Oleuropein Concentration in Four Varieties of White Fringe Trees

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Abstract

Over the past few years the emerald ash borer (EAB) insect has invaded plant species such as olive plants (*Olea Europea L.*), the privet tree (*Ligustrum obtusifolium*), and the white fringetree (*Chionanthus virginicus*) and caused significant damage. It is believed that the EAB is put off by the phenolic compound of oleuropein found in these various plants. This compound can be isolated by running a Soxhlet extraction of the plant species mentioned above and analyzed through reversed HPLC at 280 nm. distinct peak areas are then obtained and can be used to determine the concentration of Oleuropein. The overall results supported the idea that there are differences in oleuropein concentration when looking at uninfested and infested white fringe trees.

Introduction

Oleuropein is a phenolic compound that is commonly found in olive plants and is often used for medical purposes. It can also be detected in plants of the same family, such as the privet tree and the white fringe tree. Oleuropein is a common antioxidant and is the major component of olive polyphenols and it has been extensively studied for health benefits concerning a variety of ailments such as blood pressure, cancer, heart problems and an array of viral and bacterial diseases.[1] Polyphenols such as oleuropein have similar chemical structures (Figure 1), and there is a correlation between the total polyphenol concentration in olive oils and olive oil bitterness; thus, it is generally assumed

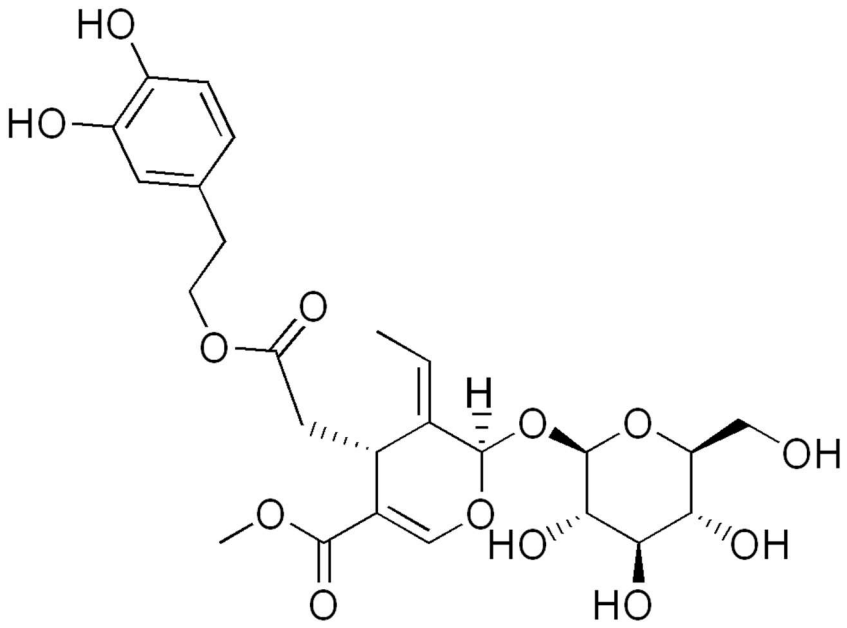

***Figure 1**. Structure of Oleuropein*

that most polyphenols have similar bitterness thresholds.[2] Research of oleuropein has been done since the late nineties, but has become more prominent in the last few years. A new area of research interest is producing oleuropein as an insecticide. When the leaves of a olive or fringe tree are mechanically damaged by herbivory and cell compartments are broken, enzymatic activity localized in organelles separate from oleuropein starts to activate oleuropein into a very strong protein denaturant.[3] Although oleuropein itself has been researched for years, it has not been studied extensively in other plants of the *Oleaceae* family besides olive trees.

The emerald ash borer (EAB) is native to many parts of Asia. Through studies of its infestation on the native trees in those Asian regions, it was determined that the EAB has a preference for hosts with low levels of oleuropein. The native range of Chinese fringetree in Asia overlaps with the range of the emerald ash borer, so the tree might possess bark defenses as a result of coevolution or selective pressures.[4] This insect has since made its way over to North America infesting and destroying many trees of the *Oleaceae* family like ash and white fringe trees. Starting in 2013, the emerald ash borer has taken North Carolina by storm. Not only is it our

newest invasive forest pest, but it is also the most capable spreader. Since its initial find in 2013, it has been found in 24 counties across the state.[5] This wood burrowing beetle uses its chemical senses to find hosts for its larvae to grow. In recent years the EAB was thought to be exclusive to the ash tree but it has since been found that they can use the white fringe tree as a host. The preference of the EAB is the ash and privet trees, but if this plant species is unavailable as a host then they seek out other hosts such as the white fringe tree. This was found through the research of Dr. Don Cipollini by examining the southwestern regions of Ohio. The white fringe tree itself can be found from Texas to New England. The first notable infestation was in 2013 in North Carolina and then again in 2014 in Yellow Springs, Ohio.[6] If a solution is not found the cycle of infestation shall continue and the EAB will evolve to find a new suitable host once its primary hosts are unavaiable.[7,8,9]

