et al., 2012). Historically, there was a diet shift for *N. lepida* to include the novel creosote in its diet; there was found to be both higher gene expression as well as gene expansions within the genome (Greenhalgh et al., 2022). However, these gene expansions may not be as helpful for detoxifying compounds from *F. californica*. While *N. lepida* has been known to shift its diet in the past, they are likely not in a place to do so in this population, given their inability to effectively detoxify known toxic PSMs or even produce the same metabolites as the experienced *N. bryanti* when in a mixture with other *F. californica* compounds, in addition to their decreased survival when forced to consume *F. californica*.

While *N. bryanti* did produce some benzoic acid, a known metabolite of benzaldehyde (Laham et al., 1988), it was at a lower amount than the specialist *N. lepida*. *Neotoma lepida* likely has a specialized CYP for this specific type of reaction, while the generalist *N. bryanti* may not, as this type of compounds is not one they normally encounter. Even though benzaldehyde was used as a proxy for the known *Prunus* compounds prunasin and amygdalin, we expect that at this point in the detoxification process they would be metabolized in a similar way (Sendker et al., 2016).

Neotoma bryanti was again more varied in its detoxification chemistry for the *Prunus* extract fraction than *N. lepida*, indicating that the generalist species detoxified even these unfamiliar compounds in multiple ways, while the specialist *N. lepida* was far more consistent. However, *N. bryanti* that had prior exposure to *P. fasciculata* showed even more variation in its detoxification chemistry compared to their naïve counterparts. Additionally, *N. bryanti* with prior exposure showed greater peak richness throughout the

assay than those without prior exposure as well as *N. lepida*. Despite previous research showing insignificant changes in CYP gene expression in *N. bryanti* exposed to *P. fasciculata* (Nielsen, 2022), there appears to be enough of a difference to influence overall detoxification chemistry. Certain modules of the *Prunus* extract fraction showed significant differences between the diet treatments, with previously exposed *N. bryanti* producing similar amounts of certain metabolites as *N. lepida*, and *N. bryanti* without prior exposure producing similar amounts of these metabolites to the *N. lepida* that were previously exposed to *F. californica*. These findings indicate shared CYPs between these species, which were upregulated or downregulated based on prior diet exposure. Previous research has shown that there are many specific CYPs that are in the genomes of both *N. lepida* and *N. bryanti*, including some which were used by both species to facilitate a diet shift onto creosote (Malenke et al., 2014).

Detoxification and maintaining diversity

Using our CYP assay, we were able to detect changes in chemistry for both individual compounds as well as the overall chemical composition of native plant extracts over time, for both *N. bryanti* and *N. lepida*. While we saw some expected patterns in the changing chemistry, with certain peaks increasing over time while others decreased, we also saw some more unexpected patterns, such as an increase followed by a decrease in concentration, indicating some sort of intermediate metabolite in the detoxification process.

In both woodrat species across both plant extracts, there are many compounds that remain stable over time. It is likely that these are either not toxic to woodrats, or at least

not toxic in low doses, or they are detoxified by other mechanisms in the body. Tolerance of low doses of varied PSMs is common especially in generalist species, and animals often facilitate this by managing the quantities of the various plants they eat (Sorensen, Heward, et al., 2005). One important strategy of detoxification aside from liver CYPs is the gut microbiome, which has been shown to be especially important for *N. bryanti* (Kohl et al., 2011; Kohl & Dearing, 2012). Previous work in this system has shown that the gut microbiome is greatly affected by a combination of host genotype and diet, which is primarily determined by habitat in the wild (Nielsen et al., 2023). Additionally, different lineages of microbes were associated with prior exposure to an unfamiliar diet for both *N. lepida* and *N. bryanti* (Nielsen, 2022). However, we do not know the specific mechanisms of detoxification carried out by the microbiome; this would be a very interesting avenue of further research.

Both *N. lepida* and *N. bryanti* detoxification had differences in detoxification chemistry based on prior exposure to the other's diet, which are likely due to differences in CYP gene expression from the prior diet treatment. However, there were also differences in detoxification chemistry that were far greater between the two woodrat species, rather than between the diet treatments. Additionally, many peaks' concentrations remained constant throughout the assays; these are likely less toxic compounds that are quickly excreted, or they are detoxified by another mechanism, such as the microbiome, which warrants further investigation. These differences in detoxification chemistry indicate a lowered ability to manage these unfamiliar diets for both species. Previous studies have also found this pattern, with a slight asymmetry where *N. bryanti* is better able to detoxify the unfamiliar *P. fasciculata* compared to *N.*

lepida on *F. californica* (Nielsen, 2022). This limited ability to shift diets, especially for the specialist *N. lepida*, poses a difficulty for these woodrats when crossing to the other side of the sharp ecotone at our study site, as the plant composition is quite different in the two habitats. This decreased detoxification ability and lowered survival of *N. lepida* consuming *F. californica* provides another filter to hybridization and interspecific gene flow, likely decreasing the number of *N. lepida* found across the ecotone, and subsequently reducing the number of hybrid individuals in the system.

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FIGURES

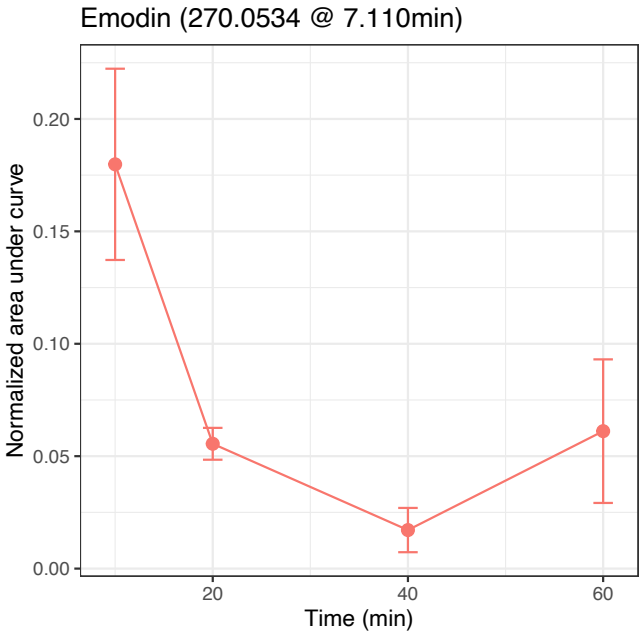


Figure 1: Biotransformation of emodin by *N. bryanti* liver CYPs over time. Mean ± SE.

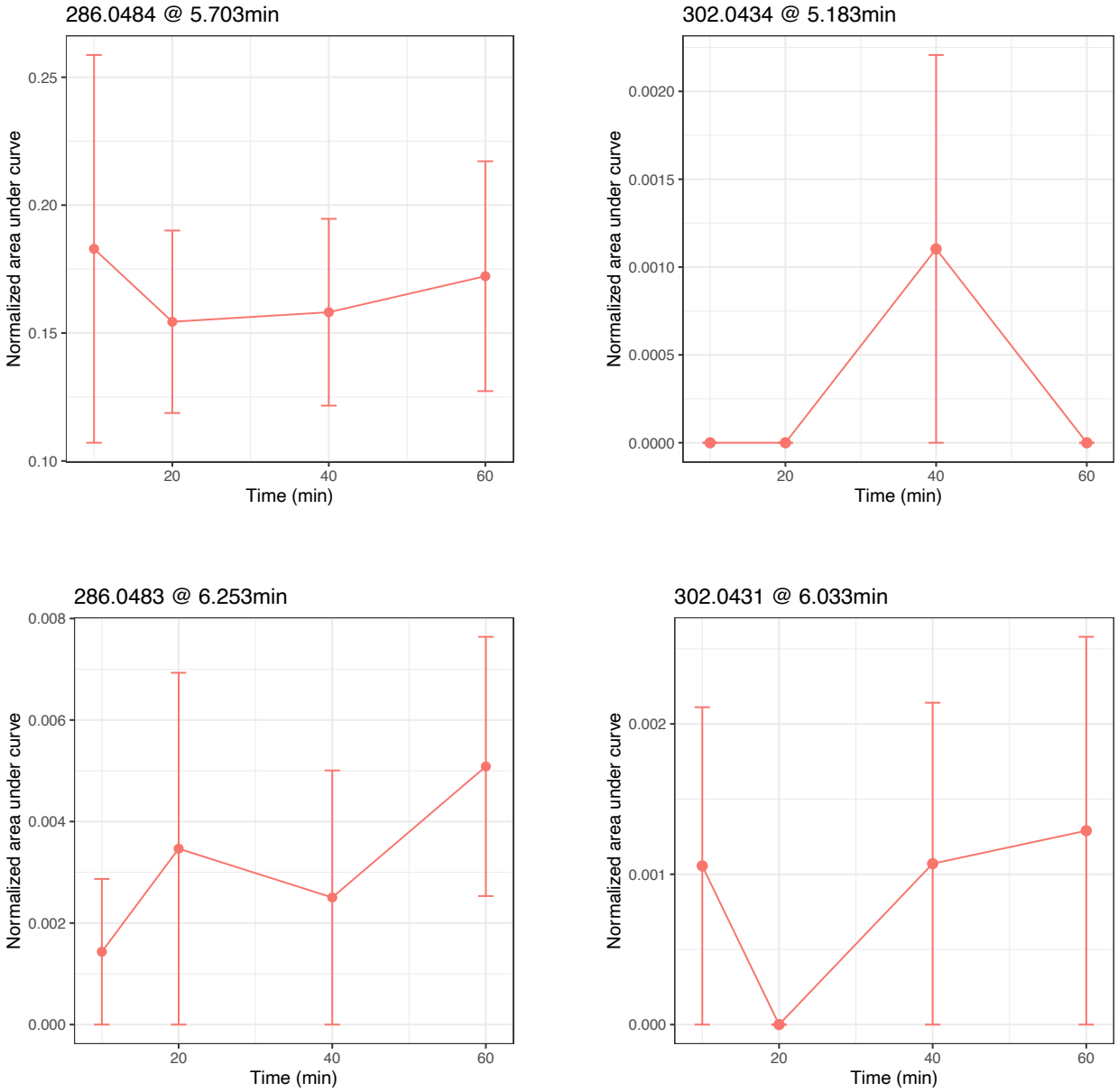


Figure 2: Biotransformation of emodin metabolites by *N. bryanti* liver CYPs over time. Mean ± SE.

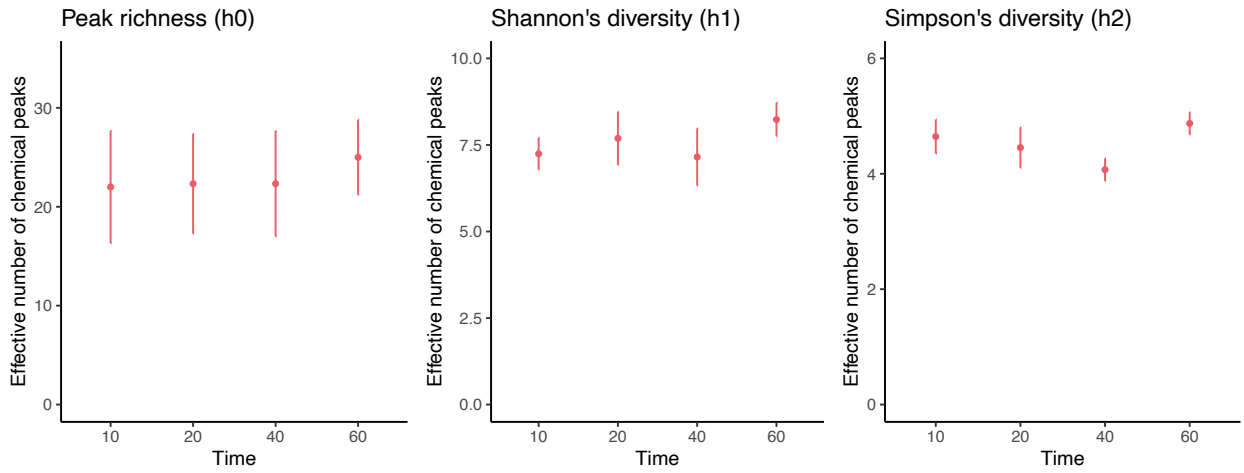


Figure 3: Hill numbers for CYP assay of *N. bryanti* on emodin assay at each time point. Mean ± SE.

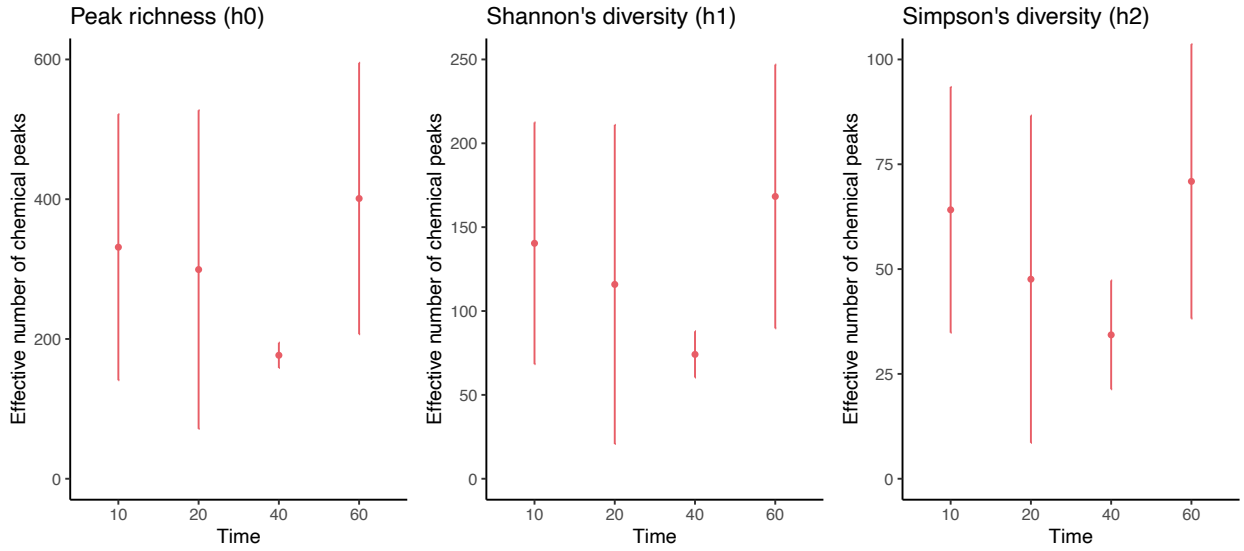


Figure 4: Hill numbers for CYP assay of *N. bryanti* on *Frangula* extract fraction assay at each time point. Mean ± SE.

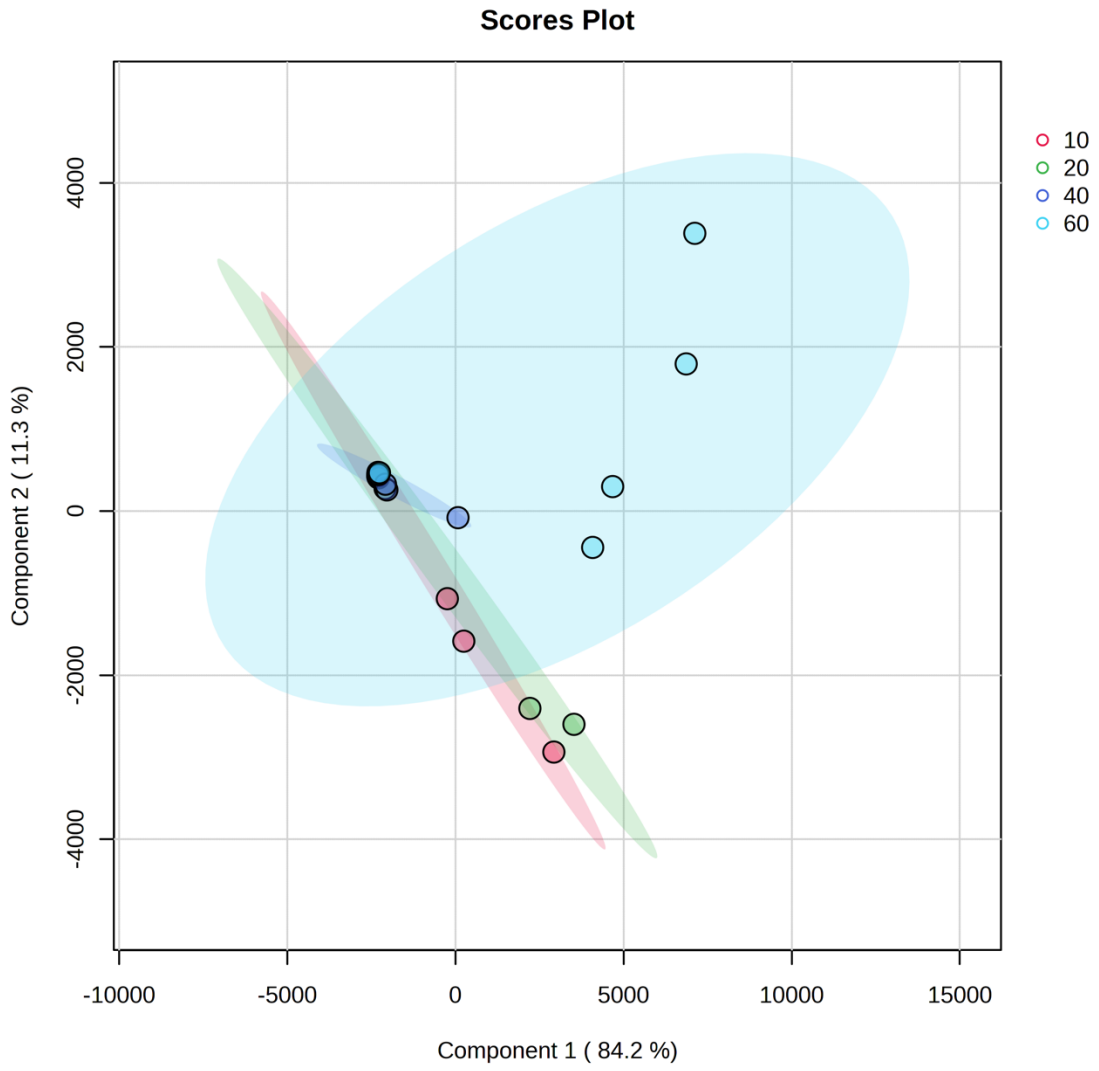


Figure 5: Partial least squares discriminant analysis for *Frangula* extract fraction CYP assay for *N. bryanti* over time.

