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DIGESTIVE STABILITY, HUMAN INTESTINAL CELL UPTAKE, AND BIOACTIVITY OF DIETARY CHLOROPHYLL DERIVATIVES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

The Degree Doctor of Philosophy in the

Graduate School of The Ohio State University

By

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*****

The Ohio State University
2001

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Food Science and Nutrition
Increased fruit and vegetable consumption has been associated with a decreased risk of specific chronic diseases. Consequently, interest in plant food phytochemicals as potential physiologically active agents has intensified. A relationship between antimutagenic activity and chlorophyll content for a number of common vegetable extracts has been made and specific natural and commercial grade derivatives have been identified as possible bioactive agents with powerful antimutagenic and antioxidative activities. While these results have been promising, very little is known regarding human digestion, absorption, and metabolism of dietary chlorophyll derivatives. Susceptibility of these pigments to heat and acid may modulate observed chemopreventive activity of native compounds as they are modified through the gastrointestinal tract.

The present study was designed to qualitatively and quantitatively investigate digestive modifications occurring to dietary chlorophylls, and to assess the potential roles these derivatives have in human health with regard to their availability, as well as antioxidant and antimutagenic activity. Both natural and commercial grade chlorophylls were subjected to two stage \textit{in vitro} digestion followed by introduction to a Caco-2 human intestinal cell culture model. Native chlorophylls quantitatively degraded to metal
free pheophytins during the *in vitro* process whereas metallo-chlorophylls such as Zn-pheophytins and Cu-chlorins were stable during simulated digestion. Both water soluble and lipophilic chlorophyll pigments were accumulated intact by monolayers of Caco-2 human intestinal cells, suggesting that these compounds may be absorbed by humans.

Predominant dietary chlorophyll derivatives demonstrated potential antioxidant activity through their ability to scavenge long-lived free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS'). Metal-free pheophytin and pyropheophytin derivatives were found to have limited antioxidant activity compared to magnesium, zinc and copper chelates, illustrating that digestive and food processing induced alterations may affect the antioxidative potential of natural chlorophyll derivatives. However, both metal-free and metallo-chlorophyll derivatives exhibited dose-dependent inhibitory activity against benzo[a]pyrene induced mutagenesis in *Salmonella typhimurium* TA100, demonstrating that strong antimutagenic activity is most likely retained during processing and digestive modification of the natural chlorophyll structure. Information gained from these studies has provided basic information critical to the understanding of the digestion, uptake, metabolism, and potential bioactivity of chlorophylls in humans. This information will facilitate the design of future investigations evaluating the association between consumption of chlorophylls and modulation of disease outcomes.
ACKNOWLEDGMENTS

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49: 2082-2089.

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Chapter 1

Review of the literature

1.1 Chlorophyll Chemistry & Function

1.1.1 Introduction & nomenclature.

Chlorophylls are bright green natural pigments found exclusively in photosynthetic plants and select bacteria. Structural similarities between chlorophyll and heme were postulated in the later half of the 19th century when Hoppe-Seyler observed that chlorophyll maintained a similar absorption spectrum as protoheme after alkaline treatment (Marks, 1969). Willstater’s elucidation of chlorophyll and heme structures a half century later clearly illustrated these similarities (Sheer, 1991). Both pigments are based on a tetrapyrrole macrocycle linked by methene bridges known as a porphyrin (Figure 1.1). This basic structure maintains a high degree of unsaturation providing an extended conjugated double-bond system which has a high metal binding capacity (Dailey, 1990). Fisher and Orth (1937) proposed a system of nomenclature based on designation of the four pyrrole rings A, B, C and D, and the methene bridges \(\alpha, \beta, \gamma, \delta\). The carbon backbone is sequentially numbered 1 through 8 beginning with pyrrole ring A as depicted in Figure 1.1. An alternative and more recent IUPAC-IUB numbering system has also become accepted for porphyrin nomenclature (Dailey, 1990). For the purpose of this review the Fisher designation will be used.
Structurally, chlorophylls are based on the porphyrin skeleton with an added fifth isocyclic ring (Ring E) joining at position 6 and γ in the Fisher numbering system. Main deviations from the porphyrin structure include substitutions of methyl (CH₃) groups at positions 1, 5 and 8; a vinyl (CH₂=CH₂) at position 2; a propionic acid group esterified to a diterpene alcohol, phytol, and a magnesium atom centrally bound by secondary valence bonds (Marks, 1969; Gross, 1991; Sheer, 1991). Five classes of chlorophylls exist naturally in plants and photosynthetic organisms: a, b, c, d, and e with the latter being only a minor derivative. Chlorophyll a and b predominate naturally in all higher plants.
while chlorophyll $c$, $d$ and $e$ derivatives are found throughout various photosynthetic algal and diatomic species including brown, red, and yellow-green algae. Chlorophyll $a$ and $b$ differ only by the presence of a formyl group on the $C_3$ carbon of chlorophyll $b$ (Figure 1.2). From these basic structures a number of chlorophyll derivatives are formed naturally or through process induced derivatization. Additionally, four classes of bacteriochlorophylls have been isolated in photosynthetic bacteria (Hendry, 2000). Bacteriochlorophyll $a$ and $b$ predominate in purple bacteria while $c$ and $d$ predominate in green and purple sulfur bacteria (Dailey, 1990; Sheer, 1991; Hendry, 2000).

Further alteration of chlorophyll structure may occur through food processing or degradation yielding a diverse array of derivatives. Modifications of this kind may include loss of the central magnesium, cleavage of phytol tail, oxidation at the $C_{10}$ carbon present on the isocyclic ring, and loss of the carboxymethyl group at the $C_{10}$ position. These reactions will be discussed in more depth in Section 1.1.5: Chlorophyll stability and degradation.
### Pigment Table

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<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
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<td>C₂H₃</td>
<td>CH₃</td>
<td>C₂H₅</td>
<td>Phytol</td>
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<td>Chlorophyll b</td>
<td>906</td>
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<td>CHO</td>
<td>C₂H₅</td>
<td>Phytol</td>
<td>a</td>
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<tr>
<td>Chlorophyll c₁</td>
<td>610</td>
<td>C₂H₃</td>
<td>CH₃</td>
<td>C₂H₅</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>Chlorophyll c₂</td>
<td>608</td>
<td>C₂H₃</td>
<td>CH₃</td>
<td>C₂H₅</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>Chlorophyll c₃</td>
<td>656</td>
<td>C₂H₃</td>
<td>COOCH₃</td>
<td>C₂H₅</td>
<td>H</td>
<td>b</td>
</tr>
<tr>
<td>Chlorophyll d</td>
<td>894</td>
<td>CHO</td>
<td>CH₃</td>
<td>C₂H₅</td>
<td>Phytol</td>
<td>a</td>
</tr>
</tbody>
</table>

**Figure 1.2.** Structural differences between major classes of natural chlorophylls in higher plants and algae.
1.1.2 Chlorophyll Ultraviolet and Visible Absorption and Fluorescence Spectra.

