USING HIGH-FIELD NMR TO IDENTIFY THE BIOACTIVE COMPOUNDS IN EXTRACTS OF BLACK RASPBERRY

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USING HIGH-FIELD NMR TO IDENTIFY THE BIOACTIVE COMPOUNDS IN EXTRACTS OF BLACK RASPBERRY

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Thesis

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ABSTRACT

Recently, food based approaches for treatment of cancer have been explored. Experimental animal research provided compelling evidence that black raspberries could inhibit the proliferation of oral, esophageal, and colon cancer cells. Studies indicated that black raspberry’s phenolic antioxidants especially the anthocyanins, which are responsible for the fruit color, may play an important chemopreventive role. Nineteen different black raspberries from different farms and cultivars were studied using a high-field nuclear magnetic resonance (NMR) spectrometer that was equipped with a cryogenic probehead. The one-dimensional $^1$H-NMR spectra were entirely integrated in 0.004 ppm intervals, which created 2101 data bins that were subjected to principal component analysis (PCA). PCA is a statistical technique for pattern recognition that reduces the information in the large data sets. Principal components (PCs) were calculated using both correlation and covariance matrices. In the correlation matrices, which were more sensitive to the minor constituents, the data was normalized by dividing the standard deviation. In the covariance method, the eigenvalues retained the original data, therefore, it gave more weight to larger values. By comparing the cumulative weighting of PCs, the first seven PCs accounted for 84% of the variability in the correlation matrices and 86% of the variability in the covariance matrices. The NMR data from 19 black raspberry samples were used as data for correlation matrices. Regression analysis against the variable of total monomeric anthocyanin content showed
that PC1, PC2 and PC5 were important in distinguishing between samples. In the covariance method, PC 1, PC 7, and PC 5 were identified as the most important. According to the absolute eigenvector values, it was found that the 1st and 2nd ranked bins correspond to the methyl peak of cyanidin 3-rutinoside and the 3rd and 4th ranked bins correspond to the methyl peak of cyanidin 3-xylosylrutinoside. Multidimensional and selective excitation NMR experiments were also used to identify the structures of bioactive components. Correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC) and selective 1D total correlation spectroscopy (TOCSY) were used to verify the peak assignments of known metabolites and to identify peaks that did not correspond to these known metabolites. Once identified, the additive or synergistic anticancer effects of active compounds can be confirmed by future cancer bioassays. This unique approach will serve as a model to others examining the chemopreventive activity of antioxidant-rich food sources.
DEDICATION

My Parents

Who support me all the time

And

My husband

Who inspires me to pursue my dreams
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CHAPTER I
INTRODUCTION

1.1 Anticancer Research in Fruit and Vegetables

The consumption of fruits and vegetables has been associated with a reduced risk for many different types of human cancer.\(^1,2\) In 1997, the World Cancer Research Fund and the American Institute for Cancer Research produced an extensive report stating that diets rich in fruits and vegetables “decrease the risk of many cancers”.\(^3\) Increasing fruit and vegetable consumption by one to two servings daily may reduce cancer risk by 30%. These protective effects are attributed to the active substances that have been shown to have anticarcinogenic properties including dietary fiber, carotenoids, vitamins C and E, selenium, glucosinolates, indoles, isothiocyanates, flavonoids, polyphenols, protease inhibitors, and plant sterols.\(^4\)

Among fruits, berries such as blackberries, black raspberries, blueberries, strawberries, cranberries and raspberries are popularly consumed in our diet in fresh and in processed forms. There is a diverse range of bioactive phytochemicals including flavanoids, condensed tannins, stilbenoids, phenolic acids and lignans. Phytochemicals are defined as substances involved in secondary plant metabolism, which exhibit a potential for modulating human metabolism in a manner favorable for the prevention of cancers. The flavonoids have demonstrated strong antioxidant activities. The flavonoid
structure is based on a 2-phenyl-benzo[a]pyrane or favane nucleus. This nucleus is defined by having a system of two benzene rings, which are connected by an oxygen-containing pyrane ring.

An oxidant may contribute to carcinogenesis both by causing mutation and by stimulating cell division. The oxidative hypothesis of carcinogenesis is that carcinogens can release free radicals to damage cells and cause these cells to undergo malignant changes. DNA contains reactive groups on its bases that are highly susceptible to free radical attack, and the oxidative DNA damage can lead to deleterious mutation. Most oxidative lesions are efficiently repaired by specific DNA glycosylases, however, unrepaired lesions accumulate with age. When the cells divide, the lesions become fixed and mutations and cancer may result.5

The mechanism of chemoprevention by berry bioactives is still being researched. For both in vitro and in vivo studies, the individual berry components or whole berry extracts have shown anticancer properties through the phytochemicals.6,7 The berry extracts exert the anticancer properties by inhibiting cell proliferation and modulating cell cycle arrest, inducing DNA repair and signal transduction, and reducing cancer cells while having little or no cytotoxic effect on normal noncancerous cells.8-10 The antioxidants in berries have been shown to act as free radical scavengers. Flavonoids act as reducing agents by donating hydrogens to free radicals and causing their removal. The flavonoids with the most hydroxyl groups connected to aromatic rings have the greatest ability to act as antioxidants.

The total antioxidant activity among different fruits can be measured using the
automated oxygen radical absorbance capacity (ORAC) assay,\textsuperscript{11,12} and the Folin-Ciocalteu procedure.\textsuperscript{13,14} Studies have shown that black raspberries have a relatively large antioxidant content among fruit.\textsuperscript{11-14} Many studies have shown that the black raspberries are able to inhibit cell mutation. Stoner et al.\textsuperscript{7,15} demonstrated that lyophilized black raspberries (LBR) could inhibit the N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in the F344 rat during initiation and postinitiation phases of carcinogenesis. Their research showed LBRs inhibited tumor progression as evidenced by significant reductions in the formation of preneoplastic esophageal lesions, decreased tumor incidence and multiplicity, and reduced cellular proliferation. In 2002, Casto et al.\textsuperscript{16} discovered that the number of 7,12-dimethylbenz(a)anthracene-induced tumors were reduced in hamsters cheek pouch by feeding them lyophilized black raspberries. Han et al.\textsuperscript{2,17} found two major chemopreventive components in black raspberries, which were ferulic acid and $\beta$-sitosterol. Their investigations demonstrated that ferulic acid and $\beta$-sitosterol could inhibit the growth of premalignant and malignant oral cells that resided in specific components, without affecting normal human oral epithelial cell lines. Recent studies showed that black raspberries inhibited the azoxymethane-induced tumors in the rat colon, and indicated that black raspberries could inhibit the progression stages of cancer.\textsuperscript{18,19} According to previous research, black raspberries contain a number of known chemopreventive agents, which play important roles to inhibit the proliferation of oral cavity, esophageal and colon cancer cells.
1.2 Basic NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy provides the most versatile and informative spectroscopic technique in modern chemical research. Additionally an NMR spectrometer is one of the most widely used instruments in analytical instrumentation. Therefore, it is important for chemists to understand NMR spectroscopy in order to advance their chemical or biochemical investigations. Before discussing the application of NMR spectroscopy in determining the components in the black raspberries, it is necessary to know different modern NMR methods.

In 1946, Bloch and Purcell discovered NMR by using samples of water and paraffin that yielded narrow and broad proton signals, foreshadowing a multitude of applications to studies in both liquid and solid states.\textsuperscript{20-23} In the recent years NMR spectroscopy has matured to include the utilization of a variety of magnetically active nuclei. The nuclei can be characterized by the nuclear spin quantum number $I$ that has value greater than or equal to zero. The atoms with $I = 0$ do not possess nuclei spin and so can not exhibit nuclear magnetic resonance. They are “NMR silent”. $^1$H and $^{13}$C, which the nuclei possess nuclei spin $I=1/2$, are the most popular nuclei for characterizing materials. Proton one dimensional NMR experiments are single pulse experiments. A pulse corresponds with turning on a radiofrequency radiation for a certain period time and then switching it off. Scheme 1.1 shows the essential elements of the single-pulse NMR experiment. A short and strong pulse is applied in the $x$-$y$ plane to excite all the nuclei and all the signals are collected simultaneously. The pulse rotates all the nuclear spin vectors from the $z$-axis into the $x$-$y$ plane, and the net magnetization signal
When the net magnetization is in the $x$-$y$ plane, it is away from the equilibrium state. Therefore, the transverse vectors will gradually disappear and simultaneously return to the $z$-axis simultaneously. This return to equilibrium is called relaxation and it causes the NMR signal to decay with time, producing the observed Free Induction Decay (FID). The collected signal is time-domain data that can be Fourier transformed to produce the corresponding frequency domain spectrum. Because the sensitivity of NMR is proportional to the magnetic field, it is necessary to use high field NMR spectrometers in order to increase signal noise ratio and obtain detailed spectra.