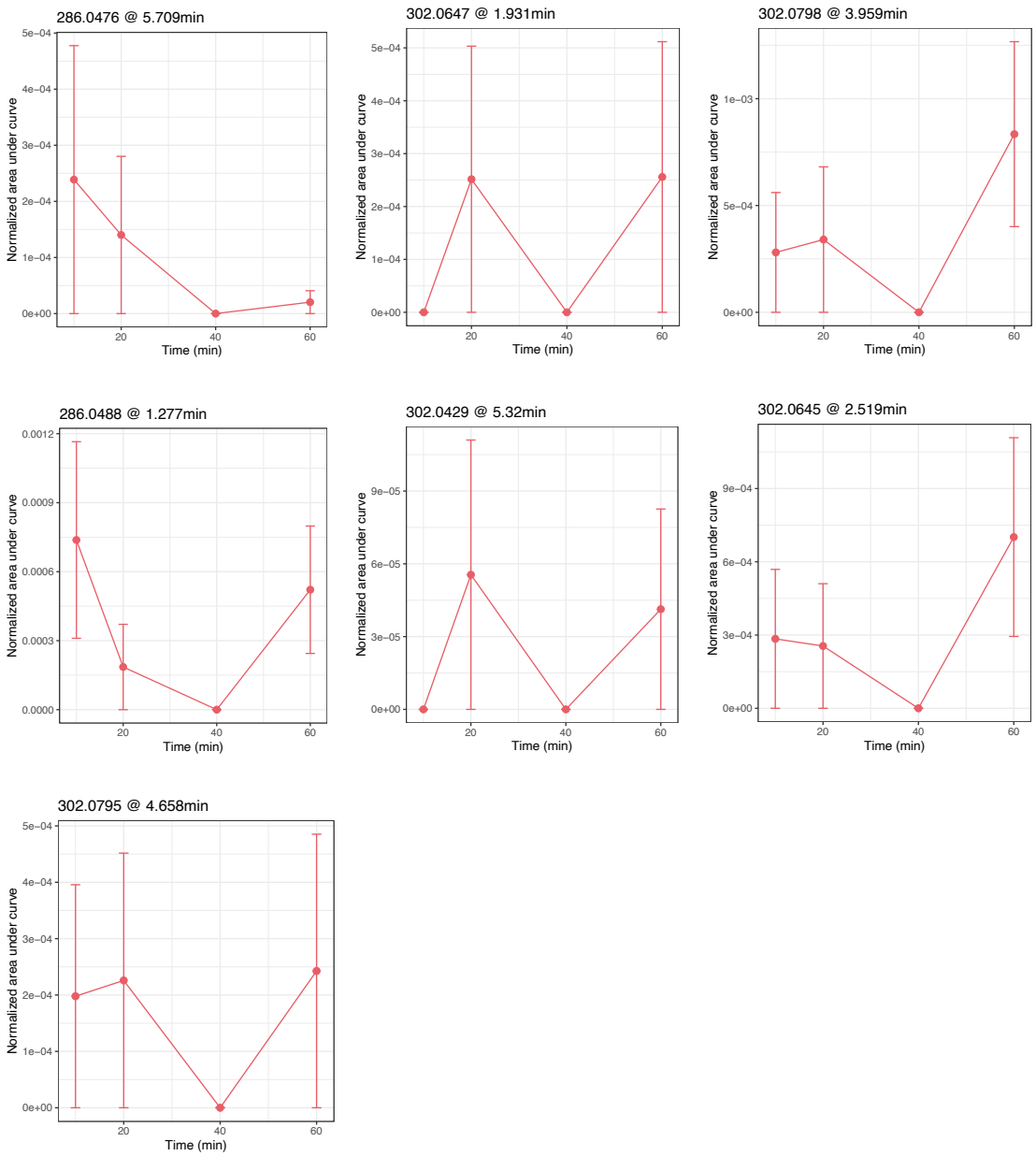


Figure 6: Biotransformation of known monohydroxylated and dihydroxylated emodin metabolite peaks from the *Frangula* extract fraction by *N. bryanti* liver CYPs over time. Mean ± SE.

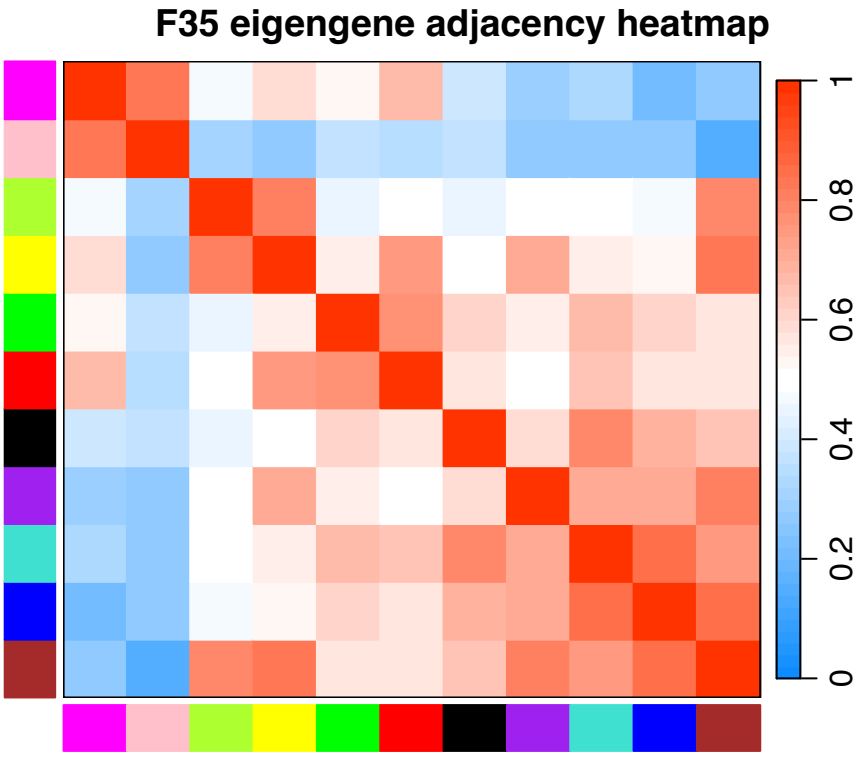


Figure 7: Eigengene adjacency heat map for *Frangula* WGCNA modules; module pairs with values closer to 1 have chemical peaks that often appear together, while module pairs with values closer to 0 have chemical peaks that do not often appear together.

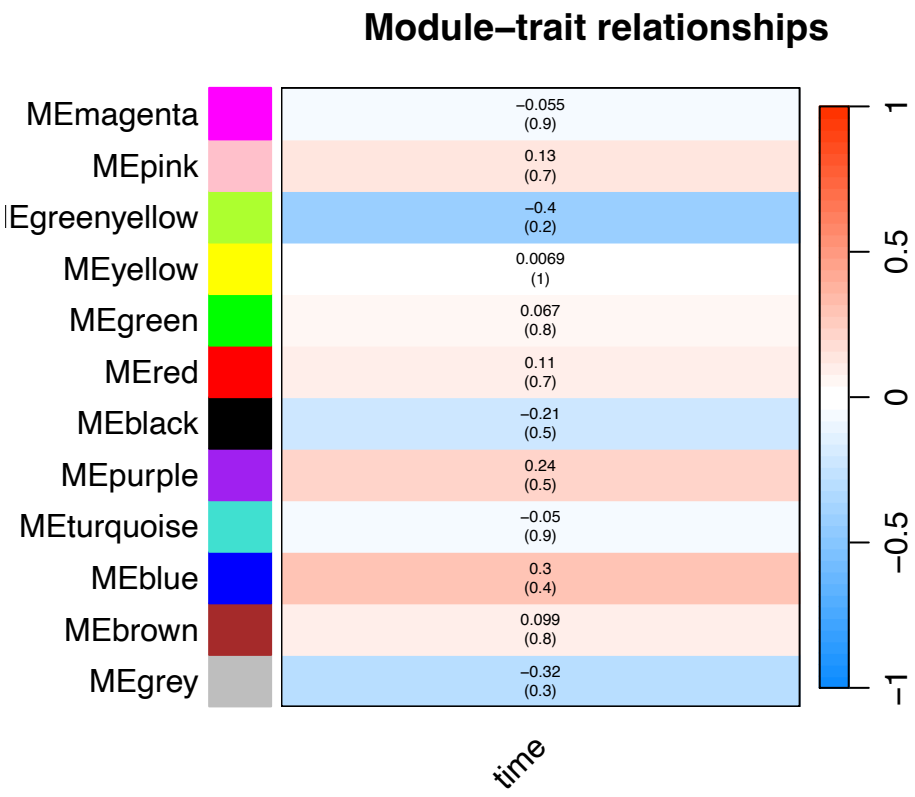


Figure 8: Module-trait relationships for *Frangula* WGCNA modules with respect to time; the modules with more positive (red) values contain chemical peaks that tend to increase over time, while modules with more negative (blue) values contain chemical peaks that tend to decrease over time.

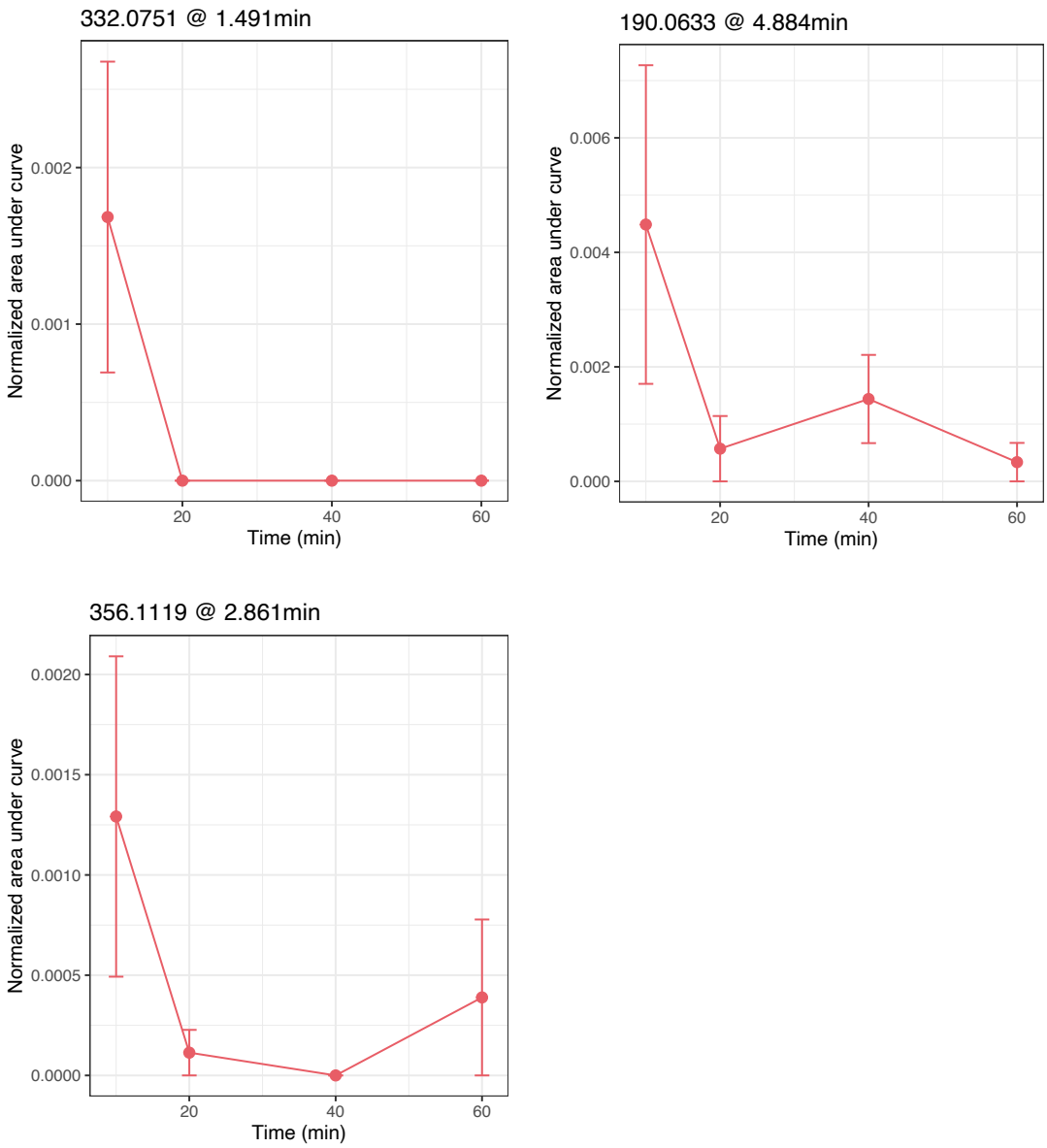


Figure 9: Biotransformation of selected peaks from the *Frangula* greenyellow WGCNA module by *N. bryanti* liver CYPs over time. Mean ± SE.

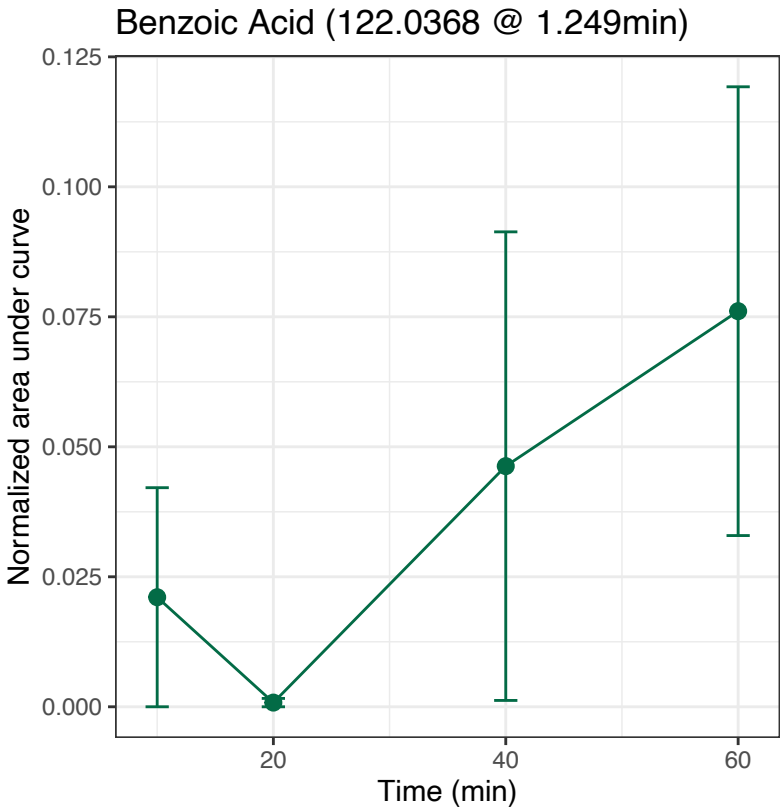


Figure 10: Biotransformation of benzaldehyde into benzoic acid by *N. lepida* liver CYPs over time. Mean ± SE.

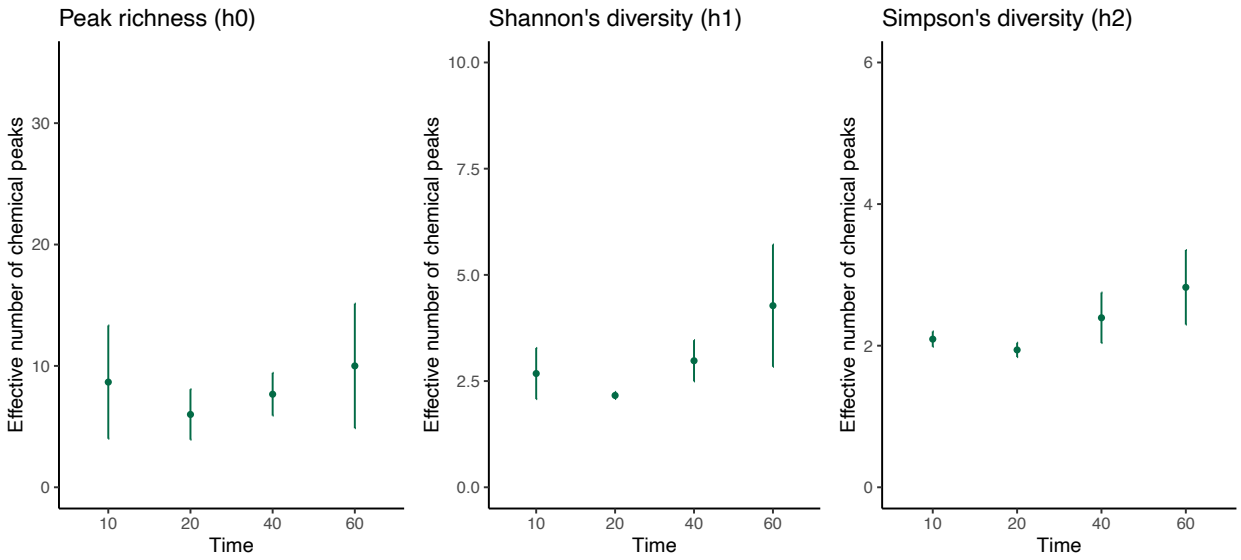


Figure 11: Hill numbers for CYP assay of *N. lepida* on benzaldehyde assay at each time point. Mean ± SE.

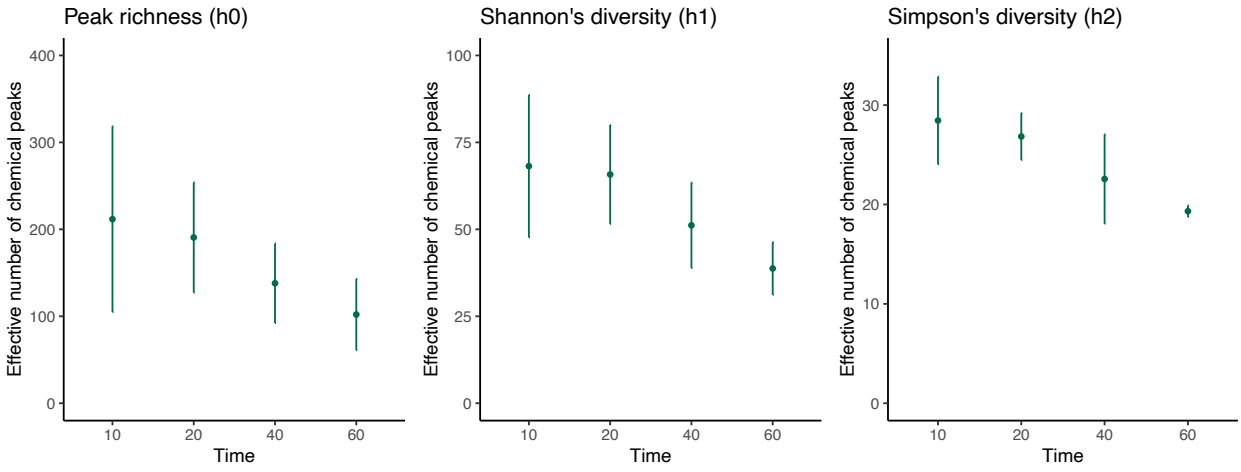


Figure 12: Hill numbers for CYP assay of *N. lepida* on *Prunus* extract fraction assay at each time point. Mean ± SE.

