Development of Fourier Transform Mid-Infrared Spectroscopy as a Metabolomic Technique for Characterizing the Protective Properties of Grain Sorghum Against Oxidation

Emily Diane Sitorius
University of Nebraska at Lincoln, emilysitorius@yahoo.com
DEVELOPMENT OF FOURIER TRANSFORM MID-INFRARED SPECTROSCOPY
AS A METABOLOMIMIC TECHNIQUE FOR CHARACTERIZING THE PROTECTIVE
PROPERTIES OF GRAIN SORGHUM AGAINST OXIDATION

by

Emily D. Sitorius

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Cellular oxidative damage has been linked to many chronic diseases including Alzheimer’s, diabetes, and cancer. Recent studies have shown that foods rich in antioxidants (i.e., grain sorghum) can protect against oxidative stress and thus reduce the risk of disease. However, a significant gap in knowledge exists in that dietary antioxidants are usually characterized by methods that determine free radical and reactive oxygen species’ scavenging capacity that are not strongly correlated to a biological system. To fully understand the health promoting properties of natural antioxidants, the study of the metabolome is essential as shifts in the biochemical phenotype, or function of a cell, will be generated. Therefore, the objective of this study was to develop a method using Fourier-Transform Mid-Infrared spectroscopy (FT-mIR) that would simultaneously monitor several cellular biochemicals in response to oxidation and dietary antioxidants. To achieve the cited research objective, sorghum polyphenols were extracted into water and characterized via Folin-Ciocalteau, aluminum chloride, and oxygen radical absorbance capacity (ORAC) methods resulting in: 109.6 ± 4.3 µg cinnamic / g flour; 22.6 ± 0.15 µg catechin/ g flour; 336 µmol Trolox/ 100 g flour. FT-mIR analysis of human intestinal epithelial cells, Caco-2, exposed to 1-40 mM of hydrogen peroxide (HP) for 16 h indicated that concentrations > 5 mM induced protein structural changes and DNA mutations. Exposure to grain sorghum extract (SE) (1-40 mg/mL) for 8 h prior to treatment with 15 mM HP for 16 h revealed minimal protection of cells with the notable exception of the phosphate containing groups, i.e., phospholipids, DNA, and RNA. At the amide III band, principle component analysis indicated that treatment with 40 mg/mL SE may actually have damaged the cells. This study showed FT-mIR to be a powerful metabolomic tool to identify optimal safe and effective dietary options for protecting cells against oxidative stress.
With Deepest Gratitude

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# TABLE OF CONTENTS

**Literature Review**

Oxidative Stress ............................................................................................................. 1

Nutraceuticals & Grain Sorghum ................................................................................... 4

Current Characterization Approaches of Dietary Natural Antioxidants ................. 9

Metabolomics ................................................................................................................. 11

Fourier-Transform Infrared Spectroscopy ..................................................................... 18

**Materials and Methods**

Sorghum Extraction and Characterization ...................................................................... 29

Cell Culture and Treatment Methodology ..................................................................... 32

Fourier Transform Infrared Spectroscopy ..................................................................... 33

Statistical Design and Analysis ..................................................................................... 35

**Results and Discussion**

Physiochemical Characterization .................................................................................. 36

FT-mIR Metabolomic Method Development .................................................................. 38

FT-mIR Oxidative Stress Studies .................................................................................... 42

FT-mIR Sorghum Extract + Oxidative Stress Studies ................................................... 62

References ...................................................................................................................... 78
LIST OF FIGURES

Figure 1: Reactive oxygen species flowcharts.................................................................2
Figure 2: Phenolic compound structures..........................................................................6
Figure 3: Flavonoid structure...........................................................................................7
Figure 4: Omics Schematic.............................................................................................13
Figure 5: Interferometer.................................................................................................21
Figure 6: Bacterial mIR Spectrum...................................................................................23
Figure 7: Biowheel and Compartment.............................................................................34
Figure 8: Control Validation............................................................................................43
Figure 9: FT-mIR Spectra: Control v. 0.2 mM HP, 0.5 h................................................45
Figure 10: 1750-700 cm⁻¹ Dendrograms, Control v. 0.2 mM HP, 0.5 h.......................46
Figure 11: FT-mIR Spectra Control v. 0.2 mM HP, Time Curve.....................................49
Figure 12: 1750-700 cm⁻¹ Dendrograms: Control v. 0.2 mM, Ti...................................50
Figure 13: FT-mIR Spectra: HP Concentration Curve, 16 h.........................................52
Figure 14: 1750-700 cm⁻¹ Dendrograms: HP Concentration Curve, 16 h.......................55
Figure 15: PCA: HP Concentration Curve, 16 h.........................................................56
Figure 16: 1645-1632 cm⁻¹ Dendrograms: HP Concentration Curve, 16 h...............57
Figure 17: 1645-1632 cm⁻¹ PCA: HP Concentration Curve, 16 h.................................58
Figure 18: 1170-1150 cm⁻¹ Dendrograms: HP Concentration Curve, 16 h..............60
Figure 19: 1170-1150 cm⁻¹ PCA: HP Concentration Curve, 16 h.................................61
Figure 20: FT-mIR Spectra: SE Concentration Curve, 15 mM HP...............................63
Figure 21: 1750-700 cm⁻¹ Dendrograms: SE Concentration Curve, 15 mM HP...........65
Figure 22: 1750-700 cm⁻¹ PCA: SE Concentration Curve, 15 mM HP..........................67
Figure 23: 1240-1180 cm⁻¹ Heterogeneity: SE Concentration Curve, 15 mM HP.........68
Figure 24: FT-mIR Spectra: 15 mg/mL SE + 15 mM HP.............................................69
Figure 25: 1750-700 cm\(^{-1}\) Dendrogram: 15 mg/mL SE + 15 mM HP............................71

Figure 26: FT-mIR Spectra: 15 mg/mL SE + 5 mM HP................................................72

Figure 27: 1750-700 cm\(^{-1}\) Heterogeneity: 15 mg/mL SE + 5 mM HP............................74
LIST OF TABLES

Table 1: Assignment of biomolecules to known functional groups..............................19

Table 2: Characterization of grain sorghum (Macia 2006) water extract......................37
LITERATURE REVIEW

Cellular Oxidative Stress: Mammalian cells are continuously exposed to oxidative stressors, which if left unprotected, can lead to several types of degenerative diseases, including coronary artery disease, Alzheimer’s, muscle degeneration, diabetes and cancer (Barnham et al, 2004; Madamanchi et al, 2004; Halliwell, 2007). The susceptibility of a biological system to oxidative stress is determined by the balance between the level of oxidant agents present in the cells and their overall antioxidant capacity (Loguercio et al, 2003). Such oxidizing agents that include free radicals and reactive oxygen species (ROS), i.e., superoxide anions (O$_2^-$), hydroxyl radicals (OH), and hydrogen peroxide (H$_2$O$_2$), can be either induced from intracellular or environmental stressors (Finkel and Holbrook, 2000). When oxidizing agents exceed the antioxidant capacity of a cell, these toxic agents are available to participate in tissue injuries, which can ultimately progress to a diseased state.

Superoxide is generated when oxygen is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the mitochondrial electron transport chain (Valko et al, 2007) (Figure 1a). When superoxide breaks down into hydrogen peroxide, hydrogen peroxide can undergo the Fenton reaction with transition metals, such as Fe$^{2+}$ and Cu$^+$, resulting in the production of hydroxyl radicals (Nakagawa et al, 2004). Hydroxyl radicals can abstract electrons from unsaturated fatty acids and create carbon-centered lipid radicals that can react with oxygen to create lipid peroxyl radicals (LOO$'$) (Valko et al, 2007) (Figure 1b).
Figure 1a: Superoxide production and subsequent breakdown to water.

Figure 1b: Lipid oxidation. (http://www.vivo.colostate.edu/)
In addition, lipid peroxyl radicals promote the formation of additional ROS, which in turn can result in protein and DNA oxidative damage. DNA mutations can then progress to uncontrolled cell proliferation and ultimately to the development of tumors (Depeint et al, 2002). Nitric oxide (NO) is another species that plays a role in the regulation of vascular, neurological, and immunological signal transduction which can become dangerous when it reacts with superoxide to form peroxynitrite (ONOO'). Peroxynitrite oxidizes and nitrosylates a variety of biomolecules that have been implicated in the pathogenesis of various diseases, including neurological and colorectal cancer (Ambs et al, 1998; Heijnen et al, 2001).

In addition to cell generated and environmental oxidants, humans are exposed to oxidized lipids through our diets with the gastrointestinal (GI) tract being the first site of impact. Fatty foods, which contain high levels of oxidized lipids, can thus off balance the antioxidant/oxidative status of the GI tract (Schaeferhenrich et al, 2003). The human body is able to combat this imbalance by activating such enzymes as superoxide dismutase, catalase, and glutathione peroxidase (Huang et al, 2005). Superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide, which is further broken down into water by catalase and glutathione peroxidase (GPx) (Finkel and Holbrook, 2000). Despite these protective mechanisms, proliferative responses in the colonic mucosa can be provoked by intrarectal hydroperoxides that are capable of overcoming cellular defenses (Bull et al, 1984; Fischer et al, 1980; Verma et al, 1980). Colorectal cancer, the second most common cause of cancer deaths in the United States,
has also been linked to hydroperoxides and other active oxygen radicals developed during lipid peroxidation (Schaeferhenrick et al, 2003). Because of the increasing concern over serious health problems caused by oxidative damages to the GI, identifying natural approaches that can reduce this stress has become a major emphasis by many researchers (Manna et al, 1997; Kuntz et al, 1999; Bestwick and Milne, 2001). In addition, the increasing public preference for natural and herbal medicines has prompted the discovery of natural anti-oxidative agents and the development of food systems rich in these agents.

**Nutraceuticals:** When our body's natural defenses fail to protect against oxidative stress, balance may be restored by the consumption of nutraceuticals that have antioxidant properties, such as enzyme cofactors (Se, Coenzyme Q10), oxidative enzyme inhibitors (aspirin, ibuprofen), transition metal chelators (EDTA), or radical scavengers (Vitamin C and E, polyphenols) (Huang et al, 2005). The term “nutraceutical” was coined in 1979 and is defined as “a food (or part of a food) that provides medical or health benefits including the prevention and/or treatment of a disease” (Biesalski, 2001). According to Wildman (2001), nutraceuticals include isoprenoid derivatives (i.e. carotenoids and tocopherols); phenolic compounds (tannins, anthocyanins, flavonoids); amino acid-based (isothiocyanates, folate, choline); carbohydrates (oligosaccharides, ascorbic acid); fatty acids (polyunsaturated fatty acids, lecithin, and conjugated linoleic acid); minerals (Ca, K, Zn); and microbials (pre- and pro- biotics).

In particular, the phenolic compounds have been shown to exert potent antioxidant capacities (Wildman, 2001; Biesalski, 2001). As secondary metabolites in
plants, phenolic compounds are not directly involved with respiration, photosynthesis, or nutrient assimilation (Blumberg and Block, 1994) but rather play primary roles in protecting a plant from insects, disease, and herbivores. Phenols also attract pollinators and thus help to ensure the propagation of the next generation in plant-to-plant competition (Wildman, 2001).