Scheme 1.1. The essential elements of a single-pulse NMR experiment: The relaxation (recovery) delay, the pulse excitation and the data acquisition time.

The $^{12}$C nucleus is magnetic silent because the spin number $I$ is zero, however, the $^{13}$C nucleus has a spin number $\frac{1}{2}$. Since the natural abundance of $^{13}$C is only 1.1% and its sensitivity detecting a single $^{13}$C is only about 1.6% of $^{1}$H, the overall sensitivity of $^{13}$C to $^{1}$H is about 1/5700. Because of the large $J_{CH}$ value (~110-320 Hz), proton-coupled carbon spectra always show complex overlapping multiplets, which are difficult to interpret. Proton broadband decoupling was developed to solve this problem. Attached
protons are irradiated and saturated at the same time with detection of $^{13}$C signals. In this way it produces a C-13 spectrum with a series of singlets, one for each different carbon environment. The removal of all $^{1}H$-$^{13}$C couplings concentrates all the carbon resonance intensity into a single line providing a significant increase in signal intensity and simplification of the spectrum.

The standardized pulse program for the proton decoupled $^{13}$C spectrum is shown in Scheme 1.2. The proton channel has the decoupler on to remove the $^{1}H$-$^{13}$C coupling, while a short powerful rf pulse excites all the $^{13}$C nuclei simultaneously.

![Scheme 1.2. Standard proton decoupled $^{13}$C NMR pulse sequence.](image)

The chemical shifts of $^{13}$C range over about 220 ppm, about 20 times that of routine proton spectra (~10 ppm). Because of the large range of carbon chemical shift, the coincidences of $^{13}$C chemical shifts are uncommon, and impurities are readily detected. The advantage of using $^{13}$C NMR spectroscopy is that the $^{13}$C chemical shift is more sensitive than $^{1}H$ chemical shift to subtle changes in its immediate electronic environment and relatively insensitive to long-range effects. These factors result in spectra free of the extensive overlap of resonances belonging to different stereo sequences. Besides $^{1}H$ and
$^{13}$C one-dimensional NMR spectroscopy, there are several other advanced 1D NMR methods such as insensitive nuclei enhanced by polarization transfer (INEPT),$^{26-28}$ attached proton test (APT)$^{29,30}$ and distortionless excitation by polarization transfer (DEPT)$^{31,32}$ etc. The purpose of the INEPT experiment is to enable non-selective pulses only. The INEPT sequence provides a method for inverting one-half of each XH doublet in a manner that is independent of its chemical shift requiring the use of non-selective pulses only. The latter two are spectral editing sequences, with APT spectra providing peaks of opposite phase for carbons with even ($N_{\text{H}} = 0,2$) and odd ($N_{\text{H}} = 1,3$) numbers of attached protons ($N_{\text{H}}$). The DEPT sequence produces signals only for protonated carbons, with editing generated by changing the flip angle of the final $^1$H pulse ($45^\circ$, $90^\circ$, or $135^\circ$). A DEPT-135 spectrum is similar in appearance to an APT spectrum (except for the absence of signals for nonprotonated carbons) with peaks of opposite phase for odd or even values of $N_{\text{H}}$. Alternatively, one can acquire a set of DEPT spectra with the three different flip angles and, by appropriate addition and/or subtraction of individual spectra, obtain an edited set of CH, CH$_2$, and CH$_3$ spectra.

High resolution NMR spectroscopy is a selective and sensitive tool to identify the compound’s structures. However, one-dimensional NMR spectra can be very complex because of the overlapping from the multiplicity and relative broadness of resonances. Since the last decade, a number of multidimensional NMR techniques have been generated and applied to the study of biocompounds. These techniques have been used to study organic structures, configurations, conformations and reaction mechanisms.$^{33-35}$ Two-dimensional NMR has been used to simplify the NMR spectra by separating signals.
into two different frequency domains. The principals underlying the generation of a two-dimensional spectrum were first presented in a lecture in 1971, and a number of years later these methods were described in a variety of applications. During the 1980s the world of NMR, and consequently the chemist’s approach to structure determination, was revolutionized by numerous two-dimensional techniques. Two-dimensional NMR spectra could map out interactions within, or sometimes between, the molecules of interest. The interactions can be separated into three categories which are related to different physical phenomena; through-bond coupling, through-space coupling and chemical exchange. The through-bond coupling can be broken down to homonuclear shift correlations and heteronuclear shift correlations, and the experiments considered are aimed at the identification and subsequent piecing together of structural fragments within a molecule. Through-space coupling provides the basis of the Nuclear Overhauser Effect employed to deduce molecular stereochemistry and conformation.

There are different two-dimensional techniques used to analyze the structures and conformations. The principal spin interactions and the main techniques are summarized in Table 1.1.

Table 1.1. The principal structures established through NMR techniques.

<table>
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<th>Principal technique(s)</th>
<th>Comments</th>
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<td>1H-1H COSY</td>
<td>Proton J-coupling typically over 2 or 3 bonds.</td>
</tr>
<tr>
<td>[ \begin{array}{c} \text{H} \downarrow \text{J} \uparrow \text{H} \ \text{X} \text{X} \end{array} ]</td>
<td>1H-1H TOCSY</td>
<td>Relayed proton J-couplings within a coupled spin system. Remote protons may be correlated provided there is a continuous coupling network between them.</td>
</tr>
</tbody>
</table>
One-bond heteronuclear couplings with proton observation.  

Long-range heteronuclear couplings with proton observation. Typically over 2 or 3 bonds when X = $^{13}$C.  

COSY only used when X-spin natural abundance > 20%. Sensitivity problems when X has low natural abundance.  

Through-space correlations. NOE difference only applicable to ‘mid-sized’ molecules with masses ca. 1-2 kDa.  

Sensitivity limited by X-spin observation. Care required to make NOEs specific in presence of proton decoupling.  

Interchange of spins at chemically distinct locations. Exchange must be slow on NMR timescale for separate resonances to be observed. Intermediate to fast exchange requires lineshape analysis.

The two dimensional sequence has preparation, evolution, mixing and detection periods (Scheme 1.3 a). Generally, there are clusters of pulses in preparation and mixing time. The detection period is entirely analogous to the detection period of one-dimensional NMR experiments. The evolution time provides the generation of the second dimension. After the detection process, the collected FID is Fourier transformed to produce a frequency domain spectrum. So in order to generate a spectrum with two dimensional frequencies $f_1$ and $f_2$, it has to sample the data as a function of two different time variables $t_1$ and $t_2$. The simplest pulse sequence for 2D NMR is that both of the preparation and mixing units of Scheme 1.3a are single 90° pulses, which is also the basic correlation spectroscopy (COSY) pulse sequence (Scheme 1.3b). The effect of this pulse sequence on the vector of proton magnetization is shown in Figure 1.1. The vector places
the equilibrium magnetization in the x-y plane along +y-axis after the first 90° pulse, after which it will precess according to the chemical shift offset. After a certain time period, $t_1$, the vector has moved through an angle of $360\nu t_1$ degrees, and then it is subjected to the second pulse. The second pulse moves the vector component along +y-axis ($M_y \cos 360 \nu t_1$) to the –z-axis, while the x-component is unaffected and keeps precessing in the transverse plane and produces the detected FID. Fourier transformation of this FID will produce the spectrum whose intensity depends on the factor $\sin 360 \nu t_1$.

![Diagram](image1)

**Scheme 1.3.** (a) The general scheme for any two-dimensional experiment. P: preparation, E: evolution, M: mixing and D: detection. (b) An illustrative two-dimensional sequence in which P and M are 90° pulses. This is the basic COSY sequence.

![Diagram](image2)

**Figure 1.1.** The action of the COSY sequence of Scheme 1.3 on a single uncoupled proton resonance.
As $t_1$ increases from zero, the resulting signal intensity also increases as $x$-magnetization has time to develop during the evolution time, reaching a maximum when it has evolved through a 90° angle. As the $t_1$ keeps increasing, the $x$-component diminishes, passes through a null, and then becomes negative according to the sine modulation, finally each of the acquired $t_2$ FID produces a series of spectra. It will produce the resonances in $f_1$ frequency domain by Fourier transforming the data in $t_1$ time domain, and so for the $f_2$ domain. Generalizing the process requires monitoring the intensity of every data point in the $f_2$ domain as a function of $t_1$ to produce a complete two-dimensional data set (Figure 1.2).