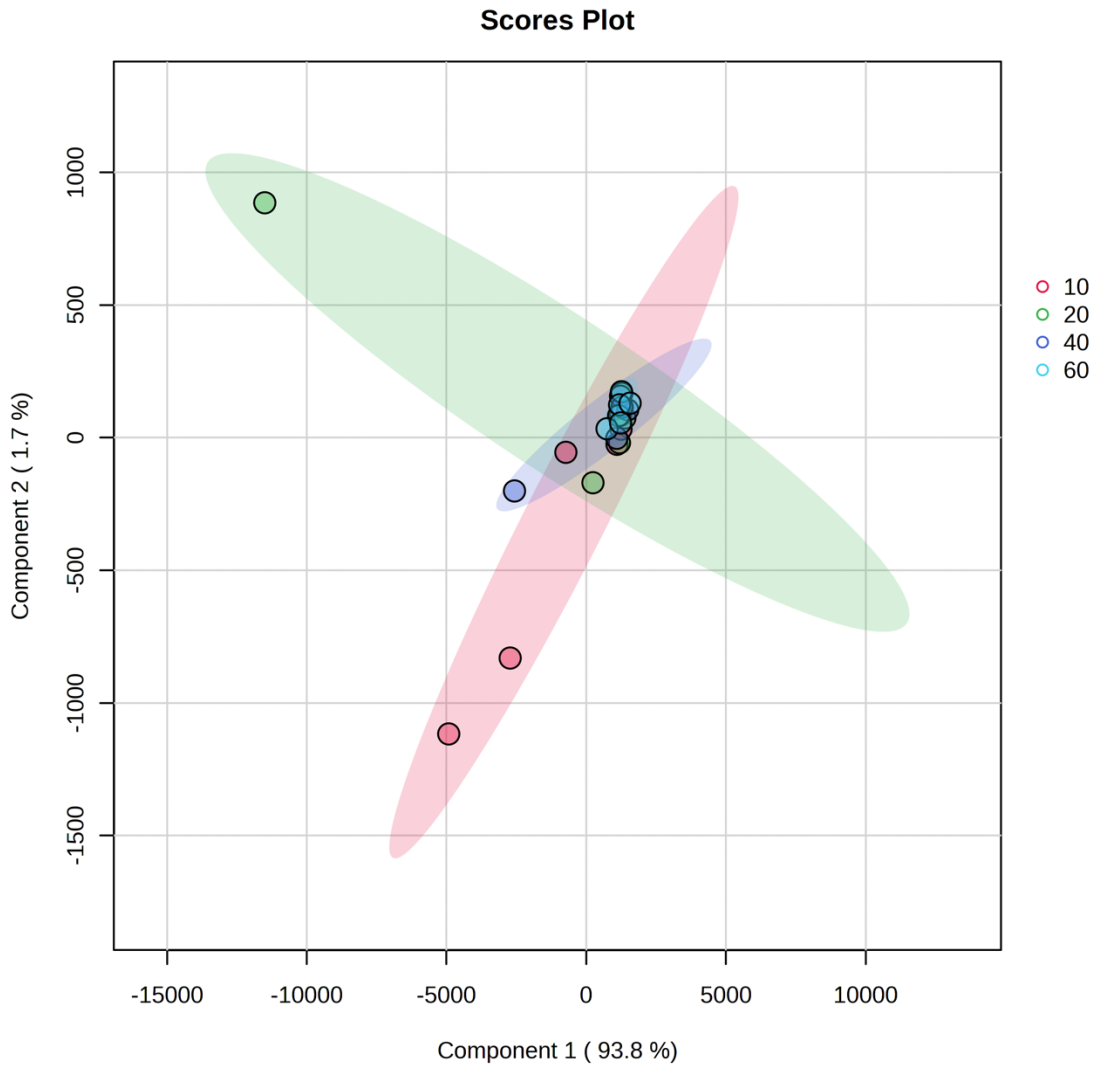


Figure 13: Partial least squares discriminant analysis for *Prunus* extract fraction CP assay for *N. lepida* over time.

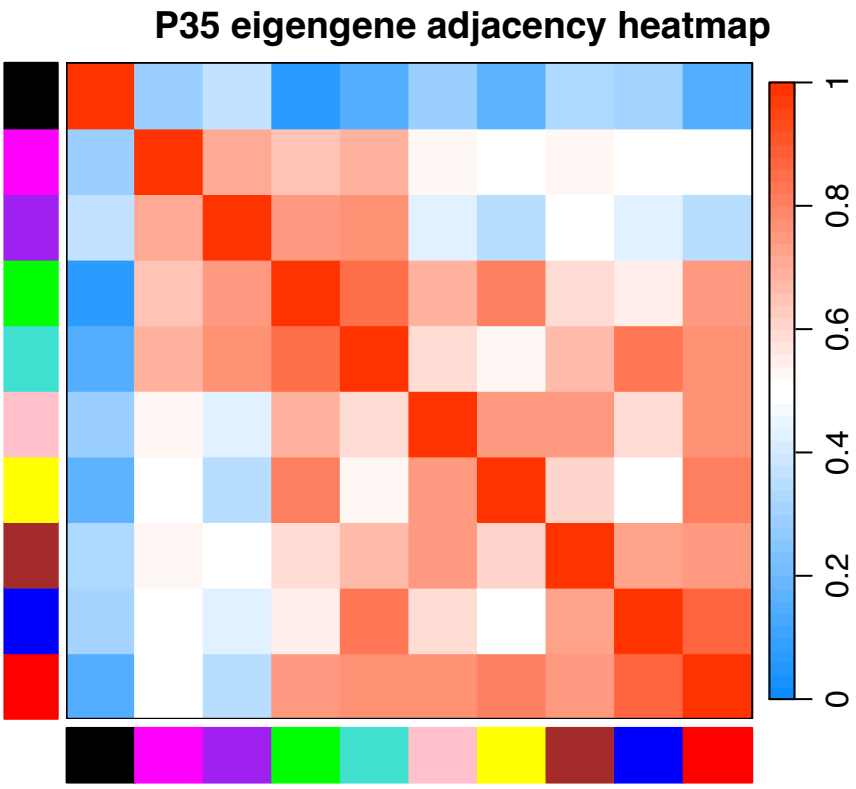


Figure 14: Eigengene adjacency heat map for *Prunus* WGCNA modules.

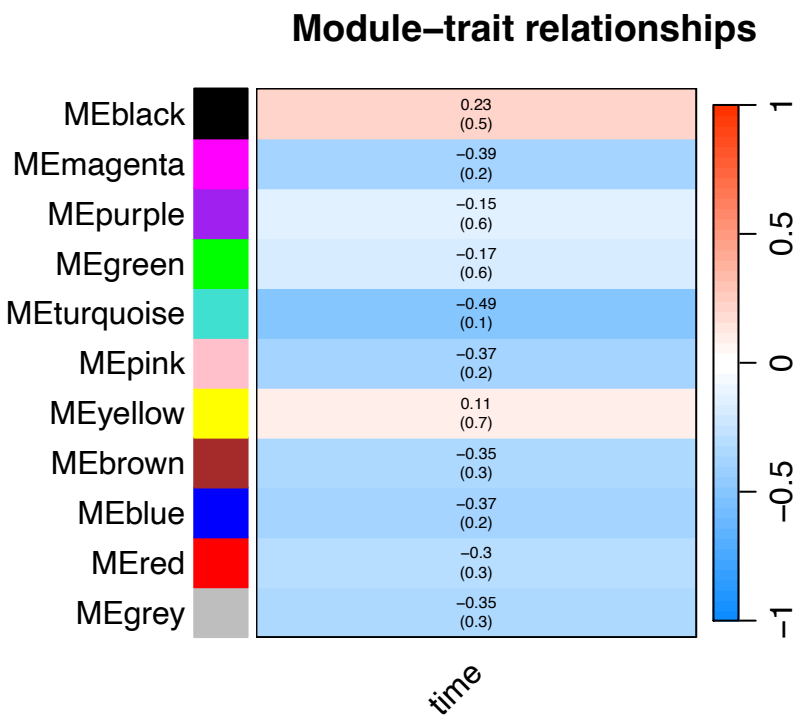


Figure 15: Module-trait relationships for *Prunus* WGCNA modules with respect to time.

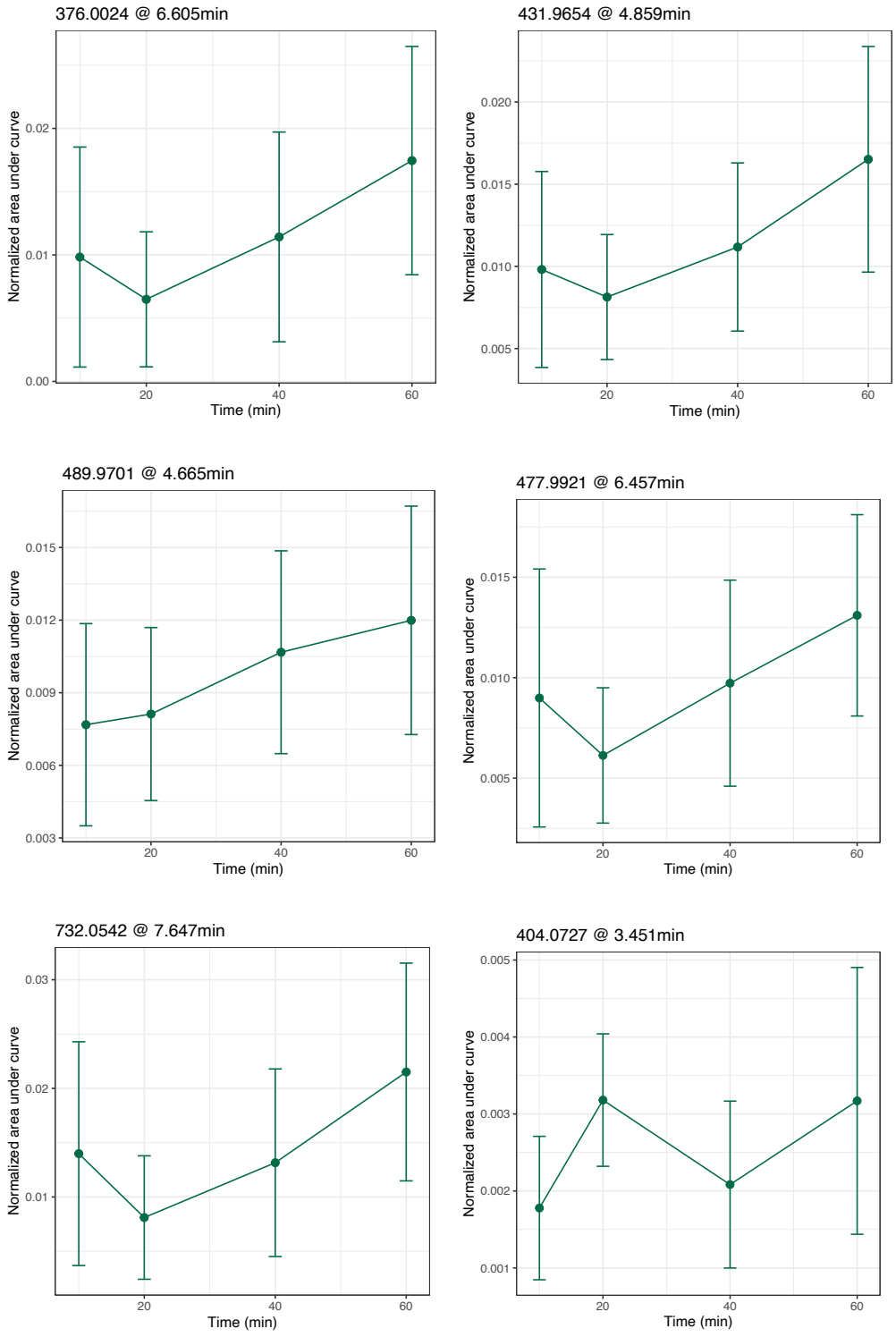


Figure 16: Biotransformation of selected peaks from the *Prunus* black WGCNA module by *N. lepida* liver CYPs over time. Mean ± SE.

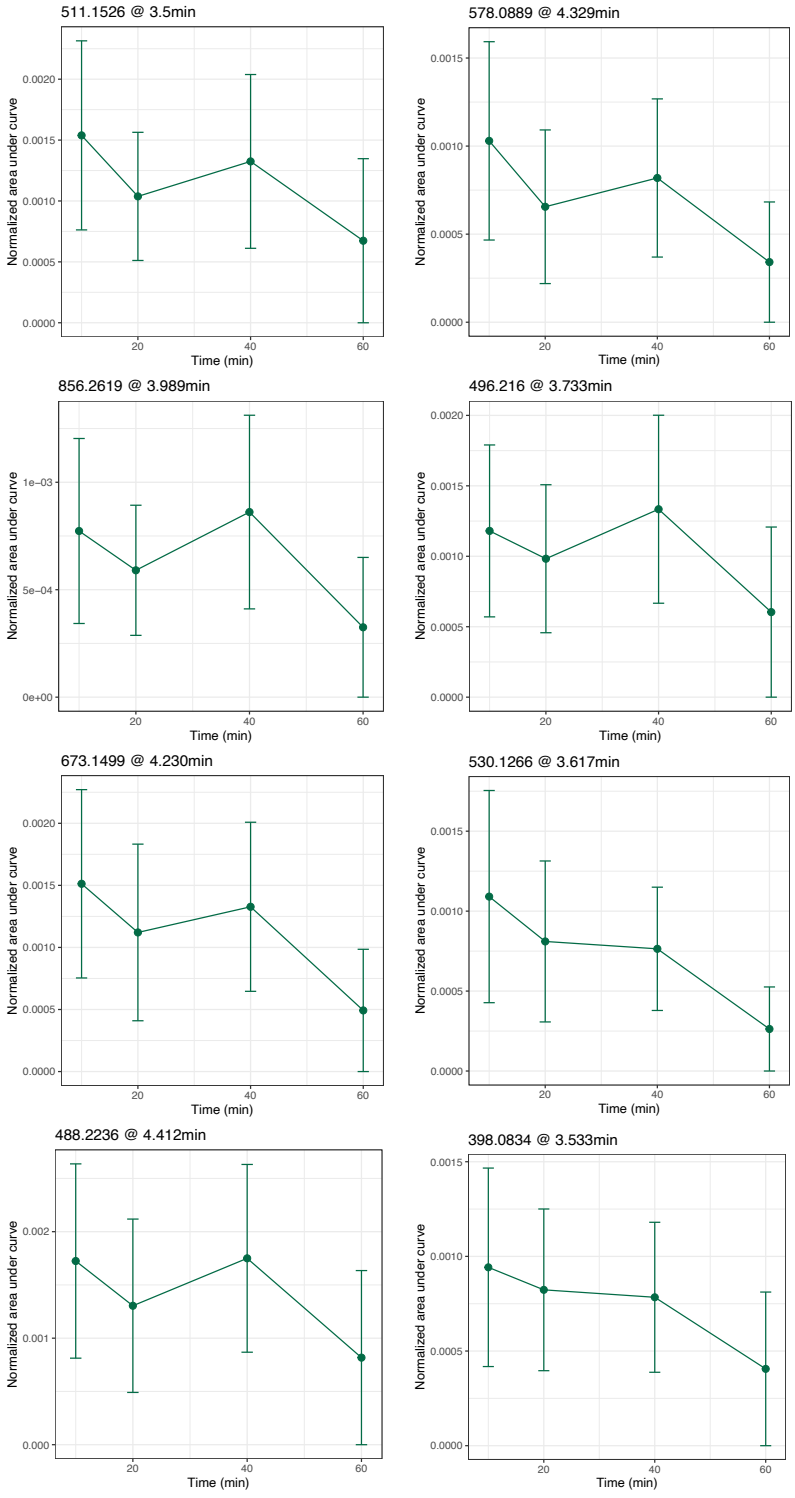


Figure 17: Biotransformation of selected peaks from the *Prunus* green WGCNA module by *N. lepida* liver CYPs over time. Mean ± SE.

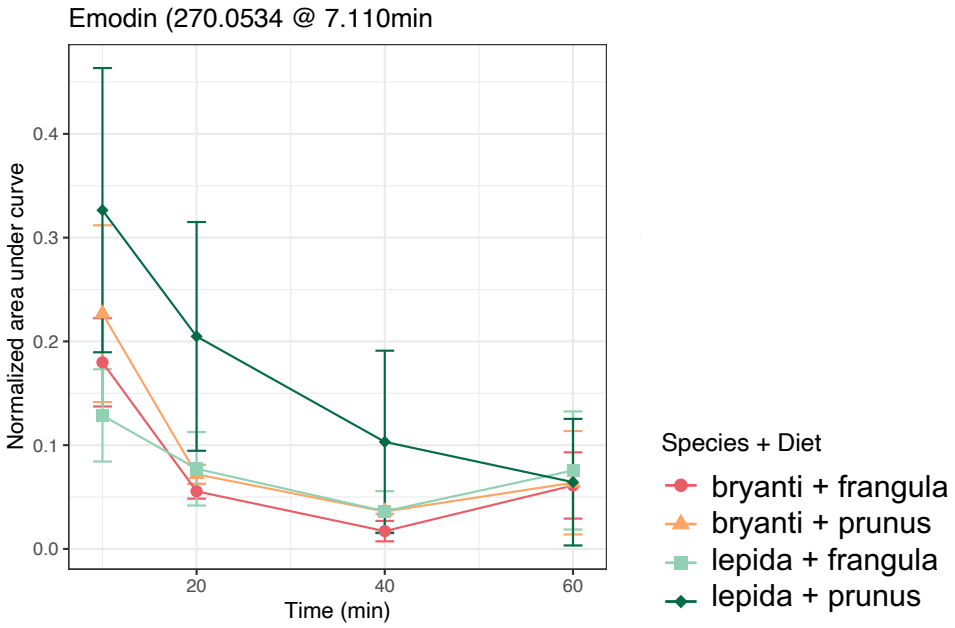


Figure 18: Biotransformation of emodin by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE.

