The Quantification and Extraction of Phenolic Acids from Blueberry Leaves Sourced from the Local Flanders and Limburg Regions as a Potential Source for Bio-active Starter Molecules

Word Count: 6,745

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21/05/2024

Deze studie werd mede mogelijk gemaakt dankzij financiële steun van het Interreg Vlaanderen–Nederland-project 'Circulaire Teelt en Chemie'

Interreg Vlaanderen-Nederland

Gefinancierd door de Europese Unie

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1. Abstract

Phenolic acids are a naturally occurring and highly varied subclass of polyphenols found within a variety of plants. Although most notably valued for their antioxidant properties and ability to impart foods with a bitter and astringent taste, there has been a growing interest in the use of phenolic acids as raw materials for the synthesis of biodegradable plastics and sustainable materials. As such, the following study served as a preliminary investigation into the extraction of phenolic acids from agricultural residues for use as bio-active starter compounds in polymer synthesis amongst other applications. The study explored which phenolic acids could be extracted from locally sourced *Vaccinium Corymbosum* blueberry leaves before quantifying their concentrations and exploring alternative extraction methods. Results showed that caffeic acid, coumaric acid, and ferulic acid could all be detected within the leaves, with caffeic acid boasting an average concentration of 0.01 – 0.05 M and coumaric acid an average concentration of 0.001 – 0.006 M respectively. Unfortunately, as ferulic acid was only detected in trace amounts, its concentration in the leaves could not be quantified.

2. Introduction

2.1 *Background*

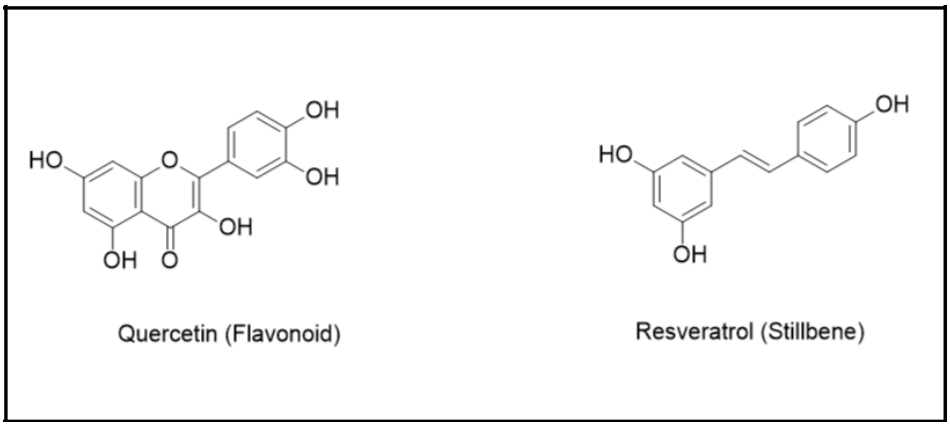


Figure 1. *Example of a Flavonoid and Stilbene*

Polyphenols, such as the flavonoid and stilbene depicted in **Figure 1**, are a highly diverse class of compounds possessing multiple phenolic rings connected through a variety of chemical bonds. As some of the most notably abundant phytochemicals, they are primarily found within plants (though certain species of algae, fungi, and bacteria are also known to possess them). Arising from the pentose phosphate, shikimate, and phenylpropanoid plant pathways, their functions in nature vary from protection against pathogens and UV radiation, to attracting pollinators and regulating oxidative stress.[1-4]

When regarding human use and consumption however, they are most commonly known for their antioxidant properties and for giving fruits and vegetables their signature sensory features (colour, taste, and smell). Anthocyanins and tannins for instance, are well-known in the agriculture and food industries for imparting upon citrus fruits and wines their vibrant colours and astringent taste. Whereas within the pharmaceutical industry, a number of polyphenol subgroups are known to possess a wide variety of beneficial physiological properties. These range from being antioxidative, anti-allergenic and anti-inflammatory, to being anti-microbial and anti-thrombotic to name a few. It should thus come as no surprise that these compounds are often further supplemented within food products and used as “lead compounds” or delivery compounds (in the form of nanoparticles) in the research sector of pharmaceutical companies.[3-8]

Certain polyphenol subgroups, however, also possess differing and more complex applications beyond dietary supplements or starter compounds for drug design. Phenolic acids for instance, are a highly promising subgroup of polyphenols. They possess a highly reactive carboxylic acid group connected to an electrophilic aromatic ring, making them susceptible to a myriad of chemical reactions.[2,9] Most interestingly, due to their structural properties, they are highly capable of being polymerized to form bio-based materials. From condensation reactions with aldehydes at the reactive hydroxyl group to form polymer chains, to electrophilic aromatic substitutions of the electrophilic ring to form polymer networks and cross-linking reactions, theoretically speaking, the topic of directly synthesizing biomaterials

from phenolic acids is indeed promising. Although a novel concept, there is a burgeoning amount of research focused on developing efficient synthesis strategies for these phenolic acid derived polymers with an emphasis on sustainability, scalability, and economic viability. Esterification of phenolic acids with alcohols to generate polymer backbones or polycondensation reactions between phenolic acids and diols/diamines to form high-molecular weight polymers are some of the more common methods found in literature. Whereas the use of the Passerini and Ugi reactions to synthesize complex polymers in a single step are of a more novel nature.[2, 9-11]

With that in mind, although there are few studies discussing the successful and optimal synthesis of such polymers from phenolic acids, there are countless examples of polyphenol derived materials. For instance, a study titled [the] "Engineering [of] functional mesoporous materials from plant polyphenol-based coordination polymers" by Feng[9] showcases the value of polyphenol derived adhesives, coatings, moulding compounds, and binders for their high heat and chemical resistance on top of their excellent mechanical properties. Similarly, there are countless other examples of plant-derived biomaterials made primarily from phenolic compounds. Bioplastics have been synthesized from polylactic acid for use in packaging and medical implants, and similarly, bioplastics comparable to conventional petroleum-based plastics have been synthesized from lignin (a complex phenolic compound found in plant cell walls).[12,13] It could thus be assumed that such applications for phenolic acids is also very viable.

This high applicability of phenolic acids within the materials industry, however, is not the only appealing aspect for these bioplastics/biomaterials. Compared to petroleum-based materials, phenolic acid derived materials would have the added benefit of being biodegradable (thus producing fewer toxic by-products in their degradation process), generating a smaller carbon footprint in their production line, and being a renewable resource as they are naturally occurring.[12,13]

2.2 *Literature Overview of Phenolic Acid Extraction*

To put things into perspective, phenolic acids are highly applicable within several fields, naturally occurring, and relatively abundant. This makes their use both highly sustainable and potentially more environmentally friendly than many alternatives. As such, it's not surprising that much interest has shifted from standard petroleum-based polymers to the extraction of phenolic compounds from plants for subsequent polymerization.[1-13] Indeed, a variety of extraction methods have been created, fine-tuned, and annexed to fully optimize the extraction of said compounds from a variety of differing plant mediums. The most common and effective techniques (as of now) come in the form of sonication, supercritical fluid extraction (SFE), solvent extractions, microwave assisted extractions (MAE), and Soxhlet extractions. Each technique possesses a varied success rate dependent on the extraction source (fruit, pomace, leaves, etc), solvent(s) used, and subclass being targeted for extraction. All of which stem from a number of common complications. Namely, the extraction of phenolic acids is made difficult by the:

1. **Complexity of plant matrices:** Plant matrices are made complex by the myriad of other compounds found within them (carbohydrates, lipids, proteins, etc). Furthermore, the concentration of phenolic acids can be highly varied depending on which plant tissues they are sourced from.

2. **Chemical diversity of phenolic acids:** The phenolic acid class is highly varied and thus differing compounds may be more or less soluble in certain solvents making selectivity difficult.

3. **Stability of phenolic acids:** Many phenolic acids are prone to degradation during the extraction process.

4. **Low extraction efficiency:** Achieving high extraction yields while minimizing solvent consumption is challenging. Furthermore, very high purity is needed to use the compounds in polymer synthesis making it even more challenging.

5. **Analytical difficulties:** Quantification of phenolic acids requires sensitive analytical techniques and is made difficult by the presence of interfering compounds and matrix effects.[3,14-17]

Nonetheless, there exists a number of published studies both investigating and optimizing the effectiveness of these techniques. In their study titled "MAE of phenolic compounds from blueberry leaves and comparison with other extraction methods" for instance, Routray and Orsat[14] compare an optimized method for the microwave assisted extraction of phenolic compounds to a standard sonication extraction. Both the MAE and sonication assisted extraction were carried out utilizing a solvent mixture of 85 or 70 % ethanol and 15 or 30 % citric acid. The sonication was performed at room temperature for an hour in the dark whereas the MAE was performed for 4-16 minutes at 800W. The results of the study not only demonstrated a strong statistical relationship between microwave power and time to the total phenolics extracted, but also showcased that total phenolics extracted via MAE was much higher (92.719 – 128.76 mg GAE/g dry weight) than that extracted from one hour of sonication (average of 97.77 mg GAE/g dry weight).[14]

In a different study, Mustafa et al[18] attempt to develop "A new HPLC-MS/MS method for the simultaneous determination of 36 polyphenols in blueberry, strawberry and their commercial products" and in the process are capable of both identifying and quantifying 12 different phenolic acids found in both strawberry and blueberry fruits and jams. With the phenolic acid content varying from 1-7 mg/kg of fresh weight (for caffeic, gallic, coumaric, and cinnamic acids), the study showcases the plausibility of extracting these compounds for subsequent use.[18]

2.3 *Research Objectives*

Based on this burgeoning new field of research and in response to the growing interest for bio-based materials sourced from green renewable sources, the Aachen-Maastricht Institute of Biobased Materials and Maastricht University have partnered with the University of Hasselt, the agricultural organization "**Compas Agro BV**", and the Research Centre for Fruit Growing, "**pcfruit**", on a new promising venture donned the

"**CTC**" project (*Circular Cultivation Chemistry Project*). The project has the aim of answering whether highly functionalized biomolecules locally sourced from sustainable plant cultivation can be increased in yield, fractionated with high purity, valorised as circular materials, and be implemented in both a sustainable and socio-economically responsible manner.

The following study, though based on this project, is of a smaller scale and focuses primarily on the quantification and extraction of phenolic acids from locally sourced blueberry leaves (as they are often residue from agricultural practices). The research question addressed in the study is: **"What phenolic acids can be extracted from local blueberry leaves and at what concentration are each of these phenolic acids found within them?"**

The study aimed to first qualitatively assess which phenolic acids can in fact be found within the provided leaf samples before attempting to quantify the concentration of each individual acid. Finally, when certain key compounds of interest were identified, the final piece of research focused on exploring alternative extraction techniques so as to further optimize the extraction methodology.

3. Materials and Methods

3.1 *Overview*

The research was conducted in three stages. Firstly, a qualitative analysis of 21 *Vaccinium Corymbosum* leaf samples was conducted to investigate which individual phenolic acids could in fact be detected post extraction. This was done using a generic acidic solvent extraction followed by an alkaline hydrolysis to both "free" the compounds of interest from the cell matrix and cleave off any sugar moieties attached to them. A subsequent analysis of each sample was then performed using liquid chromatography-mass spectrometry (LC-MS).

Next, a quantitative analysis (determination of concentration) of each sample was attempted for each compound of interest. In order to achieve this, calibration curves were created to estimate the concentrations of each compound of interest found within each sample. This was all done utilizing the photodiode array detector (PDA) of the liquid chromatography-mass spectrophotometer.

Finally, the remaining research time was spent investigating alternative extraction methods for future applications. Two different extraction methods (notably a sonication assisted extraction and a Soxhlet extraction) were performed on two duplicate samples for comparison with the generic extraction method performed in the first stage of research. The same calibration curves utilized in the second stage of research were used to analyze the results of these experiments.

3.2 *Chemicals*

Reagents, including: caffeic acid, p-coumaric acid, ascorbic acid, ethylenediaminetetraacetic acid, hydrochloric acid, and sodium hydroxide were purchased from either Sigma-Aldrich (Zwijndrecht, Netherlands) or VWR International BV (Amsterdam, Netherlands). The following solvents utilized in the study were also purchased from the same suppliers: ethyl acetate, heptane, ethanol, and methanol.

