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Biotransformation of Natural Antioxidants Osajin and Pomiferin by Cunninghamella Elegans (ATCC® 9245TM)

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BIOTRANSFORMATION OF NATURAL ANTIOXIDANTS OSAJIN AND POMIFERIN BY CUNNINHAMELLA ELEGANS (ATCC® 9245™)

by

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Stephen Blake Luis

DEDICATION

I dedicate this dissertation to my mother and father Roxanna Luis-Garcia and Stephen Luis. The importance of education is a subject that was always stressed throughout the households. They've both worked tirelessly since I was a child and have instilled in me the determination and persistence to never give up. They gave me a perfect life. I believe it only fit that I return the favor and give them a successful, determined, and dedicated son. I love you both and I hope that I've made you proud.

I would also like to dedicate this to my younger brother Caleb. I want you to know that through hard work, determination, and prayer, all things are possible.

BIOTRANSFORMATION OF NATURAL ANTIOXIDANTS OSAJIN AND POMIFERIN BY CUNNINHAMELLA ELEGANS (ATCC® 9245™)

Stephen B. Luis

University of the Incarnate Word, Year

Osajin and pomiferin, prenylated isoflavones extracted from the fruit of the osage orange tree (*Maclura pomifera*) have been reported as antioxidant compounds. This project pertains to analyzing the metabolic profile of those compounds to better understand their antioxidant properties. These two isoflavones have shown efficacy in acting as an antioxidant, cardio-protectant, anti-inflammatory, and insect repellent.

HPLC analysis of the extracts showed new, more polar compounds were formed, evidenced by peaks at lower retention times for each strain of fungi. To further investigate the metabolites produced, HPLC-guided chromatographic purification will be performed, and the pure metabolites will be analyzed through nuclear magnetic resonance and mass spectrometry.

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Introduction

Fungal biotransformation is a process in which minor to drastic structural modifications are made to chemical compounds by various species of fungi or yeast (Dube & Kumar, 2017). This process ultimately leads to the formation of molecules with a greater degree of polarity than the parent compounds. Biotransformation naturally occurs in microbes to respond to environmental changes and has recently shown to be beneficial in creating novel derivatives of various compounds without the need of animal-based model systems (Singh, 2017). The greatest benefit of biotransformation is that the original carbon skeleton of the parent material is retained with the addition of important additional functional groups. This being said, in instances where a particular compound is costly to synthesize, this process can negate said costs. This process has been used in recent years in the transformation of various hydrocarbons and pharmaceutical substances (Parshikov, et al., 2012) through enzymatic reactions of oxidation, reduction, hydrolysis, formation of carbon to carbon bonds, and introduction of various functional groups (Singh, 2017).

Fungal biotransformation is selected for analyzing the metabolism of various compounds due to these following reasons:

I. **Cost Efficiency**: Fungi can continuously be re-cultured to ensure longevity in the organism and scale up rapidly (Moody, et al., 2002).

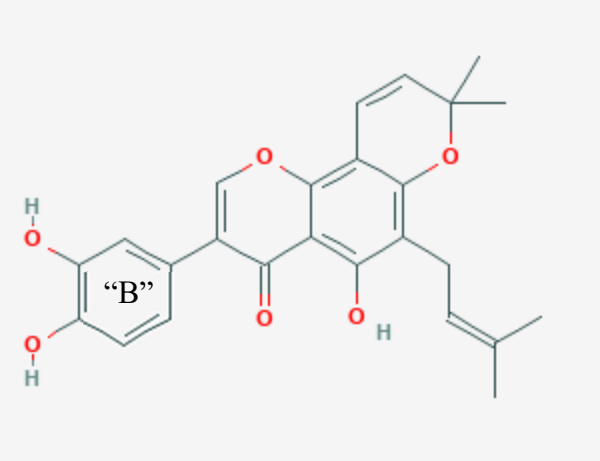
II. **Growth Rate**: Fungi has shown to have a high growth rate which increases the rate to obtain novel derivatives.

III. **Living Conditions**: It has been shown that fungi of all types can survive in even some of the harshest conditions (Kubicek, 2007).

Pomiferin and osajin

Pomiferin is a unique isoflavone that can be isolated and purified from the fruits of the *Maclura pomifera* (Osajin Orange tree). Isoflavones are isoflavonoids that are naturally occurring. These isoflavonoids are known for being phenolic in structure and have the quality of being biologically active (Kauffman, et al., 1997). When isolating pomiferin, a second molecule is found alongside it known as osajin. When examined, osajin is structurally similar to that of pomiferin; however, osajin lacks an important aromatic hydroxyl group (Figure 1). As a result of this hydroxyl group absence, it has been found that osajin is less effective as an antioxidant when compared to pomiferin (Gruber et al., 2014). Pomiferin's molecular arrangement showing the two hydroxyl groups on adjacent aromatic carbon atoms in the "B" ring is a secondary source of why pomiferin acts as a greater antioxidant. Along with this, it has been found that the structure of adjacent conjugated hydroxyl groups is common in other strong antioxidants such as vitamin C (Gruber, et al., 2014).

[Pomiferin]



[Osajin]

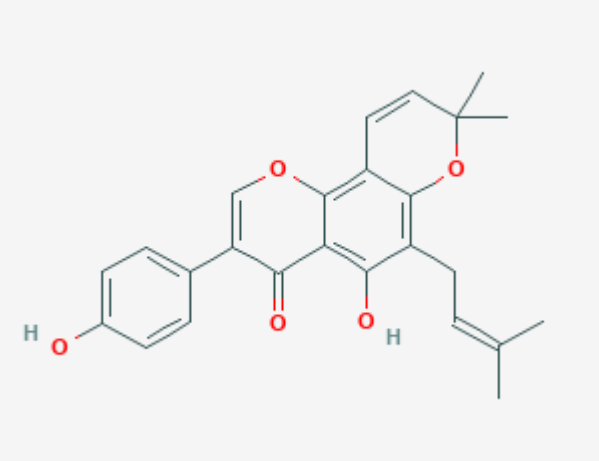


Figure 1: Chemical structure of both pomiferin and osajin.

Biotransformation of CE9245

Synthesizing a target compound is a practice that can be considered costly. At times, the cost alone may deter researchers from performing the synthesis. *Cunninghamella elegans* (ATCC 9245) (**CE 9245**) was utilized in the metabolization of quercetin, an antioxidant found in assorted produce and in red wine with structural similarities to osajin and pomiferin. Upon completion of the process, it was discovered that through biotransformation, three metabolites were obtained (Zi, Jiachen, et al., 2011). Although **CE9245** has been used in the past, the metabolization of both osajin and pomiferin has not been conducted.

