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The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman



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Abstract

Flavonoids are a class of natural products derived from plants which are incorporated into honeys through propolis, nectar and pollen. Research has shown that many honeys possess characteristic flavonoid profiles which can be used as markers for the geographical and floral origin of honey. The objective of this study has been to determine the flavonoid profile of New Zealand manuka (*Leptospermum scoparium*) honey, a honey which is internationally recognised for its unique medicinal properties. While all honeys are antibacterial due to the production of peroxides, manuka is unique because it exhibits an additional non-peroxide antibacterial activity due to high levels of methylglyoxal.

The established extraction method, which utilises Amberlite XAD-2 resin to extract phenolics from honey, has been modified to permit extraction of phenolics from samples as small as five grams, with no measurable loss of extraction reproducibility. This development opens up a much larger collection of honey samples to flavonoid profiling.

Measurement of the recovery rates for this extraction method has been further developed in this study in an attempt to account for the matrix effects of the high carbohydrate and phenolic acid content, relative to the flavonoids, of honey. This was achieved by extraction of flavonoid standards from an artificial honey matrix. The recovery rates of $10 \pm 6\%$, $16 \pm 7\%$ and $19 \pm 9\%$ for quercetin, chrysin and kaempferol respectively, were significantly lower than rates (28-60%) measured using the accepted procedure of extracting flavonoids from a simple solution. However, it should be noted that the recovery rates measured for extraction from solution were lower than those reported by other research groups. This has been partly attributed to an additional filtration step using a sacrificial HPLC column which was implemented to protect the analytical HPLC system from an unknown contaminant in the phenolic extracts.

Having evaluated the reproducibility and reliability of the modified extraction method, it was then applied to the analysis of 31 manuka honeys and 8 other non-

manuka honeys from New Zealand. The results have shown that the honeys studied have a common flavonoid profile consisting mainly of the flavanone pinocembrin and the dihydroflavonol pinobanksin. These flavonoids are derived from propolis and are a common feature of honeys from temperate regions of the Northern Hemisphere.

The manuka honeys analysed had total flavonoid content ranging between 0.59-2.24mg/100g of honey, with an average level of 1.16 ± 0.16 mg per 100g of honey. The flavonoid profile of these samples consisted mainly of pinobanksin (0.27 ± 0.04 mg/100g honey), pinocembrin (0.17 ± 0.02 mg/100g honey), luteolin (0.14 ± 0.02 mg/100g honey) and chrysin (0.13 ± 0.02 mg/100g honey), together accounting for 61% of the total flavonoid content.

Manuka honey was distinguishable from the other honeys studied by its high total flavonoid content, high luteolin content (greater than 0.05mg/100g honey) and high levels of an unidentified (unknown compound 01) which was found to elute with the flavonoids on HPLC but did not appear to be a flavonoid from its UV spectrum.

Statistical analysis showed that a positive correlation existed between the levels of unknown compound 01 in manuka honeys and their non-peroxide antibacterial activity. A similar correlation was also observed for luteolin.

The progress of this research has been hampered by the limited range of flavonoid standards available for comparison with HPLC chromatogram peaks. A total of eight flavonoids were found in manuka honey, and a further seven in the non-manuka honeys, which did not coincide with any of the flavonoid standards available. Two of unknown flavonoids were subsequently extracted from fifteen kilograms of manuka honey using Amberlite XAD-2 resin and liquid-liquid extraction, and isolated by a combination of Sephadex-LH20 column chromatography and HPLC.

Characterisation of these flavonoids was achieved using a combination of UV absorption spectroscopy, ^1H , ^{13}C and HMBC NMR spectroscopy, and LDI-TOF mass spectrometry. The isolated flavonoids were identified as pinobanksin and 8-methoxykaempferol, both flavonoids which have been previously found in honeys.

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Table of Contents

Chapter 1: Introduction.....	1
1.1 Honey	1
1.1.1 Manuka Honey	1
1.1.2 Composition of Honey.....	2
1.1.2.1 Water.....	2
1.1.2.2 Carbohydrates	2
1.1.2.3 Organics	3
1.1.2.4 Minor Components	4
1.2 Medicinal Properties of Honey	6
1.2.1 Antibacterial Activity of Manuka Honey	8
1.2.1.1 Osmotic Pressure.....	9
1.2.1.2 Acidity.....	9
1.2.1.3 Hydrogen Peroxide.....	10
1.2.1.4 1.2.1.4 Non-Peroxide Activity and Methylglyoxal	11
1.3 Flavonoids.....	15
1.3.1 Biochemistry of Flavonoids.....	16
1.3.2 Identification of Flavonoids.....	20
1.3.2.1 Ultraviolet-Visible Absorption Spectroscopy	21
1.3.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy	30
1.3.3 Analysis of Flavonoids in Honey.....	32
1.3.3.1 Flavonoids in Honey	33
1.4 Aims of the Present Research.....	39
Chapter 2: Materials and Methods	40
2.1 Materials	40
2.2 General Methods	43
2.2.1 Freeze-Drying	43
2.2.2 Evaporation.....	43
2.2.3 Statistical Analysis	43
2.3 High Performance Liquid Chromatography (HPLC)	43
2.3.1 HPLC System A	44
2.3.2 HPLC System B.....	44
2.3.2.1 Alternative HPLC Conditions Used During Early Method Development	45
2.3.2.2 Identification and Quantification	46

2.3.2.3	Cleaning the HPLC Column	47
2.3.3	HPLC System C.....	48
2.4	Well Diffusion Assay for Non-Peroxide Antibacterial Activity	49
2.4.1	Preparation of Assay Plates	49
2.4.2	Preparation of Samples and Standards.....	49
2.4.3	Antibacterial Assay.....	50
2.5	Extraction of Flavonoids from Honey for Analysis	51
2.5.1	Cleaning and Swelling of the Amberlite XAD-2 Resin.....	51
2.5.2	Testing the Flavonoid Extract of Manuka Honey for Antibacterial Activity.....	52
2.5.3	Modification of the Extraction Method to Use Smaller Honey Samples.....	53
2.5.4	Final Extraction Method Applied to Analysis of Honey Flavonoids.....	54
2.5.5	Evaluation of Extraction Method.....	55
2.5.5.1	Recovery Rate of Flavonoids Extracted from Solution.....	55
2.5.5.2	Recovery Rate of Flavonoids Extracted from Artificial Honey	56
2.5.5.3	Assessing the Reproducibility of the Extraction Method	56
2.6	Isolation of Honey Flavonoids.....	56
2.6.1	Extraction of Phenolics Using Amberlite XAD-2 Resin	56
2.6.2	Liquid-Liquid Extraction and Cleanup of the Extract	57
2.6.3	Sephadex LH-20 Separation of the Phenolic Extract.....	58
2.6.4	HPLC Separation of Individual Flavonoids.....	59
2.7	Characterisation of Isolated Flavonoids	59
2.7.1	UV-Visible Spectroscopy	59
2.7.1.1	Preparation of Spectroscopic Reagents for UV-Visible Spectroscopy.....	59
2.7.1.2	UV-Visible Spectroscopy of Isolated Flavonoids	59
2.7.2	Nuclear Magnetic Resonance Spectroscopy of Isolated Flavonoids	61
2.7.3	Mass Spectrometry of Isolated Flavonoids.....	61
Chapter 3:	<i>Results and Discussion</i>	62
3.1	The Antibacterial Activity of Fractions from the XAD-2 Extraction of Manuka Honey	62
3.2	Identification and Quantification of Flavonoids by HPLC	65
3.3	Evaluation of the Extraction Method.....	70
3.3.1	The Effect of Sample Size on Phenolic Recovery	70
3.3.2	The Recovery Rate of Flavonoids	74
3.3.3	The Reproducibility of the Extraction Method	78
3.4	The Flavonoid Profile of New Zealand Honeys.....	81
3.4.1	Manuka Honey	81

3.4.2	Comparison with Other New Zealand Honeys	88
3.4.3	The Relationship of Flavonoid Profile with the UMF Antibacterial Activity of Manuka Honey	92
3.5	Isolation of Unknown Flavonoids	95
3.6	Characterisation of Unknown Flavonoids	98
3.6.1	Flavonoid A	99
3.6.1.1	UV-Visible Spectroscopy.....	99
3.6.1.2	¹ H NMR Spectroscopy	101
3.6.1.3	LDI-TOF Mass Spectrometry	104
3.6.1.4	Final Structural Assignment of Flavonoid A	104
3.6.2	Flavonoid B	106
3.6.2.1	UV Visible Spectroscopy	106
3.6.2.2	¹ H NMR Spectroscopy	109
3.6.2.3	LDI-TOF Mass Spectrometry	111
3.6.2.4	Final Structural Assignment of Flavonoid B.....	112
Chapter 4:	Conclusions.....	114
Chapter 5:	Recommendations	118
References		118
Appendices.....		134

List of Figures

Figure 1.1: Chemical structures of some aromatic compounds found in manuka honey.	5
Figure 1.2: The enzymatic oxidation of glucose to gluconic acid and hydrogen peroxide.....	10
Figure 1.3: The structure of flavone. ^{78, 79}	15
Figure 1.4: The structural skeletons of some of the flavonoid classes. ^{79, 80}	16
Figure 1.5: The oxidation of flavonols. ⁸²	19
Figure 1.6: The formation of adducts between epicatechin and methylglyoxal (MGO). ⁸⁹	20
Figure 1.7: The conjugated systems responsible for the major ultraviolet-visible absorption peaks of flavonoids. ⁹¹⁻⁹³	22
Figure 1.8: The proposed structure of the complex formed by reaction of orthodihydroxyl groups on the flavonoid nucleus, with boric acid in the presence of sodium acetate. ^{81, 93}	26
Figure 1.9: The complexation of flavonoids with AlCl ₃ in the presence and absence of acid. ⁹³	28
Figure 1.10: The structures of some flavonoids found in honey. ^{1, 12, 94, 108}	35
Figure 2.1: The Quasi-Latin square arrangement of sample and standard wells used in the well diffusion assay of antibacterial activity.	51
Figure 3.1: Graph showing the non-peroxide antibacterial activity of the XAD-2 extraction sugar fraction, the methanol fraction at its natural concentration and concentrated 25 times, the original manuka honey and the inactive clover honey used as an artificial matrix.	63
Figure 3.2: The HPLC calibration curve of quercetin, measured at 340nm.	68
Figure 3.3: The HPLC calibration curve of kaempferol, measured at 340nm.	68
Figure 3.4: The HPLC calibration curve of pinocembrin, measured at 290nm.	69
Figure 3.5: The HPLC calibration curve of chrysin, measured at 340nm.	69
Figure 3.6: Total chromatogram area at 340nm, of the phenolics extracted from differently sized samples.	72
Figure 3.7: Area of the 15.9min phenolic peak extracted from differently sized samples, measured at 340nm....	73
Figure 3.8: Area of the 20.5min phenolic peak extracted from differently sized samples, measured at 340nm....	73
Figure 3.9: The full chromatogram of the M01 manuka honey sample, measured at 340nm.	84
Figure 3.10: The full chromatogram of the M01 manuka honey sample, measured at 290nm.	85
Figure 3.11: Expanded views of regions of the manuka honey extract chromatogram with flavonoids labelled..	85
Figure 3.12: Scatter plots of UMF non-peroxide antibacterial activity versus concentrations of individual manuka honey flavonoids.	93
Figure 3.13: Scatter plot of unknown compound 01 concentration versus UMF non-peroxide antibacterial activity.	94
Figure 3.14: Scatter plot of luteolin concentration versus UMF non-peroxide antibacterial activity.	95
Figure 3.15: A HPLC chromatogram (340nm) of fraction 8.....	96
Figure 3.16: HPLC system C chromatogram of fraction 7 showing the collected fraction.	97
Figure 3.17: HPLC system B chromatogram of flavonoid A.....	97
Figure 3.18: HPLC system C chromatogram of fraction 6 showing the collected fraction.	98
Figure 3.19: HPLC system B chromatogram of flavonoid B.....	98

Figure 3.20: The UV-Visible spectra of flavonoid A in methanol, before and after the addition of sodium methoxide.....	99
Figure 3.21: The UV-Visible spectra of flavonoid A in methanol, reacted with AlCl_3 and AlCl_3/HCl reagents.	100
Figure 3.22: The UV-Visible spectra of flavonoid A in methanol, reacted with NaOAc and $\text{NaOAc}/\text{H}_3\text{BO}_3$ reagents.	101
Figure 3.23: The ^1H NMR spectrum of flavonoid A in $\text{DMSO}-d_6$	103
Figure 3.24: The ^{13}C and ^1H NMR assignment with HMBC correlations, of 8-methoxykaempferol, the proposed identity of flavonoid A.....	105
Figure 3.25: The UV-Visible spectra of flavonoid B in methanol, before and after the addition of sodium methoxide.....	107
Figure 3.26: The UV-Visible spectra of flavonoid B in methanol, reacted with AlCl_3 and AlCl_3/HCl reagents.	107
Figure 3.27: The UV-Visible spectra of flavonoid B in methanol, reacted with NaOAc and $\text{NaOAc}/\text{H}_3\text{BO}_3$ reagents.	108
Figure 3.28: The ^1H NMR spectrum of flavonoid B in $\text{DMSO}-d_6$	110
Figure 3.29: The structure of pinobanksin, the proposed identity of flavonoid B.	112

List of Tables

Table 1.1: Average sugar composition of American floral honeys. ¹³	3
Table 1.2: The effect of oxygenation on the position of the Band I absorption peak of flavonols. ^{91, 93}	22
Table 1.3: The bathochromic shifts observed in the UV spectrum of flavonoids reacted with boric acid and sodium acetate. ^{91, 93}	27
Table 1.4: The shifts observed in the UV spectra of flavonoids in methanol and AlCl ₃ solution when acidified. ^{91, 93}	29
Table 1.5: The bathochromic shifts in the UV spectrum due to complexation of AlCl ₃ with the 3- or 5-hydroxyl groups and the 4-keto group. ^{81, 91, 93}	30
Table 1.6: Typical ¹ H chemical shifts and multiplicities for flavonoids. ^{81, 91, 93}	31
Table 2.1: The authentic chemicals used for identification and quantification	42
Table 2.2: The gradient system used for the analytical HPLC analysis of flavonoid extracts.	45
Table 2.3: The gradient system used for the analytical HPLC analysis of early flavonoid extracts.	46
Table 2.4: Preparation of quantitative flavonoid standards	47
Table 2.5: The gradient program used in the semi-prep HPLC separation of flavonoids	48
Table 2.6: The solvents used in Sephadex LH-20 chromatography of phenolic extract and the fractions collected	58
Table 3.1: The non-peroxide antibacterial activities of fractions from the XAD-2 extraction of manuka honey compared to the activity of the original honey.	62
Table 3.2: The phenolic compounds identified and quantified by HPLC.	66
Table 3.3: Adjusted peak areas measured at 340nm, of two significant peaks and the total chromatogram, for different sized sample extractions	71
Table 3.4: The recovery rate of flavonoids from an artificial honey matrix	75
Table 3.5: The recovery rate of flavonoids from a simple solution. Two standard solutions were put through the whole extraction process and one was put only through the final HPLC cleanup step.	76
Table 3.6: The results of multiple analyses of the flavonoid content of a manuka honey sample	79
Table 3.7: The flavonoid content of New Zealand manuka (<i>Leptospermum scoparium</i>) honey	82
Table 3.8: The flavonoid content of some non-manuka New Zealand honeys	89
Table 3.9: Pollen analysis data for the non-manuka samples profiled. The honeydew honey did not contain pollen and no data was available for the nodding thistle honey	90
Table 3.10: The levels of some unknown flavonoids found in non-manuka New Zealand honeys	92
Table 3.11: ¹ H NMR signals of flavonoid A	102
Table 3.12: The UV absorption spectra of 6-methoxykaempferol, 8-methoxykaempferol and flavonoid A in methanol	106
Table 3.13: ¹ H NMR signals of flavonoid B	109
Table 3.14: A comparison of the ¹³ C NMR spectra of flavonoid B and pinobanksin	113

List of Abbreviations

~	approximately
Ø	diameter
°C	degrees Celsius
%	percentage
¹ H	proton
¹³ C	carbon -13
2D	two dimensional
λ _{max}	wavelength of maximum absorption
AGEs	advanced glycation end-products
AlCl ₃	aluminium chloride
AU	absorbance units
a _w	water activity
C18	18 carbon chain
CCl ₄	carbon tetrachloride
CDCl ₃	deuteriochloroform
CE	capillary electrophoresis
C.I.	confidence interval
C.V.	coefficient of variation
Da	Daltons
DMSO	dimethyl sulfoxide
DMSO- <i>d</i> ₆	hexadeuteriodimethyl sulfoxide
DNA	deoxyribonucleic acid
<i>e. coli</i>	<i>Escherichia coli</i>
ECM	extra cellular matrix
<i>et al.</i>	<i>et alia</i>
g	gram
h	hour
H	hydrogen
H ₂ O	water
H ₃ BO ₃	boric acid
HCl	hydrochloric acid

HMBC	heteronuclear multiple bond coherence
HPLC	high performance liquid chromatography
Hz	Hertz
J	coupling constant
kg	kilogram
L	litre
LDI-TOF	laser desorption ionisation with time of flight detector
Ltd	Limited
MΩcm	mega Ohm centimetres
MALDI-TOF	matrix assisted laser desorption ionisation with time of flight detector
MECC	micellar electrokinetic capillary chromatography
MEKC	micellar electrokinetic chromatography
MeOH	methanol
mg	milligram
MGO	methylglyoxal
MHz	mega Hertz
min	minute
mL	millilitre
μL	microlitre
mM	millimolar
mm	millimetre
mm ²	millimetres squared
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
m/z	mass upon charge ratio
N ₂	nitrogen gas
NaOAc	sodium acetate
NaOMe	sodium methoxide
nm	nanometre
NMR	nuclear magnetic resonance
NPA	non-peroxide activity
<i>o</i>	<i>ortho</i>
OD	optical density

OH	hydroxyl
OMe	methoxyl
<i>p</i>	<i>para</i>
PDA	probability
pH	$-\log_{10}[\text{H}^+]$
ppm	parts per million
R ²	regression squared
RCM	radial compression module
RGD	arginine-glycine-aspartic acid
RP18	reversed phase with an 18 carbon chain
R _t	retention time
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
S.D.	standard deviation
<i>spp.</i>	species (plural)
TMS	tetramethylsilane
TMS-ether	trimethylsilyl ether
TNF	tumour necrosis factor
UMF	unique manuka factor
UV	ultra violet
UV-Vis	ultra violet and visible
vs	versus
v/v	volume per volume
WDA	well diffusion assay
w/v	weight per volume

Chapter 1: Introduction

1.1 Honey

Honey is a natural substance produced by honey bees, usually from the nectar of flowers and honeydew excreted by scale insects.¹ Honeybees make honey to use as a food store during the winter and this has been exploited by humans since ancient times. Beekeeping has been documented at least as far back as the Ancient Egyptians (2000-5000 years ago) who used honey as a medicine, as a food source and in embalming.^{2, 3}

Honey is the only known natural food which will never spoil and the only sweetening material which requires no processing to render it ready for consumption. Raw honey can be eaten straight out of the comb taken from the beehive.²

1.1.1 Manuka Honey

Manuka honey is derived from the nectar of the manuka tree, *Leptospermum scoparium*, a native of New Zealand. This nectar is produced in the flowers of the manuka tree, which are generally white in the wild and 10 to 12 mm across in diameter.⁴ Flowering takes place between September and February.⁵

The manuka plant is often colonised by scale insects which feed off the phloem of the tree and excrete honeydew. This encourages black sooty mould to grow on the bark, giving it a dark colouring.⁴ Honeybees can use this honeydew as a raw material for the production of honey and indeed, honeybees have been observed harvesting honeydew from scale insects on manuka trees.⁶ However, it is unclear as to whether this honeydew is incorporated into manuka honey. Honeydew honeys have a very complex oligosaccharide composition due to transglucosylation of the sugars by enzymes in the scale insects gut. It has been shown that while manuka honey does have higher levels of complex sugars (10%) than most floral honeys (1.5%), it clearly lacks the variety of oligosaccharides found in honeydew honeys such as Beech honeydew honey. This suggests that the sugars used to produce manuka honey are predominantly floral in origin rather than from honeydew.^{7, 8}

Manuka honey has a strong and distinctive flavour with a herby and woody character.⁹ It is also dark in colour and exhibits thixotropic behaviour similar to that found in Australian bush jelly honey and European heather honey.^{10, 11}

1.1.2 Composition of Honey

The chemical composition of honey varies depending on the floral origin, seasonal and climatic variations, and geographical origin.^{3, 12}

1.1.2.1 Water

The average water content of honey is 17.2% but it has been shown to vary between 13.4% and 22.9%.¹³ The water content of honey needs to be below 17% to prevent fermentation from occurring.¹² Nectar contains higher levels of water but evaporation of the water in honey is enhanced in the hive by bees fanning their wings to create a draft across the honeycomb.¹⁴

1.1.2.2 Carbohydrates

Over 95% of the dry weight of honey can be attributed to its sugar content. The monosaccharides glucose and fructose together make up 85-95% of the total sugar content.³ The ratio of fructose to glucose found in a honey is mostly determined by its floral source. The average ratio found in honey is 1.2:1 fructose to glucose.^{15, 16} Sucrose is also present in honey and makes up about 1% of the dry weight.¹² The remainder of the sugar content of honey is comprised of oligosaccharides, of which at least 22 have been identified in honeys.¹⁷

The oligosaccharides in honey are the result of trans-glycosylation and trans-fructosylation reactions which are assisted by enzymes in bee secretions. These reactions build up more complex sugars from the glucose and fructose monosaccharides present. These processes occur as the honey ripens and ages in the hive and even continue when the honey is stored leading to increased oligosaccharide content and decreased monosaccharide content over time.^{18, 19}

The oligosaccharides of manuka honey make up 10% of the sugar content. Significant levels of maltulose, kojibiose, turanose, nigerose, maltose, trehalose, palatinose, sucrose, erlose, panose, melezitose and maltotriose have all been identified in manuka honey.^{7, 20}

A comprehensive study of 490 American floral honeys gave the average sugar composition and ranges shown in Table 1.1.¹³

Table 1.1: Average sugar composition of American floral honeys.¹³

	Average	Range	Standard Deviation
Fructose (%)	38.38	27.25-44.26	2.07
Glucose (%)	31.28	22.03-40.75	3.03
Sucrose (%)	1.31	0.25-7.57	0.95
Reducing Disaccharides (%)	7.31	2.74-15.98	2.09
Higher Sugars (%)	1.5	0.13-8.49	1.03
Undetermined (%)	3.1	0.00-13.20	1.97

1.1.2.3 Organics

In addition to the carbohydrates, there are a large number of other organic compounds present in honey. Mono- and dibasic acids, phenolic acids, hydrocarbons and flavonoids have all been identified in honeys and in some cases can be characteristic of the floral or geographic origin.^{12, 21}

All honeys contain high levels of gluconic acid, produced via the enzymatic oxidation of glucose by glucose oxidase contained in bee secretions. This reaction also produces hydrogen peroxide. The reduced pH and hydrogen peroxide help stabilise honey against spoilage while it ripens.³

A study by Tan *et al.*²¹ found that manuka honey contained hydrocarbons (C21-C33), straight chain fatty acids (C8-C28), dibasic acids and aromatic acids. Unifloral manuka honey can be characterised by its high levels of several aromatic acids which are largely unaffected by season or geographical origin. Phenyllactic acid (**1**) and 2-hydroxy-3-(4'-methoxyphenyl)propionic acid (**2**) have an average combined concentration of 893 mg/kg and levels over 700mg/kg are considered a characteristic of manuka honey. Similarly, syringic acid (**3**) and

3,4,5-trimethoxybenzoic acid (**4**) have an average combined concentration of 64 mg/kg and levels over 35 mg/kg are characteristic. Combined acetophenone (**5**) and 2-methoxy acetophenone (**6**) levels over 20 mg/kg are also characteristic of manuka honey.²²

Cinnamic acid (**7**) and its derivatives; caffeic (**8**), coumaric (**9**) and ferulic acid (**10**) have all been found in manuka honey.^{23, 24} Yao et al²⁴ also observed high levels of gallic acid (**11**) (7.05 mg/100g) and abscisic acid (**12**) (32.8 mg/100g) in two manuka honey samples and has suggested that with further study they could be used as a floral marker for manuka honey. The same study also found the cinnamic acid derivatives mentioned previously and ellagic acid (**13**). Structures of the most significant aromatic acids in manuka honey are shown in Figure 1.1.

Honey also contains a number of flavonoids in their aglycone form. Some floral honeys have been shown to exhibit unique flavonoid profiles making flavonoid patterns a useful tool for the determining the floral and geographical origin of honeys.¹² Flavonoids, their biochemistry and their potential use in determining floral origin will be covered in Section 1.3.

1.1.2.4 Minor Components

The free amino acid content of honey is low and variable (0.26-59.65 mg/100g).²⁵ Proline is the most significant of the amino acids present, accounting for 50-85% of the total amino acid content.¹⁵ Proteins are present in higher levels than amino acids with an average of 168.6 mg/100g in honey. As with the amino acids, the protein content is highly variable and is reported to range from 57.7 to 567 mg/100g.¹⁵ This protein is of bee and plant origin, with enzymes being the most significant form of protein present. Invertase, amylase and glucose oxidase are the most common enzymes in honey but catalase and phosphatase have also been found at lower levels.^{3, 12}

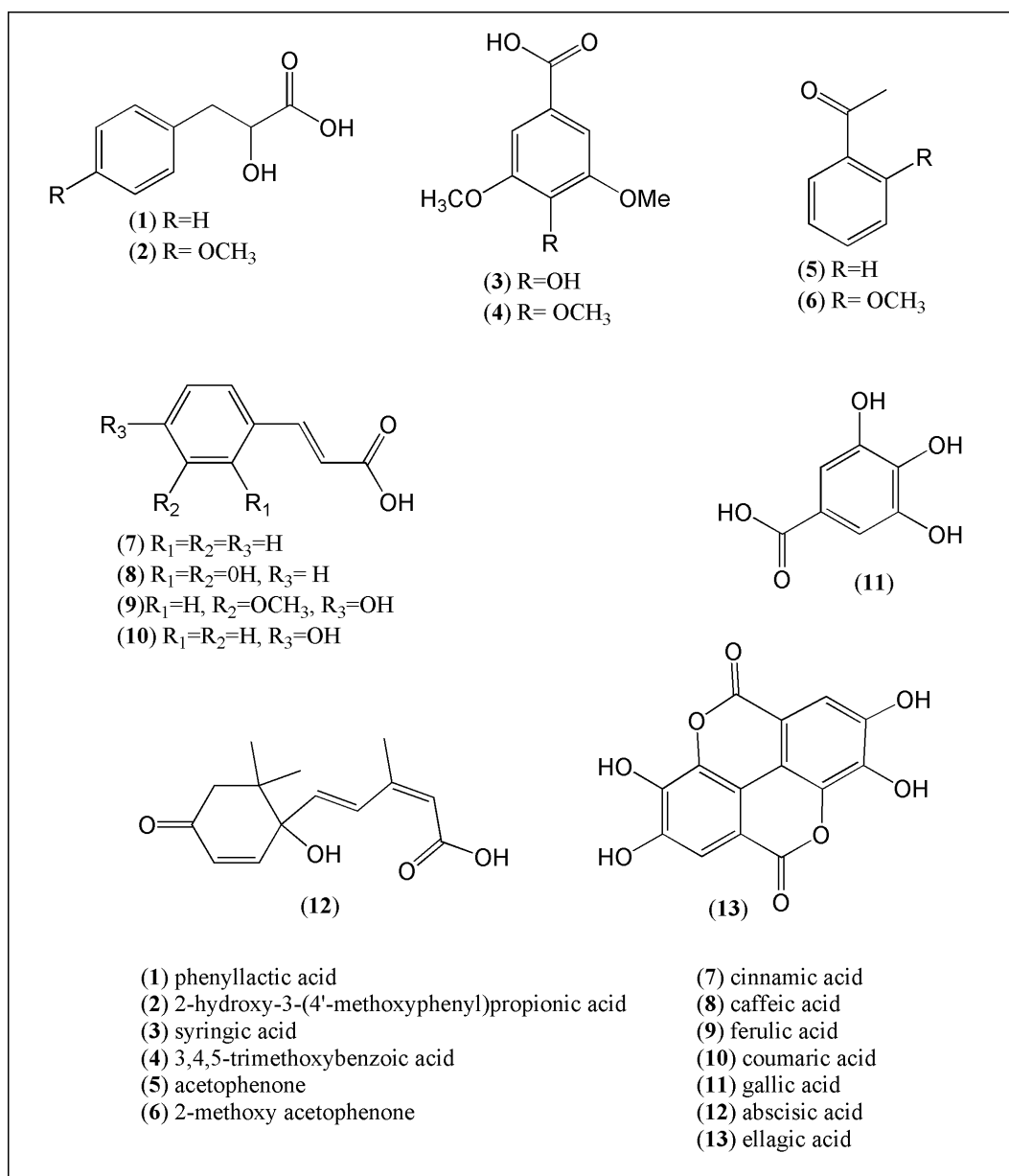


Figure 1.1: Chemical structures of some aromatic compounds found in manuka honey.

A number of minerals are commonly found in honey. These include potassium, sodium, calcium, magnesium, copper, iron, manganese, chloride, sulphur, phosphate and others. The mineral content of honey is on average 169 mg/100g but this is highly variable, ranging from 20 to 1030 mg/100g of honey.¹³ The mineral content of honey has been found to be highly dependent on environmental pollution. Levels of minerals and trace elements are generally higher in industrial areas due to pollution.¹²

Honey contains vitamins in low levels. Ascorbic acid is present at the highest concentrations with an average of 200 mg/100g found in honey. Other vitamins present include riboflavin, pantothenic acid, niacin, thiamine and pyridoxine at levels from 5.5-360 µg/100g.³

When bees collect nectar to be used in honey production, they inevitably collect pollen at the same time. This results in traces of pollen being present in honey. Melissopalnyology (pollen analysis) is the traditional method used to determine the floral origin of honeys. This involves using a microscope to identify the types of pollen present in the honey.¹²

1.2 Medicinal Properties of Honey

The use of honey as a wound dressing and medicine dates back to ancient civilisations. Like other traditional medicines it fell out of favour when antibiotics were discovered in the mid 20th century. However, the increasing problem of antibiotic resistance has resulted in a renewed interest in honey as a wound treatment.²⁶ This renewed interest has been helped by accounts of honey effectively treating chronic wound infections which have resisted other forms of treatment.²⁷⁻³¹ Honey has shown its effectiveness as a potent antibiotic agent for the treatment of infected wounds and is even being employed to prevent infection and improve healing in some wounds.^{32, 33}

The principal benefit of using honey as a dressing is its antibacterial activity against a large number of pathogenic bacteria and fungi.³⁴⁻³⁶ Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci bacterial strains show resistance to a range of antibiotics which makes their treatment problematic when they infect wounds. Clinical studies have found that honey is very effective as an antibiotic against these resistant strains.^{30, 37, 38} *Pseudomonas aeruginosa* is another resistant strain, isolated from burns, which has been treated with honey.³⁹

As well as being a potent antibacterial agent, honey also exhibits other properties which assist with wound healing. *In vitro* studies have shown that honey activates B and T-lymphocytes and phagocytes, and stimulates the release of tumour

necrosis factor α , interleukin 1 and 6 cytokines.⁴⁰ These cytokines convey signals in the immune system.⁴¹ This explains the observation that honey wound dressings increase immunity and stimulate the immune response.⁴⁰

The stimulation of the immune system, and the subsequent inflammation, by honey does not lead to long term chronic inflammation. By reducing the infiltration of inflammatory cells and providing antioxidants to scavenge free radicals, honey is able to prevent prolonged inflammation.^{42, 43} This lack of prolonged inflammation helps to reduce necrosis, scarring, oedema, pain and post-burn contracture.^{39, 42, 44}

One of the simplest ways in which honey can assist wound healing is simply by creating a physical barrier between the wound and outside sources of infection.⁴⁵ The debridement (removal of dead tissue) is also assisted by application of honey. This occurs through the activation of proteolytic enzymes which remove necrotic tissue without the need for chemical or surgical removal.⁴⁴⁻⁴⁶

Honey contains the enzyme glucose oxidase which catalyses the transformation of glucose to gluconic acid releasing hydrogen peroxide in the process.³ These low levels of hydrogen peroxide help to stimulate the growth of blood vessels (angiogenesis), fibroblasts and epithelial cells, assisting with the repair of tissue damage. The continuous supply of low level hydrogen peroxide to the wound also avoids excessive free radical generation and tissue damage caused by higher levels of hydrogen peroxide.⁴⁷

Dressing wounds with honey also helps to draw lymph through tissue with osmotic pressure. This provides a moist healing environment with increased oxygenation and nutrient supply to promote healing.⁴⁸ This moist environment enhances the rate of healing and prevents dressings from sticking to the wound, effectively allowing dressings to be changed painlessly and without damaging delicate regenerating tissue.^{42, 46}

Honey has also been observed to have a deodorising effect when used as a wound dressing. The antibacterial activity of honey helps to prevent odour producing bacteria from inhabiting the healing environment. Additionally, honey provides a

supply of glucose which can be metabolised by bacteria in place of amino acids. The lactic acid produced from the metabolism of glucose is preferable to the malodorous amines, ammonia and sulphur compounds produced by the metabolism of amino acids.⁴⁸

The principle medical application of honey is as an antibiotic wound dressing for chronically infected wounds.²⁷⁻³¹ It has also been effectively applied as a wound dressing to patients with complicating factors like immunosuppression.⁴⁹ Honey is now showing potential as a routine dressing for a range of uninfected wounds due to its ability to promote rapid healing and prevent infection.^{50, 51}

Aysan *et al.*⁵² conducted a study on rats which showed honey could be applied during abdominal surgery to prevent post-operative peritoneal adhesions. Peritoneal adhesions occur after 90% of all laparotomies and can cause intestinal obstruction, intra-abdominal abscess, ureteral obstruction and chronic abdominal pain. The rapid healing promoted by honey and the physical barrier it forms both help to prevent post-operative peritoneal adhesions and its associated problems.

Another medical application for honey which has been recently discovered is as an adhesive. Clinical studies have found honey to be very effective as an adhesive for split thickness skin grafts, being capable of holding the graft in place without the need for sutures. The antibacterial activity of honey provided an additional benefit by preventing infection.⁵³

Finally, confectionary made from UMF 15 manuka honey was found to reduce dental plaque and gingivitis when chewed after meals.⁵⁴

1.2.1 Antibacterial Activity of Manuka Honey

Several different factors, namely osmotic pressure, acidity and hydrogen peroxide, contribute to the antibacterial activity of honey of all origins. Manuka honey contains all these factors and an additional factor known as non-peroxide activity.

1.2.1.1 Osmotic Pressure

With the average composition of honey over 79% sugar and only 17.2% water, honey is super-saturated with sugars resulting in very strong interactions between sugar and water molecules.^{3, 13} This osmotic pressure limits the amount of water which is available to micro-organisms, thus inhibiting their growth. The amount of water available to micro-organisms is measured by a quantity known as water activity (a_w) with higher values reflecting higher availability of water.⁵⁵ Most species of bacteria are inhibited by conditions where a_w is below 0.94. Honey typically has a water activity within 0.47 to 0.70 a_w , well below the inhibition limit of most bacteria.⁵⁶ It has been calculated that honey exerts enough osmotic pressure to inhibit bacterial growth when diluted to levels as low as 2-12% (v/v honey in water) depending on the species of bacteria.^{34, 55}

Artificial honey is a solution of sugars in the same proportions as honey and should possess the same water activity as honey. The low water activity of undiluted artificial honey makes it effective against most species of bacteria. However, this antibacterial activity is lost when it is diluted while honey has been observed to retain its antibacterial activity when diluted. A study of 18 species of bacteria found that honey completely inhibited bacterial growth when diluted to 50% whilst artificial honey did not.³⁵

1.2.1.2 Acidity

Osmotic pressure is not the only antibacterial factor present in honey. Honey is typically acidic due to the presence of gluconic acid and gluconolactone.⁵⁵ The enzyme glucose oxidase, present in bee secretions, acts to convert glucose to gluconic acid in honey.³ This gluconic acid then spontaneously undergoes an intramolecular esterification to form gluconolactone. An equilibrium between the acid and the lactone exists which maintains a constant level of the free gluconic acid.⁵⁵ As a result the average pH of honey is 3.91¹³ but it does vary between 3.2 and 4.5.^{13, 55} The optimum pH for the growth of many pathogenic bacteria is between 7.2 and 7.4 but the minimum pH which pathogenic bacteria can tolerate is typically between 4 and 4.5. The acidity of honey is likely to be inhibiting to many bacterial species but it is unlikely to be effective in the wound environment due to the buffering capacity of body fluids neutralising the acidity of honey.⁵⁵

1.2.1.3 Hydrogen Peroxide

In most honeys it is hydrogen peroxide which accounts for all the observed antibacterial activity.⁵⁷ Hydrogen peroxide was identified by White *et al.*⁵⁸ as the major antibacterial agent in honey. Hydrogen peroxide is formed in honey by the enzyme glucose oxidase from bee secretions. This enzyme catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (Figure 1.2). The presence of hydrogen peroxide and acidity due to gluconic acid help to prevent spoilage of the honey while it ripens.^{3, 55}

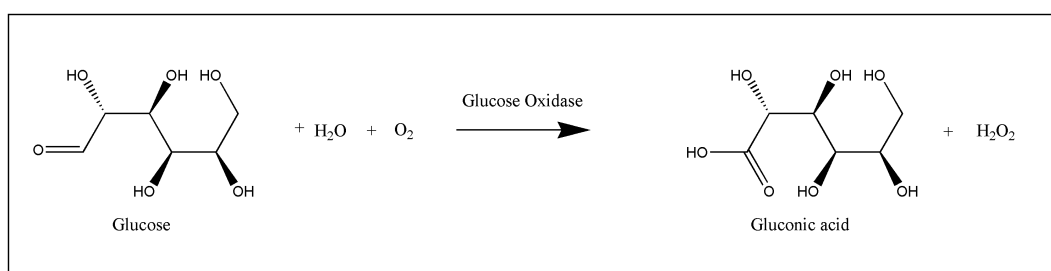


Figure 1.2: The enzymatic oxidation of glucose to gluconic acid and hydrogen peroxide.

While glucose oxidase is active while the honey matures, it is practically inactive in full strength honey.³⁴ The lack of activity in full strength honey is thought to be the result of gluconic acid, produced by the enzyme, lowering the pH to a point where the enzyme is no longer active. However, when honey is diluted the enzyme activity increases by a factor of 2,500 to 50,000 and hydrogen peroxide is produced.^{55, 59}

Honey contains a number of components which destroy hydrogen peroxide. The enzyme catalase, which catalyses the decomposition of hydrogen peroxide, occurs naturally in honey.^{60, 61} The decomposition of hydrogen peroxide to oxygen and water is also catalysed by transition metal ions and ascorbic acid present in honey. It is these components which destroy the hydrogen peroxide generated during the ripening of honey, leaving full strength honey with negligible levels of hydrogen peroxide.⁵⁵

High levels of hydrogen peroxide are known to cause DNA damage leading to cell injury and death.⁶²⁻⁶⁴ These problems of tissue damage associated with high

levels of hydrogen peroxide are not an issue when honey is used as an antibacterial agent. The activation of the glucose oxidase enzyme when honey is diluted, combined with negligible residual levels of hydrogen peroxide before dilution, make honey an effective source of hydrogen peroxide.⁵⁵ Honey can be used to supply controlled levels of hydrogen peroxide continuously over time to provide effective antibacterial activity without the tissue damage usually caused by applying hydrogen peroxide solution directly as an antibiotic.⁴⁷

The level of hydrogen peroxide antibacterial activity of honey varies considerably.^{65, 66} Variations in the levels of the hydrogen peroxide destroying components, particularly catalase, are responsible for the differences in hydrogen peroxide activity of honeys.⁵⁹

1.2.1.4 1.2.1.4 Non-Peroxide Activity and Methylglyoxal

Some honeys have been found to contain an additional antibacterial activity which cannot be attributed to osmotic pressure, acidity or hydrogen peroxide. Addition of catalase to honey results in the enzymatic decomposition of hydrogen peroxide and consequently a decrease in the peroxide antibacterial activity. While the enzymatic removal of hydrogen peroxide completely removes the activity of most honeys, some have been observed to retain significant levels of antibacterial activity. This activity has been defined as the non-peroxide activity.⁶⁷

A survey of 26 different types of monofloral New Zealand honeys found that non-peroxide activity was only present in 38% of manuka and 25% of viper's bugloss (*Echium vulgare*) honeys. Some manuka honeys in particular were observed to retain the majority of their antibacterial activity after the removal of hydrogen peroxide, suggesting that their antibacterial activity is due largely to the non-peroxide component.⁵⁷

Since the discovery of high non-peroxide activity in manuka honey in the late 1980's, there has been extensive and continuous research to try and identify the source of this activity. Mavric *et al.*⁶⁸ identified high levels of methylglyoxal (MGO) in manuka honey and showed it was responsible for the non-peroxide activity observed in manuka honeys. Using high performance liquid chromatography, MGO was detected as its quinoxaline derivative at levels

ranging from 38 to 761 mg/kg in six manuka honeys. These levels were significantly higher than those observed in 50 samples of other honey types which had an average of 3.1mg/kg and ranged between not detectable to 5.7 mg/kg.^{68, 69} A similar study determined the MGO levels for 49 manuka and 34 non-manuka honey samples directly, and using the o-phenylenediamine derivatisation to form its quinoxaline derivative. Both methods showed similar levels of MGO in manuka honey which varied from 38 to 828 mg/kg. A positive relationship between the level of MGO and the antibacterial activities of the honeys was also observed.⁷⁰

Mavric *et al.*⁶⁸ found that their six manuka honeys exhibited antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) at minimum concentrations of 15-30% (v/v with water). These levels correspond to MGO concentrations of 1.1 to 1.8 mM. The minimum concentration of MGO needed to inhibit these bacteria is 1.1mM suggesting that the high levels of MGO found in manuka honey is responsible for its non-peroxide activity. Further to this, addition of 1.9 mM MGO to an inactive forest honey gave it an antibacterial activity comparable to that of a 20% manuka honey containing a natural MGO level of 1.9 mM.⁶⁸

The identification of MGO as the component responsible for the non-peroxide activity has since been confirmed by isolation and identification of the active fraction of manuka honey.⁷⁰ Early work by Snow⁷¹ and Farr⁷² identified fractions of the reversed phase and size exclusion chromatograms of manuka honey which contained its non-peroxide activity. Using a combination of these techniques, Adams *et al.*⁷⁰ isolated 13.2 mg of the active compound from 50g of manuka honey with a high non-peroxide activity. Nuclear magnetic resonance spectroscopy and co-elution with an authentic sample of methylglyoxal were used to confirm that the active compound is MGO.

The source of the high levels of MGO found in manuka honey is unclear and the subject of ongoing investigations.

While the non-peroxide antibacterial activity of manuka honey has now been attributed to its high MGO levels, it is not known what role MGO plays in the other medicinal effects of honey detailed in Section 1.2.

Wound healing is often impaired in individuals suffering from diabetes. While the exact mechanism of this healing impairment is not known, the accumulation of advanced glycation end products (AGEs) has been implicated. MGO is a potent glycation agent and readily forms AGEs. In addition to endogenous sources of AGEs, exogenous dietary intake of AGEs such as MGO has been shown to further impair wound healing. An *in vivo* study of non-diabetic and diabetic mice on high and low AGE (*N*-carboxymethyl-lysine and MGO) diets showed that wound closure was slowest for diabetic mice on a high AGE diet. Wound closure in non diabetic mice was found to be independent of the dietary AGE intake while diabetic mice on a low AGE diet showed rates of wound closure intermediate between those of non diabetic and high dietary AGE diabetic mice. The high AGE diet decreased epithelialization, vascularity, granulation tissue formation and collagen organisation in wounds on diabetic mice. Inflammatory cell infiltration was also prolonged for diabetic mice on high AGE diets and thicker scars were observed to form. The dominant mode of wound closure was reepithelialization in mice on high AGE diets, and wound contraction for mice on low AGE diets.⁷³

AGEs have been shown to regulate a variety of inflammatory cell responses and growth-promoting events which normally occur during wound healing. However, excessive AGEs could impair normal cellular functions and wound tissue remodelling through promotion of oxidative stress.⁷³

Prolonged exposure of rats to MGO impaired wound healing and induced diabetes-like vascular damage. Impaired infiltration and reduced proliferative rates of regenerative cells was observed to cause immature granulation of wounds. A number of pro-inflammatory and profibrotic responses were also observed, including increased TNF-alpha (tumour necrosis factor-alpha) in regions of angiogenesis.⁷⁴

MGO can disrupt extra cellular matrix (ECM) interactions of endothelial cells leading to anoikis (where cell detaches from ECM and dies) of endothelial cells and decreased angiogenesis. The mechanism which causes this is binding of MGO to arginine residues at RGD and GFOGER integrin-binding sites of collagen (type IV) which are responsible for cell-cell and cell-matrix interactions, thus impairing ECM attachment of cells.⁷⁵

The toxicity of methylglyoxal has been the subject of a large number of *in vivo* and *in vitro* studies. Ghosh *et al.*⁷⁶ found that acute doses of 2 grams methylglyoxal per kilogram of body weight had no adverse effects when applied orally, subcutaneously or intravenously to mice and rats. Chronic doses of up to 1 g/kg body weight per day were also given orally, subcutaneously or intravenously to mouse, rats and dogs with no adverse effects being observed for 90 days after the treatment. However, other studies contradict these findings. A review by Kalapos⁷⁷ identified a number of studies reporting methylglyoxal to be toxic. A single dose of 800 mg methylglyoxal per kilogram of body weight, given intraperitoneally, was lethal to mice within 4 hours. The acute toxicity of methylglyoxal is not of high concern in the case of manuka honey since even manuka honeys with very high levels of MGO will not contain a lethal dose in a serving. To achieve a dose of 800 mg per kilogram would require one to consume a kilogram of UMF 30 manuka honey (MGO 800 mg/kg) for every kilogram of body weight, in a single serving.

Of much greater concern are the chronic effects of MGO. MGO is known to have a role in the development of diabetes complications and is also thought to be responsible for encephalopathy in short-bowel syndrome. The reaction of MGO with DNA can cause cancer but paradoxically it can also exert an anti-cancer effect through its cytotoxicity.^{76, 77} Clearly there is a need for further research on the toxicity of MGO and in particular the role it plays in the wound healing properties of honey.

1.3 Flavonoids

The flavonoids are a large and diverse group of phenolics with a basic $C_6-C_3-C_6$ structure in common. The two C_6 aromatic rings are designated ring A and ring B with the C_3 unit bridging them.^{78, 79}

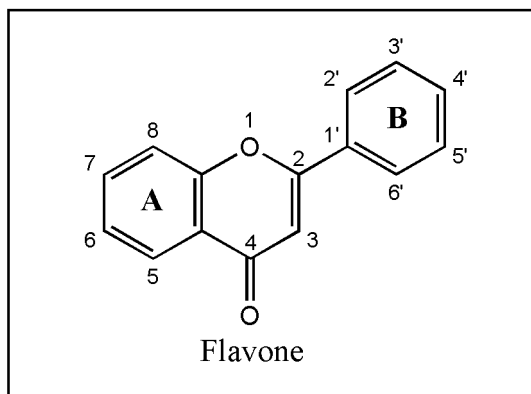


Figure 1.3: The structure of flavone.^{78, 79}

Flavonoids can be subdivided into a number of classes based on the oxidation state of the C_3 bridging unit.⁷⁹ The most significant of these classes are the flavones, their 3-hydroxy derivatives the flavonols and the anthocyanidins. Some other classes of flavonoids are the flavanones, the flavan-3-ols or catechins, the flavanonols or dihydroflavonols, the isoflavones, chalcones and aurones. The skeletal structures of these classes are shown in figure 1.4.^{79, 80} Individual compounds in these classes differ in the number and positions of substituents on the aromatic rings. Typically these substituents are hydroxy-groups which are often methylated or glycosylated.^{79, 81}

It has been observed that the majority of flavonoids found in nature exist as glycosides, with glycosylation typically occurring at the hydroxyl-groups.^{78, 79, 81}

However, there is a class of flavonoids called the C-glycosylflavonoids which have sugars linked to the aromatic skeleton through carbon-carbon bonds.⁸¹

Sugars can be present at more than one position on the flavonoid and it is not uncommon for the sugars to be di- or trisaccharides, though monosaccharides are certainly the most common.⁷⁹ While it is not unusual for extracts to contain free flavonoids as their aglycones, their presence is usually attributed to glycosidase enzymes which rapidly form the aglycone from the glycoside during extraction.⁷⁹

It is interesting to note that flavonoid glycosides have only recently been found in honey; the majority of studies finding only flavonoid aglycones in honey.¹² This is probably due to the action of amylase enzymes in bee saliva which would rapidly cleave the glycosidic linkage to liberate the flavonoid aglycones.⁸²

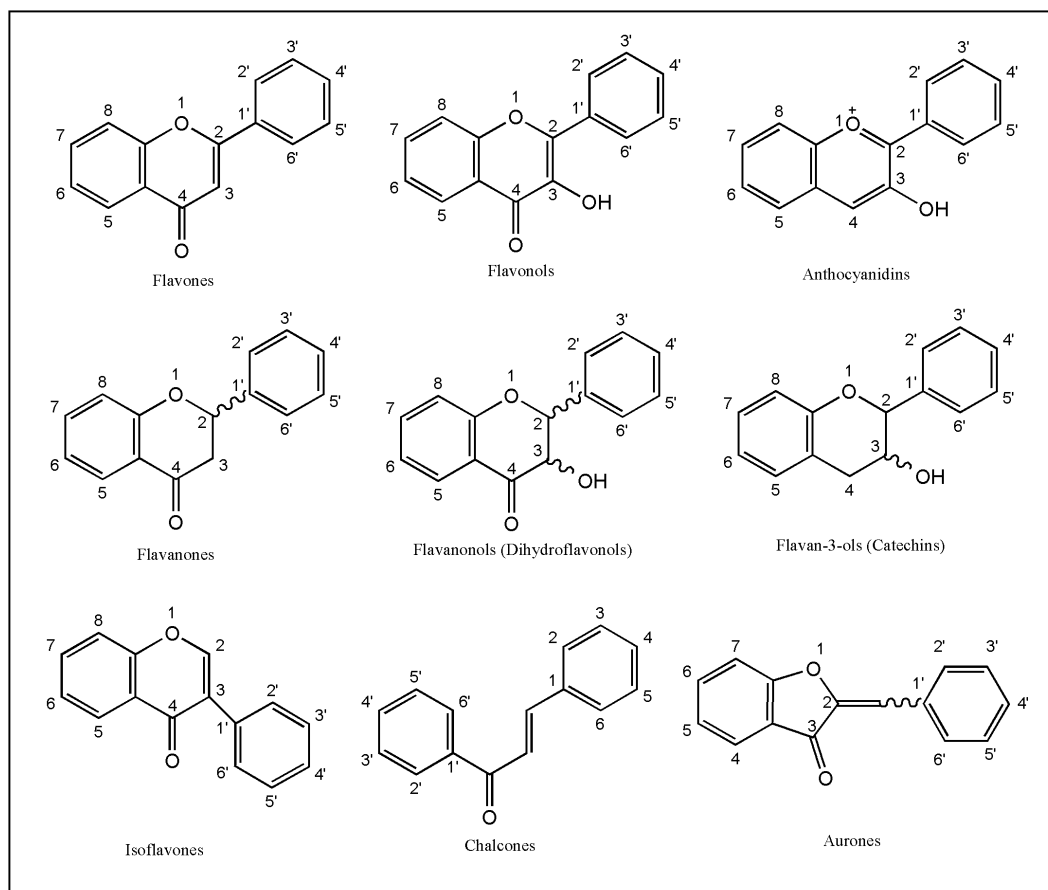


Figure 1.4: The structural skeletons of some of the flavonoid classes.^{79, 80}

1.3.1 Biochemistry of Flavonoids

The natural occurrence and biosynthesis of flavonoids is almost exclusively limited to the higher plants.^{79, 83} While there are a few reports of flavonoids in animals such as butterflies, these are most certainly the result of sequestration from plants in the diet rather than biosynthesis.^{79, 81} It has been reported however, that the mould *Aspergillus candidus* is capable of biosynthesising the flavonoid flavonin by the same route as the higher plants.⁷⁹

The biosynthesis of flavonoids occurs on a large scale, with an estimated 2% of the total carbon fixed by photosynthesis in plants being used to make flavonoids.⁷⁹

The biosynthetic origin of the C₆-C₃-C₆ lies in both the polyketide and shikimate biosynthetic pathways. Ring B and the linking C₃ unit are formed via the shikimic acid pathway whilst ring A is the result of condensation of three acetate units in the polyketide pathway.^{78, 79, 84}

Flavonoids occur in all parts of plants including the roots, stems, leaves, flowers, pollen, fruit, seeds, wood and bark.^{79, 83, 85} While particular flavonoids can be characteristic of some plant tissues, they are generally not confined to that tissue and will be found in other areas of the plant at lower levels.⁸³

The main function of flavonoids in plants seems to be as a pigment, providing colour to the plant's flowers to attract pollinators.^{78, 81, 86} The strong light absorbance of flavonoids in the ultra-violet region also allows them to act as a protective screen against harmful UV-B radiation.^{79, 81, 86}

Flavonoids can also act as protective agents against a number of attacking organisms including viruses, bacteria, fungi, herbivorous animals (both vertebrate and invertebrate) and even encroaching plants.^{79, 81, 86} This protection is afforded by both allelopathic and phytoalexin mechanisms. Allelopathic flavonoids produced by certain species of plants, such as ferns, inhibit the germination and growth of encroaching plants. Plants are also known to produce flavonoid phytoalexins in response to infection. The flavonoid phytoalexins produced are antibiotic and help fight off the invading pathogen.^{78, 81} Flavonoids are also known to alter gene expression and inhibit energy transfer in plant cells.^{82, 86}

Another important function of the flavonoids in plants is as messengers. Some plants, particularly the legumes, are able to release flavonoids from their roots into the surrounding soil. These flavonoids are taken up by nitrogen fixing bacteria where they induce expression of nod genes. Through this communication the bacteria is symbiotically incorporated into the root structure where the plant benefits from its nitrogen fixation.^{81, 82}

It is estimated that the average human ingests 0.5-2 g of flavonoids per day on a normal diet.^{82, 86} Such a large intake of relatively unknown flavonoids has led to much research into the effects of flavonoids on animal and human physiology.⁸²

Flavonoids have been reported to possess anti-inflammatory activity, oestrogenic activity, enzyme inhibition activity, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity, antiviral, antithrombotic, anaesthetic activity and cytotoxic antitumour activity.^{1, 82, 86} These activities give flavonoids the potential to prevent or treat cancers, cardiovascular disease, hypertension, allergies, vascular fragility, duodenal ulcers, gastric ulcers, diabetes, and bacterial and viral infections.^{1, 82}

Propolis (bee glue) has been recognised for its healing properties in civilisations as early as Ancient Greece where it was prescribed by Hippocrates (460-377 BC) as a topical treatment for sores and ulcers. The antimicrobial activity of propolis is attributed to its high flavonoid content, in particular the flavonoids galangin and pinocembrin.^{82, 86} Pure flavonoids and many plant extracts containing high levels of natural flavonoids have been found to possess antimicrobial activity against a variety of bacterial species including methicillin resistant *Staphylococcus aureus*.^{82, 86}

One of the most prominent and medically useful properties of the flavonoids is their ability to scavenge free radicals. Free radicals are highly reactive oxidising species such as the hydrogen peroxide radical and the hydroxyl radical. While these free radicals are produced during important physiological processes such as respiration and the immune response, left unchecked they can cause extensive cellular damage. Flavonoids can scavenge these free radicals, reducing them to non-reactive species and being oxidised themselves in the process. The oxidation of a flavonol to a *p*-quinone via a *p*-hydroquinone is shown in figure 1.5. The resulting *p*-quinone easily polymerises to an insoluble polymer which can be decomposed by the organism.⁸²

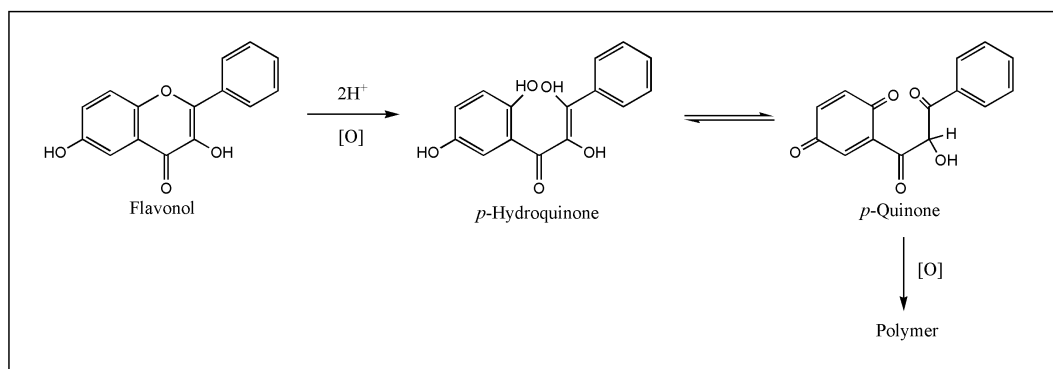


Figure 1.5: The oxidation of flavonols.⁸²

The LD_{50} for flavonoid aglycones injected into the blood of rats has been reported as $\sim 2\text{g/kg}$ of body weight.⁸² Another study measured the oral toxicity to rats of a flavonoid extract from the bracken, *Pteridium aquilinum*. It was shown that the LD_{50} was over 20.0g/kg of body weight and no mutagenic effect was observed in the sperm or the micronucleus test.⁸⁷ Consumption of flavonoids is unlikely to have an acutely toxic effect in humans. This is due to the low solubility of flavonoid aglycones in water, a short residence time in the intestine and a low coefficient of absorption (about 15%). However, there are rare cases where flavonoids can induce allergic reactions in humans.⁸²

It is inevitable that the human diet will contain flavonoids since they are ubiquitous in plants. This has certainly been the case throughout the history of the human race so it can be considered unlikely that chronic exposure to flavonoids would have serious negative health effects.⁸² However, *in vitro* studies have shown flavonoids to have a number of gene inducing, mutagenic, hormone influencing and other physiological effects which may be of some concern.^{78, 82} In spite of this it is generally considered that regular consumption of flavonoids does not pose a health risk. The half life of a typical flavonoid in the human body is only one to two hours so daily exposure to flavonoids in the diet does not cause them to accumulate in the body.⁸²

The flavan-3-ols epicatechin, epicatechin gallate and epigallocatechin gallate have been found to form adducts with C_2 , C_3 , C_4 and C_6 sugar fragments, and significantly reduce the levels of these fragments *in vitro*.^{88, 89} These sugar

fragments include methylglyoxal which has recently been identified in manuka honey as the source of its non-peroxide antibacterial activity.^{70, 88, 89} Section 1.2.1.4 highlighted a number of the physiological effects of methylglyoxal in biological systems. The observation that catechins can moderate the formation of methylglyoxal in aqueous sugar systems, and facilitate its removal as an adduct, suggests that catechins and potentially other flavonoids may play a significant role in the physiological effects of methylglyoxal in manuka honey. The adduct formation, shown in figure 1.6, was found to occur at the C6 and C8 positions of the catechin A ring. The A ring is structurally similar in all the flavonoid classes so it is feasible that this adduct formation is not restricted to the catechins.⁸⁹ It must be noted however that methylglyoxal exists at significantly higher levels (38-828 mg/kg) in manuka honey than the total flavonoid content (0.306 mg/kg) of manuka honey. It should be considered unlikely that flavonoids would be capable of eliminating the free methylglyoxal in manuka honey through adduct formation.^{24, 70}

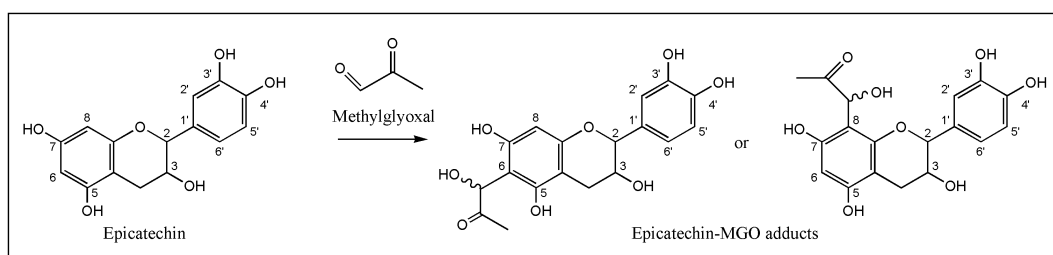


Figure 1.6: The formation of adducts between epicatechin and methylglyoxal (MGO).⁸⁹

1.3.2 Identification of Flavonoids

Traditional methods for the identification of flavonoids made use of colour reactions with reagents such as aqueous sodium hydroxide, concentrated sulphuric acid, magnesium-hydrochloric acid and sodium amalgam with acid. Colour changes observed when the flavonoid undergoes reaction with these reagents gives information on the oxidation state of the C₃ bridging unit and hence the class of the flavonoid. Variations in the colour changes can also be used to identify substitution patterns of flavonoids to a limited extent.⁹⁰

Degradative studies have also been of historical importance in the identification of flavonoids. These studies normally utilise alkaline hydrolysis with subsequent identification of the benzene ring fragments.^{79, 81, 90}

While colour reactions and degradative studies were of great importance in the early days of flavonoid chemistry, they have been superseded by spectroscopic techniques which require much less material for characterisation and yield more structural information much more quickly. The information which can be gained through ultraviolet and visible absorption (UV-Vis) and nuclear magnetic resonance (NMR) spectroscopy is enough to determine the structure of most flavonoids.^{81, 91, 92}

1.3.2.1 Ultraviolet-Visible Absorption Spectroscopy

The large degree of conjugated unsaturation in the flavonoids causes them to absorb strongly in the ultraviolet and visible regions of the electromagnetic spectrum.⁷⁹ They typically exhibit two major absorption peaks in the region of 240 to 400 nanometres. These peaks are commonly referred to as Band I (usually 300-380 nm) and Band II (usually 240-285 nm). Band I is associated with absorption due to the B-ring cinnamoyl system while Band II is the result of absorption involving the A-ring benzoyl system (see figure 1.7).^{81, 91-93}

The assignments of Band I and II is supported by the UV spectra of substituted flavonoids. Flavones and flavonols which are oxygenated in the A-ring but not in the B-ring (e.g. chrysin and galangin) tend to exhibit a pronounced Band II with only a weak Band I in their UV spectra. If the B-ring is also oxygenated (e.g. quercetin and kaempferol) then Band I is more pronounced and will appear at longer wavelengths.^{92, 93} Further evidence is found in the UV spectra of the flavanone and dihydroflavonol classes of flavonoids. These classes lack the double bond between C2-C3 of the bridging unit (see figure 1.4). As such there is no conjugation between the A- and B-ring systems. The reduction in length of the B-ring conjugated system significantly reduces the intensity of the Band I absorption so that it appears as only a shoulder or low intensity peak next to the intense Band II absorption.^{81, 93}

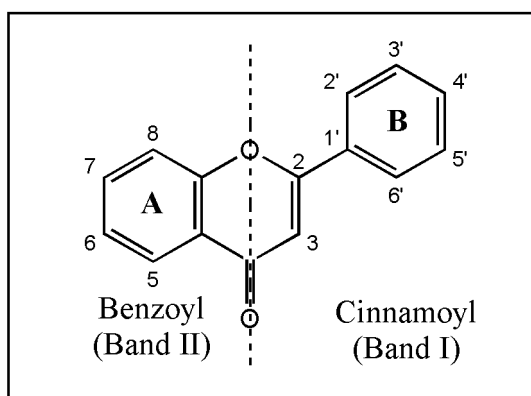


Figure 1.7: The conjugated systems responsible for the major ultraviolet-visible absorption peaks of flavonoids.⁹¹⁻⁹³

Simple inspection of the UV spectra of a flavonoid in methanol can give information about the degree of oxygenation and the substitution pattern. Increasing oxygenation of the B-ring causes a bathochromic shift (i.e. to longer wavelengths) of Band I. For example the flavonols galangin, kaempferol, quercetin and myricetin all have the same 3,5,7 oxidation pattern on their A- and C-rings but differ in the oxidation of their B-rings. These differences are enough to shift the position of the Band I peak to the wavelengths shown in table 1.2.

Table 1.2: The effect of oxygenation on the position of the Band I absorption peak of flavonols.^{91, 93}

Flavonol	Hydroxyl Positions	Band I Absorption Wavelength
Galangin	3,5,7	359 nm
Kaempferol	3,5,7,4'	367 nm
Quercetin	3,5,7,3',4'	370 nm
Myricetin	3,5,7,3',4',5'	374 nm

Changes in the B-ring oxidation pattern generally do not produce shifts in Band II but they can cause it to appear as either one or two peaks. These are designated IIa and IIb with IIa being the peak at longer wavelength. The 3',4' and to a lesser extent the 3',4',5' B-ring oxidation pattern causes flavones and flavonols to exhibit two Band II peaks (or one peak with a shoulder on the longer wavelength side).

In contrast, flavones and flavonols with the 4' B-ring oxidation pattern only show a single Band II peak.^{91, 93} In the flavanones and dihydroflavonols the oxidation pattern of the B-ring has no noticeable effect on the UV spectra.⁹³

The oxidation pattern of the A-ring has an effect on the wavelength of the Band II absorption and in the case of the flavones and flavonols, a smaller effect on the Band I absorption. Flavanones and dihydroflavonols do not exhibit significant Band I absorption peaks. The 5-hydroxyl group is particularly significant in this regard, its presence in a flavonoid increasing the wavelength of the Band II absorption by 6-17 nm and shifting Band I by 3-10 nm.^{91, 93}

Just as the oxidation pattern of the A- and B-ring of the flavonoids can have a significant effect on the UV spectra, so too the nature of the oxygen also has an effect on the spectra. The shifts mentioned previously refer to free hydroxyl groups added to or removed from the flavonoid. Methylation or glycosylation of the 3-, 5- or 4'-hydroxyl groups will produce a hypsochromic shift (i.e. to shorter wavelengths), particularly in the Band I absorption peak. Substitution of the 3-hydroxyl group produces a hypsochromic shift of 12-17 nm in Band I but this can be as large as 22-25nm in flavonols which do not contain a free 5-hydroxyl group.^{91, 93} Band II is not affected by substitution at the 3-hydroxyl group.⁹² Methylation or glycosylation of the 5-hydroxyl group produces a hypsochromic shift of 5-15 nm in both Bands I and II. Substitution of the 4'-hydroxyl group causes a hypsochromic shift of 2-10 nm in Band I while Band II may undergo smaller shifts of 2-6 nm (bathochromic) in flavones or 1-2 nm (hypsochromic) in flavonols.⁹¹⁻⁹³ Similar substitutions at other positions have little or no effect on the UV spectrum.^{91, 93}

Acetylation of a flavonoid's phenolic hydroxyl group nullifies the effect of that hydroxyl group on the UV spectrum. This effect has been utilised to great effect in the location of methoxyl groups in polyhydroxyflavones. Acetylation of the free hydroxyl groups in such a flavonoid causes its UV spectrum to approximate that of the parent methoxyflavone allowing the location of the methoxyl group to be determined.⁹¹⁻⁹³

While there is much information to be obtained simply from examination of the UV spectrum of flavonoids in methanol, there exist specific reagents which further enhance this information. These specific reagents react with one or more functional groups in the flavonoid to produce noticeable shifts in the UV spectrum.⁹¹ These reagents include sodium methoxide, sodium acetate, sodium acetate/boric acid, aluminium chloride and aluminium chloride/hydrochloric acid.⁹¹⁻⁹³

Bases such as sodium methoxide and sodium acetate are able to ionise the hydroxyl groups of flavonoids and thus change the UV spectrum. Sodium methoxide is a strong base and ionises all the free hydroxyl groups to some extent. As a result, sodium methoxide causes bathochromic shifts to the spectra of flavonoids with free hydroxyl groups. This reagent is particularly useful for detecting the presence of free 3- and 4'-hydroxyl groups. A large bathochromic shift of 40-65 nm in Band I, without a decrease in the peaks intensity, is indicative of a free 4'-hydroxyl group. Flavonols without a free 4'-hydroxyl group also show a large bathochromic shift of 50-60 nm in Band I upon addition of sodium methoxide. The shifted Band I peak is often decreased in intensity, indicating the presence of a free 3- hydroxyl group.^{81, 91, 93}

Flavonols containing free hydroxyl groups at both position 3 and 4' are unstable in alkali and their UV spectrum will consequently degenerate on addition of sodium methoxide. This will usually occur over a few minutes so it is useful to measure the UV spectrum immediately after addition of the reagent and again five minutes later to allow time for decomposition to occur. The presence of a 3,3',4'-trihydroxyl system makes the flavonol even more unstable and decomposition will be rapid. While these oxidation patterns are most commonly associated with alkali instability, other patterns may also be sensitive to alkali. The 5,6,7; 5,7,8 and 3',4',5' oxygenation patterns are worth particular mention for their instability in alkali.^{81, 91, 93}

The lack of a significant Band I absorption in the flavanones and dihydroflavonols means that sodium methoxide cannot be used to detect free 4'- hydroxyl groups in these compounds. Sodium methoxide is still a useful reagent for these classes of flavonoids since the absence of a shift in the Band II absorption is a clear

indication that the A-ring is not hydroxylated. In addition, this reagent can be used to determine the presence or absence of a free 5-hydroxyl group. A bathochromic shift of 34-50 nm indicates a 5,7-dihydroxy system while similar flavanones and dihydroflavonols without the free 5-hydroxyl group will exhibit a larger shift of 55-60 nm. In both these cases the shift is accompanied by an increase in intensity of the Band II absorption.^{91, 93}

In flavanones and dihydroflavonols the 5,6,7 and 5,7,8 oxygenation patterns are unstable in alkaline conditions and the UV spectra of such flavonoids show degeneration upon addition of sodium methoxide. Some flavanones, particularly those without a free 5-hydroxyl group, will isomerise to chalcones under alkaline conditions, resulting in a completely different UV spectrum (with a Band I peak in the 400 nm region).^{91, 93}

Sodium acetate is a weaker base than sodium methoxide. Consequently, only the more acidic of the phenolic hydroxyl groups are significantly ionised. In the flavones and flavonols these are the 3,7- and 4'-hydroxyl groups. Ionisation of hydroxyl groups at the 3 and 4' positions mainly affects the Band I absorption while ionisation of a free 7-hydroxyl group causes a bathochromic shift of the Band II absorption peak by 5-20 nm. In flavonoids without a 7-hydroxyl group, or where this group is protected by methylation or glucosylation, there is no significant change in Band II on addition of sodium acetate. This makes sodium acetate particularly useful for the detection of free 7-hydroxyl groups.^{81, 91-93} The size of this shift is severely reduced in flavones (but not flavonols) possessing oxygen substituents at the 6 and 8 positions. This is probably due to the 6- and 8-substituents reducing the acidity of the 7-hydroxyl group.⁹¹⁻⁹³ Protected 7-hydroxyl groups can be detected when the flavone or flavonol also has a 4'-hydroxyl group. Addition of sodium acetate to such flavones and flavonols with substituted 7-hydroxyl groups will exhibit a Band I shift the same or larger than that observed by reaction with sodium methoxide.⁹¹

Reaction with sodium acetate can also be used to detect free 7-hydroxyl groups in the flavanones and dihydroflavonols. When both a 7- and 5-hydroxyl group are present in a flavanone or dihydroflavonol, a bathochromic shift of 34-37 nm is

observed. This shift is increased to 51-58 nm when the 5 position is deoxygenated.^{81, 91, 93}

As is the case for sodium methoxide, addition of sodium acetate to flavonoids with alkali sensitive oxygenation patterns causes decomposition of the flavonoid and degeneration of the UV spectrum. Those groupings which caused degeneration of the UV spectra when sodium methoxide is added have a similar effect when sodium acetate is added.^{91, 93}

When flavonoids are reacted with boric acid in the presence of sodium acetate, the boric acid will chelate with orthodihydroxyl groups. This occurs at all orthodihydroxyl groups on the flavonoid nucleus except for the C-5, 6 pairing. These complexes are thought to be of the type shown in figure 1.8.^{81, 93}

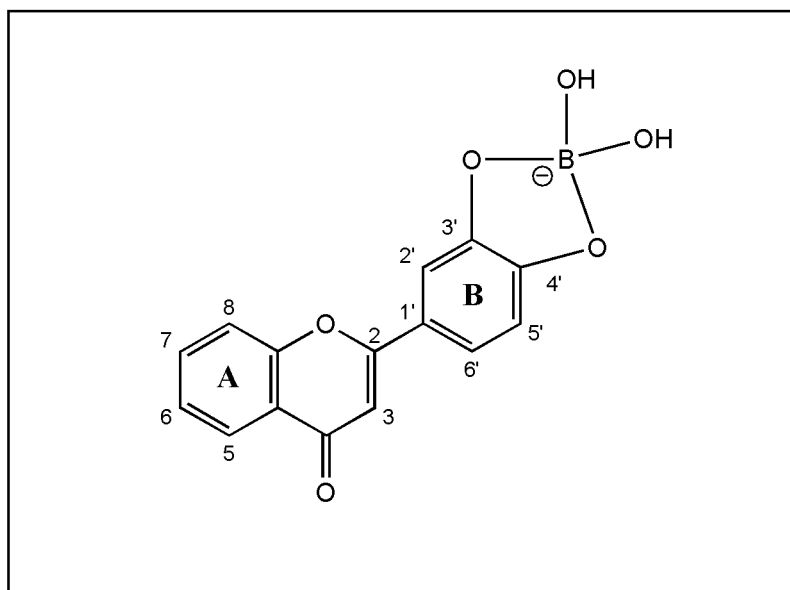


Figure 1.8: The proposed structure of the complex formed by reaction of orthodihydroxyl groups on the flavonoid nucleus, with boric acid in the presence of sodium acetate.^{81, 93}

The formation of these complexes can cause a noticeable bathochromic shift in the absorption bands of the UV spectrum. For the typical ranges of shifts observed refer to table 1.3. Orthodihydroxyl groups on the B ring of the flavanones and dihydroflavonols are not detectable by this technique due to the lack of conjugation between the two ring systems.^{91, 93}

Aluminium chloride will also form complexes with certain functional groups in the flavonoid nucleus. Those flavonoids which contain the ketone functionality at C-4 (this includes the flavones, flavonols, flavanones and dihydroflavonols) and hydroxyl groups at C-3 or C-5 will form acid-stable complexes with AlCl_3 . Orthodihydroxyl groups (other than the C-5,C-6 pair) will also complex with AlCl_3 . These orthodihydroxyl complexes are not stable under acid conditions however and will decompose to the uncomplexed flavonoid in the presence of acid. The structures and reactions of these complexes are shown in figure 1.9.^{81, 91, 93}

Table 1.3: The bathochromic shifts observed in the UV spectrum of flavonoids reacted with boric acid and sodium acetate.^{91, 93}

Flavonoid Class	Orthodihydroxyl Position	Bathochromic Shift Observed
Flavones and Flavonols	A-ring (6,7 and/or 7,8)	5-10 nm (Band I)
	B-ring	12-30 nm (Band I)
Flavanones and Dihydroflavonols	A-ring (6,7 and/or 7,8)	10-15 nm (Band II)
	B-ring	Not Detectable

The formation of aluminium complexes with the orthodihydroxyl groups of flavonoids causes bathochromic shifts in both absorption bands. Addition of acid causes these complexes to decompose and a hypsochromic shift is observed in the UV spectrum. Table 1.4 shows the characteristic hypsochromic shifts observed when a solution of AlCl_3 and a flavonoid is acidified.^{81, 91, 93} If a flavone or flavonol has orthodihydroxyl groups on both the A- and B-rings then the shifts are cumulative.⁹¹

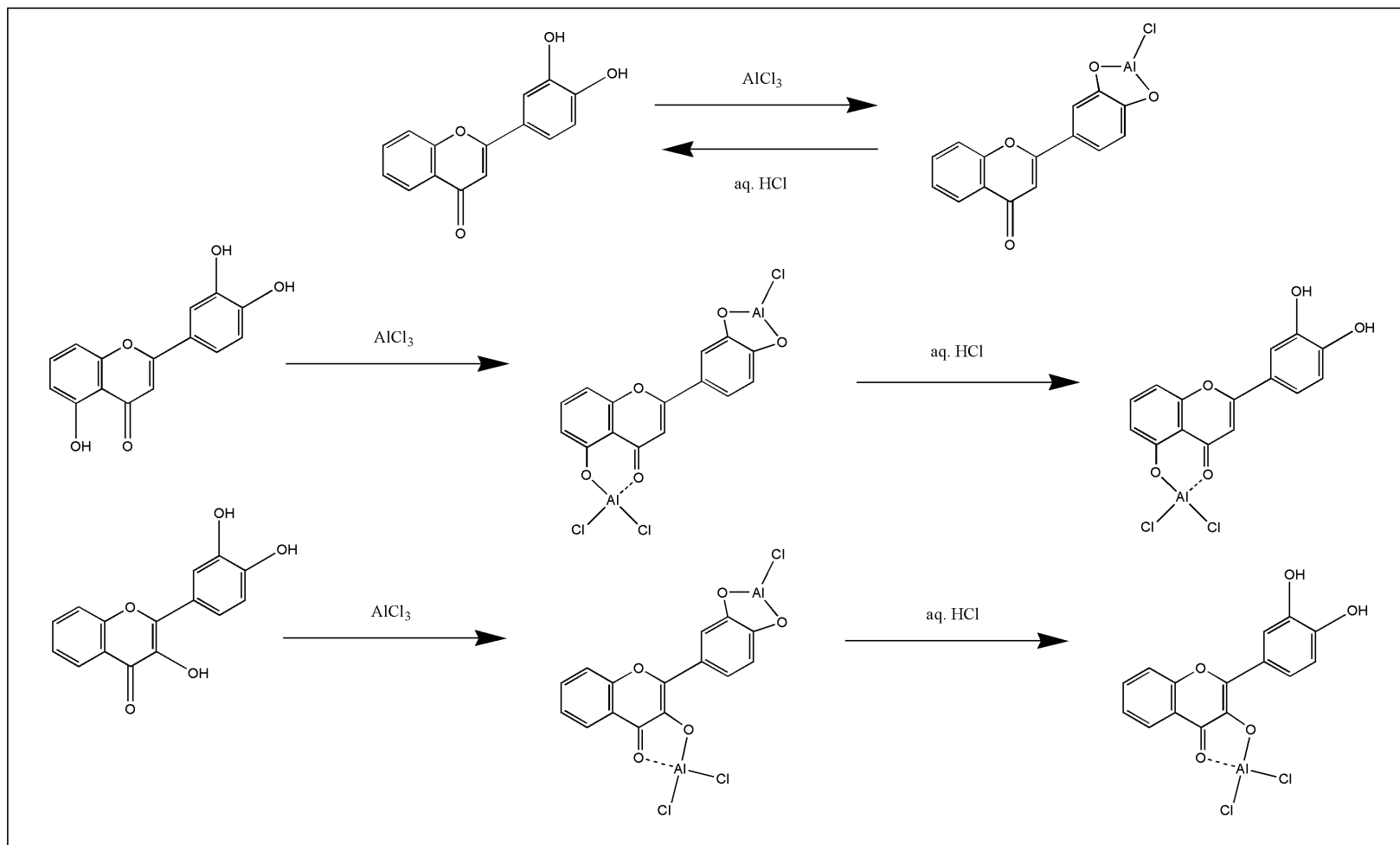


Figure 1.9: The complexation of flavonoids with AlCl_3 in the presence and absence of acid.⁹³

Table 1.4: The shifts observed in the UV spectra of flavonoids in methanol and AlCl₃ solution when acidified.^{91, 93}

Flavonoid Class	Orthodihydroxyl Position	Hypsochromic Shift of the AlCl ₃ /HCl UV Spectra Relative to the AlCl ₃ Spectra.
Flavones and Flavonols	B-ring (2 adjacent OH)	30-40 nm (Band I)
	B-ring (3 adjacent OH)	20 nm (Band I)
	A-ring (C-6,7 and C-7,8)	20-25 (Band I)
Flavanones and Dihydroflavonols	B-ring	Not Detectable in UV Spectrum
	A-ring (C-6,7 and C-7,8)	11-30 nm (Band II)

The addition of acid to a methanolic AlCl₃ solution of a flavonoid decomposes complexes between AlCl₃ and orthodihydroxyl groups. Any remaining shift in the UV spectrum, compared to the spectrum in methanol, is due to complexation between a free 3- or 5-hydroxyl group, the C-4 keto functionality and AlCl₃. The bathochromic shifts observed for these complexes are shown in table 1.5.^{81, 91, 93}

In flavones and flavonols, the complex formed with a free 3-hydroxyl is more stable than that formed with a free 5-hydroxyl group. This makes the 3-hydroxy-4-keto complex more favoured than the 5-hydroxy-4-keto complex in flavones and flavonols containing free hydroxyls at both C-3 and C-5. In the dihydroflavonols and flavanones, the 3-hydroxy-4-keto complex is actually more unstable than that formed with orthodihydroxyl groups. As a result, these complexes also decompose when acidified. They can be distinguished from the orthodihydroxyl complexes by the disappearance of the shift on addition of sodium acetate (which has no effect on orthodihydroxyl complexes).⁹¹

Table 1.5: The bathochromic shifts in the UV spectrum due to complexation of AlCl_3 with the 3- or 5-hydroxyl groups and the 4-keto group.^{81, 91, 93}

Class of Flavonoid	AlCl_3 Complex	Bathochromic Shift of AlCl_3 UV Spectra Relative to the MeOH Spectra.
Flavones and Flavonols	5-hydroxy-4-keto	35-55 nm (Band I)
	5-hydroxy-4-keto (with oxygenation at C-6)	17-20 nm (Band I)
Flavonols	3-hydroxy-4-keto	50-60 nm (Band I)
Flavanones and Dihydroflavonols	5-hydroxy-4-keto	20-26 nm (Band II)
Dihydroflavonols	3-hydroxy-4-keto	30-38 nm (Band II)

1.3.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

Both proton (^1H) and carbon (^{13}C) NMR spectroscopy is a useful technique for the characterisation of all flavonoids. Originally, the NMR study of flavonoids was limited to those which were soluble in deuteriochloroform (CDCl_3) or carbon tetrachloride (CCl_4). This confined early work to relatively non-polar flavonoids, particularly highly methylated flavonoid aglycones. The introduction of hexadeuteriodimethylsulfoxide ($\text{DMSO}-d_6$) in 1964, and the development of CCl_4 soluble trimethylsilyl ether (TMS-ether) derivatives in 1965, has opened up NMR to most naturally occurring flavonoids.^{81, 91, 93}

Most flavonoid aglycones and glycosides are sufficiently soluble in $\text{DMSO}-d_6$ to allow direct NMR analysis without the need to prepare derivatives. There are a number of disadvantages associated with using $\text{DMSO}-d_6$. The high boiling point of $\text{DMSO}-d_6$ makes the recovery of flavonoids from this solvent difficult. Some flavonoids are also known to decompose in $\text{DMSO}-d_6$ and its tendency to absorb moisture can also be problematic.^{91, 93}

The use of TMS-ether derivatisation of flavonoids to make them soluble in CCl₄ is the most widely used technique for NMR of flavonoids. All hydroxyflavonoids and their glycosides may be converted into soluble TMS-ether derivatives and no interfering signals are observed. The flavonoids can be recovered from their derivatives by treatment with aqueous methanol. Partial trimethylsilylation can also be utilised to get information about the substitution at the C-3, 6 and 8 positions, and the TMS-ethers can be easily converted into acetates or methyl ethers for more information to assist structural assignment. A limitation of the TMS-ether derivatisation is that it converts the hydroxyl protons to ethers and consequently these signals are not observed.^{91, 93}

Table 1.6: Typical ¹H chemical shifts and multiplicities for flavonoids.^{81, 91, 93}

Position	Range of Chemical Shifts (ppm)	Commonly Observed Signal Multiplicities
H-5	7.7-8.2	doublet
H-6	5.7-7.1	doublet or doublet of doublets
H-8	5.9-7.0	doublet
H-1',2',3',4',5' (B-ring)	6.7-7.9	doublets and/or doublet of doublets
H-3 (flavone)	ca. 6.3	singlet (sharp)
H-2 (flavanone)	5.0-5.5	doublet of doublets
H-3 (flavanone)	ca. 2.8	doublets of doublets
H-2 (dihydroflavonol)	4.8-5.0	doublet
H-3 (dihydroflavonol)	4.1-4.3	doublet
hydroxyl protons	ca. 9.7-12.4	singlets (may be broadened)
methoxyl protons	3.5-4.1	singlet
glucosyl protons	3.3-4.2 (H-1' further downfield)	complex

Proton NMR of flavonoids can yield significant information about oxygenation and substitution patterns. Table 1.6 shows the typical regions of the ¹H NMR spectrum where flavonoid proton signals will appear. The combination of

chemical shift and multiplicity of the standard flavonoid proton signals allows the oxygenation pattern to be determined. The natures of substitutions present in the flavonoid are also apparent from the presence of other signals such as methoxyl or glucosyl protons. Determination of where these substitutions are on the flavonoid nucleus is more complicated and requires advanced interpretation of partially trimethylsilylated derivatives or two dimensional NMR.^{81, 91, 93}

1.3.3 Analysis of Flavonoids in Honey

Honey contains a range of flavonoids with the total level being about 0.005-0.010%. These flavonoids are thought to originate from nectar, pollen which has a flavonoid content of 0.5%, and propolis which has a flavonoid content of 10%.¹²

Any analysis of the flavonoids in honey is affected by two major problems. The first of these is the high sugar content of honey which interferes with the direct and complete extraction of flavonoids.^{94, 95} Liquid-liquid extractions are hampered by the formation of inconvenient interphases due to the high sugar content.⁹⁵ The use of the non-ionic polymeric resin Amberlite XAD-2 allows the sugars and polar compounds to be removed, leaving a fraction containing flavonoids and other phenolic compounds.^{94, 95}

Phenolic acid derivatives make up the larger part of the phenolic fraction obtained from XAD-2 extraction and are part of the second problem of honey flavonoid analysis. The analysis of flavonoids is hampered by interference from other UV-absorbing compounds, including the phenolic acid derivatives.⁹⁴ Column chromatography through Sephadex LH-20 is an effective method for the separation of flavonoids from other phenolics. Observing the elution of the phenolics fraction under 360nm UV light clearly shows the phenolic acid derivatives eluting first as a blue fraction. The flavonoids elute later as a dark purple fraction.⁹⁴

There are some disadvantages associated with the use of Sephadex LH-20 to clean up phenolic fractions. Firstly, the technique is time consuming and complex, requiring a dark room setup to follow the chromatography under UV light. The separation of the flavonoids from the other phenolics is often not clear cut and a portion of the flavanones, which elute very closely to the phenolic acids, is

discarded. This prevents an accurate quantification of the flavanones being made.⁹⁵ An alternative to Sephadex LH-20 chromatography has been developed to separate the flavonoids from the other phenolics. A simple liquid-liquid extraction with diethyl ether preferentially removes the flavonoids (recovery >95% after three extractions) from an aqueous solution of the phenolic fraction, leaving the majority of the phenolic acid derivatives and any remaining polar compounds in the aqueous layer.⁹⁵ Further studies have raised concerns that this simplified procedure may only be effective when used to analyse light coloured honeys which are rich in flavonoids and contain few phenolic acid derivatives. Darker honeys tend to contain more phenolic acid derivatives and the simplified extractive technique may not be as effective as chromatography through Sephadex LH-20.⁹⁶

The principal method used to analyse flavonoid fractions is high performance liquid chromatography with detection by UV absorbance.^{1, 12, 94} Such HPLC systems are usually binary, starting with an acidified (e.g. formic acid) aqueous solvent and changing to an organic solvent such as methanol over the duration of the HPLC run. An alternative to UV detection is liquid chromatography coupled to electrospray mass spectrometry (LC-MS). This technique has the advantage of providing additional information about the structure of flavonoids from fractionation patterns, potentially allowing new flavonoids to be identified without the need for them to be isolated.¹

Capillary electrophoresis (CE) has also been used as an alternative method for the analysis of flavonoids. Micellar electrokinetic chromatography (MEKC) or micellar electrokinetic capillary chromatography (MECC) is a variation of CE which has been successfully used to separate and identify flavonoids extracted from honey.^{1, 97-99} The main disadvantage of this technique is that different conditions are required depending on which flavonoids are being targeted. An early study by Delgado *et al.* optimised three sets of chromatographic conditions for honey flavonoid analysis.⁹⁸

1.3.3.1 Flavonoids in Honey

With the exception of two recent studies, the flavonoids found in honey exist as aglycones.^{1, 12, 100, 101} Flavonoid glycosides in floral nectar are generally converted

to their aglycones by hydrolytic enzymes in the bee saliva and/or honey.^{12, 101, 102} Recently it has been reported that Acacia (*Robinia pseudoacacia*) honey contains eight rhamnose and hexose glycosides of kaempferol. It has been suggested that flavonoid rhamnosides are not hydrolysed by bee secretions like other honey glycosides (e.g. kaempferol 3-diglucoside). This is likely because rhamnosidase enzymes have not been reported in bee secretions.¹⁰¹ Another study of Lithuanian honey found the flavonoid glycoside hyperoside (quercetin-3-O-galactoside) in willow honey, and vitexin (apigenin-8-C-glucoside) in caraway and lime honey.¹⁰⁰ The discovery of flavonoid glycosides in honey significantly increases the number of flavonoids which could be investigated as markers of honey floral origin. However, it is not possible to study these glycosides using the established XAD-2 extraction method, the increased polarity of these glycosides requiring an alternative extraction techniques.¹⁰¹

The flavonoids found in honey belong to the flavone, flavonol, flavanone and dihydroflavonol classes.^{1, 12} Parent structures of these flavonoid classes are shown in figure 1.4. At least 26 flavonoids have been identified in honeys of various floral origins. The structures of some of these flavonoids are shown in figure 1.10.

Honey produced in the Northern Hemisphere is characterised by the presence of the propolis derived flavonoids. The most significant of these are pinocembrin, pinobanksin and chrysin but pinobanksin-3-acetate, galangin, galangin-3-methyl ether, pinocembrin-7-methyl ether and tectochrysin have also been identified in honey and propolis from temperate areas of Europe, North America and Asia.^{103, 104} In these areas the poplar tree is native and honey bees will preferentially harvest its bud exudates to create propolis. In contrast, honeys from most equatorial regions and Australia contain minimal levels of these propolis flavonoids. A lack of poplar trees in these areas means that bees have to find alternative sources of material for propolis, consequently incorporating different flavonoids into the honey.^{1, 103-105} Several honeys from Central and South America, and New Zealand have been found to contain flavonoids from poplar bud exudates based propolis. This is due to imported bee colonies seeking out non-native poplars which have been planted in these areas.^{1, 103, 105} These findings

show that honey flavonoid profiling can be a useful tool for the determination of the geographical origin of honeys.^{12, 103, 106, 107}

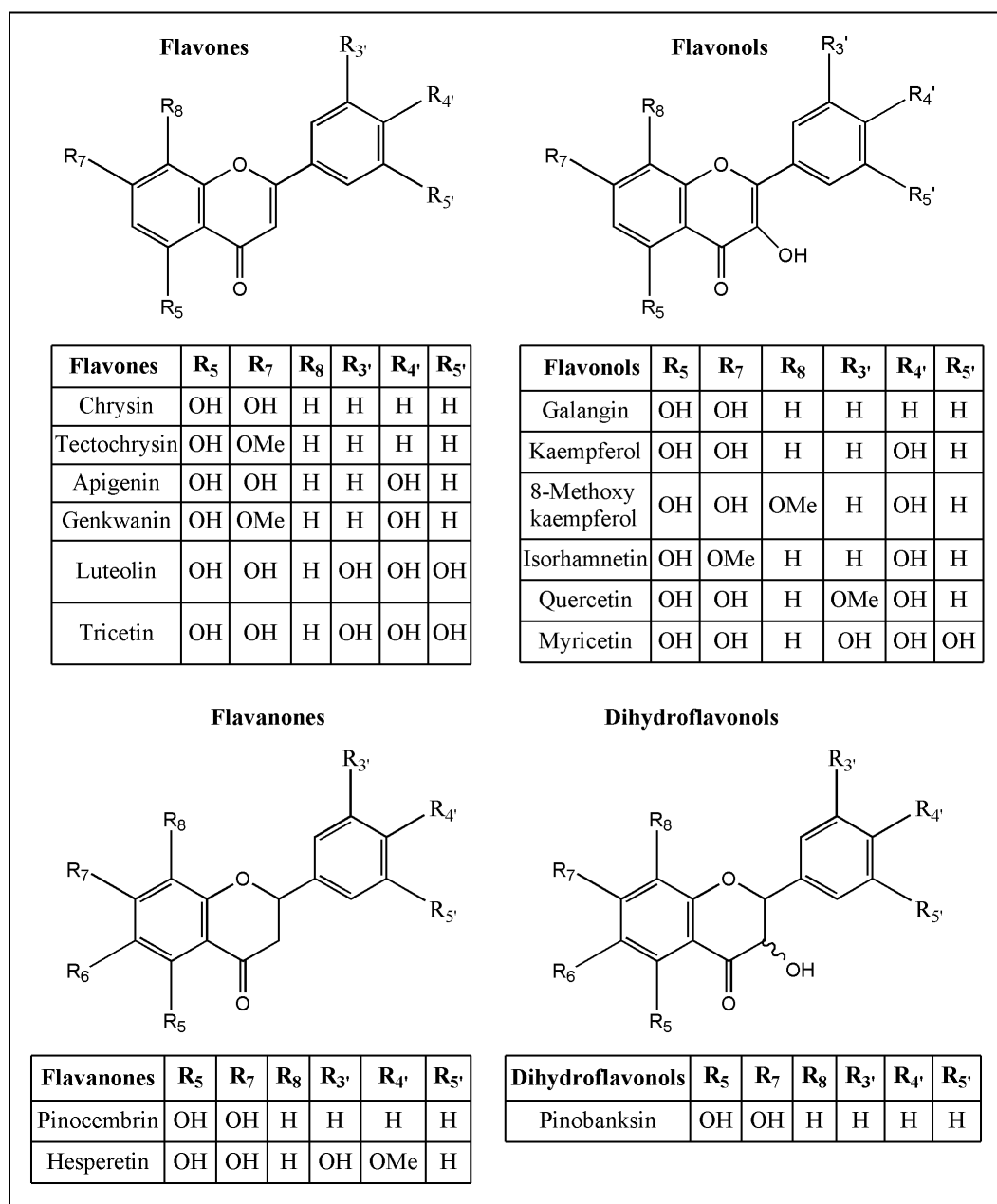


Figure 1.10: The structures of some flavonoids found in honey.^{1, 12, 94, 108}

Flavonoids have also shown potential to be marker compounds for the floral origin of honeys. The flavanone hesperetin has been identified exclusively in citrus honey and its source has been identified as hesperidin (hesperetin-7-rutinoside) in orange nectar which is hydrolysed to the aglycone by bee enzymes.^{99, 107, 109-112}

The flavonoid profile of heather honeys can be characterised by the presence of flavonoids with a trioxxygenated B ring. The most significant of these are myricetin, myricetin-3-methyl ether and myricetin-3'-methyl ether. Myricetin-3'-methyl ether, abscisic acid and ellagic acid together in honey has been suggested as a potential marker for heather honey since the only other monofloral honey they have all been found in is that of the botanically related calluna.^{96, 110, 113}

Studies have shown that French sunflower honey contains the propolis derived flavonoids pinocembrin, chrysin, pinobanksin and galangin, as well as quercetin from the sunflower nectar, as the most significant flavonoids. Consequently, high levels of quercetin have been suggested as a marker for European sunflower honey.^{104, 108, 110, 112} Australian sunflower (*Helianthus annuus*) honey also contains relatively high levels of quercetin but this flavonol is also found in other Australian honeys including tea tree (*Melaleuca quinquenervia*) honey, heath (*Banksia ericifolia*) honey, brush box (*Lophostemon conferta*) honey, crow ash (*Guioa semiglauc*) honey and several species of *Eucalyptus* honey..^{114, 115} Australian sunflower honey can be distinguished from these other honeys by a profile containing quercetin, quercetin-3,3'-dimethyl ether, myricetin and luteolin as the main flavonoids.¹¹⁴

Kaempferol-3-sophoroside has been identified as the only significant flavonoid in rosemary nectar. This glycoside is the proposed source of kaempferol in rosemary honey, which occurs at levels of 0.33-2.48 mg/kg with a coefficient of variation which is much lower than that of other flavonoids present. As a result, kaempferol is a useful marker for the floral origin of rosemary honey.^{112, 116, 117} Earlier studies had identified 8-methoxykaempferol and apigenin as potential marker flavonoids.^{99, 111}

Flavonoid profiles containing the flavonoids myricetin, tricetin, quercetin, luteolin and kaempferol are characteristic of *Eucalyptus* honeys of European and Australian origin.^{1, 105, 115, 118} In addition to these flavonoids being markers for *Eucalyptus* honeys in general, their individual levels show species-specific differences. River red gum (*Eucalyptus camaldulensis*) honeys contain tricetin as the main flavonoid marker, whereas mallee box (*Eucalyptus pilligaensis*) honeys

have luteolin as the main flavonoid marker.¹¹⁵ Bloodwood (*Eucalyptus intermedia*) honeys have myricetin and tricetin as their main flavonoids. Yapunyah, (*Eucalyptus ochrophloia*), narrow-leaved ironbark (*Eucalyptus crebra*) and black box (*Eucalyptus largiflorens*) honeys contained no myricetin, their profiles having tricetin, quercetin and/or luteolin as their main flavonoids.¹⁰⁵

Other Australian honeys including tea tree (*Melaleuca quinquenervia*), heath (*Banksia ericifolia*), brush box (*Lophostemon conferta*), crow ash (*Guioa semiglaucula*) and sunflower (*Helianthus annuus*) honeys have been found to have flavonoid profiles of myricetin, tricetin, quercetin and luteolin like the previously studied Australian *Eucalyptus* honeys. A possible reason for this similarity in profiles is that the honeys studied may be partially derived from the nectar of *Eucalyptus* trees which are abundant and provide nectar throughout the year.¹¹⁴

In an interesting development, the kaempferol glycosides kaempferol-3-*O*-(hexoxyl) robinoside-7-*O*-rhamnoside, kaempferol-3-*O*-(hexoxyl)robinoside, kaempferol-3-*O*-hexoside-7-*O*-rhamnoside, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside, kaempferol-3-*O*-robinoside and kaempferol-7-*O*-rhamnoside have been identified in acacia (*Robinia pseudoacacia*) honeys at levels of 100-800 µg/100g. These flavonoids have also been found in acacia nectar collected from the same region as the honeys.¹⁰¹ This is one of only two studies which have found flavonoid glycosides in honey. The other study found hyperoside (quercetin-3-*O*-galactoside) in Lithuanian willow honeys and vitexin (apigenin-8-*C*-glucoside) in Lithuanian caraway and lime honeys.¹⁰⁰ An earlier study of Croatian acacia honeys did not look for flavonoid glycosides but was able to identify the aglycones, quercetin, luteolin, kaempferol, apigenin, chrysin and galangin.¹¹⁹

In a study of French alder honey, 8-methoxykaempferol was the only flavonoid detected. Consequently it has been suggested as a marker for alder honey.¹¹⁰ Other honeys including lime-tree, chestnut and rapeseed honeys have been found to contain marker compounds with characteristic UV spectra. The identities of these unknowns are yet to be determined.¹¹²

Not all monofloral honeys can be distinguished by the presence of a major flavonoid. Croatian sage (*Salvia officinalis*) honey was found to contain quercetin, luteolin, kaempferol, apigenin, chrysin and galangin. These flavonoids are all found in other European honeys but it may still be possible to identify sage honeys by the relative amounts of these flavonoids and the presence of abscisic acid as a dominating constituent in the phenolic fraction.¹²⁰

In 1999 and 2000 Weston *et al.* reported that the flavonoid profile of New Zealand manuka honey was similar to that of most European honeys, containing only the propolis derived flavonoids pinobanksin, pinocembrin, chrysin and galangin at a total average level of 14.9µg/100g of honey. A total of 19 manuka honeys of varying UMF ratings from throughout the North Island of New Zealand were studied. No differences were observed in the flavonoid profile of manuka honey from different geographical regions and there was no correlation of flavonoid content with UMF rating of the honeys.^{23, 121} Similar levels of the same propolis derived flavonoids were observed in New Zealand heather (*Calluna vulgaris*), clover (*Trifolium repens*), and beech (*Nothofagus spp.*) honeydew honeys.¹²¹

A later study by Yao *et al.* has found significantly higher levels of flavonoids in Australasian *Leptospermum* honeys including New Zealand manuka honey. New Zealand manuka (*Leptospermum scoparium*) honey was found to have an average flavonoid content of 3060µg/100g of honey while Australian jelly bush (*Leptospermum polygalifolium*) honey had an average flavonoid content of 2220µg/100g of honey.²⁴ These levels were significantly higher than the 14.9µg/100g reported by Weston *et al.*¹²¹ The difference in these results has been attributed to the extraction method used by Weston *et al.* which used insufficient XAD-2 resin for the mass of honey analysed.²⁴

As well as identifying higher levels of flavonoids in manuka honey the study by Yao *et al.* also found a range of flavonoids which were not reported in the original study. The flavonoids found in manuka honey include myricetin, tricetin, pinobanksin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, 8-methoxykaempferol, pinocembrin, quercetin 3,3'-dimethyl ether, isorhamnetin, chrysin and two unidentified flavonoids. Of these flavonoids, quercetin (13.8%),

isorhamnetin (12.9%), chrysin (12.6%), luteolin (12.6%) and one of the unknown flavonoids (12.7%) were the main constituents, making up 64.6% of the total flavonoid content. Leptospermum honeys can be characterised by a flavonoid profile mainly consisting of quercetin, luteolin, quercetin 3-methyl ether, luteolin and an unknown flavonoid. Australian jelly bush honey can be distinguished from New Zealand manuka honey by its significant myricetin content. New Zealand manuka can be distinguished from jelly bush honey by the presence of chrysin and the phenolic acids, gallic acid (average of 7500µg/100g) and abscisic acid (32800µg/100g total *trans,trans*- and *cis,trans*-abscisic acids) which are present in high levels in manuka honeys. However, this study was limited to only two manuka honey samples so further work is needed to confirm a flavonoid profile for manuka honey.²⁴

1.4 Aims of the Present Research

The aim of the present research is to investigate the flavonoids in New Zealand manuka honey and how they are related to the floral origin and/or UMF level of the honey. Primary objectives are:

- Modify the established flavonoid extraction method to permit analysis of small honey samples.
- Determine whether the non-peroxide antibacterial activity of manuka honey is contained in the sugar fraction or the phenolic fraction.
- Extract and analyse the flavonoids from a range of manuka honey samples and determine if any flavonoids could be potential floral markers.
- Investigate potential relationships between the flavonoid profile of manuka honey and its non-peroxide antibacterial activity.
- Isolate and characterise any unknown flavonoids found in manuka honey.