3.3 *Leaf Samples*

All leaf samples were provided by the Dutch agricultural company "**Compas Agro BV**". Upon acquisition, samples were stored in zip-lock bags at -20 ^{O}C until utilization. All the samples analyzed were of the *Vaccinium Corymbosum* species, however, **each corresponding sample number (of which there were 21 total) indicated a difference in the growing conditions of the blueberry parent plant.** These differences in growing conditions however were not communicated.

Regarding sample preparation, initially, prior to acidic extraction, the leaf samples were dried in the oven at 40 ^{O}C for four days in aluminum pans before being crushed into a powder using a mortar and pestle. However, later samples such as numbers 113, 118, 21, 23, 25, 27, 27A, and 27B were instead frozen using liquid nitrogen before being similarly crushed. The crushing step was taken to increase the surface area of the plant material (thus increasing solvent "contact") whereas initially drying was done to minimize the effects of

excessive moisture in the sample (such as diluting the extraction solvent or competing over binding sites with the solvent).[14-17]

3.4 *Acidic Extraction*

The acidic extraction procedure performed on each sample was obtained from the University of Hasselt's research group. The method in question is in line with literature online and was already in use by the University for the determination of total phenolics and antioxidant activity.

The extraction solvent used was a solution of 80% ethanol and 1% HCl. The procedure consisted of adding the acidic solvent to the powdered leaf samples at a ratio of 1:10 weight per volume in plastic falcon tubes. The tubes were then vortexed in the dark for 30 seconds before being wrapped in aluminum foil and incubated in ice for 5 minutes (also in the dark). Next, the samples were centrifuged at 4,000 rpm and 4 ^{o}C for 30 minutes and the supernatant recovered. Prior to performing the alkaline hydrolysis, the solvent was removed via rotavapor at 38 ^{o}C to prevent degradation.

***NOTE:** As the research project was conducted in partnership with the University of Hasselt, four aliquots (of 1 ml each) were taken from 12 of the extracted samples and sent to the University for analysis of total phenolics and antioxidant activity. These samples will be marked with an asterisk in all subsequent sections.

3.5 *Basic Hydrolysis*

As a large portion of polyphenols are found in vacuoles, they are typically extracted as functional derivatives (such as methyl esters), or as conjugates with mono/polysaccharides attached to them.[2] As such, subsequent to the acidic extraction, an alkaline hydrolysis was performed on each sample based upon a similar procedure found in the following piece of literature: "Comparison of Antioxidant Capacity and Phenolic Compounds of Berries, Chokecherry, and Sea Buckthorn".[19]

The solution utilized for the hydrolysis was made by mixing 8 g of sodium hydroxide, 1 g of ascorbic acid, and 0.3 g of ethylenediaminetetraacetic acid in 100 ml of distilled water.[19] The inclusion of EDTA in the solution can be attributed to its ability to chelate metal ions, thus preventing them from oxidizing the phenolic acids. Similarly, ascorbic acid acts as a mild reducing agent and antioxidant, donating electrons to free radicals and preventing any oxidation reactions from occurring.[20] The resulting solution was neon orange in color and stored with an aluminum foil covering to minimize the oxidation of ascorbic acid.

The procedure was performed by adding the sodium hydroxide solution to the dried product of the acidic extraction in a 2:60 weight per volume ratio. The mixture was then placed in a rotary shaker at 280 rpm for 2 hours before being acidified to pH 1.5-2.5 utilizing ice cold 6 M HCl. The solution was then centrifuged at 5 ^{o}C and 4,500 rpm before being filtered over vacuum (porosity grade 4). The resulting solution was washed with heptane to remove lipids, before extracting the phenolic acids with ethyl acetate.[19] Finally, the ethyl acetate was evaporated using a Rotavapor at 38 ^{o}C to prevent degradation.

3.6 *Sample Analysis Qualitative and Quantitative*

The qualitative analysis of each sample was performed utilizing a Triple Quadrupole LC-MS equipped with an electrospray ionization source operating in both negative and positive ionization modes. The mobile phase was a mixture of (A) milli-q water and (B) acetonitrile with both being 0.1% formic acid. The flow rate was 1 mL min^{-1} with the mobile phase varying as such: 0-50 seconds, isocratic condition, 50 seconds – 5 minutes, B; 5%, 5-20 minutes, B; 5%, 20-30 minutes, B; 15%, 30-40 minutes, B; 30%, 40-42 minutes, B; 70%, 42-42 minutes and 10 seconds, isocratic condition, B; 70%, 42 minutes and 10 seconds – 43 minutes, B; 5%. All samples were dissolved in methanol prior to analysis. Example MS and PDA spectra of the results can be found in **Appendix A**.

For the quantitative analysis, two calibration curves were created utilizing the PDA of the LC-MS. The caffeic acid calibration curve was created using measurements at a lambda max of 327 nm whereas for coumaric acid a lambda max of 286 nm was utilized. The concentrations of the curves ranged from 0.000012 M to 0.006 M with both curves containing at least 5 points. All samples from which an aliquot was taken for the University of Hasselt were adjusted mathematically to account for the loss of 4 ml of the extract as such:

((h [ ]) ([ ])) – adjusted weight. (([ ]) – (4))

3.7 *Optimization Experiment 1: Sonication Assisted Acidic Extraction*

A duplicate of sample 27 was prepared for the purpose of a sonication assisted acidic extraction. This sample was donned sample 27B. Sample 27 was chosen to duplicate as it possessed fully resolved/large peaks in its PDA spectrum (thus quantification of all compounds of interest was possible) and because no aliquots were taken from it (making its calculated concentrations more accurate than other samples).

In essence, the experiment consisted of switching out the “vortex” and “incubation” step of the generic acidic extraction with sonication in hopes that this would increase the extraction efficiency of the acidic solvent. As such, the same exact procedure was used in all other steps of the extraction. The sonication was performed in the dark at circa 20 ^{O}C for 15 minutes. The extraction was also followed by an alkaline hydrolysis and was analyzed in the same manner as all other samples.

3.8 *Experiment 2: Soxhlet Extraction*

A singular Soxhlet extraction was performed on a duplicate of sample 27. The sample was donned 27A. This extraction was performed to investigate a possible alternative extraction method that would be more optimal than the generic acidic solvent extraction performed in the first stage of research. The Soxhlet extraction was performed on the powdered leaf sample for 3 hours in the dark using circa 70 ml of ethyl acetate. As it was anticipated that the extracted compounds would still be bonded to sugars, an alkaline hydrolysis was also performed on this sample before its analysis by the same method as mentioned above.

4. Results

4.1 *Qualitative Results*

Despite both literature and online databases anticipating the presence of multiple phenolic acids within *Vaccinium corymbosum* berries, there were only three phenolic acids detected after surveying 21 leaf samples. Namely, caffeic acid, coumaric acid, and ferulic acid.

Sample ID	Caffeic Acid	Coumaric Acid	Ferulic Acid
11*			
21			
23			
25			
27			
101*			
102*			
103*			
104*			
105*			
106*			
107*			
108*			
110*			
112*			
113			
114*			
115			
116			
117			
118			

Table 1. *Qualitative results of all 21 blueberry leaf samples analyzed.*

*GREEN = detected in sample with visible peak in both MS and PDA, YELLOW = present in trace amounts, RED = not detected.

***NOTE:** Samples with an asterisk are samples which had aliquots taken out of them before analysis for the University of Hasselt.

