

**Serum Metabolome and Proteome Effects of Phytoestrogenic
Dietary Supplements in Postmenopausal Women**

BY

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THESIS

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CKN

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LIST OF ABBREVIATIONS

AMBIC	ammonium bicarbonate
BC	black cohosh
CEE	conjugated equine estrogens
CHD	coronary heart disease
DG	diradylglycerolipid
EIC	extracted ion chromatogram
ESI	electrospray ionization
HPLC	high performance liquid chromatograph
HT	hormone therapy
IT-TOF	ion trap time of flight
LC	liquid chromatograph
LC-MS	liquid chromatograph mass spectrometer
LC-MS/MS	liquid chromatograph mass spectrometer mass spectrometer
<i>m/z</i>	mass to charge
NCBI	National Center for Biotechnology Information
NIH	National Institute of Health
PA	glycerophosphate
PC	glycerophosphocholine
PE	glycerophosphoethanolamine
PG	glycerophosphoglycerol
PI	glycerophosphoinositol
RC	red clover
SERMs	selective estrogen receptor modulators
TCEP	tris(2-carboxyethyl)phosphine
TG	triacylglycerol
TIC	total ion chromatogram

LIST OF ABBREVIATIONS (continued)

TMT	tandem mass tag
UIC	University of Illinois at Chicago
USDA	United States Department of Agriculture
v/v/v	volume by volume by volume
WHI	Women's Health Initiative

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SUMMARY

The efficacy of hormone therapy in reducing postmenopausal symptoms, including hot flashes, night sweats, and other women's health factors, was to be assessed by the Women's Health Initiative clinical trial. This trial was terminated early when increased risk factors associated with the hormone therapy treatment were discovered. Breast cancer, coronary heart disease, and other significant health risks were all determined to be significantly increased when taking hormone therapy. As a result, the number of women taking hormone therapy reduced drastically, despite hormone therapy significantly reducing the postmenopausal symptoms measured. The desire to identify alternative therapies for postmenopausal symptoms has grown and botanical dietary supplements have been investigated as a safe alternative to hormone therapy.

A phase II clinical trial was conducted at UIC in conjunction with the NIH. This trial measured the ability of two botanical dietary supplements, black cohosh (*Actaea racemosa*) and red clover (*Trifolium pretense*), at reducing postmenopausal symptoms relative to hormone therapy and placebo. The trial found no statistical difference, or reduced effectiveness, of the botanical supplements relative to placebo, but a large placebo effect was also observed. In order to better understand the mechanism of action of postmenopausal symptoms like hot flashes, as well as pathways affected by the treatment groups of the UIC/NIH clinical trial, the serum samples collected during the study at the six month trial point (the time point with statistical difference between the hormone therapy group and the black cohosh group from placebo and red clover) were analyzed to develop a metabolomic and proteomic profile.

The metabolomics experiments produced many hits of interest for all three treatment groups. In the red clover group, two compounds that have been previously isolated from red clover, formononetin and genistein, were identified by the database searches. These hits were tentatively confirmed by running chemical standards of those compounds and comparing the retention times. In addition several vitamin

SUMMARY (continued)

D metabolites were identified, indicating that the Vitamin D pathway may be affected by red clover treatment. The black cohosh group showed fewer hits of interest, but the hits included naturally occurring compounds not previously identified in black cohosh. The small molecule features identified in the hormone therapy group represent potential biomarkers for hot flashes or a biological pathway impacted by hormone therapy treatment.

Results from the proteomics experiments from the serum samples also produced several hits of interest. The results showed many proteins up and downregulated that function in the inflammation and metabolism pathways. The metabolism of vitamin D in particular showed hits of interest for further investigation in several treatment groups. Inflammation is a pathway of particular interest and the hits of vitronectin, ceruloplasmin, and kininogen should be investigated to identify the particular pathways causing the observed upregulation in the three treatment groups. Insulin-like growth factor binding protein 6 is of interest as it was upregulated in the hormone therapy group but downregulated in the black cohosh group. Since these groups had the opposite effects on hot flashes relative to placebo this may represent a significant biomarker of interest to identify mechanisms of action for hot flashes.

Overall this work showed the ability to generate metabolomics and proteomics data from the same samples and provided a number of hits of interest for further investigation. The validation of potential biomarkers is essential in order to further characterize the mechanism of action or effects of hot flashes, in addition to providing insight into the effects of the different treatment groups.

CHAPTER 1 BACKGROUND

1.1 Menopause and Hot Flashes

Menopause is the reduction in naturally occurring sex hormones in women that will affect every woman who lives into old age. The average age that this commences is 50 years, and this has been historically consistent.¹ As a result of advances in medicine and general health practices greatly increasing the average lifespan, many more women will spend longer periods of their lives in the postmenopausal stage. Developing a thorough understanding of the physical changes associated with menopause has therefore increased in importance, as has evaluation of potential therapies to manage menopausal symptoms. Menopause brings about many difficult changes that greatly affect the health and overall quality of life of middle aged and older women.

In menopause the amount of estrogen and progesterone produced by the body naturally decreases. This is in part because the body has aged past the fertile period of being capable of safely carrying a pregnancy to term. As the estrogen and progesterone production decreases so naturally does the production of secondary metabolites. Among these metabolites, 17 β -estradiol (estradiol) is of particular interest as the most potent endogenous ligand of the estrogen receptors.² Figure 1 displays the structure of the most common estrogen receptor ligands. Estrogen receptors have been linked to many biochemical pathways including cardiovascular function, but the classic function is as a regulator of gene transcription.²⁻⁴ As the levels of estrogens greatly impact the transcription of a variety of genes and the downstream translation of proteins, the reduction of endogenous estrogens at menopause can have a variety of effects.

The symptoms associated with menopause are varied and can have significant physical and psychological effects on postmenopausal women. One of the most easily recognized symptoms of menopause is hot flashes. During a hot flash, a woman's body temperature will rise and she will begin to

perspire. The sensation may last as short as a matter of seconds or as long as an hour.⁵ It may be a mild sensation of warmth and discomfort or it can be a painful experience that causes a loss of motor function and dizziness. These episodes can have a significant impact on the quality of life of postmenopausal women. In addition to the physical discomfort of the hot flash itself, loss of sleep, headaches, increased blood pressure, and depression or anxiety over the lack of control and knowing when the next hot flash will be are all associated detrimental outcomes that affect a significant percentage of those experiencing the symptom.⁵⁻⁷

Clinically, there is also very little understanding as to the cause and biochemical pathways associated with hot flashes. Measurement of hot flashes is primarily done through self-reporting,^{5,7-13} and relies on the subject to assess and effectively document the frequency and severity of these hot flashes. This may not always be practical, as hot flashes may occur at any time of day, and the discomfort caused by this may prevent effective self-assessment. The severity factor of these hot flashes is also subjective. To more objectively document hot flashes for research, mechanical devices have been used to record hot flashes. In the lab setting, this has been the only way to use animal models to monitor for hot flashes. The devices typically rely on the conductivity of the skin to determine the severity and duration of the hot flash.¹⁴ In animal models, surgical implantation of these devices has also been used.^{11,15,16} Some devices require activation by the participant when the hot flash commences. While some devices have been miniaturized, discomfort, sensitivity and stability still limit their effectiveness in humans. From a clinical perspective, the identification and validation of one or more biomarkers for hot flashes would allow for more accurate quantitation of their occurrences and severity, as well as help in the understanding of the biological pathways involved, possibly leading to identification of new therapies.

In addition to hot flashes, a woman undergoing menopause may experience other physical symptoms such as night sweats and vaginal dryness.¹⁷⁻¹⁹ Many women report severe migraines after entering menopause although this symptom has not been isolated from effects of treatments.^{5,20} There

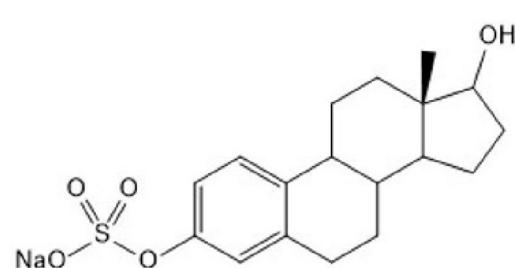
are also psychological effects that may occur. Anxiety, depression, irritability, and mood swings have all been observed, many of which are commonly attributed to lack of sleep.^{7,18,21} Overall, the quality of life of postmenopausal women can be greatly affected by these symptoms. Therefore, the development of safe and reliable treatments would greatly benefit all women. The development of these therapies depends on an understanding of the changes taking place on a chemical level and identification of the relevant chemical pathways.

1.2 Hormone Therapy and the Women's Health Initiative

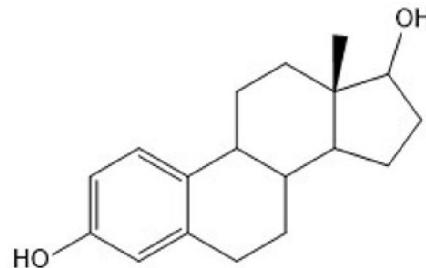
Hormone Therapy (HT) as a treatment for postmenopausal women dates back to the 1930s. Synthetic estrogens were used until the 1940s when estrogens and progestin were isolated from the urine of pregnant mares and an orally bioavailable formulation was developed.¹ Formulations of equine estrogens, still in use today, include phase 2 conjugated sulfate-metabolites, known as conjugated equine estrogens (CEE). This was the most commonly prescribed formulation during the latter half of the 20th century. Estrogen compounds commonly used in HT as well as biologically active estrogen compounds are shown in Figure 1.1. Following the early termination of the Women's Health Initiative (WHI) study, the number of women taking HRT decreased dramatically.²²

The WHI was a large-scale clinical study on the health effects of hormone therapy on postmenopausal women. The primary outcome of interest was the effect on Coronary Heart Disease (CHD), but the overall safety and benefits of the therapy were expected to be determined.²³ The study enrolled over 16,000 postmenopausal women aged 50-79 in a double-blind, clinical trial across the

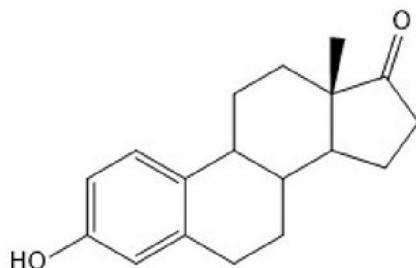
Figure 1.1 Chemical structures of characteristic endogenous and therapeutic estrogen compounds



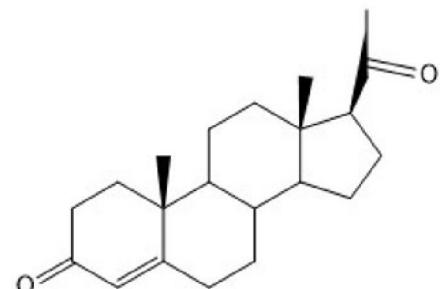
Estradiol Sulfate
Chemical Formula: $\text{C}_{18}\text{H}_{23}\text{NaO}_5\text{S}$
Exact Mass: 374.12



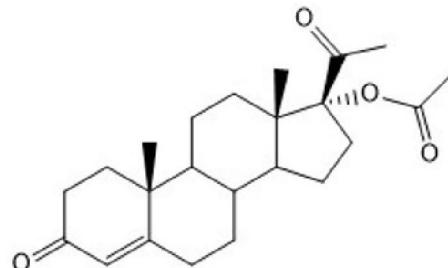
Estradiol
Chemical Formula: $\text{C}_{18}\text{H}_{24}\text{O}_2$
Exact Mass: 272.18



Estrone
Chemical Formula: $\text{C}_{18}\text{H}_{22}\text{O}_2$
Exact Mass: 270.16



Progesterone
Chemical Formula: $\text{C}_{21}\text{H}_{30}\text{O}_2$
Exact Mass: 314.22



Medroxyprogesterone acetate
Chemical Formula: $\text{C}_{23}\text{H}_{32}\text{O}_4$
Exact Mass: 372.23

United States.²³ The original planned duration of the study was 8.5 years, but the study was stopped after five years because the risk of breast cancer among women receiving HT was determined to be statistically significant.^{22,24,25} Analysis of the clinical data revealed that in addition to an increased risk of breast cancer, HT increased the risk of CHD, stroke and pulmonary embolism.^{22,25-27} There was some associated benefit in reducing the risk of hip fracture and colorectal cancer.^{25,28} In addition, HT was shown to be clinically effective at reducing hot flashes.¹² The effects and benefits of HT are still being debated and studied today, but as a result of the publication of the findings of the WHI, the use of HT has decreased, and the search for alternative therapies has intensified.

1.3 Black Cohosh

Black cohosh (*Actaea racemosa*) is a plant indigenous to the eastern United States. A map of the growth in the United States is shown in Figure 1.2 from the United States Department of Agriculture (USDA) and reprinted with permission. It is a perennial plant that blooms in summer, producing spindled flowers that are white in color as seen in Figure 1.3. The leaves of the plant grow thick and dense near the ground and long stalks grow up from the base, reaching as high as eight feet. The root of the plant is cultivated for medicinal purposes. The earliest known medicinal uses of black cohosh were focused on women's health. Records from the 18th century indicate that various tribes of indigenous North Americans used the plant as a treatment for symptoms affecting both pre- and postmenopausal women.²⁹ The medicinal use was not limited to women, however, as other historical studies indicate that the plant was also used for analgesic effects in men and women.³⁰ The use continued throughout the colonization of North America by European settlers and persisted continuously into the 20th century. In addition to controlling menopausal vasomotor symptoms, black cohosh was used as a treatment for psychological disorders in women, including postpartum depression.^{31,32} Many different formulations

Figure 1.2 Growth of black cohosh in the United States and Canada, from the USDA, reprinted with permission.³³

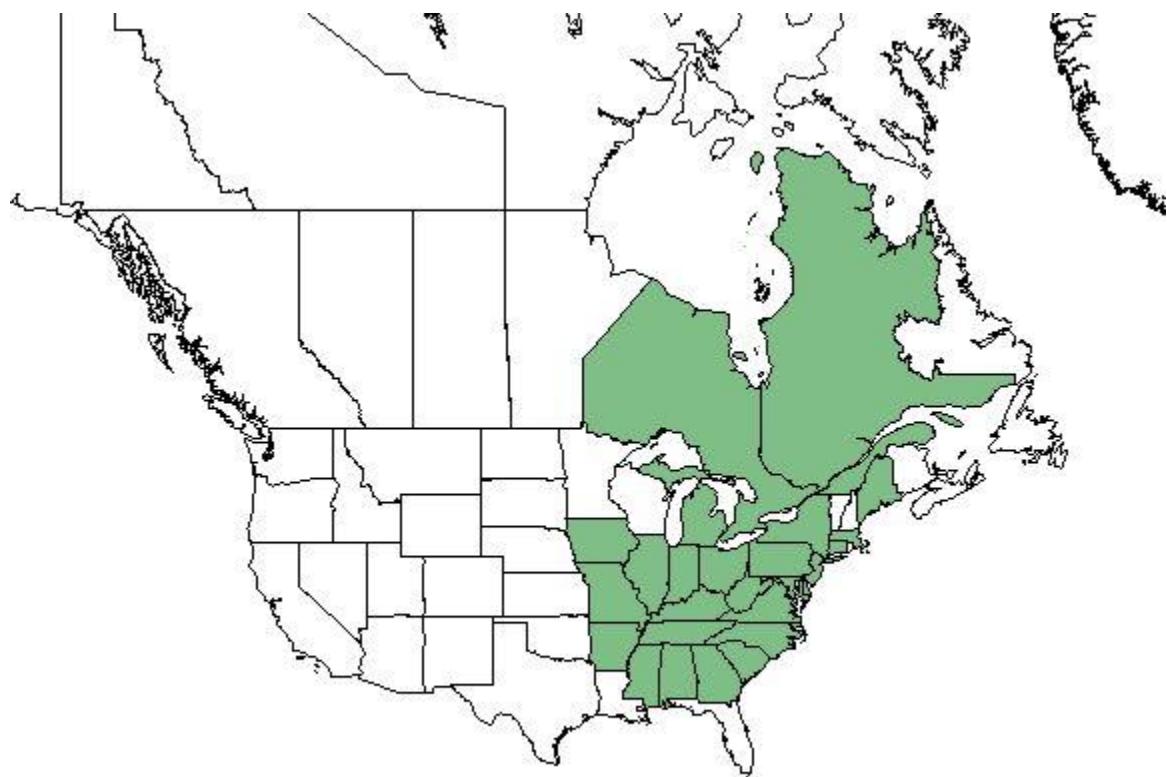


Figure 1.3 Black cohosh plant growing in a garden. From the personal collection of Richard van Breemen, used with permission.

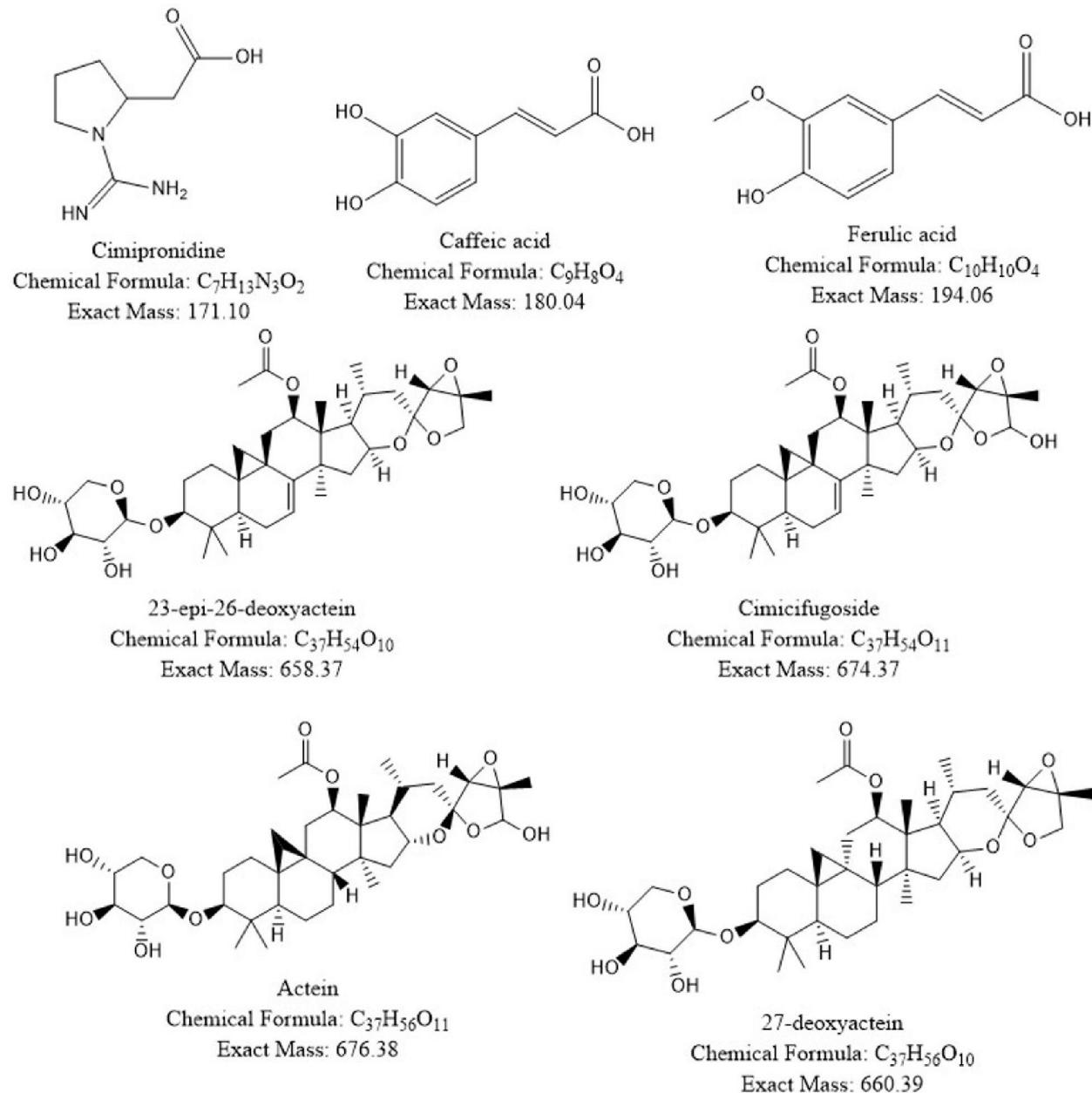


and characterization studies took place over this time, including attempts to pinpoint the activity of the plant to specific parts of the plant.^{31,34} Today, postmenopausal women regularly use the plant as a treatment for vasomotor symptoms, and black cohosh regularly appears among the top selling herbal supplements in the United States, including among the top ten in 2011.³⁵

Characterizations of the chemical components of black cohosh have largely focused on triterpenoids and phenolic acids as the major constituents of the plant. Actein and related compounds represent a significant portion of the triterpenoids, while hydroxycinnamic acids make up a majority of the phenolic acids.^{36,37} Primary and secondary metabolites of each of these classes of compounds are also present in the plant. Representative structures of many of these compounds are shown in Figure 3. In addition, a large number of nitrogenous alkaloids and secondary metabolites have been characterized and proposed to be responsible for some biological activities of the plant.³⁸ Several of these compounds are shown in Figure 1.4. However, no single compound or group of compounds in black cohosh has been linked to the estrogen receptor or other receptors that can modulate the biological effects observed in menopausal women.³⁹ While methylserotonin has been found in black cohosh and has been shown to be an estrogen receptor ligand, the compound has been shown to have almost no bioavailability, and therefore cannot be responsible for modulating hot flashes or mood.⁴⁰⁻⁴² This remains true despite ongoing experiments in vitro as well as cell culture studies.^{39,43,44}

The results of clinical trials investigating the effects of black cohosh on vasomotor symptoms in menopausal women have been mixed. The study that originated the samples used in the experiments described in this work concluded that no significant difference in effects from placebo were observed.⁹ Many other clinical trials have supported this conclusion.^{10,13,45,46} There are however a number of clinical trials that did observe a statistically significant reduction in the hot flashes observed in menopausal

Figure 1.4 Chemical formulas for characteristic triterpenoid and hydroxycinnamic acids found in black cohosh



women.^{47–50} Interestingly, many of these studies observed a significant placebo effect, and in many cases this made producing a statistically significant reduction of hot flashes difficult. As such, drawing conclusions about the efficacy of black cohosh in reducing hot flashes in postmenopausal women would be difficult at this time. Therefore, investigations into the biochemical pathways involved in hot flashes as a means of identifying biomarkers for measuring clinical response to therapy and as a means of developing effective therapeutic interventions become more important.

1.4 Red Clover

Red clover (*Trifolium pratense*) is distributed across the globe, including the Americas, Europe, Asia, and Australia. It is widely distributed across North America as seen in the map shown in Figure 1.5, from the USDA, reprinted here with permission. Red clover is a small plant, growing around to a height of less than two feet. It spreads very quickly, and can overwhelm other plants, leading it to be considered a weed in many areas. The leaves are flat and long and the flowers are pink in color, which are cultivated for medicinal use and are seen in Figure 1.5. Much like black cohosh, there is a long history of red clover use for medicinal purposes, with records of use among Native American cultures as well as ancient herbal medicines in China.⁵¹ The plant has been used as a treatment for a variety of skin disorders including eczema and psoriasis, in addition to more severe health issues like asthma and whooping cough.^{51–53} Interest in the possible effects of red clover on women's reproductive health can be traced back to the 1930s in Australia, where infertility first was noted among sheep populations that were grazing on the plant.⁵⁴ Subsequently, red clover began to be used as a treatment for hormonal issues in postmenopausal women. As a result, red clover has become widely used as a dietary supplement for postmenopausal women wishing to reduce hot flashes, in addition to other symptoms. There are also indications that

Figure 1.5 Growth of red clover in the United States and Canada, from the USDA, reprinted with permission.³³

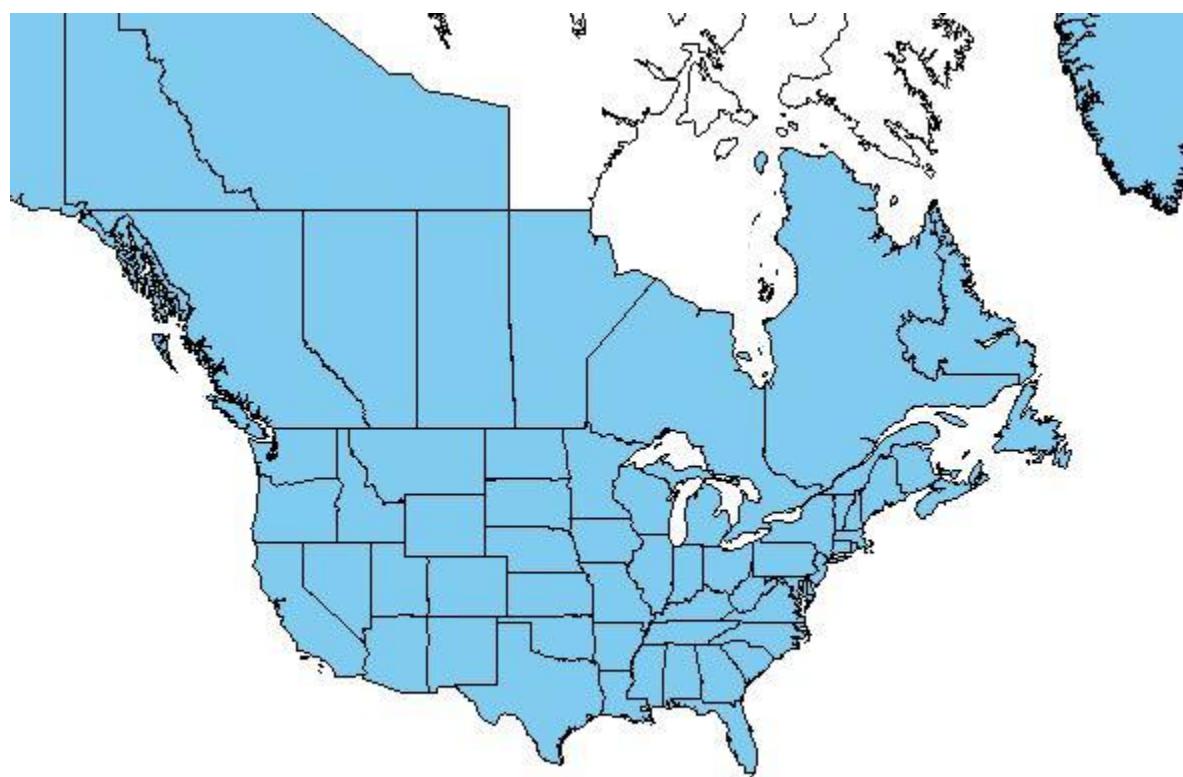


Figure 1.6 Red clover plant, from the USDA, used with permission.



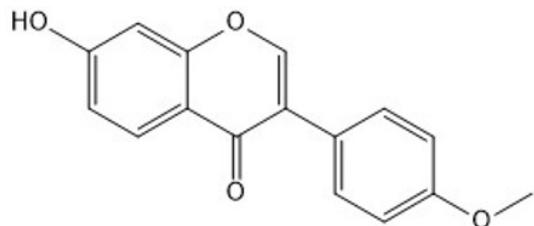
the plant improves mood disorders, including anxiety and depression, as well as vaginal and sexual health.^{21,55-57}

The compounds in red clover that are most likely responsible for a bulk of the pharmacological activities of the plant are the isoflavones. More than 30 isoflavone and isoflavone conjugates have been identified in red clover. Among these, formononetin, biochanin A, daidzen, and genistein have been the most studied due to their estrogenic or proestrogenic activities.⁵⁸⁻⁶⁰ Genistein in particular has been reported to have anticancer activity as indicated by reduction of cell proliferation in vitro and in animal models.⁶¹⁻⁶⁴ Although formononetin and biochanin A show no significant binding to the estrogen receptors, they can be enzymatically O-demethylated to form the estrogenic isoflavones daidzein and genistein, respectively.⁶⁵

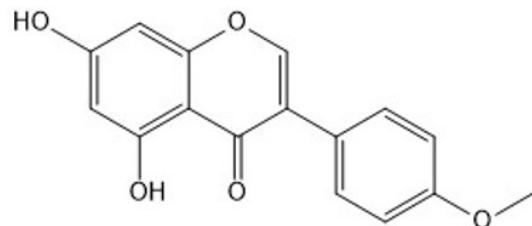
The activity of many isoflavones has been investigated and most, including those from red clover shown in Figure 1.7, have been suggested to function as selective estrogen receptor modulators (SERMs). As a result, isoflavone extracts from a variety of sources, including red clover, have become popular dietary supplements used by postmenopausal women with the intention of modulating the effects of reduced hormone production.

A large number of clinical studies on the effects of red clover, and isoflavone extracts isolated from red clover, have been conducted. Most of these focused on postmenopausal women with the clinical effect being reduction of hot flashes.^{9,56,60,66} The results of many of these studies have been a lack of efficacy in reduction of hot flashes, which is consistent with the study that provided the samples for the experiments described in this work.⁹ In most of these studies, the average number of hot flashes observed were reduced over the course of the study, but a large placebo effect was also observed, leading researchers to conclude that the effects of red clover were indistinguishable from placebo.^{9,67-70} There are however a few studies that did show a statistically significant reduction in hot flashes vs a

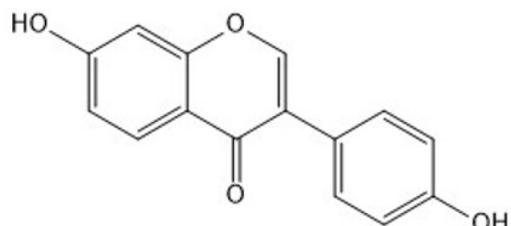
Figure 1.7 Chemical structure of characteristic isoflavone compounds from red clover.



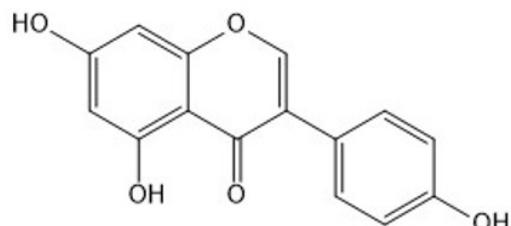
Formononetin
Chemical Formula: C₁₆H₁₂O₄
Exact Mass: 268.07



Biochanin A
Chemical Formula: C₁₆H₁₂O₅
Exact Mass: 284.07



Daidzein
Chemical Formula: C₁₅H₁₀O₄
Exact Mass: 254.06



Genistein
Chemical Formula: C₁₅H₁₀O₅
Exact Mass: 270.05

placebo control group.^{66,71} All studies concluded that the use of red clover or isoflavone extracts was safe. Interestingly, meta-analyses of clinical trials have also reached different conclusions regarding the effectiveness of red clover and isoflavones for controlling vasomotor symptoms.^{55,72} Understandably, debate remains regarding the effectiveness of isoflavones as a treatment option for postmenopausal women. As the focus of previous studies has predominantly been on hot flashes, few studies have identified other postmenopausal health issues such as mood disorders, anxiety, loss of sleep, and other problems.^{21,72} The role the red clover isoflavones might play in affecting these issues needs to be more fully investigated.

**CHAPTER 2 COMPARISON OF SERUM METABOLOMICS FROM A CLINICAL TRIAL OF
POSTMENOPAUSAL WOMEN TAKING HORMONE THERAPY, BLACK COHOSH AND RED
CLOVER**

2.1 Introduction

The Women's Health Initiative has exposed a great number of risks facing postmenopausal women. Vasomotor symptoms, metabolic irregularities, heart disease, bone fractures, and certain cancers all increase postmenopause.^{12,22,23} Even more problematic, the risks associated with HT appear to be much greater than had been realized previously. Although HT reduced vasomotor symptoms in women participating in the Women's Health Initiative study, metabolic syndrome, heart disease and cancer all carried greater risks or were not reduced through the use of estrogen or progestin.^{22,24,26,28,73,74} As a result, there is more interest than ever in finding safer therapies for the management of menopausal symptoms and risks. Hot flashes in particular are one of the more disruptive symptoms experienced by postmenopausal women. The symptom has a negative effect on the quality of life for women, due not just to the discomfort of the symptom itself but loss of sleep and associated fatigue and mood disorders.

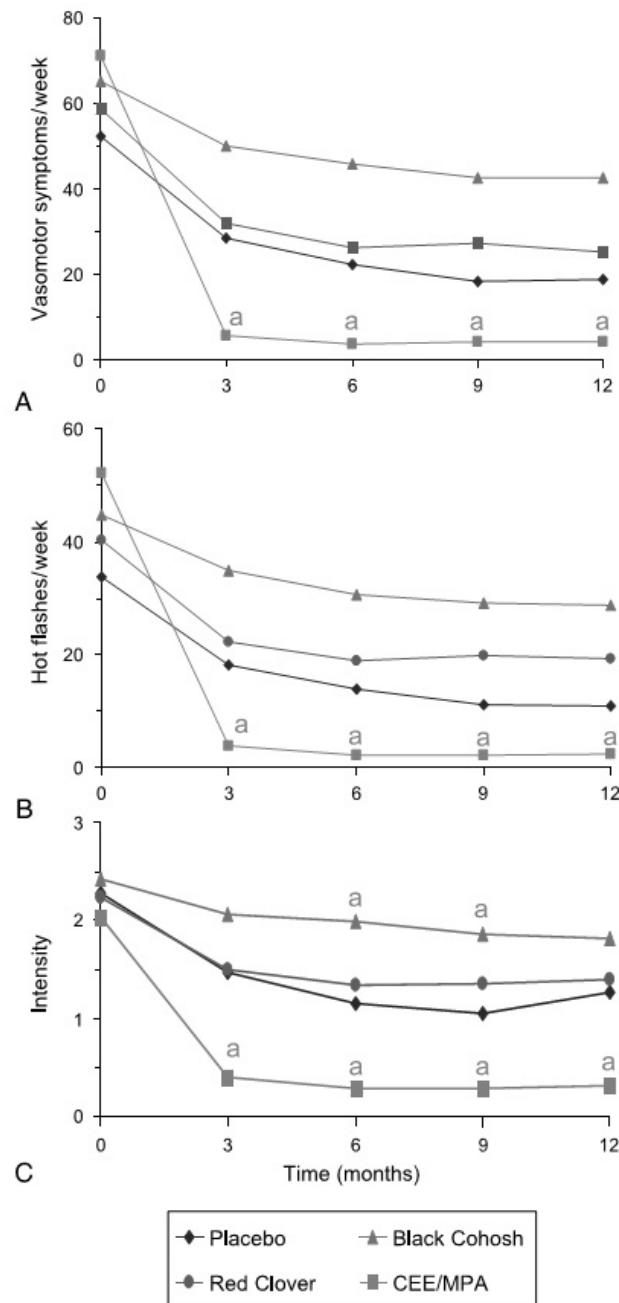
Among the most popular alternative therapies being used by postmenopausal women are black cohosh (*Cimicifuga racemosa*) and red clover (*Trifolium pratense*). The effectiveness of black cohosh and red clover at reducing postmenopausal symptoms is still under investigation. While there are positive reports showing a reduction in the number or intensity of hot flashes, there are a significant number of placebo-controlled studies that reported no statistical difference between the alternative therapies and placebo.^{9,10,47,55,56,70,71,75,76} A large placebo effect is often observed in these trials resulting in difficulty in determining the effectiveness of the therapy. For example, a phase II clinical trial on botanical supplements for reduction of hot flashes observed a 70% placebo effect and no efficacy in botanicals that could be differentiated from placebo.⁹ The results of this trial are summarized in Figure 2.1, reprinted from Menopause and used here with permission.⁹ In another, similarly designed (double-blind, placebo-

controlled) trial, a reduction of hot flashes in a group treated with red clover was observed.⁷¹ As a result, a more thorough understanding in the changes occurring in the body would be useful in understanding the biochemical pathways of interest.

The lack of suitable biomarkers for many of the health issues facing menopausal women is a limiting factor in assessing the effectiveness of any treatment option. Hot flashes in particular could benefit greatly from a method that improves the quantitative comparison of various treatments. Currently, the monitoring of hot flashes in a clinical setting is conducted in one of two ways: self-reporting^{5,7,9} or mechanical monitoring.^{14,16,77} Self-reporting relies on the subject to assess and effectively document the frequency and severity of these hot flashes. This may not always be practical, as hot flashes may occur at any time of day, and the discomfort caused by this may prevent effective self-assessment. The severity factor of these hot flashes is also subjective to the interpretation of the subject. Mechanical monitoring has limited effectiveness due to the potential for discomfort, in addition to the fact that the conductivity measured may not be directly relatable to the severity of the hot flash.¹⁴

In order to further investigate the changes in postmenopausal women subsequent to administration of hormone therapy, red clover and black cohosh serum samples from a clinical trial that was completed in 2007 were reexamined.⁹ This study concluded that no statistically significant effect (differing from placebo) was observed in hot flashes among patients receiving the botanical supplements. However the assessment was limited to self-reporting of hot flashes and analysis of specific safety biomarkers. Therefore, creation of a metabolomic profile was carried out to provide a more complete understanding of the changes occurring. Recent advances in analytical techniques allow for more rapid and sensitive analysis of these samples than was possible at the completion of the trial. Using these

Figure 2.1 "Change in vasomotor symptoms, by study group: average number of vasomotor symptoms, hot flashes plus night sweats (A); average number of hot flashes (B); and average intensity of hot flashes (C). CEE, conjugated equine estrogens; MPA, medroxyprogesterone acetate. ^aStatistically significant difference"⁹



modern techniques to compare and contrast across treatment groups might provide useful information for the design of future clinical studies of menopausal hot flashes and related symptoms.

2.2 Materials and methods

2.2.1 Chemicals and reagents

All organic solvents were HPLC grade or better and were purchased from Thermo Fisher (Hanover Park, IL). High purity water was prepared using a Millipore (Bedford, MA) Milli-Q system. Serum samples used were from the month six time point of a clinical trial on the effectiveness of four treatments to reduce hot flashes.⁹ Those treatment groups were hormone therapy (HT), red clover, black cohosh and placebo. All subjects receiving treatment were treated with capsules from the same standardized batch and recent (2015-2016) analysis of the capsules used for the red clover treatment group showed no changes in the levels of active compounds.

2.2.2 Sample Preparation

Stored at -80°C, serum samples were allowed to thaw on ice prior to 100 µL aliquots being removed for analysis. Samples used were from each of the four treatment groups at the six month time point. The six month time point was used because the level of hot flashes between the groups at this time point was large enough that the HT group was different from the other treatment groups. In order to precipitate proteins and disassociate any remaining protein-ligand complexes, 400 µL of ice cold acetone (4x volume) was added. Samples were then vortexed three times for 1 min each, pausing 1 min between repetitions. Samples were then centrifuged at 10,000 x g for 15 min in order to pellet the proteins. The top organic layer was then removed and transferred to a new sample tube. This organic layer was then evaporated to dryness using a vacuum centrifuge. Samples were then reconstituted in 100 µL of mobile

phase (see 2.2.3) and centrifuged again at 10,000 \times g for 10 min in order to remove any remaining proteins. Samples were then transferred to autosampler tubes and prepared for injection into the LC-MS system.

2.2.3 LC-MS conditions for screening runs

A Shimadzu (Columbia, MD) Prominence XR Liquid Chromatography system connected to a Shimadzu (Kyoto, Japan) IT-TOF Mass Spectrometer was used for analysis. A 10 μ L aliquot of each sample was injected onto a Phenomenex (Torrance, CA) Kinetex 2.6 μ m C₁₈ column (2.1 x 100 mm, 100 \AA). The flow rate was 350 μ L/min, and the total analysis time was 30 min. Mobile phase A was 5 mM ammonium acetate in water containing 0.1% formic acid, and mobile phase B was methanol. Gradient elution was used as follows: 5% B for 1 min; a 20 min linear gradient to 95% B; 95% B for 4 min; return to 5% B over 0.1 min; and reequilibration for 30 min before the next injection. The mass spectrometer source conditions were as follows: nebulizing gas flow 1.5 L/min; CDL temperature 200° C; heater block temperature 200° C; and detector voltage 1.65 kV. Electrospray ionization (ESI) was used to ionize the analytes in the source. The scanning events scanned from 100 to 1000 *m/z* in positive and negative mode over the entirety of the chromatographic run time. The resolving power was 10,000. The mass accuracy was ensured by calibration of the TOF using a tune solution no further than one week prior to mass measurement. In addition known standards were run prior to serum samples to ensure the mass accuracy was within 20 ppm.

2.2.4 XCMS searching parameters

Positive ion and negative ion LC-MS data files for each sample were converted into .mzxml files. These data files were uploaded to XCMS (Scripps Center for Metabolomics, La Jolla, CA) and organized by polarity and treatment group.⁷⁸ This online resource facilitated the identification and alignment of features by *m/z* value and retention time, and then compared the features and intensities among and

between the groups of data files. Groups of the same polarity were submitted for multigroup searches using the parameters “HPLC/UHD Q-TOF,” as this most closely matched the data profile for the data files submitted. Search results were then downloaded and analyzed for features of interest. Identification of features using standards run on the same LC-MS conditions was used when possible.

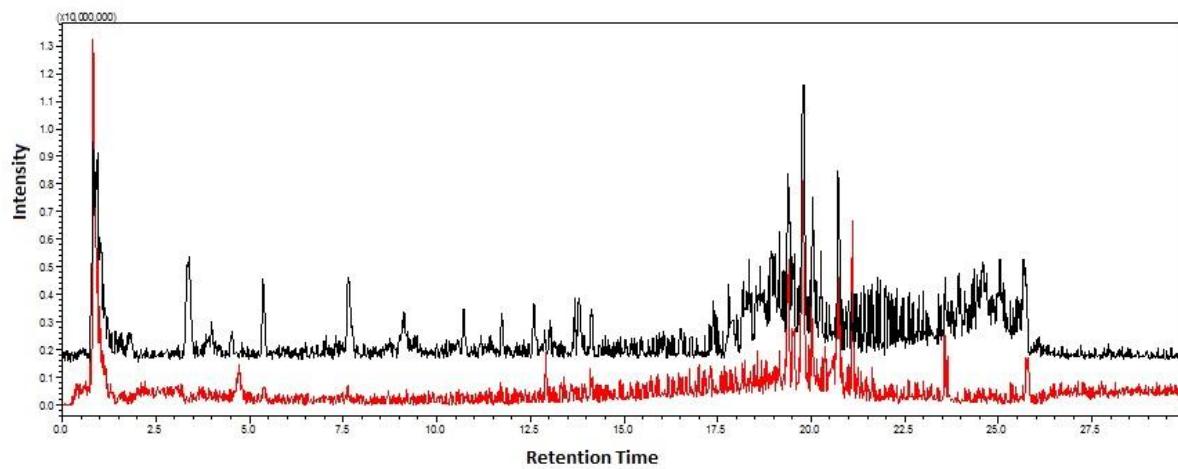
The identified features of interest are compared using one-way ANOVA testing to identify features with significant differences between the groups. The features are then searched by METLIN (Scripps Institute, La Jolla, CA) database searching, using the high resolution mass spectra and mass defects to identify potential chemical formulae and characteristic compounds corresponding to those formulae. Features with p-values less than 0.05 were considered to show a statistically significant difference between groups and were further analyzed for potential identification and potential chemical formulas by accurate mass measurement. METLIN searches were performed with a tolerance of 30 ppm and included adducts, such as sodium adducts.

2.3 Results

Sample results from the four treatment groups (placebo, hormone therapy, red clover, black cohosh) were analyzed for significant features detected using the XCMS searches. An example chromatogram from a sample is shown in Figure 2.2. The placebo group served as a negative control for all comparisons although significant features in each group were relative to the response observed in all other groups. In total 86 serum samples were analyzed by LC-MS. In addition, a subset of 16 samples were reanalyzed using the same method in order to determine if the results were reproducible. These samples provided similar results when processed through XCMS searching. Initial XCMS searches found 1039 positive ion features and 238 negative ion features in total among all the samples analyzed.

Initial results were refined to remove features that did not appear significant or did not meet criteria. For example many features indicated by XCMS eluted within the first minute of the

Figure 2.2 Total ion chromatogram (TIC) from a serum sample from the placebo treatment group. The positive ion TIC is shown offset in black, the negative ion TIC is shown in Red.



chromatographic run. These features are either noise or compounds that were not retained on the chromatographic column. This would make the confirmation of these features very difficult, and therefore, these features were instead eliminated from consideration. Likewise, there were features that eluted well after the chromatographic curve, during the reequilibration period from 24.1 to 30 min. Generally, anything that eluted after 25 min was eliminated from consideration and presumed to be either noise or insignificant features flushing off the column. Duplicates were also present in that features that differed by less than 0.1 Dalton (Da) and less than 0.1 min were considered duplicate features and reduced to one feature. In order to focus on significant features that displayed statistically significant differences between the treatment groups, the features of interest were limited to those with a p-value of less than 0.05. The resulting list of significant features included 83 positive ion features and 16 negative ion features.

The features were searched using Metlin and ChemSpider databases. Collection of high resolution accurate mass data allowed for identification of probable chemical formulas and a potential list of known chemical compounds corresponding to these accurate masses for each feature identified. An example of the data output for METLIN searching for an individual feature is presented in Figure 2.3. A tolerance of 30 ppm was used for the METLIN searches. The mass accuracy for each hit was presented by the database search output, but an evaluation of each hit may include ruling out the top hit returned (the hit with the lowest mass error). This may be the case if the top hit returned is a nonpolar compound but the elution time was very early in the chromatographic run, or if the top hit returned is a synthetic compound unlikely to be observed in human serum, i.e. a chemical dye.