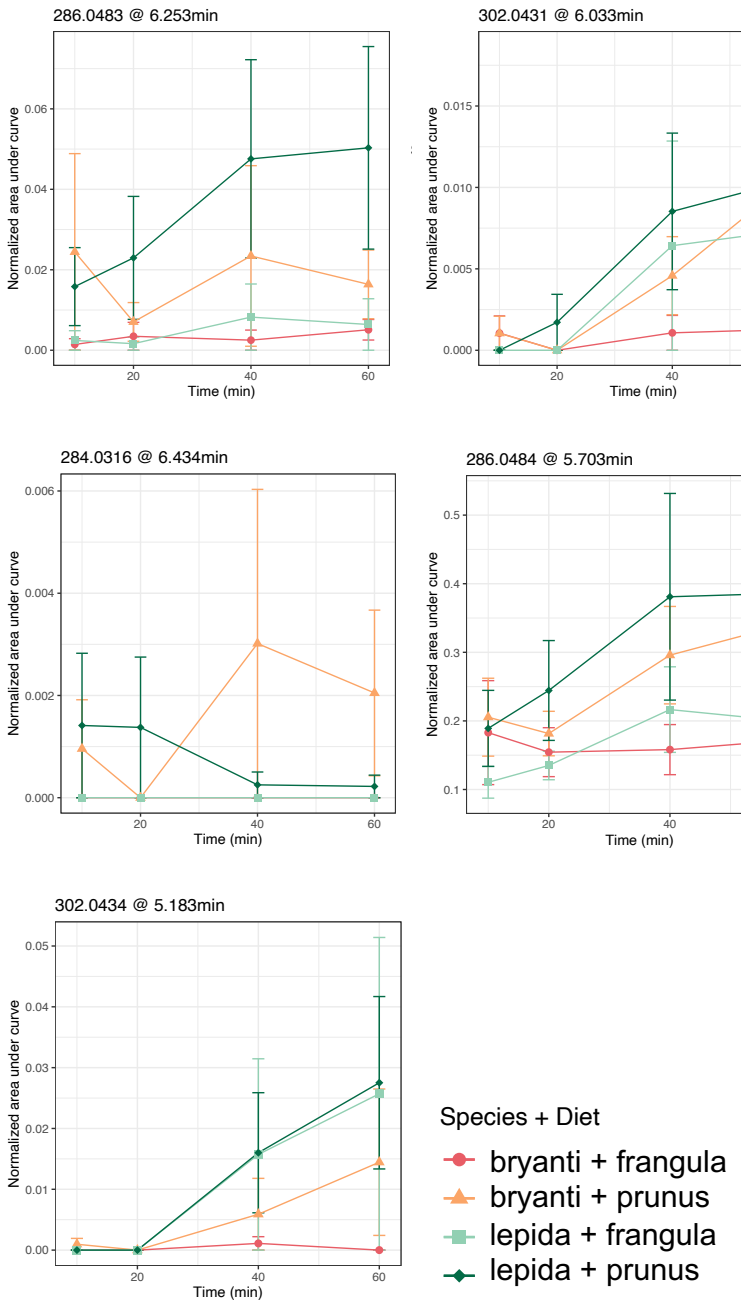


Figure 19: Biotransformation of emodin metabolites by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE.

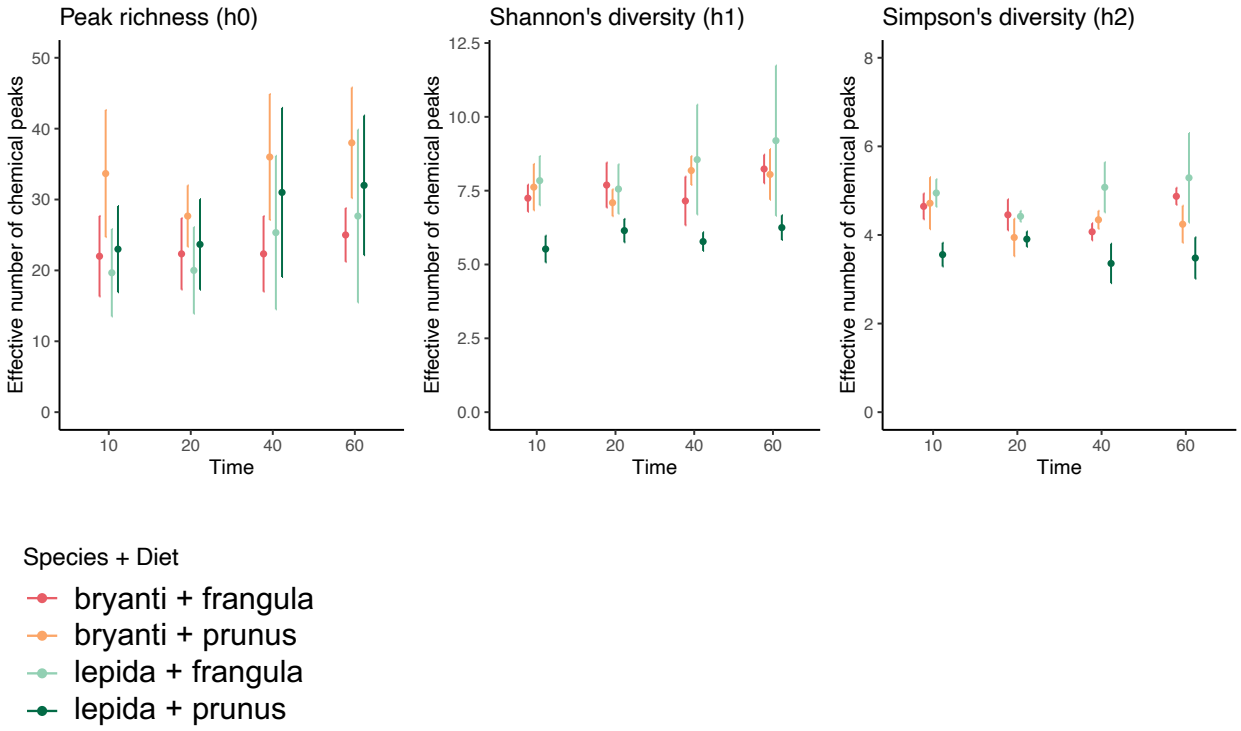


Figure 20: Hill numbers for CYP assay of *N. bryanti* and *N. lepida* on emodin CYP assay at each time point. Mean ± SE.

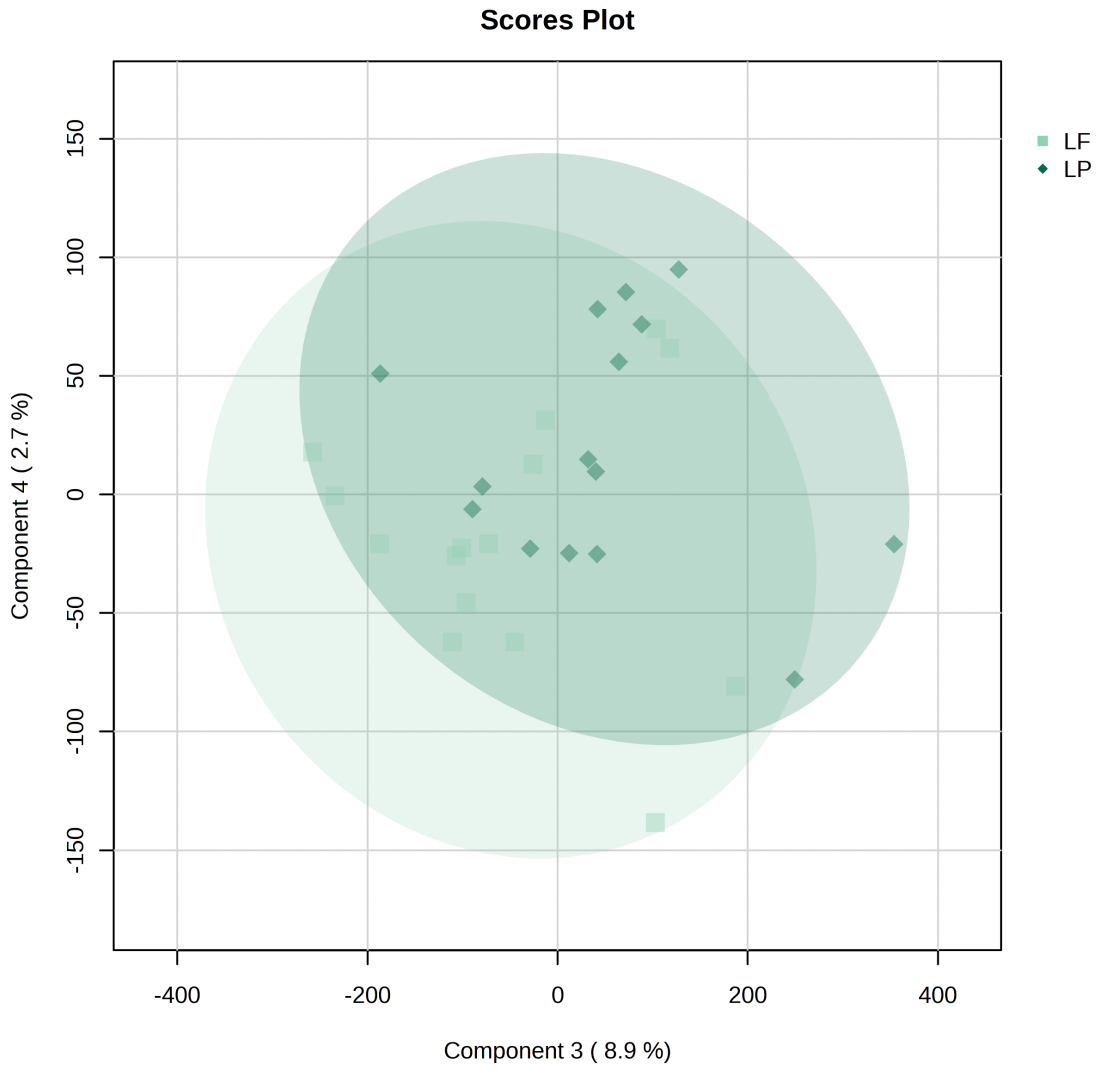


Figure 21: Partial least squares discriminant analysis for *Frangula* extract fraction for *N. lepida* including all time points of the CYP assay.

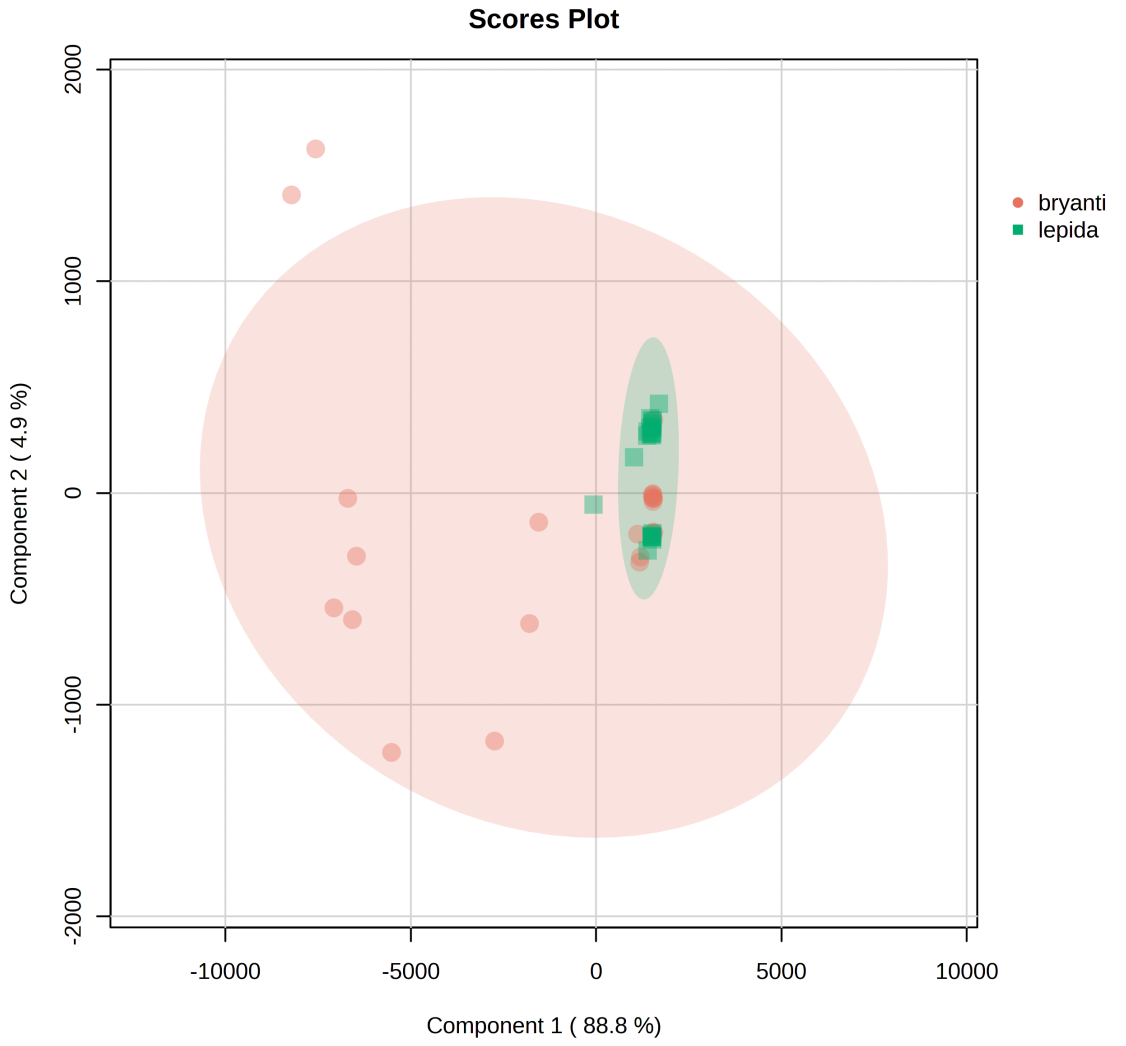


Figure 22: Partial least squares discriminant analysis for *Frangula* extract fraction for all woodrats including all time points of the CYP assay.

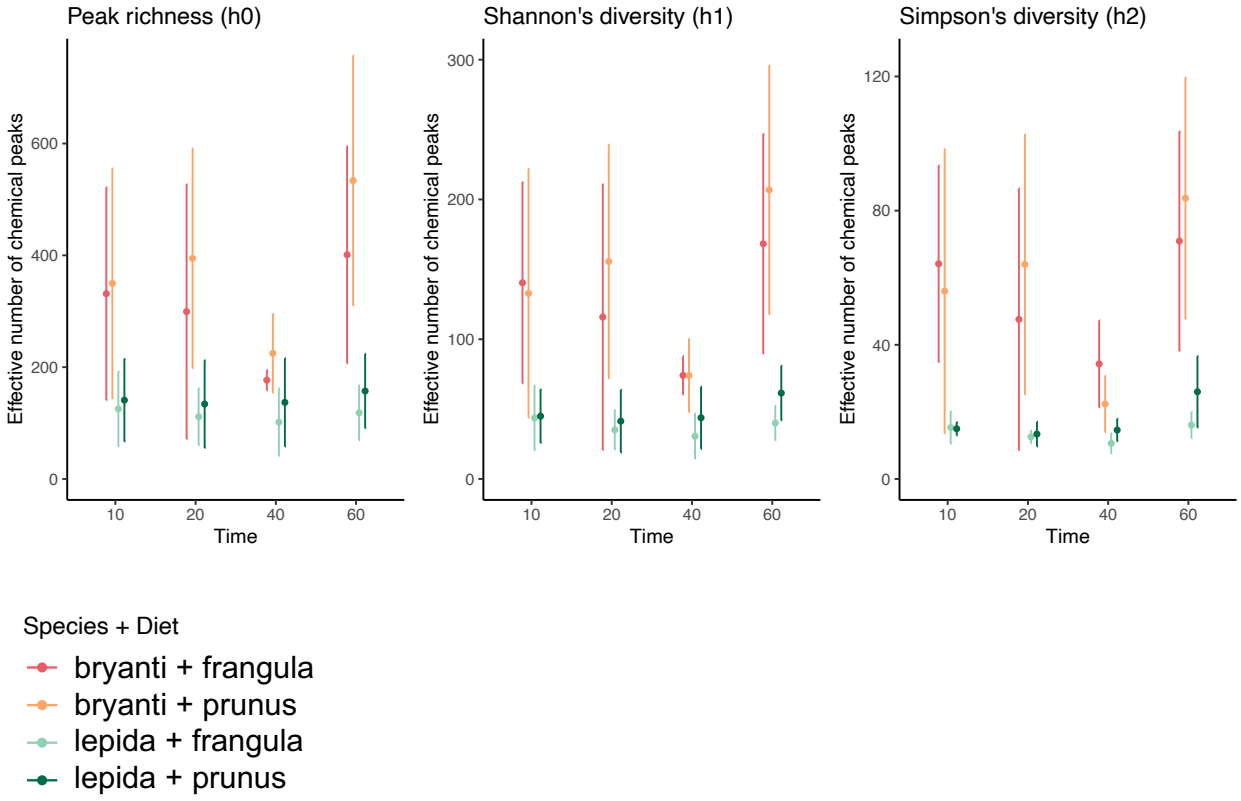


Figure 23: Hill numbers for CYP assay of *N. bryanti* and *N. lepida* on *Frangula* extract fraction CYP assay at each time point. Mean ± SE.

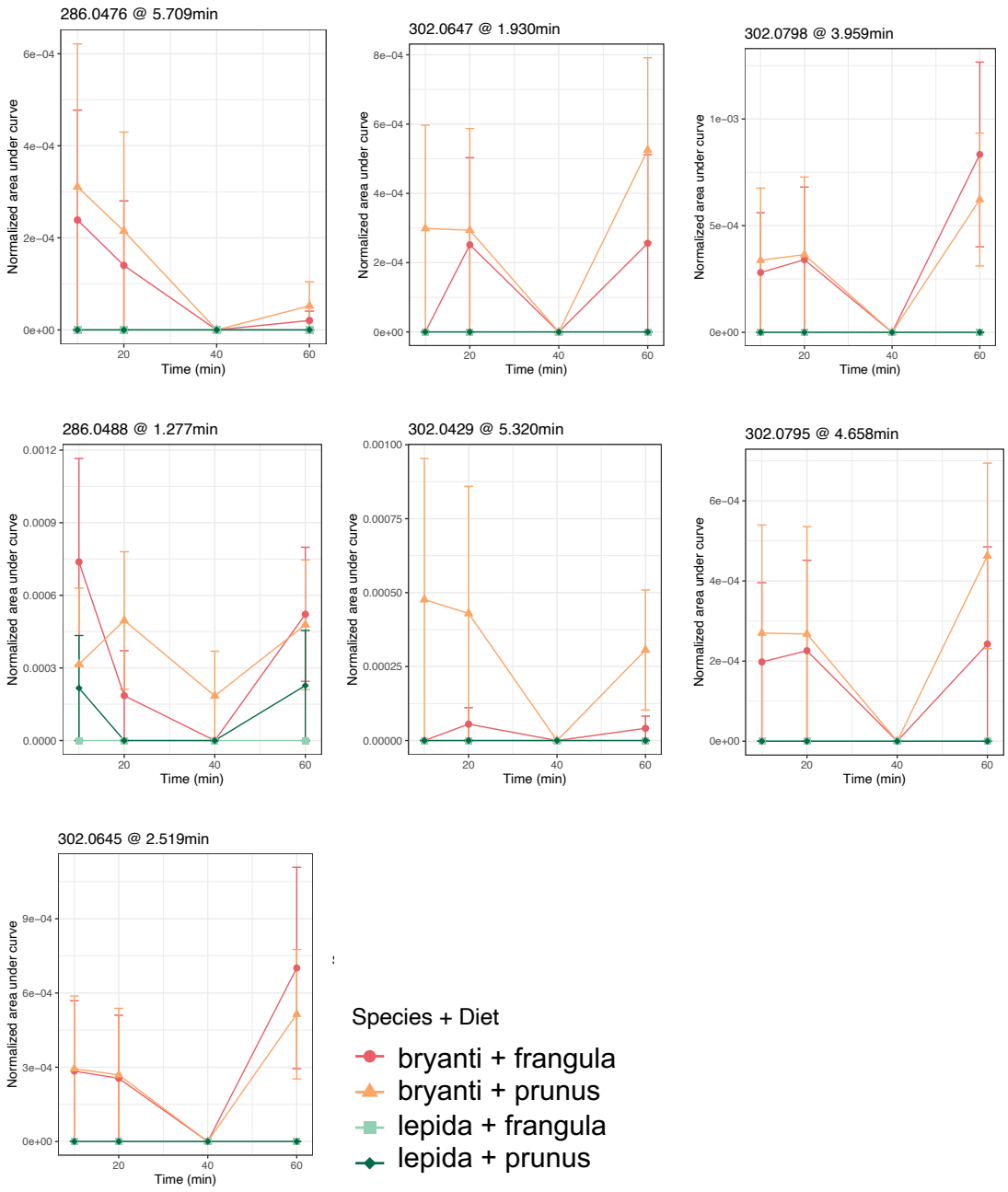


Figure 24: Biotransformation of known emodin metabolites by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE.