Working with Dr. Don Cipollini and graduate student Emily Ellison, white fringe tree samples were collected from various southwestern Ohio locations. The white fringe tree was studied, not only because it has become the next host for the EAB, but also because some trees have presented some unusual behavior. According to Ellison, the tree should typically die once infested by the EAB. However, some trees are being attacked and living or not being attacked at all according to Ellison. The concentration of oleuropein from the white fringe tree samples will be analyzed to conclude theories proposed by Dr. Cipollini and Ellison. The goal is to understand the relationship between the concentration of oleuropein in the white fringe tree samples and the level of infestation by the EAB.

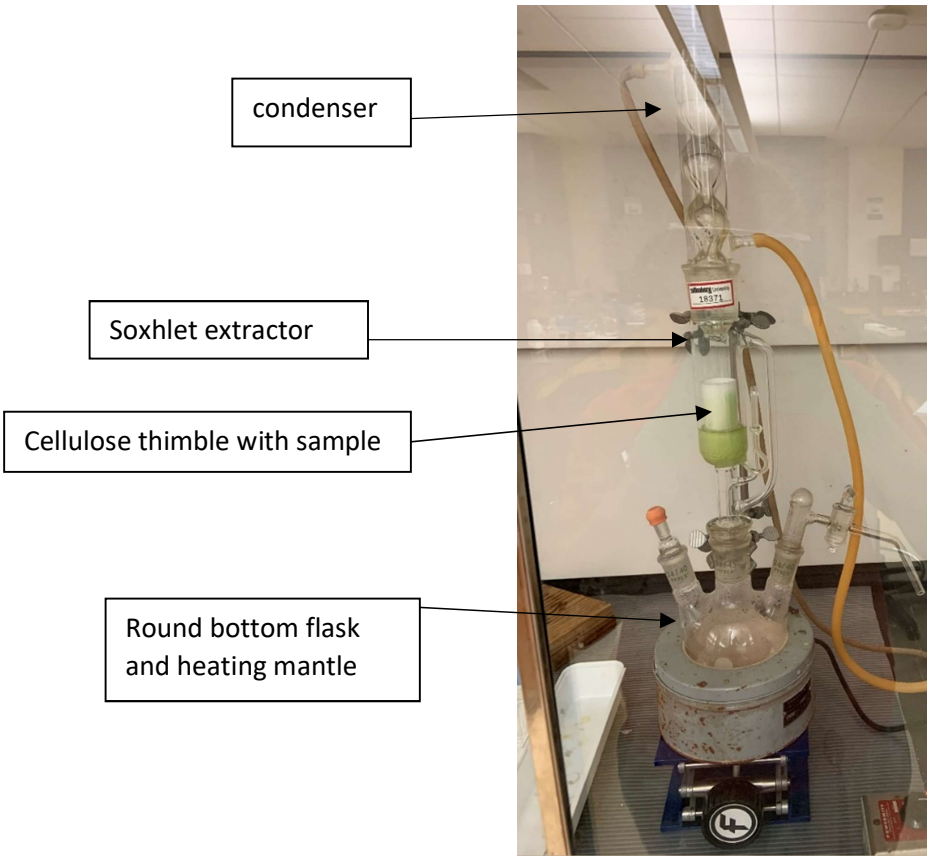

***Figure 2**. Soxhlet extraction setup for removing oleuropein from bark samples*

To extract oleuropein from a bark sample a Soxhlet extraction was used (see figure 2). A Soxhlet extractor is attached at the bottom with a round bottom flask and a condenser at the top. Each round bottom was filled with 80% ethanol and 20% DI water by mass. As the round bottom is heated by the mantle the solvent vapors travel up the outer arm of the extractor. Once in contact with the condenser, which has cold water flowing through it, the solvent vapors condense and fall into the Soxhlet extractor chamber. This chamber holds the cellulose thimble with the bark sample inside. The thimble lets the solvent saturate the walls a penetrate the sample inside. This process will continue until the chamber reaches capacity. Then the solution will empty though the siphon and back down into the round bottom flasks. This will continue to cycle through until heating is stopped.[10]