The red clover group displayed the greatest number of significant features, 25 positive ion and 2 negative ion. These hits are summarized in TABLE I. Among these, several features were not unexpected. For example, natural products known to be present in the red clover dietary supplement were among the database hits returned by the searches. The feature of m/z 269.0800 found to be higher in the red clover

Figure 2.3 Example results for METLIN database searching for a significant feature

286.1560059 m/z(285.1401 - 285.1573 daltons): 2 Metabolites [M+H]⁺

MetlinID	Mass	Δppm	Name	Formula	CAS	MS/MS
68408	285.1477	3	Cynometrine	C16H19N3O2	50656-83-2	NO
3309	285.1517	10	N-Tritylaziridine	C21H19N	26643-30-1	NO

(263.1582 - 263.1754 daltons): 7 Metabolites [M+Na]⁺

MetlinID	Mass	Δppm	Name	Formula	CAS	MS/MS
953	263.1674	2	Nortryptiline	C19H21N	72-69-5	View
1062	263.1674	2	Desmethylmaprotiline	C19H21N	5721-37-9	NO
1186	263.1674	2	didemethylmethadone(EMDP)	C19H21N	30223-74-6	NO
2184	263.1674	2	Protriptyline	C19H21N	438-60-8	View
96326	263.1685	6	Melperone	C16H22FNO		View
71497	263.1708	13	Axisothiocyanate 3	C16H25NS	59633-81-7	NO
69663	263.1746	27	Pinacidil	C13H21N5O	85371-64-8	NO

TABLE I SIGNIFICANT HITS FROM XCMS SEARCHES OF RED CLOVER SAMPLES

Polarity	pvalue	updown	m/z	RT	Chemical Formula	Example METLIN compound
POS	0.0003	DOWN	810.6807	21.65	C47H88NO7P	PE(O-20:0/22:4(7Z,10Z,13Z,16Z))
POS	0.0010	DOWN	786.6036	21.90	C50H90O6	TG(14:1(9Z)/14:1(9Z)/19:1(9Z))
POS	0.0011	DOWN	732.5579	24.27	C41H82NO7P	PC(15:0/P-18:0)
POS	0.0080	DOWN	195.0933	5.41	C13H10N2	?
POS	0.0116	DOWN	786.611	21.92	C44H84NO8P	PC(16:0/20:2(11E,14E))
POS	0.0122	DOWN	731.577	25.22	C41H79O8P	PA(16:0/22:1(11Z))
POS	0.0151	UP	521.3531	19.40	C34H48O4	Vitamin D3 metabolite
POS	0.0178	DOWN	780.5537	23.87	C43H74NO9P	PE(16:0/22:6(54Z,7Z,10Z,12E,16Z,19Z)(14OH))
POS	0.0185	UP	543.3758	19.07	C35H52O3	Ginsenoyne A linoleate
POS	0.0185	UP	573.4235	18.24	C35H56O6	22-Angeloylbarringtogenol C
POS	0.0212	DOWN	279.1844	17.38	C15H28O3	4-keto pentadecanoic acid
POS	0.0239	UP	269.085	12.62	C16H12O4	Formononetin*
POS	0.0302	UP	580.4059	18.65	C29H58NO8P	PE(12:0/12:0)
POS	0.0312	UP	499.3519	19.15	C29H48O5	Vitamin D3 metabolite
POS	0.0324	UP	587.4202	19.02	C35H54O7	18-Dehydrouronic acid 3-arabinoside
POS	0.0364	UP	246.1445	4.27	C10H19N3O4	Asparaginyl-Isoleucine
POS	0.0370	DOWN	830.5833	24.77	C48H80NO8P	PC(18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
POS	0.0380	DOWN	782.5665	24.48	C44H80NO8P	PC(16:0/20:4(5Z,8Z,11Z,14Z))
POS	0.0408	DOWN	730.5314	23.55	C40H76NO8P	PC(14:0/18:2(2E,4E))
POS	0.0408	DOWN	494.3211	18.96	C30H39NO5	?
POS	0.0408	UP	271.0462	11.14	C15H10O5	Genistein*
POS	0.0421	UP	455.3365	19.24	C26H46O6	27-Norcholestanehexol
POS	0.0450	DOWN	780.5577	23.87	C44H77O9P	PG(O-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
NEG	0.0465	DOWN	512.2995	18.57	C24H48NO6P	PC(O-16:2(9E,10E)/O:0)
POS	0.0469	UP	558.4018	18.67	C35H53NO3	Vitamin D3 metabolite
NEG	0.0497	DOWN	245.0384	7.98	C6H15O8P	Phosphatidyl glycerol

group was presumed to be formononetin. The identity was confirmed by comparison with a standard using the same LC-MS/MS conditions as the samples. The resulting chromatograms were then compared and used to confirm the identification (Figure 2.4). The same process was used to confirm the identity of genistein (feature m/z 270.0493). The peaks area measured for these features in the groups not taking red clover did show lower, but not absent, responses, and this was not entirely unexpected as a) black cohosh does not contain these isoflavones, and b) diets were not controlled in this trial so that isoflavones may have been introduced through a variety of dietary sources (FIGURE 2.5).

Several unidentified features potentially show importance for further investigation. The feature corresponding to m/z 246.1445, retention time 4.27 min, returned hits indicating that it is an amino acid or derivative of varying possible compositions. This could possibly be another signaling molecule as it is a small, polar amino acid related compound. Another potential feature of interest would be the feature of m/z 195.0948. The early elution time (5.41 min) and low molecular weight indicate that it is highly polar and might correspond a neurotransmitter or signaling hormone. Although it only returned two database hits for unrelated compounds, these possible structures probably do not correspond to the actual molecule as they were a pesticide (methurion) and a dye (9-aminoacridine).

The databases indicated that several vitamin D metabolites were potentially among the highlighted features. These features of m/z 521.2638, 499.3536, and 558.4026 were potentially metabolites related to vitamin D and all showed increased levels in the red clover group. Further investigation of these compounds will be required in order to determine if they are in fact Vitamin D metabolites, other significantly altered compounds or artifacts. Vitamin D metabolites are notoriously labile, and difficult to analyze so confirmation of these hits by additional instrumental analysis is necessary in order to positively characterize them as Vitamin D metabolites.

Figure 2.4 Extracted ion chromatograms of m/z 269.08 from A) a sample from the Red Clover treatment group and B) a standard of formononetin

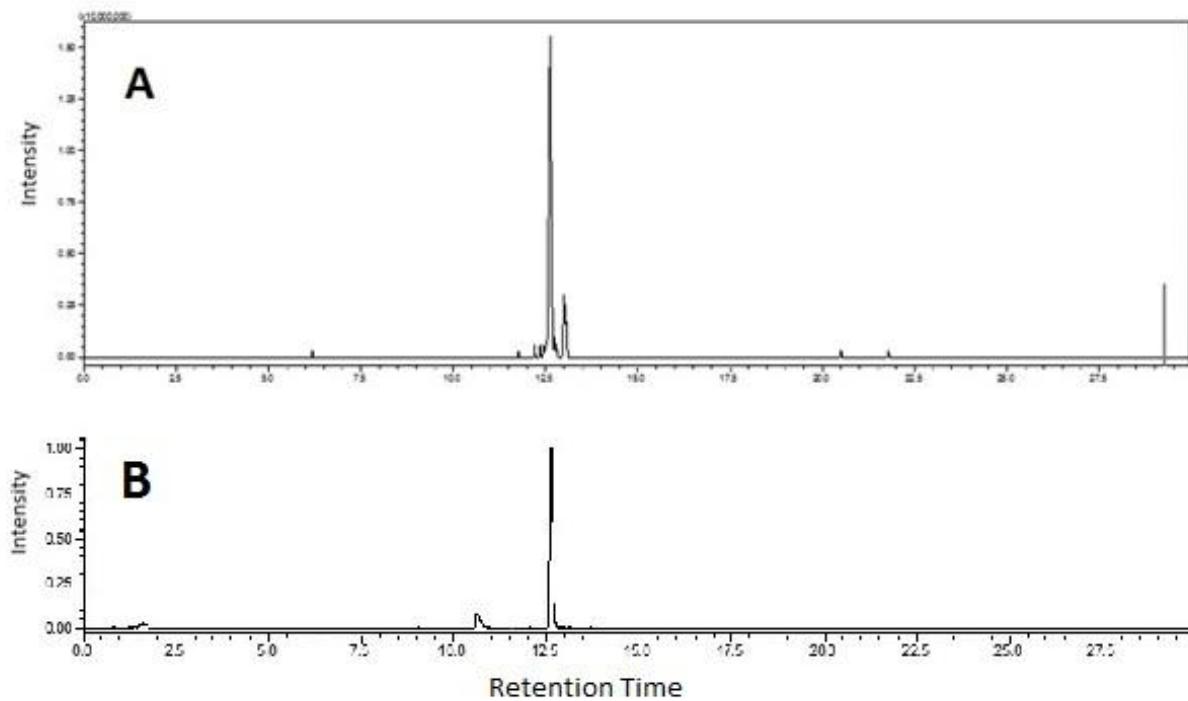
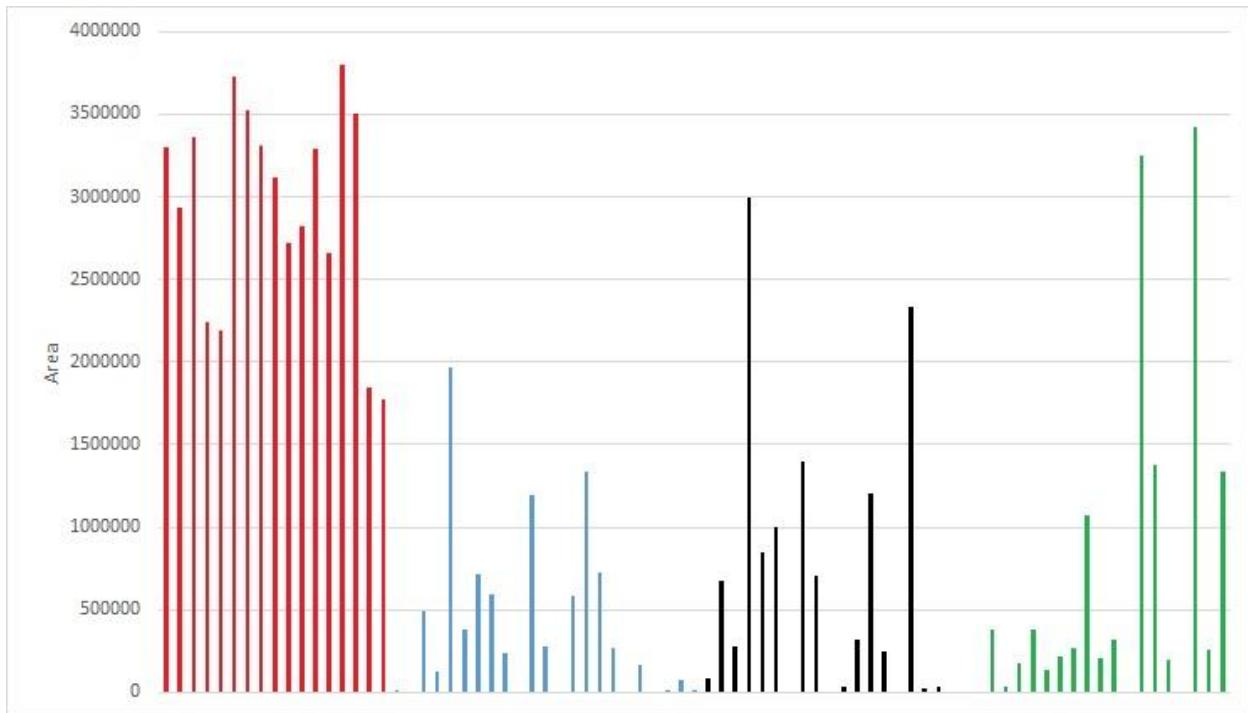


Figure 2.5 Area observed in samples for feature identified as formononetin. Treatment groups are shown as follows: red - red clover; blue - HT; black - black cohosh; green - placebo



The black cohosh group had 15 positive ion and 3 negative ion significant features identified and submitted for database searches. These hits are shown in Table II. Among the database search results, a majority (15 features) of the returned results that potentially correspond to phospholipids. The significance of these hits would require separation and identification of the individual phospholipids and additional analysis by targeted methodology that was outside the scope of this research. Unlike the red clover group, the major constituents of black cohosh were not detected as significant features. This might be the result of poor bioavailabilities of black cohosh compounds such triterpene glycosides, rapid metabolism, or lack of mass spectrometric detection.⁶⁵ It is also possible that the significance of these hits was not strong enough to be included in further analysis. Nevertheless, the presence of triterpenoids and phenolic acids was not detected during this metabolomics analysis.

Among the significant features that were detected by this analysis, of particular interest is the compound corresponding to *m/z* 523.4092. The database search for this feature indicated that it is potentially panaxydol linoleate, a known constituent of ginseng. Because this compound has not been reported to occur in black cohosh, its enhanced presence in the black cohosh group is possibly related to diet. This is also true of the compound corresponding to *m/z* 286.1560, which is possibly cynometrine, another naturally occurring botanical compound not reported to occur in black cohosh. The compound corresponding to *m/z* 393.2251 returned an interesting database hit of isodomedin. Isodomedin is a diterpene that occurs naturally but has not been detected or identified in black cohosh. This diterpene might be a metabolite or degradation product of a triterpene, or the molecule could occur naturally in black cohosh.

The hormone therapy group had several database results that are of interest for further investigation. Results of database searches are shown in Table III. Primary among these is the feature represented by *m/z* 363.1870, which returned a database hit potentially corresponding to thyrotropin releasing hormone. This compound had not been reported previously to have a role in postmenopausal

TABLE II SIGNIFICANT HITS FROM XCMS SEARCHES OF BLACK COHOSH SAMPLES

Polarity	pvalue	updown	m/z	RT	Chemical Formula	Example METLIN compound
POS	0.0099	DOWN	286.1539	8.74	C16H19N3O2	Cynometrine
POS	0.0132	UP	269.2235	20.25	C18H36O	13Z-Octadecen-1-ol
POS	0.0184	UP	780.5649	23.87	C42H70NO10P	PS(16:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))
POS	0.0260	UP	393.2722	14.12	C22H32O6	Isodomedin
POS	0.0283	UP	542.3207	18.78	C28H48NO7P	PC(20:5(5Z,8Z,11Z,14Z,17Z)/0:0)
POS	0.0295	UP	482.3299	20.35	C24H52NO6P	PC(O-8:0/O-8:0)
POS	0.0315	UP	701.5846	23.19	C40H77O7P	PA(O-20:0/17:2(9Z,12Z))
POS	0.0319	UP	522.3566	20.05	C35H54O3	Panaxyadol linoleate
POS	0.0328	UP	675.5518	22.97	C43H78O5	DG(20:1(11Z)/20:2(11Z,14Z)/0:0)
POS	0.0382	DOWN	806.5766	24.35	C41H75O13P	PI(16:0/16:2(9Z,12Z))
POS	0.0398	UP	520.3361	19.40	C26H50NO7P	PC(18:2(2E,4E)/0:0)
POS	0.0406	UP	524.3731	20.72	C25H49O9P	PG(19:1(9Z)/0:0)
POS	0.0432	UP	703.5771	23.94	C45H82O5	DG(20:0/22:3(10Z,13Z,16Z)/0:0)
NEG	0.0433	UP	480.3102	19.18	C27H43NO4	N-oleoyl tyrosine
NEG	0.0458	UP	566.3486	20.15	C30H50NO7P	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)
NEG	0.0483	DOWN	504.316	19.39	C25H43NO7	?
POS	0.0495	UP	496.3372	19.79	C23H46NO8P	PC(7:0/8:0)

TABLE III SIGNIFICANT HITS FROM XCMS SEARCHES OF HT SAMPLES

Polarity	pvalue	updown	m/z	RT	Chemical Formula	Example METLIN compound
POS	0.0005	DOWN	524.3366	20.73	C25H50NO8P	PE(10:0/10:0)
POS	0.0007	UP	363.1904	5.77	C16H22N6O4	Thyrotropin releasing hormone
POS	0.0029	DOWN	310.1768	4.63	C15H23N3O4	Lysyl-Tyrosine
POS	0.0038	DOWN	522.3385	20.05	C29H47NO7	?
NEG	0.0054	DOWN	504.3037	18.40	C25H43NO7	?
NEG	0.0054	DOWN	508.3352	20.72	C25H52NO7P	PC(O-1:0/16:0)
POS	0.0079	DOWN	482.3703	20.35	C24H52NO6P	PC(O-8:0/O-8:0)
POS	0.0088	DOWN	701.5171	23.19	C39H73O8P	PA(18:1(11Z)/18:1(11Z))
POS	0.0088	UP	364.2126	11.76	C22H25N3O2	?
POS	0.0122	DOWN	544.3266	19.38	C28H50NO7P	PC(20:4(5Z,8Z,11Z,14Z)/0:0)
NEG	0.0142	DOWN	566.3327	20.05	C30H50NO7P	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)
POS	0.0167	UP	732.5681	24.27	C40H78NO8P	PC(14:0/18:1(9E))
POS	0.0233	DOWN	286.1891	8.74	C17H23N3O	Pyrilamine
POS	0.0277	UP	756.5262	23.93	C39H76NO9P	PS(P-16:0/17:0)
POS	0.0459	DOWN	246.1763	4.27	C11H23N3O3	Valyl-Lysine
POS	0.0460	DOWN	809.629	24.77	C47H85O8P	PA(22:0/22:4(7Z,10Z,13Z,16Z))
POS	0.0465	DOWN	394.2902	14.13	C23H39NO4	PGH2-EA
NEG	0.0471	DOWN	481.296	19.45	C27H46O5S	26-hydroxycholesterol 3-sulfate

symptoms and therefore might represent a new lead for investigation. However comparison to a standard of thyrotropin releasing hormone indicates that this is not the compound represented by this feature. Interestingly, this feature was also slightly upregulated in the red clover group. Structural determination of this hit must first be determined before further investigation.

There were several hits among the hormone therapy group potentially corresponding to small molecule signaling molecules. The feature corresponding to *m/z* 286.1891 returned a database search result of pyrilamine, a histamine receptor agonist. This compound had a lower response across the hormone therapy group compared to all other groups. The hits corresponding to *m/z* 310.1768 and 246.1763 returned hits of varying amino acid composition, and the retention times indicate that they are small, polar compounds.

2.4 Discussion

The use of serum samples that had been frozen at -80°C for several years presented several challenges. The deep freezing process preserved many compounds present in the samples but degradation of others most likely resulted in the inability to detect some compounds that varied across each treatment group. Degradation might also have reduced the overall fold difference observed for each feature. Therefore, the features and compounds identified through these experiments most likely represent only a subset of those that might be observed using fresh samples. Nevertheless, the features identified here are still significant. The p-value was used as the most reliable measure of the significance of the differences between the treatment groups for each feature.

The serum metabolome profile generated by these experiments produced several significant hits of interest. Other metabolomics studies on postmenopausal women focused on compounds or effects not monitored directly here. For example several studies identified specific amino acids in the serum metabolome as being up or downregulated in postmenopausal women.^{79,80} Amino acid profiles are

generated through different chromatographic techniques than those employed in these experiments. Another study identified many different lipids and lipid metabolism pathways that were observed to be modulated by menopause.⁸¹ The differentiation and characterization of lipid profiles requires targeted MS-MS settings that were not used here, although the hits generated in these experiments did potentially include many lipids.

The presence of several compounds that were known to be present in the treatment group provided evidence that the experiments conducted did result in the identification of relevant compounds. Formononetin and genistein are both major constituents of red clover, and both of these compounds were successfully identified in the serum samples from that group. The metabolites of these compounds are either represented by unidentified features or they degraded and were not detected during analysis. The non-detection of biochanin A is surprising as this is the major metabolite of formononetin, while genistein is the major metabolite of daidzein. This internal inconsistency is not fully understood, however it may be that the undetected isoflavones were overwhelmed by other signals and more direct analysis of these compounds would result in their detection. The red clover group provided the greatest number of hits, and several compounds of interest were identified for further study. Targeted analysis of many of these compounds should produce more detailed tandem mass spectra for additional characterization and then identification.

Many small, polar compounds gave hits in database searches and were in agreement with the low retention times corresponding to these compounds. Several of these compounds returned database hits of many different combinations of amino acids and substituted amino acid derivatives. As a result, the characterization of these compounds might be more suitable for normal phase chromatographic analysis to provide better separation and more detailed tandem mass spectra. Proper identification would require the acquisition of chemical standards for comparison while contributing to the metabolome database.

Overall, the black cohosh group displayed a large proportion of significant hits corresponding to phospholipids while the red clover group returned more results for botanical compounds and smaller molecules. Whether this is the result of actual differences between the plant metabolomes or changes occurring within the study (diet, degradation, etc.) remains to be seen. The potential lipid hits are intriguing as all groups identified several lipid hits. These in turn could provide insight into the effects of the botanical supplements in comparison to the hormone therapy group, and in turn the efficacy of these treatments at reducing hot flashes. The hormone therapy group served as a positive control but also returned several hits that warrant further investigation. Among all the hits returned by the database searches, thyrotropin releasing hormone was the most promising as a potential biomarker for hot flashes, however this compound was ruled out as possible identification of this feature by analysis of a standard. Positive identification of this hit is required in order to investigate the pathway affected, which would also provide other potential biomarkers and targets of interest for therapeutic treatment. Identification of more hits in the HT group represent more leads for investigation and potential biomarkers. More targeted studies on these samples would generate the most productive information for biomarkers of postmenopausal symptoms. These experiments provided a view of the metabolome that was intentionally broad in order to be able to further refine future investigations and contribute to the overall understanding of the metabolome of postmenopausal women undergoing treatment.

Chapter 3 Quantitative Serum Proteomics from a Clinical Trial of Postmenopausal Women Taking Hormone Therapy, Black Cohosh and Red Clover

3.1 Introduction

Development of effective therapies for postmenopausal women has been hindered by a lack of known biomarkers and targets of action. The most effective intervention, hormone therapy, was shown by the Women's Health Initiative to carry many serious risks with use including an increased risk of breast cancer and cardiovascular ailments.^{23,26,82} As a result, many women are seeking alternatives to hormone therapy such as botanical dietary supplements. Despite the increasing demand for safer, effective alternatives to hormone therapy, biochemical pathways and targets for new therapies to manage postmenopausal symptoms, in particular hot flashes, remain largely unknown.⁸³ Research into the effectiveness of therapies is limited due to the lack of suitable biomarkers for assessment. Current clinical trials rely primarily on self-reporting, a technique that requires the patient to properly assess and categorize each event.^{5,18} There are many limitations with the accuracy of self-reporting of menopausal symptoms such as hot flashes. A suitable biomarker or set of biomarkers would therefore be useful for determining the efficacy of experimental therapies and to guide research into potential new drugs for the management of menopausal symptoms.

Black cohosh and red clover are two botanical supplements that have been used by postmenopausal women for hot flash symptoms.⁶⁵ The effectiveness of these supplements is still not clear as clinical trials have shown varying results from an effective reduction of symptoms to no statistical difference from placebo.^{9,17,46,56,75,76} As a result, the usefulness of these supplements is still being investigated. A major cause of the lack of clear results is a large placebo effect observed in many of these trials, including the phase II clinical trial that provided the samples for this work.⁹ In that trial, a 70% placebo effect was observed, and had a placebo not been correctly employed red clover would have been shown to be 70% as effective as HT, as shown in Figure 2.1 from the original paper and reprinted here

with permission.⁹ Since the measurement of interest is often self-reported hot flashes, strong reduction in hot flashes due to the placebo effect often causes a lack of clarity regarding investigational therapies. A more definitive quantitative outcome such as a biomarker measurement could help elucidate the true differences between treatment groups and placebo.

The secretion of proteins and peptides into the bloodstream from diseased or damaged cells form the secretome, and along with transporter proteins and other signaling proteins/peptides, form the serum proteome. Analysis of the secretome provides an understanding of the protein levels within the cell in their present state.⁸⁴ In the case of menopause, the secretome of women experiencing hot flashes compared with the secretome of women not suffering hot flashes might provide insight into the mechanisms and biochemical changes causing this symptom.

Previously, the secretome has been surveyed for the discovery of biomarkers for diseases ranging from cancer⁸⁵ to sleep apnea in children.⁸⁶ Analysis of the serum proteome of postmenopausal women could lead to the discovery of biomarkers for vasomotor symptoms and will provide insight into the cellular changes occurring during menopause. Previous proteomics studies of menopausal women have produced only limited proteomes (378 and 611 proteins),^{87,88} and investigations of the available biomarkers have not confirmed their applicability to long term study of symptoms.^{15,16,89-91} The clinical specimens to be evaluated in this study were de-identified and banked following a randomized, placebo-controlled and double-blind 12-month intervention in of 84 postmenopausal women.⁹

3.2 Materials and methods

3.2.1 Chemicals and reagents

All organic solvents were HPLC grade or better and were purchased from Thermo Fisher (Hanover Park, IL). Protein depletion spin columns and amine-reactive Tandem Mass Tag (TMT) reagents were purchased from the Pierce division of Thermo Fisher. High purity water was prepared using a Millipore (Bedford, MA) Milli-Q system. Human serum for evaluation was provided by the UIC/NIH Center for Botanical Dietary Supplements Research. Stored at -80° until use in this dissertation study, the serum samples represented de-identified material remaining after completion of a 12-month clinical trial of red clover, black cohosh, placebo, and Prempro for the management of menopausal hot flashes in women.⁹

3.2.2 Sample Preparation

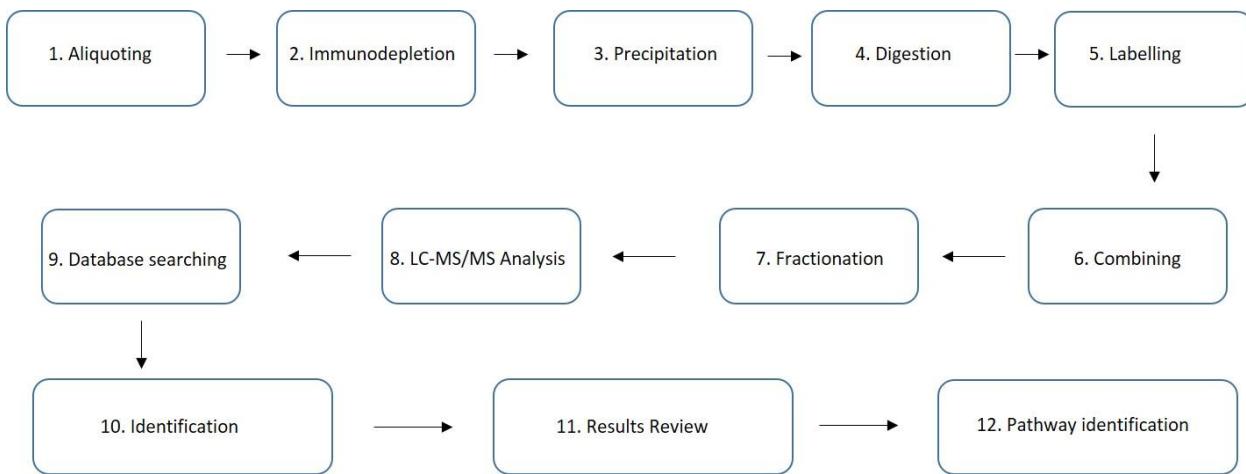
3.2.2.1 Solution Preparation

Solutions were prepared freshly on the day of the experiments and dissolved in high purity water. Ammonium bicarbonate (AMBIC) (100 mM) was prepared by dissolving 79 mg in 10 mL of water. Urea (10 M) was prepared by dissolving 600 mg in 1 mL water. Calcium chloride (500 mM) solution was prepared by dissolving 73.5 mg in 1 mL water. Tris(2-carboxyethyl)phosphine (TCEP) (100 mM) was prepared by dissolving 28.7 mg in 1 mL water, and 500 mM Iodoacetamide was prepared by dissolving 23 mg in 250 μ L water.

3.2.2.2 Immunodepletion and Digestion

A schematic of the sample preparation steps is shown in Figure 3.1. Serum samples from the clinical trial of menopausal hot flashes were allowed to thaw on ice prior to analysis. Serum (aliquots of 2 μ L each) from five different women of the same treatment group were combined for each analysis. Based on an estimate of 60 to 80 μ g of protein per 1 μ L of serum, the total of 10 μ L of serum per

Figure 3.1 Scheme for workflow of protein sample generation, preparation and analysis.



analysis should generate between 600 and 800 µg of protein. Three separate analyses of 10 µL pooled serum were carried out, representing 15 samples from each of the four treatment groups.

Each 10 µL combined serum sample was applied to a Thermo Pierce Top 12 Abundant Protein Spin Column and incubated at room temperature for 60 min, with vortexing every 5 min (Step 2 in Figure 3.1). After incubation, the columns were centrifuged at 1000 rcf for 2 min. Approximately 500 µL of sample was collected. A 100 µL aliquot was transferred to a new sample tube, and cold acetone (400 µL) was added to the sample in order to precipitate the proteins (Step 3 in Figure 3.1). Samples were vortexed 3 times for 1 min each, with a rest of 1 min between repetitions, and then centrifuged for 15 min at 10,000 $\times g$. The organic solvent layer was removed by pipette and discarded. The protein precipitate was resuspended in 80 µL of water/AMBIC/urea (1:1:8, v/v/v).

Samples were reduced by adding 3 µL of TCEP (100 mM) and incubation at room temperature for 30 min. A 1.2 µL aliquot of iodoacetamide (500 mM) was added to each sample followed by incubation in the dark at room temperature for 30 min. LysC (10 µL of 0.1 µg/ µL) was added to each sample, which was then incubated for 6 hr at 37° C. Aliquots (200 µL) of AMBIC (100 mM) was added to each sample followed by 3.5 µL of calcium chloride solution (500 mM). Trypsin (10 µL, 0.1 µg/ µL) was added to each sample followed by incubation overnight at 37° C (Step 4 in Figure 3.1).

After tryptic digestion, 41 µL of a unique Thermo Pierce TMT labelling reagent was added to each sample followed by incubation at room temperature for 1 hr (Step 5 in Figure 3.1). TMT labelling reagent adds a unique, isobaric tag to up to six different peptide samples for quantitative analysis. Each treatment group was labelled using a unique TMT labelling reagent, giving it a unique mass tag. Aliquots (150 µL) of each labelled sample were then combined to make a mixed sample (Step 6 in Figure 3.1). This sample mixture was then fractionated overnight using an Agilent (Agilent Technologies, Santa Clara, CA) pH 4-10 High Resolution OffGel strip and an Agilent OffGel Fractionator (Step 7 in Figure 3.1). Initially, 24 fractions

were collected, and every three fractions were combined resulting in 8 final sample fractions for subsequent analysis.

3.2.3 LC-MS/MS conditions

A Dionex (Columbia, MA) 3000 Liquid Chromatography system connected to a Thermo LTQ-Velos Pro Orbitrap mass spectrometer was used for analysis (Step 8 in Figure 3.1). For each sample, a 10 μ L injection was applied to an Agilent Zorbax 300SB-C₁₈ column (150 mm x 75 μ m, 3.5 μ m). A flow rate of 250 μ L/min was used with a linear gradient from mobile phase A (water containing 5 mM ammonium acetate and 0.1% formic acid) to mobile phase B (methanol) as follows: 5% B hold for 5 min; 5% to 65% B over 65 mins; 65% to 90% B over 5 min; hold at 90% B for 10 min; and reequilibration at 5% B for 5 min. The MS settings scanned from 400-1800 *m/z* at a resolution of 30,000 and a collision energy of 35 eV. Data-dependent MS/MS analysis was used. The ions are filtered to select multiply charged species and the top ten most intense ions from the scan are sent for MS/MS analysis. The MS/MS settings scanned from 100-2000 at a resolution of 7500.

3.2.4 Data Processing

LC-MS/MS files were converted to .mgf files and submitted to a Mascot search engine (Step 9 in Figure 3.1). Settings for searches allowed identification with in 1.2 Da for the peptide mass and 0.6 Da for the MS/MS ions. A decoy database of reversed sequences was used to help detect false identifications. The database used was the NCBI_Human (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MA) proteome database. Only hits with a false discovery rate below 5% and a p-value < 0.05 were accepted for analysis. In addition protein hits were filtered to only include those that had more than two peptides identified from the protein sequence by the Mascot search engine. Quantitation results were filtered to remove hits with a %RSD greater than 20. Finally only proteins with

a Mascot search engine algorithm score of more than 100 were reviewed (Steps 10 and 11 in Figure 3.1). Biological pathways for the protein hits of interest were identified by submitting identified proteins to the NCBI Biosystems search engine (<http://www.ncbi.nlm.nih.gov/biosystems>) (Step 12 in Figure 3.1).

3.3 Results

The serum samples analyzed were from the four treatment groups of the phase II clinical trial: placebo, HT, red clover and black cohosh. The six-month time point was used as this time point displayed statistical difference between the hormone therapy group (positive control) and the other treatment groups. Black cohosh also had a statistically higher number of hot flashes at the six-month time point. This time point provided the greatest differentiation between the treatment groups.

3.3.1 Method development

The initial experimental design attempted to utilize the OFFGEL isoelectric focusing instrument to fractionate the extracted protein samples without prior immunodepletion (Step 2 in Figure 3.1) of the most abundant serum proteins. Fractionation of digested proteins could have potentially separated the abundant peptides such that the individual fractions containing the abundant peptides could be isolated while the overall number of protein hits would remain high. Previous studies have shown OFFGEL fractionation to be efficient and compatible with labelling reagents for quantitation.^{92,93}

However, the resulting experiments showed that the abundant peptides did not focus into individual fraction wells, and as a result, the resulting database searches returned primarily serum albumin and immunoglobulins. The lower abundance peptide signals of interest were lost in the overwhelming signal from these abundant serum proteins. The database searches (Step 9 in Figure 3.1) resulted in the identification of only 103 proteins (not including abundant proteins such as serum albumin and immunoglobulins). As a result, immunodepletion (Step 2 in Figure 3.1) was determined to be

necessary in order to visualize a greater number of serum proteins. An LC-MS chromatogram illustrating the effect of the effect of immunodepletion is shown in Figure 3.2.

High resolution OFFGEL kits (24 wells) were used for this experiment. The effectiveness of the isoelectric focusing separation was evaluated to determine if it was necessary to analyze each of the 24 fractions from each experiment using LC-MS/MS or if wells could be combined without compromising protein identification. In order to assess this possibility, a serum sample was prepared for analysis as described in the Methods section. The OFFGEL fractions 8-14 were separated into aliquots and analyzed individually or in combined samples of both two and three wells. The results indicated that combining the fractions from three wells resulted in a less than 10% reduction in the number of protein hits (266 hits for the combined sample vs. 284 hits for the same individual wells). It was determined that three wells would be combined for these experiments in order to reduce overall analysis time and instrument cost.

3.3.2 Sample Results

An LC-MS chromatogram from a protein sample prepared according to Figure 3.1 is shown in Figure 3.3. The database search results of the LC-MS/MS analyses of the combined OFFGEL fractions, representing all four treatment groups, returned 289 protein hits excluding decoy results. In order to compare results between groups the protein must be detected in the placebo group, used as the baseline control for ratios, and the treatment group. Among these, 44 proteins displayed differing levels among the serum samples among any of the four UIC/NIH Center for Botanical Dietary Supplements hot flashes clinical trial treatment groups. Several of these results were subsequently excluded as their false discovery rates were well above the normal limit of 0.05. After eliminating these hits with high false discovery rates, 31 protein hits remained that displayed varying levels among the different treatment groups. These results are displayed in TABLE IV.

Figure 3.2 LC-MS base peak chromatogram of a serum protein sample without immunodepletion (above, black) and with immunodepletion (below, red).

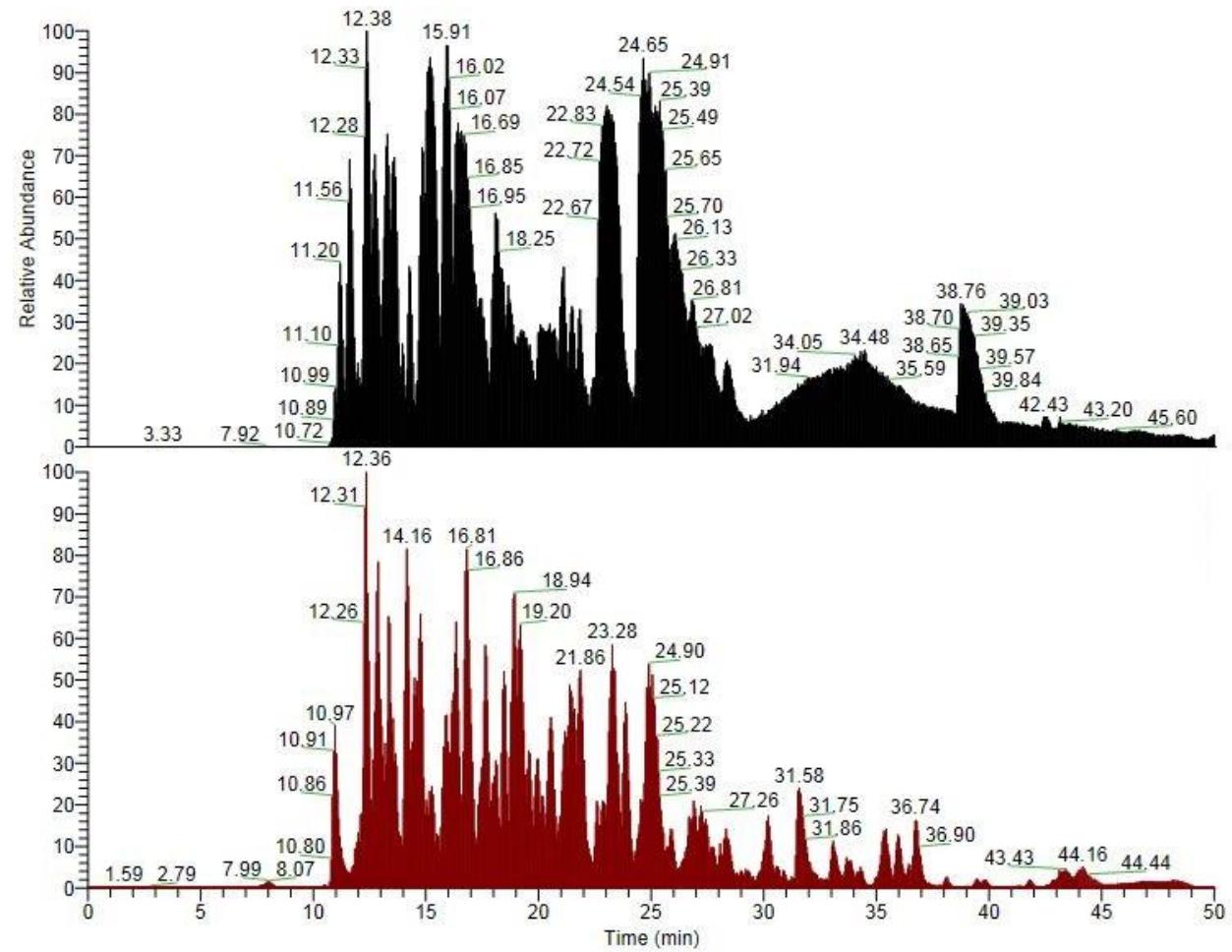


Figure 3.3 LC-MS chromatogram from a combined serum sample containing all four treatment groups

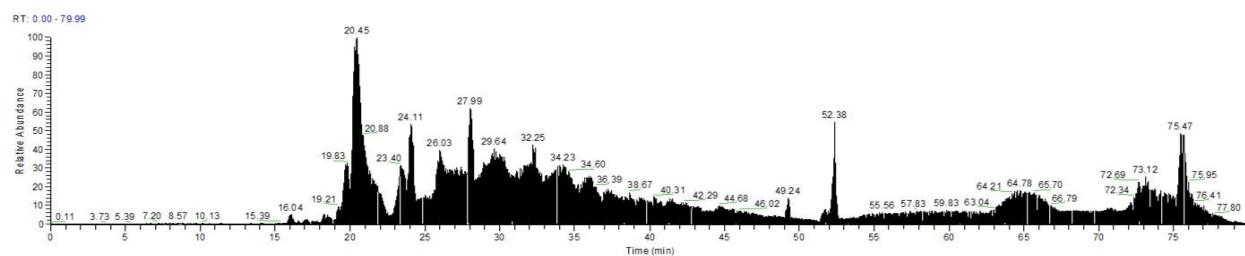


TABLE IV PROTEIN HITS AND OBSERVED RATIOS TO PLACEBO GROUP FOR SERUM PROTEIN SAMPLES

Pathway	Protein	HT Ratio	BC Ratio	RC Ratio
Inflammation	Vitronectin	2.70	1.36	1.45
	Kininogen	2.23	1.33	1.65
	Plasminogen	2.18	1.53	
	Vitamin K-dependent protein S	1.93		1.62
	Ceruloplasmin	1.80		
	Platelet basic protein	1.67		
	Thrombospondin-1	1.58		
	Coagulation factor X	1.43		
	Fibronectin	1.19		
	Matrix metalloprotease 2	0.40		
Metabolism	Hyaluronan-binding protein 2			1.40
	Histidine-rich glycoprotein			1.31
	Insulin-like growth factor binding protein 1	2.05	1.37	1.74
	Insulin-like growth factor binding protein 6	1.76	0.74	
	Vitamin D binding protein	1.67	1.57	1.78
	Apolipoprotein A-IV	1.51		0.76
	Apolipoprotein A-I	1.24	1.27	1.47
	Apolipoprotein E	0.82	0.79	
	Transthyretin	0.41		
Immune Response	Afamin		1.47	
	Pregnancy zone protein			1.48
Osteogenesis	Leucine-rich alpha-2-glycoprotein	1.71		1.47
	Complement Factor B	1.12		
Vessel morphogenesis	Fetuin B	1.43	1.10	
	Macrophage stimulating protein 1	1.39		
Cell Growth	Inhibin, beta E	1.30		
Vasoconstriction	Angiotensinogen	1.57		1.39

3.3.2.1 Hormone Therapy (Prempro)

In the clinical trial, the HT group receiving the standard of care for the management of menopausal symptoms, Prempro, showed a 95% reduction in hot flashes.⁹ As expected, the HT group also displayed the greatest variation from the placebo control group in terms of the number of proteins found to have increased or decreased response. Protein results are summarized in Table V. In total, 25 proteins were identified as either upregulated or downregulated relative to the levels of these same proteins in the control group (TABLE IV). This level of response varied from 2.72 to 0.40 fold variation. Among the strongest responses observed were vitronectin, insulin-like growth factor binding protein 1, kininogen, and matrix metalloprotease 2. The extracted ion chromatogram of a peptide hit from vitronectin is shown in Figure 3.4 and the MS-MS spectra of the peptide hit is shown in Figure 3.5.

Many of these proteins have related functional pathways and are grouped by identified pathways in TABLE IV. As indicated, proteins relating to inflammation, metabolism, immune response, and osteogenesis were all identified showing up and downregulation in the hormone therapy group. The inflammation and metabolism pathways showed the most protein hits.

3.3.2.2 Black cohosh

The black cohosh group showed some variations from the placebo group, but these effects were less pronounced than those observed in the hormone therapy group. These results are detailed in TABLE VI. Overall, the magnitude of variation of the proteins found to be significantly different in both the black cohosh and hormone therapy groups was less in the black cohosh group. In addition, the direction of change in regulation was not consistent across both groups. Of note is that while the insulin-like growth factor binding protein 1 was upregulated as in the hormone therapy group, the insulin-like

TABLE V: SUMMARY OF MEASUREMENTS FOR HT PROTEIN HITS

Pathway	Protein	HT Ratio	x1	x2	x3	stdev	rsd
Inflammation	Vitronectin	2.70	2.97	2.74	2.4	0.29	0.11
	Kininogen	2.23	2.44	1.85	2.39	0.33	0.15
	Plasminogen	2.18	2.47	2.06	2.01	0.25	0.12
	Vitamin K-dependent protein S	1.93	1.71	1.76	2.31	0.33	0.17
	Ceruloplasmin	1.74	1.67	1.57	1.98	0.21	0.12
	Platelet basic protein	1.67	1.3	1.87	1.85	0.32	0.19
	Thrombospondin-1	1.61	1.41	1.69	1.72	0.17	0.11
	Coagulation factor X	1.43	1.65	1.17	1.47	0.24	0.17
	Matrix metalloprotease 2	0.44	0.46	0.49	0.39	0.05	0.11
Metabolism	Insulin-like growth factor binding protein 1	2.03	2.31	2.07	1.7	0.31	0.15
	Insulin-like growth factor binding protein 6	1.76	1.79	1.61	1.89	0.14	0.08
	Vitamin D binding protein	1.67	1.38	1.77	1.85	0.25	0.15
	Apolipoprotein A-IV	1.56	1.76	1.44	1.48	0.17	0.11
	Apolipoprotein A-I	1.29	1.1	1.45	1.33	0.18	0.14
	Apolipoprotein E	0.82	0.73	0.91	0.83	0.09	0.11
	Transthyretin	0.41	0.44	0.55	0.44	0.06	0.13
Immune Response	Leucine-rich alpha-2-glycoprotein	1.71	1.57	1.85	1.71	0.14	0.08
	Complement Factor B	1.12	1.22	0.99	1.14	0.12	0.10
Osteogenesis	Fetuin B	1.42	1.32	1.26	1.67	0.22	0.16
	Macrophage stimulating protein 1	1.39	1.26	1.6	1.3	0.19	0.13
Cell Growth	Inhibin, beta E	1.30	1.19	1.36	1.37	0.10	0.08
Vasoconstriction	Angiotensinogen	1.57	1.76	1.64	1.3	0.24	0.15

Figure 3.4 Extracted ion chromatogram of m/z 501.95, identified as a peptide from the protein vitronectin

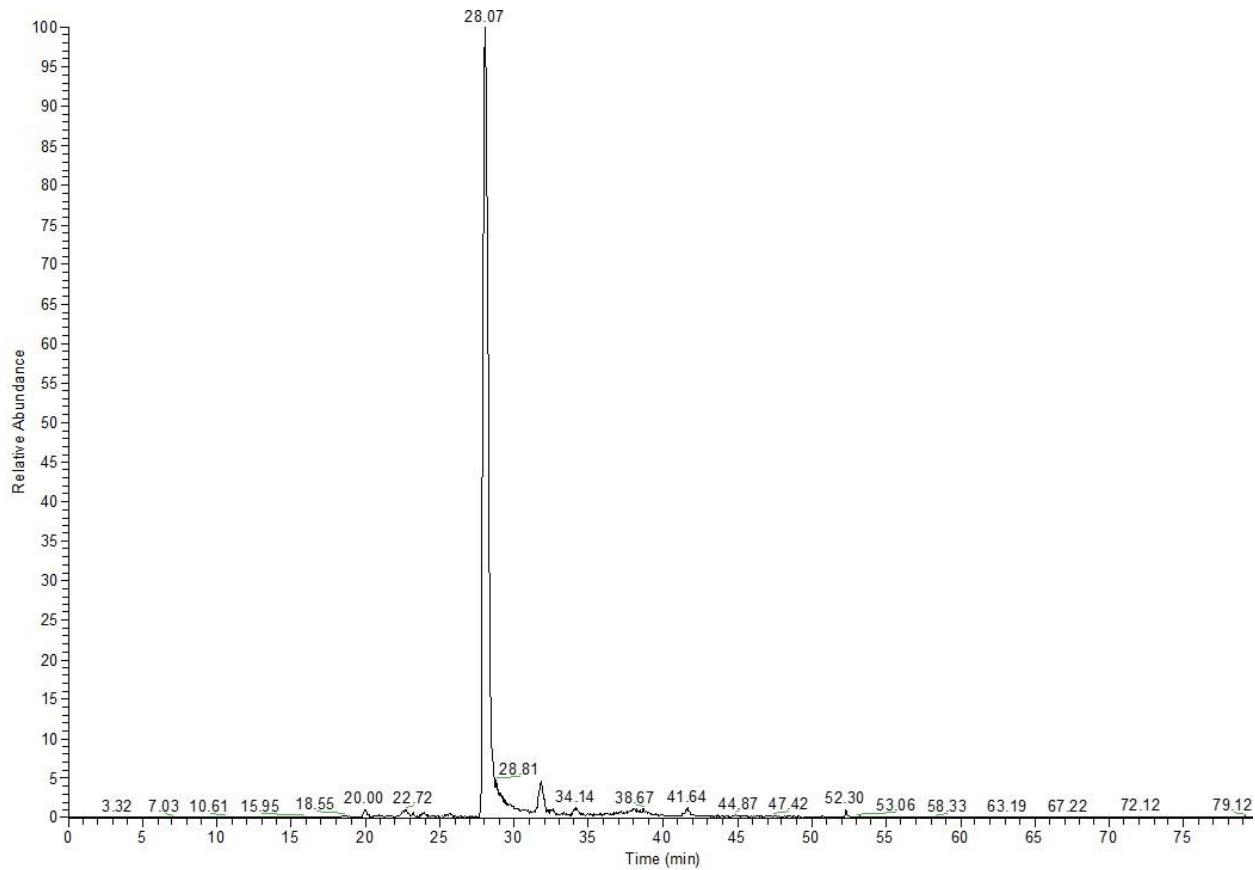


Figure 3.5 Mascot peptide hit results for a peptide sequence (IYISGMAPRPSLAK) identified as part of Vitronectin.