Chapter 2: Materials and Methods

2.1 Materials

Thirty two manuka honey samples with non-peroxide antibacterial activities ranging between very low to 27.3 UMF, were kindly supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand). UMF measurements of these manuka honeys were provided by Dr Christopher Adams from previous research carried out in this research group.⁷⁰ A bulk sample of active manuka honey (6kg, from Haddrell's of Cambridge, New Zealand) was used for the optimisation and testing of the extraction method. Fifteen kilograms of manuka honey was sourced from Natures Country Gold (Hamilton, New Zealand) for use in the isolation of flavonoids. Eight non-manuka New Zealand honeys and accompanying data were kindly supplied by Airborne Honey Ltd. (Leeston, New Zealand). These included one sample each of rata (*Metrosideros spp.*), rewarewa (*Knightia excelsa*), beech (*Nothofagus spp.*) honeydew, clover (*Trifolium spp.*), kamahi (*Weinmannia racemosa*), viper's bugloss (*Echium vulgare*), thyme (*Thymus vulgare*) and nodding thistle (*Carduus nutans*) honeys. All honeys were stored in a cold room (5-8°C) when not being used.

Solvents used in this study were methanol, acetonitrile, water, dimethyl sulfoxide and diethyl ether. Methanol used in the extraction of flavonoids was redistilled from drum grade while the methanol used for HPLC was of HPLC grade and supplied by either Scharlau or Ajax Finechem (as was available). For spectroscopy of flavonoids, HPLC grade Multisolv methanol from Scharlau was used since the Ajax HPLC methanol absorbed more strongly in the region of 200-260 nm. Acetonitrile used was of HPLC grade and obtained from Burdick & Jackson.

Water used in the extraction of flavonoids was deionised and obtained from a Crystal Pure Ultra Pure Water System. Higher grade Milli-Q water was used as a solvent in the HPLC system. This was obtained from a Barnstead E-pure system (18.2 MΩcm). Dried and purified diethyl ether was obtained from a Pure Solv Solvent Purification System (Model: PS-SD-5) as needed and used promptly.

The dimethyl sulfoxide used for the cleaning of HPLC systems was of spectrophotometric grade and supplied by Aldrich Chemical Company. Deuterated dimethyl sulfoxide- d_6 (99.9 atom% D containing 0.03% v/v TMS), obtained from Sigma-Aldrich, was used as the solvent for NMR analysis of isolated flavonoids.

The authentic chemical compounds used for identification and quantification in this study are shown in table 2.1 along with their source. D(-)-fructose (analytical grade), glacial acetic acid (analytical grade) and concentrated hydrochloric acid were obtained from Ajax Finechem. Sucrose (analytical grade), D(+)-glucose (analytical grade) and sodium metal (sticks in paraffin) were obtained from BDH. Boric acid (reagent grade) was obtained from May and Baker while aluminium chloride (reagent grade) was obtained from Merck. Sodium hydroxide (flaked and purified for nitrogen determination) was obtained from J. T. Baker Chemical Co.

XAD-2 Amberlite resin (pore size 9 nm, particle size 0.3-1.2 mm) used in the extraction of flavonoids from honey was obtained from Sigma-Aldrich. Sephadex LH-20 resin was obtained from Amersham Pharmacia Biotech AB.

Tryptic soy broth was obtained from Bacto™ and nutrient agar was sourced from Difco. Catalase (from Bovine liver, 2950 units/mg) was purchased from Sigma-Aldrich while AnalaR grade phenol was purchased from BDH. Freeze dried cultures of *Staphylococcus aureus* (ATCC 9144) were obtained from Environmental Science and Research Ltd and used as a bead culture for producing cultures in liquid media.

Table 2.1: The authentic chemicals used for identification and quantification

Common Name	Chemical Name	Source
Caffeic acid	3(3,4-Dihydroxyphenyl)-prop-2-enoic acid	1
(+)-Catechin hydrate (98%)	3,5,7,3',4'-Pentahydroxyflavane (2R, 3S)	2
Chlorogenic acid (predominantly trans)	3-Caffeoyl quinic acid	2
Chrysin	5,7-Dihydroxyflavone	2
<i>trans</i> -Cinnamic acid (99+%)	<i>trans</i> -3-Phenylprop-2-enoic acid	2
p-Coumaric acid	3(4-Hydroxyphenyl)-prop-2-enoic acid	1
(-)-Epicatechin (97%)	3,5,7,3',4'-Pentahydroxyflavane (2R, 3R)	2
Galangin	3,5,7-Trihydroxyflavone	2
Gallic acid (97%)	3,4,5-Trihydroxybenzoic acid	1
Isorhamnetin (pure)	3,5,7,4'-Tetrahydroxy-3'-methoxyflavone	3
Kaempferol (pure)	3,5,7,4'-Tetrahydroxyflavone	3
Luteolin	5,7,3',4'-Tetrahydroxyflavone	1
Myricetin (approx 85%)	3,5,7,3',4',5'-Hexahydroxyflavone	1
Naringin (pure)	5,4'-Dihydroxyflavanone-7- <i>O</i> -rhamnoglycoside	3
Pinocembrin	5,7-Dihydroxyflavanone	1
Quercetin dihydrate (98%)	3,5,7,3',4'-Pentahydroxyflavone	2
(+)-Rutin hydrate (95%)	5,7,3',4'-Tetrahydroxyflavone-3- <i>O</i> -rutinoside	2
Syringic acid (98%)	3,5-Dimethyl-4-hydroxybenzoic acid	2

1 = Sigma-Aldrich Inc., 2 = Aldrich Chemical Company, Inc., 3 = Indofine Chemical Company Inc.

2.2 General Methods

2.2.1 Freeze-Drying

A Labconco Bulk Tray Drier was used to freeze-dry samples when required. Liquid samples were frozen with liquid nitrogen prior to freeze-drying while solid samples were dried without freezing. Samples were left on the freeze drier for 20-48 hours depending on the amount of drying required.

2.2.2 Evaporation

Large volumes of liquid were reduced using two rotary evaporators. The first system comprised a Büchi Rotavapor R-200, Büchi Heating Bath B-490 and a Büchi Vac[®] V-500 vacuum pump. The second system was an Eyela rotary evaporator equipped with an Eyela Water Bath SB-650 and a Büchi Vac[®] V-500 vacuum pump. Both systems were run with their water bath set to 40°C.

Once liquid volume had been reduced enough to fit in a sample vial, further evaporation was achieved using a blow down block. This system consisted of a Pierce Reacti-Therm Heating Module set to heat samples to 40°C, and a Pierce Reacti-Vap Evaporating Unit which blew nitrogen gas over the samples.

2.2.3 Statistical Analysis

Statistical analysis was performed by Dr Ray Littler (Department of Statistics, University of Waikato, Hamilton, New Zealand) using Minitab statistical software.

2.3 High Performance Liquid Chromatography (HPLC)

Three different HPLC systems were used in this study and were referred to as systems A, B and C.

2.3.1 HPLC System A

This system was used as a coarse separation to filter honey phenolic extracts prior to analytical analysis by HPLC. This coarse separation was necessary to protect HPLC system B which was used for the quantitative analysis of honey phenolics.

HPLC system A consisted of a Waters 515 pump and 996 PDA detector (240-400nm), Alltech Elite Degassing System and a Rheodyne 7725i injector fitted with a 5mL loop. A Waters reversed phase C18 column (Nova-Pak® C18 4µm 8x100mm Radial-Pak™) was fitted inside a Waters radial compression module (RCM 8x10) and protected by a guard cartridge (Waters Resolve™ C18 Guard-Pak HPLC Precolumn Insert) which was replaced as required.

The system was operated isocratically with 2mL/min of methanol. Extracts were injected into this system and the elution of phenolics was followed by their UV absorbance (240-400nm maximum absorbance plot). A fraction was collected as appropriate to the extract type being filtered. Small extracts, such as those in the analysis of honey phenolic profiles, were collected as a fraction between 1.0min and 7.0min. Higher loading rates, such as those used to filter bulk phenolic extracts, required a larger fraction to be collected to recover the phenolics.

2.3.2 HPLC System B

Flavonoid extracts were analysed using HPLC system B. System B was a binary HPLC system operated using Waters Empower Pro software (Waters Empower 2, build number 2154). Two Waters 515 HPLC pumps were operated remotely with their flows being combined through a mixer (Grace Binary Large Volume Mixer SS Housing with 350µL Mixer Cartridge). This mixer setup was required to suppress noise generated by the mixing of eluents at high pressure.

Samples and standards were warmed (40°C) and injected manually using a Rheodyne 7725i injector system fitted with a Rheodyne loop (5µL). Separation of extracts was achieved using a Waters Symmetry Shield™ octadecylsilane HPLC column (RP18, 5µm, 3.0x250mm). A matching guard column (Waters Universal Sentry™ Guard

SymmetryShield™ Column in a Waters Universal Sentry™ Guard Column Holder) was fitted to protect the analytical column. This guard column did not need to be replaced during the course of the analysis of samples.

A binary gradient was operated on this system. Solvent A was Milli-Q water and methanol (5% v/v) to prevent microbial growth, acidified with acetic acid (0.075% v/v). Solvent A was prepared in batches (1L) which were sonicated (20min) to assist removal of dissolved gasses prior to using in the HPLC. Solvent B was methanol. A degasser (Waters In-Line Degasser AF) was also utilised in the HPLC system to further remove dissolved gasses. The gradient shown in table 2.2 was run at a constant combined flow rate (0.3mL/min). Detection was achieved using a Waters 996 Photodiode Array Detector (240.0-400.0nm).

Table 2.2: The gradient system used for the analytical HPLC analysis of flavonoid extracts.

Time (min)	Solvent A % ¹	Solvent B % ²
0	70	30
15	70	30
20	40	60
60	0	100
70	0	100
73	70	30
78	70	30

¹ Acetic acid (0.075% v/v) and methanol (5% v/v) in Milli-Q water

² Methanol

2.3.2.1 Alternative HPLC Conditions Used During Early Method Development

Early extraction method development was analysed using an alternative set of HPLC conditions. The same HPLC instrument was utilised but several changes were made to the operating conditions. Firstly, acetonitrile was used instead of methanol as

solvent B. The total flow rate (0.4mL/min) was also different and the gradient program was that described in table 2.3.

The HPLC conditions were later changed after the column became contaminated and had to be cleaned. After cleaning it was found that the strong solvent had to be changed to methanol to achieve an acceptable peak shape. This change of solvent necessitated the change of flow rate and gradient program.

Table 2.3: The gradient system used for the analytical HPLC analysis of early flavonoid extracts.

Time (min)	Solvent A %¹	Solvent B %²
0	77	23
3	77	23
4	67	33
30	30	70
30.1	0	100
33	0	100
36	77	23
44	77	23

¹ Acetic acid (0.075% v/v) and methanol (5% v/v) in Milli-Q water

² Acetonitrile

2.3.2.2 Identification and Quantification

Peaks in the HPLC chromatograms were identified by comparison of the retention time and UV spectra with those of authentic standards. Where authentic standards were not available the peak had to be isolated and characterised according to the methods described in Sections 2.6 and 2.7.

Quantification of the flavonoids was achieved by comparing their absorbance in the HPLC chromatograms to four external standards. Pinocembrin at 290nm was used to quantify all flavanones and dihydroflavonols. Chrysin at 340nm was used to quantify flavones and flavonols with unsubstituted B rings. Similarly, kaempferol at 340nm

was used for those flavones and flavonols with a singly oxygenated B ring, while quercetin at 340nm was used for all other flavones and flavonols.

Pinocembrin ($3.25 \pm 0.02 \text{mg}$), chrysin ($2.91 \pm 0.02 \text{mg}$), kaempferol ($2.47 \pm 0.02 \text{mg}$) and quercetin dihydrate ($2.99 \pm 0.02 \text{mg}$) were weighed out and dissolved in HPLC grade methanol ($5.00 \pm 0.05 \text{mL}$). This 0.5mg/mL stock solution was sonicated (15min) to ensure complete dissolution and then diluted to create six standards using the scheme shown in table 2.4. These standards were analysed on HPLC system B using the method described in Section 2.3.2.1. A calibration curve of peak area versus concentration was created and subsequently used to quantify the flavonoids in samples analysed by HPLC.

Table 2.4: Preparation of quantitative flavonoid standards.

Volume of 0.5mg/mL Stock Solution (mL)	Volume Solution Made Up To (mL)	Approximate Concentration of the Standard (mg/mL)
$0.0200 \pm 0.0002 \text{mL}$	$2.000 \pm 0.004 \text{mL}$	0.005
$0.04 \pm 0.0002 \text{mL}$	$2.000 \pm 0.004 \text{mL}$	0.01
$0.1000 \pm 0.0002 \text{mL}$	$2.000 \pm 0.004 \text{mL}$	0.025
$0.2000 \pm 0.0002 \text{mL}$	$2.000 \pm 0.004 \text{mL}$	0.05
$0.400 \pm 0.002 \text{mL}$	$2.000 \pm 0.004 \text{mL}$	0.1
$1.000 \pm 0.002 \text{mL}$	$2.000 \pm 0.002 \text{mL}$	0.25

2.3.2.3 Cleaning the HPLC Column

During the course of this research it was found that certain components of honey persisted in the HPLC column, causing the quality of the chromatography to be severely reduced. When the column became contaminated in this way the peaks of flavonoids became broadened and resolution was reduced.

The most effective method for cleaning the HPLC column once it was contaminated was found to be multiple injections of dimethyl sulfoxide ($200 \mu\text{L}$). These could be run using the gradient system being used for the experiment or alternatively they

could be injected into a constant flow of methanol. The injections were repeated until the chromatography was restored to optimal condition (observed using a standard mixture of flavonoids).

2.3.3 HPLC System C

HPLC system C was a semi-preparative system used to isolate flavonoids from bulk extracts of honey phenolics. The system consisted of two Waters 515 pumps, an Alltech Elite Degassing System, Rheodyne 7725i injector fitted with sample loop (500 μ L), and a Waters 996 photodiode array detector. A reversed phase octadecylsilane Waters Xterra Prep column (PR18, 10 μ m, 7.8mmx300mm) was used in this system without a guard column.

A binary mobile phase system the same as that used for the analytical HPLC (Section 2.3.2) was used for the semi-prep HPLC. This consisted of solvent A which was Milli-Q water acidified with acetic acid (0.075% v/v) and contained methanol (5% v/v) to prevent microbial growth; and solvent B which was straight HPLC methanol. An overall flow rate (4mL/min) was maintained throughout the gradient program shown in table 2.5.

Table 2.5: The gradient program used in the semi-prep HPLC separation of flavonoids.

Time (min)	Solvent A % ¹	Solvent B % ²
0.0	60	40
5.0	60	40
10.0	50	50
60.0	20	80
60.2	0	100
65.0	0	100
67.0	60	40
70.0	60	40

¹ Acetic acid (0.075% v/v) and methanol (5% v/v) in Milli-Q water

² Methanol

Bulk phenolic extracts were centrifuged (5000rpm, Heraeus Sepatech Medifuge) prior to injection onto the semi-prep HPLC system at high loading rates (up to 150 μ L). Fractions were collected from the photodiode array outlet as appropriate.

2.4 Well Diffusion Assay for Non-Peroxide Antibacterial Activity

2.4.1 Preparation of Assay Plates

Nutrient agar (23.0g) was dissolved in distilled water (1.0L) and then divided into 150mL amounts which were sterilised in an autoclave. The prepared flasks of nutrient agar were stored at room temperature until required, at which point the flasks were steamed in a water bath (30min, 100°C) to sterilise and liquefy the agar. The agar was then transferred to another water bath (30min, 50°C) to cool to a temperature which was tolerable to the bacterial culture.

Cultures of *Staphylococcus aureus* were prepared by inoculating tryptic soy broth (30g/L) with *S. aureus* bead culture and incubating (18h, 37°C). The *S. aureus* culture was diluted with tryptic soy broth until it had an optical density of 0.5AU. The optical density was measured using a Thermo Spectronic Helios γ spectrophotometer (540nm) with tryptic soy broth used as a blank.

Square assay plates (Corning® 431111 sterile bioassay dish, 245mmx245mmx18mm) were prepared on a level surface. Nutrient agar (150mL, 50°C) was inoculated with *S. aureus* culture (100 μ L, 0.5AU optical density as prepared above) and carefully poured into the assay plate. Once the plates had solidified they were stored upside-down overnight at 4°C.

2.4.2 Preparation of Samples and Standards

A 10% stock solution of phenol (10g phenol/100mL distilled water) was diluted to give phenol standards at 2, 3, 4, 5, 6 and 7% (w/v). These standards were stored at 4°C in the dark for up to one month before being replaced.

Whole honey samples, fractions added to clover honey and syrupy fractions were prepared by weighing the sample (ca. 2.10g) and dissolving in distilled water (2.1mL). The resulting 50% (w/v) solutions were incubated (37°C, 30min) to soften the honey and then stirred until homogenous.

50% (w/v) solutions of honey/syrup samples were further diluted by combining equal volumes (1:1) of sample with catalase solution (41mg/20mL distilled water).

2.4.3 Antibacterial Assay

Wells (8mm Ø) were punched into the agar plate in a regular 8x8 grid using a flamed, cooled cork borer and a sterilised inoculating needle was used to remove the agar wells. The 8x8 grid of wells were filled with samples and standards according to a *Quasi*-Latin square of 16 numbered wells repeated 4 times over the plate (once in each pair of rows and columns) as shown in figure 2.1. The arrangement of samples in this template distributes the samples over the plate to remove bias due to edge effects.

Samples (prepared as above) and standards were placed in each well (100µL) at their allocated positions. The plates were then incubated (18h, 37°C) before measuring the clearance zones around wells using a digital calliper (Mitutoyo 500.321).

The clearing zones for each sample were squared and compared with a standard curve of squared clearing zone versus % (w/v) phenol standard concentration. This allowed the equivalent phenol concentration of the 25% (w/v) honey solution to be determined. The phenol equivalent antibacterial activity of undiluted honey was calculated by multiplying by a conversion factor of 4.65 to account for the dilution (4x) and the density of honey (~1.4g/mL).

1	13	8	12	3	16	9	6	Row
7	2	10	5	14	11	4	15	
14	11	4	15	1	13	8	12	
3	16	9	6	7	2	10	5	
10	5	14	11	4	15	1	13	
8	12	3	16	9	6	7	2	
4	15	1	13	8	12	3	16	
9	6	7	2	10	5	14	11	
Column								

Figure 2.1: The *Quasi*-Latin square arrangement of sample and standard wells used in the well diffusion assay of antibacterial activity.

2.5 Extraction of Flavonoids from Honey for Analysis

The method used to extract flavonoids from honey is a variation of that developed by Ferreres *et al.*⁹⁴ This method has been modified over the course of this study to allow antibacterial testing of fractions and a reduction of the sample mass required from fifty grams to five grams.

2.5.1 Cleaning and Swelling of the Amberlite XAD-2 Resin

Amberlite XAD-2 resin was cleaned by soaking in methanol overnight. This was considered enough to desorb any contaminants which could later elute into the flavonoid fraction. When the resin became contaminated with large particulates or

microbial growth it was cleaned up by swirling the resin in methanol and decanting off the contaminants with methanol. This procedure allowed the resin to be cleaned up with minimal losses of the resin. Smaller particulates were separated from the resin by filtering the resin on cotton cloth over a sieve which allowed the fine particulates to be washed out of the resin.

Removal of the resin from solvents was achieved by filtering it on a Büchner funnel. While this would not completely dry the resin, it was considered enough to remove excess solvent.

Before using the resin for extractions it was left to soak overnight in a 50% solution of methanol and water. This allowed the resin to swell to its working volume. This step was only necessary when the resin was dry. Provided it was kept covered in solvent at all times (except when filtering on the Büchner funnel to remove excess solvent) it could be reused without having to swell the resin overnight.

2.5.2 Testing the Flavonoid Extract of Manuka Honey for Antibacterial Activity

Active manuka honey (Comvita UMF30+, ca. 50g) was mixed with acid water (250mL, adjusted to pH 2 with HCl) and stirred (20min) until homogenous. The solution was filtered through cotton wool to remove particulates. A glass column (Ø2cmx70cm) of swelled XAD-2 resin (187g dry weight) was prepared by flushing with Milli-Q water (400mL) to remove methanol residues. The filtered solution was then poured into the glass column of XAD-2 resin and held (30min) before being dripped through and the eluent collected (the aqueous fraction). The column was washed with acid water (250mL) and then Milli-Q water (300mL) to elute the sugars and other polar constituents. The aqueous column washings were collected and combined with the previously collected aqueous fraction.

Sodium hydroxide solution (1.0000 ± 0.0002 mol/L) was added to the aqueous fraction to neutralise the acid water used in the extraction. Sufficient sodium hydroxide solution was added to increase the pH of the aqueous fraction to that of

honey diluted to the same degree with water (pH 3.5 ± 0.1). This fraction was then concentrated to a syrupy consistency under vacuum at 35°C.

Phenolics were eluted from the resin by washing with methanol (400mL). The eluent was collected and concentrated under vacuum at 35°C before being made up to 2mL with Milli-Q water. Half of this phenolic fraction (1mL) was added to clover honey (ca. 25g) which had been previously shown to have no detectable non-peroxide antibacterial activity. This permitted the contribution that the phenolic made to the non-peroxide antibacterial activity of honey to be measured. The remaining half of the phenolic fraction was added to clover honey (1g) to provide a test of the non-peroxide antibacterial activity of the phenolic fraction at a concentration twenty five times that in the original honey. The extraction of honey fractions for activity testing was done in duplicate.

The manuka honey fractions, active manuka honey (Comvita UMF30+) and inactive clover honey were assayed for non-peroxide antibacterial activity according to the methods describe in Section 2.4.

2.5.3 Modification of the Extraction Method to Use Smaller Honey Samples

The original method⁹⁴ called for fifty grams of honey to be extracted. This was not feasible in a study of manuka honey, due to limitations on the amount of samples available, so the method has been modified to allow the extraction of flavonoids from five grams of honey.

Active manuka honey (ca. 50g, 20g and 5g) was weighed out and dissolved in five parts acid water (pH 2 with HCl). The solution was stirred until homogenous then filtered through cotton wool to remove particulates. A glass column (Ø2cmx70cm) of swelled XAD-2 resin (187g dry weight) was washed with acid water (150mL) to remove methanol. The honey solutions were then loaded onto the column and held there (1h) before being eluted.

The resin was washed with acid water (250mL) and then Milli-Q water (300mL) to elute the polar compounds. The phenolics were eluted with methanol (650mL) until the eluent was no longer coloured yellow. The eluent was collected and concentrated to dryness under vacuum at 40°C before resuspending in Milli-Q water (5mL) and extracting with diethyl ether (5mLx3). The resulting ether extract was concentrated to dryness under N₂ gas at 40°C and redissolved in methanol (2mL) before filtering (0.25µm syringe filter). The filtered extract was then concentrated to dryness under N₂ gas at 40°C and made up to its volume with methanol (500µL for 50g extracts, 200µL for 20g extracts and 50µL for 5g extracts). The extracts were then analysed on HPLC system B using the alternative conditions described in Section 2.3.2.1.

2.5.4 Final Extraction Method Applied to Analysis of Honey

Flavonoids

To extract flavonoids the honey sample (ca. 5g) was mixed with acid water (50mL, adjusted to pH 2 with HCl) and stirred until homogenous. The resulting honey solution was filtered through cotton wool to remove solid particles.

Clean swelled XAD-2 resin (30g) was removed from the methanol, in which it was stored, on a Büchner funnel, and transferred to the filtered honey solution using acid water (50mL) to rinse the resin out of the funnel. The filtered honey solution and resin was stirred (1h) by a magnetic stirrer, before being poured into a glass column (Ø2.5cmx40cm).

The excess honey solution was run out of the column until the resin was just covered with solvent. The resin was then washed with acid water (250mL) followed by deionised water (300mL) to remove sugars and polar constituents. The phenolics were eluted with distilled methanol (400mL) and collected.

The XAD-2 resin was left in the column, soaking in methanol, when not in use. When it was time to do another extraction the resin was removed by inverting the column and washing the resin out with methanol (methanol was found to be much more effective than water which caused the resin to stick to glass). The resin was

then washed with acid water and partially dried under vacuum on a Büchner funnel to make it ready for the next extraction.

The phenolic fraction was concentrated to dryness under vacuum at 40°C, resuspended in deionised water (5mL) and extracted with diethyl ether (5mLx3). The resulting ether extract was concentrated to dryness under N₂ gas at 40° and redissolved in HPLC grade methanol (200µL).

The extract was then submitted to a coarse separation on HPLC system A (described in Section 2.3.1) to filter out contaminants which were harmful to the analytical HPLC (system B). The 200µL phenolic extract was injected onto the reversed phase HPLC system A and the fraction eluting between 1.0min and 7.0min was collected. The fraction recovered from the HPLC was concentrated under vacuum at 40°C and redissolved in HPLC grade methanol (50µL) for analytical HPLC analysis on system B, as described in Section 2.3.2.

2.5.5 Evaluation of Extraction Method

2.5.5.1 Recovery Rate of Flavonoids Extracted from Solution

To determine the extraction efficiency of the flavonoid extraction method a standard mixture containing quercetin dihydrate, kaempferol, pinocembrin and chrysin (0.5mg/5mL for each compound) was dissolved in methanol (0.5mL) and extracted using the method described in Section 2.5.4 as though it was a 5g sample of honey. This test was carried out in duplicate.

The recovery rate of the reversed phase HPLC cleanup step (HPLC system A) developed in this study was assessed using a standard mixture of quercetin dihydrate (0.91mg), kaempferol (0.60mg), pinocembrin (0.88mg) and chrysin (0.56mg) dissolved in HPLC grade methanol (200µL). The resulting standard solution was injected into the reversed phase HPLC system A as described in Section 2.3.1 and the recovered fraction (eluting 1.0-7.0min) was concentrated to dryness under vacuum at 40°C. The dried extract was redissolved in methanol (5mL) and the recovery rate

determined by quantitative HPLC analysis on system B, using the method described in Section 2.3.2.

2.5.5.2 Recovery Rate of Flavonoids Extracted from Artificial Honey

An artificial honey was prepared in order to assess the impact of matrix effects on the recovery rate of the extraction method. Chrysin (1.7mg), kaempferol (2.04mg), quercetin dihydrate (4.25mg), p-coumaric acid (12.54mg), gallic acid (2.77mg), caffeic acid (1.49mg) and chlorogenic acid (1.73mg) were weighed out and thoroughly mixed together. Sucrose (5.7820g), D(+)-glucose (72.2727g) and D(-)-fructose (80.2469g) were added to the phenolic mixture and shaken to mix thoroughly. Deionised water (35mL) was added and the resulting syrup was warmed to 44°C with warm tap water, and shaken until the artificial honey had a homogenous consistency. This artificial honey was then extracted according to the method described in Section 2.5.4.

2.5.5.3 Assessing the Reproducibility of the Extraction Method

Eleven extractions of the same active manuka honey (supplied by Haddrell's of Cambridge, New Zealand) were carried out over several weeks. The quantitative results of these extractions were compared to assess the precision and reproducibility of the extraction method.

2.6 Isolation of Honey Flavonoids

2.6.1 Extraction of Phenolics Using Amberlite XAD-2 Resin

Unknown compounds were isolated from 15kg of manuka honey. Batches of honey (ca. 1kg) were dissolved in acid water (pH 2 with HCl, ca. 3L) and stirred until homogenous. Clean and swelled Amberlite XAD-2 resin (500g) was added to the honey solution and stirred (1 hour) by a magnetic stirrer. The resin and honey solution slurry was then poured into a glass column (Ø4cmx120cm) and the honey solution drained out. The resin was washed with acid water (2L) and deionised water (1L) to remove the sugars and other polar compounds. All the aqueous washings and

the honey solution were collected and later subjected to a second extraction on XAD-2 resin.

The phenolics were eluted from the resin by washing with distilled methanol (600mL) and then deionised water (1L). It was observed during this study that the previously reported washing method which used 750mL of methanol was insufficient to recover all phenolics. Increasing the volume of the methanol wash was not effective so an additional washing with deionised water was implemented. This water washing was intended to make any remaining phenolics adsorb back onto the resin so that they could be eluted with the next batch of extracted phenolics

The column was inverted and deionised water flushed through to wash the resin out of the column. The resin was then partially dried on a Büchner funnel and added back to the aqueous washings to increase the recovery of flavonoids by extracting those compounds not recovered during the first treatment. In this extraction process the honey was not filtered prior to extraction because it was found that 1kg batches of honey quickly overloaded the cotton wool filters used for the analytical extraction of honey samples. This resulted in fine particulates building up in the resin over time which reduced the flow rate of the XAD-2 column. This problem was resolved by washing the resin with deionised water over a cotton cloth filter which permitted the contaminating particulates to pass through. The clean resin could then be scraped off the cloth and partially dried on a Büchner funnel to make it ready for the next extraction.

The phenolic extracts were combined and concentrated under vacuum at 40°C until the extract reached a syrupy consistency.

2.6.2 Liquid-Liquid Extraction and Cleanup of the Extract

The combined phenolic extract obtained from the XAD-2 resin extraction was resuspended in Milli-Q water (150mL). The extract was then filtered through cotton wool to remove solids which had not dissolved. The filtrate was then subjected to eight extractions with diethyl ether (150mL). The ether extracts were combined and concentrated to dryness under vacuum at 40°C.

The extract was redissolved in distilled methanol (40mL) and centrifuged (5000rpm, Heraeus Sepatech Medifuge) to separate out a white waxy solid. The supernatant was then run through HPLC system A (described in Section 2.3.1) to filter out contaminants. The extract was injected in batches (1mL) with the eluent being continuously collected and combined. The combined eluent from the HPLC injections was concentrated to dryness under vacuum at 40°C.

2.6.3 *Sephadex LH-20 Separation of the Phenolic Extract*

The flavonoids were separated from the phenolic acids by chromatography on a Sephadex LH-20 column according to the method described by Bohm.⁸¹

The extract was dissolved in distilled methanol (40mL) and poured into a column of Sephadex LH-20 (Ø4cmx30cm) which had been pre-soaked in 30% (v/v) distilled methanol in water. The phenolics were then eluted and collected using increasing stronger methanol solutions as shown in table 2.6. The flow rate was increased by applying a light head pressure with N₂ gas.

Table 2.6: The solvents used in Sephadex LH-20 chromatography of phenolic extract and the fractions collected.

Fraction Number	Solvent Strength (% methanol v/v in water)	Volume of Solvent Used (mL)
1	30	250
2	30	250
3	60	250
4	60	250
5	80	250
6	80	250
7	100	250
8	100	250
9	100	250

2.6.4 HPLC Separation of Individual Flavonoids

Individual flavonoids were isolated from the extract using the semi-preparative HPLC system C, as describe in Section 2.3.3. The extract was centrifuged (5000rpm, Heraeus Sepatech Medifuge) prior to injection onto the semi-prep HPLC system at high loading rates (up to 150 μ L). Fractions were collected from the photodiode array outlet to isolate targeted flavonoids

The flavonoids isolated from the semi-prep HPLC system were subjected to a further fraction collecting step on the analytical HPLC described in Section 2.3.2. For this work the sample loop size was increased from 5 μ L to 200 μ L to prevent sample losses.

2.7 Characterisation of Isolated Flavonoids

2.7.1 UV-Visible Spectroscopy

2.7.1.1 Preparation of Spectroscopic Reagents for UV-Visible Spectroscopy

Spectroscopic reagents were prepared according to the methods published by Mabry *et al.*⁹³ Anhydrous sodium acetate (Panreac, analytical grade) was ground to a powder and stored in a labelled vial. Boric acid (May and Baker, reagent grade) was ground to a powder and stored in a labelled vial. Powdered boric acid was added to methanol (20mL, Scharlau HPLC grade) until the solution was saturated. Sodium metal (1.8825g, BDH, sodium sticks in paraffin) was cut into small pieces and washed with petroleum spirits (X4, drum grade). The sodium metal pieces were added cautiously to methanol (75mL, Scharlau HPLC grade) to create a sodium methoxide solution. Aluminium chloride (2.5800g, Merck, reagent grade) was added to methanol (52mL, Scharlau HPLC). Concentrated hydrochloric acid (21mL, Ajax, 36%) was diluted with distilled water (42mL). All reagent solutions were stored in Schott bottles at room temperature in the dark when not being used.

2.7.1.2 UV-Visible Spectroscopy of Isolated Flavonoids

UV-Visible spectra of isolated flavonoids were recorded on a Varian Cary 100 Scan UV-Visible Spectrophotometer. The spectrophotometer was operated and results

processed using Cary WinUV Scan Application software (version 3.00(182)). Spectra were recorded in a quartz cuvette (1cmx1cmx4.5cm) which did not absorb over the spectral region of interest.

A small amount of the isolated flavonoid (ca. 1mg) was dissolved in HPLC grade methanol (10mL) to create a stock solution. The UV-visible spectrum was taken of this solution at a rapid scan rate (600nm/min) to check the optical density. The concentration of the flavonoid solution was then adjusted so that the optical density of the major absorption peak (between 250-400nm) was within 0.6 to 0.8AU.

The spectrum of the flavonoid stock solution (2-3mL) was measured at a normal scan speed (50nm/min) over the 200-500nm region. Additional spectra were measured over the regions of peak maxima at a reduced scan rate (10nm/min) to determine the wavelength of maxima accurately.

Sodium methoxide solution (3 drops) was added to the cuvette of flavonoid in methanol. The spectrum was then recorded immediately at a normal scan rate. After five minutes the spectrum was re-recorded to check for decomposition of the flavonoid. The solution in the cuvette was then discarded.

Aluminium chloride solution (6 drops) was then added to fresh flavonoid stock solution (2-3mL) in the cuvette. The spectrum was then recorded at a normal scan rate. Hydrochloric acid (3 drops) was then added to the cuvette containing aluminium chloride and flavonoid in methanol. The spectrum was recorded again and the solution discarded.

An excess of powdered anhydrous sodium acetate was added to fresh flavonoid stock solution (2-3mL) in the cuvette and shaken. The powdered sodium acetate formed a layer (1-2mm) at the bottom of the cuvette. The spectrum was recorded immediately and again after ten minutes to check for decomposition.

Finally, an excess of powdered anhydrous boric acid was added with shaking to saturate the sodium acetate and flavonoid solution. The spectrum was then recorded

and the solution discarded. In cases where the flavonoid decomposed in the presence of sodium methoxide, a different method was used to obtain a boric acid/sodium acetate spectrum. Boric acid solution (5 drops) was added to fresh flavonoid stock solution (2-3mL) which was quickly saturated with powdered sodium acetate and the spectrum recorded immediately.

2.7.2 Nuclear Magnetic Resonance Spectroscopy of Isolated Flavonoids

NMR spectra of isolated flavonoid samples were obtained using a Bruker Avance DRX fitted with a 400MHz Bruker Spectrospin superconducting magnet (52mm). Proton and carbon 1D experiments were carried out using a 5mm dual $^{13}\text{C}/^1\text{H}$ probe head. Samples were thoroughly dried by freeze drying over several days and dissolved in dimethylsulfoxide- d_6 for NMR analysis. Operation of the NMR and processing of spectra were performed using Topspin software (Bruker). ^1H , ^{13}C and HMBC experiments were carried out.

2.7.3 Mass Spectrometry of Isolated Flavonoids

Mass spectrometry was performed by Jonathon Puddick using a Bruker Daltronics autoflex II MALDI-TOF mass spectrometer. The spectrometer was operated in reflector mode (acceleration voltage 19kV, reflector voltage 20kV).

Isolated flavonoid samples were dissolved in methanol and spotted onto an anchorchip (600 μm , Bruker) and the methanol evaporated to leave a neat sample. Calibration of the spectrometer was against 2,5-dihydroxybenzoic acid ions (153.02Da and 307.05Da) laid on a neighbouring sample spot. A pulsed ion extraction (60ns) and suppression (to 100Da) were used to obtain mass spectra of the samples in negative ion mode.

Chapter 3: Results and Discussion

3.1 The Antibacterial Activity of Fractions from the XAD-2 Extraction of Manuka Honey

Activity testing of fractions obtained from the XAD-2 extraction of manuka honey was carried out to determine whether the non-peroxide antibacterial activity was eluted with the phenolics or the sugars. Antibacterial activity was measured for the aqueous fraction of the extraction which contained the sugars and other polar compounds, and the methanol fraction which contained the phenolics including the flavonoids and phenolic acids. As a comparison, the activity of the original manuka honey and a sample of inactive clover honey, which was used as an artificial matrix for the methanol fractions, were tested. The results of the activity testing are shown in table 3.1 and illustrated in the graph (figure 3.1).

Table 3.1: The non-peroxide antibacterial activities of fractions from the XAD-2 extraction of manuka honey compared to the activity of the original honey.

	Non-Peroxide Antibacterial Activity (% Phenol Equivalents)
Aqueous Sugar Fraction	30.5 ± 0.7^a
MeOH Phenolic Fraction 1:1	No Detectable Activity ^a
MeOH Phenolic Fraction 25:1	37 ± 1^b
Manuka Honey	30.6 ± 0.8^a
Clover Honey	No Detectable Activity ^b

^aMean and 95% confidence interval from 48 determinations.

^bMean and 95% confidence interval from 32 determinations.

It is clear that the aqueous fraction of the extraction, with an activity of 30.5 ± 0.7 % phenol equivalents, accounts for the majority of the antibacterial activity of manuka honey (activity of 30.6 ± 0.8 % phenol equivalents). The methanol fraction, which contained the flavonoids, did not exhibit any detectable antibacterial activity at the concentration it occurs in whole manuka honey. The methanol fraction did exhibit an activity of 37 ± 1 % phenol equivalents when concentrated to levels twenty five times that which it naturally occurs in honey.

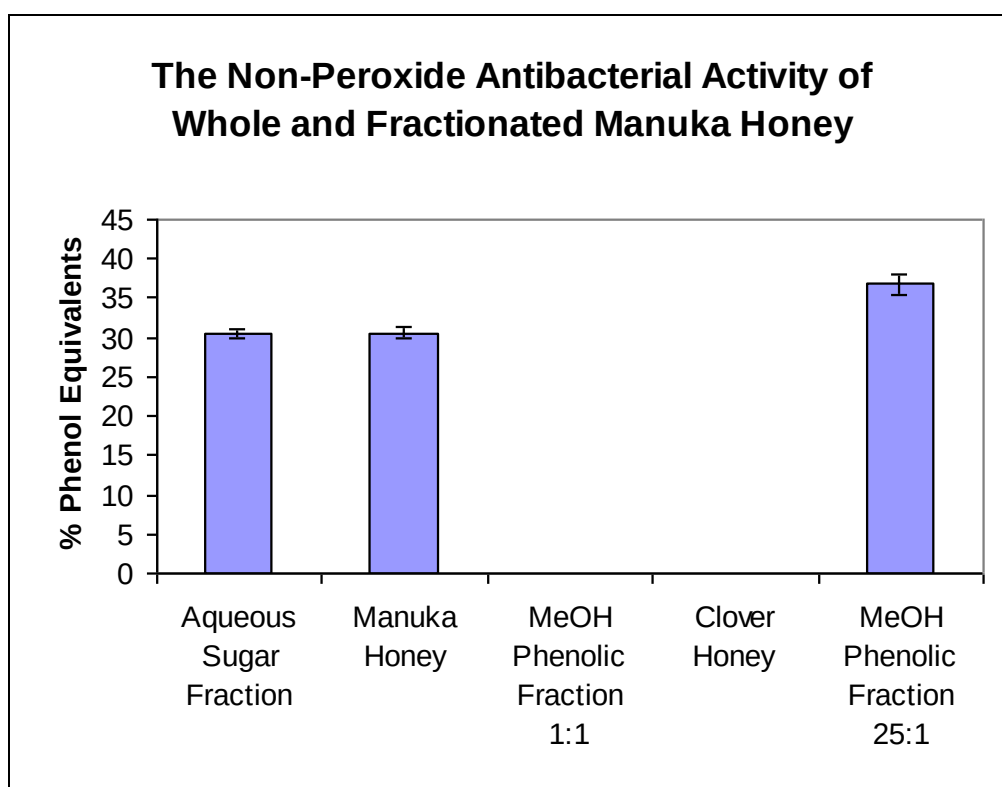


Figure 3.1: Graph showing the non-peroxide antibacterial activity of the XAD-2 extraction sugar fraction, the methanol fraction at its natural concentration and concentrated 25 times, the original manuka honey and the inactive clover honey used as an artificial matrix.

Weston *et al.*²³ reported a similar result in 1999. Using the XAD-2 extraction method developed by Ferreres *et al.*⁹⁴, carried out under neutral conditions, the activity of antibacterial activity of carbohydrate and phenolic fractions were determined. It was found that all the antibacterial activity of manuka honey eluted with the carbohydrate fraction while the phenolics showed no significant activity.

The results of the present study and the early work by Weston *et al.*²³ have shown that the antibacterial activity of manuka honey elutes with the carbohydrates in the XAD-2 extraction technique. This is not surprising since methylglyoxal, a sugar decomposition product, has been identified as responsible for the non-peroxide antibacterial activity of manuka honey.^{68, 70} Sharing common structural features

with carbohydrates, methylglyoxal would be expected to elute with them and the other polar constituents of honey.

While it is apparent that the phenolics do not make a significant contribution to the antibacterial activity of manuka honey, the present study has also shown that the phenolic fraction of manuka honey is antibacterial, if only when concentrated. Antibacterial activity is not the only biological activity which has been attributed to the flavonoids. These natural products have been shown to have a range of activities including anti-inflammatory activity, oestrogenic activity, enzyme inhibition activity, antiallergic activity, antioxidant activity, vascular activity, antiviral, antithrombotic, anaesthetic activity and cytotoxic antitumour activity.^{1, 82, 86} The flavonoids may not make a significant contribution to the antibacterial activity of manuka honey but they may still contribute to other therapeutic properties of honey.

The biggest problem usually associated with the bioassay for non-peroxide antibacterial activity has always been the reproducibility of the antibacterial assay. Variation is seen between the results measured by different analysts, on different days, on different agar plates and even within the same plate. A range of factors such as temperature, agar thickness, materials used and well positions cause an uncertainty level of the order of 1 % phenol equivalents in all activity measurements. In the present study an effort has been made to account for these variations. Two extractions on XAD-2 resin were carried out to provide two sets of fractions for activity testing. These fractions were then tested on two separate occasions over four agar plates. The use of the *Quasi*-Latin square distribution of wells is also standard practice in the antibacterial assay of honey and serves to average out effects associated with the position of individual wells on the agar plate (e.g. wells at the edge show less activity). The large number of wells used for each sample in this study (48 for the carbohydrate and 1:1 phenolic fractions, and manuka honey; 32 for the 25:1 phenolic fraction and clover honey) has allowed the variations to be averaged out giving the 95% confidence intervals shown in table 3.1 (calculated using a t distribution).

3.2 Identification and Quantification of Flavonoids by HPLC

Flavonoids in HPLC chromatograms were identified by their co-elution with authentic standards whenever possible. The collection of standards available for comparison with HPLC peaks was limited and in many cases the peaks could not be identified beyond classifying them as flavonoids based on their UV spectra. Identification of these unknown flavonoids required them to be isolated, characterised and then used as a standard for identification of HPLC flavonoid peaks. This has restricted the progress of the present study. Other research groups have built up extensive collections of isolated flavonoids over time, with which they can compare their HPLC chromatograms of honey phenolics. Such research groups have a distinct advantage in this area of research since some of the flavonoids, such as pinobanksin a common flavonoid in honey, are not easy to source commercially. Table 3.2 shows the flavonoid peaks, their retention time and their UV absorption spectra maxima along with the means by which they were identified (by comparison with standard or isolation and characterisation).

Four standards were used for the quantitative analysis of flavonoids by HPLC. Chrysin (at 340nm) was used as a standard for the flavones and flavonols with an unsubstituted B-ring. This included the flavonol galangin, the flavone chrysin and their derivatives. Similarly, kaempferol (at 340nm) was used as the standard for quantifying those flavones and flavonols with a single oxygen substituent on the B-ring. Pinocembrin (at 290nm) was used to quantify the flavanones and dihydroflavonols while quercetin (at 340nm) was used as a standard for all other flavones and flavonols. The standards used to quantify each flavonoid peak are shown in table 3.2. Calibration curves showing the HPLC peak area versus the concentration of these compounds are shown in figures 3.2, 3.3, 3.4 and 3.5.

Table 3.2: The phenolic compounds identified and quantified by HPLC.

Name	Identification ^a	Quantification ^b	R _t (min) ^c	λ _{max} (nm) ^d
Flavonoids found in manuka honey samples.				
Pinobanksin	I	P	33.9	292
Unknown Compound 01	NA	Q	34.4	342
Unknown Flavonoid 01	NA	P	38.3	286
Quercetin	S	Q	38.7	367, 256
Luteolin	S	Q	39.3	350, 255
Unknown Flavonoid 02	NA	P	40.4	292
8-Methoxykaempferol	I	K	41.2	375, 272
Pinocembrin	S	P	41.5	290
Unknown Flavonoid 03	NA	Q	42.4	360, 258
Unknown Flavonoid 04	NA	K	43.7	361, 253
Isorhamnetin	S	K	44.3	373, 255
Kaempferol	S	K	45.5	364, 255
Unknown Flavonoid 05	NA	P	46.4	291
Chrysin	S	C	46.7	314, 269
Galangin	S	C	50.0	360, 266
Unknown Flavonoid 06	NA	C	50.6	311, 269
Flavonoids found in non-manuka honey samples.				
Unknown Flavonoid 07	NA	P	31.1	289
Unknown Flavonoid 08	NA	P	32.0	294
Unknown Flavonoid 09	NA	P	33.2	288
Unknown Flavonoid 10	NA	C	47.9	351, 268
Unknown Flavonoid 11	NA	C	48.3	322, 260
Unknown Flavonoid 12	NA	C	48.6	349, 268
Unknown Flavonoid 13	NA	C	52.1	349, 268

^a The method used to identify the compound: S – authentic standard, I – isolation and characterisation and NA- not identified.

^b The quantification standard used for the compound: Q – quercetin, K – kaempferol, P – pinocembrin and C – chrysin.

^c The retention time of the compound on HPLC System B

^d The peak maxima in the UV absorbance spectrum (240-400nm) of the compound under HPLC System B conditions.

HPLC detection of flavonoids was carried out at two different wavelengths due to the differences in the UV-Visible absorption spectra of the flavanones and dihydroflavonols compared with the flavones and flavonols. The flavone and flavonol class of flavonoids exhibit two major absorption bands with Band I usually occurring between 300 to 380nm, and Band II usually occurring between 240 to 285nm. The flavanones and dihydroflavonols do not exhibit a Band I absorption, having only the Band II absorption. Thus it is necessary to detect the flavanones and dihydroflavonols at a wavelength within their Band II absorption for the best sensitivity. For this experiment the wavelength of 290nm was chosen to be consistent with the previous work on honey flavonoids by Ferreres *et al.*⁹⁵

While it would also be possible to detect the flavones and flavonols at this wavelength, an alternative wavelength of 340nm was chosen. The particular choice of 340nm is also consistent with the work of Ferreres *et al.*⁹⁵, allowing the results of this study to be compared to with published work.²⁴ The choice of a detection wavelength within the Band I absorption region has the added benefit of improving the sensitivity of the analysis. The Band I absorption of flavones and flavonols typically occurs at lower wavelengths than 290nm and they only absorb weakly at this wavelength. Analysis at 340nm also reduces interference from phenolic acid derivatives which absorb strongly at 290nm.

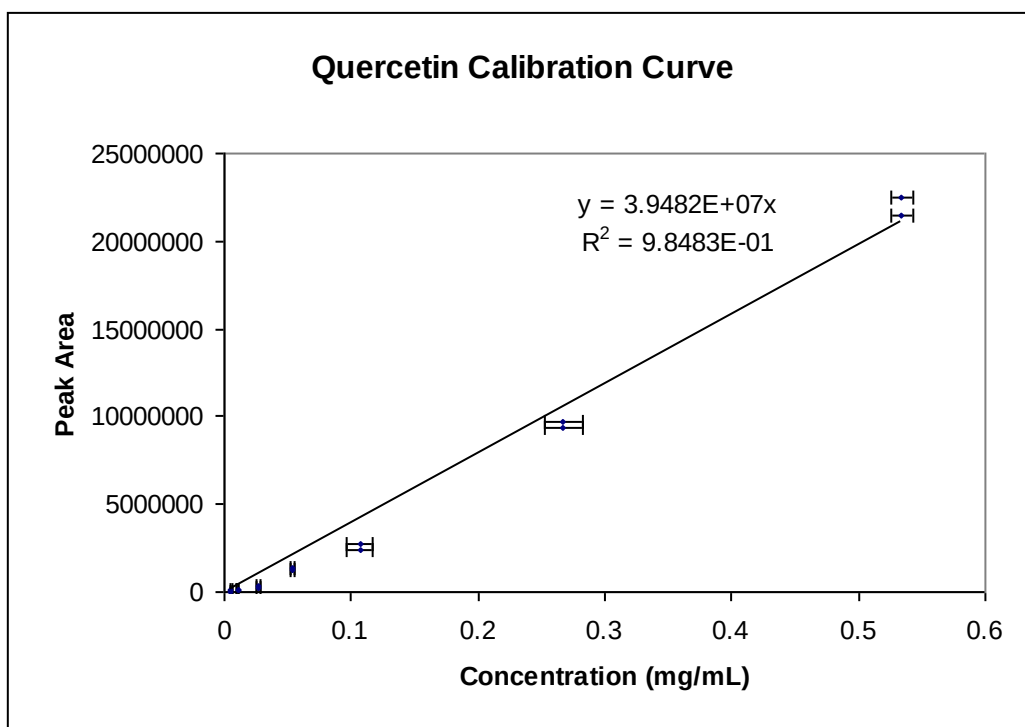


Figure 3.2: The HPLC calibration curve of quercetin, measured at 340nm.

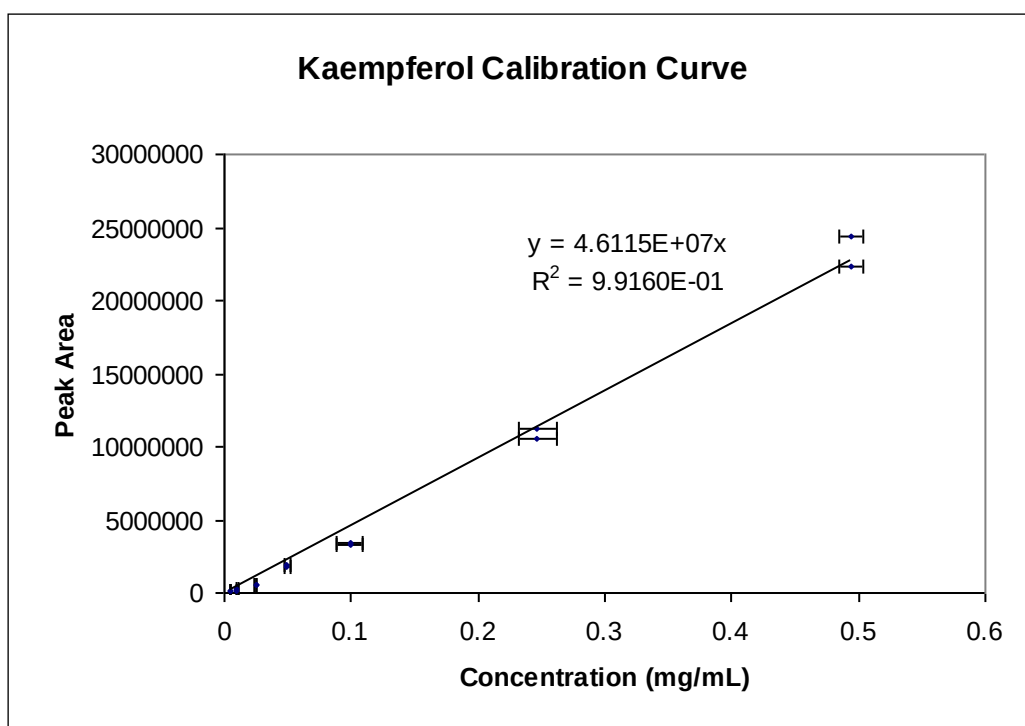


Figure 3.3: The HPLC calibration curve of kaempferol, measured at 340nm.

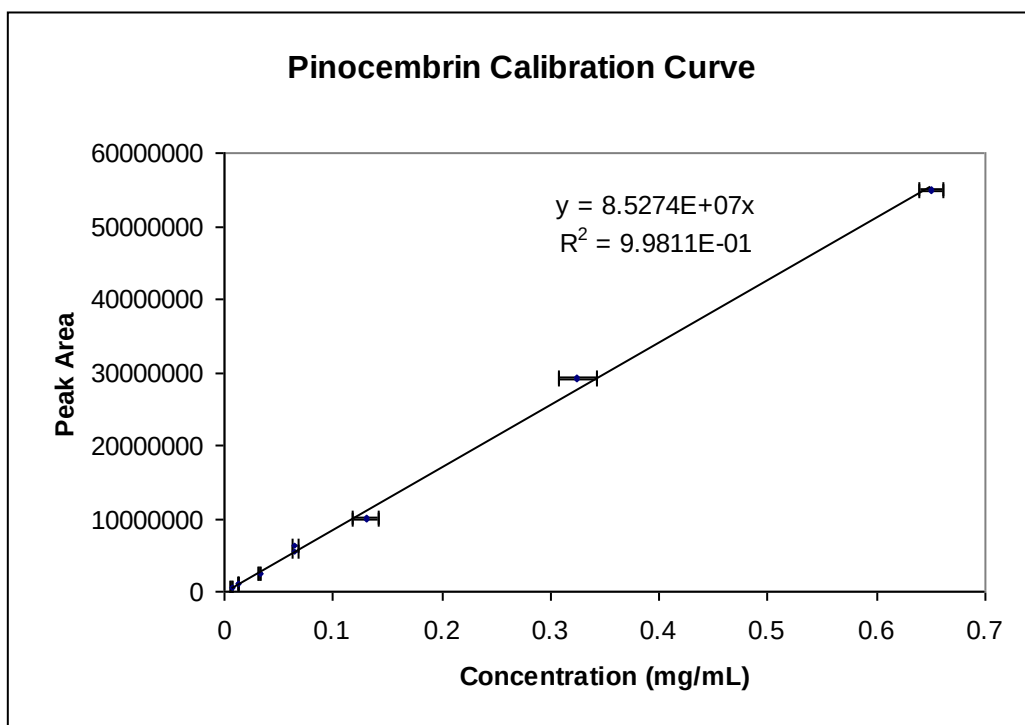


Figure 3.4: The HPLC calibration curve of pinocembrin, measured at 290nm.

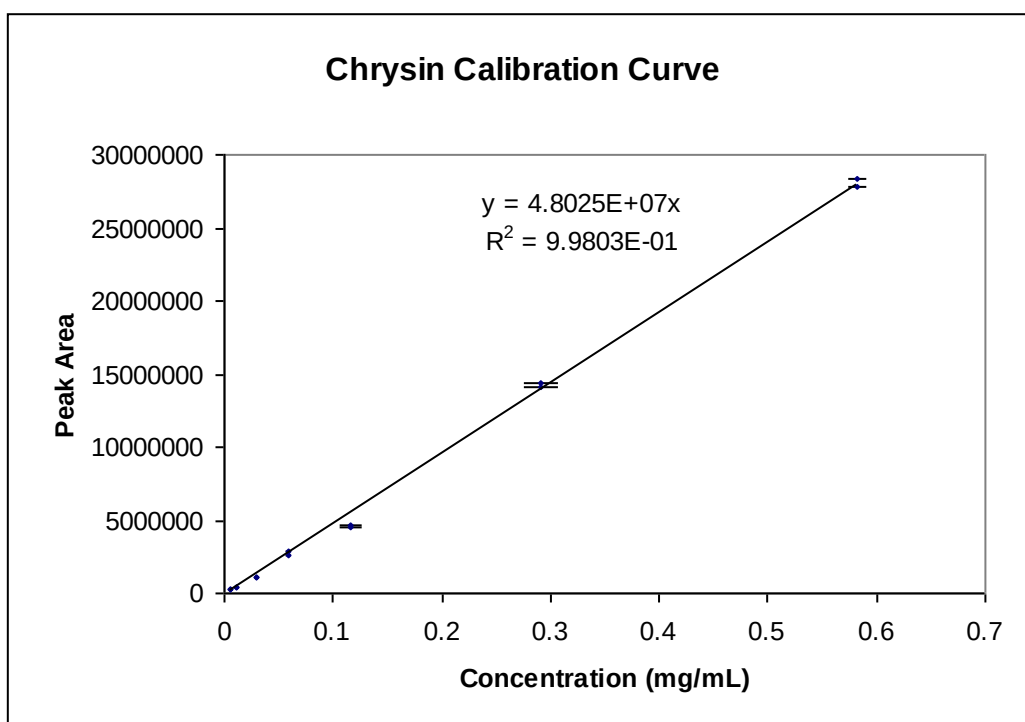


Figure 3.5: The HPLC calibration curve of chrysin, measured at 340nm.

A problem which was frequently observed during this study was precipitation of standards. Left overnight in a methanol solution, the flavonoids would often precipitate out. This made quantitative analysis difficult since the concentration of flavonoid standards was uncertain and it could not be guaranteed that the sample extracts were homogenous. To minimise this problem all standards and samples were warmed to 40°C prior to analysis by HPLC. This was enough to redissolve any precipitates and ensure the solution was homogenous.

Precipitation of polyphenolic compounds appears to be a common problem. In the brewing industry chill haze is a precipitate that forms when beer is chilled. Polyphenolics are a major contributor to this haze and much effort is made to remove them during the brewing process.⁸¹

3.3 Evaluation of the Extraction Method

3.3.1 *The Effect of Sample Size on Phenolic Recovery*

The Chemistry Department of the University of Waikato has a large collection of manuka honey samples which were made available for this study. Unfortunately, the majority of these samples had only about 10 grams of honey remaining. This was not enough for extraction by the XAD-2 method established by Ferreres *et al.* which uses at least 50 grams of honey.⁹⁴ This study has attempted to scale down the XAD-2 extraction method to permit extraction of honey phenolics from samples as small as 5 grams.

In order to determine the effect of sample size on phenolic recovery, multiple extractions of 50, 20 and 5 gram samples of the same manuka honey were carried out under the same conditions and the extracts were analysed by HPLC. Table 3.3 shows the peak areas at 340nm for the peaks eluting at 15.9 and 20.5 minutes, as well as the total area of the chromatogram at 340nm. The peak areas have been adjusted to account for small weight differences between samples of the same nominal weight. At these early stages of the research project it was not deemed necessary to identify and quantify the individual flavonoids in the honey extracts. Comparison of the total area of the chromatogram and the areas of two significant flavonoid peaks was used to assess the effect of sample size on honey phenolic recover.

Table 3.3: Adjusted peak areas measured at 340nm, of two significant peaks and the total chromatogram, for different sized sample extractions.

Sample Size (g)	Peak Area (at 340nm)		
	15.9min Peak	20.5min Peak	Total Chromatogram
50	$(3.4 \pm 1.3) \times 10^6$	$(1.4 \pm 0.8) \times 10^6$	$(1.7 \pm 0.8) \times 10^8$
20	$(3.3 \pm 0.8) \times 10^6$	$(1.6 \pm 0.3) \times 10^6$	$(1.9 \pm 1.0) \times 10^8$
5	$(3.4 \pm 1.6) \times 10^6$	$(1.4 \pm 0.7) \times 10^6$	$(2.1 \pm 1.0) \times 10^8$

All the results showed a high level of variability but it is apparent that the sample size does not have a significant effect on the recovery of honey phenolics by the XAD-2 extraction method. Calculated 95% confidence intervals for the average total chromatogram area at 340nm were $(1.7 \pm 0.8) \times 10^8$ for the 50g extractions, $(1.9 \pm 1.0) \times 10^8$ for the 20g extractions and $(2.1 \pm 1.0) \times 10^8$ for the 5g sample extractions. These results all agree within the limits of uncertainty, showing that the recovery of honey phenolics from a 5g sample is no better or worse than from a 50g or 20g sample. It is also clear from the large uncertainty in these confidence intervals that the variation in honey phenolic recovery between samples of the same nominal mass is much more significant than that between samples of different nominal mass. The results for the two peaks studied show similar large variations which are independent of the nominal sample mass.

The peak areas of individual extractions are shown in figures 3.6, 3.7 and 3.8. These graphs illustrate that the peak area and its variation are not significantly affected by the size of the honey sample extracted. This result is not surprising since the XAD-2 extraction is a form of solid phase extraction. In solid phase extraction it would normally be expected that the recovery of target analytes would be greater when smaller samples are extracted. This is because matrix effects are a common problem with interfering compounds (for example the phenolic acids in the present study) occupying the surface of the solid phase.¹²² Thus, when large samples are analysed there is a risk that the solid phase will be saturated with interfering compounds, which will prevent the complete recovery

of the target analyte. Smaller samples are less likely to saturate the solid phase with interfering compounds and should allow more complete recovery of the honey phenolics. The fact that the recovery did not increase as the sample size decreased shows that saturation of the solid phase is not a problem for 50g extractions.

While the high level of uncertainty in these results is a concern, it is not sufficient reason to restrict the XAD-2 extraction method to honey samples of 50g or larger since the variability in the recovery of phenolics is independent of the sample size. Extracting phenolics from the smaller sample size of 5g meant that a much larger stock of samples could be analysed. Many of these samples could not have been extracted if 50g of honey was required.

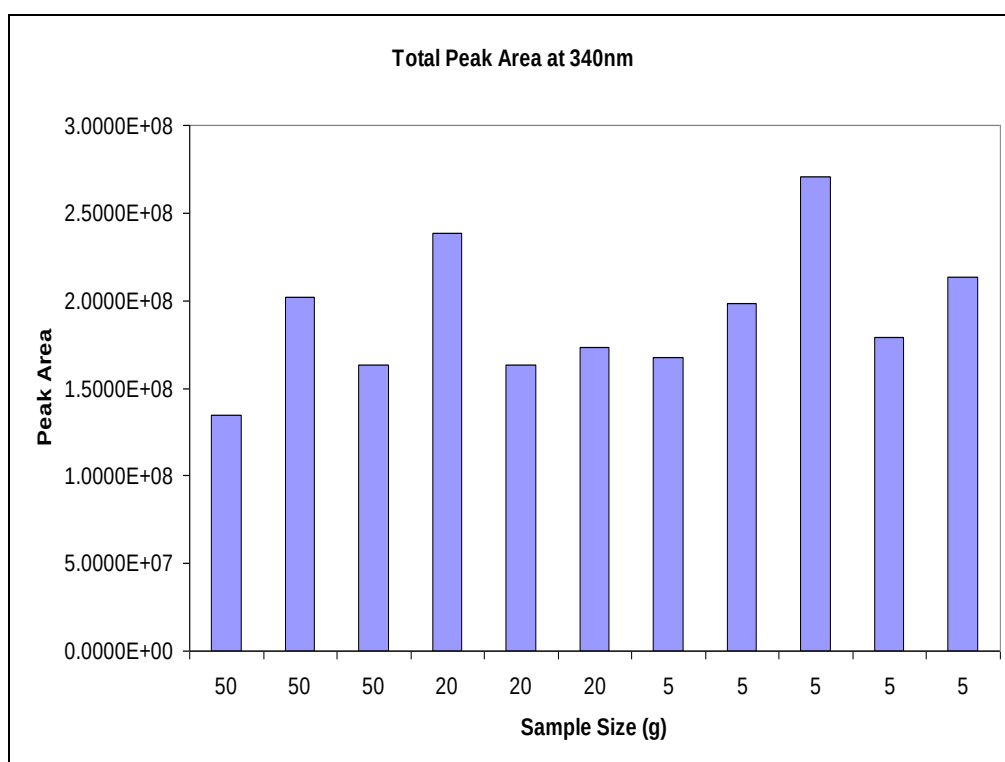


Figure 3.6: Total chromatogram area at 340nm, of the phenolics extracted from differently sized samples.

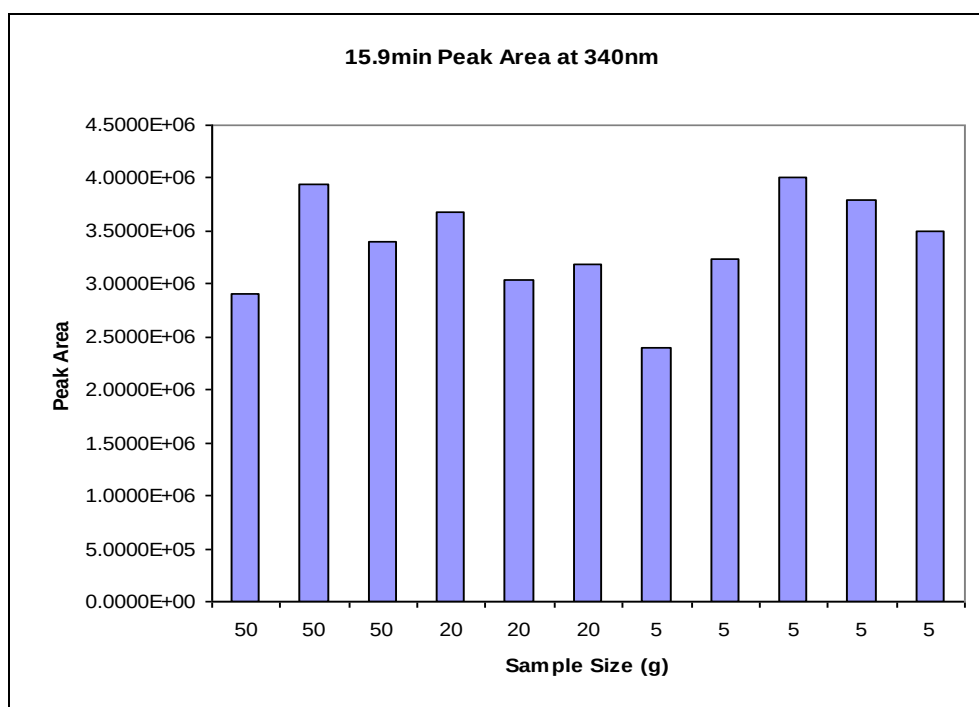


Figure 3.7: Area of the 15.9min phenolic peak extracted from differently sized samples, measured at 340nm.

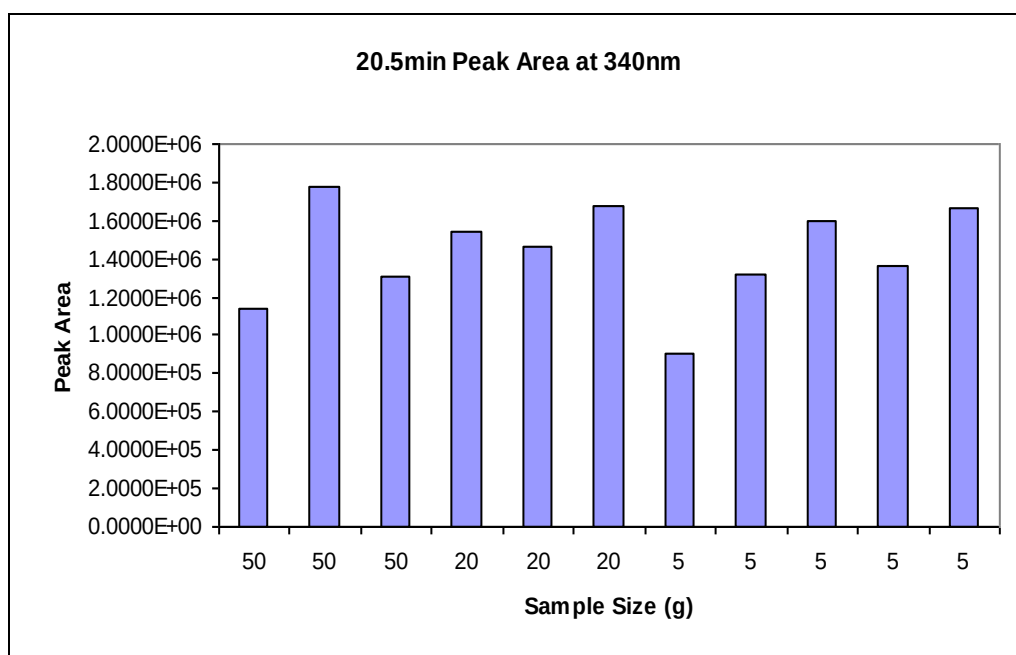


Figure 3.8: Area of the 20.5min phenolic peak extracted from differently sized samples, measured at 340nm.

3.3.2 The Recovery Rate of Flavonoids

It had been previously reported, by those who developed the XAD-2 extraction method for honey phenolics, that the technique recovered honey phenolics at more than 80%.^{1, 24, 104, 107} An experiment was carried out in order to assess the recovery rate of flavonoids for the modified extraction method developed in this study. To test the recovery rate of flavonoids an artificial honey, a mixture of glucose, fructose, sucrose and water, was prepared with known levels of quercetin, kaempferol and chrysin added. The phenolic acids *p*-coumaric acid, chlorogenic acid, gallic acid and caffeic acid were also added. This artificial honey was considered a good model for the complex matrix that is real honey. Extraction of the phenolics from this artificial honey allowed the recovery rates shown in table 3.4 to be determine.

The results show that the extraction method employed in this study is very inefficient at recovering the flavonoids from an artificial honey matrix. Quercetin was only recovered at an average rate of $10 \pm 6\%$ while kaempferol and chrysin were recovered at the slightly higher rates of $16 \pm 7\%$ and $19 \pm 9\%$ respectively. These results are significantly lower than the $>80\%$ recovery rate reported by other groups.^{1, 24, 104, 107} This could possibly be due to the more sophisticated method used to test the recovery rate in this study. The previously reported work does not specify how the recovery rate was determined. Work by D'Arcy reported recovery rates of 38% for quercetin, 88% for chrysin and 92% for hesperetin. These were measured by simply mixing a methanol solution of flavonoid standards with XAD-2 resin.¹ Honey is a complex matrix and no matrix effects were accounted for in the earlier work. The present study has attempted to account for the effects of the honey matrix by measuring the recovery of flavonoids from an artificial honey matrix. It is possible that other components of the honey including the carbohydrates and phenolic acids could interfere with the extraction of flavonoids and reduce their recovery rate.

Table 3.4: The recovery rate of flavonoids from an artificial honey matrix.

Sample Name	% Recovery Rate		
	Quercetin	Kaempferol	Chrysin
AA Ext Eff	16.38%	16.91%	15.33%
AB Ext Eff	3.38%	6.68%	10.63%
AC Ext Eff	17.69%	21.82%	24.87%
AD Ext Eff	9.13%	7.80%	10.86%
AF Ext Eff	15.40%	27.92%	36.85%
AG Ext Eff	4.07%	14.58%	14.34%
AH Ext Eff	4.87%	14.36%	21.25%
Mean	10%	16%	19%
S.D.	6%	7%	9%
95% C.I.	6%	7%	9%

The large amount of carbohydrates could potentially carry some of the flavonoids through the XAD-2 extraction, preventing them from binding to the resin and causing them to elute with the aqueous fraction. Honey also contains a lot of phenolic acids which also adsorb onto the resin. If there are sufficient phenolic acids in the honey then they can overload the resin, preventing flavonoids from being adsorbed and thus reducing their recovery.

A large degree of variance was observed in these results. The standard deviation was greater than 40% of the mean for all three of the flavonoid recovery rates determined. A possible reason for this variance was thought to be inconsistencies in the artificial honey. It is very difficult to prepare a homogenous artificial honey due to its syrupy nature. Despite the great care taken in this study to mix the dry ingredients together thoroughly before adding water, it is possible that the flavonoids are not evenly spread throughout the artificial honey. This would explain why the recovery rate appeared to be so highly variable since higher recovery rate results would come from artificial honey with higher levels of flavonoids (compared to the average level for the whole honey).

To further investigate the possibility that matrix effects may be limiting the recovery rate of flavonoids, the recovery rate was measured using the method described by D'Arcy.¹ Flavonoid standards were dissolved in methanol and mixed with XAD-2 resin to extract the phenolics. Two such standard solutions were put through the whole extraction process, including filtration on HPLC

system A. The recovery rates of the quercetin, pinocembrin, kaempferol and chrysin from these standard solutions are shown in table 3.5. The table shows an additional result which was measured for a standard solution which was put through the cleanup step on HPLC system A.

Table 3.5: The recovery rate of flavonoids from a simple solution. Two standard solutions were put through the whole extraction process and one was put only through the final HPLC cleanup step.

Extraction Type	% Recovery Rate			
	Quercetin	Pinocembrin	Kaempferol	Chrysin
Whole Extraction 1	38	60	39	45
Whole Extraction 2	28	48	34	35
HPLC Cleanup Step	60	88	66	75

The recovery rate for quercetin was measured at 38 and 28% in two tests of the entire extraction process. These values were well short of the >80% recoveries reported by the Tomas-Barberan *et al.*^{24, 104, 107} They do however compare well with the average quercetin recovery rate of 38% reported by D'Arcy.¹ Both results are within the range of 16-65% measured by D'Arcy.

Kaempferol was recovered at a rate of 39 and 35% in this experiment. These recovery rates are similar to quercetin which is not unsurprising given their structural similarities (kaempferol has one less hydroxyl group). These values are below the >80% recovery rate reported by in the literature.^{24, 104, 107}

The other flavone studied, chrysin, was recovered at rates of 45 and 35%. Both measurements were again well below the >80% reported in the literature. D'Arcy reported recovery rates in the range of 85-99%, much higher than those measured in the present study.^{1, 24, 104, 107}

The other flavonoid recovery rate measured in this study was that of pinocembrin. Pinocembrin is a flavanone while the others were flavones and flavonols. The difference in flavonoid class had a noticeable effect on the recovery rate. The flavanone pinocembrin was recovered at rates of 60 and 48% which were significantly higher than those found for the flavones and flavonols studied. The recovery rate of the flavanone was still outside the > 80% rate reported in the

literature. While D'Arcy¹ did not measure the recovery rate of pinocembrin, recovery rates of 84-95% were reported for the flavanone hesperetin. These rates are significantly higher than those found in the present study.^{1, 24, 104, 107}

These recovery rates were much higher than those measured for the flavonoids in an artificial honey matrix. This suggests that matrix effects may have an influence on the recovery of flavonoids. More research is needed to confirm this however, since the low recovery of flavonoids from the artificial honey could still be due to an inhomogeneous distribution of flavonoids in the artificial honey.

With the exception of quercetin, the extraction method used in this study was not achieving the same recovery rates reported by other groups. The HPLC cleanup step that was introduced in this study was identified as an area where loss of phenolics could occur. This cleanup step was implemented after it was found that running phenolic extracts through the analytical HPLC caused the chromatography conditions to deteriorate as an unidentified contaminant built up on the column and interfered with analysis. This contaminant was removed by injecting dimethyl sulfoxide into the system and the cleanup step was implemented to filter out the contaminant before extracts were analysed. The recovery of flavonoids from this step was measured by running a methanol solution, containing measured levels of flavonoids, through the cleanup step and measuring the levels remaining post cleanup.

The results of the cleanup recovery test are shown in table 3.5. The recovery rates were found to be 60, 88, 66 and 75% for quercetin, pinocembrin, kaempferol and chrysin respectively. These results show that significant amounts (up to 40%) of the flavones and flavonols are lost in the cleanup step. The flavanone pinocembrin appeared to be less affected by the cleanup step, only losing 12%. Clearly the HPLC cleanup step is making a significant contribution to the loss of flavonoids in the extraction method. The cleanup step was kept in the extraction method despite this loss because it was very effective at protecting the analytical HPLC column. Contamination problems which were a constant recurring problem before the implementation of the cleanup step did not occur once the samples were cleaned up on the sacrificial reversed phase HPLC column.

The low recovery rates found in this experiment are a concern. If only 28% of some flavonoids are being recovered then there is significant uncertainty in the quantitative levels of flavonoids reported for honeys analysed in this study. It is highly likely that the actual levels of flavonoids will be higher than those measured in this study.

3.3.3 The Reproducibility of the Extraction Method

The experiments evaluating the modified XAD-2 extraction method discussed in Sections 3.3.1 and 3.3.2 showed that the extraction method gave results with relatively high levels of variability. To investigate this variability and assess the reproducibility of the method, a bulk manuka honey sample was extracted eleven times over several weeks. The results of these analyses are shown in table 3.6.

The total flavonoid content was observed to vary within the range of 0.64 to 1.20mg/100g with a standard deviation of 0.18mg/100g. This standard deviation equates to a coefficient of variation of 19% showing that the results are subject to a high level of variability. The standard deviations for most of the individual flavonoid contents showed a similar level of variability with the coefficients of variation being between 10 and 37% for pinobanksin, luteolin, pinocembrin, isorhamnetin, chrysin and the unknown flavonoids 01-06.

The kaempferol, galangin and unknown compound 01 contents showed higher levels of variation with coefficients of variation as large as 87, 73 and 115%. The higher level of variation in the kaempferol and galangin levels, compared to the other flavonoids, is probably due to a combination of small peak areas and overlap with other closely eluting peaks. This made integration of these peaks difficult and could have contributed to the high level of variation observed. The kaempferol peak was particularly difficult to integrate and could not be identified in 7 of the 11 extractions studied due to its small peak area and overlap with the isorhamnetin and unknown flavonoid 05 peaks. The unknown compound 01 content variability cannot be explained by small peak areas and overlapping peaks. This compound produced large, resolved peaks in most of the manuka

Table 3.6: The results of multiple analyses of the flavonoid content of a manuka honey sample.

Extraction Number	Concentration of flavonoids (mg/100g of honey) ^a																
	PB	UN01	F01	Q	L	F02	8MK	PC	F03	F04	IRM	K	F05	C	G	F06	Total
A	0.223	0.020	0.061		0.090	0.017		0.102	0.009	0.013	0.023		0.013	0.082	0.025	0.016	0.695
B	0.317	0.049	0.091		0.099	0.025		0.208	0.011	0.022	0.035		0.039	0.140	0.037	0.051	1.123
C	0.207	0.023	0.039			0.020		0.164	0.007	0.015	0.025		0.021	0.095	0.003	0.017	0.635
D	0.250	0.098	0.077		0.082	0.021		0.180	0.008	0.021	0.027		0.029	0.123	0.052	0.039	1.008
E	0.270	0.048	0.053		0.083	0.025		0.171	0.015	0.018	0.030	0.003	0.017	0.097	0.042	0.022	0.895
F	0.328	0.070	0.086		0.099	0.023		0.204	0.012	0.025	0.038	0.010	0.021	0.130	0.054	0.030	1.130
G	0.328	0.191	0.082		0.090	0.028		0.192	0.009	0.021	0.037	0.007	0.022	0.121	0.037	0.030	1.195
A1	0.305	0.007	0.051		0.075	0.017		0.182	0.012	0.012	0.028		0.022	0.102	0.008	0.018	0.838
B1	0.332	0.010	0.041		0.105	0.020		0.185	0.018	0.016	0.031		0.039	0.119	0.010	0.038	0.963
A2	0.359	0.013	0.062		0.097	0.030		0.231	0.015	0.017	0.035		0.027	0.149	0.012	0.026	1.072
B2	0.290	0.007	0.058		0.086	0.019		0.176	0.015	0.013	0.035	0.026	0.031	0.128	0.007	0.028	0.919
Mean	0.292	0.049	0.064		0.091	0.022		0.181	0.012	0.017	0.031	0.011	0.026	0.117	0.026	0.029	0.952
S.D. ^b	0.049	0.056	0.018		0.009	0.004		0.032	0.003	0.004	0.005	0.010	0.008	0.021	0.019	0.011	0.179
95% C.I. ^c	0.033	0.037	0.012		0.006	0.003		0.022	0.002	0.003	0.003	0.007	0.006	0.014	0.013	0.007	0.120
C.V.(%) ^d	16.7	114.6	28.2		10.3	19.0		17.9	29.2	23.4	16.2	87.2	32.6	17.6	73.0	36.7	18.8
% ^e	30.6	5.1	6.7		9.5	2.3		19.1	1.2	1.8	3.3	1.2	2.7	12.3	2.7	3.0	

^a PB – pinobanksin (3,5,7-trihydroxyflavanone), UN01 – unknown compound 01, F01 – unknown flavonoid 01, Q – quercetin (3,5,7,3',4'-pentahydroxyflavone), L – luteolin (5,7,3',4'-tetrahydroxyflavone), F02 – unknown flavonoid 02, 8MK – 8-methoxykaempferol (3,5,7,4'-tetrahydroxy-8-methoxyflavone), PC – pinocembrin (5,7-dihydroxyflavone), F03 – unknown flavonoid 03, IRM – isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), F04 – unknown flavonoid 04, K – kaempferol (3,5,7,4'-tetrahydroxyflavone), F05 – unknown flavonoid 05, C – chrysin (5,7-dihydroxyflavone), G – galangin (3,5,7-trihydroxyflavone), F06 – unknown flavonoid 06.

^b Standard deviation. ^c 95% confidence interval for 11 determinations

^d Coefficient of variation (standard deviation/mean x 100%).

^e Individual flavonoid level as a percentage of total flavonoid content.

samples studied (see Section 3.4.4). While it did occur in lower levels (compared to other manuka honeys) in the particular sample used for this experiment, it still gave a large peak which equated to 5.1% of the total flavonoid content. An alternative reason for the large variation in results for this compound could be that it is not a flavonoid. The UV spectrum of unknown compound 01 shows a single absorbance with a maximum absorption occurring at 342nm. This spectrum does not show the expected Band I and II absorptions of the flavones and flavonols; or the single Band II absorption of the flavanones and dihydroflavonols. Thus it is probably not a flavonoid and should not be expected to have the same extraction properties as the flavonoids.

While the majority of the flavonoids showed less variation than kaempferol, galangin and unknown compound 01, they still showed a significant level of variation which will have an impact on the results of this study. Possible reasons for this variation are:

- The recovery rates measured in Section 3.3.2 show that, at best, the extraction method only recovers 60% of the flavonoids and recovery rates were a lot less in most cases. A consequence of low recovery rates is lower concentrations of flavonoids in the phenolic extract. If the flavonoids were recovered at higher rates then flavonoid content would be higher and the variation should be less significant.
- The quality and history of the XAD-2 resin used in this study was not known. It is feasible that the resin was not extracting at its peak performance. This would explain the low recovery rates and contribute to variation in extraction results.
- An important source of variation in this experiment is the human operator. The extraction of phenolics from honey is a complicated procedure involving many steps, some of which are quite difficult. Transferring the XAD-2 resin from beaker to column, and vice versa, should be a simple procedure but it is hampered by the tendency of the resin to stick to glass when in aqueous conditions. While every effort was made to standardise the extraction method it is inevitable that minor differences would occur between extractions, ultimately contributing to the large variation seen in results.

This experiment has shown that a single extraction of a honey sample is unlikely to provide a reliable estimate of the flavonoid profile. The standard deviations were on average, 20% of the mean values (ignoring kaempferol, galangin and unknown compound 01). For the purposes of this study, an uncertainty estimate of 20% will be applied to all flavonoid contents from analysis of a single sample.

3.4 The Flavonoid Profile of New Zealand Honeys

The flavonoid profile was determined for New Zealand manuka (*Leptospermum scoparium*) honey and a range of other New Zealand honeys.

3.4.4 Manuka Honey

A total of 31 different manuka honey samples were analysed to determine a flavonoid profile for manuka honey. The results of this study are shown in table 3.7. The total flavonoid content of manuka honey was found to be, on average, $1.16 \pm 0.16\text{mg}/100\text{g}$ of honey. The total flavonoid content varied between 0.59 and $2.24\text{mg}/100\text{g}$ with a standard deviation of $0.46\text{mg}/100\text{g}$. This is several orders of magnitude higher than the $0.0147\text{mg}/100\text{g}$ total flavonoid content reported by Weston *et al.*¹²¹ The result found in this study is much closer to that reported by Yao *et al.*²⁴ ($3.06\text{mg}/100\text{g}$ of honey). Yao *et al.* have previously explained that the differences in these results are probably due to incomplete extraction of honey flavonoids by Weston *et al.*^{23, 121} Martos *et al.*¹⁰⁴ has reported that at least 100g of Amberlite XAD-2 should be used for extraction of phenolics from 100g of honey.¹⁰⁴ In the study by Weston *et al.*¹²¹, only 54-60g of resin was used to extract phenolics from 150-236g of honey.^{23, 121} In the present study, 30g of XAD-2 was used to extract phenolics from 5g samples of honey. This was a considerably higher ratio of resin to honey than used by Yao *et al.* (150g XAD-2 for 100g of honey).²⁴ Despite the large excess of resin used in this study, the recovery rates of flavonoids were found to be 60% at most, and a lot less for most of the flavonoids studied (see Section 3.3.2). The low recovery rate of flavonoids experienced in this study is considered to be the reason for the differences in flavonoid content measured between this study and that by Yao *et al.*²⁴

Table 3.7: The flavonoid content of New Zealand manuka (*Leptospermum scoparium*) honey.

Sample	UMF	Concentration of flavonoids (mg/100g of honey) ^a																
		PB	UN01	F01	Q	L	F02	8MK	PC	F03	F04	IRM	K	F05	C	G	F06	Total
ACT5+	7.7	0.343	0.109	0.058	0.000	0.114	0.037	0.013	0.200	0.013	0.016	0.020	0.017	0.006	0.102	0.027	0.009	1.083
UMF10	11.1	0.313	0.152	0.065	0.020	0.130	0.036	0.017	0.213	0.013	0.029	0.026	0.019	0.009	0.118	0.052	0.016	1.229
UMF15	15.8	0.365	0.126	0.071	0.031	0.163	0.040	0.025	0.206	0.023	0.046	0.030	0.038	0.014	0.207	0.083	0.014	1.482
UMF20	17.1	0.392	0.240	0.062	0.115	0.138	0.042	0.004	0.231	0.018	0.092	0.034	0.146	0.012	0.442	0.051	0.016	2.036
UMF25	22.9	0.224	0.205	0.043	0.040	0.169	0.028	0.008	0.128	0.023	0.036	0.030	0.072	0.005	0.246	0.020	0.007	1.285
UMF30	27	0.200	0.279	0.039	0.016	0.147	0.015	0.010	0.112	0.013	0.016	0.016	0.012	0.006	0.081	0.021	0.008	0.990
BD12.5	13.8	0.585	0.420	0.094	0.037	0.268	0.040	0.036	0.337	0.030	0.044	0.042	0.027	0.016	0.168	0.072	0.020	2.235
BD15.2	17.7	0.297	0.188	0.039	0.049	0.121	0.023	0.020	0.157	0.018	0.024	0.024	0.072	0.004	0.151	0.033	0.011	1.231
BD21.1	20	0.166	0.229	0.047	0.006	0.202	0.027	0.010	0.129	0.015	0.020	0.017	0.013	0.008	0.081	0.038	0.013	1.021
BD28.8	27.3	0.144	0.254	0.058	0.002	0.111	0.022	0.014	0.137	0.010	0.016	0.016	0.006	0.012	0.086	0.023	0.011	0.923
M01	25.3	0.062	0.130	0.027	0.013	0.110	0.015	0.005	0.070	0.007	0.018	0.009	0.007	0.004	0.075	0.034	0.008	0.594
M03	23.6	0.262	0.232	0.075	0.014	0.334	0.062	0.019	0.227	0.020	0.040	0.025	0.022	0.020	0.168	0.078	0.024	1.622
M05	17.5	0.279	0.183	0.068	0.020	0.160	0.033	0.009	0.146	0.023	0.032	0.028	0.021	0.006	0.103	0.043	0.008	1.162
M09	17.7	0.240	0.138	0.082	0.043	0.170	0.043	0.016	0.127	0.020	0.042	0.017	0.016	0.003	0.070	0.030	0.005	1.062
M10	13.9	0.310	0.274	0.066	0.051	0.172	0.045	0.016	0.197	0.018	0.037	0.031	0.019	0.010	0.104	0.053	0.013	1.417
M11	14.8	0.254	0.291	0.064	0.000	0.133	0.047	0.011	0.191	0.015	0.029	0.023	0.016	0.009	0.090	0.042	0.010	1.225
M12	14.9	0.197	0.123	0.048	0.010	0.174	0.058	0.000	0.142	0.007	0.025	0.015	0.012	0.009	0.082	0.047	0.011	0.960
M14	10.9	0.141	0.088	0.060	0.012	0.051	0.026	0.005	0.110	0.006	0.017	0.009	0.006	0.003	0.043	0.016	0.006	0.599
M15	15.3	0.125	0.158	0.067	0.035	0.211	0.035	0.015	0.102	0.012	0.022	0.013	0.016	0.003	0.052	0.021	0.005	0.894
M17	14.2	0.273	0.105	0.112	0.000	0.082	0.063	0.000	0.176	0.000	0.012	0.013	0.000	0.003	0.038	0.017	0.005	0.899
M18	14.2	0.336	0.092	0.129	0.000	0.092	0.098	0.009	0.312	0.017	0.017	0.025	0.000	0.010	0.099	0.054	0.011	1.302
M20	1	0.340	0.039	0.034	0.017	0.029	0.018	0.083	0.239	0.010	0.017	0.017	0.011	0.003	0.051	0.016	0.004	0.927
M21	9.2	0.291	0.073	0.081	0.000	0.047	0.029	0.004	0.186	0.000	0.010	0.014	0.000	0.006	0.047	0.017	0.008	0.813

Continued on page 78.

Table 3.7 (Continued from page 77): The flavonoid content of New Zealand manuka (*Leptospermum scoparium*) honey.

Sample	UMF	Concentration of flavonoids (mg/100g of honey) ^a																
		PB	UN01	F01	Q	L	F02	8MK	PC	F03	F04	IRM	K	F05	C	G	F06	Total
M22	8.3	0.235	0.075	0.075	0.000	0.051	0.017	0.000	0.164	0.000	0.008	0.014	0.000	0.007	0.046	0.015	0.005	0.711
M23	9.3	0.211	0.087	0.052	0.000	0.064	0.010	0.002	0.129	0.006	0.008	0.011	0.002	0.003	0.032	0.011	0.004	0.631
M26	7.3	0.325	0.004	0.056	0.017	0.128	0.023	0.007	0.185	0.014	0.018	0.017	0.016	0.004	0.064	0.022	0.004	0.904
M27	7.3	0.276	0.063	0.054	0.000	0.171	0.050	0.011	0.248	0.011	0.026	0.024	0.021	0.016	0.133	0.054	0.018	1.178
M29	8	0.284	0.081	0.059	0.000	0.048	0.026	0.007	0.177	0.005	0.012	0.015	0.031	0.005	0.060	0.022	0.005	0.837
M32	5.9	0.269	0.059	0.057	0.000	0.098	0.057	0.008	0.181	0.007	0.009	0.015	0.004	0.007	0.048	0.018	0.003	0.841
M34	4.5	0.309	0.051	0.044	0.000	0.131	0.061	0.003	0.018	0.004	0.012	0.021	0.008	0.005	0.063	0.020	0.004	0.754
NCG		0.264	0.064	0.049	0.000	0.144	0.045	0.005	0.198	0.007	0.016	0.019	0.021	0.004	0.061	0.024	0.005	0.926
Mean		0.267	0.157	0.061	0.024	0.136	0.038	0.012	0.174	0.013	0.029	0.022	0.032	0.008	0.131	0.035	0.010	1.155
S.D. ^b		0.098	0.096	0.021	0.043	0.062	0.018	0.014	0.064	0.008	0.029	0.009	0.051	0.005	0.132	0.020	0.005	0.461
95% C.I. ^c		0.036	0.034	0.008	0.015	0.022	0.006	0.005	0.023	0.003	0.010	0.003	0.018	0.002	0.047	0.007	0.002	0.164
C.V. (%) ^d		36.9	61.3	34.7	175.8	45.7	47.4	120.7	36.6	60.8	99.5	43.3	161.5	60.3	100.4	56.4	56.8	39.9
% ^e		23.1	13.6	5.3	2.1	11.8	3.3	1.0	15.1	1.2	2.5	1.9	2.7	0.7	11.4	3.0	0.8	

^a PB – pinobanksin (3,5,7-trihydroxyflavanone), UN01 – unknown compound 01, F01 – unknown flavonoid 01, Q – quercetin (3,5,7,3',4'-pentahydroxyflavone), L – luteolin (5,7,3',4'-tetrahydroxyflavone), F02 – unknown flavonoid 02, 8MK – 8-methoxykaempferol (3,5,7,4'-tetrahydroxy-8-methoxyflavone), PC – pinocembrin (5,7-dihydroxyflavone), F03 – unknown flavonoid 03, IRM – isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), F04 – unknown flavonoid 04, K – kaempferol (3,5,7,4'-tetrahydroxyflavone), F05 – unknown flavonoid 05, C – chrysin (5,7-dihydroxyflavone), G – galangin (3,5,7-trihydroxyflavone), F06 – unknown flavonoid 06.

^b Standard deviation. ^c 95% confidence interval from 31 determinations.

^d Coefficient of variation (standard deviation/mean x 100%).

^e Individual flavonoid level as a percentage of total flavonoid content.

The flavonoid profile of the manuka honeys studied mainly consisted of pinobanksin ($0.27 \pm 0.04\text{mg}/100\text{g}$ honey), pinocembrin ($0.17 \pm 0.02\text{mg}/100\text{g}$ honey), luteolin ($0.14 \pm 0.02\text{mg}/100\text{g}$ honey) and chrysin ($0.13 \pm 0.02\text{mg}/100\text{g}$ honey). Together these main flavonoids represent 61% of the total flavonoid content ($1.16 \pm 0.16\text{mg}/100\text{g}$ honey). Unknown compound 01, while not a flavonoid, was also found at relatively high levels, representing an average of 14% of the total flavonoid content. Eleven other flavonoids including quercetin, 8-methoxykaempferol, isorhamnetin, kaempferol, galangin and the unknown flavonoids 01-06, occurred at lower levels in the manuka honeys studied. Chromatograms of a manuka honey sample (at 340nm and 290nm) are shown in figures 3.9 and 3.10. Expanded views of the regions where flavonoids eluted are shown in figure 3.11. The large unresolved peaks seen in the 290nm chromatogram at 22 and 26 minutes are phenolic acid derivatives. These were too concentrated in the extracts studied so no attempt was made to quantify them.

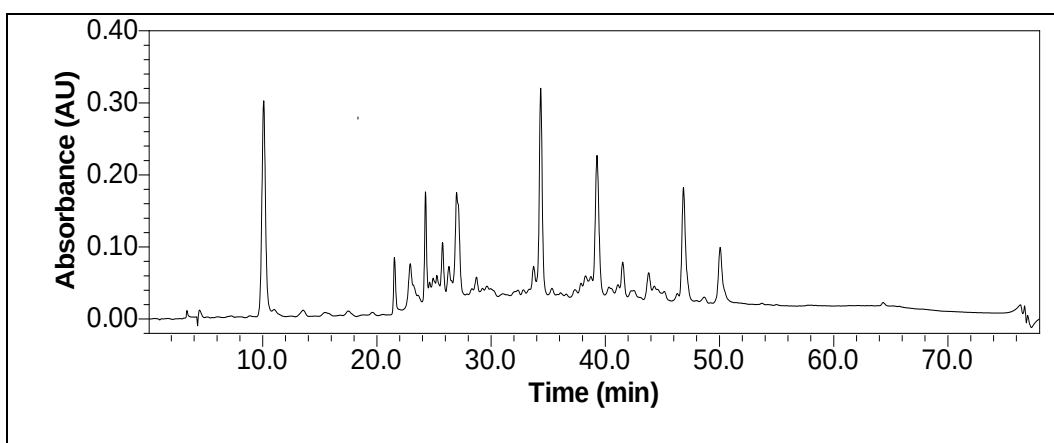


Figure 3.9: The full chromatogram of the M01 manuka honey sample, measured at 340nm.

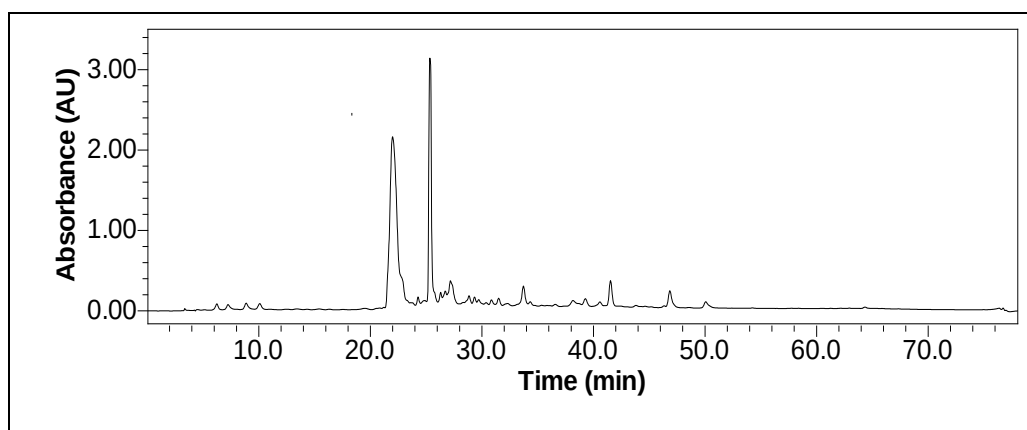
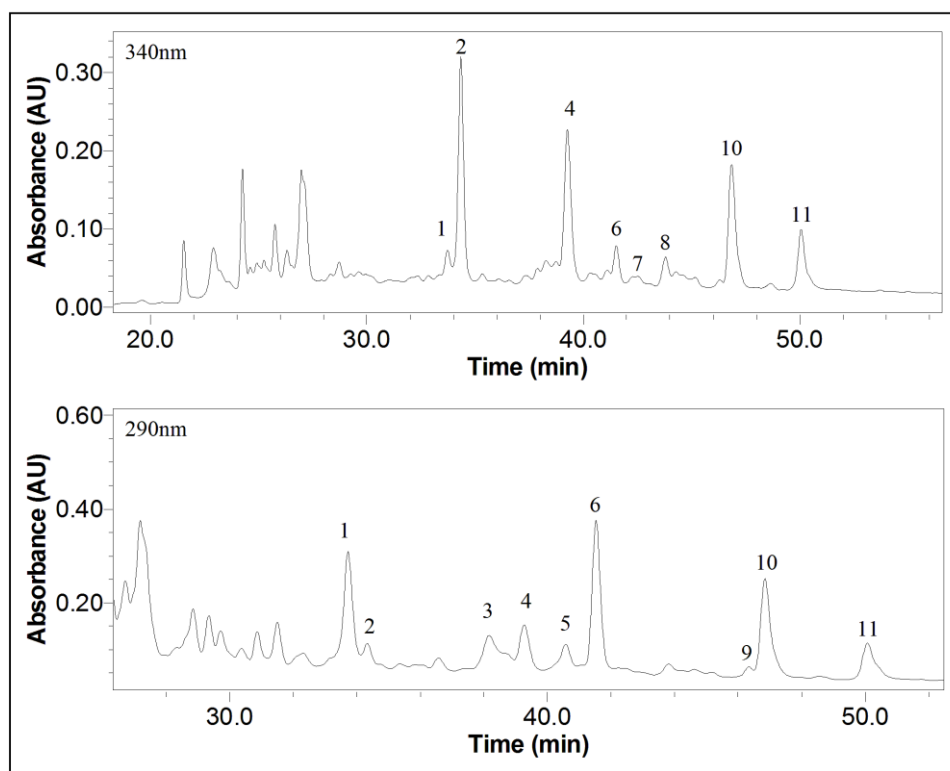


Figure 3.10: The full chromatogram of the M01 manuka honey sample, measured at 290nm.



Flavonoids are: (1) pinobanksin, (2) unknown compound 01, (3) unknown flavonoid 01, (4) luteolin, (5) unknown flavonoid 02, (6) pinocembrin, (7) unknown flavonoid 03, (8) unknown flavonoid 04, (9) unknown flavonoid 05, (10) chrysin and (11) galangin.

Figure 3.11: Expanded views of regions of the manuka honey extract chromatogram with flavonoids labelled.

The flavonoid results show similarities and differences when compared to previous studies of the flavonoid profile of manuka honey. Weston *et al.*^{23, 121} found only the propolis derived flavonoids pinocembrin, pinobanksin, chrysin and galangin in a study of 19 manuka honeys. All these flavonoids were identified in the 31 manuka honeys analysed in the present study. The present study has found eleven flavonoids in manuka honey which were not observed by Weston *et al.*^{23, 121}

As explained earlier, the study by Weston *et al.*^{23, 121} under-reported the levels of flavonoids in manuka honey due to problems with the extraction method used. The results of the present study show a stronger resemblance to those of Yao *et al.*²⁴ which identified quercetin (13.8%), isorhamnetin (12.9%), an unknown flavanone (12.7%), chrysin (12.6%) and luteolin (12.6%) as the main components of two manuka honey samples.²⁴ The present study has also identified chrysin and luteolin as two of the main components (after pinocembrin and pinobanksin). In contrast however, isorhamnetin and quercetin were only found at low levels in the present study. Pinobanksin, which was the main component of the manuka honey flavonoids in the present study, was not observed in the two manuka honey samples analysed by Yao *et al.*²⁴

A possible reason for the low levels of isorhamnetin and quercetin found in the present study is a low recovery rate of these flavonoids by the extraction method. It is interesting to note that, in this study, the flavones luteolin, and chrysin were found at levels over three times that of their flavonol counterparts, quercetin and galangin. It is possible that the presence or absence of a 3-hydroxyl group on the flavonoid has a significant effect on its recovery rate, with higher rates being achieved for those flavonoids without 3-hydroxyls. The results of the recovery rate tests described in Section 3.3.2 show a similar result with the flavone chrysin having slightly higher recovery rates (35-45%) than the flavonols quercetin (28-38%) and kaempferol (34-39%). When the experiment was designed it was thought that the oxygenation of the flavonoid B-ring would have a greater effect on the recovery rate since it is the oxidation pattern of the B-ring which has the greatest effect on HPLC retention times. This lead to a selection of standards with different levels of B-ring oxygenation for the recovery rate testing. In hindsight, it would have been useful to include luteolin and galangin in the recovery rate

tests. This would have allowed the effect of hydroxylation at C-3, on the recovery rate, to be measured by comparison with quercetin and chrysin respectively.