Figure 1.2. The Fourier transformation of a two-dimensional spectrum.

The total correlation spectroscopy (TOCSY) experiment has been widely used because of its high sensitivity. It involves addition of an isotropic mixing period instead of the second 90° pulse in the basic COSY sequence. The mixing pulse is achieved by a spin-lock pulse that is a composite pulse mixing scheme known as MLEV-17 (Scheme
1.4). The effect of chemical shift is completely eliminated during the spin lock sequence and all the spins in the same spin system are temporarily equivalent. In the presence of spin-lock pulse, the nuclei are strongly coupled to each other in the whole spin system. In this way the pulse sequence can provide correlations among all coupled nuclei in the same spin system rather than between two directly coupled nuclei. The extent of magnetization transfer within a coupled spin system is determined by the sizes of coupling constants and by the length of mixing time. With longer mixing time, the spectrum will have more crosspeaks and correlations. Therefore, by acquiring a series of TOCSY spectra with increasing mixing times, one can observe the successive transfer of magnetization from one proton to others within the sequence of coupled protons. This experiment is particularly suited for the identification and assignment of protons in individual monosaccharide units of a complex polysaccharide.

Scheme 1.4. The TOCSY pulse sequence based on the MLEV-17 mixing scheme. The MLEV sequence is bracketed by short, continuous-wave, spin-lock trim pulses to provide pure-phase data.
As an alternative to the collection of a full 2D spectrum, the 1D analogue can prove advantageous in some circumstances.\textsuperscript{38,39} Compared to the 2D spectra, the time to collect the 1D equivalent is quicker, and it requires less storage space with higher resolution. These features are particularly attractive when specific information is needed, especially for small to medium size molecules. Selective 1D TOCSY presents an especially useful example of this type of experiment since the acquisition of a series of selective 1D TOCSY spectra take much less time than a corresponding 2D TOCSY spectra. Generally, the selective 1D TOCSY pulse sequence begins by selectively exciting one resolved resonance in the spectrum and using it for all the subsequent magnetization transfer. There have been different methods to generate 1D TOCSY with or without gradients, and a general scheme using the MLEV-17 sequence is shown in Scheme 1.5. After the excitation spins, the magnetization transfer is initiated with the mixing period. The only resonances that show up in the resulting spectra are those that have received the magnetization from the source spin. The experiment produces 1D subspectra for discrete spin systems in the molecules, especially showing multiplet structures that were overlapped or buried in the standard 1D NMR spectrum. Furthermore, the higher digital resolution of 1D analogue may resolve ambiguities arising from crosspeak overlap in 2D spectra.

Scheme 1.5. The general sequence for selective 1D TOCSY. Any selective 90° pulse can
be used to selectively excite the target resonance.

In 1977, the $^1$H-$^{13}$C heteronuclear $J$-resolved (HET2DJ) experiment was designed to directly detect $^{13}$C chemical shifts in one dimension and to resolve the $^1$H-$^{13}$C $J$ coupling in the second dimension. The $^{13}$C-$^1$H heteronuclear chemical shift correlation (HETCOR) experiment could correlate $^{13}$C and $^1$H chemical shifts among directly bonded proton and carbon atoms. To produce this two-dimensional shift correlation experiment, the variable $t_1$ evolution period is added immediately after the initial proton excitation and prior to the polarization transfer step so that the detected $^{13}$C signal becomes modulated by the proton chemical shift as a function of $t_1$ (Scheme 1.6). During this, $^1$H-$^1$H and $^1$H-$^{13}$C coupling will also evolve, leading to the appearance of both homonuclear and heteronuclear splittings in $f_1$, reducing signal intensities. The removal of $^1$H-$^{13}$C coupling can be achieved by refocusing $^1J_{CH}$ with the insertion of a 180° carbon pulse at the midpoint of $t_1$ to reduce resonance offset effects.

Scheme 1.6. The 2D shift correlation experiment (HETCOR) is derived from the INEPT sequence by the addition of the $t_1$ evolution period to encode proton chemical shifts prior to polarization transfer. The 180° carbon pulse at the midpoint of $t_1$ refocuses heteronuclear coupling evolution and thus provides $^{13}$C decoupling from $^1$H in $f_1$. 
The high-sensitivity of modern correlation techniques often provides a fast method to determine chemical shifts of the X-nucleus indirectly and avoids the need for the direct observation altogether. Therefore, the heteronuclear multiple quantum correlation (HMOC)\textsuperscript{43} and heteronuclear single quantum correlation (HSQC)\textsuperscript{44} represent the primary $^1\text{H}-^1\text{C}$ 2D techniques in structural organic chemistry\textsuperscript{45} especially since the advent of pulse field gradients (PFGs). Pulse field gradients were first adopted into HMQC experiments to accomplish the coherence selection in 1991.\textsuperscript{46,47} PFGs are used as a tool to select those signals deemed interesting and suppressing those which are not, and speed up the 2D NMR experiments. The PFGs cause the elimination of the signals from the protons bond to $^{12}\text{C}$ in the presence of detected signals from $^1\text{H}-^1\text{C}$ (suppressing ratio $\approx 1:100$); even the selection of natural abundance signals from $^1\text{H}-^1\text{C}-^1\text{C}$ fragments becomes feasible.\textsuperscript{48,49}

The basic HMQC sequence is simple, comprising only four rf pulses (Scheme 1.7). Considering only one simple $^1\text{H}-^1\text{C}$ spin pair, the sequence starts with proton excitation followed by the evolution of proton magnetization under the influence of the one-bond carbon-proton coupling. During the period $\Delta$, antiphase proton magnetization develops and will be transferred to the coupled partner by the action of a subsequent rf pulse. The first pulse on the carbon channel generates proton-carbon multiple quantum coherence. This coherence is a combination of both heteronuclear double- and zero-quantum coherences, as represented on the coherence transfer pathway of Scheme 1.7. Since the coherences contain terms for both transverse proton and carbon magnetization, they will evolve under the influence of both proton and carbon chemical shifts. To remove the
effect of proton shifts during $t_1$, a spin-echo is incorporated by placing a proton $180^\circ$ pulse at the midpoint of $t_1$, so by the end of the evolution time these shifts have refocused and thus have no influence in $f_1$. Evolution of the carbon shifts is unaffected by the proton pulse, so these remain to produce the desired frequency labeling. The final carbon pulse then reconverts the multiple-quantum coherence back to observable single-quantum proton magnetization. When collecting the FID decoupling on the carbon channel is generally applied to remove proton-carbon coupling. The second $\Delta$ period is inserted to refocus the proton-carbon coupling, which avoids the cancellation of the antiphase proton satellites.

Scheme 1.7. The HMQC sequence and associated coherence transfer pathway. The $\Delta$ periods are set the $1/2 \, J_{\text{CH}}$ to enable defocusing and subsequent refocusing of the one-bond heteronuclear coupling.

The heteronuclear single-quantum correlation experiment (HSQC) differs from HMQC in that only single-quantum magnetization of the heteronuclear spin evolves during $t_1$ period (Scheme 1.8), rather than $^1\text{H}$-$\text{X}$ multiple-quantum coherence. The transverse heteronuclear magnetization is generated by polarization transfer from the
attached protons via the INEPT sequence. The X-nucleus magnetization evolves during $t_1$ with the proton 180° pulse at its midpoint refocusing $^1\text{H}$-X coupling evolution, thus decoupling the $^1\text{H}$-X interaction so that only heteronuclear chemical shifts modulations remain in $f_1$. Following $t_1$, the heteronuclear magnetization is transferred back to the protons by a reverse INEPT step to produce in-phase proton magnetization for detection in the presence of X-spin decoupling. The basic two-step phase cycle is analogous to that of HMQC requiring inversion of the first 90° X pulse with associated inversion of the receiver.

![Scheme 1.8](image)

Scheme 1.8. The HSQC experiment and associated coherence transfer pathway. The experiment uses the INEPT sequence to generate transverse X magnetization which evolves and is then transferred back to the proton by an INEPT step in reverse.

HSQC produces similar interactions to those of the HMQC experiment; the crosspeaks do not contain homonuclear $^1\text{H}$-$^1\text{H}$ couplings along $f_1$. This is because only X-nucleus magnetization evolves during $t_1$ which is not influenced by homonuclear proton couplings. HSQC has advantages over HMQC for small molecules when the X nucleus spectrum is poorly dispersed. The greatest disadvantage of HSQC is the many pulses it uses, especially the 180° pulses on the heteronuclear channel. The intensity is lost from rf inhomogeneity, pulse miscalibration or off-resonance excitation. Such losses
could be minimized by careful probe tuning and the use of composite or adiabatic 180° pulses.