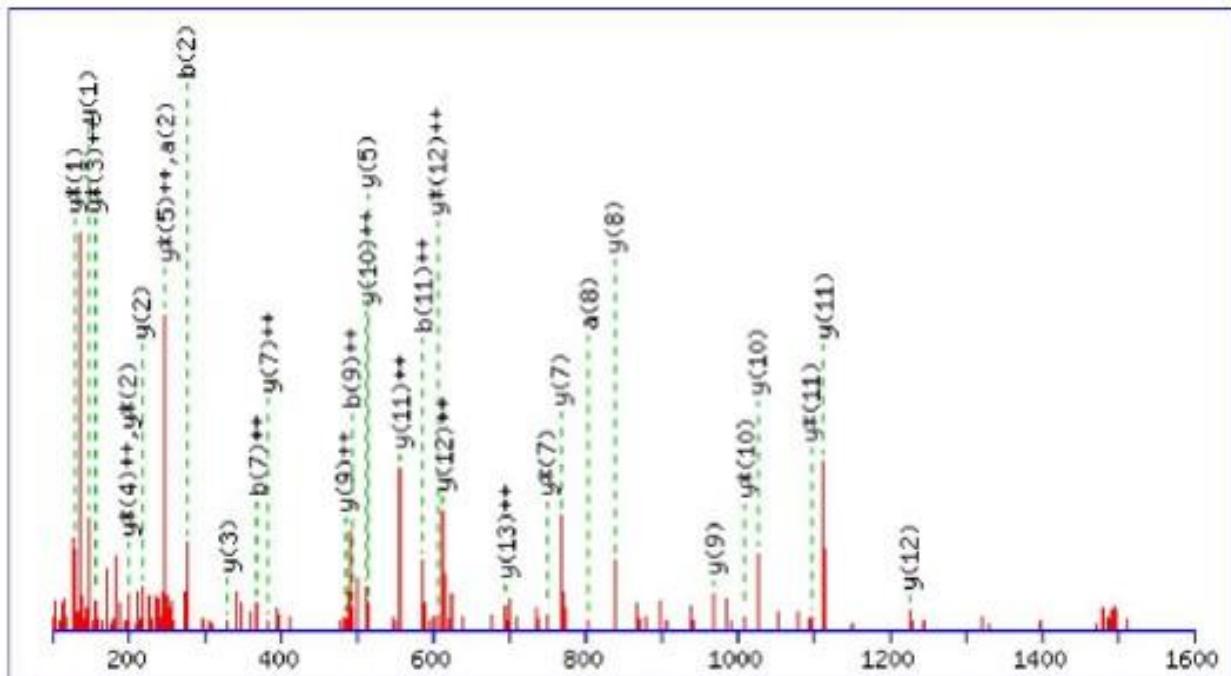


TABLE VI: RESULTS OF PROTEIN HITS FOR BLACK COHOSH SAMPLES

Pathway	Protein	BC Ratio	x1	x2	x3	stdev	rsd
Inflammation	Vitronectin	1.36	1.58	1.27	1.22	0.20	0.14
	Kininogen	1.33	1.3	1.4	1.29	0.06	0.05
	Plasminogen	1.54	1.42	1.8	1.41	0.22	0.14
Metabolism	Insulin-like growth factor binding protein 1	1.37	1.24	1.45	1.41	0.11	0.08
	Insulin-like growth factor binding protein 6	0.74	0.8	0.72	0.7	0.05	0.07
	Vitamin D binding protein	1.57	1.43	1.75	1.52	0.17	0.11
	Apolipoprotein A-I	1.27	1.09	1.49	1.23	0.20	0.16
	Apolipoprotein E	0.79	0.66	0.79	0.91	0.13	0.16
	Afamin	1.46	1.37	1.51	1.5	0.08	0.05
Osteogenesis	Fetuin B	1.10	1.26	1.17	0.95	0.16	0.14
Vessel morphogenesis	Transgelin 2	1.53	1.84	1.41	1.35	0.27	0.17

growth factor binding protein 6 was slightly downregulated instead of being upregulated as in the hormone therapy group.

There were a small number of other protein hits in the black cohosh treatment group showing significant variation from the placebo group that were not observed to have variation in the hormone therapy group. These hits include transgelin 2 and afamin. The magnitude of variation of these proteins was 53.2 and 47.1% increase respectively.

3.3.2.3 Red Clover

Many proteins were observed to vary in the red clover treatment group relative to the placebo group that also changed in both the black cohosh and hormone therapy groups. The magnitude of variation for these proteins was less than that observed in the hormone therapy group and was not always consistent in the direction of regulation. Apolipoprotein A-IV was upregulated in the HT samples but downregulated in the red clover samples. These results are summarized in TABLE VII. Only two protein hits were seen to show variation in the red clover group and not the hormone therapy group: hyaluronan-binding protein and pregnancy zone protein. The magnitude of variation for these proteins was only 39.6 and 33.7% respectively.

3.4 Discussion

The protein hits observed in the hormone therapy samples were generally consistent with published studies.⁸⁸ Significantly fewer protein hits were identified in this study compared with previous work,⁸⁸ most likely as a result of the age of the samples and the possibility that some degradation had occurred prior to analysis. Most of the proteins identified as having variation were previously identified in proteomic studies on postmenopausal women.⁸⁸ Protein hits not previously identified in literature as being up or down regulated in postmenopausal women treated with HT were: transthyretin,

TABLE VII: RESULTS OF PROTEIN HITS FROM RED CLOVER SAMPLES

Pathway	Protein	RC Ratio	x1	x2	x3	stdev	rsd
Inflammation	Vitronectin	1.45	1.34	1.63	1.37	0.16	0.11
	Kininogen	1.63	1.58	1.84	1.46	0.19	0.12
	Vitamin K-dependent protein S	1.62	1.79	1.62	1.45	0.17	0.10
	Hyaluronan-binding protein 2	1.40	1.42	1.3	1.49	0.10	0.07
	Histidine-rich glycoprotein	1.31	1.57	1.24	1.13	0.23	0.17
Metabolism	Insulin-like growth factor binding protein 1	1.74	1.89	1.89	1.43	0.27	0.15
	Vitamin D binding protein	1.78	1.65	2.06	1.64	0.24	0.13
	Apolipoprotein A-IV	0.76	0.89	0.71	0.68	0.11	0.15
	Apolipoprotein A-I	1.47	1.69	1.28	1.45	0.21	0.14
	Pregnancy zone protein	1.48	1.22	1.46	1.77	0.28	0.19
Immune Response	Leucine-rich alpha-2-glycoprotein	1.47	1.56	1.3	1.55	0.15	0.10
Vasoconstriction	Angiotensinogen	1.39	1.29	1.34	1.53	0.13	0.09

thrombospondin-1, and fibronectin. As shown in TABLE IV the largest number of proteins have functions in the biological pathways of inflammation and metabolism. The magnitude of change observed in this study was often less but was generally in agreement with the direction of regulation for individual proteins as reported in previous studies.

The protein hits identified in the red clover and black cohosh treatment groups were generally consistent with those observed in the hormone therapy group. Ceruloplasmin, vitamin D binding protein, and apolipoprotein A-I in particular were consistent across all three compared treatment groups relative to placebo. The magnitude of change in the black cohosh and red clover groups was typically lower than that observed in the hormone therapy group, indicating that the effect of change on the overall proteome was lower. Of particular note are the protein hits identified as being up or downregulated in the botanical treatment groups but not in the hormone therapy group. In the red clover group these were hyaluronan-binding protein and pregnancy zone protein (PZP). It is important to investigate these proteins and pathways for possible mechanisms of action for the red clover constituent compounds. Hyaluronan-binding proteins are known to be involved in inflammation pathways. Pregnancy zone protein is typically involved in endopeptidase activity but may indicate that some hormonal regulation pathway is being affected.

In the black cohosh group, vitamin D binding protein and afamin were hits of interest. Vitamin D is a widely studied biological signaling molecule affecting many different functional pathways. Investigations into the effects of black cohosh on vitamin D levels would be required in order to further characterize the effect black cohosh has on these pathways. Afamin is another protein that has vitamin D activity so investigations into these pathways are of paramount interest.

The hits of interest for further investigation as possible biomarkers for hot flashes (and other menopausal symptoms) in the inflammation pathway are vitronectin and kininogen. These proteins showed much higher upregulation than in the botanical treatment groups. This may reflect a greater

reduction of the pathways causing hot flashes or a potential effect of reduced hot flashes. The individual pathways need to be more closely investigated in order to determine the importance of these proteins. Insulin-like growth factor 6 is a hit of particular interest. The protein was upregulated in the HT group and downregulated in the black cohosh group. The black cohosh group had more hot flashes than the placebo group (as shown in Figure 2.1) and therefore Insulin-like growth factor 6 represents a potential pathway of investigation. This protein regularly functions to regulate the metabolism of other proteins so the specific effect related to hot flashes would need in depth, targeted research to identify the protein-protein interactions that would cause these effects.

Studies have indicated that black cohosh may prevent bone resorption and prevent osteoporosis.⁹⁴⁻⁹⁶ Fetuin B was only slightly upregulated in the black cohosh group. This protein has been identified as having a role in osteogenesis and bone resorption. Vitamin D has also been shown to prevent bone resorption as well.⁹⁷⁻¹⁰⁰ As shown in Table IV, the upregulation of Fetuin B and Vitamin D binding protein was also observed in the HT group. These proteins represent hits of interests for identifying the possible mechanism for prevention of bone loss by black cohosh. In the UIC/NIH clinical trial that supplied the samples for this work the bone resorption observed in black cohosh was slightly improved in black cohosh from placebo but this was not consistent across all time points.⁹ More investigation into these pathways would be of interest for potential treatments for osteoporosis.

Validation of any of the identified potential biomarkers of interest would require more in depth study of the levels observed. Absolute quantitation of these proteins would be possible through the use of isotopically labelled internal standards and an LC/MS-MS capable of sensitive measurement over a large dynamic range, such as a triple quadrupole instrument. Analysis across all time points of sample collection in this study would provide valuable insight into the levels of proteins as related to the observed response of hot flashes and other symptoms. Additional studies would provide fresh samples that would

presumably allow for identification of many more hits of interest, in addition to more accurate measurements of these proteins.

Overall, this work demonstrated the ability to characterize and identify potentially significant changes to the serum proteome after treatment with botanical dietary supplements or hormone therapy intended for the management of menopausal hot flashes in women. The hormone therapy group showed good agreement with previously published data.⁸⁸ The botanical treatment groups showed similar protein hits as the hormone therapy group but reduced magnitude of the effects. In both treatments, proteins differing from those identified in the hormone therapy group were observed to have variation from placebo, representing potential pathways of investigation. The age of the samples used might have resulted in a reduced number of protein hits from those observed in previous studies. Nevertheless, significant, new data was generated that could potentially help guide future studies.

**CHAPTER 4 EVALUATION OF PROTEOMICS AND METABOLOMICS OF SERUM
SAMPLES FROM A CLINICAL TRIAL ON BOTANICAL SUPPLEMENTS IN
POSTMENOPAUSAL WOMEN**

4.1 Introduction

A Phase II clinical trial to determine the efficacy and safety of the botanical supplements black cohosh and red clover was completed.⁹ This trial randomized participants into four groups: placebo, hormone therapy, black cohosh, and red clover. The major outcomes of interest were the reduction of postmenopausal symptoms, primarily hot flashes. Analysis of the patient records revealed that the therapies were statistically similar to placebo, and that only hormone therapy could be shown to reduce intensity and frequency of hot flashes. The hot flashes were measured by self-reporting from the individuals participating in the study, however in order to analyze for safety biomarkers, serum and urine samples were collected once a month. These biological samples were analyzed for the biomarkers and then kept frozen at -80° C.

In order to further characterize the results, an analysis of the serum metabolome and proteome of the frozen specimens from the clinical trial was undertaken. Serum samples have been shown to contain many important biomarkers for disease states and symptoms.^{86,101–104} The purpose of the metabolomic and proteomic experiments was to identify biomarkers and biological pathways of interest for postmenopausal symptoms. The biochemical causes of hot flashes are not well characterized, and as such, the mechanism of action for any potential therapy is unknown.^{5,6,18,73,83} The identification of any biological pathway affected by the treatments could potentially be used to identify avenues for drug development and biological monitoring for efficacy.

In order to develop the metabolomics and proteomic profiles, high resolution LC-MS scanning was carried out. The use of scan functions allows for an untargeted approach to identify a wide range of analytes in an individual chromatographic run. The instruments used in these experiments are able to perform these scan functions at a much faster rate than possible at the time of the completion of the clinical trial. A Shimadzu IT-TOF mass spectrometer was used to develop the metabolomics profile. This instrument was chosen because the ion trap allows for efficient scanning of ionized species and the time of flight analyzer allows for high resolution mass spectra to be obtained. This instrument also performs rapid polarity switching, allowing for the collection of positive and negative ionized species in the same experiment. The proteomic profile was developed using a high resolution Thermo Fisher Orbitrap mass spectrometer. This instrument has been used to develop very large proteomic profiles of sample types as varied as human cells to plants.¹⁰⁵⁻¹⁰⁷

4.2. Methods

4.2.1 Metabolomics analysis

The methods for sample preparation and instrumental analysis for serum samples for metabolomics was described in depth in section 2.2. Briefly, serum samples were treated with organic solvent to precipitate proteins and then analyzed on a Phenomenex (Torrance, CA) Kinetex 2.6 μ m C₁₈ column (2.1 x 100 mm, 100 \AA) with Shimadzu Prominence HPLC pumps connected to a Shimadzu IT-TOF mass spectrometer. Negative and positive ion electrospray mass spectra were collected using scan mode.

4.2.2. Proteomics analysis

The methods for sample preparation and instrumental analysis for serum samples for proteomics were described in depth in section 3.2. Briefly, serum samples were treated with cold organic solvent to

precipitate proteins. The proteins were resuspended and digested using trypsin. Digested samples were treated with TMT tags for quantitative analysis and then combined. The combined sample was fractionated using an Agilent OFFGEL Fractionator. The fractions were then analyzed using an Agilent Zorbax C₁₈ column on Dionex UltiMate 3000 pumps connect to a Thermo Orbitrap mass spectrometer.

4.2.3. Database searches

Data files obtained from the proteomics measurements in section 4.2.2. were converted to mzxml files and submitted to XCMS in order to identify potential small molecules and metabolites of interest. This database uses high resolution MS data to identify features based on signal intensity, chromatography and spectra, then identifies potential hits within chosen search parameters. The results obtained from the search were also processed through the Metlin database and links to important relevant information (biological, spectroscopic, etc.) were provided. Data files converted to mzxml files were also submitted to Mascot search engine to identify proteins contained within the samples. The files were combined into a single mzxml file prior to submission in order to eliminate redundant hits in the returned protein list. Quantitative data were also returned from the database search.

XCMS results were then submitted to the MetaboAnalyst 3.0 (www.metaboanalyst.ca) search engine to identify affected pathways from the metabolomics data.¹⁰⁸ Biological pathways are identified based on the chemical formula and potential compounds corresponding to features of the data submitted. The results were filtered for pathways that showed a p >0.05. The proteins identified by the Mascot search engine were then submitted to the NCBI Biosystems search engine. This search engine identifies the biological pathways that submitted proteins have a function in. These two database search results were then compared in order to identify common pathways and determine the pathways that are most likely to be affected by the treatment groups studied.

4.3 Results

4.3.1 Hormone Therapy Group

Metabolomics results and proteomics results summarizing the findings from Chapter 2 and Chapter 3 for the HT group are summarized in TABLE VIII. Among the small molecule features of interest was potentially thyrotropin releasing hormone. This hormone has been identified as showing significant changes in levels among those with thyroid disorders, in addition to several other clinical diagnoses including depression, bipolar disorder and sleep deprivation.¹⁰⁹⁻¹¹¹ While this hit was ruled out after analysis of a known standard, the protein hit of kininogen is an associated protein of bradykinin. Bradykinin is a peptide with similar neurotransmitter pathway functions as thyrotropin releasing hormone.

There are several more small molecule features that require further investigation and characterization. There are early eluting compounds of possible amino acid structure that could be signaling molecules that would be of interest to determine the signaling pathway affected. Another small molecule that returned a database hit was pyrilamine, a common antihistamine. It is unlikely that this is in fact pyrilamine as a medication appearing in one group as a significant hit seems unlikely. Therefore, further characterization of this hit is necessary to identify the compound correctly.

The proteomics results were largely in agreement with previously published results.⁸⁸ The identified proteins were largely from inflammation, metabolism and osteoporosis pathways. Investigation into the individual proteins that have been identified as relating to inflammation pathways are the best targets for further study on the causes and effects of hot flashes. Identification of the pathways most

TABLE VIII: SUMMARY OF HITS FROM HORMONE THERAPY GROUP

Small Molecules		Proteins	
Up	Down	Up	Down
PS(P-16:0/17:0)	PE(10:0/10:0)	Vitronectin	Matrix metalloprotease 2
PC(14:0/18:1(9E))	PG(19:1(9Z)/0:0)	Vitamin D binding protein	Transthyretin
	Lysyl-Tyrosine	Plasminogen	Apolipoprotein E
	3,4-Didehydro- β -carotene	Macrophage stimulating protein 1	
	PC(O-1:0/16:0)	Kininogen	
	PC(O-8:0/O-8:0)	Insulin-like growth factor binding protein 6	
	PA(18:1(11Z)/18:1(11Z))	Insulin-like growth factor binding protein 1	
	PC(20:4(5Z,8Z,11Z,14Z)/0:0)	Inhibin, beta E	
	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	Fetuin B	
	Pyrilamine	Complement Factor B	
	Valyl-Lysine	Ceruloplasmin	
	PA(22:0/22:4(7Z,10Z,13Z,16Z))	Angiotensinogen	
	PGH2-EA	Vitamin K-dependent protein S	
	26-hydroxycholesterol 3-sulfate	Vitamin K-dependent protein S	
		Thrombospondin-1	
		Platelet basic protein	
		Leucine-rich alpha-2-glycoprotein	
		Fibronectin	
		Coagulation factor X	
		Apolipoprotein A-IV	
		Apolipoprotein A-I	

affected by hormone therapy may prove to provide the most insight into the mechanism that causes hot flashes in patients, and therefore the best target for biological monitoring.

The MetaboAnalyst pathway analysis for metabolomics data identified several pathways of interest. The various lipids identified by XCMS were processed by MetaboAnalyst, which identified the glycerophospholipid metabolism, glycerolipid metabolism, and alpha-linolenic acid metabolism pathways as having common metabolites present in the features. The glycerophospholipid metabolism pathway search results are shown in Figure 4.1. All of the identified metabolites in the pathways were downregulated in the HT group, indicating that the pathways in question are likely to be downregulated in the patients receiving HT treatment.

4.3.2. Red Clover

The metabolomics and proteomics results for the red clover group are summarized in Table IX. The metabolomics results for women treated with red clover returned many significant features. Among those of particular interest were isoflavones and vitamin D metabolites. Isoflavones are naturally occurring compounds present in red clover. Vitamin D metabolites potentially indicate an increased response in the vitamin D receptor pathway. Proteomics results indicated a mild increase in the Vitamin D associated protein afamin. The effect of red clover and isolated isoflavones on vitamin D levels has not been measured. The effects of soy isoflavones have been studied on menopausal women but the association with the vitamin D pathway is unclear.^{112,113} Vitamin D supplementation is common among postmenopausal women, but the increased levels among particular groups indicate that the effects observed in this work is less likely to be a result of diet or supplementation.^{25,112,113} Furthermore, the women in this botanical dietary supplement study were cautioned not to consume any other supplements

Figure 4.1 MetaboAnalyst pathway results for glycerophospholipid metabolism from HT small molecule hits. Pathway objects in red represent hits from XCMS search results.

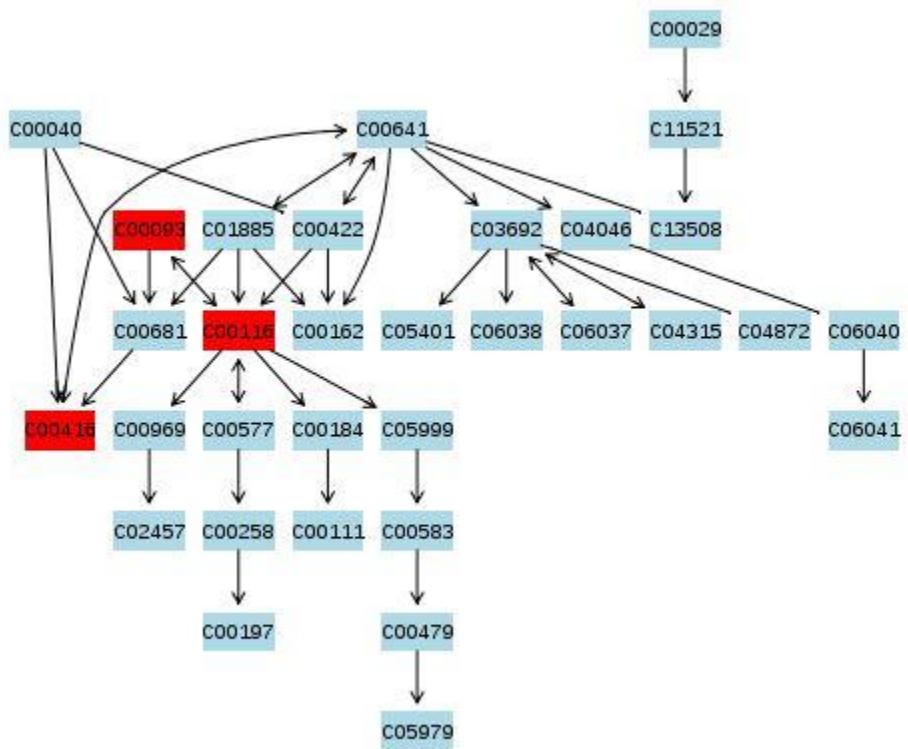


TABLE IX: SUMMARY OF HITS FROM RED CLOVER GROUP

Small Molecules		Proteins	
Up	Down	Up	Down
Vitamin D3 metabolite	PA(22:1(11Z)/22:2(13Z,16Z))	Vitronectin	Apolipoprotein A-IV
Ginsenoyne A linoleate	PE(O-20:0/22:4(7Z,10Z,13Z,16Z))	Kininogen	
22-Angeloylbarringtogenol C	TG(14:1(9Z)/14:1(9Z)/19:1(9Z))	Vitamin K-dependent protein S	
Formononetin	PC(15:0/P-18:0)	Hyaluronan-binding protein 2	
PE(12:0/12:0)	PC(16:0/20:2(11E,14E))	Histidine-rich glycoprotein	
Vitamin D3 metabolite	PA(16:0/22:1(11Z))	Insulin-like growth factor binding protein 1	
18-Dehydrouronic acid 3-arabinoside	PE(16:0/22:6(54Z,7Z,10Z,12E,16Z,19Z)(14OH))	Vitamin D binding protein	
Asparaginyl-Isoleucine	4-keto pentadecanoic acid	Apolipoprotein A-I	
Genistein	PC(18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Pregnancy zone protein	
27-Norcholestanehexol	PC(16:0/20:4(5Z,8Z,11Z,14Z))	Leucine-rich alpha-2-glycoprotein	
Vitamin D3 metabolite	PC(14:0/18:2(2E,4E))	Angiotensinogen	
	PG(O-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))		
	PC(O-16:2(9E,10E)/0:0)		
	Phosphatidyl glycerol		

during the study. Other proteomics hits observed in serum from women in the red clover arm of the study were similar to those in the hormone therapy group. However, the effects were less than those observed in the hormone therapy group. These included proteins identified to affect inflammation and metabolism.

MetaboAnalyst database search results identified the Vitamin D metabolism pathway as being affected based on features identified by XCMS. This is consistent with what was observed in both the proteomics and metabolomics data sets. The other pathway identified was glycoprophospholipid metabolism. The lipids identified by the XCMS search results were all downregulated, just as in the HT group. More targeted lipidomics studies would need to be performed in order to: 1) accurately identify the lipids affected by treatments, and 2) accurately determine the level of effect observed. These pathways require more careful investigation in given the effects observed in this study.

4.3.3. Black Cohosh

The metabolomics and proteomics results from the black cohosh group are summarized in TABLE X. The results from the serum samples for the black cohosh treatment arm of the study showed features potentially corresponding to diterpenoid compounds. These may be directly from the plant itself or metabolites of other terpenoid structures. There were several other naturally occurring compounds indicated in the database searches of significant features. These naturally occurring compounds have not previously been identified in black cohosh extracts. Further investigation into these compounds is necessary to determine, 1) if the hit in question can indeed be confirmed to be a naturally occurring compound; and 2) the compounds originated from the black cohosh dietary supplement. Since diet was not controlled in this study, it is possible that another source was responsible for these hits. However, if originating from a different dietary source, it seems unlikely that these compounds would then result in

TABLE X: SUMMARY OF HITS FROM BLACK COHOSH GROUP

Small Molecules		Proteins	
Up	Down	Up	Down
13Z-Octadecen-1-ol	Cynometrine	Vitronectin	Insulin-like growth factor binding protein 6
PS(16:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))	PI(16:0/16:2(9Z,12Z))	Kininogen	Apolipoprotein E
Isodomedin		Plasminogen	
PC(20:5(5Z,8Z,11Z,14Z,17Z)/0:0)		Insulin-like growth factor binding protein 1	
PC(O-8:0/O-8:0)		Vitamin D binding protein	
PA(O-20:0/17:2(9Z,12Z))		Apolipoprotein A-I	
Panaxydol linoleate		Afamin	
DG(20:1(11Z)/20:2(11Z,14Z)/0:0)		Fetuin B	
PC(18:2(2E,4E)/0:0)		Transgelin 2	
PG(19:1(9Z)/0:0)			
DG(20:0/22:3(10Z,13Z,16Z)/0:0)			
N-oleoyl tyrosine			
PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)			
PC(7:0/8:0)			

an upregulation only in the black cohosh group. The MetaboAnalyst searches identified two pathways potentially affected by black cohosh: glycerophospholipid metabolism and alpha-linolenic acid metabolism. The significant difference is that these metabolites identified in these pathways were upregulated in the black cohosh group but downregulated in the HT group.

The proteomics results indicated that Vitamin D associated proteins were shown to be upregulated as a result of black cohosh treatment. There is little research to indicate that black cohosh has effects on the Vitamin D receptor, although osteoprotective effects have been reported.⁹⁴ Whether this is a direct result of the treatment or a byproduct of another affected pathway cannot be determined from this investigation. Further study on the effects of individual compounds in black cohosh on the Vitamin D receptor and Vitamin D bioregulated pathways are required in order to fully understand the importance of these findings.

4.4. Discussion

4.4.1. Findings

There are several important findings from the experiments detailed in this work. The methodology utilized provides a framework for experiments to generate initial, untargeted metabolomic and proteomic profiles from the same sample set. Although the experiments here were performed on serum samples, the methodology could very easily be applied to different biological samples, including tissues and other biological fluids. This provides a general profile that can be used to generate hits for further investigation or potential leads to guide future, more targeted profiling. The analysis of small molecules by fast scanning, high resolution mass spectrometry allows for metabolomics to be generated that represent a wide variety of compound types. The metabolomics hits discovered in this experiment

range from small, polar peptides to large, nonpolar lipids. More focused analysis for either one of these compound types should generate even more hits.

The hits identified in these work provide more understanding of the effects of the treatment groups on the biological profile. In the hormone therapy group, several small molecule features of interest were identified. The investigation of the biological significance of these molecules for postmenopausal symptoms represents a promising lead toward developing a better understanding of the biochemical changes as a result of menopause, and potential development of a biomarker for hot flashes. This would be consistent with the proteomics results which showed significant hits among many proteins associated with inflammation. Further investigation into these proteins should provide a more concise understanding into which inflammation pathways are most affected by hot flashes, and in turn, how to monitor and treat these pathways.

The MetaboAnalyst searches indicated that glycerophospholipid metabolism, glycerolipid metabolism, and alpha-linolenic acid metabolism were all downregulated in the HT group relative to placebo. This is consistent with previously published studies that indicated these pathways are upregulated in postmenopausal plasma samples.⁸¹ The downregulation in the HT group could reflect the reduction in hot flashes relative to placebo group, making these significant pathways for biomarker identification.

The small molecule features among the patients treated with red clover contained many isoflavones. This was expected as isoflavones are present in red clover in large abundance and are often considered to be the source of the biological effects of the plant. The significance of these hits is that it demonstrated the effectiveness of the extraction and that the presence of small molecules in the patient samples was relatable to the treatment. The other small molecule hits among the red clover group of primary interest were a variety of Vitamin D metabolites. Several Vitamin D metabolites were indicated

and provide some indication that this pathway was affected by red clover. This is further supported by the proteomics data, which showed an upregulation of afamin, a Vitamin D related protein. Metaboanalyst database searching indicated that Vitamin D metabolism is a pathway affected by red clover treatment. The activity of red clover on the vitamin D receptor is a potential avenue of interest for future research. In addition, Metaboanalyst database searching identified glycerophospholipid metabolism as potentially being downregulated in the red clover group, similar to the hormone therapy group.

The black cohosh group did not show as many significant small molecules for novel pathways of biological activity, however several naturally occurring compounds were represented that have not previously been associated with black cohosh. A further investigation into the presence of these compounds in the plant could indicate significant new leads responsible for biological activity. The pathway analysis indicated an opposite effect from the HT group in the lipid pathways identified. These pathways are therefore very important for investigation given the significantly increased number of hot flashes observed in the black cohosh group.⁹ The protein hits from the patients receiving black cohosh of greatest interest were Vitamin D related compounds. Vitamin D activity in black cohosh has not been fully investigated and could represent a new biological pathway of relevance.

4.4.2. Limitations of investigations

The experiments detailed in this work were performed using serum samples from a clinical trial that had been completed in 2009.⁹ After analysis for safety biomarkers, the samples were stored at -80° C. The freezing of samples preserved them as best as possible, and this analysis showed that valuable information could be derived from experimentation. However, degradation of samples occurs even at such low temperatures. These studies were limited to those small molecules and protein hits that did not

degrade and were able to be detected. If fresh samples had been used instead, the same hits of interest should have been detected along with additional hits representing less stable molecules. The protein hits were developed by comparing responses relative to the other groups. Any protein hit identified in these studies were required to appear in the identified group and the control group for comparison. This resulted in a lower number of hits of interest being identified as many proteins were identified in only one group that potentially degraded so much in the control group as to render it unidentifiable by database searching.

4.4.3. Recommendations for future work

4.4.3.1. Validation and quantitation of small molecule hits

The hits of interest generated by the small molecule analysis (Chapter 2) require further investigation and validation as biomarkers. This would entail several steps. First, the identities of the hits need to be confirmed. Obtaining standards of this compound and comparison of retention times and tandem mass spectra is recommended. Upon successful identification, a quantitative method could be developed using a sensitive quantitative instrument, for example, a triple quadrupole mass spectrometer. Isotopically labelled standards of the compound in question would need to be obtained and used as internal standards for accurate quantitative data to be generated. Development of this method would allow the analysis of these compounds to be completed for all samples in this study, across all twelve months of data collection. This would provide a clearer picture of the levels in serum across the full study, and provide clarity on the significance of the hit in question.

4.4.3.2. Further metabolomics investigations

Metabolomics analysis utilizing the Shimadzu IT-TOF mass spectrometer generated several small molecule hits of interest. Many of those were identified by database searches as being amino acid or amino acid derivatives. This class of compounds might be better analyzed using normal phase chromatography such as HILIC, as this type of chromatography uses a polar stationary phase to retain the highly polar amino acids. This type of analysis could potentially identify many more amino acid or peptide hits, as well as other significant polar small molecules such as neurotransmitters. This would provide significant information on the effects of hormone therapy and the botanical treatments on many biological pathways, as the signaling molecules for nearly all biological pathways require small polar signaling peptides. This analysis would require an altered method of sample cleanup, however, as these polar small molecules will not dissolve into the organic layer as described in Chapter 2.2.

4.4.3.3. Analysis of lipid profiles

Many hits generated during the analysis of the metabolomics samples were identified by database searches to be lipids. Phospholipids and fatty acids were both identified as corresponding to features submitted for searching. The differentiation between many of these lipid molecules requires different chromatographic conditions so it was not within the scope of this research to accomplish this task. However, it would be beneficial as other published metabolomics studies have identified many lipids as hits of interest among menopausal women.⁸¹ An in depth analysis of the lipid profile from the clinical trial samples would provide additional information for postmenopausal women in addition to the effect that the treatment groups may have on these compounds.

4.4.3.4. Absolute Quantitation of Proteins

The protein hits and proteomic profile identified in Chapter 3 were quantitated using TMT labelling tags. These tags allowed a quantitative comparison to a defined base level. In the case of this experiment, the comparison was relative to the placebo control group. This relative quantitation is useful for generating hits of interest and a relative profile, but the identification of these proteins of interest allows for more rigorous quantitative methods to be developed. Absolute quantitation of protein levels is accomplished through the synthesis of isotopically labelled peptides of interest as internal standards. Several peptides of a given protein are selected, and a quantitative method using a sensitive instrument can be produced to show absolute concentrations in patient samples. This will allow for a faster workflow to obtain quantitative data. Samples from all time points of collection can be easily processed, and the concentration levels over time across all groups can be generated to help validate hits of interest and further characterize the effects of treatment groups on selected protein levels.

4.4.3.5. Further studies

The experiments performed here detail a method for development of comparative metabolomic and proteomic profiles for serum samples from clinical trials. Application of these methods should result in lists of hits of interest to pursue further and help clarify affected pathways and potential mechanisms of action for various treatments. Analysis of samples at a time closer to collection should generate much more detailed profiles and even greater numbers of hits of interest. A new clinical trial of the treatment groups would provide more information and pathways of interest in addition to providing additional confirmation of the hits discussed in this work. The analysis of a detailed profile of proteins and small molecules from a single sample is a valuable tool in discovering pathways of interest and mechanisms of

action for observed effects that require further characterization in order to develop more effective therapies and tools for monitoring.

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APPENDICES

APPENDIX A: HRMS SPECTRA OF METABOLOMICS FEATURES

Figure A1: MS Spectra for feature of m/z 810.6307

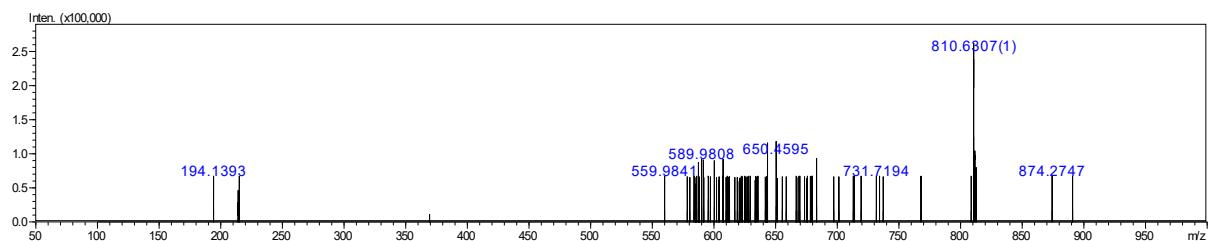
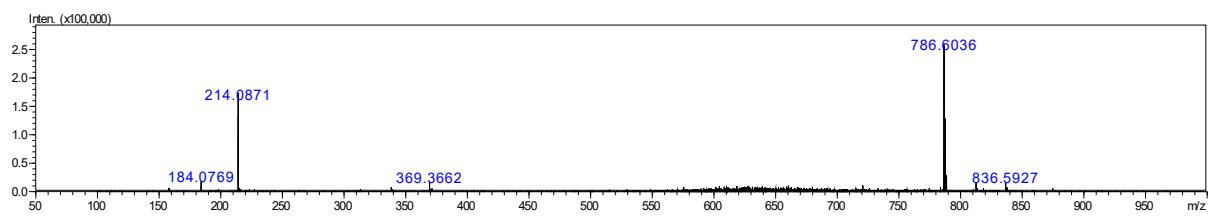
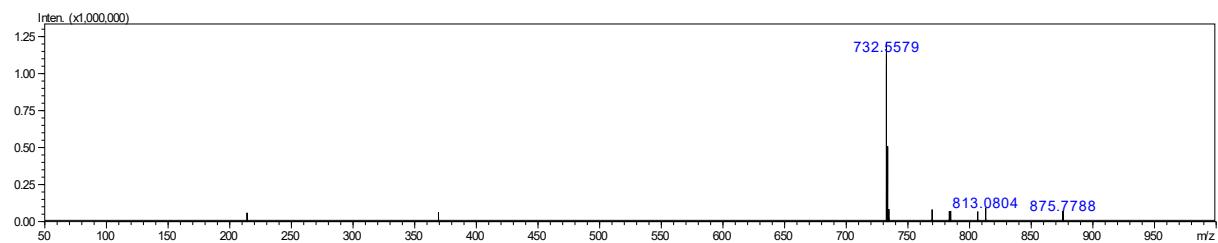
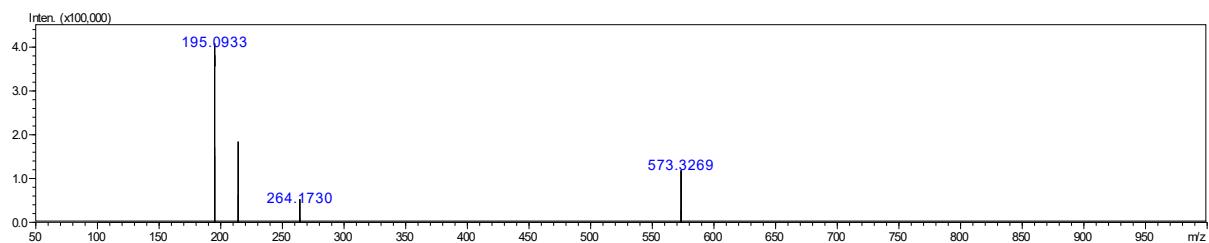


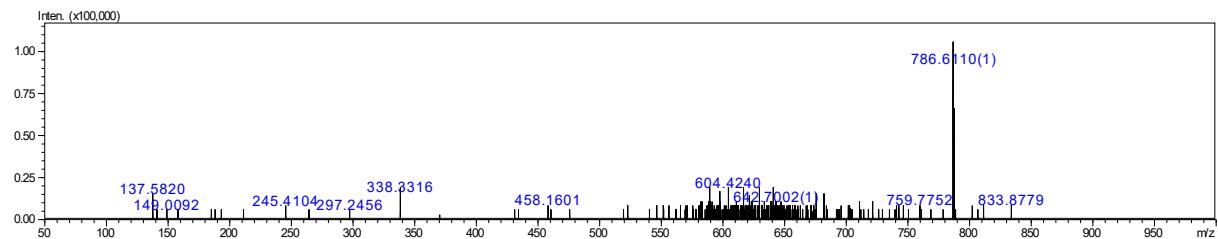
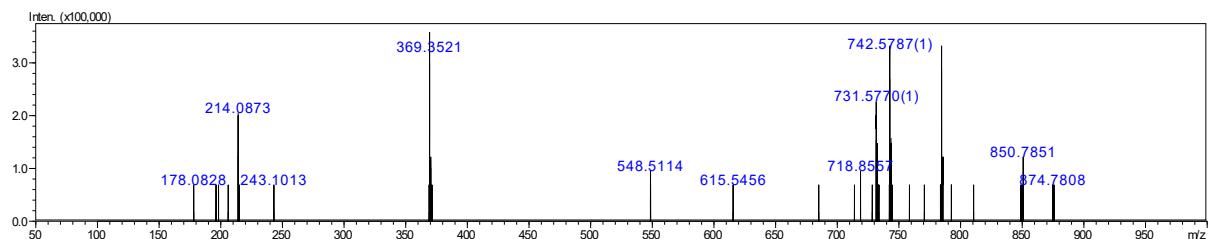
Figure A2: MS Spectra for feature of m/z 786.6036



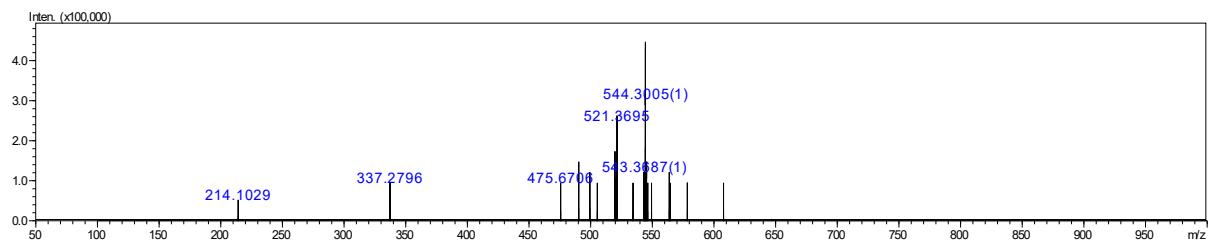
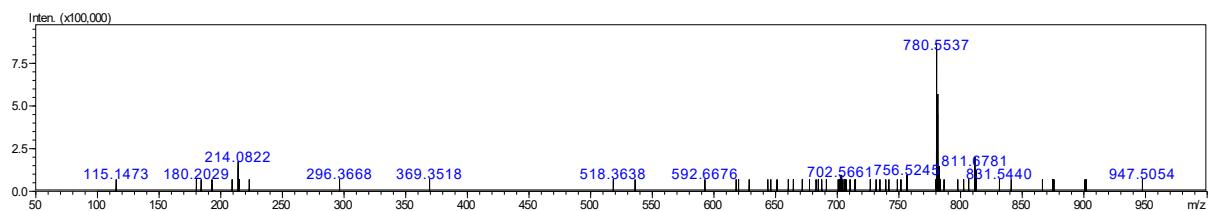
APPENDIX A (continued)

Figure A3: MS Spectra for feature of m/z 732.5579Figure A4: MS Spectra for feature of m/z 195.0933

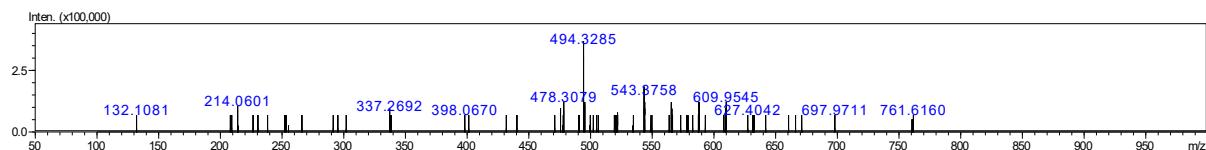
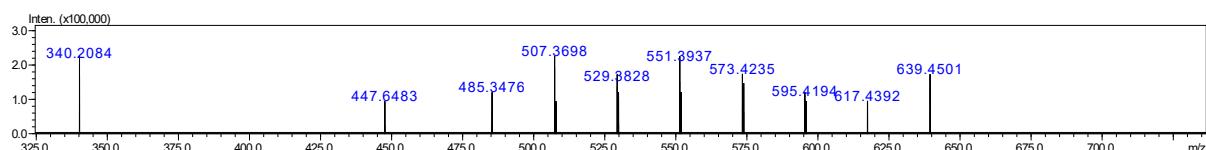
APPENDIX A (continued)

Figure A5: MS Spectra for feature of m/z 786.6110Figure A6: MS Spectra for feature of m/z 731.5770

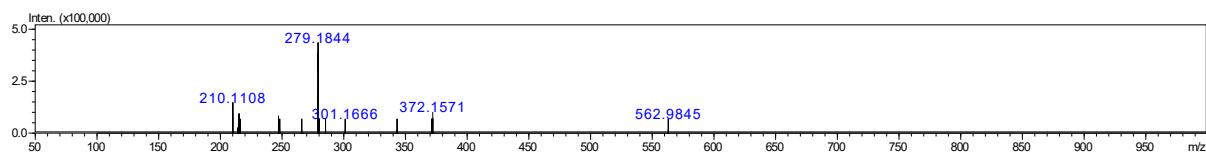
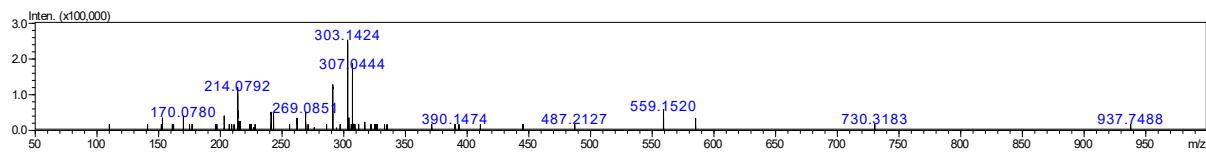
APPENDIX A (continued)

Figure A7: MS Spectra for feature of m/z 521.3695Figure A8: MS Spectra for feature of m/z 780.5537

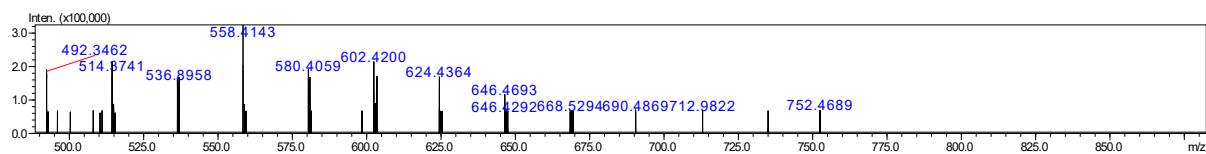
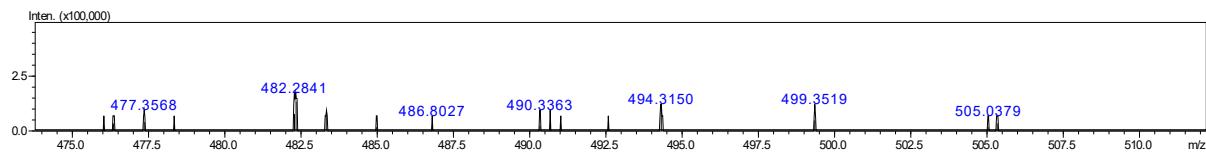
APPENDIX A (continued)

Figure A9: MS Spectra for feature of m/z 543.3758Figure A10: MS Spectra for feature of m/z 573.4235

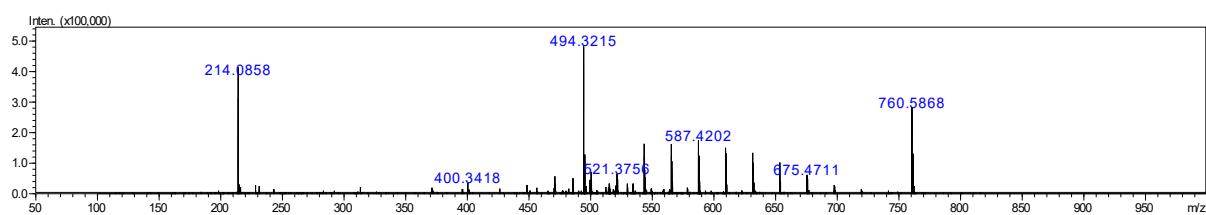
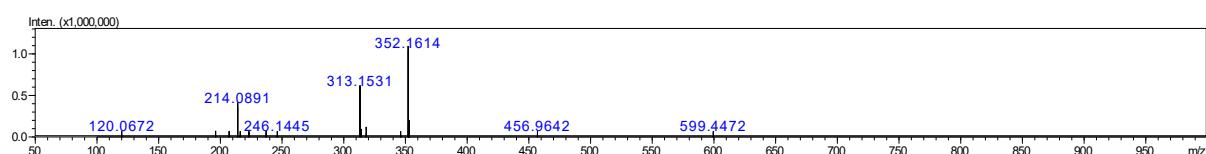
APPENDIX A (continued)