The green color associated with chlorophylls is a result of their strong absorbance of light in both red and blue regions of the visible absorption spectra as shown in Figure 1.3 (Gross, 1991). Optical absorption spectra for all porphyrin derivatives can be described in terms of Gouterman's four-orbital model (Sheer, 1991). Chlorophyll's spectra is a result of a combination of one electron promotions between the two highest (e_{gx} and e_{gy} \pi^*) and two lowest molecular orbitals (a_{1u} and a_{2u} \pi). Red, or Q, bands result from differences in these promotions, while the near UV Soret bands arise from their sum (Gouterman, 1978). Typically, chlorophylls are characterized by strong absorbance in both Soret (22,000-40,000 cm\(^{-1}\)) and Q (12,500-20,000 cm\(^{-1}\)) bands while maintaining elevated molar extinction coefficients between 10^4 and 10^5 M\(^{-1}\)cm\(^{-1}\) (Nonomura et al., 1997).

Electronic state plays critically into absorption spectra allowing for minor changes in porphyrin structure to significantly alter the observed electronic absorption spectra (Nonomura et al., 1997). A summary of the UV-Vis absorption characteristics for major chlorophyll derivatives is shown in Table 1.1.
Figure 1.3. Ultraviolet and visible absorption spectra of chlorophyll $a$ and $b$. 
<table>
<thead>
<tr>
<th>Derivative</th>
<th>Solvent</th>
<th>Soret band</th>
<th>Q band</th>
<th>Other maxima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll <em>a</em></td>
<td>DE</td>
<td>410, 430</td>
<td>662 (91.0)</td>
<td>578</td>
</tr>
<tr>
<td>Chlorophyllide <em>a</em></td>
<td>DE</td>
<td>410, 430</td>
<td>662 (54.2)</td>
<td>578</td>
</tr>
<tr>
<td>Pheophytin <em>a</em></td>
<td>PE</td>
<td>408</td>
<td>667</td>
<td></td>
</tr>
<tr>
<td>Pyropheophytin <em>a</em></td>
<td>DE</td>
<td>409</td>
<td>667</td>
<td></td>
</tr>
<tr>
<td>Pheophorbide <em>a</em></td>
<td>DE</td>
<td>408</td>
<td>667 (41.6)</td>
<td></td>
</tr>
<tr>
<td>Zn-Pheophytin <em>a</em></td>
<td>AC</td>
<td>423</td>
<td>653 (90.3)</td>
<td></td>
</tr>
<tr>
<td>Zn-Pheophorbide <em>a</em></td>
<td>AC</td>
<td>423</td>
<td>653 (90.9)</td>
<td></td>
</tr>
<tr>
<td>Cu-Pheophytin <em>a</em></td>
<td>AC</td>
<td>421</td>
<td>648 (67.9)</td>
<td></td>
</tr>
<tr>
<td>Cu-Pheophorbide <em>a</em></td>
<td>AC</td>
<td>421</td>
<td>648 (69.3)</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll <em>b</em></td>
<td>DE</td>
<td>430, 453</td>
<td>642 (72.8)</td>
<td>549</td>
</tr>
<tr>
<td>Chlorophyllide <em>b</em></td>
<td>DE</td>
<td>430, 453</td>
<td>642 (32.9)</td>
<td>549</td>
</tr>
<tr>
<td>Pheophytin <em>b</em></td>
<td>PE</td>
<td>413, 434</td>
<td>655 (37.3)</td>
<td>520, 550</td>
</tr>
<tr>
<td>Pyropheophytin <em>b</em></td>
<td>DE</td>
<td>435</td>
<td>656</td>
<td></td>
</tr>
<tr>
<td>Pheophorbide <em>b</em></td>
<td>DE</td>
<td>413, 434</td>
<td>655 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Zn-Pheophytin <em>b</em></td>
<td>AC</td>
<td>446</td>
<td>634 (60.2)</td>
<td></td>
</tr>
<tr>
<td>Zn-Pheophorbide <em>b</em></td>
<td>AC</td>
<td>446</td>
<td>634 (59.0)</td>
<td></td>
</tr>
<tr>
<td>Cu-Pheophytin <em>b</em></td>
<td>AC</td>
<td>438</td>
<td>627 (67.9)</td>
<td></td>
</tr>
<tr>
<td>Cu-Pheophorbide <em>b</em></td>
<td>AC</td>
<td>438</td>
<td>627 (69.3)</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll <em>c1</em></td>
<td>PY</td>
<td>444</td>
<td>640 (35.0)</td>
<td>578</td>
</tr>
<tr>
<td>Chlorophyll <em>c2</em></td>
<td>PY</td>
<td>448</td>
<td>642 (31.8)</td>
<td>579</td>
</tr>
<tr>
<td>Chlorophyll <em>c3</em></td>
<td>DE</td>
<td>451-452</td>
<td>626</td>
<td>585-586</td>
</tr>
<tr>
<td>Chlorophyll <em>d</em></td>
<td>DE</td>
<td>447</td>
<td>688</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Absorption maxima of main chlorophyll derivatives in organic solvents. Compiled from Hendry (2000); Sheer (1991); Ward et al. (1994); and Jones et al. (1968). Molar extinction coefficients are given in parentheses (x 10^3). Solvents: DE = Diethyl Ether; AC = Acetone; PY = Pyridine.
Chlorophyll $a$ spectrum is characterized by a strong absorbance in both Soret ($\sim 432$ nm) and Q ($\sim 663$ nm) regions. Chlorophyll $b$ differs from $a$ by slight shifts in both Soret and Q bands ($\sim 453$ nm and $646$ nm respectively) resulting from the replacement of the C$_3$ methyl group with a formyl group, thereby altering the conjugated $\pi$ system (Sheer, 1991). Removal of the central magnesium atom results in a shift in both Soret and Q bands for pheophytin derivatives and the appearance of new bands as is evident for pheophytin $b$ (Table 1.1). Removal of the phytol tail, (e.g., chlorophyllides and pheophorbides), has minimal effect on overall absorbance spectra since it does not contribute significantly to the $\pi$ electron structure (Sheer, 1991).