The 1H-X heteronuclear correlation matrices presented so far are not able to provide assignments for non-protonated and ambiguous carbon assignments when proton resonances exactly overlap. The alternative way is to establish correlations between carbons and neighboring protons over more than one bond, as is accomplished with the proton-detected heteronuclear multiple bond correlation (HMBC) experiments.50,51 The HMBC experiment involves proton-carbon connectivities through couplings over two or three bonds. The ability to identify 1H-13C correlations across carbon-proton linkages presents a wealth of information on the molecular skeleton.

The sequence of HMBC is in essence similar to the HMQC sequence (Scheme 1.9). The tuning of the experiment is achieved by setting the \( \Delta \) preparation period to a sufficiently long time to allow the small long-range proton-carbon couplings to evolve to produce the antiphase displacement of vectors required for the subsequent generation of heteronuclear multiple-quantum coherence. During this long \( \Delta \) period, homonuclear 1H-1H couplings also evolve and introduce phase distortions to the observed crosspeaks. Therefore, the absolute value display mode is widely used for HMBC spectra to avoid the phase distortions. For sensitivity reasons, the refocusing \( \Delta \) period of HMQC is omitted in HMBC so that long-range heteronuclear couplings are antiphase at the start of \( t_2 \), precluding the application of 13C-decoupling. The introduction of pulsed field gradients successfully suppresses the 1H-12C signals which could mask the long-range satellites, along with the dramatic reduction of \( t_1 \)-noise.
1.3 NMR Spectroscopy and Pattern Recognition Techniques

NMR spectroscopy is usually the method of choice for natural product structure determination and it is not surprising that this technique has come to the fore in plant metabolomics. The data requirements for metabolomics are the qualitative and quantitative analysis of the maximum number of metabolites with the highest achievable throughput. Most metabolomics laboratories use a variety of spectroscopic techniques to identify and quantify the complete set of metabolites in plants as quickly as possible, and without bias. NMR has a number of advantages over other analytical methods currently being used. NMR is non-destructive, and spectra can be recorded from cell suspensions, tissues, and even whole plants, as well as from extracts and purified metabolites. The sample preparation is relatively simple when compared to other analytical methods. NMR offers an array of detection schemes that can be tailored to the nature of the sample, and the desired qualitative or quantitative information on the components of metabolic network. Thus it is possible to use NMR to analyze the metabolite compositions of tissue extracts, determine the structure of a novel metabolite, demonstrate the existence of a particular metabolic pathway in vivo, and localize the distribution of a metabolite in a tissue. A high sample throughput with little instrument drift is readily achieved.
Metabolite screening requires maximum sensitivity with a broad compound coverage. For NMR, this usually means that only the most sensitive and commonly occurring magnetic nucleus (i.e. $^1$H) is observed. However, more information on topics such as metabolite flux can be obtained with other nuclei, particularly $^{13}$C and $^{15}$N.

Fingerprinting techniques involve collecting spectra of unpurified solvent extracts under standardized conditions and ignoring the problem of making individual assignments of peaks in the resulting complex NMR spectra. Multivariate statistical methods such as principal component analysis (PCA) are used to compare sets of spectra, and to identify clusters of similarity or difference so that conclusions can be drawn about the classification of individual plant samples. The identities of metabolites responsible for differences between classes can be investigated from loadings plots generated by PCA and related techniques. In outline, metabolite fingerprinting involves sorting datasets into categories so that conclusions can be drawn about the classification of individual samples. Typically, the starting point is a principal component analysis of the digitized spectrum, and this may be sufficient to divide the sample set into a number of categories. Subsequently, it investigates the variables that are very important in distinguishing between the samples, which leads back to the NMR signals and the metabolites. Thus the approach has the great advantage of avoiding the time-consuming process of assigning every signal before it is necessary. When that point is reached, attention is focused on those parts of the spectrum that are most relevant to the problem. The approach also has the advantage of being rapid, and it is largely unbiased in detecting the metabolites in the sample, especially the minor components. All the advantages make NMR fingerprinting
an attractive analytical technique for defining metabolic phenotypes.

For example, NMR fingerprinting with multivariate analysis of the data has been used to identify and classify the maize seeds into different classes according to changes in metabolites. Baker et al. used NMR fingerprinting to examine three kinds of transgenic wheat which were grown in two different fields over three years. Multivariate analysis of the data collected from extracts of flour showed that there was a stronger influence of site and year than there was due to genotype. They concluded that the growth environment had a significant effect on plant metabolome. In a study of apple juices, it was shown that the ¹H-NMR spectra could be used to distinguish between three types of apple with a success rate of up to 100% under favorable conditions. This study was largely based on principal component analysis; inspection of the loadings identified sucrose and malate as the major basis for the classification. Bailey et al. compared commercial feverfew and demonstrated that NMR is a non-selective analytical technique that can pick out anomalous or unusual samples, as well as the power of multivariate data analysis for discriminating between sets of similar spectra.

This research was designed to take the advantage of the metabolomic approach with statistical analyses, but without the initial identification of all of the chemical constituents. The study was focused on employing NMR techniques along with chemical or biological assays to identify specific compounds and explain the complex interactions that are responsible for the activity of crude extract assays. The advantages of this approach are that NMR spectroscopy could reveal substantial variability in the metabolic composition of plants and multivariate analysis could identify the interesting features of a
spectrum prior to making assignments to every single peak. The overall goal is to develop a model system to correlate the bioactive compounds in plants with their chemoprotective properties. The premise of this modeling is that NMR spectroscopy provides a fast, non-destructive, accurate and reproducible method to screen comprehensive compositional data from plant materials, and the principal component analysis and multivariate regression analysis can lead to identification of specific signals that are associated with the active compounds, avoiding the interpretation of the entire complex 1D NMR spectra.

Based on the previous research, in this study black raspberries were chosen to develop a model system. Recent research has provided solid evidence that among different fruits black raspberries (BRs) contain higher content of chemopreventive compounds. Black raspberries have also inhibited the proliferation of cancer cells such as oral, esophagus and colon cancer. Additionally, a database has been developed as to the black raspberry antioxidant and phenolic constituents, especially the content of anthocyanins. Once the constituents with bioactive properties are identified, the additive or synergistic anticancer effects of active compounds could be confirmed by additional cancer bioassays. This unique approach could serve as a model to examine the chemopreventive activity of other antioxidant-rich food sources.
CHAPTER II
EXPERIMENTAL

2.1. Materials

Caffeic acid, chlorogenic acid, quercetin dihydrate, quercetin 3-glucoside, gallic acid, ellagic acid, trifluoroacetic acid, methanol-d₄ (99.8 atom % D), trifluoroacetic acid-d (99.5 atom % D) and tetramethylsilane were purchased from Sigma-Aldrich. The chloride salts of cyanidin 3-glucoside (cy 3-glc), cyanidin 3-rutinoside (cy 3-rut), cyanidin 3-sambubioside (cy 3-sam) and pelargonidin 3-glucoside (pel 3-glc) were purchased from Polyphenols Laboratories AS (Sandnes, Norway). The C₁₈ Solid Phase Extraction columns were purchased from Alltech Associates, Inc. (Deerfield, IL).

2.2. Sample Preparation, Chemical Assays and Biochemical Analyses

Nineteen ‘Bristol’, ‘Jewel’, and ‘Mac Black’ BR samples were obtained from either commercial farms or from OARDC field plots in July-August, 2004. All the samples were frozen at –30°C. Samples were thawed at room temperature (≈20°C), combined with doubly-distilled water (2:1 v:v) and homogenized in a blender. The resultant slurry was centrifuged (12000 g) for 30 minutes to separate the juice from the pulp. Juice samples were refrozen at –30°C until analyzed. Chemical assays were used to measure the levels of soluble solids, titratable acidity levels, total phenolic content and total...
monomeric anthocyanins levels. Two different biochemical analyses were conducted. The ferric reducing ability of plasma (FRAP) analysis was conducted according to Benzie and Strain, and the ability of standards and fruit extracts to scavenge the DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical was measured by using the method of Brand-Williams et al.60

2.3. NMR Sample Preparation

Black raspberry samples for NMR examination were processed to remove free sugars by separation on SPE columns. Alltech C18 SPE columns were equilibrated with 25 mL of 0.1% TFA in doubly-distilled H2O after washing the column with 25 mL of 0.1% HPLC grade methanol. Four milliliter aliquots of black raspberry juice, acidified with 0.1 % TFA, were loaded onto individual columns and washed with an additional 25 ml of acidified water to remove free sugars. Anthocyanins and other phenolics were eluted with 25 mL of acidified methanol. Methanol was removed from the eluted materials under N2 at 30°C and water was then removed by lyophilization. Dried samples were sealed in glass tubes capped with Teflon-lined caps and stored at -80°C.