Figure A11: MS Spectra for feature of m/z 279.1844Figure A12: MS Spectra for feature of m/z 269.085

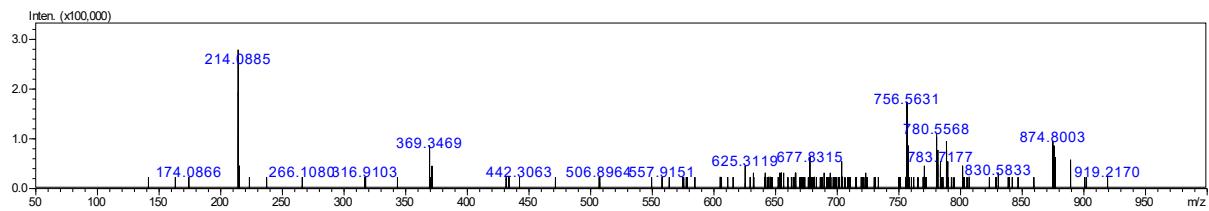
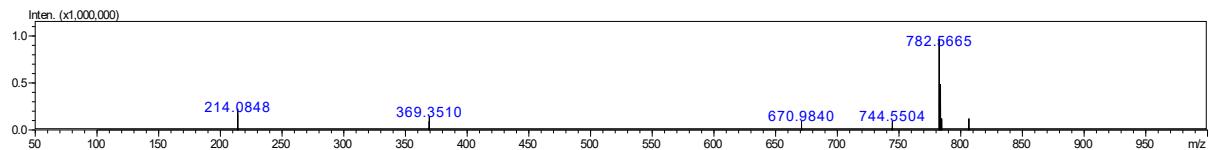
APPENDIX A (continued)

Figure A13: MS Spectra for feature of m/z 580.4059Figure A14: MS Spectra for feature of m/z 499.3519

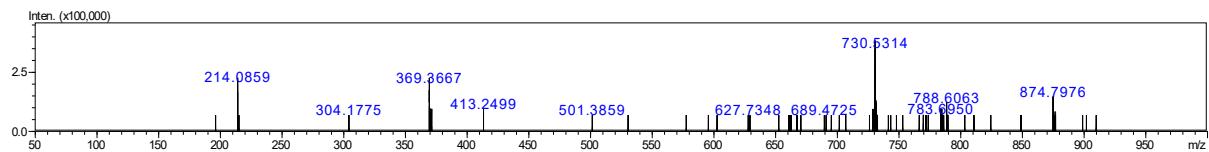
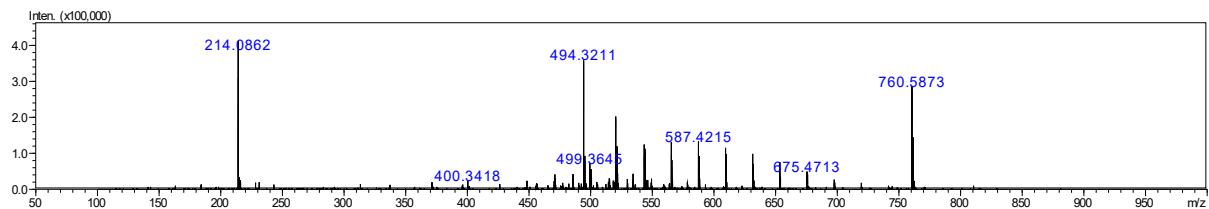
APPENDIX A (continued)

Figure A15: MS Spectra for feature of m/z 587.4202Figure A16: MS Spectra for feature of m/z 246.1445

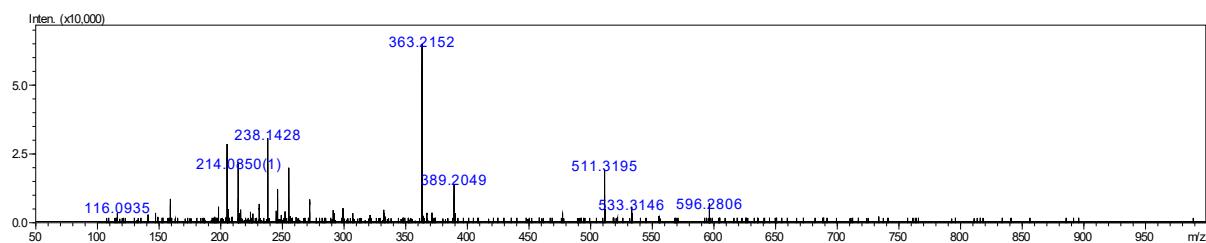
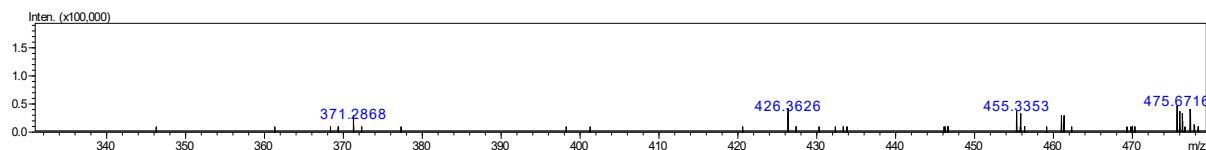
APPENDIX A (continued)

Figure A17: MS Spectra for feature of m/z 830.5833Figure A18: MS Spectra for feature of m/z 782.5665

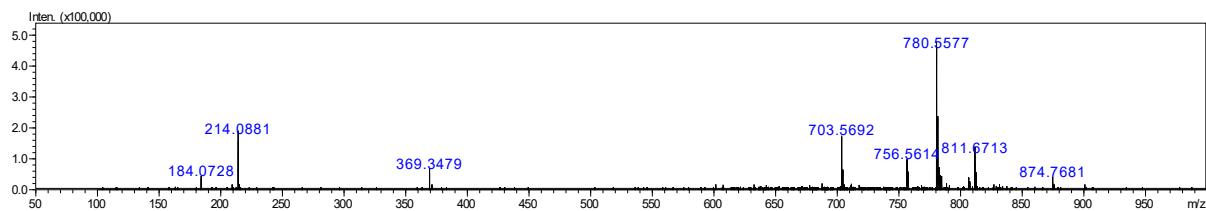
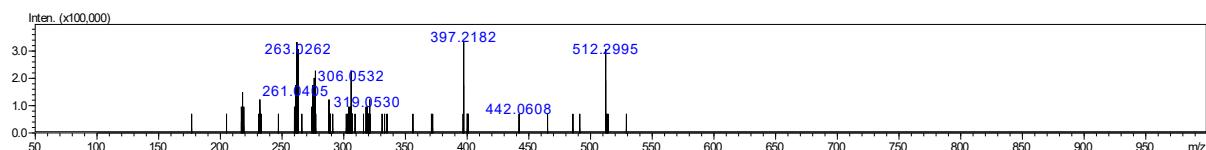
APPENDIX A (continued)

Figure A19: MS Spectra for feature of m/z 730.5314Figure A19: MS Spectra for feature of m/z 494.3211

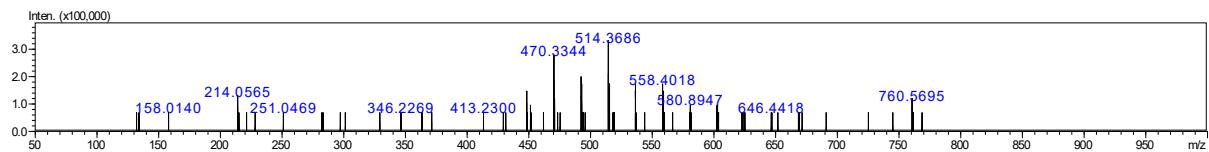
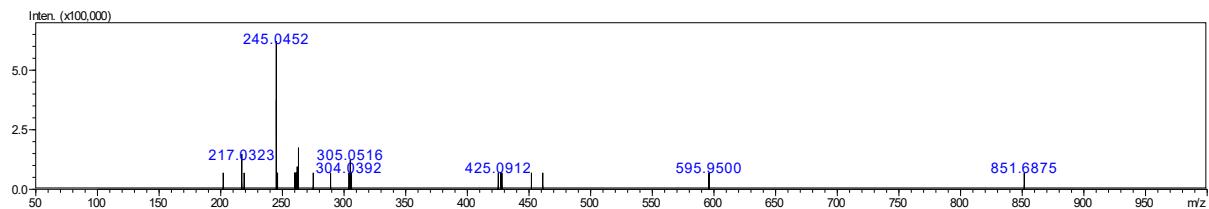
APPENDIX A (continued)

Figure A21: MS Spectra for feature of m/z 271.0462Figure A22: MS Spectra for feature of m/z 455.3353

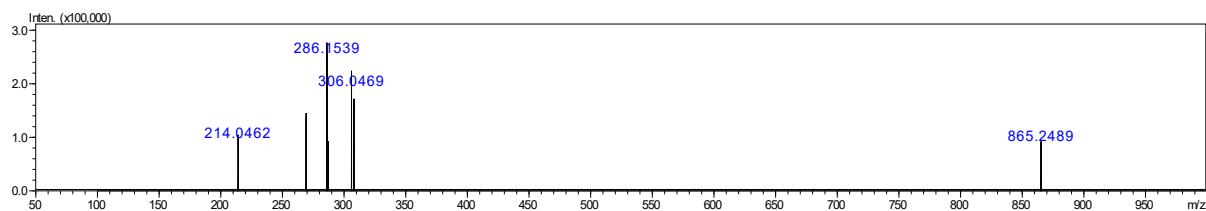
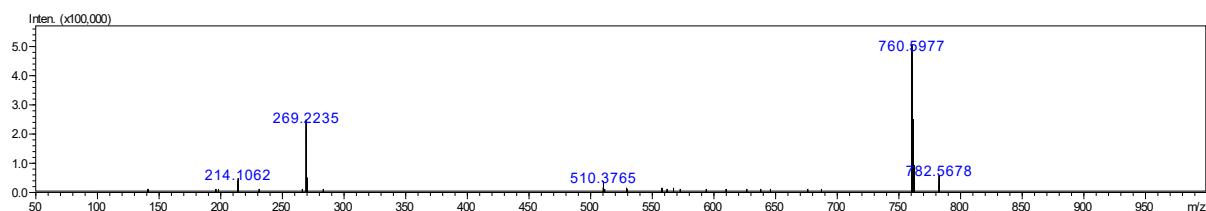
APPENDIX A (continued)

Figure A23: MS Spectra for feature of m/z 780.5577Figure A24: MS Spectra for feature of m/z 512.2995

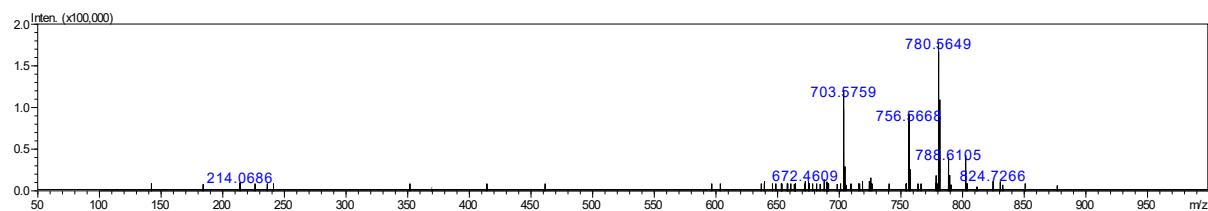
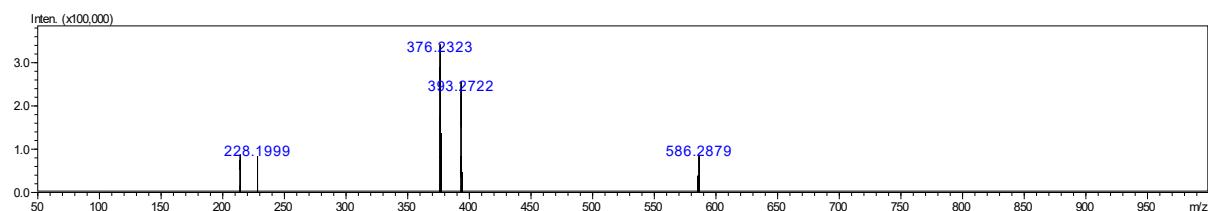
APPENDIX A (continued)

Figure A25: MS Spectra for feature of m/z 558.4018Figure A26: MS Spectra for feature of m/z 245.0452

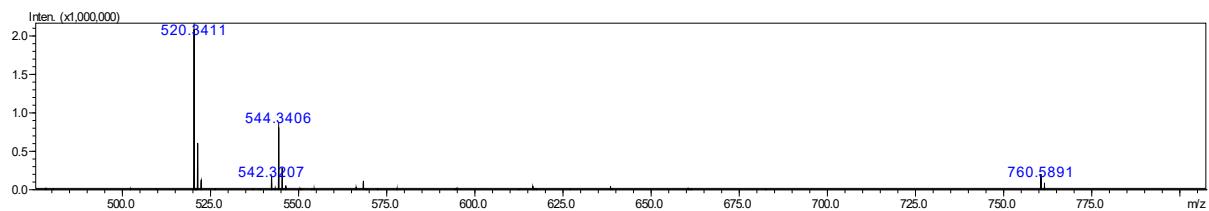
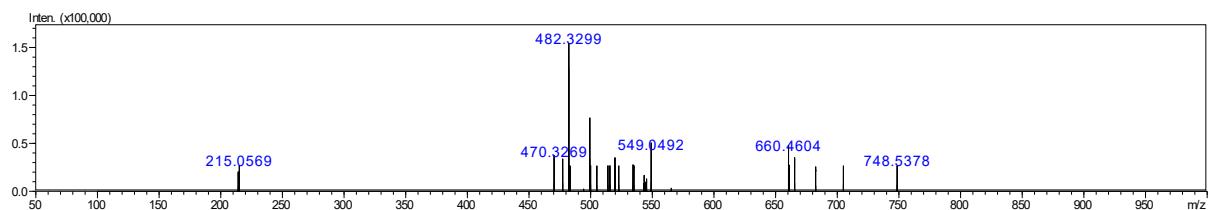
APPENDIX A (continued)

Figure A27: MS Spectra for feature of m/z 286.1539Figure A28: MS Spectra for feature of m/z 269.2235

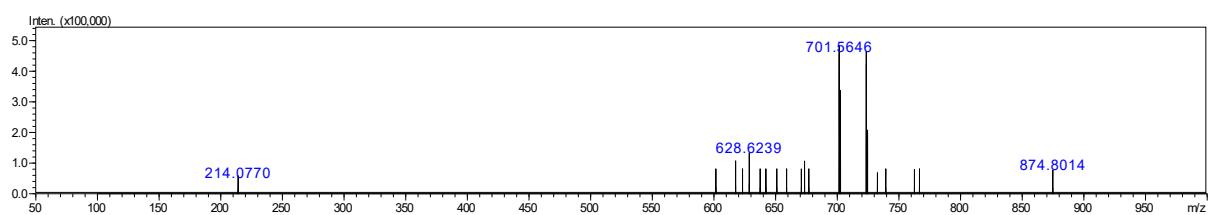
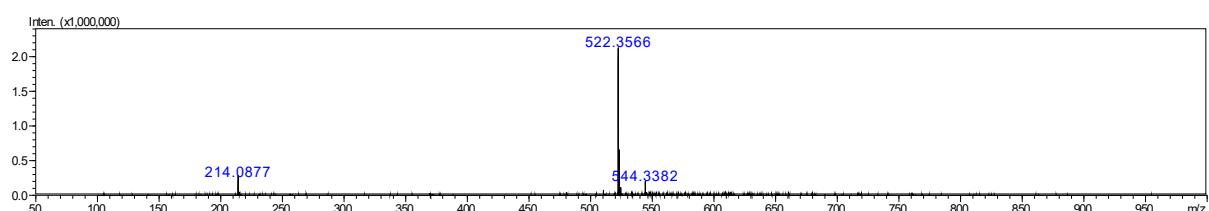
APPENDIX A (continued)

Figure A29: MS Spectra for feature of m/z 780.5649Figure A30: MS Spectra for feature of m/z 393.2722

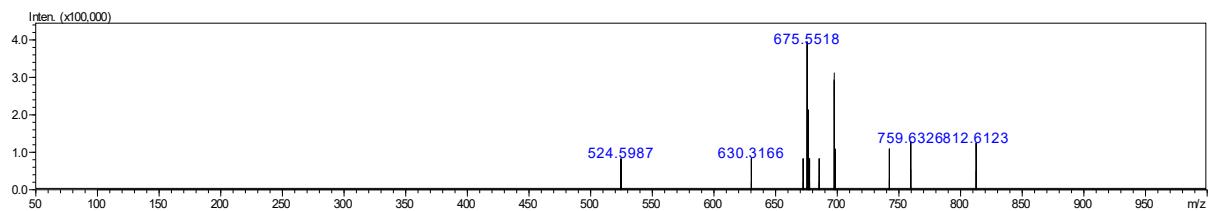
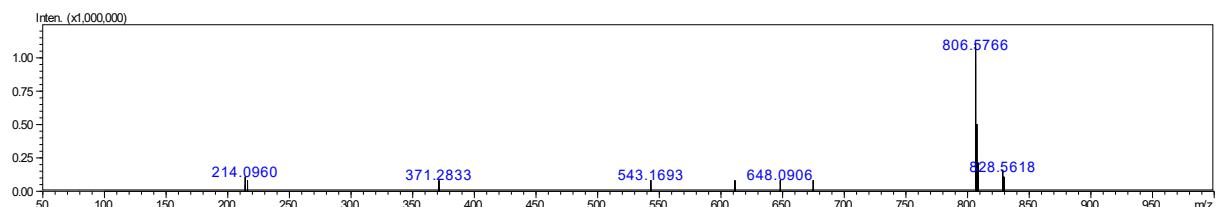
APPENDIX A (continued)

Figure A31: MS Spectra for feature of m/z 542.3207Figure A32: MS Spectra for feature of m/z 482.3299

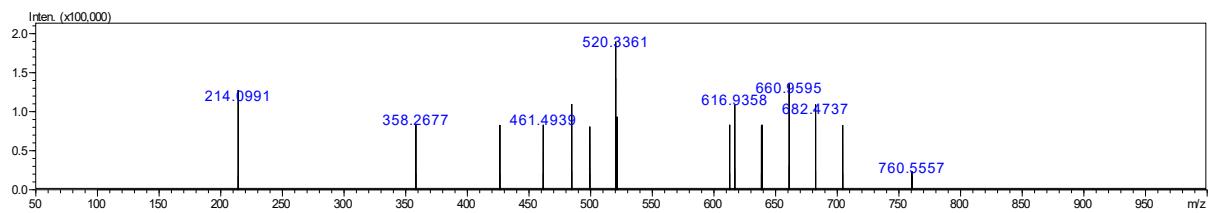
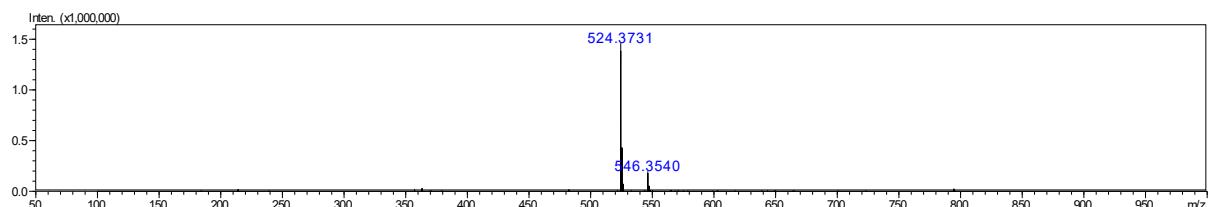
APPENDIX A (continued)

Figure A33: MS Spectra for feature of m/z 701.5846Figure A34: MS Spectra for feature of m/z 522.3566

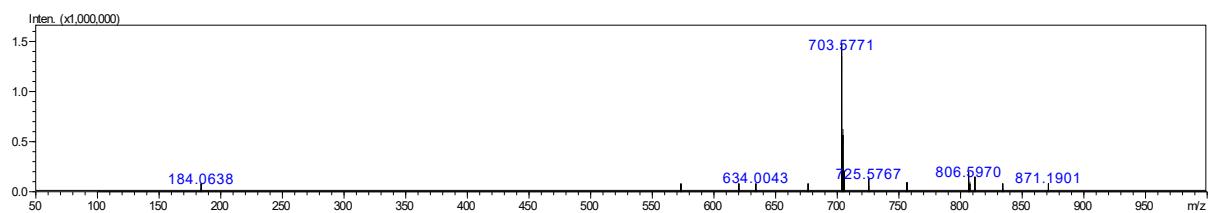
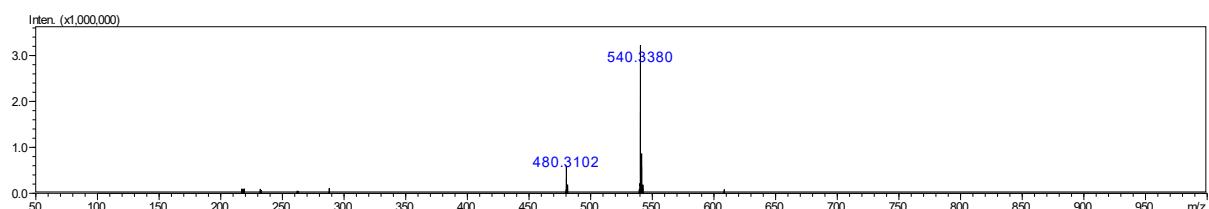
APPENDIX A (continued)

Figure A35: MS Spectra for feature of m/z 675.5518Figure A36: MS Spectra for feature of m/z 806.5766

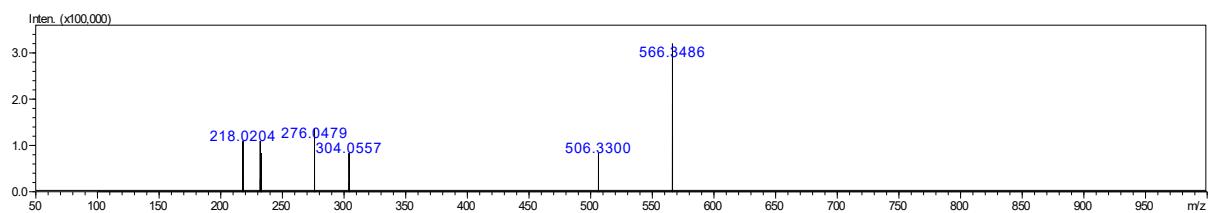
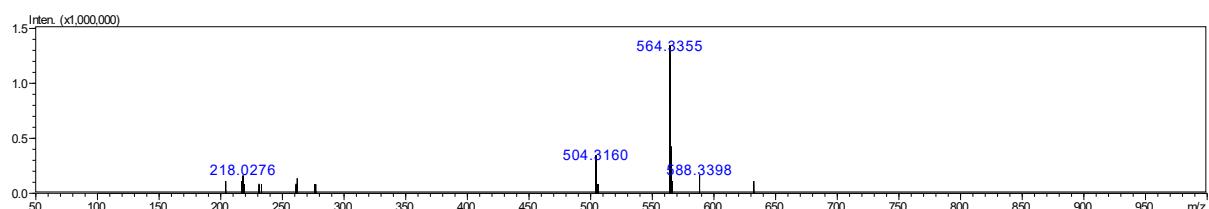
APPENDIX A (continued)

Figure A37: MS Spectra for feature of m/z 520.3361Figure A38: MS Spectra for feature of m/z 524.3731

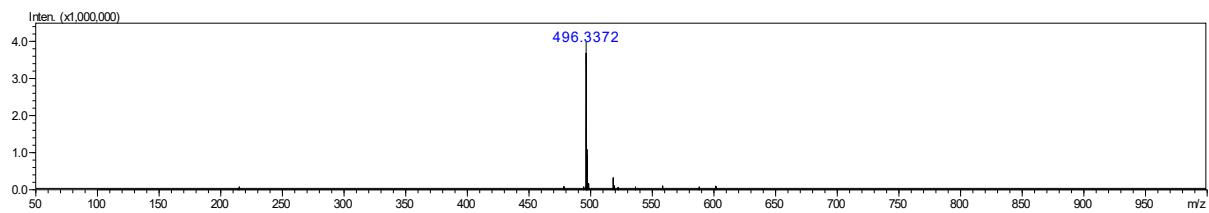
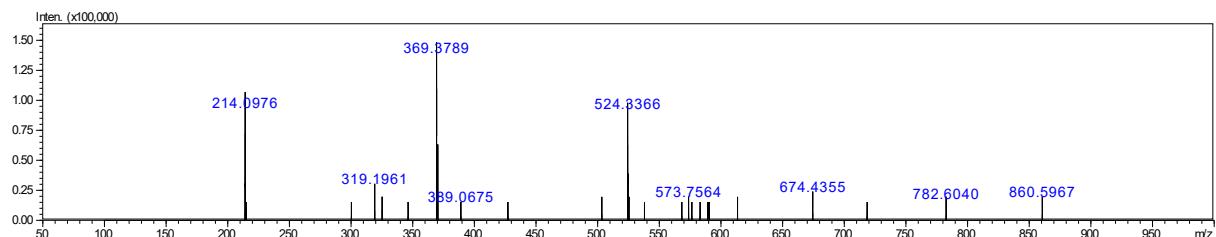
APPENDIX A (continued)

Figure A39: MS Spectra for feature of m/z 703.5771Figure A40: MS Spectra for feature of m/z 480.3102

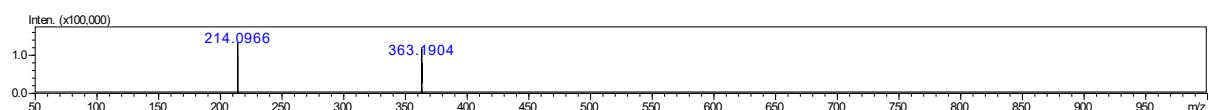
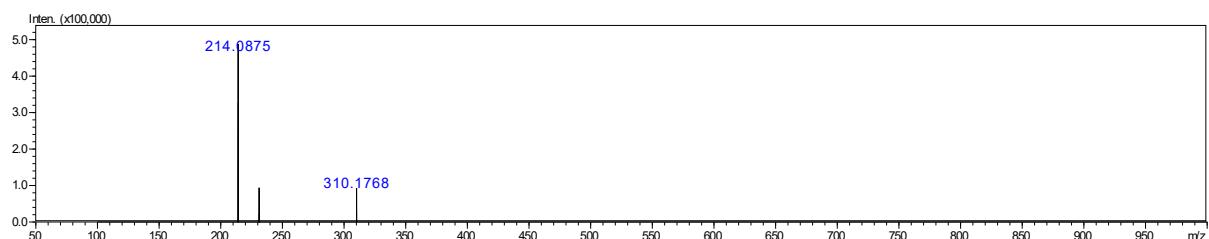
APPENDIX A (continued)

Figure A41: MS Spectra for feature of m/z 566.3486Figure A42: MS Spectra for feature of m/z 504.3160

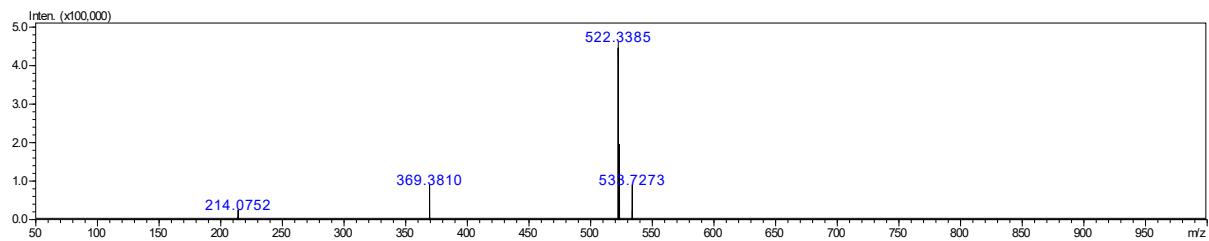
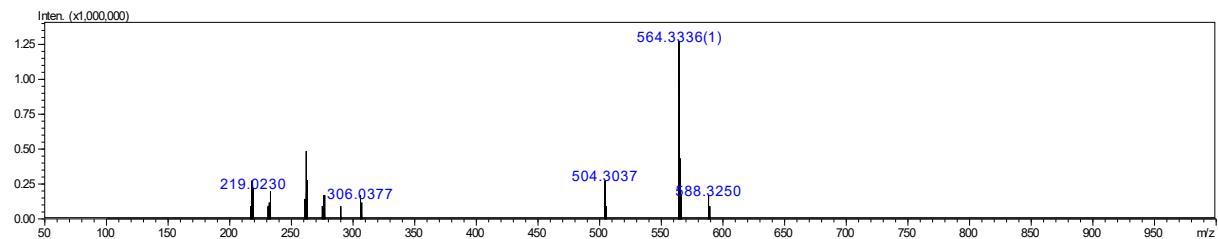
APPENDIX A (continued)

Figure A41: MS Spectra for feature of m/z 496.3372Figure A42: MS Spectra for feature of m/z 524.3366

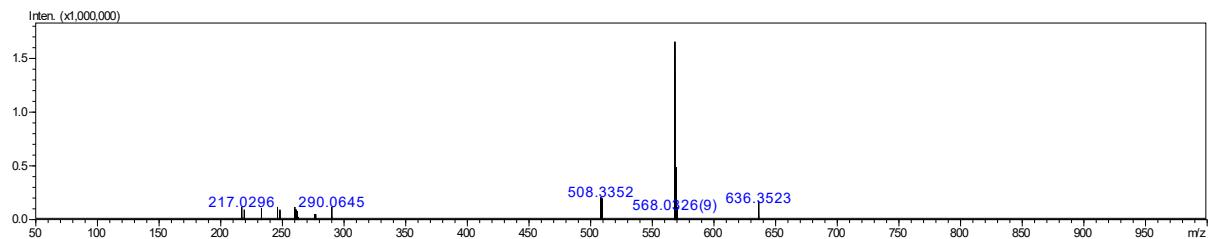
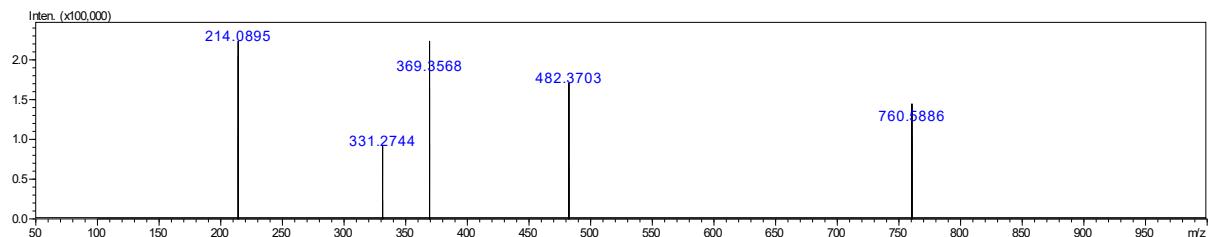
APPENDIX A (continued)

Figure A43: MS Spectra for feature of m/z 363.1904Figure A44: MS Spectra for feature of m/z 310.1768

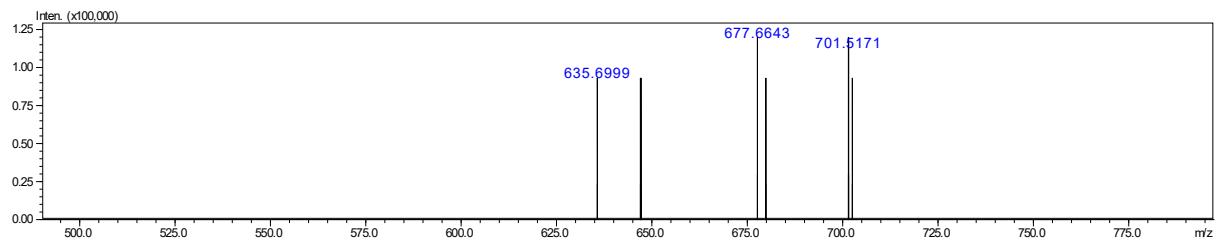
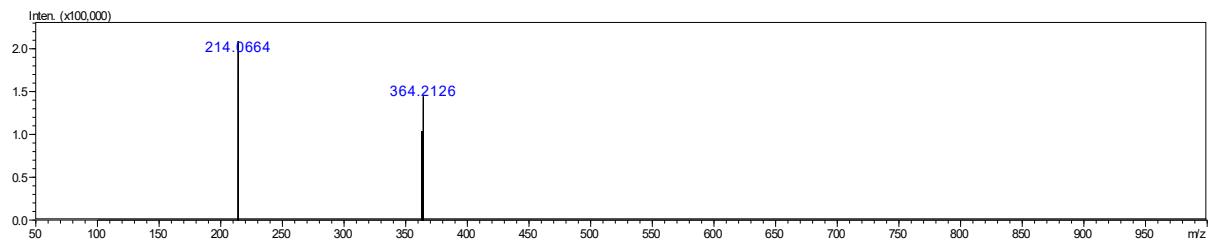
APPENDIX A (continued)

Figure A45: MS Spectra for feature of m/z 522.3385Figure A46: MS Spectra for feature of m/z 504.3037

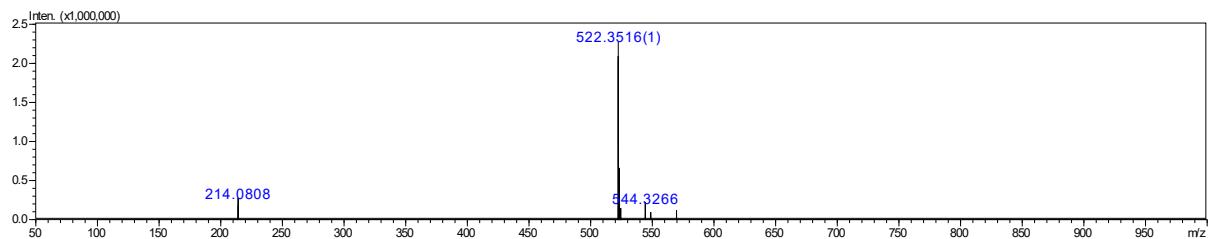
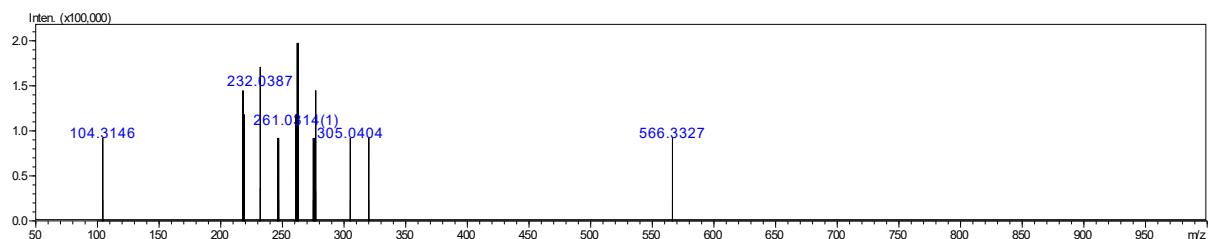
APPENDIX A (continued)

Figure A47: MS Spectra for feature of m/z 508.3352Figure A48: MS Spectra for feature of m/z 482.3703

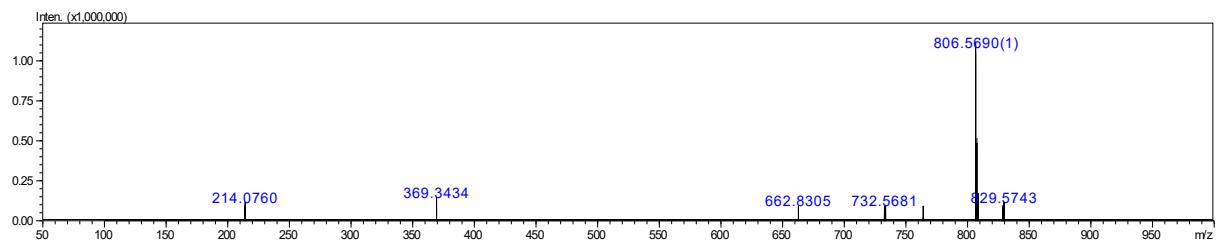
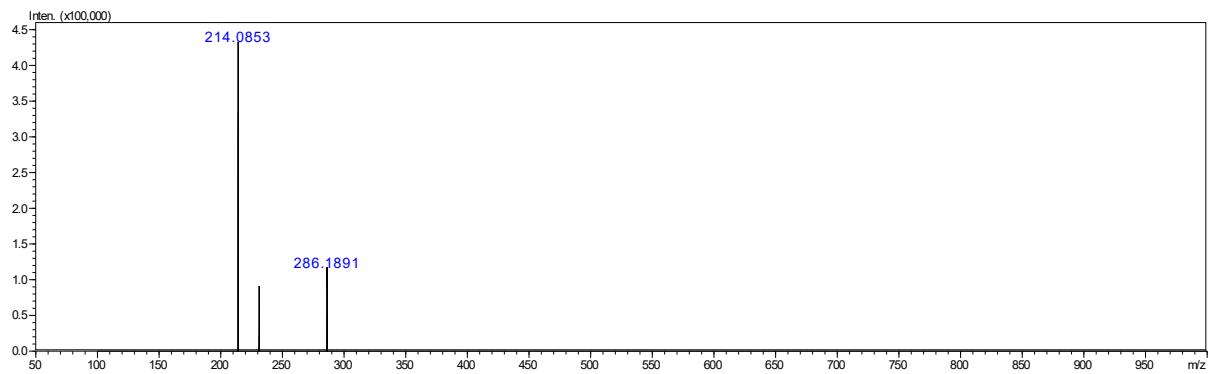
APPENDIX A (continued)

Figure A49: MS Spectra for feature of m/z 701.5171Figure A50: MS Spectra for feature of m/z 364.2126

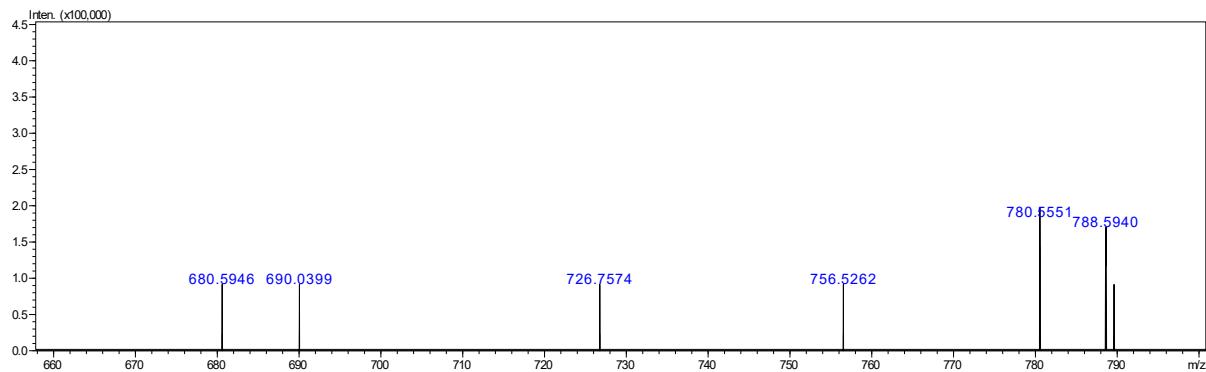
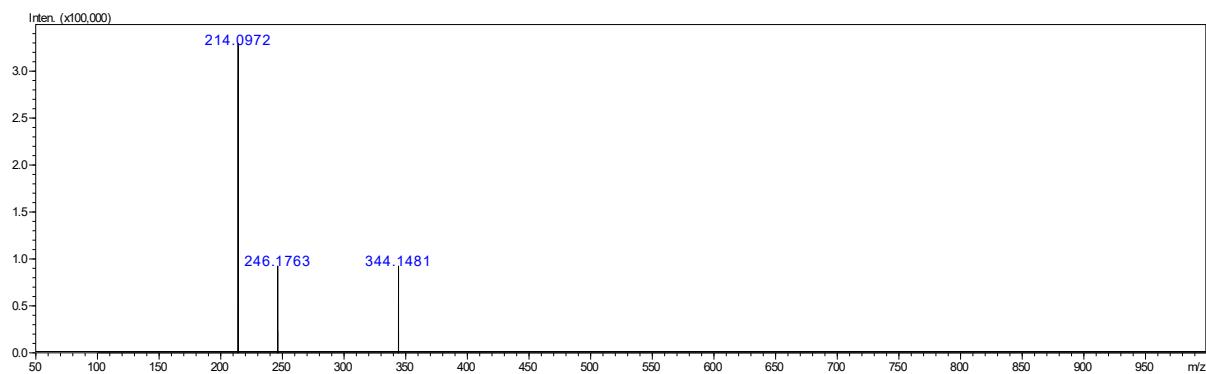
APPENDIX A (continued)

Figure A51: MS Spectra for feature of m/z 544.3266Figure A52: MS Spectra for feature of m/z 566.3327

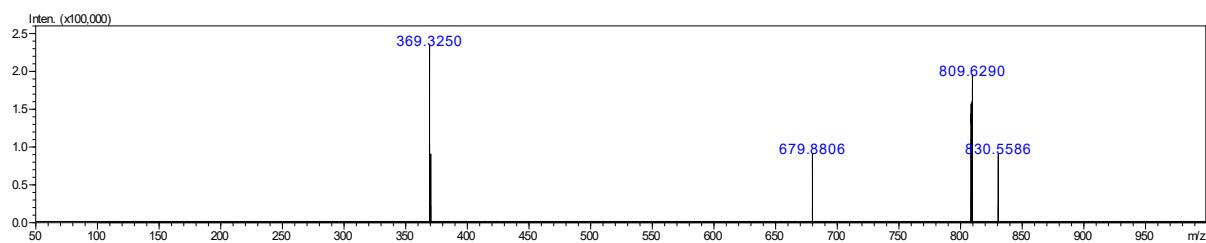
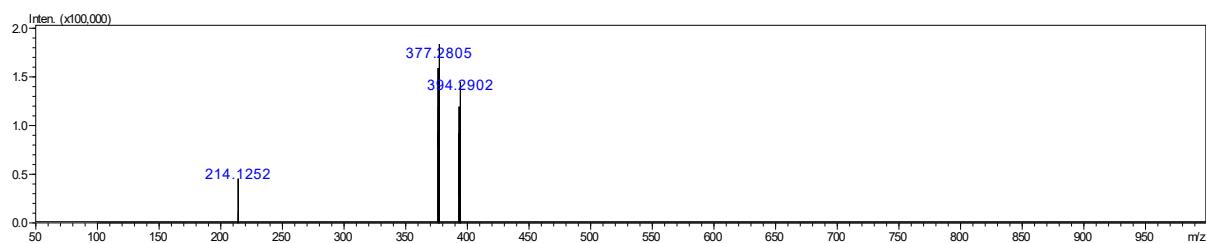
APPENDIX A (continued)

Figure A53: MS Spectra for feature of m/z 732.5681Figure A54: MS Spectra for feature of m/z 286.1891

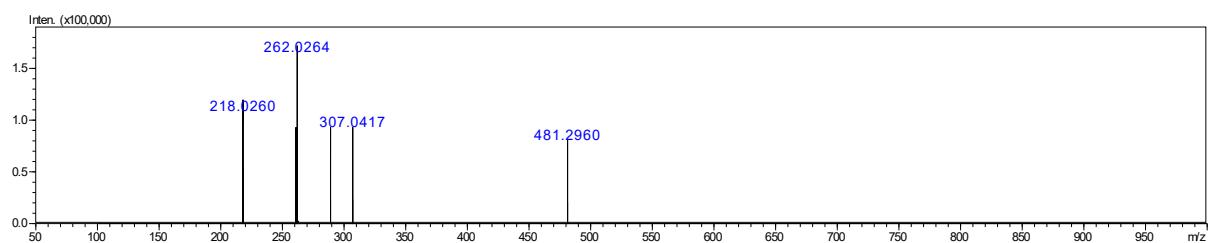
APPENDIX A (continued)

Figure A55: MS Spectra for feature of m/z 756/5262Figure A56: MS Spectra for feature of m/z 246.1763

APPENDIX A (continued)

Figure A57: MS Spectra for feature of m/z 809.6290Figure A58: MS Spectra for feature of m/z 394.2902

APPENDIX A (continued)

Figure A59: MS Spectra for feature of m/z 481.2960

APPENDIX B: MS/MS SPECTRA OF PEPTIDE HITS

Figure B1: MS/MS spectra of peptide identified as vitronectin FEDGVLDPDYPR

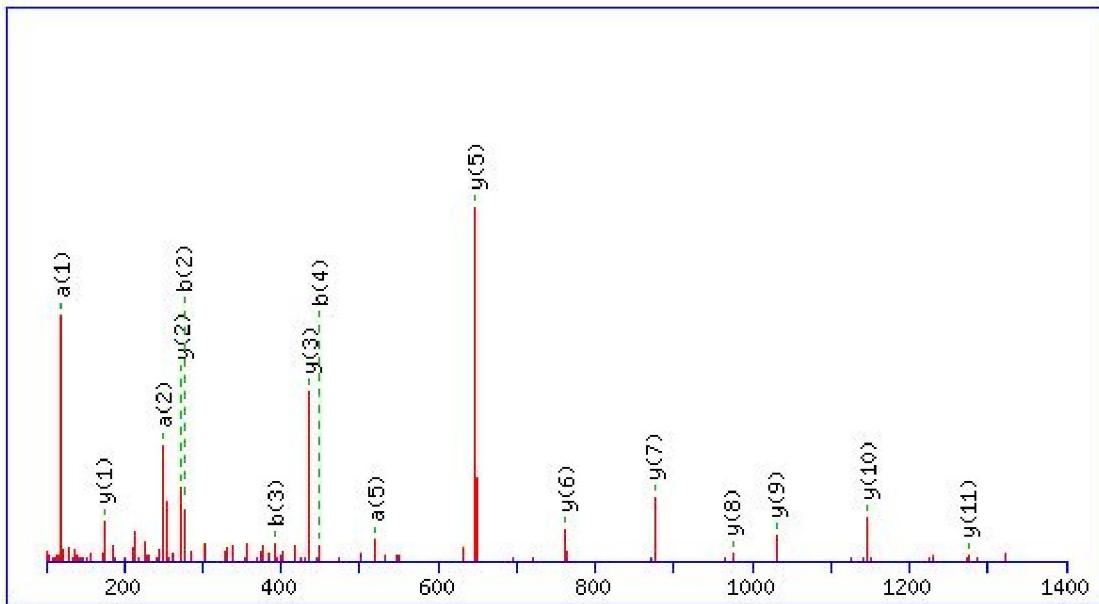
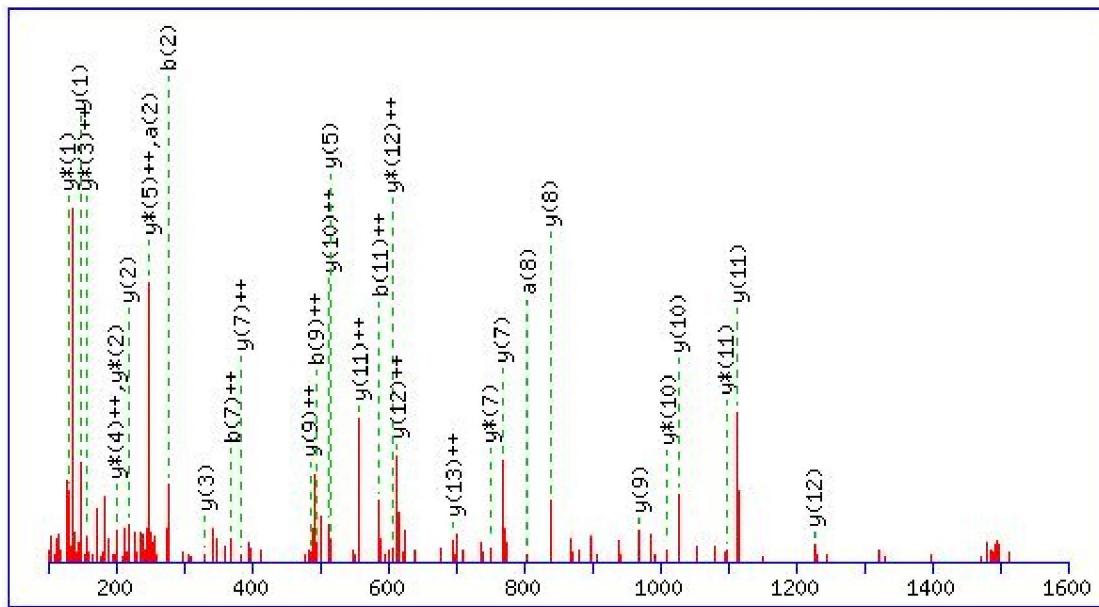