As shown in Figure 2, the phenolic compounds include phenolic acids and various classes of flavonoids, including flavonols, isoflavones, anthocyanins, etc. Phenolic compounds are synthesized in plants by the removal of the amino group from phenylalanine producing trans-cinnamic acid (Wildman, 2001). Polyphenols are then synthesized when a phenol group is added to cinnamic acid to form para-coumaric acid, which in turn is modified by the addition of 3-malonyl CoA to form a ring structure. More ring structures and phenol groups combine to produce flavonoids and finally anthocyanins, which are the most polymerized phenols (Wildman, 2001).

Phenols protect against oxidation in both plants and humans by donating the hydrogen from their hydroxyl group to act as a free radical scavenger. The antioxidant activity of a flavonoid is thereby dependent on the arrangement of the functional groups about the nuclear structure, which consists of three aromatic rings labeled A, B, and C (Figure 3a), as well as subtle changes in the nuclear structure itself. The structure that provides the most stability after hydrogen donation favors higher antioxidative activities. Studies have shown that flavonoids containing OH groups at C-3’ and C-4’ on the B ring
Figure 2: Phenolic compounds (Apak et al, 2007)
Figure 3a: A, B, C rings of flavonoid structure (Heim et al, 2002)

Figure 3b: Quercetin aglycone and Quercetin-3-glucoside
(biotec.or.th; http://www.chemicalbook.com)
and at C-3, C-5 and C-7 on the AC ring as well as a double bond at C-2,3 and a carbonyl at C-4 are the most effective scavengers of oxygen and nitrogen radicals while methylation of any hydroxyl results in drastically reduced scavenging activities (Heijnen et al, 2001; Heim et al, 2002; Depeint et al, 2002). The antioxidant activity of phenolic compounds is also positively correlated with pH as the electron donating capacity of the phenolic OH increases with dissociation (Mukai et al, 1997).

The ability of any dietary antioxidant to deliver its protective benefits is ultimately dependent on its degree of bioavailability in the human system. If an oxidized lipid is deep within a membrane or not exposed to an aqueous environment, a lipid soluble compound such as a carotenoid would be the most bioavailable antioxidant. However, the polar-based phenolic compounds must either be transported across the cell membrane or be modified to be absorbed (Depeint et al, 2002). Considering that flavonoids exist in nature mainly as glycosides, i.e., the phenolic hydrogens are substituted to a sugar moiety (Figure 3b), they are able to pass intact through the digestive tract to the large intestine where the glycosides are hydrolyzed to their aglycone counterparts. The flavonoids are then solubilized as micelles and absorbed by the large intestine (Tereo, 1999).

The consumption of phenol containing foods has been linked to protecting the GI tract from several disorders. Depeint et al (2002) showed that quercetin had potent anti-proliferation properties against the human colon cancer cell line, HT-29. This health
benefit is especially relevant to the intestinal epithelium, as it has one of the highest cell turnover rates in the body (48-72 h) (Aw, 1999). If cell growth can be inhibited by a natural antioxidant until the diseased cells are removed via routine apoptotic exfoliation, healthy epithelial cells can emerge and take their place. In another study, Manna et al. (1997) demonstrated that hydroxytyrosol, a phenolic compound produced by olives, was able to protect the human colon cancer cell line, Caco-2, against hydrogen peroxide and xanthine/xanthine oxidase induced oxidative stress. The isoflavones genistein and daidzein, commonly found in soy, have also been credited for their ability to protect against GI cancer (Fritz et al, 1998; Setchell and Cassidy, 1999). It was further demonstrated that genistein was able to inhibit hydroxyl radical induced lipid peroxidation while daidzein was more effective in reducing superoxide anion induced lipid peroxidation (Toda and Shirataki, 1999).

**Current Characterization Approaches of Dietary Natural Antioxidants:** The antioxidative capacity of nutraceuticals is typically determined by several colorimetric methods, including 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH*), Trolox-equivalent antioxidant capacity TEAC), and oxygen radical absorbance capacity (ORAC) (Adom and Liu, 2002; Awika and Rooney, 2004; Tabart et al, 2009). Although these tests are rapid and inexpensive, most are only able to measure a compound’s ability to scavenge a particular radical (DPPH*, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid linoleate (ABTS*), superoxide, hydroxyl) that in some cases are not biologically significant and/or produce inconsistent results. Using the TEAC, DPPH*, and ORAC
tests, Tabart et al (2009) showed that each method resulted in different antioxidative capacities when applied to naringenin and gallocatechin. As expected, the ORAC generated the highest value for both compounds as this test measures all oxygen radicals rather than just one radical. Similar outcomes have been shown for multiple natural systems indicating that comparisons cannot be made pending the standardization of a given method for multiple compounds and matrices (Antolovich et al, 2002; Arnao, 2000; Frankel, 1993). Huang et al (2005) stated that these in vitro chemical tests “bear no similarity to biological systems” and that “any claims about the bioactivity of a sample based solely on these assays…would be exaggerated, unscientific, and out of context”. Nonetheless, a list of the antioxidant capacities of commonly consumed foods has been published by the United States Department of Agriculture based on ORAC values (USDA, 2007) and is frequently used as a point of reference.

In an effort to more accurately characterize the antioxidative properties of nutraceutical compounds, cell culture model systems have also been used. One accepted in vitro model for the intestinal lining is the human colon adenocarcinoma cell line, Caco-2, as these cells display many of the morphological and functional properties of in vivo intestinal mucosa (Meunier et al, 1995). Originally used to evaluate drug transport, this system is now being applied to oxidatively-related studies of natural compounds. Specifically, Wijeratne et al (2005, 2006) evaluated the response of Caco-2 cells when exposed to different concentrations of hydrogen peroxide and lipid hydroperoxides using indicators of cellular damage (cell viability, membrane integrity, and cellular antioxidant
enzyme activity). At oxidizer levels of 100-250 µM, cell viability was maintained but membrane integrity was significantly affected, which was attributed to efficient colon cell antioxidant enzyme mechanisms. After distinguishable oxidative effects were detected, Wijeratne and Cuppett (2007) exposed the cell line to the isoflavones genistein and daidzein to determine their ability to protect against lipid hydroperoxide induced oxidative damage. Treatment with both compounds resulted in higher antioxidant enzyme capacity and membrane integrity. Peng and Kuo (2003) also used Caco-2 cells to investigate the protective capacity of various flavonoid compounds (quercetin, luteolin, and (-)-epigallocatechin gallate (EGCG)) against hydrogen peroxide/Fe²⁺ induced lipid peroxidation. By measuring malondialdehyde (MDA) levels to determine lipid peroxidation, the researchers showed that quercetin and luteolin significantly decreased MDA levels at concentrations < 10 µmol but EGCG neither positively nor negatively affected the MDA levels.

While the above cited strategies have increased our understanding of the antioxidative properties of natural systems, it ignores the enormous complexity of nutraceuticals. Foods are not purified compounds acting on a single target, but rather complex mixtures that may affect many biochemical pathways. Because the expectation of any health promoting dietary agent is to consistently provide specific benefits without causing other disorders, methods must be developed that are capable of detecting subtle and temporal changes in multiple regulatory processes of complex biological systems.
Such quantitative and comprehensive measurements can only be achieved via the technological advances driven by the genomic era.

**Metabolomics:** The field of genomics has had tremendous achievements in the past two decades. The first whole genome sequencing of a living organism, the bacteria *Haemophilus influenza* (1,830,000 base pairs (bp)), was accomplished in 1995 (Goodacre et al, 2004) followed by the sequencing of the first plant genome, *Arabidopsis thaliana*, (157,000,000 bp), in 2001 (Fiehn, 2002). Finally, the human genome (3,200,000,000 bp) project was completed in 2003 (genomics.energy.gov). As these landmarks were reached, scientists set out to determine downstream outcomes of the sequenced genes, by on transcriptomics (the study of the expression of the genes as a whole) and proteomics (the study of the complete set of proteins) (Dettmer and Hammock, 2004).

The holistic study of these molecular mechanisms and their products have since been used to provide a snapshot of a biological system making, it possible to track the progression from a healthy to diseased state, but post-genomic studies have further shown that they may not follow the previously held linear model (Figure 4). Regulation (up or down) of a gene does not necessarily affect the phenotype because changes only reflect the potential of function (Bouche and Bouchez, 2001). Shifts in the proteome also may not result in a relative shift in the phenotype because metabolite pools are controlled by a complex enzyme network and by the effectors (i.e. mRNA, metabolites) that act upon these proteins (Raamsdonk et al, 2001). Conversely, changes to single markers in the genome or proteome can result in intricate downstream fluxes (Roessner et al, 2001).
Figure 4: Omics schematic  (Goodacre, 2005)
Therefore, characterizing natural anti-oxidative agents based solely on their ability to affect gene or protein markers may not account for possible impacts, negative and positive, to global metabolism.

Oliver and co-workers (1998) were one of the first groups to recognize the need for the holistic study of metabolism and are credited for the concept of the metabolome. According to Hollywood et al (2006), metabolomics is the study of the metabolome “[which is] the quantitative complement of all the low molecular weight molecules (typically <3000 m/z) present in cells in a particular physiological or developmental state”. Eisenreich and Bacher (2007) defined it more concisely as “the systematic study of the unique chemical fingerprints that specific cellular processes leave behind”. Ultimately, metabolites are the products of up-stream regulatory processes. Changes in the metabolome are thus closest to the physiological phenotype of a biological system (Figure 4). Therefore, metabolic shifts at any given point in time and/or in response to environmental stressors, such as oxidation, represent the functional level of the cell more appropriately (Dunn and Ellis, 2005). As an example, preliminary work completed in our laboratory showed that resveratrol, (a reported anti-inflammatory agent present in grapes) failed to prevent the up-regulation of select pro-inflammatory genes at the dosage levels used but was able to protect metabolism of a typical inflammatory model system (Gries, personal communication, 2008). Another advantage to metabolomics is that metabolic pathways between organisms are highly conserved allowing for the use of model
systems, although studies have yet to be completed to test this hypothesis (Goodacre, 2005).

Despite these attributes, metabolomics has not progressed to the extent of the other "omics" because this discipline has largely been ignored by the scientific community, which is due in part to lack of agreement to what exactly constitutes a metabolite. As metabolomics evolves, sharing of information is essential, i.e., via online metabolomics databases (Goodacre, 2005), to unify metabolomic terminology. Another disadvantage to metabolomics as opposed to other "omic" fields, that target compounds of the same basic chemistry, is that the metabolome encompasses such broad classes of molecules as lipids, amino acids, carbohydrates, nucleotides, organic acids, and vitamins (Goodacre, 2005). Significant challenges remain as a result of the heterogeneity of the molecules, the dynamic range of the measuring technique and the limited sample throughput of the instruments. At present, it is technologically impossible to measure the entire metabolome of any system (e.g., cells, urine, blood, tissue, etc.) with a single analysis. Metabolomics has thus been divided into two general categories: profiling (targeted) or fingerprinting (non-targeted) (Dunn and Ellis, 2005).