4.2 *Quantitative Results*

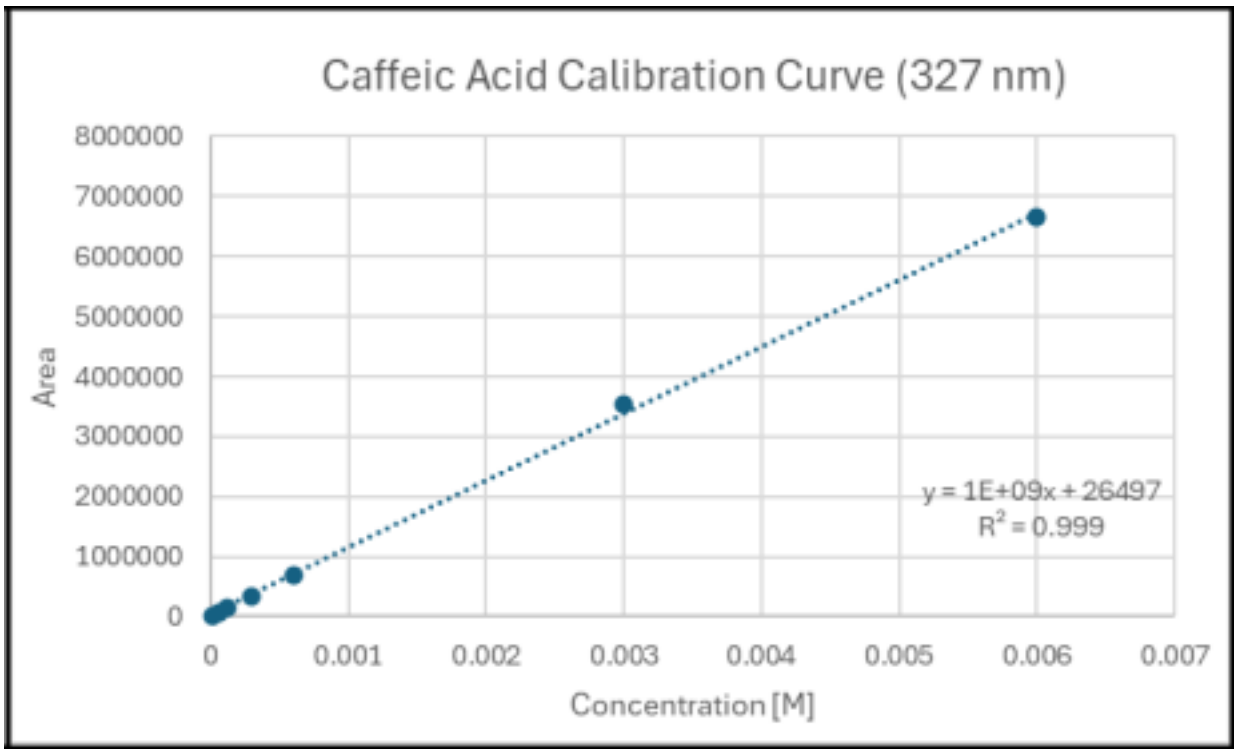


Figure 2. *Calibration curve for caffeic acid (lambda max: 327 nm)*

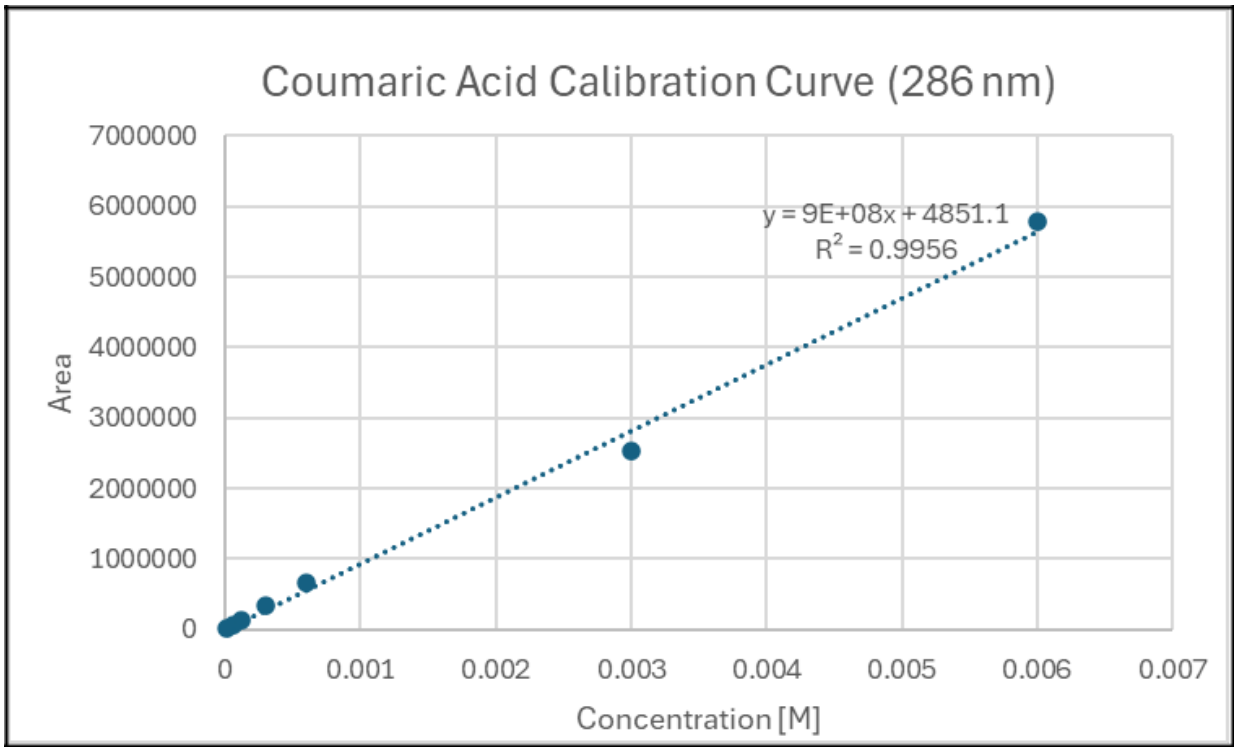


Figure 3. *Calibration curve for coumaric acid (lambda max: 286 nm)*

Sample ID	Wet Weight [g]	Concentration of Caffeic Acid [M]	Concentration of Coumaric Acid [M]	Weight of Caffeic Acid [mg]	Weight of Coumaric Acid [mg]	% Weight of Caffeic Acid to Sample Weight	% Weight of Coumaric Acid to Sample Weight
11*	1.7591	0.015315959	0.002572011	5.518646	0.843863	0.31	0.05
21	1.6528	0.0205692	0.001433452	9.264367	0.587885	0.56	0.035
23	1.6608	0.000905115	0.005401854	0.407664	2.2153989	0.025	0.13
25	1.7945	0.009662084	0.001528154	4.351803	0.6267237	0.24	0.035
27	1.6004	0.005506394	0.003825871	1.984064	1.2552476	0.12	0.08
101*	1.6346	0.007108415	0.017374966	2.561304	5.7006324	0.16	0.35
102*	1.7952	0.030430138	0.001904999	10.96459	0.6250198	0.61	0.035
103*	1.7867	-	0.002949855	-	0.4839157	-	0.027
104*	1.6801	-	0.017677045	-	4.3498073	-	0.26
105*	1.7706	-	0.000372864	-	0.0611673	-	0.0035
106*	1.6404	-	0.003007838	-	0.9868553	-	0.06
107*	1.6768	-	0.021076566	-	5.1863306	-	0.31
108*	1.6395	0.027722737	0.004875116	9.989057	1.5994994	0.61	0.1
110*	1.6328	0.004163269	0.002178823	3.000218	1.42972	0.18	0.09
112*	1.7201	0.031329765	0.00167917	28.22185	1.3773166	1.64	0.08
113	2.0986	0.014474192	0.006222058	5.215341	2.0414236	0.25	0.1
114*	1.7933	0.016450862	0.003190919	5.927575	1.0469234	0.33	0.06
115	1.6301	0.004540688	0.000974	3.272201	0.6391285	0.2	0.04
116	2.0465	0.02184409	0.005151668	15.74173	3.3804691	0.77	0.2
117	1.7613	0.054948509	0.004654181	19.79905	1.5270116	1.12	0.09
118	1.8503	0.0371933	0.035283175	13.401	11.576219	0.72	0.63

Table 2. *Quantitative results of all 21 blueberry leaf samples.*

***NOTE:** Samples with an asterisk are samples which had aliquots taken out of them before analysis for the University of Hasselt. These values were adjusted mathematically to account for this as shown in the methods section.

***NOTE:** Samples highlighted yellow will be considered outliers and not factored in the discussion as their areas in the PDA were higher than the range covered by the calibration curves making their quantification very inaccurate.

The highest concentration of caffeic acid was found in sample 102, whereas the lowest concentration of caffeic acid was found in sample 23. Overall, the concentrations of caffeic acid ranged from as low as 0.0009 M to as high as 0.03 M with the majority of samples falling within the range of 0.01 – 0.05 M. Regarding % weight, samples 108 and 102 boasted the highest ratio of caffeic acid weight to sample wet weight and sample 23 boasted the lowest. The range in which caffeic acid made up the wet weight of leaf samples was thus 0.025 % to 0.61 %. However, on average, caffeic acid made up 0.1 – 0.6 % of the sample wet weight.

The highest concentration of coumaric acid was found in sample 113, whereas the lowest concentration of coumaric acid was found in sample 105. Overall, the concentrations of coumaric acid ranged from as low as 0.0004 M to as high as 0.0062 M with the majority of samples falling within the range of 0.001 – 0.006 M. Regarding % weight, sample 101 boasted the highest ratio of coumaric acid weight to sample wet weight and sample 105 boasted the lowest. The range in which coumaric acid made up the wet weight of leaf samples

was thus 0.0035 % to 0.35 %. However, on average, coumaric acid made up 0.027 - 0.35 % of the sample wet weight.

4.3 Alternative Extraction Techniques

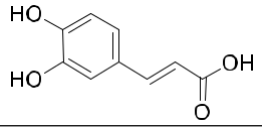
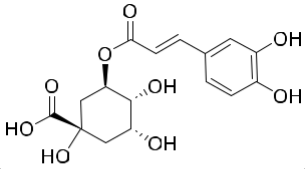
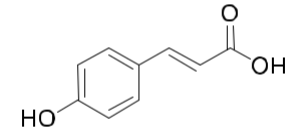
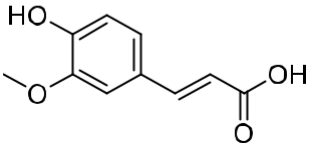
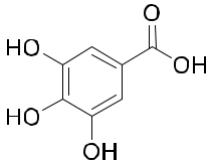
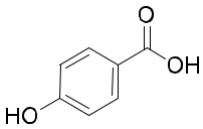
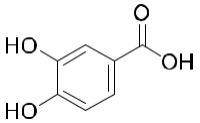
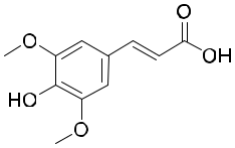
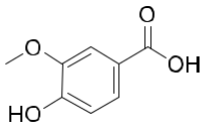
Sample ID	Wet Weight [g]	Concentration of Caffeic Acid [M]	Concentration of Coumaric Acid [M]	Weight of Caffeic Acid [mg]	Weight of Coumaric Acid [mg]	% Weight of Caffeic Acid to Sample Weight	% Weight of Coumaric Acid to Sample Weight
27	1.6004	0.005506394	0.003825871	1.984064	1.2552478	0.12	0.078
27A	0.6086	0.00067014	0.006201721	0.242465	2.034751	0.04	0.33
27B	1.0728	0.002141912	0.003901265	1.157661	1.919976	0.11	0.18

Table 3. *Quantitative results of Soxhlet extraction (A), sonication assisted extraction (B), and generic acidic solvent extraction of sample 27.*

5. Discussion

5.3 *Analysis of Qualitative Results*

According to literature, there are at least 10 different phenolic acids that can be found within both the fruit and leaves of *Vaccinium Corymbosum* blueberry plants.[21-26] These have been summarized in the following table along with a brief commentary on their general stability:

Phenolic Acid	Structure	Stability	Degradation Factors
Caffeic Acid		Relatively unstable, particularly sensitive to oxidation and light exposure.	Light, oxygen, high temperatures.
Chlorogenic Acid		Moderately unstable, degrades readily upon exposure to light and heat	Heat, light, enzymatic activity
Coumaric Acid		Relatively unstable, prone to oxidative degradation	Light, oxygen
Ferulic Acid		More stable compared to caffeic and chlorogenic acids (can still degrade under prolonged exposure to light and oxygen)	Light, oxygen
Gallic Acid		Generally stable under normal conditions (can degrade with high heat and extreme pH levels)	Heat, pH changes
p-Hydroxybenzoic Acid		Relatively stable but can degrade from prolonged light exposure	Light
Protocatechuic Acid		Fairly stable under normal conditions but can degrade with light and oxygen exposure	Light, oxygen
Sinapic Acid		Fairly stable but can degrade when exposed to light and oxygen for extended periods	Light, oxygen
Vanillic Acid		Fairly stable but can degrade when exposed to light for extended periods	Light

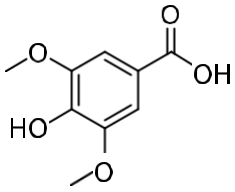
Syringic Acid		Relatively stable but can degrade from prolonged light and oxygen exposure	Light, oxygen

Table 4. *List of common phenolic acids found in the Vaccinium Corymbosum species and their individual stability.*[27-29]

After analyzing 21 different leaf samples, however, only three different phenolic acids were detected. These were caffeic acid, coumaric acid, and ferulic acid. Of these three, coumaric acid was found in the most samples (100% of the samples analyzed) and caffeic acid was found in all but 3 samples (namely, samples 104, 105, and 107). Ferulic acid on the other hand was only found in trace amounts, and even so, only 5 of the analyzed samples were found to contain these traces (samples 11, 101, 102, 113, and 118). As for the reason why these results do not match theoretical expectations found in literature, there could be a variety of possible explanations:

1. **Degradation of compounds:**

As summarized in **Table 4.,** a large number of the phenolic acids found within *Vaccinium Corymbosum* plant matter are prone to degradation whether it be oxygen, pH, light, or temperature induced.[27-29] Although the methods used to extract said compounds attempted to mitigate and prevent the exposure of samples to various degradation factors, it is possible that degradation still occurred at various steps of the extraction and hydrolysis procedure. Aside from this, it is possible that the hydrolysis procedure itself performed on the samples was too harsh and degraded any compounds present in the samples in lower amounts. The opposite is also true where perhaps the hydrolysis procedure was too mild and the compounds are in fact present within the sample but still bound to sugar glucosides or appearing as methylated esters.

That being said, there are a few different factors that combat this theory. Firstly, when inspecting **Table 4.,** we can see that of the 10 phenolic acids discussed, the most unstable ones prone to degradation are: caffeic acid, chlorogenic acid, and coumaric acid. Two of these, namely caffeic acid and coumaric acid (as chlorogenic acid was only detected prior to basic hydrolysis), were part of the only phenolic acids detected. As these two are not only more unstable than the remaining phenolic acids, but also similarly susceptible to light and oxygen, it is unlikely that degradation is the cause for a low number of phenolic acids being detected. In fact, when reading other studies utilizing alkaline hydrolysis to "free" polyphenol compounds from their sugar moieties, we see that the alkaline hydrolysis procedure employed is neither harsh nor mild. In their study of how "Ultrasound-alkaline combined extraction improves the release of bound polyphenols from pitahaya (*Hylocereus undatus* 'Foo-Lon') peel: Composition, antioxidant activities and enzyme inhibitory activity", Zhong et al[29] successfully utilize a 6 M NaOH

solution (compared to the 2 M solution used in this method) to identify a number of phenolic acids (including those mentioned above).

2. Growth and harvest conditions of parent plants:

30-32

As previously stated in the methods, each individual number associated to a sample correlate to a different growing and harvest condition of the parent plant. These differing growing conditions could include soil make-up, exposure to UV radiation, and plant maturity before harvest to name a few. It is thus important to note that all these different factors have a large impact on phenolic acid content.

30-32

For instance, plant maturity plays a large role in phenolic acid content. In a number of studies, it has been found that younger plants earlier in their development cycle often boast higher phenolic acid content. This is due to the needs of the plant being different. At this stage of development, plants are more susceptible to environmental stressors, predation by herbivores, and pathogens. Thus, as phenolic acids play a crucial role in defending plants from said factors, it is thus logical that phenolic acid content would be higher. With this in mind, many studies disagreed that as the plant matures, there is a decrease in phenolic acid content and an increase in anthocyanin content as the plants function switches from defense to propagation through animals and insects. It is thus possible to extrapolate this logic to leaves as within the *Vaccinium Corymbosum* species and many more, phenolic acid content in fruits and leaves are correlated and comparable.

This would mean that the lower number of phenolic acids detected in the samples could also be a result of the maturity of the leaves.