Figure B2: MS/MS spectra of peptide identified as vitronectin IYISGMAPRPSLAK



APPENDIX B (continued)

Figure B3: MS/MS spectra of peptide identified as vitronectin DVWGIEGPIDAAFTR

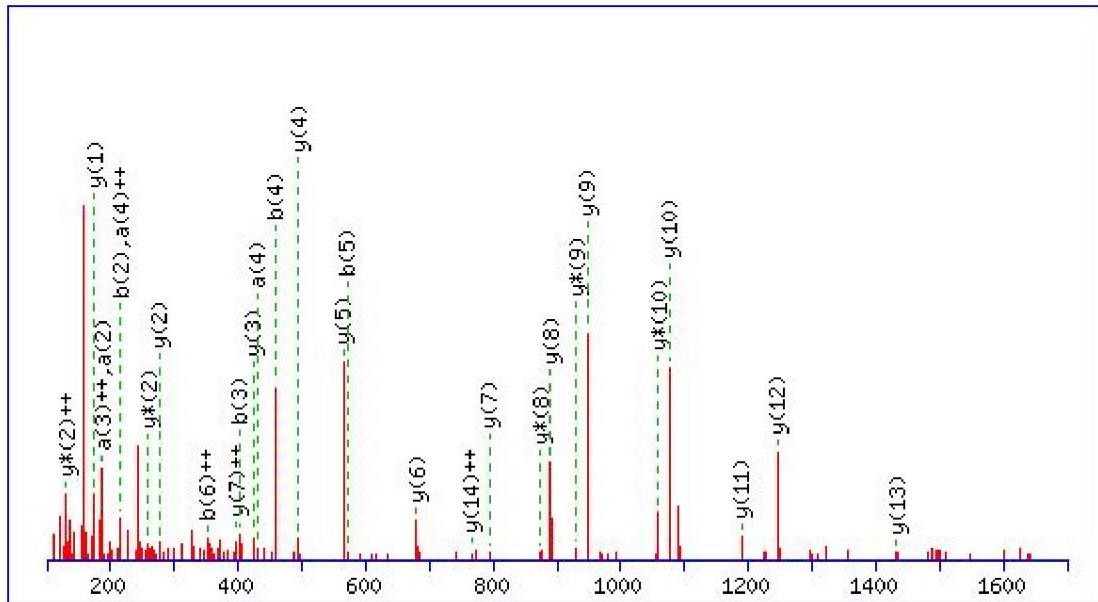
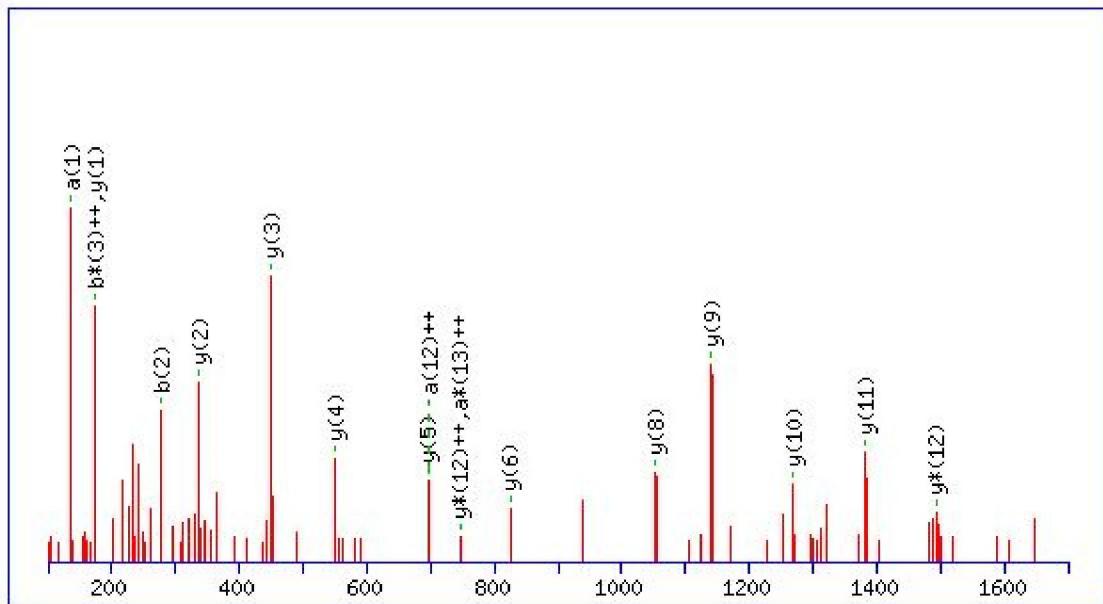


Figure B4: MS/MS spectra of peptide identified as kininogen YNSQNQSNQFVLYR



APPENDIX B (continued)

Figure B5: MS/MS spectra of peptide identified as kininogen DIPTNSPELEETLTHTITK

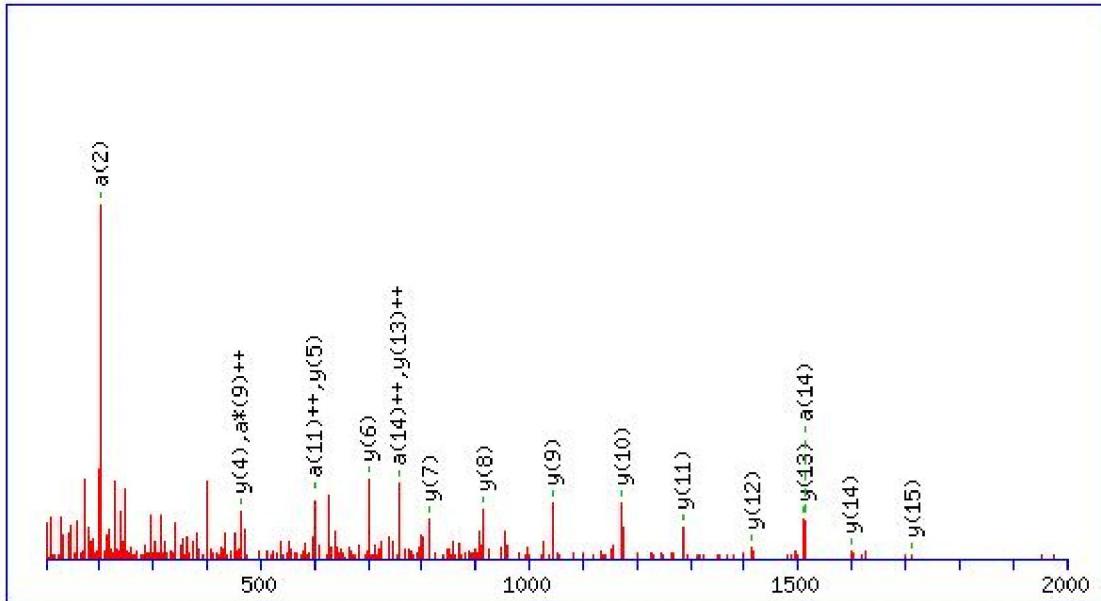
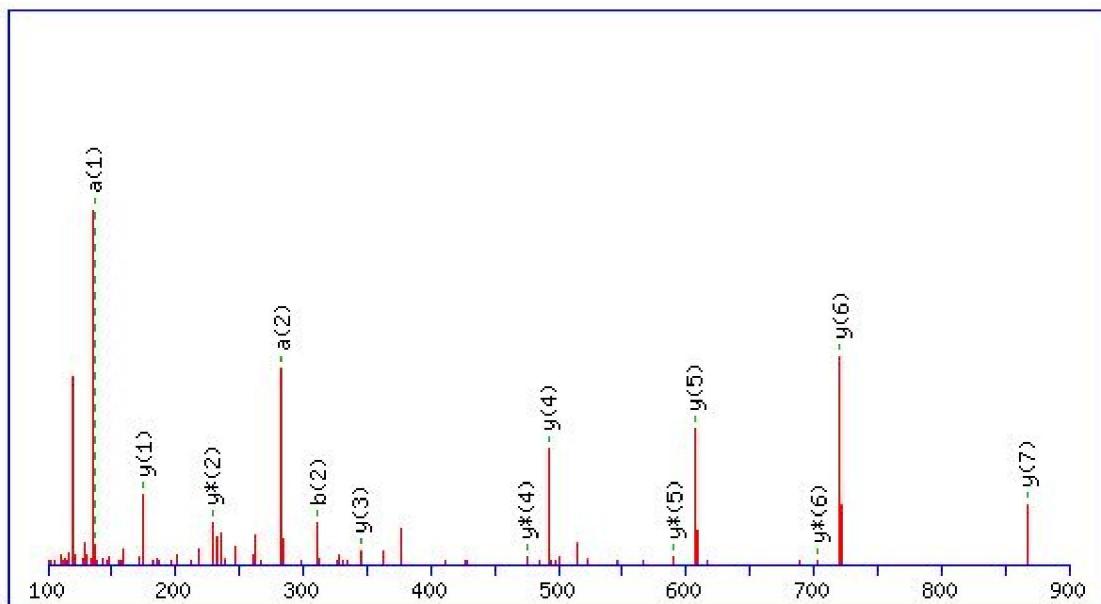


Figure B6: MS/MS spectra of peptide identified as kininogen YFIDFVAR



APPENDIX B (continued)

Figure B7: MS/MS spectra of peptide identified as plasminogen NPDGDVGGPWCYTTNPR

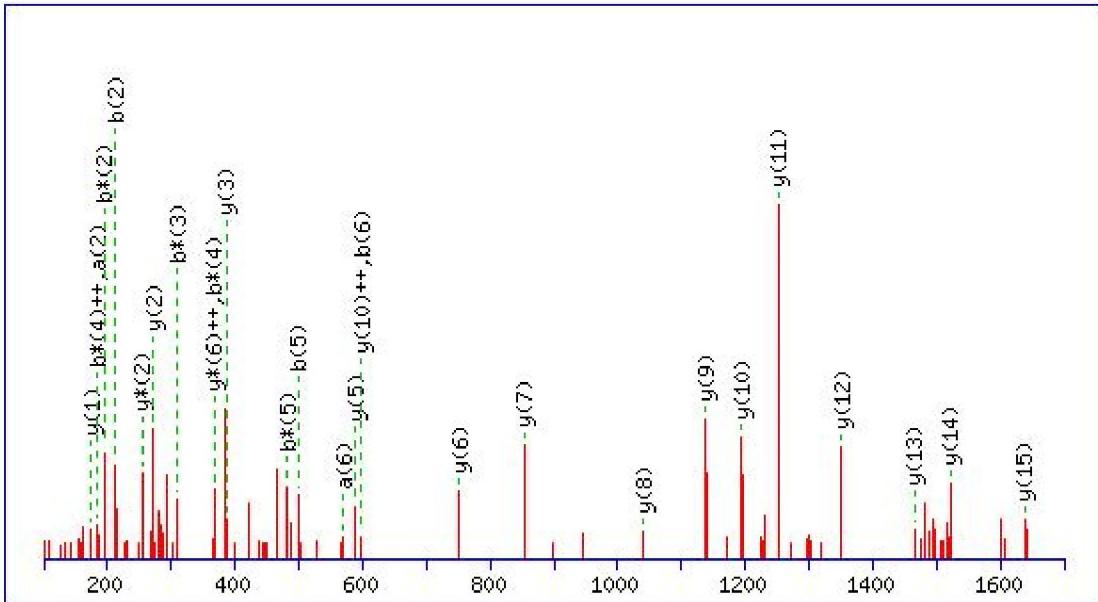
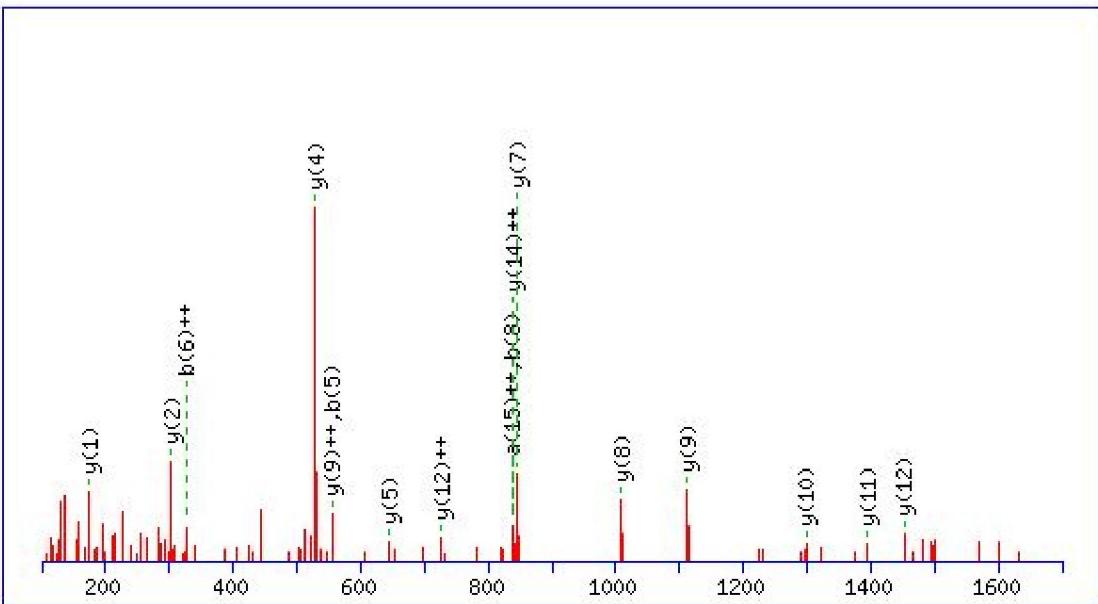


Figure B8: MS/MS spectra of peptide identified as plasminogen NPDNDPQGPWCYTTDPEKR



APPENDIX B (continued)

Figure B9: MS/MS spectra of peptide identified as plasminogen ATTVTGTPCQDWAAQEPHR

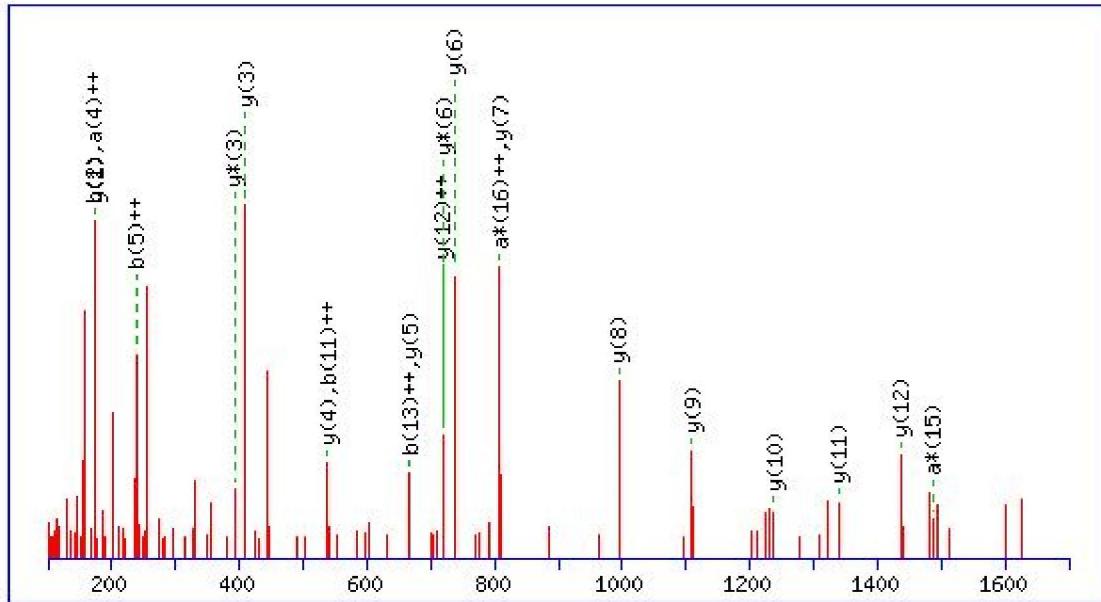
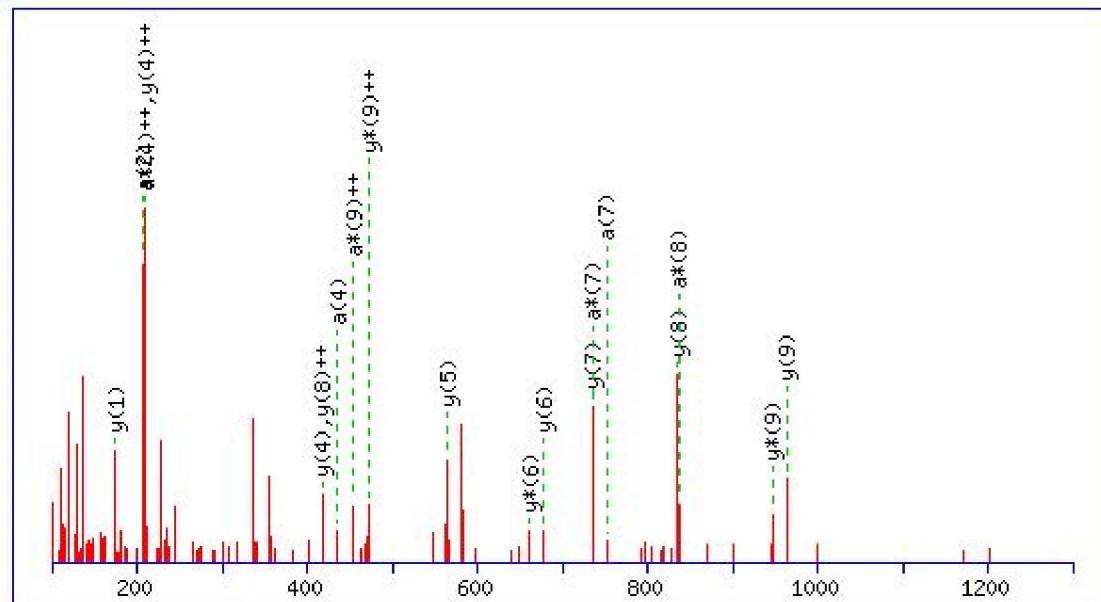


Figure B10: MS/MS spectra of peptide identified as vitamin k dependent protein s SFQTGLFTAAR



APPENDIX B (continued)

Figure B11: MS/MS spectra of peptide identified as vitamin k dependent protein s
SEELAALSSQQPEKVMAK

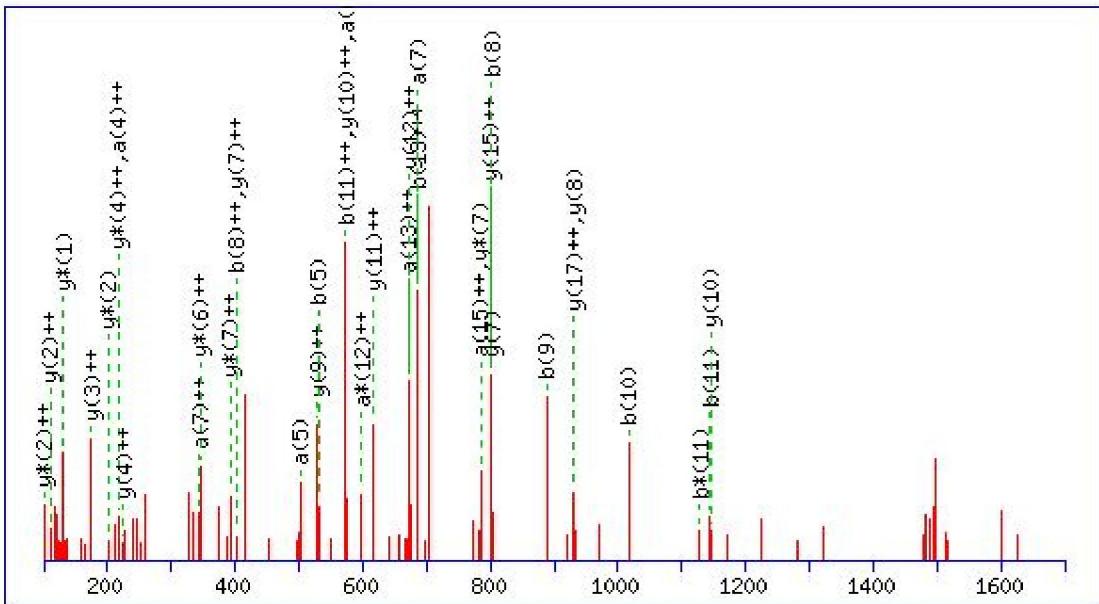
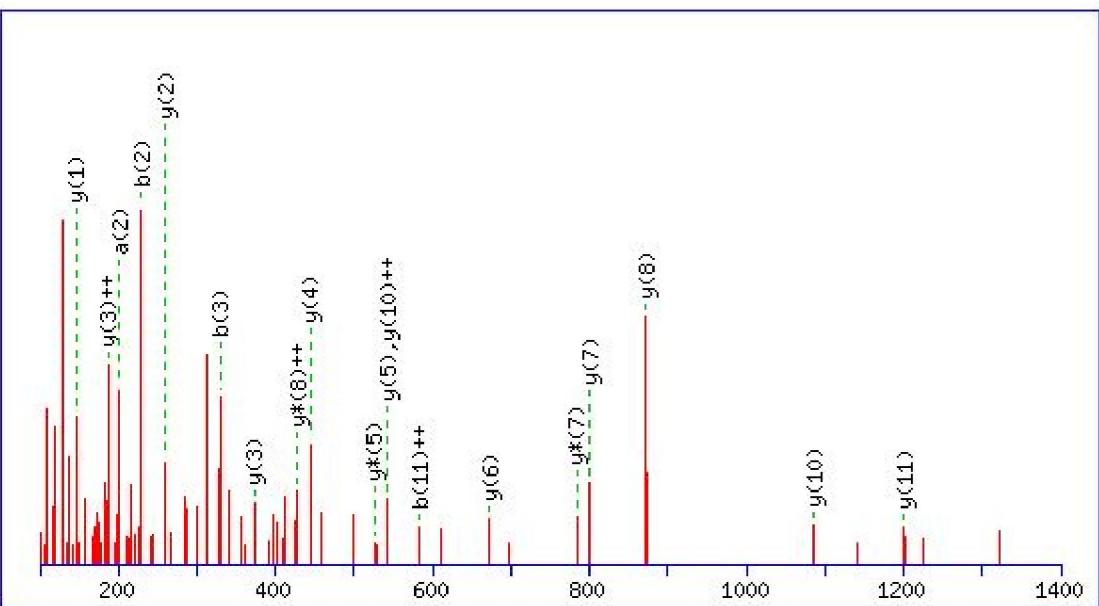


Figure B12: MS/MS spectra of peptide identified as vitamin k dependant protein s LDTLAQEVALLK



APPENDIX B (continued)

Figure B13: MS/MS spectra of peptide identified as ceruloplasmin GAYPLSIEPIGVR

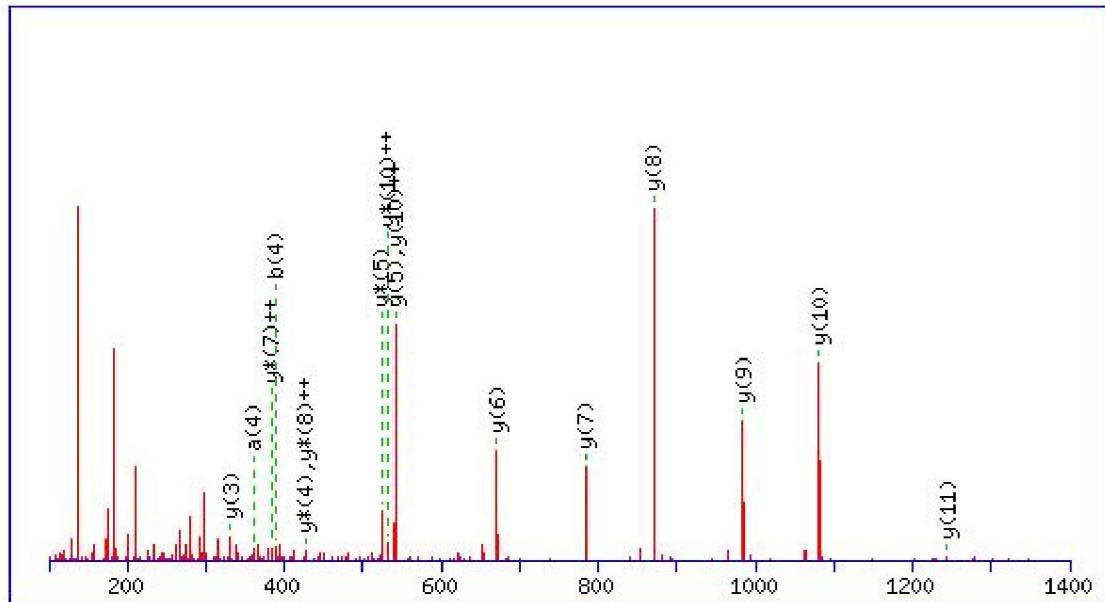
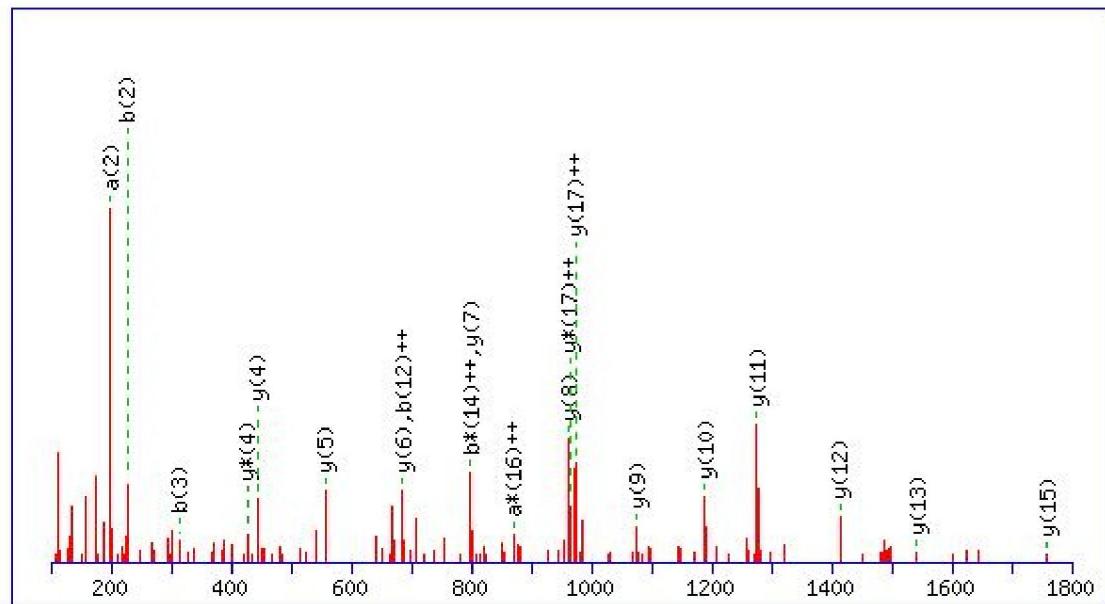


Figure B14: MS/MS spectra of peptide identified as ceruloplasmin LISVDTEHSNIYLQNGPDR



APPENDIX B (continued)

Figure B15: MS/MS spectra of peptide identified as ceruloplasmin DVDKEFYLFP-TVFDENESLLLEDNIR

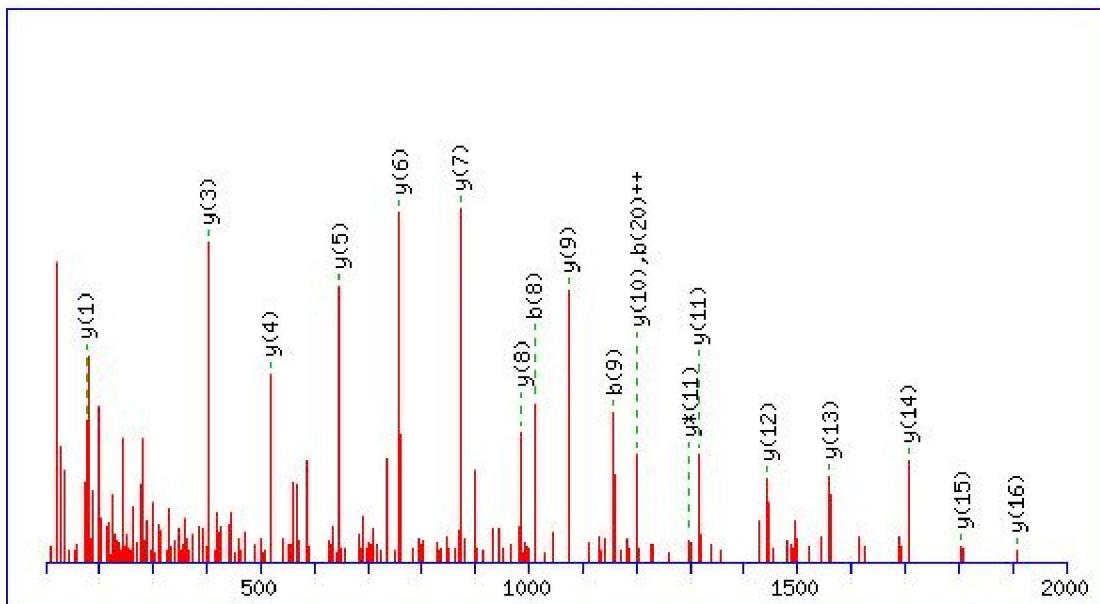
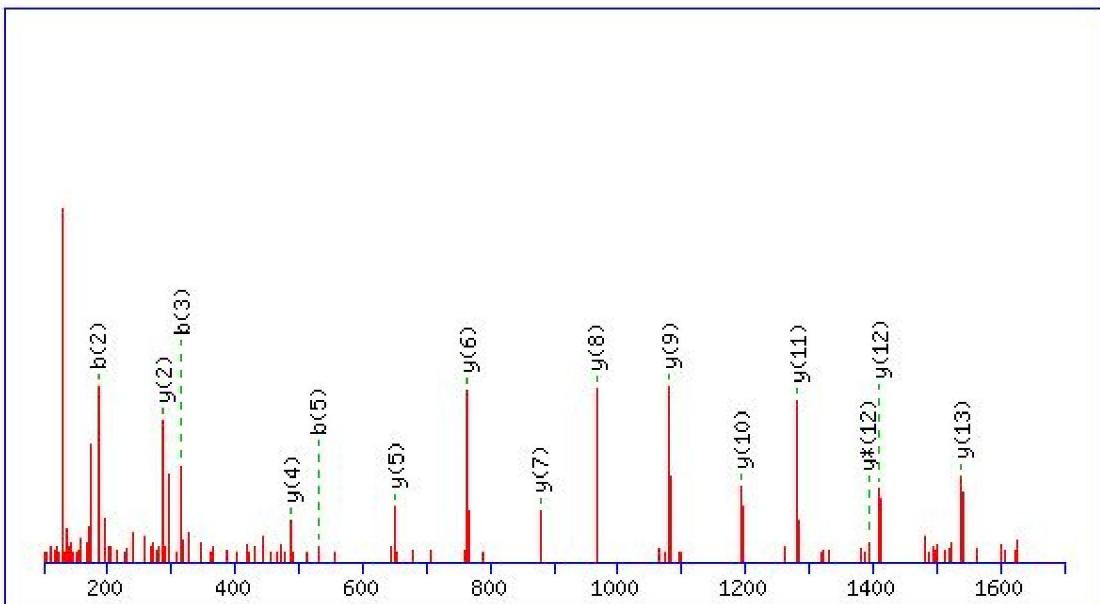


Figure B16: MS/MS spectra of peptide identified as platelet basic protein GKEESLDSDL-YAELR



APPENDIX B (continued)

Figure B17: MS/MS spectra of peptide identified as platelet basic protein AEAESLYQSKYELQITAGR

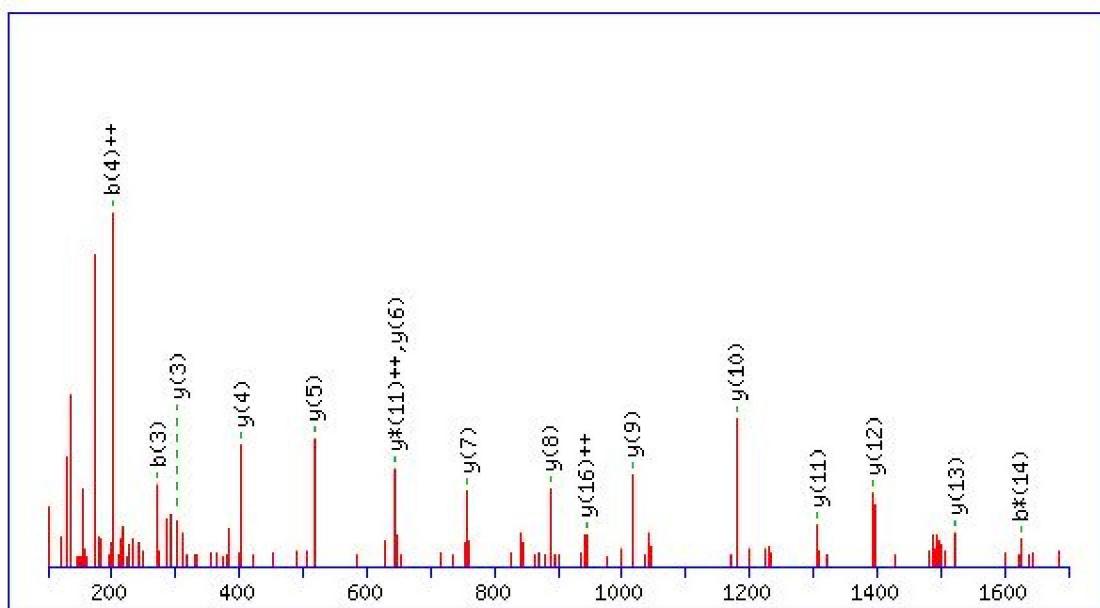
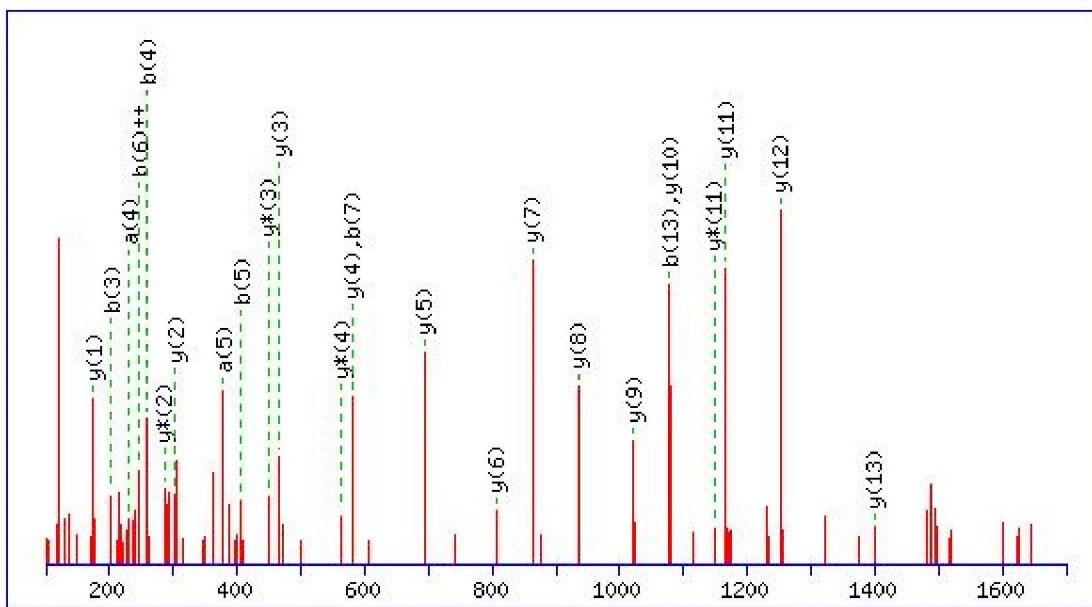


Figure B18: MS/MS spectra of peptide identified as platelet basic protein SGGGFSSGSAGIINYQR



APPENDIX B (continued)

Figure B19: MS/MS spectra of peptide identified as thrombospondin TIVTTLQDSIR

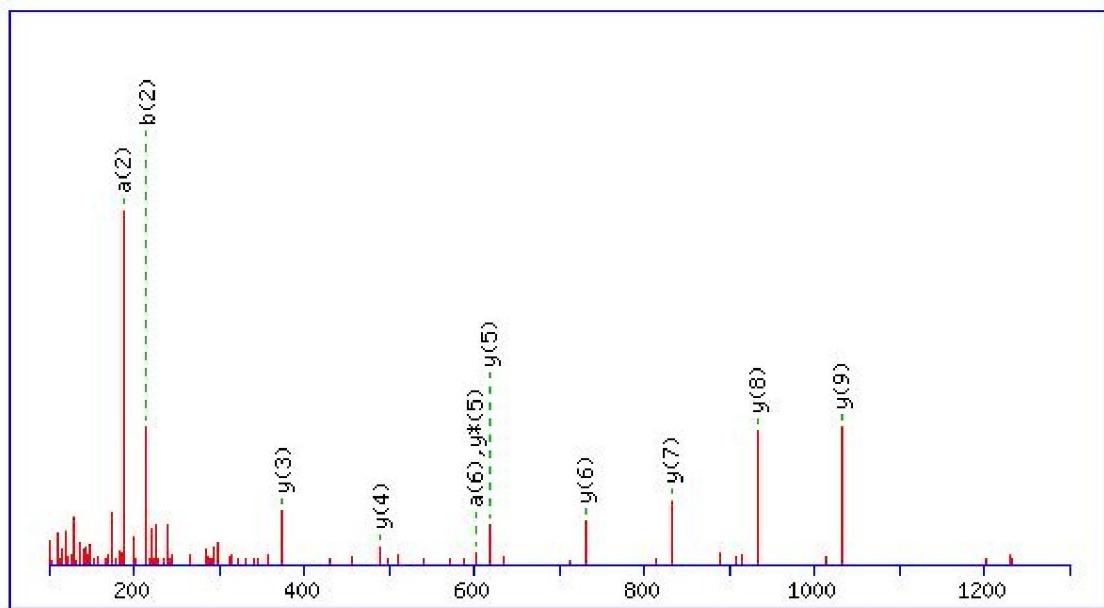
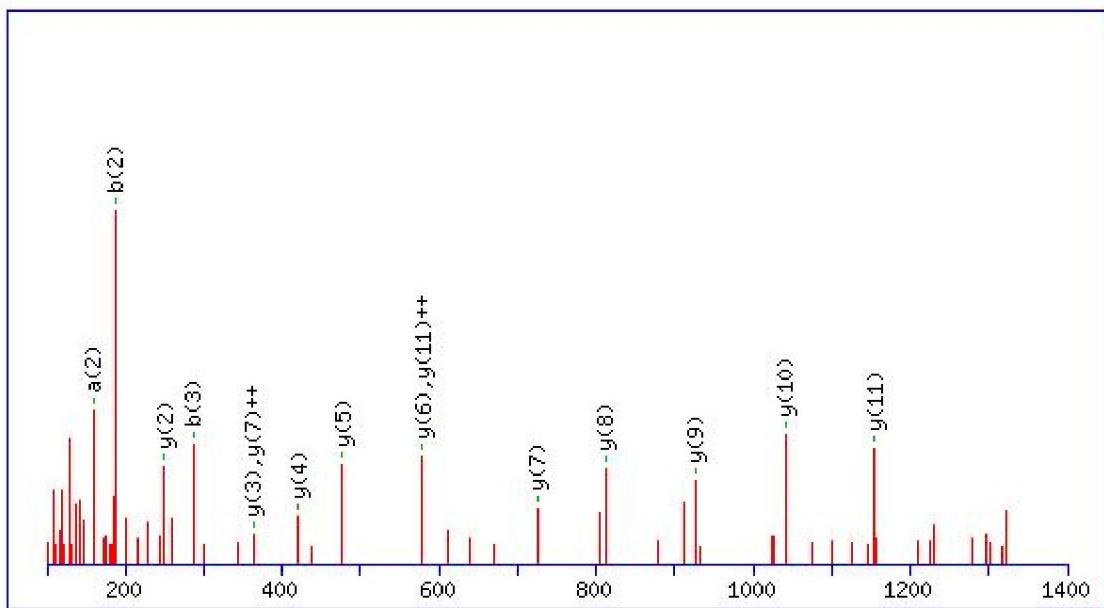


Figure B20: MS/MS spectra of peptide identified as thrombospondin GEVLDNSFTGGICK



APPENDIX B (continued)

Figure B21: MS/MS spectra of peptide identified as thrombospondin
HQGTITVNEEGTQATTVTTVGFMPPLSTQVR

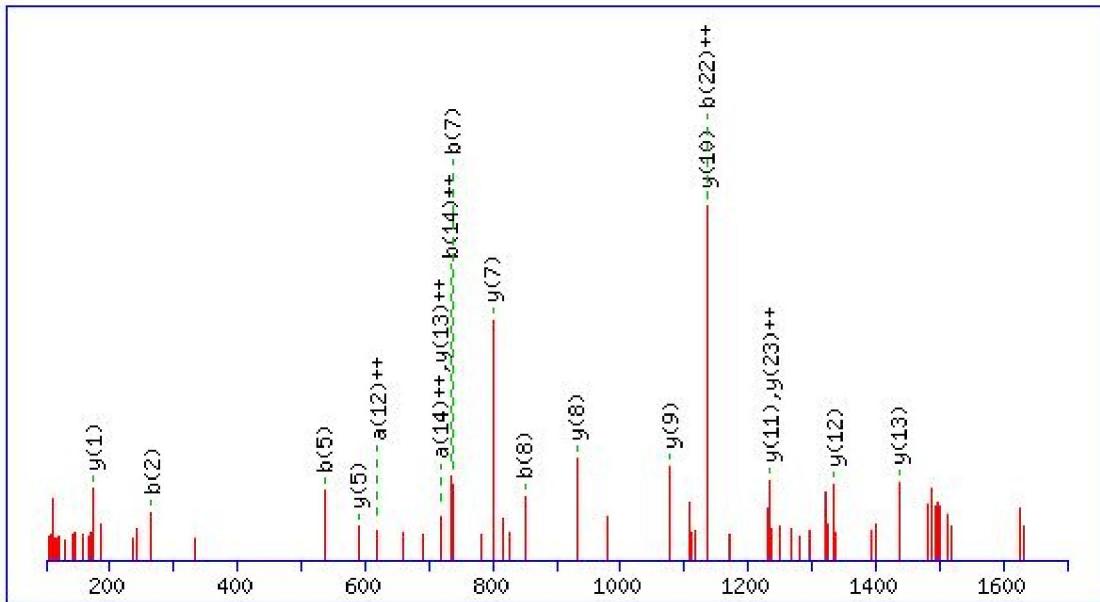
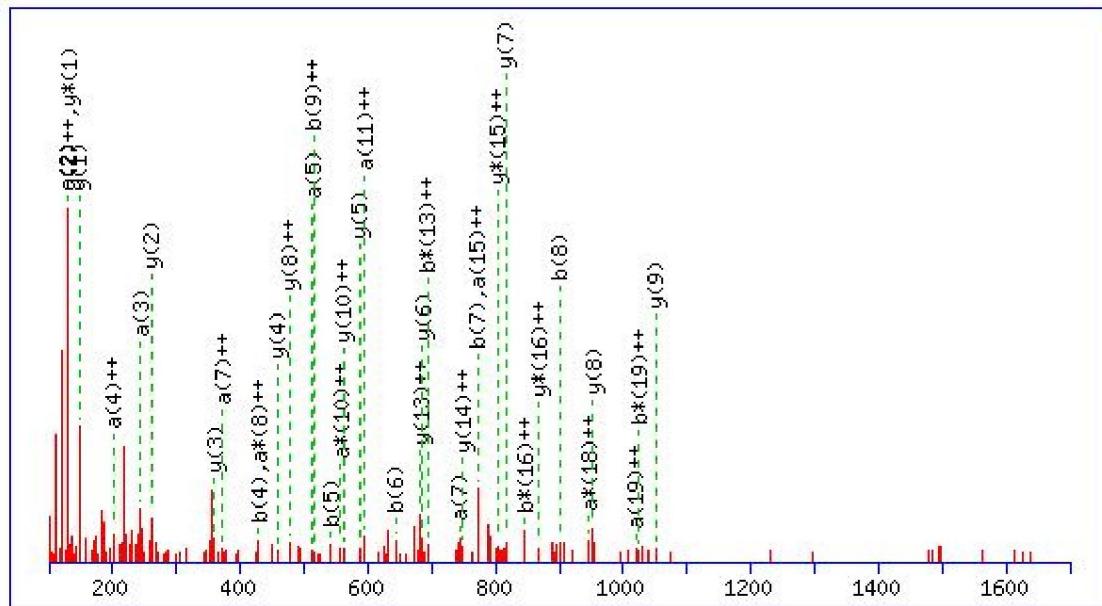


Figure B22: MS/MS spectra of peptide identified as coagulation factor x VGDRNTEQEEGGEAVHEVEVVIK



APPENDIX B (continued)

Figure B23: MS/MS spectra of peptide identified as coagulation factor x FDEFFSEGCAPGSK