Profiling methods target metabolites based on similar characteristics, such as chemistries (amino acids versus fatty acids), pathway of origin (tricarboxylic acid or eicosanoid), or biological relevance (specific metabolic biomarkers). Specific metabolites are then identified and quantified relative to a given treatment or disease status using separation techniques followed by selective detection modes. For example,
Hertog et al (1997) used high performance liquid chromatography with electrochemical detection to selectively target the metabolite 8-oxo-7, 8-dihydro-2’-deoxyguanosine in the urine of human subjects to determine the antioxidant protective capabilities of high or low habitual fruit and vegetable consumption. Patrignani and Tacconelli (2005) applied gas chromatography coupled with mass spectrometry to determine F2-isoprostanes present in blood and urine samples for monitoring non-enzymatic lipid peroxidation. F2-isoprostanes are one of four products of the isoprostane lipid peroxidation pathway, which has been linked to many diseases related to oxidative stress such as acute coronary syndromes, Alzheimer's disease, cystic fibrosis, and diabetes mellitus (Davi et al, 2004). Szuchman et al (2008) successfully used liquid chromatography interfaced to mass spectrometry to analyze for covalently bound linoleic acid and tyrosine, N-linoleoyl tyrosine (LT), as a metabolic marker of oxidative stress in the blood of diabetic and hypercholesterolaemic (Hc) patients versus healthy controls.

Alternatively, non-targeted metabolomic methods are used to obtain as many metabolites as possible and/or fingerprints of biological samples in response to a given treatment (Dunn and Ellis, 2005). For the former case, shifted metabolites in terms of appearance/disappearance or abundance relative to a control may be identified by targeted metabolomic methods. In both cases, however, the entire data set or a sub-set can be analyzed by pattern recognition approaches to determine if the responses between treatments groups cluster together or apart relative to each other. Hierarchical cluster analysis is one of the most commonly applied analysis method used for non-targeted
metabolomics (Mordehai et al, 2003; Perromat et al, 2002; Salman et al, 2002). This pattern recognition test is an unsupervised technique that examines the interpoint distances between the data and presents the information in the form of a two-dimensional plot known as a dendrogram. Cluster analysis methods, such as Ward's algorithm, are used to form the clusters of data based on their nearness in high dimensional row space with a D value representing their relative closeness to each other. The dendrogram thereby displays the dimensional row spaces in an interpretable pattern (Salman et al, 2002). Principle component analysis (PCA) is yet another typically used unsupervised pattern recognition tool that is able to correlate the relationships between the samples and several of their quantitative variables/components. PCA defines new components as linear combinations of original variables, allowing for subsequent classifications. The data are presented in two or three-dimensional maps with axes defined by the components (Melin et al, 1999).

Using pattern recognition approaches coupled with nuclear magnetic resonance (NMR) as a metabolomic fingerprinting method, Ludwig et al. (2009) evaluated the feasibility of detecting early stages of colorectal cancer. Although NMR spectra was difficult to evaluate due to overlapping signals and limited sensitivity, the spectra was adequately deconvoluted with PCA. As a result, the researchers were able to distinguish samples obtained from colorectal cancer patients with healthy controls by using 1) the first principle component of first derivative spectra and 2) the second principle component of first derivative spectra.
One of the most extensive metabolomic fingerprinting studies completed to date was reported by Denkert et al (2008). Using gas chromatography-time of flight mass spectrometry, a non-selective detection mode, fingerprints of colon carcinomas and healthy tissues obtained from the same patient resulted in the detection of 206 metabolites, 107 of which could be identified by chemical structure and metabolite name. In addition, metabolites from all major pathways were detected, including metabolites from the Kreb's cycle intermediates, from purine and pyrimidine metabolism, and from the lipid, sugar, amino acid metabolic pathways. Based upon PCA analysis of the data, the metabolomic method was clearly able to distinguish between cancer and normal tissue. Only one out of forty-five samples was assigned to the wrong cluster, and in a blind study the metabolite signature correctly predicted the status of tissue at sensitivity and specificity of 95%. Hierarchical clustering analysis also proved to be a robust pattern recognition tool. Of the 107 metabolites identified, 82 were significantly different, 25 of which were up-regulated in cancer tissue.

*Fourier Transform Mid Infrared Spectroscopy (FT-mIR):* Infrared spectroscopy (IR) is a technique that is considered a metabolomic fingerprinting method owing to its ability to simultaneously monitor carbohydrates, lipids, fatty acids, proteins, phosphate-containing molecules and polysaccharides of intact cells and other biological samples. Although this group of compounds contains molecules that are not small metabolites, IR is been an accepted metabolomic tool because it is able to provide biochemical fingerprints of biological systems (Dunn and Ellis, 2005). When a sample absorbs IR
light, energy transitions occur in the various molecules that present as bond stretching, rocking, and bending (Kenkel, 1994). Molecules or groups of compounds (Table 1) can then be identified based on the IR wavelength absorbed by these chemical bonds and functional groups. The infrared electromagnetic regions can further be separated into the near, mid, and far infrared region corresponding to 12500-4000, 4000-650, and 650-100 cm\(^{-1}\), respectively.

As opposed to conventional IR spectroscopy, which uses a grating based monochromator to scan one wavelength at a time, Fourier Transform infrared (FT-IR) incorporates a Michelsen interferometer (Figure 5). This devise handles the absorbed light by splitting the infrared beam in two, sending it to both a moveable mirror and a fixed mirror. After the mirrors reflect the light and resend it to the splitter, constructive or deconstructive waves are created based upon the distance between the splitter and the moveable mirror as compared to the distance between the fixed mirror and the splitter. The recombined infrared beam travels through the detector where an interferogram is produced. The inverse function of the interferogram (Fourier transform) is then obtained to generate a spectrum, which is typically expressed in wavenumbers (cm\(^{-1}\)) (Chamberlain, 1971).

Two advantages of FT-IR as compared to conventional IR spectroscopy are increased sensitivity and faster spectra collection times (Kenkel, 1994). The latter attribute is particularly advantageous for metabolomics as other methods, such as mass
Table 1: Assignment of biomolecules to known functional groups

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Functional group</th>
<th>Possible Biomolecule Contributors</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000-2800</td>
<td>deformation and stretching vibrations of CH₃, CH₂ and CH groups</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>1658</td>
<td>α-helical structure</td>
<td>Amide I</td>
</tr>
<tr>
<td>1638, 1628</td>
<td>β-structure</td>
<td>Amide I</td>
</tr>
<tr>
<td>1546</td>
<td>α-helical structure</td>
<td>Amide II</td>
</tr>
<tr>
<td>1480-1430</td>
<td>deformation and stretching vibrations of CH₃, CH₂ and CH groups</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>1413-1387</td>
<td>weak NH vibrations and CH in-plane deformations</td>
<td>Carbohydrates, DNA/RNA backbone, proteins</td>
</tr>
<tr>
<td>1242-1236</td>
<td>Phosphodiester backbone region, asymmetric</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>1200-1000</td>
<td>C-O-C, C-O dominated by ring vibrations</td>
<td>Polysaccharides within cell wall</td>
</tr>
<tr>
<td>1080</td>
<td>Phosphodiester backbone region, symmetric</td>
<td>DNA, RNA, Phospholipids</td>
</tr>
<tr>
<td>986</td>
<td></td>
<td>Polysaccharides within cell wall</td>
</tr>
</tbody>
</table>

(Yu and Irudayaraj, 2005)
Figure 5: Michelson Interferometer. Infrared light exits the source, travels to a beam splitter, is reflected by the mirrors and recombines before passing through the sample and on to the detector. (http://teaching.shu.ac.uk/)
spectrometry (1-3 m), NMR (minutes-hours) and chromatography (10-30 m), require longer analysis times. In addition, sample preparation is minimal for FT-IR analysis as extraction of the components or chemical derivatization is usually not needed. However, a significant disadvantage of FT-IR as a metabolomic fingerprinting method is that without prior dehydration of the sample intense water bands can be prevalent in the mid-IR region (4000-650 cm\(^{-1}\)) (Dunn and Ellis, 2005; Hollywood et al, 2006).

Nonetheless, FT-IR in the mid-region (FT-mIR) has been shown to exhibit rich spectra of intact cells as well as other biological samples (Melin et al, 1999). As shown in Figure 6, the FT-mIR spectrum of a cell system can be divided into five distinct windows, each representing a specific biochemical region. Window 1 (3000 to 2800 cm\(^{-1}\)) contains asymmetric and symmetric stretching vibrations of CH\(_3\) and CH\(_2\) that correspond to fatty acid groups. Window 2 (1700-1500 cm\(^{-1}\)) contains the amide I and amide II peaks that are primarily due to C=O stretching and N-H bending vibrations, respectively. Window 3 (1500-1200 cm\(^{-1}\)) contains a mix of functional groups including rocking CH\(_2\), stretching P=O, and methylene groups attributed to amide III, fatty acids, and phosphate carrying groups, such as DNA, nucleotides, and phospholipids. Window 4 (1200-900 cm\(^{-1}\)) consists mainly of bands exhibited by carbohydrates and the DNA/RNA backbone whereas window 5 (900-500 cm\(^{-1}\)) is known as the fingerprint region. Although the latter region has an overwhelming amount of bands that make assignment to specific functional
Figure 6: Bacterial mK spectrum. Window 1 (3000-2800 cm⁻¹) contains fatty acids; Window 2 (1750-1500 cm⁻¹) contains amide I and II; Window 3 (1500-1200 cm⁻¹) contains lipids, carbohydrates and proteins; Window 4 (1200-900 cm⁻¹) contains phosphate carrying groups such as DNA/RNA and phospholipids; Window 5 (1200-500 cm⁻¹) is the fingerprint region.
groups difficult, it is very specific from organism to organism and has been used for bacteria classification purposes (Melin et al, 1999; Yu and Irudayaraj, 2005).

Because the FT-mIR spectra of intact cells are complex resulting in highly convoluted profiles, spectral preprocessing of the data is usually required to enhance resolution and the signal to noise resulting in more molecular structural details (Burrueta et al, 2007). The simplest form of pre-processing is to apply a cluster analysis method to select spectral regions of the raw data. In most cases, more pre-processing steps are needed to eliminate other superflous features. The most common approaches include one or a combination of the following A) obtaining the average of spectra obtained from replicate analysis of samples, B) variance scaling to eliminate outliers, C) normalizing the spectra to a given band or internal standard, D) smoothing in order to remove noise without broadening peaks excessively, and E) obtaining the the first or second derivative spectra to reduce random fluctuations in the baseline or spectra slope, respectively (Burrueta et al, 2007; Perromat et al, 2003). After the appropriate pre-processing method has been optimized, cluster analysis is then applied to the spectra to distinguish shifts in the entire spectral region, select windows, and even specific bands (Salman et al, 2002).