Chlorophyll fluorescence spectra are mirror images of absorbance spectra and result from transition of energy from the lowest excited singlet state attained by direct Q band absorption and relaxation from Soret band absorption to the ground state (Sheer, 1991). Strong fluorescence of these compounds is indicative of retention of the configuration and vibrational energies upon excitation (Birks et al., 1963). Quantum yield and fluorescence lifetime of chlorophyll derivatives is dependent on both solvent and the presence of molecular oxygen ranging from $\phi = 0.30-0.35$ for chlorophyll $a$ to $\phi = 0.06-0.12$ for chlorophyll $b$, with fluorescence lifetimes between 5.5-6.5 nanoseconds (Sheer, 1991). Longer lifetimes have been noted with higher concentrations of chlorophyll resulting from self-absorbance (Birks, 1970).

Non-covalent associations may alter chlorophyll absorption and fluorescence spectra as evident with $in vivo$ association in plant protein complexes (Gross, 1991; Sheer, 1991). Absorption spectra can be further altered by structural changes, dielectric
properties of solvent, and self association at higher pigment concentration, (Schwartz & Lorenzo, 1990; Sheer, 1991). Solvent extract composition, temperature, pigment aggregation and concentration may also significantly impact chlorophyll fluorescence (Schwartz & Lorenzo, 1990).

1.1.3 Chlorophyll Biosynthesis.

An estimated 11 million tons of chlorophyll is produced annually by terrestrial and aquatic photosynthetic species (Hendry, 2000). Biosynthesis of these natural pigments has been the subject of numerous investigations through the later half of the twentieth century (Granick, 1950; Granick, 1961; Rebeiz & Castelfranco, 1973; Rebeiz, 1982). These sequential reactions as outlined by Beale & Weinstein (1990) are depicted in Figure 1.4. The initial step in biosynthesis of tetrapyrrole compounds is the formation of 5-aminolevulinic acid (ALA), which provides backbone carbon and nitrogen atoms for the porphyrin structure. ALA is formed either through the Shemin pathway by the action of ALA synthetase catalyzing the condensation of succinyl CoA with glycine, or from the backbone of glutamate through the C-5 pathway (Gross, 1991). Formation of the basic pyrrole is accomplished by asymmetrical condensation of two ALA molecules forming prophobilinogen (PBG). Polymerization and subsequent cyclization of PBG gives rise to the intermediate uroporphyrinogen III. Three stages of side-chain modifications form the central intermediate protoporphyrin IX. Although uroporphyrinogen III is considered the universal precursor for all porphyrins, protoporphyrin IX is thought to be the key branch point between plants (chlorophylls) and animals (heme) (Dailey, 1990).
Figure 1.4. Biosynthetic pathway of chlorophyll \(a\). Key enzymes: (1) ALA dehydratase, (2) PGB deaminase, (3) Uroporphyrinogen III synthase, (4) Uroporphyrinogen decarboxylase, (5) Coproporphyrinogen oxidase, (6) Protoporphyrinogen oxidase, (7) Mg chelatase, (8) Mg-protoporphyrin methyl transferase, (9) MPE oxidative cyclase, (10) NADPH protochlorophyllide oxidoreductase, (11) Chlorophyllase.
Insertion of the central magnesium atom by Mg-chetalase follows in a highly specific process which excludes other metals, such as iron and zinc that may easily be inserted into the tetrapyrrole backbone non-enzymatically (Rüdiger, 1997). Formation of the isocyclic ring E, and final esterification with the characteristic hydrophobic phytol tail result in the formation of chlorophyll \( a \) (Dailey, 1990).

Biosynthesis of chlorophyll \( b \) has been thought to occur directly from chlorophyll \( a \). It has been suggested that transformation of the \( C_3 \) methyl group on ring B to a formyl group is driven by molecular oxygen (Rüdiger, 1997), and that the intermediate in this process is a hydroxymethyl chlorophyll (Dailey, 1990). Conversion is thought to occur after esterification of chlorophyllide \( a \), although there is evidence of transformation prior to esterification since chlorophyllide \( b \) has been identified in leave chloroplasts (Gross, 1991; Rüdiger, 1997). This area of chlorophyll research is continuously being reevaluated as new analytical methodologies highlight the discovery of new chlorophyll derivatives (Spooner et al., 1995; Küpper et al., 1998; Hyvärinen et al., 1998; Takaichi et al., 1999).

1.1.4 Photosynthesis.

Chlorophylls have often been referred to as the pigments of life. This concept arises from their primary function as the main photosynthetic pigment for all green plants, photosynthetic algae, and bacteria (Sheer, 1991). Chlorophylls capture light energy and aid in its conversion into chemical energy through a two-stage oxygen evolving process (Stryer, 1995). Photosynthetic reactions occur primarily in cellular
plastids known as chloroplast. These organelles are typically oblate structures approximately 7µm long and 3µm thick having both outer and inner membranes (Gross, 1991; Stryer, 1995). The interior of the chloroplast contains the stroma matrix including the membrane structure thylakoids. Thylakoids stack together to form grana which are connected together by stroma lamella. The photosynthetic apparatus is embedded into the thylakoid membrane in two distinct, but intimately linked, photosystems (Blankenship & Hartman, 1998; Gross, 1991).

Photosynthesis is initiated at photosystem II (PSII), a multisubunit membrane protein complex including chlorophyll. Absorption of light energy results in the splitting of water and release of oxygen (Alberts et al., 1994; Barber & Kühlbrandt, 1999). This process generates excited electrons which are passed through the PSII electron transport chain in a mode analogous to the mitochondrial process (Stryer, 1995). The end result is the generation of a proton gradient across thylakoid membranes utilized by ATP synthase in ATP synthesis (Alberts et al., 1994; Stryer, 1995). Photosystem I (PSI) completes the light energy transfer and further illumination provides a second excitation which is passed through iron-sulfur clusters, resulting in reduction of NADP⁺ to NADPH (Alberts et al., 1994; Stryer, 1995).