Being categorized as a flavonoid, isoflavones were known for their beneficial effects on health long before they were identified as the effective compounds. Recent findings have shown that the anti-inflammatory properties of flavonoids prevented the synthesis and biological activities of different pro-inflammatory mediators, such as prostaglandins E2, F2 and thromboxane A2 (Rathee, et al., 2009). As with all types of medication, the body progressively develops a resistance to drugs when continuously taken. With the production of new derivatives that display anti-inflammatory attributes, newer types of anti-inflammatory medications could be produced utilizing these newly formed osajin and pomiferin derivatives in order to combat the effects of progressive drug resistance.

CE9245 is a genus of filamentous fungi found within soil and various plant materials. To date, it is known to display efficacy in catalyzing various reactions on organic compounds consisting of phase I (oxidative) and phase II (conjugative) biotransformation mechanisms. Of the many *Cunninghamella* strains, **CE9245** has been previously used as an *in vitro* model for mammalian drug metabolism due to a striking similarity (Zi, Jiachen, et al., 2011).

The purpose of this study is to analyze the metabolization of two isoflavones, osajin, and pomiferin, by the fungus **CE9245**. These isoflavones are plant secondary metabolites which may have estrogenic properties (Dilek, et al,. 2017), anti-inflammatory activity (Abourashed et al., 2015), and even showing efficacy when acting as a repellent towards insects (Peterson et al., 2001). HPLC analysis of the crude extract of fungal broth, after supplementation with osajin and pomiferin, showed that the compounds were consumed after 7 days, with the appearance of peaks with lower retention time, indicating higher polarity, consistent with the formation of probable metabolites. The compounds responsible for those peaks will be isolated and submitted for characterization through nuclear magnetic resonance (NMR) and mass spectrometry (MS). Aside from **CE9245**, testing was conducted on *Umbelopsis ramanniana* (Möller) Gams (ATCC® 9628™) and *Aspergillus fumigatus* fresnius (ATCC® 204305™). These other strains yielded similar results.

Aims

Aim 1: Investigation of the viability of biotransformation of osajin and pomiferin by *Cunninghamella elegans* (ATCC® 9245™).

Aim 2: Establishment of an analytic methodology for assessing the biotransformation of osajin and pomiferin by *C. elegans* 9245.

Materials and methods

Drugs

Osajin and pomiferin were obtained from the dehydrated fruit of *Maclura pomifera*, also known as an Osage orange. The fruits were coarsely ground, soaked overnight with ethyl acetate and filtered through Whatman no.1 filter paper. The solid residue was rinsed three times each

with 300mL of ethyl acetate and filtered. The filtrate was then concentrated using a rotary evaporator. (Tsao et al., 2003).

The concentrate was purified through column chromatography running isocratic with 30% ethyl acetate in hexanes through normal phase silica gel, yielding approximately twice the amount of pomiferin in grams compared to osajin. On TLC - thin layer chromatography (ethyl acetate/hexanes 1:1), pomiferin exhibited a Rf = 0.32 and osajin Rf = 0.5.

Strain, media, and culture conditions

The fungal strain that was utilized is *C. elegans* 9245 (ATCC® 9245™). Plates were stored at room temperature within a Kewaunee Interceptor fume hood and grown by streaking a piece of the mycelium onto Difco™ potato dextrose (PD) agar [4.0g potato starch, 20.0g dextrose, 15.0g agar] and incubated. Flasks (250mL) containing 50mL of HiMedia yeast malt broth (YM broth) [5.0g/L peptone, 3.00g/L yeast extract, 3.00g/L malt extract, and 10.00g/L dextrose (glucose)] were then inoculated with a loopful of mycelia growth from the PD agar plates and stored to grow within a Thermo Scientific MaxQ 6000 shaker (125 rpm) at room temperature for 48 hours. This strain, **CE9245**, grows as pea-like orbs within the media. Following the 48 hour incubation period, each flask was inoculated into a larger flask containing 450mL of YM broth and once again incubated, but for an extended period of five days. The other strains utilized, *Umbelopsis ramanniana* (Möller) Gams, and *Aspergillus fumigatus* fresnius were maintained as above.

Administering osajin or pomiferin to the flask

After a five-day incubation period, each flask was administered a solution containing 5mL of acetone and 200mg of either osajin or pomiferin. This mixture is then given to a flask using a 5cc syringe, and 0.2μm syringe filter**.** Each flask is then allowed to continue incubating at 125rpm on a shaker for seven days until full metabolization of the isoflavone.

Vacuum filtration of fungal flask

Following the seven-day incubation for the flask to undergo metabolization, vacuum filtration is performed on the flask to remove any large pieces of fungi and solely obtain the broth prior to extraction. This filtered broth contains the possible metabolites. The flask containing both the fungi, and the broth is poured through Whatman no.1 filter paper to isolate the broth from fungal growth.

Ethyl acetate extraction, roto evaporation, and HPLC analysis

Using a 1L separatory funnel, 100mL of ethyl acetate was poured into the glassware following the vacuum filtered fungal broth. This was repeated in triplicate, each with 100mL of ethyl acetate. Once both liquids were transferred to the separatory funnel, the funnel was vigorously shaken to merge the two liquids. The separatory funnel was then allowed to rest on a holder and wait for the layers to dissociate. After each extraction, the clear ethyl acetate layer was drained into a separate 500mL flask. Each extraction required a new 100mL of ethyl acetate, whereas the fungal broth will be reused. Upon completion of three extractions, each time draining the clear layer into the 500mL flask, roughly 300mL of ethyl acetate should be obtained. At this point, the fungal broth may be appropriately discarded. After the triplicate process, 5g of sodium sulfate was added to the flask containing 300mL of ethyl acetate and allowed to rest for 15 minutes. Excess sodium sulfate was then filtered out of the extract by pouring it through a single sheet of filter paper into a 500mL pear shaped flask. This was then evaporated with the use of a Buchi model R rotavapor system. The product was then analyzed by an UltiMate 3000 HPLC system on a X-terra RP8 column (Injection volume: 10uL), guard column: PRP-1 LC method, with a gradient consisting of 20% acetonitrile in 2% acetic acid to 100% acetonitrile in 16 minutes, 100%

acetonitrile for 2 minutes, back to 20% acetonitrile in 2 minutes, with a total run time of 20 minutes.