In the late 1990’s, researchers began using FT-mIR to monitor the biochemical fingerprint of mammalian cells for studying cell cycle and death responses for healthy versus diseased cells. Holman et al (2000) showed increases in the DNA/RNA spectral region (window 4) during replication (S phase) and an overall increase in absorption during mitosis (G2/M stage) as compared to the initial growth and normal metabolic role
stages (G\textsubscript{1}). The ability and sensitivity of FT-mIR to detect cell cycle stages was confirmed by Mourant et al in 2003. By monitoring changes in glycogen responses via cluster analysis of 1500 FT-mIR spectra of cancerous and normal cervical cells, Diem et al (2002) also showed that precancerous cells had a greater absorption in the glycogen region due to the presence of a higher number of immature cells.

From an oxidative stress perspective, Melin et al (1999) used FT-mIR to study the effect of ascorbic acid-induced free radicals on \textit{Deinococcus radiodurans}, i.e., a radiation-resistant strain of bacteria. Comparison of bacteria samples treated with 0, 10, 40, and 150 mM ascorbic acid showed changes in several regions ranging between 1700-900 cm\textsuperscript{-1} (W2-4). Based on the zero order spectra (pre-processed without derivatization), subtle shifts in the protein and phospholipid structure were detected with increasing ascorbic acid concentrations. The second derivative spectra showed more dramatic differences resulting in highly clustered groups relative to treatment levels. While the 10 mM spectra were not different from the 0 mM, new bands appeared in the spectra of the 40 mM treated cells with more significant changes occurring in the spectra of the 150 mM treated samples, i.e., especially in windows 3 and 4. The authors (Melin et al, 1999) attributed these results to the low level of polyunsaturated fatty acids in the \textit{D. radiodurans} membrane. The data from 1700-900 cm\textsuperscript{-1} was further analyzed by hierarchical classification of the first derivative spectra. A dendrogram generated by application of Ward's algorithm produced a cluster corresponding to 40-150 mM ascorbic acid and another cluster divided into two subclusters representing 0-10 mM and 10-40
mM ascorbic acid. Based on these results, Melin et al (1999) concluded that levels < 10 mM ascorbic acid were not toxic to *D. radiodurans* but 40 mM treatments induced oxidative damage while concentrations of 150 mM resulted in substantial damage.

Using FT-mIR, Perromat et al (2002) studied the effects of reactive oxygen species generated by gamma radiation on *Micrococcus luteus* bacterial cells. At levels > 0.70 kGy, significant effects occurred in the 1485-900 cm<sup>-1</sup> region of the second derivative spectra, which contains bands arising from lipids, cell wall polysaccharides, and nucleic acids. Because *M. luteus* contains an intrinsic carotenoid that is associated with membrane lipids, the researchers hypothesized that this antioxidant could protect against radical induced membrane damage. Therefore, *M. luteus* was reincubated for 1-24 h after radiation exposure to allow for evaluation of a mutagenic response. Both hierarchical cluster analysis and PCA of the 985-900 cm<sup>-1</sup> region produced clusters of the controls and irradiated cells versus cells reincubated for > 5 h, which indicated a reorganization of biomolecules due to reincubation (Perromat et al, 2002).

In 2004, Lasch et al used three different clustering methods to analyze FT-mIR spectra obtained from colorectal samples consisting of healthy epithelial, goblet, muscle, blood vessels, and cancerous epithelial cells. The researchers ascertained that data preprocessing was essential for multivariate data analysis and that the Ward’s algorithm was the optimal clustering method of the three tested algorithms in terms of tissue structure differentiation. After preprocessing the spectra by baseline correction, vector normalization, and taking the second derivative, the data showed that cancerous
epithelials contained more phosphate bands as compared to healthy epithelials, which was attributed to higher division rates in cancerous cells (Lasch et al, 2004). 

Carmona et al (2008) used FT-mIR to study lipid peroxidation and protein structures of different rat brain exposed to oxidative stress induced by nicotine and amphetamine. Based on past research, the 900-800 cm$^{-1}$ region was examined for a distinguishable peroxide band. After PCA analysis of the second derivative spectra, the authors were able to identify the band at 873 cm$^{-1}$ as the O--O vibrational mode from peroxides. Carmona et al (2008) proposed that the ratio of peak height at 873 cm$^{-1}$ relative to that of the lipid chain CH$_2$ rocking peak at 720 cm$^{-1}$ could be used to quantitatively measure lipid peroxidation. To test this hypothesis, the researchers monitored the intensity increases of the 873 cm$^{-1}$ band in response to increasing amphetamine concentrations. The results showed that the peroxide changes could be detected by the second derivative FT-mIR spectra as was also supported by using other biochemical methods.

Despite advances in FT-mIR as a metabolomic method to study oxidation, application of this technique for characterizing the capability of natural antioxidants to protect against oxidative stress has yet to be reported. Considering that current methodologies used to monitor natural antioxidants may not be biologically relevant and/or may not account for possible toxic effects of the food systems, FT-mIR may be able to bridge this information gap by providing a global snapshot of cell status in response to treatments with a natural antioxidant.
RESEARCH OBJECTIVE & SPECIFIC AIMS

The objective of this application was to develop Fourier Transform Mid Infrared Spectroscopy (FT-mIR) as a metabolomic fingerprinting tool to study the protective properties of natural antioxidants at the cellular/molecular level. The following specific aims were completed to satisfy the objective of this project.

**Specific Aim 1:** To characterize the physiochemical and antioxidative properties of sorghum based extract using classical approaches. The assessed need for this specific aim was that such methods will provide information on the natural antioxidants present in the sorghum extract that may be responsible for an oxidative protective response. The subsequent information was expected to be also used as a point of reference for developing the FT-mIR method.

**Specific Aim 2:** To develop a FT-mIR method for monitoring the biochemical fingerprint of a model mammalian cell system in response to oxidative stress. The assessed need for this specific aim was that an in vitro method capable of detecting the effects of oxidation on the biochemical fingerprint of a complex mammalian line must be demonstrated before natural antioxidants can be fully understood. The Caco-2 cell line was selected as it is a viable model for oxidative stress. FT-mIR was selected to fulfill this assessed need as it is a robust and selective analytical tool for measuring significant changes in the physiological state of a cell.
**Specific Aim 3:** To apply the parameters established for the FT-MIR method, as developed in Specific Aim 2, to a model mammalian cell system in response to a natural antioxidant, i.e., sorghum phenols. The assessed need for this specific aim was that an in vitro method is required that is capable of detecting the protective effect of natural antioxidants on the biochemical fingerprint of complex mammalian systems. It was expected that this information would provide a link between the physiochemical properties of a natural antioxidant system and a biological outcome that can be linked to human health.

**MATERIALS & METHODS**

**Preparation of Sorghum Extracts:**

Macia 2006 grain sorghum provided by Dr. Curtis Weller, Department of Biological Systems Engineering, University of Nebraska-Lincoln, was ground into a fine consistency. The homogenized sample (~20 g) was combined with 50 mL of nanopure water and mixed for at least 1 h at 25 °C. The suspension was centrifuged at 25 °C for at least 15 min and the supernatant collected. To remove proteins, the supernatant was passed through a Whatman 1 (Whatman International Ltd., Maidstone, England) filter followed by an Amicon Ultra 30K (Millipore, Billerica, MA). The extract was evaporated to 1-3 mL using a rotavap, transferred to 1-3 microcentrifuge tubes and evaporated to dryness using a Labconco Centrivap concentrator at 37 °C. The residues were weighed and re-suspended in 1.0 mL of nanopure water. The concentration was
determined for the stock samples based on an average yield of 30 mg extract/g sorghum. The extract was labeled and stored at 15 to 20 °C until further use.

**Characterization of Sorghum Extracts (SE):**

**Total Phenols:** The Folin-Ciocalteu method was used to determine total phenol levels in the sorghum extract as described by Singleton and Rossi (1965). Briefly, the stock extract was diluted with water as needed for the analysis to ensure that the phenolic content was within the linear region of the calibration curve (16-250 µg/mL). A sample aliquot (100 µL) was combined with 100 µL of Folin-Ciocalteu reagent provided by Sigma (St. Louis, MO) and 4.5 mL of nanopure water. After 3 min of shaking, 0.3 mL of 2 % (w/v) sodium carbonate was added and the samples were incubated at 25 °C for 2 h with intermittent shaking. Detection of the phenols was achieved with a Beckman Coulter DU 800 Spectrophotometer (Fullerton, CA) set at wavelength 760 nm. Trans-cinnamic served as the standard and total phenols, results were expressed in mg trans-cinnamic/g sorghum powder.

**Total Flavonoids:** To quantify total flavonoids, the stock extract was diluted with water as needed to ensure that the phenolic content was within the linear region of the calibration curve (4-250 µg/mL). The diluted stock extract (125 µL) was combined with 37.5 µL of 5 % (w/v) sodium nitrite and 0.625 mL of nanopure water and allowed to react for 4-6 min according to Adom and Liu (2002). Then, 75 µL of 10 % (w/v) aluminum chloride was added to the test sample. After another 5-7 min, 0.25 mL of 1.0 M sodium hydroxide and 0.4 mL of nanopure water was added. The mixture was
vortexed and an aliquot was monitored at a wavelength of 510 nm. Catechin hydrate was used as a standard to generate a calibration curve and total flavonoids were expressed as mg catechin / g sorghum powder.

**Antioxidative Capacity:** The antioxidative capacity of the sorghum extract was determined by the oxygen radical absorbance capacity (ORAC) method as described by Huang et al (2002). A stock solution of standard was prepared by dissolving 0.01 g of Trolox in 10 mL of 75 mM potassium phosphate buffer, pH 7.4. Standard dilution concentrations ranged from 0.46–62.50 µg/mL. The sample stock solution was prepared for this assay as described in the total phenols section. The fluorescent probe, fluorescein (8.16 x 10⁻⁵ mM), was incubated with the standards and samples for 10 min, 3 min of which was with shaking. After incubation, the reaction was activated by adding 153 mM 2, 2'-azobis (2-amidinopropane) hydrochloride, i.e., the radical initiator. All samples / standards were prepared in 96 well plates and monitored with a BMG Labtech FLUOstar Optima microplate reader (Durham, NC). The fluorescence was measured every 1.5 min at an excitation and emission wavelength of 485 nm and 520 nm, respectively, until the decreasing fluorescence values plateaued. From these data, the area under the curve (AUC) and Net AUC were calculated as follows:

1.) \[ \text{AUC} = 0.5 + \frac{R_2}{R_1} + \frac{R_3}{R_1} + \ldots + 0.5\frac{R_n}{R_1} \]

\[ R_1 = \text{fluorescence reading at the initiation of the reaction} \]

\[ R_n = \text{last measurement} \]

2.) \[ \text{Net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}} \]
Net AUC vs. trolox (µg/mL) standard curves were then generated and an ORAC value expressed as µmol Trolox / 100 g sorghum flour.