Analysis of the extracted oleuropein was performed through high performance liquid chromatography (HPLC). Reverse phase HPLC takes samples and separates them based on their polarity. The samples were injected into the HPLC and passed through a C18 column bonded with silica (see figure 3). This C18 column acts as the HPLC's stationary phase. The solvents of acetic acid buffer and methanol flow through the column and act as the mobile phase. The interaction of the analyte between the two phases means that, based on the compound polarity, it will elute out at a specific time during the run. This period of elution is known as the retention time and is affected by the sample's polarity. The solution that is outputted from the column was

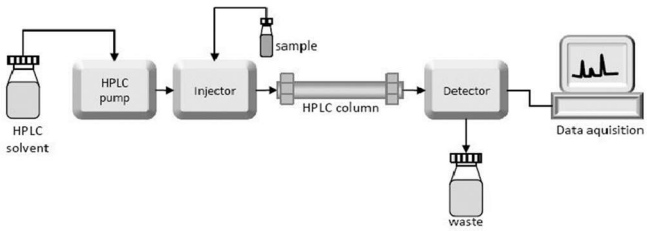

***Figure 3**. High performance liquid chromatography process*

then run through the UV-Vis detector. To make sure we obtain the information for our desired compound a specific wavelength was set at which the oleuropein will be absorbed. Based on literature and previous research the method was set at a wavelength of 280 nm[11], a ramping method of solvents and a total run time 12 minutes. Together the integrated peak from absorption and the retention time values can then be compared to a standard calibration curve to accurately determine the amount of oleuropein in the bark sample. Using this extraction technique, we will compare oleuropein concentrations between four types of white fringe trees to see the correlation of the EAB selecting the white fringe tree as a host.

Experimental

Standard

A standard curve was made as reference for the concentration of oleuropein in the samples. There was a total of six standards at concentrations of: 100, 250, 500, 1,000, 1,500, and 2,000 ppm. Standards were made by dissolving 0.05 g of solid oleuropein in 5 mL of methanol and diluting in a 25 mL volumetric flask with DI water. A serial dilution was then used to achieve the remaining concentrations. Each was obtained with transfer pipets and 10 mL volumetric flasks. The completed standards were run though HPLC and UV-Vis for analysis. For HPLC a method was created where the run was 12 minutes using 10 g/L acetic acid buffer at pH 3.5 as solvent A and pure methanol as solvent B. The solvent gradient was set to ramp from 80% solvent A and 20% solvent B and reversed percentages at the 6-minute run time; this was saved under the file name “Oleuropein.m”. Using this method, the oleuropein elutes at the 6.3-minute mark. Once all runs were complete, if the HPLC gave an inaccurate baseline, they were made manually in the data analysis window. The peaks were then integrated, and the resulting areas were used to create a calibration curve. These standards were run once a day for a maximum of four days to ensure that the calibration curve remained accurate.

Plant Samples

All samples were given to the research team at Wittenberg from Dr. Cipollini and Emily Ellison from Wright State University in Dayton, Ohio. A handsaw was used to cut the branch above the collar tissue so as not to harm the tree, or the main stem was cut and collected if the tree was too young to have five-centimeter branches. The saw was disinfected with 100% ethanol before and after each use to avoid contamination. Cut stems were stored upright in five-

***Figure 4**. samples dried in aerated fume hood for two weeks*

gallon buckets with the ends submerged in water to facilitate water uptake. A disinfected utility knife was used to extract approximately one gram of bark tissue off the top of each stem of fringe tree, which was dried on a laboratory bench before being sent to the research team at Wittenberg University to determine oleuropein concentrations in the bark tissue (see figure 4).[12] Some samples were dried for two additional weeks once received at Wittenberg. Each sample was weighed and recorded before entering the Soxhlet extraction.

Extraction Process

Bark samples were broken up by hand and weighed to approximately 1 g. The broken-up samples were then inserted into a cellulose thimble and placed into the Soxhlet extractor. A 250 mL round bottom flask was filled with 125 mL of 80% ethanol and 20% DI water by mass (104 mL of ethanol and 21 mL of DI water). The condenser was then attached, and cold water was then circulated through it. The heating mantle was then turned on and left for two hours and forty minutes to reflux. Once finished, the heating mantle was turned off and the round bottom flask

was left to cool while the other pieces of the extraction were dismantled and cleaned for the next extraction.