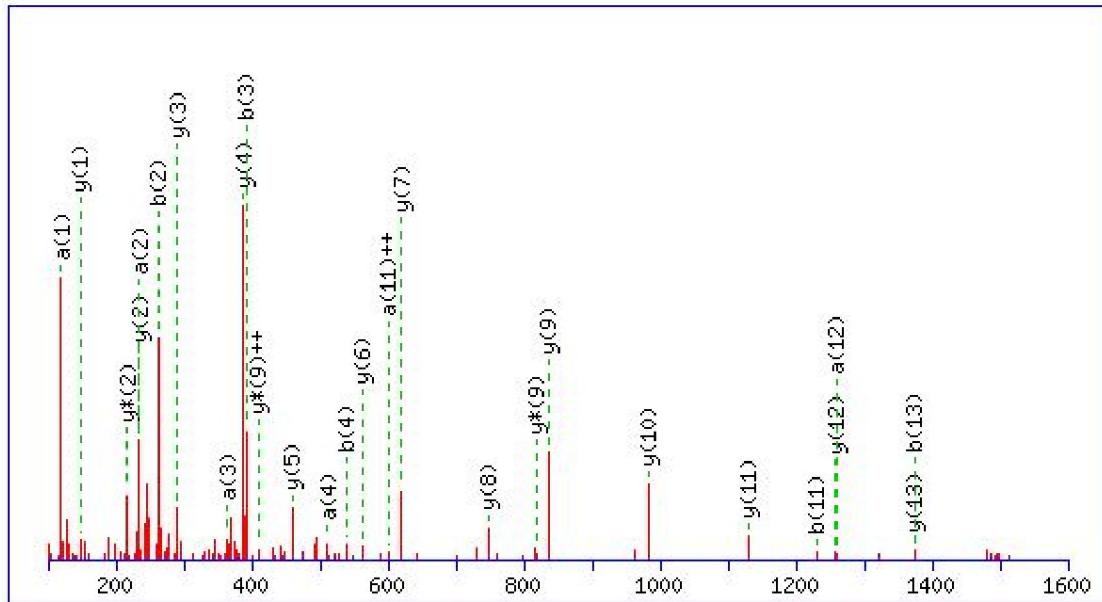
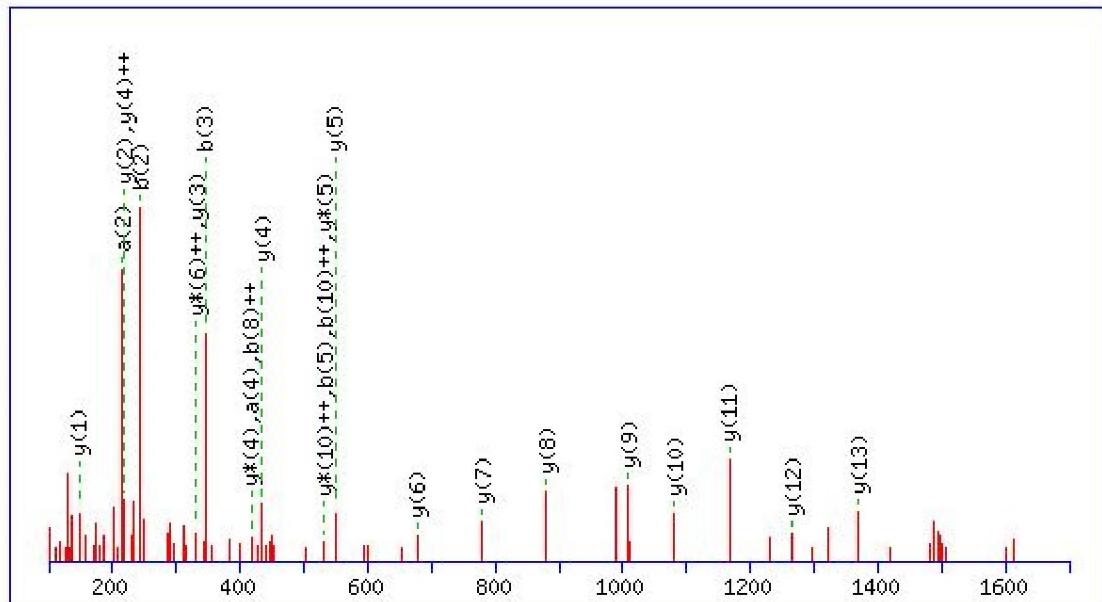


Figure B24: MS/MS spectra of peptide identified as coagulation factor x IECVSAETTEDCIAK



APPENDIX B (continued)

Figure B25: MS/MS spectra of peptide identified as fibronectin TKTETITGFQVDAVPANGQTPIQR

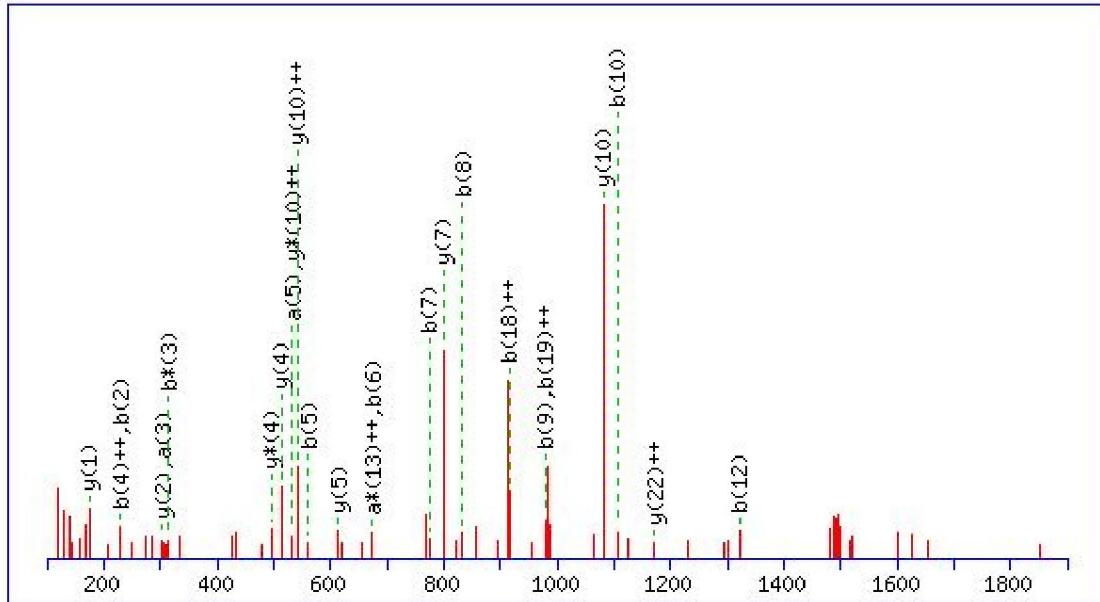
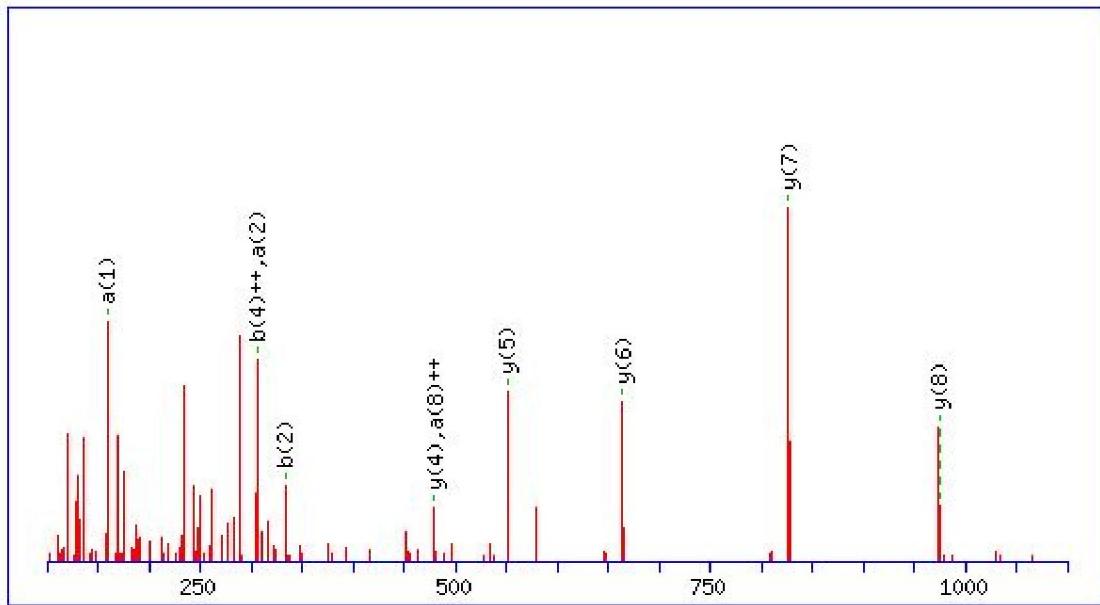


Figure B26: MS/MS spectra of peptide identified as fibronectin WFYIASAFR



APPENDIX B (continued)

Figure B27: MS/MS spectra of peptide identified as fibronectin LNSQRLVFNRPFLMFIVDNNILFLGVNRP

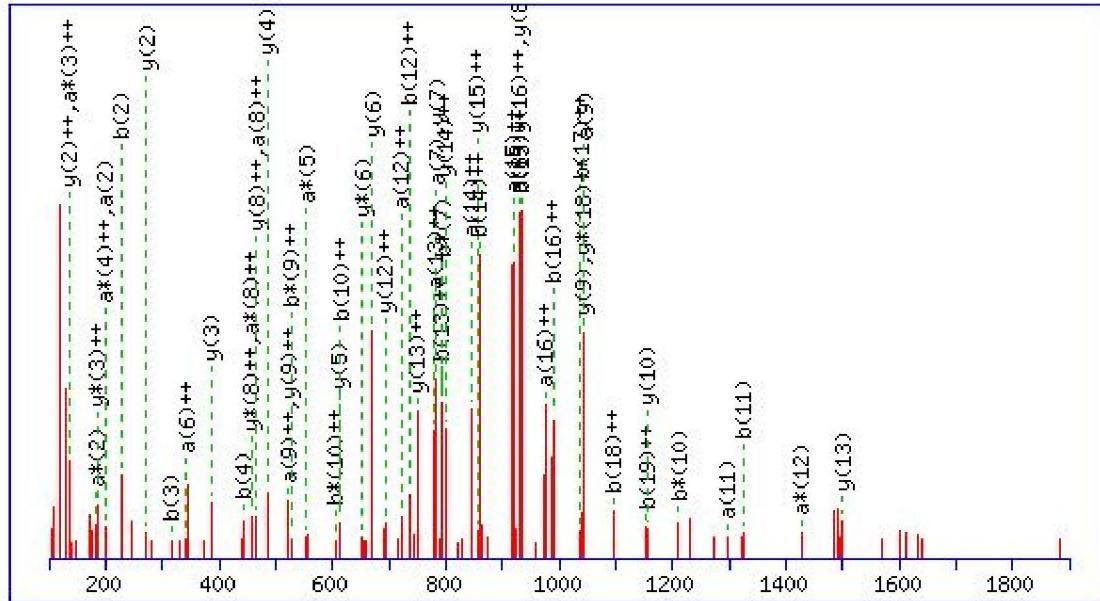
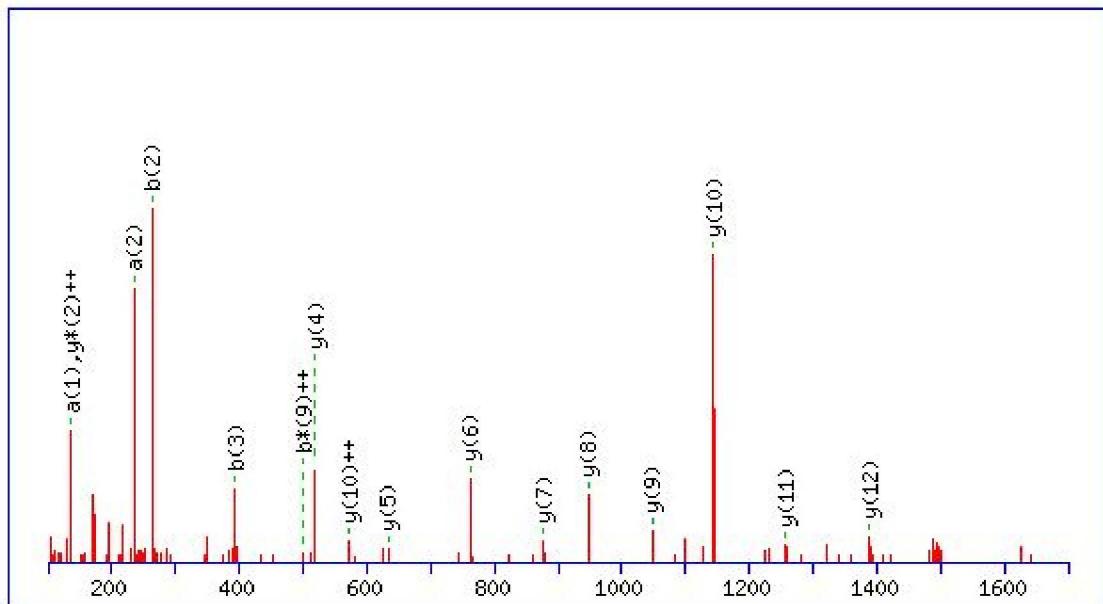


Figure B28: MS/MS spectra of peptide identified as matrix metalloprotease 2 YVMLPVADQDQCIR



APPENDIX B (continued)

APPENDIX B (continued)

Figure B29: MS/MS spectra of peptide identified as matrix metalloprotease 2 VMPICLPSKDYAEVGR

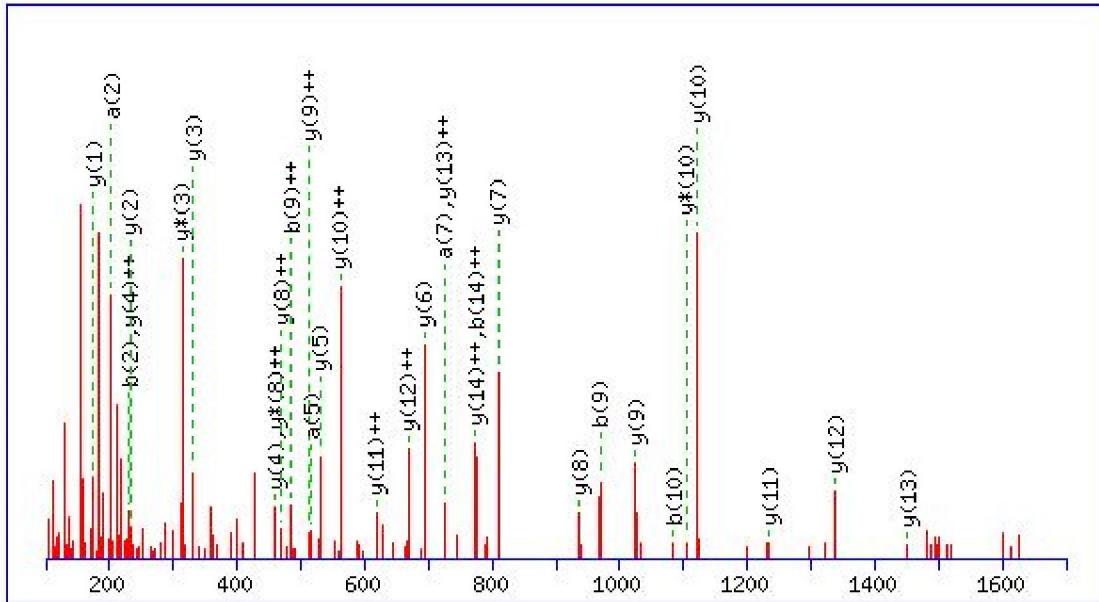
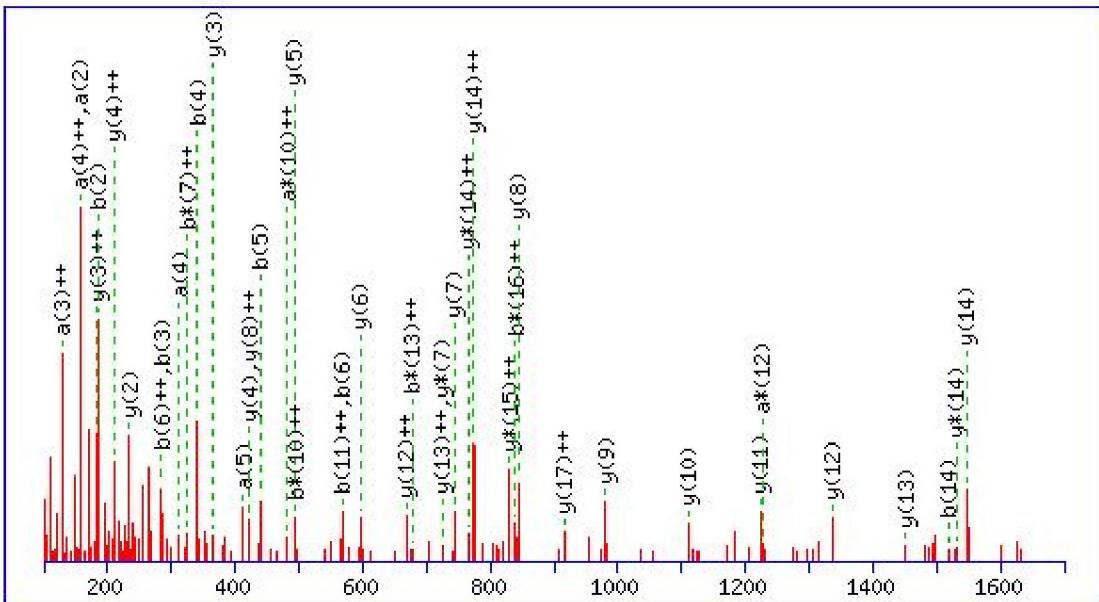


Figure B30: MS/MS spectra of peptide identified as matrix metalloprotease 2 SPVGVQPILN



APPENDIX B (continued)

Figure B31: MS/MS spectra of peptide identified as hyaluron binding protein LYLVQGTQVYVFLTK

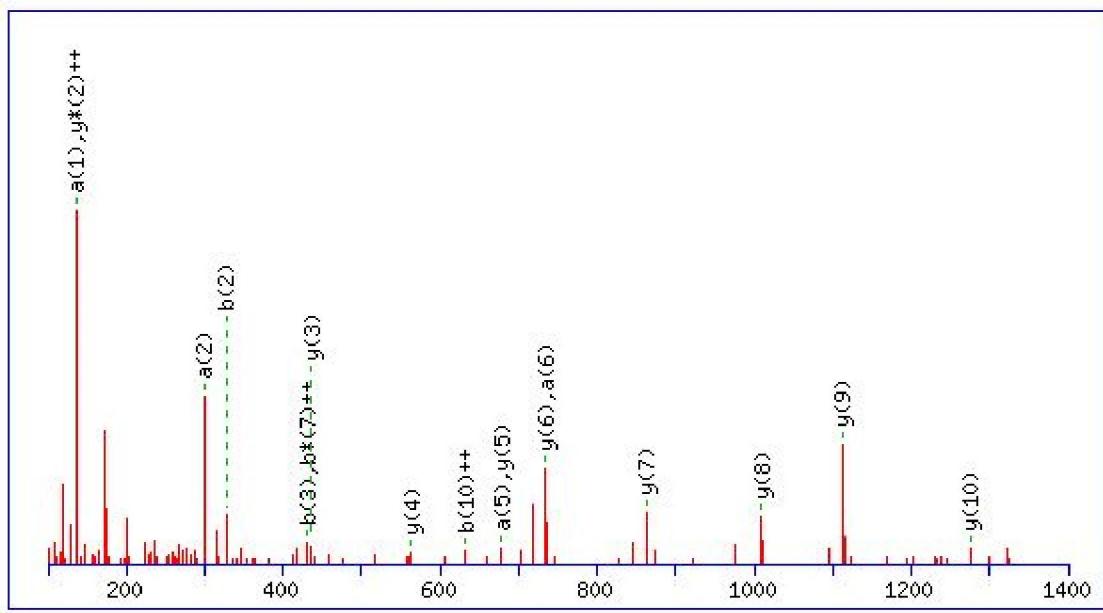
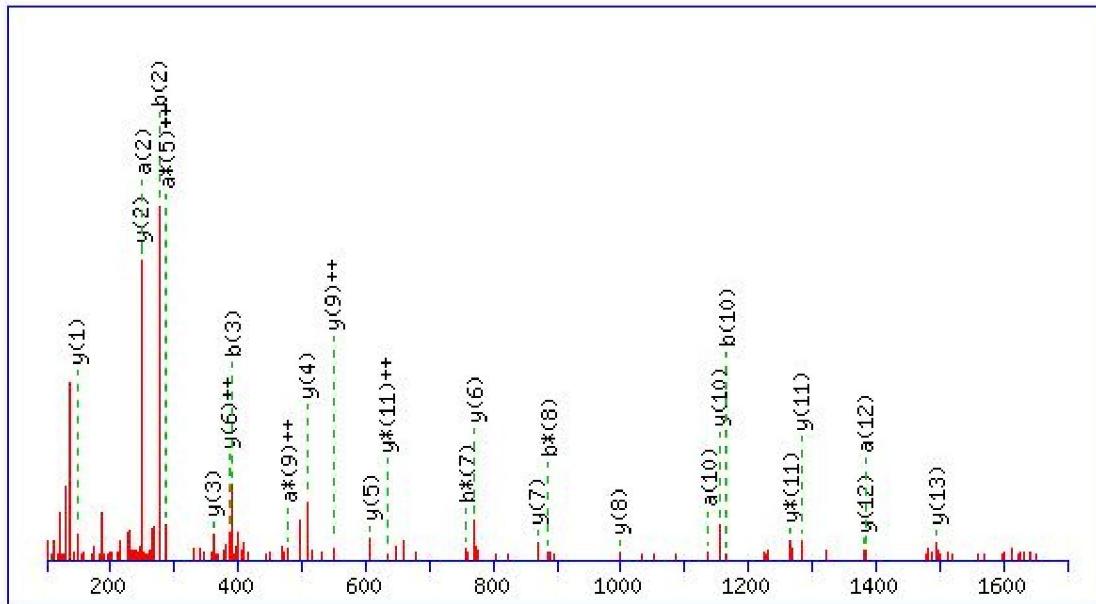


Figure B32: MS/MS spectra of peptide identified as hyaluron binding protein LYLVQGTQVYVFLTK



APPENDIX B (continued)

Figure B33: MS/MS spectra of peptide identified as hyaluron binding protein LLQDEFPGIPSPLDAAVECHR

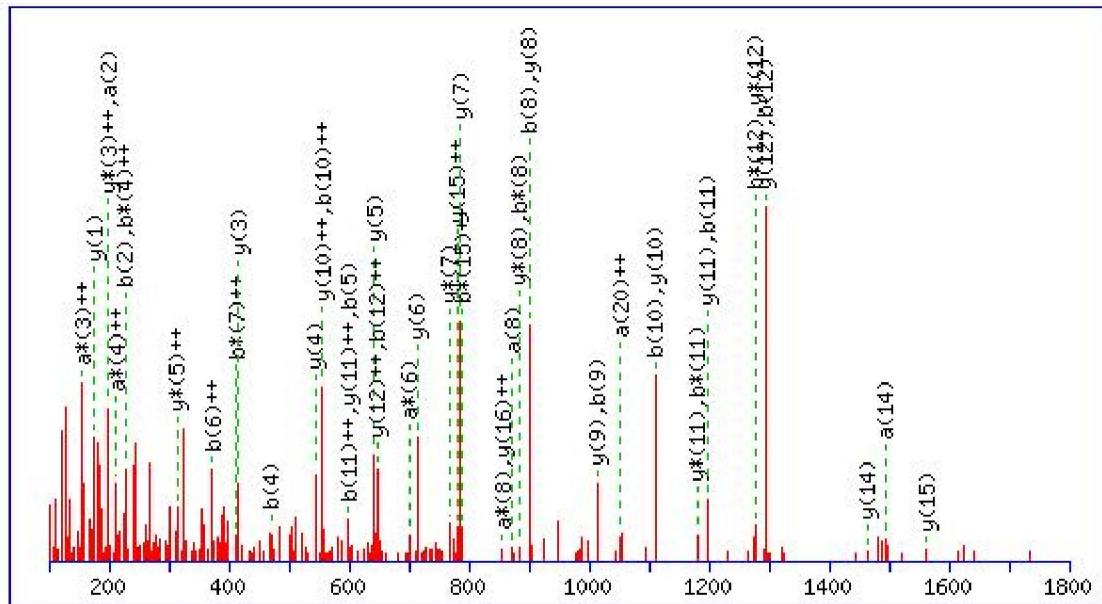
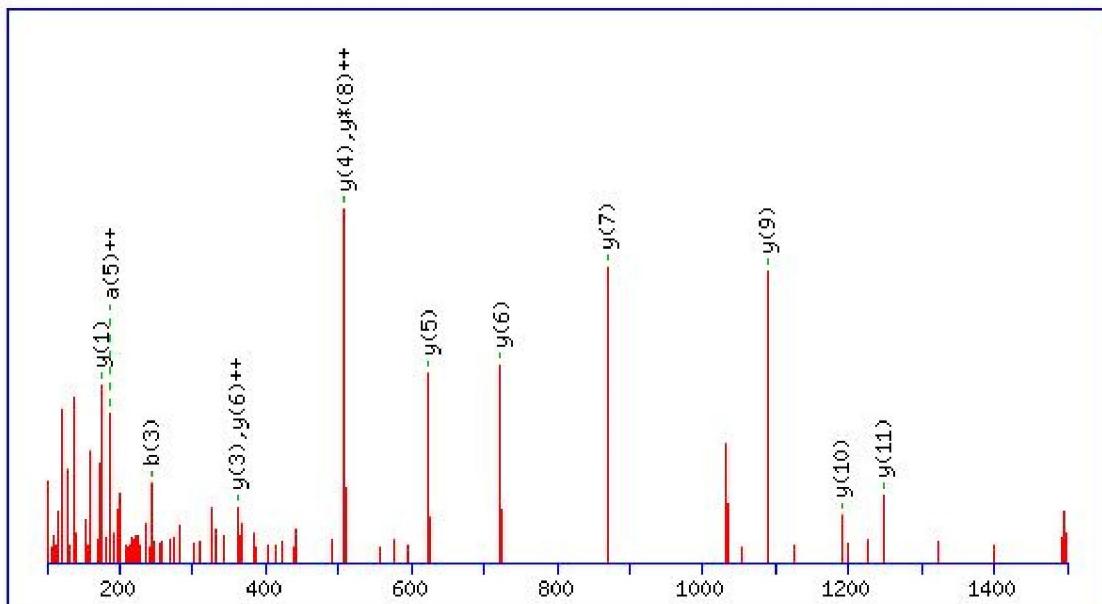


Figure B34: MS/MS spectra of peptide identified as histidine rich glycoprotein GGEGTGYFVDFSVR



APPENDIX B (continued)

Figure B35: MS/MS spectra of peptide identified as histidine rich glycoprotein DSPVLIDFFEDTER

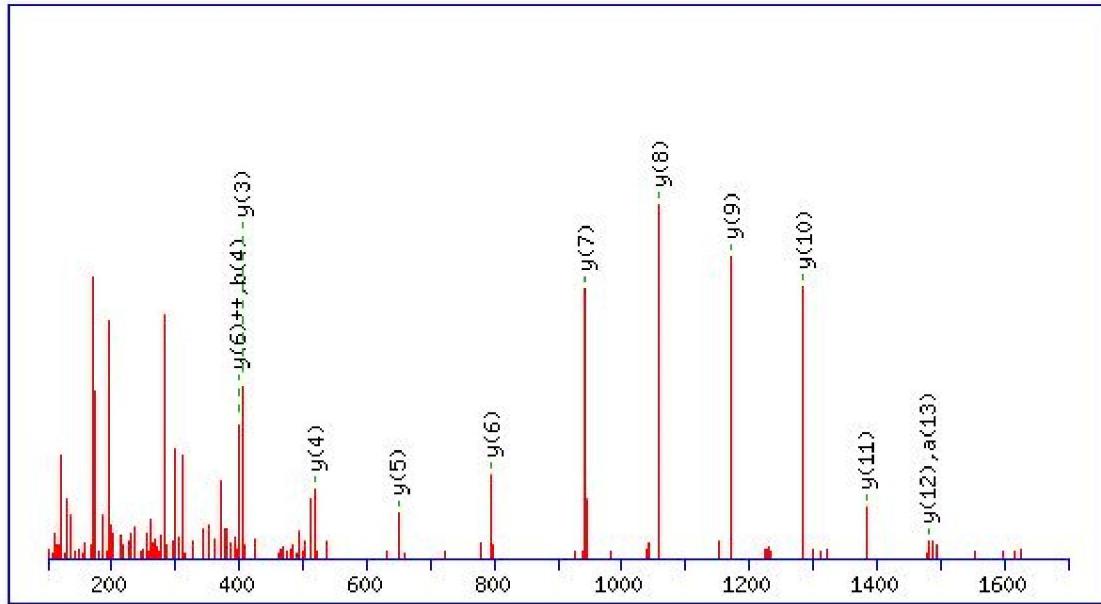
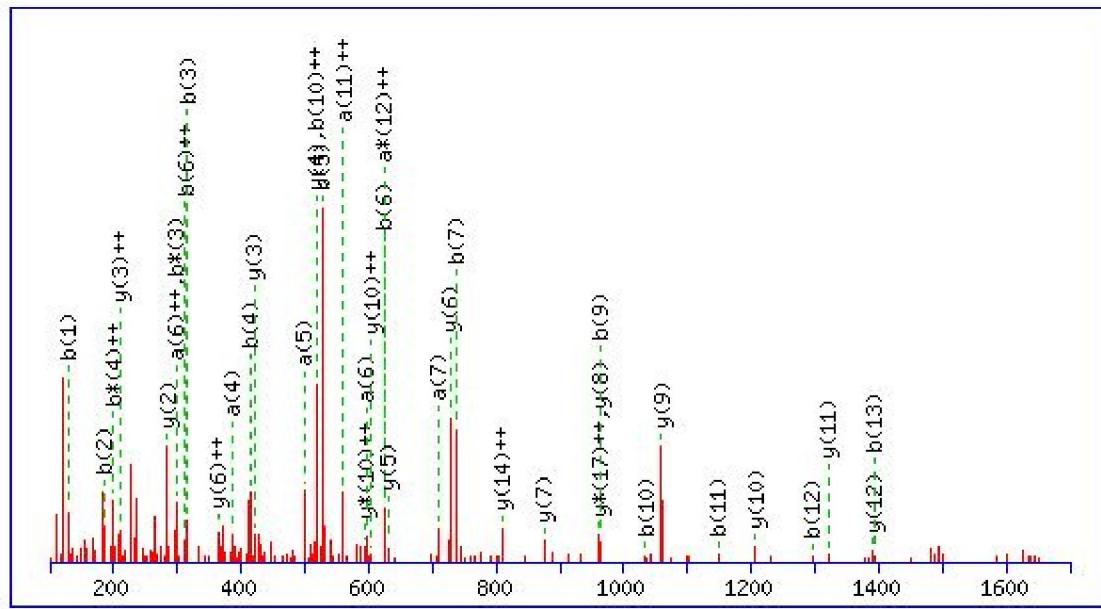


Figure B36: MS/MS spectra of peptide identified as histidine rich glycoprotein KGEVLPLPEANFPSFPLPHHK



APPENDIX B (continued)

Figure B37: MS/MS spectra of peptide identified as insulin like growth factor binding protein 1
YVTSAAPMPEPQAPGR

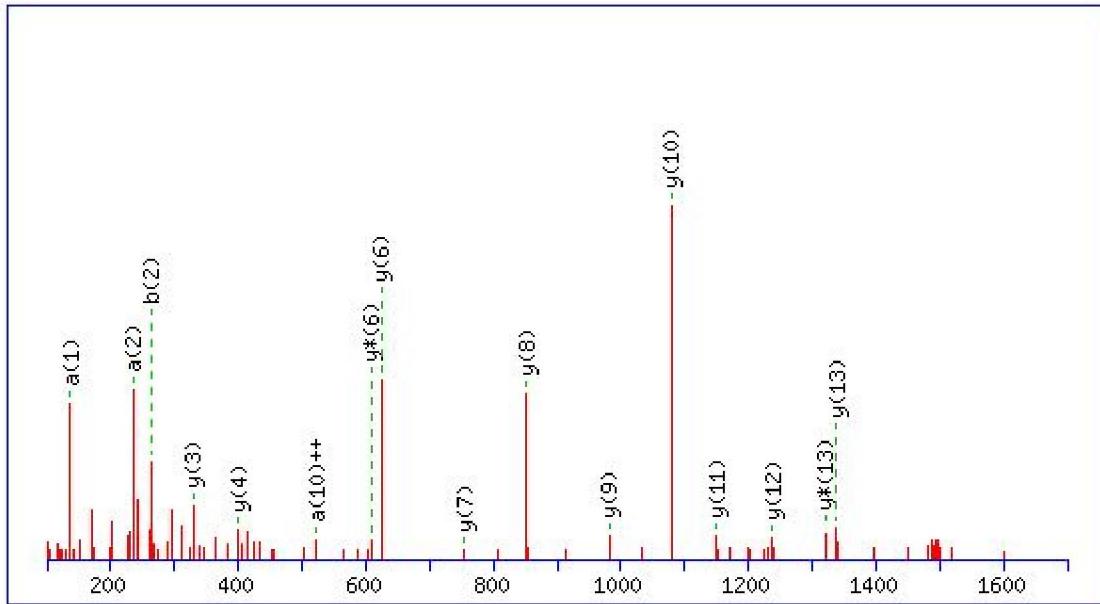
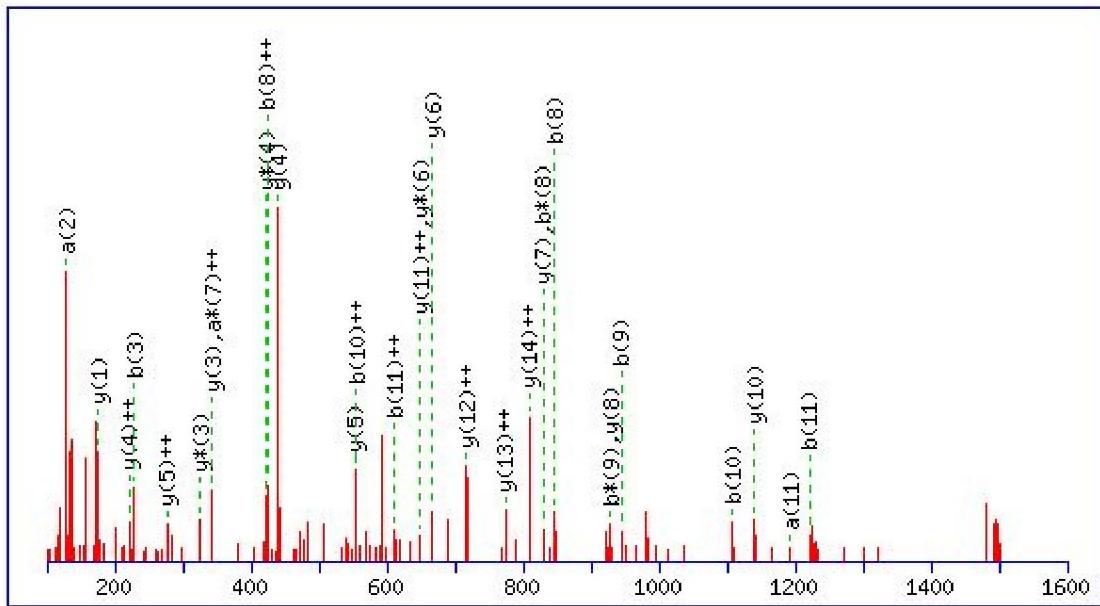


Figure B38: MS/MS spectra of peptide identified as insulin like growth factor binding protein 1
GVALHRPDVYLLPPAR



APPENDIX B (continued)

Figure B39: MS/MS spectra of peptide identified as insulin like growth factor binding protein 1
QIQVSWLR

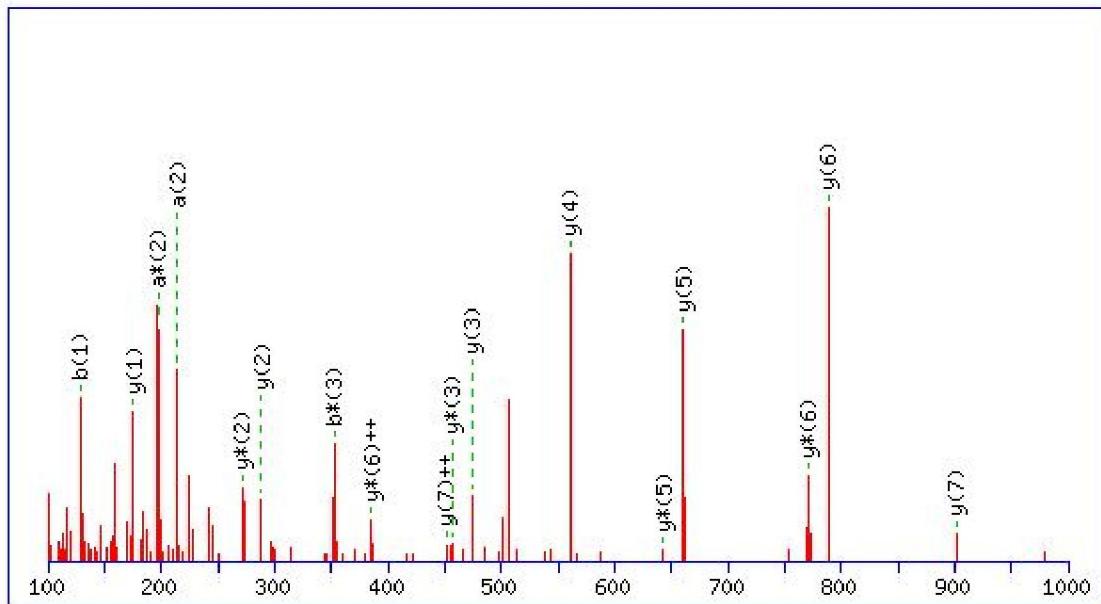
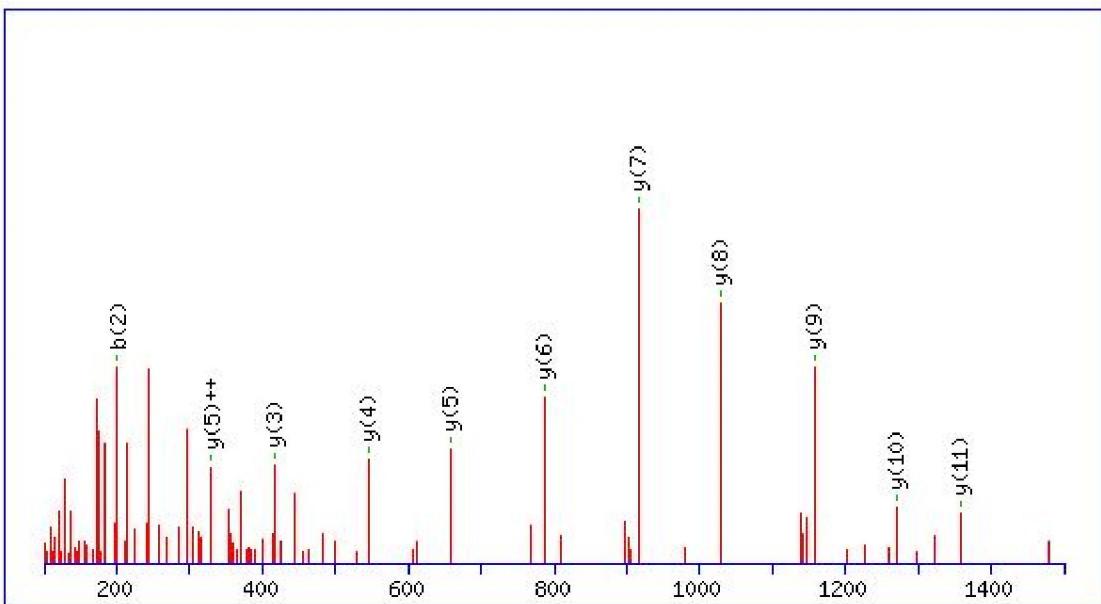


Figure B40: MS/MS spectra of peptide identified as insulin like growth factor binding protein 6
LSLEIEQLELQR



APPENDIX B (continued)

Figure B41: MS/MS spectra of peptide identified as insulin like growth factor binding protein 6
NWRDPDQTDSLGSYLSHIANVER

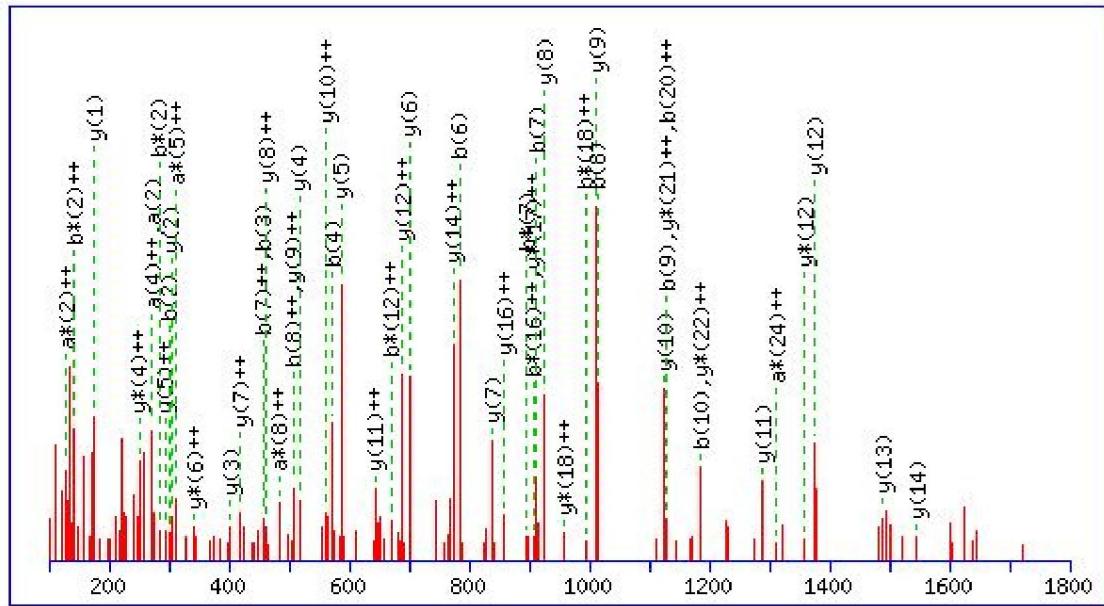
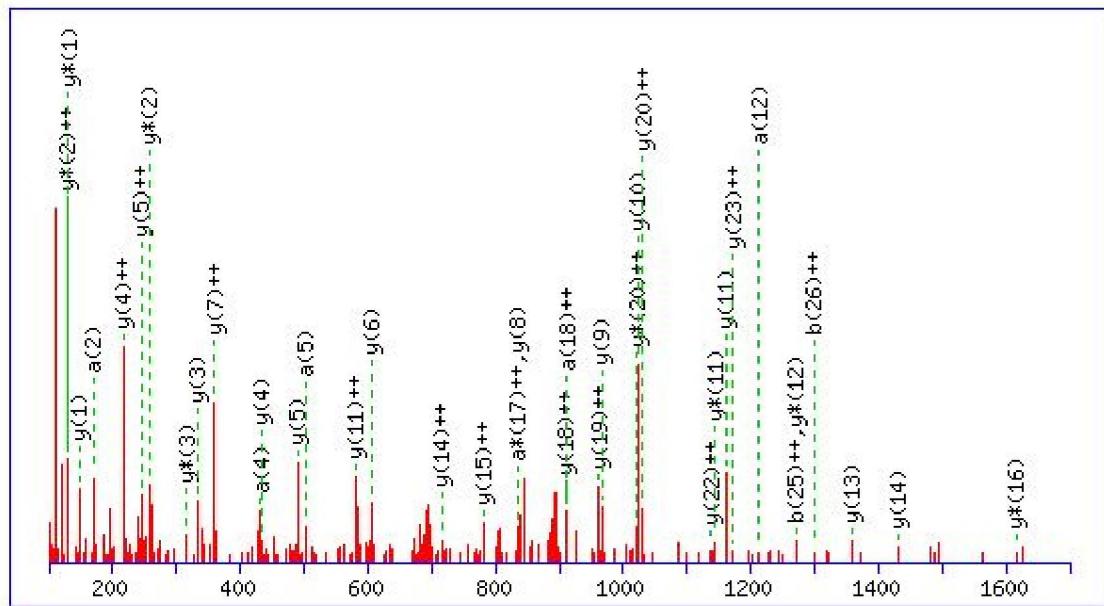


Figure B42: MS/MS spectra of peptide identified as insulin like growth factor binding protein 6
VPFDAATLHTSTAMAAQHGMDDDGTGQK



APPENDIX B (continued)

Figure B43: MS/MS spectra of peptide identified as vitamin d binding protein VMDKYTFELSR

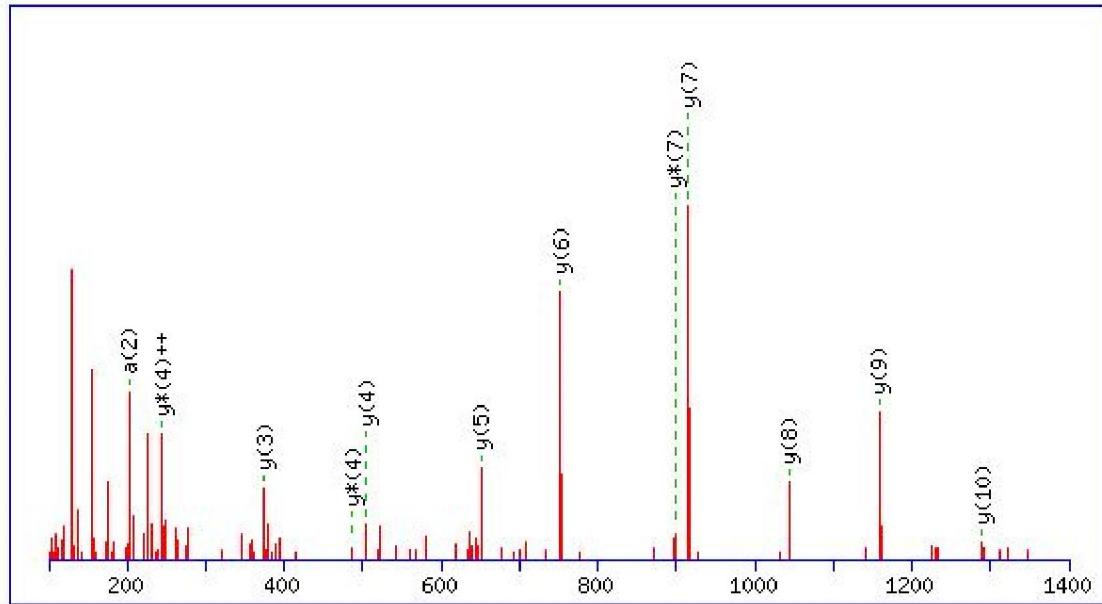
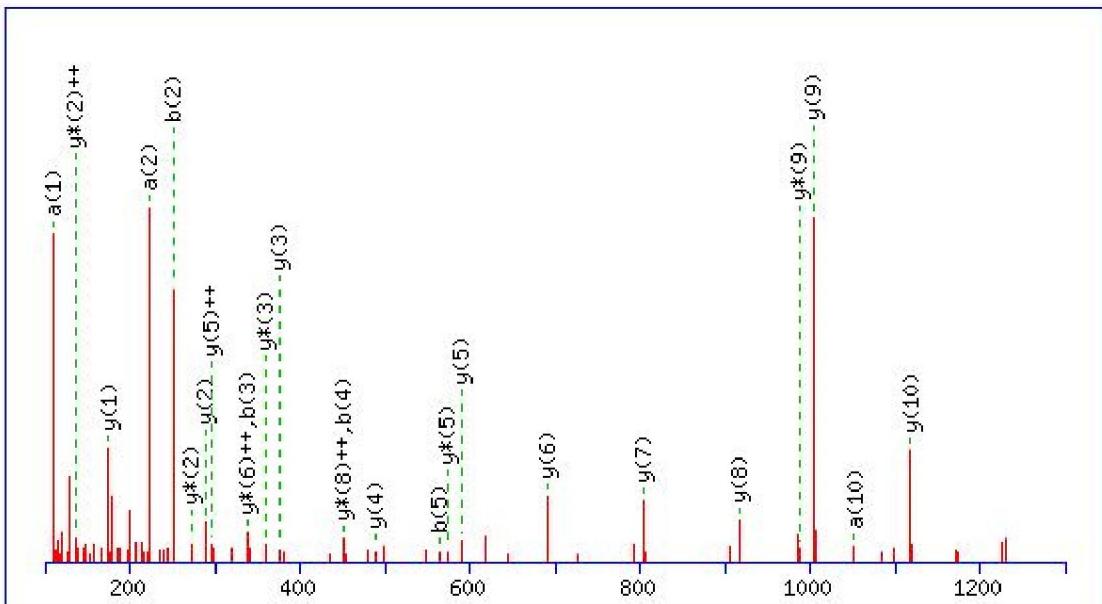