The exact structure of PSII and PSI are unknown and currently the subject of intense research (Barber & Kühlbrandt, 1999; Race & Hind, 1997; Boekema et al., 1998; Bialek-Bylka, 1995; Allen & Williams, 1998; Jianmin et al., 1995). In general, PSII and PSI differ in the nature of their electron acceptor, with PSII being a pheophytin-plastoquinone and PSI utilizing iron-sulfur clusters (Allen & Williams, 1998). Both photosystems possess antenna chlorophyll–protein complexes responsible for harvesting
light energy and transferring excited electrons to reaction center complexes (Alberts et al., 1994). A chlorophyll \(a/b\)-protein complex has been determined to be a major component of the antenna complex (Gross, 1991). Collected energy is rapidly transferred to adjacent chlorophyll complexes until it reaches the photochemical reaction center containing a unique pair of chlorophylls (Allen & Williams, 1998).

The PSII reaction center is a multi-subunit complex containing two main protein centers with bound cofactors. A chlorophyll \(a\) complex absorbing light at 680 nm (P680) in this center is excited to a singlet state which rapidly passes its electron to an adjacent pheophytin molecule forming a radical pair. The nature of this transfer mechanism is hotly debated with the predominant view favoring a sequential mode between the chlorophyll pair and pheophytin (Zusman & Beratan, 1998). Electron transfer continues from this pair to bound plastoquinones. The final stage involves reduction of plastoquinones by a \(\text{Mn}_4\text{O}\) cluster creating the proton gradient utilized in ATP generation (Barber & Kühlbrandt, 1999).

1.1.5 Chlorophyll Stability and Degradation.

The orderly and programmed natural biochemical process of chlorophyll decomposition known as senescence catabolizes nearly one billion pounds of photosynthetic pigment annually (Heaton & Marangoni, 1996). This phenomena is most evident in the fall as leaves loose their bright green colors and has been extensively researched, although comprehension of the biochemical pathways remains incomplete (Kufner, 1980; Heaton & Marangoni, 1996). Excluding their fate in senescing tissue,
chlorophylls associated with the plant structure are quite stable considering the harsh photodegradative environment (Hendry, 2000). The same stability does not characterize isolated chlorophylls or chlorophylls in foods subjected to processing and preparation including thermal treatment and acidification (Schwartz & Lorenzo, 1990).

Degradation of chlorophylls during food processing of green fruits and vegetables has been thoroughly studied and is the subject of a number of reviews (Simpson, 1985; Schwartz & Lorenzo, 1990; Heaton & Marangoni, 1996). A brief summary of main degradative reactions encountered during food processing is depicted in Figure 1.5.

Chlorophylls are extremely sensitive to physical and chemical changes encountered during food processing. These changes contribute to the discoloration of green vegetable tissue to olive brown during thermal processing and/or acidification. This color loss is primarily the result of replacing the centrally chelated magnesium atom by two atoms of hydrogen producing metal free pheophytin derivatives (Mackinney & Weast, 1940; McFeeters & Schandral, 1968; Schwartz & Lorenzo, 1990). More severe heat treatments, e.g. canning operations, may cause a loss of C10 decarboxymethoxyl moiety forming derivatives known as pyrochlorophyllins (Schwartz et al., 1981; Schwartz & von Elbe, 1983). Pyrochlorophyll derivatives that have been decarboxymethoxylated but having retained the central magnesium atom have also been isolated from both steamed and microwave processed spinach leaves (Teng & Chen, 1999).
Figure 1.5. Major chlorophyll degradation and derivatization reactions occurring during food processing operations.
Milder processing conditions can also derivatize native chlorophyll. Formation of C\textsubscript{10} epimers of the native chlorophyll molecule occurs during freezing, drying and mild heating such as blanching (Katz et al., 1968; Schwartz & Lorenzo, 1990; Sheer, 1991). While other conversions affect final product color, epimer formation has no detrimental effect since their spectral properties are identical to those of their parent chlorophyll molecule (Sheer, 1991). Enzymatic removal of the esterified phytol by chlorophyllase results in the formation of water soluble chlorophyllide derivatives (Schwartz & Lorenzo, 1990). These derivatives are often generated when mild thermal treatment such as blanching are utilized which activates chlorophyllase (Canjura et al., 1991; von Elbe & Schwartz, 1996). Further thermal processing or acidification results in formation of metal-free water soluble derivatives known as pheophorbides and pyropheophorbides (Schwartz & Lorenzo, 1990; Sheer, 1991).

Thermal degradation kinetics of chlorophyll have been the subject of numerous studies. Conversion of chlorophylls to pheophytins follows a first order kinetic model with chlorophyll \textit{a} degrading between two and seven times faster than chlorophyll \textit{b} (Mackinney & Joslyn, 1941; LaJollo et al., 1971; Canjura et al., 1991). Experimental activation energies range between 7.5 to 16.0 kcal/mol (Schwartz & Lorenzo, 1990), and were found to increase proportionally with temperature (Weemaes et al., 1999). Further conversion of pheophytins to pyropheophytins was determined to be pseudo-first order with degradation of pheophytin \textit{b} being between 25 and 40\% faster than pheophytin \textit{a}. Activation energies for pheophytin \textit{a} and \textit{b} degradation were calculated to 20.7 and 15.7 kcal/mol respectively (Schwartz & von Elbe, 1983).
The importance of color to final perceived quality of food products combined with the labile nature of chlorophyll derivatives have provided the impetus to preserve native green vegetable appearance. Numerous advances have been made in both the processing and formulation of thermally processed green vegetables in an effort to control chlorophyll deterioration. Use of continuous flow and high-temperature-short-time (HTST) processing minimizes heating requirements and result in improved pigment retention (Schwartz & Lorenzo, 1989; Schwartz & Lorenzo, 1990; von Elbe & Schwartz, 1996). Addition of alkalizing agents such as calcium hydroxide has been utilized in an attempt to neutralize organic acids, thereby stabilizing the magnesium chlorophyll complex (Blair, 1940). However, both HTST and acid neutralization have met with limited long term success prompting the need for more effective methods of color stabilization (von Elbe & Schwartz, 1996).