Dried black raspberry samples (30-40 mg) were then solubilized in 1 mL methanol-d4 and trifluoroacetic acid-d (95/5 v/v), filtered through glass wool and placed in 5 mm NMR tubes for analysis on a 750 MHz NMR spectrometer. Tetramethylsilane was used as an internal reference standard. All samples were stored at 4 °C prior to analysis. The sample temperature was held at 25 °C while collecting NMR spectra.
2.4. NMR Investigation

$^1$H and 2D NMR spectra were obtained using a Varian Inova 750 MHz spectrometer with a Varian triple resonance $^1$H{${}^{13}$C/$^{15}$N} pulsed field gradient (PFG) cryoprobe. The probe is optimized for $^1$H observations with the capability for simultaneous or single irradiation of $^{13}$C and/or $^{15}$N frequencies. The $^2$H frequencies are derived from a cooled coil for the most efficient lock performance, deuterium pulses, and decoupling. These probes are equipped with a high-performance, actively-shielded z-axis gradient. $^{13}$C-NMR spectra were obtained using a Varian Inova 400 MHz spectrometer with 5mm $^1$H,$^{19}$F/$^{15}$N-$^{31}$P switchable probe. Switchable probe is a direct detect X-nucleus probe and has excellent $^{13}$C sensitivity. Spectra were obtained from extracts of black raspberry in methanol-d$_4$. All NMR spectra were processed with Varian’s VNMR software on Sun workstations.

2.4.1. Acquisition of 1D NMR Spectra

The $^1$H spectrum was obtained with the following parameters: a 7.5 $\mu$s (90°) $^1$H pulse width, a 9599.2 Hz spectral window, a 2.73 s acquisition time, 128 transients, and 5s relaxation delay. The data were zero filled to 132k and exponentially weighted with 0.5 Hz line-broadening before Fourier transformation. The 1D $^{13}$C-NMR spectra were obtained with a 5mm switchable probe, with an 8.3 $\mu$s (90°) $^{13}$C pulse width, a 23068 Hz spectral window, a 1.31s acquisition time, 13312 transients, and 2s relaxation delay. The spectra were obtained with continuous WALTZ-16 modulated decoupling. The data were zero filled to 132k and exponentially weighted with 2 Hz line-broadening before
2.4.2. Acquisition of PFG-HSQC and HMBC 2D NMR Spectra

The 2D HSQC NMR spectra were obtained with a 90° pulse widths for $^1$H and $^{13}$C of 7.5 µs and 12.5 µs, a 1 s relaxation delay, $\Delta = 3.6$ ms ($1/2J$, based on $^1J_{CH} = 140$ Hz) and a 0.085s acquisition time (with $^{13}$C GARP decoupling$^{61}$). Sixteen transients were averaged for each of 1024 increments during $t_1$ using the States method$^{62}$ of phase sensitive detection. The evolution time was incremented to provide the equivalent of a 38461 Hz spectral width in the $f_1$ dimension, and an 8381.4 Hz spectral width was used in the $f_2$ dimension. The PFG pulses were 2.0 and 1.0 ms in duration and had amplitudes of 0.188 and 0.0941 T/m, respectively. The experiment time was about 10 hours. Data was zero filled to a 4k × 4k matrix before Fourier transformation. The pulse sequence for the HSQC experiment is shown in Figure 2.1.

![Figure 2.1](image)

Figure 2.1. The gradient selected HSQC sequence for multiplicity editing within the 2D correlation experiment. Setting $\Delta = 1/2J$ inverts $XH_2$ responses relative to those of $XH$ and $XH_3$.

The 2D HMBC NMR spectra were collected using the pulse program shown in Figure 2.2 with the following parameters: a 90° pulse widths for $^1$H and $^{13}$C of 7.5 µs and
14 µs, a 1s relaxation delay, $\Delta = 1.8$ ms (1/4$J_{\text{CH}} = 140$ Hz) and a 0.122s acquisition time. Twenty-four transients were averaged for each of 1024 increments during $t_1$ with a spectra window of 44223 Hz in the $f_1$ dimension and 8381.4 Hz in the $f_2$ dimension. HMBC spectra were obtained with $\tau_{\text{mb}}$ delays of 0.080s to allow the long-range heteronuclear antiphase magnetization to evolve from multiple-bond $^nJ_{\text{CH}}$ to HMBC correlation. The PFG pulses were 2.0 ms in duration and had amplitudes of 0.188 and 0.140 T/m respectively. Data was zero filled to a 4k × 4k matrix before Fourier transformation.

Figure 2.2. The HMBC sequence with incorporation of pulsed field gradients.

2.4.3. Acquisition of 1D Selective TOCSY Spectra

The 1D selective TOCSY NMR spectra were collected on a Variana Inova 750 MHz spectrometer, using a Varian $^1\text{H}/^2\text{H}/^{13}\text{C}/X$ (X tunable between the frequencies of $^2\text{H}$ and $^{31}\text{P}$) 5 mm PFG probe. The pulse sequence consisted a 90° pulse-z gradient-selective 180° pulse (Gaussian)-z gradient train, to achieve selective excitation of the target peak, followed by an MLEV17 TOCSY spin lock (Figure 2.3).$^{63-65}$ The relaxation delay and mixing time were set to 1s and 100 ms respectively without presaturation. The
PFG pulses were 2.0 ms in duration and had amplitudes of 0.0938 T/m. A total of 128 transients were averaged, resulting in a total experiment time of 12 minutes. The data were zero filled to 132k and exponentially weighted with a 2 Hz line-broadening before Fourier transformation.

Figure 2.3. Pulse sequence for 1D selective TOCSY.

2.5. Statistical Models

\(^1\text{H}\)-NMR spectra of 19 berry samples were first baseline corrected. To quantify the variations, the integrated areas (termed as bins) were obtained in 3 Hz (0.004 ppm) intervals from 0.664 ppm (498 Hz) to 9.548 ppm (7161 Hz). Any region containing solvent signals and suppressed signals were excluded from the analysis. The excluded regions included solvent signal from 3.292 ppm (2496 Hz) to 3.360 ppm (2520 Hz), and the suppressed signal from 4.812 ppm (3609 Hz) to 5.212 ppm (3909 Hz). The 2101 bins from each berry sample were subjected to principal component analysis carried out with MINITAB software (Release 14.1 version), using both covariance and correlation matrices. PCA entails the computation of eigenvectors of a matrix \((X^TX)/(n-1)\), where \(X\) contains \(n\) observations entered into the matrix row-wise. If the \(X\) is mean-centered (column means subtracted), then \((X^TX)/(n-1)\) is the covariance matrices. If the data in \(X\) are standardized (mean-centered and columns scaled to unit variance), then \((X^TX)/(n-1)\)
is the correlation matrices. The number of PCs to be used for a given model was
determined using Scree plots. With only nineteen samples, eighteen was the largest
number of principal components possible. The principal components were calculated for
each sample and correlations between the principal components and the biochemical
variables were then calculated. Each of the 19 samples was evaluated to determine which
samples were most extreme for each principal component.