Results and discussion

To date, the metabolic activity of **CE9245** on osajin or pomiferin has been analyzed by HPLC, Negative controls of **CE9245** and the secondary strains *Umbelopsis ramanniana* (Möller) Gams and *Aspergillus fumigatus* fresnius have been produced without the introduction of either antioxidant in order to confirm the absence of interference peaks from nutrients from the provided broth.

Fungal biotransformation of pomiferin by CE9245

Fungal biotransformation of pomiferin by **CE9245** over seven days produced possible metabolites as shown in the HPLC chromatogram. These possible metabolites are indicated at the 2, 4, and 7 minute marks by the red boxes (Figure 2). The larger peak identified at the 14 minute mark is unmetabolized pomiferin. As indicated by the presence of peaks at a lower retention time, these results are consistent with the formation of metabolites.

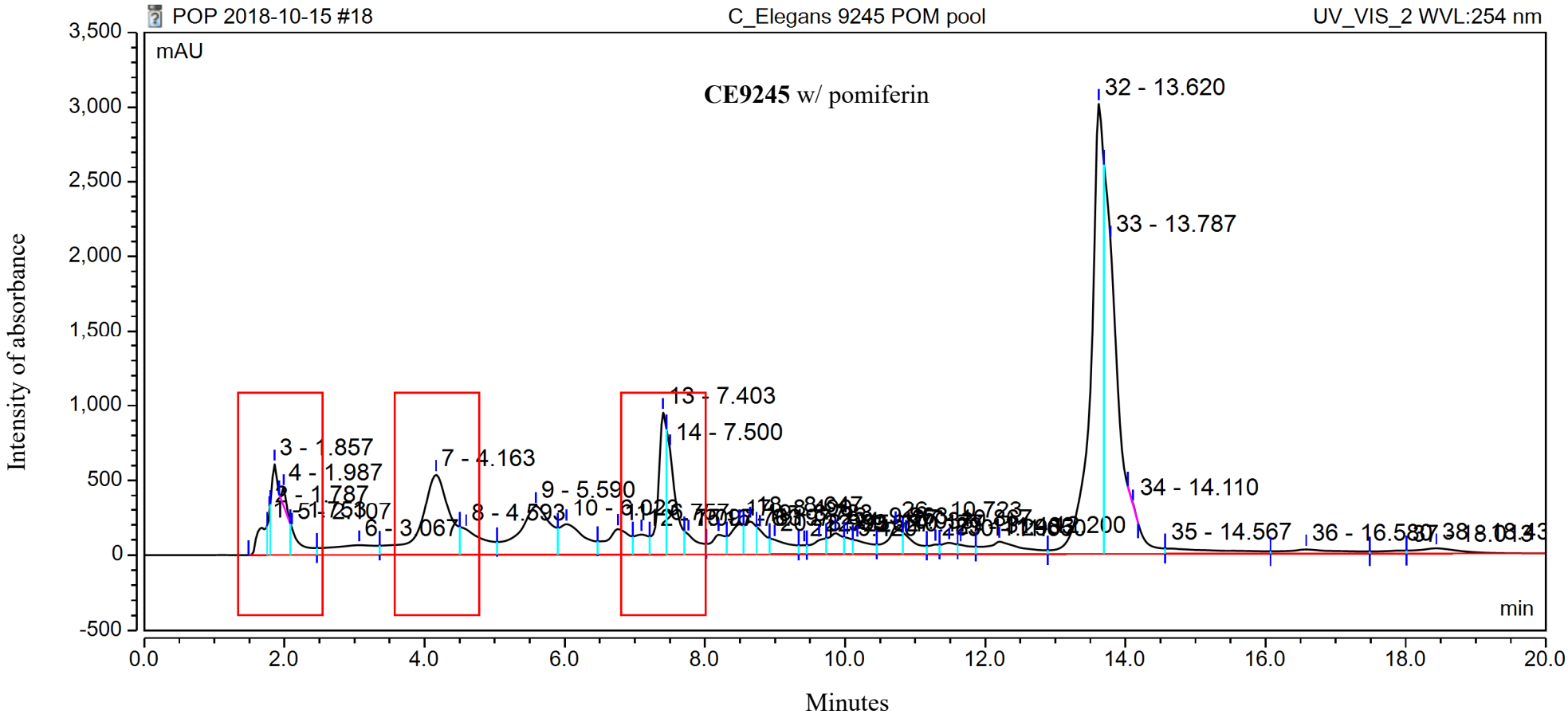


Figure 2: HPLC analysis and fungal metabolism of **CE9245** with pomiferin by UltiMate 3000 HPLC system.

Fungal biotransformation of osajin by CE9245

Over a seven-day period, fungal biotransformation of pomiferin by CE9245 produced possible metabolites, as can be seen in the HPLC chromatogram. As indicated by the red boxes, these possible metabolites are displayed at the 2, 4, and 8 minute marks (Figure 3). The larger peak identified at the 15-minute mark is unmetabolized osajin. As indicated by the presence of peaks at a lower retention time, these results are consistent with the formation of metabolites.