**Cell Culture and Treatment Methodology:**

*Hydrogen Peroxide (HP) Treated Caco-2 Cells:* Human colon cancer cells (Caco-2) were selected as the model mammalian GI cell line because studies have shown Caco-2 cells to be a proven model mammalian system for monitoring oxidative stress (Wijeratne et al., 2005; 2006). Caco-2 cells provided by the American Type Culture Collection (ATCC) (Rockville, MD) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1.5 g/L sodium bicarbonate, 1 % nonessential amino acid (NEAA), 50 units/mL penicillin with 50 µg/mL streptomycin, 1 % L-glutamine and 20 % fetal bovine serum according to Wijeratne and Cuppett (2006). The cells were seeded on "day one" at a concentration of 5 x 10^5 cells/well onto collagen coated 96 well plates and maintained at 5 % CO₂, 95 % air at 37 °C by using a CO₂ water jacketed incubator (Thermo Fisher Scientific Inc., Forma Series II 3110, Waltham, MA). The medium was replaced after 24 h on day 2 and day 3. Later in day 3 or on day 4, the culture medium was removed and the cells were treated with different concentrations of hydrogen peroxide (HP) in DMEM supplemented with 1 % L-glutamine and 1 % NEAA and for
different exposure periods as described in the Results and Discussion Section. Cells that were not treated with HP served as the control.

*Sorghum Treated (SE-) Caco-2 Cells:* Caco-2 cells were cultured as described above but on Day 3, 48 h after seeding, the medium was replaced with fresh DMEM supplemented with 1 % L-glutamine, 1 % NEAA and a water extract of whole grain sorghum kernel extract. Treatment concentrations are presented in the Results and Discussion Section.

*Sorghum + Hydrogen Peroxide (SE+HP)Treated Cells:* Caco-2 cells were cultured as described above, with sorghum treatment 48 h after seeding followed by HP treatment at 56 h after seeding. Treatment concentrations are presented in the Results and Discussion Section.

**Fourier Transform Infrared Spectroscopy:**

*Preparation of Biofilms:* Immediately after the specified treatment exposure time, the medium and loose cells from each treatment group were pooled into microcentrifuge tubes from the plate wells. The remaining cells were collected by adding 40 μL of trypsin to each well and incubating for 5 min at 37 °C and 5 % CO₂ or until they had detached from the plate. All cells and media were pooled and centrifuged at 2000 rpm for 10 min at 4 °C, the medium was decanted from each treatment sample and the remaining pellet was washed 3 times and re-suspended with 0.1 X phosphate buffered saline (PBS).
The optical density at 600 nm was then determined for each treatment sample and the suspension was adjusted with 0.1 X PBS to an OD of approximately 1.15. Samples (20 µL) were each applied in triplicate to a Bruker Optics zinc selenide (ZnSe) 15 window biowheel (Billerica, MA) (Figure 7a) and dried under moderate vacuum (bar) at 23-27 °C for ≈ 45 minutes until a transparent biofilm had formed.

**FT-mIR Analysis:** Temporal changes in the FT-mIR spectra of untreated and treated cells were evaluated and compared. The biowheel sample was inserted into the mid-IR sample compartment of a Bruker Equinox 55 FT-MIR equipped with a deuterated triglycine sulfate detector (Figure 7b). A spectrum of each biofilm was obtained over the entire mid-IR region (4000-500 cm\(^{-1}\)) at a spectral resolution of 4 cm\(^{-1}\). To increase signal to noise, each spectrum were collected as an average of 64 scans, apodized with the Blackman-Harris 3-Term function and then Fourier-transformed. A background spectrum of the ZnSe window was also obtained and subsequently subtracted from each sample spectrum. To reduce fluctuations due to spectra slope, second derivative spectra were obtained using a 9 smoothing point Savitsky-Golay algorithm. Recording of spectra, data storage, and all other spectral manipulations were performed using the Bruker Opus 4.0 Software.
Figure 7a: Biowheel

Figure 7b: Biowheel compartment
Statistical Design and Analysis:

Culture Design: Culturing followed a randomized complete block design blocking for 12 replicates.

FT-mIR Spectral Analysis: Spectra were compared using Ward's algorithm for hierarchical grouping or cluster analysis (Bruker Opus 4.0 Software). Principle component analysis was performed using The Unscrambler 9.7 Software.

RESULTS AND DISCUSSION

Specific Aim 1: Physiochemical Characterization

Grain sorghum is a rich source of phenolic compounds, including such compounds as tannins; ferulic, sinapic, and p-coumeric acids; the flavonoids luteolin and naringenin; and 3-deoxyanthocyanins (Dykes et al, 2005). Because of concerns regarding poor digestibility, low protein content and the perceived unfavorable flavor, grain sorghum is used primarily as cattle feed in the United States (Chibber et al, 1980). However, grain sorghum is an accepted and widely used food source throughout the world, particularly in third world countries. Epidemiological data accumulated on these populations have shown reduced risks for GI tract based cancers with grain sorghum consumption (Chen et al, 1993; Isaacson, 2005; Van Rensburg, 1981), which may be due in part to the presence of antioxidant agents (Awika and Rooney, 2004).

As a result of these studies and previous work completed in our laboratory with grain sorghum lipid based systems (Carr et al, 2005; Hwang et al, 2004; Zbasnik et al, 2009), polar based samples extracted from the Macia 2006 grain sorghum line served as
the antioxidant containing natural system for this study. Prior to use, the extracts were characterized for total phenol, total flavonoid content, as well as antioxidative capacity using the ORAC assay. As shown in Table 2, the extracts contained 0.11 +/- 0.00 mg t-cinnamic acid/ g (phenols) and 0.02 +/- 0.00 mg catechin/ g sorghum (flavanoids), while the ORAC value was 336 μmol Trolox/100 g flour. To put these values in perspective to other food systems, the hydrophilic ORAC value for fresh tarragon was reported to be 15542; blueberries, 6520; red table wine, 3873; yellow sweet corn, 593; carrots, 355; and cucumbers, 214 μmol TE/100 g (USDA, 2007).

The phenolic content obtained in our laboratory was comparable to white grain sorghum content (100 mg gallic acid / 100 g) reported by the USDA but the ORAC value was significantly lower (2100 μmol TE / 100 g). As previously discussed, polyphenols are secondary metabolites in plants that are affected by seasonal changes, i.e., temperature, rain, nutrient levels in soil, and can vary greatly in quantity and type from one season to the next (Wildman, 2001), which may account for the differences between the two values. It is also common practice to extract phenols using a single type of solvent, but this solvent and the overall extraction procedure used may differ between laboratories resulting in extracts containing different types and levels of phenols (Awika, et al, 2003; Dykes et al, 2005; Huang et al, 2005). It also must be noted that while phenols and flavonoids are usually credited for the hydrophilic ORAC values, other components, such as minerals, small peptides, and water soluble vitamins, may be present.
**Table 2:** Characterization of grain sorghum (Macia 2006) water extract.

<table>
<thead>
<tr>
<th>Phenols</th>
<th>Flavonoids</th>
<th>ORAC</th>
</tr>
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<tbody>
<tr>
<td>mg $t$-cinnamic/g flour</td>
<td>mg catechin/g flour</td>
<td>$\mu$mol Trolox/100 g flour</td>
</tr>
<tr>
<td>0.11 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>336</td>
</tr>
</tbody>
</table>
in polar based extraction, which in turn can enhance or compromise the overall antioxidant capacity (Adom and Liu, 2002; Awika et al, 2003).

**Specific Aim 2: FT-mIR Metabolomic Method for Monitoring Oxidative Stress in Caco-2 Cells**

**FT-Method Development**

_Solvent selection:_ The literature has demonstrated the importance of consistent and precise experimental protocol, i.e. incubation time, medium, temperature, harvest, and sample preparation, to obtain reproducible FT-mIR data from biological systems (Melin et al, 1999; Diem et al, 1999; Holman et al, 2000). In a cell cycle study of human lung fibroblasts, Holman et al (2000) showed that high quality cell monolayers were needed to produce optimal FT-mIR data. To ensure that homogeneous biofilms were consistently prepared, the treated cells were washed two times with PBS, pipetted onto gold-coated glass slide pieces, and spread out with a pipette tip. The researchers were also careful to apply a consistent cell density to the slides, as well as to control temperature and humidity. Another group, Diem et al (1999), noted that large spectral differences in the DNA bands were dependant on cell growth cycle. As a result, the DMEM culture medium was removed from the cell culture immediately after the treatment period was completed to stop cell growth and inhibit metabolism (ATCC, 2009).

Accordingly, an appropriate solvent for our study was selected based upon three criteria: that the solvent could not induce cell death, influence the spectra, or support
post-harvest metabolism. Based on results presented by Diem et al (1999), the DMEM growth medium was removed from the cell culture as soon as the treatment period was completed. Because phosphate buffered saline (PBS) can be adjusted to a physiological pH and had been successfully used for developing the Caco-2 oxidative model (Wijeratne and Cuppett, 2005; 2006), it was the logical cell suspension solvent for the next step, i.e., biofilm preparation. To ensure that PBS did not influence the spectra, 20 μL of Caco-2 cells suspended in 1x PBS was applied to 15 windows of a bio-wheel and dried under vacuum. At this concentration, the salt in the PBS crystallized resulting in non-uniform films and noisy spectra (data not shown). PBS concentrations ranging from 0x-1x PBS were thus evaluated. Homogenous biofilms were produced with PBS concentrations less than 1x resulting in FT-mIR spectra with high signal to noise ratios. Using a light microscope at 10x power, it was further determined that cell death was not induced at any of these concentrations. Therefore, a concentration of 0.1x PBS solutions was used throughout the studies to prepare the biofilms. No nutrients were added to the PBS and the cell suspension was kept at 4 °C until applied to the wheel to inhibit post-harvest metabolism as much as possible. Once dried, the biofilm was examined using a light microscope to confirm that cells dried in a monolayer.

Optimizing Optical Density (OD): Previous work within our group completed with bacteria (Plantz, personal communication, 2008) has shown that high and consistent signal to noise ratios were only obtained when the spectral peak absorbance of the amide I peak (1695-1610 cm⁻¹, Figure 6, window 2) was between 0.60 and 0.90. To
standardize this parameter for Caco-2 cells, an optimal optical density (OD) at 600 nm was determined by suspending the cells in different volumes of PBS and applying the suspension to the biofilm windows using different volumes. The associated OD and transfer volume were then compared to the absorbance of amide 1 peak produced by the resulting biofilm. An optimum absorbance was thus achieved using 20 μL cell suspensions with an OD of 1.15.

**Spectra pre-processing technique:** The FT-mIR literature has also shown that signals exhibited by cellular systems can be adequately resolved by pre-processing baseline corrected raw data followed by normalization to the amide I peak and then by obtaining the second derivative spectra (German et al, 2006; Lasch et al, 2002; Mordehai et al, 2003; Yu and Irudayaraj, 2005). To ensure that this approach could be applied to our data, a variety of preprocessing techniques were evaluated on FT-mIR data obtained from Caco-2 cells, untreated or treated with hydrogen peroxide (HP). These parameters included vector normalization of the entire mIR region, smoothing with different points, first and second derivative spectra and various combinations of the pre-processing parameters followed by evaluation with hierarchical clustering analysis and PCA. Evaluation criteria included degree of 1) treatment clustering 2) heterogeneity based on (D) values and 3) % variance described by PC 1. After assessing the different combinations, it was confirmed that the cited literature approach was the optimal pre-processing method for our data (German et al, 2006; Lasch et al, 2002; Mordehai et al, 2003; Yu and Irudayaraj, 2005). As discussed previously, hierarchical cluster analysis
and PCA are proven chemometric methods for analyzing the pre-processed FT-mIR spectra and thus were applied to the pre-processed data generated from this study (Carmona et al, 2008; German et al, 2006; Lasch et al, 2002; Mordehai et al, 2003; Yu and Irudayaraj, 2005).