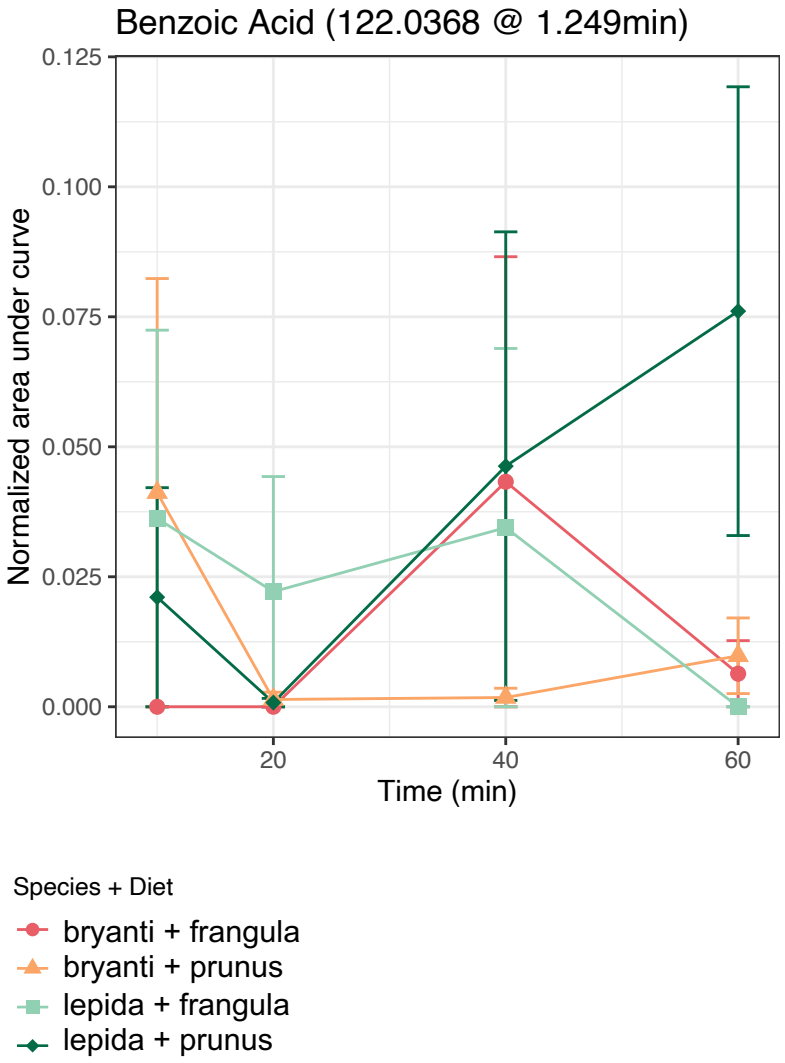


Figure 25: Biotransformation of benzaldehyde into benzoic acid by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE.

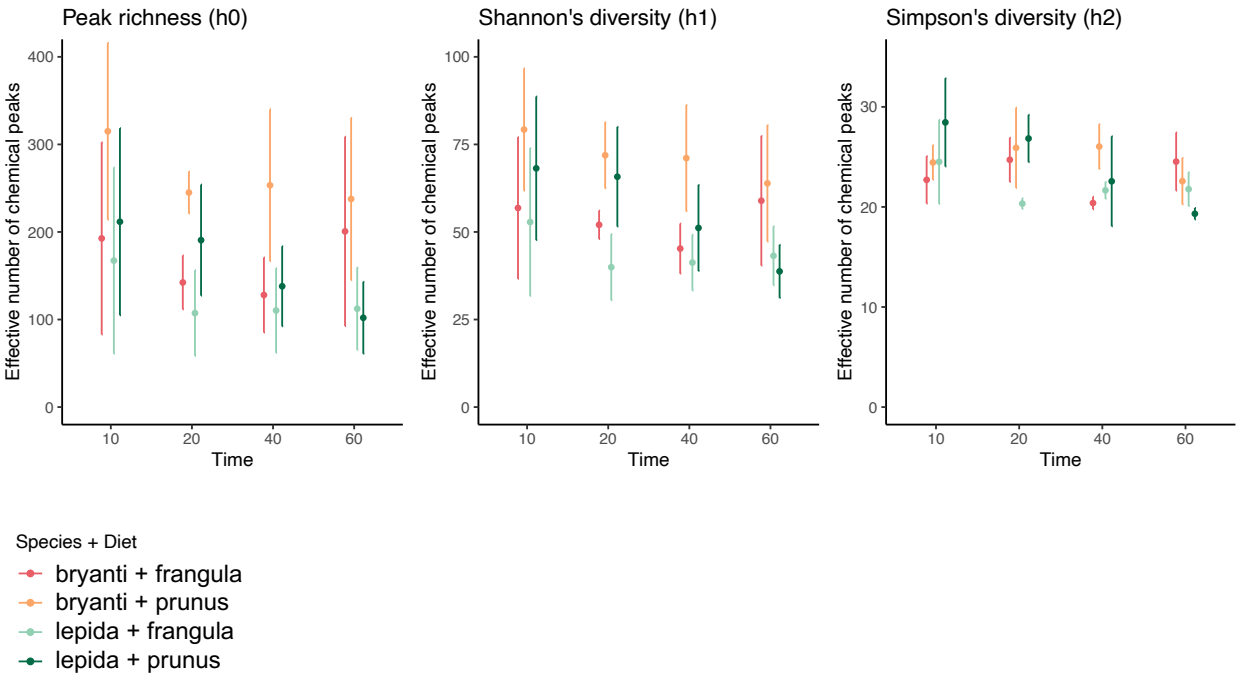


Figure 26: Hill numbers for CYP assay of *N. bryanti* on *Prunus* extract fraction at each time point. Mean ± SE.

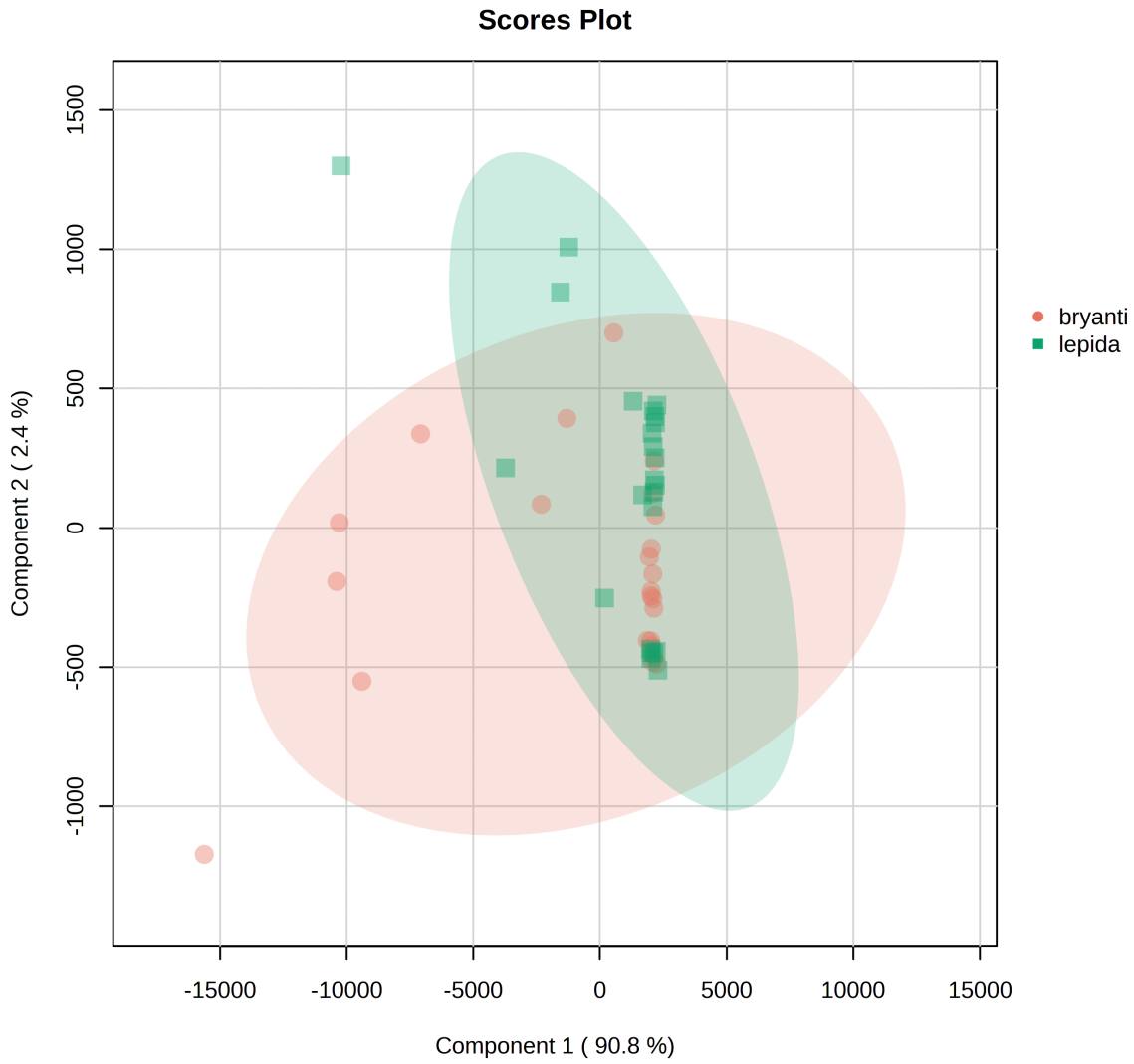


Figure 27: Partial least squares discriminant analysis for *Prunus* extract fraction for all woodrats including all time points of the CYP assay.

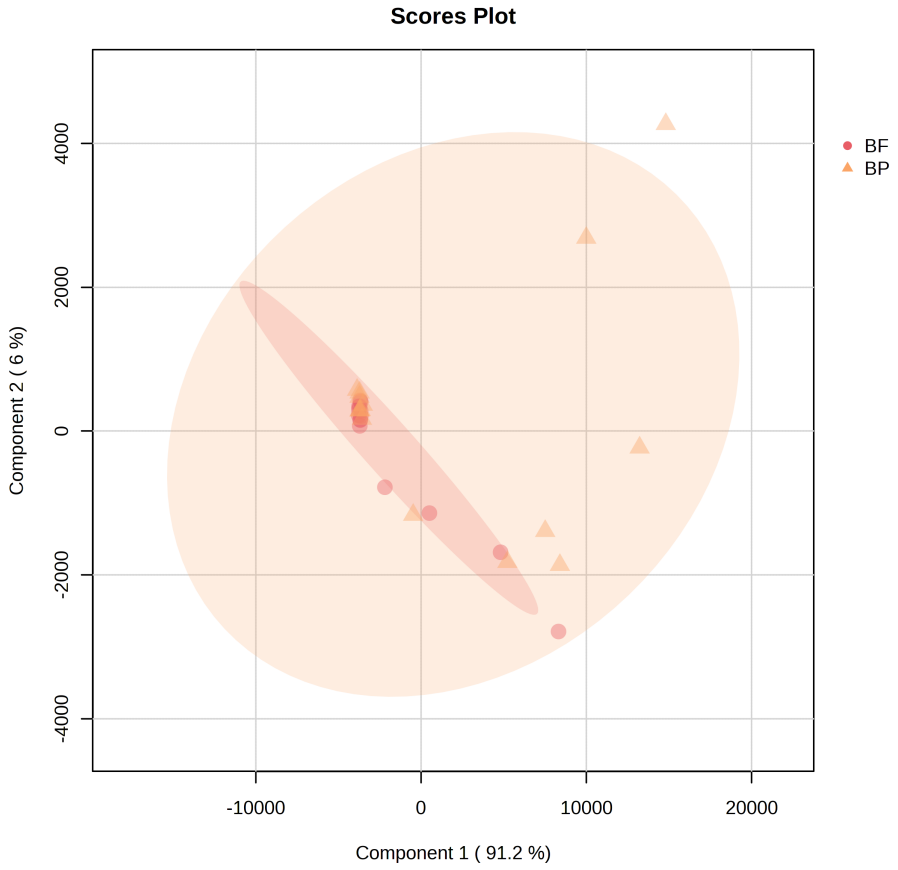


Figure 28: Partial least squares discriminant analysis for *Prunus* extract fraction for *N. bryanti* including all time points of the CYP assay.

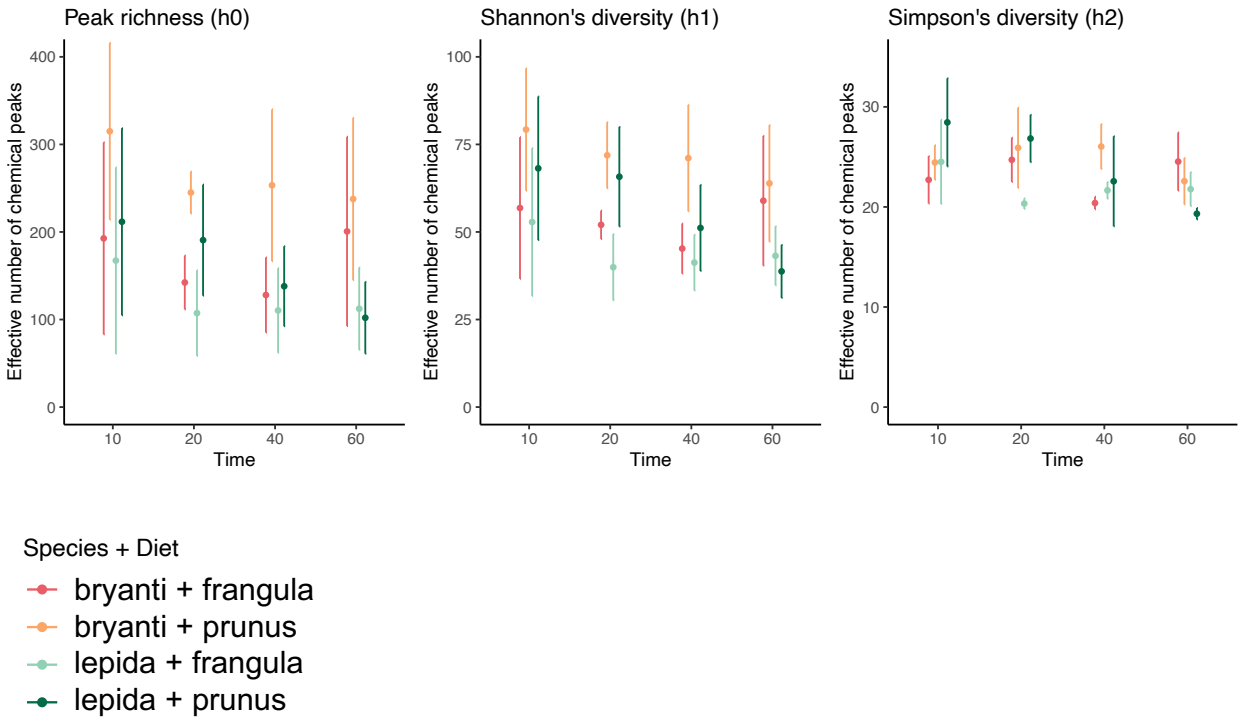


Figure 29: Hill numbers for CYP assay of *N. bryanti* on *Prunus* extract fraction at each time point. Mean ± SE.

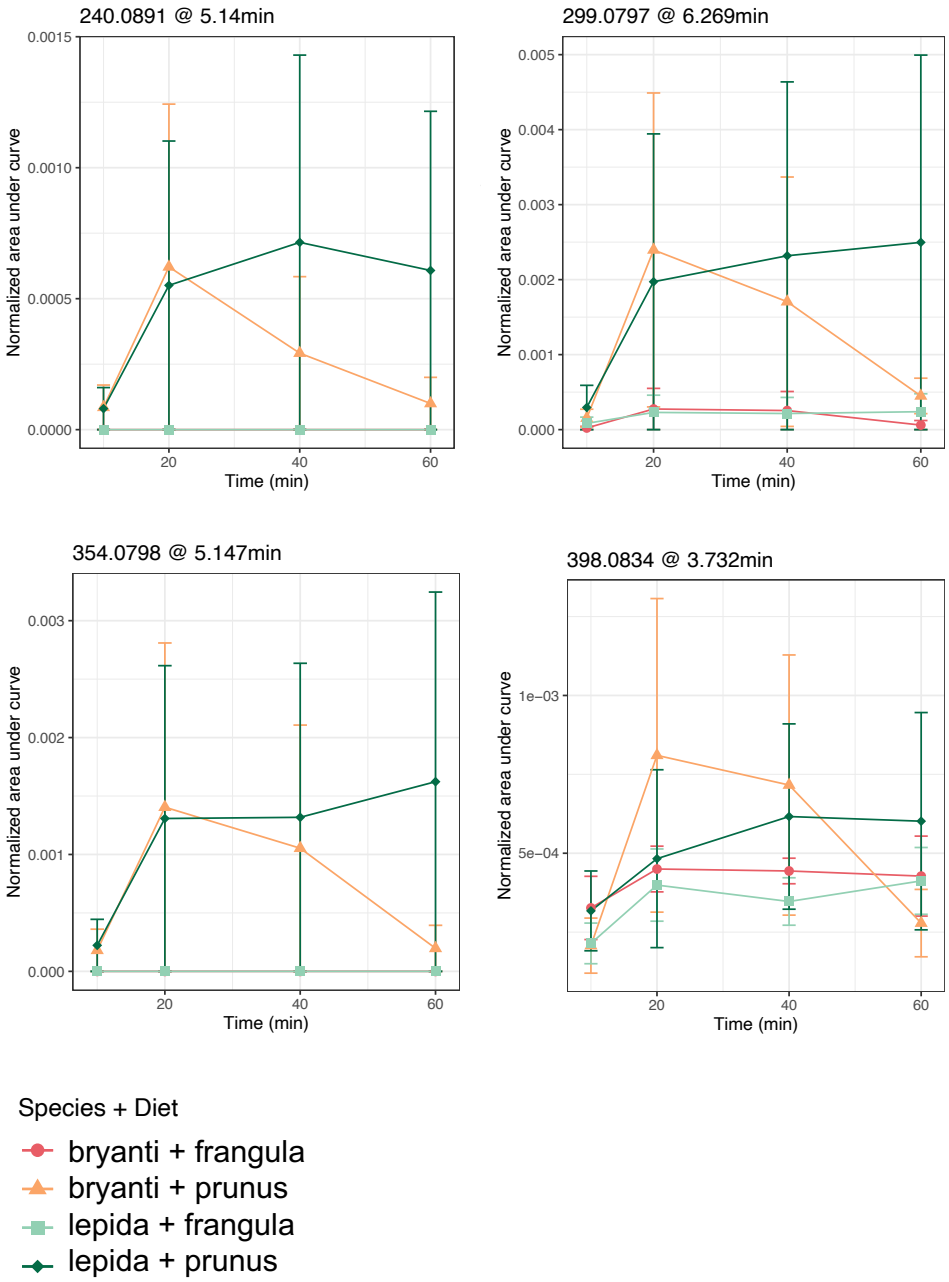


Figure 30: Biotransformation of selected peaks from the *Prunus* black WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found significant differences between diet treatments (F=5.27, p=0.0264).