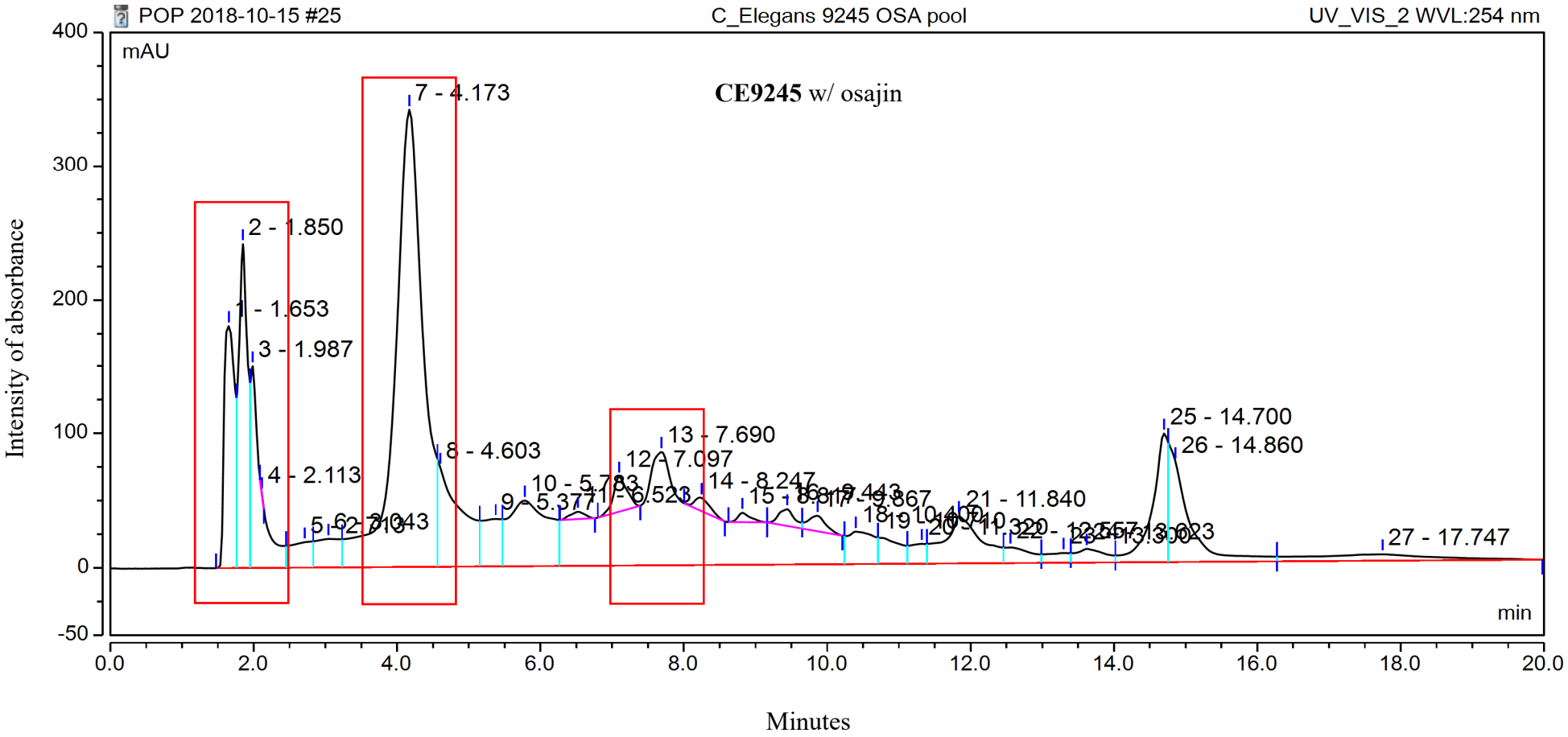


Figure 3: HPLC analysis and fungal metabolism of **CE9245** with osajin by UltiMate 3000 HPLC system.

Negative control of CE9245

The chromatogram in figure 4 is a negative control of **CE9245.** The purpose of these results is to display that the obtained peaks within the previous chromatograms could be nothing other than formed metabolites. The array of peaks that can be seen from the 1.5 minute mark onwards is either residual ethyl acetate obtained from the extraction, or pieces of the fungal membrane, with a negligible area under the curve which does not interfere with probable metabolite peaks obtained.

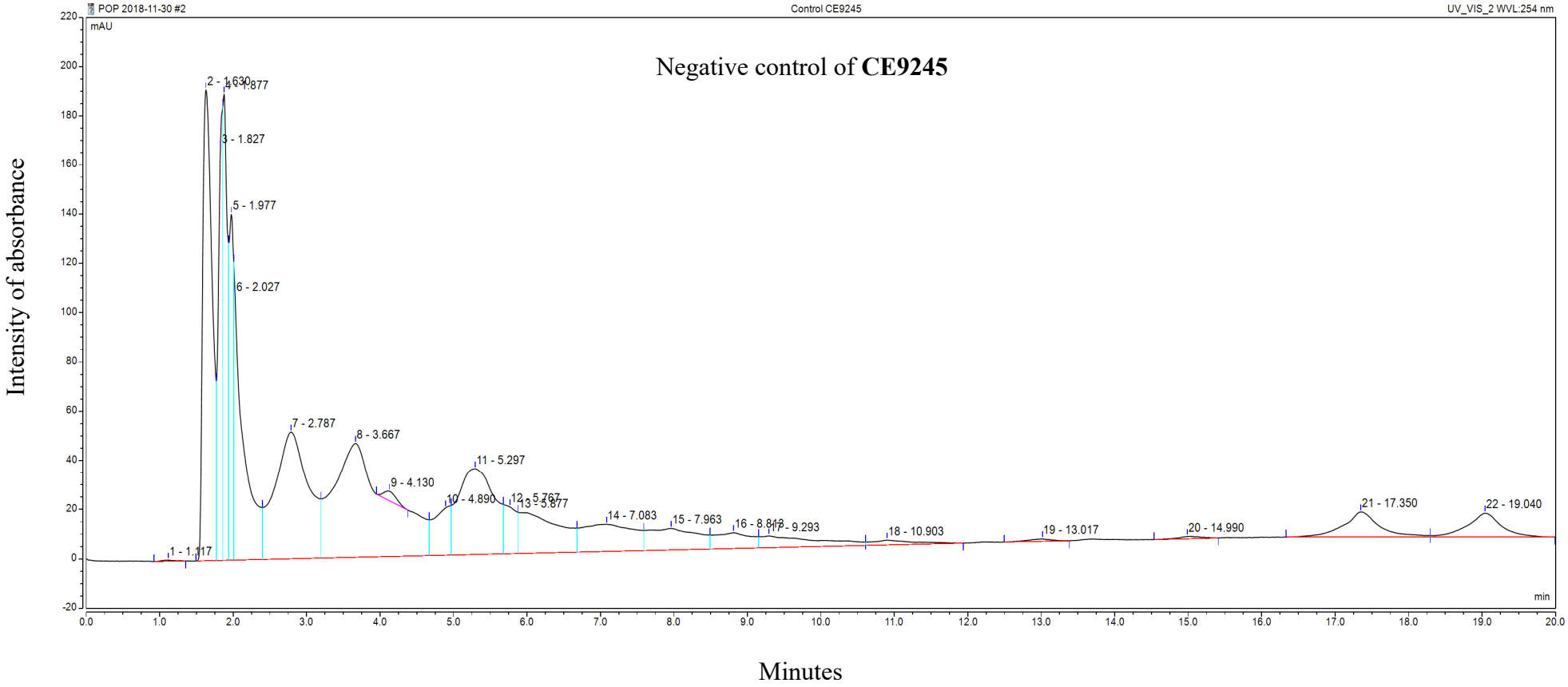


Figure 4: A negative control of CE9245 by UltiMate 3000 HPLC system.