Overall, it cannot be stated confidently that these local *Vaccinium Corymbosum* leaves only possess three types of phenolic acids within them. The samples should all be re-analyzed at least two more times to minimize any human errors that might have occurred throughout the extraction and hydrolysis procedures. Furthermore, the hydrolysis procedure should be tested on a sample or two to determine optimal parameters. The NaOH solution concentration should be varied alongside the ratio of each antioxidative reagent and reaction time. The ratio of solid to solution could also be varied similarly. Furthermore, more information on the plant growing conditions should be acquired as younger leaves harvested after a period of high stress and metabolic activity would theoretically give rise to more phenolic acid content whereas older leaves would not.[30-32]

5.4 *Analysis of Quantitative Results*

The quantitative results of all 21 leaf samples in **Table 2.** showed that the concentration of caffeic acid on average ranged from 0.01 – 0.05 M. Whereas the concentration of coumaric acid ranged from 0.001 – 0.006 M. However promising these results appear to be, it is important to first compare them to available literature.

Although there aren't many studies quantifying the exact concentrations of individual phenolic acids (especially within leaves), the following table is a summary of the average phenolic acid concentrations found in three different applicable studies:

Study	Sample Type	Caffeic Acid Concentration	Coumaric Acid Concentration
1	Blueberry fruit	1.73 ± 2.7 mg/kg fresh weight	2.11 ± 2.6 mg/kg fresh weight
2	Blueberry Leaves	0.5 – 7.4 mg/g dry weight	0.1 – 1.2 mg/g dry weight
3	Blueberry Leaves	1.5 – 5.2 mg/g dry weight	0.3 – 1.5 mg/g dry weight
Average Results (Omitting outliers)	Blueberry Leaves	0.41 – 6.1 mg/g wet weight	0.035 – 3.5 mg/g wet weight

Table 5. *Phenolic acid concentration range compared to three other studies.*[18,33,34]

***Study 1:** "A new HPLC-MS/MS method for the simultaneous determination of 36 polyphenols in blueberry, strawberry and their commercial products and determination of antioxidant activity".[18]

***Study 2:** "Blueberry leaves from 73 different cultivars in southeastern China as a rich source of phenolics".[34]

***Study 3:** "Phenolic Compounds and Antioxidant Properties of Field-Grown and In Vitro Leaves, and Calluses in Blackberry and Blueberry".[33]

When looking at **Table 5**., it can be seen that the concentrations are in fact comparable to what can be found in literature. That being said, there are some outliers within the data set that would merit from being re-done to increase the robustness of these findings.

5.5 *Analysis of Alternative Extraction Techniques*

The alternative methods of extraction performed on sample 27 showed no significant difference between the generic acidic solvent extraction employed on sample 27 and the sonication assisted extraction employed on sample 27B. That being said, the experiment served as a confirmation of the overall accuracy of the quantification method utilized. The caffeic acid concentration of sample 27 is only circa 0.003 M higher than that of sample 27B whereas the coumaric acid concentration of both samples are nearly identical. It should be noted that more coumaric acid (weight wise) was "obtained" from the sonication assisted extraction with a % coumaric acid weight to sample wet weight of 0.18 (as opposed to 0.078 % for sample 27).

The Soxhlet extraction performed on sample 27A was deemed "incomplete" upon inspection of both the mass spectrum and PDA of said sample (provided in **Appendix B**) as there is a clear large chlorogenic acid peak still present in both spectra. This would indicate that the basic hydrolysis of the sample was not successful and thus not all the chlorogenic acid was hydrolyzed into caffeic acid. Along that vein, it is also possible that any coumaric acid glucosides have also not been completely hydrolyzed.

5.6 *Implications and Future Directions*

The results found in this study seem to indicate that the extraction of phenolic acids from blueberry leaves is both feasible and promising. With caffeic acid making up 0.1 – 0.6% and coumaric acid making up 0.027 – 0.35 % of sample wet weight, it is very feasible to upscale the extraction process and generate a large amount of bio-active starter molecules. However, there are still a number of challenges that still need to be addressed:

1. **Increasing robustness of data:**

   Currently, although the results are promising and indicate a high plausibility for the use of phenolic acids extracted from leaves for various applications, the data set lacks much robustness. Firstly, each sample should be analyzed, and their phenolic acid concentrations quantified at least two more times. Furthermore, in order to further increase the robustness of the calibration curves used, an internal standard should be utilized for the next batch of samples.

2. **Separation of phenolic acids:**

   Post acidic solvent extraction and alkaline hydrolysis, the samples were stored as a mixture of caffeic acid, coumaric acid, ferulic acid, and various anthocyanins. As it stands, without a method to separate each of these components from the mixture, it will remain impossible to utilize these compounds for any further applications. As such, a next step in the research project could be to experiment with a variety of separation techniques to acquire high purity fractions. Some techniques that could be looked into might include thin-layer chromatography (TLC) paired with preparative high-performance liquid chromatography (HPLC) or, if a greener approach was desired, a supercritical fluid extraction (SFE).

3. **Alternative greener extraction method:**

   As it stands, the whole extraction method utilized in this study is not very “green”. A large amount of solvent (particularly ethyl acetate) is required to extract the phenolic acids from even a small quantity of extract (about 60 – 100 ml for 1.6 g of wet leaf). As such, alternative methods of extractions, or at least alternative solvents could be looked into to mediate the negative effects of such high solvent consumption.

6. Conclusion

In essence, the extraction of phenolic acids from blueberry leaves is in fact feasible and promising. Caffeic acid and coumaric acid were both detected in high enough concentrations to warrant their extraction and use as bio-active starter molecules from a sustainable source. However, an appropriate separation technique ensuring fraction purity must be developed to make this process possible and a greener extraction technique involving less solvent consumption and removal is necessary to further make this endeavor more compelling.

7. Critical Self-Reflection

During the four-month long research period, I was challenged to deepen my theoretical knowledge and understanding of both organic and analytical chemistry, as well as to quickly pick-up new lab techniques in my arsenal of skills. This proved to be a difficult endeavor to manage while simultaneously working on the written thesis, however, I believe I came out of the project with a better understanding of how to conduct research and manage a project from beginning to end.

An area of the research project that I found quite challenging was adhering to my writing schedule. Initially, I had chosen to schedule a week at the end of my research period to dedicate to writing, with all the time before then being primarily dedicated to conducting experiments. I believed that a week to write up the more prominent sections of my report (the results, discussion, and reflection) would be sufficient as I assumed I would undoubtedly be able to write all the other smaller sections in the evenings after my lab days. This proved to be a gross underestimation of the workload. My research required me to be in the lab almost every weekday from 9am to 5pm leaving little time for writing in the evenings when you factor in travel time and having to read papers in preparation of running new experiments or procedures. In the end, by the time my one week scheduled for writing came around, I had far more than three sections to complete and had to scramble to have enough content to submit for a rough draft. In retrospect, I should have scheduled at least two separate writing periods, one period half-way through the research project, and another at the end. This would have significantly lowered the amount of stress I experienced from this mistake and perhaps contributed to the overall quality of the written thesis.

Similarly, an area which I initially struggled with but eventually adapted to, was the dynamic nature of researching a novel topic. In a university lab practical, students are confronted with trialed and tested reactions/experiments that do not challenge students to think about and perhaps re-evaluate their approach to the experiment. There were a number of times during my research however, where I encountered a problem or found unexpected results that challenged me to re-evaluate how I had chosen to conduct my research. Priorities often changed and certain methods and procedures needed to be amended or stopped altogether. It was initially quite stressful coming into the lab with a preconceived notion of what you needed to get done on that day only to have your results send you straight back to the drawing board to come up with a new approach. A good example of this occurred right at the beginning of my research period when my first task was to extract the phenolic acids from my leaf samples and identify them all using LC-MS. The issue was that although all the literature pointed towards a simple acidic solvent extraction being sufficient for this, neither I nor my supervisor could detect any phenolic acids in our samples. We thus concluded that they were perhaps present in the samples but still methylated or attached to sugar moieties. So, we then found a procedure to conduct a basic hydrolysis on our samples and hoped that this would solve the problem. Unfortunately, even after this, we still could not detect the acids. It was not until I looked into the various components of our solution (such as ascorbic acid and EDTA) and what their purposes were in the reaction that we were able to conclude that we were not putting in enough ascorbic acid and thus inadvertently degrading our

compounds. After encountering quite a few of these types of situations, I feel I have now acquired the right skills and mindset to deal with them effectively.

Although there were a lot of aspects of the research project that I found myself challenged by, there were also a great number of skills I already possessed that I was able to further nurture and refine thanks to this experience. One of which being the ability to multitask and run multiple experiments concurrently without sacrificing the quality of my results. As my research required me to perform extractions on a number of different leaf samples to build a robust data pool, I found myself having to manage and keep track of the extraction of multiple samples all at once. Oftentimes I would have four samples I was performing the acidic solvent extraction on and another four that I was performing the basic hydrolysis on. I am very impressed with the aptitude I showed in being able to manage all of these at once and find that my ability to maximize my time in the lab has grown tremendously. Along with this, I also believe I have developed a higher degree of "finesse" in the lab when it comes to performing common chemistry operations. For instance, through my daily supervisor, I learned many tips and tricks (to add to some I already knew) on how to maximize the efficiency of a procedure or to speed up a process without compromising results.

Overall, this has been a very fruitful four months that I believe has both given me the experience and equipped me with the skills to further myself in the field of chemistry. This has been a great simulation of what research in the "real world" would look like and I am very grateful for all the guidance and support I received throughout the process.

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9. Appendices

8.1 *Appendix A: Example mass and PDA spectra of leaf Samples after acidic extraction and basic hydrolysis*