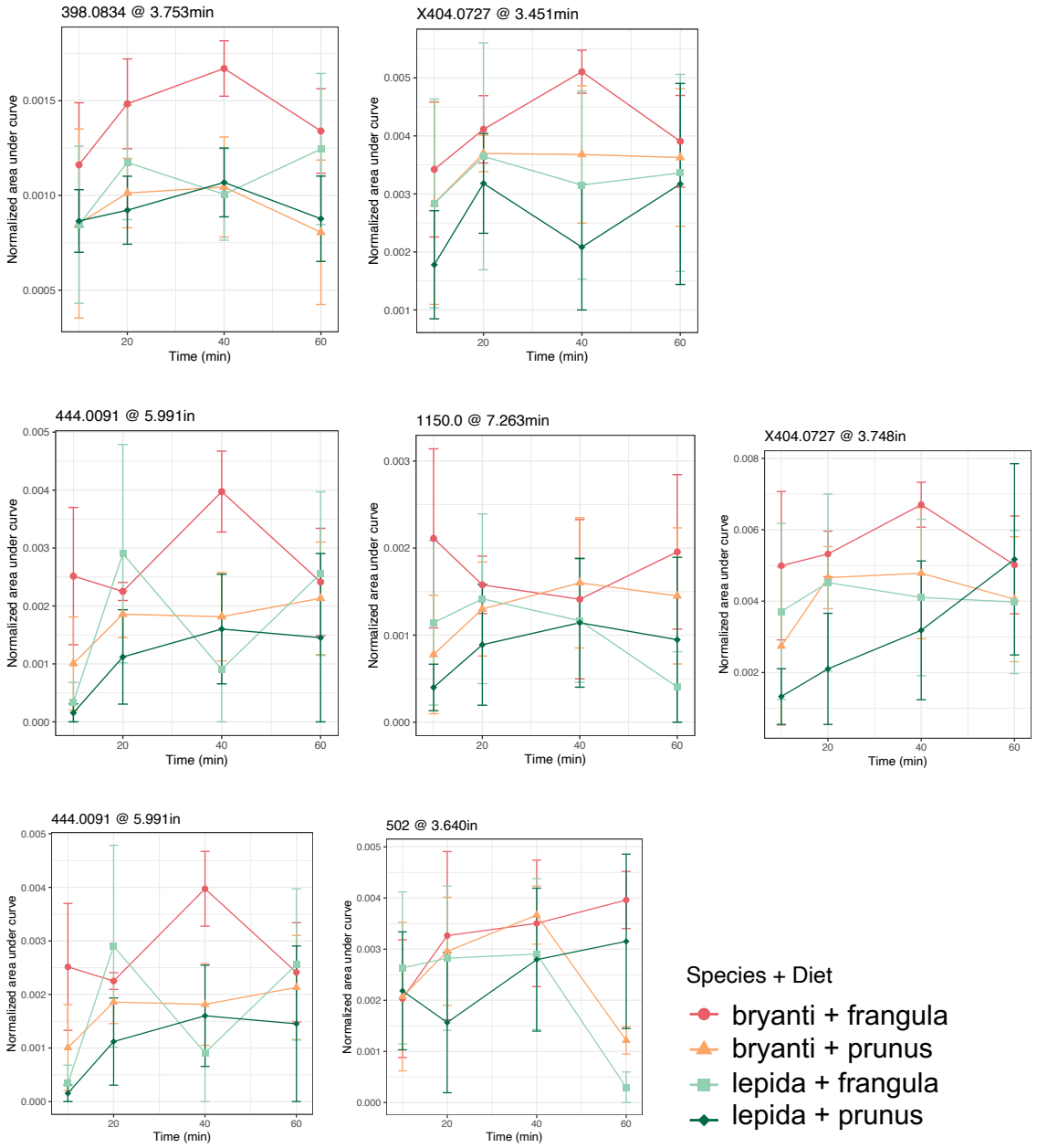


Figure 31: Biotransformation of selected peaks from the *Prunus* green WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found differences between species and diet (F=3.096, p=0.0853; F=2.729, p=0.1055).

SUPPLEMENTAL MATERIAL

Peak	Comp 1
286.215@7.4299946	0.47091
770.227@4.7149973	0.41937
770.2264@4.3999968	0.40195
756.2114@4.173002	0.34959
566.2951@4.3150015	0.27912
740.2163@4.3110023	0.24039
580.274@3.807002	0.19206
518.2377@3.674	0.18302
634.2821@4.3150015	0.16831
578.2577@3.674	0.16467
462.1389@3.8200018	0.16011
506.2741@4.2590013	0.15484
386.1225@3.0490017	0.1505

Peak	Comp 2
770.227@4.7149973	-0.32403
770.2264@4.3999968	-0.30648
756.2114@4.173002	-0.27655
566.2951@4.3150015	-0.23146
740.2163@4.3110023	-0.19095
634.2821@4.3150015	-0.16848
580.274@3.807002	-0.15572

Table S1: Selected PLS-DA loadings for *N. bryanti* CYP assay on *Frangula* extract over time.

Peak	Comp 1
386.1945@3.7200007	-0.68673
610.1546@4.231997	-0.466
610.1543@4.231997	-0.42302
500.1882@3.7200007	-0.16292
313.1171@2.3590007	-0.14794

Peak	Comp 2
500.1882@3.7200007	-0.53912
484.1927@4.427001	-0.30447
484.1935@4.388	-0.24211
313.116@1.804	-0.23714
449.1907@3.7200007	-0.23175
402.1531@3.6139994	-0.21197
610.1546@4.231997	-0.20482
678.1407@4.233001	-0.15741
409.0992@3.7050004	-0.15103

Peak	Comp 2
313.1171@2.3590007	0.40099
610.1543@4.231997	0.29267
386.1945@3.7200007	0.20802
448.1606@3.4989965	0.19013

Table S2: Selected PLS-DA loadings for *N. lepida* CYP assay on *Prunus* extract over time.

Peak	Comp 1
770.2264@4.3999968	0.5033
756.2114@4.173002	0.48665
740.2163@4.3110023	0.36861
770.227@4.7149973	0.31013
634.2821@4.3150015	0.29019
838.2144@4.3989987	0.27598
286.215@7.4299946	0.19674
566.2951@4.3150015	0.17708
620.2665@4.257001	0.15404

Peak	Comp 2
245.9931@4.1359987	-1.4818
770.2264@4.3999968	-0.26583
489.9701@4.665	-0.24515
756.2114@4.173002	-0.21072
497.9953@7.437005	-0.20756
740.2163@4.3110023	-0.1949
838.2144@4.3989987	-0.19322
491.988@5.5460014	-0.16685
439.9736@4.196002	-0.15575

Table S3: Selected PLS-DA loadings for CYP assay for *N. lepida* on *Frangula* extract over time.

Peak	Comp 1
286.215@7.4299946	0.83579
770.227@4.7149973	0.79965
770.2264@4.3999968	0.7369
756.2114@4.173002	0.64349
566.2951@4.3150015	0.54788
740.2163@4.3110023	0.44261
580.274@3.807002	0.38404
518.2377@3.674	0.35507
578.2577@3.674	0.3155
634.2821@4.3150015	0.31241
462.1389@3.8200018	0.30781
506.2741@4.2590013	0.3071
386.1225@3.0490017	0.28772
568.2301@3.672999	0.26941
650.3146@3.985999	0.26104
556.2656@4.3150015	0.25783
784.2433@4.938998	0.2476
402.1532@3.5850008	0.2348
552.2785@3.446999	0.22514
466.1121@1.236	0.21068
534.2683@3.8029997	0.20587

Peak	Comp 2
770.2264@4.3999968	-0.46602
756.2114@4.173002	-0.39169
770.227@4.7149973	-0.38378
740.2163@4.3110023	-0.27701
634.2821@4.3150015	-0.26695
286.215@7.4299946	-0.18082
566.2951@4.3150015	-0.1477
838.2144@4.3989987	-0.13066
620.2665@4.257001	-0.12863
386.1225@3.0490017	-0.11982
784.2433@4.938998	-0.11368
576.1305@3.8229985	-0.10267

Table S4: Selected PLS-DA loadings for CYP assay on all woodrats on *Frangula* extract over time.

Peak	Comp 1
386.1945@3.7200007	-0.81011
610.1546@4.231997	-0.42769
610.1543@4.231997	-0.27482
313.1171@2.3590007	-0.15467

Peak	Comp 2
386.1945@3.7200007	-0.39464
172.0197@2.042	-0.1544
416.2056@4.3900027	-0.12035

Peak	Comp 2
610.1543@4.231997	1.1597
610.1546@4.231997	0.51039
500.1882@3.7200007	0.45439
245.9932@4.1289988	0.31746
678.1407@4.233001	0.3052
448.1606@3.4989965	0.23944
732.0512@7.6449986	0.16737
732.0542@7.6469994	0.15425

Table S5: Selected PLS-DA loadings for CYP assay on all woodrats on *Prunus* extract over time.

Peak	Comp 1
386.1945@3.7200007	0.80368
610.1546@4.231997	0.40261
610.1543@4.231997	0.2227
313.1171@2.3590007	0.15092

Peak	Comp 2
610.1543@4.231997	-1.0759
448.1606@3.4989965	-0.14006
500.1882@3.7200007	-0.1209

Peak	Comp 2
313.1171@2.3590007	0.216
386.1945@3.7200007	0.17342

Table S6: Selected PLS-DA loadings for CYP assay on *N. bryanti* on *Prunus* extract over time.

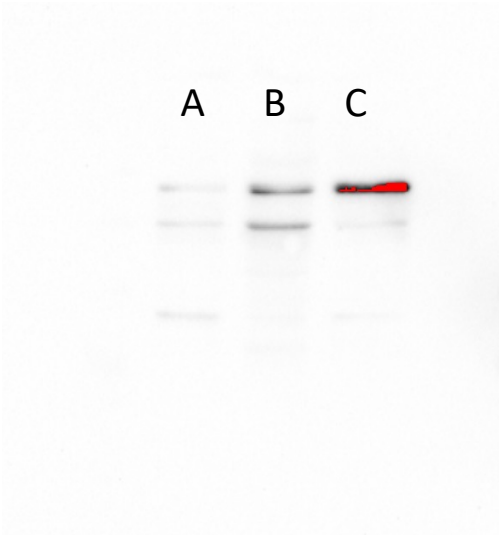


Figure S1: Example western blot for CYP verification; column A refers to the liver homogenate, column B is the S9 fraction (supernatant in first centrifugation), and column C is the final microsomes. The highest row is CYP antibodies, while the row below is VDAC, showing a higher concentration of CYPs throughout the microsomal isolation process.

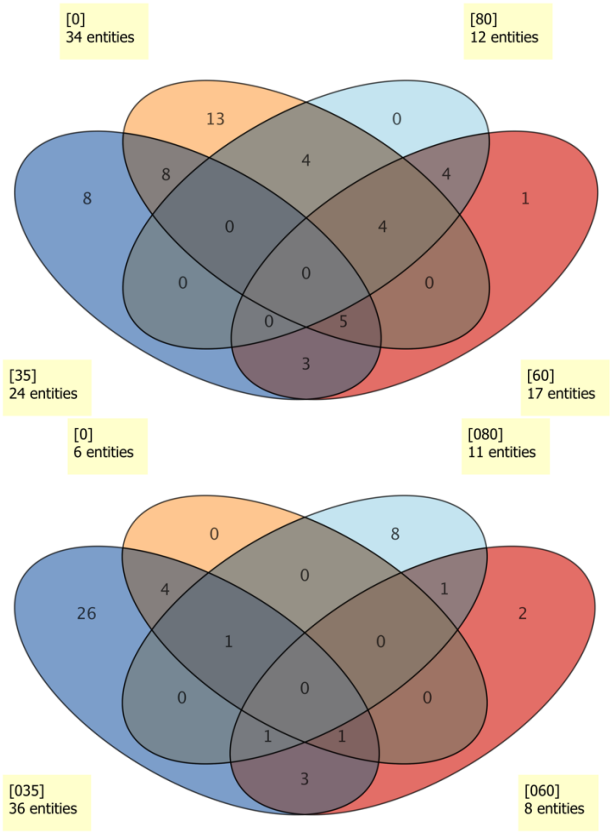


Figure S2: Venn Diagrams showing the number of unique entities in the original frangula (left) and prunus (right) extract fractions; both of the 35% fractions showed high numbers of unique entities. While F0 had more unique entities, we decided on the *Frangula* fraction for our CYP assays due to the presence of emodin.

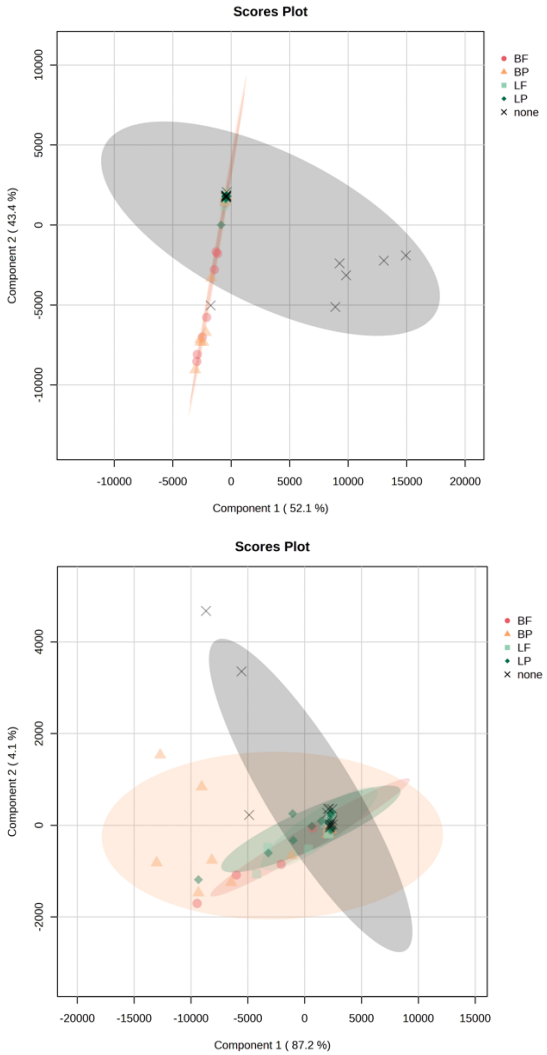


Figure S3: Partial least squares discriminant analyses for *Frangula* (left) and *Prunus* (right) extract fractions for all time points including lab rats in addition to woodrats.

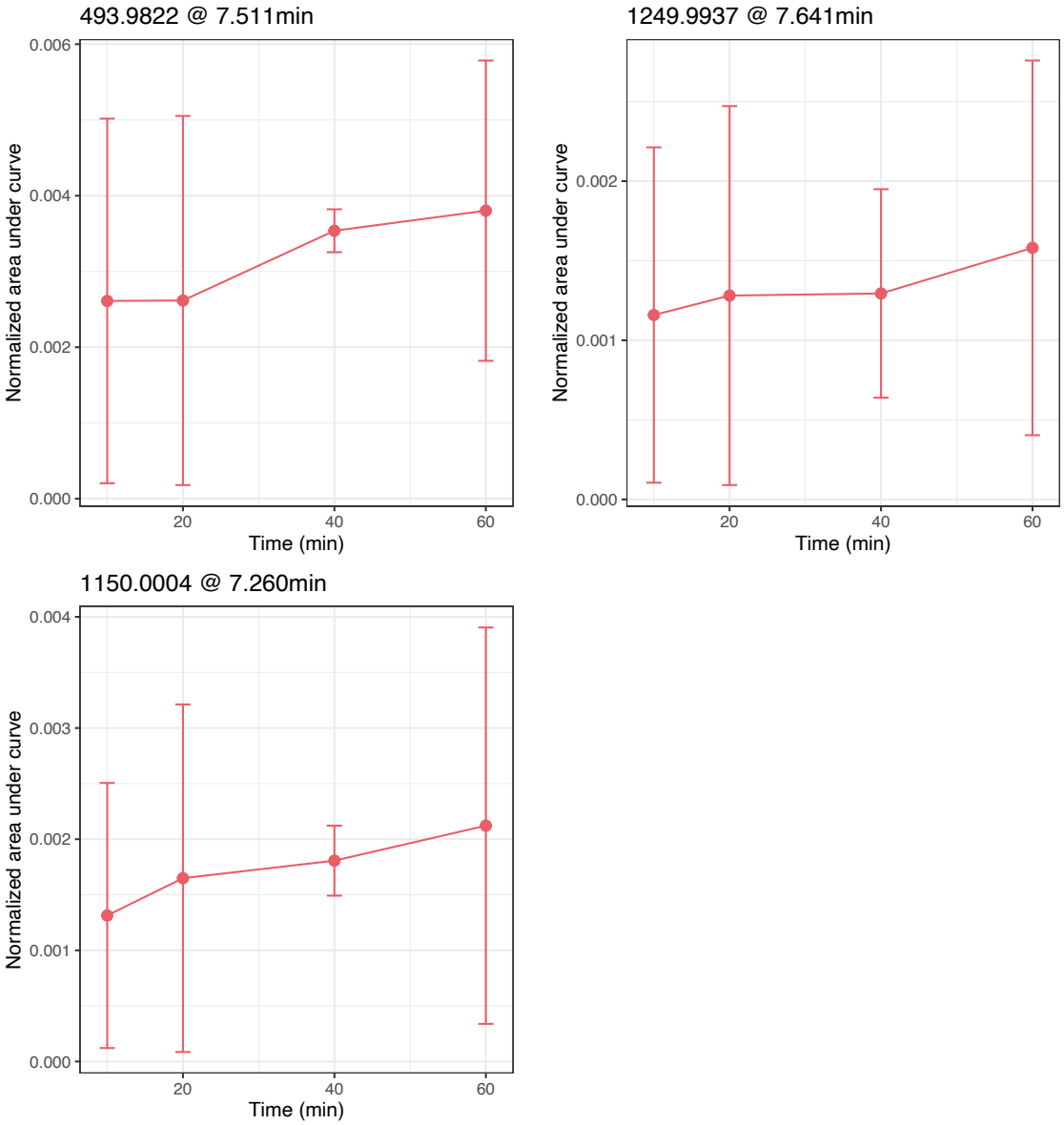


Figure S4: Biotransformation of selected peaks from the *Frangula* pink WGCNA module by *N. bryanti* liver CYPs over time. Mean ± SE.