**FT-mIR Performance Qualification:** After the preliminary development steps were completed, the method was applied to 68 untreated Caco-2 samples collected from five different days. The spectra were baseline corrected followed by vector normalization to the amide I peak. After an average and standard deviation (SD) from 1750-700 cm⁻¹ region were obtained, 2 x the standard deviation (SD) spectrum was then added to and subtracted from the average spectrum, (i.e., mean +/- 2SD) (Figure 8). Each individual spectrum was examined as an outlier based upon whether they fell within the mean +/- 2 SD boundaries (95%), resulting in 4 of 68 being discarded as outliers. This low number of outliers confirmed that biofilms were reproducible and that pre-processing parameters were satisfactory.

**FT-mIR Oxidative Stress Studies**

**Control versus 0.2 mM HP: Treatment time of 0.5 h:** The method as developed was then applied to Caco-2 cells treated with HP to identify effects due to oxidative stress. Spectra (7-8 per sample) were collected of samples treated with 0.2 mM HP for 0.5 h on two consecutive days to achieve two replicates. As shown by Figure 9a, FT-mIR of the zero order spectra (pre-processed but not derivatized) exhibited large bands at 1650 and 1545 cm⁻¹, which have been attributed to the protein amide I and
amide II functional groups, respectively (Holman et al, 2000). A broad band at 3000-2800 cm\(^{-1}\) due to antisymmetric stretching vibrations of CH\(_2\) and CH\(_3\) in fatty acids (data not shown) (Mordehai et al, 2003) was also present in the spectra. In addition, smaller peaks were exhibited that have been linked to lipid esters (1740 cm\(^{-1}\)), the lipid methylene bending mode (1457-60 cm\(^{-1}\)), symmetric bending mode of branched methyl groups in lipids, carbohydrates, and proteins (1400-02 cm\(^{-1}\)), amide III (1302 cm\(^{-1}\)), asymmetric stretching vibrations of PO\(_2\)\(^{-}\) in phospholipids or nucleic acids (1240 cm\(^{-1}\)), DNA/RNA backbones (1155 cm\(^{-1}\)), symmetric stretching vibrations of PO\(_2\)\(^{-}\) in DNA/RNA (1088 cm\(^{-1}\)) and nucleotides (970 cm\(^{-1}\)) (Yu and Irudayaraj, 2005). No apparent differences occurred in either amplitude or absorbance over most of the spectral region for the zero order spectra but the second derivative spectra showed a treatment effect for the 854 cm\(^{-1}\) band (Figure 9b). Although this region (window 5) contains multiple bands that have been attributed to the ring structure in nucleotides, the peroxide band at 870 cm\(^{-1}\) (Carmona et al, 2008) is also in close proximity. The difference at 854 cm\(^{-1}\) was amplified resulting in a large trough at 854 cm\(^{-1}\) in the control spectra and a peak at 871 in HP spectra (Figure 9b, a-b).

Pre-processing the spectra to the second derivative revealed no other differences in response to HP treatment but cluster analysis of the individual spectra of the 1750-700 cm\(^{-1}\) range resulted in two distinct clusters (Figure 10a). With a few exceptions, cluster 1 contained the 0.2 mM HP and untreated cells from one day while cluster 2 contained samples prepared on the next day. Review of the within day clusters did show minimal
Figure 8: Control Validation. Sixty-eight control spectra from five different days within average +/- 2 x standard deviation upper and lower limits. Four spectra were outliers.
Figure 9: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm$^{-1}$ region: A) original spectra B) second derivative spectra. Hydrogen peroxide treatment time was 0.5 h while treatment concentrations were as follows: a) 0 mM b) 0.2 mM. C) Cluster analysis was performed using the second derivative spectra in the 1750-700 cm$^{-1}$ spectral range.
Figure 10: Heterogeneity of Caco-2 cells treated for 0.5 h at 0 or 0.2 mM hydrogen peroxide. Cluster analysis using the Ward’s algorithm was performed on the second derivative of the A) individual spectra B) average spectra in the 1750-700 cm⁻¹ spectral range.
clustering by treatment (2 consecutive spectra) but the averaged spectra resulted in the same two clusters (Figure 10b), which indicated that the treatment level was not high enough to overcome the clustering by day effect. Panteleeva et al (2002) noted that small differences between passage numbers (due to day) of the same cell line became resolved in second derivative spectra and thus concluded that controls have to be taken from identical passages as treatments.

Cluster analysis of the individual spectra in the 860-850 cm\(^{-1}\) region resulted in two clusters (data not shown), the first with HP and untreated spectra from both days, the second with three control spectra from the first day. The larger cluster was further broken down into six subgroups, separating first by day and then by improved (4 consecutive spectra) treatment clustering. The data demonstrated that the peak at 854 cm\(^{-1}\) may be a point of interest for treatment effect in future studies.

Work completed by Wijeratne et al (2005) showed that Caco-2 cells treated with 0.2 mM HP for 0.5 h did not decrease in viability but the lipid membrane and DNA damage were affected, as determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactic acid dehydrogenase (LDH), and the Comet assay, respectively. The results also showed that the antioxidative enzymes catalase and glutathione peroxidase were upregulated for the treatment time and levels (Wijeratne et al, 2005). Up regulation of these enzymes at levels able to overcome the HP concentration and exposure time may be protecting the cells, which may account for the similarities between the control and treated cells in this study (Wijeratne et al, 2005).
Therefore, it was determined that either higher concentrations of HP or longer treatment times were needed to induce a significant treatment effect.

Control versus 0.2 mM HP, Treatment time course: Spectra (3-4 per sample) collected for samples treated for 0, 0.5, 1, 4, and 20 h with 0.2 mM HP are shown in Figure 11a. With increasing HP treatment times, an overall smoothing effect was accompanied by decreasing peak heights that were more evident in the second derivative spectra (Figure 11b). The most significant spectral differences were detected for the 1743, 1731, 1688, 1632, 1290, 1227, 1156, 1095, 907, 857 cm\(^{-1}\) bands, suggesting that the lipid esters, amide I (B-turns, B-sheets), amide III, phospholipids, and DNA and RNA, respectively, were affected with increasing HP treatment times (Yu andIrudayaraj, 2005). The peaks at 1612, 907, 857 cm\(^{-1}\) have not been assigned to specific functional groups, but as previously noted, the region from 900-800 cm\(^{-1}\) is known to contain multiple nucleotide peaks, i.e., C=C, C=N, C-H (Carmona et al, 2008). The peaks at 1632 and 1227 cm\(^{-1}\) disappeared for treatment times of 4 h and longer while the 1039 cm\(^{-1}\) band split into two peaks with apexes at 1046 and 1034 cm\(^{-1}\) for the 1, 4, and 20 h treatments. The latter region is attributed to the C-O-C, and C-O ring vibrations in carbohydrates (Yu and Irudayaraj, 2005).

Cluster analysis of individual spectra (Figure 12a) resulted in two major groups (or clades), the first containing the untreated sample spectra, all of the 0.5 h sample spectra, and one spectrum each of the remaining treatments whereas the second clade contained the untreated spectra, 1, 4, and 20 h. However, cluster analysis of averaged
Figure 11: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm\(^{-1}\) region: A) zero order spectra, i.e., based line corrected followed by normalization B) second derivative spectra. Hydrogen peroxide treatment concentration was 0.2 mM while treatment times were as follows: a) 0 h, b) 0.5 h, c) 1 h, d) 4 h, e) 20 h.
**Figure 12:** Heterogeneity of Caco-2 cells treated with 0.2 mM hydrogen peroxide for 0, 0.5, 1, 4, or 20 h. Cluster analysis using the Ward’s algorithm was performed on the second derivatives of the A) individual spectra B) average spectra in the 1750-700 cm\(^{-1}\) spectral range.
spectra (Figure 12b) produced two clades, the first containing the control and the 0.5 h treated samples, the second containing 1, 4, and 20 h. The second clade also had two subgroups that separated according 1 h and the 4 / 20 h treatments. In combination with the second derivative spectra data, the clustering of the 4 and 20 h samples suggests that these treatment times are more similarly impacting the Caco-2 cells relative to the 1 h treatment. Because the 20 h treated samples clustered more closely to the control than the 4 h samples, which may be representative of the fact that certain peak amplitudes (1688, 857 cm⁻¹) appeared to be recovering to the level of control peaks, a treatment exposure time of 16 h, i.e., a treatment time between 4 and 20 h, was selected to determine the effects of different HP concentrations.

*HP concentration curve, Treatment time of 16 h:* Spectra (3-4 per treatment) were then collected on two consecutive days of samples treated with different concentrations of HP (2, 5, 15, and 40 mM) for 16 h. In order to reduce variability due to growth time, the control was maintained for 24 h. As expected, spectral differences were evident using a 16 h treatment time especially for the 15 and 40 mM HP treated cells (Figures 13a-b). Similar to the time course study, a general decrease in amplitude in the 1642 and 1535 cm⁻¹ bands occurred with increasing treatment strength indicating that proteins were being impacted by oxidative stress (Figure 13b). At 15 mM, a peak (1619 cm⁻¹) in the protein region disappeared while this peak as well as the 1642 and 1535 cm⁻¹ bands were absent in the 40 mM treated sample. The 1588, 1096, 1007, and 985 cm⁻¹ bands were present in all the treatment groups but decreased with increasing HP
Figure 13: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm$^{-1}$ region: A) zero order spectra B) second derivative spectra. Hydrogen peroxide treatment time was 16 h while treatment concentrations were as follows: a) 0 mM, b) 2 mM, c) 5 mM, d) 15 mM, e) 40 mM.
concentration. In addition, the 1412 cm\(^{-1}\) peak was apparent in the control but presented as only a smooth bump for all of the HP treated samples. Although the peak at 868 cm\(^{-1}\) is more defined for the control and 2 mM sample, the trough at 854 cm\(^{-1}\) was deeper for the control compared to any of the HP treatments (Figure 13b). In some cases, relative peak shifts, shape changes, and new peak appearances occurred with higher treatment levels. A peak at 1634 cm\(^{-1}\) appeared in the spectrum of the > 15 mM treated samples while a peak at 1613 cm\(^{-1}\) was apparent in the samples treated with > 5 mM HP levels.