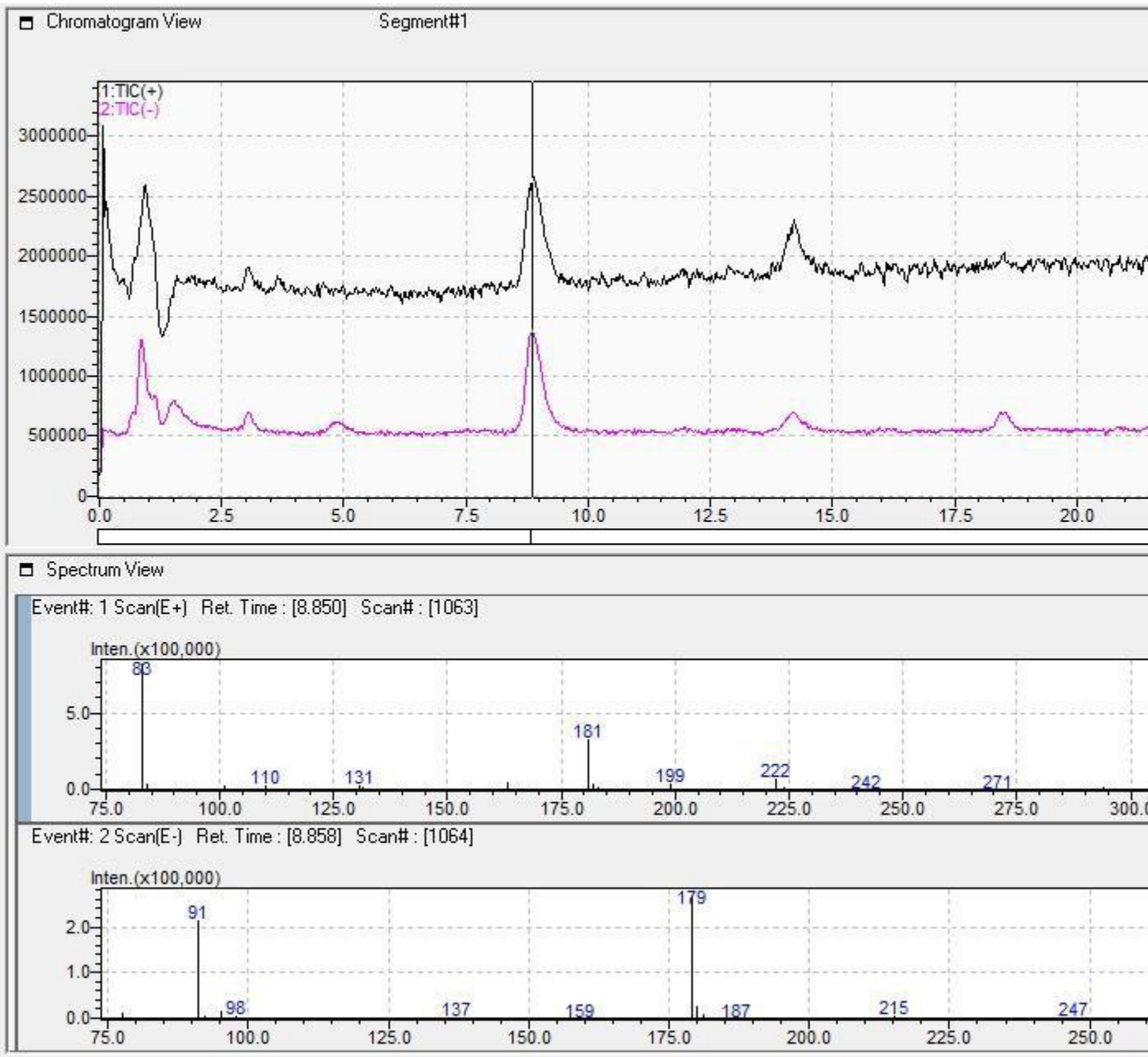

Figure A1. *Mass spectrum of sample 113 (centered on caffeic acid peak)*

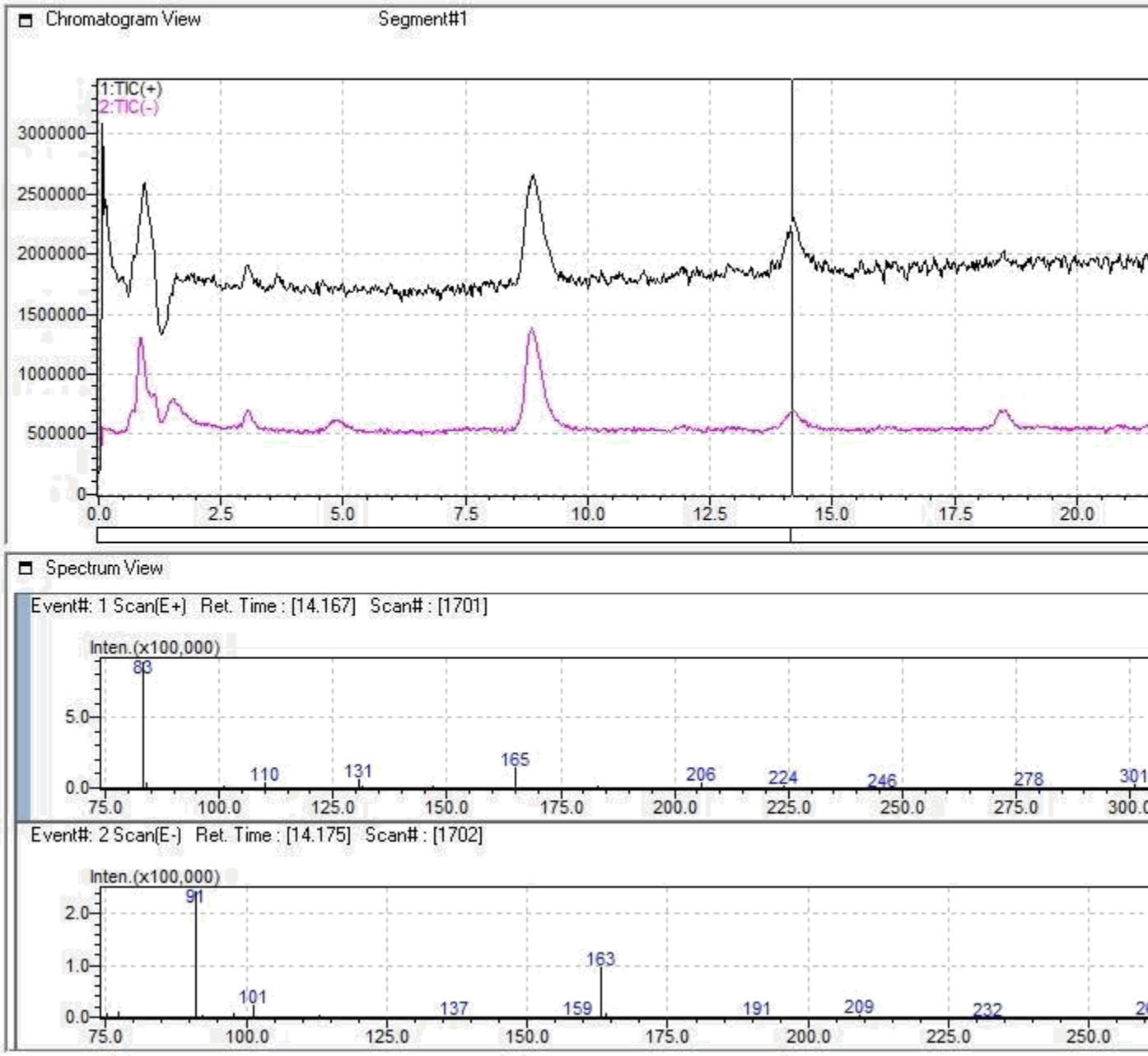

Figure A2. *Mass spectrum of sample 113 (centered on coumaric acid peak)*

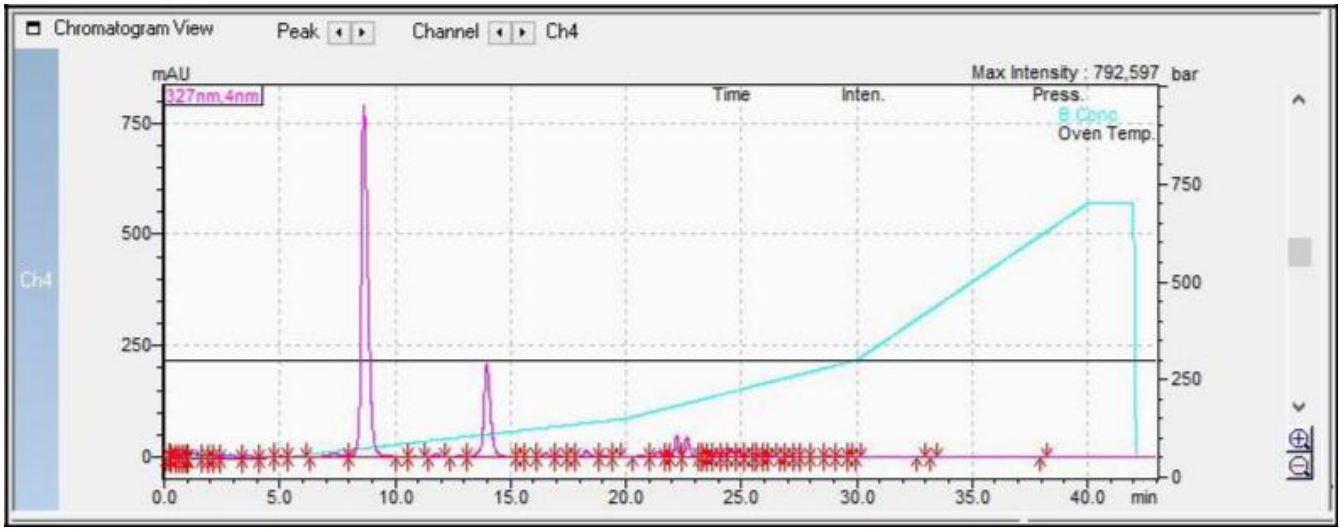

Figure A3. *PDA spectrum of sample 113 (327 nm)*

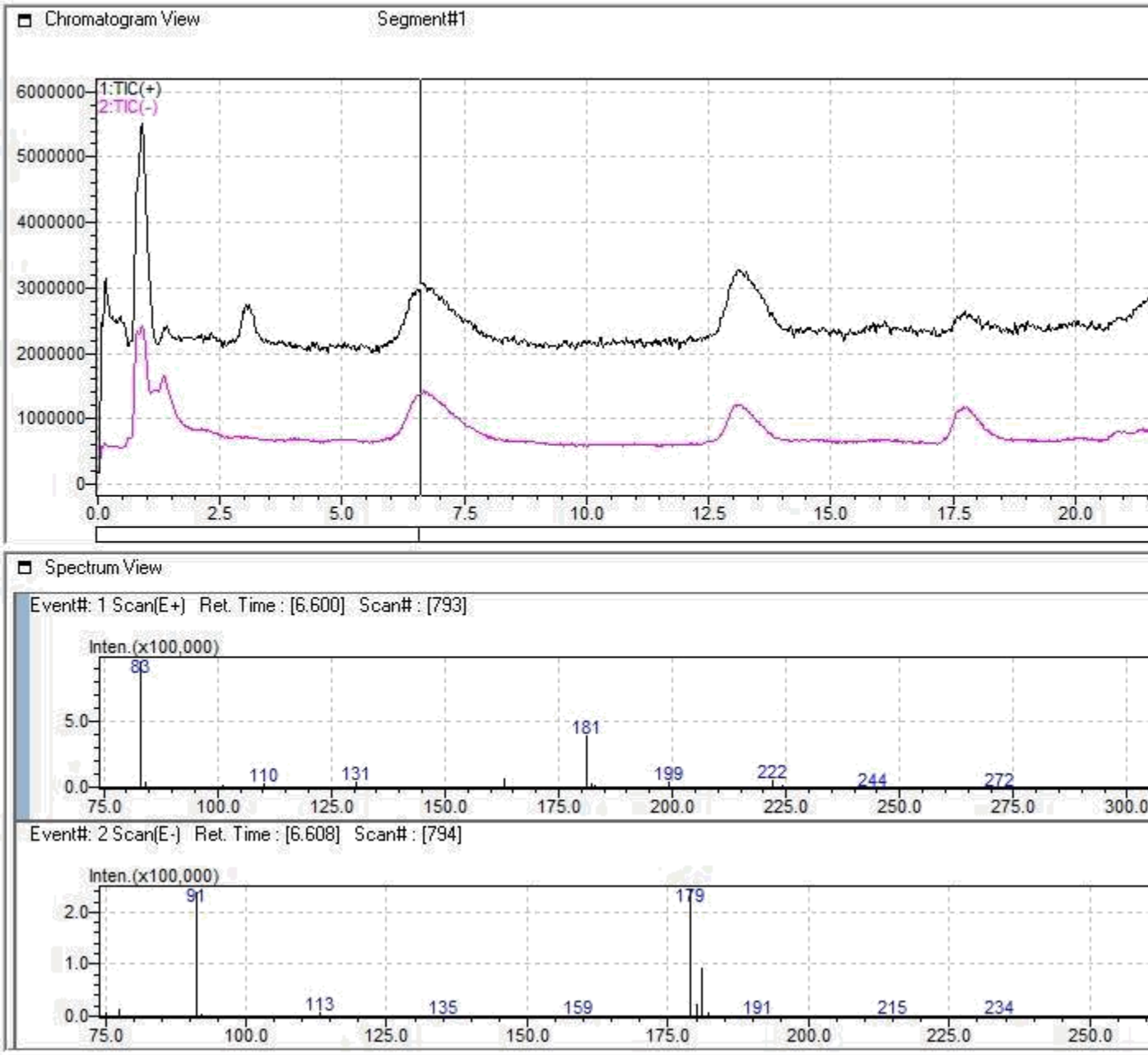