***Fungal biotransformation of pomiferin by Umbelopsis ramanniana* (Möller) Gams**

As seen in the HPLC chromatogram, fungal biotransformation of pomiferin by *Umbelopsis ramanniana* (Möller) Gams after seven days produced metabolites. The red box at the 8-minute mark indicates formed metabolites (Figure 5). The higher peak identified at the 14 minute mark is unmetabolized pomiferin. As indicated by the presence of peaks at a lower retention time, these results are consistent with the formation of metabolites.

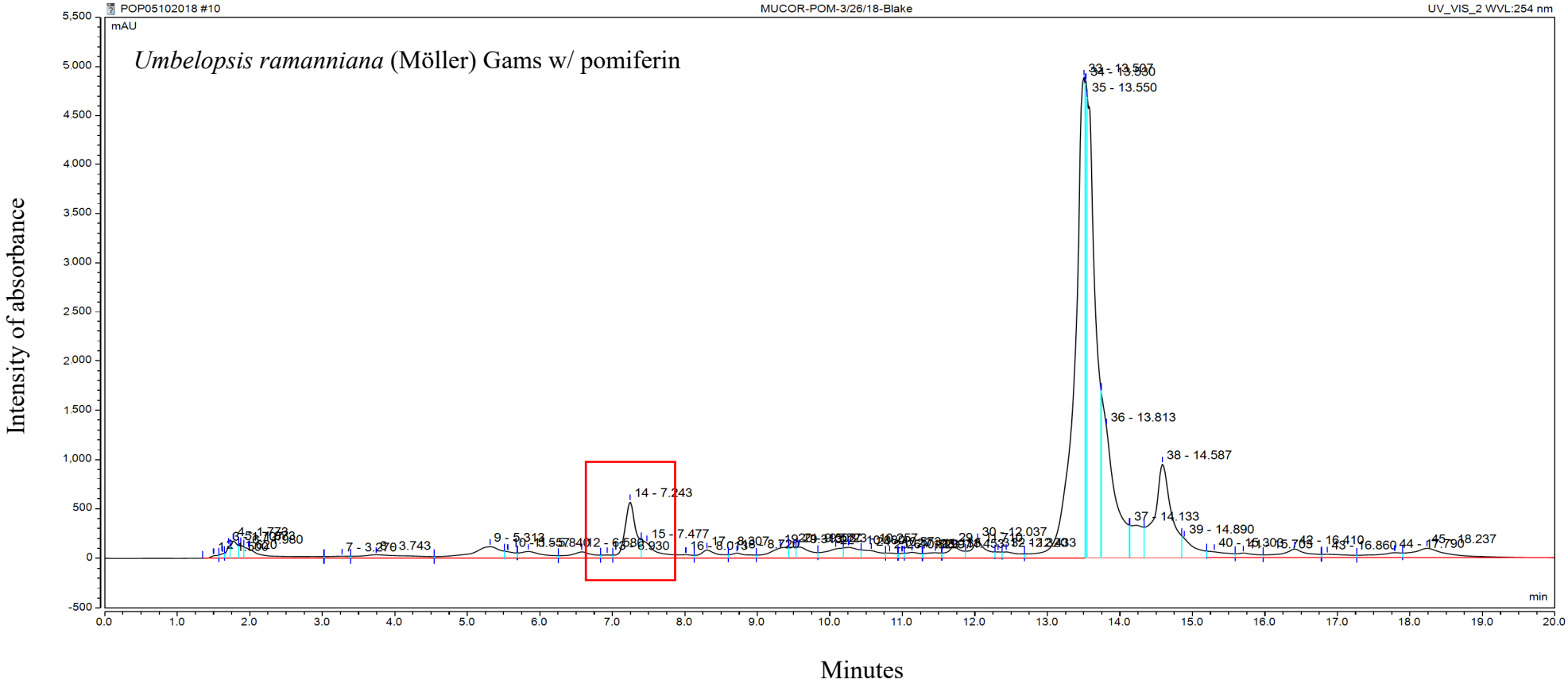


Figure 5: HPLC analysis and fungal metabolism of *Umbelopsis ramanniana* (Möller) Gams with pomiferin by UltiMate 3000 HPLC system.

***Fungal biotransformation of osajin by Umbelopsis ramanniana* (Möller) Gams**

Fungal biotransformation of pomiferin by *Umbelopsis ramanniana* (Möller) Gams over seven days formed potential metabolites as shown in the HPLC chromatogram. These possible metabolites are displayed at the 2 and 5.5-minute mark by the red box (Figure 6). The greater peak identified at the 14.5-minute mark is unmetabolized osajin. These results are consistent with the formation of metabolites, as indicated by the presence of peaks at a lower retention time.