The high recovery rate observed for the flavanone pinocembrin (48-60%) also suggests that the oxidation level of the C-3 link of flavonoids may have an effect upon recovery rate. In the present study of 31 manuka honeys, a total of six unidentified flavonoids were found at mostly low levels. From UV-spectra recorded on the photodiode array HPLC detector, it was found that three of the unidentified flavonoids were either flavanones or dihydroflavonols. Of these two classes of flavonoids, only pinocembrin, pinobanksin and hesperetin have been previously identified in honeys. Pinocembrin and pinobanksin were identified as other peaks in the manuka honey chromatogram, and it is unlikely that hesperetin will be in manuka honey since it has been exclusively found in citrus honey, for which it is an established floral marker. The three unidentified flavanone and dihydroflavonols in the present study are therefore not likely to have been observed in honey before. It is possible that the modified extraction method used in this study preferentially extracted the flavanones and dihydroflavonols over the flavones and flavonols, the difference between these classes being the oxidation level of the C-3 link

In their study of two manuka honeys, Yao *et al.*²⁴ observed two unknown flavonoids which they could not identify. These two flavonoids had not been previously observed in any of the honeys they had profiled, except for Australian jelly bush (*Leptospermum polygalifolium*). The Australian jelly bush is closely related to the New Zealand manuka tree, with both species belonging to the *Leptospermum* genus. The research group of Tomas-Barberan are arguably the world leaders in honey phenolic profiling and have an extensive catalogue of honey flavonoids with which to compare their unknowns. The unknown flavonoids that they found in *Leptospermum* honeys therefore have great potential to be floral markers for these honeys. The UV absorption spectra presented for these unknowns²⁴ show the single Band I absorptions characteristic of the flavanone and dihydroflavonol classes of flavonoids.²⁴ Three of the unidentified flavonoids found in the present study show UV absorption spectra characteristic of these flavonoid classes. Flavanones and dihydroflavonols do not show the same variation in their UV absorption spectra as do the flavones and flavonols.

This, and the different HPLC conditions used in the two studies, makes it difficult to correlate the unknown flavonoids of the present study with those reported by Yao *et al.*²⁴

3.4.5 Comparison with Other New Zealand Honeys

For a comparison with manuka honey, a selection of eight other New Zealand honeys were analysed to determine flavonoid profiles. This selection included rata (*Metrosideros spp.*), rewarewa (*Knightia excelsa*), beech (*Nothofagus spp.*) honeydew, clover (*Trifolium spp.*), kamahi (*Weinmannia racemosa*), viper's bugloss (*Echium vulgare*), thyme (*Thymus vulgare*) and nodding thistle (*Carduus nutans*) honeys. The flavonoid profiles of these honey samples are shown in table 3.8.

Only one sample of each type was analysed so the results cannot be considered proof of unique markers for the non-manuka honey types. They do however serve as a useful comparison with manuka honey. With the exception of the viper's bugloss sample, all the honeys show a common flavonoid profile with manuka honey. The main components of this profile are pinobanksin and pinocembrin with a number of other flavonoids also being common to all the New Zealand honey types. Pinocembrin and pinobanksin were also found in viper's bugloss honey at significant levels but isorhamnetin and unknown flavonoid 04 were the main constituents.

Pollen analysis data was supplied with the non-manuka samples and is shown in table 3.9. The pollen analysis shows that, with the exception of the thyme honey, the non-manuka honeys studied were highly monofloral. This suggests that the common flavonoid profile observed in these honeys cannot be due to common nectar sources. It is more likely that the common flavonoid profile is derived from propolis. This is not surprising since pinocembrin and pinobanksin, along with chrysin, galangin and tectochrysin, have been reported as the main constituents of honeys produced in temperate areas of the Northern Hemisphere. These flavonoids accumulate in the bud exudates of poplars and are incorporated into honeys through propolis.¹⁰⁴ While *Populus* species are not native in New Zealand they have been imported and it appears that imported bee colonies seek

Table 3.8: The flavonoid content of some non-manuka New Zealand honeys.

Honey Type	Concentration of flavonoids (mg/100g of honey) ^a																
	PB	UN01	F01	Q	L	F02	8MK	PC	F03	F04	IRM	K	F05	C	G	F06	Total
Rata	0.027	0.009	0.006		0.017	0.006		0.042		0.002	0.004		0.005	0.025	0.005	0.005	0.156
Rew arew a	0.239	0.008	0.012	0.007	0.004	0.017	0.013	0.189	0.009	0.013	0.018	0.009	0.004	0.061	0.024	0.008	0.656
Honeydew	0.065	0.036	0.012		0.023	0.002	0.020	0.066		0.009	0.013	0.004	0.002	0.016	0.016		0.291
Clover	0.214		0.039			0.059	0.016	0.246		0.017	0.019	0.010	0.004	0.047	0.013	0.006	0.705
Kamahi	0.029	0.001	0.006		0.016	0.011		0.045		0.004	0.009		0.002	0.027	0.004	0.002	0.155
Vipers Bugloss	0.028	0.005	0.006	0.002	0.010	0.012	0.009	0.030	0.008	0.044	0.094	0.010	0.006	0.016	0.010	0.004	0.298
Thyme	0.127		0.004	0.019	0.015	0.007	0.023	0.070		0.031	0.086	0.021	0.004	0.031	0.011	0.005	0.488
Nodding Thistle	0.297		0.035		0.059	0.068	0.020	0.262	0.013	0.148	0.000	0.019	0.005	0.068	0.022	0.006	1.044
Manuka Honey Mean ^b	0.267	0.157	0.061	0.024	0.136	0.038	0.012	0.174	0.013	0.029	0.022	0.032	0.008	0.131	0.035	0.010	1.155
95% C.I. ^c	0.036	0.034	0.008	0.015	0.022	0.006	0.005	0.023	0.003	0.010	0.003	0.018	0.002	0.047	0.007	0.002	0.164

^a PB – pinobanksin (3,5,7-trihydroxyflavanone), UN01 – unknown compound 01, F01 – unknown flavonoid 01, Q – quercetin (3,5,7,3',4'-pentahydroxyflavone), L – luteolin (5,7,3',4'-tetrahydroxyflavone), F02 – unknown flavonoid 02, 8MK – 8-methoxykaempferol (3,5,7,4'-tetrahydroxy-8-methoxyflavone), PC – pinocembrin (5,7-dihydroxyflavone), F03 – unknown flavonoid 03, IRM – isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), F04 – unknown flavonoid 04, K – kaempferol (3,5,7,4'-tetrahydroxyflavone), F05 – unknown flavonoid 05, C – chrysin (5,7-dihydroxyflavone), G – galangin (3,5,7-trihydroxyflavone), F06 – unknown flavonoid 06.

^b Mean individual flavonoid level in manuka honey. ^c 95% confidence interval, from 31 determinations, of the individual flavonoid level in manuka honey.

out these exotic trees as a source of resinous material for producing propolis.¹⁰³ A study by Markham *et al.* identified pinobanksin, pinocembrin, chrysin, galangin, pinobanksin-3-acetate, pinocembrin-7-methyl ether, chrysin -7-methyl ether and galangin-7-methyl ether as the flavonoids in New Zealand propolis collected from beehives.¹²³ The results of the present study indicate that propolis derived flavonoids are a common feature of all the New Zealand honeys studied.

Table 3.9: Pollen analysis data for the non-manuka samples profiled. The honeydew honey did not contain pollen and no data was available for the nodding thistle honey.

Honey Type	% of Total Pollen Count From Nominal Floral Source
Rata	79.1
Rewarewa	38.3
Clover	87.5
Kamahi	85.1
Viper's Bugloss	87
Thyme	18

Despite the common propolis-derived flavonoids of all the honey types studied, it may still be possible to distinguish manuka honey from other New Zealand honeys. The total flavonoid content of manuka honey was relatively high at 1.16 ± 0.16 mg/100g of honey. Of the other honey types studied only the nodding thistle honey sample had a similar total flavonoid content of 1.04 mg/100g of honey. The other honey samples contained total flavonoid contents between 0.71 and 0.16 mg/100g of honey. These apparent differences in total flavonoid content could be used, in conjunction with other results, to distinguish manuka honey from other types of honey.

While luteolin was present in seven of the eight non-manuka honey types studied, the levels were lower than half the average luteolin content found in manuka honey

($0.136 \pm 0.022\text{mg}/100\text{g}$ honey). The unknown compound 01 is also a potential marker for manuka honey. It was found in five of the other honey types studied but at levels less than $0.04\text{mg}/100\text{g}$ of honey. In contrast, manuka honey had an average unknown compound 01 content of $0.16 \pm 0.03\text{mg}/100\text{g}$ honey.

Luteolin (in conjunction with other flavonoids) has been previously identified as part of unique flavonoid profiles for Australian *Eucalyptus* honeys and several other Australian honey types.^{24, 105, 114, 115} Yao *et al.*²⁴ have suggested that Australasian *Leptospermum* honeys may be distinguished from these other honeys by the lack of tricetin in their flavonoid profile and the presence of an unknown flavanone instead.²⁴

The presence of high levels of luteolin and unknown compound 01, along with a high total flavonoid content are potential marker compounds for manuka honey. Further investigation is needed to identify unknown compound 01 before it can be confirmed as a marker for manuka honey.

In addition to the six unknown flavonoids found in manuka honey, a further seven were found in the non-manuka honey samples which did not coincide with any of the standards available. Table 3.10 shows the levels of these six unknown flavonoids in non-manuka honeys. These flavonoids were not observed in the manuka honeys studied but may still have been present at low levels. The chromatogram of the phenolic extract of manuka honey was complex and had large levels of phenolic acids which hindered the analysis of flavonoids eluting early in the flavonoid profile. Unknown flavonoids 07-09 in particular would have eluted in this region so it is possible that they were in manuka honey but were not detected under the noise of this region. The flavonoid profile of these other New Zealand honey types should be investigated using a larger number of samples to determine if they are potential floral markers.

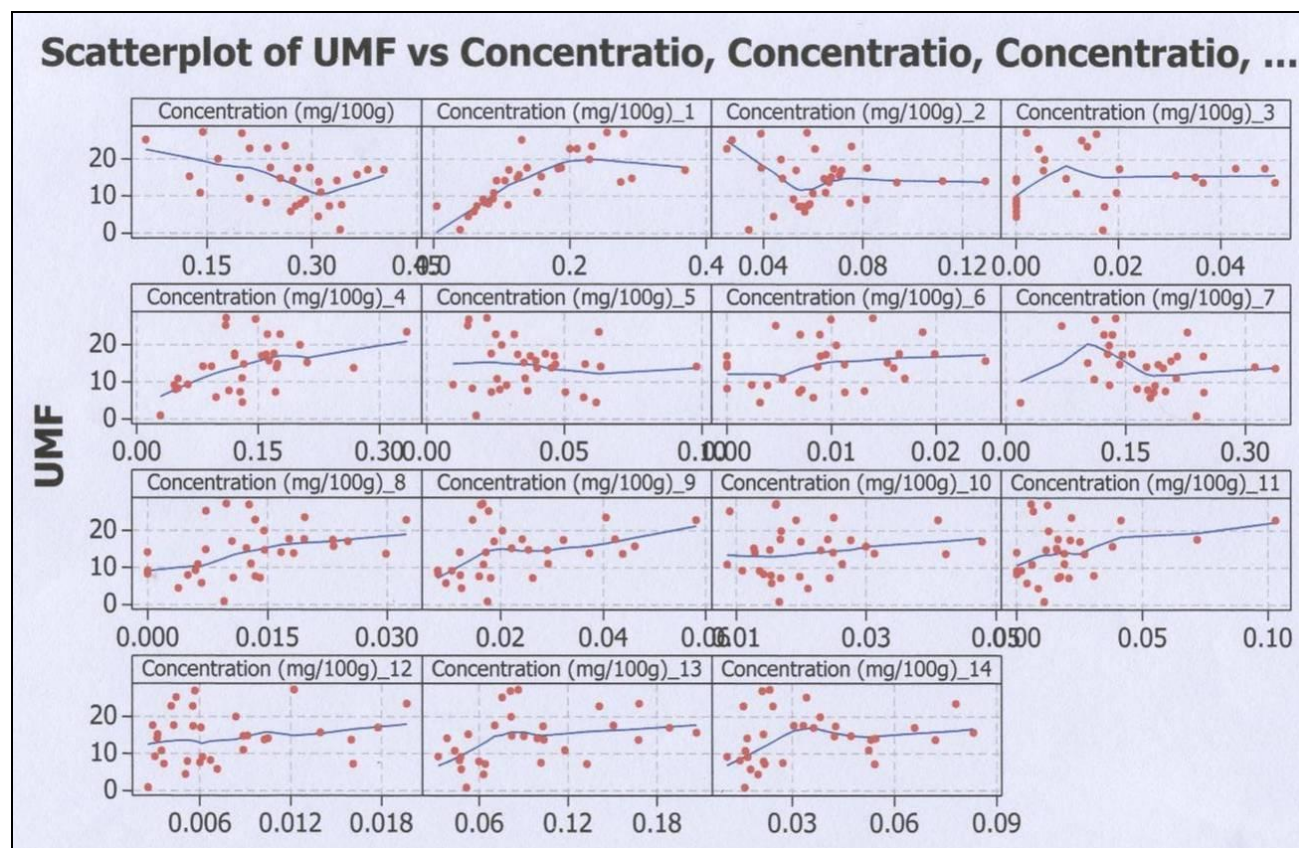
Table 3.10: The levels of some unknown flavonoids found in non-manuka New Zealand honeys.

Honey Type	Concentration of flavonoids (mg/100g of honey) ^a						
	F08	F08	F09	F10	F11	F12	F13
Rata			0.002				
Rew arew a		0.022					
Honeydew		0.007					
Clover		0.006	0.008				
Kamahi					0.002		
Vipers Bugloss				0.004		0.005	0.004
Thyme	0.024	0.008	0.002				
Nodding Thistle	0.003	0.011	0.008				

3.4.6 The Relationship of Flavonoid Profile with the UMF

Antibacterial Activity of Manuka Honey

As well as investigating the flavonoid profile of manuka honey for potential floral markers, there was also an interest in determining if there was any relationship between flavonoid profile and non-peroxide antibacterial activity. This source of this activity has previously been identified as high levels of methylglyoxal.^{68, 70} It has recently been reported that the methylglyoxal in manuka honey is formed from dihydroxyacetone which has been traced to the nectar of manuka flowers.¹²⁴ It has been known for some time that the dilution of manuka honey with nectar from other sources proportionally decreases the antibacterial activity. A large range of *L. scoparium* varieties exist and it has been postulated that the nectar of these varieties produce different levels of antibacterial activity in honeys derived from them.¹²⁵ It is feasible that any manuka nectar derived flavonoids in manuka honey will show a positive correlation with antibacterial activity. Statistical analysis was used to compare the flavonoid profiles of the manuka honeys to their UMF non-peroxide antibacterial activity. Scatter plots of UMF versus the concentration of individual manuka honey flavonoids are shown in figure 3.12



Top row (left to right): pinobanksin, unknown compound 01, unknown flavonoid 01, quercetin.

Second row (left to right): luteolin, unknown flavonoid 02, 8-methoxykaempferol, pinocembrin.

Third row (left to right): unknown flavonoid 03, unknown flavonoid 04, isorhamnetin, kaempferol.

Bottom row (left to right): unknown flavonoid 05, chrysin, galangin.

Figure 3.12: Scatter plots of UMF non-peroxide antibacterial activity versus concentrations of individual manuka honey flavonoids.

The best relationship was observed between UMF and the levels of unknown compound 01. Figure 3.13 shows an enlarged scatter plot of UMF versus unknown compound 01 concentration. A straight line of best fit applied to the data appeared to go through the 20% uncertainty limits of most of the data points. This result suggests that there may be a relationship between UMF and unknown compound 01. Future work should focus on identifying unknown compound 01.

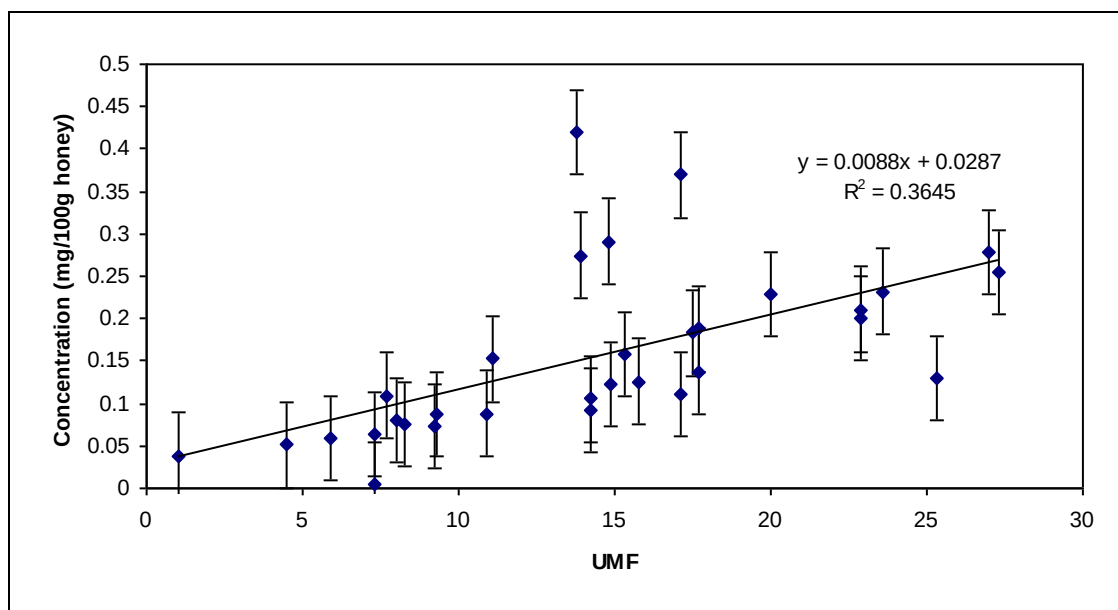


Figure 3.13: Scatter plot of unknown compound 01 concentration versus UMF non-peroxide antibacterial activity.

Luteolin also showed a potential linear relationship with UMF. This is illustrated in figure 3.14 which shows luteolin concentration versus UMF non-peroxide activity. The linear relationship between these variables is not as strong as that for unknown compound 01 (R^2 of 0.2268 compared with an R^2 of 0.3645 for unknown compound 01) but the line of best fit still passes within the uncertainty limits of most results.

These linear relationships support the identification of luteolin and unknown compound 01 as marker compounds for manuka honey. UMF non-peroxide antibacterial activity is largely restricted to manuka honey (some viper's bugloss honeys also possess this activity however). The recent discovery that this activity originates from dihydroxyacetone in the nectar of manuka flowers¹²⁴ suggests that

higher incorporation of manuka nectar into manuka honey may cause higher levels of antibacterial activity. As a consequence of this, it can be expected that flavonoids derived from manuka nectar will also be concentrated in highly active honeys.

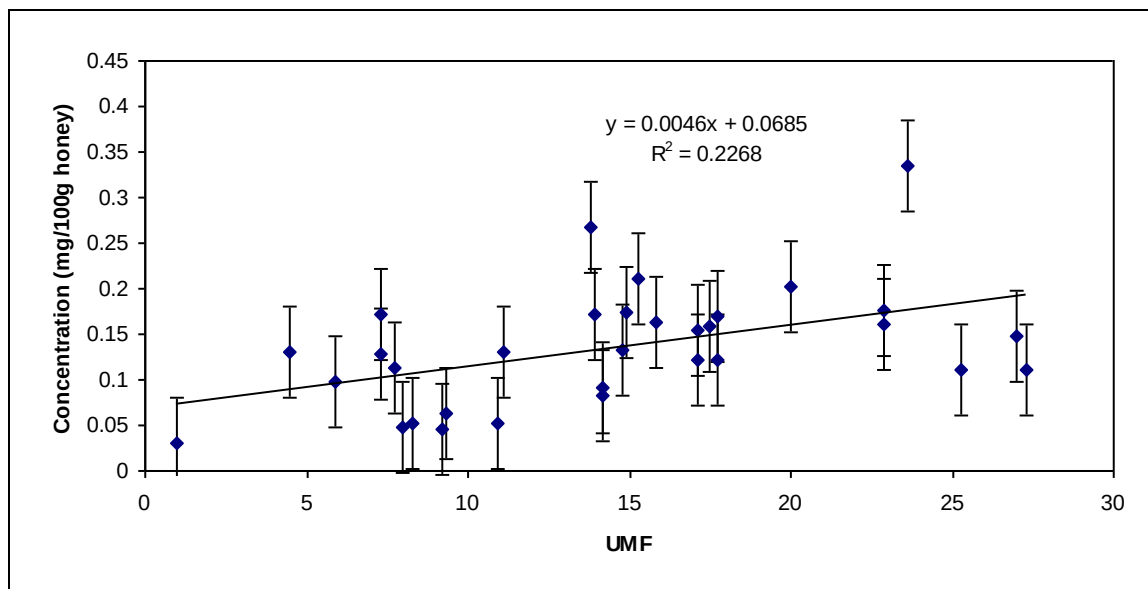


Figure 3.14: Scatter plot of luteolin concentration versus UMF non-peroxide antibacterial activity.

3.5 Isolation of Unknown Flavonoids

One of the problems which had to be overcome in this study was a limited range of authentic flavonoid standards. These standards were needed to identify the flavonoids in manuka honey by their co-elution and matching UV absorbance spectra in chromatograms of the honey phenolic extracts. A number of flavonoid peaks were identified in the HPLC chromatograms which did not coincide with the available authentic standards. To identify these unknown flavonoids it was necessary to isolate them as individual compounds and characterise them using the methods described in Sections 2.6 and 2.7.

Isolation of flavonoids proceeded with the extraction of phenolics from 15kg of manuka honey on Amberlite XAD-2 resin. The phenolic extract obtained from this procedure contained a large amount of insoluble material which was removed by extracting the phenolics from an aqueous solution with diethyl ether. This afforded an

extract containing flavonoids and phenolic acid derivatives, the phenolic acid derivatives being present in much greater quantities. Separation of the flavonoids from the phenolic acids was achieved by chromatography on Sephadex-LH20 resin. The phenolic acids eluted early, in a weak solvent while the flavonoids were concentrated in fractions 6 to 9. Figure 3.15 shows the chromatogram of fraction 8, recorded at 340nm on HPLC system B. It is clear that the fraction is largely free from the phenolics which elute in the first 30 minutes of the chromatography.

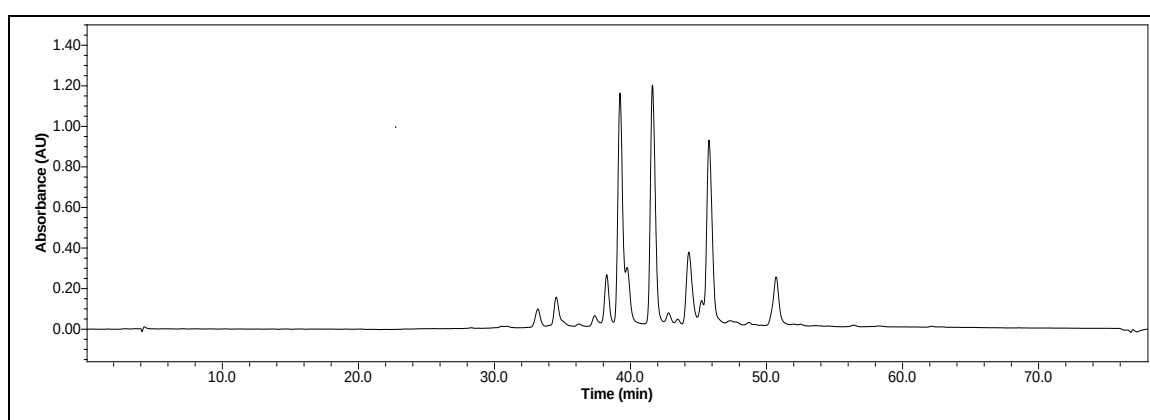


Figure 3.15: A HPLC chromatogram (340nm) of fraction 8.

While there are a large number of flavonoids in fractions 6 to 9, many more than were detected in the analytical study of manuka honey flavonoids, time limitations meant that only two peaks were isolated in sufficient quantity for characterisation.

Flavonoid A was obtained from fraction 7 by collecting the major peak eluting between 23.0-25.9min on the semi-prep HPLC system C (as shown in figure 3.16). This peak was further purified on HPLC system B where it eluted at 42.0 minutes. A chromatogram of the final purified flavonoid A is shown in figure 3.17.

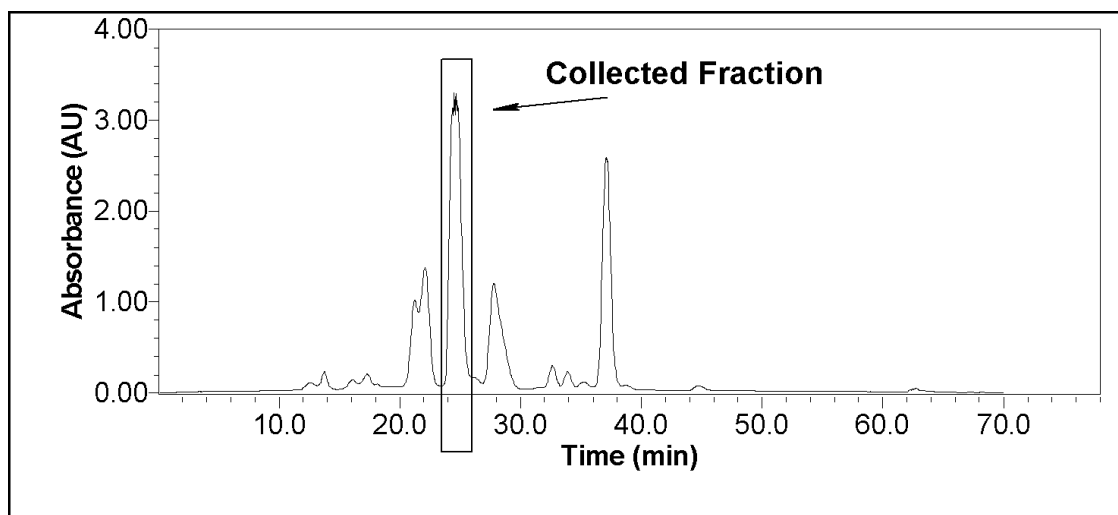


Figure 3.16: HPLC system C chromatogram of fraction 7 showing the collected fraction.

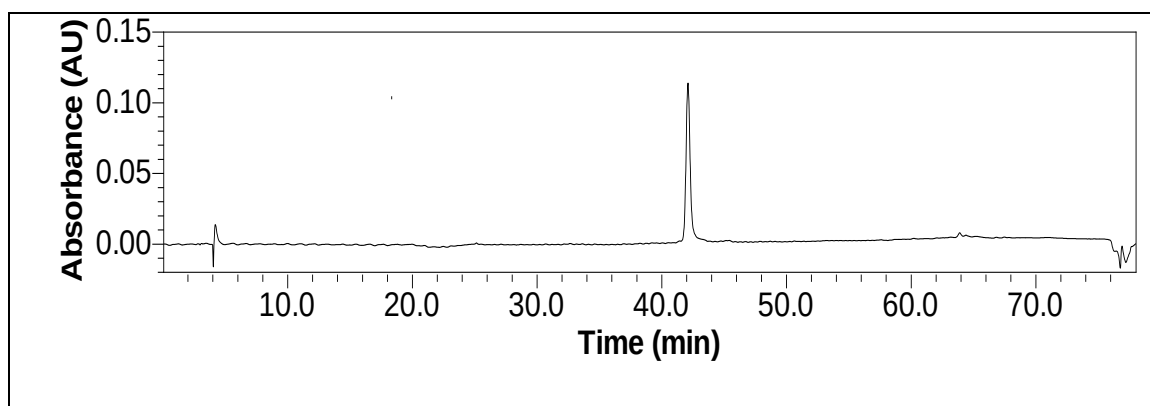


Figure 3.17: HPLC system B chromatogram of flavonoid A.

Flavonoid B was obtained from the 15.5-17.0min peak (on HPLC system C) of fraction 6. The collected fraction is shown in figure 3.18. As with flavonoid A, this flavonoid was also purified on HPLC system B. A chromatogram of the final purified flavonoid B, eluting at 34.8min is shown in figure 3.19.

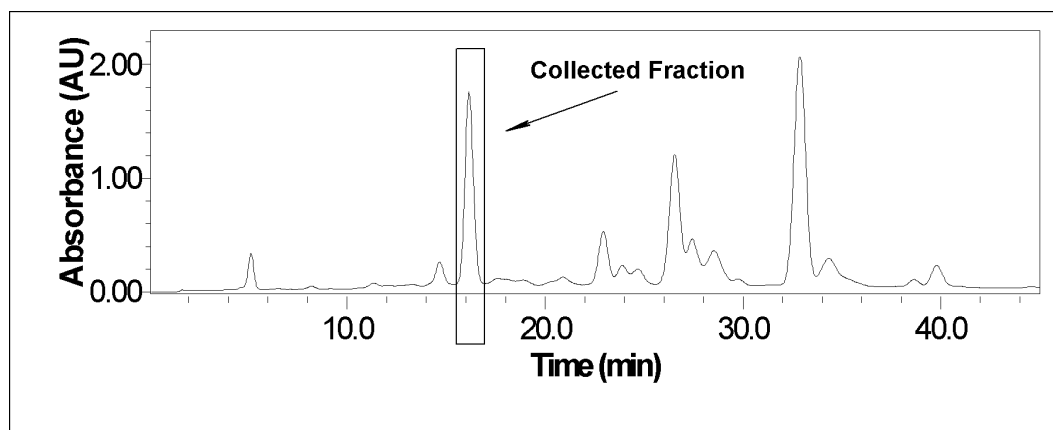


Figure 3.18: HPLC system C chromatogram of fraction 6 showing the collected fraction.

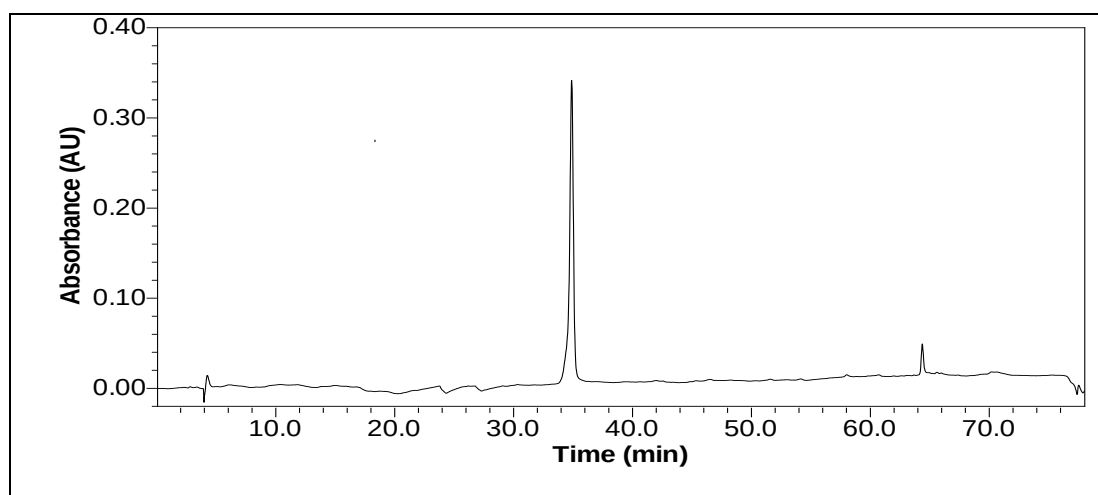


Figure 3.19: HPLC system B chromatogram of flavonoid B.

3.6 Characterisation of Unknown Flavonoids

The two flavonoids isolated in this study were characterised using a combination of UV-Visible absorption spectroscopy, NMR spectroscopy and LDI-TOF mass spectrometry.

3.6.7 Flavonoid A

3.6.7.1 UV-Visible Spectroscopy

The UV spectra of flavonoid A in methanol and after the addition of sodium methoxide are shown in figure 3.20. The methanol spectrum showed a Band II absorption at 271.1nm, a Band I absorption at 373.4nm and an additional minor absorption peak at 322.5nm. The presence of both Band I and Band II absorptions showed that flavonoid A belongs to the flavone or flavonol classes (rather than the flavanone or dihydroflavonol classes which have only Band II absorptions). The location of Band I in the region of 352-385nm is consistent with a flavonol rather than a flavone (Band I: 304-350nm).

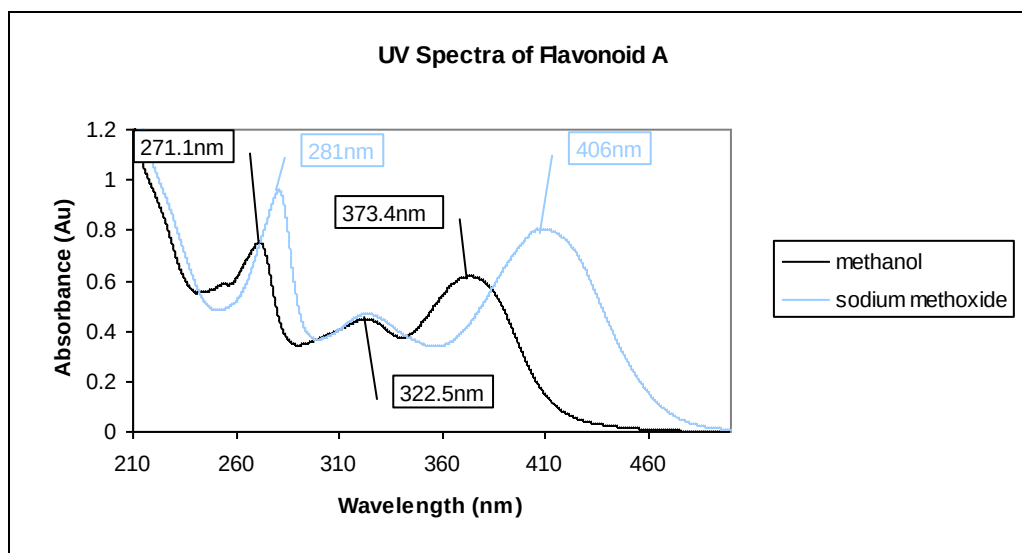


Figure 3.20: The UV-Visible spectra of flavonoid A in methanol, before and after the addition of sodium methoxide.

Further information about flavonoid A can also be gathered from its UV absorption spectrum in methanol. A summary of this information is as follows:

- Band II was a single peak without high wavelength shoulders. This suggested that the B-ring did not have a 3',4'- or 3',4',5'- oxygenation pattern.
- The long wavelength of the absorption bands suggests that flavonoid A is highly oxygenated and probably has a 5-hydroxyl.

Addition of sodium methoxide caused bathochromic shifts of Band I (32nm) and Band II (9nm) with an increase in intensity being observed for both peaks. The increased intensity of Band I was suggestive of a free 4'-hydroxyl, though the shift was smaller than expected (40-65nm). No decomposition was observed when the sodium methoxide spectrum was re-recorded after five minutes, showing that flavonoid A did not possess an oxygenation pattern unstable in alkali. Thus the presence of 3' and 4'-hydroxyls were ruled out.

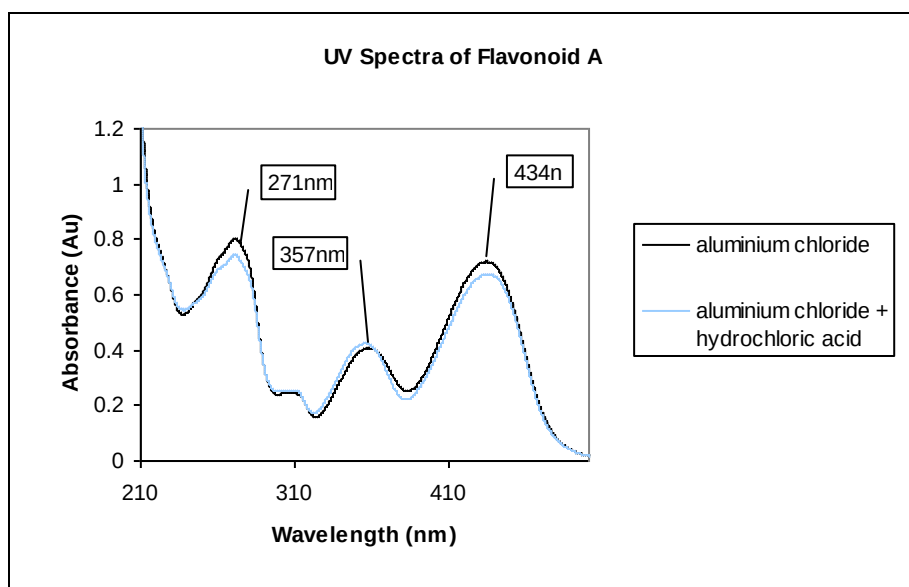


Figure 3.21: The UV-Visible spectra of flavonoid A in methanol, reacted with AlCl₃ and AlCl₃/HCl reagents.

The UV spectra of flavonoid A reacted with AlCl₃ is shown in figure 3.21. Addition of this reagent caused a bathochromic shift (60nm) of Band I to 434nm. This shift is due to the formation of aluminium complexes with *o*-dihydroxyl, 3-hydroxyl-4-keto and/or 5-hydroxyl-4-keto functionalities. Acidification with HCl did not change the spectra (also shown in figure 3.21) so it can be concluded that flavonoid A does not contain any *o*-dihydroxyl groups since the acid conditions disrupt only this type of complex. The large bathochromic shift remaining (60nm) was consistent with complexation of a 3-hydroxyl-4-keto group (50-60nm). This complex is formed preferentially to the 5-hydroxyl-4-keto complex so a 5-hydroxyl-4-keto group is still possible in flavonoid A.

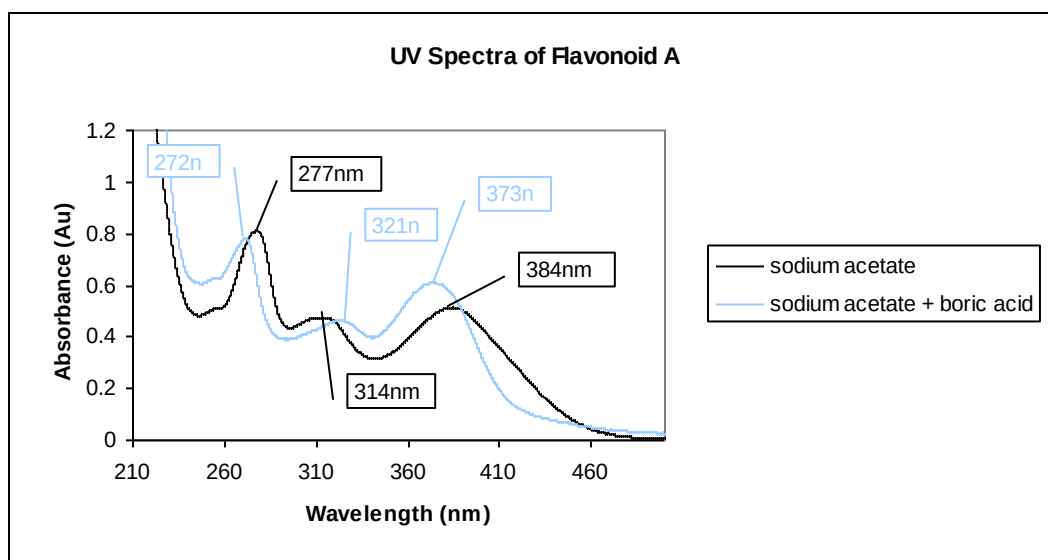


Figure 3.22: The UV-Visible spectra of flavonoid A in methanol, reacted with NaOAc and NaOAc/H₃BO₃ reagents.

The spectrum of flavonoid A reacted with sodium acetate is shown in figure 3.22. The bathochromic shift (6nm) of Band I to 277nm is consistent with a free 7-hydroxyl group (5-20nm shift). Addition of H₃BO₃ caused the spectrum to return to that of flavonoid A in methanol. This indicated that flavonoid A did not have any *o*-dihydroxyl groups, except possibly at the C-5,6 position which does not form complexes with H₃BO₃.

In summary, the UV spectral analysis yielded the following information about flavonoid A:

- Highly oxygenated, probably with hydroxyls at the 3, 5, 7 and 4' positions.
- No *o*-dihydroxyl groups, except possibly at the 5, 6 position.

3.6.7.2 ¹H NMR Spectroscopy

An assigned ¹H NMR spectrum of flavonoid A, in DMSO-*d*₆, is presented in figure 3.23. The spectrum is remarkably simple due to the small number of protons present in flavonoid A and the limited short range coupling between them. Table 3.11 shows the chemical shift, multiplicity, integral and relative integral of the signals belonging to flavonoid A.

Firstly, the doublet of doublets centred at 6.99 and 8.09ppm occur in the region of the spectrum usually associated with B-ring protons (6.7-7.9ppm). The doublet of doublet multiplicity pattern of these signals is consistent with a B-ring which is only substituted at the 4'-position. The ^3J coupling constant (9.2Hz) for the 6.99 and 8.09ppm doublets was the same for both signals confirming that they are coupling to each other. The constant compares well with the 8.5Hz coupling constant reported for flavonoids with a 4'-oxygenated B-ring.⁹³

Table 3.11: ^1H NMR signals of flavonoid A.

Label	Chemical Shift (ppm)	Multiplicity	Relative Integral
a	3.85	s	3
b	6.30	s	1
c	6.99	d	2
d	8.09	d	2
e-h	9.46	s	1
e-h	10.15	s	1
e-h	10.66	s	1
e-h	12.18	s	1

The large singlet at 3.85ppm is characteristic of a methoxyl group (3.5-4.1ppm) and the integral confirms that the proton environment producing this signal is a methyl group. The other important signal in this spectrum is the singlet at 6.30ppm which belongs to a proton on the A-ring of flavonoid A.

The lack of any long range W coupling in this signal is further proof that all but one of the A-ring positions are substituted. These results suggest a flavonoid with a highly substituted A-ring. The remaining flavonoid A signals belong to the hydroxyl protons. There are four such signals in the spectrum providing further confirmation (along with the methoxyl signal) that the flavonoid is highly substituted.

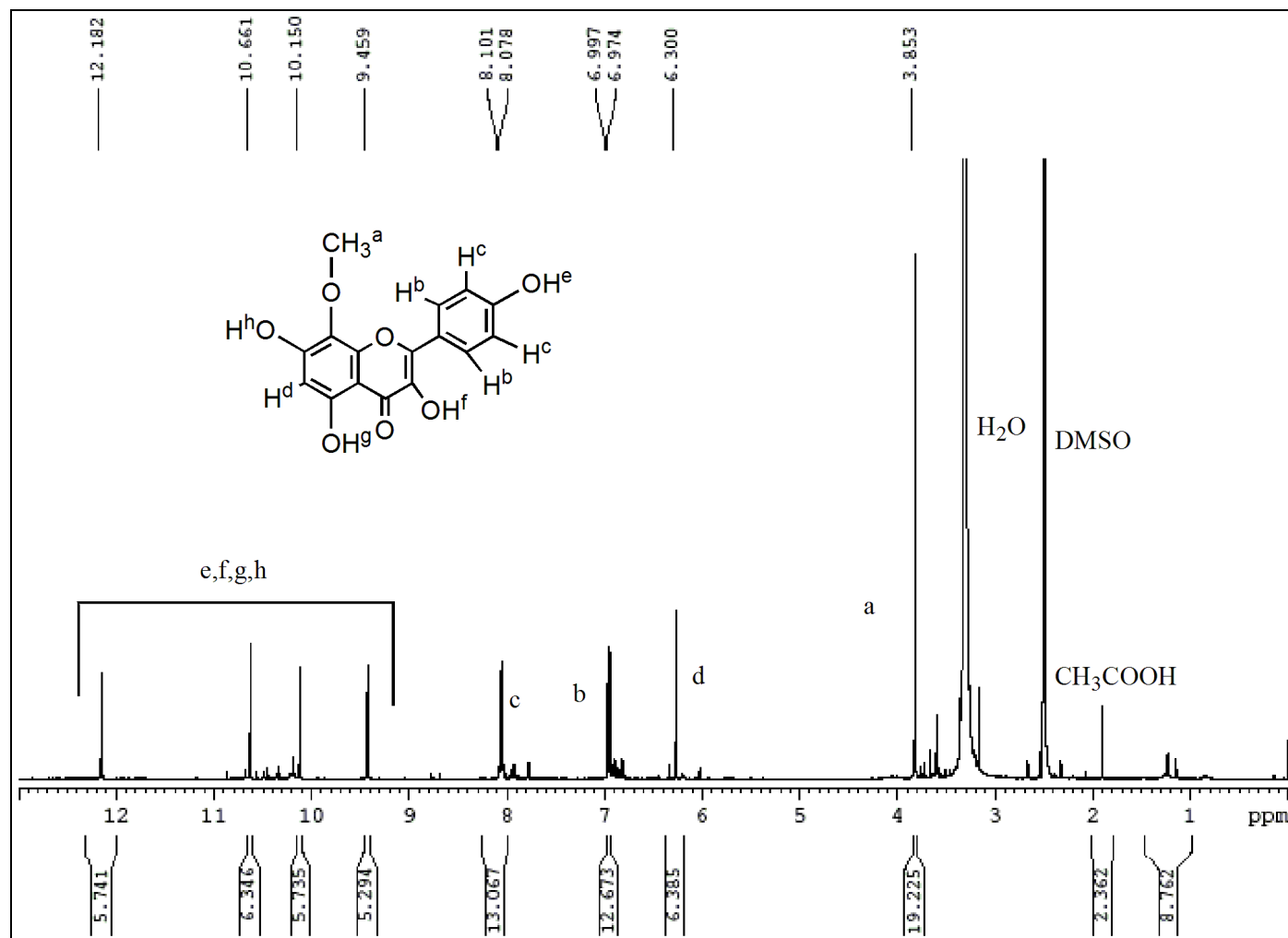


Figure 3.23: The ¹H NMR spectrum of flavonoid A in DMSO-*d*₆

3.6.7.3 LDI-TOF Mass Spectrometry

The highly conjugated nature of the flavonoids makes them ideal for mass spectral analysis by LDI-TOF. Their strong absorbance of UV light meant that a matrix was not required to acquire a mass spectrum of flavonoid A. The mass spectrum of flavonoid A produced a major ion with m/z of 315.079. This is consistent with a tetrahydroxy-monomethoxyflavone ($C_{16}H_{12}O_7$) which has a molecular mass of 316.06 and would produce an $[M-H]^-$ ion at m/z 315.06.

3.6.7.4 Final Structural Assignment of Flavonoid A

From the UV spectra, NMR spectrum and MS data it was deduced that flavonoid was a flavonol with a B-ring monosubstituted at the 4'-position; and a trisubstituted A-ring. Three of these substitutions were simple hydroxyl groups while the fourth was a methoxyl group. Since it was identified as a flavonol by UV analysis there was also a free 3-hydroxyl group.

UV analysis showed that flavonoid A did not contain any o-dihydroxyl groups, except possibly at the 5,6-position. It also showed that the flavonoid hydroxyls at the 5,7 and 4'-positions. The doublet of doublets in the NMR spectrum showed that the methoxyl was not on the B-ring and must therefore have been on the A-ring at either the 6 or 8-position. These results leave two possible structures for flavonoid A: 8-methoxykaempferol or 6-methoxykaempferol. Table 3.12 shows UV absorbance spectral maximums for these compounds which have reported previously.

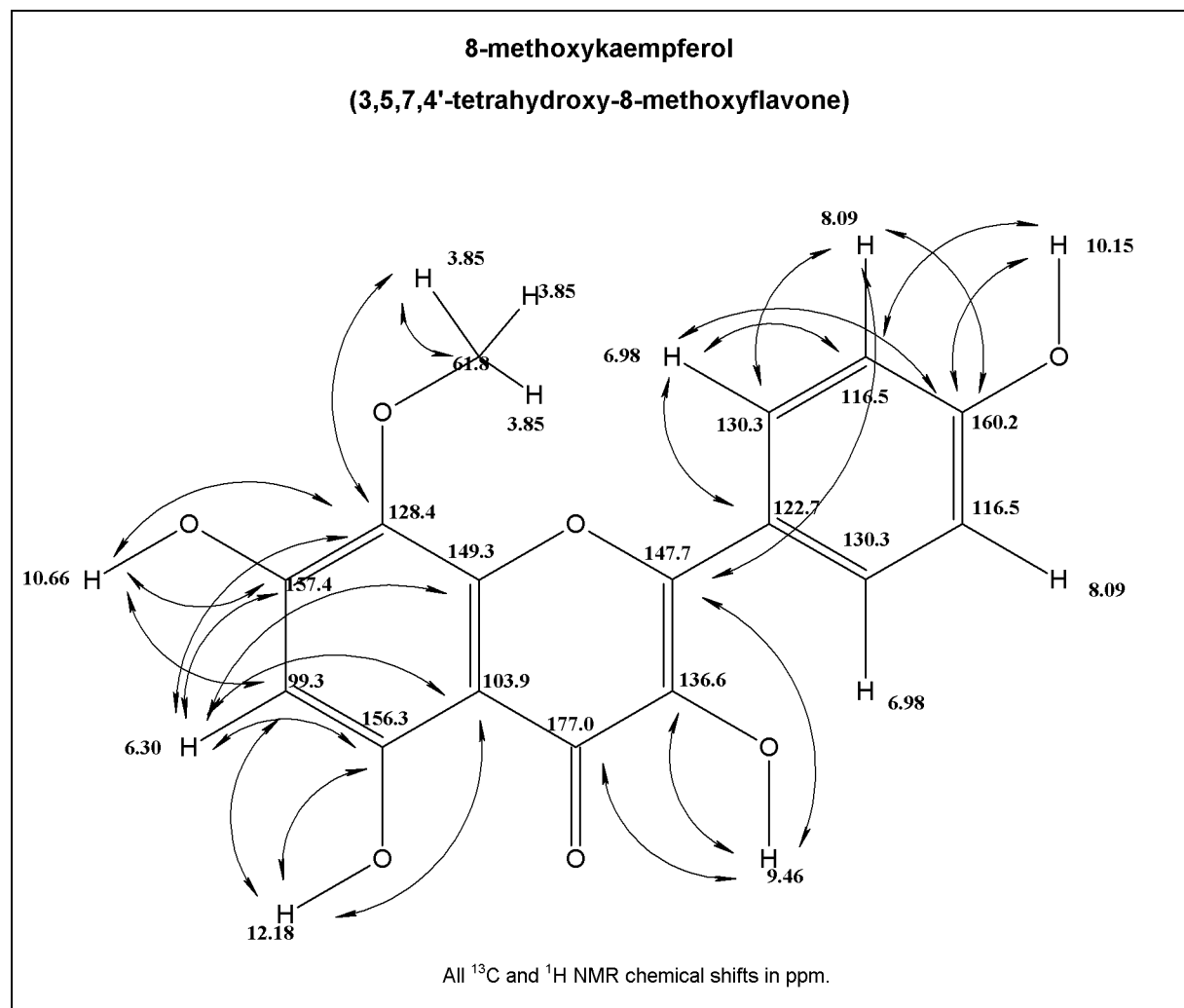


Figure 3.24: The ¹³C and ¹H NMR assignment with HMBC correlations, of 8-methoxykaempferol, the proposed identity of flavonoid A

Table 3.12: The UV absorption spectra of 6-methoxykaempferol, 8-methoxykaempferol and flavonoid A in methanol.

Absorption Maxima (nm)		
6-methoxykaempferol ¹²⁶	8-methoxykaempferol ⁹⁴	Flavonoid A
256sh	257sh	
269	274	271.0
	325	322.5
366	375	373.4

The UV spectrum of flavonoid A most closely resembles that of 8-methoxykaempferol but the three spectra are very similar and flavonoid A cannot be conclusively characterised. 8-methoxykaempferol has been previously identified in honeys, while 6-methoxykaempferol has not been reported in honey. Thus it would seem that identifying flavonoid A as 8-methoxykaempferol is a sensible conclusion. Further proof, in the form of a ¹³C NMR spectrum and HMBC correlation experiment, were obtained and confirmed that flavonoid B is 8-methoxykaempferol. The structure of 8-methoxykaempferol, along with the ¹³C and ¹H NMR shifts and HMBC correlations, is shown in figure 3.24.

3.6.8 Flavonoid B

3.6.8.1 UV Visible Spectroscopy

The UV spectra of flavonoid B in methanol and after the addition of sodium methoxide are shown in figure 3.25. The methanol spectrum showed a Band II absorption at 291.6nm, and no Band I absorption. The absence of a Band I absorption showed that flavonoid B belongs to the flavanone or dihydroflavonol classes (rather than the flavone or flavonol classes which have both Band I and Band II absorptions).

Addition of sodium methoxide caused a bathochromic shift (25nm) to 326nm with an increase in intensity being observed. The bathochromic shift on addition of sodium methoxide showed that the A-ring was hydroxylated. The small size of this shift (25nm) suggested that free hydroxyls were present at both the 5 and 7-positions (expect a 55-60nm shift if there is no free 5-hydroxyl). No decomposition was

observed so flavonoid B probably does not have an unstable 5,6,7 or 5,7,8-oxygenation pattern.

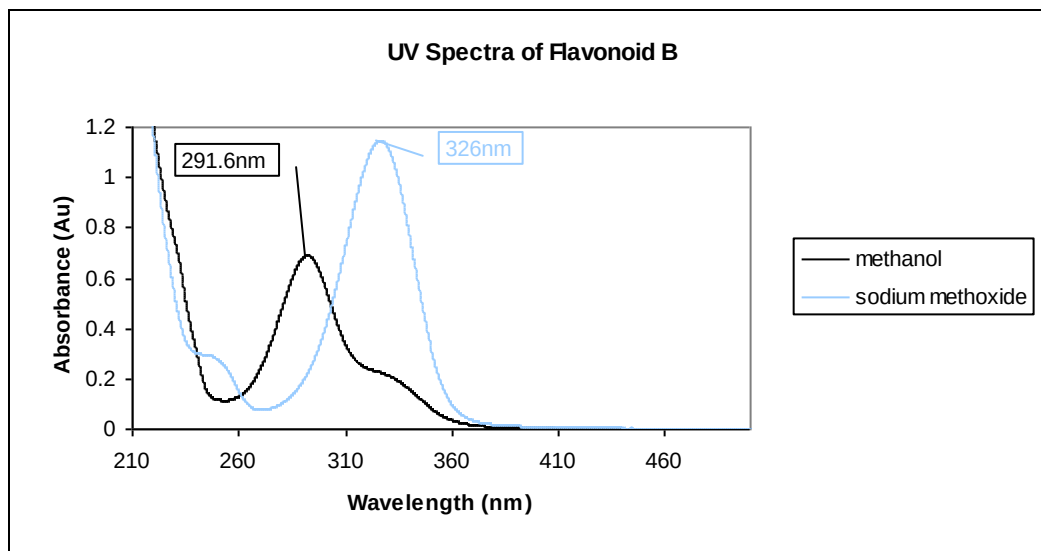


Figure 3.25: The UV-Visible spectra of flavonoid B in methanol, before and after the addition of sodium methoxide.

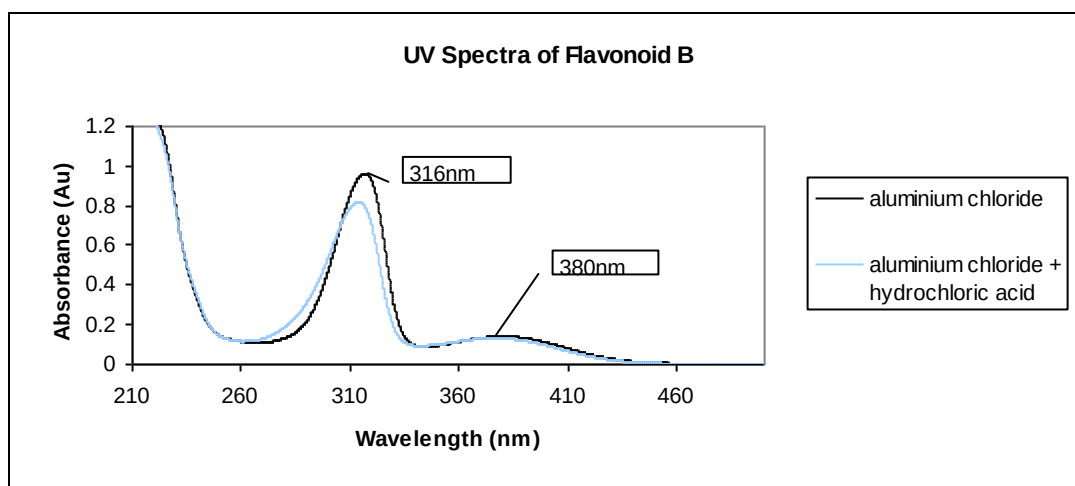


Figure 3.26: The UV-Visible spectra of flavonoid B in methanol, reacted with AlCl_3 and AlCl_3/HCl reagents.

The UV spectra of flavonoid B reacted with AlCl_3 are shown in figure 3.26. Addition of this reagent caused a bathochromic shift (24nm) of Band II to 316nm. This shift is

due to the formation of aluminium complexes with *o*-dihydroxyl, 3-hydroxyl-4-keto and/or 5-hydroxyl-4-keto functionalities in the A-ring. Acidification with HCl did not change the spectra (also shown in figure 3.26) so it can be concluded that flavonoid B does not contain any *o*-dihydroxyl groups in the A-ring since the acid conditions disrupt only this type of complex. The presence of B-ring *o*-dihydroxyl groups could not be determined since there is no Band-I absorption. The bathochromic shift remaining (25nm) was consistent with complexation of a 5-hydroxyl-4-keto group (20-26nm). The 3-hydroxyl-4-keto group complex is less stable than the 5-hydroxyl-4-keto group complex which is formed preferentially. A consequence of this is that the presence or absence of a 3-hydroxyl could not be determined by UV analysis.

The spectrum of flavonoid B reacted with sodium acetate is shown in figure 3.27. The bathochromic shift (34nm) of Band II to 326nm is consistent with free hydroxyls at both the 5 and 7-positions (34-37nm shift). Addition of H_3BO_3 caused the spectrum to return to that of flavonoid B in methanol. This indicated that flavonoid B did not have any *o*-dihydroxyl groups on the A-ring (except possibly at the 5,6-position). It is not possible to identify B-ring *o*-dihydroxyl groups in flavanones and dihydroflavonols by UV analysis.

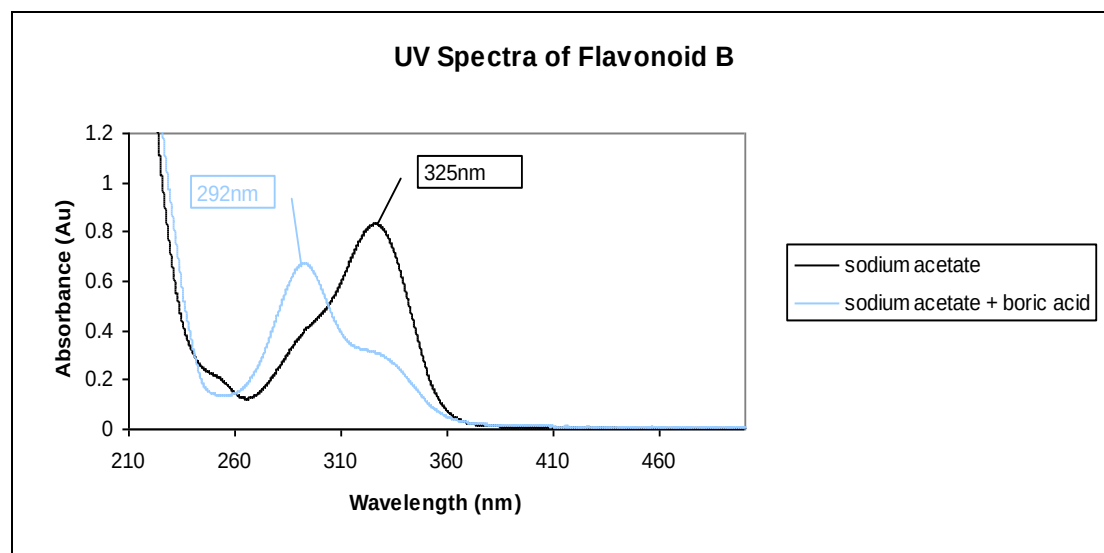


Figure 3.27: The UV-Visible spectra of flavonoid B in methanol, reacted with NaOAc and NaOAc/ H_3BO_3 reagents.

In summary, the UV spectral analysis yielded the following information about flavonoid B:

- Free hydroxyls at the 5 and 7-position.
- No *o*-dihydroxyl group at the 7,8-position and therefore no free 8-hydroxyl.

3.6.8.2 ^1H NMR Spectroscopy

An assigned ^1H NMR spectrum of flavonoid B, in $\text{DMSO-}d_6$, is presented in figure 3.28. Table 3.13 shows the chemical shift and multiplicity of the signals belonging to flavonoid B.

The two doublets centred at 4.58ppm and 5.14ppm was characteristic of the dihydroflavonol 3-H and 2-H protons. A flavanone in contrast would be expected to have a H-3 signal further upfield at about 2.8ppm. These doublets had matching ^3J coupling constants (11.2Hz) confirming that these doublets arise from vicinal proton environments. The large coupling constant is characteristic of a 1,2-diaxial proton arrangement. This shows that the dihydroflavonol is in the R, R-absolute configuration at C-2 and C-3.⁹³

Table 3.13: ^1H NMR signals of flavonoid B.

Label	Chemical Shift (ppm)	Multiplicity
a-c	7.36-7.44	2 nd order
d	5.14	d
e	4.58	d
f	5.85	d of d
g	7.51	d of d
h	5.80	Under f signal
i or j	10.15	s

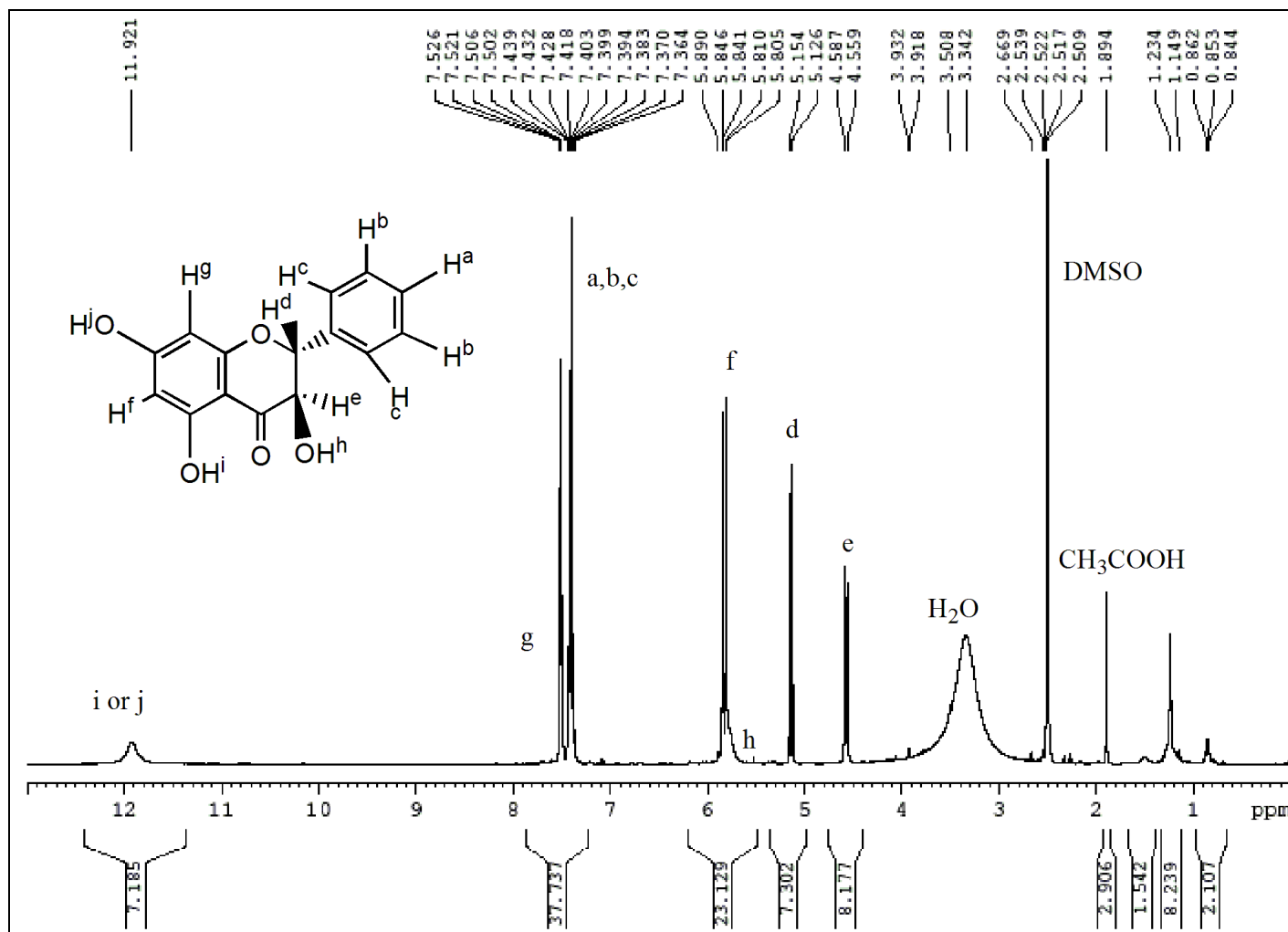


Figure 3.28: The ¹H NMR spectrum of flavonoid B in DMSO-*d*₆.

Two doublets of doublets were also observed in the spectrum. These were centred at 5.85ppm and 7.51ppm, within the region associated with A-ring protons. The appearance of these signals as doublets of doublets was unexpected since the UV results had suggested that the A-ring was hydroxylated at the 5 and 7-positions. This would leave the H-6 and H-8 protons which were expected to show long range W coupling with each other by appearing as doublet signals. The observation of the doublet of doublet signal multiplicity can be explained by coupling of H-6 and H-8 with the hydroxyl protons at the 5 and 7-positions. Normally coupling with hydroxyls is not observed due to rapid exchange of the hydroxyl proton with water. The DMSO- d_6 used in this study was of a high grade which contained very little water. The small water peak observed in the spectrum is due to traces of water in the dried flavonoid B. Under such conditions it is possible to observe coupling between the hydroxyl and aromatic protons.¹²⁷

The spectrum showed one well separated hydroxyl signal at 10.15ppm and what appeared to be a second under the 5.85ppm doublet of doublet signal. For a dihydroflavonol with hydroxyls at the 5 and 7-position the NMR spectrum would be expected to show three hydroxyl signals. It is possible that the third signal has been shifted downfield, out of the analysed range, by hydrogen bonding with the DMSO- d_6 solvent.¹²⁷

A complex second order pattern is observed between 7.36-7.44ppm which was attributed to the B-ring protons. The complexity of this signal suggested that the B-ring of flavonoid B was unsubstituted.

3.6.8.3 LDI-TOF Mass Spectrometry

The LDI-TOF mass spectrum of flavonoid B showed two principal ions (m/z 271.371 and m/z 272.366). The more intense m/z 271.371 ion has been attributed to the $[M-H]^-$ ion while the other m/z 272.366 ion is thought to be a radical $[M]^\bullet$ ion. These results were consistent with a trihydroxyflavanone ($C_{15}H_{12}O_5$) which has a molecular weight of 272.07.

3.6.8.4 Final Structural Assignment of Flavonoid B

From the UV and ^1H NMR spectroscopy, and mass spectrometry carried out on flavonoid B the following structural features have been identified:

- Flavonoid B is a dihydroxyflavonol.
- The A-ring has free hydroxyls at the 5 and 7-positions.
- The B-ring is probably not substituted.
- There is no hydroxyl group at the 8-position.
- Flavonoid B has three hydroxyl groups.
- Flavonoid B has the R,R-absolute configuration at C-2 and C-3.

From this information it was deduced that flavonoid B was 3,5,6-trihydroxyflavanone, more commonly known as pinobanksin. The structure of this dihydroflavonol is shown in figure 3.29. It is a common flavonoid in honey which originates from poplar bud exudates derived propolis.^{12, 103}

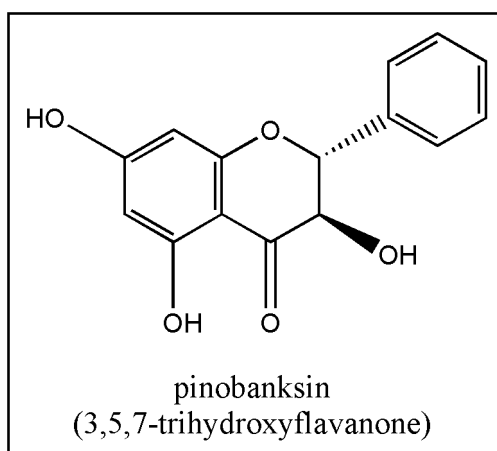


Figure 3.29: The structure of pinobanksin, the proposed identity of flavonoid B.

There was sufficient flavonoid B isolated to achieve a good ^{13}C NMR analysis. The chemical shifts recorded in this analysis are shown in table 3.14 along with reported chemical shift values for pinobanksin from the literature.¹²⁸ The chemical shift values show a good correlation confirming that flavonoid B is pinobanksin.

Table 3.14: A comparison of the ^{13}C NMR spectra of flavonoid B and pinobanksin.

Carbon Number	Pinobanksin (in CDCl_3) ¹²⁸	Flavonoid B (in $\text{DMSO}-d_6$)
2	83.5	83.7
3	72.5	72.3
4	196.0	197.5
5	163.6	164.2
6	96.9	97.4
7	167.5	169.5
8	96.0	96.4
9	163.0	163.2
10	100.5	100.7
1'	130.5	129.4
2'	127.6	128.1
3'	128.6	128.8
4'	129.2	129.0
5'	128.6	128.8
6'	127.6	128.1

Chapter 4: Conclusions

All the non-peroxide antibacterial activity of manuka honey was found to elute with the carbohydrate fraction when phenolics were extracted from honey using Amberlite XAD-2 resin. The phenolic fraction did not exhibit any measurable activity at its natural concentration but did show significant antibacterial activity when concentrated (see Section 3.1). This showed that the phenolics in honey do possess some biological activity but are not a major contributor to the non-peroxide antibacterial activity.

Modifications were made to the established XAD-2 method¹⁰⁴ for extraction of phenolics from honeys. Measurements of the phenolic content recovered by extraction of 50, 20 and 5g samples showed that recovery of phenolics was unaffected by the sample size extracted. The changes made to the method reduced the sample size required from 50g to only 5g (Section 3.3.1). This opened up a larger range of manuka honey samples, which would otherwise have been considered too small, to be extracted and analysed for flavonoid profiling.

Measurement of the recovery rates of flavonoids raised some concerns about the reliability of the XAD-2 phenolic extraction method. Extraction of flavonoid standards from an artificial matrix resulted in recovery rates of $10 \pm 6\%$ for quercetin, $16 \pm 7\%$ for kaempferol and $19 \pm 9\%$ for chrysin (see Section 3.3.2). Higher recovery rates of 28-60% were obtained for flavonoids extracted from simple methanol solutions using the methods established in the literature.¹ This result has raised the possibility that matrix effects have been reducing the recovery of flavonoids by the extraction method, with the recovery rates reported in the literature not reflecting the true rates.

The extraction rates measured in this study (28-60%) were lower than the $>80\%$ previously reported in the literature¹⁰⁴, suggesting that the extraction method employed in this study needs further optimization to maximize the recovery of phenolics. One aspect of the extraction method which is known to have reduced the

recovery rates of flavonoids was a coarse separation on a sacrificial HPLC column, used instead of filtering to remove contaminants from the phenolic extracts. Recovery rates of flavonoids from this step were found to be 60% for quercetin, 88% for pinocembrin, 66% for kaempferol and 75% for chrysin (see Section 3.3.2).

The results of the recovery rate assessment and general observations in the later analysis of manuka honey flavonoids have suggested that the recovery rate of flavonoids may be more affected by the class of the flavonoid (i.e. the oxidation state of the C3 linkage) than its overall polarity. The flavanones were observed to be recovered at the highest rates while the flavonols were recovered at the lowest rates and consequently only occurred at trace levels when honey extracts were analysed. The flavones appeared to be recovered at higher rates than the flavonols, but not the flavanones.

Repeated extractions were carried out on a single honey sample to assess the reproducibility of the extraction method (see Section 3.3.3). Extractions were found to be highly variable and several reasons, including low recovery rates and complicated extraction procedures, were suggested to explain the variability. Estimates of mean flavonoid content, for a particular sample, could be made by applying an uncertainty of 20% to the measurement from a single extraction of that sample.

Analysis of the phenolic extracts from 31 samples of New Zealand manuka (*Leptospermum scoparium*) honey allowed a common flavonoid profile to be established for this honey type (see Section 3.4). The total flavonoid content of manuka honey was found to range between 0.59-2.24mg/100g of honey, with an average of 1.16 ± 0.16 mg per 100g of honey. The flavonoid profile of manuka honey was found to mainly consist of pinobanksin (0.27 ± 0.04 mg/100g honey), pinocembrin (0.17 ± 0.02 mg/100g honey), luteolin (0.14 ± 0.02 mg/100g honey) and chrysin (0.13 ± 0.02 mg/100g honey), which together represented 61% of the total flavonoid content. With the exception of luteolin, these flavonoids are known to originate in propolis derived from poplar bud exudates and are a common feature of honeys from temperate areas of the Northern Hemisphere.¹⁰³

Eleven other flavonoids were found in the manuka honeys studied, including eight which did not coincide with the available authentic standards. Two of these unknown flavonoids were subsequently isolated using a combination of XAD-2 extraction, liquid-liquid extraction, Sephadex-LH20 chromatography and HPLC (see Section 3.5). Characterisation of these flavonoids was achieved by UV absorption spectroscopy, ^1H and ^{13}C NMR spectroscopy, and LDI-TOF mass spectrometry (see Section 3.6). These results lead to the identification of the unknown flavonoids as 8-methoxykaempferol and pinobanksin, two flavonoids which have been previously reported in other many other honeys.^{1, 12, 103, 111, 118}

Phenolic extracts from eight samples of non-manuka, New Zealand honeys were analysed and their flavonoid profiles compared with that of manuka honey (see Section 3.4.5). All the honeys studied showed similarities in their flavonoid profile, with pinocembrin and pinobanksin being the major constituents. This shows that propolis is the main source of flavonoids in these New Zealand honeys. Since propolis is the same in all types of floral honey, these propolis derived flavonoids are of no use in determining floral origin of honeys.

A further seven flavonoids were found in the non-manuka honeys which did not coincide with the standards available and consequently could not be identified. While they only occurred at low levels they also showed some variations with floral origin. It is possible that some of these flavonoids could be useful floral markers.

Manuka honey can be distinguished from the other New Zealand honeys studied (see Section 3.4.5) by a combination of its high total flavonoid content, high luteolin content (higher than 0.05mg/100g of honey) and high levels of an unknown compound 01 which did not coincide with the standards available. This compound was found to elute with the flavonoids at high levels ($0.16 \pm 0.03\text{mg}/100\text{g}$ of honey) in manuka honey. It was clear from the UV absorption spectrum that this compound was not a flavonoid but further characterisation could not be achieved.

Statistical analysis showed that a positive correlation existed between the concentration of unknown compound 01 and the non-peroxide antibacterial activity in

manuka honeys. A similar relationship was observed for the luteolin levels in the manuka honeys studied (Section 3.4.6). These correlations confirm the usefulness of unknown compound 01 and luteolin as marker compounds for manuka honey. The correlation between these flavonoids the non-peroxide antibacterial activity of manuka honey could prove useful in future investigations into the source of this activity.

Chapter 5: Recommendations

The results of this study have raised a number of concerns about the reliability and reproducibility of the XAD-2 phenolic extraction method. The low recovery rates experienced in this study did not compare well to some of those reported in the literature.^{24, 104} To investigate the cause of these low recovery rates the extraction method needs to first be carried out exactly as described by these other researchers, and the flavonoid recovery rate measured. If the recovery rate is not >80% then the following steps should be taken to attempt to improve the recovery rate:

- The XAD-2 resin should be thoroughly cleaned by soxhlet extraction with methanol and acetone. D'Arcy¹ has previously described a method for this purification of the resin.
- If cleaning the resin fails to improve the recovery rate then new resin should be used.
- The volume of solvents required to optimise the recovery of flavonoids should be determined.

Once the above recommendations have been carried out the recovery rate of flavonoids from a simple methanol solution should be comparable to those values reported by other groups.^{1, 104} If the recovery rates are still low in comparison then it should be considered that the recovery rates have been over estimated in the literature.

This will provide a solid foundation from which to investigate the effects of matrix and flavonoid type on recovery rate. Measurement of the recovery rate of flavonoids from an artificial honey should be repeated on an optimised XAD-2 extraction system and compared with rates for flavonoids from simple methanol solutions. This should allow the effect of the honey matrix on flavonoid recovery to be determined.

The recovery rates of the flavones and their corresponding flavonols should be compared to assess the effect of the 3-hydroxyl on the extraction efficiency. This was not tested in the present study but the observation that flavones were more

abundant than their corresponding flavonols in the extracts analysed suggests that the flavones may be preferentially extracted. This preferential extraction is a problem when the concentrations of individual flavonoids in honey are being compared since the flavonols will be under-represented.

With the XAD-2 extraction method optimised for extraction from 50g samples it would be useful to re-investigate the effect of sample size on the extraction process. The benefit of being able to analyse small honey samples make this modification of the extraction method worthwhile, provided it does not adversely affect the reproducibility of the extraction. The results of the present study showed that the variation in extraction results was large for all the sample weights extracted. If this variability could be reduced then there may be a significant difference between the results of 5g and 50g honey extractions.

The collection of flavonoid standards should be increased to allow identification of more flavonoids by comparison of retention time and UV absorbance spectra. Some of the flavonoids found in honey are difficult to source commercially (e.g. pinobanksin) and will have to be isolated, purified and characterised so that they may be used as qualitative standards for future research.

Six flavonoids were found in most of the manuka honeys, and a further seven in the non-manuka honeys which did not coincide with the known flavonoids for which we had authentic standards. It is possible that these flavonoids could be unique floral markers for New Zealand monofloral honeys if future research is able to isolate and identify them to confirm their uniqueness.

While only two flavonoids were isolated in the present study, the process of extracting and isolating these flavonoids from a bulk sample of manuka honey has yielded several fractions which are rich in flavonoids. These fractions should contain the six unknown flavonoids found in the manuka honeys studied. They may potentially contain many more interesting flavonoids which were not sufficiently concentrated to observe in the analytical study of manuka honey flavonoids. Future research should

isolate and characterise the unknown flavonoids from these fractions so that they may be added to the collection of flavonoid standards and utilised in future work.

The unknown compound 01 in particular needs to be isolated and characterised. This compound showed a positive correlation with UMF antibacterial activity so its identity is of interest to the manuka honey industry. It is possible that this unknown compound 01 may be a precursor of the dihydroxyacetone form which methylglyoxal derives. Once it has been identified it is recommended that a future study search for the source of the compound in the nectar of manuka flowers. It would also be worthwhile searching for luteolin and its glycosides in manuka nectar since it appeared to be a marker for manuka honey. If the luteolin in manuka honey was derived from the nectar then the nectar should contain glycosides of luteolin which are hydrolysed by bee enzymes to form the aglycone in honey.

Future analyses of the phenolic profiles of honey should be carried out on an LC-MS system rather than HPLC with photodiode array detection. A combined UV absorption spectrum, molecular mass and fragmentation pattern recorded on such a system has the potential to allow many more unknown flavonoids to be identified rapidly without the need for isolation and characterisation.

References

1. D'Arcy, B. R. *Antioxidants in Australian floral honeys - identification of health-enhancing nutrient components*; Australian Government Rural Industries Research and Development Corporation: Barton, May 2005, 2005.
2. Coulston, A. M., Honey...how sweet it is! *Nutrition Today* **2000**, 35, (3), 96-100.
3. White, J. W., Honey. In *The hive and the honey bee.*, Graham, J. M., Ed. Dadant & Sons: Hamilton, Illinois, 1992; pp 869-925.
4. Ward, C., Kanuka and manuka. *Forest and Bird* 2000, pp 24-27.
5. Salmon, J. T., *Native New Zealand flowering plants*. Reed Books: Auckland, 1991.
6. Morales, C. F., Margarodidae (Insecta:Hemiptera). *Fauna of New Zealand / Ko te Aitanga Pepeke o Aotearoa* **1991**, 21, 16-17.
7. Weston, R. J.; Brocklebank, L. K., The oligosaccharide composition of some New Zealand honeys. *Food Chemistry* **1999**, 64, (1), 33-37.
8. White, J. W. J.; Riethof, M. L.; Subers, M. H.; Kushnir, I. *Composition of American honeys.*; Washington D. C., 1962.
9. Crane, E., The flowers honey comes from. In *Honey: A Comprehensive Survey*, Crane, E., Ed. Heinemann: London, 1976; pp 3-76.
10. Airborne Honey Ltd Manuka honey. <http://www.airborne.co.nz/manuka.htm> (1/01/2009),
11. White, J. W. J., Physical characteristics of honey. In *Honey: A Comprehensive Survey*, Crane, E., Ed. Heinemann: London, 1979; pp 207-239.
12. Anklam, E., A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry* **1998**, 63, (4), 549-562.

13. White, J. W. J.; Subers, M. H.; Schepartz, A. I., The identification of inhibine. *American Bee Journal* **1962**, 102, 430-431.
14. National Honey Board Honey and honey bees: the incredible story. <http://www.honey.com/consumers/kids/beefacts.asp> (01/01/09),
15. White, J. W., Honey. *Advances in Food Research* **1978**, 4, 287-374.
16. White, J. W., Detection of honey adulteration by carbohydrate analysis. *Journal of the Association of Official Analytical Chemists* **1980**, 63, 11-18.
17. Doner, L. W., The sugars of honey - A review. *Journal of the Science of Food and Agriculture* **1977**, 28, (5), 443-456.
18. Maurizio, A., From the raw material to the finished product: honey. *Bee World* **1962**, 43, 66-81.
19. White, J. W. J.; Riethof, M. L.; Kushnir, I., Composition of honey vi. the effect of storage on carbohydrates, acidity and diastase content. *Journal of Food Science* **1961**, 26, 63-71.
20. Wu, J. A chemical investigation of oligosaccharides in some New Zealand unifloral honeys. University of Waikato, Hamilton, 2000.
21. Tan, S. T.; Holland, P. T.; Wilkins, A. L.; Molan, P. C., Extractives from New Zealand honeys. 1. White clover, manuka and kanuka unifloral honeys. *J. Agric. Food Chem.* **1988**, 36, (3), 453-60.
22. Wilkins, A. L.; Lu, Y.; Molan, P. C., Extractable organic substances from New Zealand unifloral manuka. *J. Apic. Res.* **1993**, 32, (1), 3-9.
23. Weston, R. J.; Mitchell, K. R.; Allen, K. L., Antibacterial phenolic components of New Zealand manuka honey. *Food Chemistry* **1999**, 64, (3), 295-301.
24. Yao, L.; Datta, N.; Tomas-Barberan, F. A.; Ferreres, F.; Martos, I.; Singanusong, R., Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand *Leptospermum* honeys. *Food Chem.* **2003**, 81, (2), 159-168.

25. White, J. W., The protein content of honey. *Apicultural Research* **1978**, 17, 234-238.
26. Molan, P. C., Honey as a topical antibacterial agent for treatment of infected wounds. *World Wide Wounds* **2001**, 10.
27. Gethin, G.; Cowman, S., Case series of use of manuka honey in leg ulceration. *International Wound Journal* **2005**, 2, (1), 10-15.
28. Visavadia, B. G.; Honeysett, J.; Danford, M., H., Manuka honey dressing: An effective treatment for chronic wound infections. *British Journal of Oral and Maxillofacial Surgery* **2006**, doi:10.1016/j.bjoms.2006.09.013.
29. Chambers, J., Topical manuka honey for MRSA-contaminated skin ulcers. *Palliative Medicine* **2006**, 20, 557.
30. Cooper, R. A.; Molan, P. C.; Krishnamoorthy, L.; Harding, K. G., Manuka honey used to heal a recalcitrant surgical wound. *European Journal of Clinical Microbiology & Infectious Diseases* **2001**, 20, (10), 758-759.
31. Natarajan, S.; Williamson, D.; Grey, J.; Harding, K. G.; Cooper, R. A. *Healing of an MRSA-colonized, hydroxyurea-induced leg ulcer with honey*; Wound Healing Research Unit, University of Wales College of Medicine, Heath Park, Cardiff, UK.: England: United Kingdom, 2001; pp 33-6.
32. Molan, P. C., The antibacterial activity of honey .2. Variation in the potency of the antibacterial activity. *Bee World* **1992**, 73, (2), 59-76.
33. Khan, F. R.; Abadin, Z. U.; Rauf, N., Honey: nutritional and medicinal value. *Int. J. Clin. Pract.* **2007**, 61, (10), 1705-1707.
34. Molan, P. C., The antibacterial activity of honey .1. The nature of the antibacterial activity. *Bee World* **1992**, 73, (1), 5-28.
35. Wahdan, H. A., Causes of the antimicrobial activity of honey. *Infection* **1998**, 26, (1), 26-31.

36. Efem, S. E. E.; Udoh, K. T.; Iwara, C. I., The antimicrobial spectrum of honey and its clinical significance. *Infection* **1992**, 20, (4), 227-229.
37. Cooper, R. A.; Molan, P. C.; Harding, K. G., The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. *J Appl Microbiol* **2002**, 93, (5), 857-63.
38. Cooper, R. A.; Molan, P. C.; Harding, K. G., Antibacterial activity of honey against strains of *Staphylococcus aureus* from infected wounds. *J R Soc Med* **1999**, 92, (6), 283-5.
39. Cooper, R. A.; Halas, E.; Molan, P. C., The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns. *J Burn Care Rehabil* **2002**, 23, (6), 366-70.
40. Abuharfeil, N.; Al-Oran, R.; Abo-Shehada, M., The effect of bee honey on the proliferative activity of human B- and T-lymphocytes and the activity of phagocytes. *Food and Agricultural Immunology* **1999**, 11, 169-177.
41. Tonks, A. J.; Cooper, R. A.; Jones, K. P.; Blair, S.; Parton, J.; Tonks, A., Honey stimulates inflammatory cytokine production from monocytes. *Cytokine+* **2003**, 21, (5), 242-247.
42. Molan, P. C., The role of honey in the management of wounds. *Journal of Wound Care* **1999**, 8, 415-418.
43. Oryan, A.; Zaker, S. R., Effects of topical application of honey on cutaneous wound healing in rabbits. *J. Vet. Med. A* **1998**, 45, 181-188.
44. Subrahmanyam, M., Topical application of honey in treatment of burns. *British Journal of Surgery* **1991**, 78, (4), 497-498.
45. Efem, S. E. E., Clinical observations on the wound healing properties of honey. *British Journal of Surgery* **1988**, 75, (7), 679-681.
46. Lord, A., Sweet healing. *New Scientist* **2000**, 168, (2259), 32.

47. Bang, L. M.; Bunting, C.; Molan, P., The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. *Journal of Alternative & Complementary Medicine* **2003**, 9, (2), 267-273.
48. Dunford, C.; Molan, P. C., Using honey as a dressing for infected skin lesions. *Nursing Times* **2000**, 96, 7-9.
49. Simon, A.; Sofka, K.; Wiszniewsky, G.; Blaser, G.; Bode, U.; Fleischhack, G., Wound care with antibacterial honey (Medihoney) in pediatric hematology-oncology. *Support Care Cancer* **2006**, 14, 91-97.
50. Lusby, P. E.; Coombes, A.; Wilkinson, J. M., Honey: a potent agent for wound healing? *J Wound Ostomy Continence Nurs* **2002**, 29, (6), 295-300.
51. Lusby, P. E.; Coombes, A. L.; Wilkinson, J. M., A comparison of wound healing following treatment with lavandula x allardii honey or essential oil. *Phytotherapy Research* **2006**, 20, 755-757.
52. Aysan, E.; Ayar, E.; Aren, A.; Cifer, C., The role of intra-peritoneal honey administration in preventing post-operative peritoneal adhesions. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **2002**, 104, 152-155.
53. Emson, I. M., A different and safe method of split thickness skin graft fixation: Medical honey application. *Burns* **2007**, 33, 782-787.
54. English, H. K. P.; Pack, A. R. C.; Molan, P. C., The effects of manuka honey on plaque and gingivitis: a pilot study. *J Int Acad Periodontol* **2004**, 6, (2), 63-7.
55. Molan, P., The antibacterial properties of honey. *Chemistry in New Zealand* **1995**, (July), 10-14.
56. Ruegg, M.; Blanc, B., The water activity of honey and related sugar solutions. *Lebensmittel-Wissenschaft and Technologie* **1981**, 14, 1-6.
57. Allen, K. L.; Molan, P. C.; Reid, G. M., A survey of the antibacterial activity of some New Zealand honeys. *J Pharm Pharmacol* **1991**, 43, (12), 817-22.

58. White, J. W., Jr.; Subers, M. H.; Schepartz, A. I., The identification of inhibine. *Am. Bee J.* **1962**, 102, 430-1.
59. White, J. W., Jr.; Subers, M. H.; Schepartz, A. I., The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochim. Biophys. Acta, Spec. Sect. Enzymol. Subj.* **1963**, 73, (1), 57-70.
60. Gillette, C. C., Honey catalase. *J. Econ. Entomol.* **1931**, 24, 605-6.
61. Schepartz, A. I.; Subers, M. H., Catalase in honey. *J. Apicult. Res.* **1960**, 5, (1), 37-43.
62. Cochrane, C. G., Cellular injury by oxidants. *Am. J. Med.* **1991**, 91, (3C), 23S-30.
63. Simon, R.; Scoggin, C.; Patterson, D., Hydrogen peroxide mediates the toxic effect of oxygen radicals on human fibroblasts. *Chest* **1981**, 80, (1, Suppl.), 45.
64. Simon, R. H.; Scoggin, C. H.; Patterson, D., Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. *J. Biol. Chem.* **1981**, 256, (14), 7181-6.
65. Allen, K. L.; Molan, P. C.; Reid, G. M., A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmacology* **1991**, 43, (12), 817-822.
66. Molan, P. C.; Smith, I. M.; Reid, G. M., A comparison of the antibacterial activities of some New Zealand honeys. *Journal of Apicultural Research* **1988**, 27, (4), 252-256.
67. Molan, P. C.; Russell, K. M., Non-peroxide antibacterial activity in some New Zealand honeys. *Journal of Apicultural Research* **1988**, 27, (1), 62-67.
68. Mavric, E.; Wittmann, S.; Barth, G.; Henle, T., Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol. Nutr. Food Res.* **2008**, 52, (4), 483-489.

69. Weigel, K. U.; Opitz, T.; Henle, T., Studies on the occurrence and formation of 1,2-dicarbonyls in honey. *Eur. Food. Res. Technol.* **2004**, 218, 147-151.
70. Adams, C. J.; Boulton, C. H.; Deadman, B. J.; Farr, J. M.; Grainger, M. N. C.; Manley-Harris, M.; Snow, M. J., Isolation by HPLC and characterization of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydr. Res.* **2008**, 343, (4), 651-659.
71. Snow, M. J. An investigation into the non-peroxide antibacterial activity of manuka honey. The University of Waikato, Hamilton, 2001.
72. Farr, J. M. An investigation into some properties of the non-peroxide antibacterial activity of manuka honey. The University of Waikato, Hamilton, 2005.
73. Peppas, M.; Brem, H.; Ehrlich, P.; Zhang, J.; Cai, W.; Li, Z.; Croitoru, A.; Thung, S.; Vlassara, H., Adverse effects of dietary glycotoxins on wound healing in genetically diabetic mice. *Diabetes* **2003**, 52, 2805-2813.
74. Berlanga, J.; Cibrian, D.; Guillen, I.; Freyre, F.; Alba, J. S.; Lopez-Saura, P.; Merino, N.; Aldama, a.; Quintela, A. M.; Triana, M. E.; Montequin, J. F.; Ajamieh, H.; Urquiza, D.; Ahmed, N.; Thornalley, P. J., Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. *Clinical Science* **2005**, 109, (1), 83-95.
75. Dobler, D.; Ahmed, N.; Song, L.; Eboigbodin, K. E.; Thornalley, P. J., Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes* **2006**, 55, 1961-1969.
76. Ghosh, M.; Talukdar, D.; Ghosh, S.; Bhattacharyya, N.; Ray, M.; Ray, S., In vivo assessment of toxicity and pharmacokinetics of methylglyoxal. Augmentation of the curative effect of methylglyoxal on cancer-bearing mice by ascorbic acid and creatine. *Toxicology and Applied Pharmacology* **2006**, 212, 45-48.

77. Kalapos, M. P., Methylglyoxal in living organisms. Chemistry, biochemistry, toxicology and biological implications. *Toxicology Letters* **1999**, 110, 145-175.
78. Walker, J. R. L., *The biology of plant phenolics*. Edward Arnold Limited: London, 1975.
79. Britton, G., *The biochemistry of natural pigments*. Cambridge University Press: Cambridge, 1983.
80. Bohm, B. A., Chalcones, aurones and dihydrochalcones. In *The flavonoids*, Harborne, J. B.; Mabry, T. J.; Mabry, H., Eds. Chapman and Hall: London, 1975; pp 442-504.
81. Bohm, B. A., *Introduction to flavonoids*. Harwood Academic Publishers: Amsterdam, 1998.
82. Havsteen, B. H., The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics* **2002**, 96, (2-3), 67-202.
83. Geissman, T. A., The occurrence of flavonoid compounds in nature. In *The chemistry of flavonoid compounds*, Geissman, T. A., Ed. The Macmillan Company: New York, 1962; pp 1-5.
84. Birch, A. J., Biosynthesis of flavonoids and anthocyanins. In *The chemistry of flavonoid compounds*, Geissman, T. A., Ed. The Macmillan Company: New York, 1962; pp 618-625.
85. Seshadri, T. R., Isolation of flavonoid compounds from plant materials. In *The chemistry of flavonoid compounds*, Geissman, T. A., Ed. The Macmillan Company: New York, 1962; pp 6-33.
86. Cushnie, T. P. T.; Lamb, A. J., Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, 26, (5), 343-356.
87. Chen, C.-w.; Chen, N.-f.; Zhang, L.; Zhou, Y.-b.; Li, Y., Studies on the acute toxicity and the mutagenicity of flavonoids extracts from *Pteridium aquilinum* var. *latiusculum*. *Aibian, Jibian, Tubian* **2008**, 20, (2), 157-159.

88. Noda, Y.; Peterson, D. G., Structure-reactivity relationships of flavan-3-ols on product generation in aqueous glucose/glycine model systems. *J. Agric. Food Chem.* **2007**, 55, (9), 3686-3691.
89. Totlani, V. M.; Peterson, D. G., Epicatechin carbonyl-trapping reactions in aqueous maillard systems: identification and structural elucidation. *Journal of Agricultural and Food Chemistry* **2006**, 54, (19), 7311-7318.
90. Venkataraman, K., Methods for determining the structure of flavonoid compounds. In *The chemistry of flavonoid compounds.*, Geissman, T. A., Ed. The Macmillan Company: New York, 1962; pp 70-106.
91. Markham, K. R.; Mabry, T. J., Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. In *The flavonoids.*, Harborne, J. B.; Mabry, H.; Mabry, T. J., Eds. Chapman and Hall: London, 1975.
92. Jurd, L., Spectral properties of flavonoid compounds. In *The chemistry of flavonoid compounds.*, Geissman, T. A., Ed. The Macmillan Company: New York, 1962; pp 107-155.
93. Mabry, T. J.; Markham, K. R.; Thomas, M. B., *The systematic identification of flavonoids*. Springer-Verlag: New York, 1970.
94. Ferreres, F.; Tomas-Barberan, F. A.; Gil, M. I.; Tomas-Lorente, F., An HPLC technique for flavonoid analysis in honey. *J. Sci. Food Agric.* **1991**, 56, (1), 49-56.
95. Ferreres, F.; Tomas-Barberan, F. A.; Soler, C.; Garcia-Viguera, C.; Ortiz, A.; Tomas-Lorente, F., A simple extractive technique for honey flavonoid HPLC analysis. *Apidologie* **1994**, 25, (1), 21-30.
96. Ferreres, F.; Andrade, P.; Tomas-Barberan, F. A., Flavonoids from Portuguese heather honey. *Z. Lebensm.-Unters. Forsch.* **1994**, 199, (1), 32-7.
97. Delgado, C.; Talou, T.; Gaset, A., Capillary electrophoresis: Application and development in the domain of the food science and agroresource industry. *Spectra Anal.* **1994**, 23, (181), 42-6.

98. Delgado, C.; Tomas-Barberan, F. A.; Talou, T.; Gaset, A., Capillary electrophoresis as an alternative to HPLC for determination of honey flavonoids. *Chromatographia* **1994**, 38, (1-2), 71-8.
99. Ferreres, F.; Blazquez, M. A.; Gil, M. I.; Tomas-Barberan, F. A., Separation of honey flavonoids by micellar electrokinetic capillary chromatography. *J. Chromatogr., A* **1994**, 669, (1-2), 268-74.
100. Ceksteryte, V.; Kazlauskas, S.; Racys, J., Composition of flavonoids in Lithuanian honey and beebread. *Biologija* **2006**, (2), 28-33.
101. Truchado, P.; Ferreres, F.; Bortolotti, L.; Sabatini, A. G.; Tomas-Barberan, F. A., Nectar flavonol rhamnosides are floral markers of acacia (*Robinia pseudacacia*) honey. *J. Agric. Food Chem.* **2008**, 56, (19), 8815-8824.
102. Zborowski, J., Flavonoids in bee honeys. II. Rutin and quercetin. *Pszczelnicze Zesz. Nauk.* **1968**, 12, (1-2), 75-83.
103. Tomas-Barberan, F. A.; Ferreres, F.; Garcia-Viguera, C.; Tomas-Lorente, F., Flavonoids in honey of different geographical origin. *Z. Lebensm.-Unters. Forsch.* **1993**, 196, (1), 38-44.
104. Martos, I.; Cossentini, M.; Ferreres, F.; Tomas-Barberan, F. A., Flavonoid composition of Tunisian honeys and propolis. *J. Agric. Food Chem.* **1997**, 45, (8), 2824-2829.
105. Yao, L.; Jiang, Y.; D'Arcy, B.; Singanusong, R.; Datta, N.; Caffin, N.; Raymont, K., Quantitative high-performance liquid chromatography analyses of flavonoids in Australian *Eucalyptus* honeys. *J. Agric. Food Chem.* **2004**, 52, (2), 210-214.
106. Ferreres, F.; Ortiz, A.; Silva, C.; Garcia-Viguera, C.; Tomas-Barberan, F. A.; Tomas-Lorente, F., Flavonoids of La Alcarria honey. A study of their botanical origin. *Z. Lebensm.-Unters. Forsch.* **1992**, 194, (2), 139-43.
107. Tomas-Barberan, F. A.; Ferreres, F.; Garcia-Viguera, C.; Tomas-Lorente, F., Flavonoid analysis in the determination of the geographical and botanical origin of honey. *Bull. Liaison - Groupe Polyphenols* **1992**, 16, (Pt. 2), 233-6.

108. Sabatier, S.; Amiot, M. J.; Tacchini, M.; Aubert, S., Identification of flavonoids in sunflower honey. *J. Food Sci.* **1992**, 57, (3), 773-4, 777.
109. Ferreres, F.; Garcia-Viguera, C.; Tomas-Lorente, F.; Tomas-Barberan, F. A., Hesperetin: a marker of the floral origin of citrus honey. *J. Sci. Food Agric.* **1993**, 61, (1), 121-3.
110. Soler, C.; Gil, M. I.; Garcia-Viguera, C.; Tomas-Barberan, F. A., Flavonoid patterns of French honeys with different floral origin. *Apidologie* **1995**, 26, (1), 53-60.
111. Tomas-Barberan, F. A.; Ferreres, F.; Blazquez, M. A.; Garcia-Viguera, C.; Tomas-Lorente, F., High-performance liquid chromatography of honey flavonoids. *J. Chromatogr.* **1993**, 634, (1), 41-6.
112. Tomas-Barberan, F. A.; Martos, I.; Ferreres, F.; Radovic, B. S.; Anklaam, E., HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. *Journal of the Science of Food and Agriculture* **2001**, 81, (5), 485-496.
113. Ferreres, F.; Andrade, P.; Gil, M. I.; Tomas-Barberan, F. A., Floral nectar phenolics as biochemical markers for the botanical origin of heather honey. *Z. Lebensm.-Unters. Forsch.* **1996**, 202, (1), 40-4.
114. Yao, L.; Jiang, Y.; Singanusong, R.; D'Arcy, B.; Datta, N.; Caffin, N.; Raymont, K., Flavonoids in Australian *Melaleuca*, *Guioa*, *Lophostemon*, *Banksia* and *Helianthus* honeys and their potential for floral authentication. *Food Res. Int.* **2004**, 37, (2), 166-174.
115. Martos, I.; Ferreres, F.; Yao, L.; D'Arcy, B.; Caffin, N.; Tomas-Barberan, F. A., Flavonoids in monospecific *Eucalyptus* honeys from Australia. *J. Agric. Food Chem.* **2000**, 48, (10), 4744-4748.
116. Gil, M. I.; Ferreres, F.; Ortiz, A.; Subra, E.; Tomas-Barberan, F. A., Plant phenolic metabolites and floral origin of rosemary honey. *J. Agric. Food Chem.* **1995**, 43, (11), 2833-8.
117. Ferreres, F.; Juan, T.; Perez-Arquillue, C.; Herrera-Martache, A.; Garcia-Viguera, C.; Tomas-Barberan, F. A., Evaluation of pollen as a source of kaempferol in rosemary honey. *J. Sci. Food Agric.* **1998**, 77, (4), 506-510.