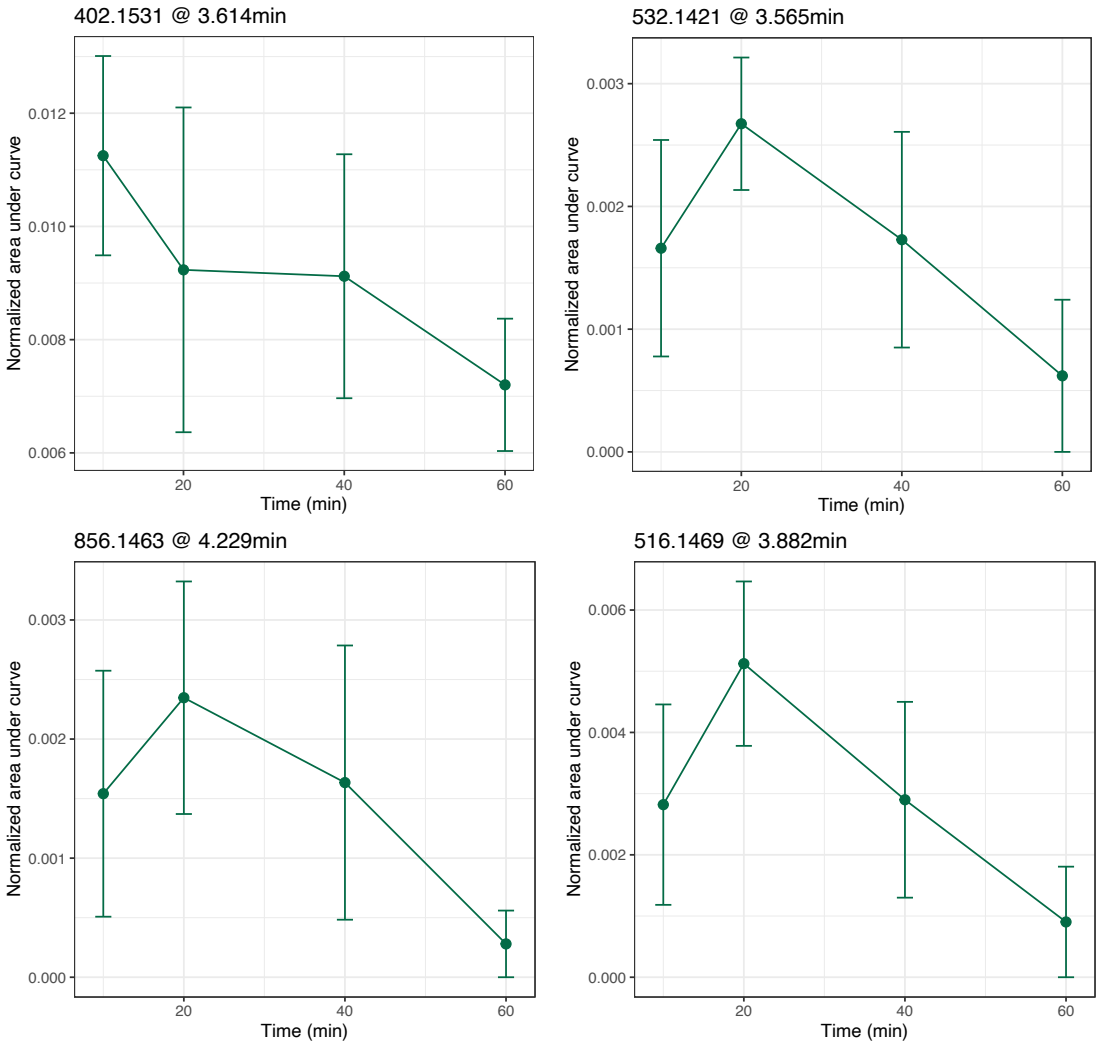


Figure S5: Biotransformation of selected peaks from the *Prunus* magenta WGCNA module by *N. lepida* liver CYPs over time. Mean ± SE.

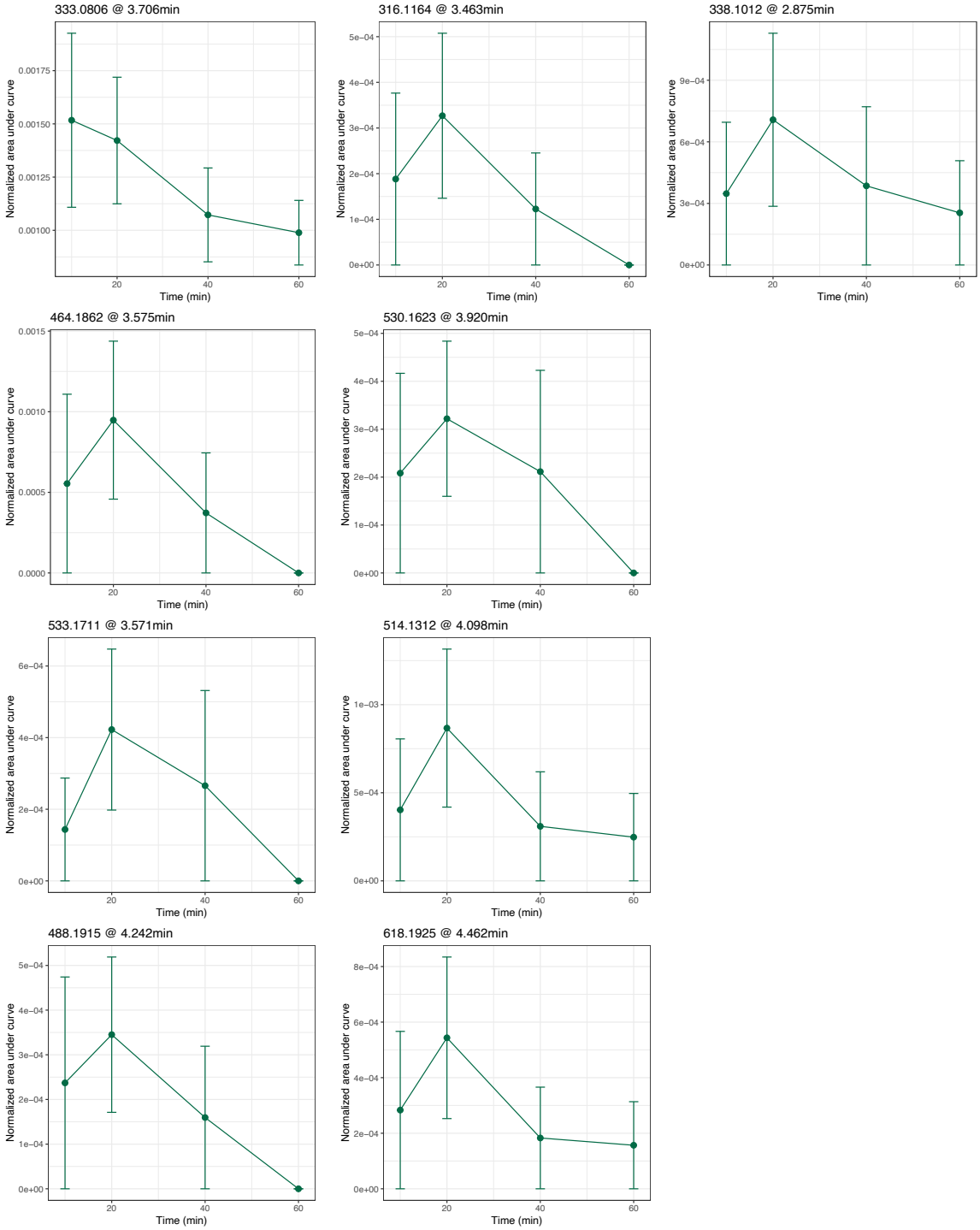


Figure S6: Biotransformation of selected peaks from the *Prunus* red WGCNA module by *N. lepida* liver CYPs over time. Mean ± SE.

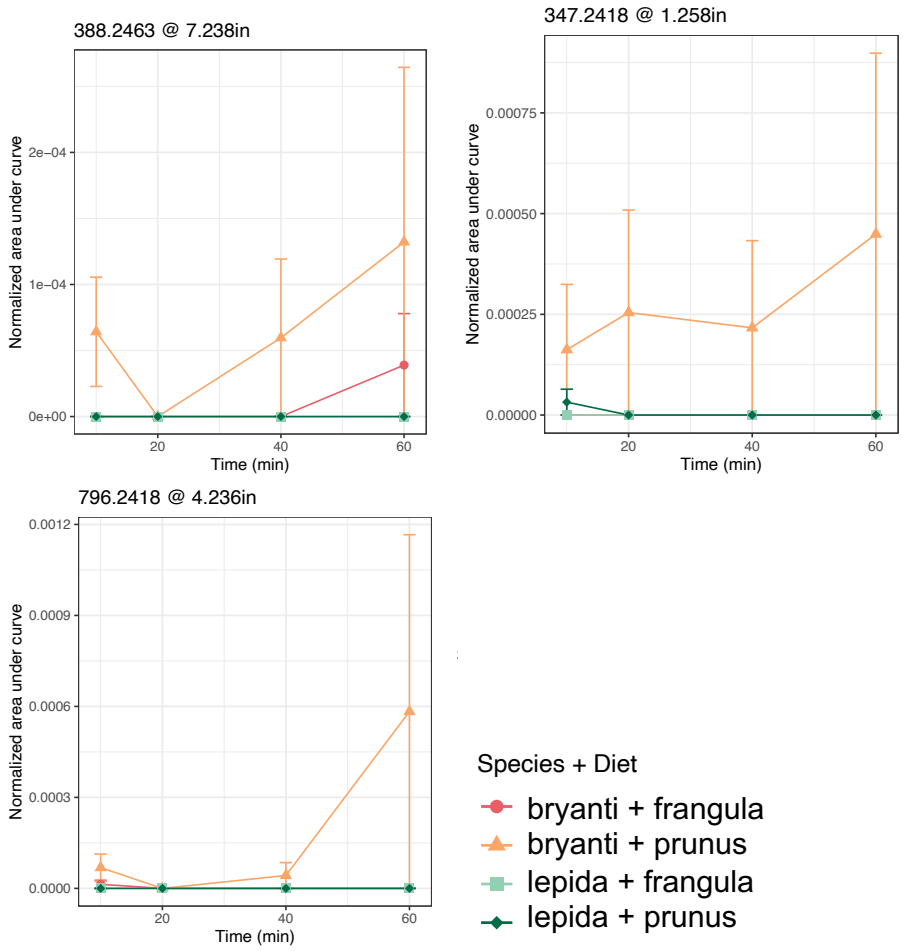
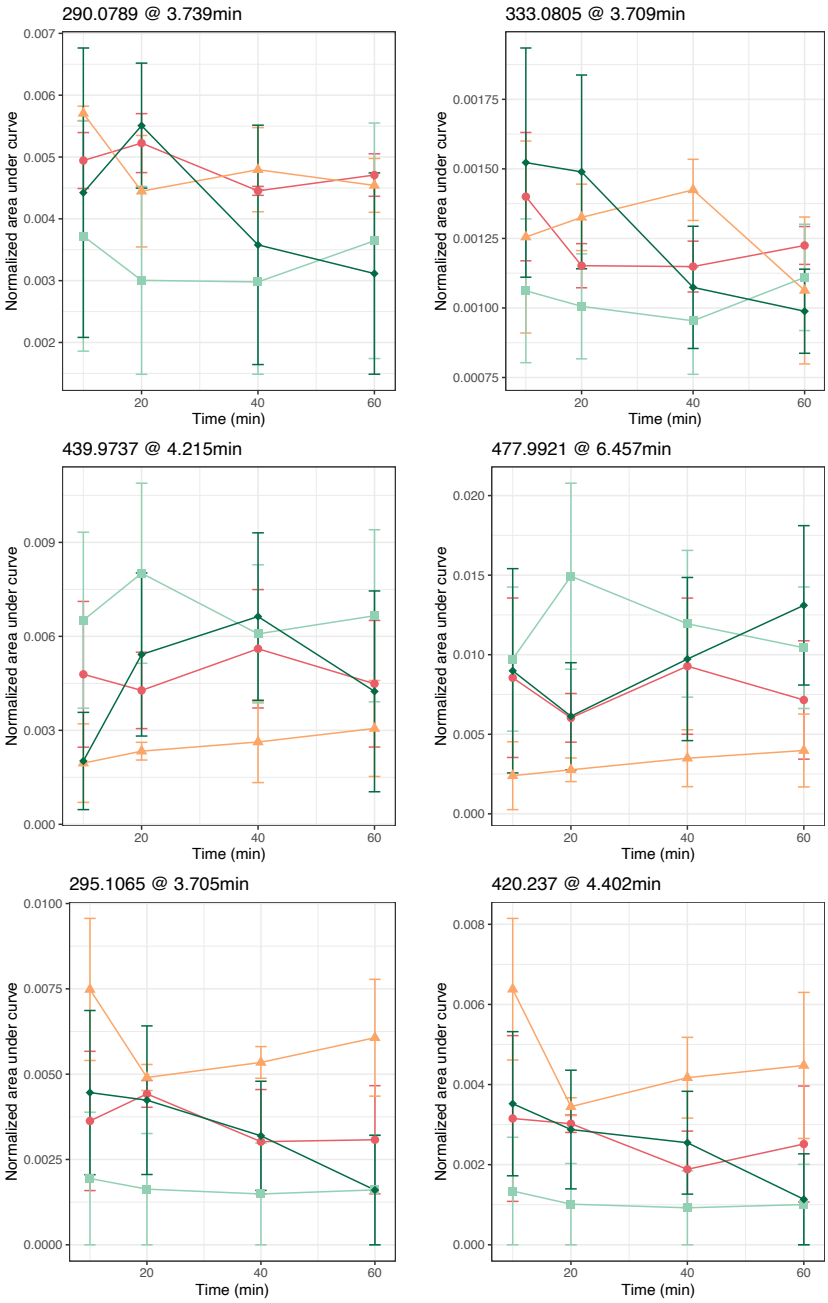


Figure S7: Biotransformation of selected peaks from the *Prunus* pink WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found differences between species and diet (F=3.489, p=0.0683; F=2.886, p=0.963).



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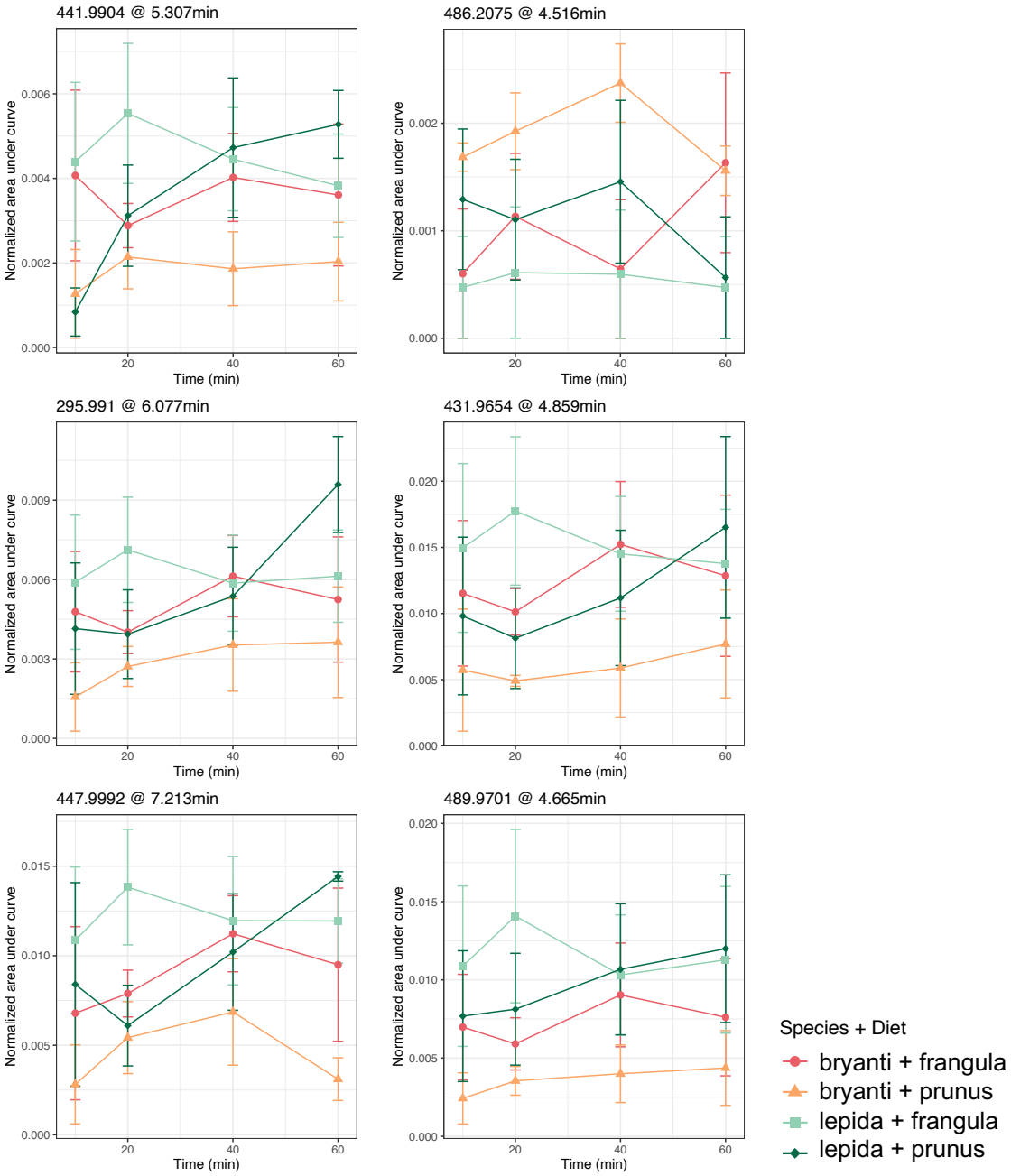

Figure S8: Biotransformation of selected peaks from the *Prunus* yellow WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found significant differences between both species and diet treatments (F=7.983, p=0.00702; F=5.299, p=0.02603).