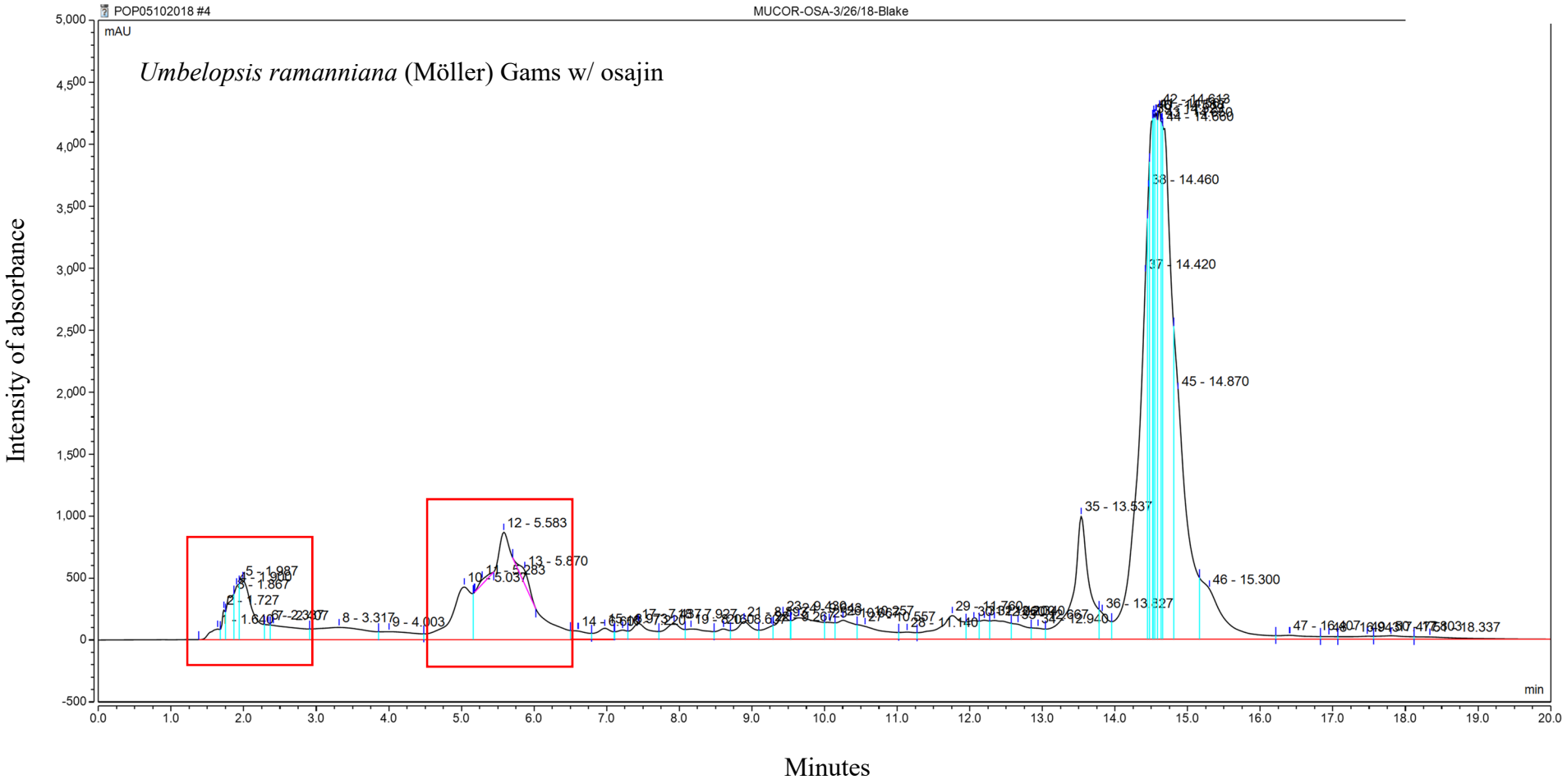


Figure 6: HPLC analysis and fungal metabolism of *Umbelopsis ramanniana* (Möller) Gams with osajin by UltiMate 3000 HPLC system.

***Negative control of Umbelopsis ramanniana* (Möller) Gams**

The following chromatogram is a negative control of *Umbelopsis ramanniana* (Figure 7). The purpose of these results is to display that the obtained peaks within the previous chromatograms could be nothing other than formed metabolites. The array of peaks that can be seen from the 1.5 minute mark onwards is either residual ethyl acetate obtained from the extraction, or pieces of the fungal membrane.

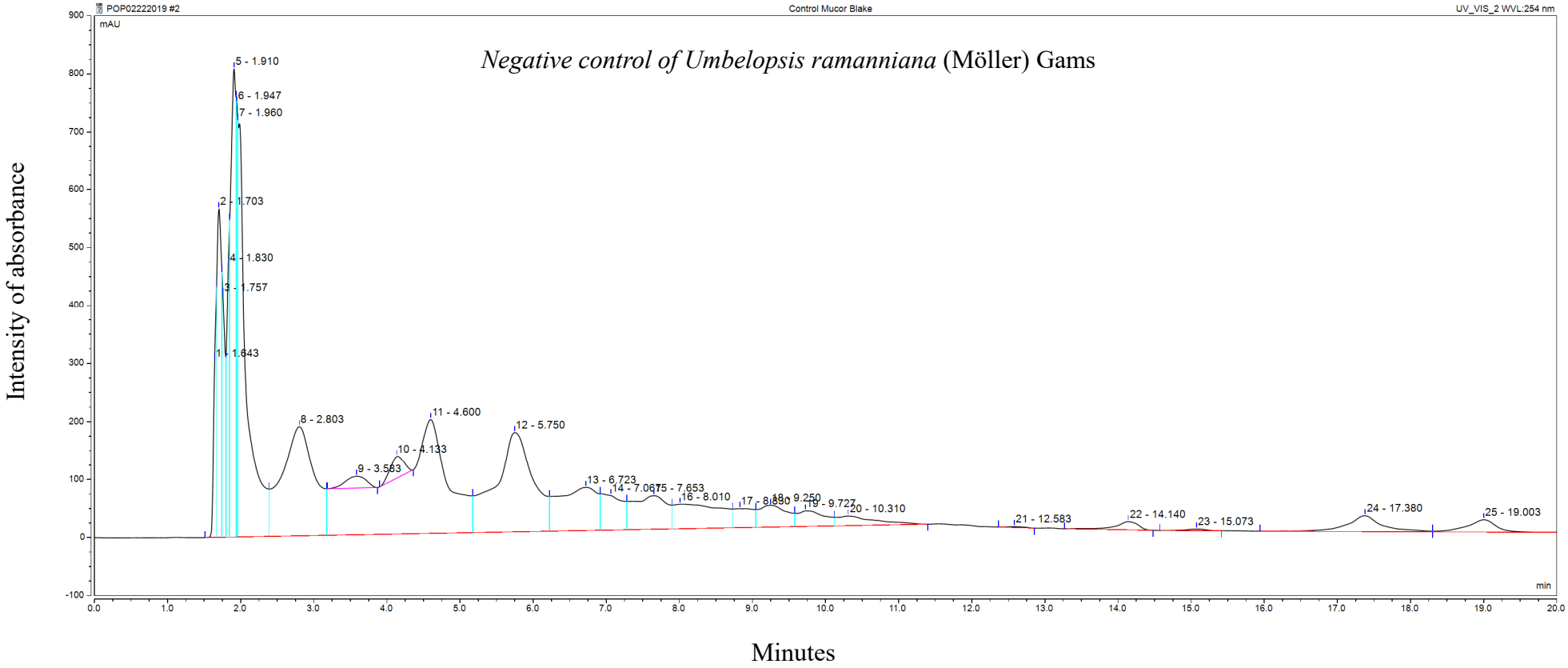


Figure 7: A negative control of *Umbelopsis ramanniana* by UltiMate 3000 HPLC system.

***Fungal biotransformation of pomiferin by Aspergillus fumigatus* fresnius**

The fungal biotransformation of pomiferin by *Aspergillus fumigatus* fresnius over seven days produced possible metabolites as identified in the HPLC chromatogram. The red box indicates these potential metabolites at the 6-minute mark (Figure 8). The larger peak identified at the 14.5 minute mark is unmetabolized pomiferin. As indicated by the presence of peaks at a lower retention time, these results are consistent with the formation of metabolites.