118. Martos, I.; Ferreres, F.; Tomas-Barberan, F. A., Identification of flavonoid markers for the botanical origin of eucalyptus honey. *J. Agric. Food Chem.* **2000**, 48, (5), 1498-1502.
119. Kenjeric, D.; Mandic, M. L.; Primorac, L.; Bubalo, D.; Perl, A., Flavonoid profile of Robinia honeys produced in Croatia. *Food Chemistry* **2007**, 102, (3), 683-690.
120. Kenjeric, D.; Mandic, M. L.; Primorac, L.; Cacic, F., Flavonoid pattern of sage (*Salvia officinalis* L.) unifloral honey. *Food Chem.* **2008**, 110, (1), 187-192.
121. Weston, R. J.; Brocklebank, L. K.; Lu, Y., Identification and quantitative levels of antibacterial components of some New Zealand honeys. *Food Chemistry* **2000**, 70, (4), 427-435.
122. *Bulletin 910 Guide to Solid Phase Extraction*; Supelco: Bellefonte, PA, 1998.
123. Markham, K. R.; Mitchell, K. R.; Wilkins, A. L.; Daldy, J. A.; Lu, Y., HPLC and GC-MS identification of the major organic constituents in New Zealand propolis. *Phytochemistry* **1996**, 42, (1), 205-211.
124. Adams, C. J.; Manley-Harris, M.; Molan, P. C., The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research* **2009**, doi: 10.1016/j.carres.2009.03.020.
125. Stephens, J. M. C. The factors responsible for the varying levels of UMF in manuka (*Leptospermum scoparium*) honey. University of Waikato, Hamilton, 2006.
126. Mishio, T.; Honma, T.; Iwashina, T., Yellow flavonoids in *Centaurea ruthenica* as flower pigments. *Biochemical Systematics and Ecology* **2006**, 34, 180-184.
127. Silverstein, R. M.; Webster, F. X.; Kiemle, D. J., *Spectrometric identification of organic compounds*. 7th ed.; John Wiley & Sons: Hoboken, New Jersey, 2005.
128. Agrawal, P. K.; Thakur, R. S.; Bansal, M. C., Flavonoids. In *Carbon-13 NMR of flavonoids.*, Agrawal, P. K., Ed. Elsevier: Amsterdam, 1989.

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman



THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

The University of Waikato

2009

Antibacterial Activity Testing of Extraction Fractions					
Plate Number	Equivalent % Phenol				
	Aqueous	MeOH 1:1	MeOH 25:1	Manuka Honey	Clover Honey
1	30.8	ND	42.8	32.3	ND
	35.9	ND	39.7	34.8	ND
	30.7	ND	33.6	33.0	ND
	31.2	ND	33.8	33.3	ND
	31.5	ND	43.1	35.4	ND
	28.0	ND	33.4	34.8	ND
	31.2	ND	44.1	35.7	ND
	30.0	ND	35.8	31.4	ND
	33.8	ND		31.8	
	32.5	ND		35.8	
	30.1	ND		33.0	
	30.3	ND		27.9	
2	31.2	ND	30.8	28.6	ND
	28.6	ND	36.4	27.9	ND
	29.0	ND	35.4	28.5	ND
	27.6	ND	34.2	27.8	ND
	29.1	ND	35.3	30.5	ND
	33.0	ND	36.8	31.0	ND
	29.1	ND	35.8	29.1	ND
	27.7	ND	34.7	28.6	ND
	30.8	ND		31.0	
	27.1	ND		33.9	
	30.5	ND		27.9	
	28.4	ND		28.2	
3	39.6	ND	35.2	35.7	ND
	28.5	ND	39.5	29.4	ND
	30.0	ND	35.5	25.8	ND
	32.5	ND	42.0	31.3	ND
	28.8	ND	39.7	33.3	ND
	32.3	ND	31.5	28.1	ND
	28.5	ND	43.1	34.5	ND
	35.5	ND	38.8	28.6	ND
	36.9	ND		33.3	
	31.1	ND		30.1	
	26.8	ND		35.2	
	31.7	ND		29.4	
4	30.8	ND	31.6	28.6	ND
	29.5	ND	38.4	28.0	ND
	29.6	ND	36.7	28.3	ND
	29.7	ND	40.4	26.7	ND
	28.7	ND	30.8	30.5	ND
	28.3	ND	37.9	28.5	ND
	30.3	ND	34.5	29.2	ND
	29.9	ND	37.3	25.9	ND
	29.5	ND		29.1	
	30.3	ND		29.6	
	28.5	ND		28.7	
	31.1	ND		28.4	
Mean	30.5	#DIV/0!	36.8	30.6	#DIV/0!
St Dev.	2.5	#DIV/0!	3.7	2.8	#DIV/0!
95% Conf. Limit	0.7	#DIV/0!	1.3	0.8	#DIV/0!
ND= no detectable activity					

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
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by

Benjamin Jade Deadman



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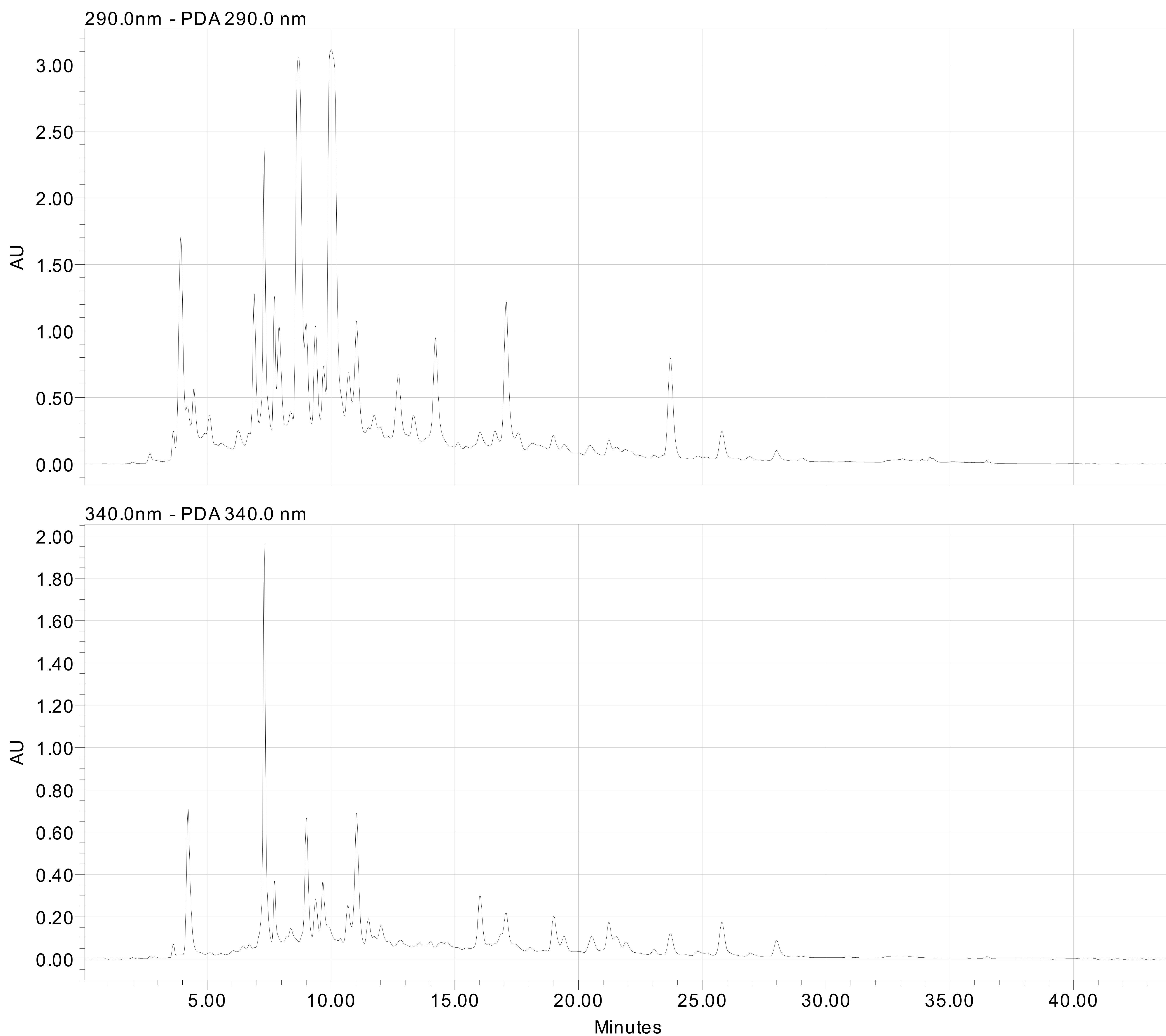
The University of Waikato

2009

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160408**

**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



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Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

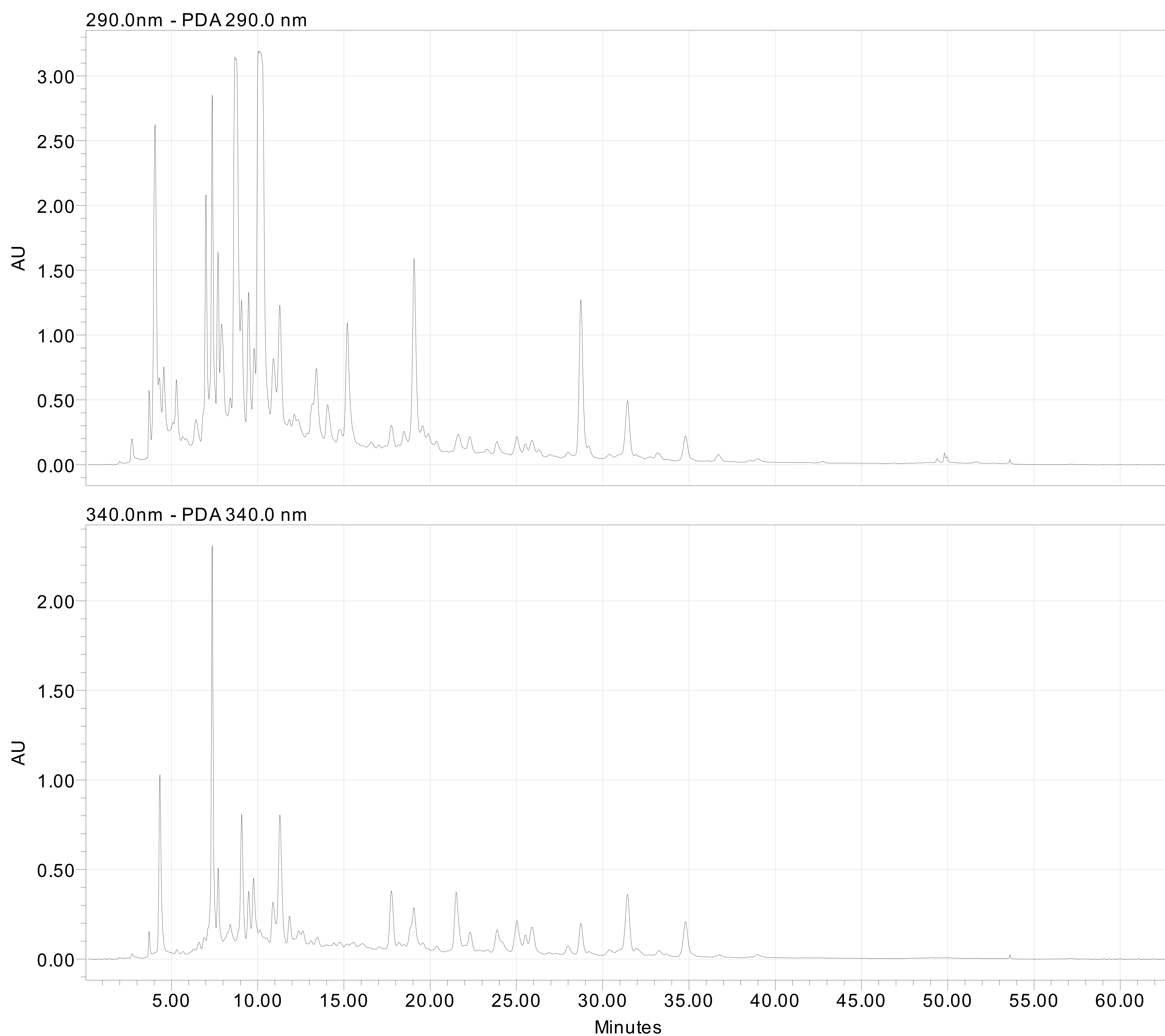
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Project Name: Chris Adams\Ben

Date Printed:

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170408****Testing the Effect of Sample Size on
Extraction Method****Chromatograms**

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Date Acquired: 5/27/2008 3:25:39 PM NZST

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Report Method ID 3207

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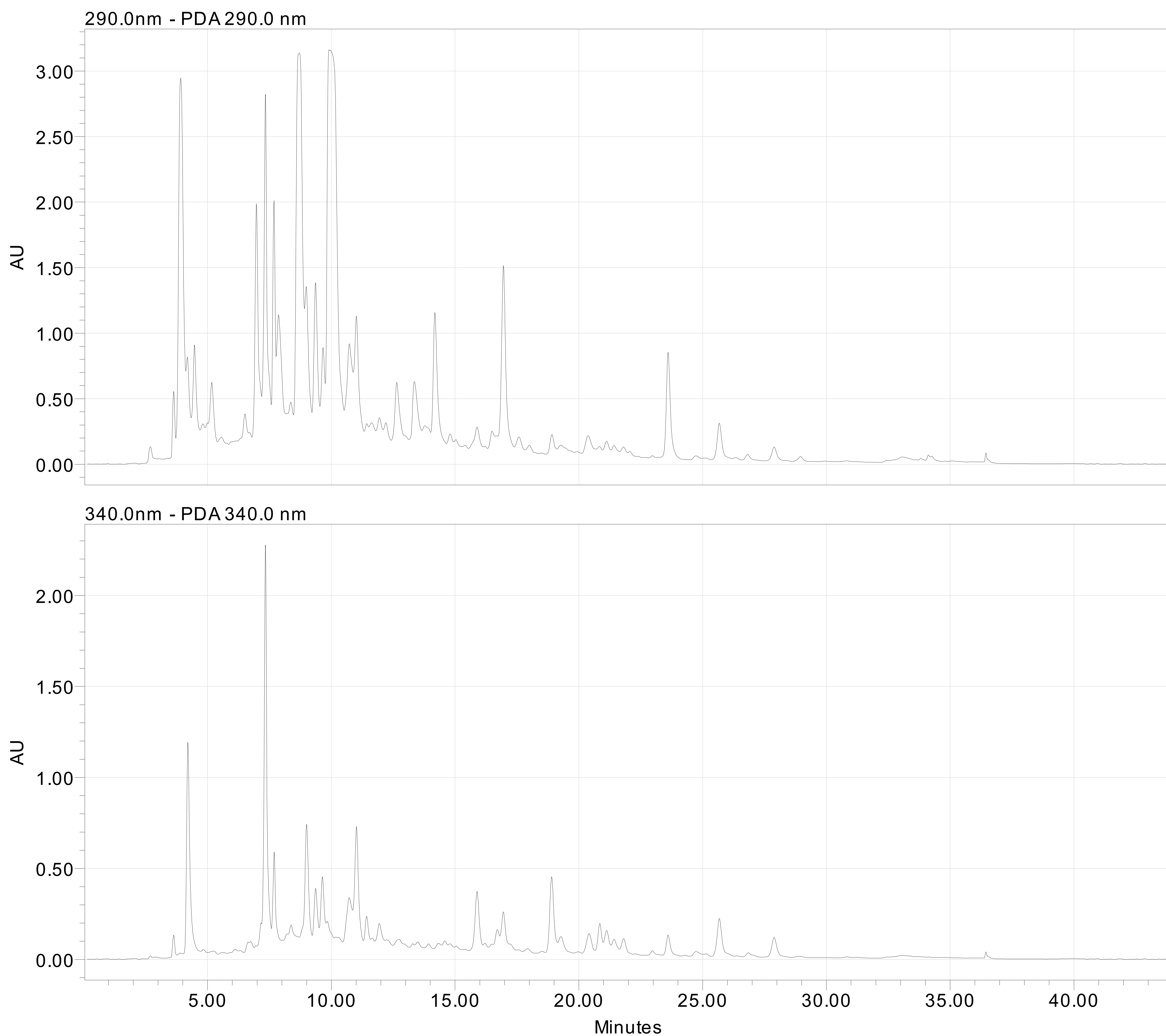
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**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



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Report Method ID 3207

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Date Printed:

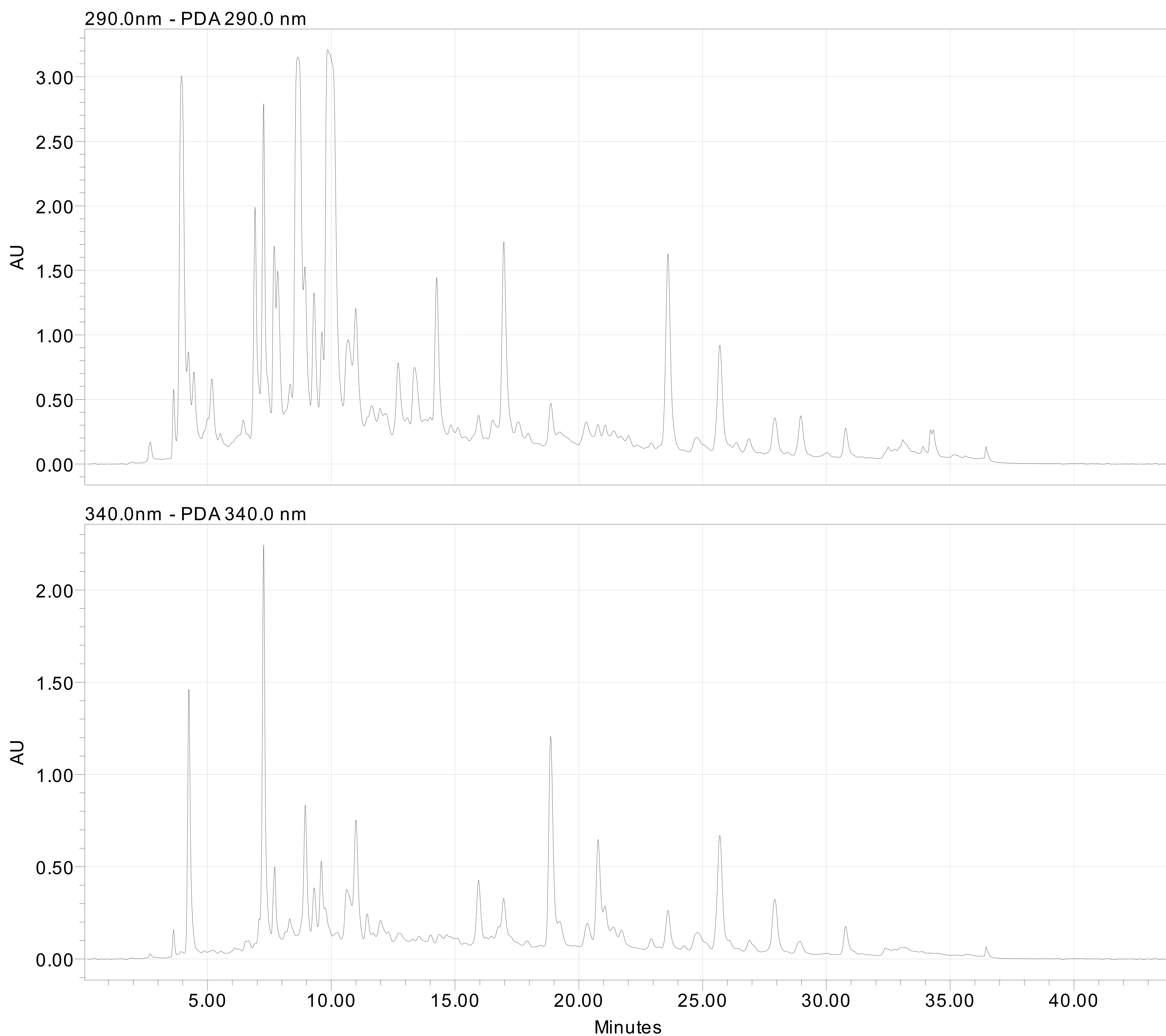
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**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



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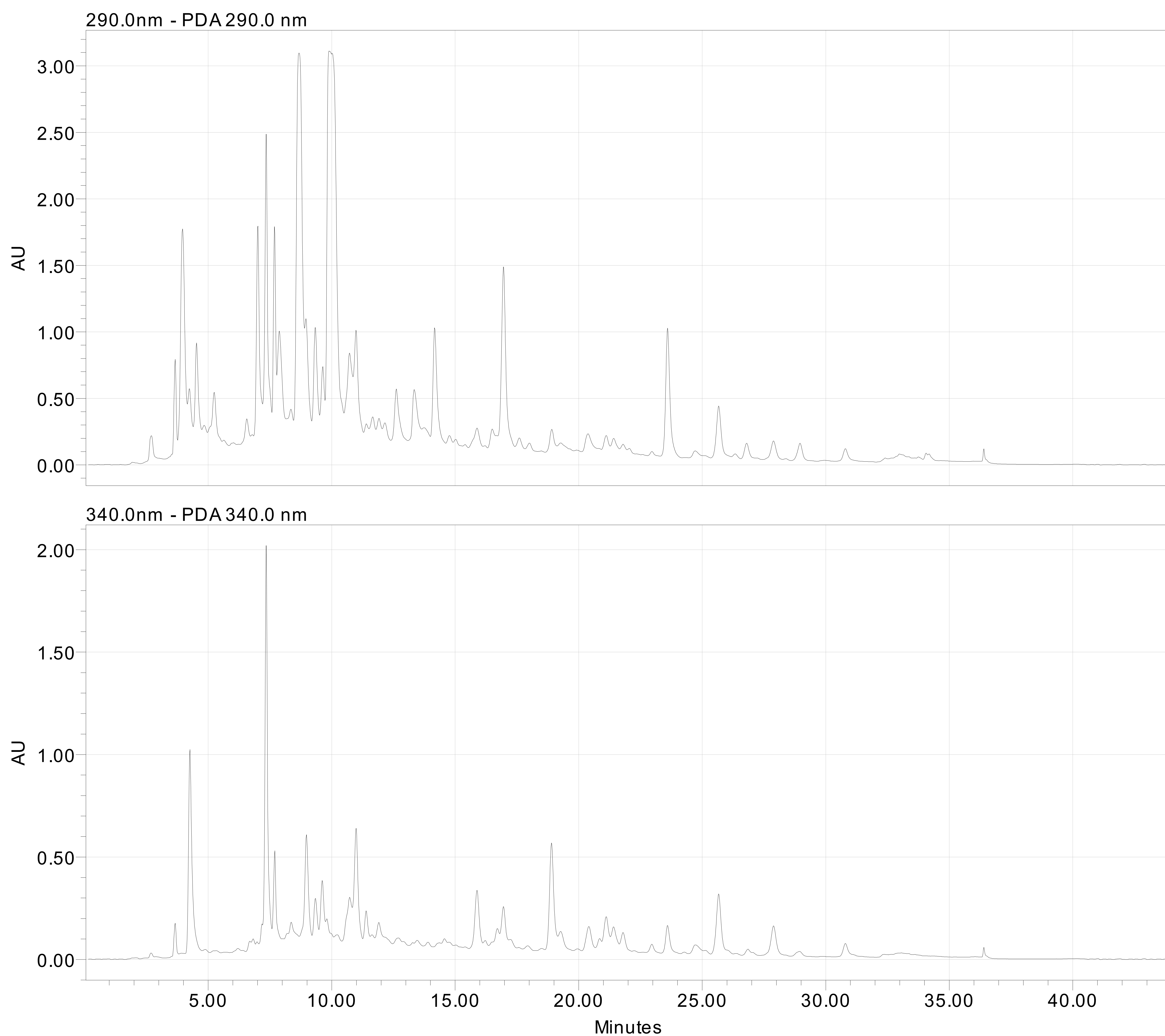
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**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 5/15/2008 3:07:10 PM NZST

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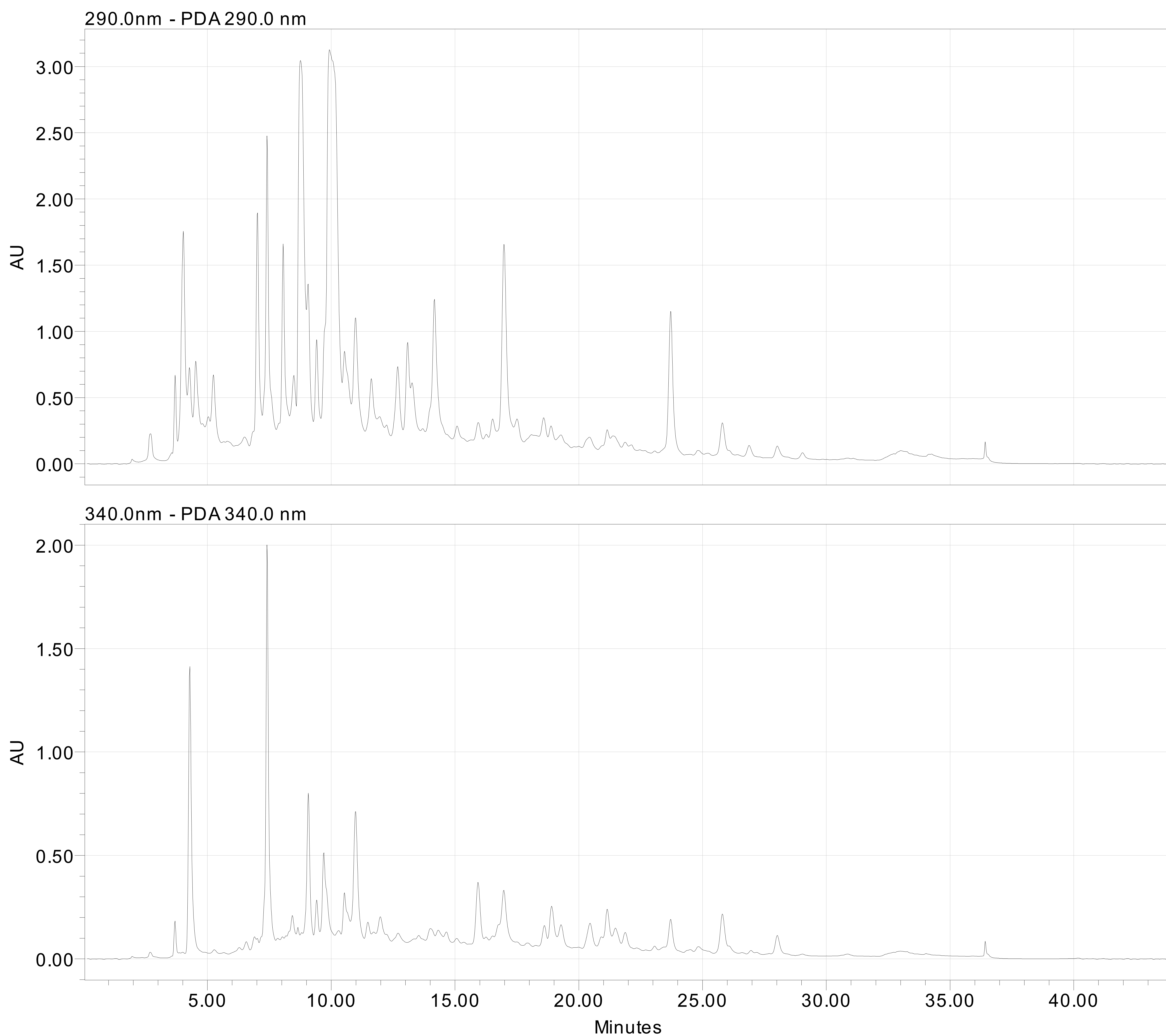
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**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



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Report Method ID 3207

Page: 1 of 1

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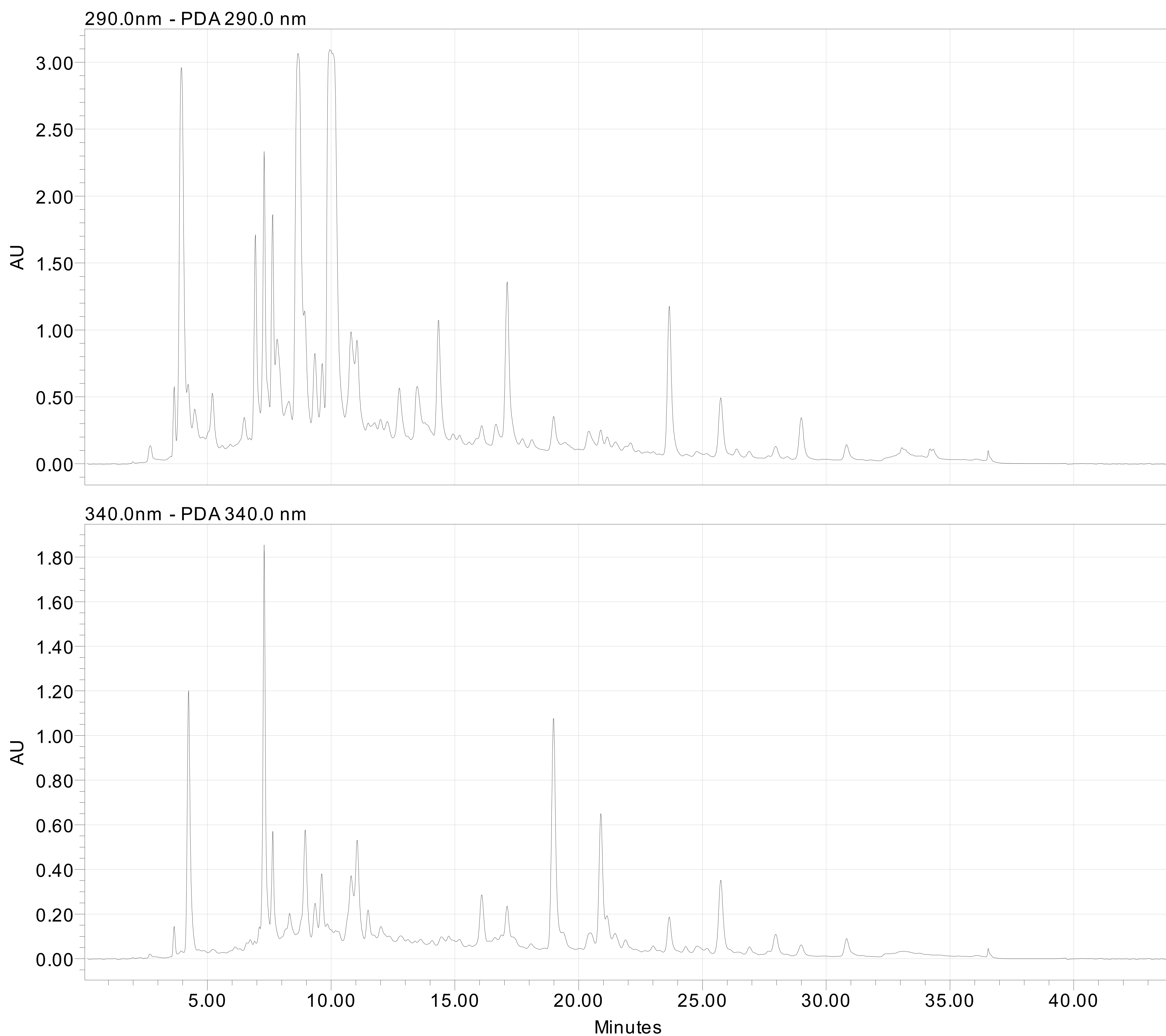
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Chromatograms



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Date Acquired: 5/20/2008 3:11:43 PM NZST

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Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

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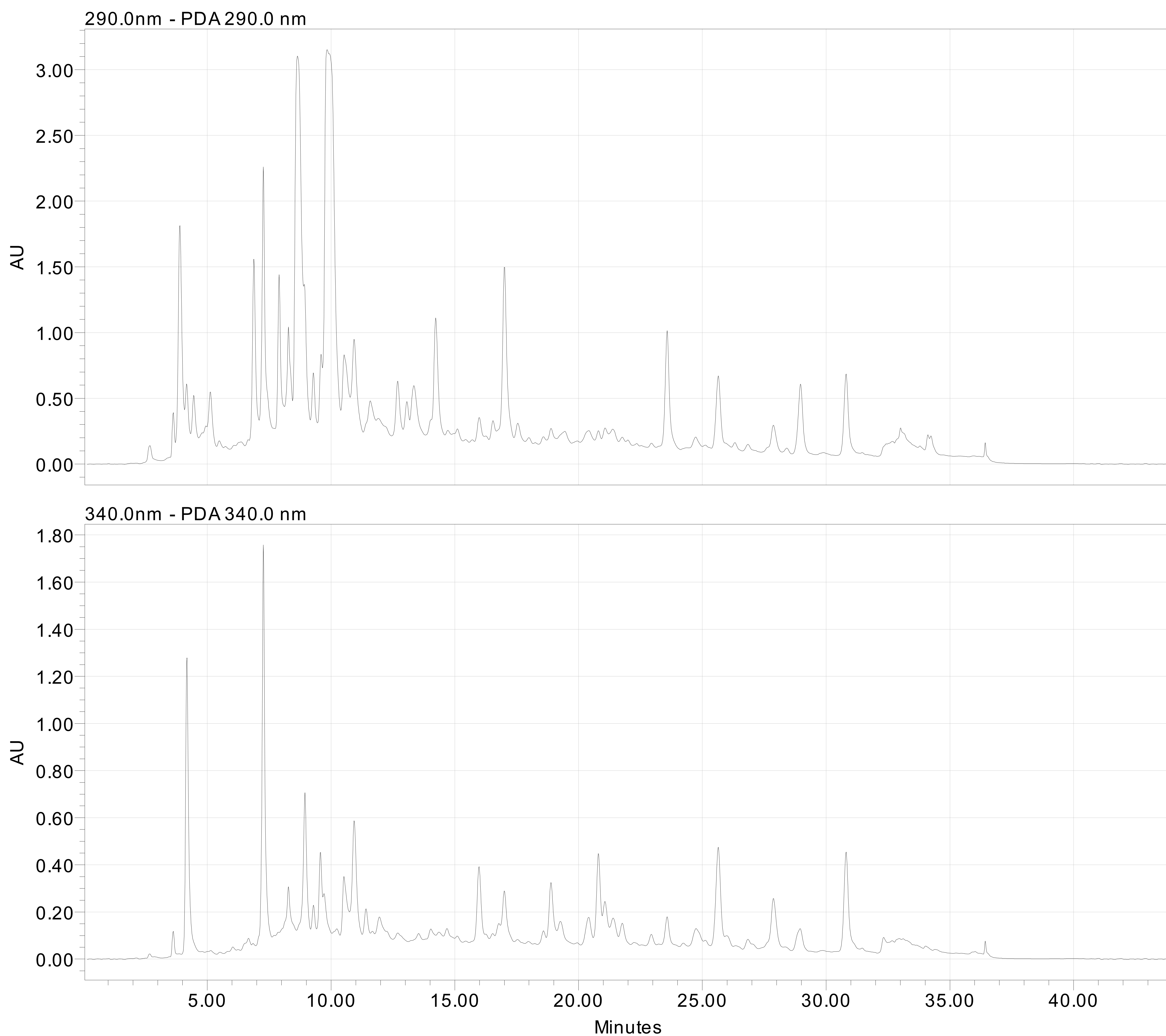
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Chromatograms



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Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

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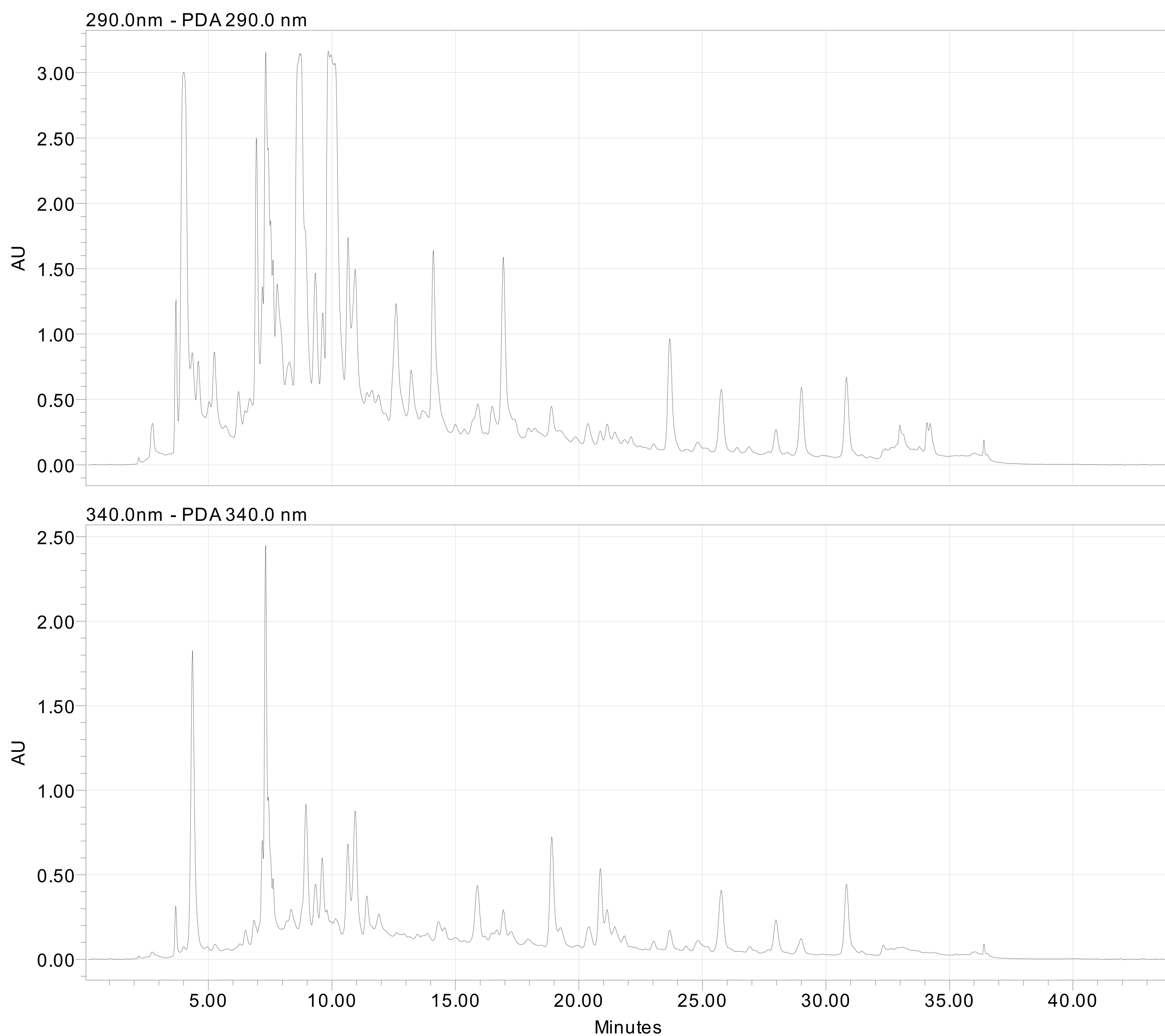
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Chromatograms

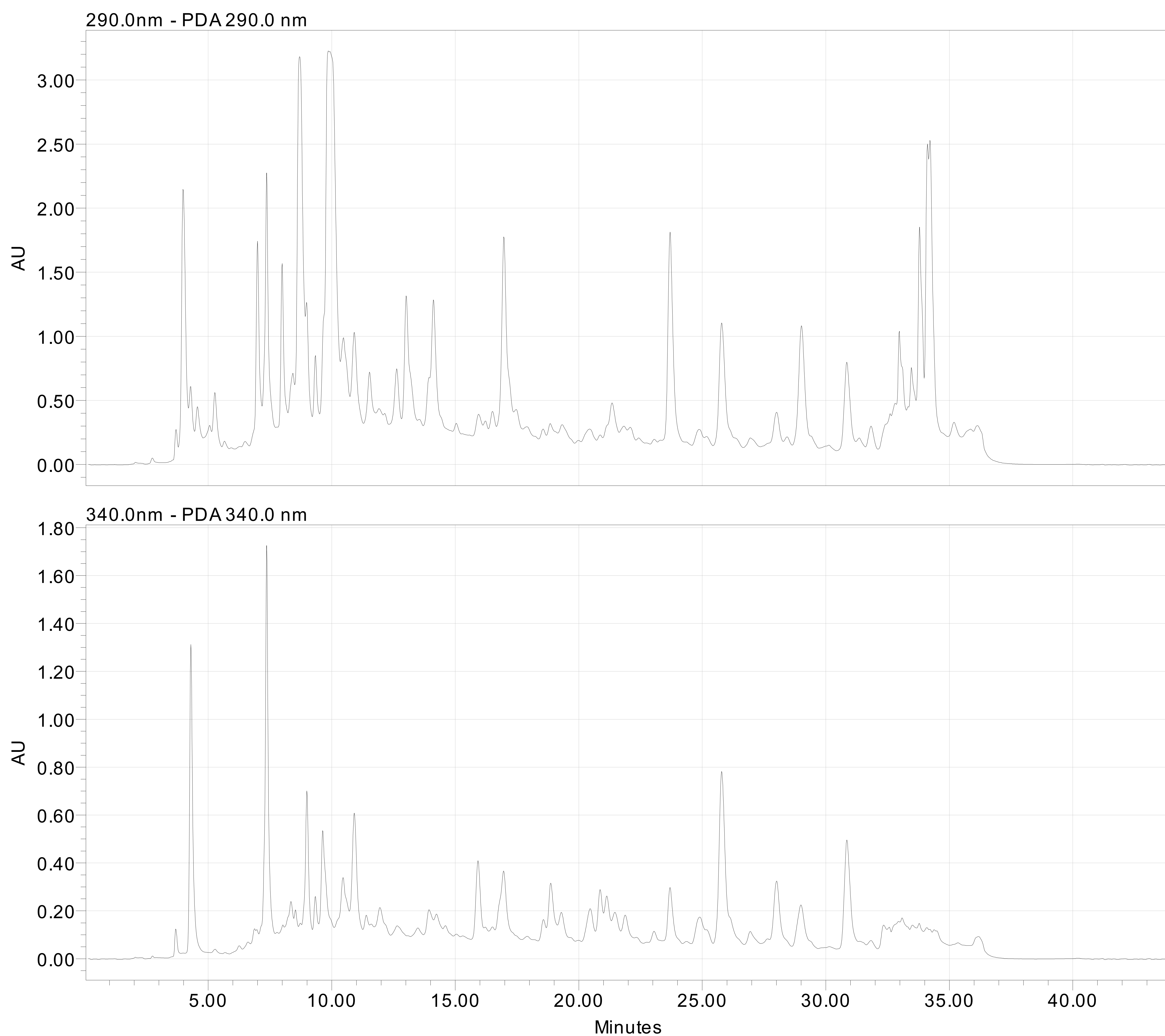


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Reported by User: System
Report Method: Honey Flavonoid Chromatogram
Report Method ID 3207
Page: 1 of 1

Project Name: Chris Adams\Ben
Date Printed: 3/31/2009
6:11:19 PM NZ

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SXAD2 5g****Testing the Effect of Sample Size on
Extraction Method****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 5/26/2008 1:35:02 PM NZST

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Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

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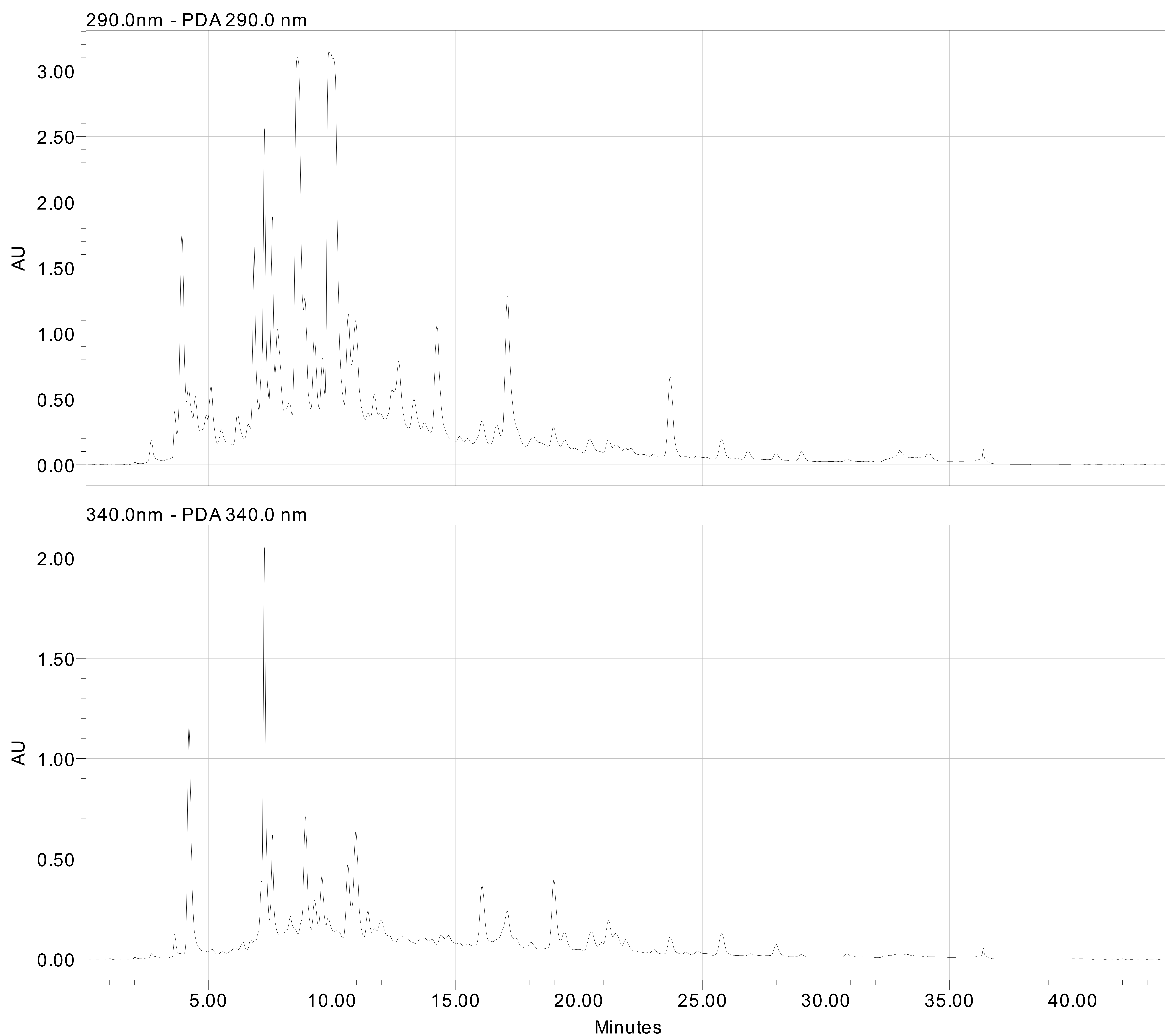
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**Sample Name: M110 160508A
LXAD2 5g**

**Testing the Effect of Sample Size on
Extraction Method**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 5/26/2008 12:45:58 PM NZST

Instrument Method Name: Chris OPD 2007

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

6:14:33 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman



THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

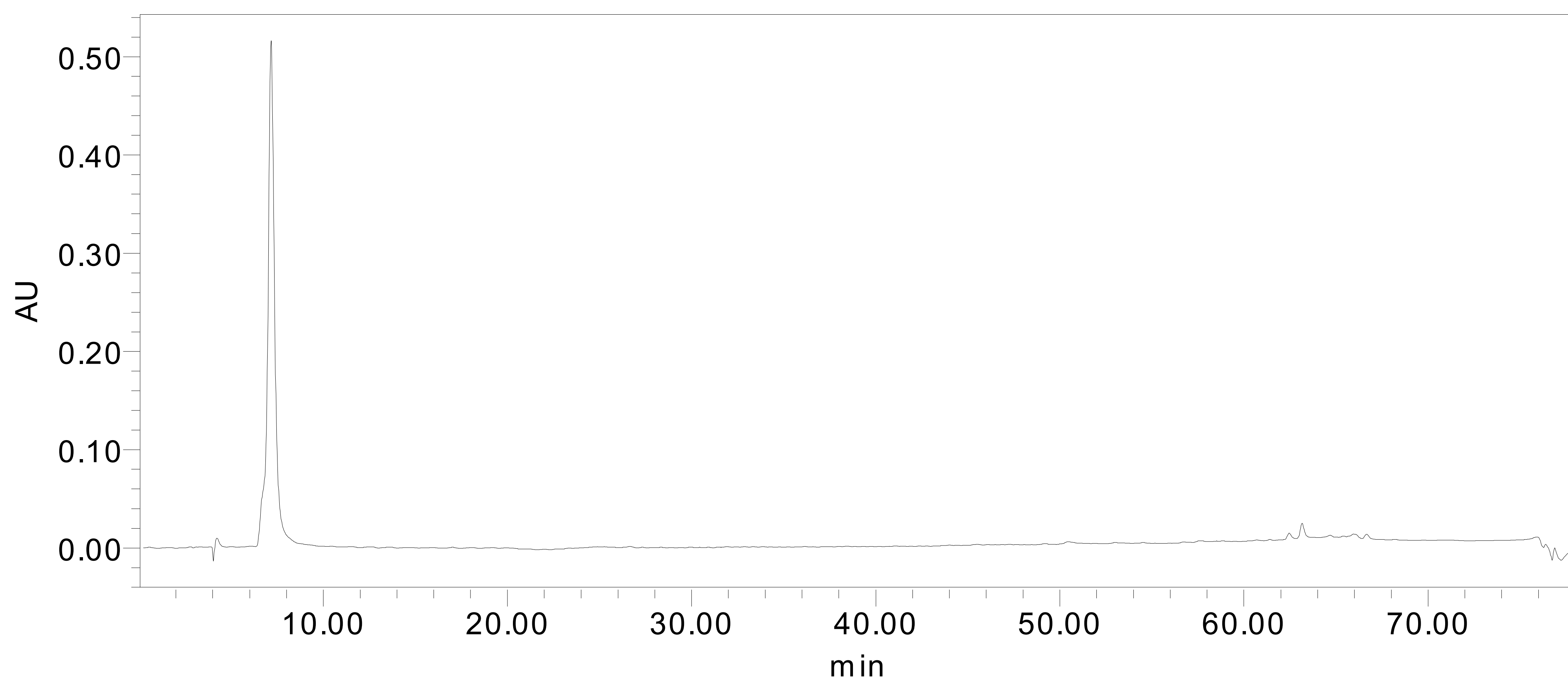
The University of Waikato

2009

S A M P L E I N F O R M A T I O N

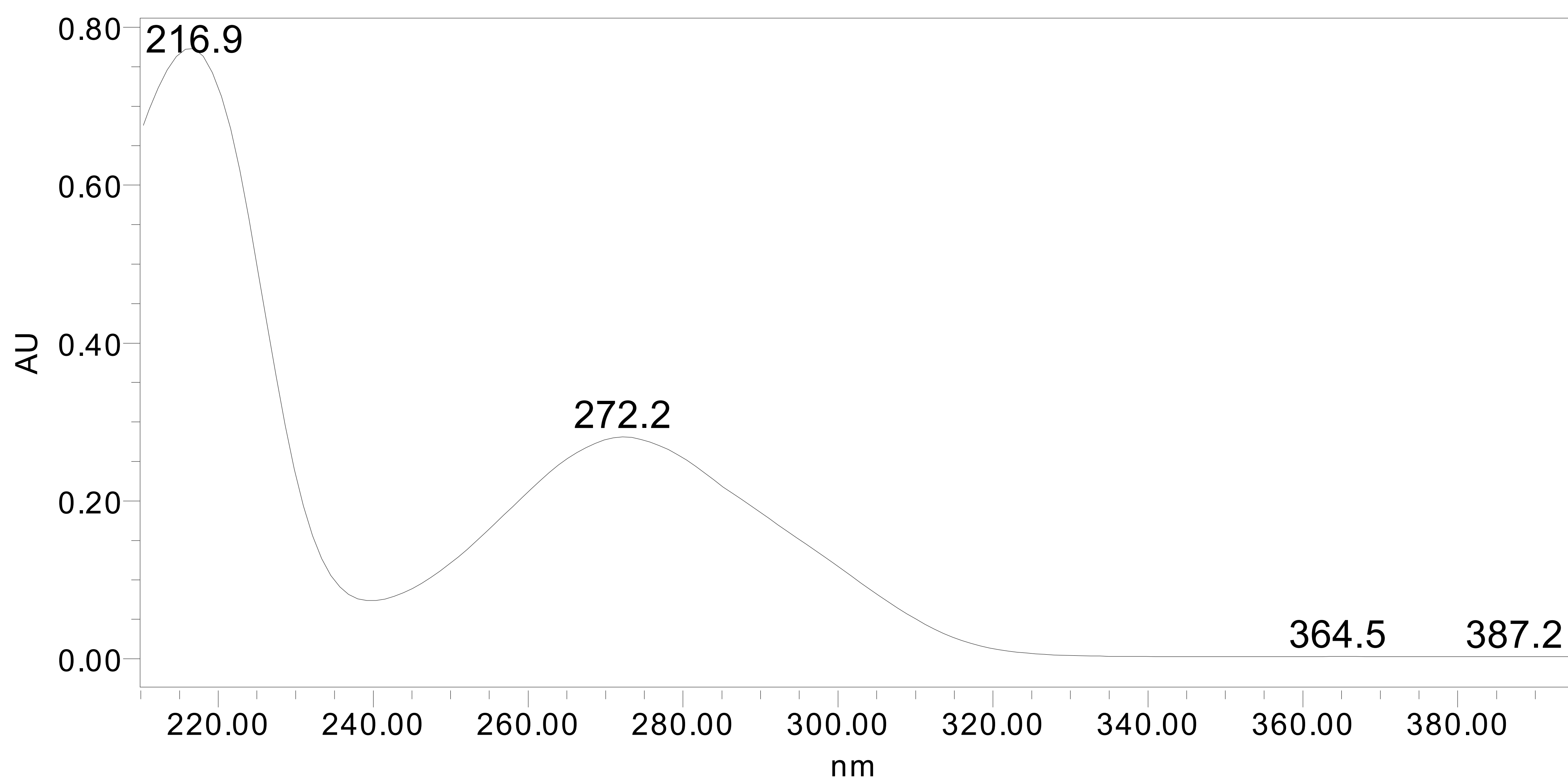
Sample Name: Gallic Acid	Acquired By: System
Sample Type: Standard	Date Acquired: 12/10/2008 1:01:32 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 2	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

290nm Chromatogram



—— 996 Extraction at 290 nm

UV Absorption Spectrum

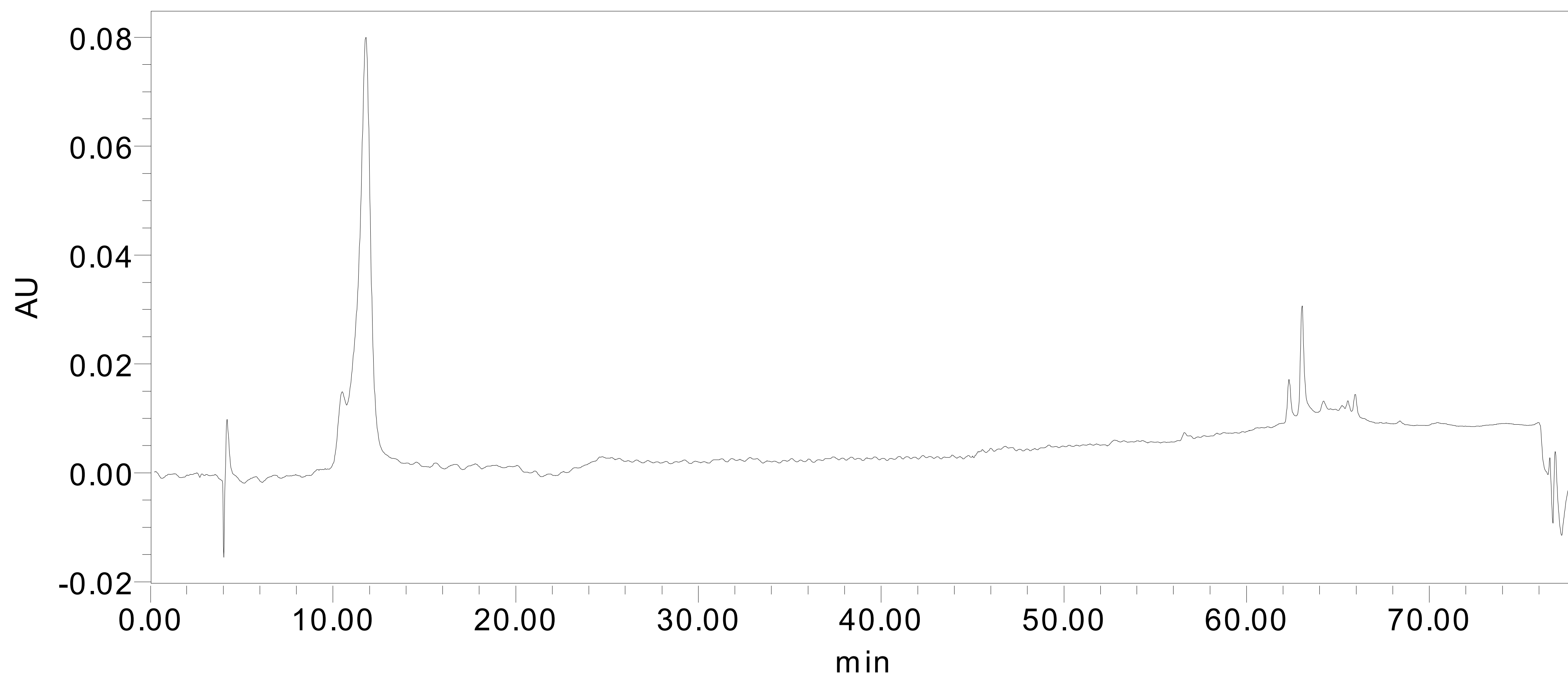


—— Time: 7.400 min

S A M P L E I N F O R M A T I O N

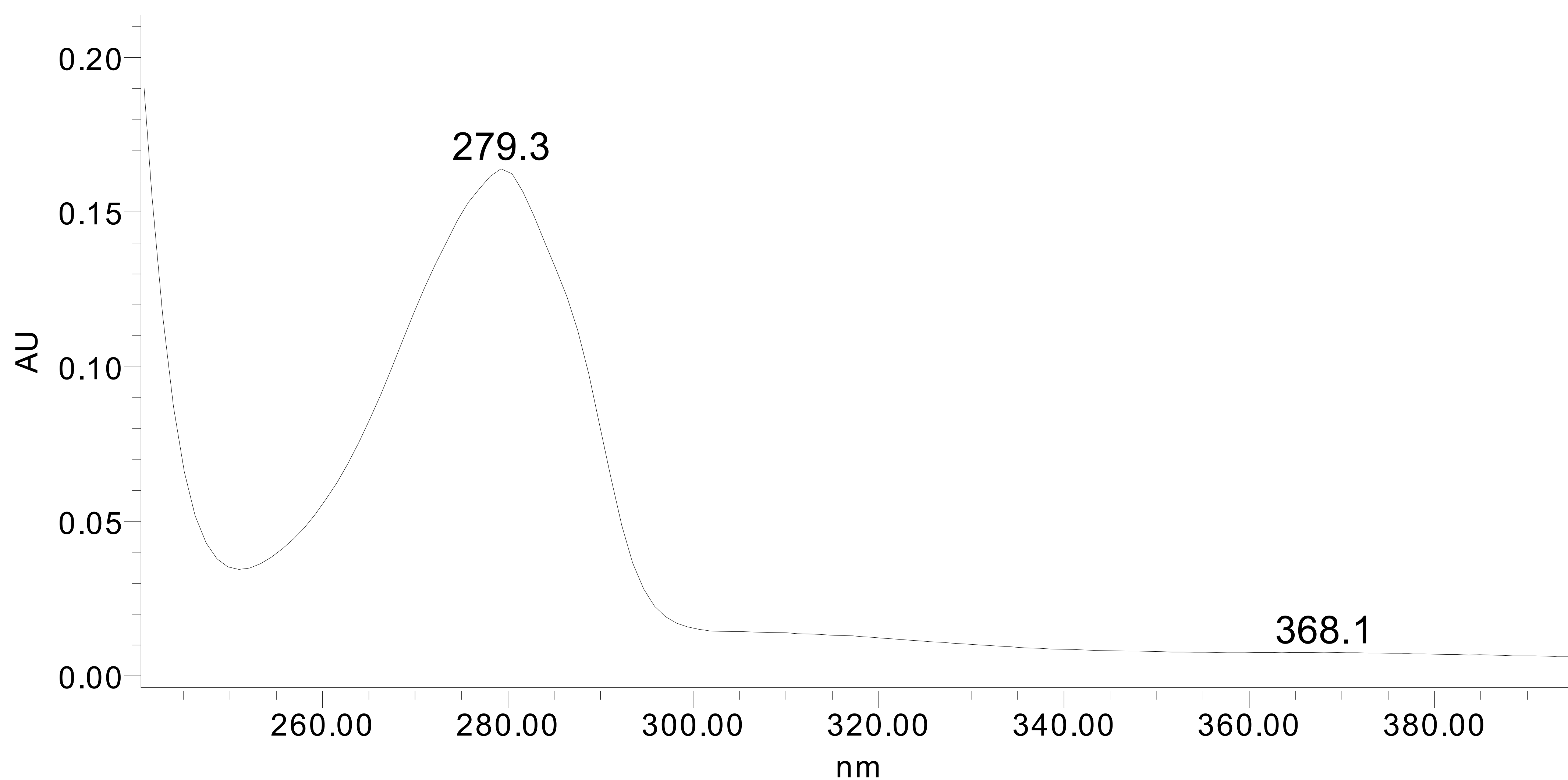
Sample Name: (+)-Catechin Hydrate Sample Type: Unknown Vial: 1 Injection #: 1 Injection Volume: 5.00 ul Run Time: 78.0 Minutes Sample Set Name:	Acquired By: System Date Acquired: 1/14/2009 11:20:42 AM NZDT Acq. Method Set: Symmetry MeOH 3 a Date Processed: **** Processing Method: **** Channel Name: **** Proc. Chnl. Descr.: ****
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290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum

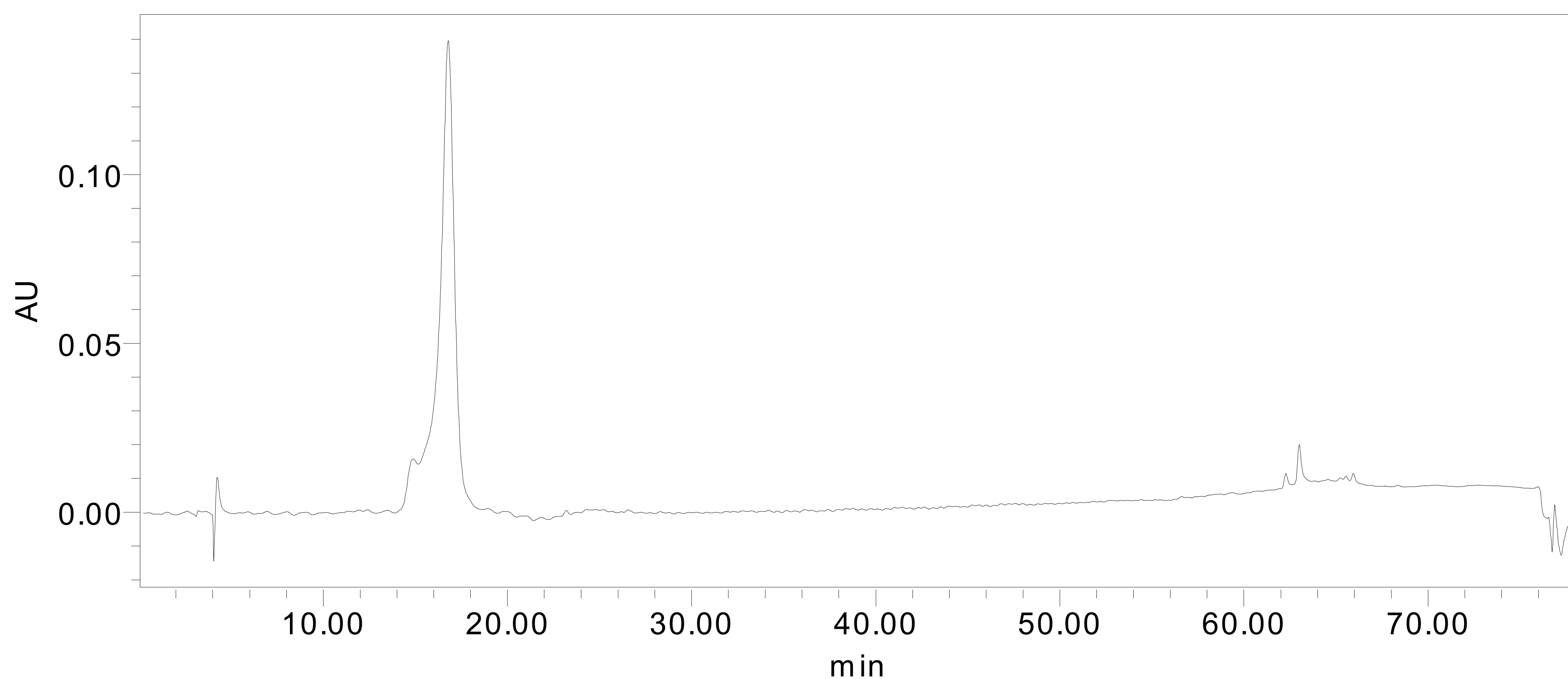


— Time: 11.780 min

SAMPLE INFORMATION

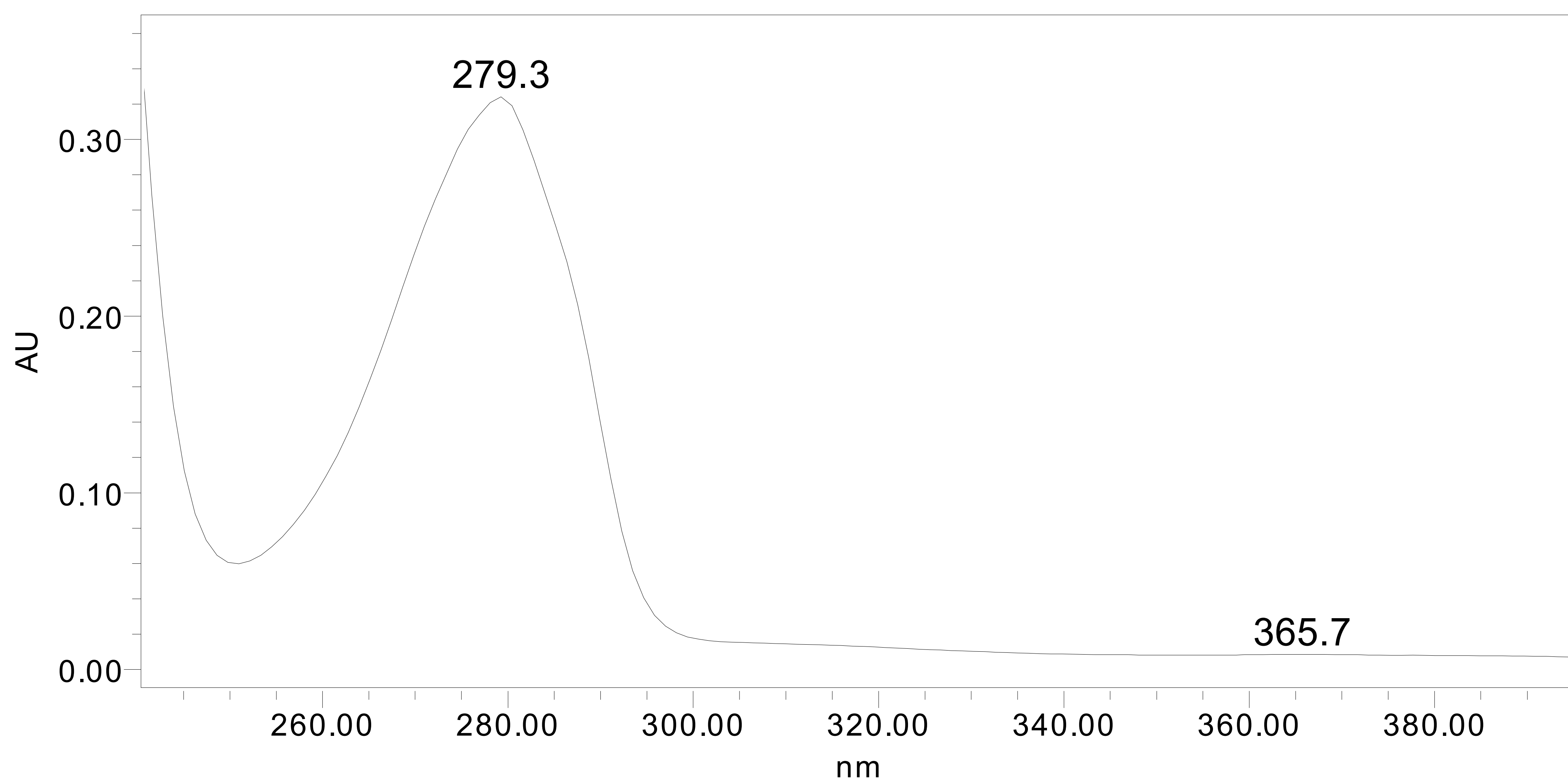
Sample Name: (-)-Epicatechin	Acquired By: System
Sample Type: Unknown	Date Acquired: 1/14/2009 12:43:25 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 2	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum

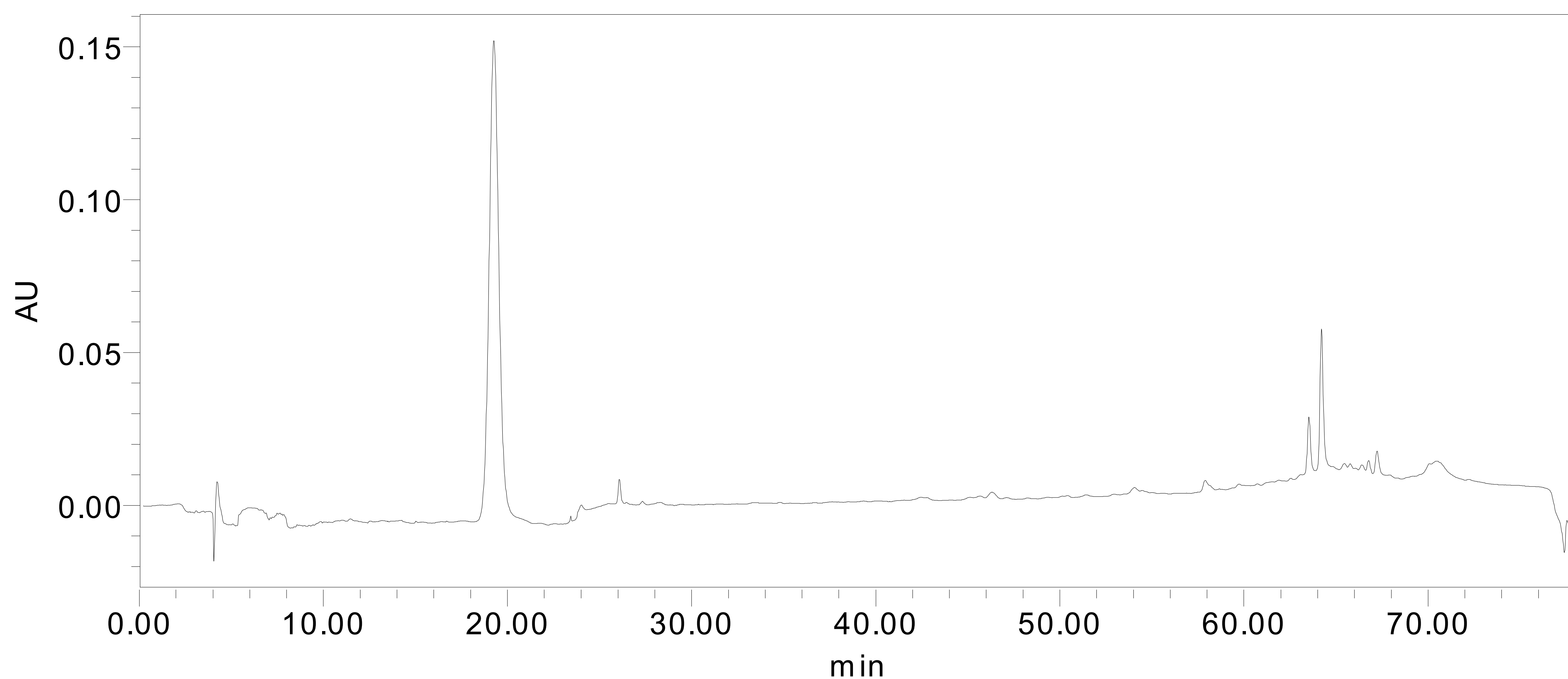


— Time: 16.800 min

SAMPLE INFORMATION

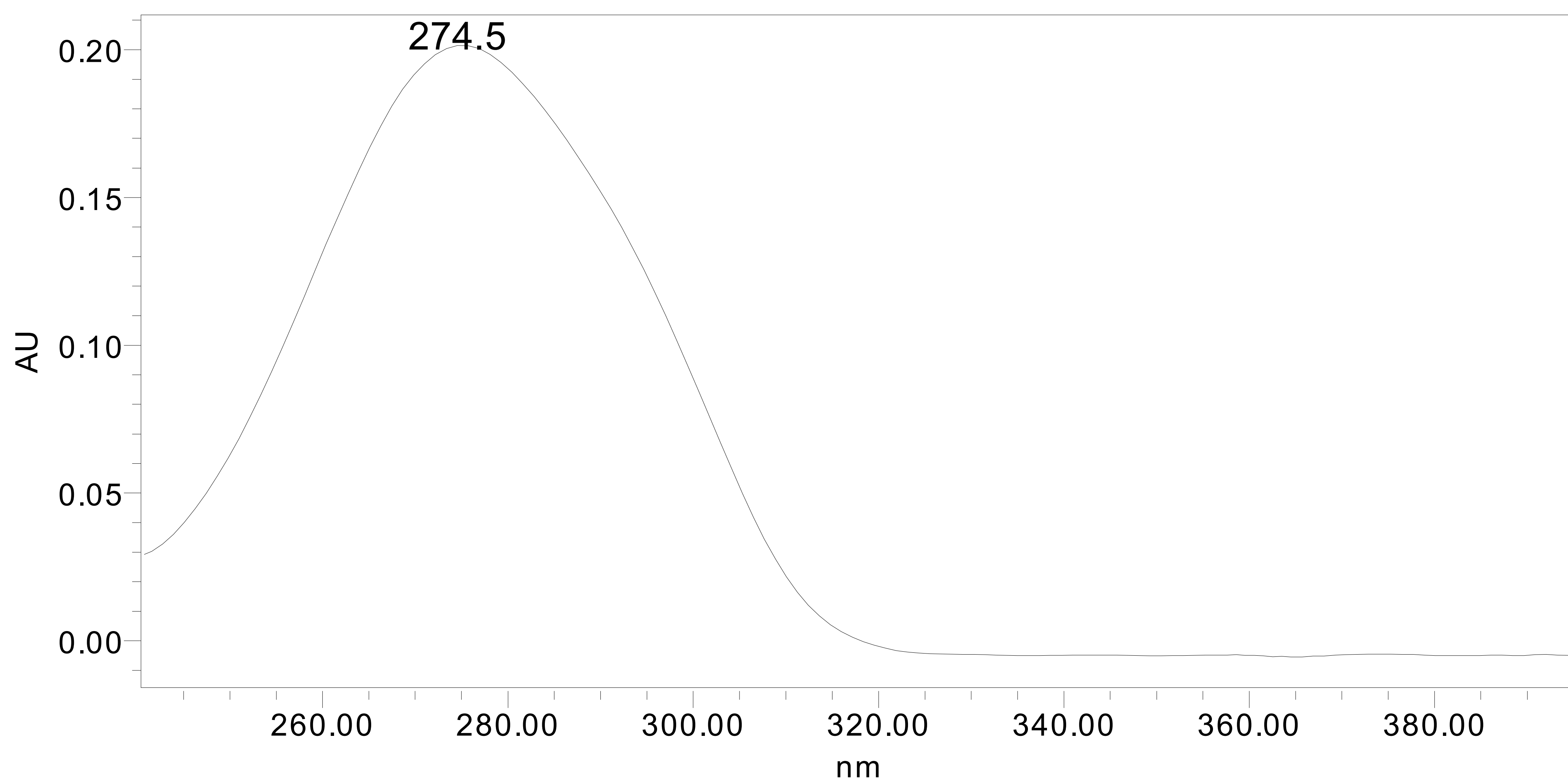
Sample Name:	Syringic acid	Acquired By:	System
Sample Type:	Standard	Date Acquired:	3/12/2009 10:56:15 AM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	1	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum

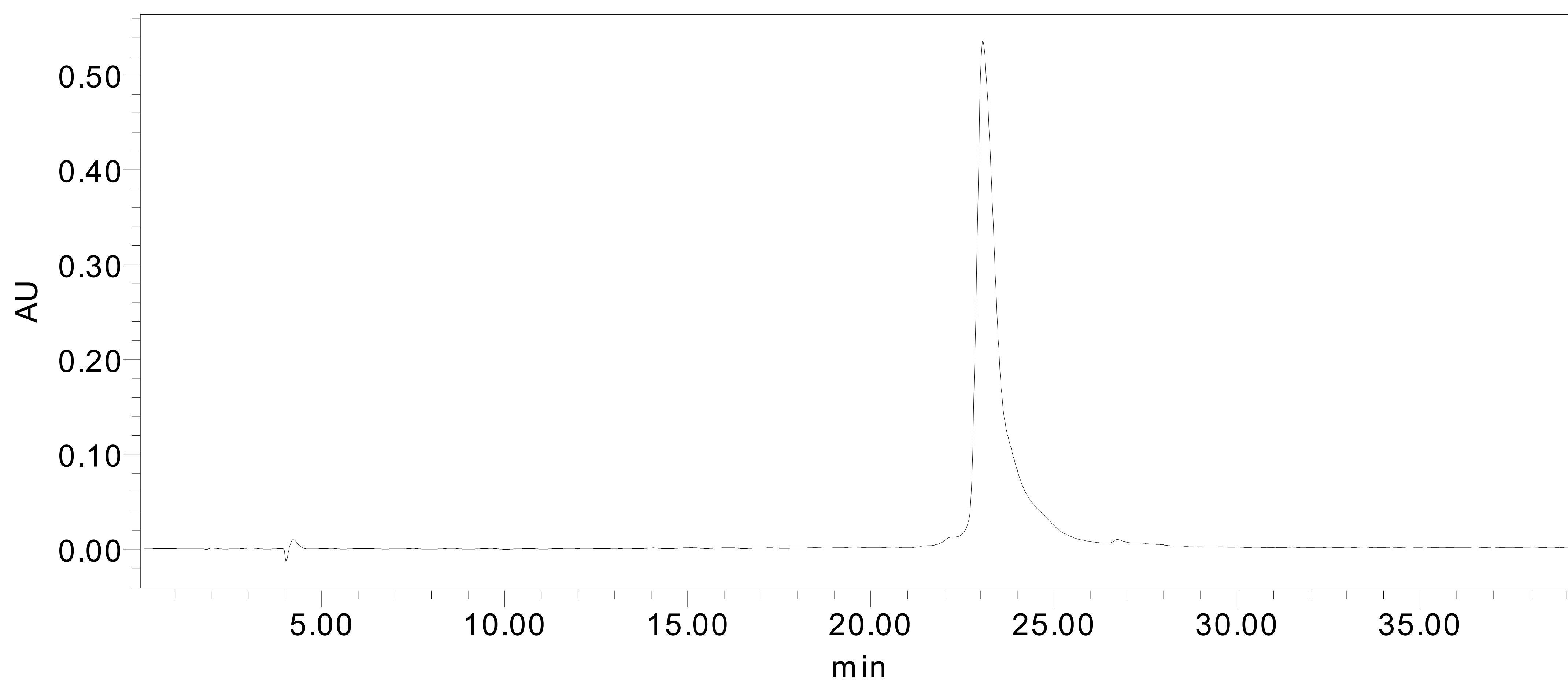


— Time: 19.270 min

SAMPLE INFORMATION

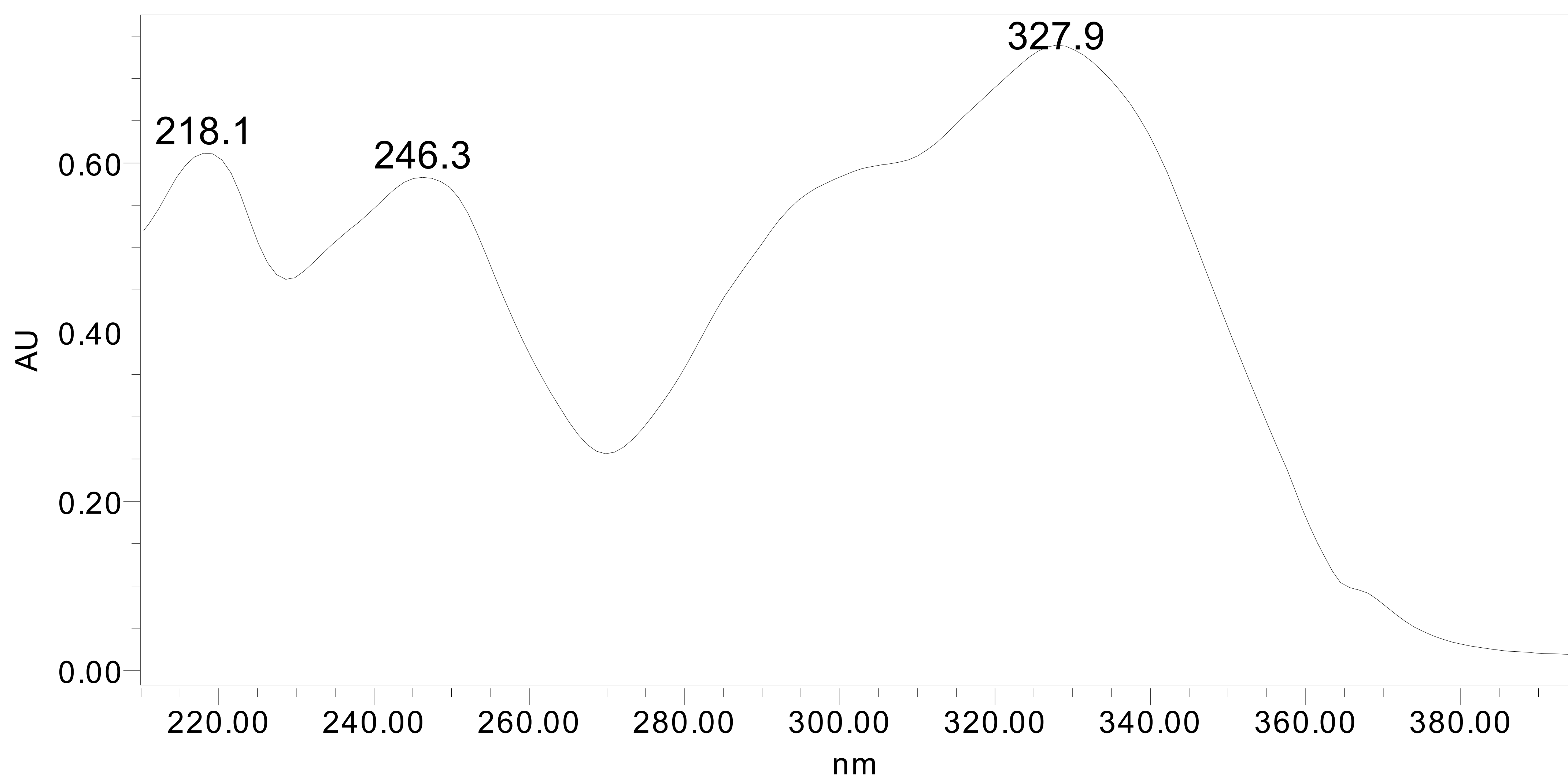
Sample Name:	Chlorogenic Acid	Acquired By:	System
Sample Type:	Standard	Date Acquired:	12/9/2008 3:37:05 PM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	2	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

290nm Chromatogram



996 Extraction at 290 nm

UV Absorption Spectrum

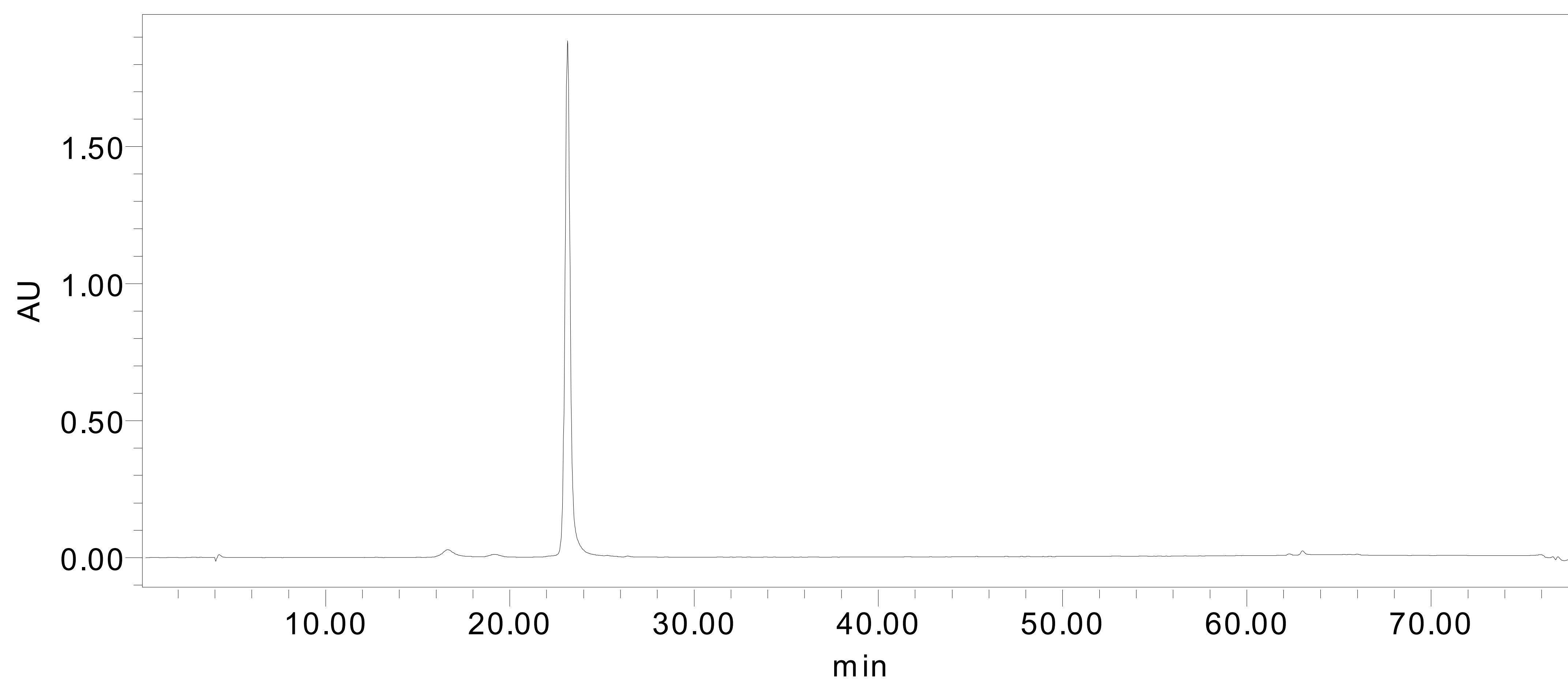


Time: 23.000 min

SAMPLE INFORMATION

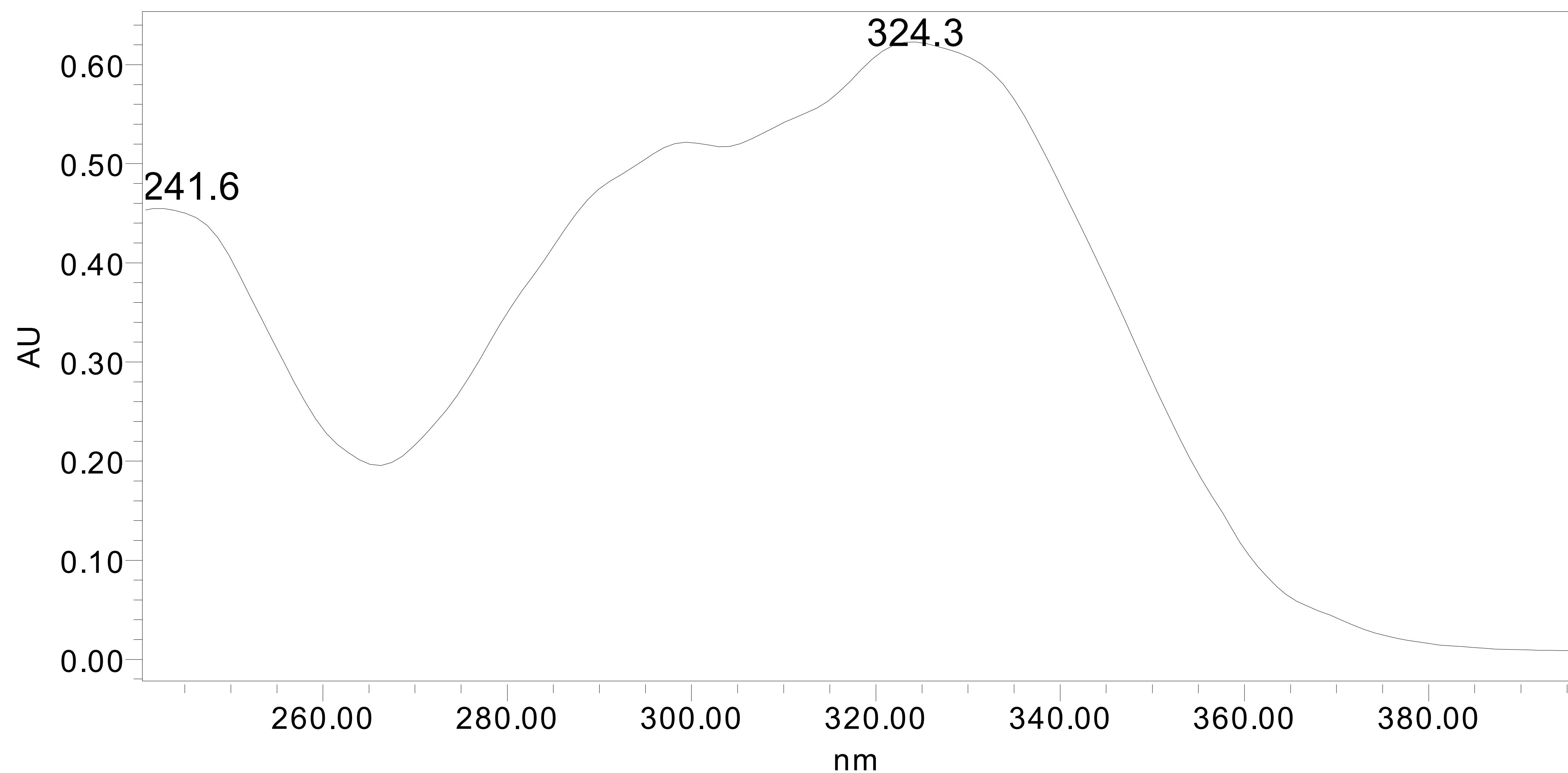
Sample Name:	Caffeic Acid	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/8/2009 2:21:43 PM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	3	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

290nm Chromatogram



996 Extraction at 290 nm

UV Absorption Spectrum

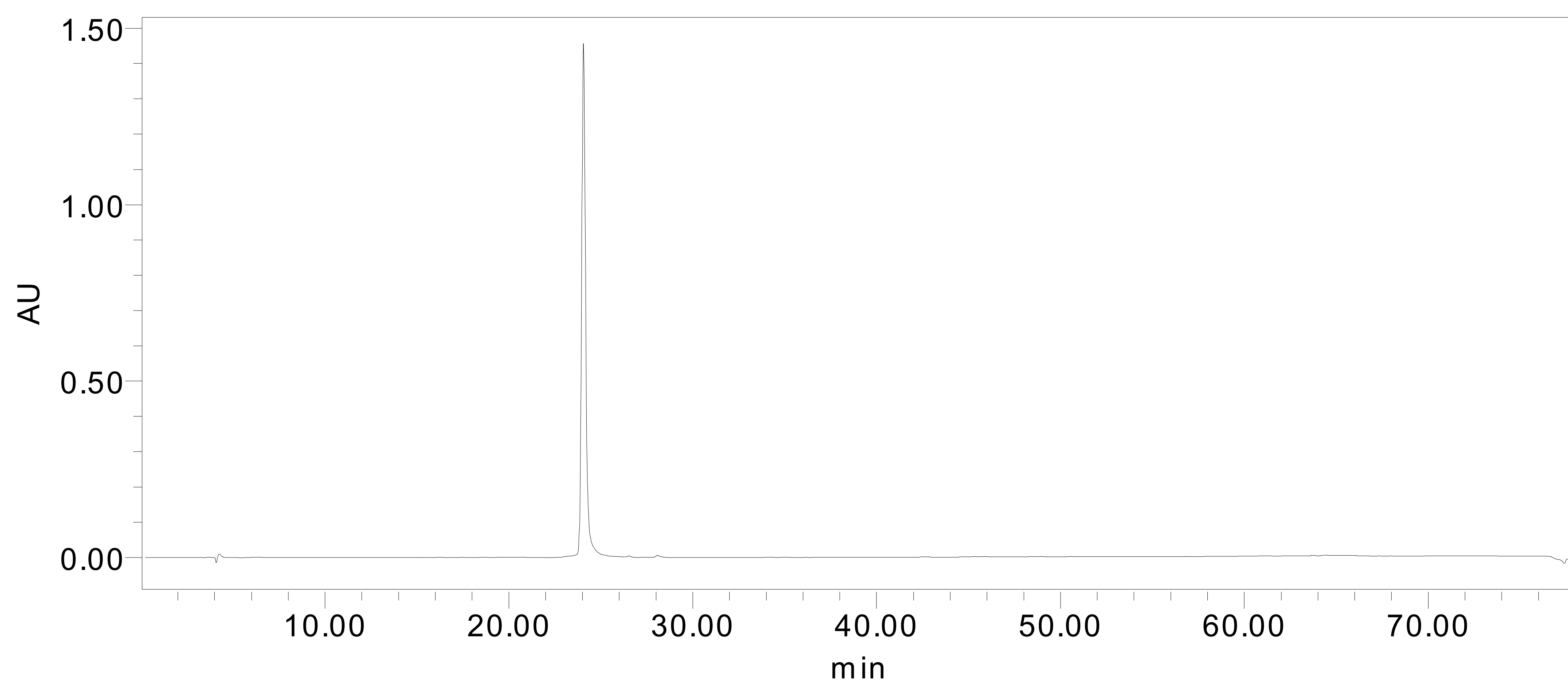


Time: 23.350 min

SAMPLE INFORMATION

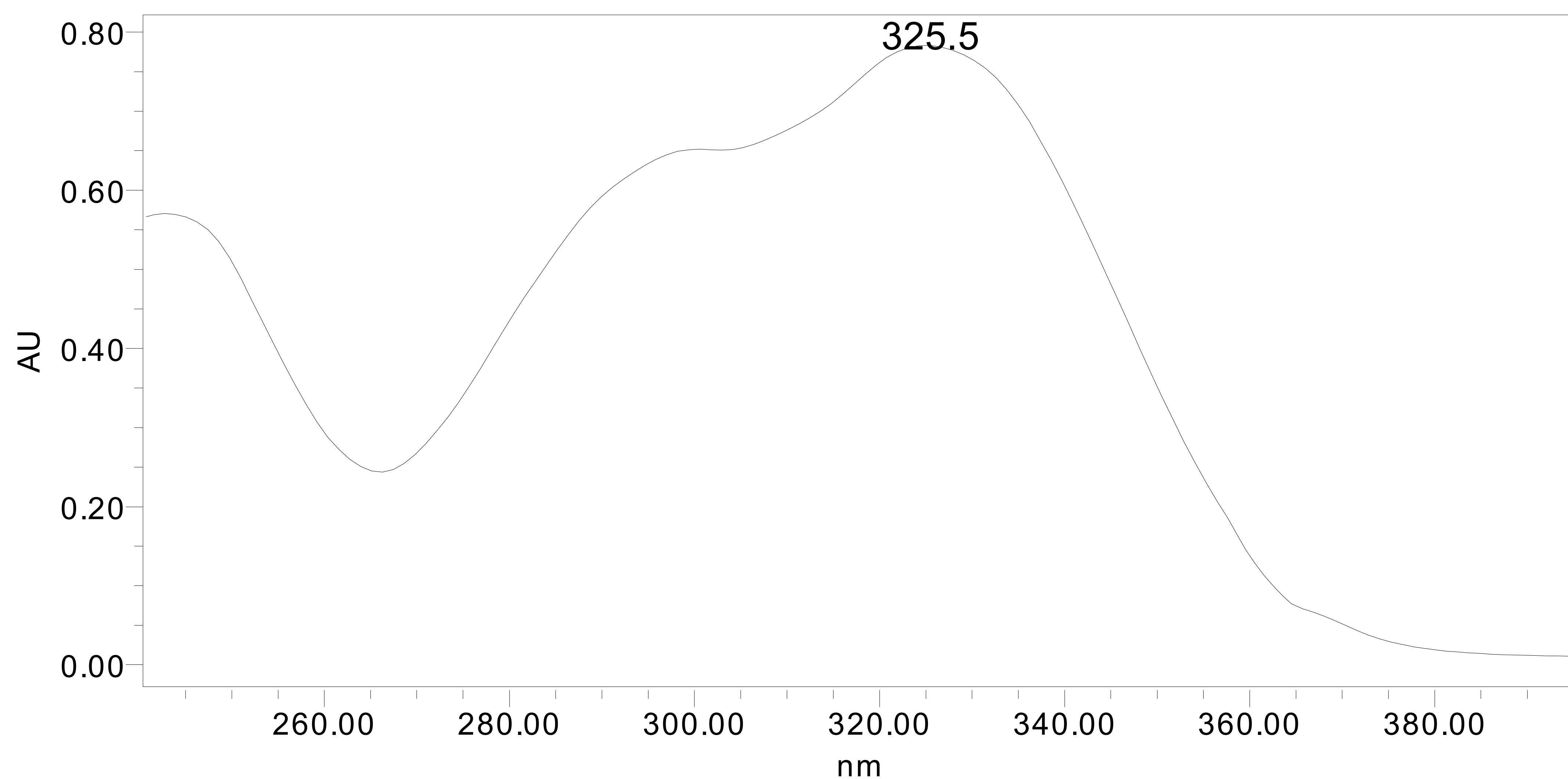
Sample Name:	3,4-Dihydroxycinnamic acid	Acquired By:	System
Sample Type:	Standard	Date Acquired:	3/11/2009 2:04:12 PM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	2	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

340nm Chromatogram



996 Extraction at 340 nm

UV Absorption Spectrum

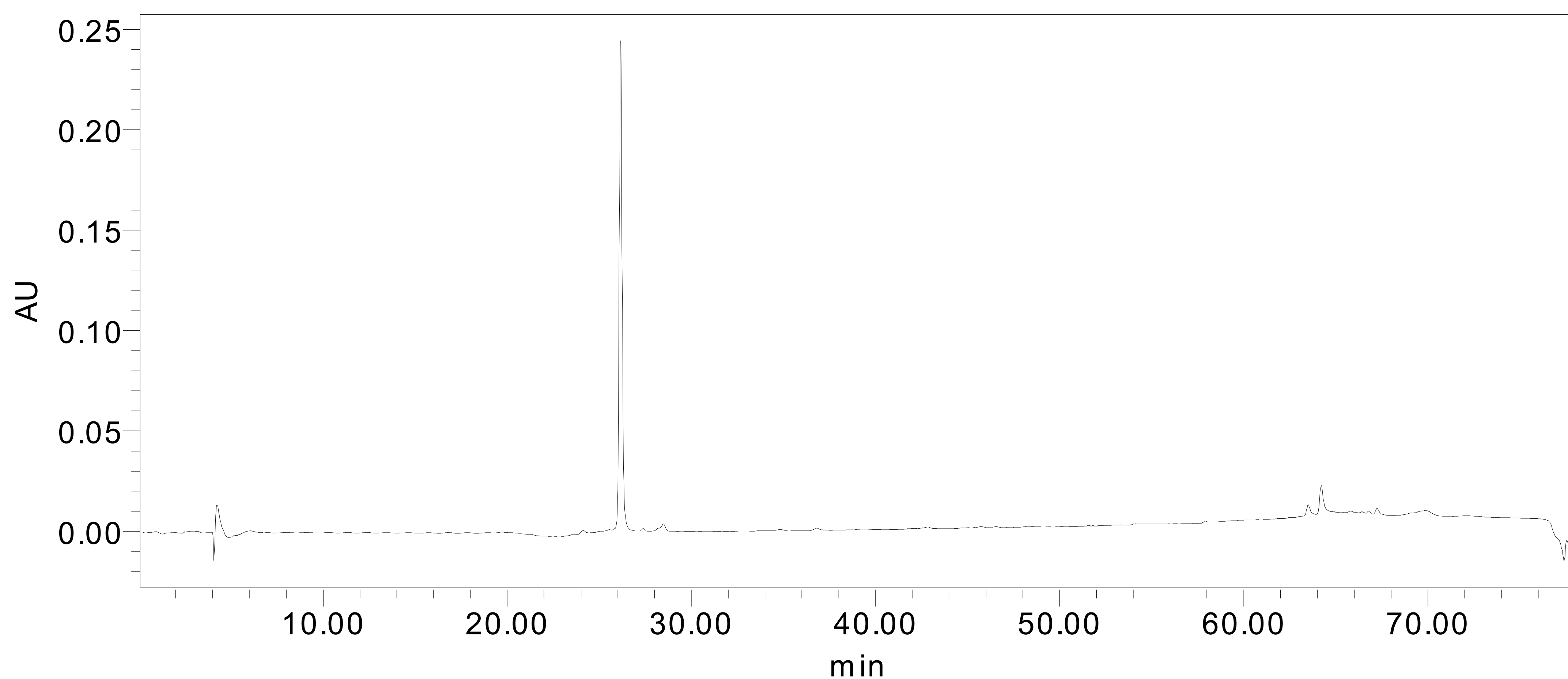


Time: 24.190 min

SAMPLE INFORMATION

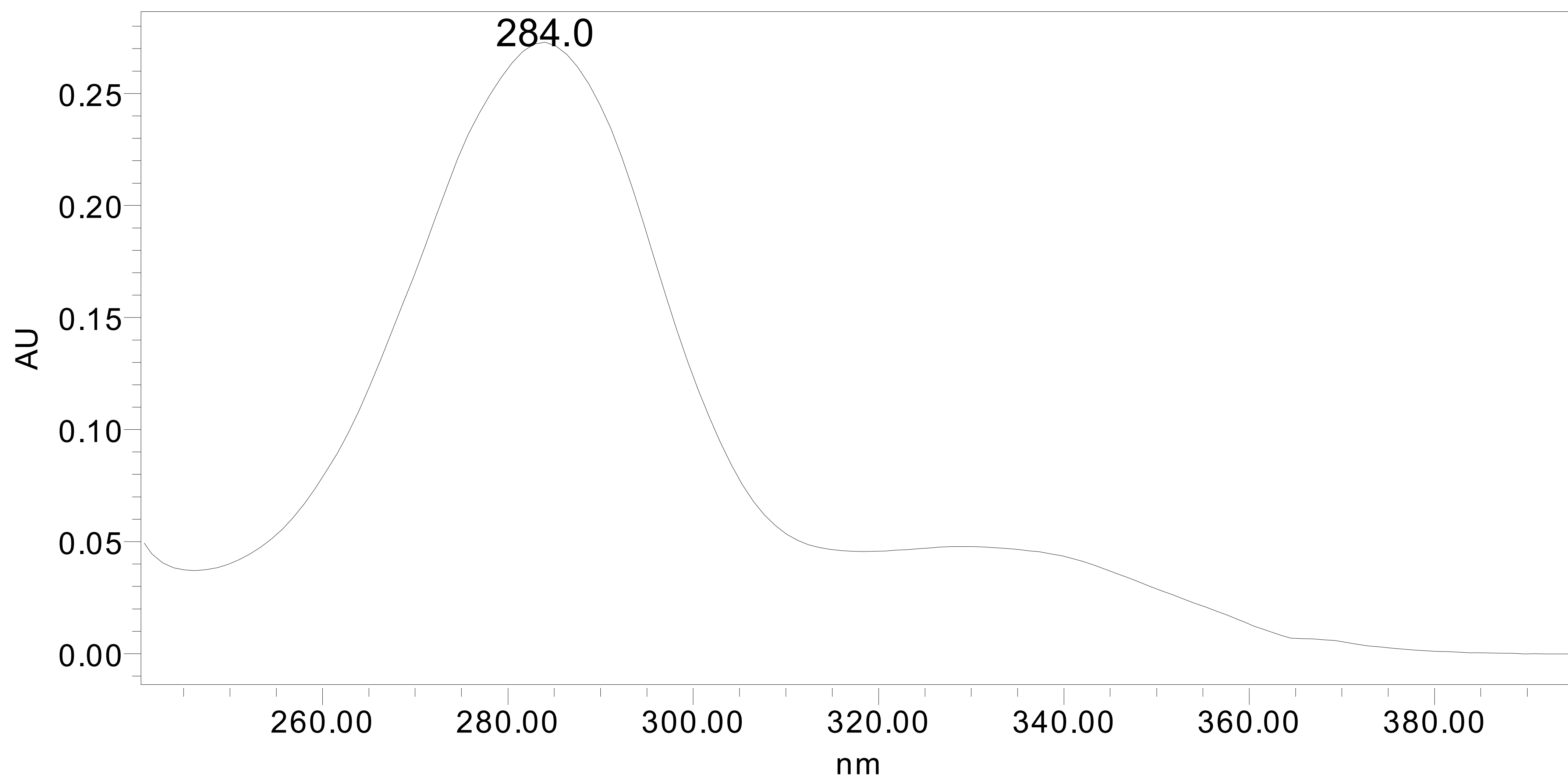
Sample Name:	Naringin	Acquired By:	System
Sample Type:	Standard	Date Acquired:	3/12/2009 12:19:43 PM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	2	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