Figure A4. *Mass spectrum of sample 118 (centered on caffeic acid peak)*

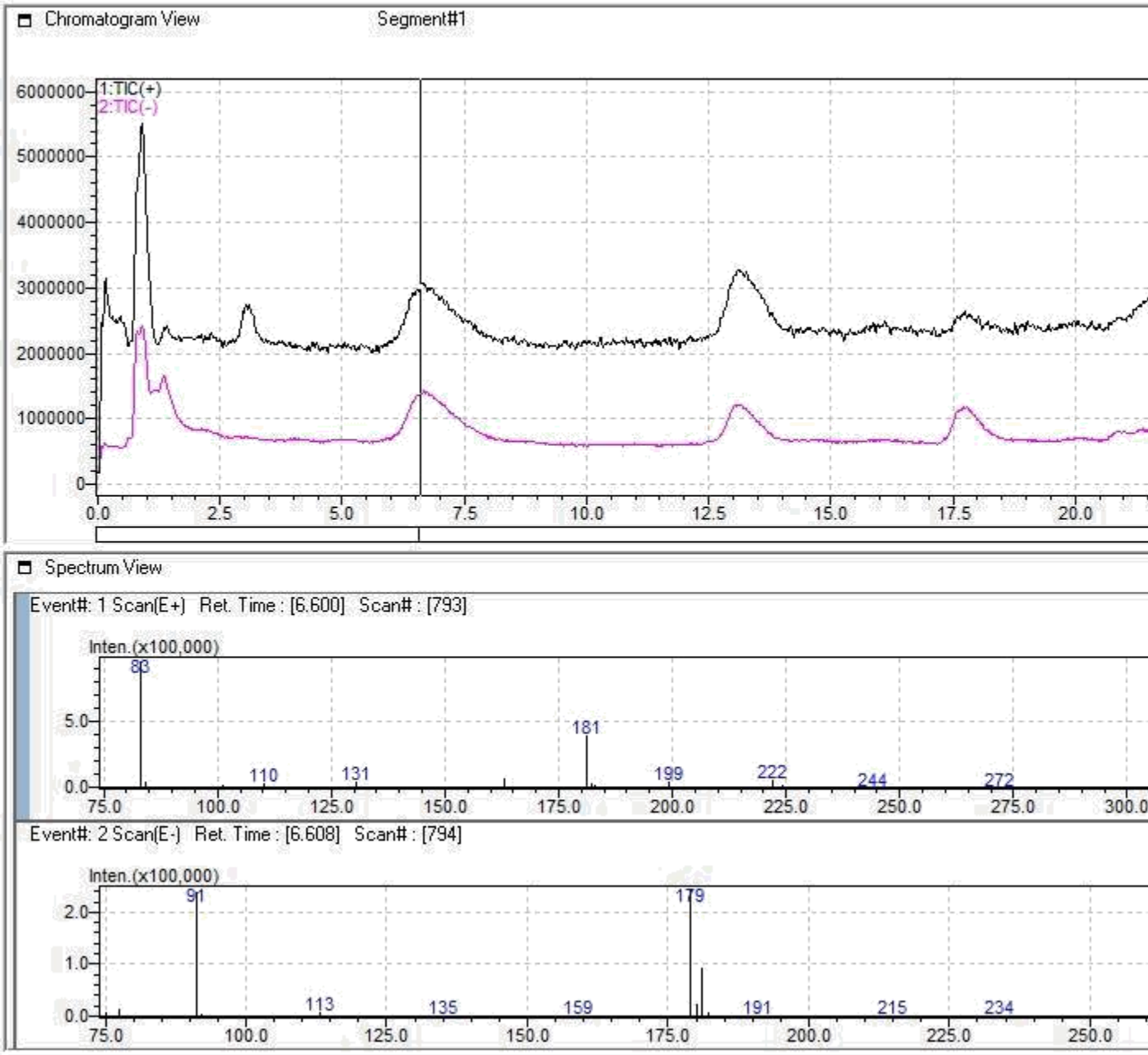

Figure A5. *Mass spectrum of sample 118 (centered on coumaric acid peak)*

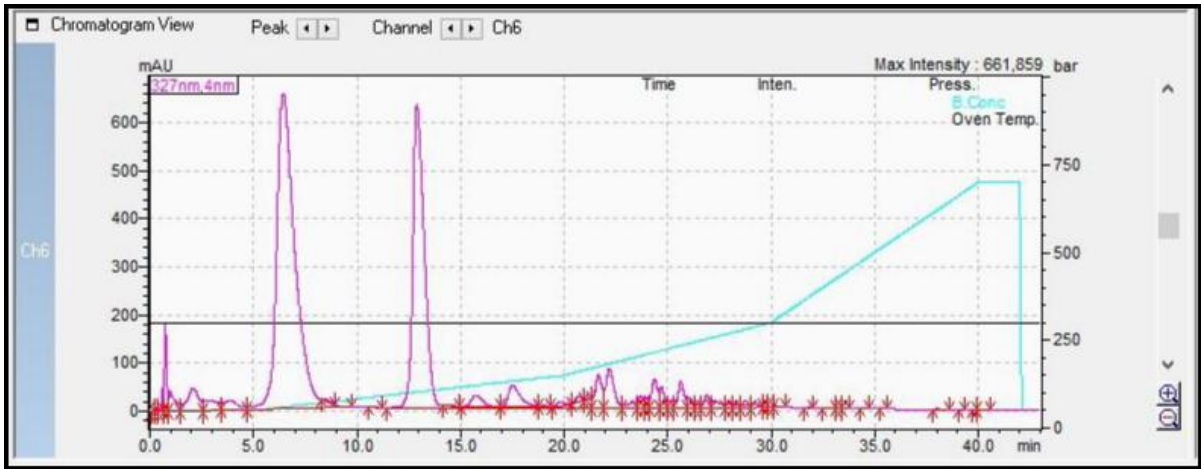

Figure A6. *PDA spectrum of sample 118 (327 nm)*

8.2 *Appendix B: Mass and PDA spectra for alternatively extracted samples (27, 27A, 27B)*