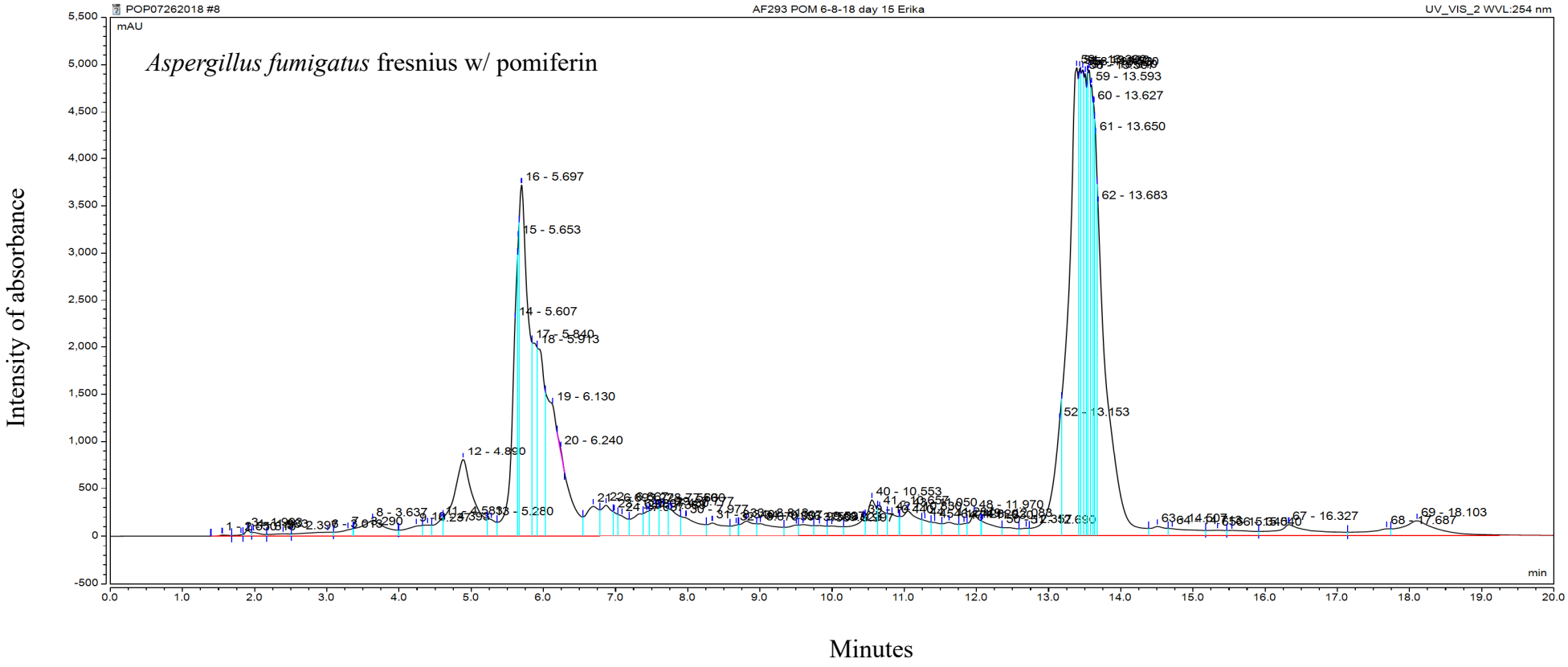


Figure 8: HPLC analysis and fungal metabolism of *Aspergillus fumigatus* fresnius with pomiferin by UltiMate 3000 HPLC system.

***Fungal biotransformation of osajin by Aspergillus fumigatus* fresnius**

As shown in the HPLC chromatogram, fungal biotransformation of pomiferin by *Aspergillus fumigatus* fresnius over seven days produced possible metabolites. These possible metabolites are indicated at the 6-minute mark by the red box (Figure 9). The unboxed peak identified at the 14.5-minute mark is unmetabolized osajin. These results are consistent with the formation of metabolites, as indicated by the presence of peaks at a lower retention time.