Fishenbach (1943) and Schanderl et al. (1965) first noticed formation, increased thermal stability, and the color similarities of zinc and copper pheophytins to natural chlorophylls. Since then many efforts have been directed toward understanding the formation of these zinc and copper chlorophyll complexes for the purpose of color stabilization in processed foods. Zinc and copper pheophytin \( a \) and pyropheophytin \( a \) derivatives are rapidly formed by the addition of zinc and copper salts to commercially canned vegetables prior to thermal treatment in a process known as re-greening (Sheer, 1991). Jones et al. (1977) studied the formation of copper and zinc derivatives and found that complexation is favored at salt concentrations between 1-10 fold the pigments molar equivalents. The Crown Cork & Seal Company Inc. has commercialized this canning technology for use with green beans under the trade name VERI-GREEN\(^\text{®} \) (Segner et al.,
1984). Currently this process is widely utilized in color preservation of canned green beans. von Elbe et al. (1986) investigated the composition of VERI-GREEN® beans and determined that they consisted primarily of zinc-pheophytin $a$ and zinc-pyropheophytin $a$. Subsequently it was shown that formation of zinc complexes was dependent on chlorophyll species, pH, temperature and ion concentration (LaBorde & von Elbe, 1990; Tonucci & von Elbe, 1992). Efficient formation of zinc-pheophytins and zinc-pyropheophytins in peas subjected to a continuous flow aseptic processing system raises the possibility that color preservation by metallo-chlorophyll formation may be extended beyond simple canning to more advanced processing techniques (Canjura et al., 1999).
1.2 Chlorophyll Analysis.

1.2.1 Extraction Techniques

Extraction of chlorophyll pigments from plant tissue requires great care due to the sensitivity of these pigments to oxygen, heat and light. All procedures and sample handling should be carried out in clean glassware, under subdued light, and with fresh solvents (Schwartz, 1998). Fresh extracts of chlorophyll are extremely sensitive to degradative reactions, and should therefore ideally be analyzed immediately. For storage, extracts may be dried, and placed at sub zero temperatures under a nitrogen or argon atmosphere and protected from light (Gross, 1991; Schwartz, 1998).

The lipophilic nature of these pigments makes them ideally suited for complete quantitative extraction by organic solvents such as acetone and petroleum ether. Generally, extraction of chlorophyll from plant tissue is accomplished by homogenization of fresh tissue with acetone or ether. Calcium carbonate is usually added to buffer the matrix, minimizing artifactual pheophytinization caused by release of organic acids (Schwartz, 1998). The homogenate is subsequently filtered to obtain the chlorophyll rich acetone fraction (AOAC, 1995). Using a separatory funnel, chlorophyll pigments can be transferred into other organic solvents, e.g. ether, in preparation for spectrophotometric or chromatographic analysis/isolation.

1.2.2 Spectrophotometric Methods

Early assessment of chlorophyll content in plant tissue was based on their strong electronic absorption spectra described previously (Section 1.1.2: Chlorophyll Ultraviolet and visible absorption and fluorescence spectra). Arnon (1949) developed
an early method based on measurement of 80% acetone plant extracts for absorption
contribution of chlorophyll $a$ and $b$. Absorbance of the extract was measured at different
wavelengths and simultaneous equations were constructed based on extinction
coefficients for each derivative's unique electronic absorption maxima.

During the 50 years numerous methods have been developed utilizing this
approach. White et al. (1965) utilized equations for simultaneous determination of
chlorophyll, pheophytin and pheophorbide derivatives in plant tissues. Jones et al. (1977)
described methods for the spectrophotometric estimation of zinc pheophytins in complex
mixtures with both chlorophylls and pheophytins. AOAC recognizes a
spectrophotometric assay for the determination of chlorophyll $a$ and $b$ components in
plant extracts (AOAC, 1995). Pigments are extracted into acetone and subsequently
partitioned into ether. Absorbance readings are then taken at 660.0 and 642.5 nm and
chlorophyll concentrations are calculated by the following equation:

\begin{align*}
(1) \quad \text{TOTAL Chlorophyll} & = 7.12 A_{660.0} + 16.8 A_{642.5} \\
(2) \quad \text{Chlorophyll } a & = 9.93 A_{660.0} - 0.777 A_{642.5} \\
(3) \quad \text{Chlorophyll } b & = 17.6 A_{642.5} - 2.81 A_{660.0}
\end{align*}

Limitations of these more traditional assays are encountered when multiple
derivatives are present, chlorophyll concentration is low, and the chlorophyll $a/b$ ratio
exceeds 5-8 (Lichtenthaler, 1987; Porra, 1989; Sheer, 1991). Ogawa & Shibata (1965)
developed a method for the sensitive and accurate determination of chlorophyll $b$
specifically for extracts with a high $a/b$ ratio. This method is based on the conversion of
minor components of chlorophyll b to an oxime derivative with a strong absorbance in the red region. The procedure of Laval-Martin (1985) can be used to determine chlorophyll a and b plus total pheophytins by first reading at 663 nm and then following the absorbance at 642 nm after acidification of the 80% acetone extract, thereby taking advantage of the rapid pheophytinization of chlorophyll a relative to b.

1.2.3 Open Column and Thin Layer Chromatography.

While traditional photometric methods for the analysis of chlorophyll are rapid and reliable, difficulties arise when complex pigment mixtures are encountered. Chromatographic techniques are well-suited for determination of mixtures of photosynthetic pigments. Open column methods based on gravity or vacuum assisted flow have enjoyed wide application and success due to their inexpensive nature and excellent resolving power that allows both qualitative and quantitative separation. Tswett (1906a; 1906b) was the first to use column chromatography of both calcium carbonate and sugar solid phases to provide separation of carotenoids and chlorophyll a and b pigments from chloroplast. Since then, numerous adsorbents have been used for the separation of chlorophyll derivatives, including calcium carbonate (Tswett 1906b), talcum (Zscheile, 1934), and activated magnesium (Van Norman, 1957). However, sucrose in the form of powdered sugar (3% starch) has enjoyed the widest application.
Many of these methods have been thoroughly reviewed (Strain & Svec, 1969). Sweeney & Martin (1958) achieved separation of chlorophylls $a$ and $b$ by elution of plant extracts with benzene, petroleum ether and acetone on a powdered sugar column. More recently, these methods have found great use as an efficient preparative step in the separation of chlorophyll $a$ and $b$ from their diastereomers (Schaber, 1985).