Figure B44: MS/MS spectra of peptide identified as vitamin d binding protein HLSLLTTLNSR



APPENDIX B (continued)

Figure B45: MS/MS spectra of peptide identified as vitamin d binding protein
HQPQEFPTYVEPTNDEICEAFR

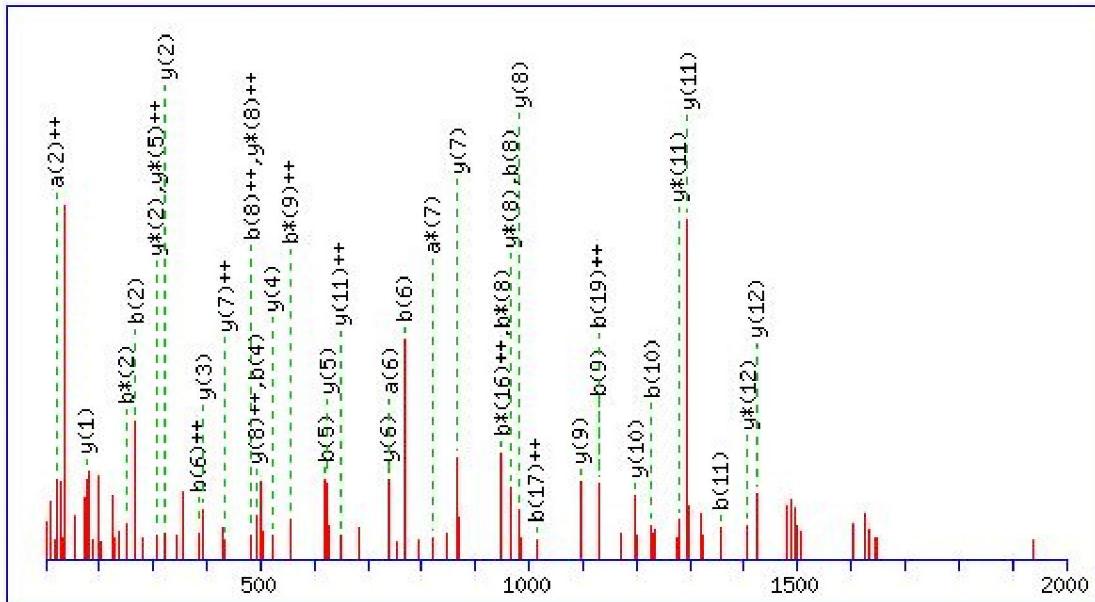
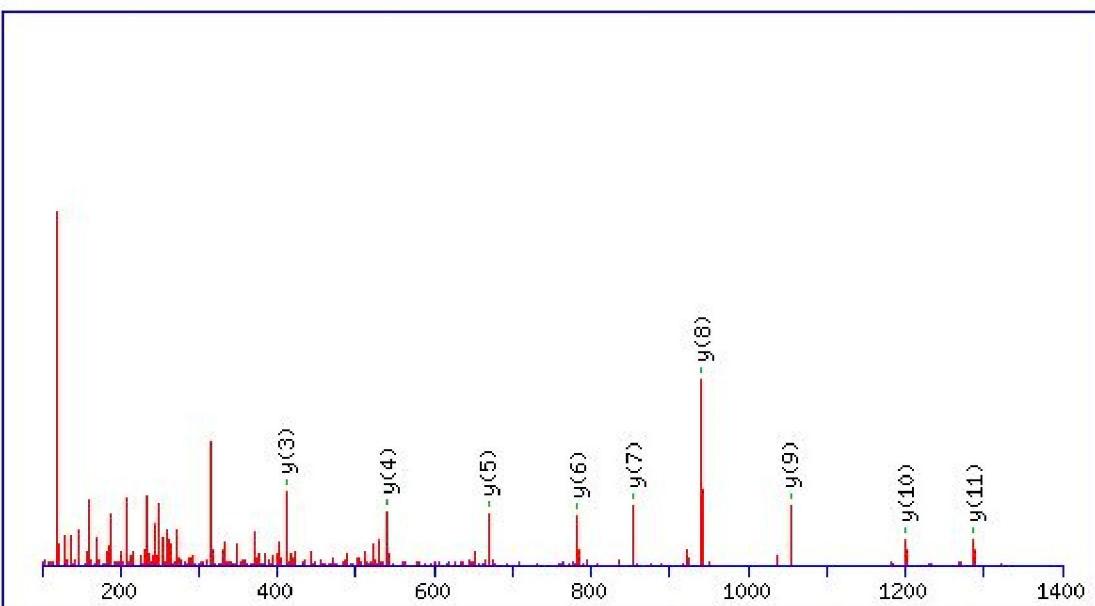


Figure B46: MS/MS spectra of peptide identified as apolipoprotein a1 VSFLSALEEYTK



APPENDIX B (continued)

Figure B47: MS/MS spectra of peptide identified as apolipoprotein a1 DSGRDYVSQFEGSALGK

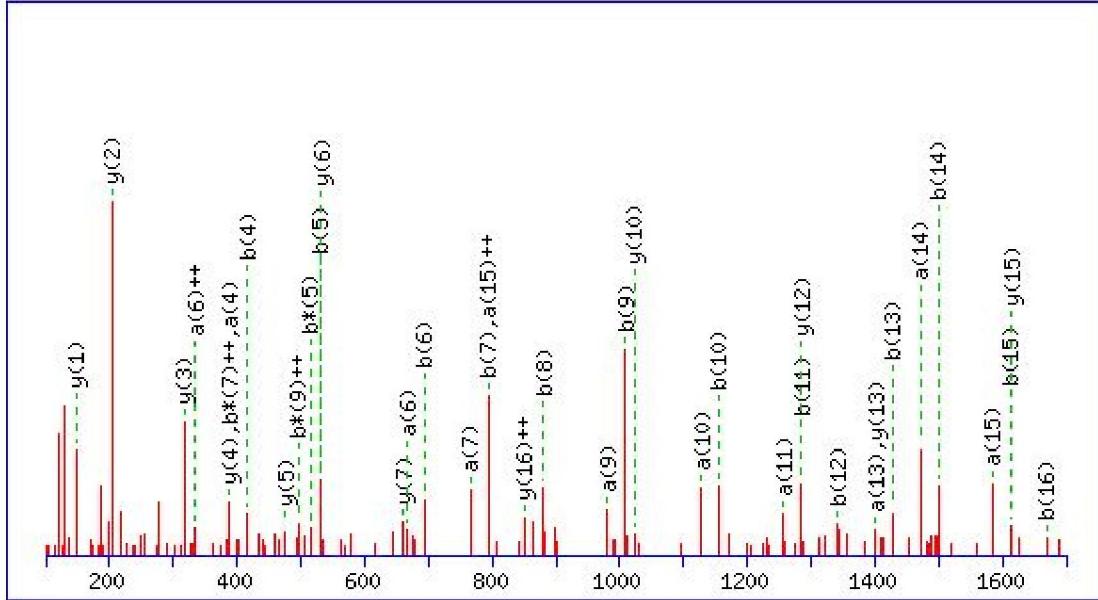
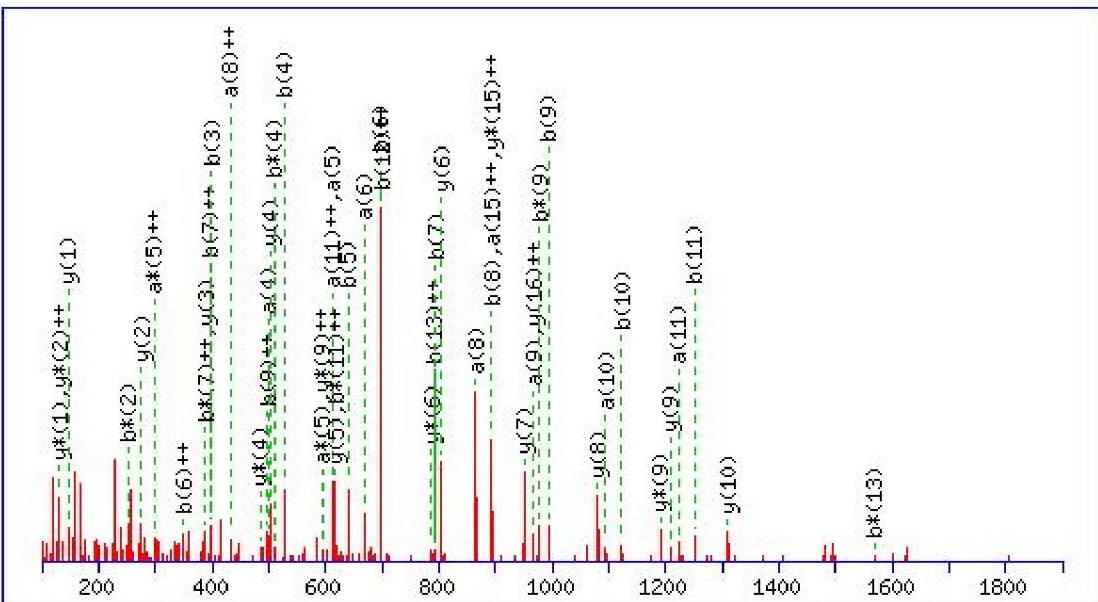


Figure B48: MS/MS spectra of peptide identified as apolipoprotein a1 LREQLGPVTQEFWDNLEK



APPENDIX B (continued)

Figure B49: MS/MS spectra of peptide identified as apolipoprotein a4 LKEEIGKELEELR

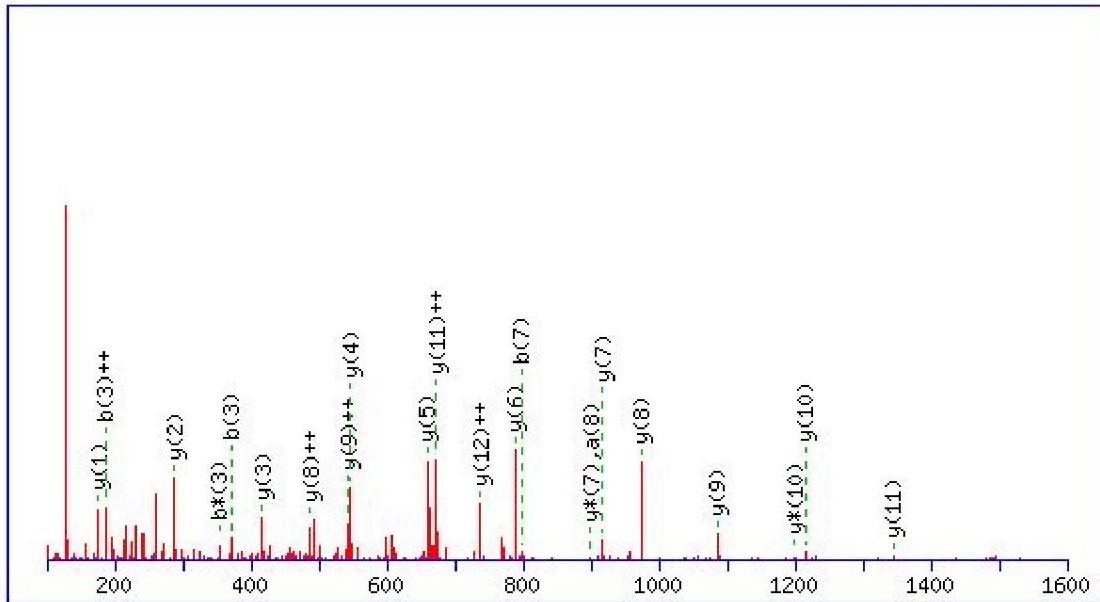
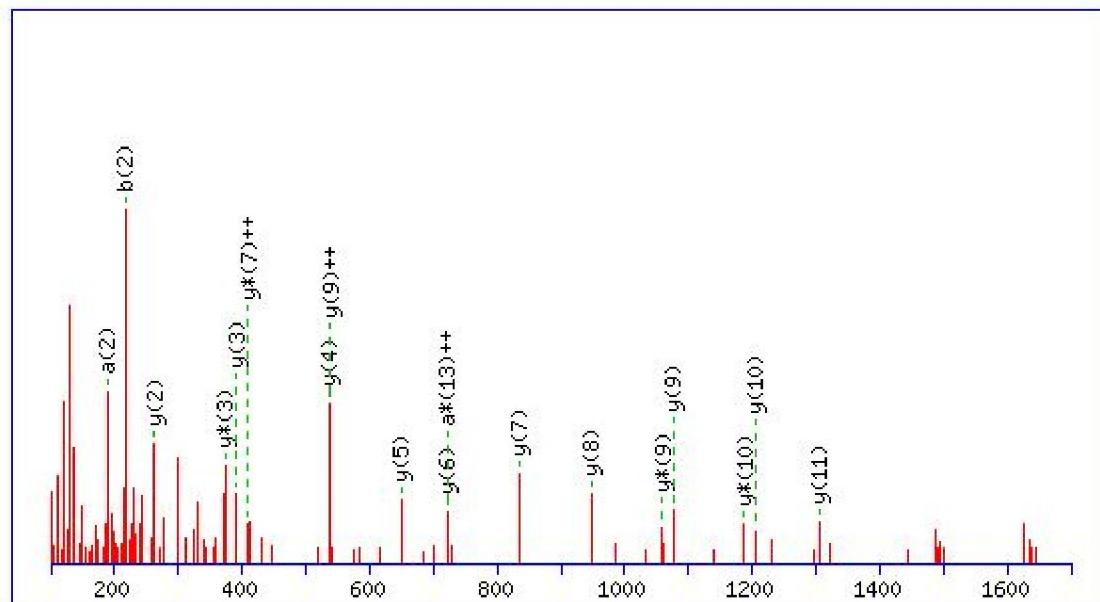


Figure B50: MS/MS spectra of peptide identified as apolipoprotein a4 SELTQQQLNALFQDK



APPENDIX B (continued)

Figure B51: MS/MS spectra of peptide identified as apolipoprotein a4 DKVNSFFSTFK

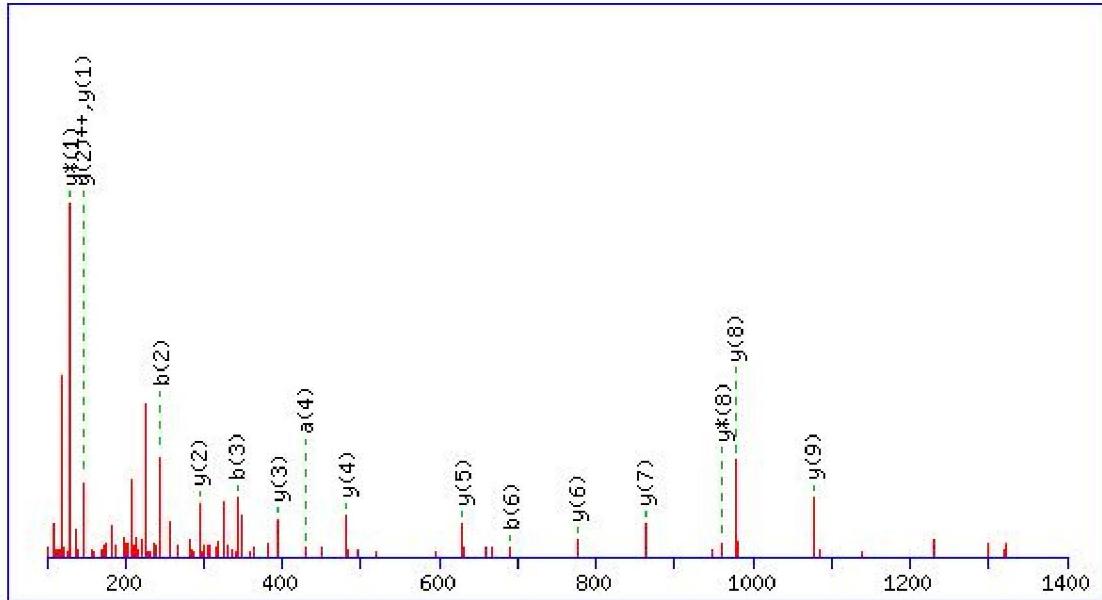
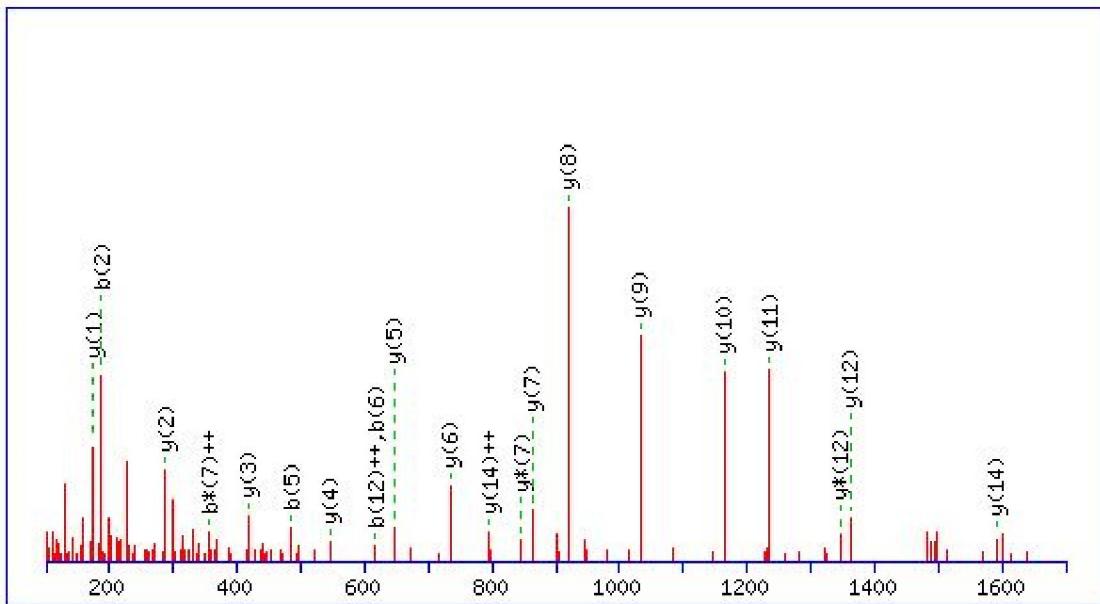


Figure B52: MS/MS spectra of peptide identified as apolipoprotein e GEVQAMLGQSTEELR



APPENDIX B (continued)

Figure B53: MS/MS spectra of peptide identified as apolipoprotein e SELEEQLTPVAEETR

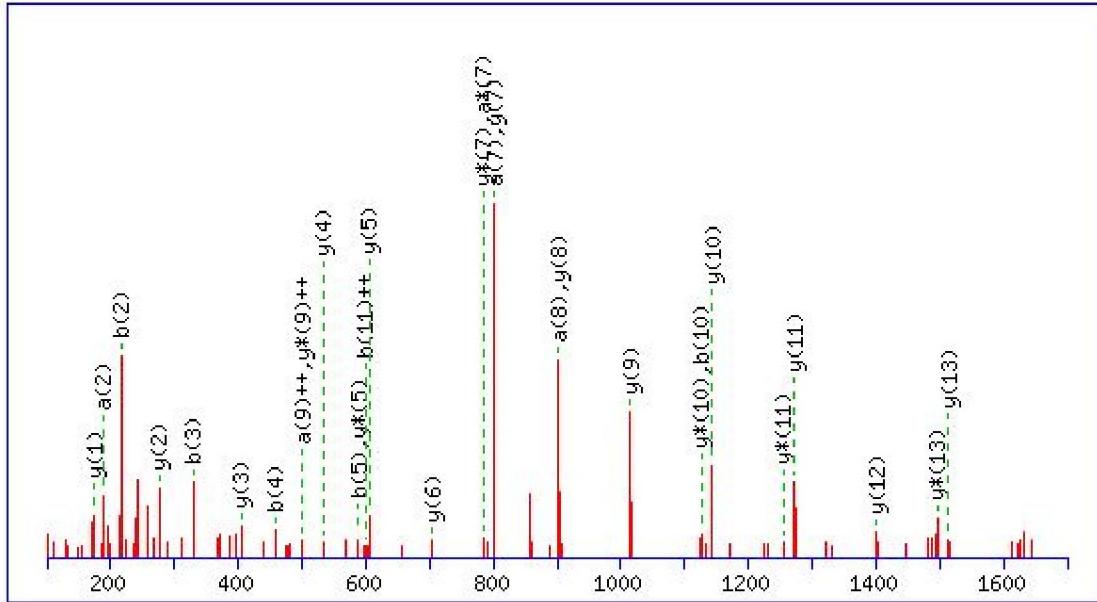
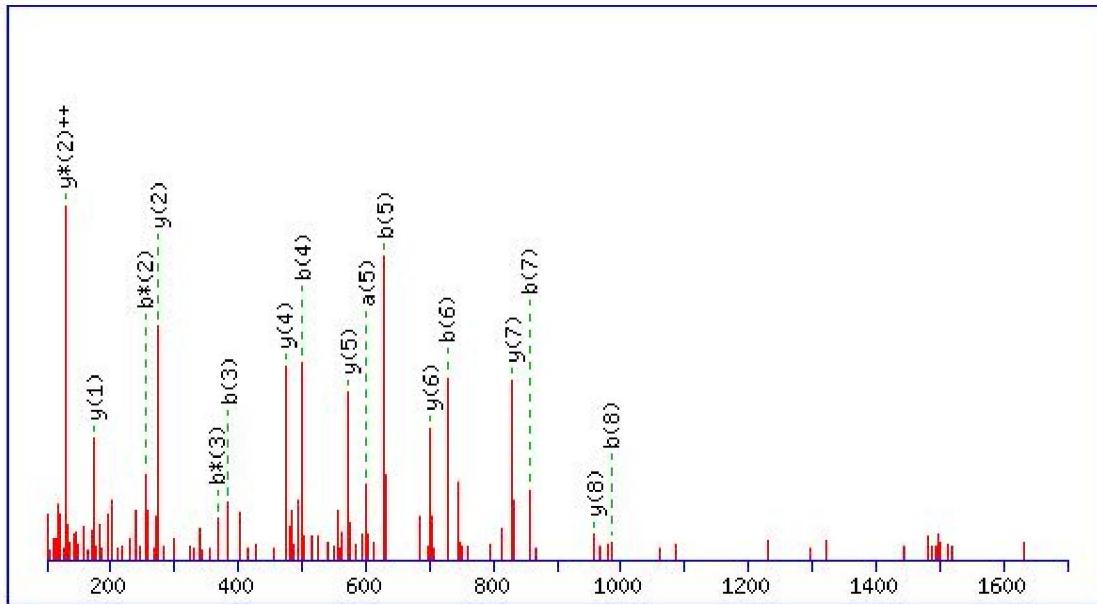


Figure B54: MS/MS spectra of peptide identified as apolipoprotein e DRLDEVKEQVAEVR



APPENDIX B (continued)

Figure B55: MS/MS spectra of peptide identified as transthyretin ALGISPFHEHAEVVFTANDSGPR

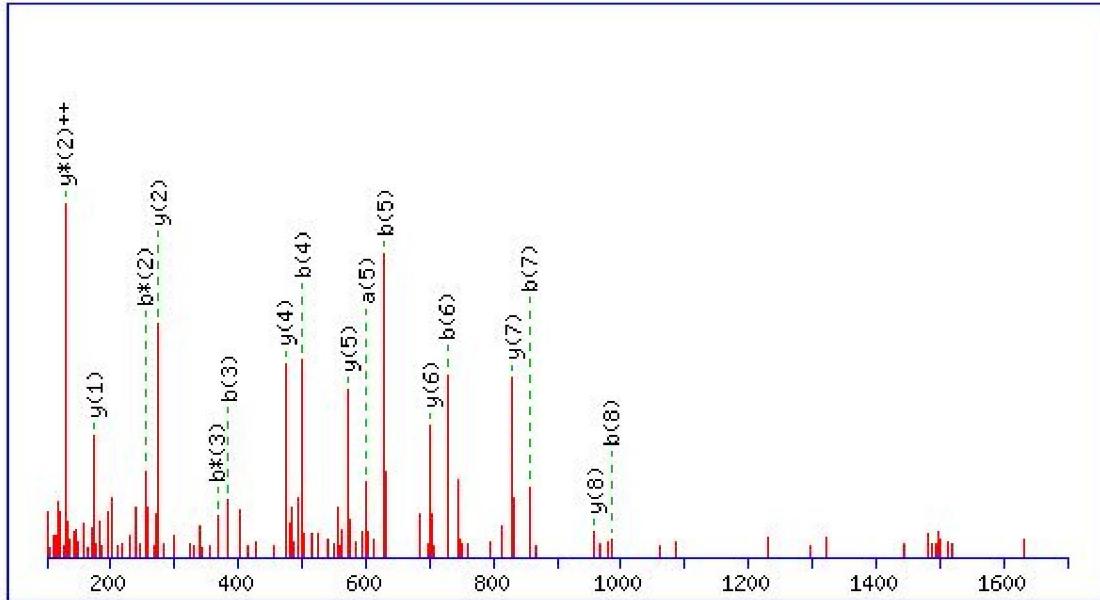
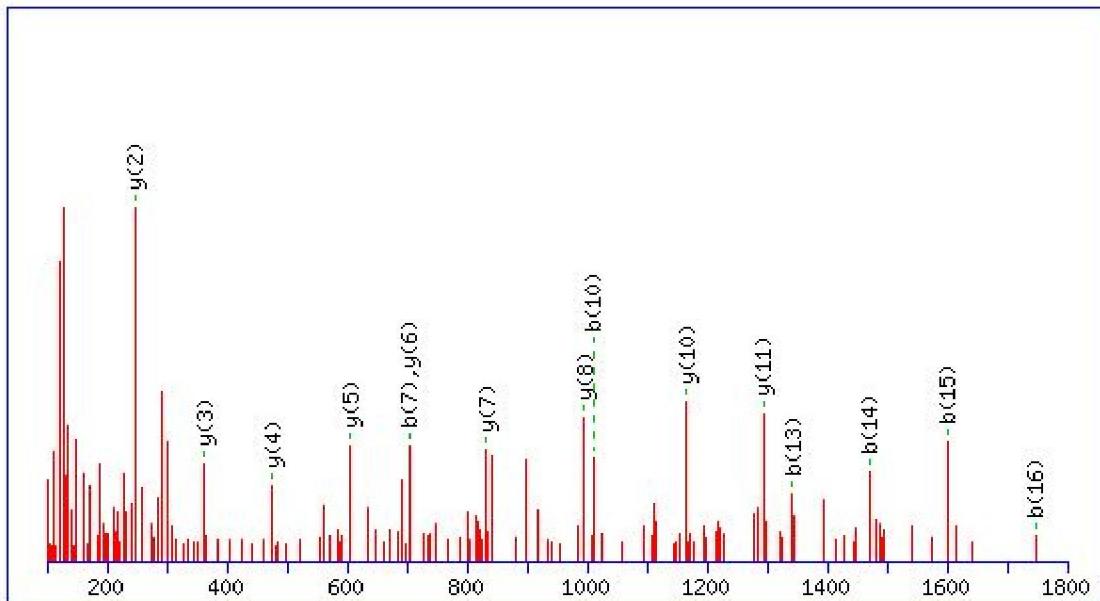


Figure B56: MS/MS spectra of peptide identified as transthyretin TSESGELHGLTTEEFVEGIYKVEIDTK



APPENDIX B (continued)

Figure B57: MS/MS spectra of peptide identified as transthyretin ADDTWEPEFASGK

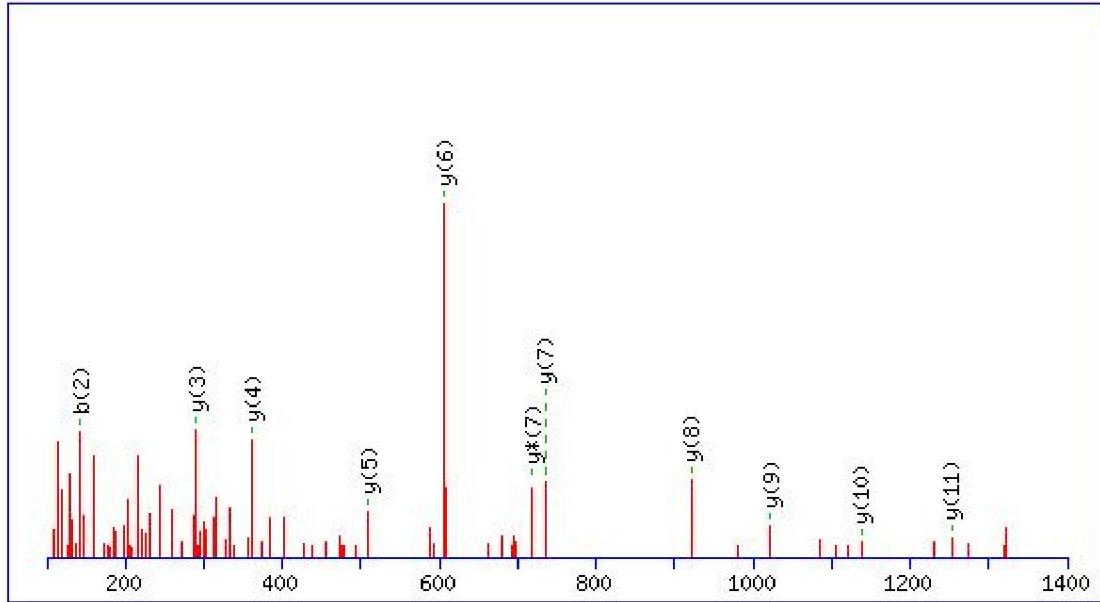
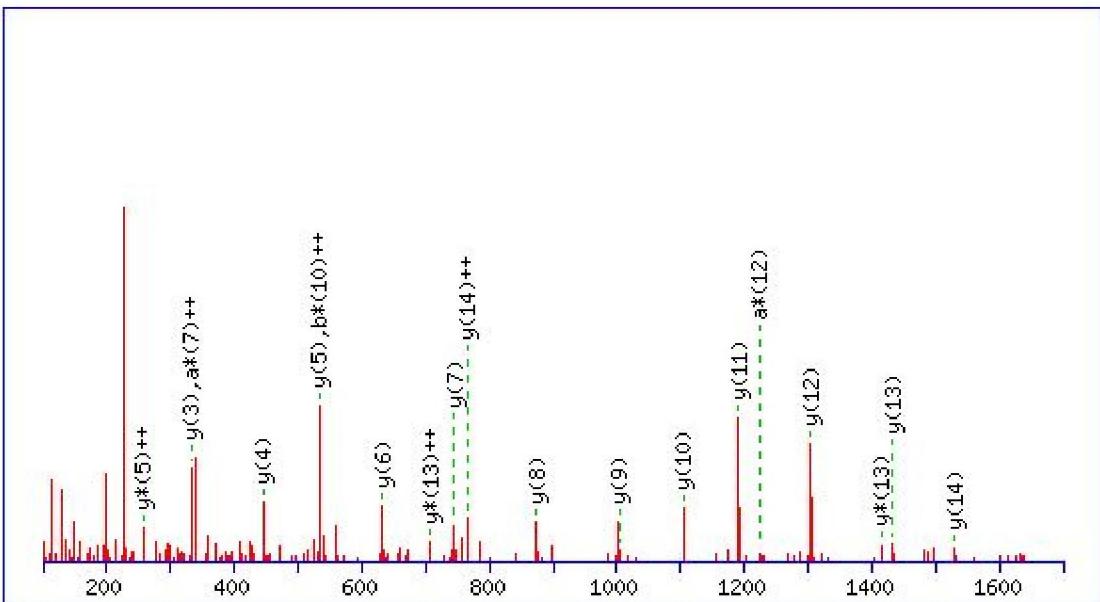


Figure B58: MS/MS spectra of peptide identified as afamin IAPQLSTEELVSLGEK



APPENDIX B (continued)

Figure B59: MS/MS spectra of peptide identified as afamin RNPFVFAPTLTVAHFEEVAK

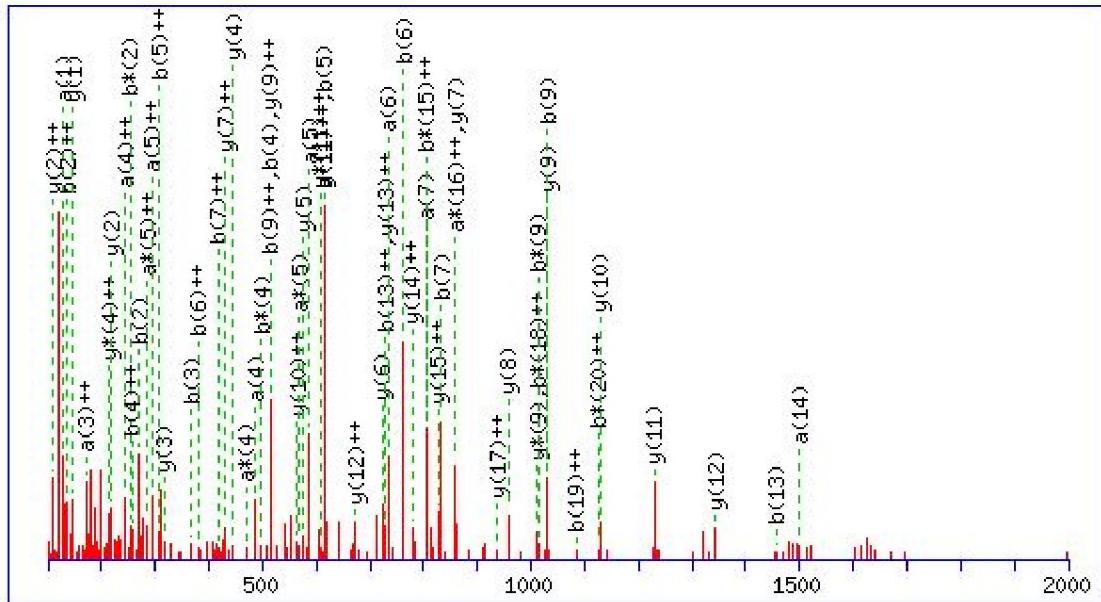
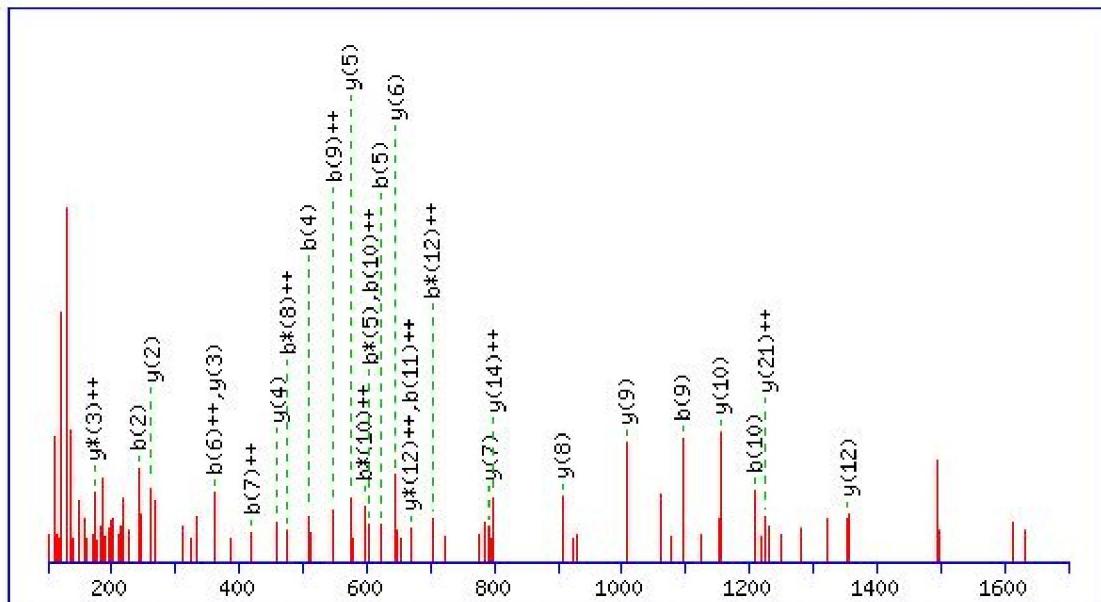


Figure B60: MS/MS spectra of peptide identified as afamin LKHELTDEELQLSFTNFANVVDK



APPENDIX B (continued)

Figure B61: MS/MS spectra of peptide identified as pregnancy zone protein MVSGFIPLKPTVK

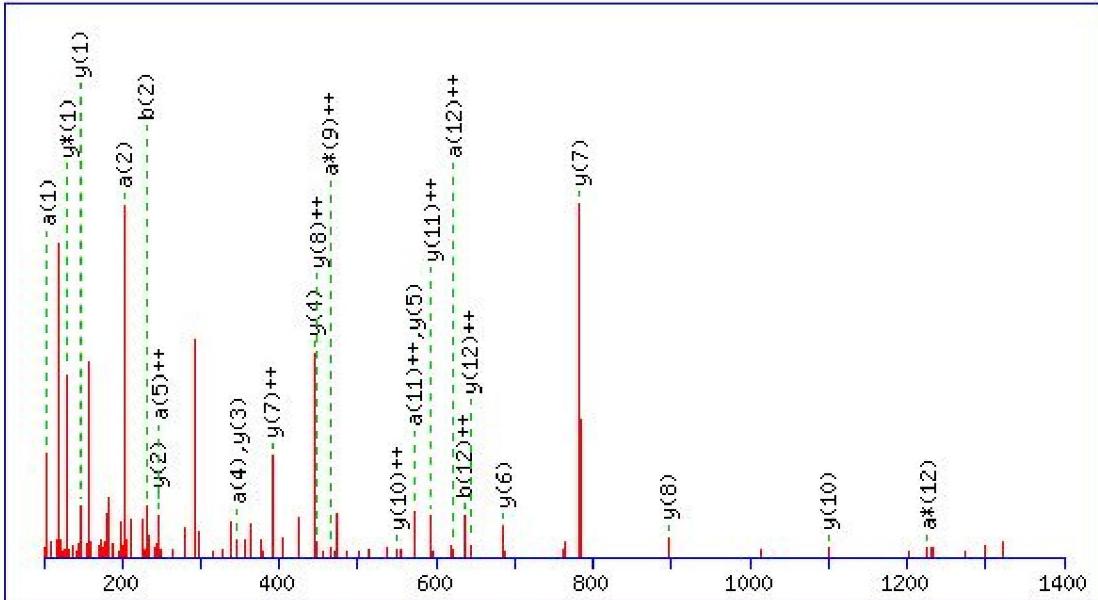
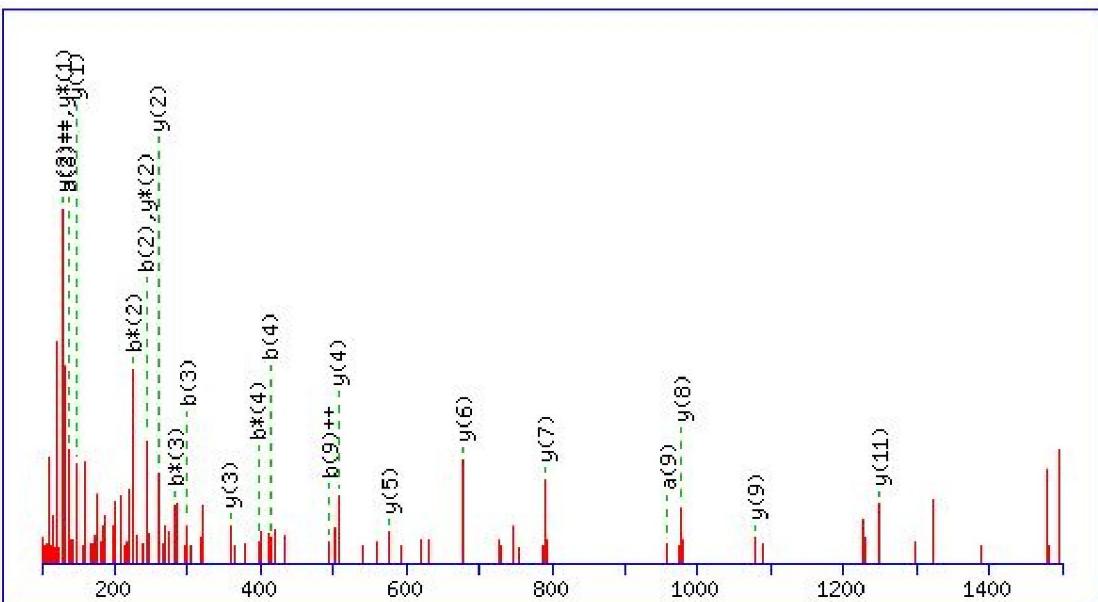


Figure B62: MS/MS spectra of peptide identified as pregnancy zone protein NQGNTWLTAFLVK



APPENDIX B (continued)

Figure B63: MS/MS spectra of peptide identified as pregnancy zone protein DLLLPQPDLR

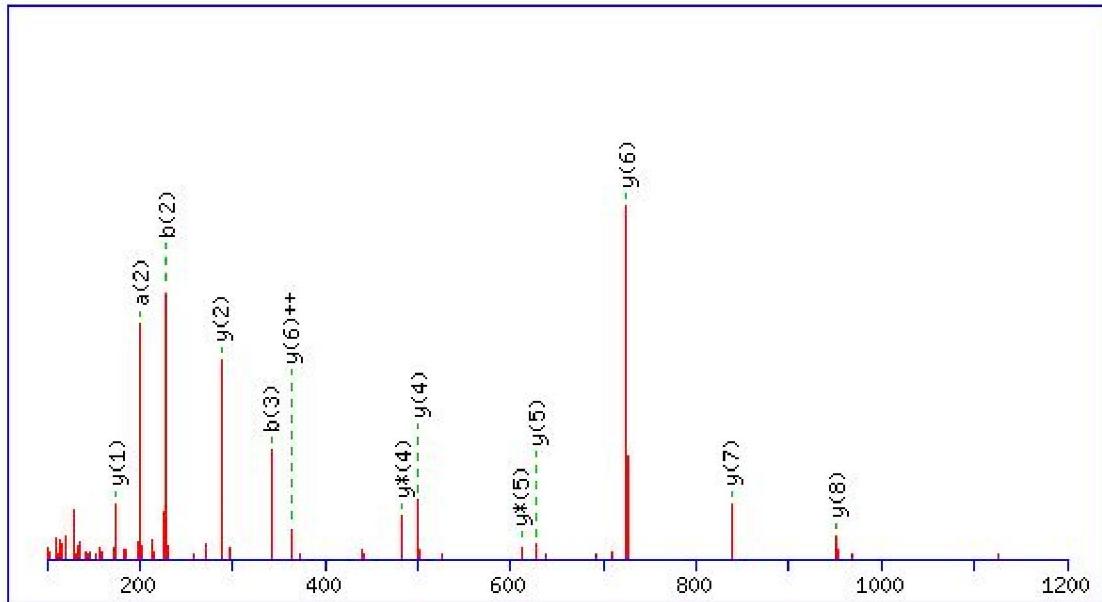
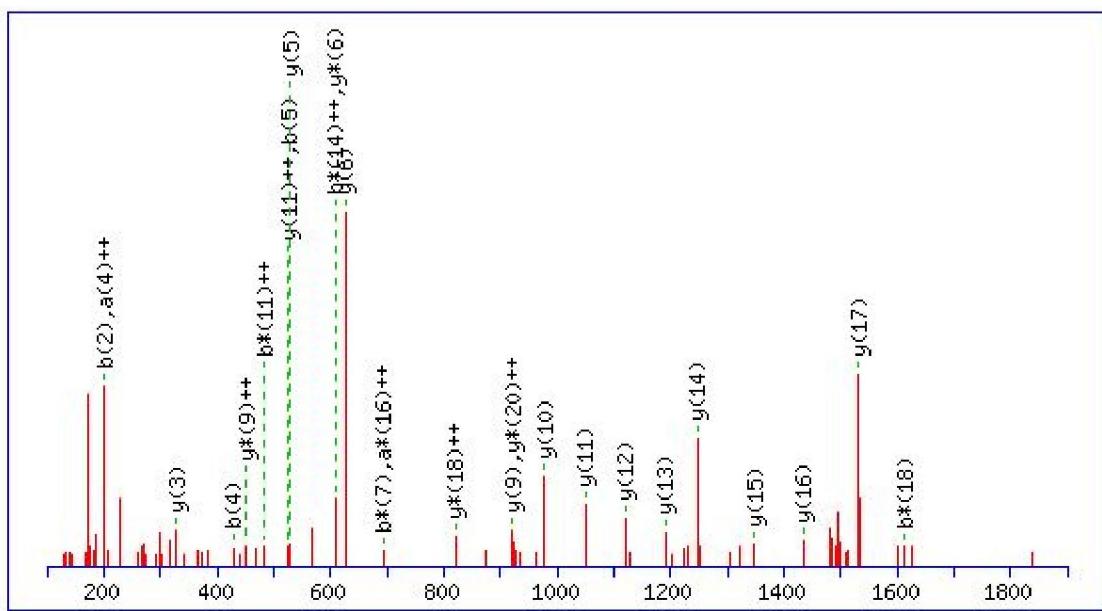


Figure B64: MS/MS spectra of peptide identified as leucine rich glycoprotein TVVQPSVGAAAGPVVPPCPGR



APPENDIX B (continued)

Figure B65: MS/MS spectra of peptide identified as leucine rich glycoprotein EHAVEGDCDFQLLK