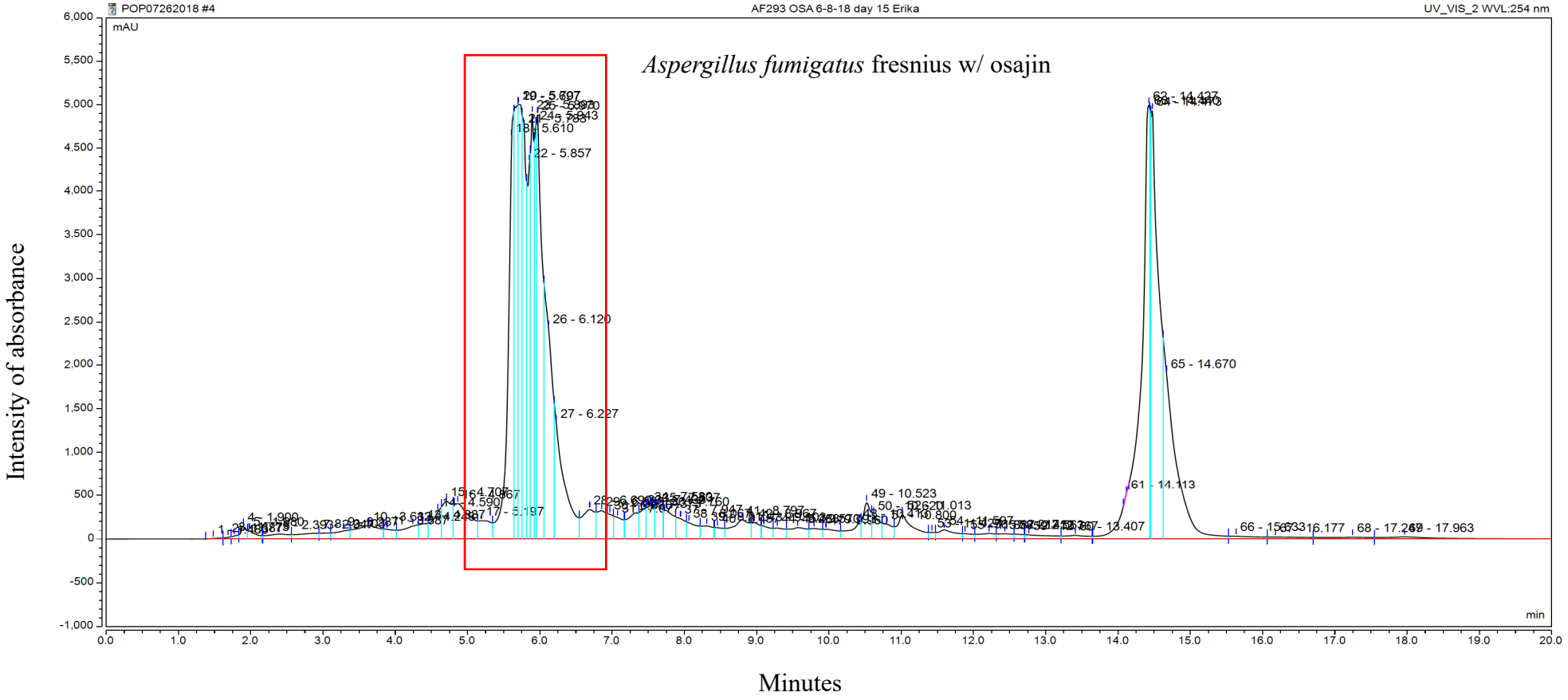


Figure 9: HPLC analysis and fungal metabolism of *Aspergillus fumigatus* fresnius with osajin by UltiMate 3000 HPLC system.

Negative control of Aspergillus fumigatus fresnius

The following chromatogram is a negative control of *Aspergillus fumigatus fresnius*. The purpose of these results is to display that the obtained peaks within the previous chromatograms could be nothing other than formed metabolites. The array of peaks that can be seen from the 1.5 minute mark onwards is either residual ethyl acetate obtained from the extraction, or pieces of the fungal membrane.

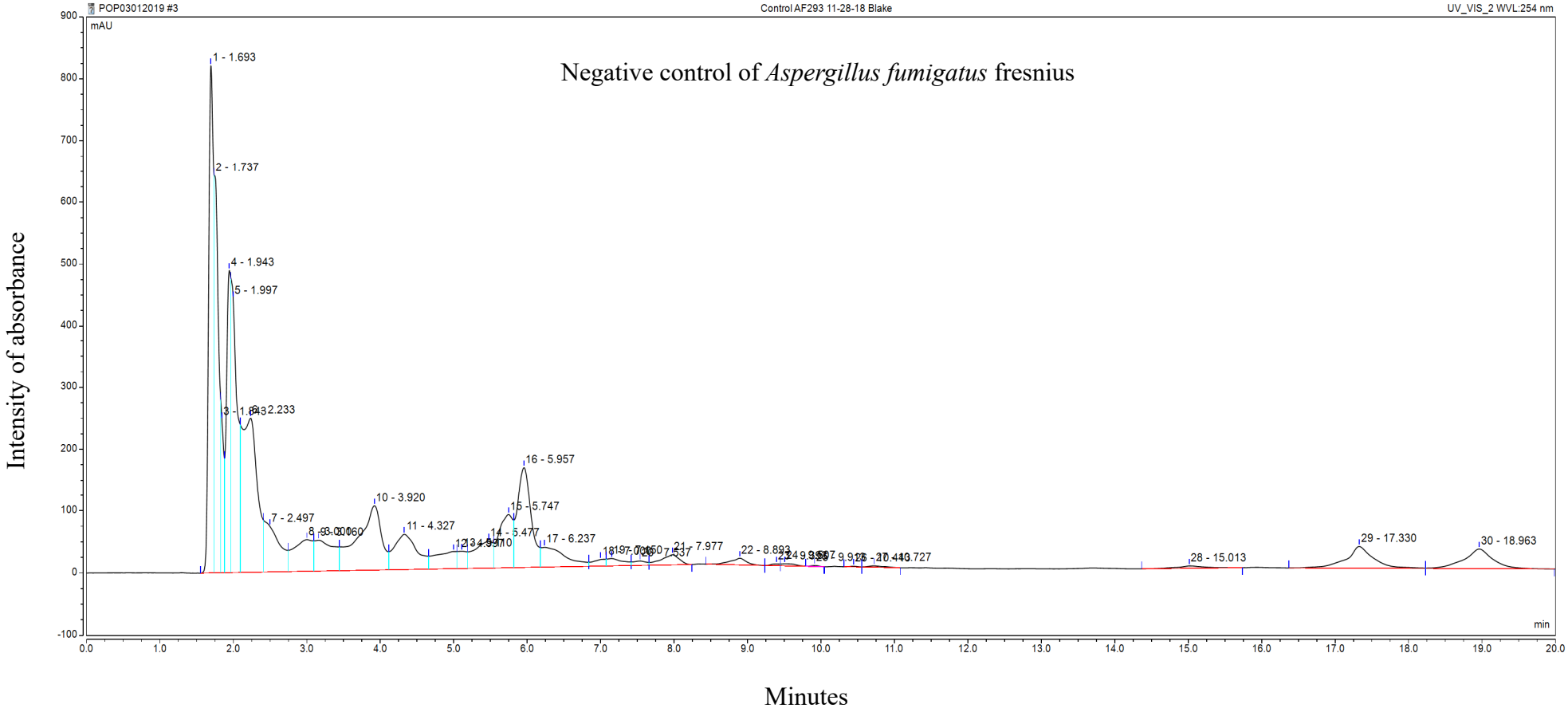


Figure 10: A negative control of *Aspergillus fumigatus* fresnius by UltiMate 3000 HPLC system.

Conclusion

The expected results of this project is the production and possible applications of osajin and pomiferin derivatives/metabolites. Additionally, this project will offer evidence that fungi can be used to bio-transform specifically osajin and pomiferin into useful metabolites. This project will also provide both the proof of concept and a viable method of obtaining osajin and pomiferin derivatives.

Formation of metabolites from CE9245

To date, all metabolic activity of **CE9245** with both pomiferin and osajin has been documented and recorded. As observed in figure 3, it can be said that **CE9245** metabolized osajin more efficiently than that of pomiferin. Within figure 2, at the 15 minute mark, there is still a significant amount of unmetabolized pomiferin that is still present compared to that of figure 3 where almost all of the administered osajin has been metabolized.

Formation of metabolites by supporting strains Umbelopsis ramanniana (Möller) Gams *and Aspergillus fumigatus* fresnius

When observing the chromatograms of the supporting strains that were administered osajin and pomiferin, (*Umbelopsis ramanniana* and *Aspergillus fumigatus*), it can be said that both osajin and pomiferin were metabolized successfully by these strains. This is indicated by the presence of possible metabolites (the peaks) at the assorted minute marks on the chromatograms.

Future directions

With the current obtained results, we do know that metabolites have been produced. The next step is to send the metabolites for NMR analysis to determine the metabolic structures that have been produced.

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