Thin layer chromatography (TLC) is a logical extension of open column, often utilizing similar adsorbents such as sucrose. TLC is based on utilization of glass plates coated with very thin layers of adsorbent material, which is developed by organic solvents. Colman & Vishniac (1964) described separation of chlorophylls $a$ and $b$ by thin layers of sucrose. Methods were subsequently developed for separation of multiple chlorophyll derivatives including pheophytins and chlorophyllides (Bacon, 1965; Schaltegger, 1965). While TLC and open column methods remain useful, the development of high performance methods has offset their prominence in modern chlorophyll separation and analysis.

1.2.4 High Performance Liquid Chromatography (HPLC).

High performance liquid chromatography (HPLC) has been the predominant separation technique during the past 20 years. The fast, accurate and reproducible nature of these methods make them ideal for both research and quality assurance needs. Both normal and reverse phase methods have been developed for analysis of chlorophylls and their derivatives (Schwartz & Lorenzo, 1990).
Over the last few decades, numerous procedures employing an octadecyl-bonded stationary phase (C_{18}) have appeared in the literature. These reverse phase methods have predominated mainly because of their ease of use, aqueous mobile phases, and wide commercial availability of the stationary phase, making published methodology extremely transferable and reproducible. Eskins et al. (1977) developed an excellent method for chlorophyll \(a\) and \(b\) analysis in fruit. Mantoura & Llewellyn (1983) analyzed fruits and vegetables for chlorophyll and carotenoids and their degradation products. However, these methods are often found to be complex, labor intensive, and time consuming with long run times. In a classic method, Schwartz et al. (1981) developed a C_{18} reverse phase separation for 12 chlorophyll derivatives including chlorophylls \(a\) and \(b\) and their corresponding pheophytins and pyropheophytins within 25 minutes. Gradient elution based on methanol:water and ethyl acetate also allowed the effective separation of chlorophyll C_{10} epimers. Shioi et al. (1983) further applied C_{18} HPLC for the separation of novel chlorophyll derivatives such as protochlorophyll \(a\) and bacteriochlorophyll \(a\) esters.

The broad range of polarity offered by chlorophylls is a true analytical challenge. When chromatographed on a reversed phase column, non-esterified chlorophyll derivatives such as chlorophyllides disappear during successive analysis (Shioi et al., 1984). Inclusion of buffer salts such as ammonium acetate in the mobile phase is often useful as it provides a proton equilibrium suitable for ionogenic chlorophyllides and pheophorbides (Almela et al., 2000). Mínquez-Mosquera & Gandul-Rojas (1995) utilized a reverse-phase C_{18} method with ion pairing to investigate the polar chlorophyll
derivatives generated by alkaline treatment. Canjura & Schwartz (1991) successfully applied both normal and reverse phase techniques to the broad separation of both polar chlorophyllide and pheophorbide, as well as lipophilic chlorophyll and pheophytin derivatives.

Great effort has been given to simultaneous separation of chlorophylls and carotenoids from a variety of fruit and vegetable tissues. Minguez-Mosquera et al. (1992) described a method to resolve these pigments from virgin olive oil quantifying 17 species including 7 chlorophyll derivatives. Gandul-Rojas & Minguez-Mosquera (1996) further utilized these methods to characterize carotene and chlorophyll pigments in Spanish olive varieties. Application of a reversed-phase method based on methanol, acetonitrile and hexane for analysis of bean extracts successfully chromatographed xanthophyll, chlorophyll and carotene pigments (Lopez-Hernandez et al., 1993). Khachik et al. (1986) successfully separated and identified 20 separate chlorophyll and carotenoid species in green vegetables such as broccoli, spinach and kale utilizing both isocratic and gradient elution parameters on a Microsorb C18 column. Cano (1991) modified the method of Schwartz et al. (1981) for the separation of chlorophyll and carotenoid pigments from kiwi fruit cultivars. Effective resolution of 31 chlorophyll and carotenoid species was achieved from seaweed using a reverse-phase C18 column with a mild gradient based on methanol, acetone, and ammonium acetate (Hegazi et al., 1998).

Metallo-chlorophyll derivatives such as zinc, copper, and iron derivatives present different analytical challenges as their prevalence in the food supply increases. Schwartz (1984) developed reverse phase C18 methods for separation of copper and zinc pheophytins. Minguez-Mosquera et al. (1996) applied both thin layer and reverse-phase
HPLC for the determination of copper complexes of oxidized chlorophyll derivatives.
Inoue et al. (1994) demonstrated the usefulness of an isocratic C\textsubscript{18} method with methanol and acetic acid elution for separation of major components of commercial grade sodium copper chlorophyllin. Nonomura et al. (1996) further developed C\textsubscript{18} reverse phase methods based on acetonitrile-phosphate buffer (pH 2) for resolution of iron(III)chlorophyllin components including Fe(III)pheophorbide \textit{a}, Fe(III)chlorin \textit{e\textsubscript{6}} and Fe(III)chlorin \textit{e\textsubscript{4}}.

1.2.5 Methods of Detection.
Post column detection of chlorophyll derivatives is often accomplished by ultraviolet and visible spectroscopic techniques that take advantage of the strong absorption spectra of these pigments. While these methods have enjoyed wide application (Schwartz et al., 1981; Khachik et al., 1986; Mínguez-Mosquera et al., 1992), a major advance was made with the introduction of photodiode array detection (PDA). Multi-channel photodiode array detection allows for simultaneous monitoring of multiple wavelengths resulting in the generation of on-line electronic absorption spectra of a compound as it elutes from the HPLC column (Bramely, 1992). Because of the unique absorption spectra of individual chlorophyll derivatives, these techniques have enjoyed wide application for tentative identification of components from complex mixtures and extracts (Canjura & Schwartz, 1991; Mínguez-Mosquera & Gandul-Rojas, 1995; Hegazi et al., 1998; Almela et al., 2000).
When a higher degree of sensitivity and selectivity is required, fluorescence and mass selective detectors may be applied. Fluorescence has been widely utilized because of its ease of use and picomole limits of detection (Shioi et al., 1983; Shioi et al., 1984; Bidigare et al., 1985; Almela et al., 2000). Mass spectrometry has also proven to be both a sensitive and efficient way to identify numerous chlorophyll derivatives. van Breemen et al. (1991) utilized both fast atom bombardment (FAB) and tandem mass spectrometry (MS/MS) for the structural characterization and mass determination of numerous derivatives including chlorophylls, chlorophyllides, pheophytins, pheophorbides, pyropheophytins, and Zn-pheophytins. Hyvärinen & Hynninen (1999) further utilized FAB-MS for identification of chlorophyll b allomers. HPLC with tandem MS/MS was recently applied for identification of Cu(II)-chlorin ethyl ester in human sera (Egner et al., 2000).
1.3 Chlorophyll Biological Activity.