Melin et al (1999) noted a similar increase in intensity with ascorbic acid concentration in this region, which they attributed to conformational changes in proteins. In addition, the peak and trough at 1168 and 1155 cm\(^{-1}\) (DNA/RNA backbones) changed in appearance as the peak appeared to slide down into the trough, although there was no actual shifting with increasing treatment levels. This trough was also deeper in the control compared to any of the HP-treated samples. Similarly, Perromat et al (2002) reported a change in peak intensity at 1155 cm\(^{-1}\) in response to increased radiation exposure, which were assigned as the C-O-C vibrations in DNA backbones. The authors hypothesized that strand breakage occurred as a result of radiation damage.

As shown in Figure 13b, the second derivative spectra exhibited a peak at 1033 cm\(^{-1}\) for the control, 2 and 5 mM treated cells that shifted to 1037 cm\(^{-1}\) for the 15 and 40 mM samples while the 905 and 845 cm\(^{-1}\) bands for the control, 2 and 5 mM samples changed in appearance as compared to those exhibited by the 15 and 40 mM samples. Collectively, these peaks (1033-905 cm\(^{-1}\)) are in the phosphate region (Lasch et al, 2002;
Mordehai et al, 2003) and have been associated with DNA, RNA, and phosphorylated proteins. Mordehai et al (2003) showed similar changes in the spectra of white blood cells in response to bacterial infection, which may suggest that bacteria exert a stress similar to oxidation.

The cited spectral changes were significant across the entire 1750-700 cm\(^{-1}\) region as evidenced by the hierarchical cluster analysis of both the individual and average spectra (Figures 14a-b). For the averaged spectra, two major clades resulted with the control, 2, and 5 mM samples distinctly separating from the 15 and 40 mM samples. For the individual spectra, the 15 mM and 40 mM treatments clustered by treatment and then by day whereas the control, 2 and 5 mM treatments clustered by day and then by treatment. A two-dimensional (2D) PCA map of individual spectra supported the dendrogram results (Figure 15a) as the control, 2, and 5 mM treatments generally clustered in the positive PC 1 region while the 15 and 40 mM samples clustered in the negative PC 1 region.

Cluster analysis of average spectra in the 1645-1632 cm\(^{-1}\) range, which contains the amide I bond of \(\beta\)-sheets (Yu and Irudayaraj, 2005), resulted in the same clustering (Figure 16b) as the individual spectra of the 1750-700 cm\(^{-1}\) region (Figure 14a). A 3D PCA plot of this region provided more information (Figure 17b) showing that the untreated cell spectra grouped in the negative PC 1 and negative PC 3. Cells treated with 2 mM HP were predominantly negative of PC 2 but scattered on both sides of PC 1 and PC 3. The overlapping control and 2 mM HP spectra suggest that at least in this region
Figure 14: Heterogeneity of Caco-2 cells treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the A) individual spectra and B) average spectra in the 1750-700 cm\(^{-1}\) spectral range. The red numbers indicate one or more spectra in a cluster may be more homogenous with a spectrum in another cluster.
Figure 15: Principle Component Analysis of Caco-2 cells in the 1750-700 cm\(^{-1}\) region treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the individual spectra. A) Two dimensional plot of principle component 1 (horizontal axis) versus PC 2 (vertical axis) B) Three dimensional plot of PC 1 (x axis) x PC 2 (yl axis) x PC 3 (z axis).
Figure 16: Heterogeneity of Caco-2 cells treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the A) individual spectra and B) average spectra in the 1645-1632 cm\(^{-1}\) spectral range. The red numbers indicate one or more spectra in a cluster may be more homogenous with a spectrum in another cluster.
Figure 17: Principle Component Analysis of Caco-2 cells in the 1645-1632 cm$^{-1}$ region treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the individual spectra. A) Two dimensional plot of principle component 1 (horizontal axis) versus PC 2 (vertical axis) B) Three dimensional plot of PC 1 (x axis) x PC 2 (y axis) x PC 3 (z axis).
(1645-1632 cm\(^{-1}\)), the 2 mM HP treatment did not impact the cells. Although overlapped by the control and 2 mM data, the 5 mM HP spectra clearly grouped in the positive part of PC 1, negative of PC 2, and negative of PC 3. The 15 and 40 mM HP spectra were for the most part separated from all other treatments. These results suggest that treatment levels of 15 and 40 mM HP significantly altered the amide I bond of \(\beta\)-sheets. As \(\beta\)-sheets are responsible for the secondary structure of proteins (Melin et al, 1999), it is reasonable to conclude that at these levels, HP is damaging the conformational structure of proteins in Caco-2 cells.

Cluster analysis of the 1170-1150 cm\(^{-1}\) range, which contains the various bonds \([\delta(COP), \delta(COH), \nu(CC)]\) attributed to DNA and RNA backbones (Perromat et al, 2002; Yu and Irudayaraj, 2005), resulted in similar clustering with the exception that the untreated cells grouped more closely to the 5 mM cells (Figures 18a-b). However, the 2D PCA plot (Figure 19) shows the 5 mM samples clustering separating from the other treatments. The controls and 2 mM HP spectra are positive of PC1 and negative of PC 2 whereas the spectra of 5 mM HP cells were negative of PC 1 and negative of PC 2. Lastly, the 15 and 40 mM HP spectra were all positive of PC 2, and scattered on both sides of PC 1. The control and 2 mM HP treatment were more separated from the 15 and 40 mM HP while the 5 mM HP shared characteristics of both groups. These results support the second derivative spectra in that the 5 mM treatment had a shallower trough at 1155 cm\(^{-1}\) band compared to the lower treatments but the appearance of the peak at 1168 cm\(^{-1}\) had not changed compared to the higher concentrations of HP. The
Figure 18: Heterogeneity of Caco-2 cells treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the A) individual spectra and B) average spectra in the 1170-1150 cm⁻¹ spectral range. The red numbers indicate one or more spectra in a cluster may be more homogenous with a spectrum in another cluster.
**Figure 19:** Principle Component Analysis of Caco-2 cells in the 1170-1150 cm$^{-1}$ treated with hydrogen peroxide for 16 h and 0, 2, 5, 15, or 40 mM hydrogen peroxide. Cluster analysis was performed using the second derivatives of the individual spectra. Two dimensional plot of principle component 1 (horizontal axis) versus PC 2 (vertical axis).
dendrograms and PCAs collectively indicate that a HP concentration of between 5 mM and 15 mM inflicted significant oxidative stress to the Caco-2 cells. As the 5 mM HP samples clustered with the untreated cells in a few cases, it was decided to treat the cells with 15 mM HP in the next stage of the project, which was to determine with the ability of grain sorghum to protect the biochemical fingerprint of oxidatively stressed Caco-2 cells.

**Specific Aim 3: FT-mIR Sorghum Extract + Oxidative Stress Studies**

**SE concentration curve, 15 mM HP:** Spectra (3-4 per treatment) were obtained of samples treated with SE concentrations of 0, 1, 5, 10, and 40 mg/mL for 8 h followed by 15 mM HP for an additional 16 h. As shown in Figure 20a, the 1318 peak (amide III) shifted to 1304 cm\(^{-1}\) with increasing levels of SE treatments while the 40 mg/mL SE + HP spectrum contained a larger peak at 1318 cm\(^{-1}\). This effect also occurred in the HP time study with a shift to lower frequencies with increasing incubation times (Figure 11a). The band at 1162 cm\(^{-1}\) (DNA/RNA backbones) in the control shifted to 1154 cm\(^{-1}\) in all of the SE + HP and HP spectra. Lastly, the peak at 970 cm\(^{-1}\) (DNA/RNA backbones) was more defined in SE + HP spectra as compared to the untreated and HP spectrum (Figure 20a).

The second derivative spectra amplified a number of additional spectral differences (Figure 20b). For the amide I and amide II region, the most notable changes occurred in the 1689, 1645 and 1632 cm\(^{-1}\) bands. For example, the 1689 cm\(^{-1}\) was smaller for the control and the 10 mg/mL SE + HP spectra but largest in the 1 and 40
Figure 20: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm\(^{-1}\) region: A) zero order spectra B) second derivative spectra. Cells were treated with a) control media b) 1 mg/mL SE + 15 mM HP c) 5 mg/mL SE + 15 mM HP d) 10 mg/mL SE + 15 mM HP e) 40 mg/mL SE + 15 mM HP f) 15 mM HP. Control cells were treated for time = 24 h, SE + HP for time = 8 + 16 h, and HP for time = 16 h.
mg/mL SE + HP samples as well as for the 15 mM HP treated spectra. For the 1645 cm\(^{-1}\), the control and 1 mg/mL SE + HP spectra exhibited a peak that diminished for cells treated with higher concentrations of SE + HP and HP only. Although the control and the 1 mg/mL SE + HP did not contain 1632 cm\(^{-1}\), this band appeared in response to higher treatments of SE treated samples. Comparison of these results with HP concentration data (Figure 13b) showed similar effects for the both the 1645 and 1632 cm\(^{-1}\) bands. All the spectra contained a peak at the 1310 cm\(^{-1}\) (amide III) except for the 40 mg/mL SE + HP spectra, which had a trough at 1318 cm\(^{-1}\) in its place (Figure 20b). Cluster analysis of the 1321-1304 cm\(^{-1}\) region supported the spectral data resulting in two clades, one containing the 40 mg/mL SE + HP data and the other containing the remaining treatments including the HP without the sorghum (data not shown). However, the HP spectrum exhibited a peak at 1224 cm\(^{-1}\) (phospholipids) that was not present in the control or in any other treatment group or was present in other oxidation spectra. At this time, we do not have an explanation for this band. In the nucleotide region, the double peak at 1007/983 cm\(^{-1}\) was smaller in the 5, 10, and 40 mg/mL SE + HP treatments compared to the control, 1 mg/ml SE + HP, or the HP treatments suggesting that the higher concentrations of SE are affecting the cells differently than the peroxide alone. Moreover, the general topography of window 4 (900-800 cm\(^{-1}\)) was different for the 5 and 40 mg/mL SE + HP and HP samples while the 1 and 10 mg/mL SE + HP spectra were more similar to the control (Figure 20b).
Hierarchical cluster analysis of the 1750-700 cm⁻¹ region of the averaged spectra resulted in two major clades, i.e., the control versus all other treatments (Figure 21a) with the 5 and 10 mg/mL SE + HP clustering together relative to the 1, 40 mg/mL SE + HP and 15 mM HP spectra. These results show that although the SE treatments were unable to completely prevent oxidative damage at the concentration tested, the 5 and 10 mg/mL may have partially protected the cells. The three dimensional PCA plot of the individual spectra (Figure 22) supports the hierarchical cluster data in that the control, 1, 5, and 10 mg/mL SE + HP group separately from 40 mg/mL SE + HP and 15 mM HP. Combined with second derivative spectral changes, the results also indicate that the 40 mg/mL SE may be more harmful than helpful in protecting cells from the oxidative stress of cells, particularly affected the amide III band (1308 cm⁻¹).

Further application of the pattern recognition tests to the 1240-1180 cm⁻¹ region of the second derivative data indicated that in this region, all of the SE concentrations tested were able to protect against oxidative stress inflicted by 15 mM HP. As shown in Figure 23, cluster analysis and 2D PCA of the 1240-1180 cm⁻¹ resulted in two major clusters with 15 mM HP separating from all the other treatments. It must be noted that the 2D PCA accounted for 90 %+ of the variability and thus the data was not analyzed with 3D PCA. The most prominent band (1240 cm⁻¹) in this region is due to the asymmetric stretching P=O bond attributed to phospholipids (Yu and Irudayaraj, 2005).