290nm Chromatogram



996 Extraction at 290 nm

UV Absorption Spectrum

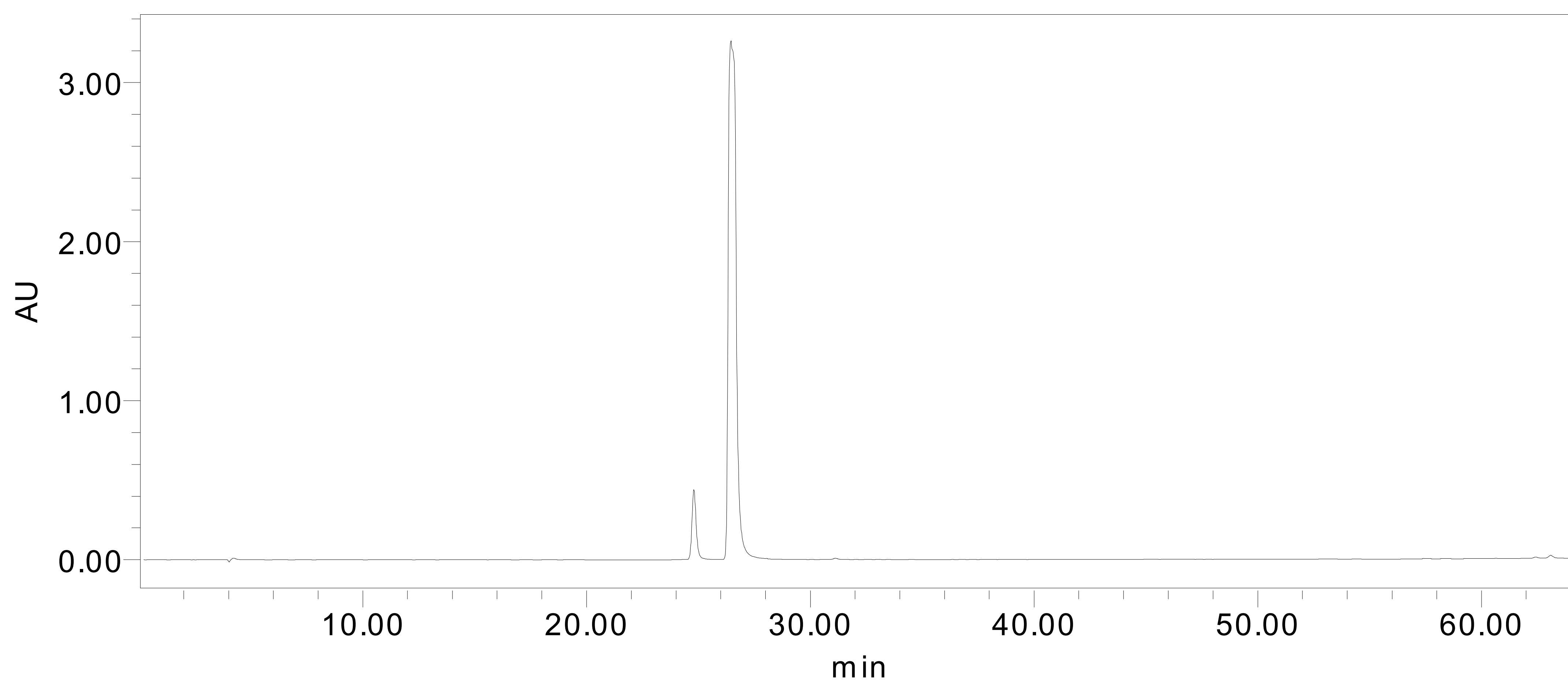


Time: 26.150 min

S A M P L E I N F O R M A T I O N

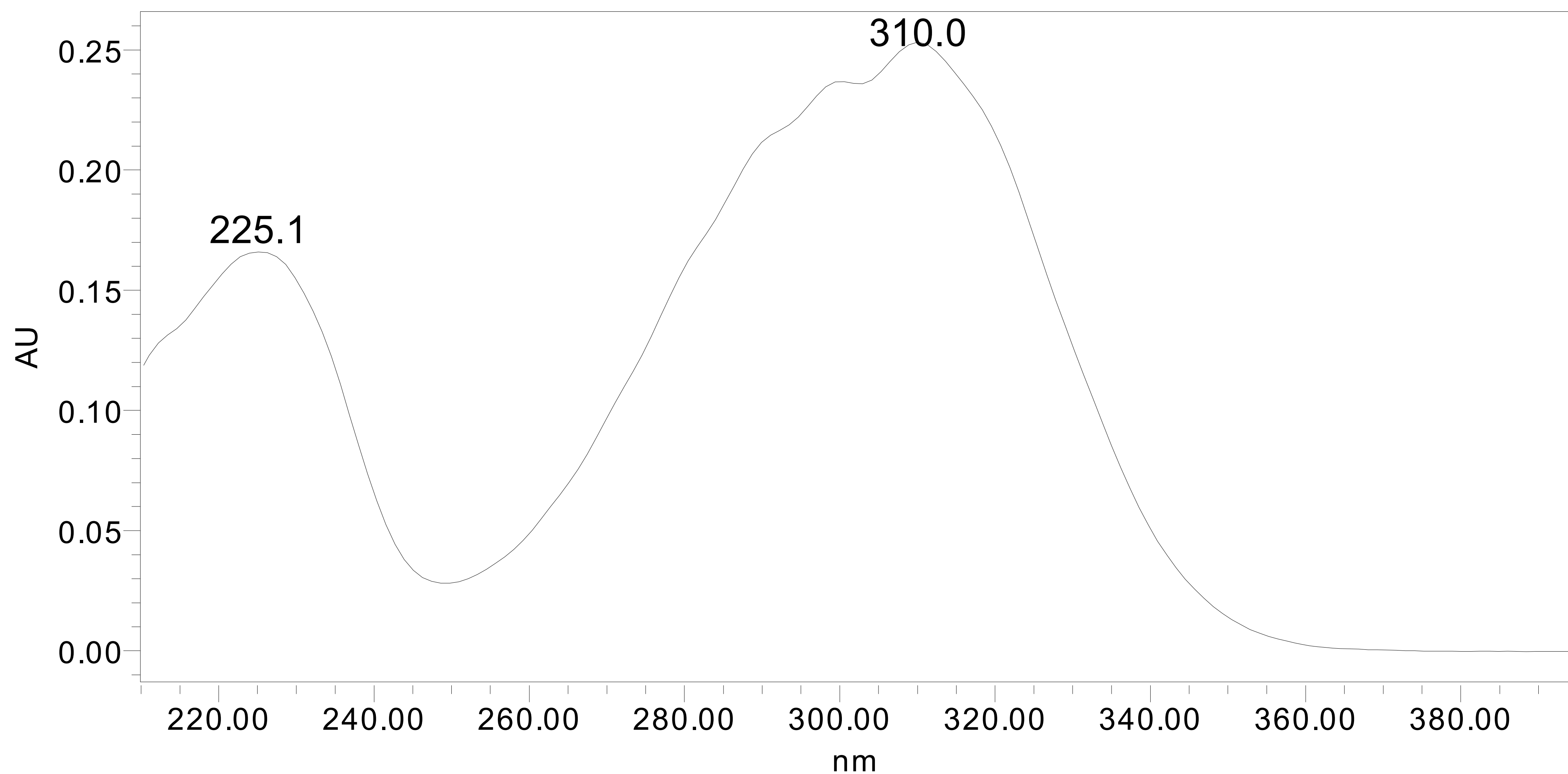
Sample Name: p-Coumaric Acid Sample Type: Standard Vial: 1 Injection #: 3 Injection Volume: 5.00 ul Run Time: 78.0 Minutes Sample Set Name:	Acquired By: System Date Acquired: 12/10/2008 3:17:38 PM NZDT Acq. Method Set: Symmetry MeOH 3 a Date Processed: **** Processing Method: **** Channel Name: **** Proc. Chnl. Descr.: ****
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290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum

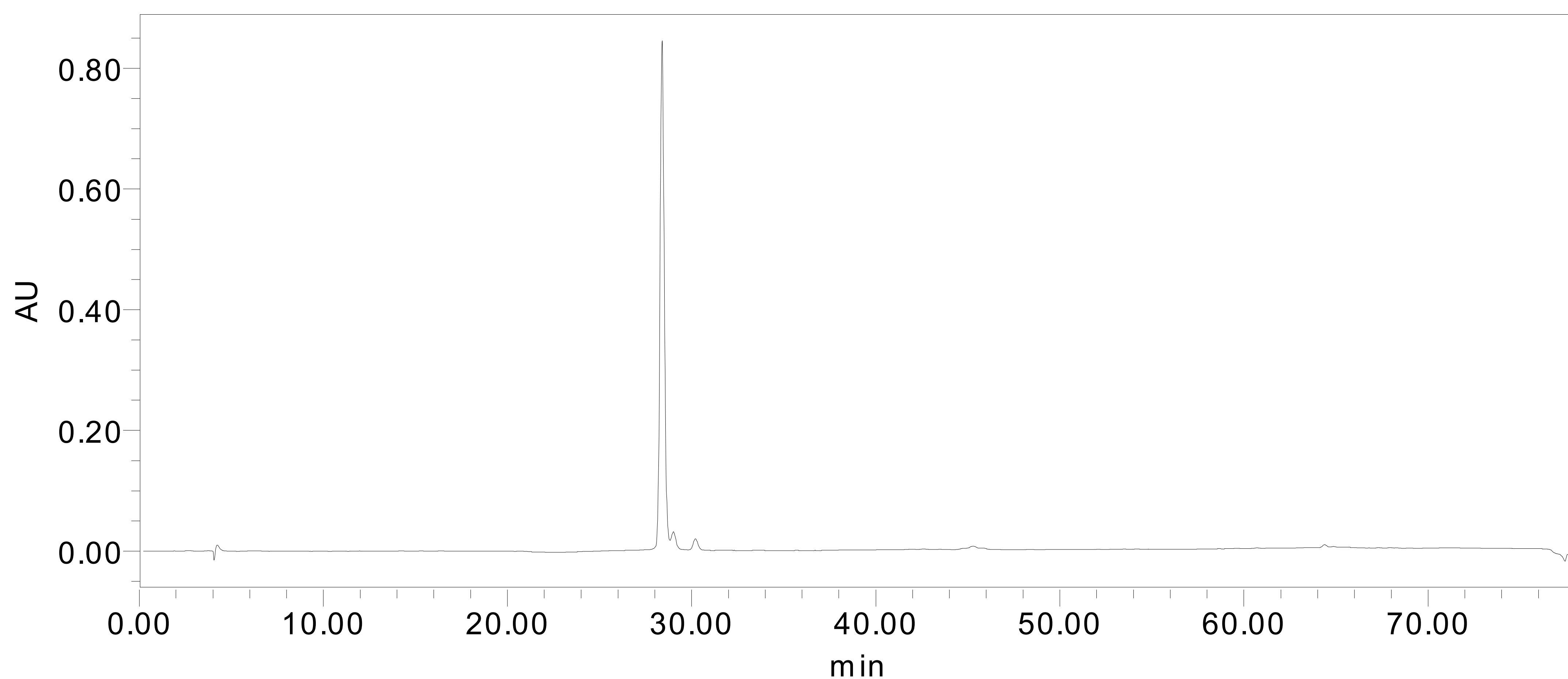


— Time: 26.900 min

S A M P L E I N F O R M A T I O N

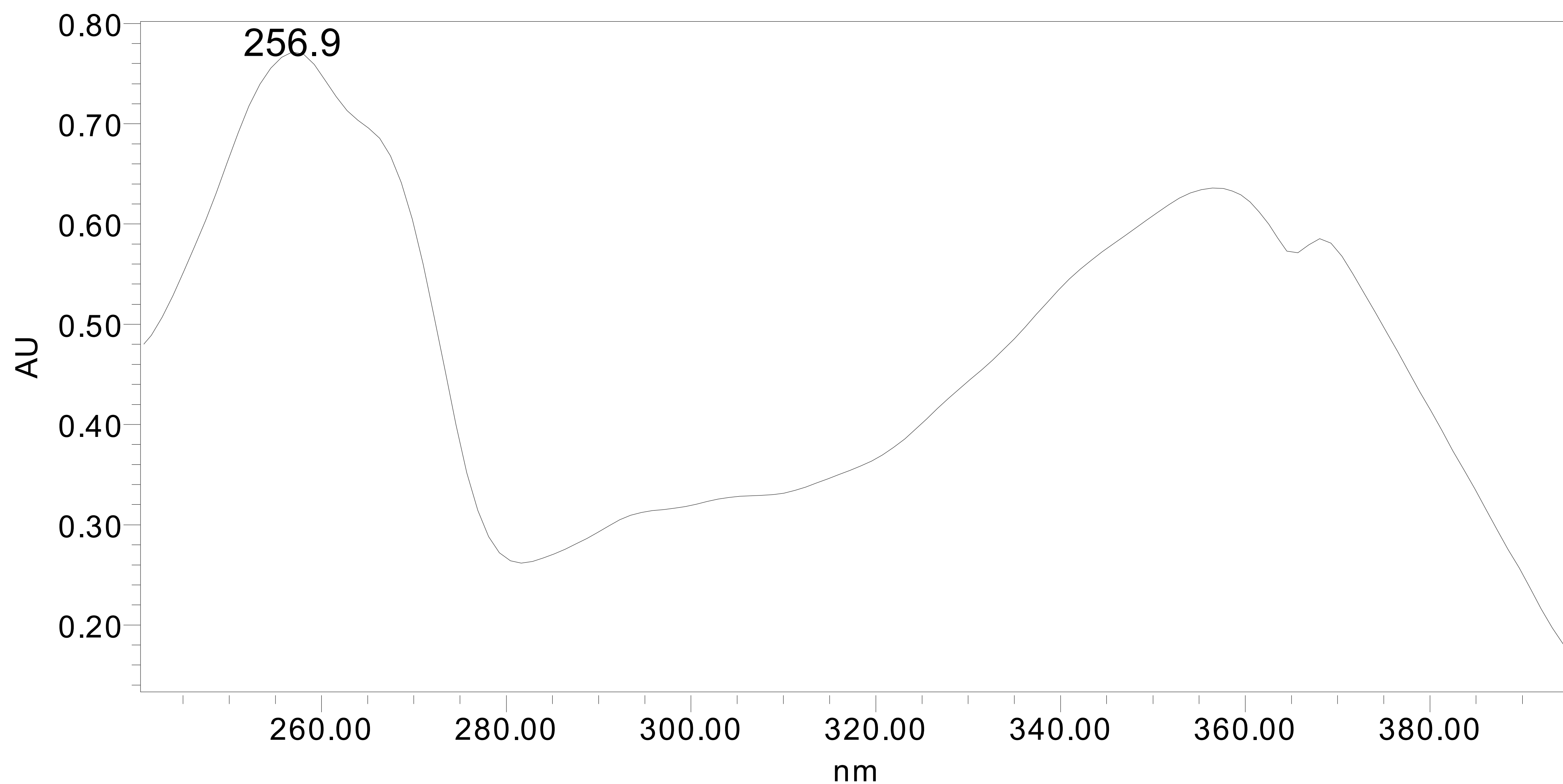
Sample Name: (+)-Rutin	Acquired By: System
Sample Type: Standard	Date Acquired: 3/11/2009 12:36:58 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 1	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum



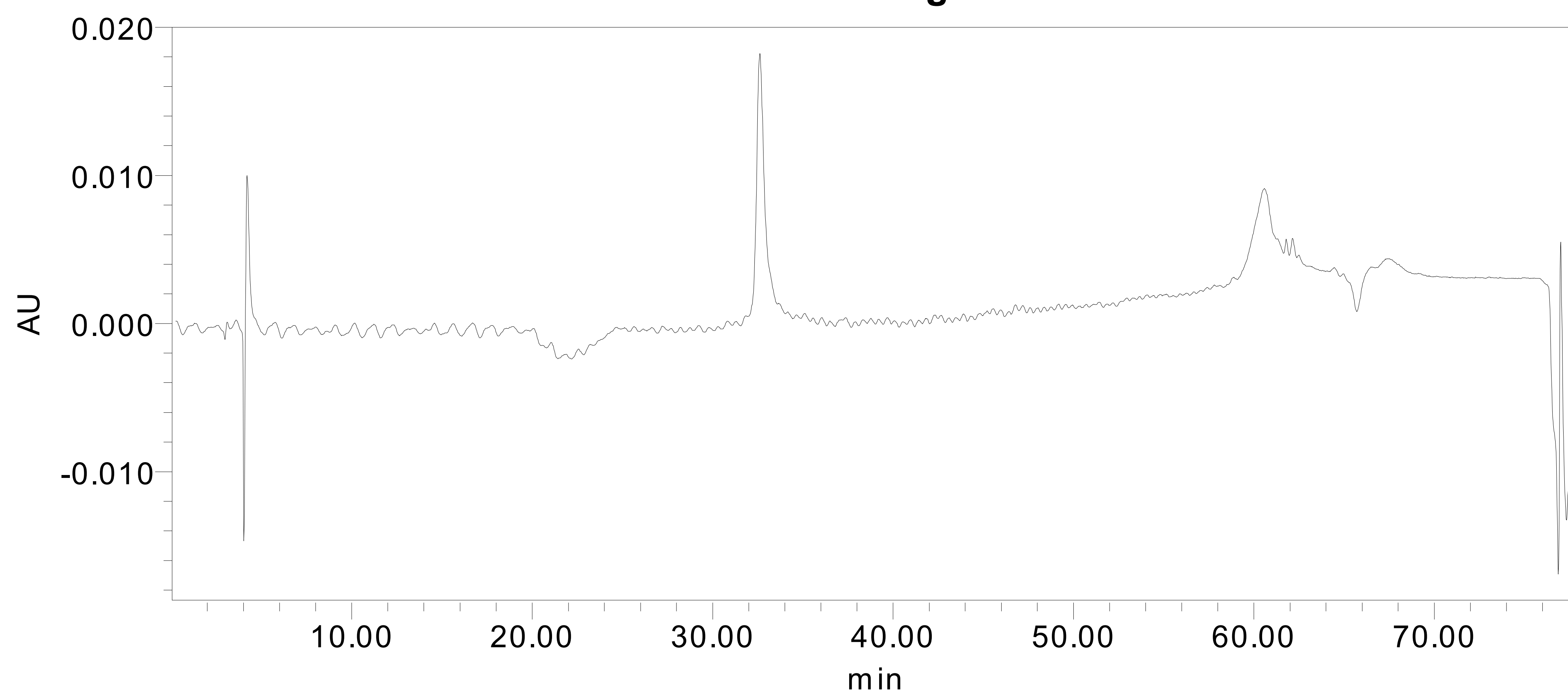
— Time: 28.300 min

SAMPLE INFORMATION

Sample Name: Myricetin
Sample Type: Unknown
Vial: 1
Injection #: 4
Injection Volume: 5.00 ul
Run Time: 78.0 Minutes
Sample Set Name:

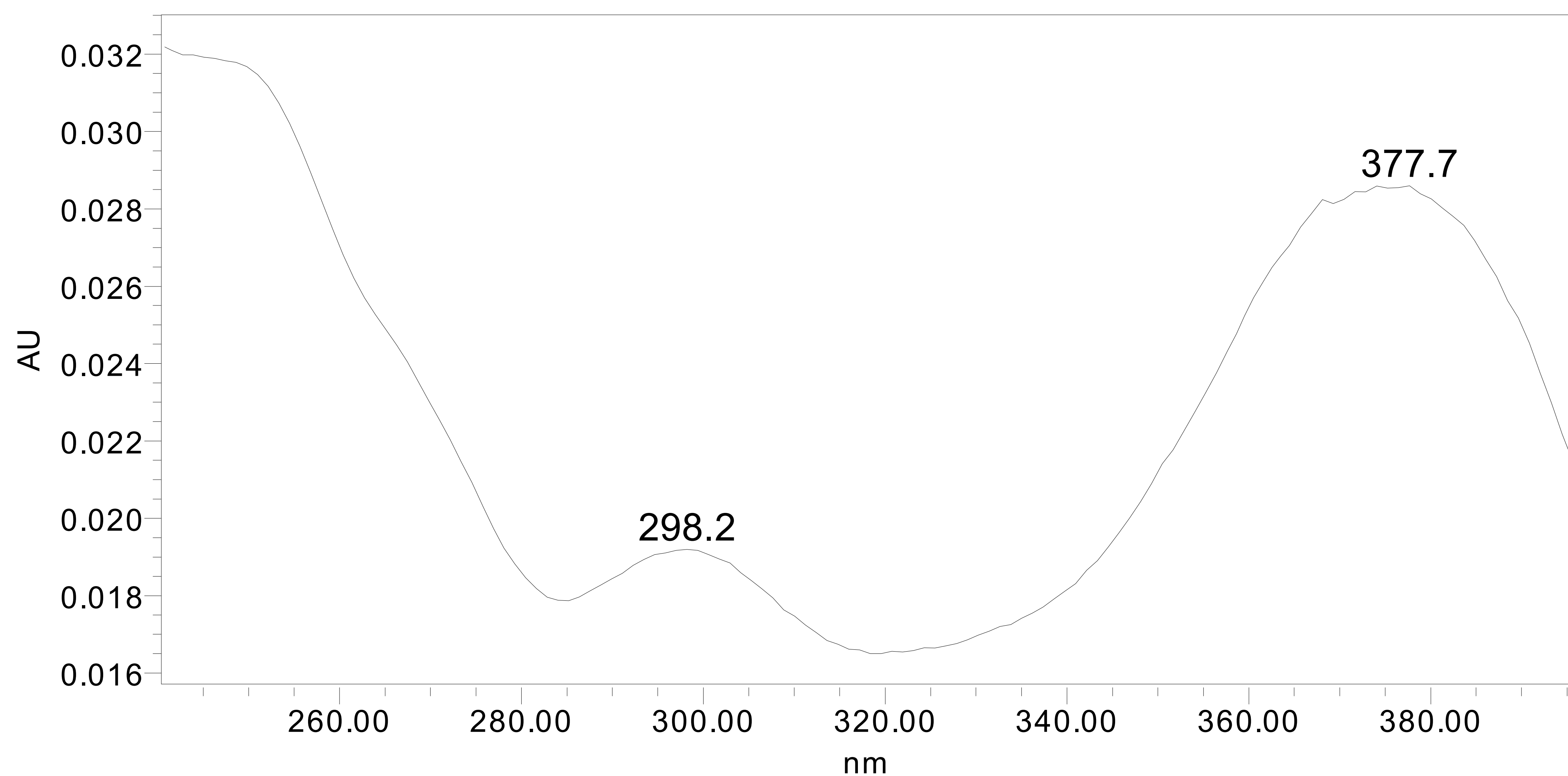
Acquired By: System
Date Acquired: 1/13/2009 3:52:11 PM NZDT
Acq. Method Set: Symmetry MeOH 3 a
Date Processed: ****
Processing Method: ****
Channel Name: ****
Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum

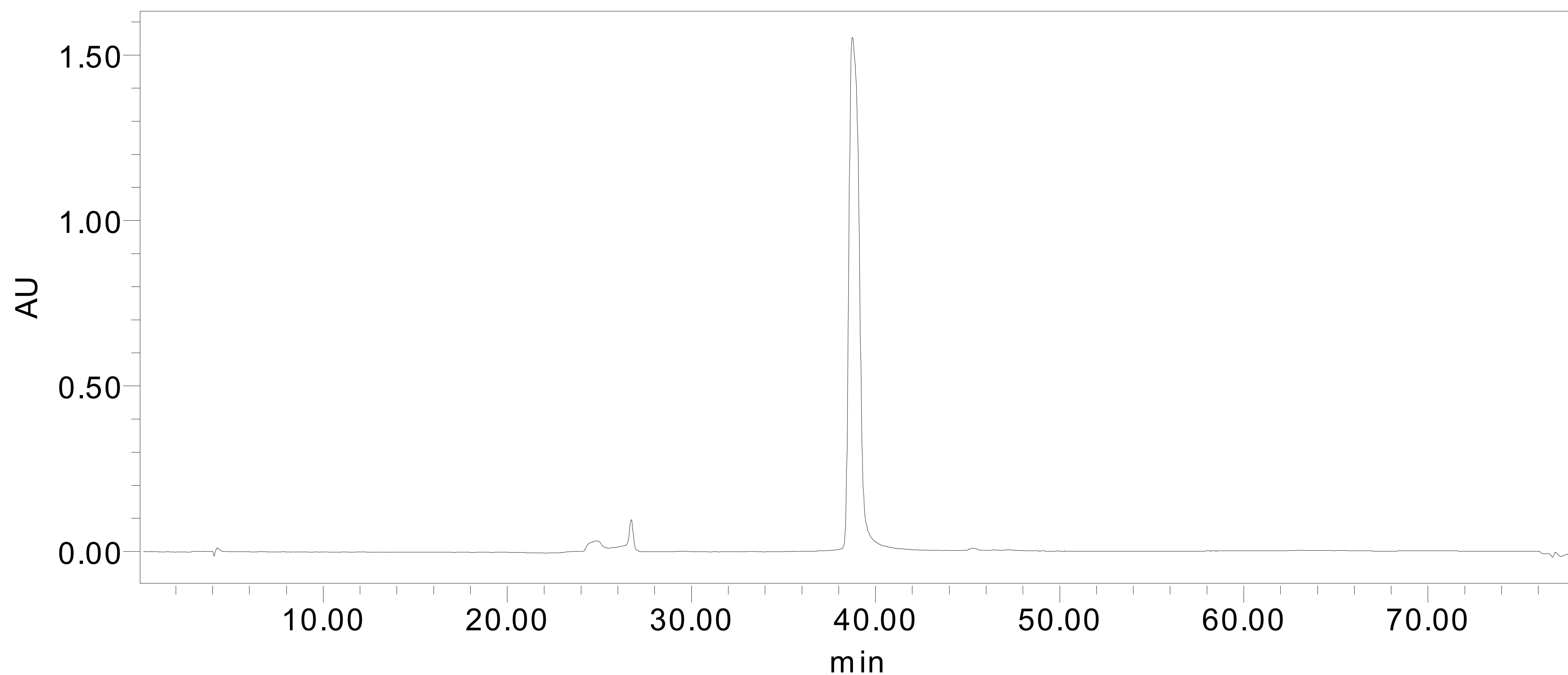


— Time: 32.600 min

SAMPLE INFORMATION

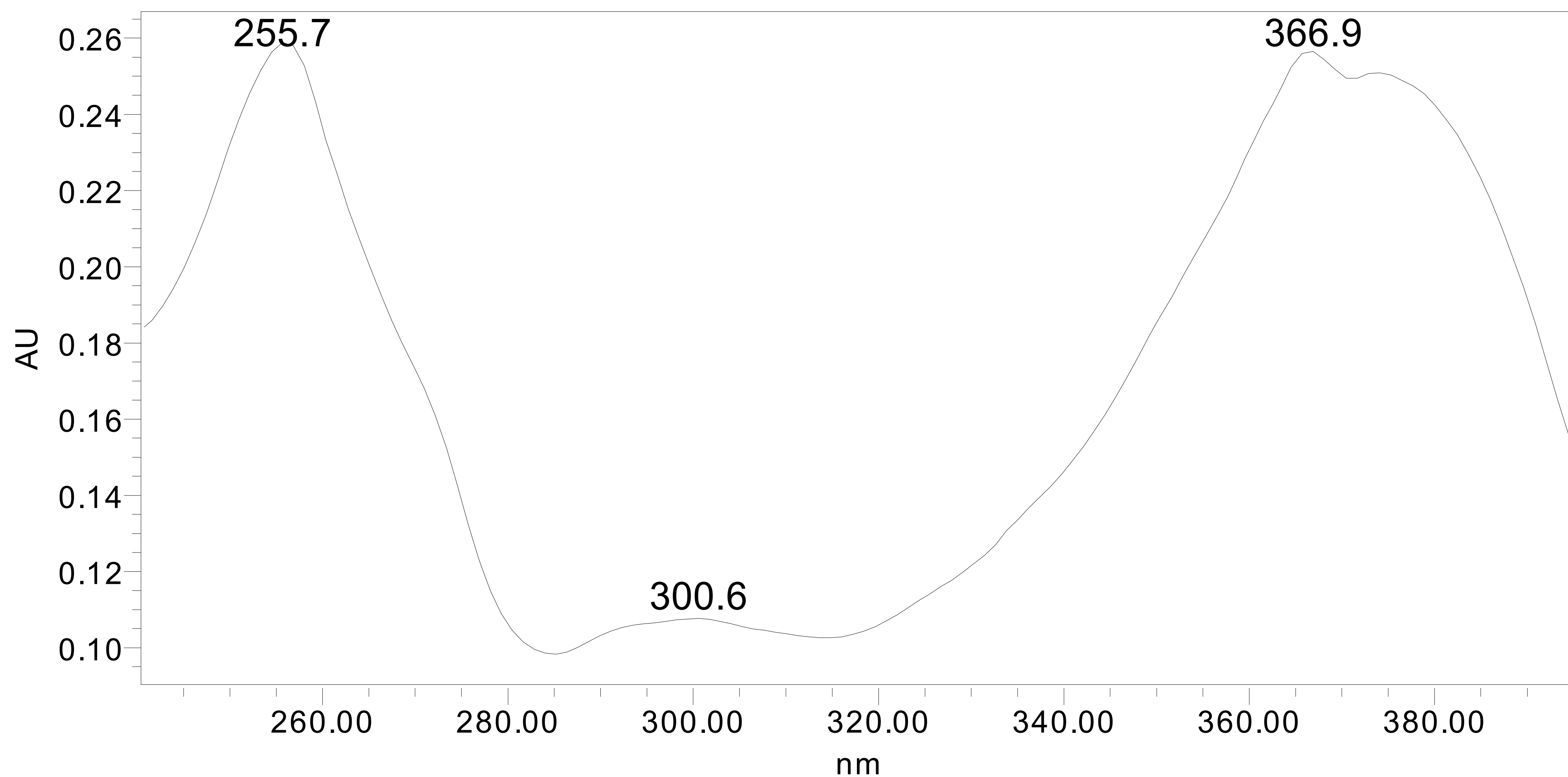
Sample Name: Quercetin	Acquired By: System
Sample Type: Unknown	Date Acquired: 1/8/2009 3:41:56 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 4	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum

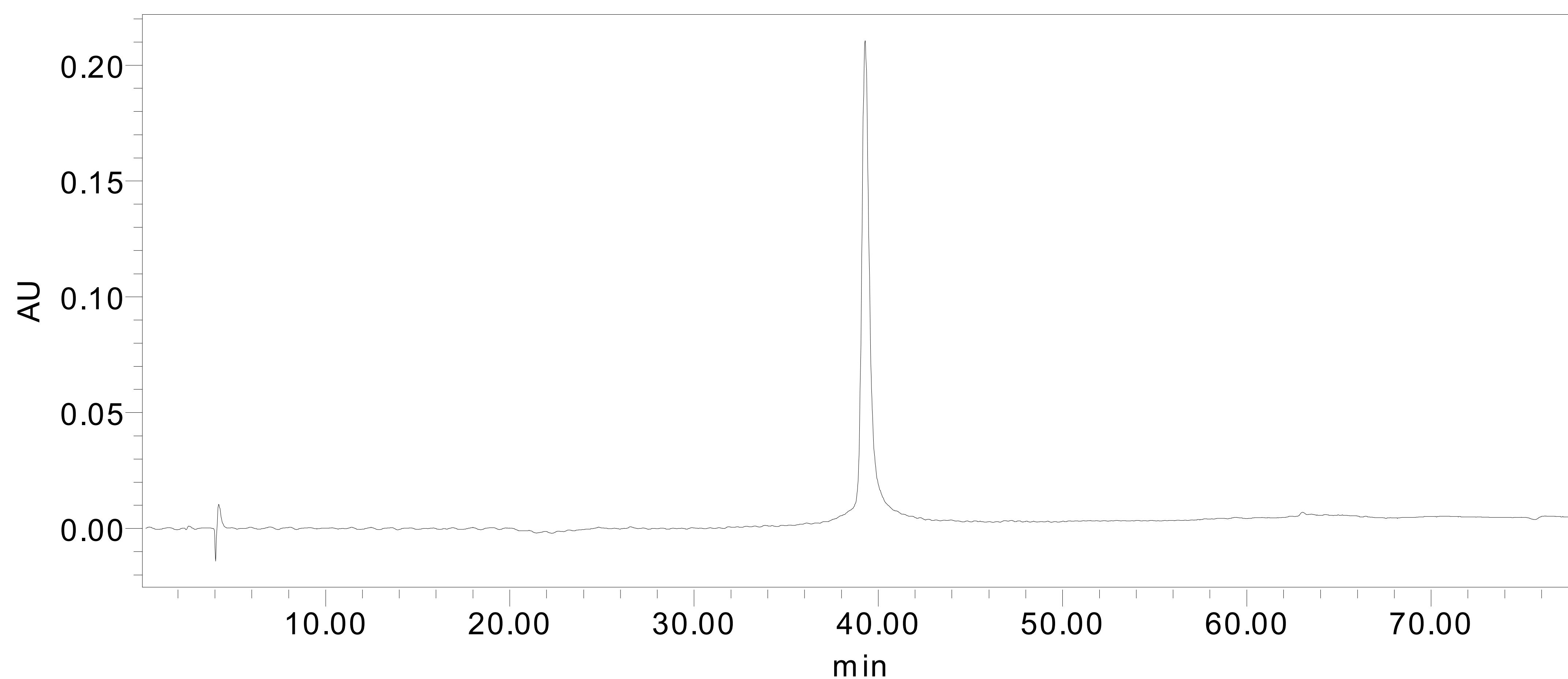


— Time: 38.400 min

SAMPLE INFORMATION

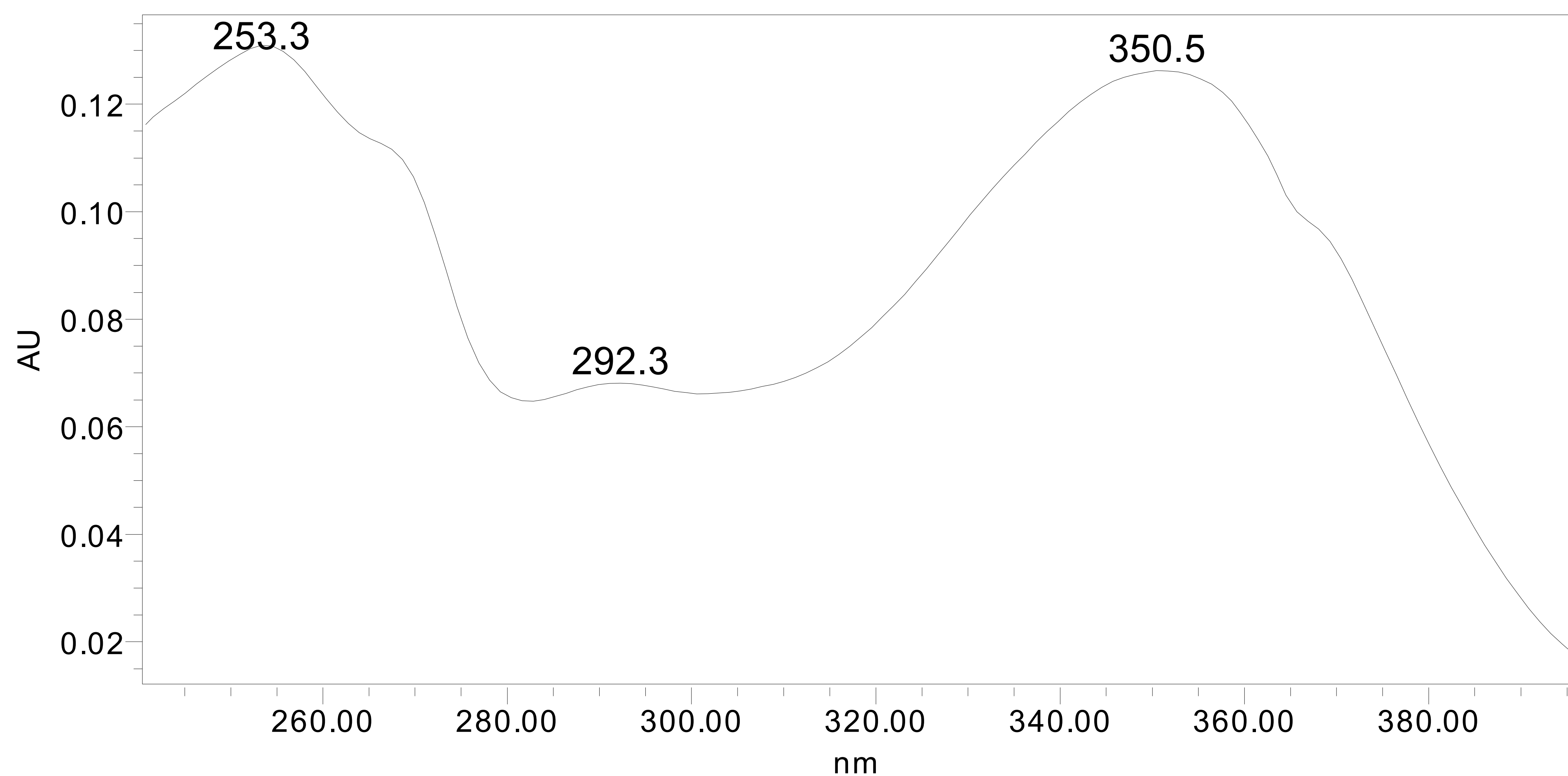
Sample Name:	Luteolin	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/7/2009 4:12:21 PM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	2	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum

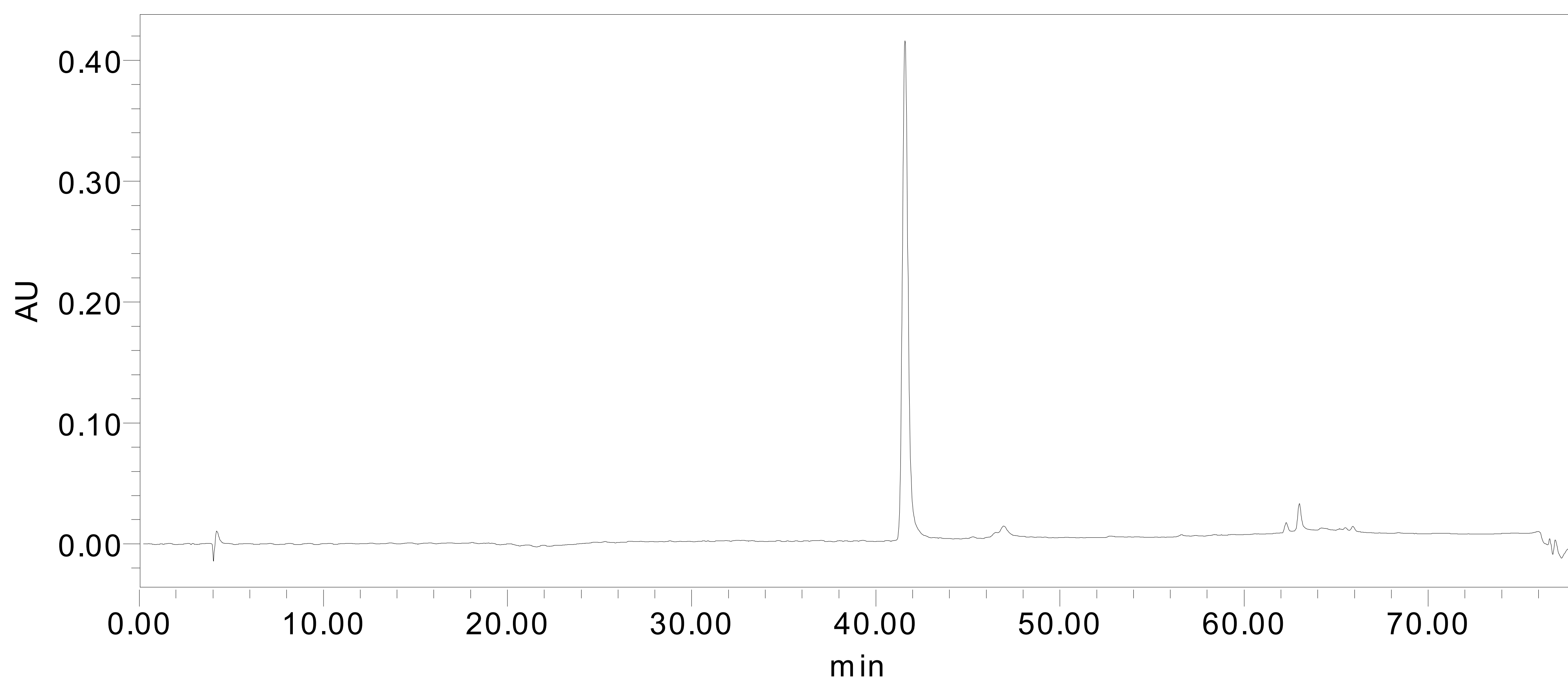


— Time: 39.500 min

S A M P L E I N F O R M A T I O N

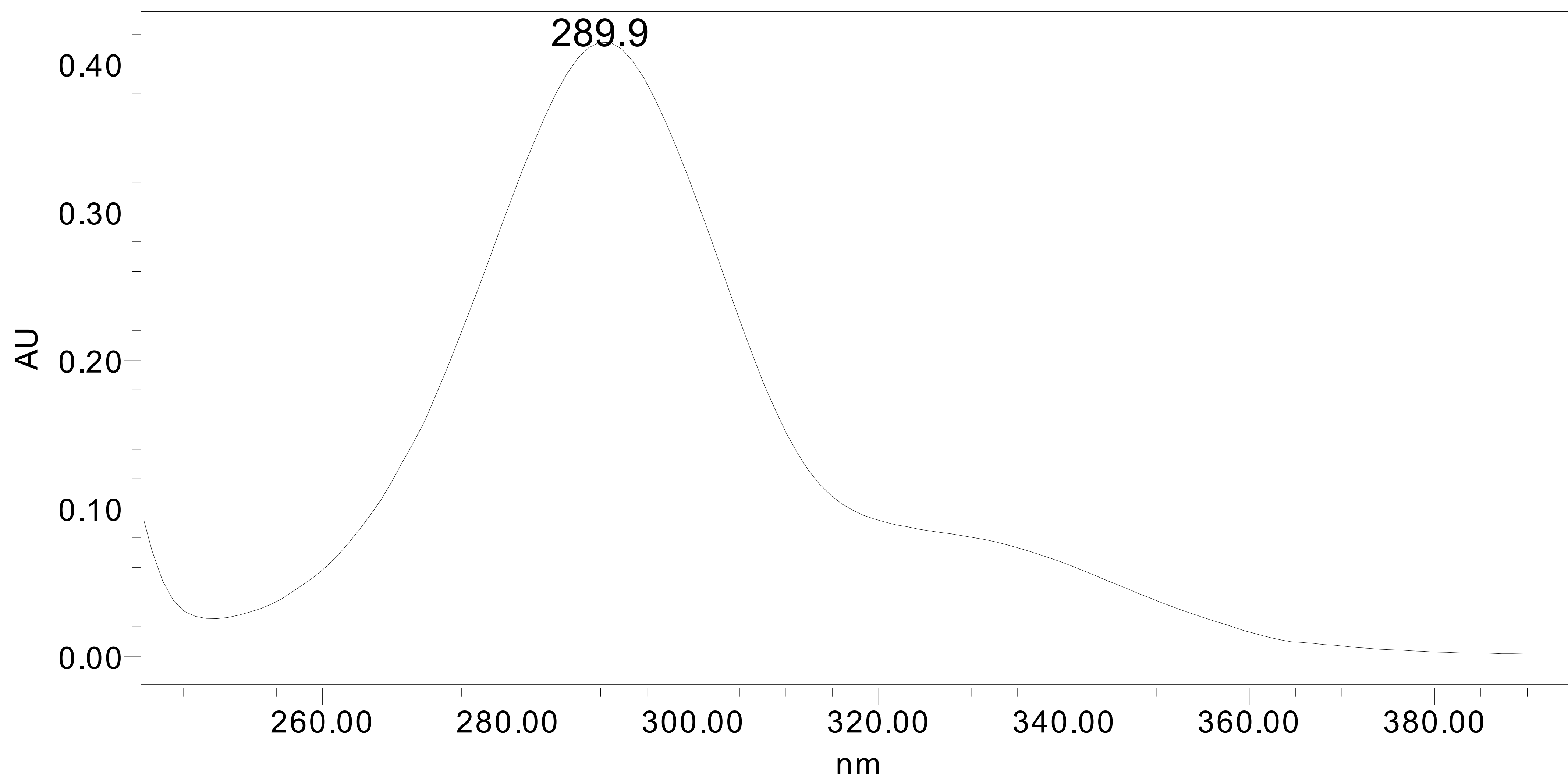
Sample Name:	Pinocembrin	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/13/2009 11:31:35 AM NZDT
Vial:	1	Acq. Method Set:	Symmetry MeOH 3 a
Injection #:	1	Date Processed:	****
Injection Volume:	5.00 ul	Processing Method	****
Run Time:	78.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****

290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum

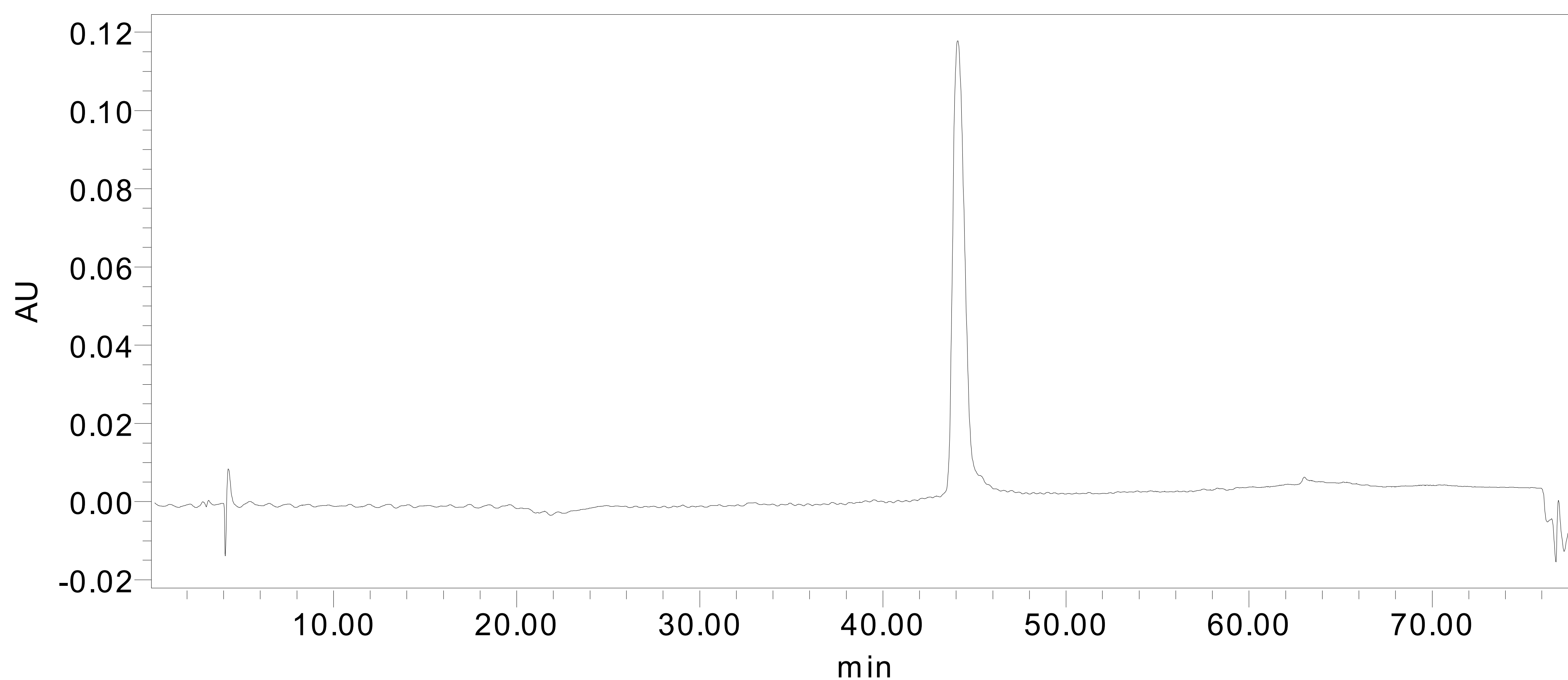


— Time: 41.600 min

SAMPLE INFORMATION

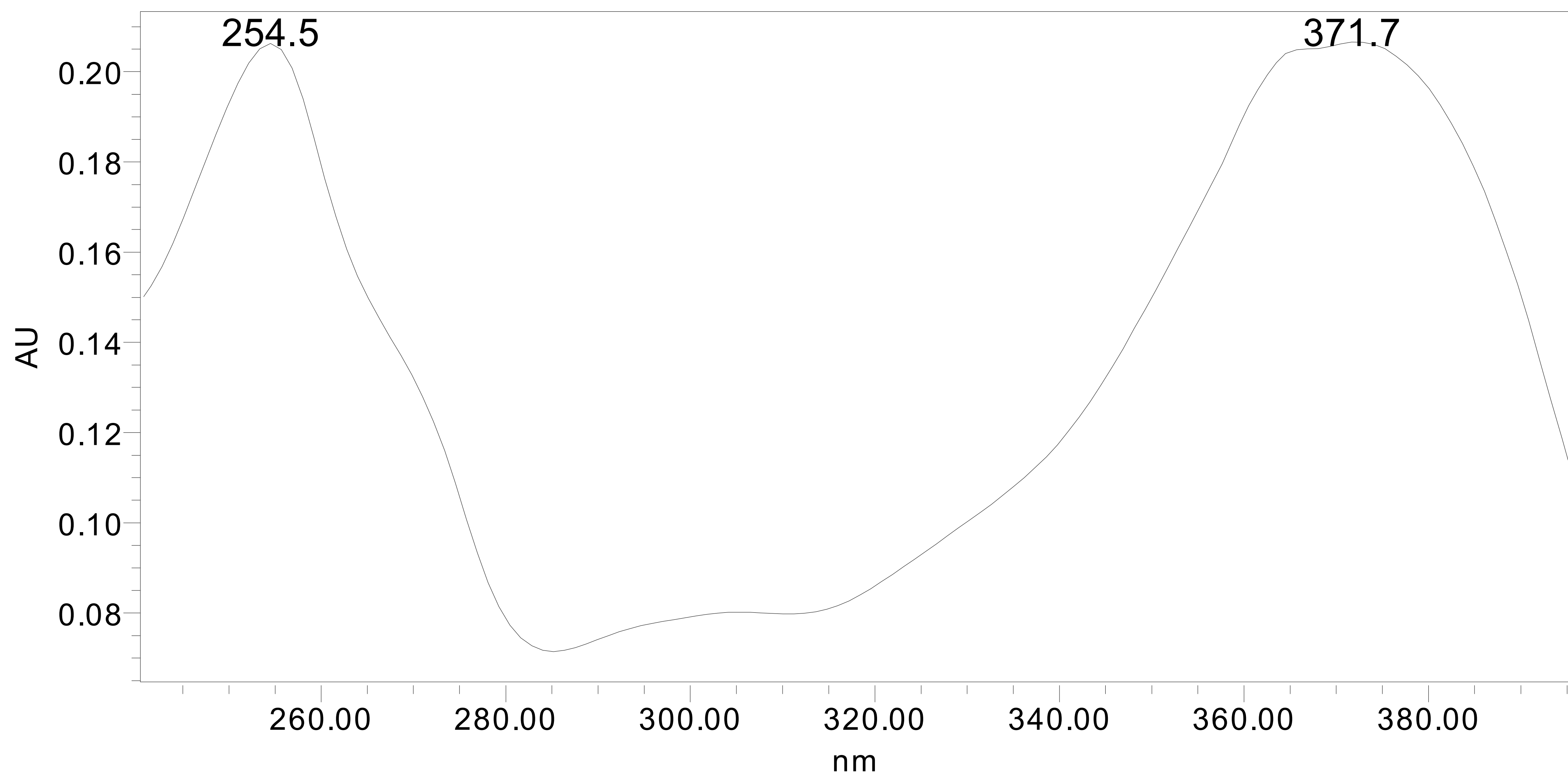
Sample Name: Isorhamnetin	Acquired By: System
Sample Type: Unknown	Date Acquired: 1/12/2009 12:42:25 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 2	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum

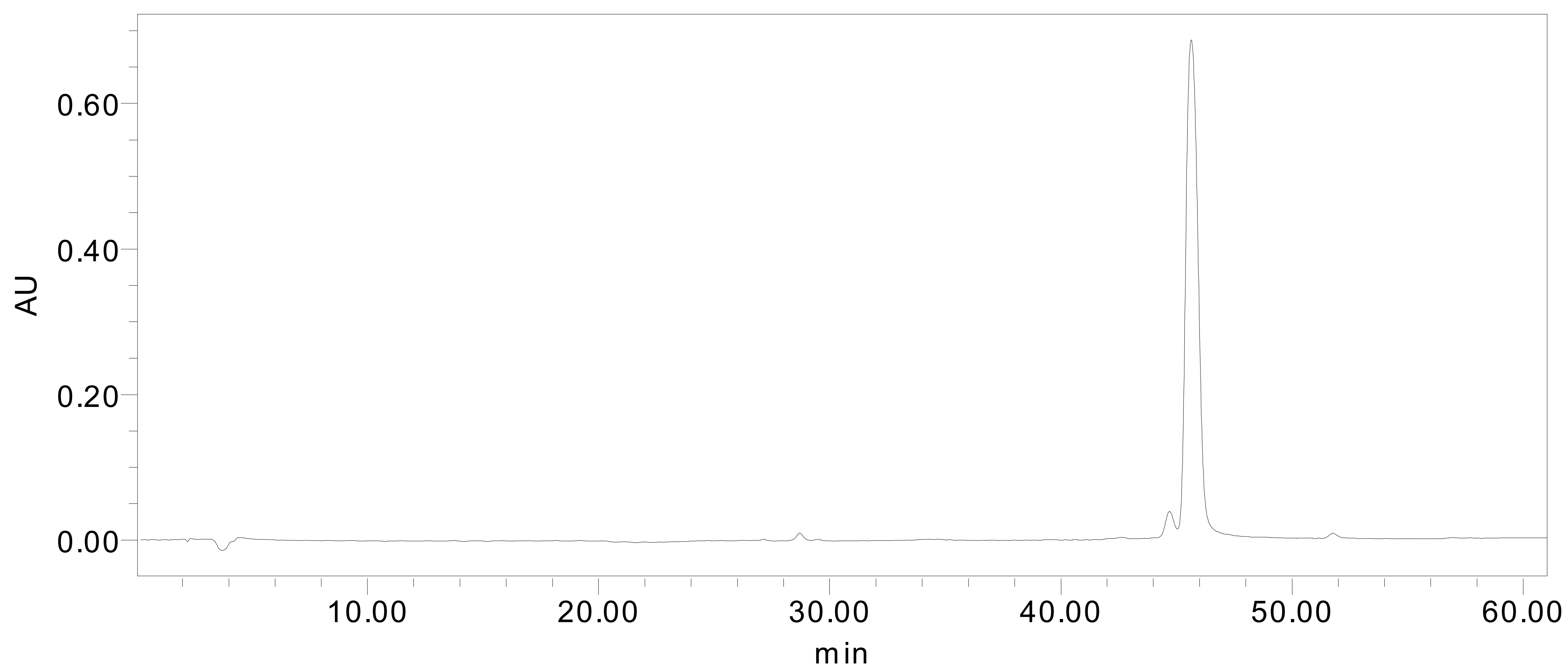


— Time: 44.090 min

S A M P L E I N F O R M A T I O N

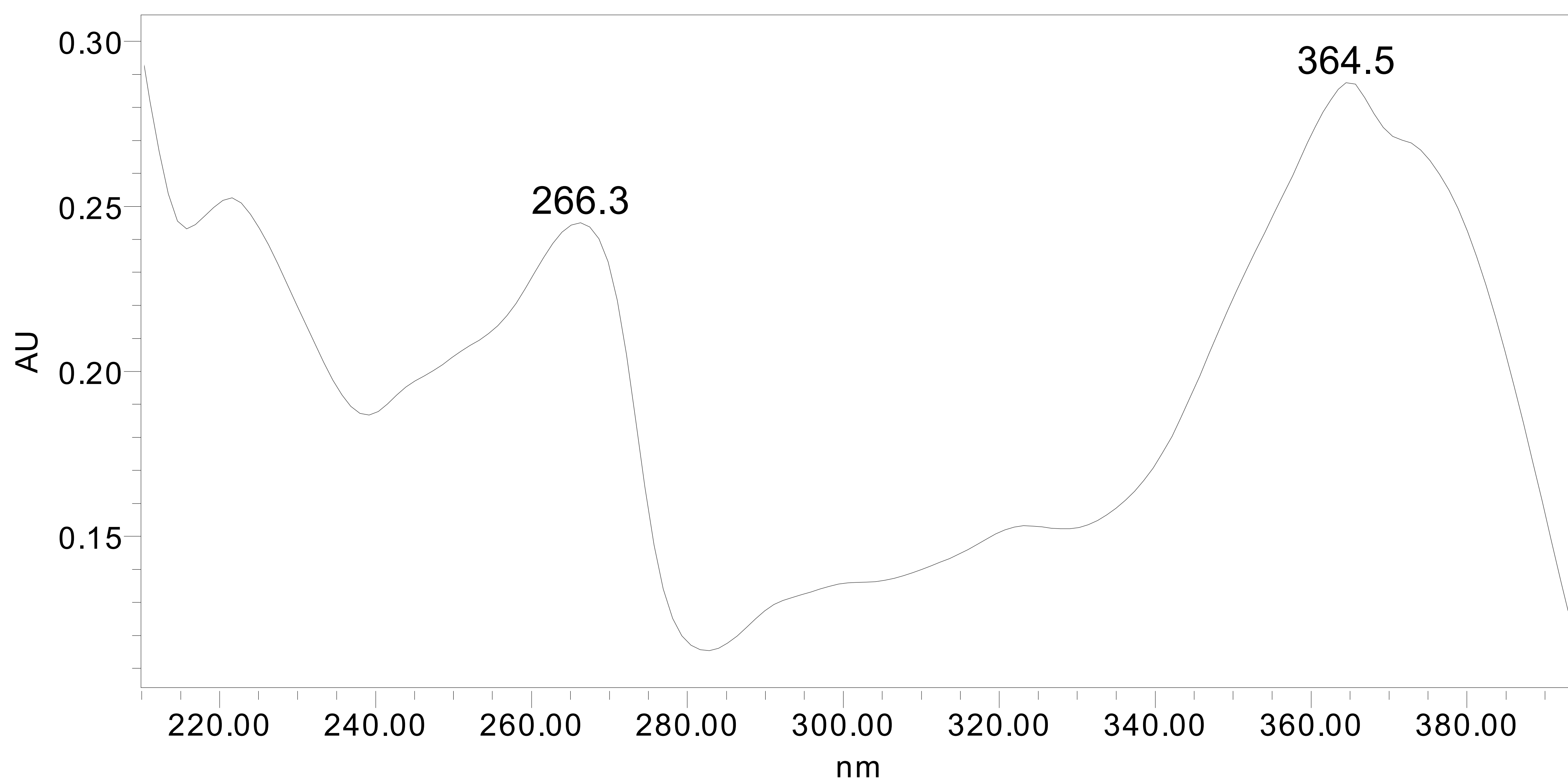
Sample Name: Kaempferol	Acquired By: System
Sample Type: Standard	Date Acquired: 12/9/2008 4:18:23 PM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 3	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum



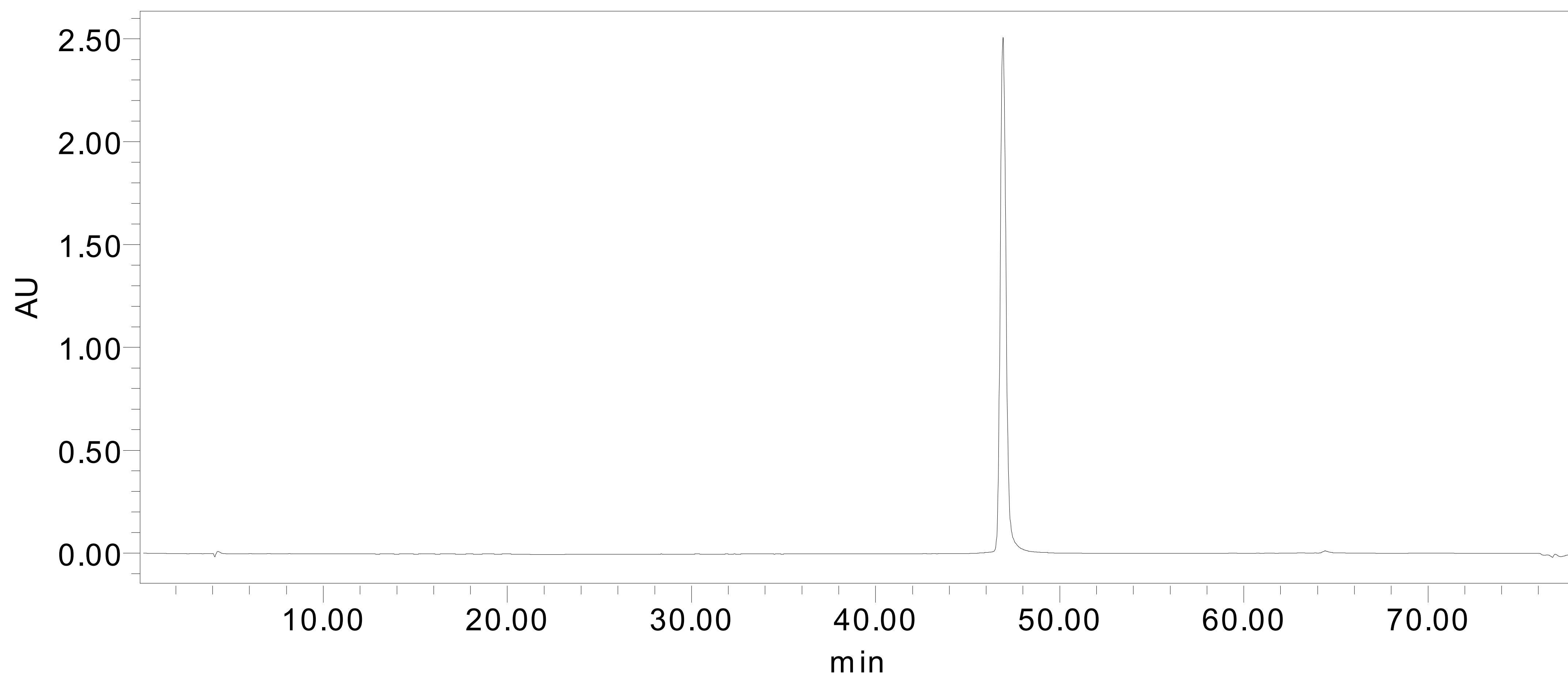
— Time: 45.300 min

SAMPLE INFORMATION

Sample Name: Chrysin
Sample Type: Unknown
Vial: 1
Injection #: 4
Injection Volume: 5.00 ul
Run Time: 78.0 Minutes
Sample Set Name:

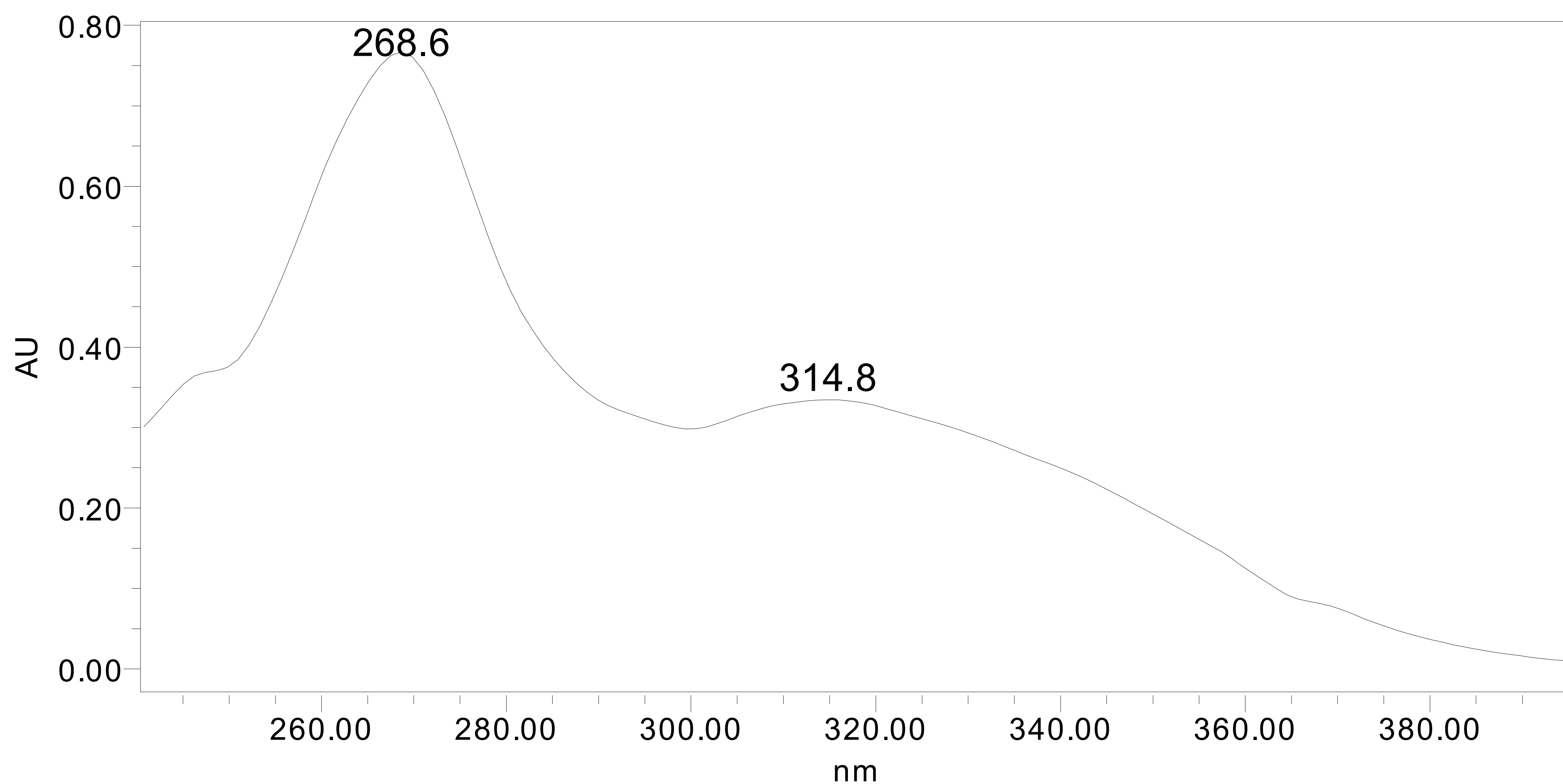
Acquired By: System
Date Acquired: 1/12/2009 3:21:48 PM NZDT
Acq. Method Set: Symmetry MeOH 3 a
Date Processed: ****
Processing Method: ****
Channel Name: ****
Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum

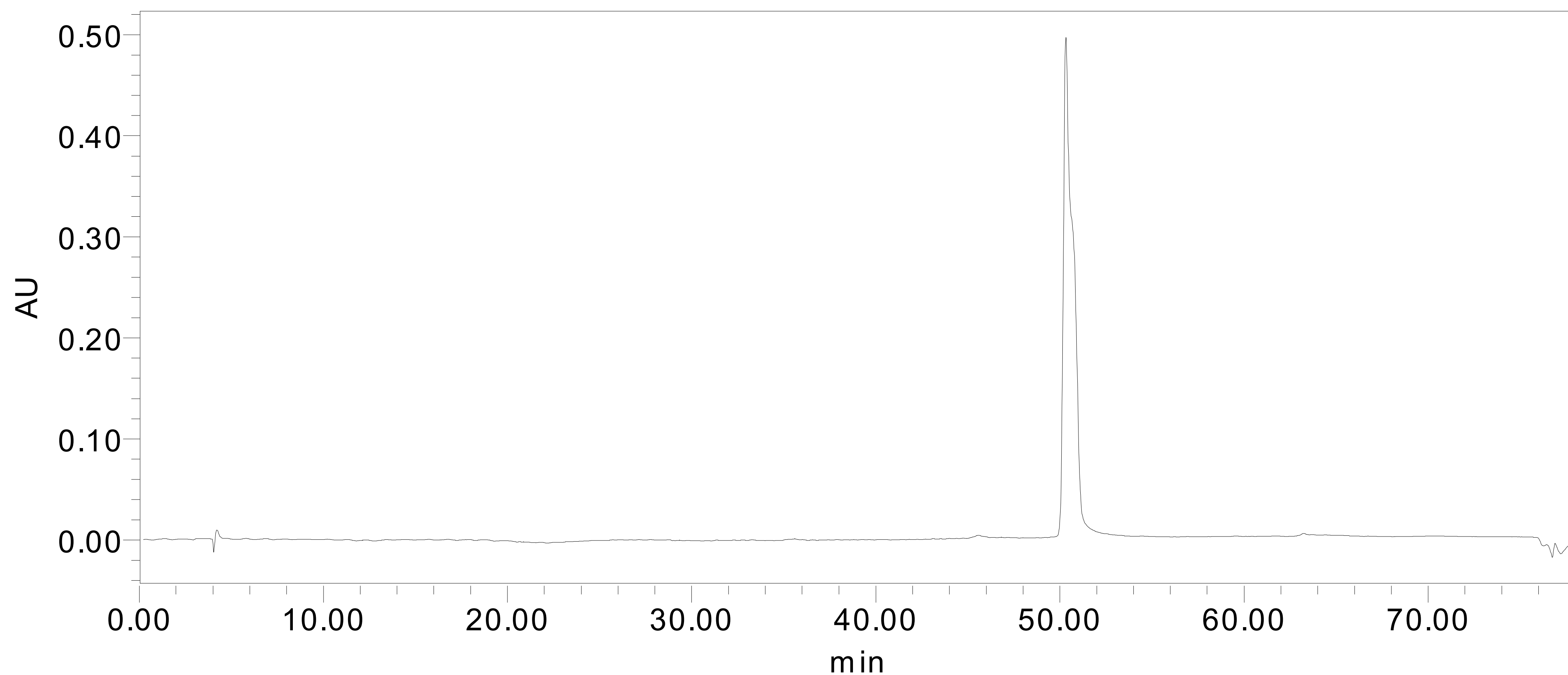


— Time: 47.260 min

SAMPLE INFORMATION

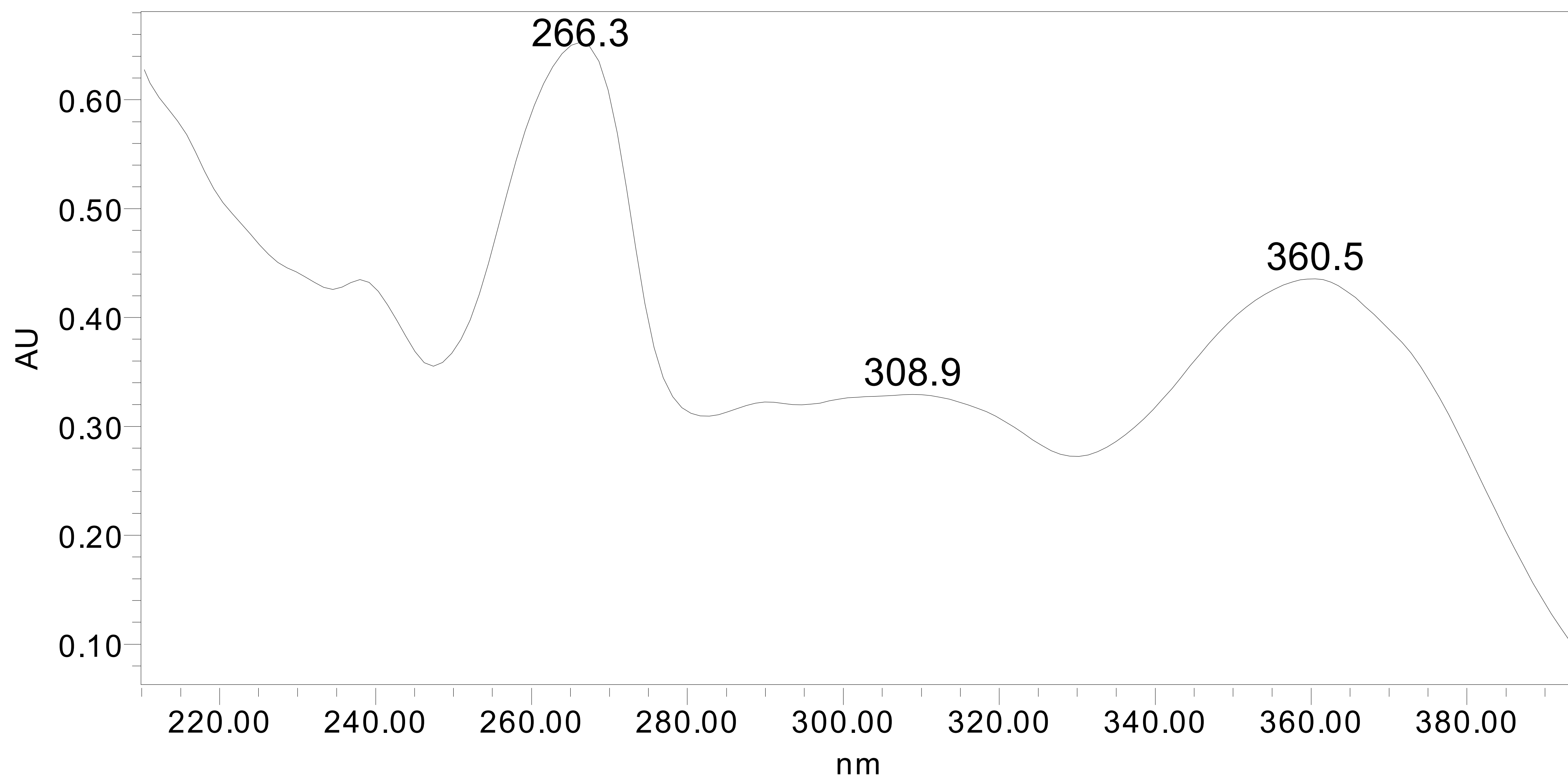
Sample Name: Galangin	Acquired By: System
Sample Type: Standard	Date Acquired: 12/10/2008 11:08:16 AM NZDT
Vial: 1	Acq. Method Set: Symmetry MeOH 3 a
Injection #: 1	Date Processed: ****
Injection Volume: 5.00 ul	Processing Method: ****
Run Time: 78.0 Minutes	Channel Name: ****
Sample Set Name:	Proc. Chnl. Descr.: ****

340nm Chromatogram

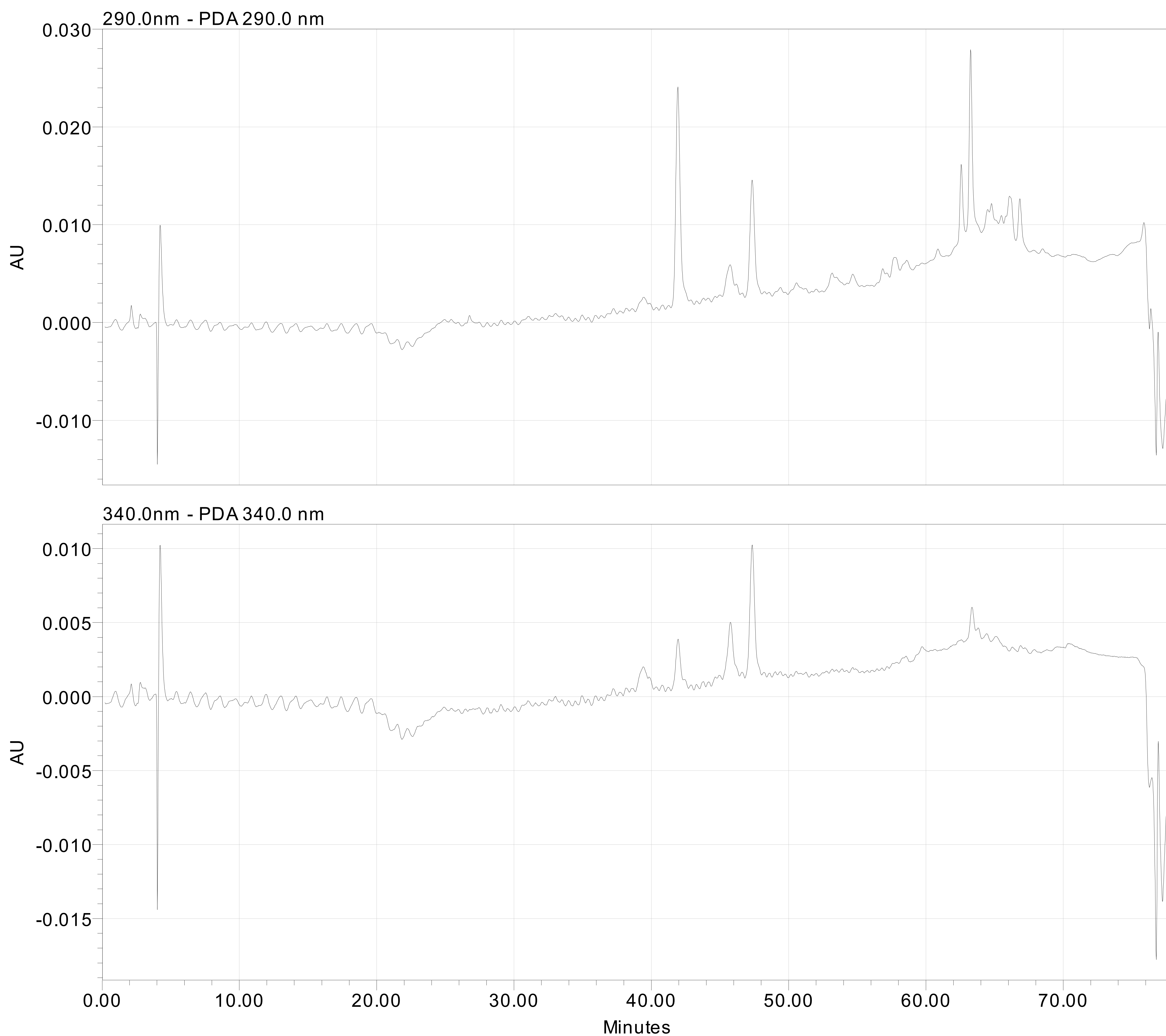


— 996 Extraction at 340 nm

UV Absorption Spectrum



— Time: 50.200 min

Sample Name: 0.005mg/mL**Quantitative Flavonoid Standards****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/11/2008 12:05:33 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

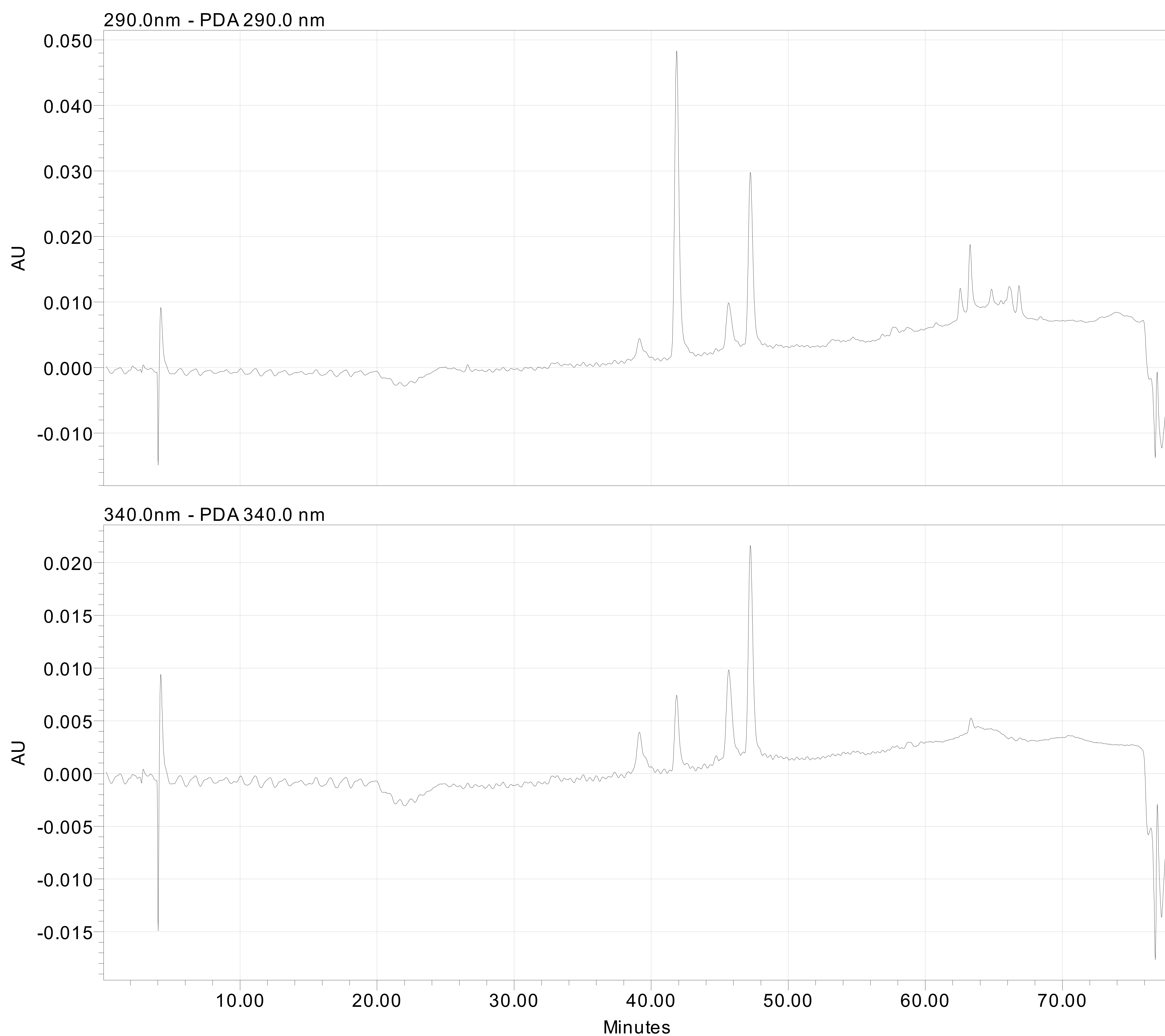
3/31/2009

5:52:26 PM NZ

Sample Name: 0.01mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/11/2008 1:32:25 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

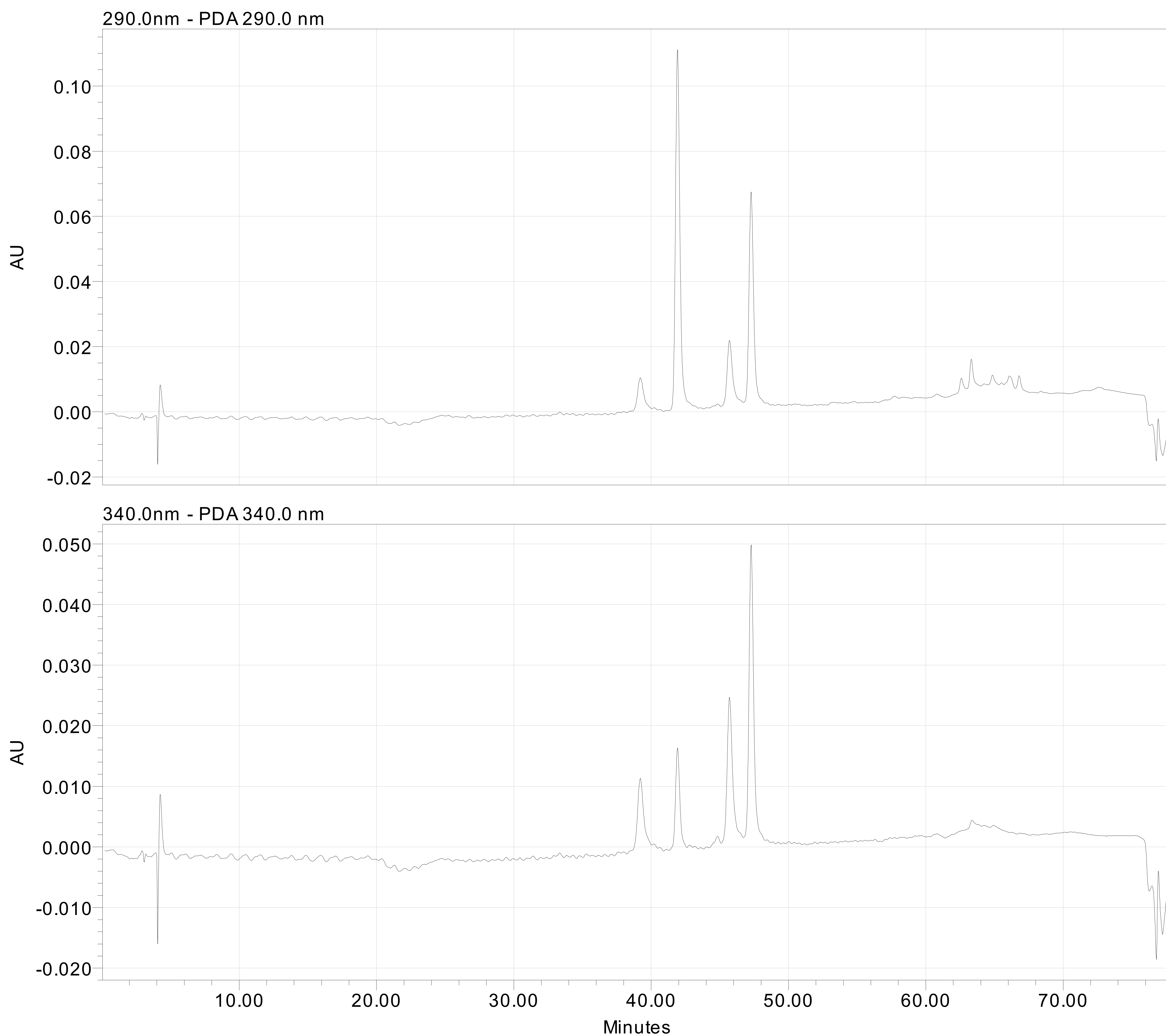
3/31/2009

5:52:07 PM NZ

Sample Name: 0.025mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/11/2008 2:53:38 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

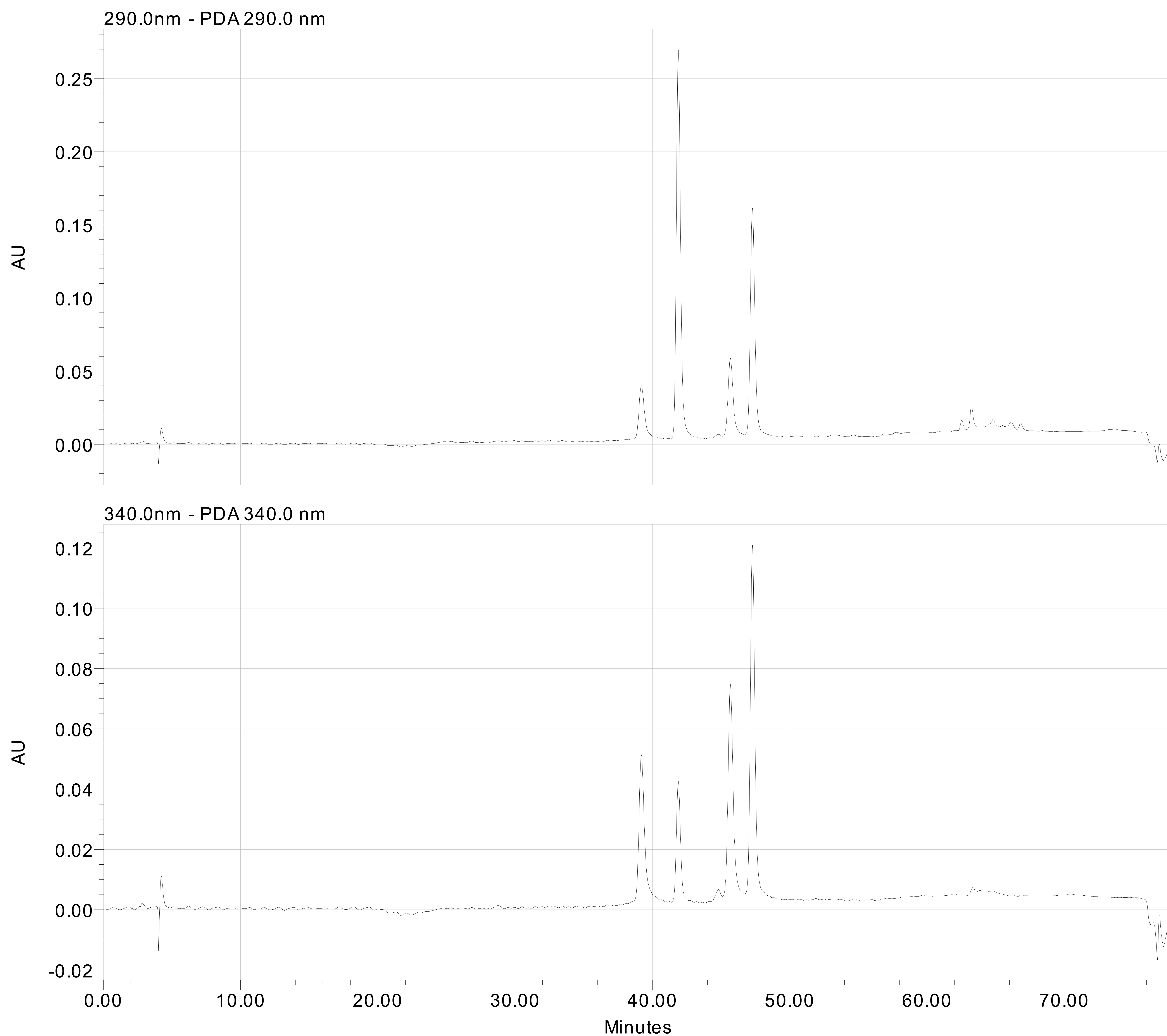
3/31/2009

5:51:50 PM NZ

Sample Name: 0.05mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/12/2008 11:30:45 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

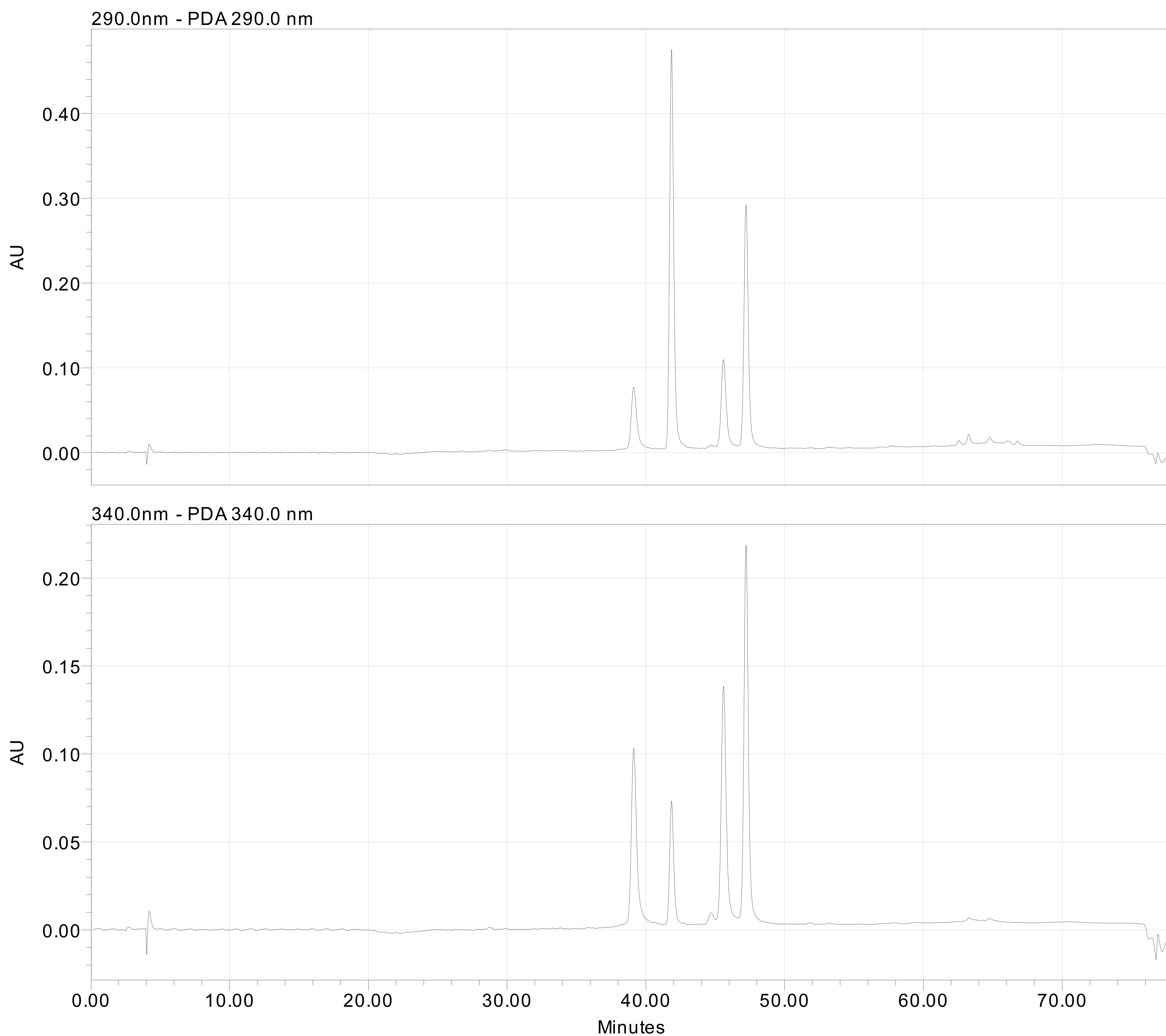
3/31/2009

5:51:32 PM NZ

Sample Name: 0.1mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/12/2008 1:08:59 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

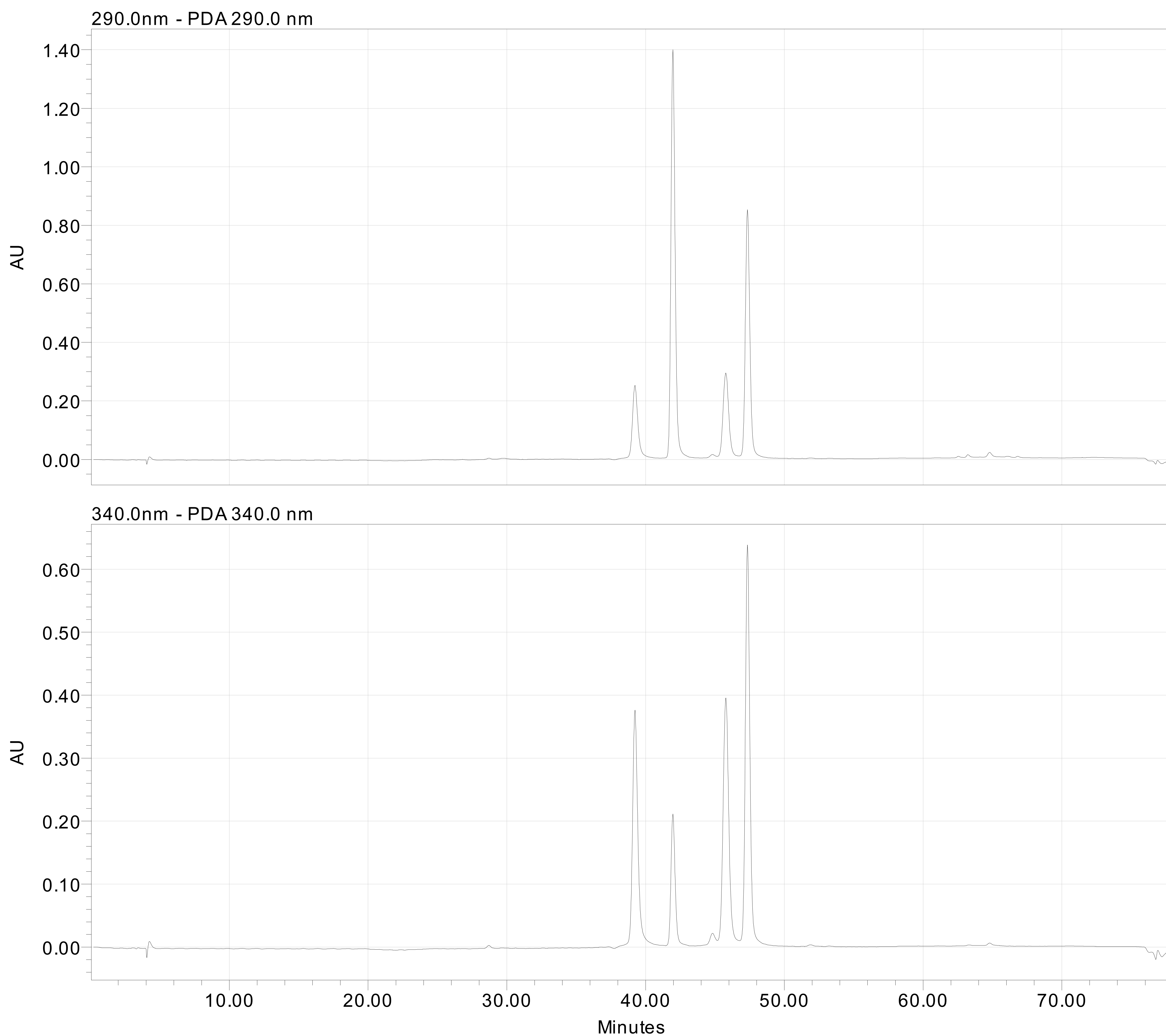
3/31/2009

5:51:10 PM NZ

Sample Name: 0.25mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/12/2008 2:29:07 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