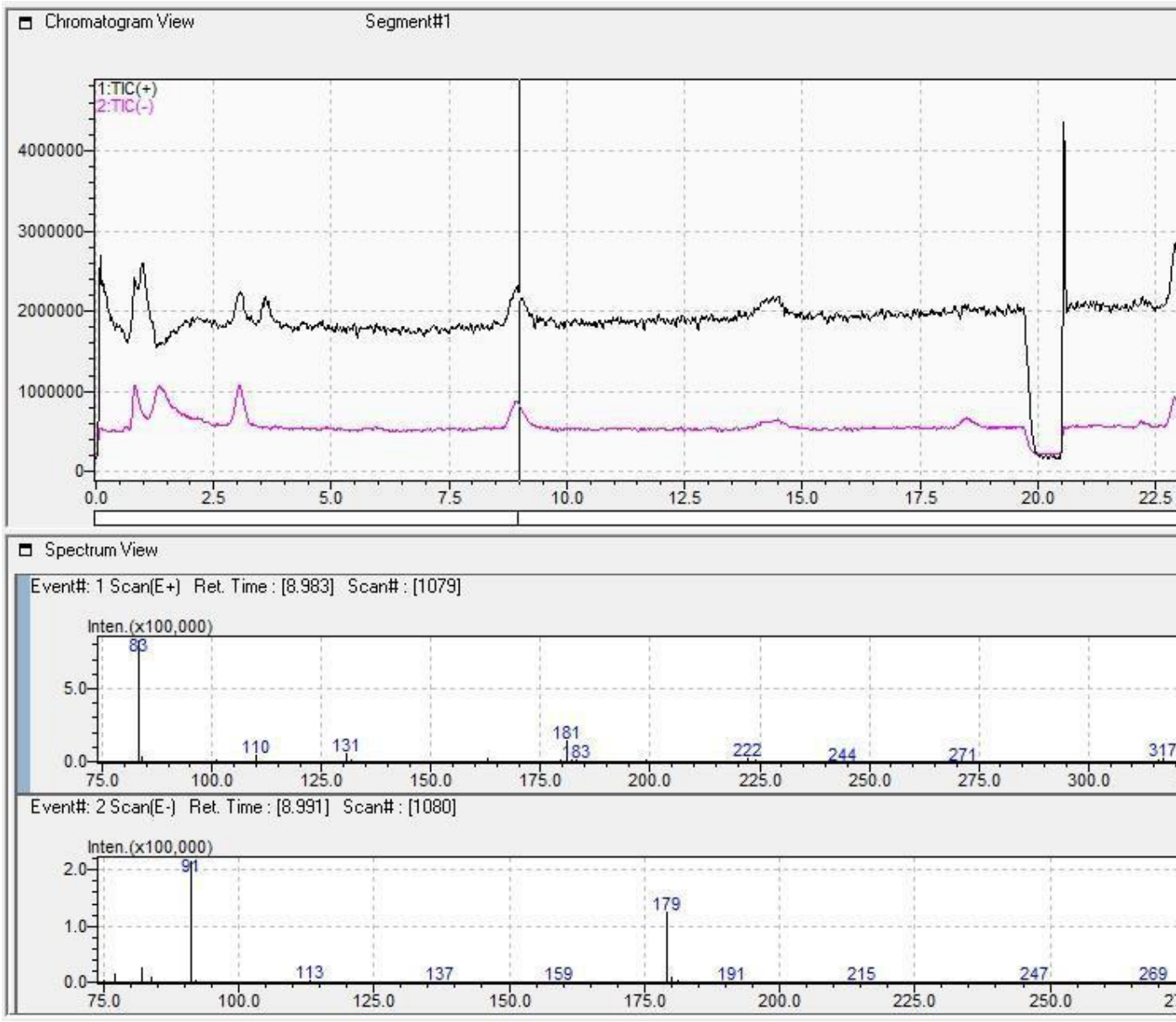

Figure B1. *Mass spectrum of sample 27 (centered on caffeic acid peak)*

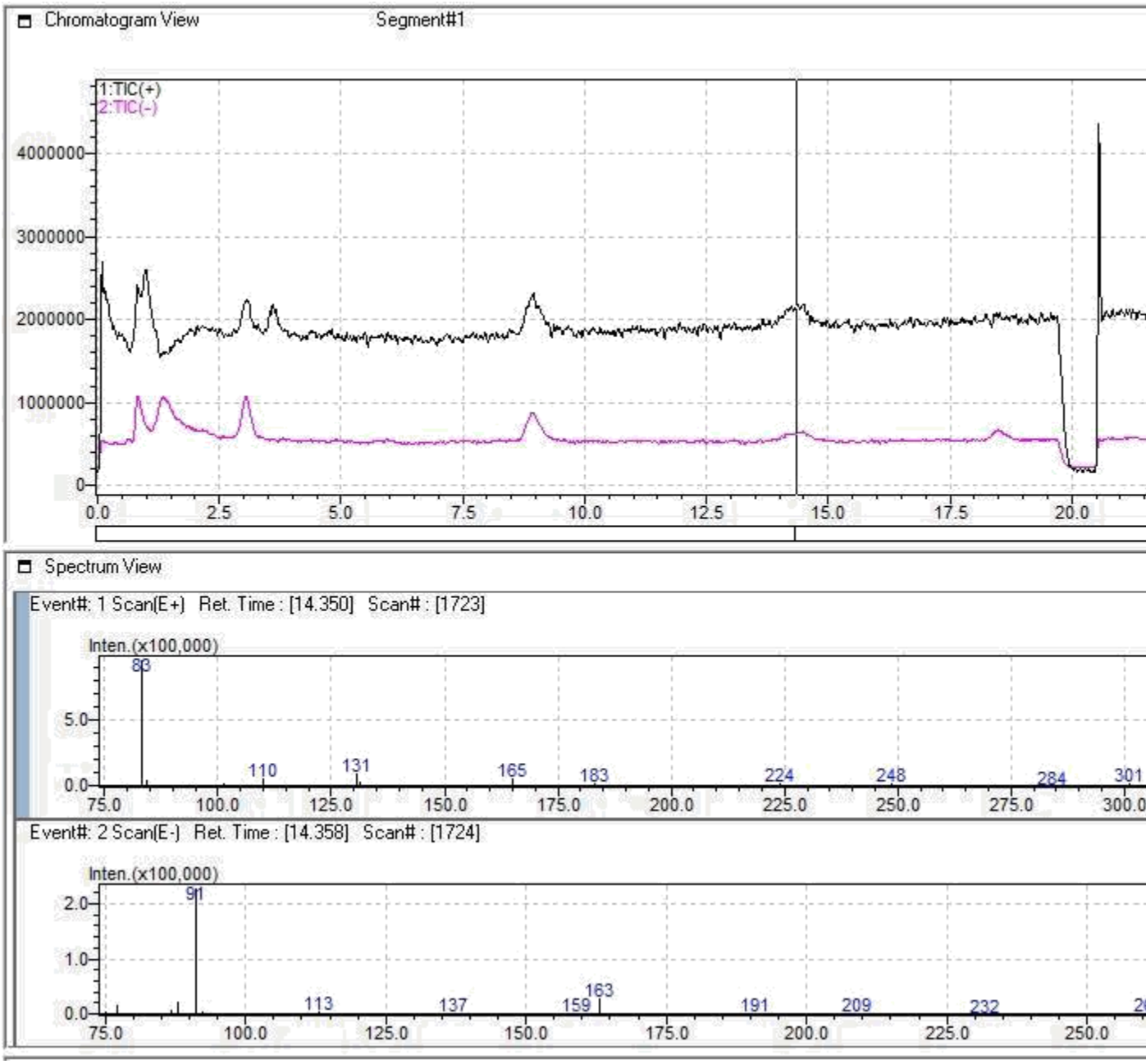

Figure B2. *Mass spectrum of sample 27 (centered on coumaric acid peak)*

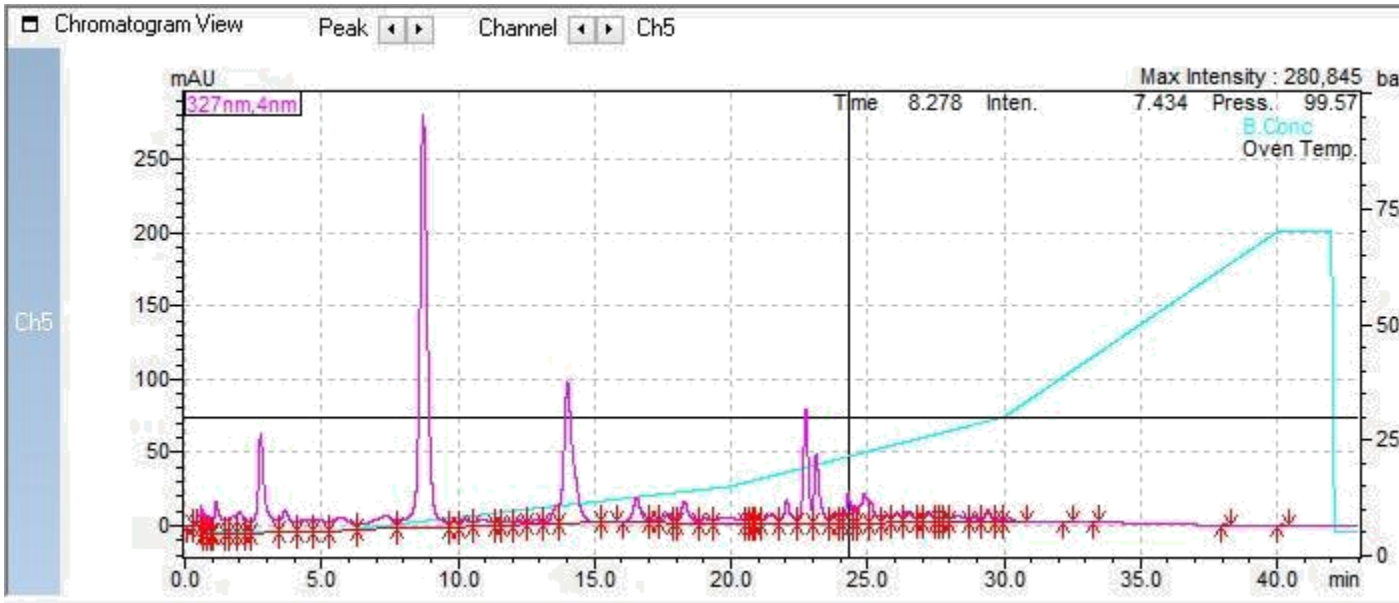

Figure B3. *PDA spectrum of sample 27 (327nm)*

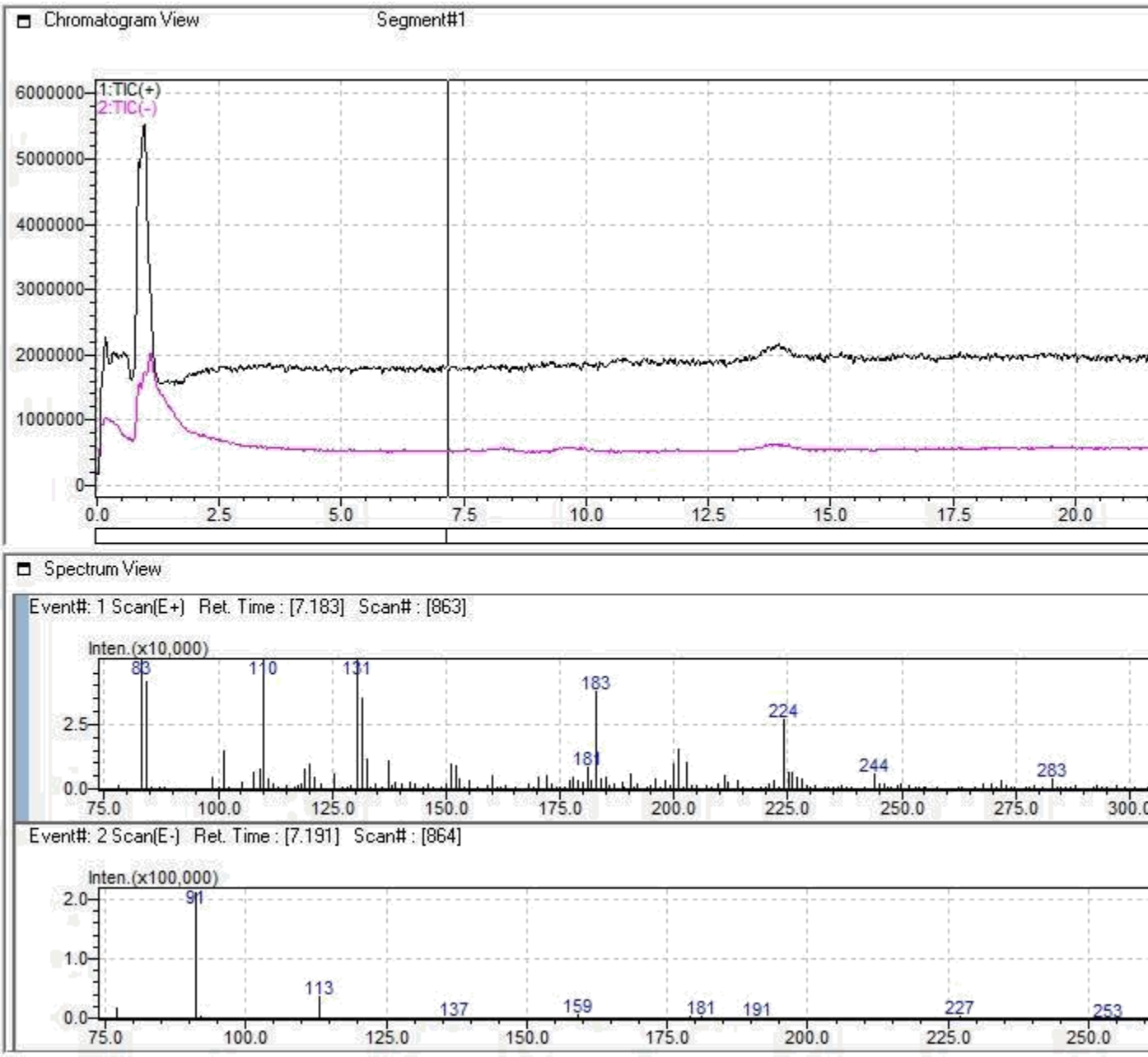

Figure B4. *Mass spectrum of sample 27A (centered on caffeic acid peak)*

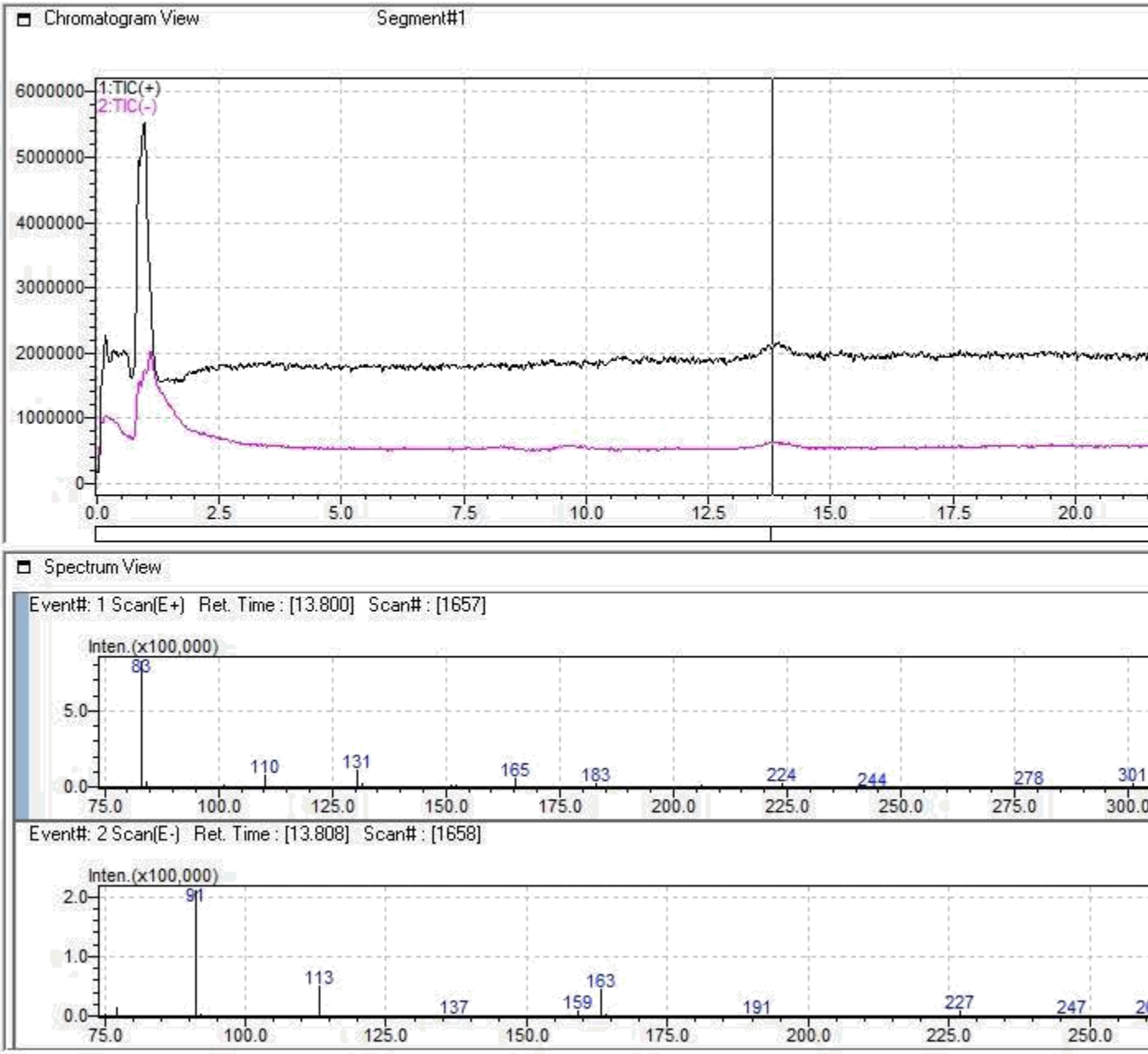