Best subsets regression analysis was conducted with MINITAB software (Release
14.1 version) using the PCs as predictors to develop multiple regression equations for the
chemical and biological variables. The bins associated with the regression model were
used to identify the regions of the NMR spectra of black raspberry samples that most
likely accounted for the regression relationship. Once the important areas of the $^1$H NMR
spectra were determined, the individual compounds associated with the peaks were
identified by 2D NMR analyses.
CHAPTER III
RESULT AND DISCUSSION

3.1. Compositions and Structures of Berry Bioactives

Over the past few decades, with the advent of highly sensitive analytical methods, knowledge of the compositions of berry fruits has rapidly expanded allowing researchers to establish phytochemical profiles or “chemical fingerprints” of these fruits. These bioactive phytochemicals have been identified as flavanoids (anthocyanins, flavonols, and flavanols), condensed tannins (PAs), hydrolyzable tannins (ETs and GTs), stilbenoids, phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and lignans.\textsuperscript{66,67} Different skeletal structures of these compounds produce unique biological properties for each class that affects the absorption, distribution, metabolism, and excretion in humans.\textsuperscript{68,69} The structural diversity of berry bioactives can be observed by the different types and oxidation levels of their heterocyclic ring, their hydroxylation substitution patterns, the existence of stereoisomers, their glycosylation by various sugars, acylation by organic and phenolic acids, and by conjugation with themselves to form polymers etc.
Of the numerous phytochemicals found in berries, the anthocyanins are probably the best known. Anthocyanins are the pigments that contribute to bright red, blue, purple, violet, and intermediate red-purple in berries and many other fruits, vegetables, and grains.\textsuperscript{70,71} Anthocyanins occur naturally in fruits and vegetables as glycosides. The deglycosylated or aglycone forms of anthocyanins are known as anthocyanidins. Several hundred anthocyanins are known varying in the basic anthocyanidin skeleton, with the six most common being cyanidin (the most ubiquitous), delphinidin, pelargonidin, malvidin, petunidin, and peonidin (Figure 3.1 and Table 3.1). However, the most common anthocyanidin that exists in black raspberries is cyanidin. Besides the

![Figure 3.1. Structure of anthocyanidin skeletons: with different substituents R$_1$, R$_2$ and R$_3$, representing different anthocyanidin skeletons.](image)

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin (Pg)</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>271</td>
</tr>
<tr>
<td>Cyanidin (Cy)</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>287</td>
</tr>
<tr>
<td>Delphinidin (Dp)</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>303</td>
</tr>
<tr>
<td>Peonidin (Pn)</td>
<td>OMe</td>
<td>OH</td>
<td>H</td>
<td>301</td>
</tr>
<tr>
<td>Petunidin (Pt)</td>
<td>OMe</td>
<td>OH</td>
<td>OH</td>
<td>317</td>
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<tr>
<td>Malvidin (Mv)</td>
<td>OMe</td>
<td>OH</td>
<td>OMe</td>
<td>331</td>
</tr>
</tbody>
</table>
variations in the anthocyanidin skeleton, structural diversification is further achieved by
the identity, extent, and position at which glycosides and acyl groups are attached to the
skeleton. The most common glycosides attached on anthocyanins are glucose, galactose,
rhamnose, and arabinose usually as 3-glycosides or 3,5-diglycosides. Based on the NMR
studies, we found that cyanidin 3-glucoside (cy 3-glc), cyanidin 3-rutinoside (6-O-L-
rhamnosyl-D-glucoside) (cy 3-rut), cyanidin 3-sambubioside (2-O-D-xylosyl-D-
glucoside) (cy 3-sam), and cyanidin 3-xylosylrutinoside (2-O-D-xylosyl-6-O-L-
rhamnosyl-D-glucoside) (cy 3-xylrut) were the major anthocyanins in black raspberries.
There also was a minor amount of pelargonidin 3-rutinoside (pg 3-rut) in black raspberry.
The structures of all anthocyanins discovered in black raspberry are shown in the Figure
3.2.
Figure 3.2. The structures of anthocyanins found in the black raspberry: (a) Cyanidin 3-glucoside; (b) Cyanidin 3-rutinoside; (c) Cyanidin 3-xylosylrutinoside; (d) Cyanidin 3-sambubioside, (e) Pelargonidin 3-rutinoside.

The 19 different samples from different commercial farms and cultivars in Ohio State were studied. The 19 samples were from four different cultivars and seven different farms. Table 3.2 gives detailed information about the 19 samples and Table 3.3 shows the commercial farm locations in Ohio.
3.2. Identification of Black Raspberry Components by NMR Spectroscopy

High resolution NMR spectroscopy was used to identify the components in the black raspberries. The $^1$H-NMR and $^{13}$C-NMR spectra were used to identify the protons on the anthocyanidin skeleton and some protons on the sugars such as H1’, H1” and the methyl peak on rhamnose, etc. Multidimensional NMR was also employed to obtain the structure information on the different anthocyanins.

3.2.1. Increase of Sensitivity and Resolution

Compared to other analytical methods, NMR has the advantage of unequalled reproducibility and stability. However, there are limitations for life sciences because of the low sensitivity of the method. Therefore, it is always desirable to increase the sensitivity and resolution of NMR experiments. In the past decade, there have been continuous and amazing improvements of NMR since it was adopted as an analytical technique. There are several options that can have a major influence on NMR spectra.
with respect to the analysis of plant metabolic analysis.

There are two key hardware effects that can influence the sensitivity and resolution: the magnetic field strength, and the design of the probehead that accommodates the sample and generates the NMR signals. The magnetic field strength ($B_o$) available now ranges from 4.6 to 21 Tesla, which corresponds to $^1$H resonance frequencies of 200-900 MHz. The resolution, sensitivity of detection and signal-to-noise ratio are proportional to $B_o$, $B_o^2$, $B_o^{3/2}$ respectively, so it is advantageous to collect the spectra at high field strength. High magnetic fields are particularly beneficial for metabolic profiling, because the improved resolution could give more detailed information from the spectra. Therefore, a 750 MHz NMR spectrometer was used for proton detected experiments.

Achievement of high fields can be limited by technical and economic factors. Probehead design has a marked influence on the achievable sensitivity at a particular field strength. The sensitivity of any spectroscopic method depends on the signal-to-noise ratio. For NMR, the random thermal motions of electrons in the detection coil and in the first amplifier stage, which create random voltages, cause the noise. Theory shows that the root mean square noise voltage is linearly related to the coil’s resistance and to the square root of its absolute temperature. Therefore, the leading manufacturers have commercialized the concept of a cryogenic cooled detection coil.\textsuperscript{72} The sensitivity advantage of a cryoprobe results from the cooling of the receiver coil and its associated circuitry to cryogenic temperature ($\approx 25$K). Several hundred cryogenic probes are currently in use for a wide variety of research involving both large and small molecules.\textsuperscript{73}

In the beginning the probes were mainly used to record the spectra of biological
macromolecules, but recently the cryogenic probeheads have become more widespread. A recent analysis of alkaloid metabolism in cultured plant cells benefited from a substantial gain in sensitivity by using a cryogenic probehead in a 500 MHz magnet. Figure 3.3 shows the same region of $^1$H-NMR spectra of berry extract sample 9, which were run on a normal PFG triple resonance probe (Figure 3.3 a) and a cryoprobe (Figure 3.3 b). As the figure shows, the signal-noise ratio of the $^1$H-NMR spectrum run on a cryoprobe was increased by 2~3 fold compared to the spectrum of the same sample ran on a normal triple resonance PFG probe. In this way, the spectra were able to provide us more detailed and substantial information about minor peaks, which was helpful in principal component analysis.

Figure 3.3. $^1$H-NMR Spectra (750MHz) for berry extract sample 9 from a normal probe (a), and cryoprobe (b).

3.2.2. One dimensional NMR Spectra

The one dimensional $^1$H-NMR spectrum of black raspberry extract and the expanded regions are shown in Figure 3.4. The peaks were referenced relative to the TMS peak (0 ppm). The presence of cy 3-glc, cy 3-rut, cy 3-sam, cy 3-xylrut, and pg 3-rut was confirmed by comparison of the $^1$H- and $^{13}$C-NMR chemical shifts with those of
known standards and published data.  

On the basis of chemical shifts and coupling-patterns, the signals of AMX system at 7.03, 8.03, 8.28 ppm were assigned to H5’, H2’ and H6’ respectively. The 2H AX system at 6.90 and 6.68 correspond to H8 and H6, and the singlets around 9.00 ppm are from H4 on the anthocyanidin skeleton. The H4 proton peaks are considerably downfield compared to the sugar region. The H4 region shows four different resonances, which correspond to at least four different anthocyanins peaks from downfield to upfield: cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside (Figure 3.4 b). The 1H-NMR spectrum shows signals of four anomeric protons appearing at 5.46, 5.29, 4.78, 4.65 ppm respectively. They are the chemical shifts of H1″ on glucose, H1‴ on the xylose attached to glucose, and H1IV on the rhamnose attached to glucose. Two different chemical shifts are observed for H1″, which is caused by different structures of the anthocyanins. From Figure 3.2, it is obvious that in some anthocyanins the 2″-OH group of xylose causes the chemical shifts of H1″ of glucose to shift downfield (Figure 3.4 c). Therefore, the H1″ on cy 3-xylrut and cy 3-sam has the chemical shift of 5.46 ppm, and the H1″ on cy 3-glc and cy 3-rut has a chemical shift of 5.29 ppm.
Figure 3.4. The $^1$H-NMR spectrum of Sample 8: (a) whole spectrum (-0.6 ppm ~ 10.4 ppm), (b) expansion of H-4 region, (c) expansion of the anomeric protons region.