1.3.1 Medical Utilization of Chlorophyll.

Use of natural chlorophyll for "general health and healing" is believed to have started over 150 years ago when Verdeil hypothesized the close similarities of the green plant pigment to another important porphyrin, heme (Rystan, 1947). While numerous chlorophyll derivatives exist, the water soluble commercial grade form known as sodium copper chlorophyllin (Figure 1.6) has been the most investigated exhibiting a wide range of beneficial biological activities (Kephart, 1955).

Figure 1.6. Two main chlorophyllin derivatives identified in commercial grade sodium copper chlorophyllin.
Sodium copper chlorophyllin is synthesized from a crude natural chlorophyll extract by treatment with methanolic sodium hydroxide followed by replacement of the central magnesium atom with a heavy metal such as copper (Kephart, 1955). The final mixture is composed of numerous chlorin type compounds derived from natural chlorophyll, including two main components referred to as Cu(II)chlorin e₄ and Cu(II)chlorin e₆ (Chernomorsky, 1994; Dashwood, 1997; Chernomorsky et al., 1997). Cell regeneration, wound healing, and anti-inflammatory properties in response to sodium copper chlorophyllin treatment have been observed in both model systems and human studies (Larato & Pfau, 1970; Edwards, 1954; Bowers, 1947). This crude commercial mixture was found to have inhibitory activity against antibody mediated allergic reactions in mice (Nagai et al., 1983). Intravenously administered chlorophyllin inhibited the deposition and growth of calcium oxalate crystals in rats, demonstrating a role in the prevention of calcium oxalate urolithiasis (Tawashi et al., 1980). Another property of copper chlorophyllin is that of internal deodorizer. When given orally to geriatric patients, chlorophyllin significantly reduced foul odor and flatulence (Young & Beregi, 1980). Sodium copper chlorophyllin has been recognized as safe by the U.S. Food and Drug Administration and is approved for use as an internal deodorizer and coloring agent at levels not to exceed 0.1% of the total product formulation.

1.3.2 Use of Chlorophylls in Photodynamic Chemotherapy.

Photodynamic therapy (PDT) is a medical technique utilizing a combination of light and drug resulting in a cytotoxic or modifying effect on cancerous tissue (Sternberg et al., 1998). Typically, a photosensitizer is either ingested or injected into the body, for
accumulation in rapidly dividing cells such as those associated with cancerous tissues. Following accumulation, the photosensitizer is elevated into an excited triplet state by absorption of UV light. Energy transfers between the excited triplet sensitizer and molecular oxygen ($^3$O$_2$) results in the formation of reactive oxygen species including singlet oxygen ($^1$O$_2$) which acts as the principle cytotoxic agent (Henderson et al., 1992).

Candidates for PDT agents must conform to a specific set of standards as described by Sternberg et al. (1998). The drug must have minimal toxicity in the dark. When administered, it must accumulate rapidly in cancerous tissue for subsequent light activation. PDT agents must be capable of undergoing efficient intersystem crossing (ISC) upon light excitation. This forbidden or lower probability transition in electronic state allows for the transformation of the PDT agent from the exited singlet to a radical triplet state. Once in the excited triplet state the PDT agent reaches its maximum effectiveness by virtue of cytotoxic $^1$O$_2$ generation via triplet-triplet annihilation with $^3$O$_2$. Ideally, this process of PDT drug activation would occur with red or near-infrared light, as longer wavelengths have deeper penetration capacity for more effective treatment of deep non-operable tumors. Finally, rapid clearance from the body is crucial to limit secondary damage to exposed skin.

Chlorophyll derivatives have long been reported to be efficient sensitizers of $^1$O$_2$ (Endo et al., 1984; Lee & Min, 1988; Telfer et al., 1994). This property has often led to significant quality defects in vegetable oils as residual chlorophyll and pheophytin act as prooxidants when these food systems are exposed to light (Terao & Matsushita, 1977; Endo et al., 1984). Quenching of these reactive oxygen species for improvement of vegetable oil flavor quality, has been the focus of numerous studies (Lee and Min, 1988;
Lee and Min, 1990; Jung & Min, 1991). However, it is their effectiveness as photosensitizers that have made chlorophyll derivatives attractive candidate drugs for PDT. A number of natural and commercial chlorophyll derivatives have been investigated for their potential use in PDT including pyropheophorbide (Henderson et al., 1997), bacteriochlorin a (van Iperen et al., 1995), crude chlorophyllins (Nourse et al., 1988), isochlorin e₄ (Stenberg et al., 1998), chlorin p₆ (Kessel et al., 1995), and methylpheophorbide a (Mayhew et al., 1993). Photofrin® is a porphyrin-based PDT drug which has recently gained global approval as the first widely utilized PDT drug. As identification and synthetic modification of chlorophyll derivative progress, their applicability to the growing PDT field is likely to increase.

1.3.3 Chlorophyll Antimutagenic, Antigenotoxic and Chemopreventative Activity.

During the past 25 years, the nutrition-health paradigm of foods being the source of essential nutrients to sustain life has evolved into one in which foods are also recognized to provide physiological benefits for the prevention or management of different disease states. This shift is due in part to the body of epidemiological evidence associating diets rich in fruits and vegetables with the reduced risk of developing certain types of diseases including cancer (Wattenberg, 1990; Willett, 1994; Michaud et al., 1999; Slattery et al., 2000). Experimental evidence corroborating epidemiological studies has also been accumulating (Tang & Edenharder, 1997; Yoshikawa et al., 1996;
Ito et al., 1998). Chlorophyll and its various derivatives are believed to be among a number of compounds in fruits and vegetables that are potentially responsible for such associations (Waters et al., 1996; Hayatsu et al., 1988; Chernomorsky & Segelman, 1988; Dashwood et al., 1998; Laï et al., 1980; Tang & Edendharder, 1997).