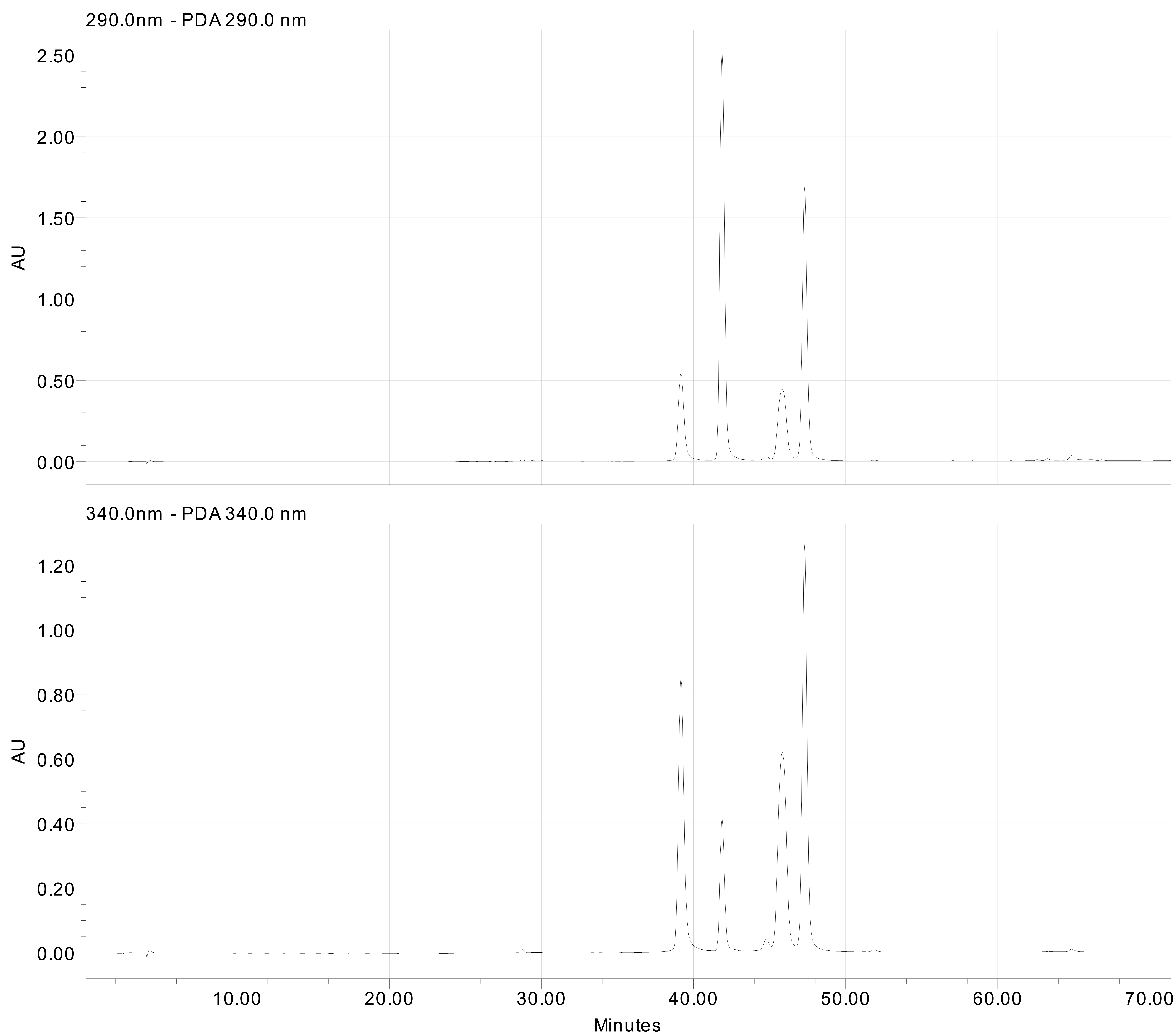
3/31/2009

5:49:40 PM NZ

Sample Name: 0.5mg/mL

Quantitative Flavonoid Standards

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/12/2008 3:50:08 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:49:10 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman



THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

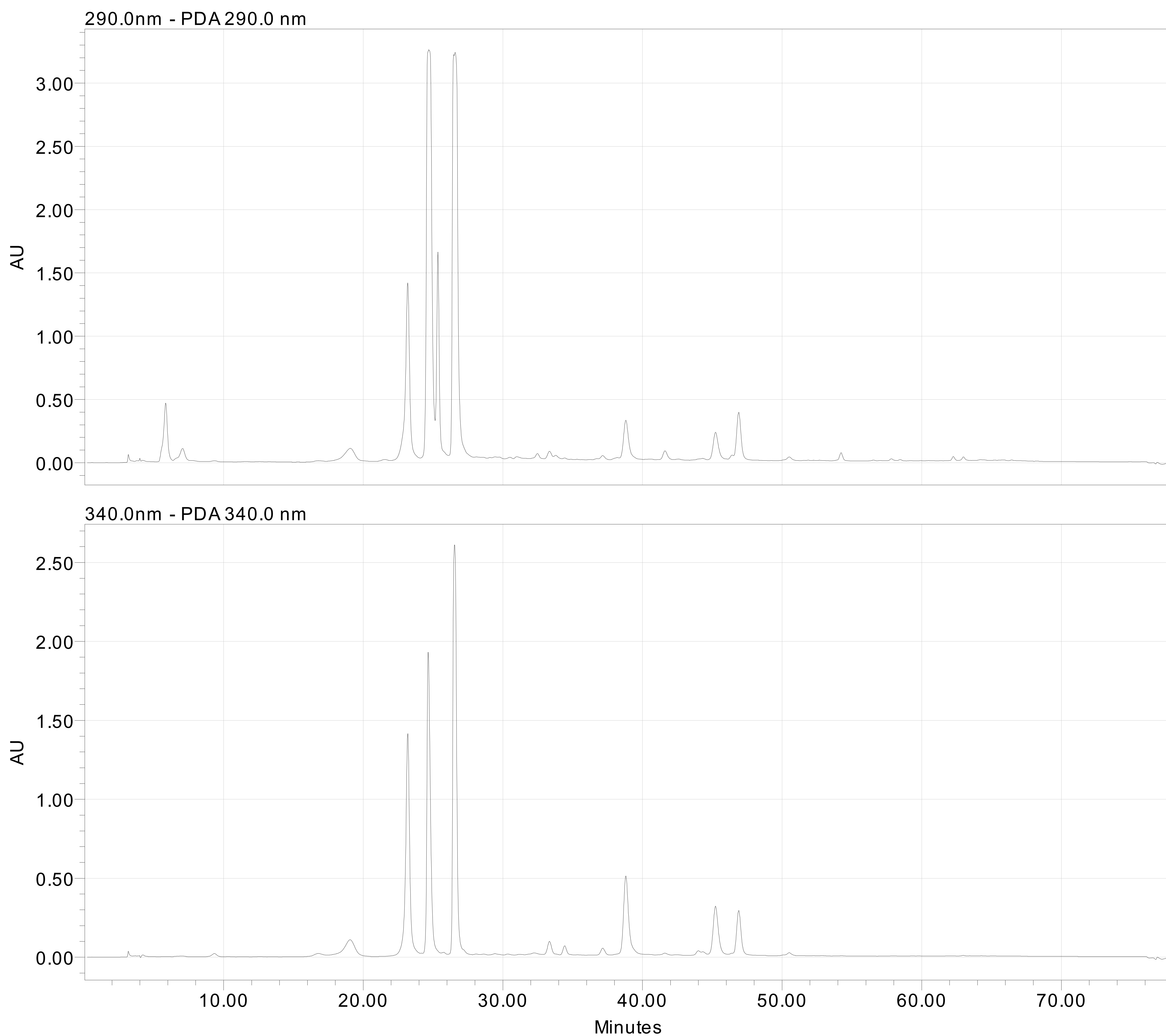
The University of Waikato

2009

Sample Name: CC 111208 AA Ext Eff

**Extraction of Flavonoids from Artificial Honey
Extraction 1**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/16/2008 11:43:50 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

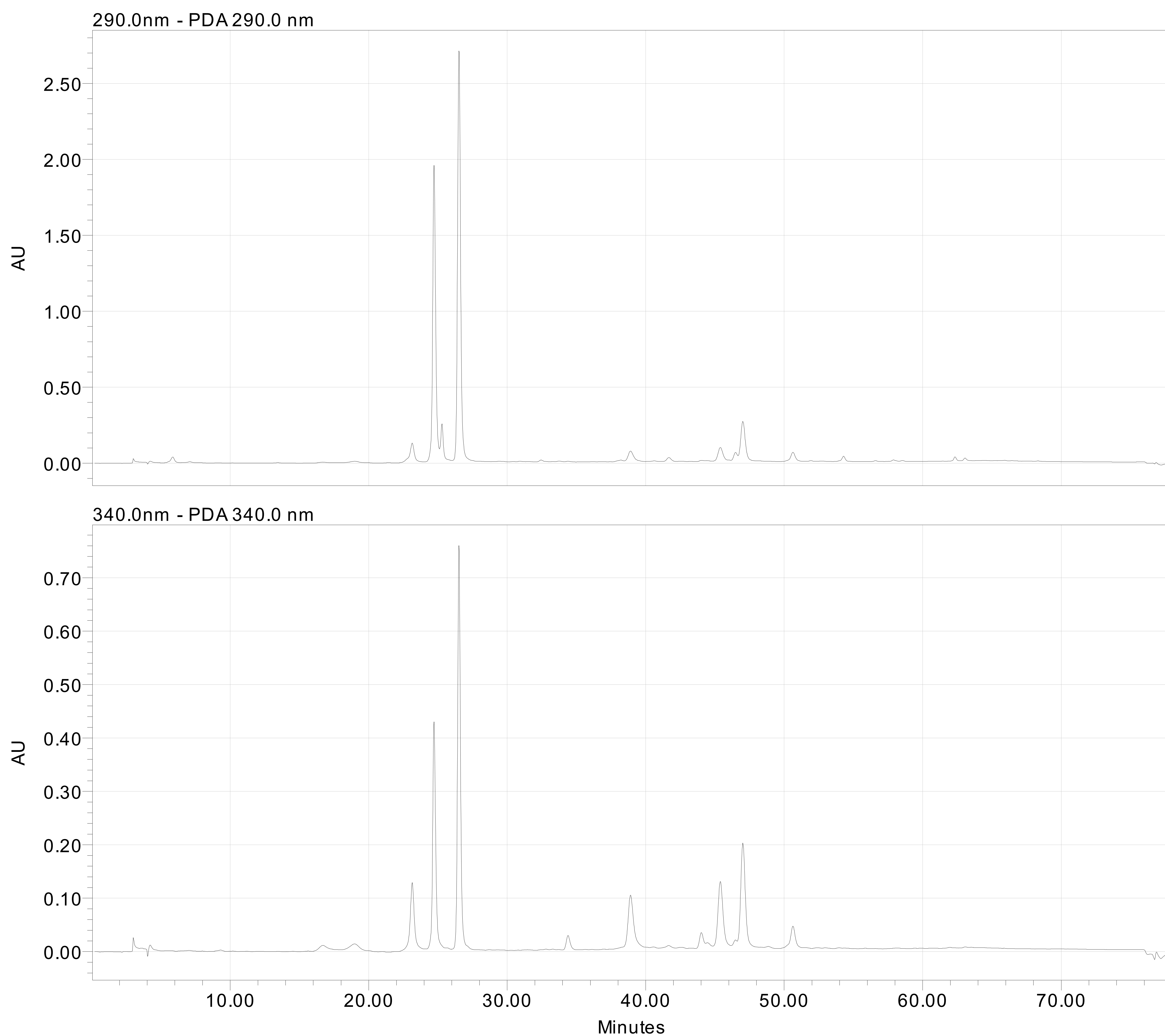
3/31/2009

5:15:30 PM NZ

Sample Name: CC 111208 AB Ext Eff

**Extraction of Flavonoids from Artificial Honey
Extraction 2**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/16/2008 1:12:19 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

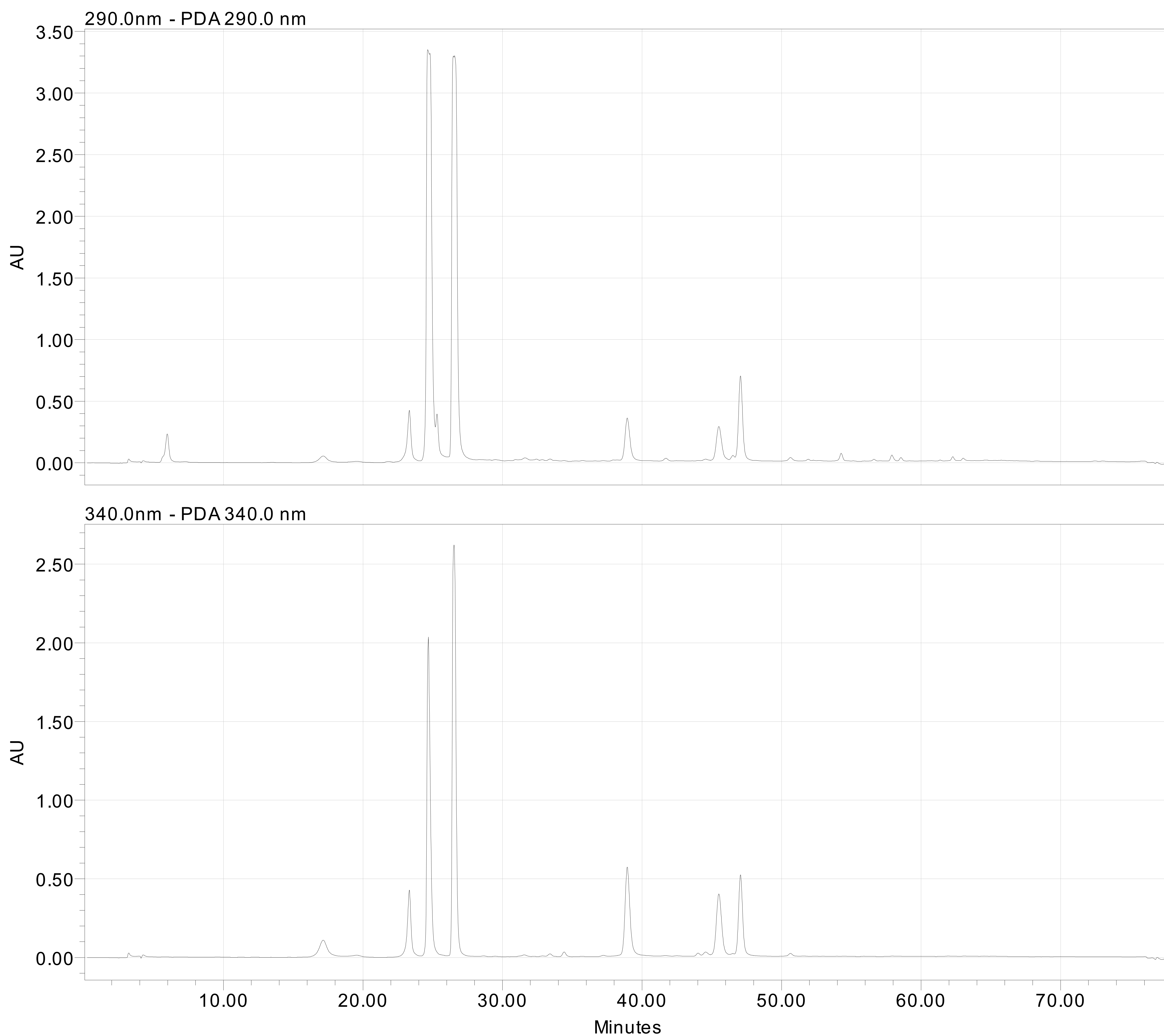
3/31/2009

5:15:03 PM NZ

Sample Name: CC 111208 AC Ext Eff

Extraction of Flavonoids from Artificial Honey Extraction 3

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/18/2008 2:33:30 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

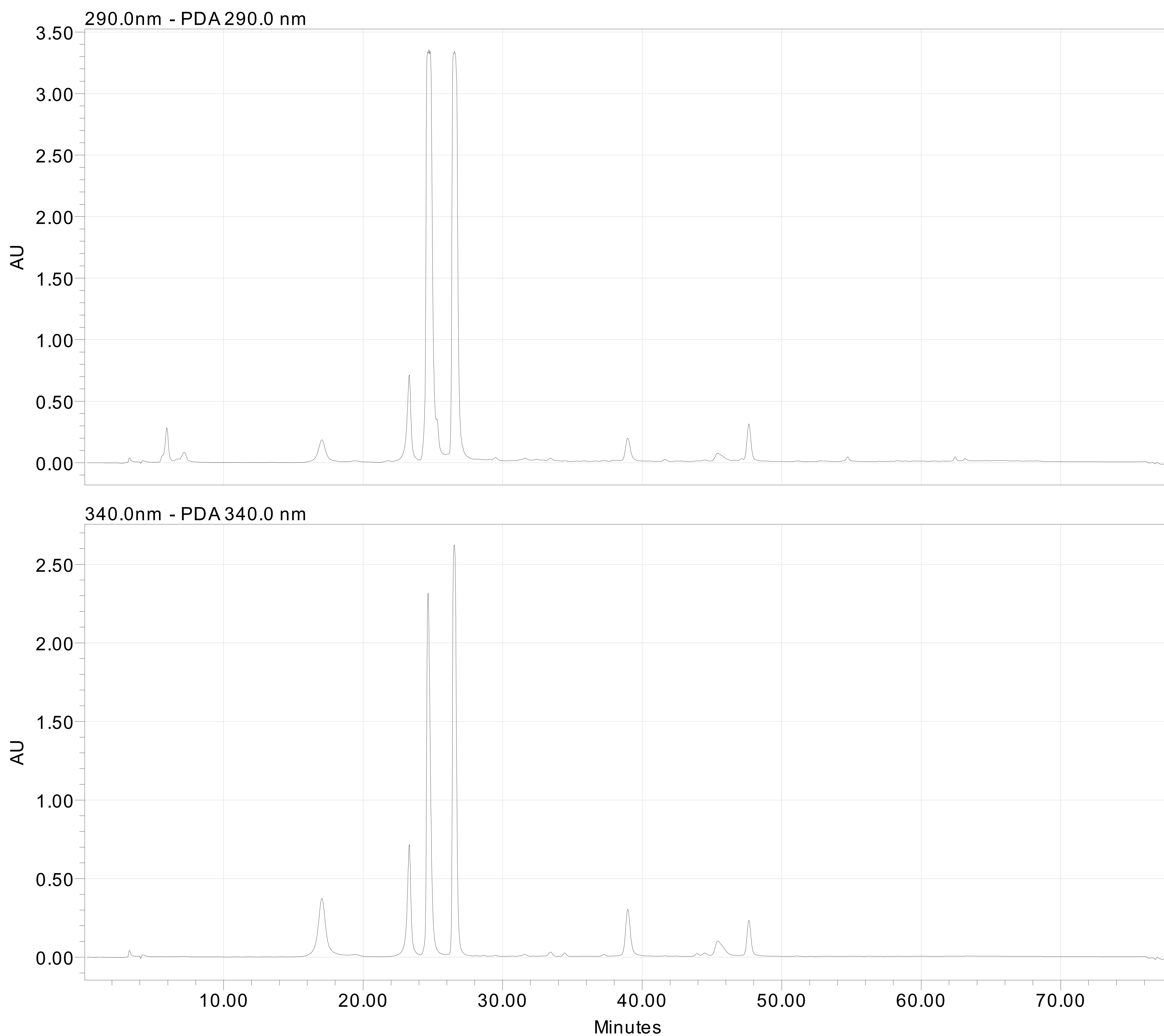
3/31/2009

5:14:29 PM NZ

Sample Name: CC 111208 AD Ext Eff

**Extraction of Flavonoids from Artificial Honey
Extraction 4**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/18/2008 3:54:22 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

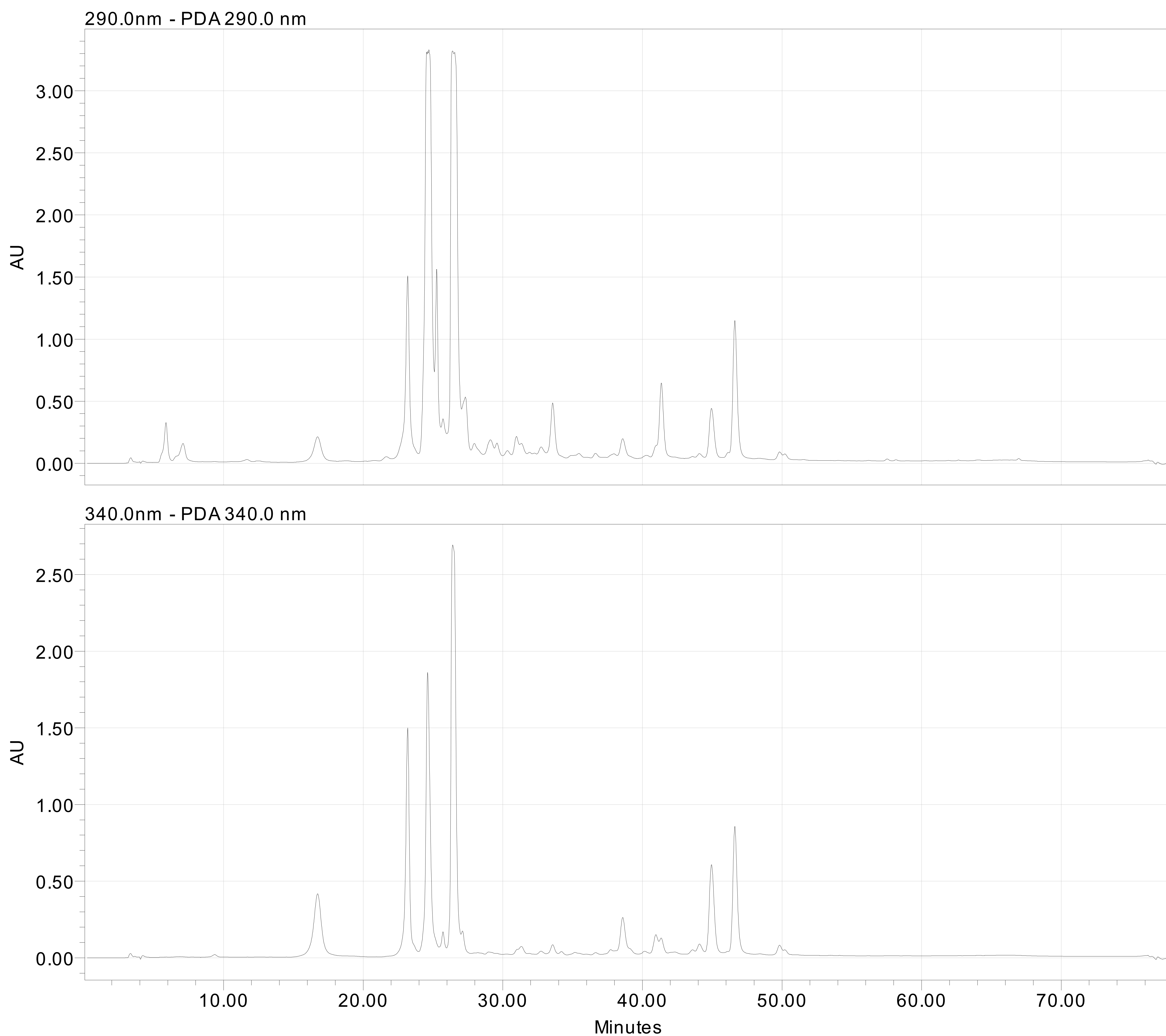
3/31/2009

5:13:47 PM NZ

Sample Name: CC 171209 AF

**Extraction of Flavonoids from Artificial Honey
Extraction 5**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/28/2009 12:03:11 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

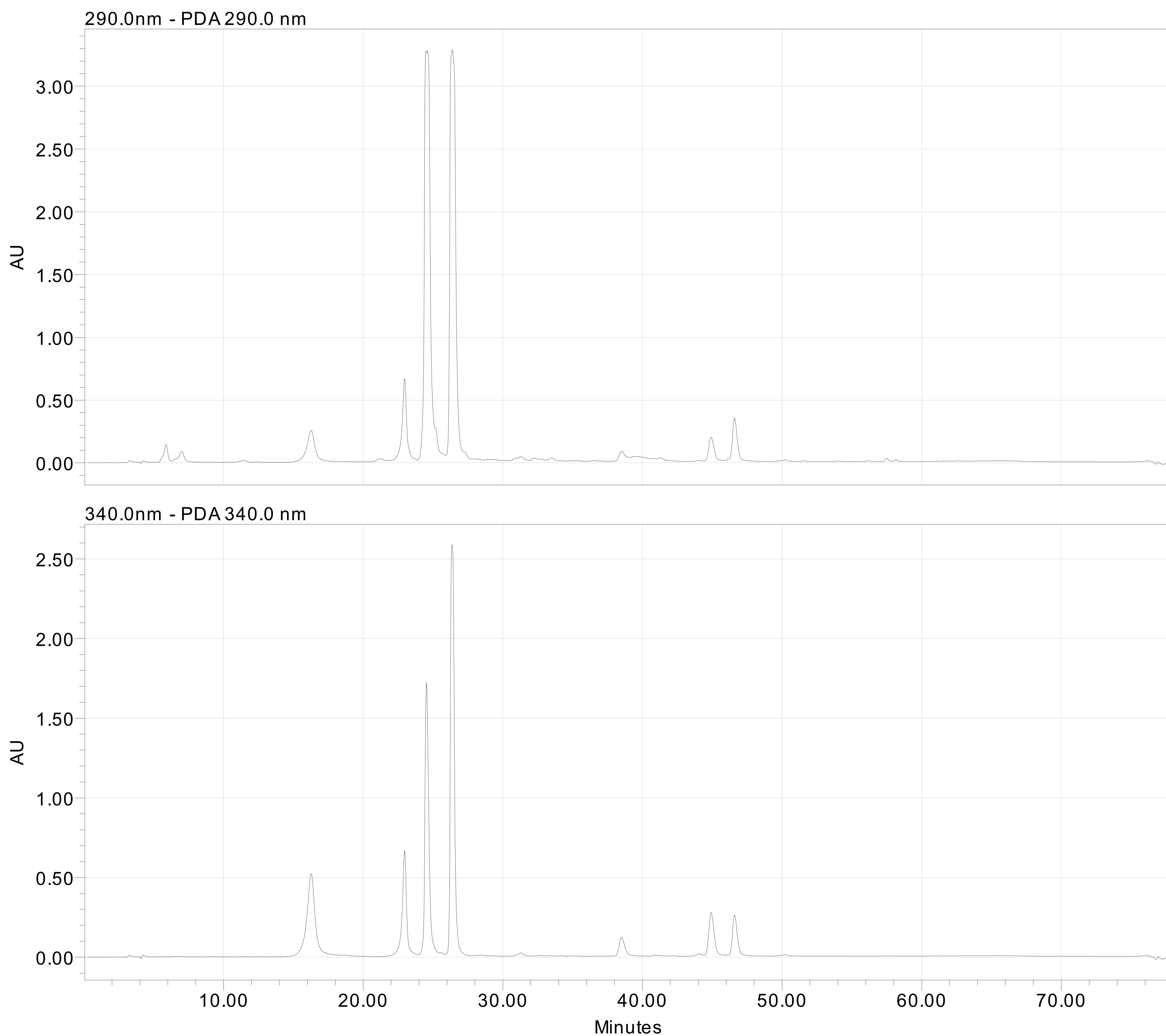
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:16:22 PM NZ

Sample Name: CC 050109 AG**Extraction of Flavonoids from Artificial Honey
Extraction 6****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/28/2009 2:46:11 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

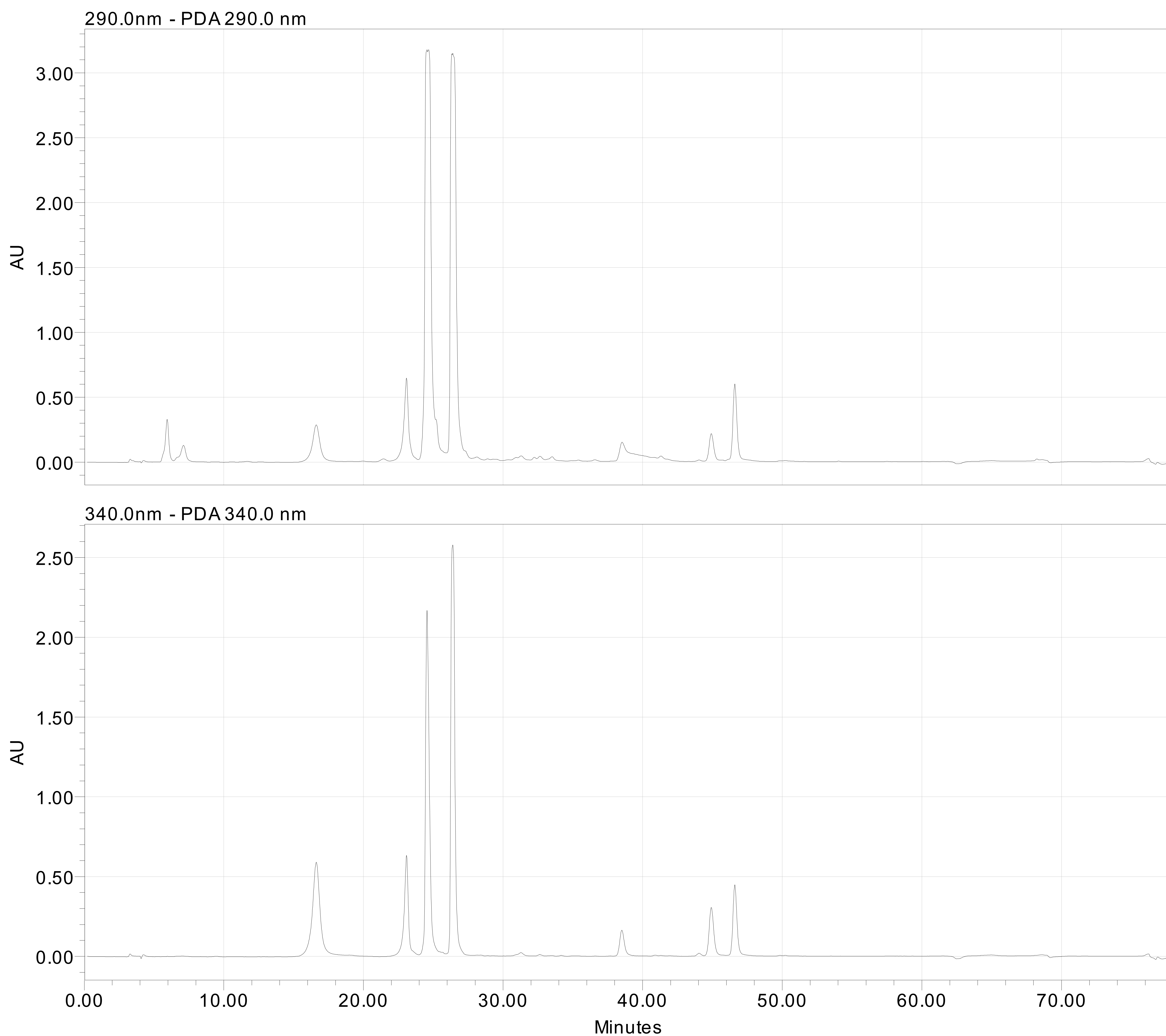
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:17:39 PM NZ

Sample Name: CC 050109 AH**Extraction of Flavonoids from Artificial Honey
Extraction 7****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/28/2009 1:23:08 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

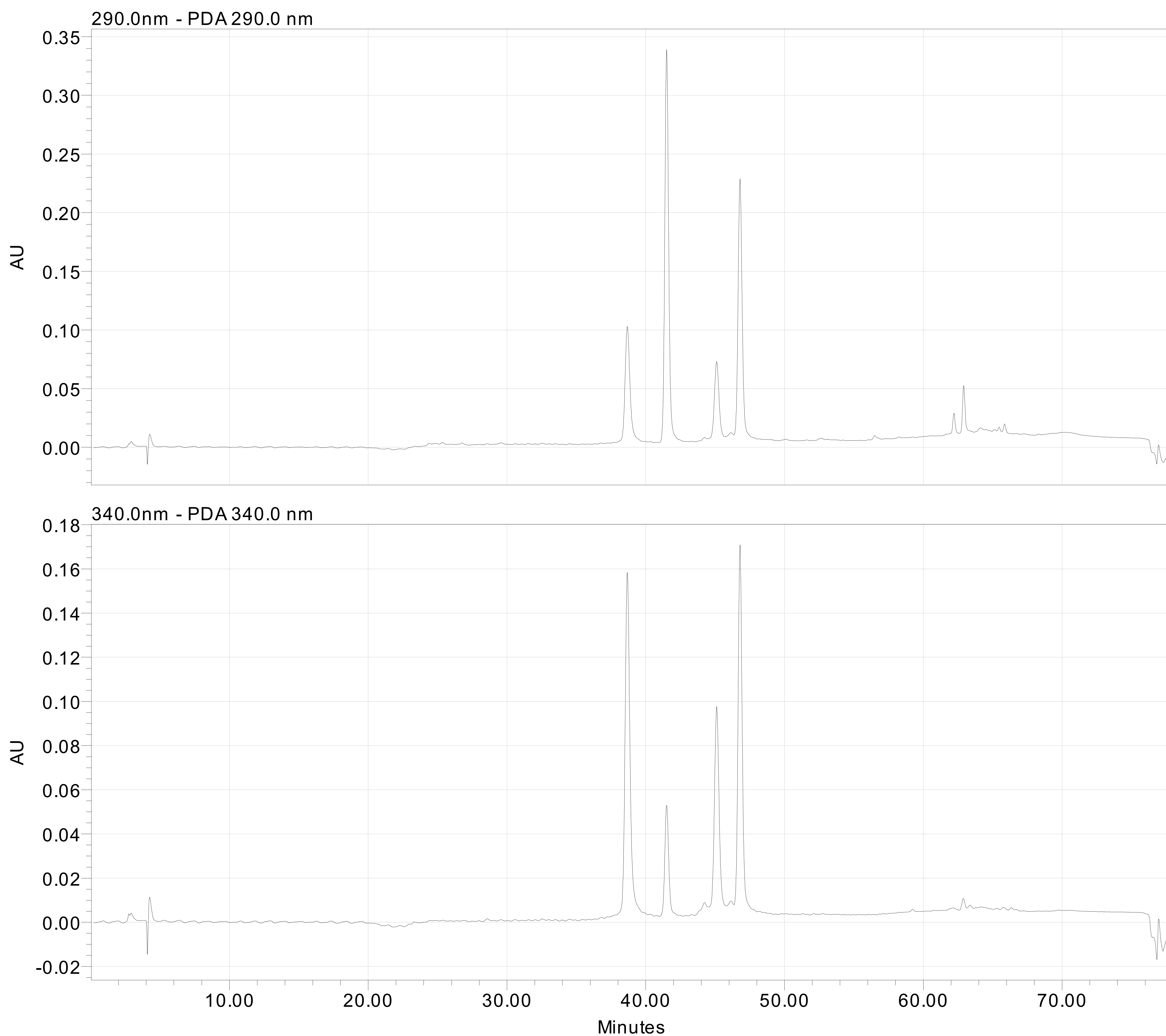
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:16:55 PM NZ

Sample Name: CC 200209 Mix3**Recovery Rate Test of Extraction Method
Extraction 1****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/20/2009 1:16:26 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

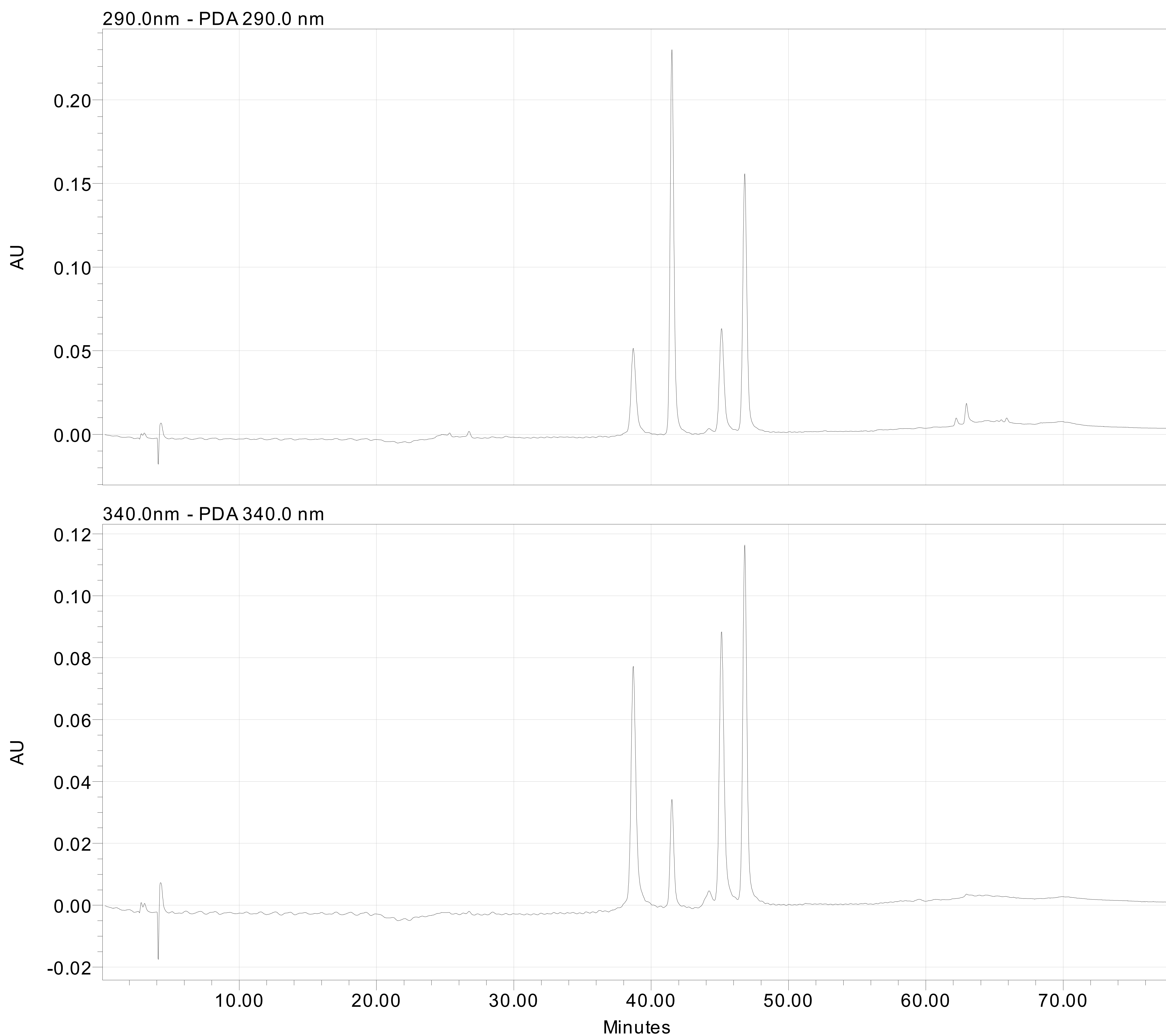
3/31/2009

4:35:49 PM NZ

Sample Name: CC 200209 Mix4

**Recovery Rate Test of Extraction Method
Extraction 2**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/20/2009 2:36:12 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

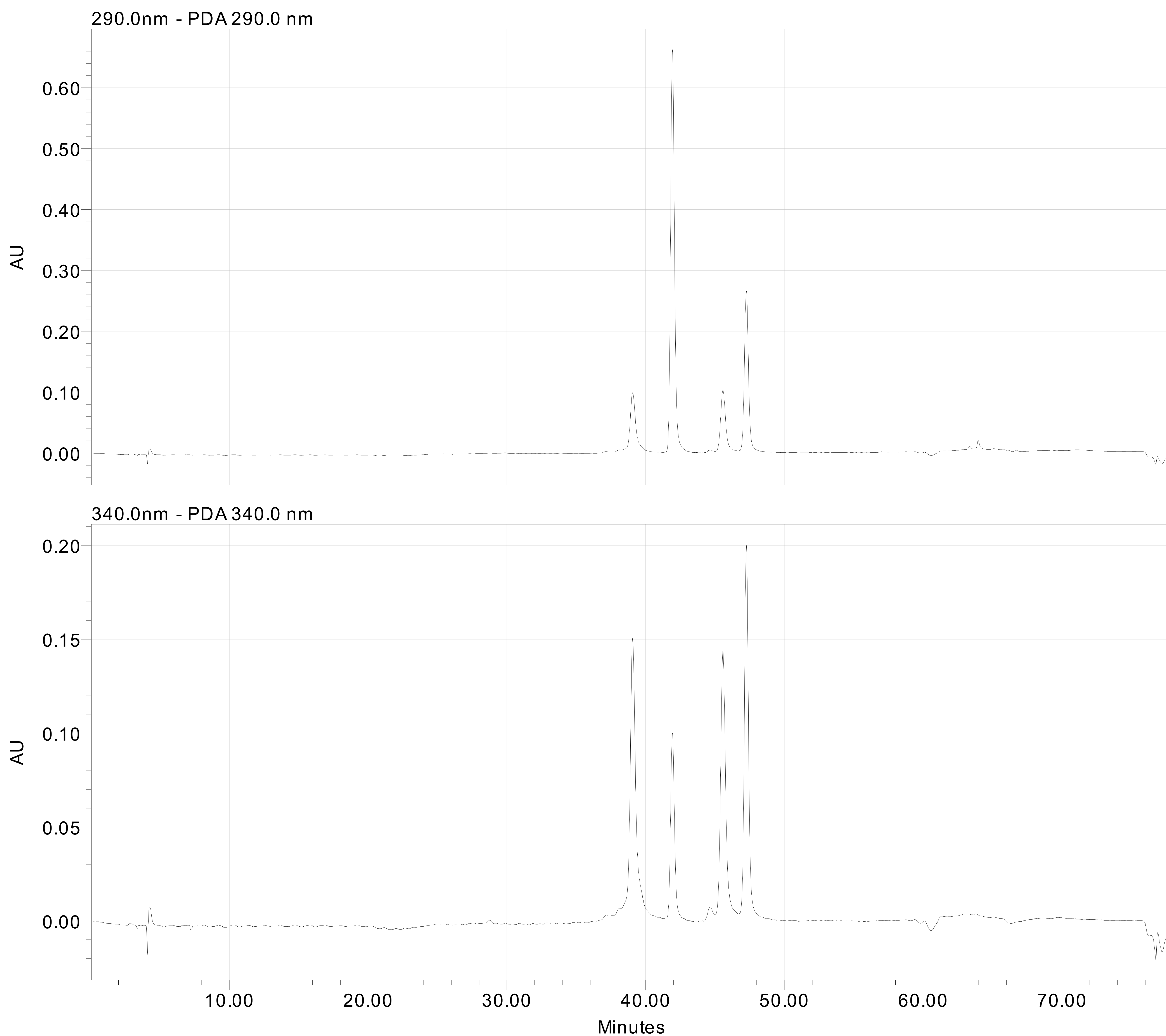
3/31/2009

4:36:50 PM NZ

**Sample Name: BD 230209 Cleanup
Recovery Test**

Recovery Rate Test of HPLC Cleanup Method

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/23/2009 1:13:54 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

4:13:58 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman



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Te Whare Wānanga o Waikato

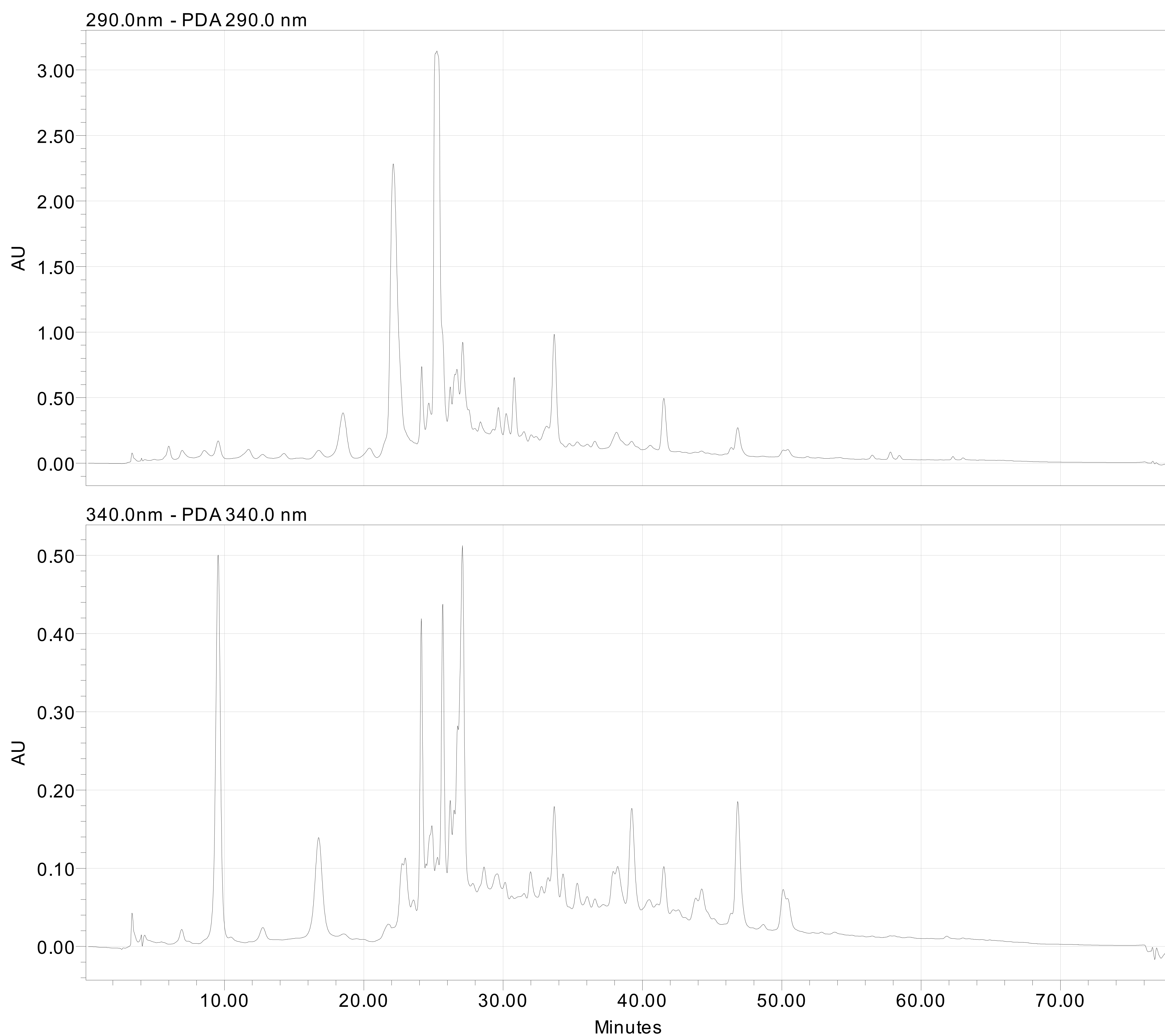
The University of Waikato

2009

Sample Name: CC 211108 A

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/16/2008 2:31:49 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

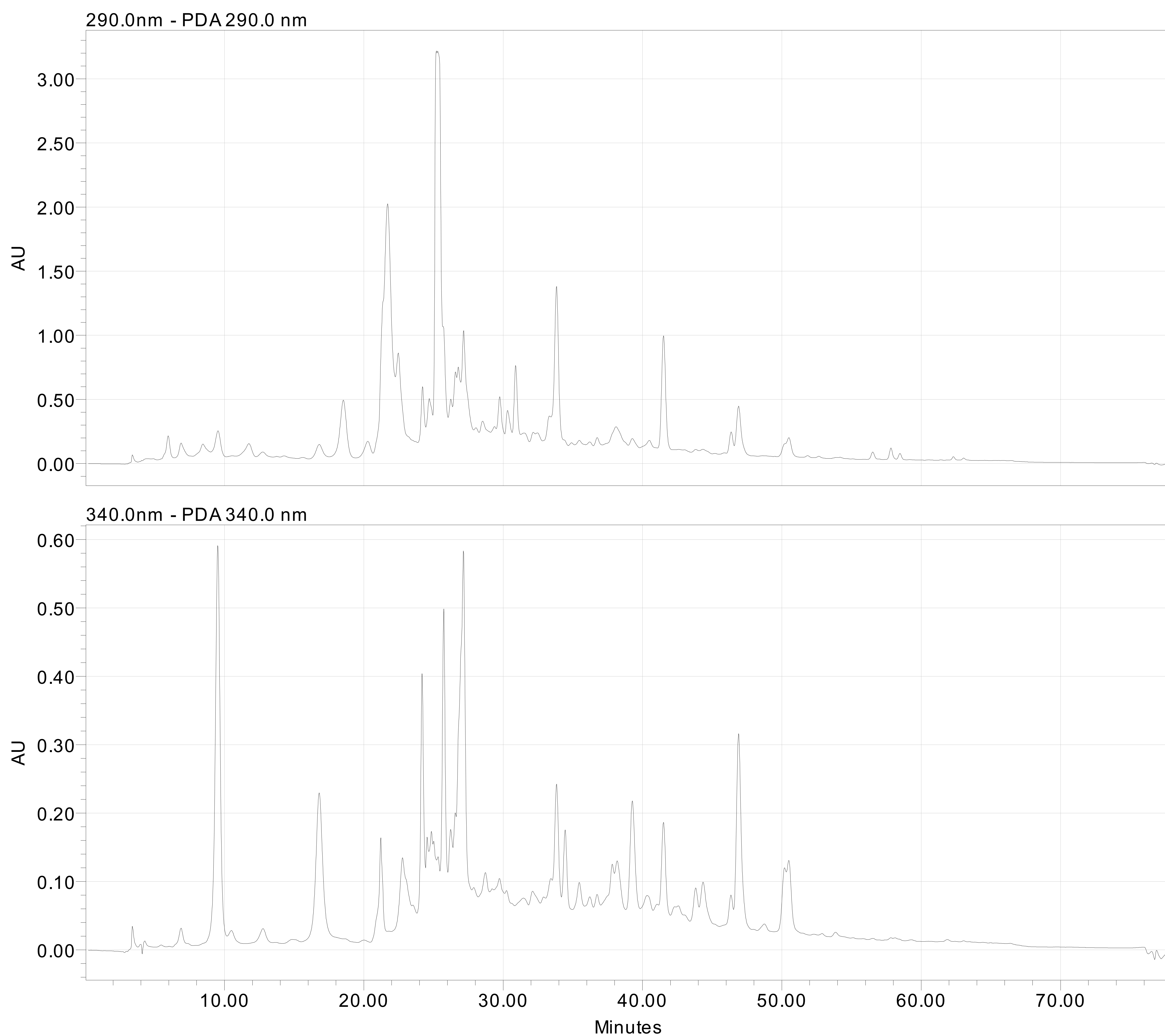
3/31/2009

5:32:54 PM NZ

Sample Name: CC 211108 B

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/16/2008 3:52:13 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

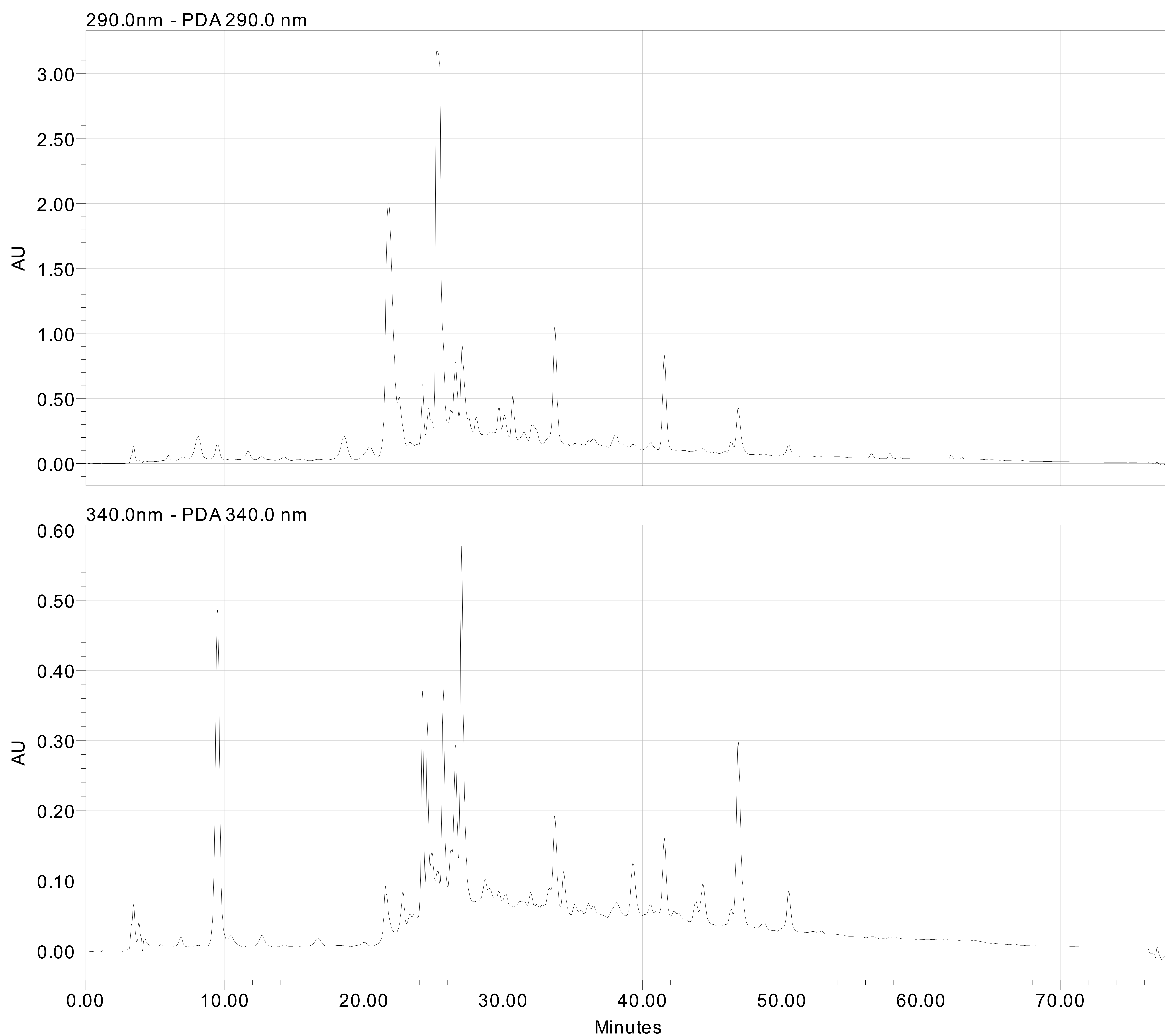
3/31/2009

5:33:20 PM NZ

Sample Name: CC 211108 C

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/18/2009 2:32:24 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

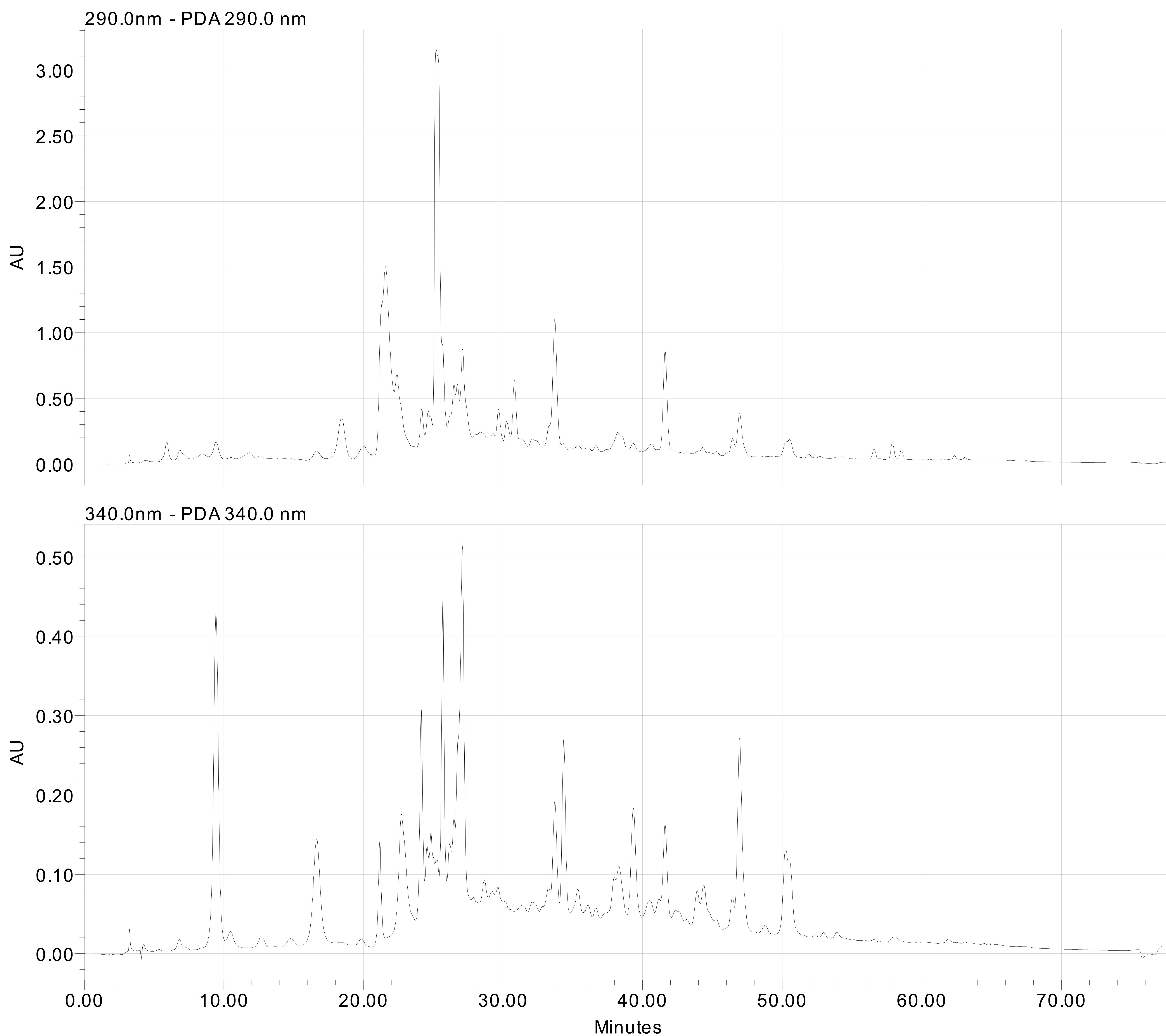
3/31/2009

5:41:49 PM NZ

Sample Name: CC 261108 D

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/16/2008 5:13:43 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

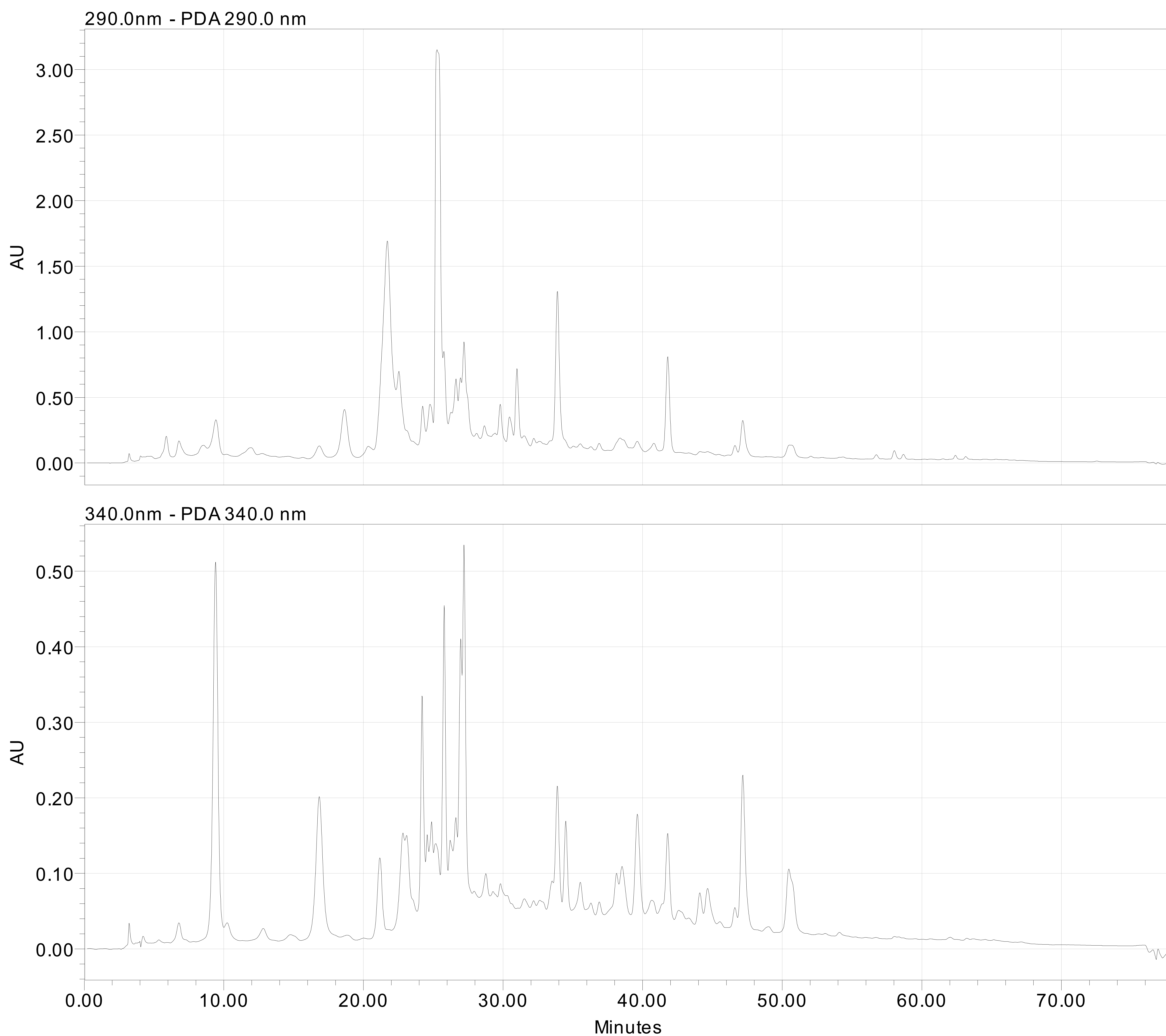
3/31/2009

5:33:48 PM NZ

Sample Name: CC 261108 E

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/17/2008 11:21:07 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

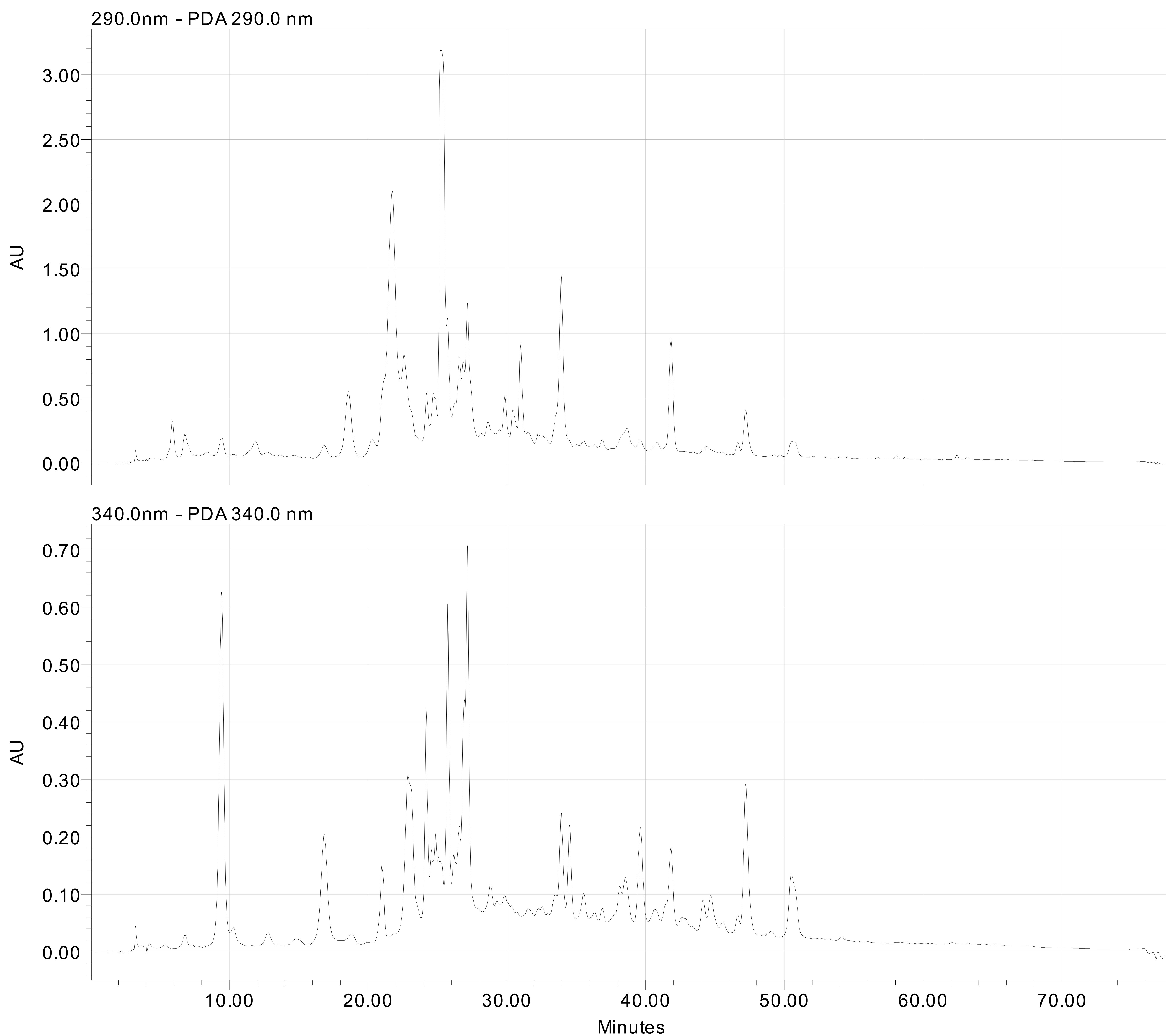
3/31/2009

5:34:12 PM NZ

Sample Name: CC 281108 F

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/17/2008 12:53:58 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

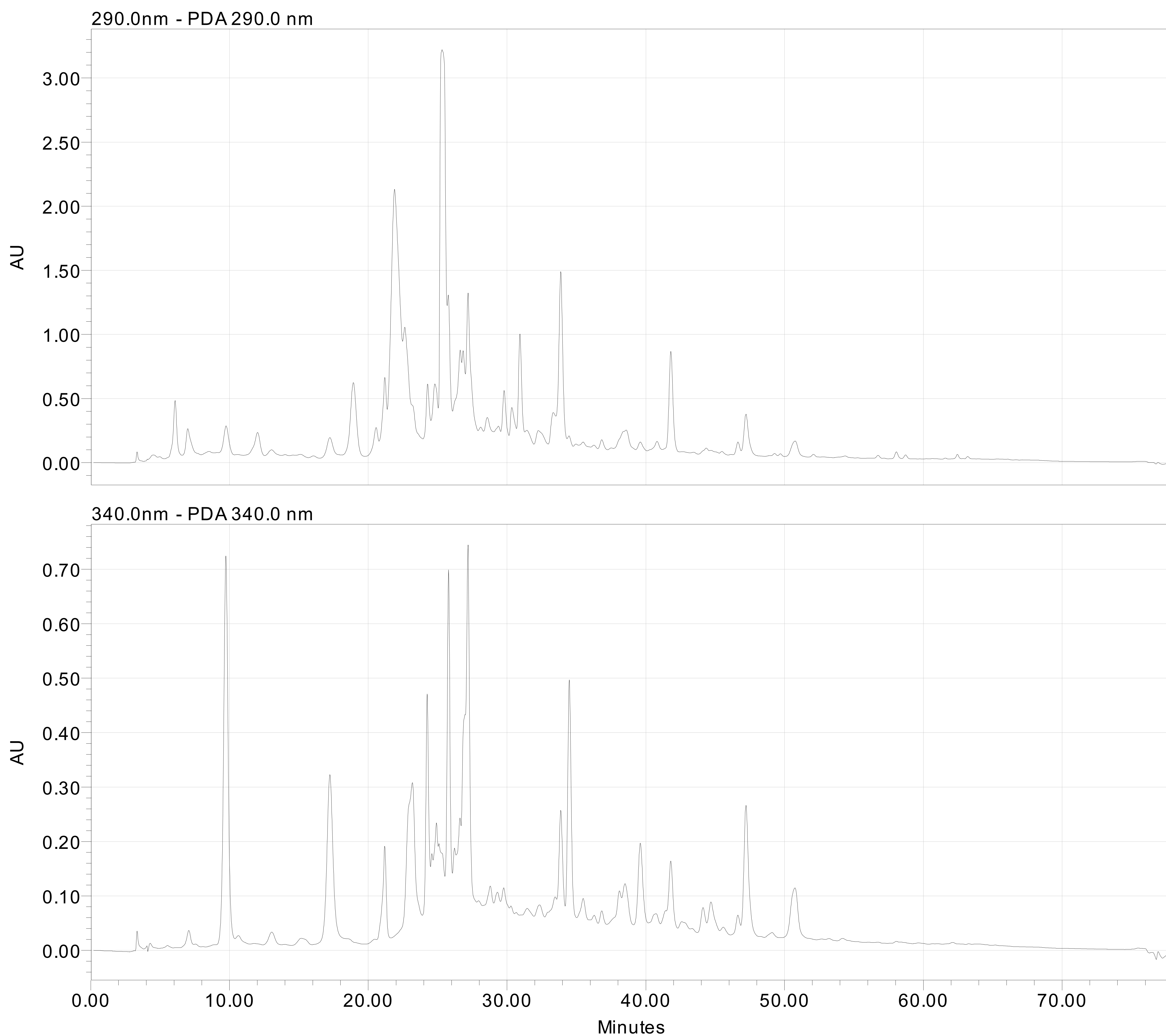
3/31/2009

5:34:30 PM NZ

Sample Name: CC 281108 G

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/17/2008 2:13:20 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

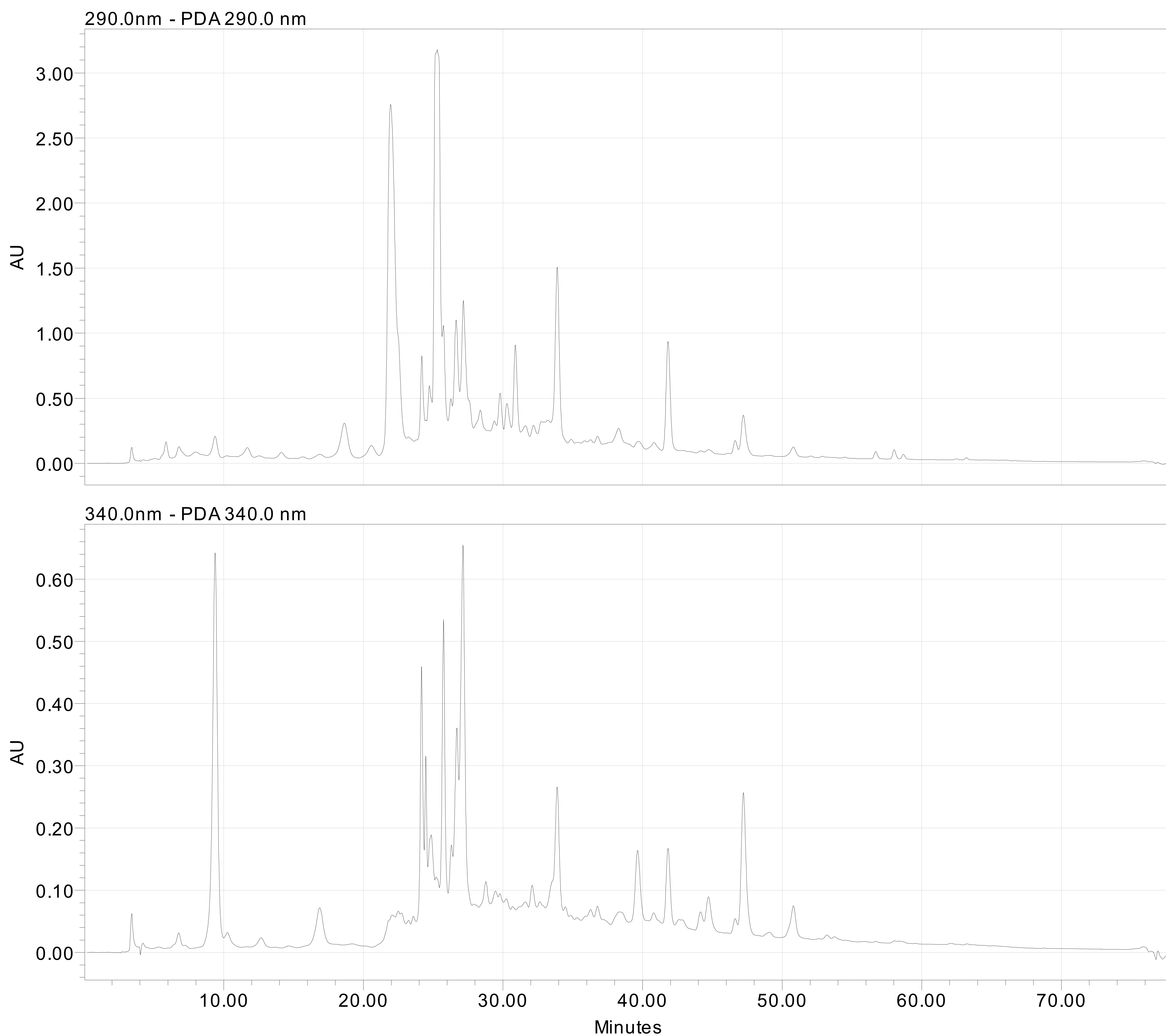
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:34:48 PM NZ

Sample Name: BD 220908 A1**Repeated Extraction of M110
Manuka Honey Sample****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/17/2008 3:41:05 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

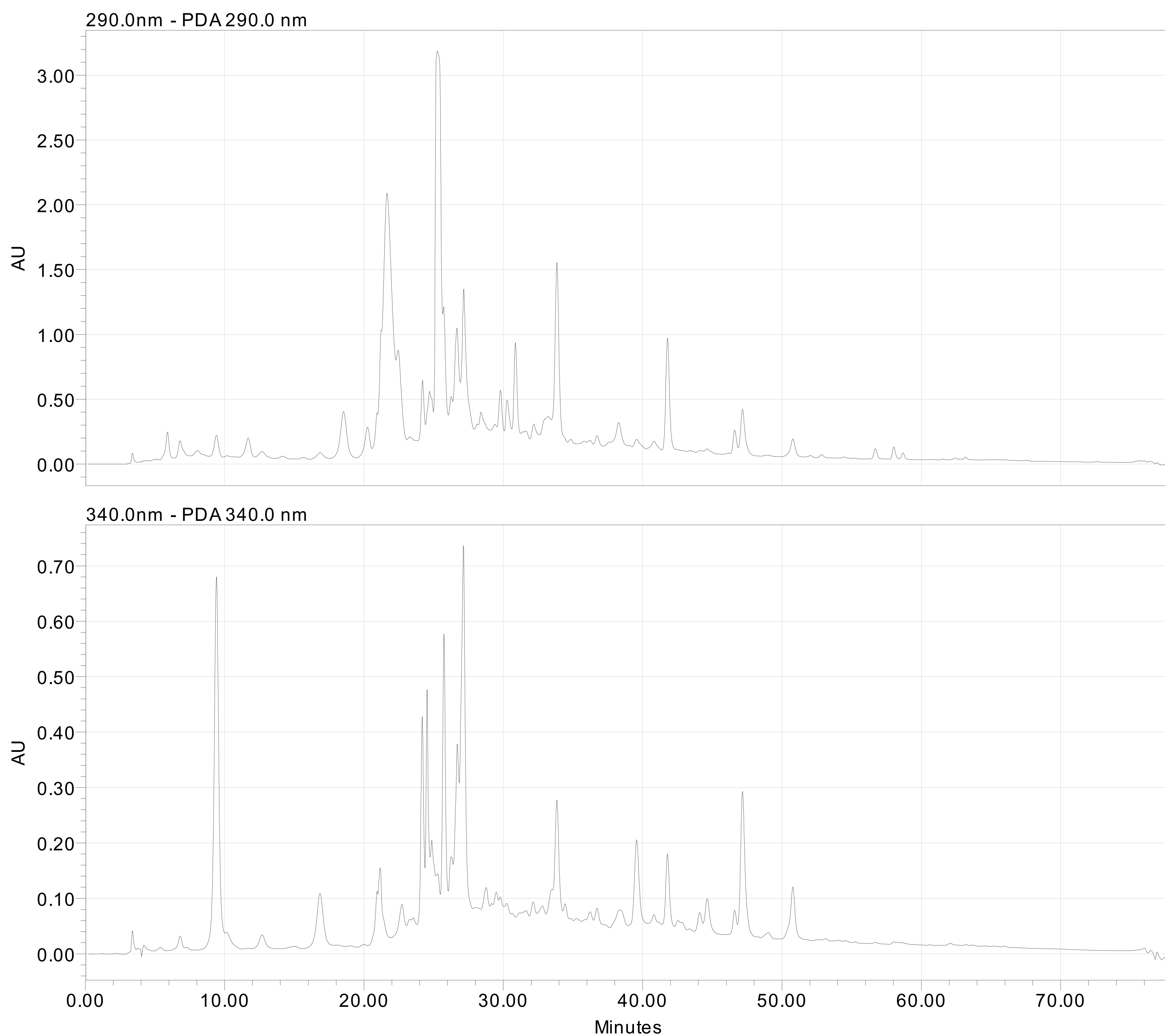
3/31/2009

5:36:06 PM NZ

Sample Name: BD 220908 B1

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/17/2008 5:06:36 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

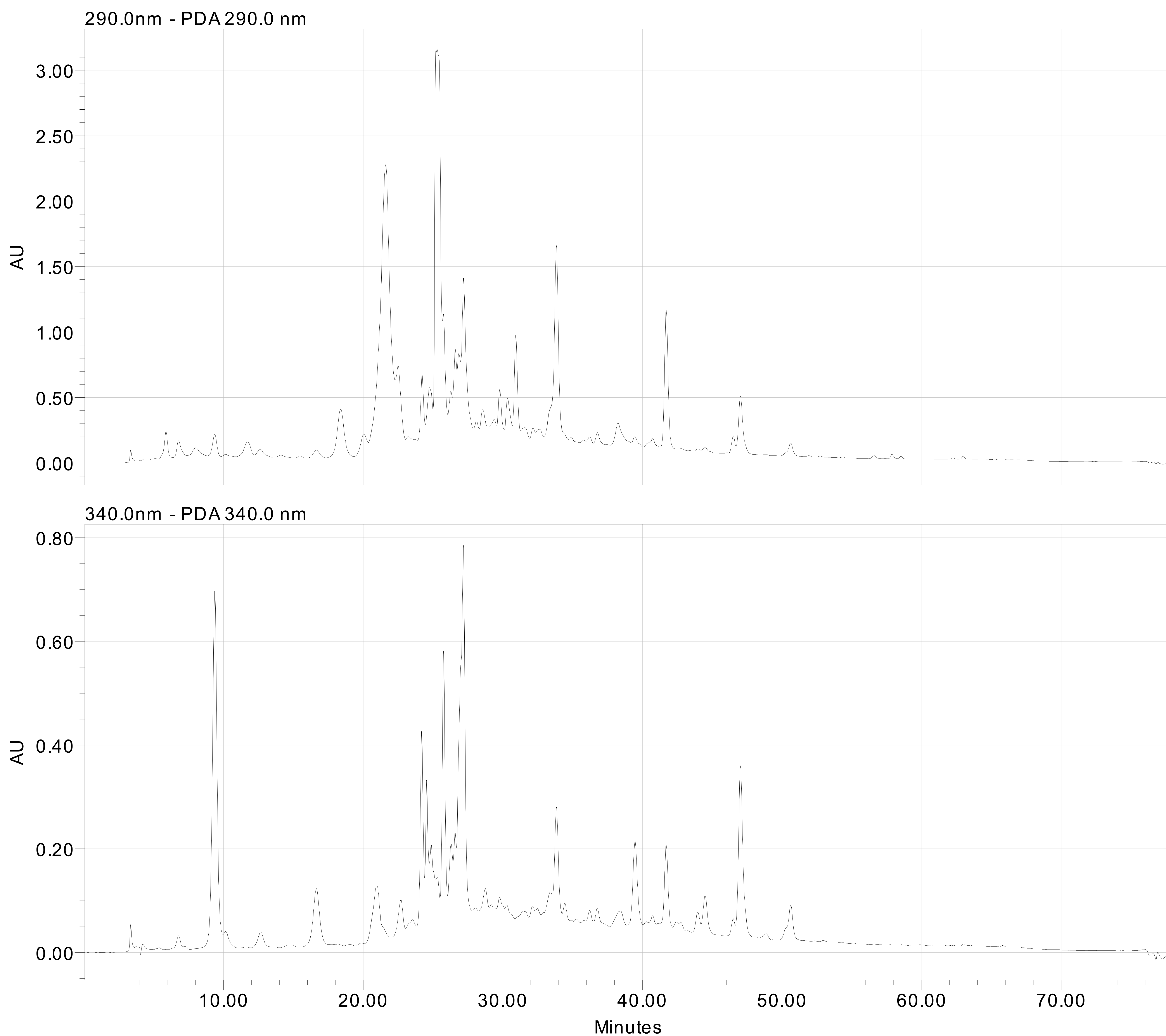
3/31/2009

5:36:38 PM NZ

Sample Name: BD 021008 A2

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/18/2008 11:53:16 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

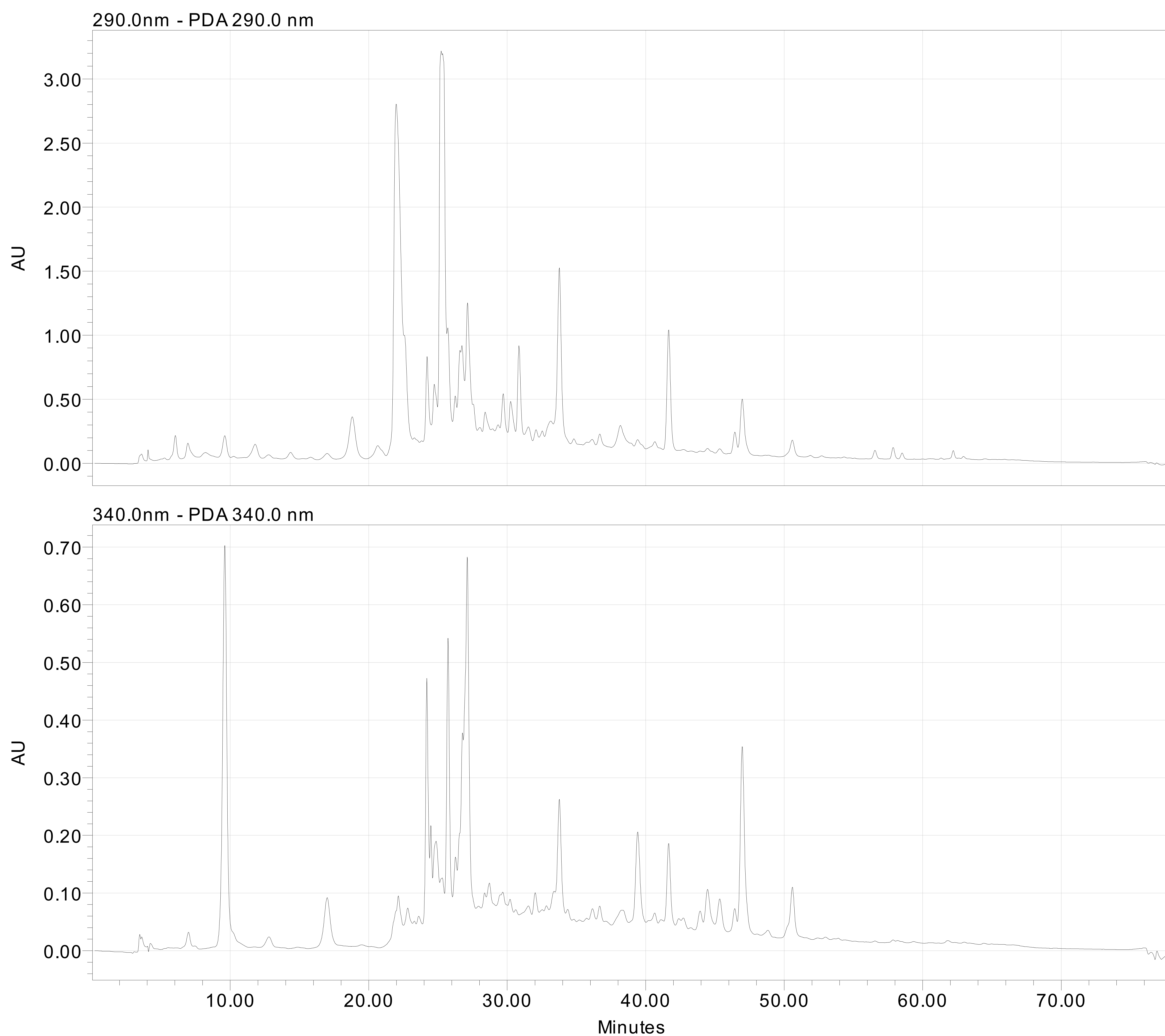
3/31/2009

5:37:05 PM NZ

Sample Name: BD 021008 B2

**Repeated Extraction of M110
Manuka Honey Sample**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 12/18/2008 1:12:40 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:37:32 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

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by

Benjamin Jade Deadman



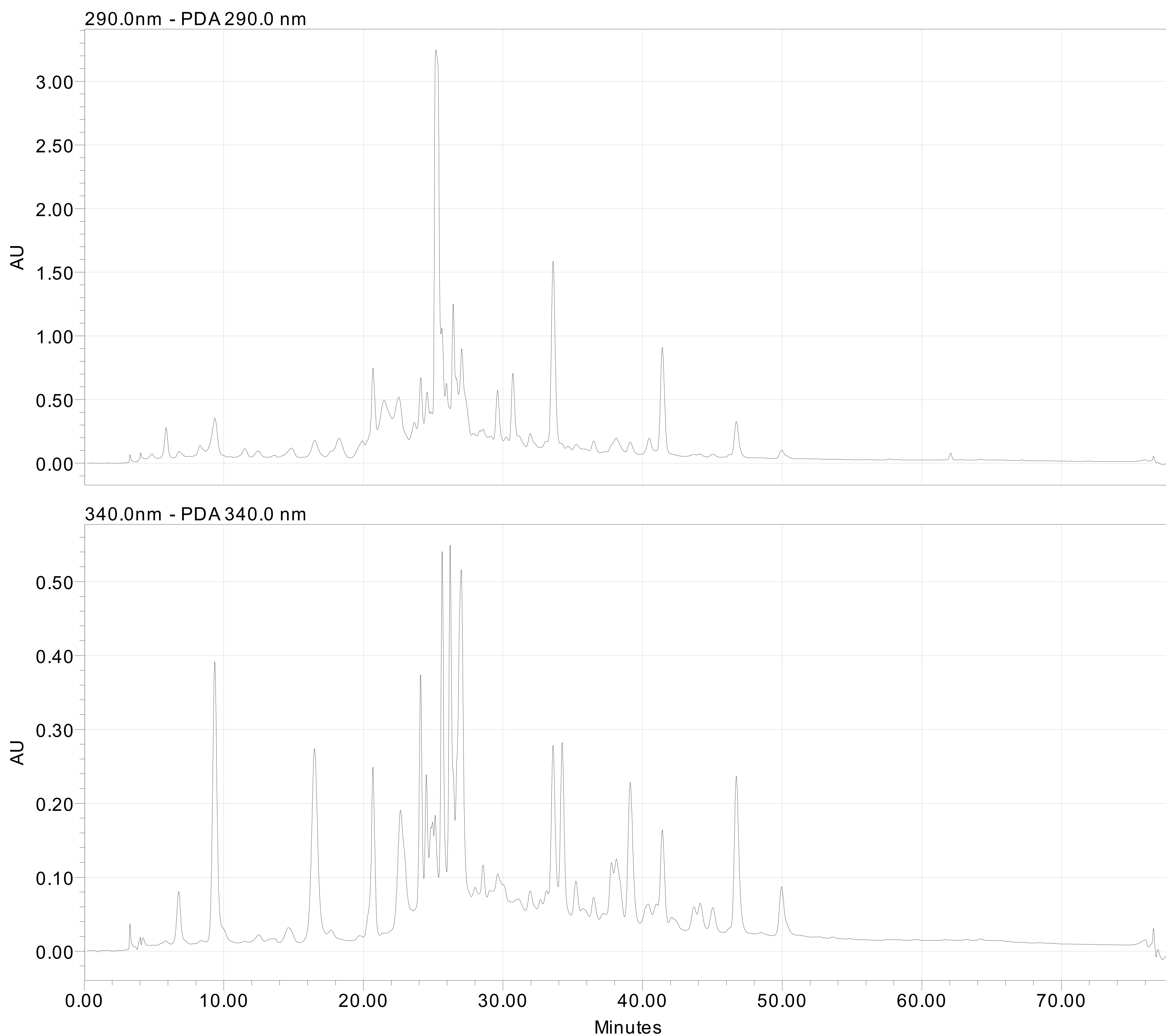
THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

The University of Waikato

2009

Sample Name: CC 130109 ACT5+ Manuka Honey UMF 7.7

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/30/2009 3:22:38 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

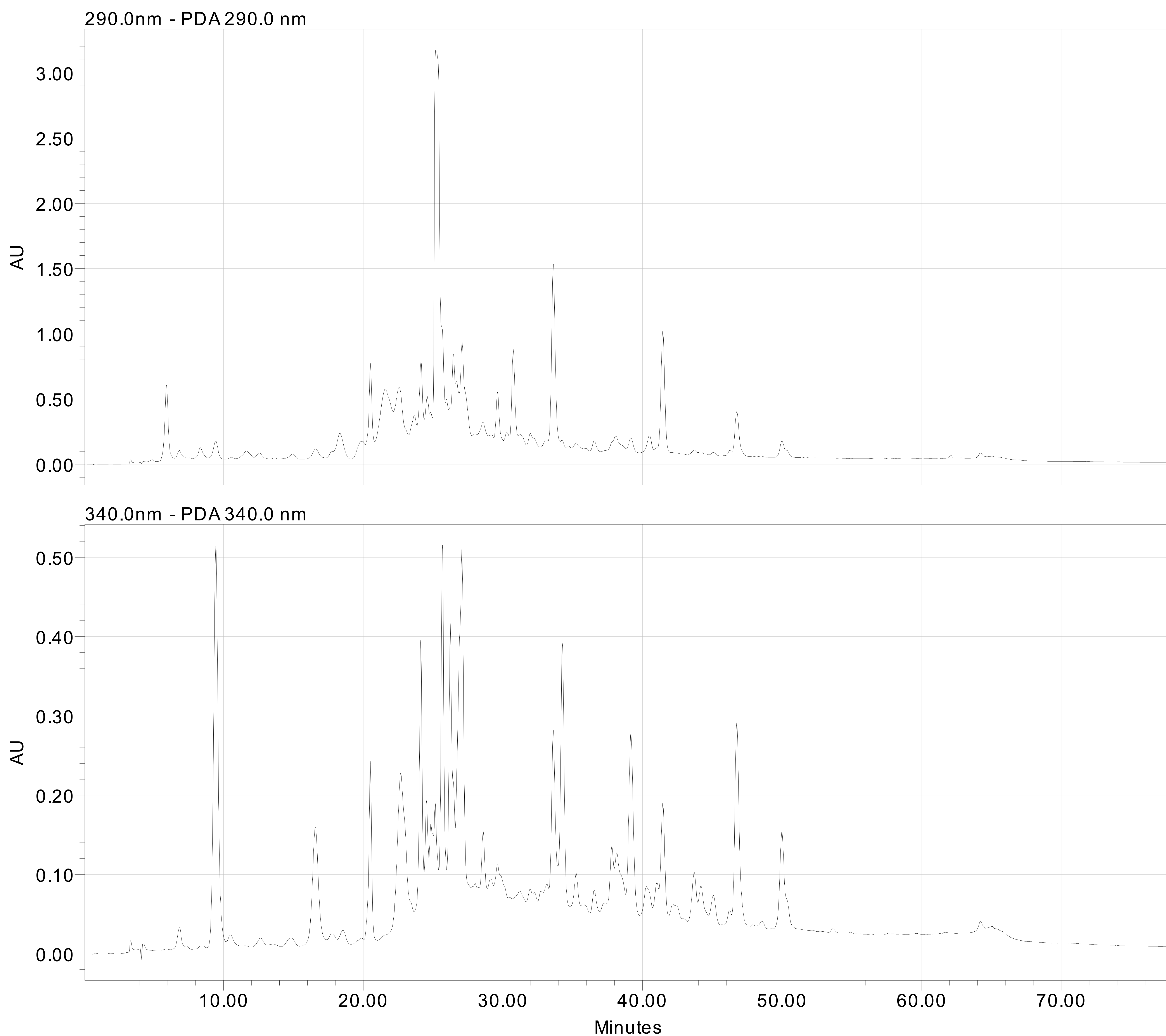
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:58:43 PM NZ

Sample Name: CC 140109 UMF10 Manuka Honey UMF 11.1**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/30/2009 4:45:17 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

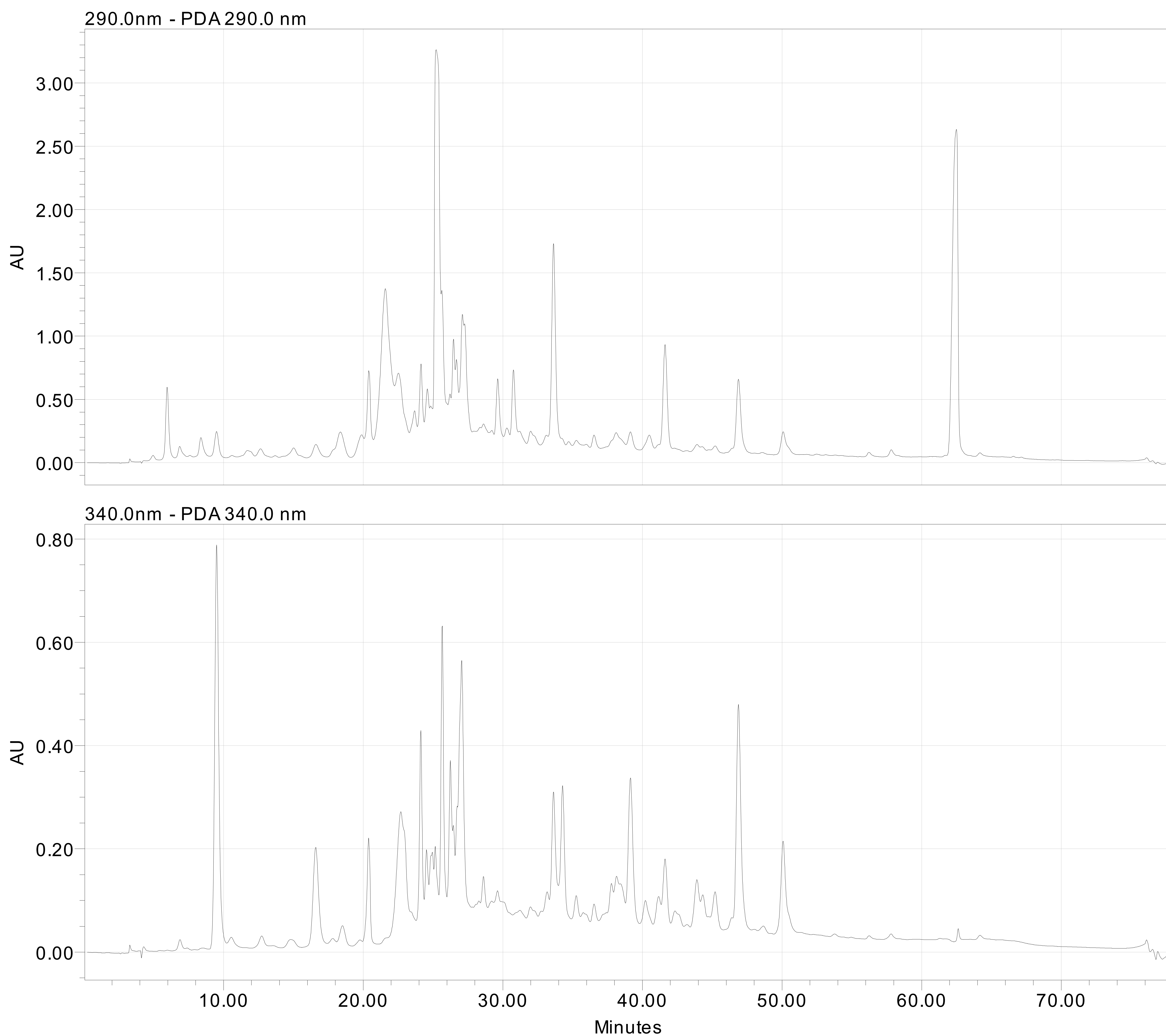
Date Printed:

3/24/2009

3:57:52 PM NZ

Sample Name: CC 130109 UMF15 Manuka Honey UMF 15.8

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/30/2009 1:55:44 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

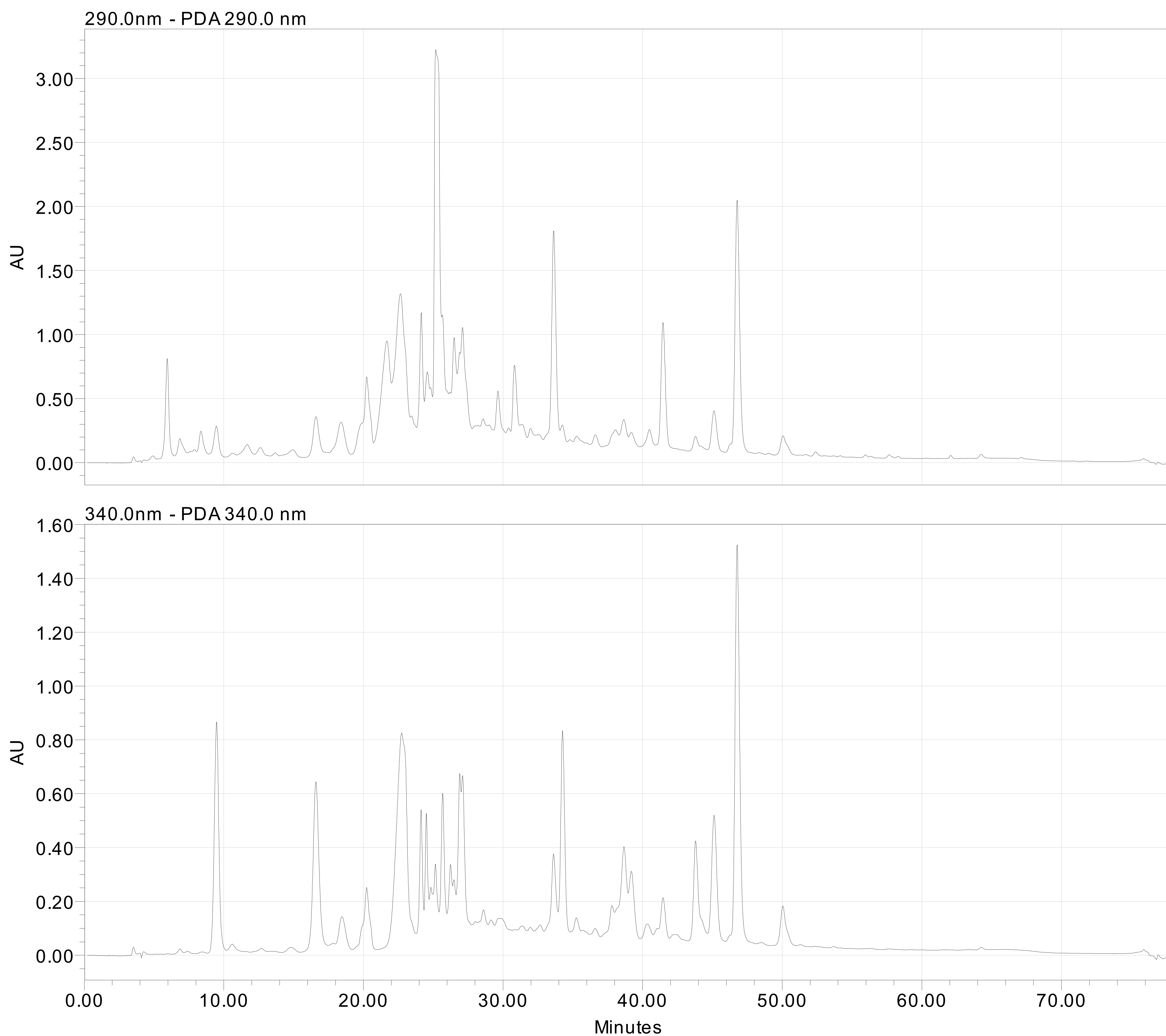
Date Printed:

3/24/2009

3:59:25 PM NZ

Sample Name: CC 080109 UMF20A Manuka Honey UMF 17.1

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/29/2009 4:25:06 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

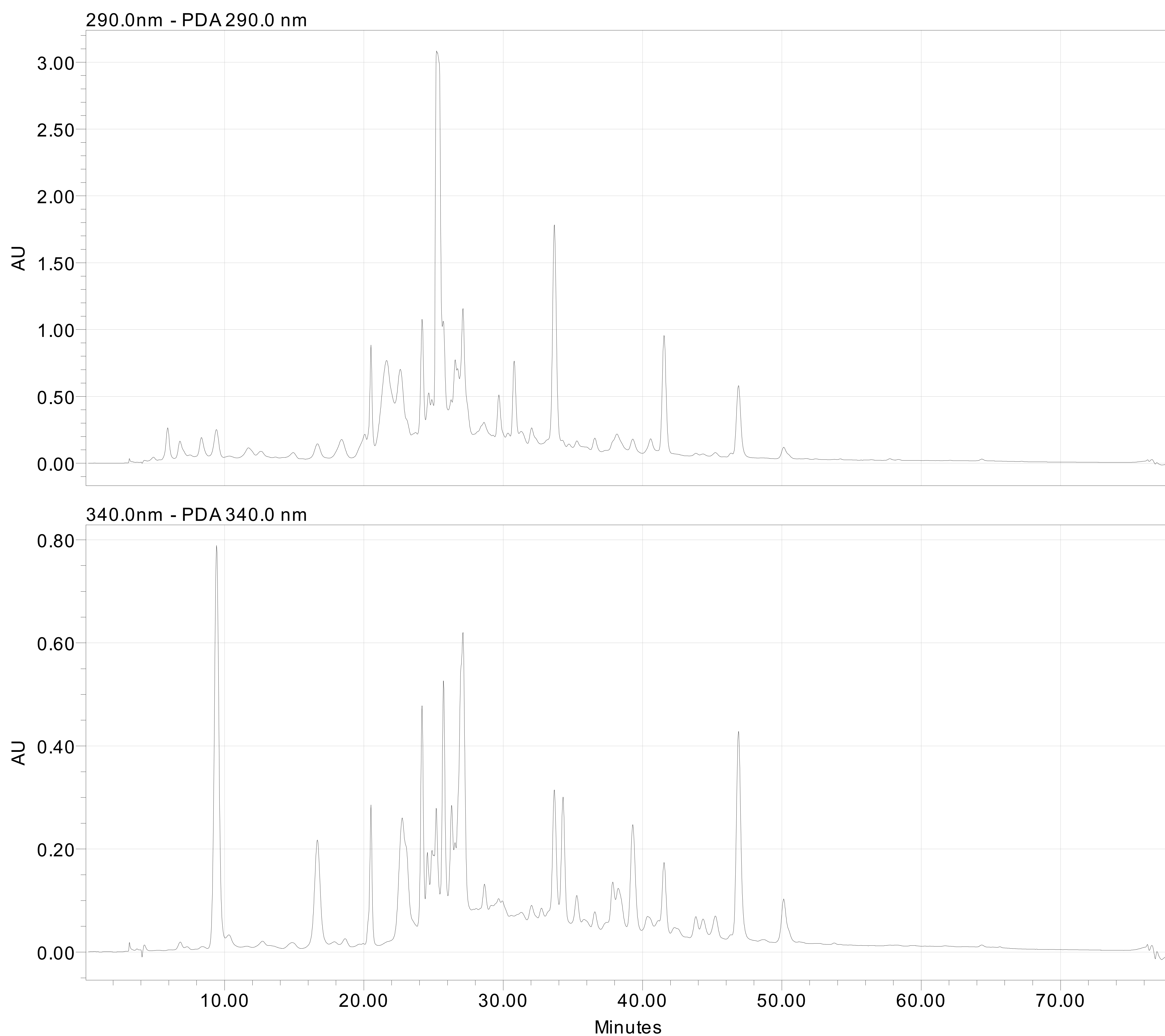
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