15 mg/mL SE + 15 mM HP: Considering that 5 and 10 mg/mL SE + HP were clustering apart from the 15 mM HP group and the other SE treatments (Figure 24),
Figure 21: Heterogeneity of Caco-2 cells treated with a) control media  b) 1 mg/mL SE + 15 mM HP  c) 5 mg/mL SE + 15 mM HP  d) 10 mg/mL SE + 15 mM e) 40 mg/mL SE + 15 mM  f) 15 mM HP. Control cells were treated for time = 24 h, SE + HP for time = 8 + 16 h, and HP for time = 16 h. Cluster analysis was performed using the second derivatives of the A) average spectra  B) individual spectra PC 1 x PC 2 in the 1750-700 cm\(^{-1}\) spectral range.
**Figure 22:** Principle Component Analysis of Capo-2 cells in the 1750-700 cm⁻¹ region treated with a) control media b) 1 mg/mL SE + 15 mM HP c) 5 mg/mL SE + 15 mM HP d) 10 mg/mL SE + 15 mM HP e) 40 mg/mL SE + 15 mM HP f) 15 mM HP. Three dimensional plot of PC 1 (x axis) x PC 2 (y axis) x PC 3 (z axis) using the second derivatives of the individual spectra. Each spectrum is depicted by a circle of color a) purple b) green c) pink d) blue e) black f) red.
Figure 23: Heterogeneity of Caco-2 cells treated with a) control medium b) 1 mg/mL SE + 15 mM HP c) 5 mg/mL SE + 15 mM HP d) 10 mg/mL SE + 15 mM e) 40 mg/mL SE + 15 mM f) 15 mM HP. Cluster analysis was performed using the second derivatives of the A) average spectra B) individual spectra PC 1 x PC 2 in the 1240-1180 cm$^{-1}$ spectral range.
Figure 24: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm⁻¹ region: A) zero order spectra B) second derivative spectra. Cells were treated with a) control media, time = 24 h b) 15 mg/mL SE, time = 24 h c) 15 mg/mL SE + 15 mM HP, time = 8 h + 16 h d) 15 mM HP, time = 16 h.
indicating that they may be partially protecting the cells, 15 mg/mL of SE was then used to determine if this slightly higher concentration could provide more protection to cells exposed to 15 mM HP. A negative control (0 mg/mL SE - HP) as well as a positive control (15 mg/ml SE – HP) were also included in this study. As shown in Figure 24a, the zero order spectra of the four treatments were similar in most of the windows with notable peak shifts at 1304-1307 cm\(^{-1}\) (amide III), 1170-1154 cm\(^{-1}\) and 986-968 cm\(^{-1}\) (DNA/RNA backbones) for the negative / positive control vs the SE + HP and HP treated cells. Comparison of the second derivative spectra showed that the SE + HP spectral fingerprint was more similar to that of HP than either control (Figure 24b). The most significant difference between the controls occurred for the 1306 cm\(^{-1}\) band as the former sample had a downward slope that was not present in the latter. However, cluster analysis of the averaged spectra yielded two clades: 1) SE + HP and HP and 2) SE and untreated cells (Figure 25) indicating that 15 mg/mL SE did not inflict any damage but it was not able to protect against oxidation for the entire 1750-700 cm\(^{-1}\) region.

15mg/mL SE + 5 mM HP: The next test was conducted to determine whether 15 mg/mL SE could protect cells against oxidative stress inflicted by a lower concentration of HP (5 mM). Similar to the previous study, the amide III peak (~1310 cm\(^{-1}\)) was again affected by the HP treatment as shown by the zero order spectra (Figure 26a). In most cases, the positive control as well as the SE + HP spectra were similar to the untreated cells with the exception of certain DNA/RNA backbone bands (1160 and 971 cm\(^{-1}\)) as the SE + HP spectrum more closely resembled the 5 mM HP. Pre-processing to the second
Figure 25: Heterogeneity of Caco-2 cells treated with a) control medium, time= 24 h  b) 15 mg/mL SE, time=24 h  c) 15 mg/mL SE + 15 mM HP, time= 8 h + 16 h d) 15 mM HP, time= 16 h. Cluster analysis was performed using the second derivatives of the average spectra.
Figure 26: FTIR spectra obtained from Caco-2 cells in the 1750-700 cm$^{-1}$ region: A) zero order spectra B) second derivative spectra. Cells were treated with a) control media, time=24 h  b) 15 mg/mL, time=24 h SE  c) 15 mg/mL SE + 5 mM HP, time = 8 h +16 h  d) 5 mM HP, time=16h.
derivative resulted in peaks with higher amplitudes at 1731 and 1721 cm$^{-1}$ in the SE and SE + HP treated cells, an effect not detected in previous studies (Figure 26b). (Bands in this area correspond to lipid esters (1741 cm$^{-1}$) and RNA/DNA (1708 cm$^{-1}$) (Yu and Irudayaraj, 2005)). In the amide I and II region, the band at 1619 cm$^{-1}$ in the negative control and SE spectra shifted to 1613 cm$^{-1}$ with respect to the SE + HP and HP spectra. Indeed, many bands of the SE + HP and HP spectra were more similar to each other than compared to the other treatments including bands at 1587 cm$^{-1}$ (amide II), 1447 cm$^{-1}$ (lipids, proteins), 1413 cm$^{-1}$ (lipids, carbohydrates, proteins), 1008, 983, 921, and 844 cm$^{-1}$ (DNA, RNA, and nucleotides). Review of the spectral data alone again indicated that that 15 mg/mL SE was not able to prevent oxidative damage inflicted by 5 mM HP except perhaps at 1587 and 1310 cm$^{-1}$.

Hierarchical cluster analysis of the average spectra in the 1750-700 cm$^{-1}$ range supported the spectral data resulting in two major clusters: control versus all other treatments (Figure 27a). Principle component analysis, however, showed that 15 mg/mL SE may possibly be protecting against oxidative stress. As depicted in Figure 27b, the 3D PCA plot of individual spectra separated into three clusters that contained 1) untreated and SE + HP spectra, 2) HP spectra, and 3) SE spectra.
Figure 27: Heterogeneity of Caco-2 cells treated with a) control media, time=24 h b) 15 mg/mL, time=24 h SE c) 15 mg/mL SE + 5 mM HP, time = 8 h +16 h d) 5 mM HP, time=16h. Cluster analysis was performed using the second derivatives of the A) average spectra B) individual spectra PC 1 x PC 2 x PC 3 in the 1750-700 cm⁻¹ spectral range. Each spectrum is depicted as a circle of color a) black b) red c) blue d) green.
CONCLUSIONS

Caco-2 cells proved to be a valid model for monitoring oxidative stress in mammalian intestinal cells. Moreover, FT-mIR analysis is a rapid, high throughput method capable of obtaining a global picture of the biochemical fingerprint in response to oxidative stress. In these studies, FT-mIR spectroscopy was developed to investigate the effect of oxidative stress on Caco-2 cells, which included increasing HP concentrations and treatment times. As determined by hierarchical cluster analysis of the second derivative spectra, cells treated with 0.2 mM for 0.5 h did not show significant differences from the control but changes could be detected with increasing exposure times. When examining the 1750-700 cm\(^{-1}\) region for cells treated with different concentrations of HP for 16 h, the 2 mM and 5 mM HP samples clustered more closely with the control indicating that these HP treatment levels had less impact on the cells compared to the 15 mM and 40 mM HP treatment levels. However, at 1645-1632 cm\(^{-1}\) and 1170-1150 cm\(^{-1}\), the PCA data showed three clusters, 1) untreated and 2 mM HP spectra 2) 5 mM HP and 3) 15 and 40 mM HP. The range from 1645-1632 cm\(^{-1}\) is located in the greater amide I band and contains peaks specific to secondary protein structure. The range from 1170-1150 cm\(^{-1}\) contains peaks specific to DNA and RNA backbone structure. From these results, we can conclude that oxidative stress is altering the cellular biochemical fingerprint, specifically protein structure and DNA mutations at concentrations of 5, 15, and 40 mM.
As the bench top method (ORAC) showed that grain sorghum polyphenol extract had anti-oxidative properties, the FT-mIR metabolomic method was applied as a means of detecting the protective effect of natural antioxidants on the biochemical fingerprint of Caco-2 cells. Hierarchical cluster analysis of second derivative spectra (1750-700 cm\(^{-1}\)) of cells pretreated for 8 h with a SE concentration curve, followed by 16 h treatment with 15 mM HP resulted in two major clades, i.e., the control versus all other treatments with the 5 and 10 mg/mL SE + HP clustering together relative to the 1, 40 mg/mL SE + HP and 15 mM HP spectra. The three dimensional PCA plot of the individual spectra partially support the hierarchical cluster data in that the control, 1, 5, and 10 mg/mL SE + HP group separately from 40 mg/mL SE + HP and 15 mM HP. Further application of the pattern recognition tests to the phospholipid (1240-1180 cm\(^{-1}\)) region of the second derivative data indicated that all of the SE concentrations tested were able to protect against oxidative stress inflicted by 15 mM HP. Collectively, these results show that although the SE treatments were unable to completely prevent oxidative damage at the concentrations tested, but the 5 and 10 mg/mL may have partially protected the cells, e.g. the phosphate containing groups without inflicting other types of responses. Combined with second derivative spectral changes, the results also indicate that the 40 mg/mL SE may be more harmful than helpful in protecting cells from the oxidative stress of cells, particularly the amide III band.
As determined by hierarchical cluster analysis of the second derivative spectra, cells treated with 15 mg/mL SE followed 15 mM HP did not show significant differences from 15 mM HP, with both negative and positive controls clustering separately from the treatments. While reducing the HP concentration to 5 mM resulted in spectra similar to the 15 mg/mL SE + 15 mM HP study, different clustering patterns were determined. Hierarchical cluster analysis of the second derivative spectra in the 1750-700 cm⁻¹ range resulted in two major clusters: control versus all other treatments. The principle component analysis (3D) plot separated the individual spectra into three clusters that contained 1) untreated and SE + HP spectra, 2) HP spectra, and 3) SE spectra, indicating that 15 mg/mL SE may possibly be protecting against oxidative stress. From these results, we can conclude that FT-mIR is able to detect subtle and temporal biochemical shifts in a model mammalian cell line, Caco-2, as a result of treatment with hydrogen peroxide-induced oxidative stress as well as with grain sorghum polar extract.

However, future work should be completed to determine specific protective levels and exposure times of sorghum relative to different HP levels and incubation times. The FT-mIR method should then be applied to test different cultivars of sorghum as well as other natural systems and even purified nutraceuticals in response to dietary oxidants, such as lipid hydroperoxides, as a means to identify and develop optimal dietary options for protecting the GI tract against oxidative damage while safeguarding consumers from potential adverse effects.
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