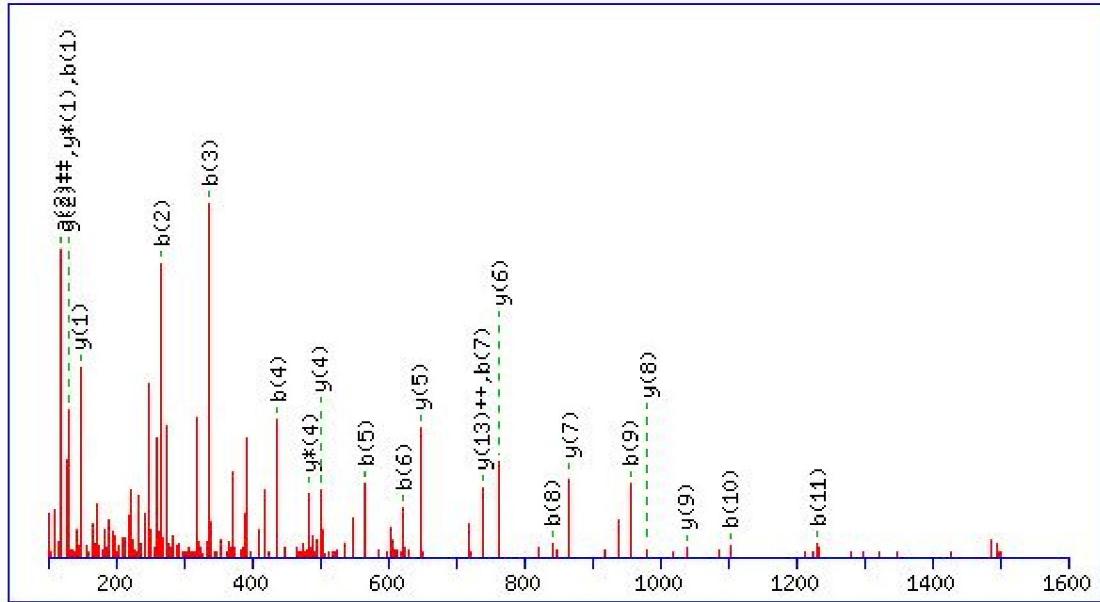
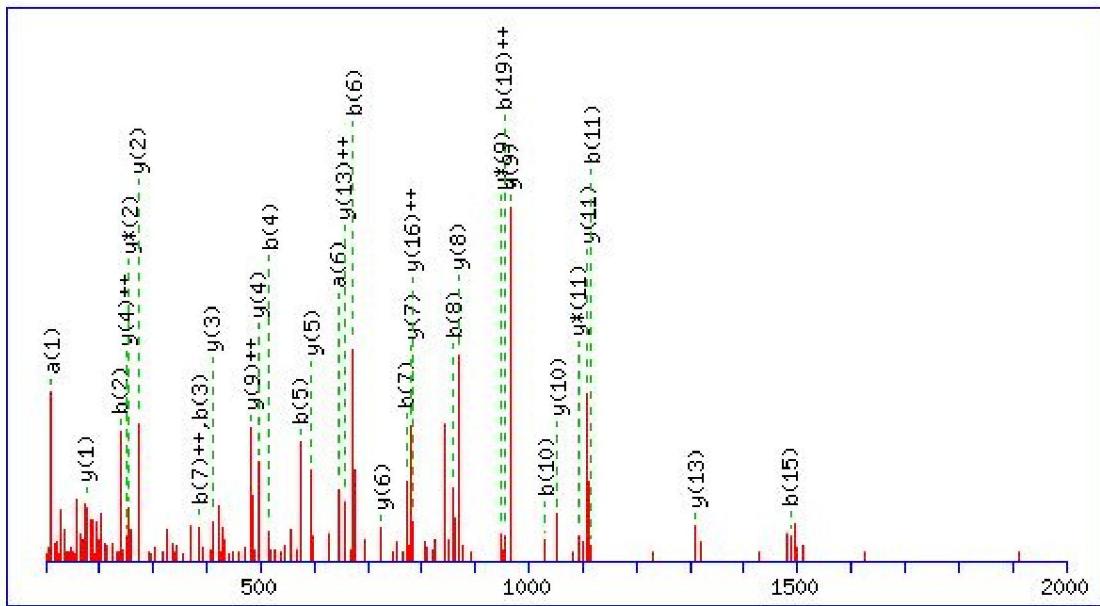


Figure B66: MS/MS spectra of peptide identified as leucine rich glycoprotein HTFMGVVSLGSPSGEVSHPR



APPENDIX B (continued)

Figure B67: MS/MS spectra of peptide identified as complement factor b YGLVTYATYPK

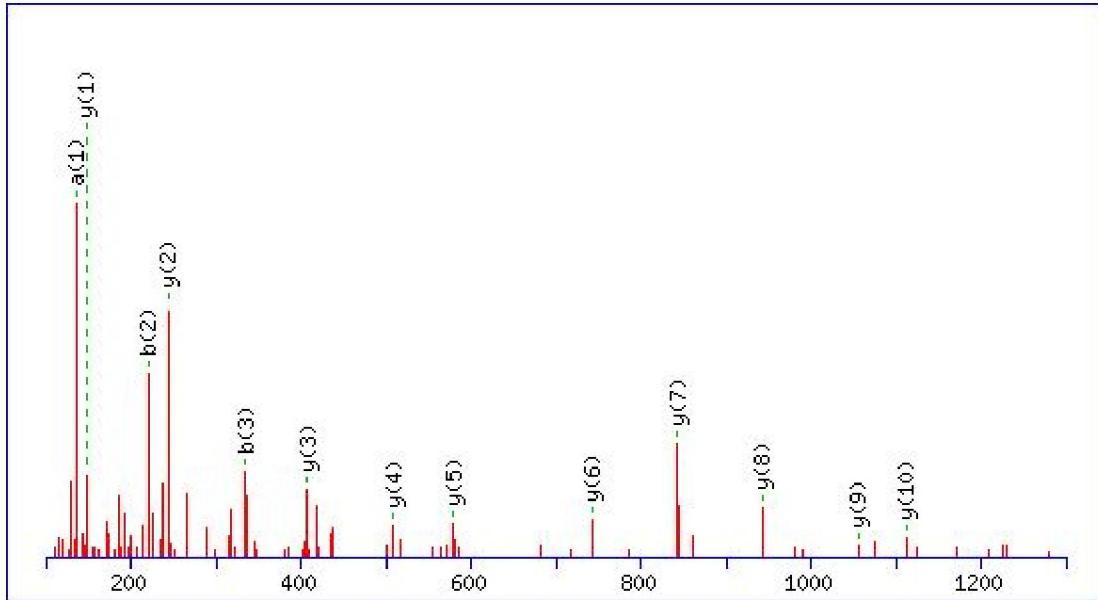
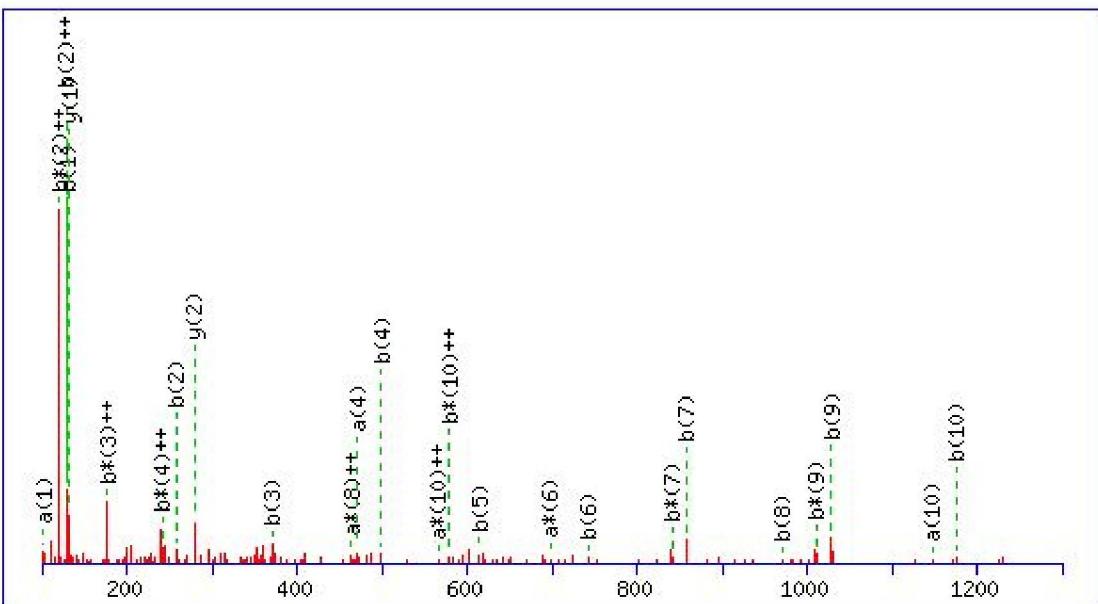


Figure B68: MS/MS spectra of peptide identified as complement factor b EKLQDEDLGFL



APPENDIX B (continued)

Figure B69: MS/MS spectra of peptide identified as factor b DFHINLFQVLPWLK

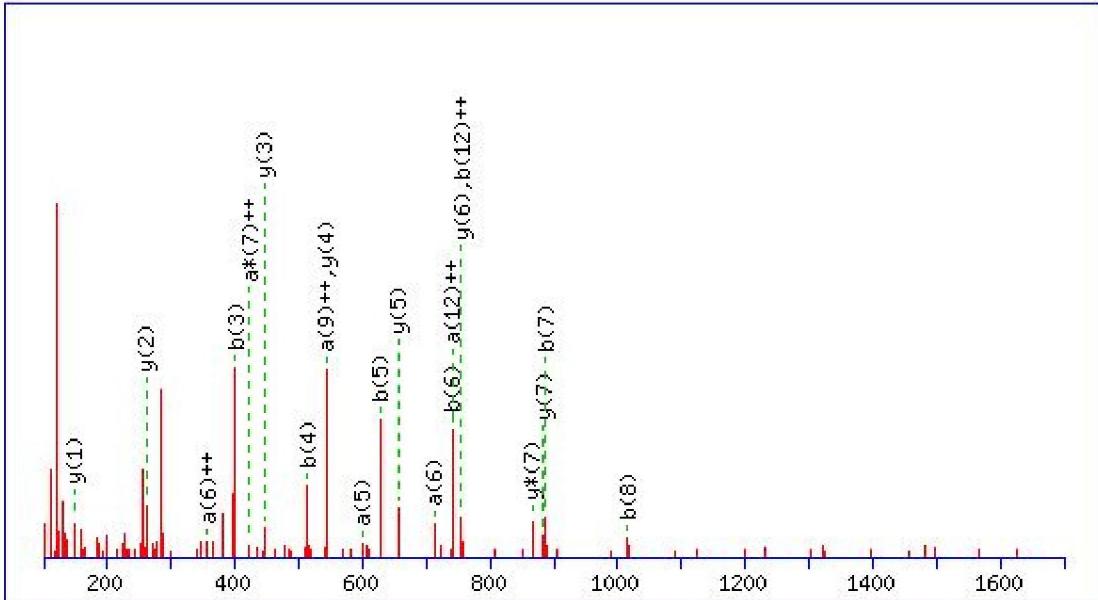
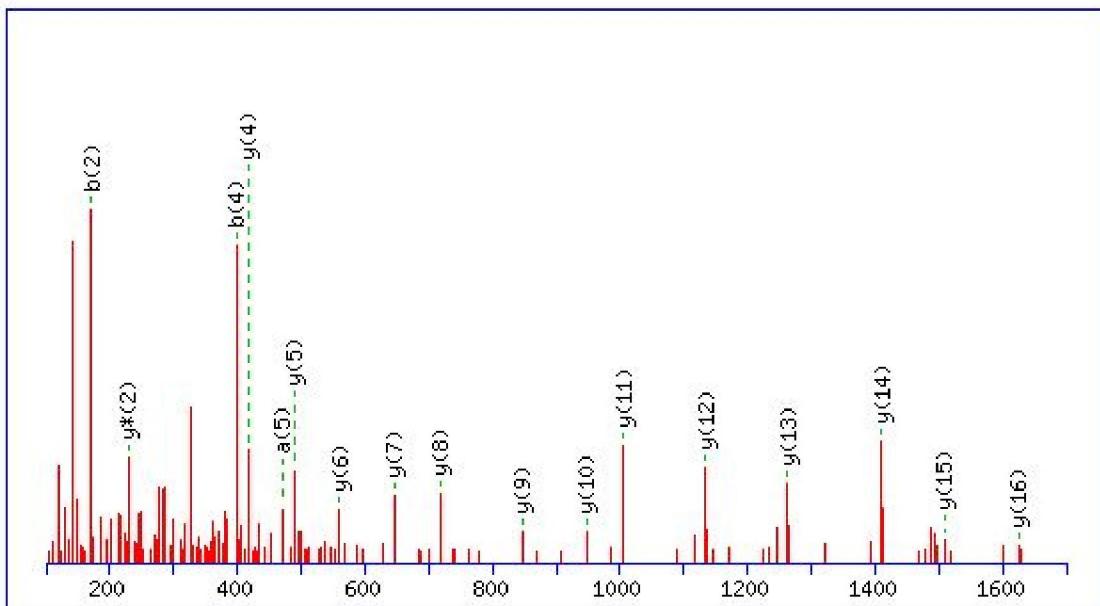


Figure B70: MS/MS spectra of peptide identified as fetuin b AVLDVFEEGTEASAATAVK



APPENDIX B (continued)

Figure B71: MS/MS spectra of peptide identified as fetuin b LYGSEAFATDFQDSAAK

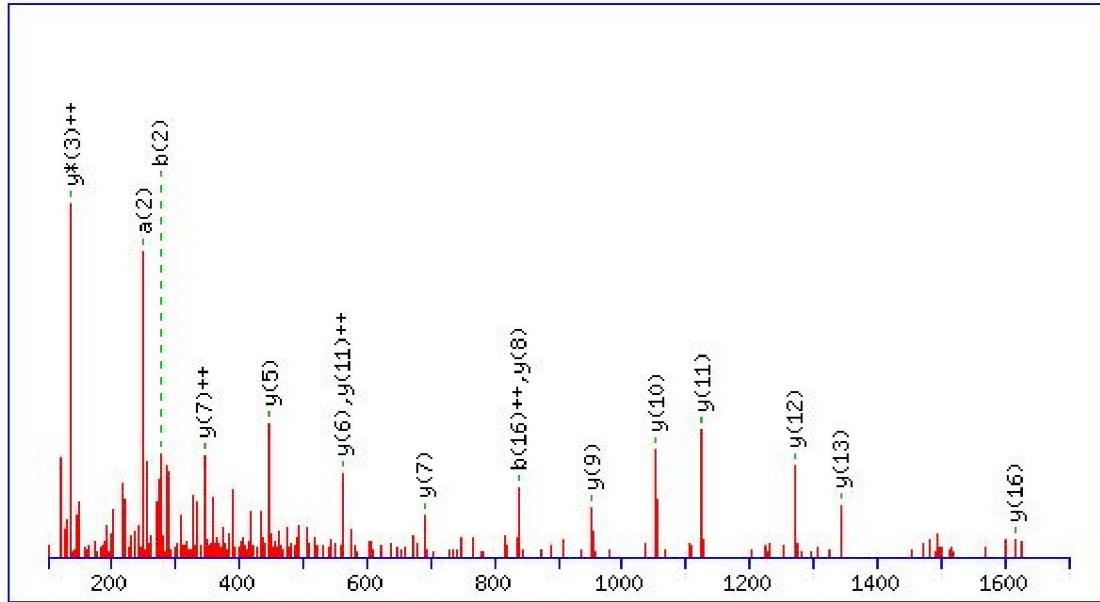
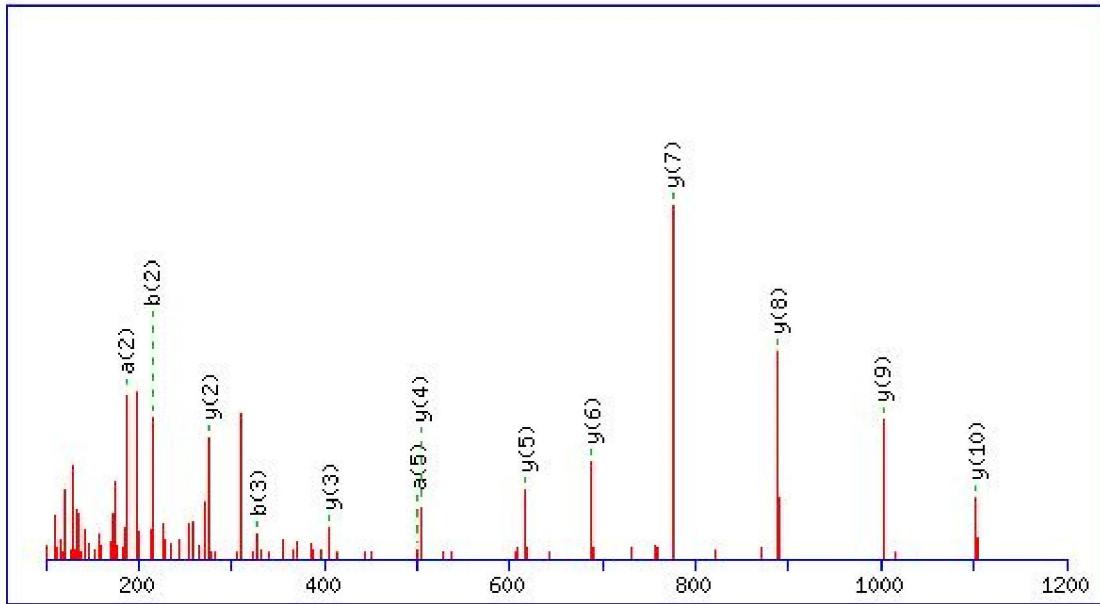


Figure B72: MS/MS spectra of peptide identified as fetuin b ITLLSALVETR



APPENDIX B (continued)

Figure B73: MS/MS spectra of peptide identified as macrophage stimulating protein 1
VFSNGADLSGVTEEAPLK

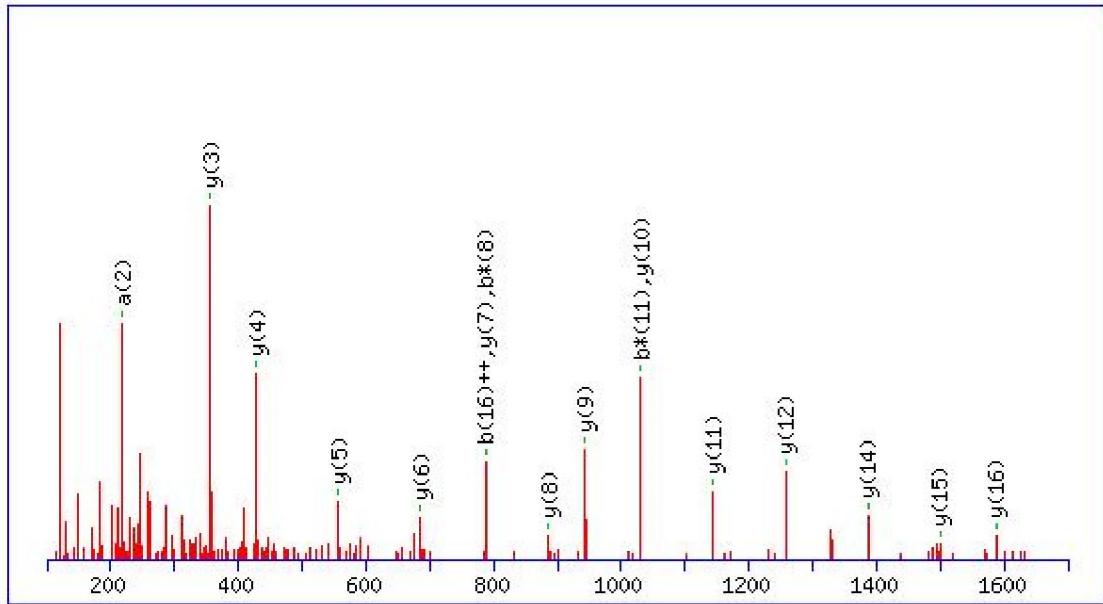
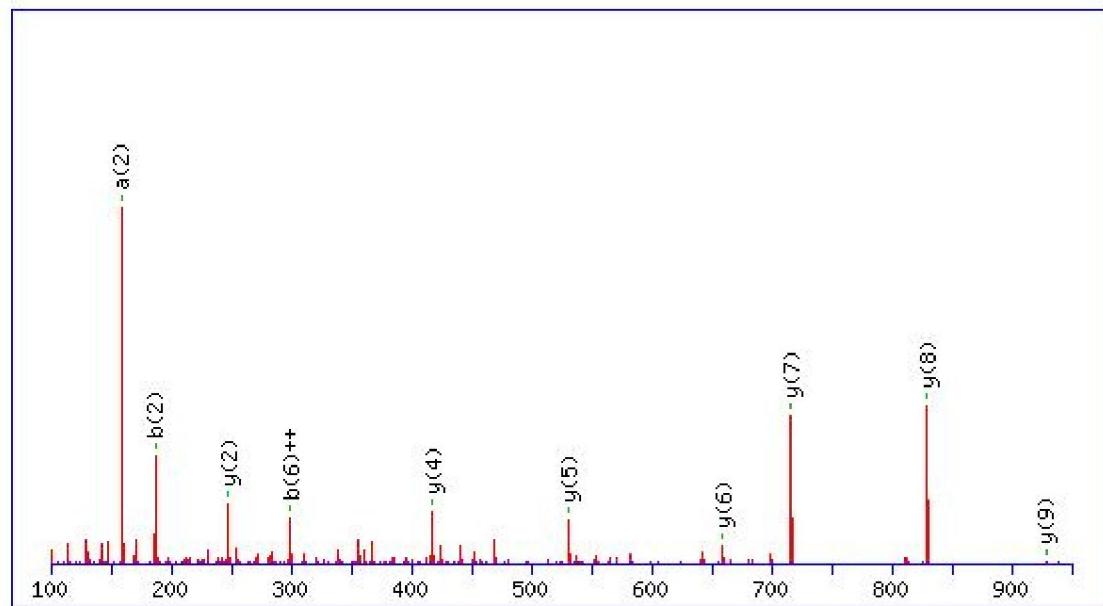


Figure B74: MS/MS spectra of peptide identified as macrophage stimulating protein 1 SVLGQLGKIT



APPENDIX B (continued)

Figure B75: MS/MS spectra of peptide identified as macrophage stimulating protein 1 ITPNLAEFAFSLYR

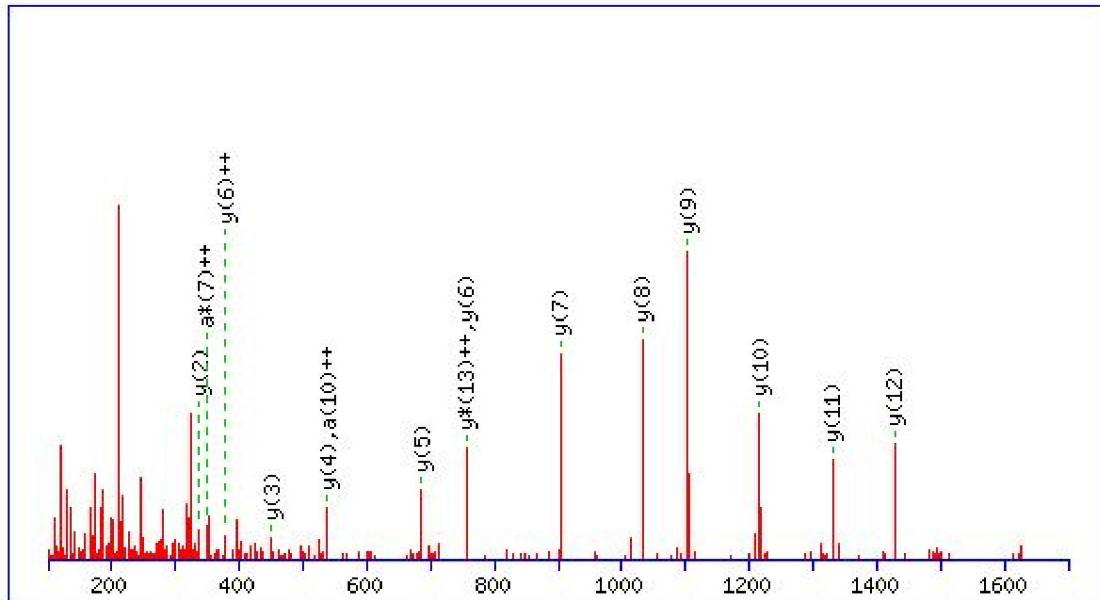
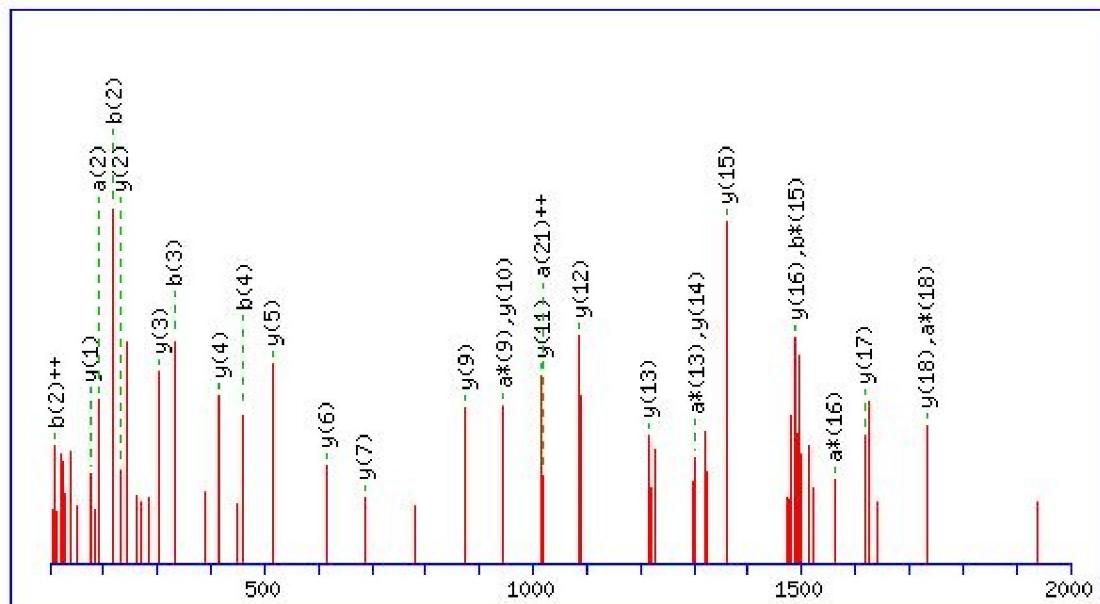


Figure B76: MS/MS spectra of peptide identified as transgelin 2 AFLEVNEEGSEAAASTAVVIAGR



APPENDIX B (continued)

Figure B77: MS/MS spectra of peptide identified as transgelin 2 FRIEDGFSLK

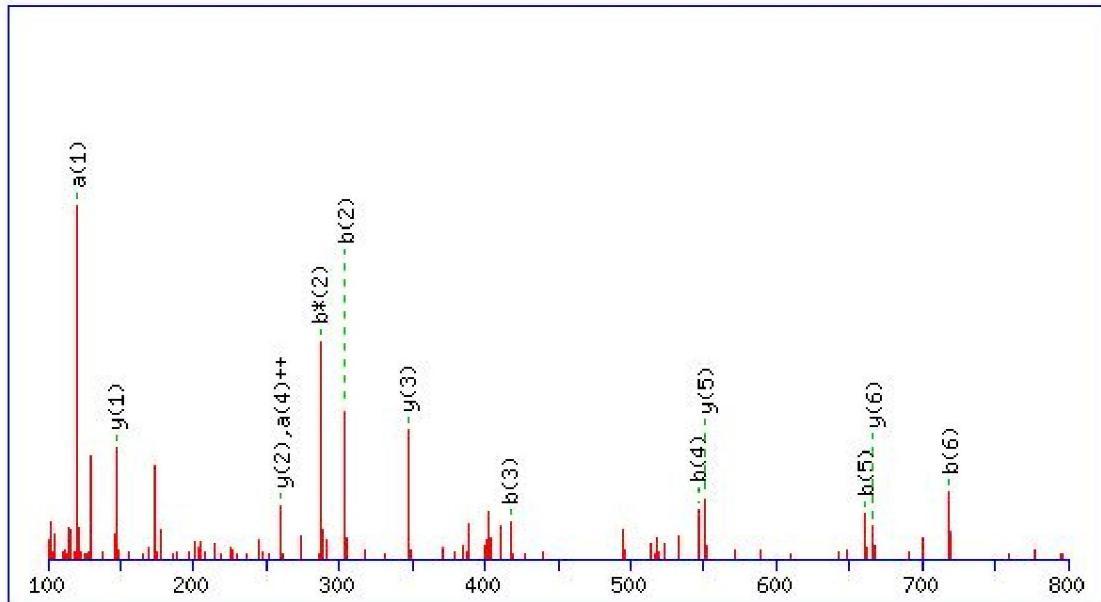
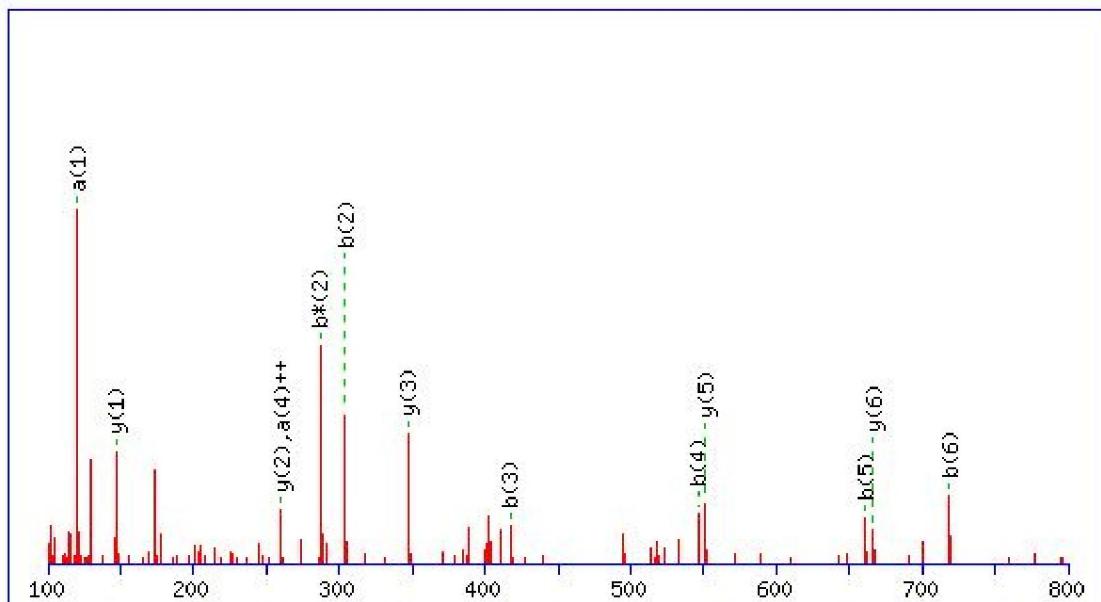


Figure B78: MS/MS spectra of peptide identified as transgelin 2 FRIEDGFSLK



APPENDIX B (continued)

Figure B79: MS/MS spectra of peptide identified as inhibin YVTSAPMPEPQAPGR

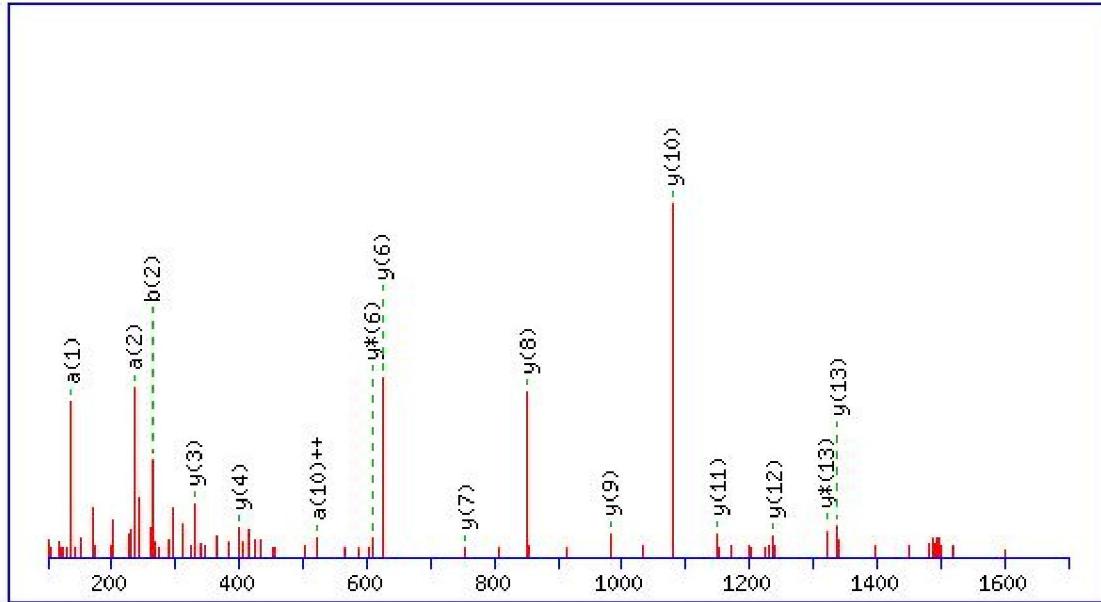
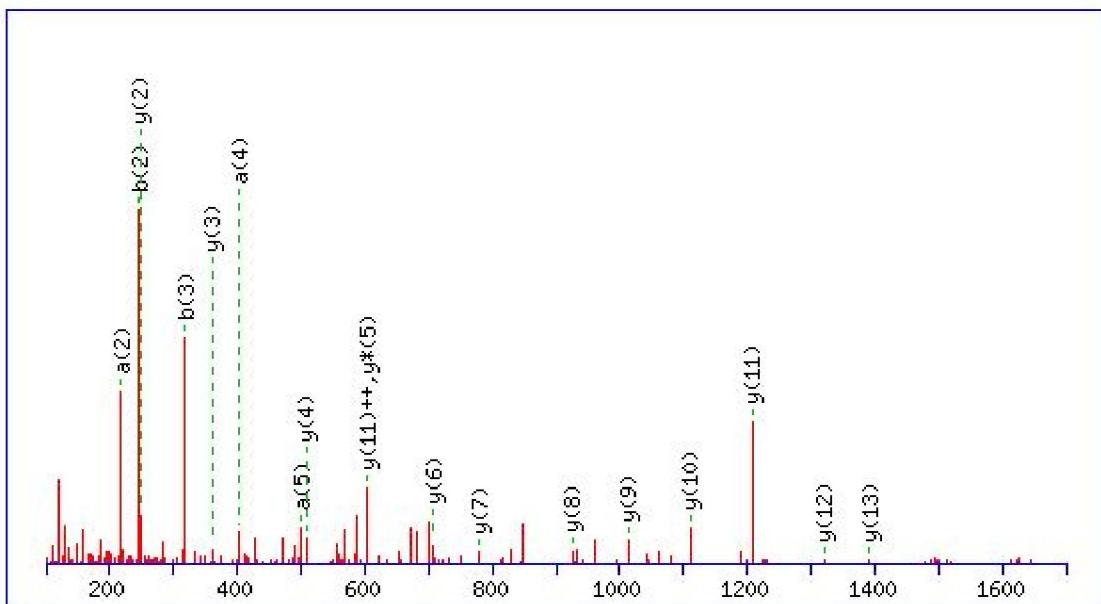


Figure B80: MS/MS spectra of peptide identified as inhibin VFAIPPSFASIFLTK



APPENDIX B (continued)

Figure B81: MS/MS spectra of peptide identified as inhibin GVALHRPDVYLLPPAR

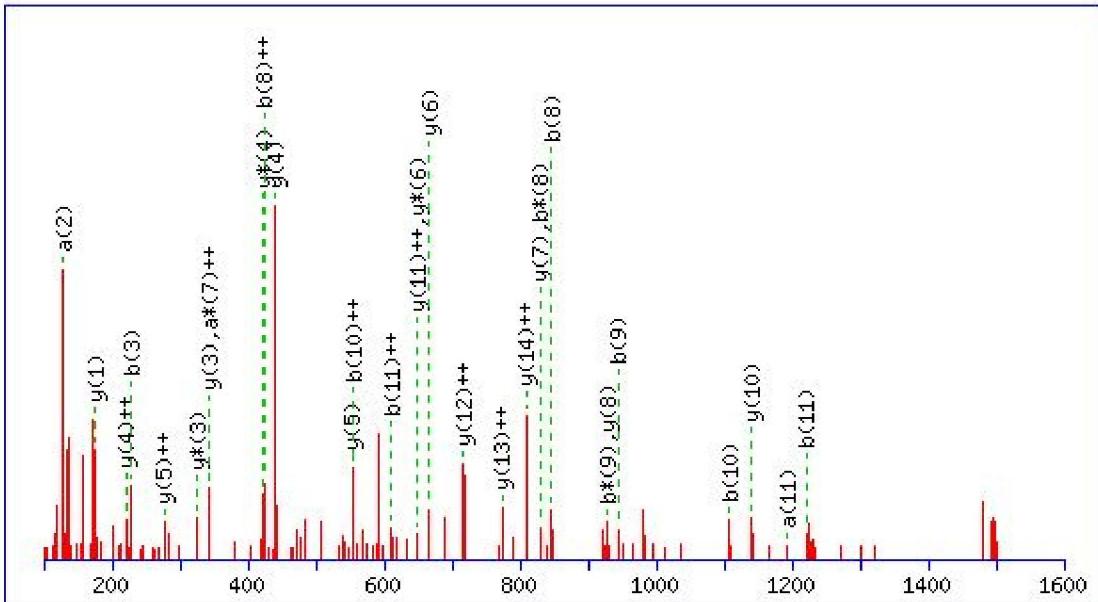
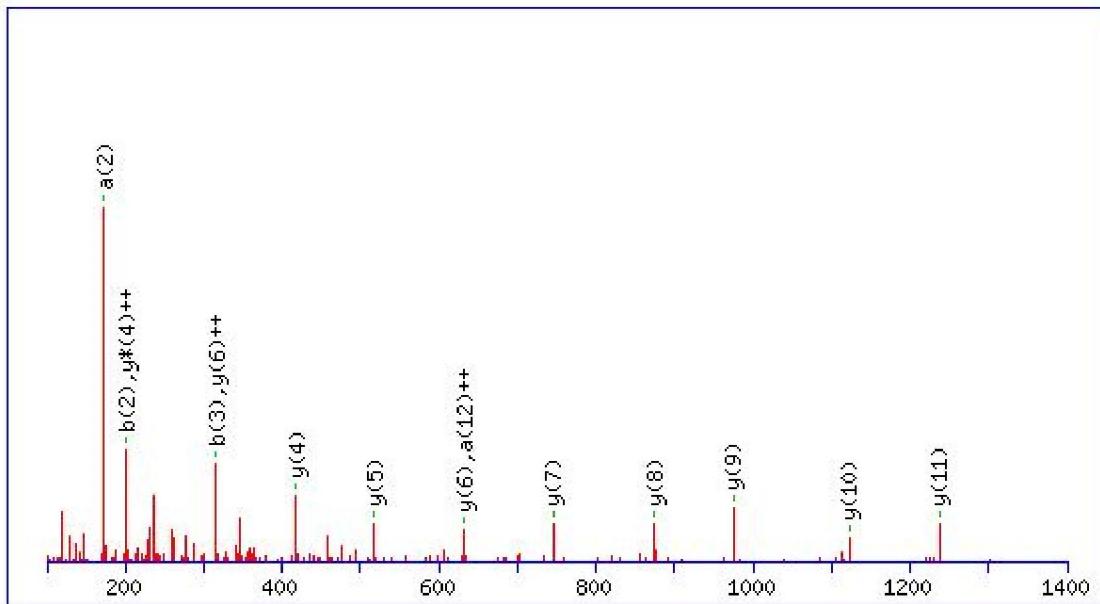


Figure B82: MS/MS spectra of peptide identified as angiotensinogen SLDFTELDVAAEK



APPENDIX B (continued)

Figure B83: MS/MS spectra of peptide identified as angiotensinogen ALQDQLVLVAAK

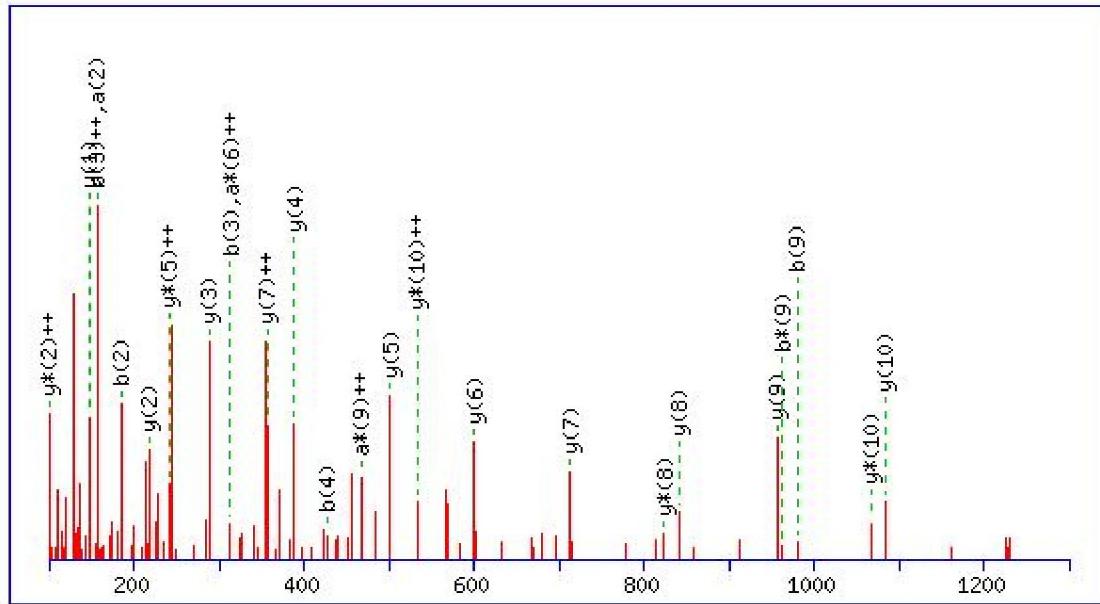
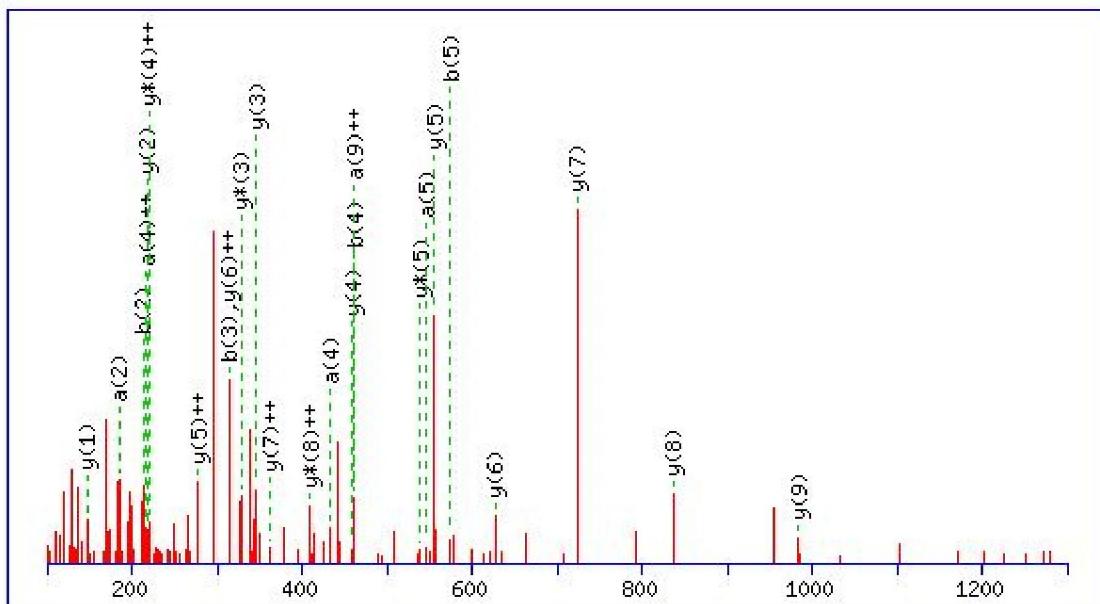


Figure B84: MS/MS spectra of peptide identified as angiotensinogen DPTFIPAPIQAK



VITA

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EDUCATION

Ph.D Medicinal Chemistry anticipated 2016

University of Illinois-Chicago College of Pharmacy

Concentrations: Analytical Chemistry, Proteomics, Metabolomics

Research: *Serum Metabolome and Proteome Effects of Phytoestrogenic Dietary Supplements in Postmenopausal Women*

M.S. Forensic Sciences 2006

University of Illinois-Chicago College of Pharmacy

Concentrations: Toxicology, Drug Chemistry

Research: *The Analysis of PCB Congeners in Air Samples from PCB Transformers for Identification and Quantification*

B.S. Biology: Cell and Molecular 2004

Winona State University College of Science and Engineering

Concentrations: DNA Analysis; Analytical Chemistry

Research: *The Analysis of Plant DNA Isolated from Northern White Cedar Using RAPD-PCR*

B.S. Criminal Justice

2004

Winona State University College of Liberal Arts

Concentrations: Forensic Sciences, Investigations

Internship: *Milwaukee County Sheriff's Department, Summer 2002*

PROFESSIONAL EXPERIENCE

MS Tech Support Specialist 2015-present

Shimadzu Scientific Instruments, Inc., Columbia, MD

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Research Resources Center, University of Illinois at Chicago

Analyst 2007-2010

Animal Forensic Toxicology Laboratory, University of Illinois at Chicago

Consultant 2006-2007

Mills Consulting, Inc., Oak Park, IL

Chemical Analyst 2001-2004

South Eastern Minnesota Analytical Service, Winona State University

RESEARCH EXPERIENCE

Dr. Richard van Breemen's Lab University of Illinois at Chicago	<i>2010-present</i>
Dr. William Mills' Lab University of Illinois at Chicago	<i>2004-2006</i>

RESEARCH SUBMITTED AND POSTER PRESENTATIONS

- *The Analysis of Plant DNA Isolated from Northern White Cedar using RAPD-PCR*
- *The Analysis of PCB Congeners in Air Samples from PCB Transformers for Identification and Quantification*
- *Combining OFFGEL Electrophoresis with Chip-Based HPLC to Expedite Shotgun Proteomics*, MIKI 2013
- *Investigation of Serum Metabolome Changes in Postmenopausal Women After Administration of Phytoestrogenic Dietary Supplements*, MSACL 2014
- *Investigation of Serum Metabolome Changes in Postmenopausal Women After Administration of Phytoestrogenic Dietary Supplements*, UIC College of Pharmacy Research Day 2014
- *Investigation of Serum Metabolome Changes in Postmenopausal Women After Administration of Phytoestrogenic Dietary Supplements*, ASMS 2014

PROFESSIONAL MEMBERSHIPS

- American Academy of Forensic Sciences 2004-2006
- American Society of Mass Spectrometry 2011-current
- Chicago Mass Spectrometry Discussion Group 2013-2015

AWARDS

- University Fellowship, University of Illinois-Chicago 2010
- MSACL Conference Young Investigator Award, San Diego, CA 2014