Two other tests were run using this extraction technique. The first was a test using old olive leaf samples and running the extraction for five hours taking samples every fifteen minutes. This was to observe the amount of oleuropein extracted over time. The second was a decay test of a white fringe tree bark sample. This was to see if the amount of time between sample collection from the main tree, to drying and then running affects the overall oleuropein amount in that sample.

HPLC Analysis

The stored samples were then taken to HPLC and analyzed. The same method used for the standards was used to analysis all samples. The chromatograms were then integrated through the HPLC program or reintegrated with a new manual baseline. This information was then taken and entered into excel and the concentration of oleuropein in each sample was determined by using the calibration curves.

Results

Absorbance Versus Time

At the beginning of research as test was done to see if the procedure for the Soxhlet extraction could be updated. This was done by running last year's samples in a Soxhlet extraction for five hours taking samples every fifteen minutes for analysis. These samples were then analyzed with UV-Vis absorption spectra at wavelengths 200, 240, and 282 nm. These values were then placed into a graph plotting absorbance versus time (Figure 5). This determined the best time to run each Soxhlet extraction, and at which wavelength to use for HPLC analysis.

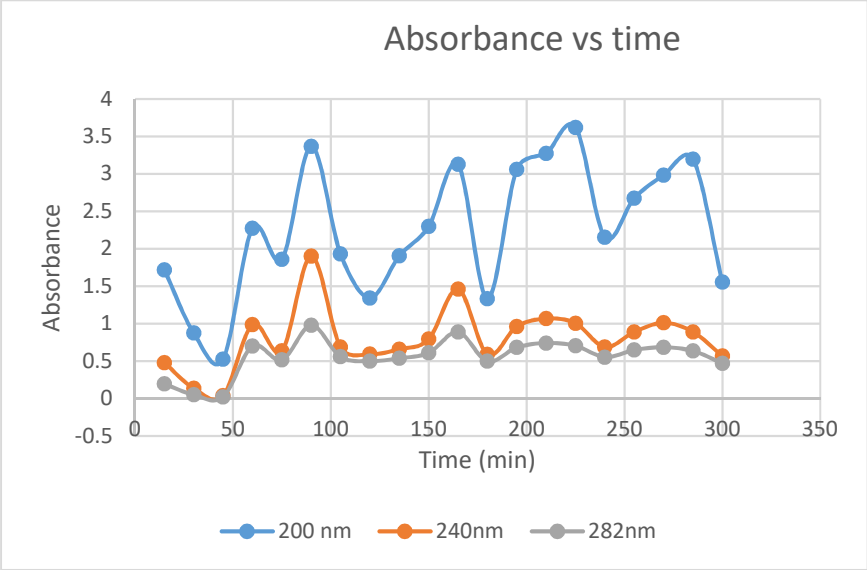

***Figure 5**. Sample 149 analysis via UV-Vis of five-hour Soxhlet extraction*

With this data, it can be concluded that wavelengths 240 nm and 282 nm are suitable for detecting oleuropein. The time of 160 minutes was chosen because compared to the 240 nm plot, this time and the 90-minute point were the most similar in absorbance. For this research thisvwas a slight change from the previous 240 minutes.

Decay Test

The decay test was run with a white fringe tree bark sample every three to seven day for a total of six data points. This information indicates the effect of time between sample collection and processing and informs us that a potential new handling of samples could be used in further research.