Figure B5. *Mass spectrum of sample 27A (centered on coumaric acid peak)*

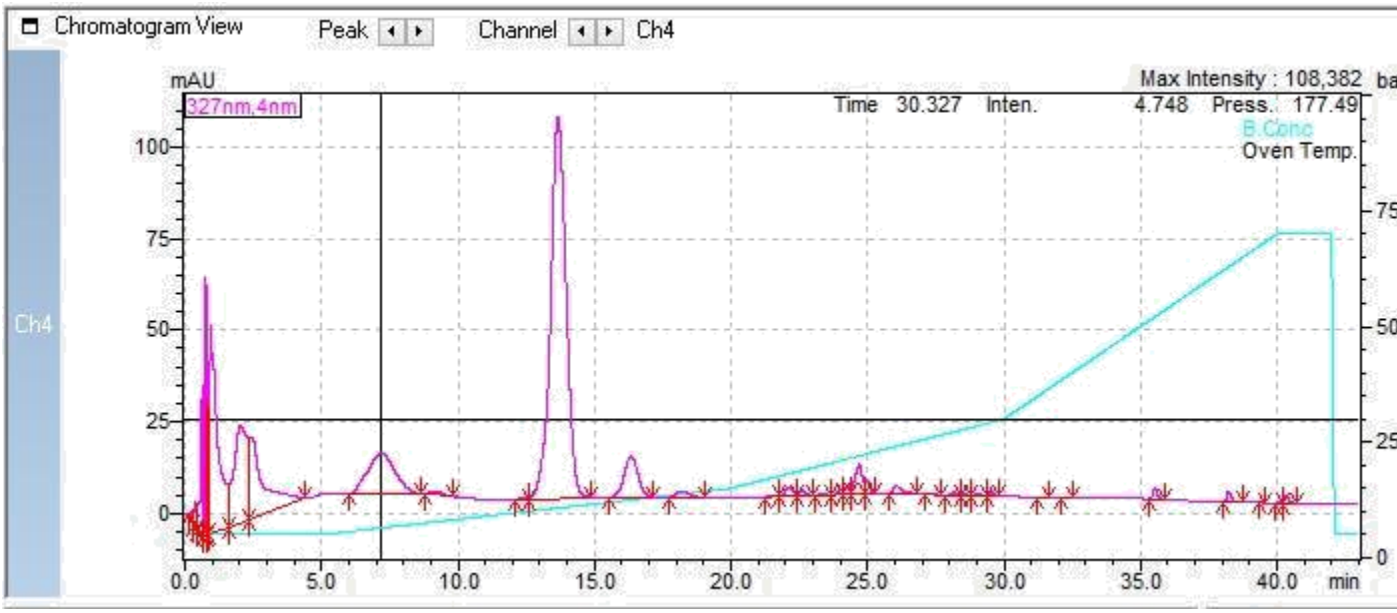

Figure B6. *PDA spectrum of sample 27A (327nm)*

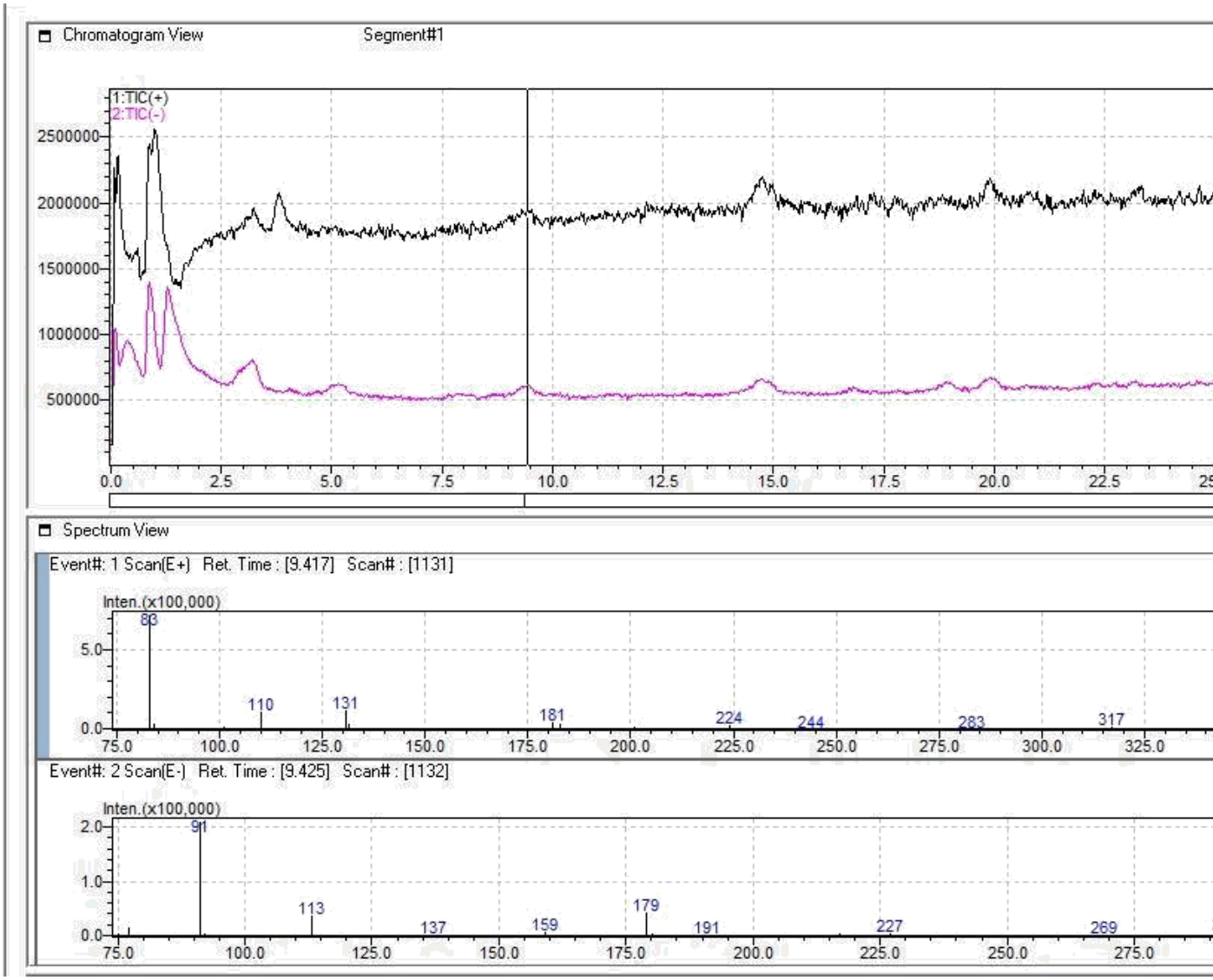

Figure B7. *Mass spectrum of sample 27B (centered on caffeic acid peak)*

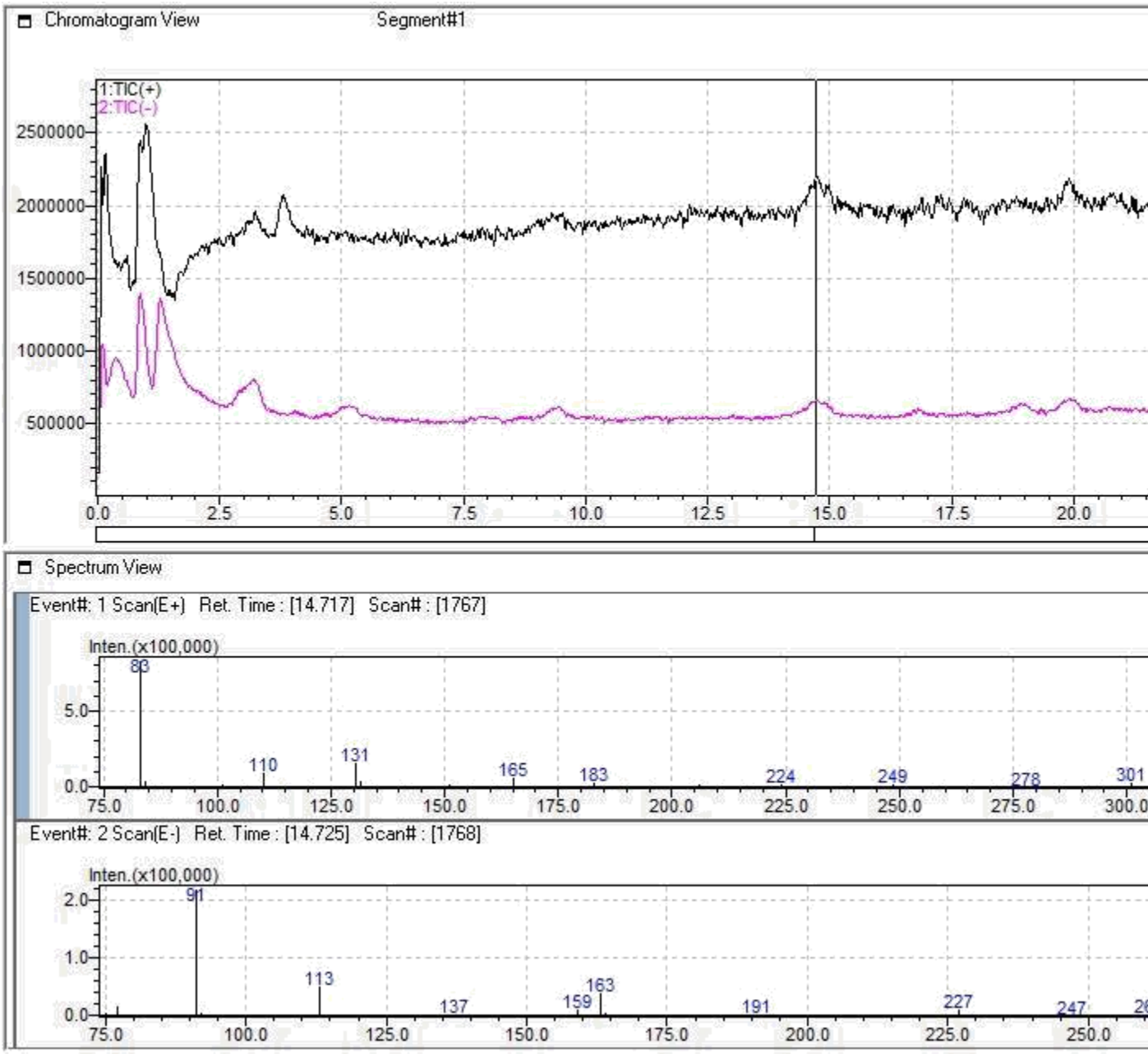

Figure B8. *Mass spectrum of sample 27B (centered on coumaric acid peak)*

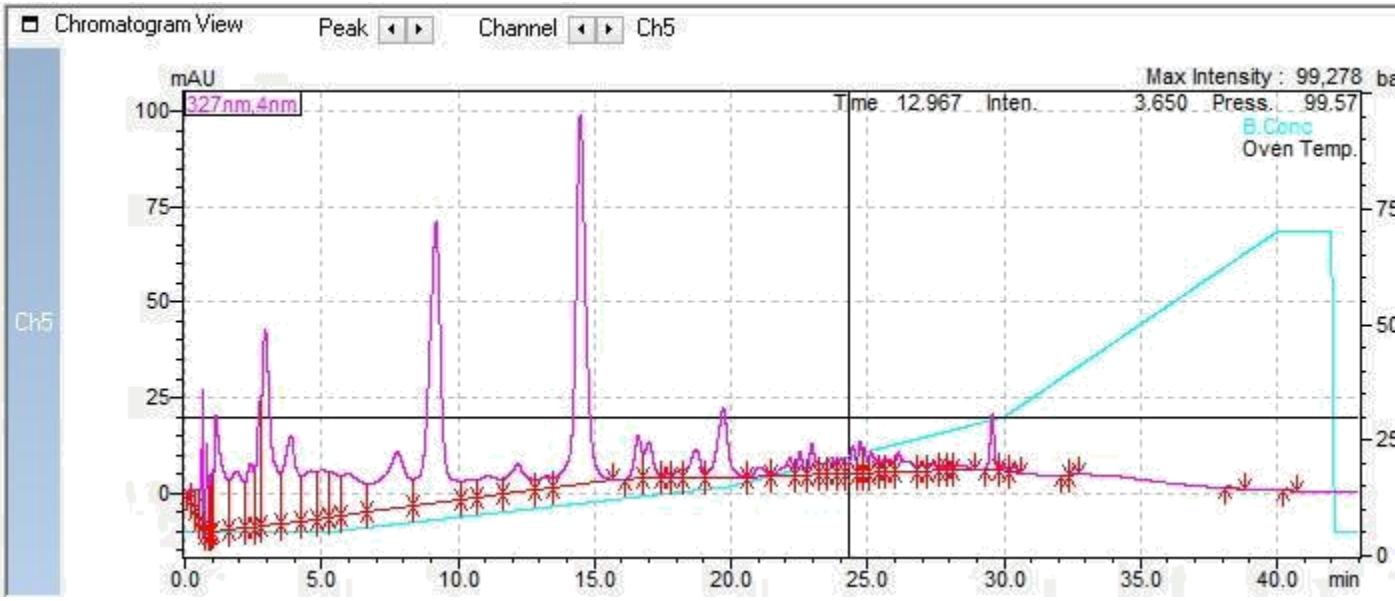

Figure B9. *PDA spectrum of sample 27B (327 nm)*