The $^{13}$C-NMR chemical shifts observed for the extract of black raspberry were similar to the published data.$^{75-78}$ The C7 resonated at very low field 170.43 ppm compared to the rest of the oxygenated aromatic carbons in anthocyanins. The oxygen
cation in the adjacent ring is responsible for the downfield shift of C7 carbon. The $^{13}\text{C}$ chemical shifts of the anthocyanidin skeleton and glucose were identified based on previous references. The carbon chemical shifts are shown in Table 3.4.

Table 3.4. $^{13}\text{CNMR}$ chemical shifts for the berry extract sample.

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3.2.3. Multidimensional NMR Spectra

Multidimensional NMR experiments such as HSQC and HMBC were used to study the linkage of sugars to anthocyanidins. From the HSQC spectrum, there were three crosspeaks in the H4 region, which were H4/C4 on cy 3-glc (9.02/137.10), H4/C4 on cy 3-rut (8.94/136.30), H4/C4 on cy 3-xylrut (8.86/135.51). In the corresponding HMBC spectrum, the H4 on each anthocyanin showed correlations with C-2, C-5, C-4a, C-8a, respectively. Using the H4 on cy 3-rut as an example, the HMBC spectrum of sample 3 showed crosspeaks between H4/C2 (8.94/163.90), H4/C5 (8.94/158.62), H4/C8a (8.94/157.28), H4/C3 (8.94/145.20), H4/C4a (8.94/112.82), which elucidated the connections of H-4 with different carbons 2 or 3 bonds away. The correlation of H1''/C3 (5.46/144.92) verified that the glucose is bonded to the cyanidin at the C3 –OH group on
cy 3-rut. At the same time, the crosspeaks showed the correlation of H1''/C3 (5.29/145.27) on cy 3-xylrut and the correlation of H1''/C3 (5.27/145.16) on cy 3-glc (Figure 3.5). The 2D spectrum also verified that H1'' had different proton chemical shifts in different anthocyanins. Also, the correlation of the first proton on rhamnose H1 IV/C6'' (4.65/67.8) showed the connection between the glucose and rhamnose at 6'' -CH₂OH group.
3.3. Principal Component Analysis of the 1D NMR Spectra

Because the one-dimensional NMR spectra were complex, it was difficult to interpret all the peaks. The complication of the NMR spectra was reduced, and the interpretation of biological influences was made easier by using multivariate-computer based pattern recognition methods. Spectra can be analyzed either in full resolution or more often after a primary data reduction step, whereby each spectrum is digitized into integrated regions in certain spectral intervals. The most general way to analyze the spectral data was principal component analysis (PCA). PCA reduces a large number of original variables to a smaller number of principal components that are linear combinations of the original ones. In this research, to quantify the variations between

Figure 3.5. Selected regions of HSQC and HMBC NMR spectra of sample 3B, (a) H4 region, (b) H1″ and H1IV region.
different samples, integrated areas were obtained from the ¹H-NMR spectra at 3 Hz intervals from 0.664 ppm to 9.548 ppm. Figure 3.6 shows a small portion of the spectrum where peaks for H4 of the anthocyanins are observed. The integration values and the chemical shifts under the curve are shown in the table. The integrated areas for each of the BR samples were subjected to PCA. The data matrix is the input for the PCA procedure using statistical software, which presents the option of performing PCA on both covariance matrices and correlation matrices.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Bin #} & 1.31 & 1.52 & 1.53 & 1.54 & 1.55 \\
\hline
\text{Hz} & 6708 - 6711 & 6703 - 6706 & 6702 - 6705 & 6809 - 6702 & 6966 - 6959 \\
\text{area} & 0.480542 & 0.209322 & 1.190009 & 2.053280 & 0.226471 \\
\hline
\end{array}
\]

Figure 3.6. Integrated areas at 3 Hz intervals for a selected region of the black raspberry 750 MHz ¹H-NMR spectrum.

In the covariance matrices, the total variability of the original variables equals the total variability of the PCs. The first PC corresponds to the first eigenvalue and thus explains the largest percentage of the total variability in the data. The \( n \)th PC has the largest variance of all the remaining \( (n+1, n+2, \ldots) \) PCs and is not correlated with the previously obtained \( (n-1) \)th. The PCs were also obtained from the eigenvectors of the
estimated correlation matrix of the original variables. In this case, the variables are
standardized, and therefore, their variances are all equal to 1. The sum of the
eigenvectors is equal to the total number of variables. The quantities obtained from
covariance matrices are generally different from the ones derived from correlation
matrices. An advantage of the covariance matrices is that the eigenvectors retain the
scale of the original data. Those variables with larger variances will have a larger impact
on the weights of the PCs. In contrast, the eigenvectors obtained by the correlation
matrices are usually very unfamiliar in appearance. However, an advantage of
correlation matrices is that the PCA is influenced by all spectral features equally.
Consequently, the correlation matrices can be useful when minor constituents, with small
spectral contributions, are of primary interest. For both methods, a scree plot was used to
identify the PCs of interest. The scree plot is the sum of the magnitudes of the variability
contributed by each of the principal components in sequential order.\textsuperscript{80-82} Figure 3.7
shows the scree plot comparing the cumulative weighting for the PCs calculated by the
covariance and correlation matrices. According to the scree plot, the first seven PCs
accounted for 84% of the variability in the correlation matrices and 86% of the variability
in covariance matrices.
The NMR integration data from the 19 black raspberry samples were used to compute principal components by the correlation matrices, and multivariate analysis was conducted using the total monomeric anthocyanin content as a variable. About 74% of the variation in total anthocyanin content in the black raspberry samples is accounted for by a 3 factor regression model: 2266 - 19.3 PC 1 + 22.1 PC 2 - 14.0 PC 5 with $R^2 = 78.1\%$. The top bins for PCs 1, 2 and 5 were then analyzed with respect to their NMR spectra and black raspberry sample groupings. Minor and some known anthocyanin peaks were identified to contribute to the variability in specific BR samples.

The principal components were also calculated by the covariance method, and regression equations were calculated using the same variables. For total anthocyanin content, PC 1 was identified as the most important, followed by PC 7, 5, 8 and 4. BR samples 3, 13, 11, 19 and 16 were identified as important. When the top 10% of the bins in PC 1 were ranked according to their absolute eigenvector values, it was found that the
1st and 2nd ranked bins corresponded to known peaks for the methyl group in cyanidin 3-rutinoside and the 3rd and 4th ranked bins corresponded to the methyl group in cyanidin 3-xylosylrutinoside. The 1st and 3rd ranked bins in PC 7 corresponded to the H4 in cyanidin 3-xylosylrutinoside and cyanidin 3-rutinoside, and the 2nd, 5th and 6th ranked bins were related to the 2’ anthocyanin proton regions. Figure 3.8 shows the methyl and H4 regions of 1H-NMR spectra for black raspberry samples 3, 11, and 13.

Figure 3.8. (a) 1H-NMR spectra of selected methyl regions showing highest ranked bins in PC 1. (b) 1H-NMR spectra of selected H4 regions showing highest ranked bins in PC 7; from top to bottom, the spectra were from sample 13, sample 11 and sample 3.
Best subsets regression was also computed using values for antioxidant capacity and the principal components. A two factor regression equation using PC 7 and PC 5 was computed with $R^2 = 64.9\%$. BR samples 11 and 3 were the most important ones, and samples 18, 19, 13 and 16 were the second most important ones. Bins corresponding to peaks for polyphenolic compounds other than anthocyanins were also found in the top 10% ranked bins. For example, the 4th ranked bin in PC 7 and the 14th ranked bin in PC 5 corresponded to a peak associated with quercetin. Total phenolic values were also used as regression variables with the principal components yielding a two factor regression equation using PC 7 and PC 11 with $R^2 = 58.1\%$. BR samples 3 and 2 were identified as the most important ones. Many NMR regions for specific polyphenolic compounds protons were also highly ranked, similar to the regression analysis for antioxidant capacity. For example, the 2nd ranked bin in PC 11 corresponded to a distinctive peak associated with quercetin.