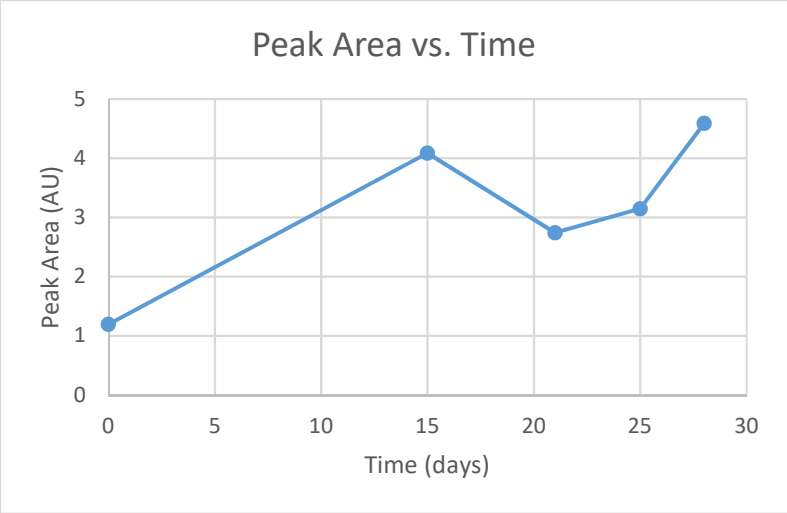

Figure 6. Peak area versus time; results of decay testing

Standard Curve

Once the areas from the six standards were integrated by the HPLC program, a plot was made with the integrated peaks with respect to the standards concentration. In all runs done of the standards, it became apparent that any sample with a concentration of 6 ppm or lower would not be detectable and therefore unable to be analyzed.

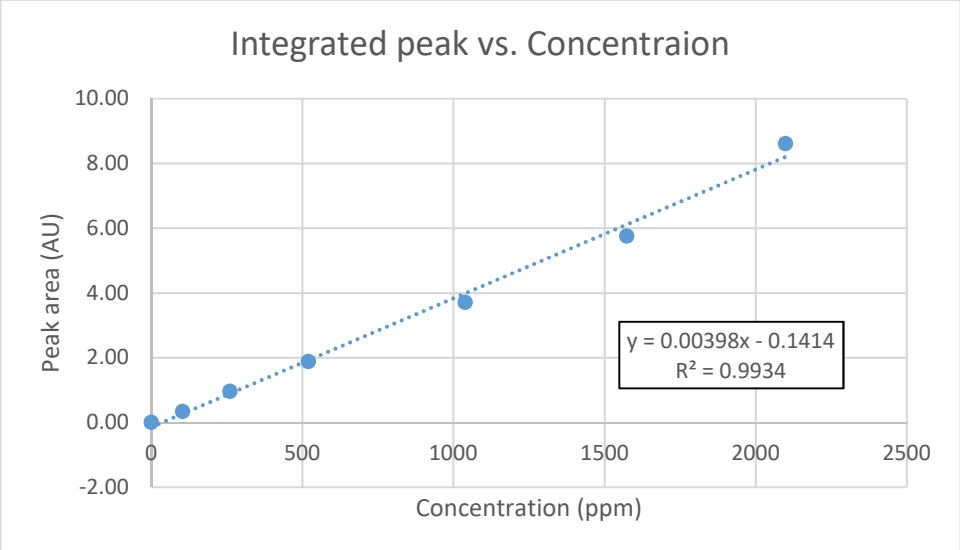

Figure 7. Oleuropein standard curve from HPLC integrated peaks; 0.00398 ± 0.00014

Oleuropein Concentrations

After doing a Soxhlet extraction followed by HPLC of each sample, the peak areas were integrated. Then with the equation provided by the calibration curve (Figure 7) was then used to find the concentration of oleuropein in each sample. The concentration found from the calibration curve was found in ppm and then converted to total grams of oleuropein. This was then divided by the sample amount weighed for the Soxhlet extraction to give a final value in grams of oleuropein per sample mass. (All samples can be found in appendix I)

Table 1. Oleuropein Concentrations in White Fringe Tree Samples

SAMPLE NUMBER	CONCENTRATION (PPM)	G OLEUROPEIN/ SAMPLE (G)
100	1887.98	0.3014
110	785.52	0.0811
154	717.67	0.0890
162	1028.26	0.1082
201	363.56	0.0525
205	1429.11	0.1469
251	757.06	0.0778

Conclusion

All the sample were categorized into four different types of white fringe trees; ornamental uninfested, ornamental infested, wild uninfested, and wild infested. Based on the grams of oleuropein per sample of all the samples, it is evident that the ornamental, uninfested trees have the highest amounts of oleuropein. Whereas, the lowest amounts can be found in the wild infested tree samples. This can be easily explained by looking at the conditions of each sample type. Ornamental trees that have not been previously infested likely have high enough