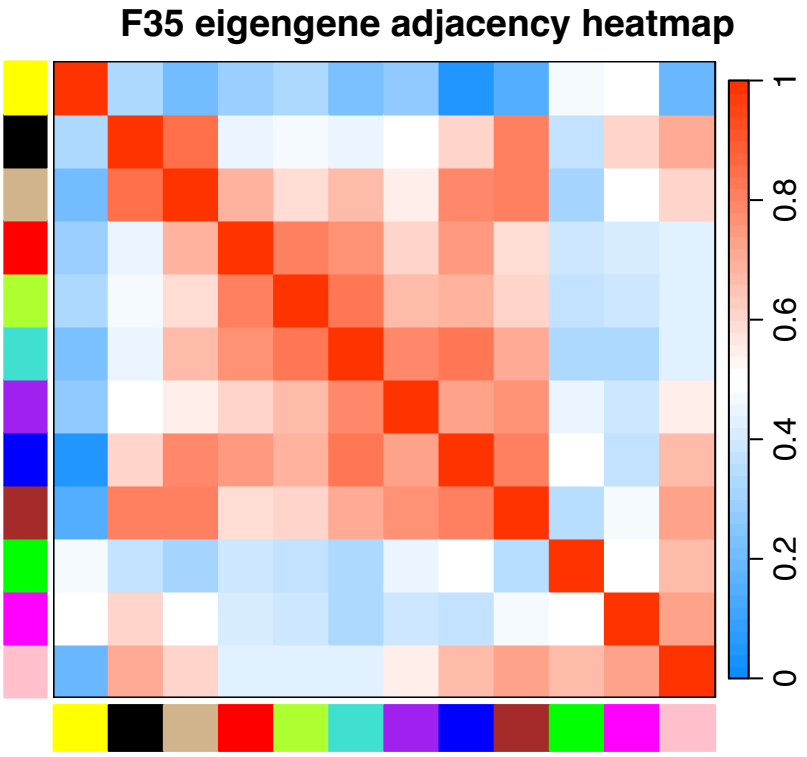

Figure S9: Eigengene adjacency heat map for *Frangula* WGCNA modules.

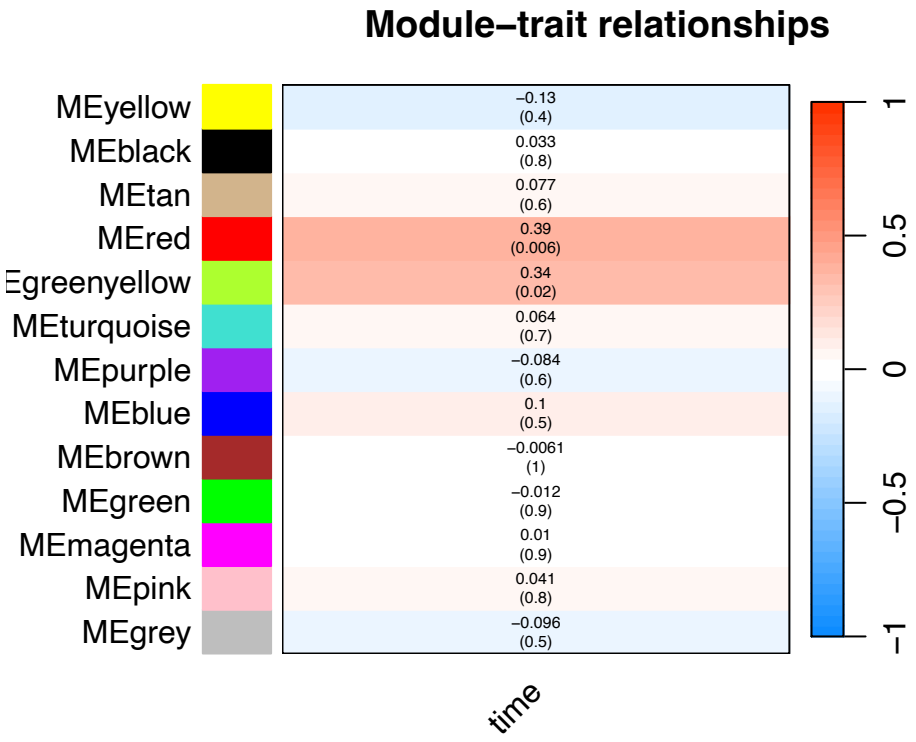

Figure S10: Module-trait relationships for *Frangula* WGCNA modules with respect to time.

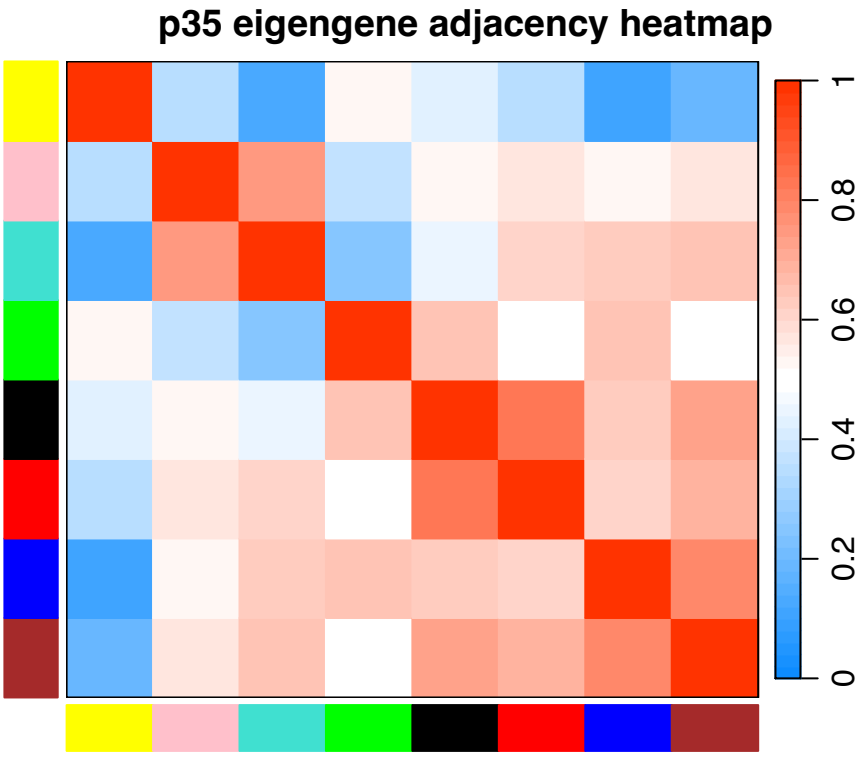

Figure S11: Eigengene adjacency heat map for *Prunus* WGCNA modules.

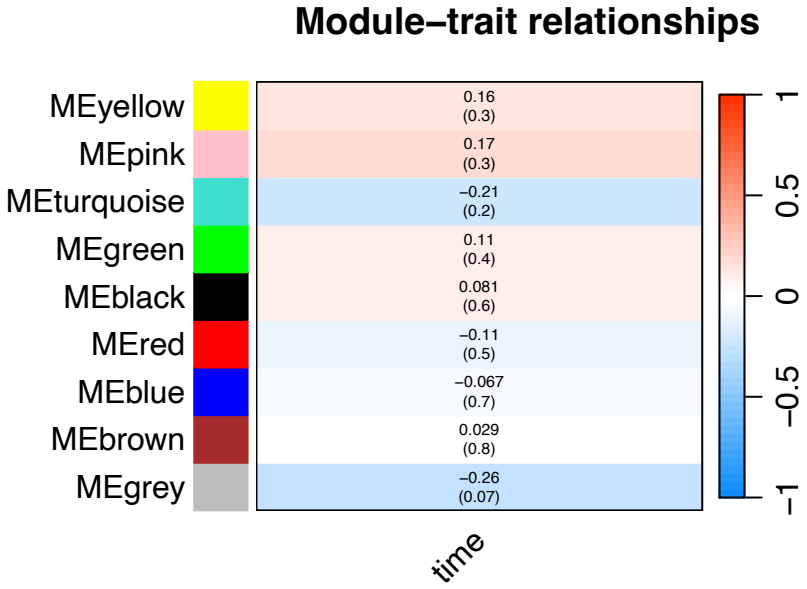

Figure S12: Module-trait relationships for *Prunus* WGCNA modules with respect to time.

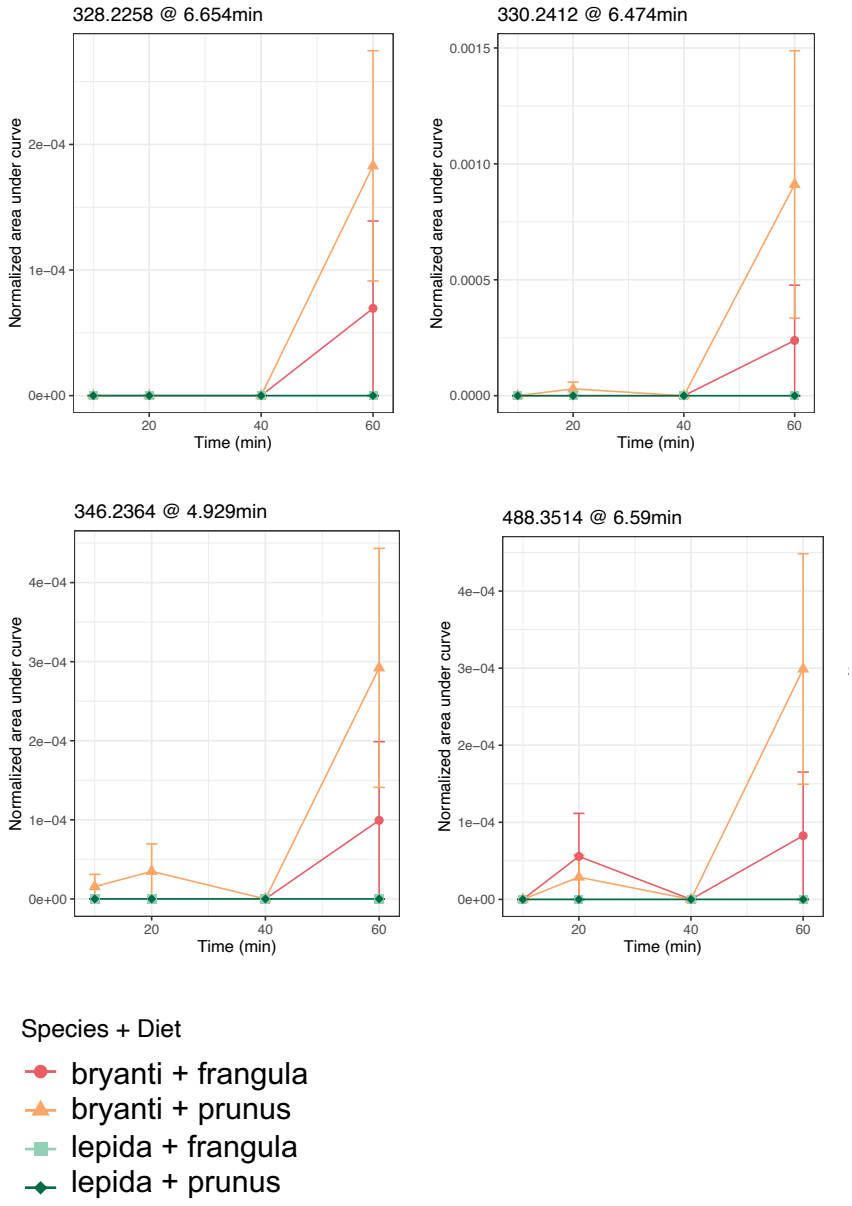

Figure S13: Biotransformation of selected peaks from the *Frangula* greenyellow WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found differences between species (F=3.273, p=0.060).

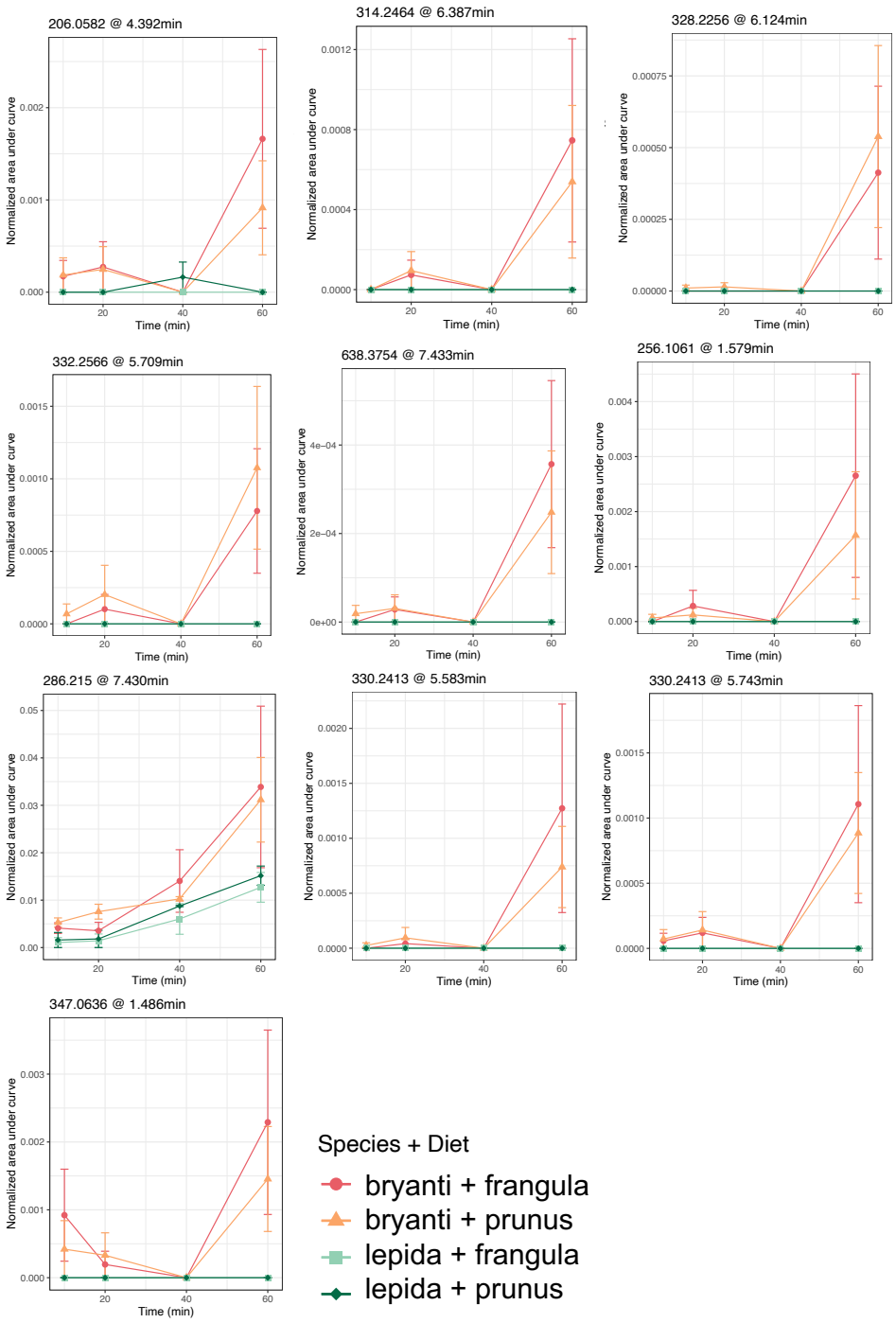

Figure S14: Biotransformation of selected peaks from the *Frangula* red WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found significant differences between species (F=5.62, p=0.0221).

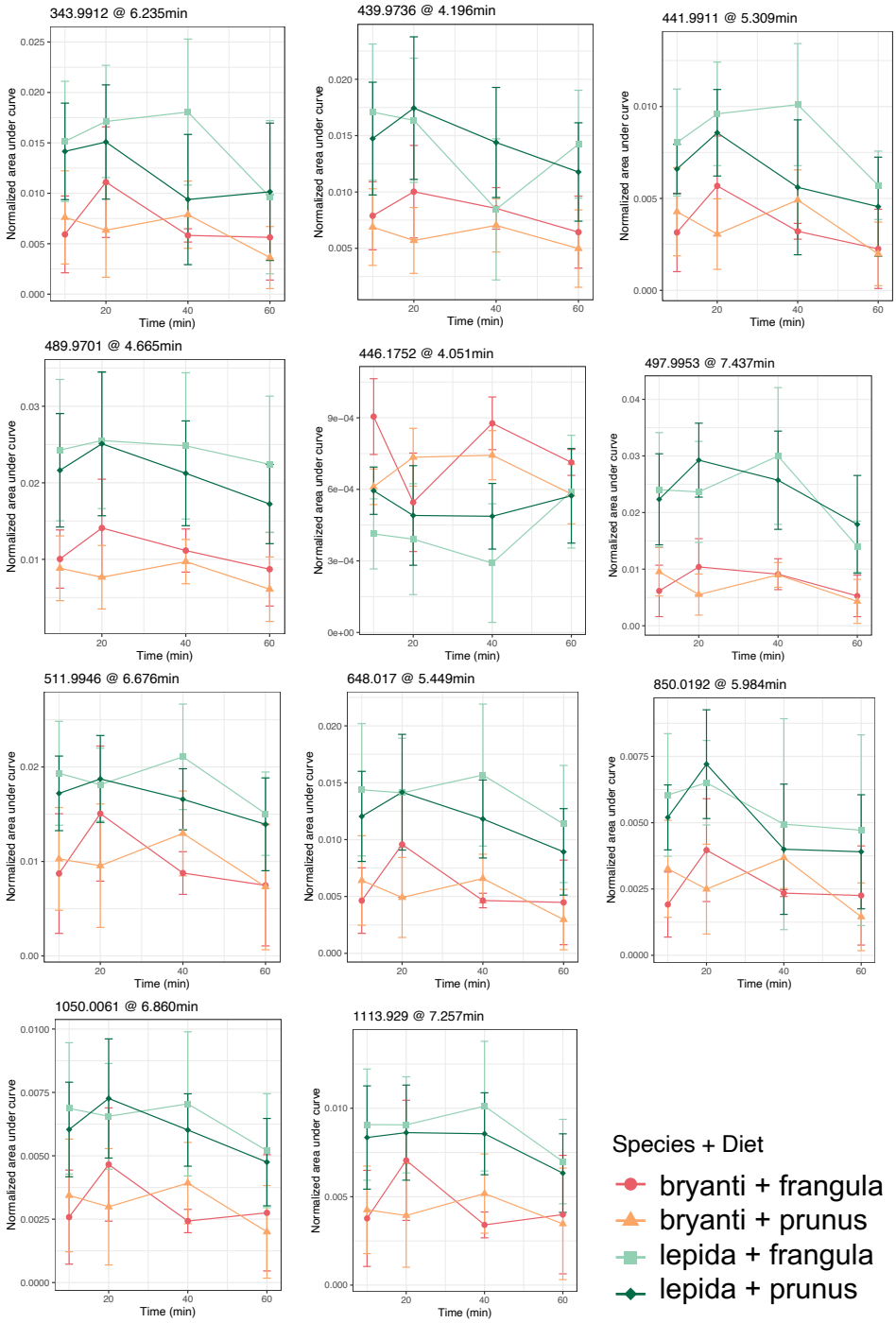

Figure S15: Biotransformation of selected peaks from the *Frangula* yellow WGCNA module by *N. bryanti* and *N. lepida* liver CYPs over time. Mean ± SE. A two-way analysis of variance was performed on the module eigenvalues and found significant differences between species (F=14.905, p=0.000359).