3.4. Content of Anthocyanins in Different Samples

The 19 different samples harvested in 2004 were screened for anthocyanins levels. The principal component analysis could correlate the NMR spectra with anthocyanin components in 19 different black raspberries. Figure 3.9 shows the different anthocyanin constituents in the 19 samples, which were obtained by integrating the H4 region of the 19 black raspberry spectra. From this chart we can tell that the two main anthocyanins in black raspberry were cy 3-rut and cy 3-xylrut. Our work demonstrated that $^1$H-NMR spectra can be used to quantify the relative distributions of the anthocyanins and thereby
show the variability among black raspberry samples.

![Anthocyanin constituents of 19 different black raspberry samples harvested in 2004.](image)

Figure 3.9. Anthocyanin constituents of 19 different black raspberry samples harvested in 2004.

3.5. Verification of HPLC Fractions

In order to test the chemopreventive properties of each anthocyanin in the black raspberry, in this study, HPLC was used to separate and identify the components. Figure 3.10 shows the HPLC peak separations that were identified as cyanidin 3-sambubioside, cyanidin 3-glucoside, cyanidin 3-xylosylrutinoside, cyanidin 3-rutinoside, and pelargonidin 3-rutinoside in sequence respectively. Because the pelargonidin 3-rutinoside was only an extremely minor component of black raspberry, only the first four HPLC fractions were analyzed by NMR spectroscopy. $^1$H-NMR, multidimensional NMR (HSQC and HMBC), and selective 1D TOCSY were employed to identify the anthocyanin in each HPLC fraction.
Figure 3.10. Separation and verification of BR anthocyanins by HPLC. From peaks 1 to 5 they were cy 3-sam, cy 3-glc, cy 3-xylrut, cy 3-rut, and pg 3-rut respectively.

According to the principal component analysis, the methyl, H1″ and H4 regions in the ¹H-NMR spectra are associated with the top ranked bins. Figure 3.11 shows the different regions of the ¹H-NMR spectra from the different HPLC fractions. From Figure 3.11a, it was obvious that the first two HPLC fractions do not have methyl signals, and that HPLC fractions 3 and 4 contain cy 3-xylrut and cy 3-rut, respectively. In the H1″ region (Figure 3.11b), the chemical shifts were around 5.46 ppm, which indicated that cy 3-sam or cy 3-xylrut are present in fractions 1 and 3, respectively. According to the study shown in Figure 3.11a, the cy 3-sam was in the HPLC fraction 1 and cy 3-xylrut was in the HPLC fraction 3. The analysis of the peaks around 5.29 ppm was similar to the peaks near 5.46 ppm, so the cy 3-glc was in HPLC fraction 2 and cy 3-rut was in the HPLC fraction 4. The Figure 3.11c shows the H4 region of ¹H-NMR spectra for the different HPLC fractions. There is only one peak with a different chemical shift in each of the HPLC fractions. The NMR studies verified that our HPLC method was successful in separating the various anthocyanins, which could be collected in a virtually pure state.
Wu et al. identified the anthocyanins in black raspberry using HPLC-ESI/MS/MS. According to their studies, the main anthocyanins in black raspberries were cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-rutinoside, and cyanidin 3-sambubioside-5-rhamnoside. This observation was different from the component found in HPLC fraction 3 (cyanidin 3-xylosylrutinoside) in our studies. Multidimensional NMR was employed to identify the structure of the component in HPLC fraction 3. The anomeric proton signals in the $^1$H-NMR spectrum appeared considerably downfield compared to other sugar resonances, and thus the integration ratio for the doublets at 5.47, 4.77 and 4.65 ppm defined the glucose : xylose : rhamnose ratio as 1:1:1. Figure 3.12 shows the HSQC and HMBC of the component in HPLC fraction 3. Starting from the singlet at 8.86 ppm, the observed crosspeak at 8.86/135.26 ppm in the HSQC spectrum shows the H4/C4 correlation, which was used to assign the C4 chemical shift as 135.26 ppm. The cross-peak at H1′′′/C2″ (4.78/82.54) indicates that the xylose is connected to C-2″ of the glucose ring, while the rhamnosyl residue is attached to the 6″ –OH position H1IV/C6″ (4.64/68.72) of the glucose. Therefore, the identity of the component in HPLC fraction 3 is proven to be cyanidin 3-xylosylrutinoside.
Figure 3.12. The HSQC and HMBC spectra of HPLC fraction 3, and the structure of cy 3-xyllrut. The arrows on the structures show the correlations found in the HMBC spectra. (a) The correlations H4/C2, C3, C5, C8a and C4a; the correlation H1″/C3 indicate the glucose is connected at the 3 –OH position; (b) The correlations of H1‴/C2‴ and H1‴/C6‴ indicate the sugar-sugar linkages.

Selective 1D TOCSY was used to help assign the $^1$H chemical shifts. Selective 1D TOCSY was developed about twenty years ago, and was used to isolate individual chemical shifts from the same spin system. However, the intensities of the TOCSY
peaks were strongly affected by the mixing time and relaxation times of the target spin system. When increasing the mixing time, the intensity of the selective inversion peak decreased while the intensities of other peaks within the same spin system increased. Also increasing relaxation time could increase the intensity of whole spectrum. Longer shaped pulse durations in the selective TOCSY pulse sequence will narrow the selective excitation band, thus increasing the selectivity to the target spin system, however, increasing the shaped pulse duration results in a significant decrease in the intensity of the TOCSY peaks. The pulse sequence used in the selective TOCSY was shown in Figure 2.3. Initially, all the protons were excited by a hard 90° pulse and are subsequently dephased by a gradient pulse G1; a selective Gaussian 180° pulse inverts the desired protons, and the second gradient pulse G2 refocuses the selected protons and continues to dephase the rest of the protons. After the second gradient pulse, an MLEV-17 spin lock is used which does not introduce a further change of coherence. The last 90° pulse is out of phase all the desired proton spins to x-y plane before detection.

The shaped pulse used in the experiment is a 180° refocusing for all the desired pulses, the duration and shape of the pulse was determined by the bandwidth of the selected peak. Generally, narrowing the excitation frequency bandwidth lengthens the pulse duration.
Figure 3.13. $^1$H-NMR (d) and Selective 1D TOCSY of HPLC fraction 3; the selective peak is at (a) 4.06 ppm ($H_2^{''}$), (b) 5.47 ppm ($H_1^{''}$), (c) 1.15 ppm ($H_6^{IV}$).

Figure 3.13 d shows the normal $^1$H-NMR spectra of HPLC fraction 3, and three different peaks were selected for excitation, which were $H-6^{IV}$, $H-2^{''}$ and $H-1^{''}$. When the selected peaks were the protons on glucose (Figure 3.13 a, b), the resonances of all the other protons in the same spin system are observed. When the methyl peak of rhamnose was excited (Figure 3.13 c), the spectrum shows the other four protons of the rhamnose ring. It is obvious that when the target excitation peak is well separated from other peaks, the neighboring peaks would not interfere with detection of the target spin system resonances, and the signal to noise ratio is high. Our work demonstrates that selective 1D TOCSY experiments can be used to analyze anthocyanins and other phenolics that are present in black raspberry.
With the help of selective 1D TOCSY and 2D heteronuclear experiments (HSQC and HMBC), the assignment of $^1$H chemical shifts was accomplished using mainly the 1D $^1$H-NMR experiment. We also extracted the coupling constants from $^1$H-NMR spectra of the components in four HPLC fractions, which are shown in Table 3.5.

Table 3.5. $^1$H NMR chemical shifts of different HPLC fractions in CD$_3$OD, with coupling constants and description of signals (s-singlet, d-doublet, dd-doublet of doublet, m-multiplet).

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CHAPTER IV
CONCLUSION

Our studies verified that NMR spectroscopy can provide a fast, non-destructive, accurate and reproducible method to comprehensively screen the anthocyanin compositions of black raspberry. The use of 1D $^1$H and $^{13}$C NMR spectroscopy enabled the reliable assignments of anthocyanin peaks in the extracts of black raspberry, and multidimensional NMR was used to identify the structures of the different anthocyanins. The 1D 1H-NMR spectra were subjected to principal component analysis that could identify chemical groups that varied among samples. PCA coupled with multivariate analysis using bioactivity indicators were valuable for determining why highly variable black raspberry samples act as chemoprotective agents. This work demonstrated that our modified metabolomic methods can be used to correlate variables such as total monomeric anthocyanin content with BR samples and NMR results, validating our approach to identifying BR compounds with potential chemopreventive effects. The constituents with bioactive properties could be identified by combining high-field NMR spectroscopy and multivariate statistical modeling. This unique approach will serve as a model to examine the chemopreventive activity of antioxidant-rich food sources.
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