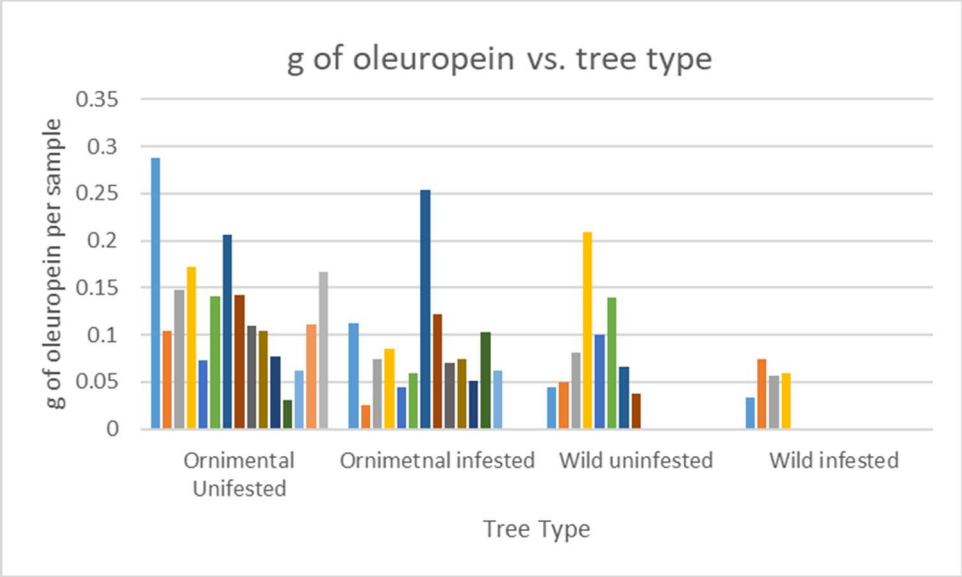

***Figure 8**. Bar chart of a samples based on tree type*

concentrations such that the chemical sense of the EAB do not want to lay eggs and grow larvae there. Also, due to the type being ornamental it can be assumed that the tree was raised in a nursery where the tree may be bred to be more resistant to the EAB. The results about the wild infested trees indicate that they are more likely to be attacked due to the lower levels of oleuropein. The fact that the tree had been attacked by the EAB before tells us that it was a suitable host of new larvae if the privet or ash tree is unavailable.

In terms of the overall method of research, there were some aspects of the experiment that were more precise and accurate than others. This was the first year a decay test and absorbance versus run time was tested on samples. Looking specifically at absorbance versus time, this was aimed to modify the length of the Soxhlet extraction. Normally, the extractions would run for four hours. However, literature varies from how long each extraction should take to obtain the maximum amount of oleuropein. Even at this new time of 160 minutes, there was still some discrepancy between runs so out of three runs the average was taken and then the grams of oleuropein per sample was solved for. The decay test was aimed to see of the amount of time a bark sample sits and repeatedly gets tested affects the concentration of oleuropein. As this test has some very raw data it is hard to say if it was successful or not. This test should be run again in the future to see if the method for handling these samples should change.

As of today, all the values presented have shown no correlation to the behaviors of the EAB according to Ellison. This is supported with calculations done with a T-test showing no significant difference between average oleuropein concentrations between tree types. There is however a connection between oleuropein concentrations in infested and uninfested white fringe trees. We are unsure of why this is and looking into ways that the research can be altered to yield better results.

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12. Emily’s method

Appendix I

This table is all the samples that were run during the ten total weeks of research. It includes oleuropein concentrations and grams of oleuropein per sample. Samples 100-115 are ornamental uninfested, sample 150-163 are ornamental infested, sample 200-207 are wild uninfested, and samples 250-253 are wild infested white fringe trees.

Sample #	Oleuropein concentration in ppm	g of Oleuropein/ sample
100	1887.98	0.301
101	809.24	0.109
102	1314.32	0.155
103	1762.13	0.181
104	745.12	0.076
105	1427.51	0.147
106	2101.16	0.217
107	1453.10	0.150
108	1115.77	0.114
109	1061.13	0.110
110	785.52	0.081
111	313.69	0.032
112	480.87	0.065
113	1131.29	0.116
114	1651.37	0.176
115	674.09	0.083
150	898.19	0.117
151	199.91	0.026
153	749.91	0.077
154	717.67	0.089
155	412.79	0.046
156	598.78	0.062
157	2582.82	0.266
158	857.51	0.128
159	711.13	0.073
160	757.60	0.078
161	521.04	0.054
162	1048.26	0.108

163	628.44	0.065
200	375.05	0.046
201	363.56	0.052
202	828.26	0.085
203	1026.01	0.218
204	1018.15	0.105
205	1429.11	0.147
206	675.19	0.070
207	380.30	0.039
250	338.77	0.035
251	757.06	0.078
252	582.49	0.060
253	603.12	0.062