4:01:21 PM NZ

Sample Name: CC 120109 UMF20B Manuka Honey UMF 17.1**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/30/2009 11:01:06 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

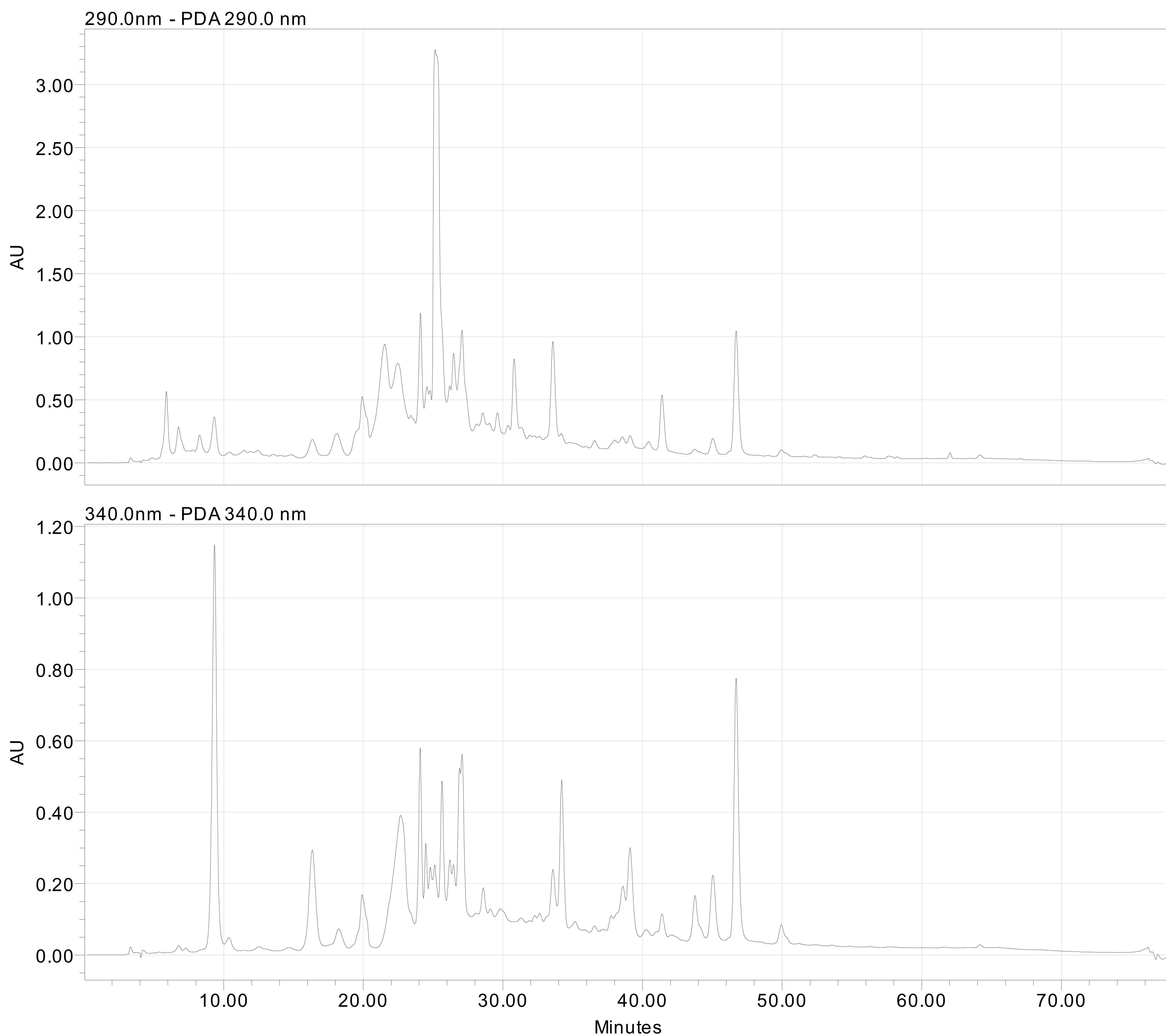
Date Printed:

3/24/2009

4:00:49 PM NZ

Sample Name: CC 080109 UMF25A Manuka Honey UMF 22.9

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/29/2009 3:04:24 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

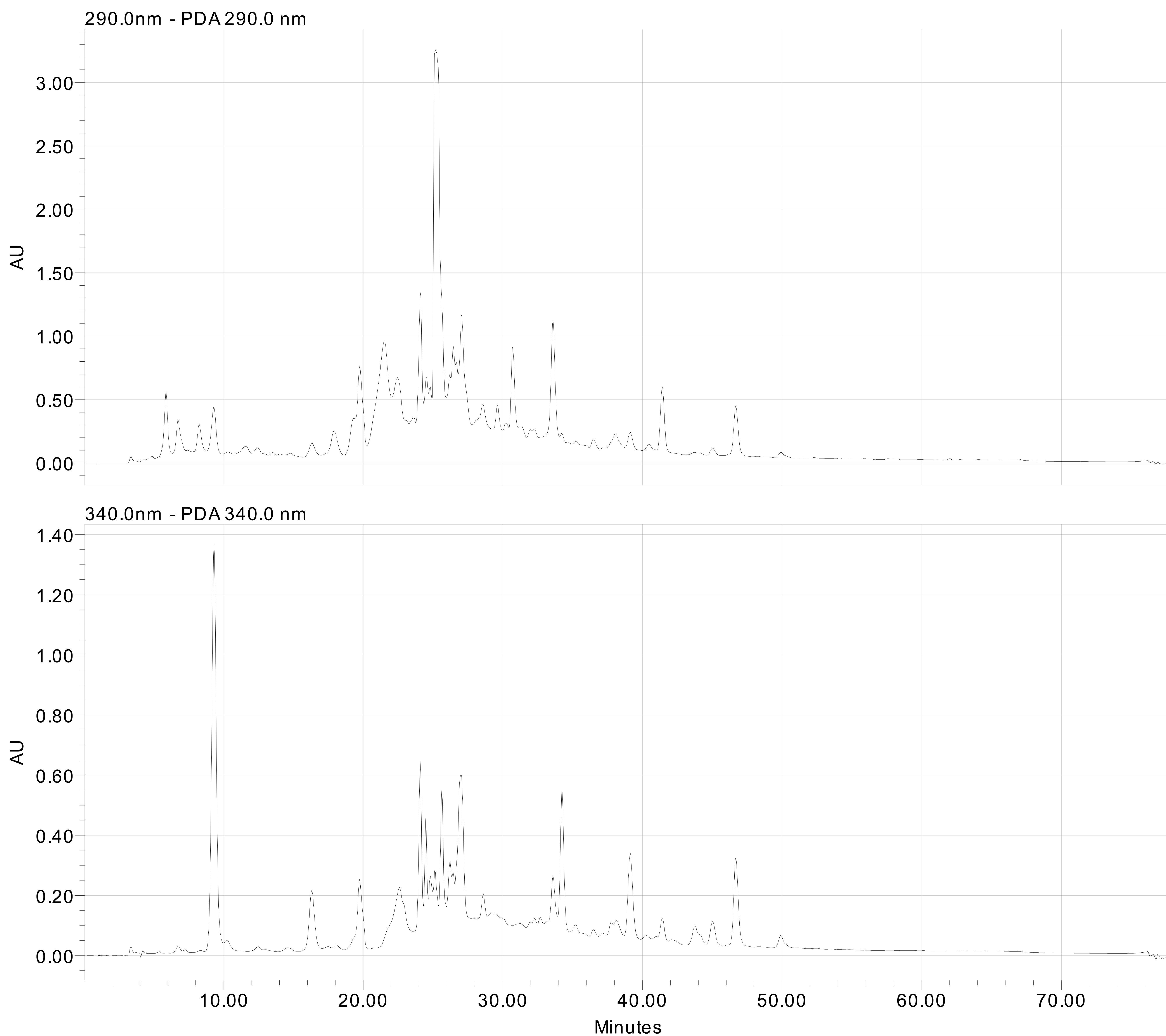
Date Printed:

3/24/2009

4:02:23 PM NZ

Sample Name: CC 120109 UMF25B Manuka Honey UMF 22.9

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/30/2009 12:35:10 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

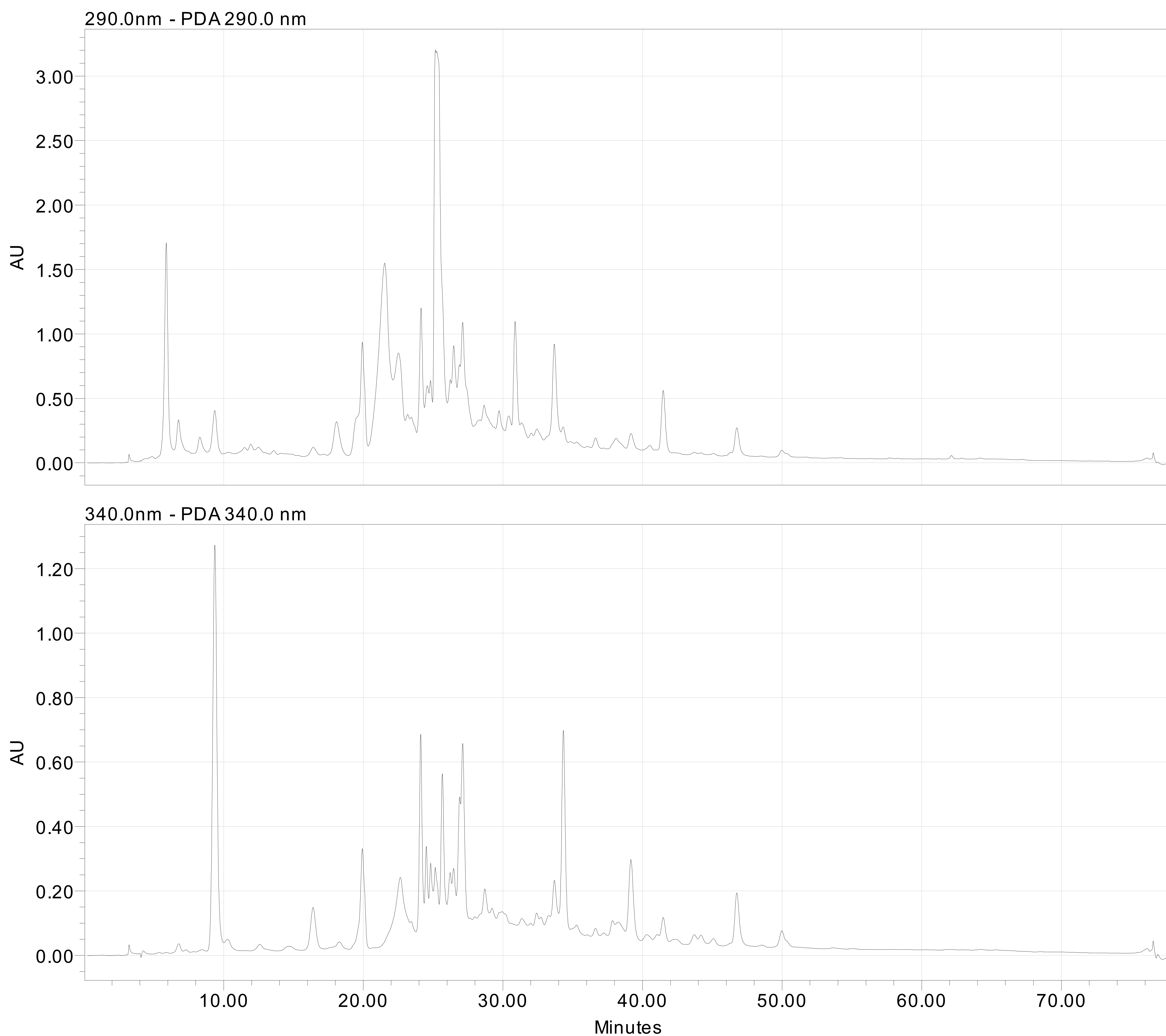
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

4:00:04 PM NZ

Sample Name: CC 140109 UMF30 Manuka Honey UMF 27**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/2/2009 12:25:10 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

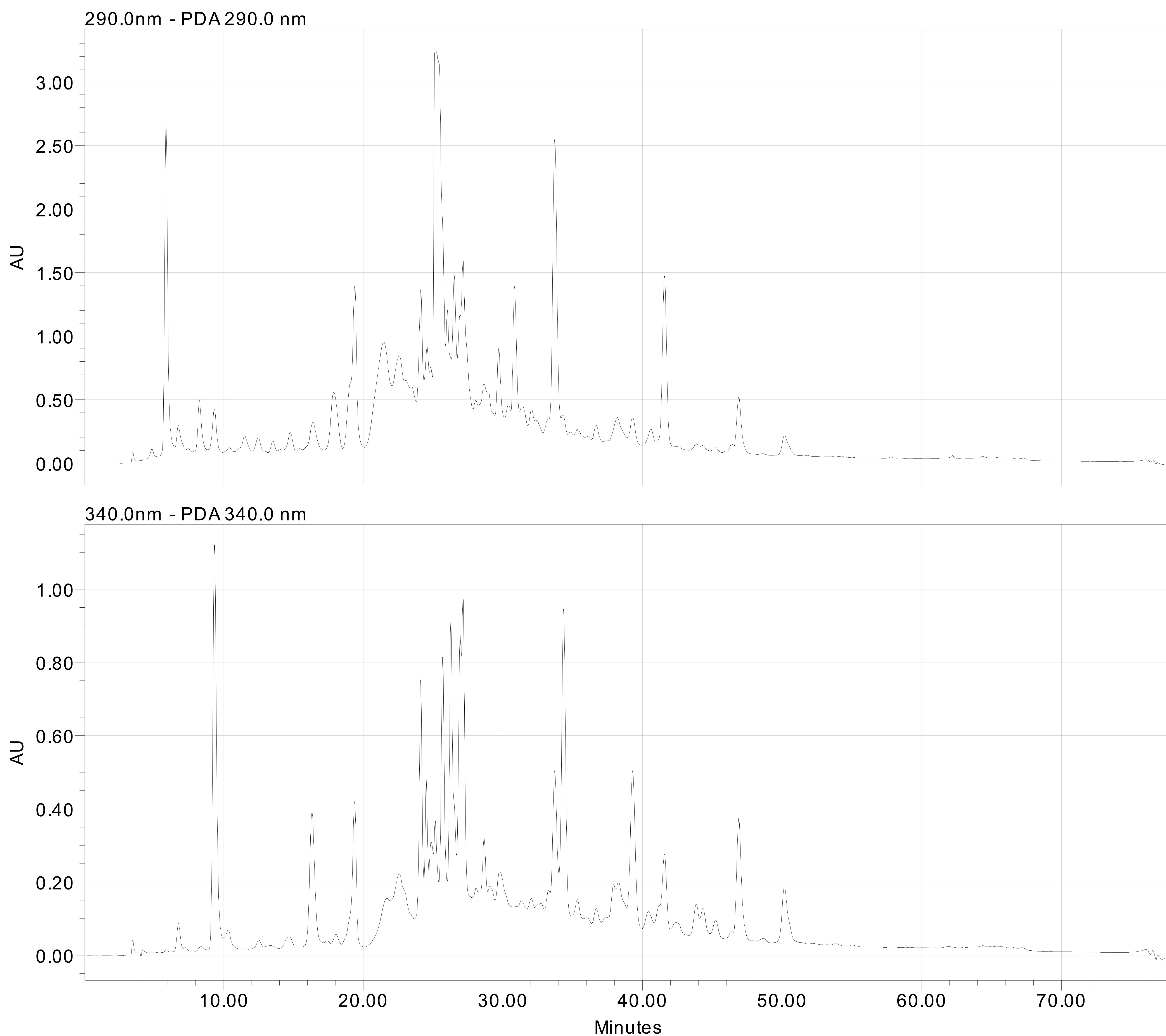
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:57:08 PM NZ

Sample Name: CC 150109 BD12.5 Manuka Honey UMF 13.8**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/2/2009 2:18:47 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

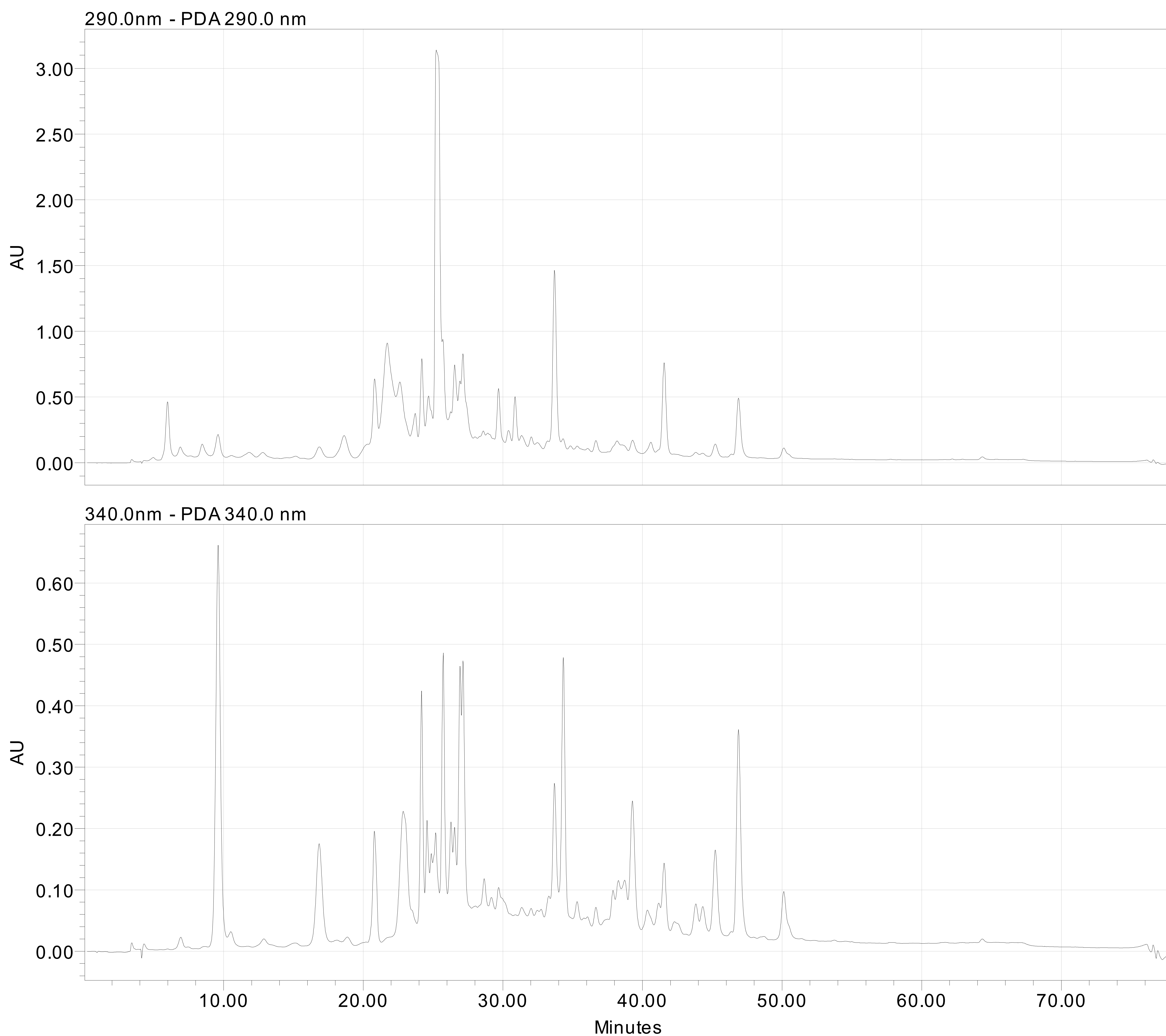
Date Printed:

3/24/2009

3:45:09 PM NZ

Sample Name: CC 150109 BD15.2 Manuka Honey UMF 17.7

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/2/2009 3:40:09 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

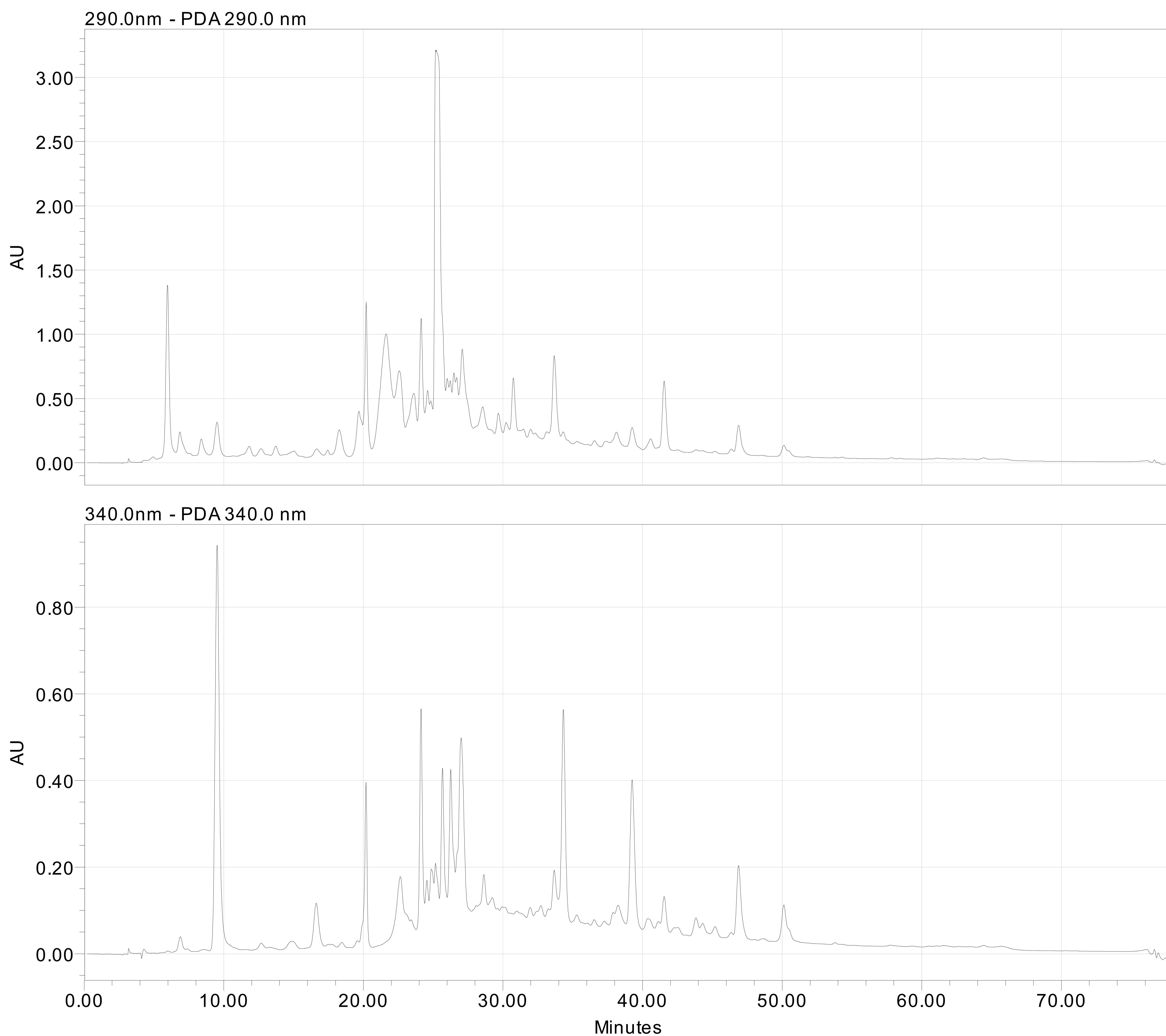
Date Printed:

3/24/2009

3:44:26 PM NZ

Sample Name: CC 190109 BD21.1 Manuka Honey UMF 20

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/4/2009 4:09:55 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

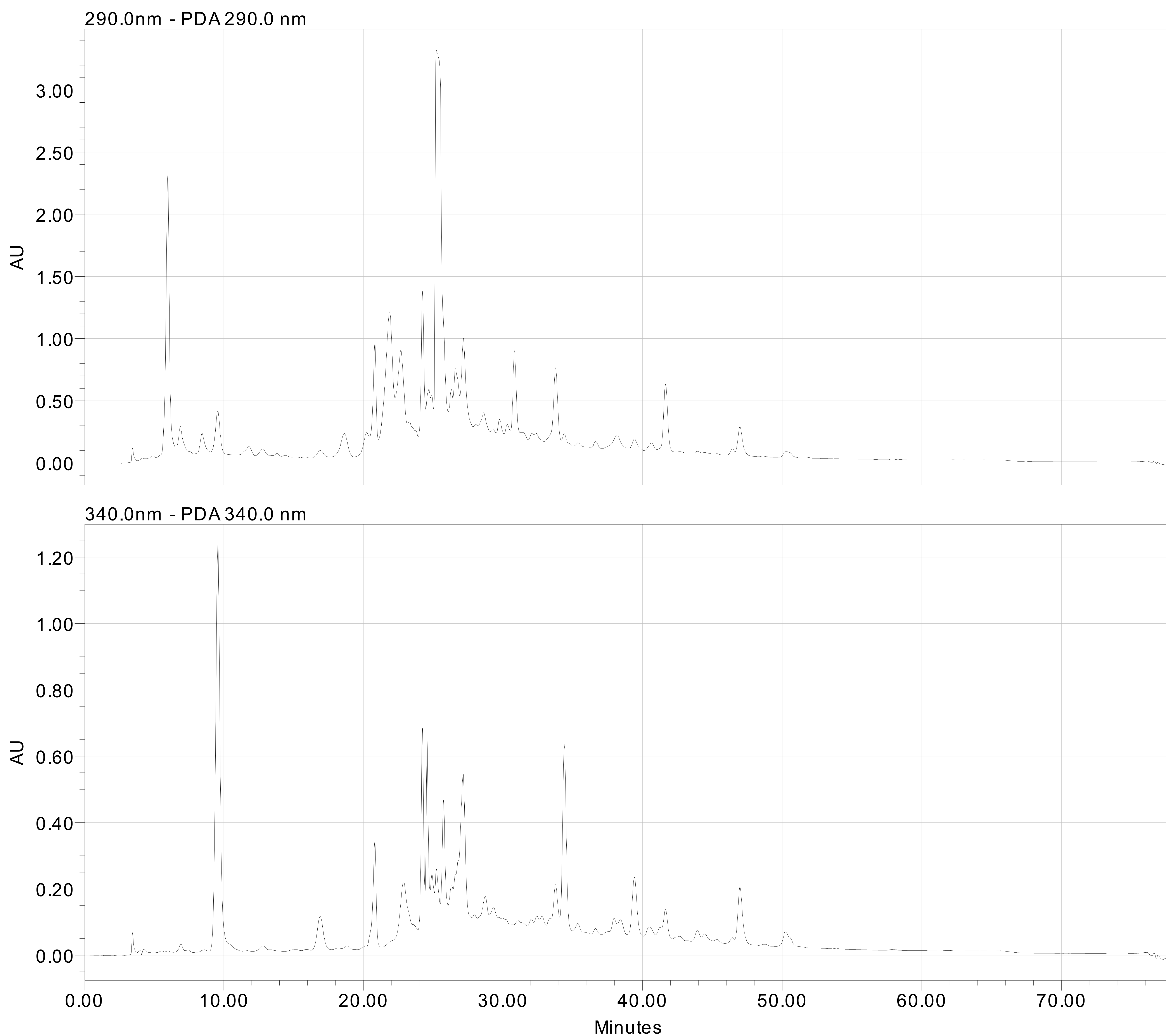
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:39:08 PM NZ

Sample Name: CC 190109 BD28.8 Manuka Honey UMF 27.3**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/4/2009 5:30:11 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

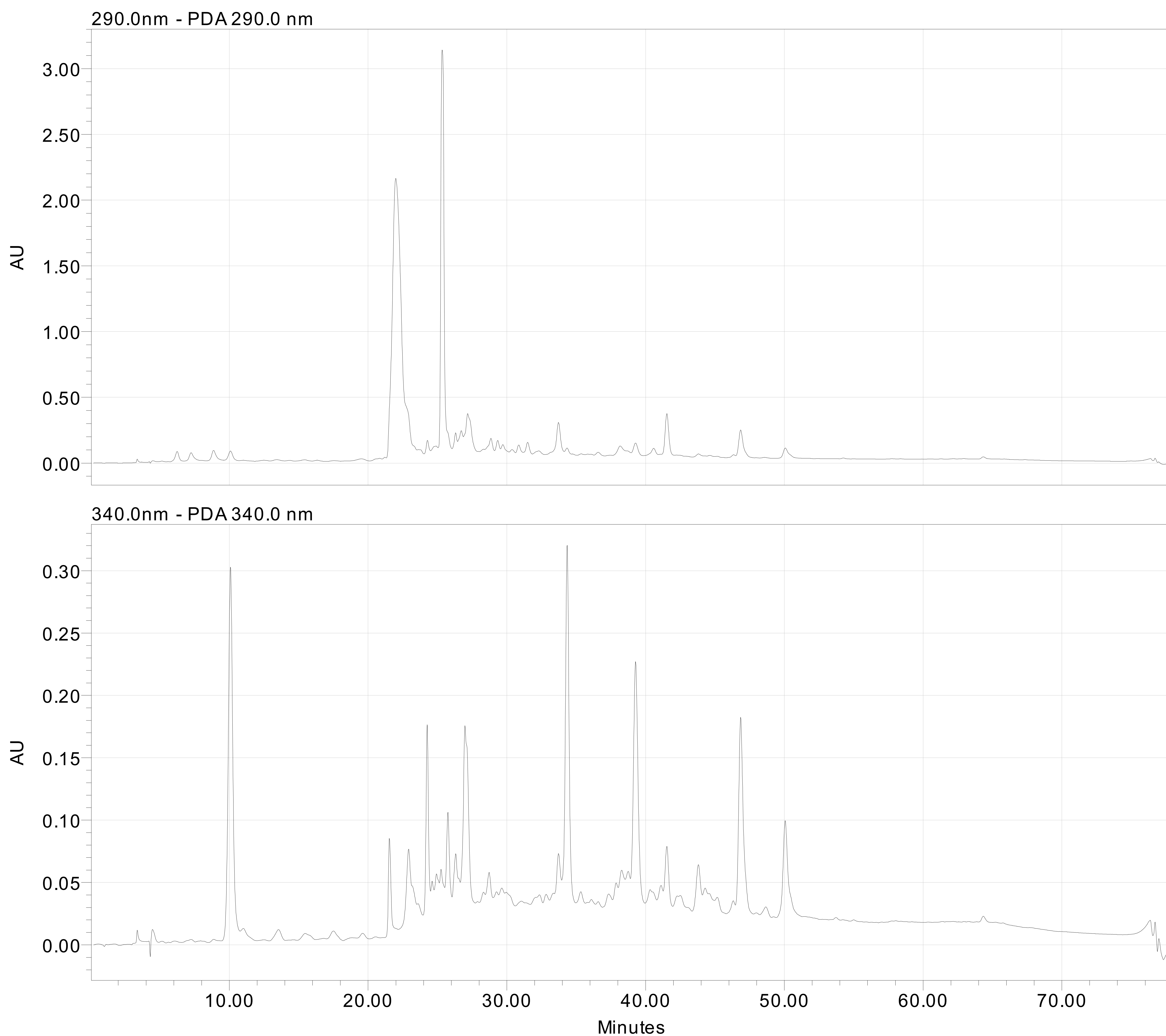
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:38:21 PM NZ

Sample Name: CC 200109 M01**Manuka Honey****UMF 25.3****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/3/2009 12:34:18 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

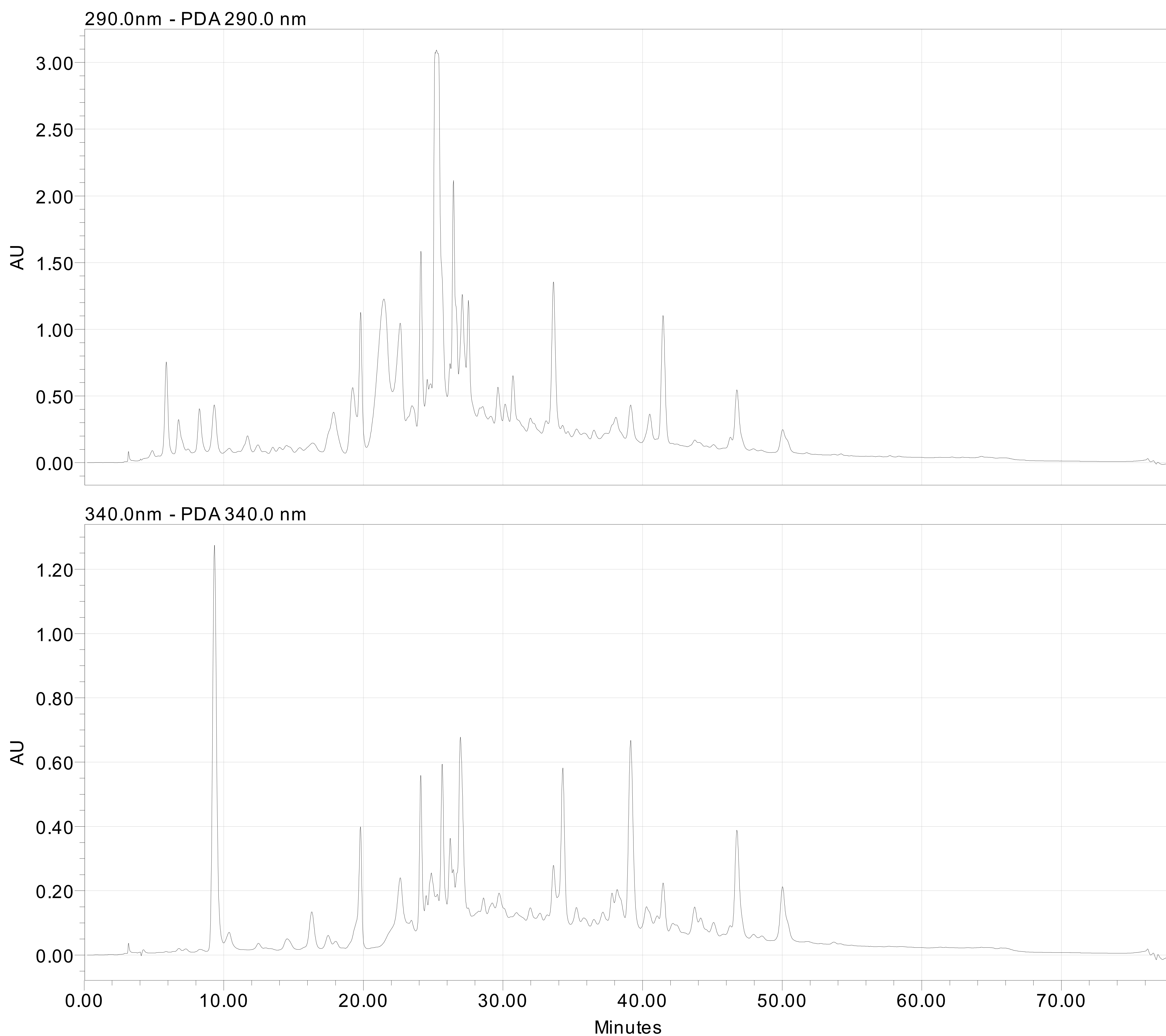
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:42:53 PM NZ

Sample Name: CC 200109 M03**Manuka Honey****UMF 23.6****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/3/2009 11:07:51 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

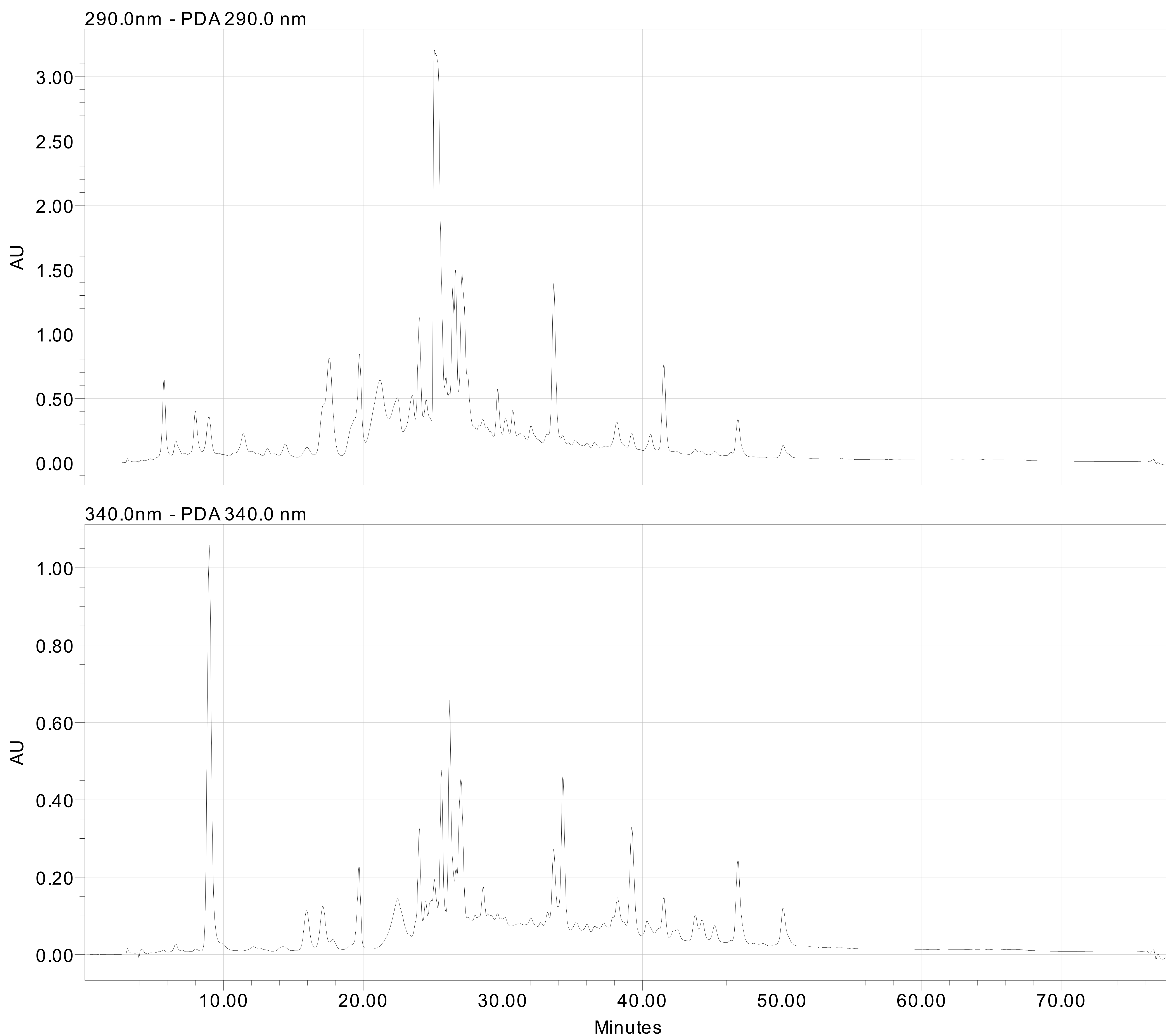
3:43:35 PM NZ

Sample Name: CC 210109 M05

Manuka Honey

UMF 17.5

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/3/2009 2:07:30 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

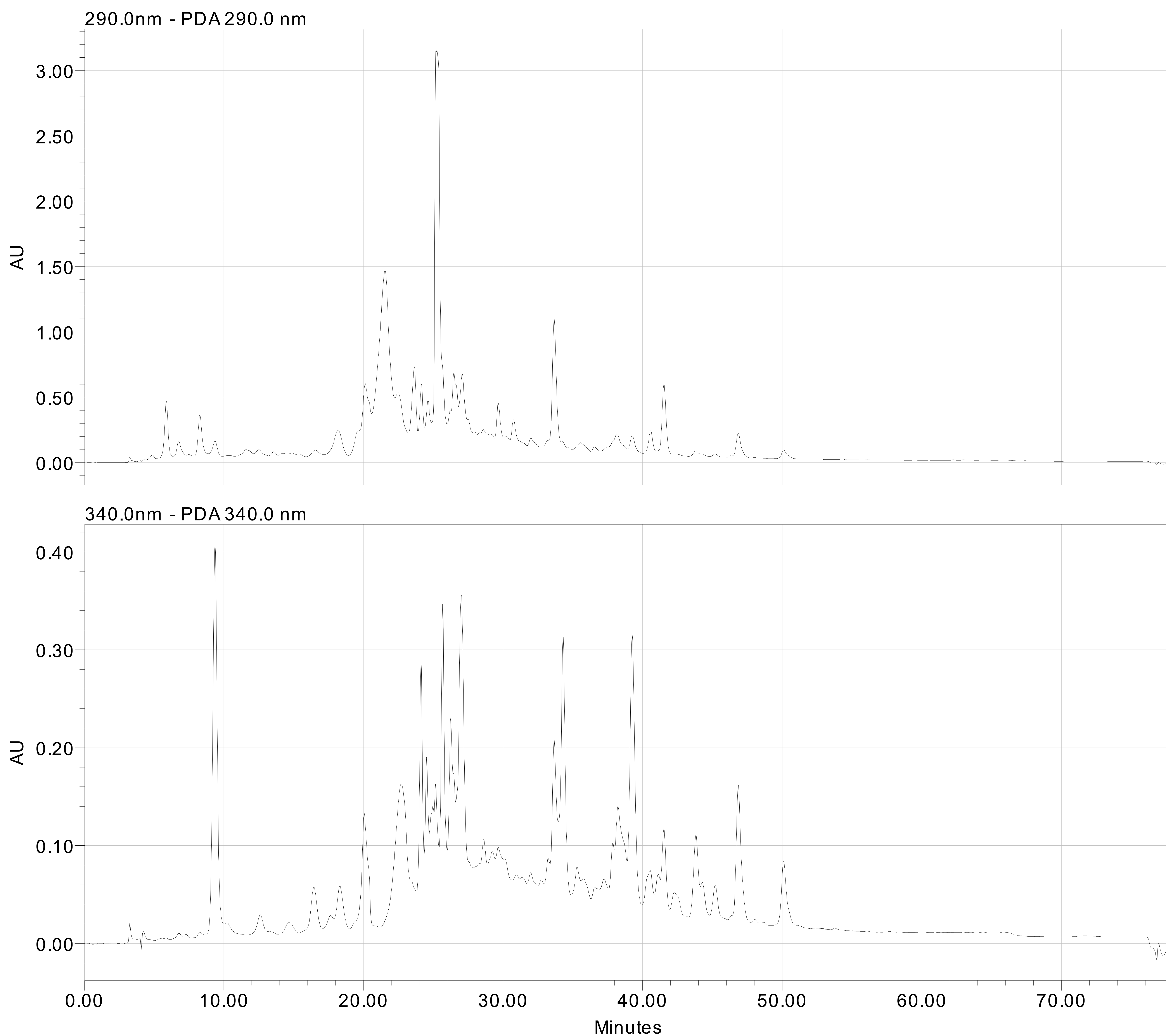
3:42:11 PM NZ

Sample Name: CC 220109 M09

Manuka Honey

UMF 17.7

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/4/2009 12:47:20 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

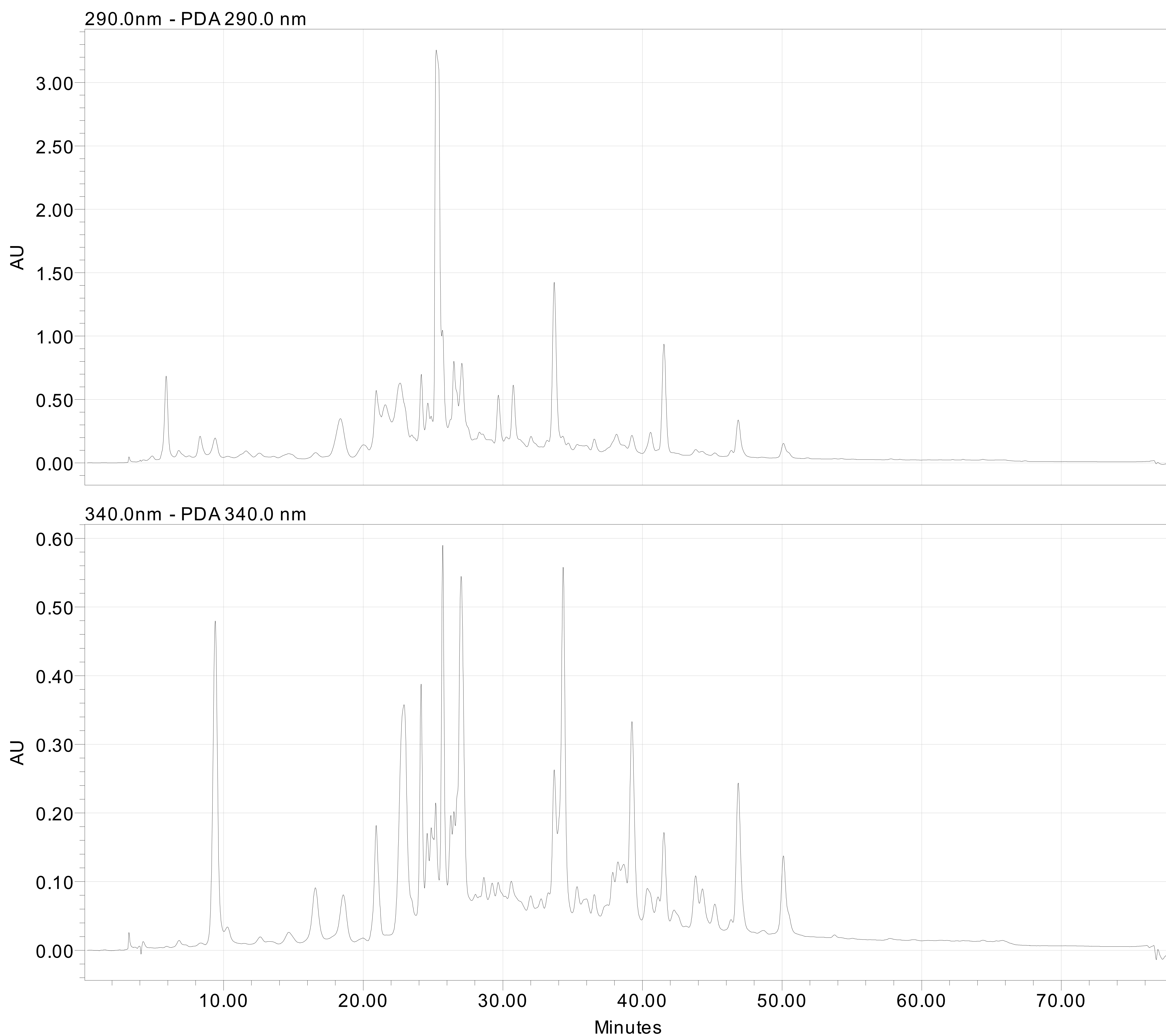
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:40:42 PM NZ

Sample Name: CC 210109 M10**Manuka Honey****UMF 13.9****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/4/2009 2:49:25 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

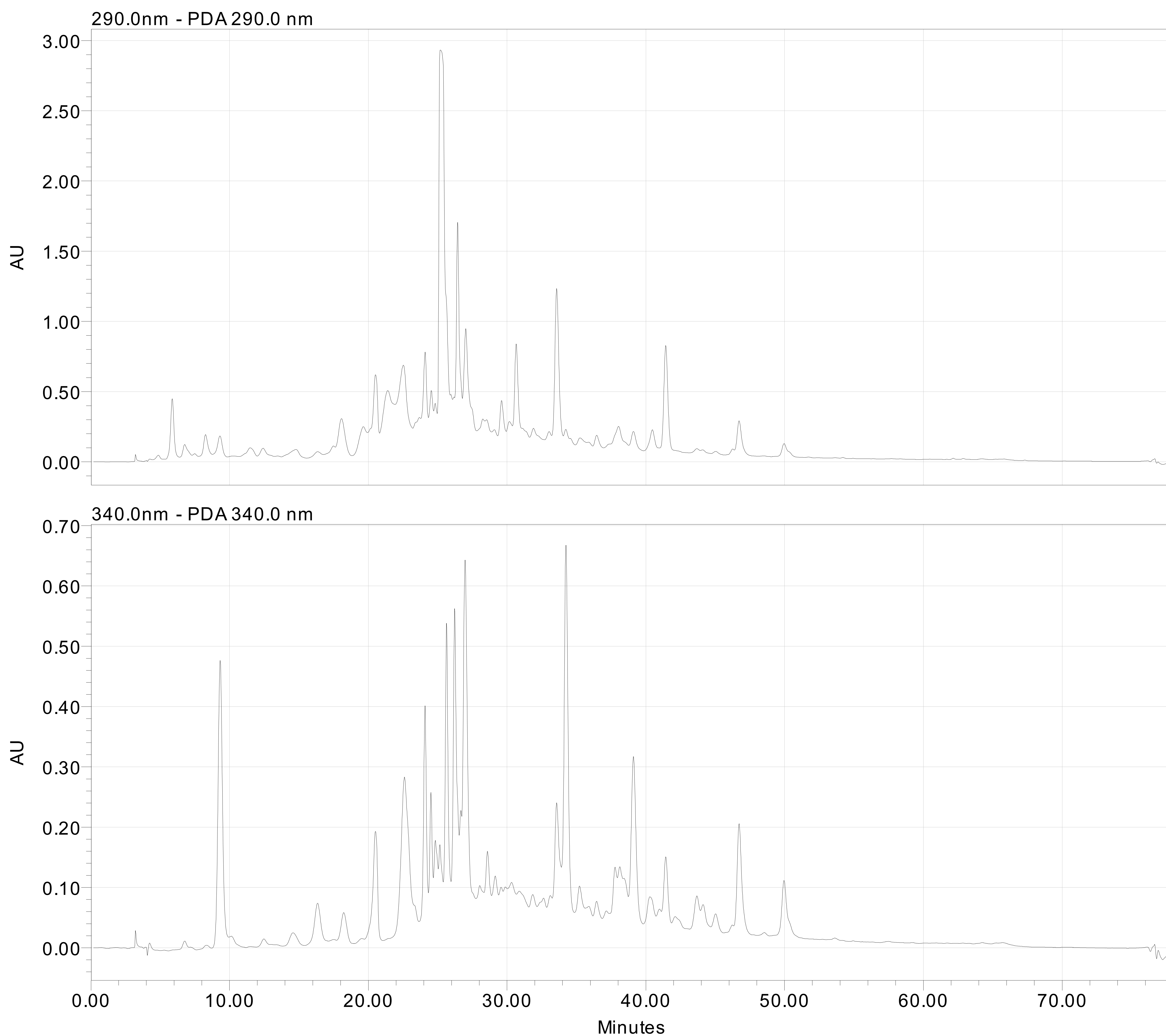
3:39:51 PM NZ

Sample Name: CC 220109 M11

Manuka Honey

UMF 14.8

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/4/2009 10:52:18 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

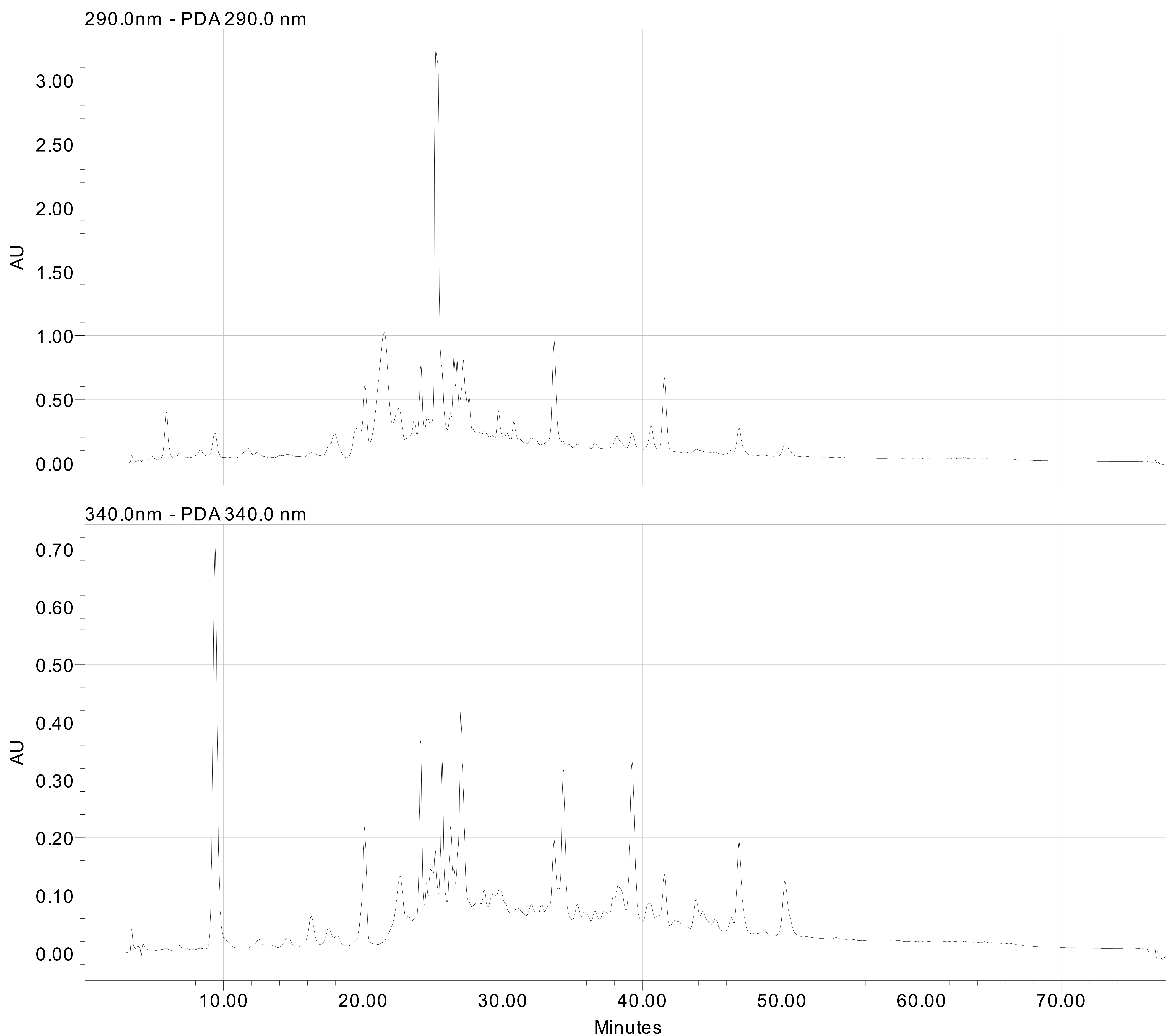
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:41:24 PM NZ

Sample Name: CC 280109 M12**Manuka Honey****UMF 14.9****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/10/2009 3:28:35 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

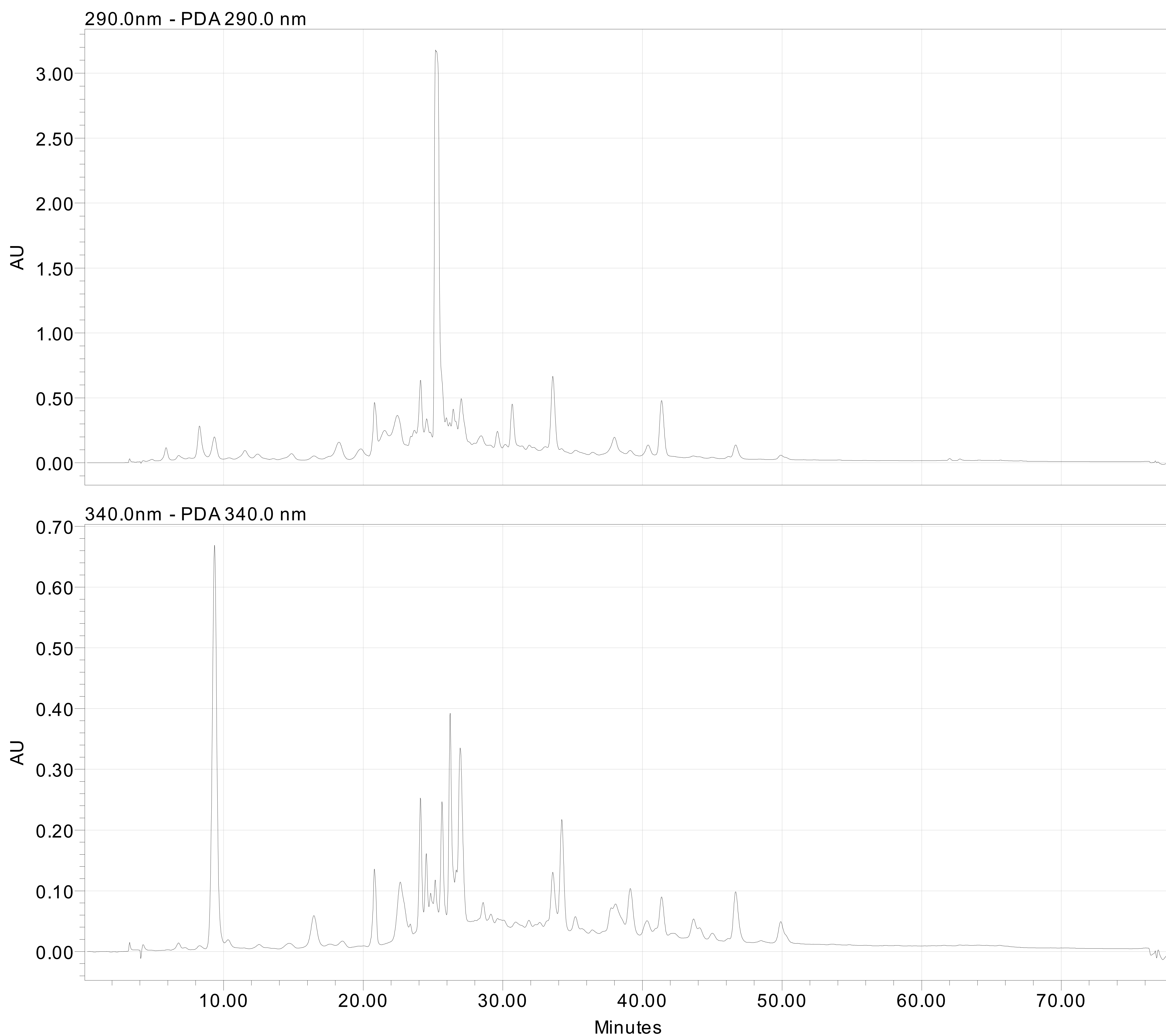
3:27:32 PM NZ

Sample Name: CC 230109 M14

Manuka Honey

UMF 10.9

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/5/2009 12:34:35 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

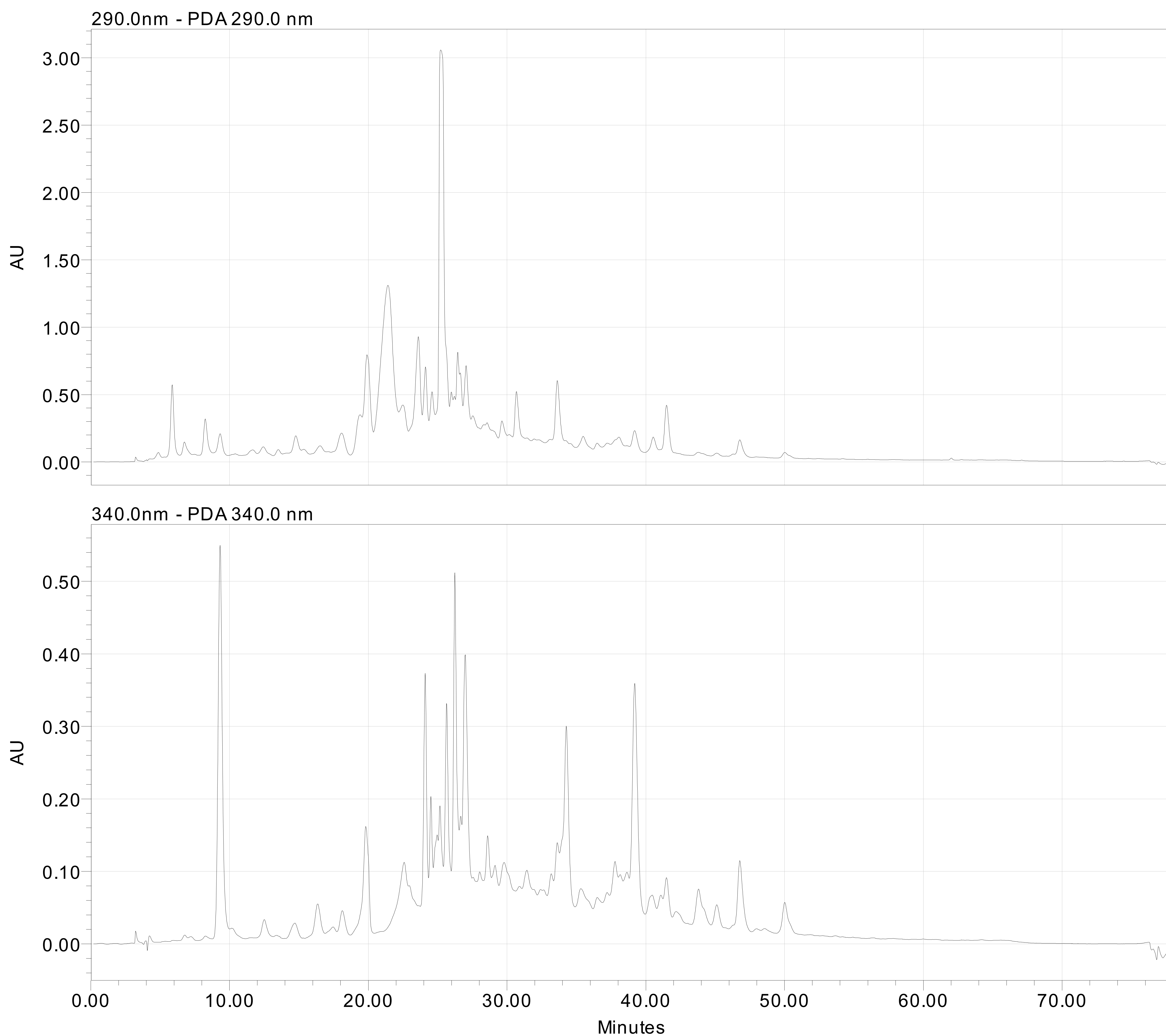
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:36:55 PM NZ

Sample Name: CC 230109 M15**Manuka Honey****UMF 15.3****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/5/2009 10:49:36 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

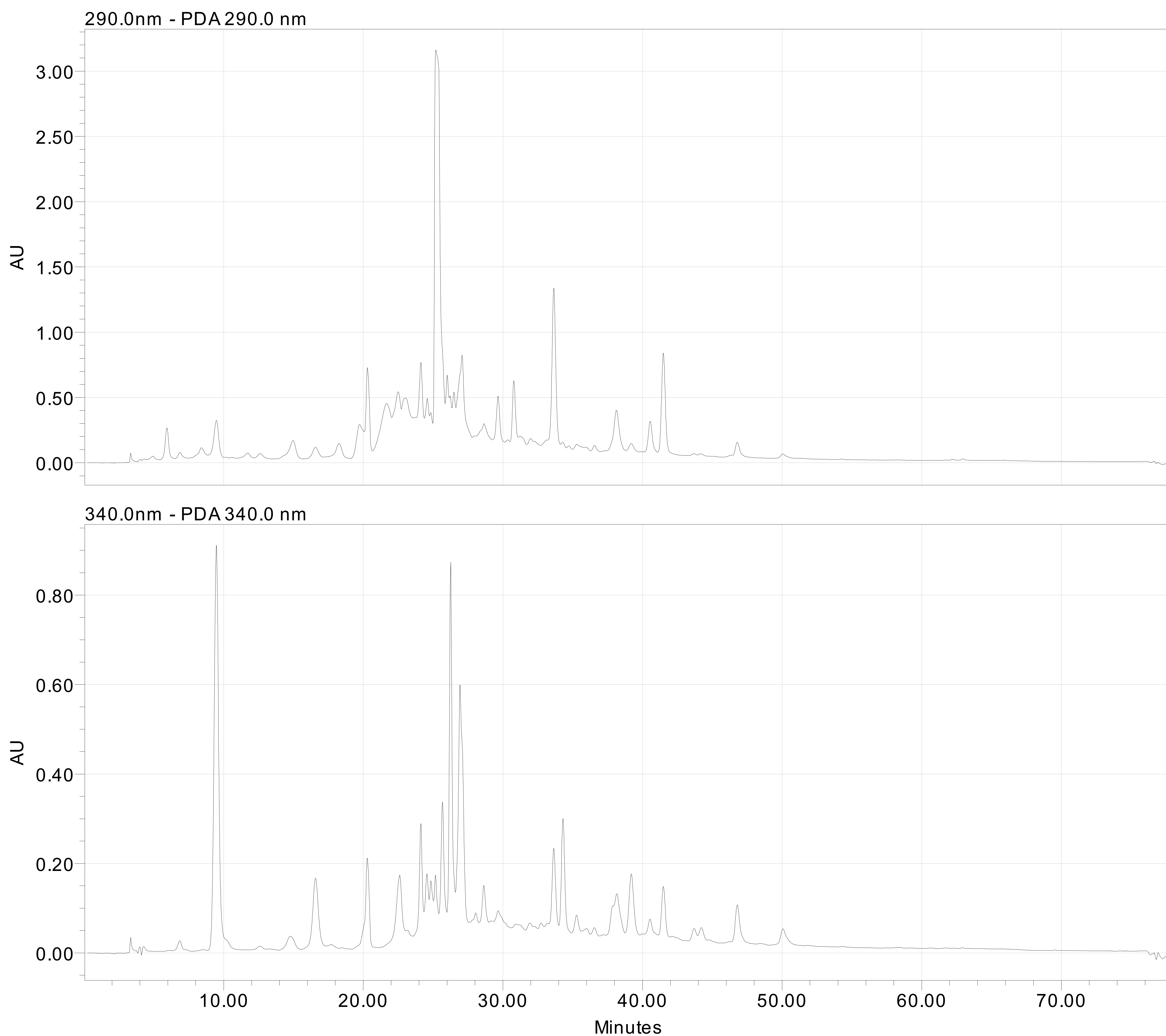
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:37:32 PM NZ

Sample Name: CC 280109 M17**Manuka Honey****UMF 14.2****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/10/2009 2:04:45 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

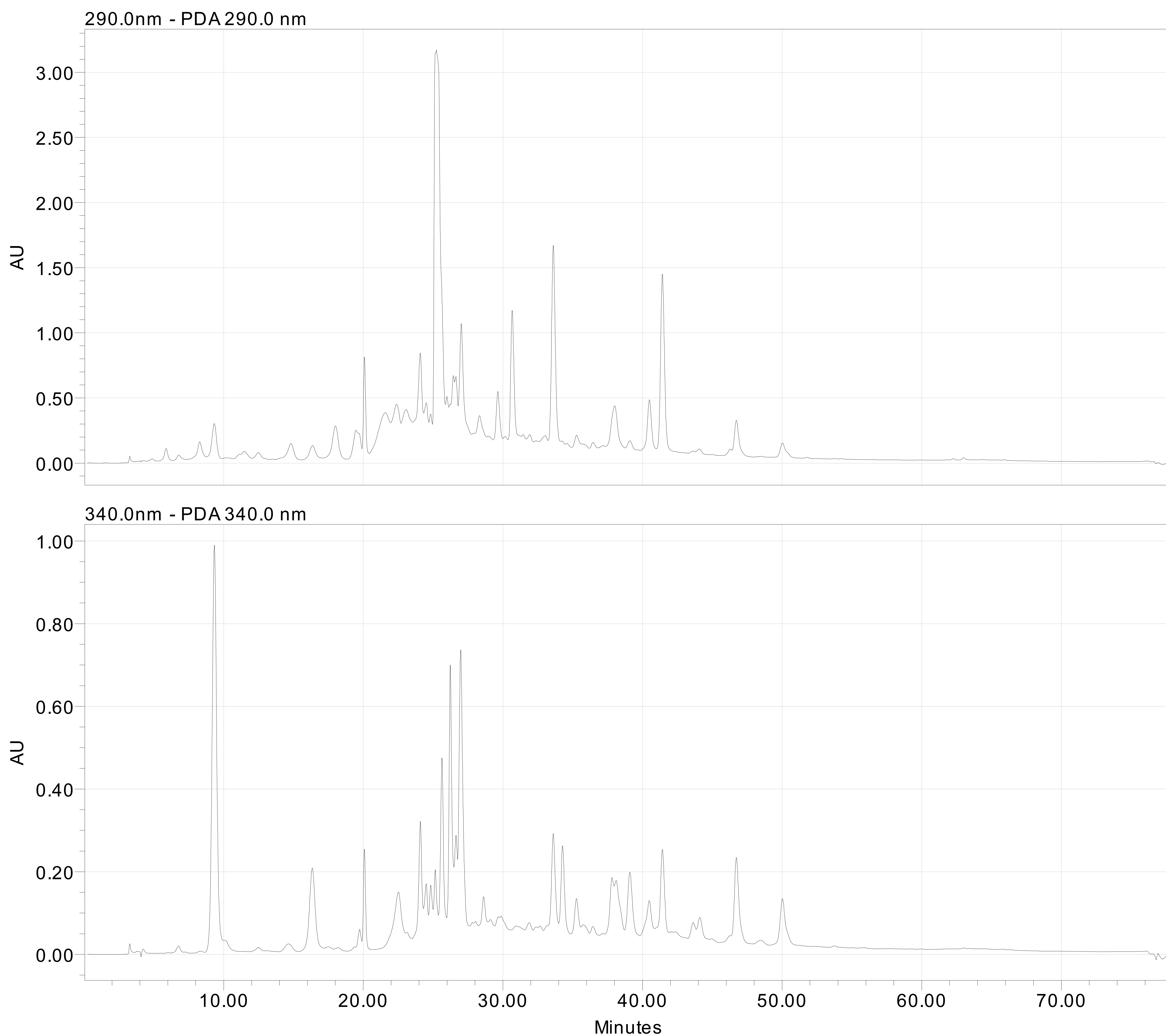
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:28:24 PM NZ

Sample Name: CC 290109 M18**Manuka Honey****UMF 14.2****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/11/2009 11:46:28 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

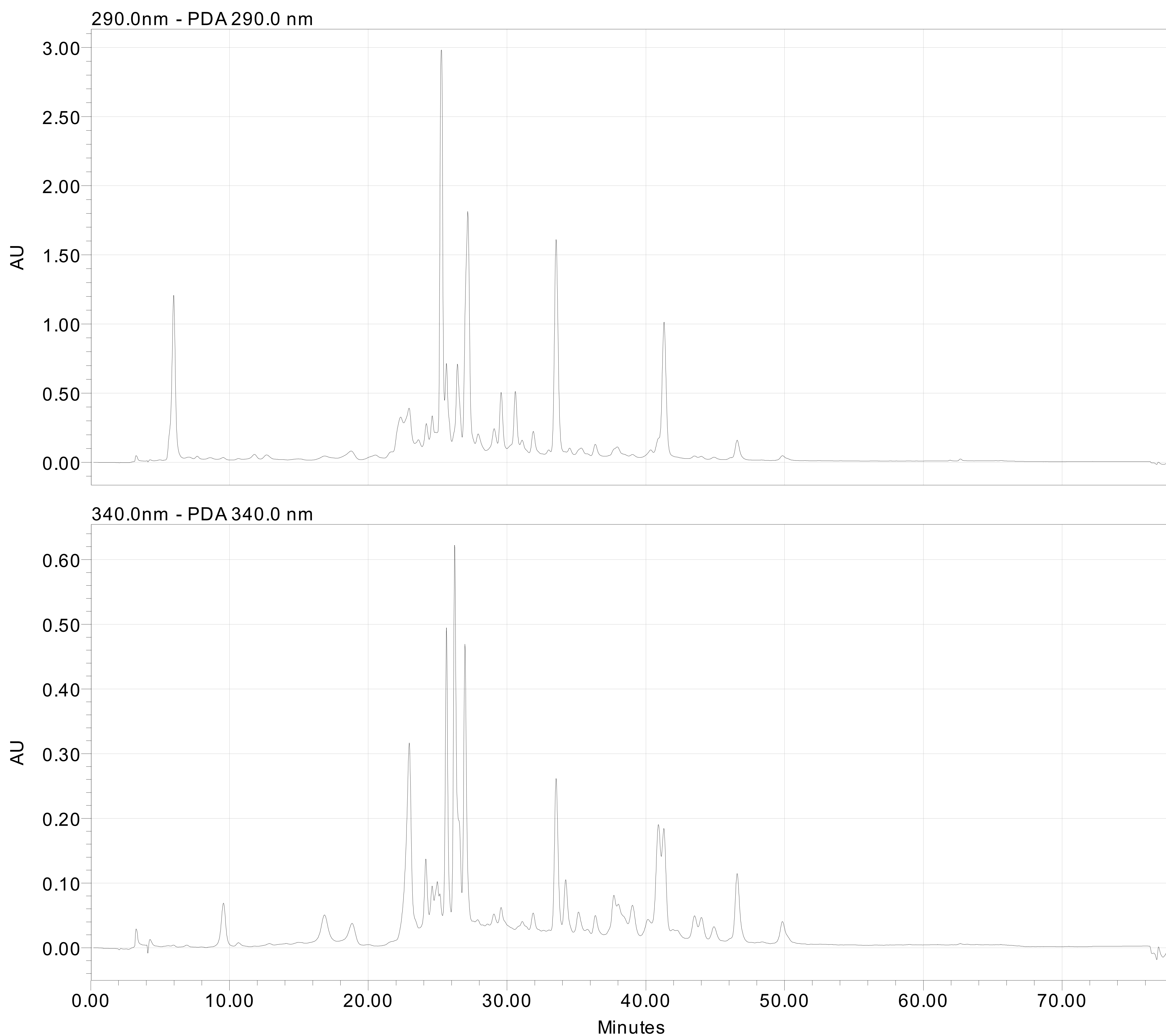
3:26:11 PM NZ

Sample Name: CC 040209 M20

Manuka Honey

Low UMF

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/12/2009 1:18:11 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

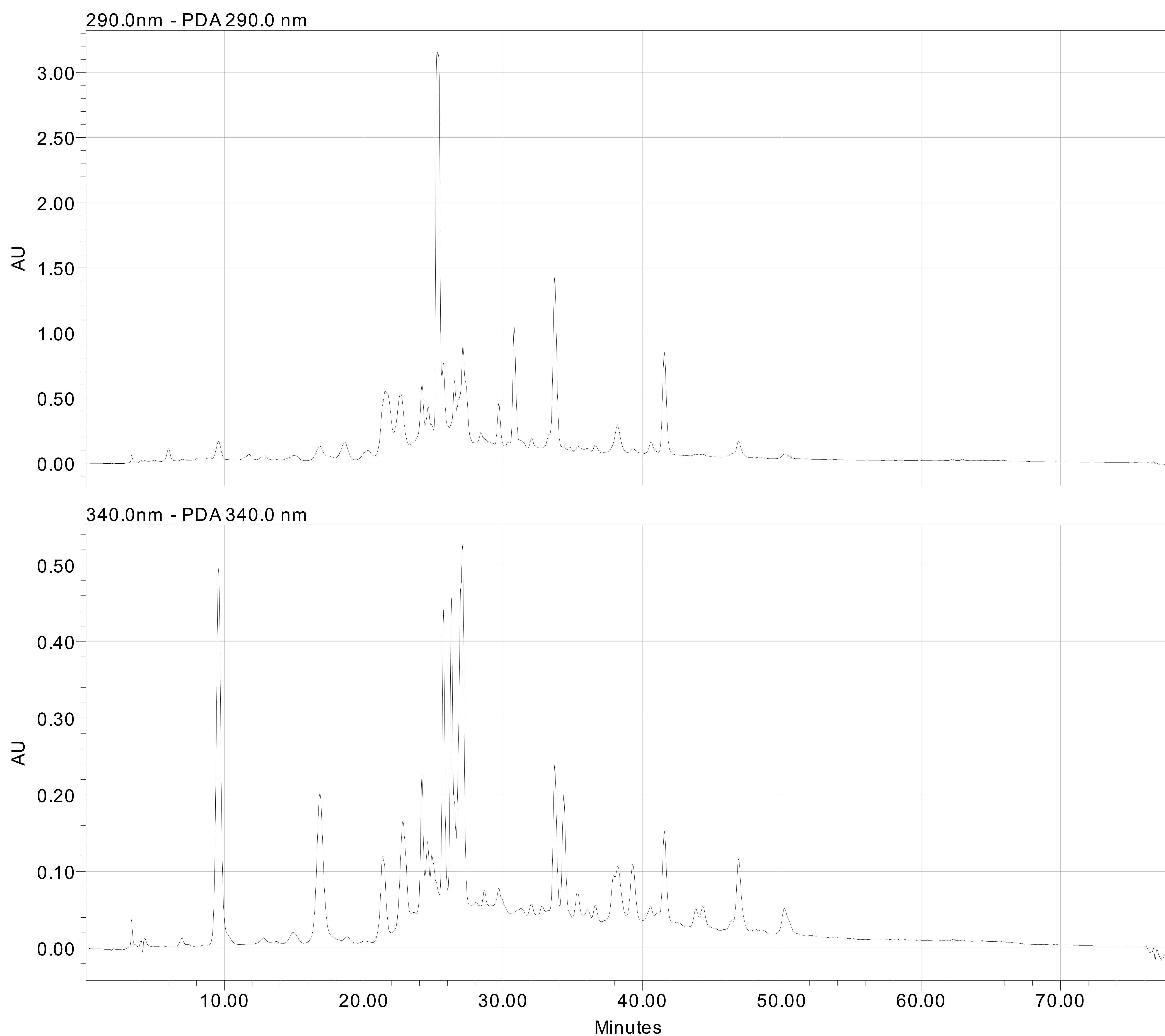
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:22:16 PM NZ

Sample Name: CC 270109 M21**Manuka Honey****UMF 9.2****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/10/2009 12:42:50 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

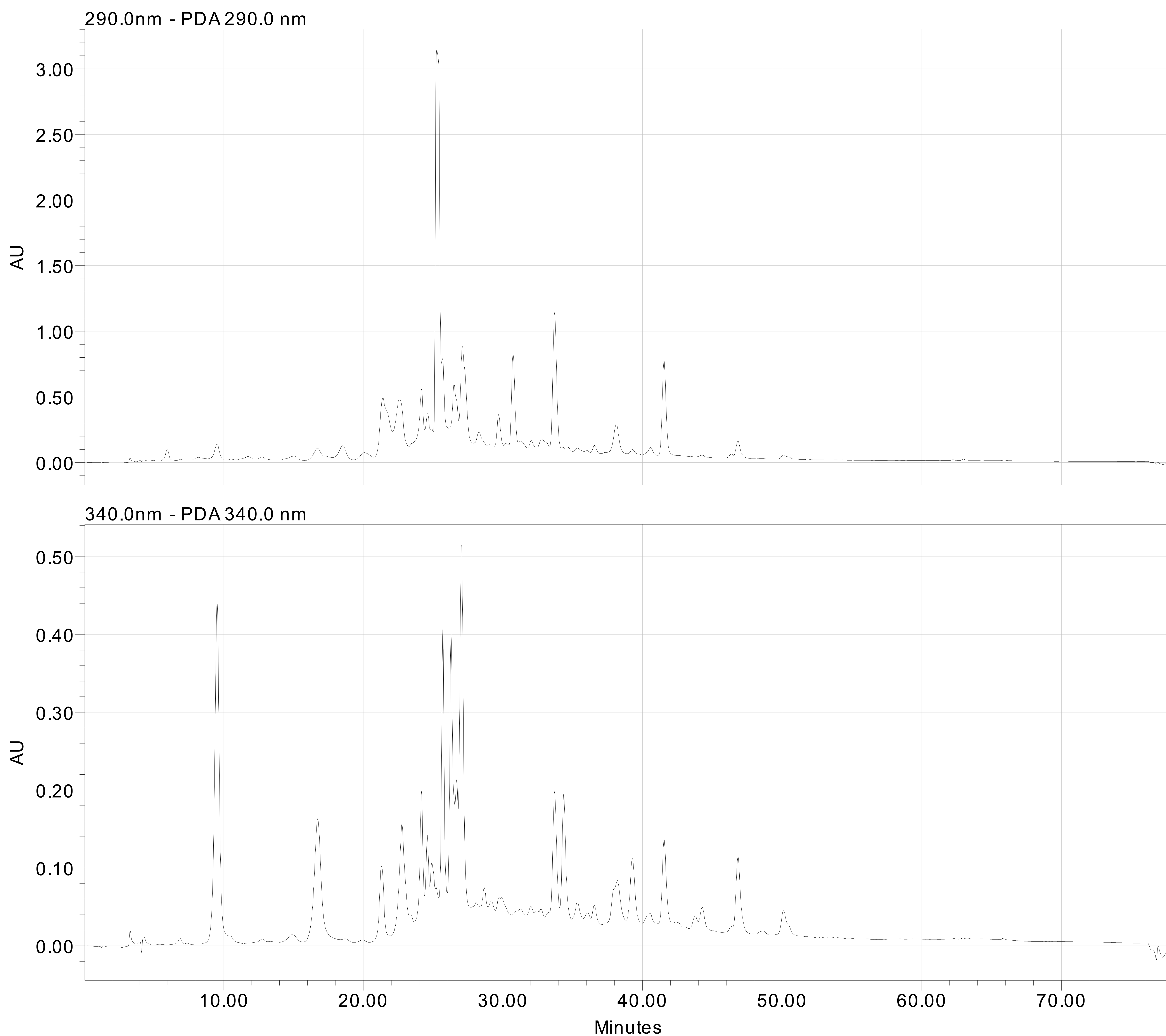
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:29:25 PM NZ

Sample Name: CC 290109 M22**Manuka Honey****UMF 8.3****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/11/2009 1:07:25 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

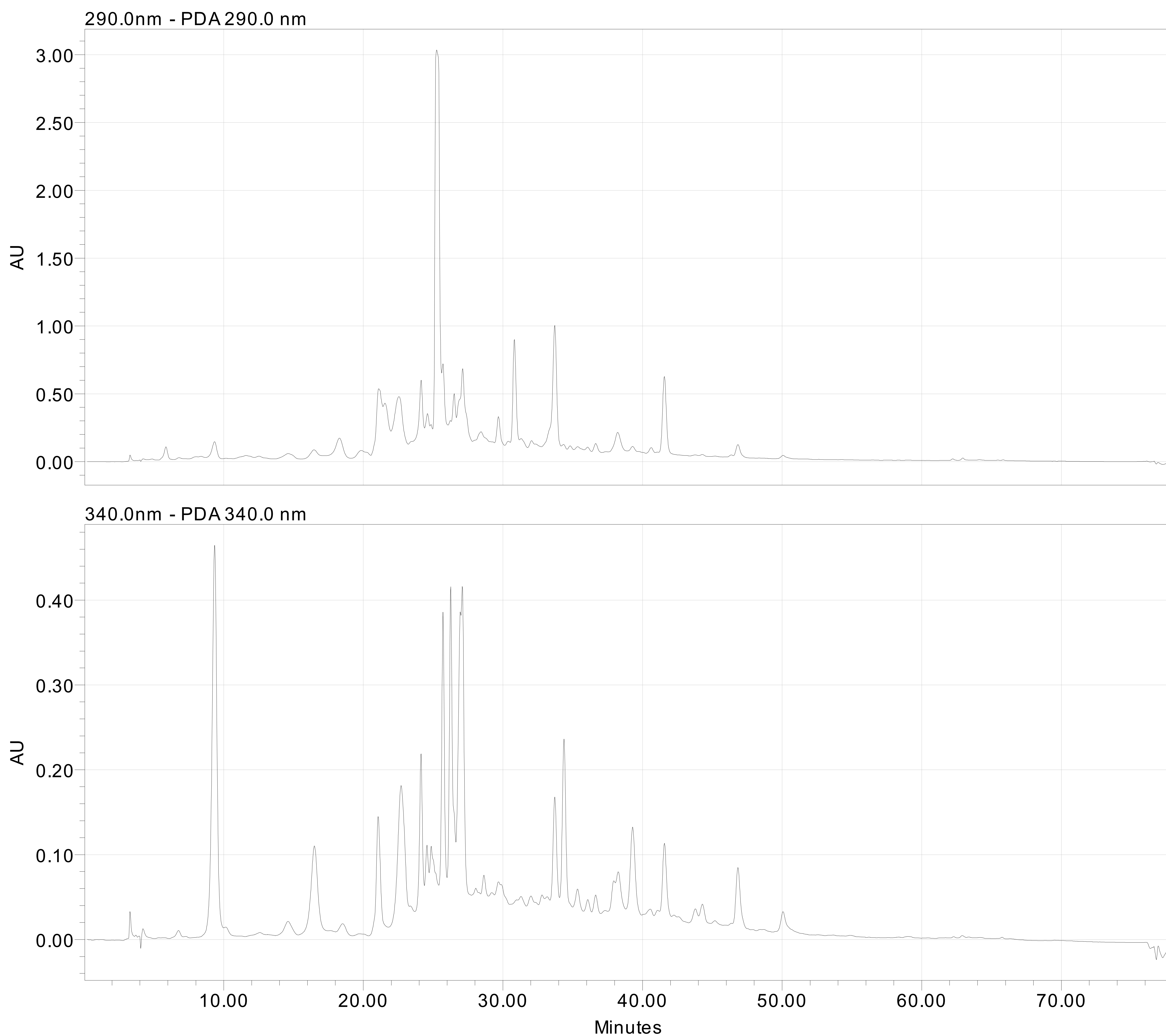
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:25:15 PM NZ

Sample Name: CC 270109 M23**Manuka Honey****UMF 9.3****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/10/2009 11:22:43 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

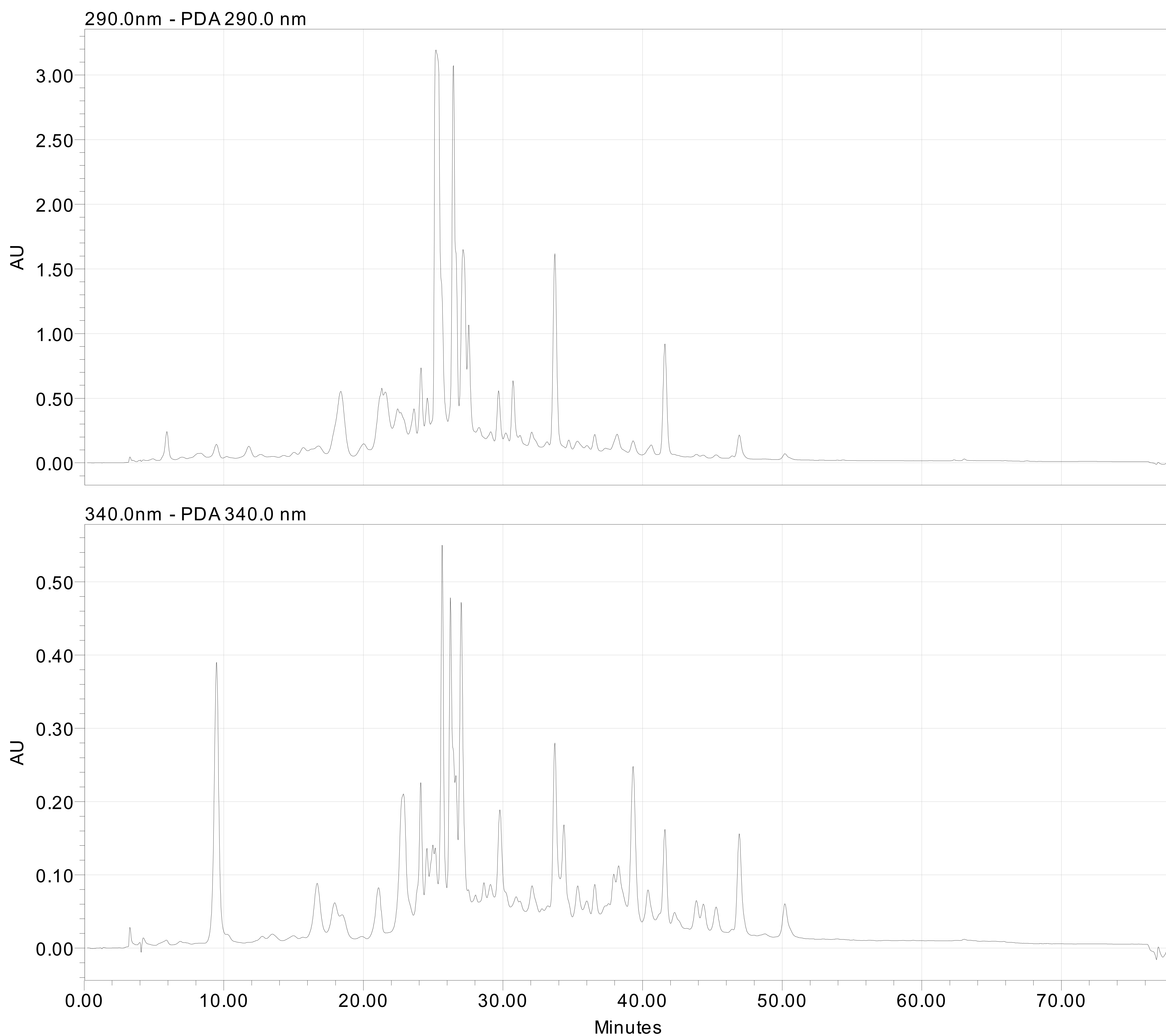
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:30:02 PM NZ

Sample Name: CC 300109 M26**Manuka Honey****UMF 7.3****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/11/2009 2:32:14 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

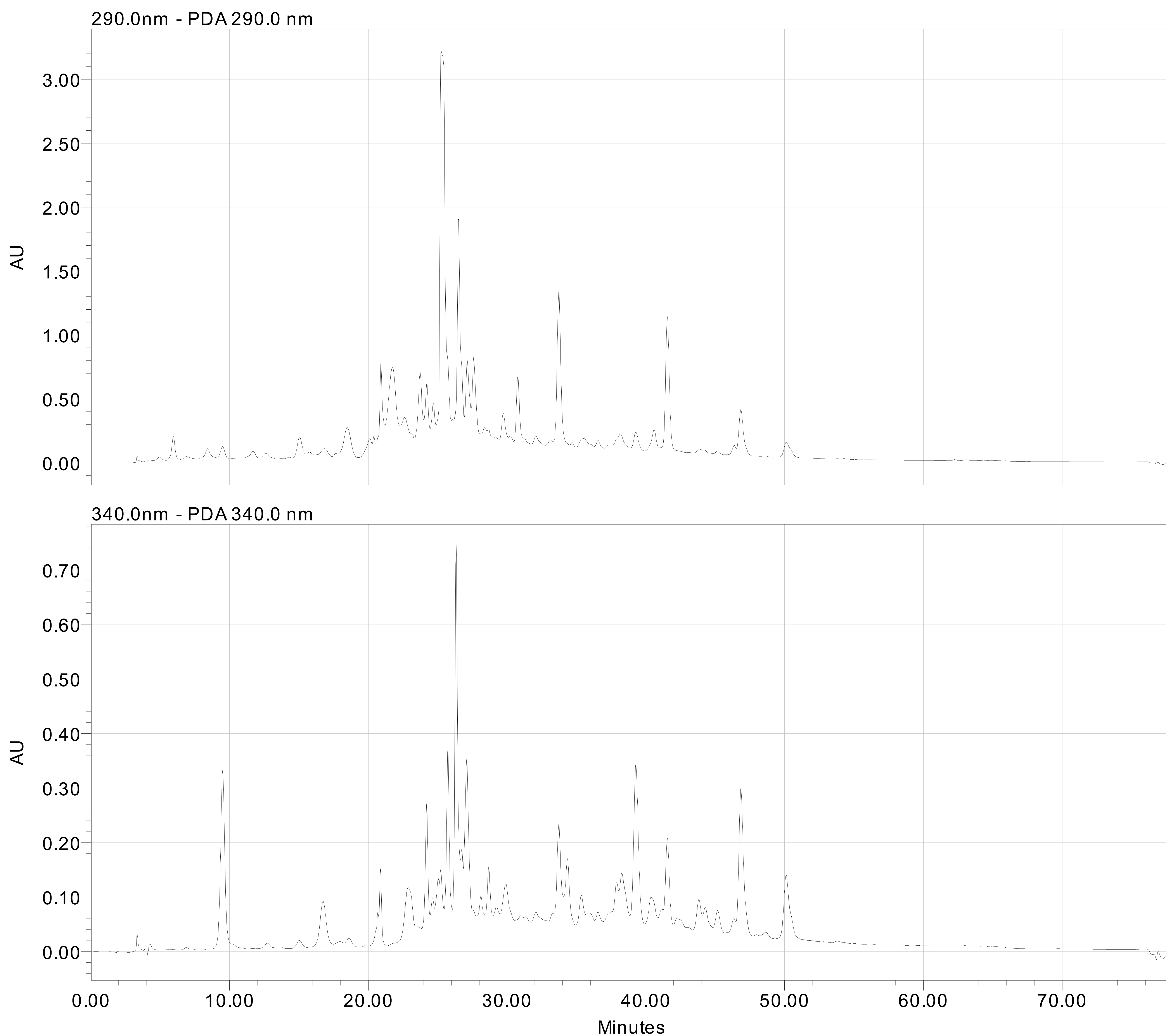
3:24:07 PM NZ

Sample Name: CC 300109 M27

Manuka Honey

UMF 7.3

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/11/2009 3:52:59 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

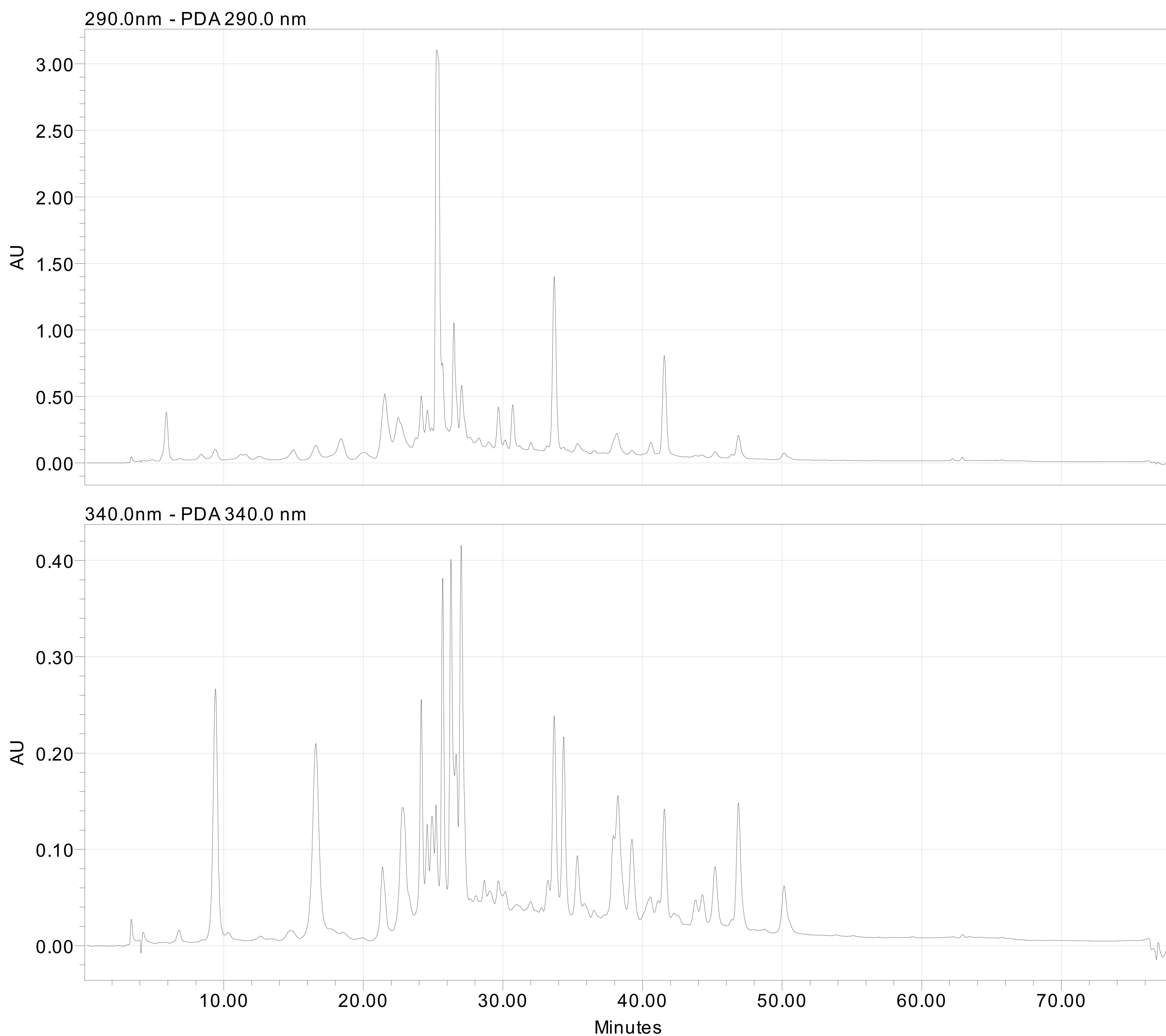
3:23:36 PM NZ

Sample Name: CC 020209 M29

Manuka Honey

UMF 8

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/12/2009 11:57:42 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

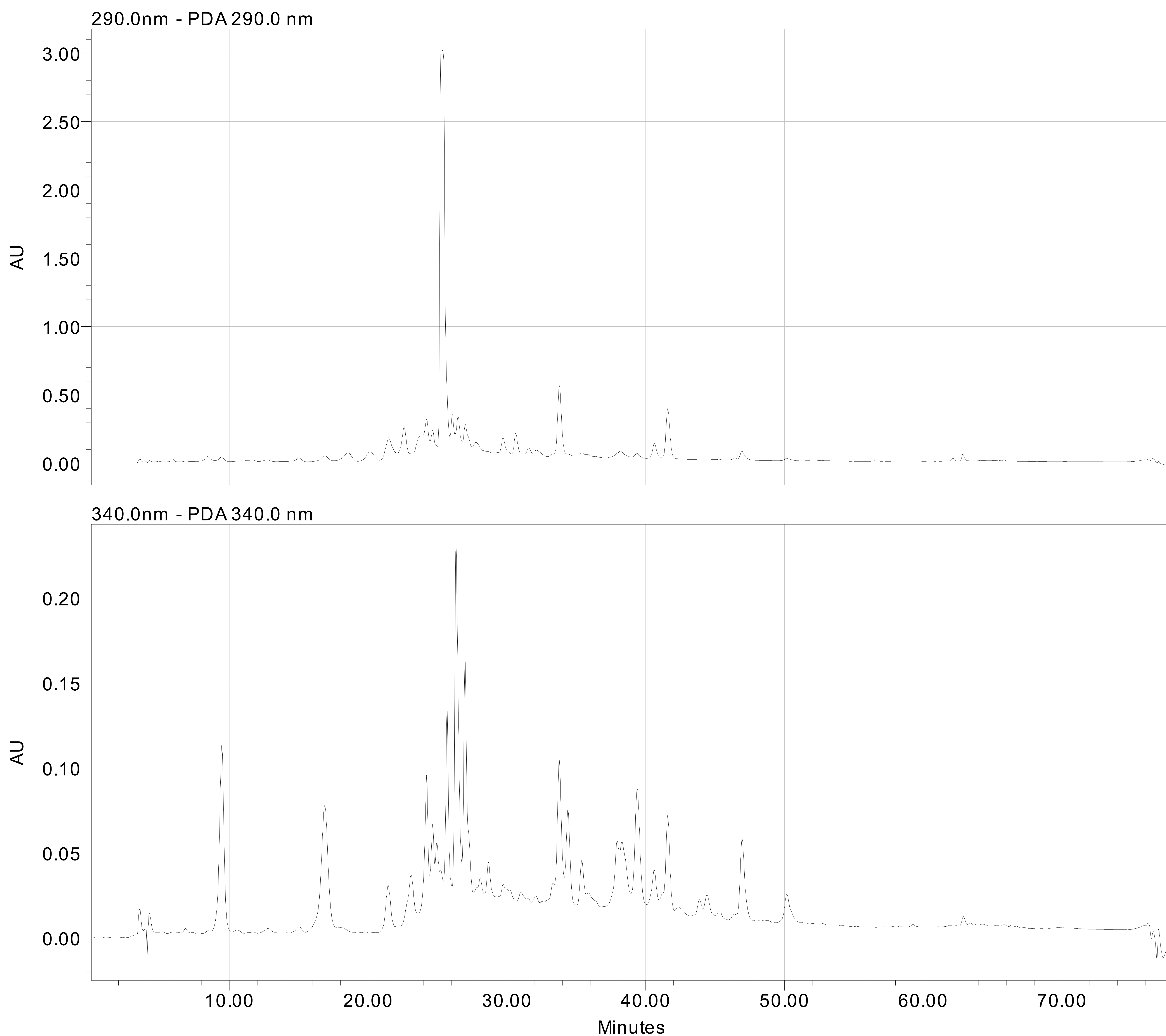
3:22:51 PM NZ

Sample Name: CC 040209 M32

Manuka Honey

UMF 5.9

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/16/2009 11:44:45 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

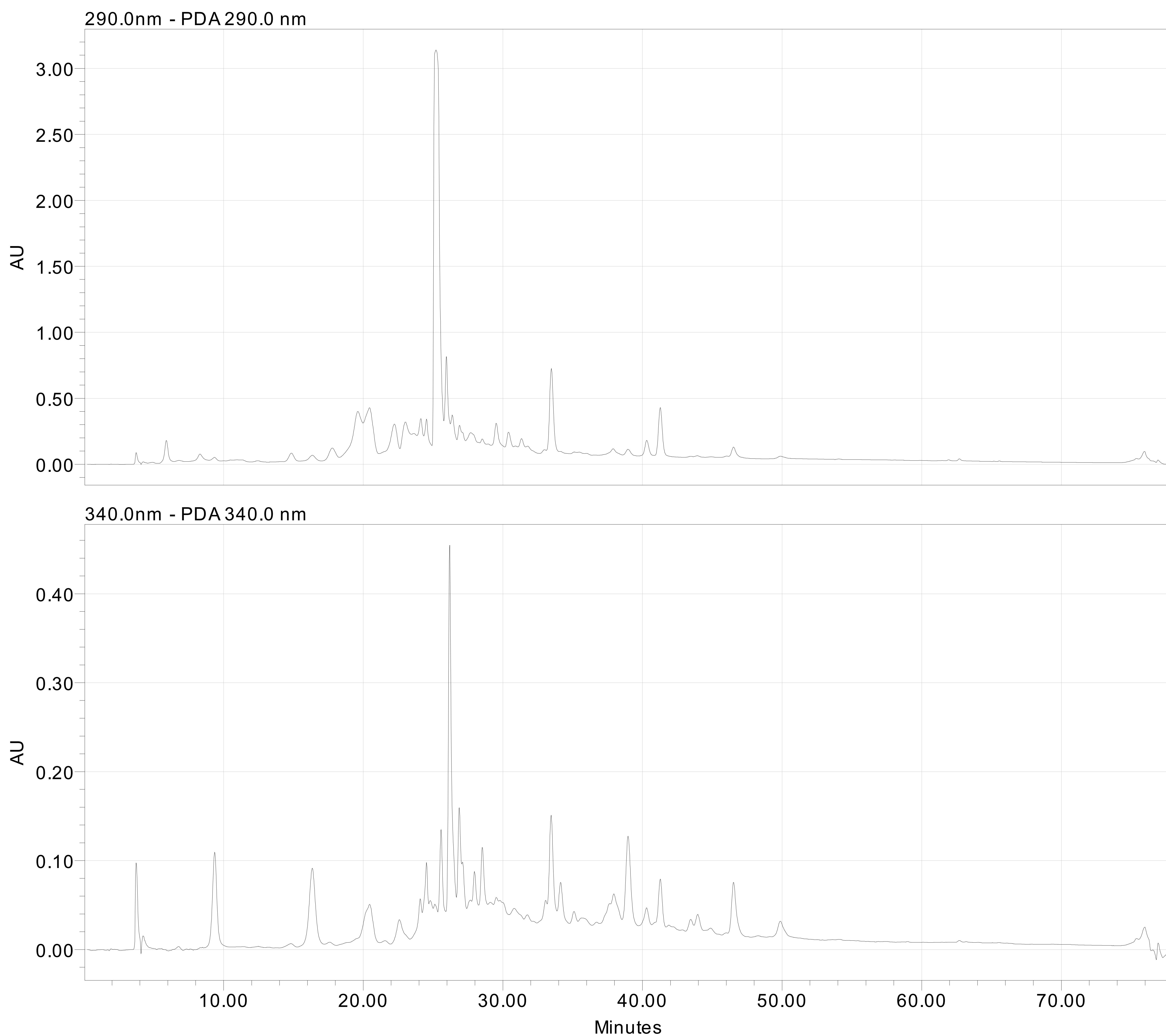
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:14:18 PM NZ

Sample Name: CC 040209 M34**Manuka Honey****UMF 4.5****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/12/2009 2:41:08 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

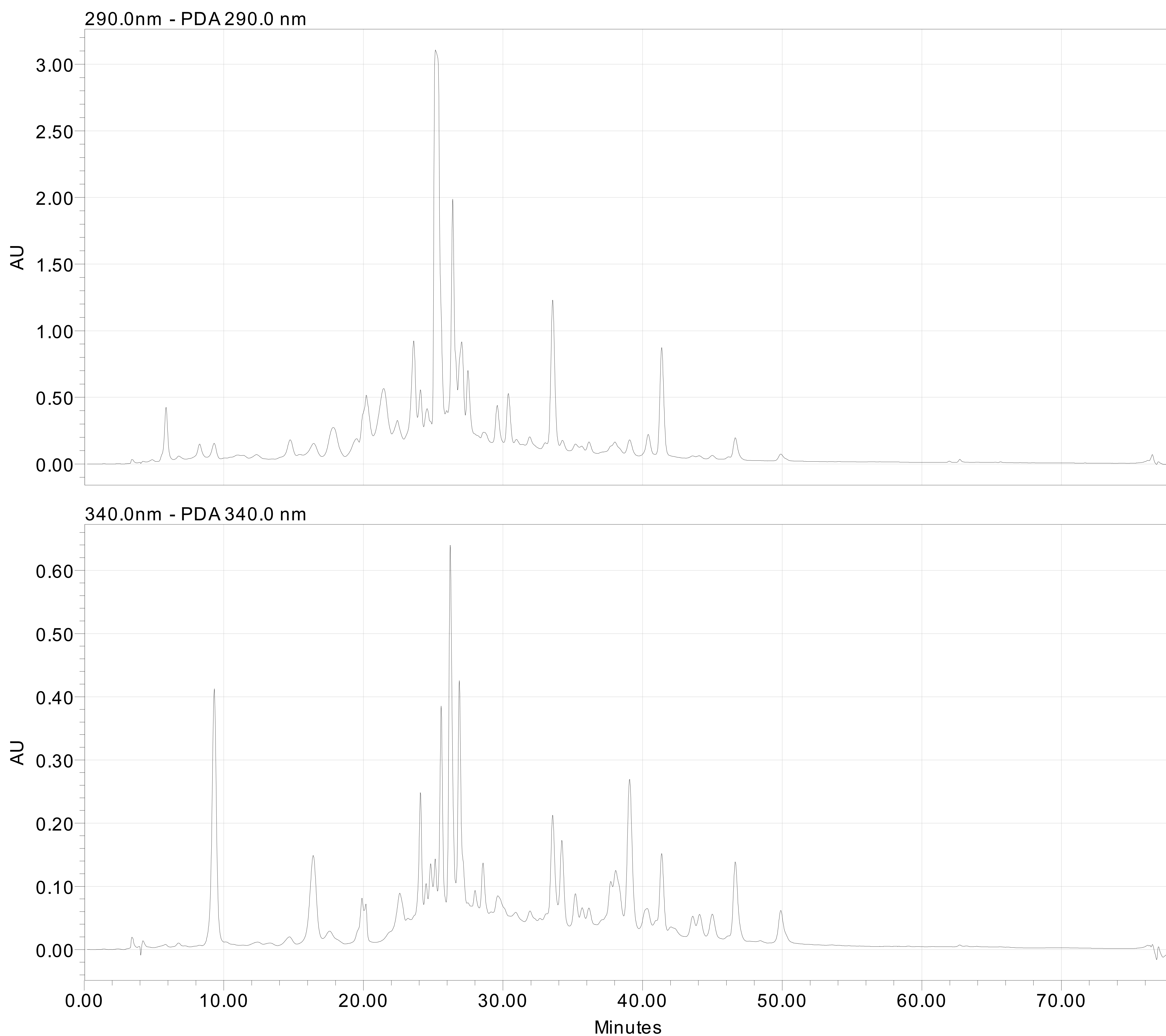
3:21:34 PM NZ

Sample Name: CC 040209 NCG

Manuka Honey

Low UMF

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/13/2009 10:20:22 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:20:42 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

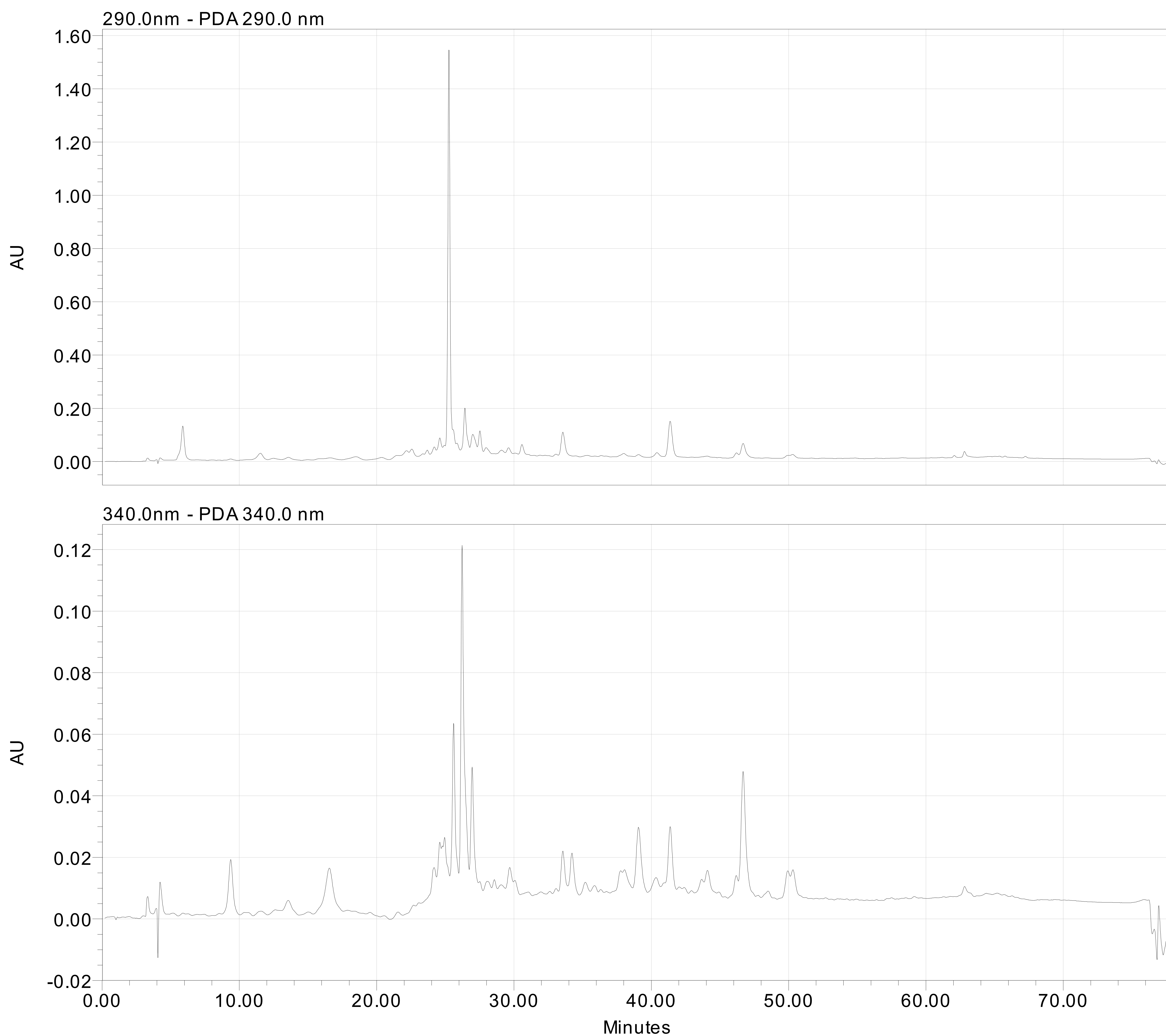
Benjamin Jade Deadman



THE UNIVERSITY OF
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Te Whare Wānanga o Waikato

The University of Waikato

2009

Sample Name: CC 050209 19708 Rata Honey**Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/16/2009 3:20:39 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

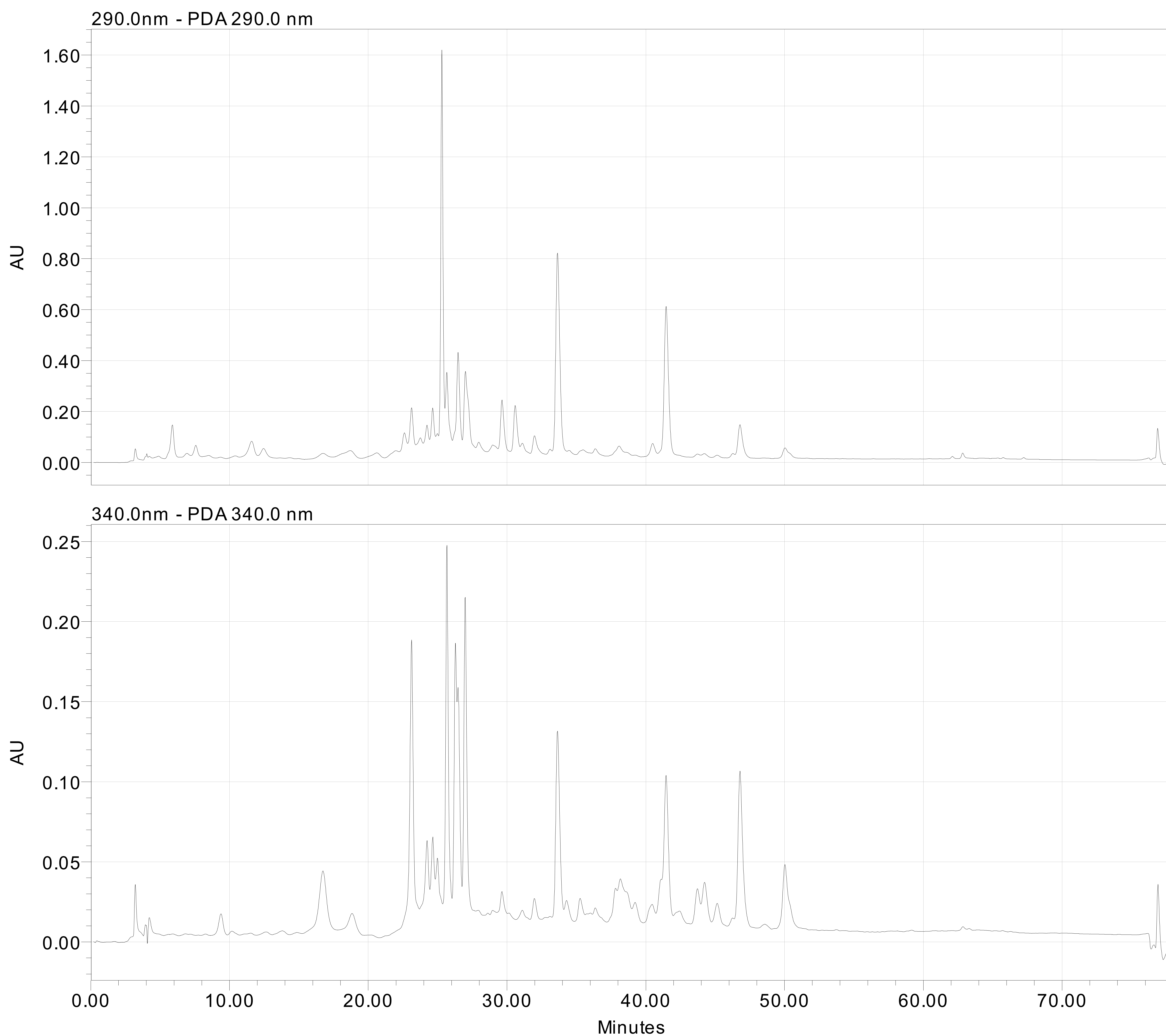
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:08:32 PM NZ

Sample Name: CC 050209 21850**Rewarewa Honey****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/16/2009 1:27:28 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

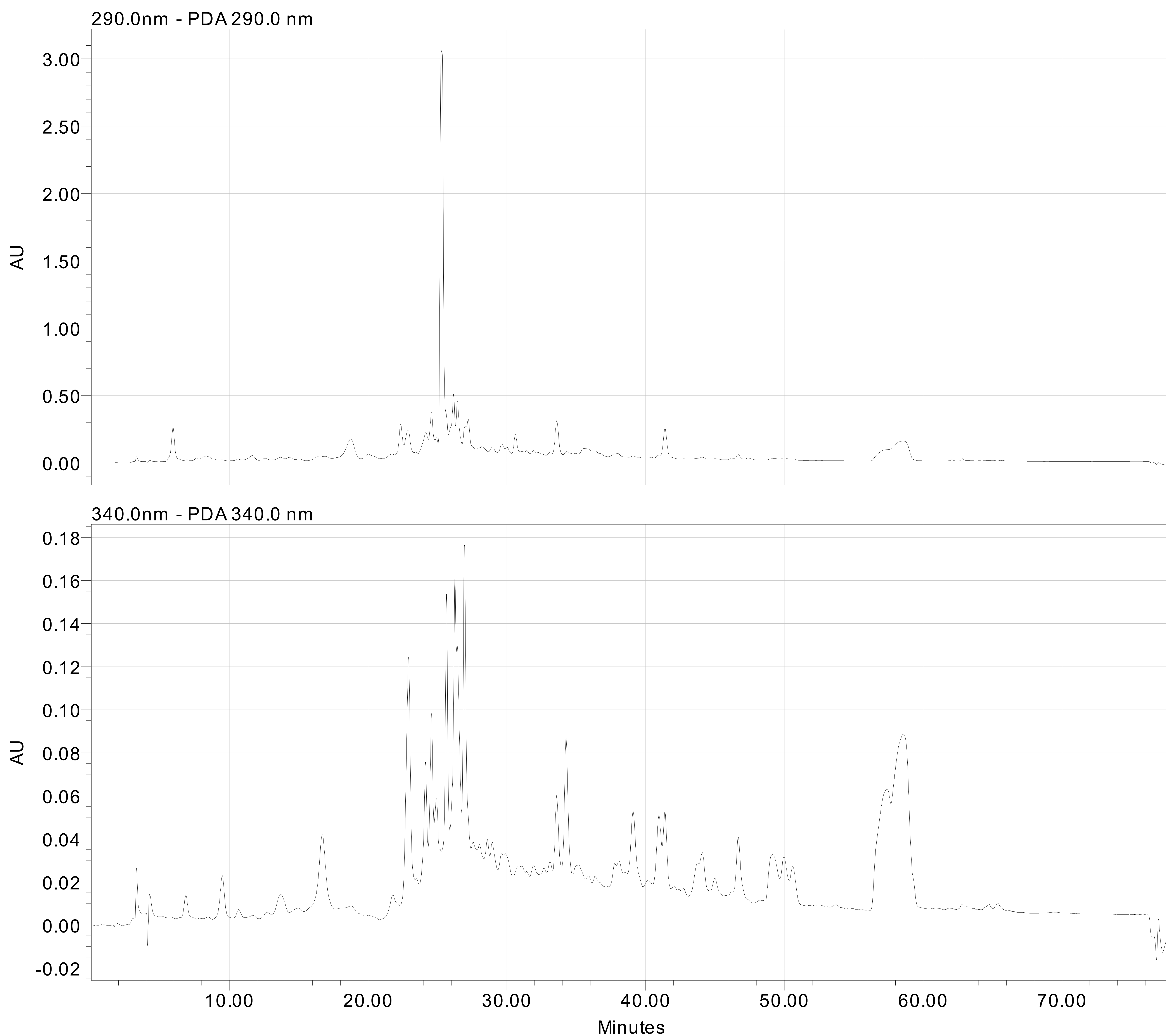
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:09:44 PM NZ

Sample Name: CC 100209 20681**Honeydew Honey****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/17/2009 1:32:40 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

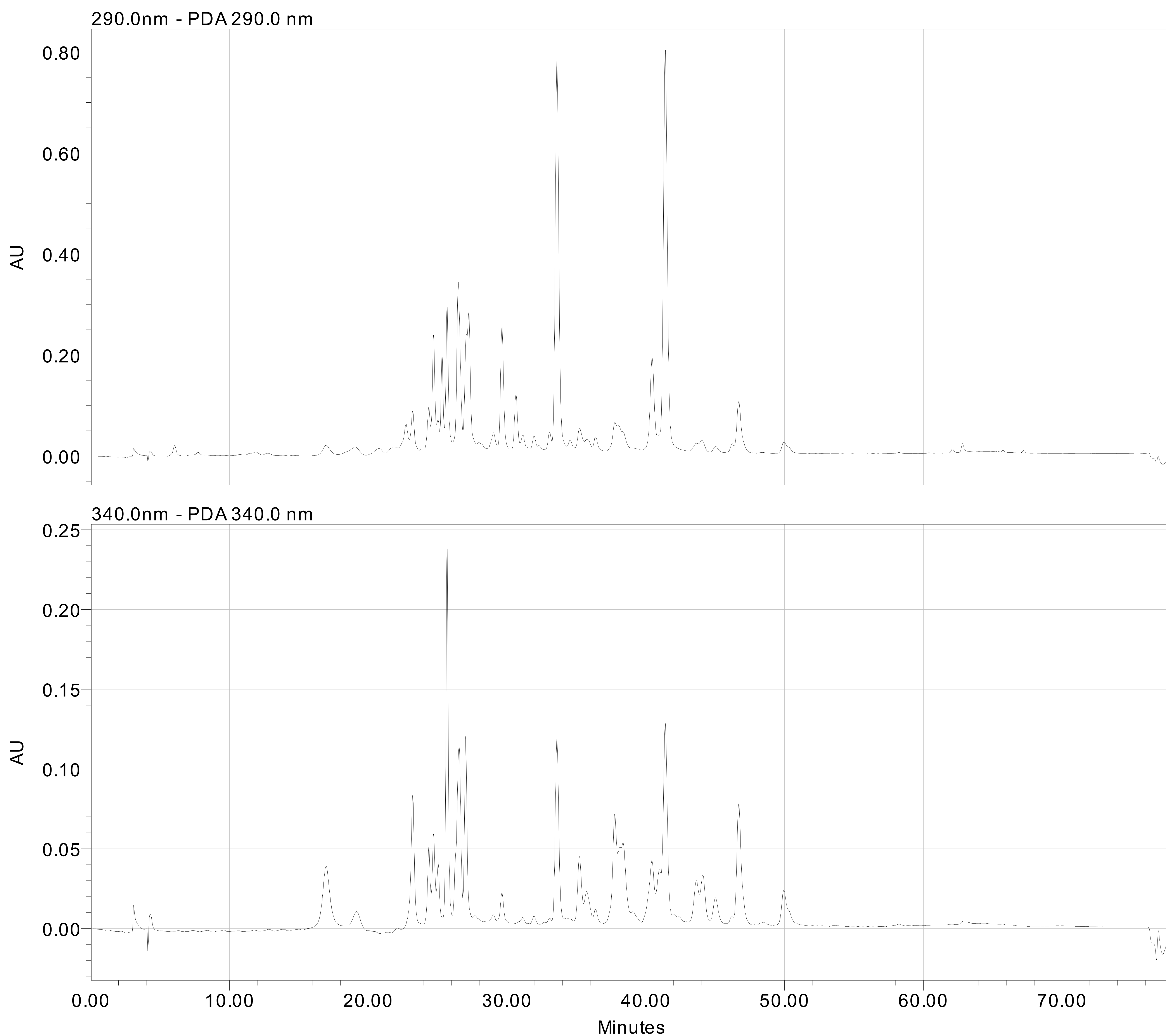
Date Printed:

3/24/2009

3:06:52 PM NZ

Sample Name: CC 100209 21947 Clover Honey

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/17/2009 2:52:10 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

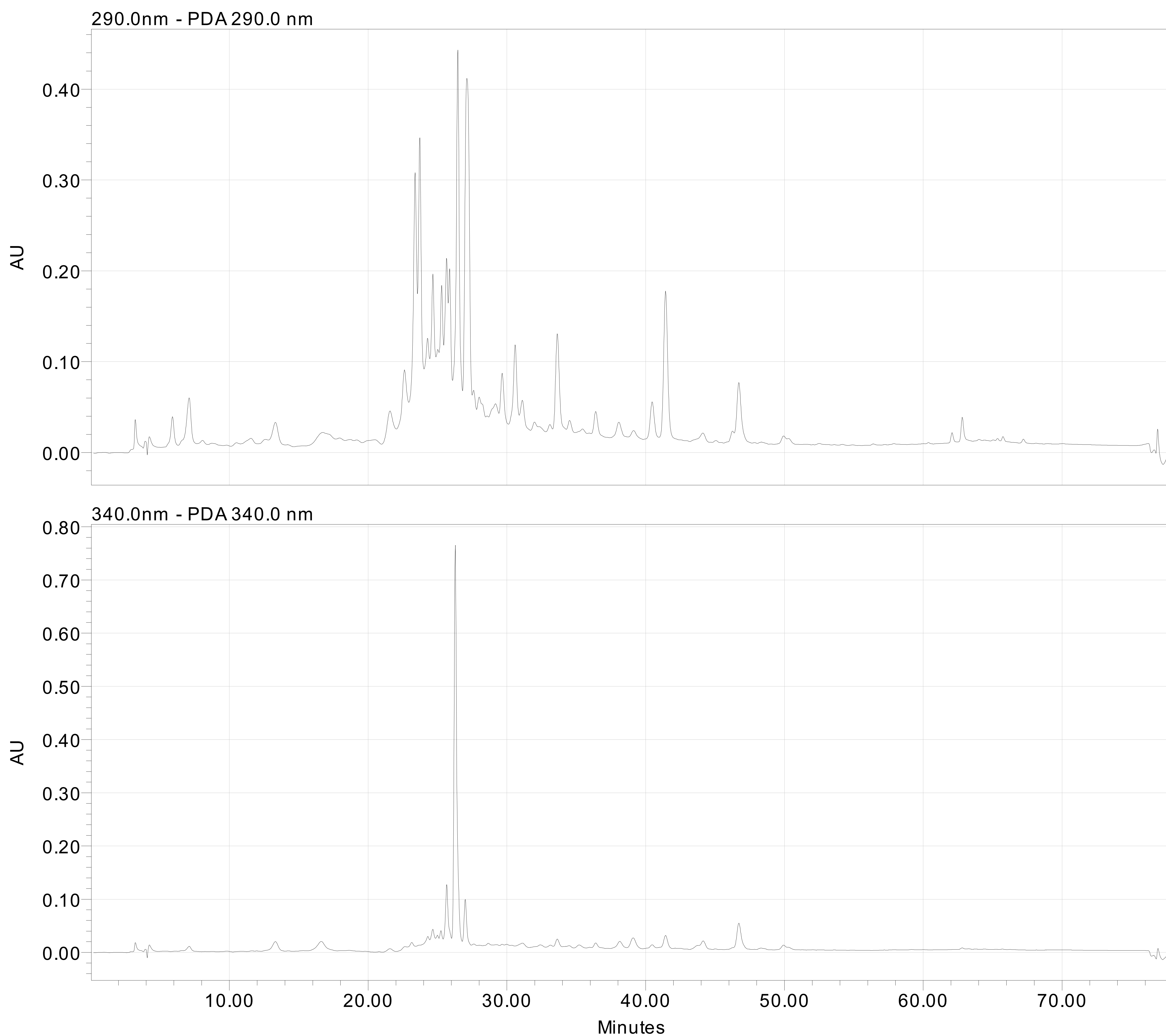
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:05:48 PM NZ

Sample Name: CC 100209 20256**Kamahi Honey****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/17/2009 12:09:38 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3082

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

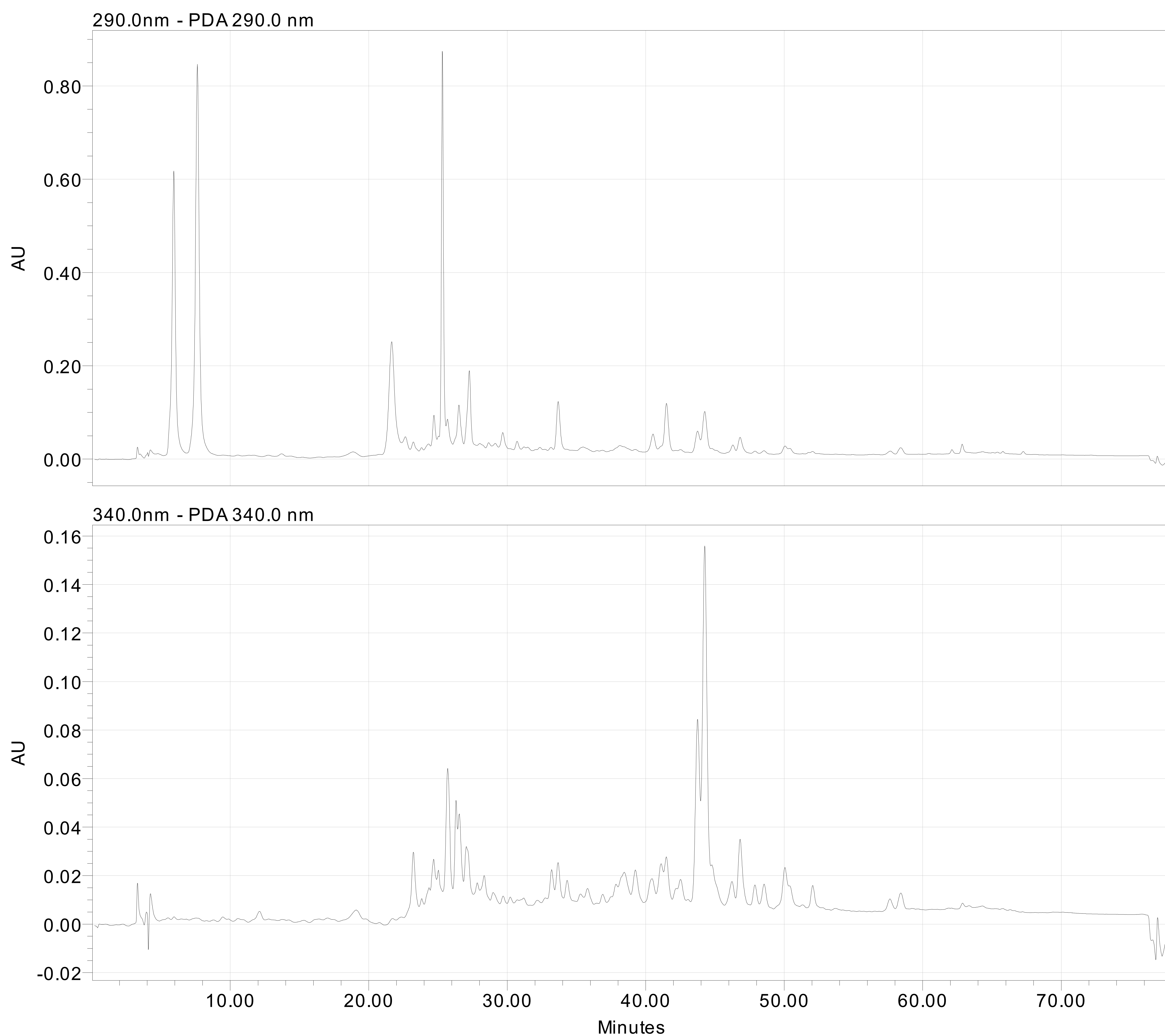
3/24/2009

3:07:41 PM NZ

Sample Name: CC 100209 19520

Viper's Bugloss Honey

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/17/2009 4:26:40 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3081

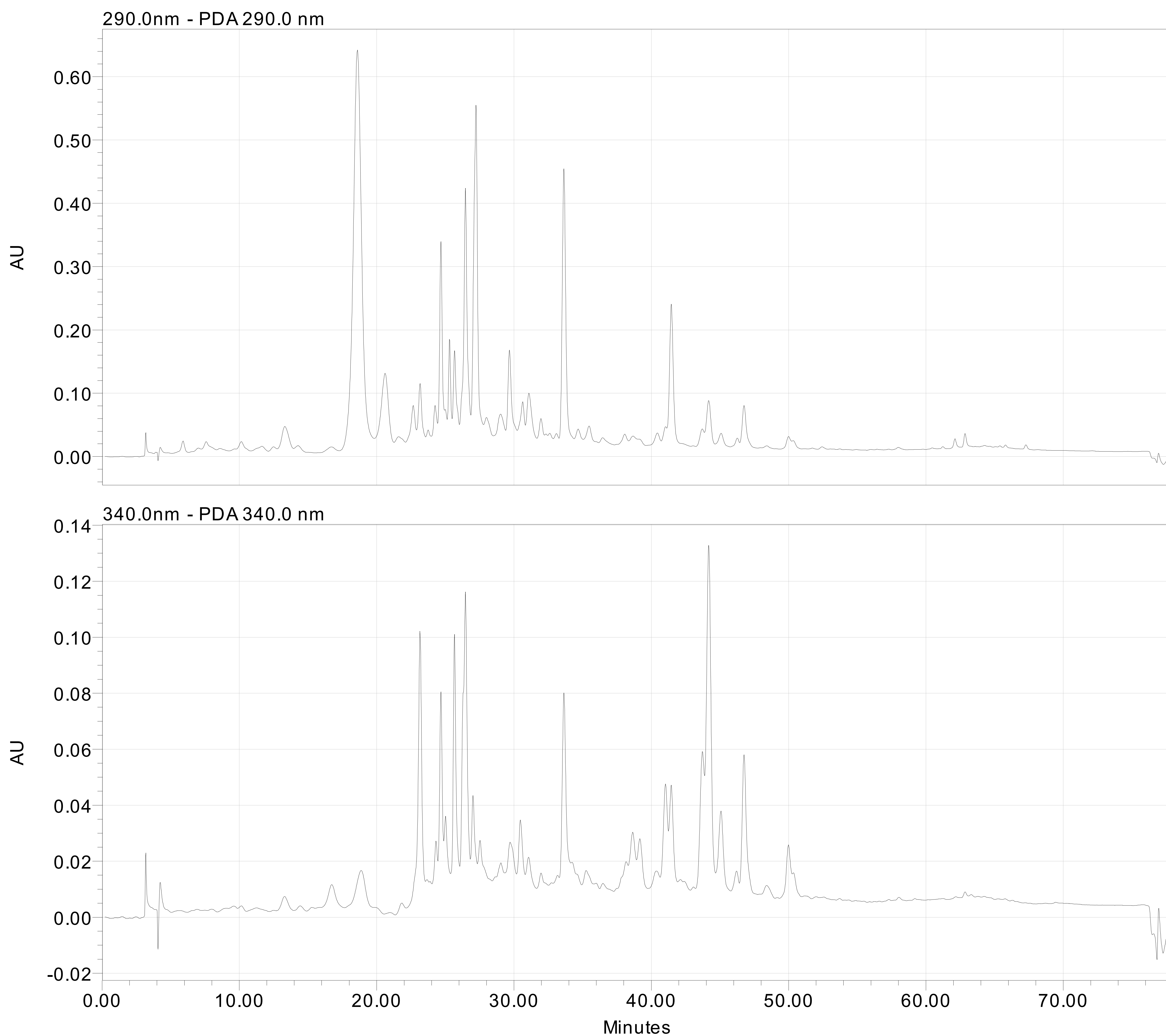
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:04:53 PM NZ

Sample Name: CC 110209 20238**Thyme Honey****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/18/2009 1:08:14 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3080

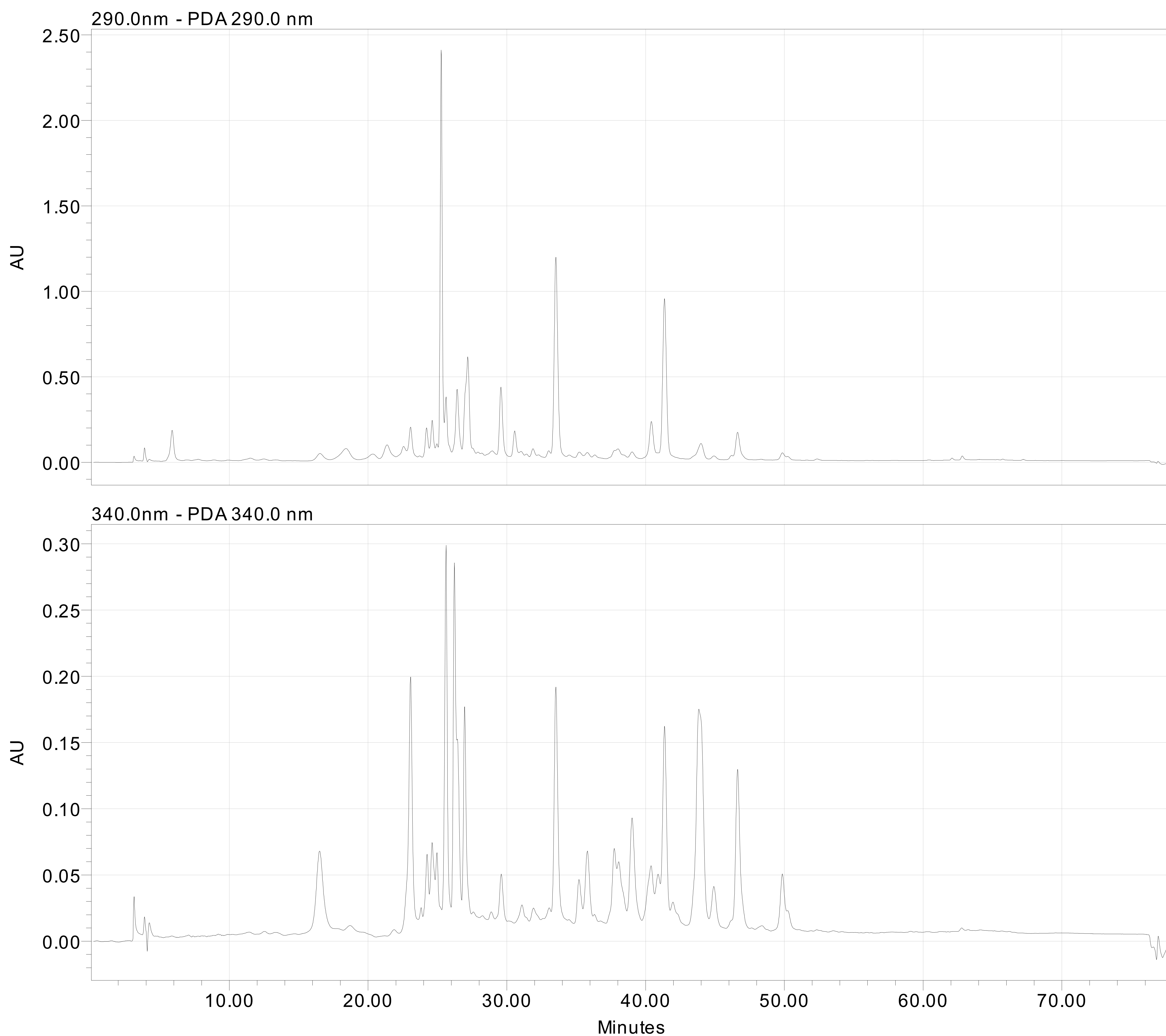
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:03:45 PM NZ

Sample Name: CC 110209 21020**Nodding Thistle Honey****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 2/18/2009 11:32:15 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3080

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/24/2009

3:02:05 PM NZ

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

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by

Benjamin Jade Deadman



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WAIKATO
Te Whare Wānanga o Waikato

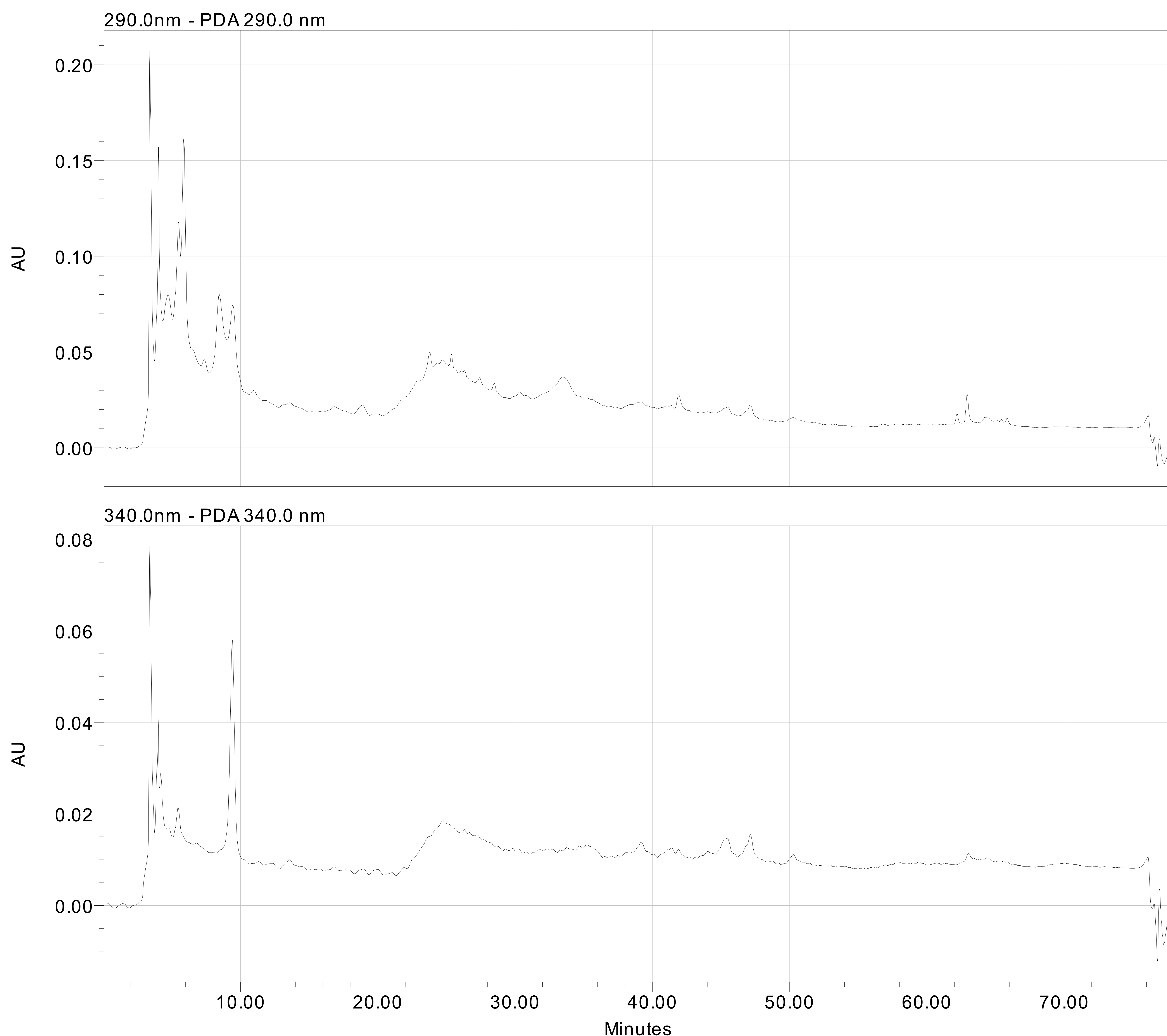
The University of Waikato

2009

**Sample Name: SephadexLH20
Fraction 1**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/20/2009 11:10:05 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

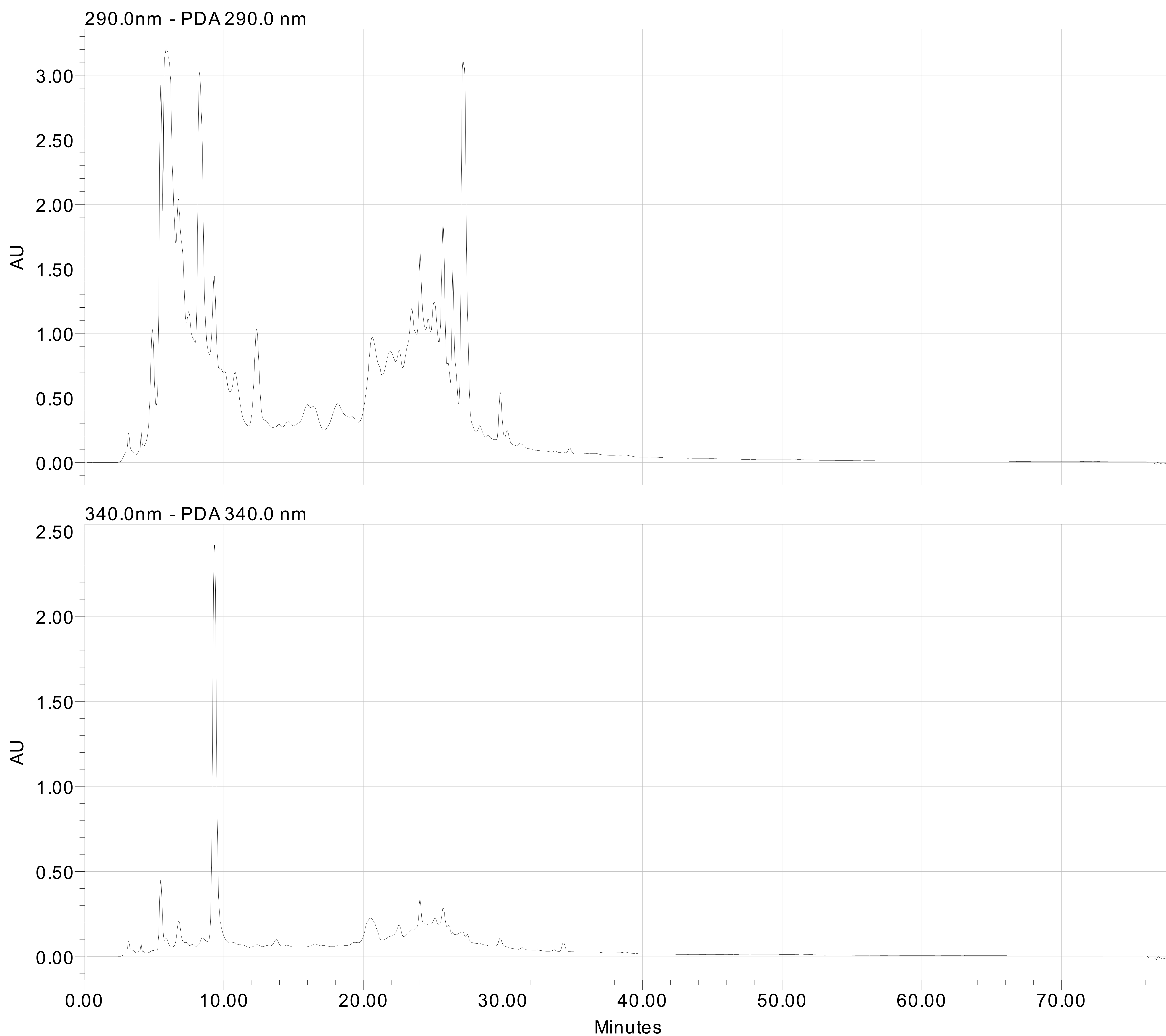
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:27:24 PM NZ

**Sample Name: SephadexLH20
Fraction 2****Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/20/2009 12:33:01 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

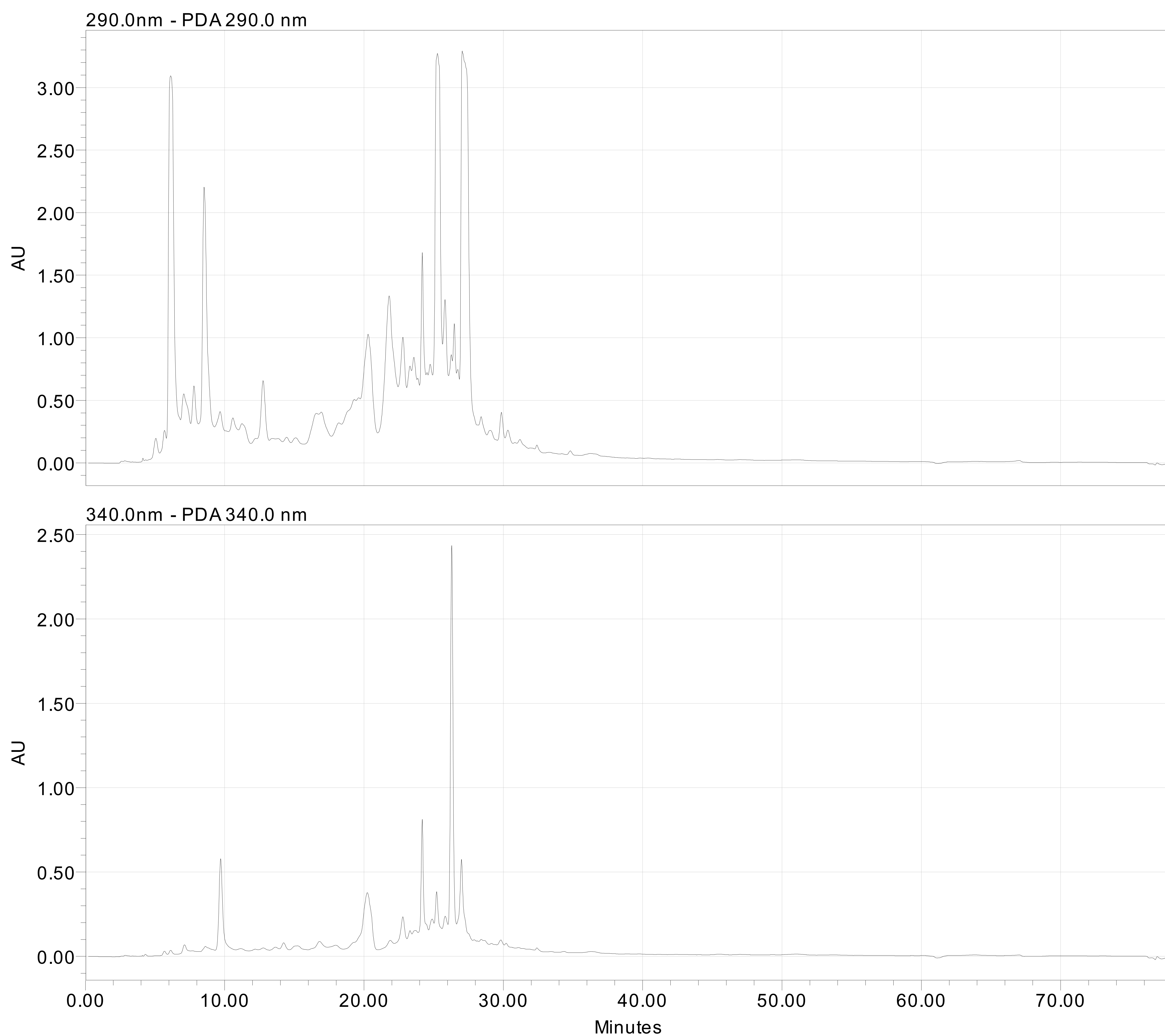
3/31/2009

5:27:06 PM NZ

**Sample Name: SephadexLH20
Fraction 3**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/20/2009 3:24:38 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

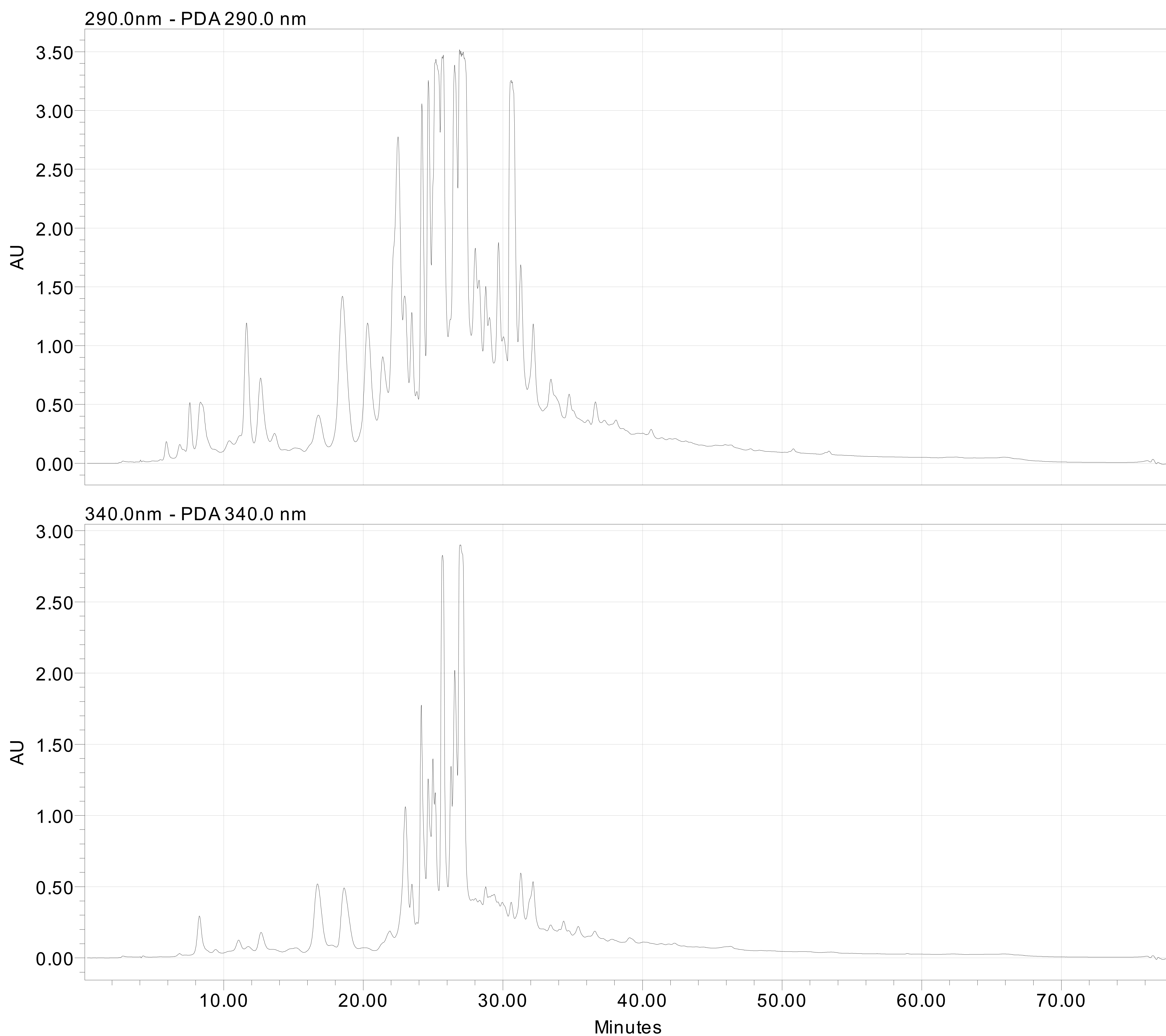
3/31/2009

5:26:16 PM NZ

**Sample Name: SephadexLH20
Fraction 4**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/20/2009 2:05:46 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

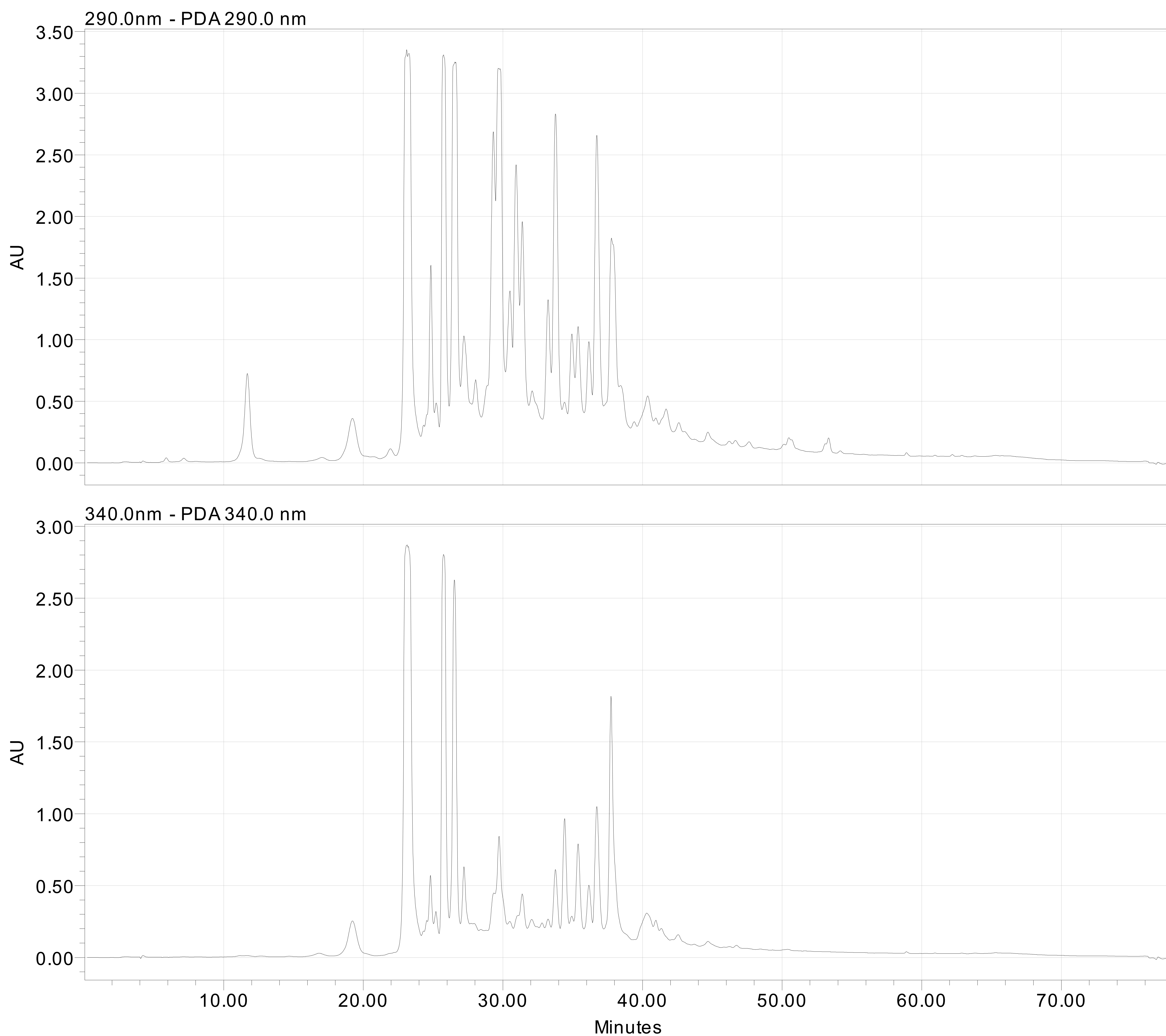
3/31/2009

5:26:37 PM NZ

**Sample Name: SephadexLH20
Fraction 5**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/21/2009 11:15:19 AM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

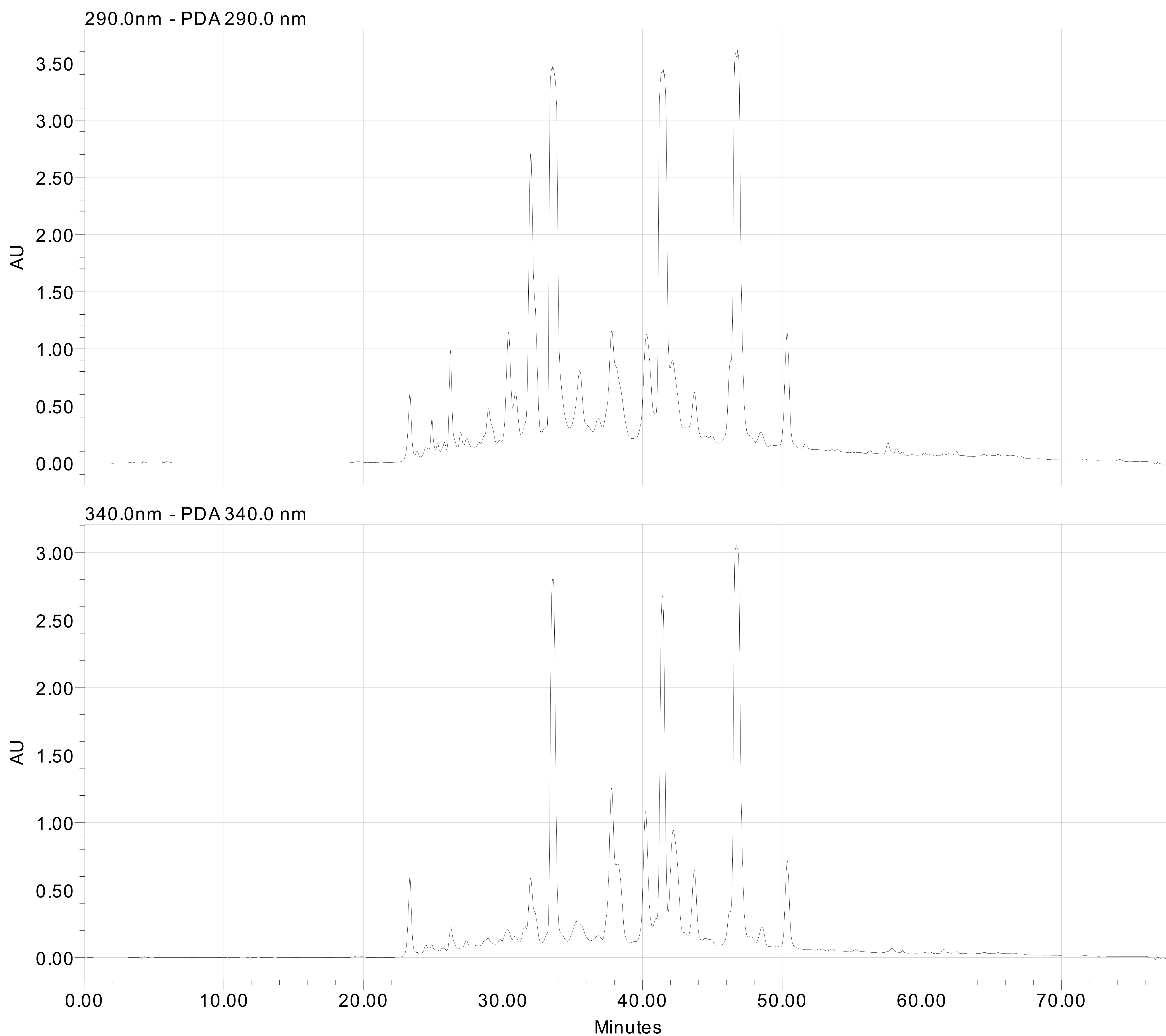
3/31/2009

5:25:58 PM NZ

**Sample Name: SephadexLH20
Fraction 6**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/21/2009 12:35:35 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

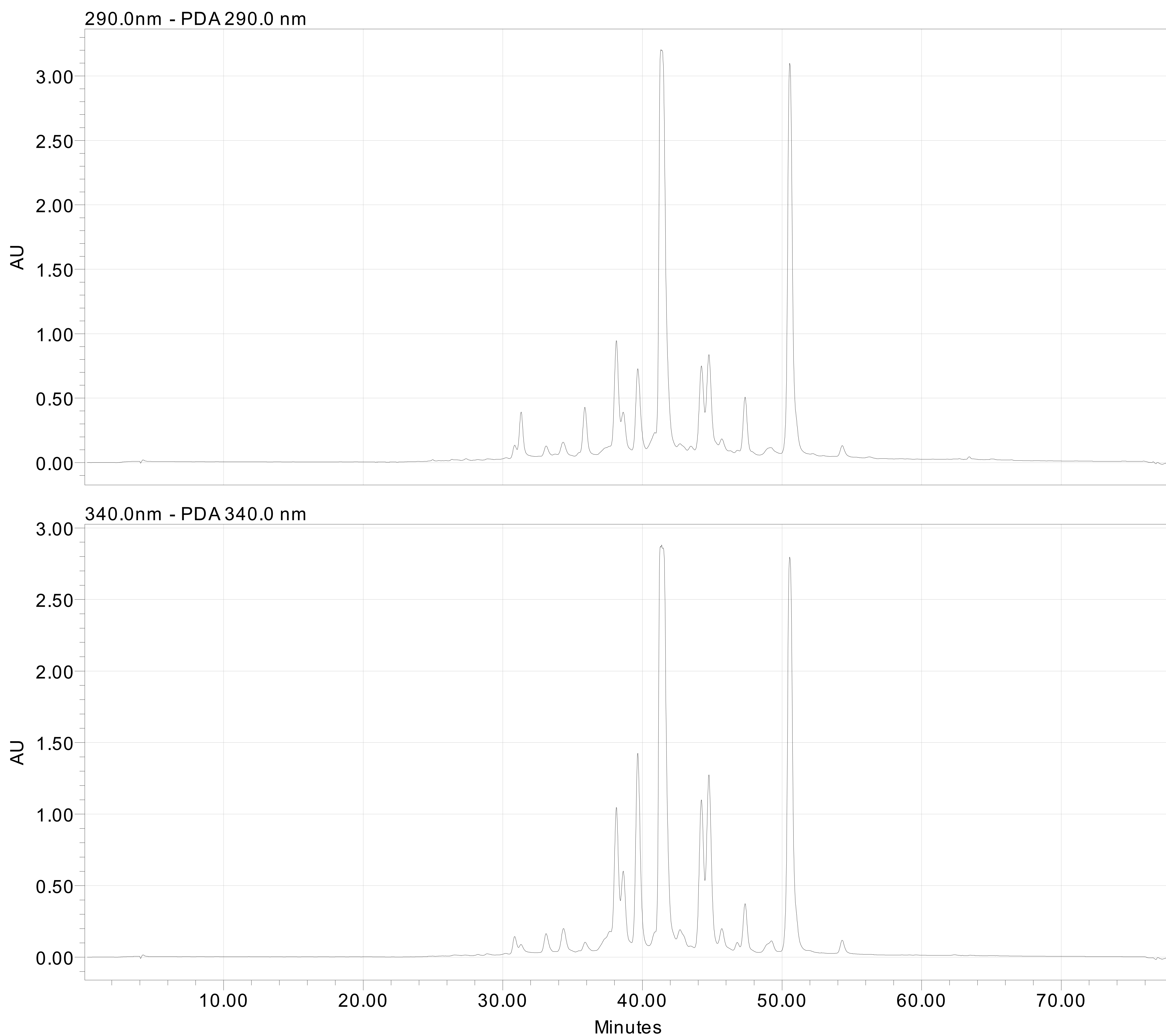
Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

5:25:36 PM NZ

**Sample Name: SephadexLH20
Fraction 7****Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography****Chromatograms**

Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/18/2009 2:09:44 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

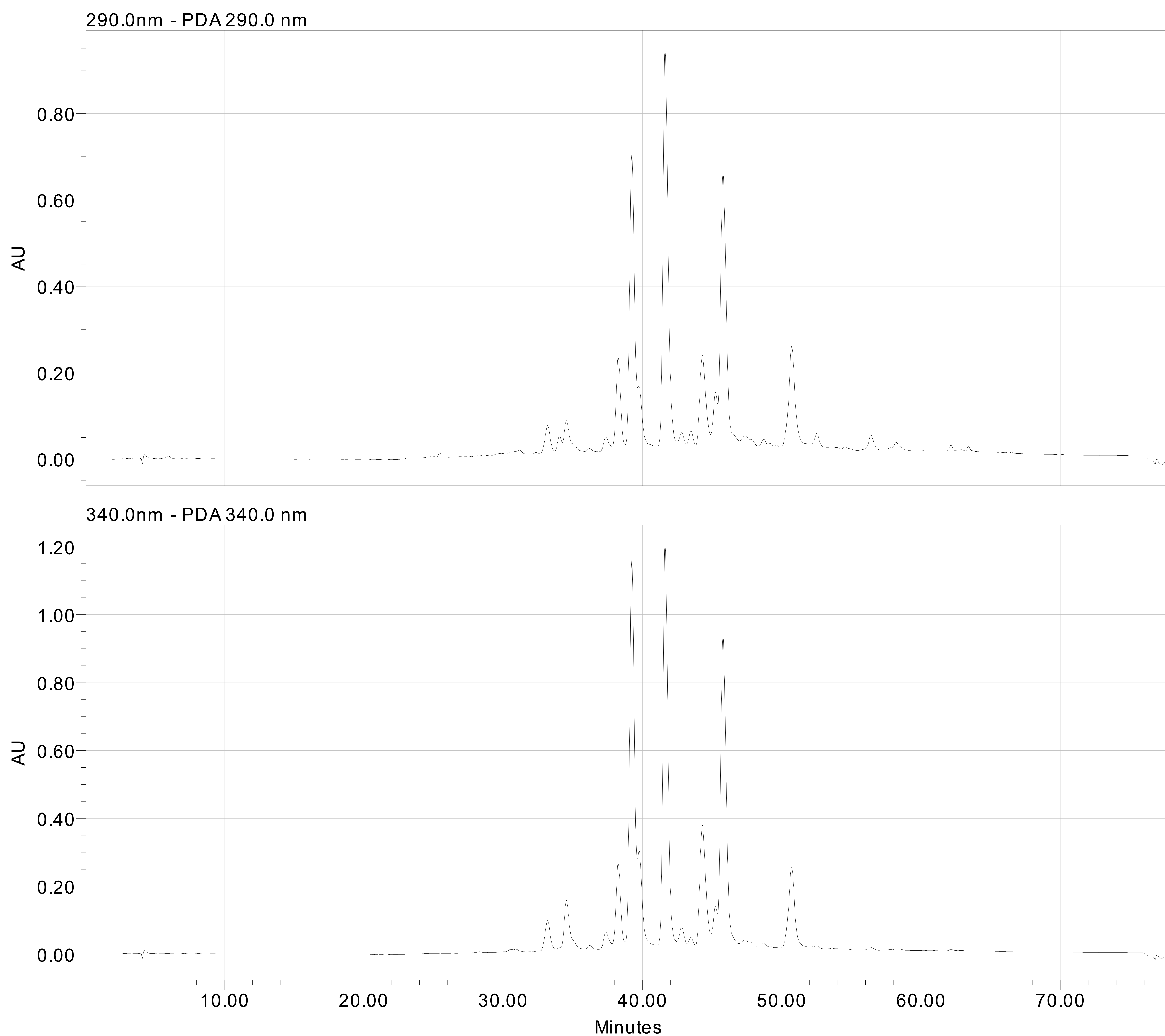
3/31/2009

5:28:48 PM NZ

**Sample Name: SephadexLH20
Fraction 8**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/18/2009 3:33:58 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

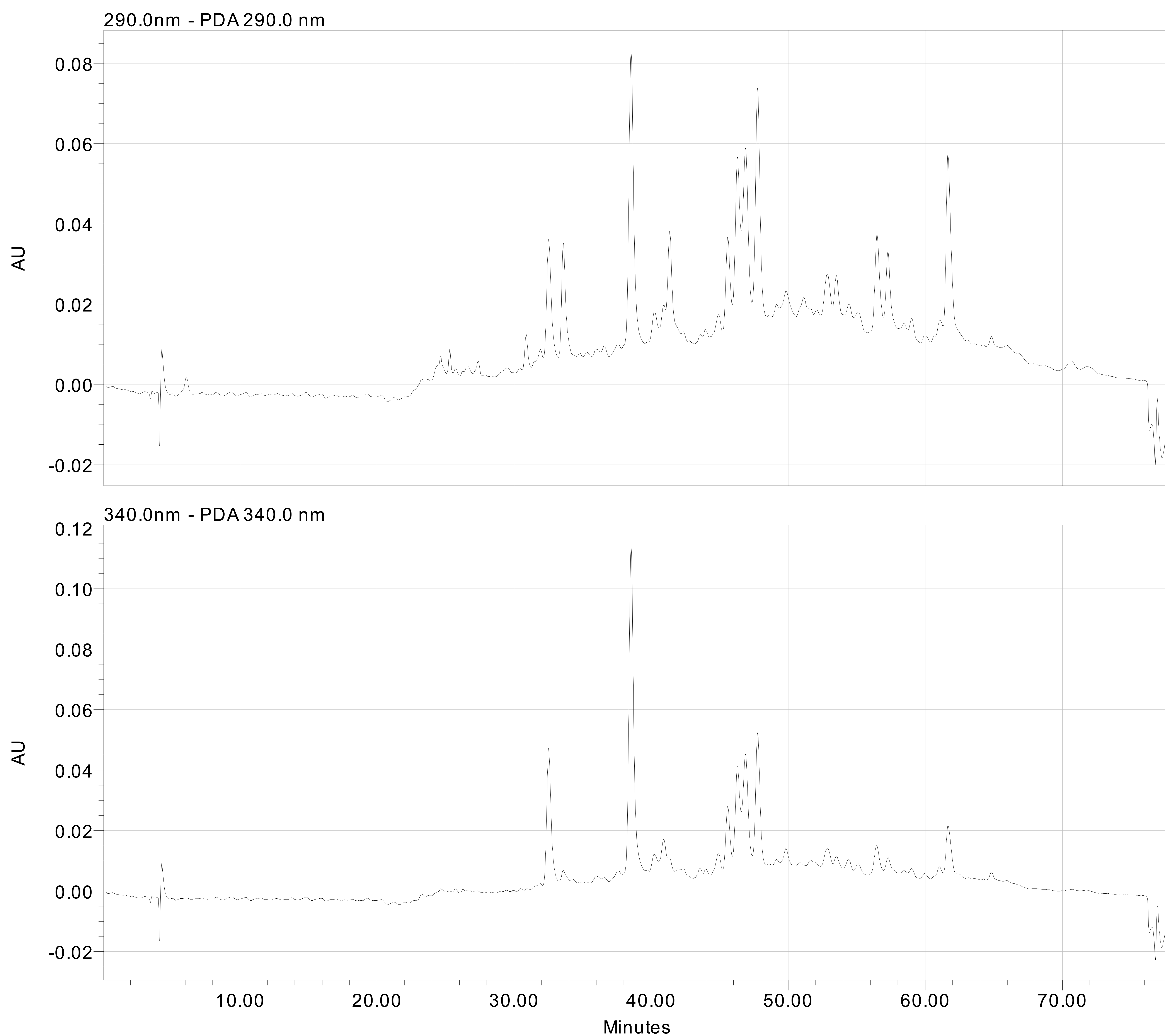
3/31/2009

5:28:32 PM NZ

**Sample Name: SephadexLH20
Fraction 9**

**Fractionation of Bulk Honey Phenolic Extract by
Sephadex-LH20 Chromatography**

Chromatograms



Empower Node: Hplc-a

System Name: Waters 2007

Date Acquired: 1/21/2009 1:54:34 PM NZDT

Instrument Method Name: Symmetry MeOH 3 a

Reported by User: System

Report Method: Honey Flavonoid Chromatogram

Report Method ID 3207

Page: 1 of 1

Project Name: Chris Adams\Ben

Date Printed:

3/31/2009

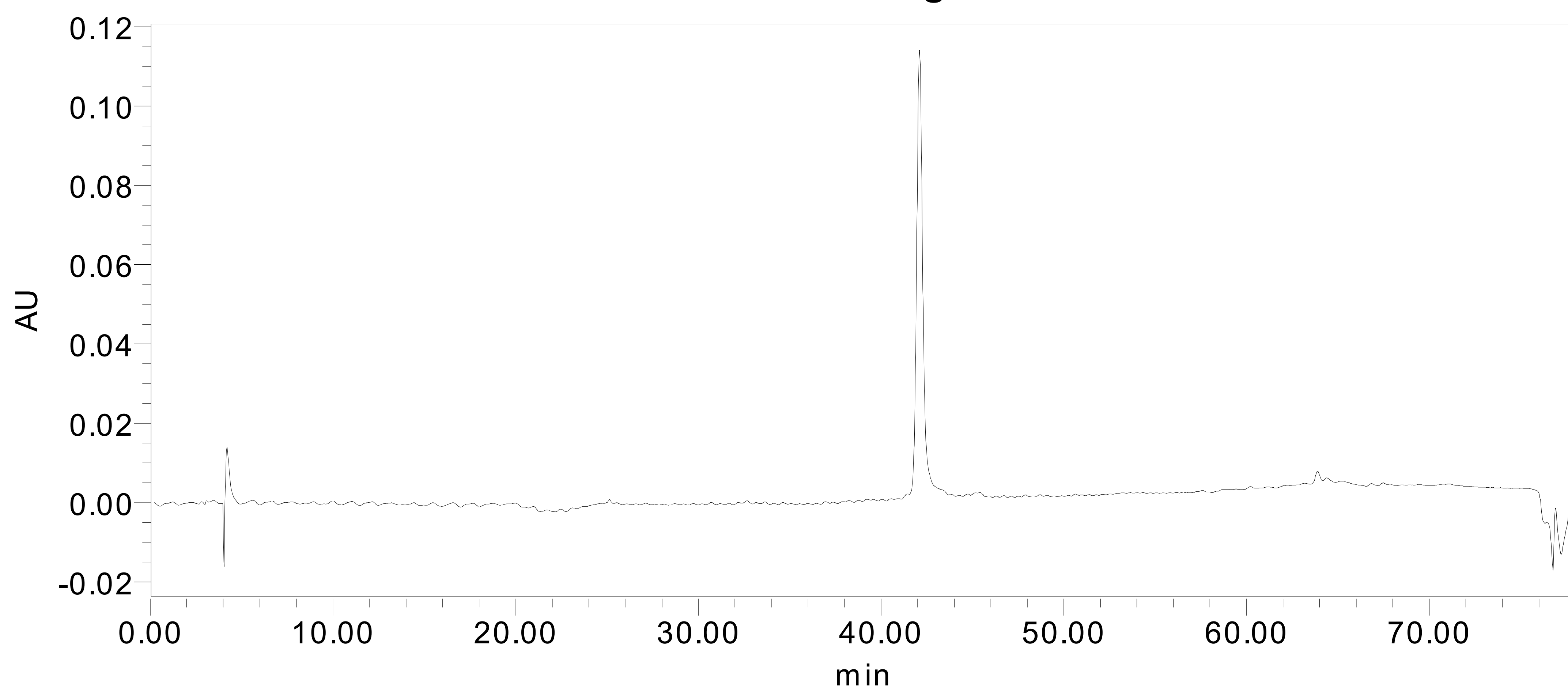
5:24:57 PM NZ

S A M P L E I N F O R M A T I O N

Sample Name: Flavonoid A
Sample Type: Standard
Vial: 1
Injection #: 2
Injection Volume: 5.00 ul
Run Time: 78.0 Minutes
Sample Set Name:

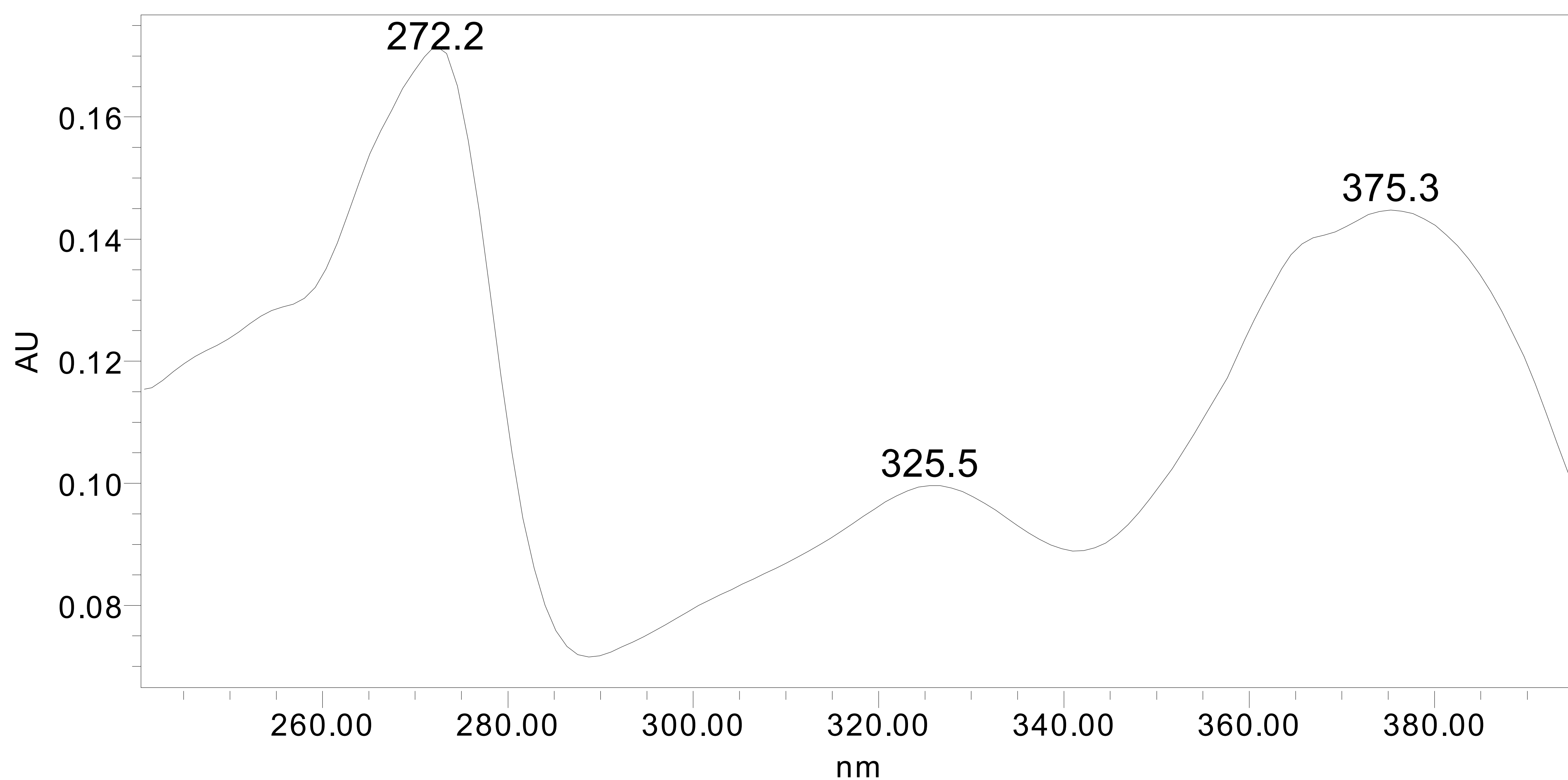
Acquired By: System
Date Acquired: 3/29/2009 3:26:03 PM NZST
Acq. Method Set: Symmetry MeOH 3 a
Date Processed: ****
Processing Method: ****
Channel Name: ****
Proc. Chnl. Descr.: ****

340nm Chromatogram



— 996 Extraction at 340 nm

UV Absorption Spectrum



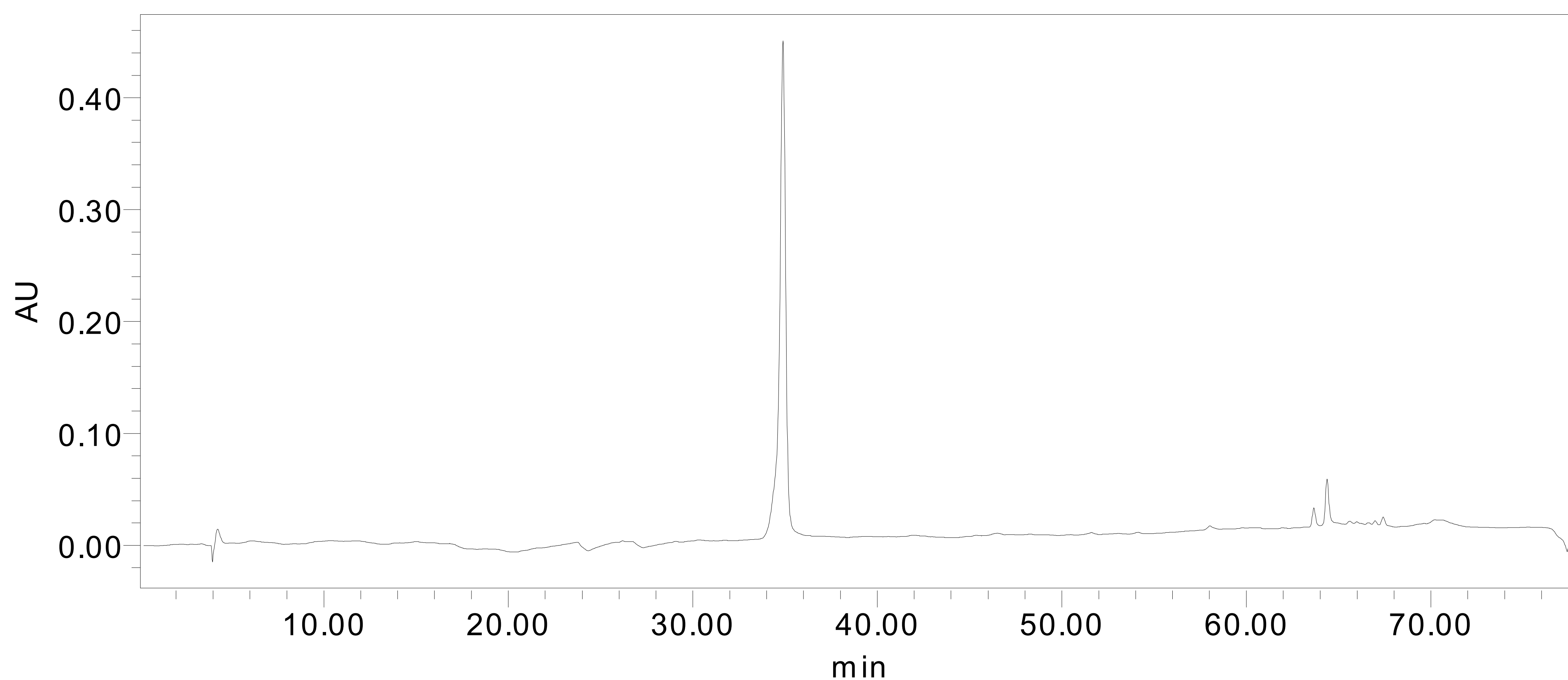
— Time: 42.000 min

SAMPLE INFORMATION

Sample Name: Flavonoid B
Sample Type: Standard
Vial: 1
Injection #: 1
Injection Volume: 5.00 ul
Run Time: 78.0 Minutes
Sample Set Name:

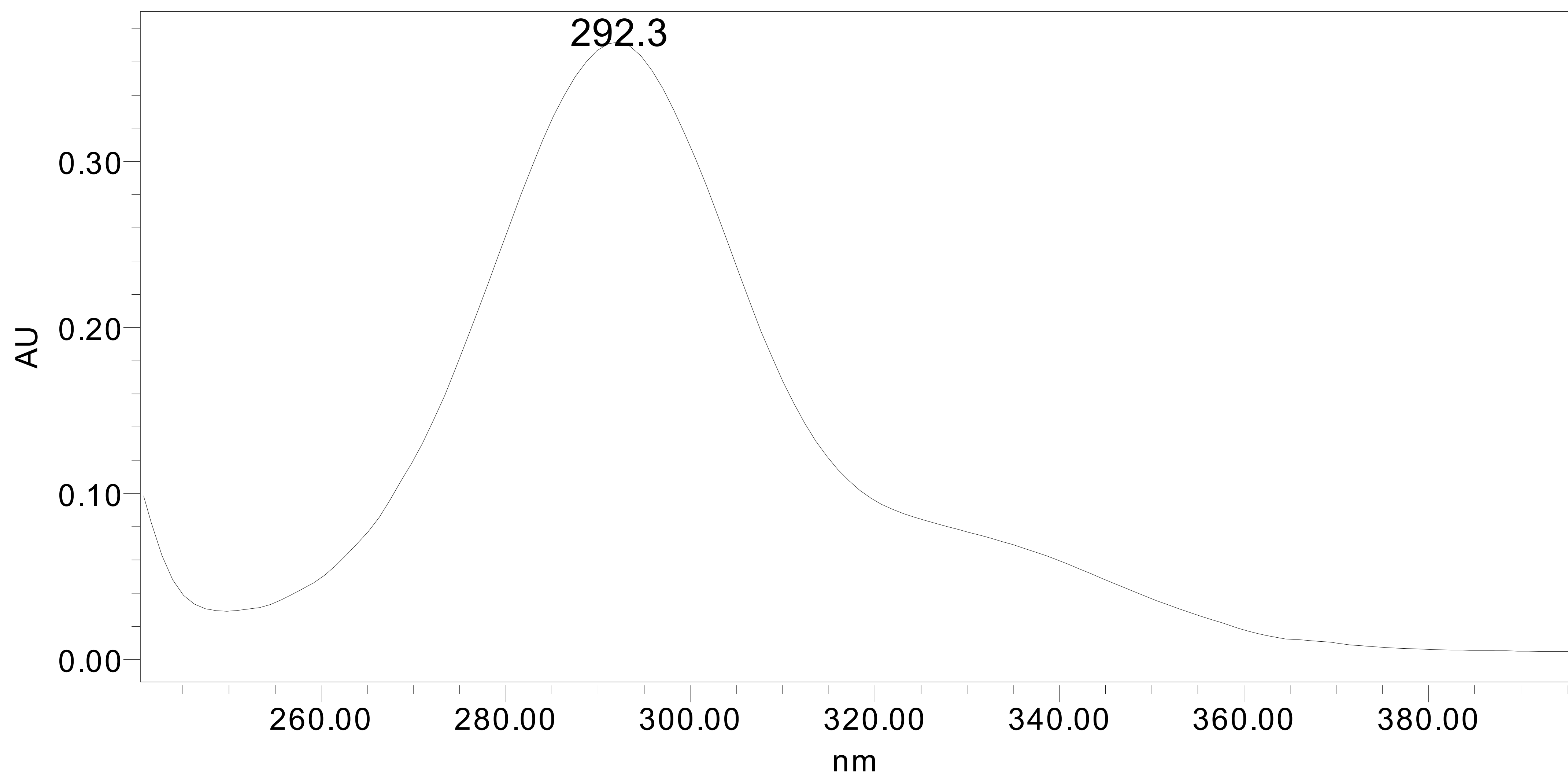
Acquired By: System
Date Acquired: 3/16/2009 1:04:27 PM NZST
Acq. Method Set: Symmetry MeOH 3 a
Date Processed: ****
Processing Method: ****
Channel Name: ****
Proc. Chnl. Descr.: ****

290nm Chromatogram



— 996 Extraction at 290 nm

UV Absorption Spectrum



— Time: 34.800 min

The Flavonoid Profile of New Zealand Manuka Honey

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Chemistry at

The University of Waikato

by

Benjamin Jade Deadman

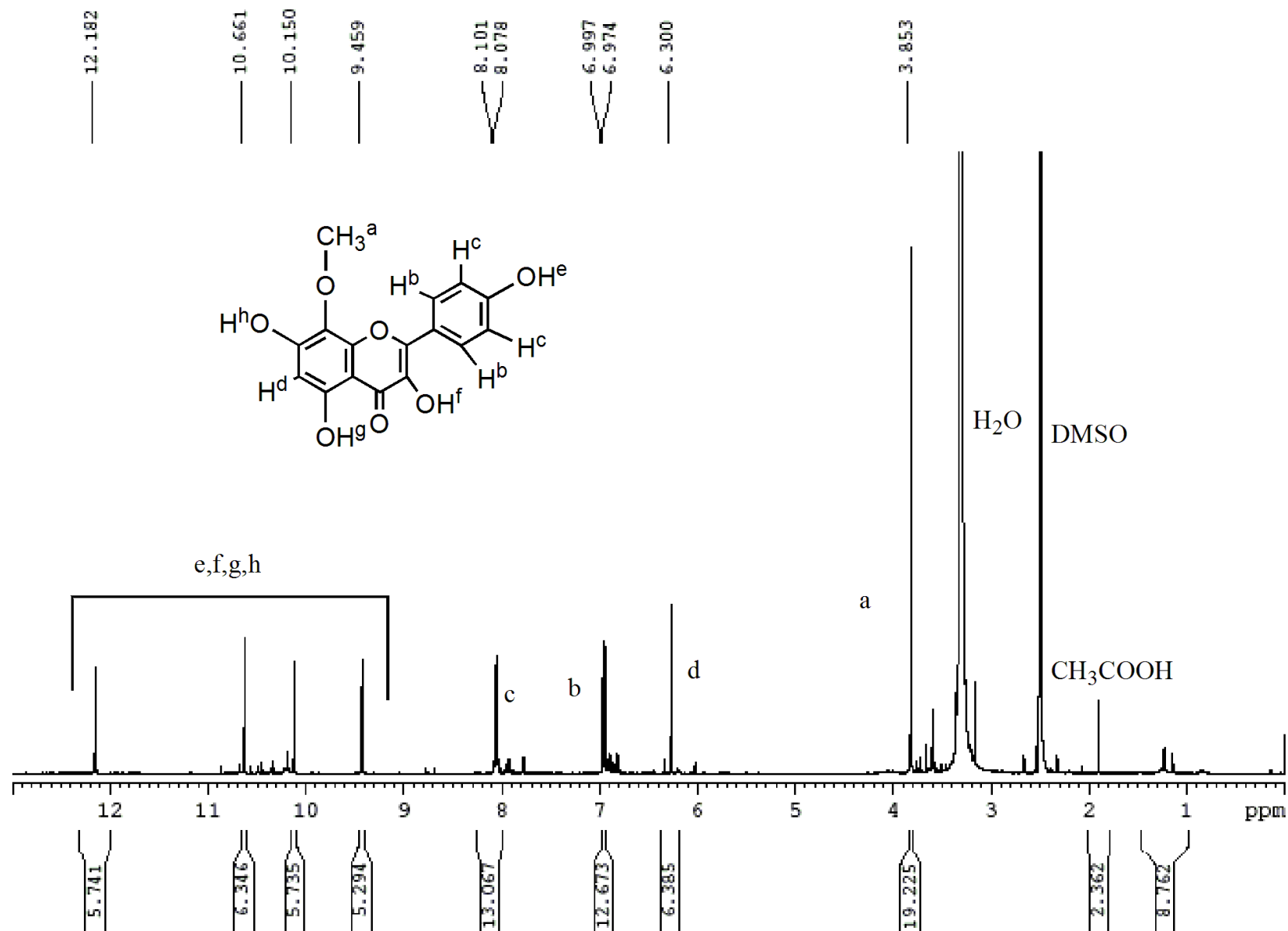


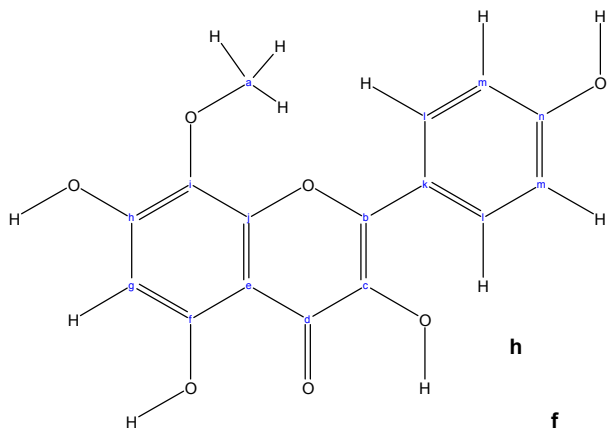
THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

The University of Waikato

2009

41.2 min peak 1H NMR





d

j

b

n

f

h

c

j

i

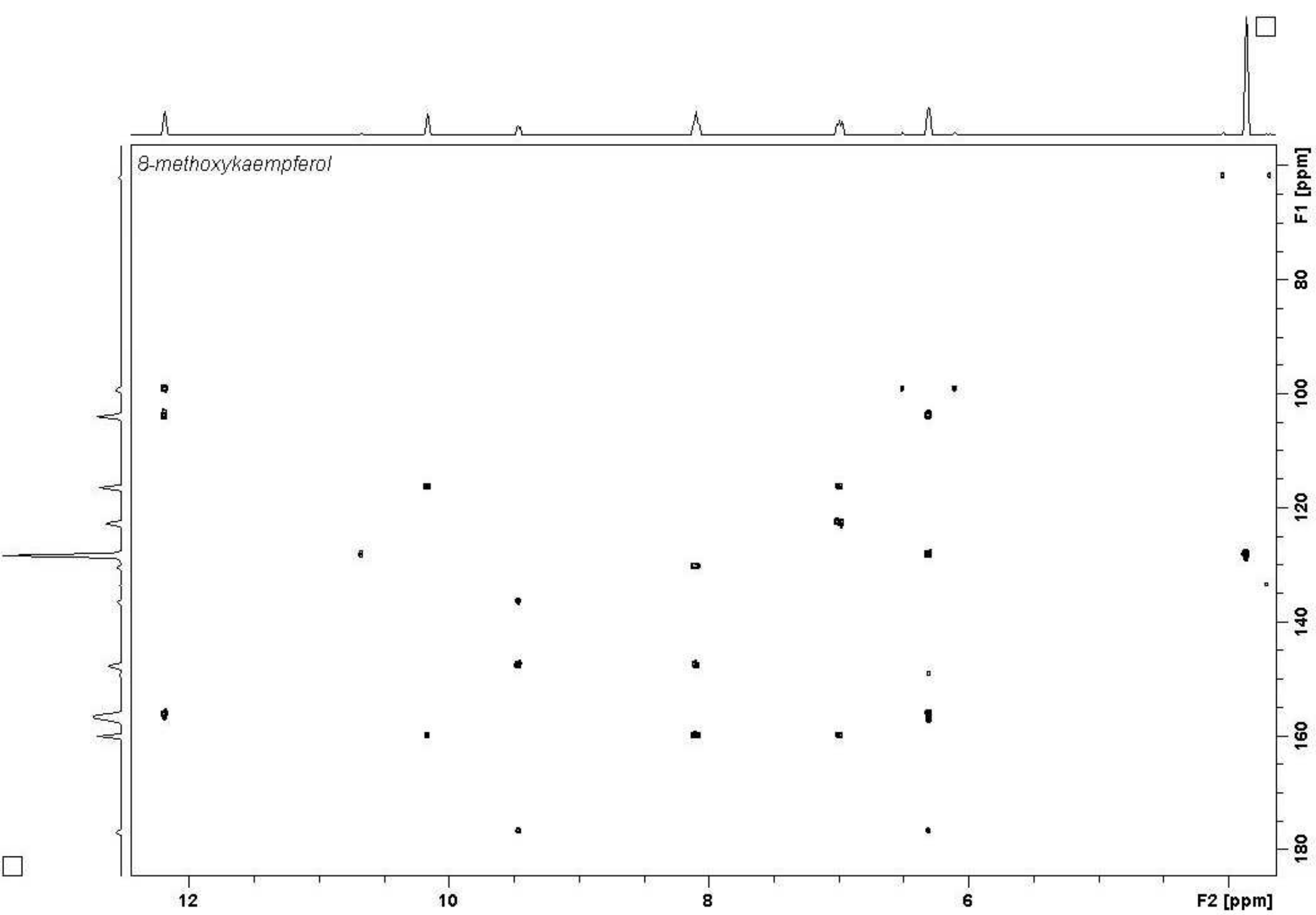
e

g

a

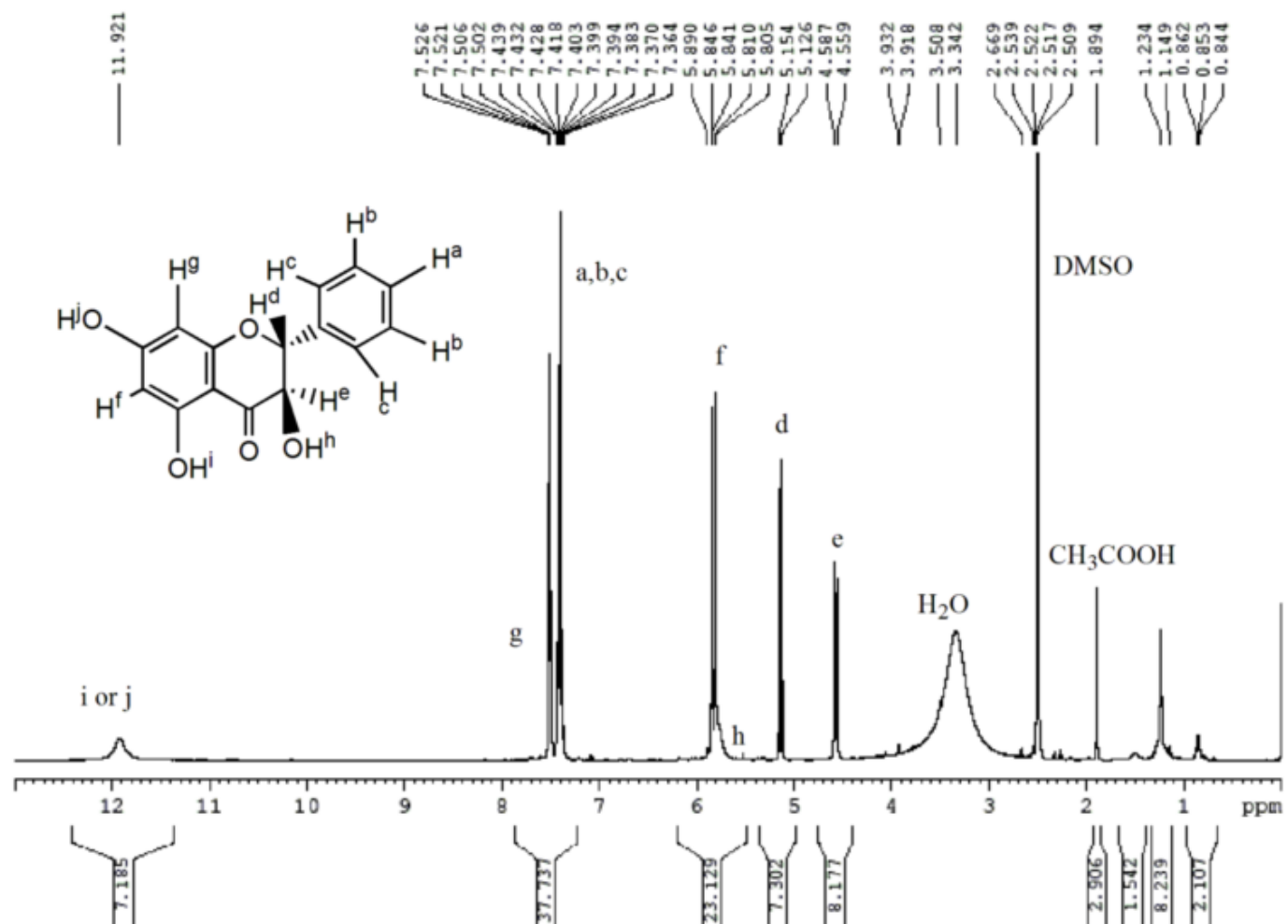
m

l



¹H NMR Spectrum of Flavonoid B

34.8min peak ¹H NMR



^{13}C NMR Spectrum of Flavonoid B

34.8 min peak ^{13}C NMR