Chemopreventive effects of chlorophyll derivatives have been extensively studied with particular emphasis on their antimutagenic activity against numerous dietary and environmental mutagens. A number of these studies have utilized bacterial mutagenicity assays focusing on commercially derived sodium copper chlorophyllin as reviewed by Dashwood et al. (1998), Sarkar et al. (1994), Hayatsu et al. (1988) and Hayatsu et al. (1993). Waters et al. (1996) reviewed nearly 100 in vitro antimutagenic studies involving sodium copper chlorophyllin and a wide variety of both dietary and environmental mutagens. A comparative summary of the antimutagenic activities of chlorophyllin, retinol and N-acetylcysteine highlights the potential protective role of copper chlorophyllin (Figure 1.7). It was concluded from this review that sodium copper chlorophyllin was as effective as other known antimutagens.
Figure 1.7. Summary of in vitro antimutagenic protective effects of chlorophyllin, retinol and N-acetylcysteine against typical dietary and environmental mutagens. AFB1 — aflatoxin B1; B[a]P = benzo[a]pyrene; Has = Heterocyclic amines. I — Inhibitory; E = Enhancement; NE = No Effect. Results marked with * indicate those confirmed in vivo. Adapted from (Waters et al., 1996).

The studies of Trewel & van der Hoeven (1985) illustrated the antimutagenic activity of copper chlorophyllin against benzo[a]pyrene and cigarette smoke condensate, thereby demonstrating effectiveness against complex mixtures of mutagens. Ong et al. (1986) demonstrated the ability of sodium copper chlorophyllin to produce a 90% to 100% reduction in the mutagenicity of extracts from a variety of complex dietary and environmental mixtures including fried beef, cigarette smoke, tobacco snuff, coal dust and emission residue. A comparative study found copper chlorophyllin to be a more effective antimutagen against environmental and dietary mutagens in a Salmonella typhimurium TA98 assay than retinol, β-carotene, vitamin C and E (Ong et al., 1989). Further work demonstrated the antimutagenic activity against both direct and indirect acting mutagens including heterocyclic amines 3-amino-1-methyl-5H-pyrido-[4,3-
b]indole and 2-amino-3-methylimidazo[4,5-f]quinoline, (Dashwood et al., 1991), chromium (VI) oxide (Olvera et al., 1993), aflatoxin B₁ (Dashwood et al, 1991), benzo[a]pyrene (Romert et al., 1992; Arimoto et al., 1995), benzo[a]pyrene-7,8-dihydrodiol-9-10-epoxide (Surh, 1998), 3-methylchboroanthracene (Chernomorsky et al., 1997), and 7,12-dimethylbenzo[a]anthracene (Chung et al., 1999).

Natural chlorophyll derivatives have also been investigated with regard to antimutagenic/antigenotoxic activity. Lai et al. (1980) found a positive correlation between the antimutagenic activity of common vegetable extracts and their chlorophyll content. In the same study, solutions of sodium copper chlorophyllin demonstrated similar inhibitory activity to that of the natural extracts. Pheophorbide a was identified as an anti-tumor agent from leaves of the water herb Neptunia oleracea (Nakamura et al., 1996a). Inhibitory effects of pheophorbide a on skin tumor promotion were further demonstrated in ICR mice (Nakamura et al., 1996b). Natural chlorophyll isolated from Chlorella was found to inhibit the mutagenicity of N-hydroxyl Trp-P-2 (Negishi et al., 1989). Using a Drosophila wing spot assay, Negishi et al. (1997) found natural spinach chlorophylls had antigenotoxic activity. Okai et al. (1996) demonstrated inhibition of β-galactosidase activity indicative of mutagen induced umu C gene expression in Salmonella typhimurium TA 1535/pSK 1002 cells by chlorophyll a isolated from red algae (Porphyra tenera). Both chlorophyll a and b exhibited dose-dependent protection against 6-sulfooxymethylbenzo[a]pyrene, an ultimate metabolite of benzo[a]pyrene, in V79 cells (Chung et al., 1997).
Metal-free chlorophyll derivatives such as pheophytins and pheophorbides have also exhibited significant suppressive activity in *S. typhumirium* and *E. coli* tester bacteria (Yoshikawa, et al. 1996; Okai & Higashi-Okai, 1997). Pheophorbide α inhibited tumor promotor induced activation of Epstein-Barr virus (Nakamura et al., 1996b). Chemomorsky et al. (1999) further illustrated antimitogenic activity of metal free chlorophyll derivatives pheophytin, pyropheophytin, and pheophorbide toward both direct and indirect acting mutagens. Myeloma cell multiplicity was effectively inhibited by these metal-free chlorophyll derivatives with the more water-soluble pheophorbide possessing the highest activity.

The demonstrated antimitogenic activity has led to investigation of potential anticarcinogenic activity of dietary chlorophyll derivatives *in vivo*. Dietary sodium copper chlorophyllin blocks aflatoxin B₁-induced hepatocarcinogenesis in rainbow trout by retarding DNA-binding by the fungal product (Breinholt et al., 1995a; Dashwood et al., 1991). Likewise, sodium copper chlorophyllin reduced benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide induced skin carcinogenesis in ICR mice (Park & Surh, 1996). Chung et al. (1999) found that orally administered copper chlorophyllin inhibited both tumor promotion and progression of papillomagenesis in 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoyl-phorbol-13-acetate induced mouse skin carcinogenesis. Dietary chlorophyllin significantly reduced the average number of liver neoplasms and proliferation but not overall liver cell cancer incidents in a diethylnitroamine-phenobarbital hepatocarcinogenesis model (Sugie et al., 1996).
However, chlorophyllin acted as a promoter of colon carcinogenesis in rats treated with dimethylhydrazine (Nelson, 1992). These results may have been due to potential impurities in the commercial grade SCC (Chernomorsky et al. 1997).

*In vivo* studies with natural chlorophylls have been limited by the cost involved with isolation and purification of specific derivatives and chemical instability of purified derivatives (Dashwood et al., 1998a). However, pilot studies have addressed a number of questions regarding *in vivo* chemopreventive effects. Harttig & Bailey (1998) reported that natural chlorophylls from spinach inhibited dibenzo[a,l]pyrene-DNA adduct formation in rainbow trout liver. Furthermore, chlorophyll a protected against DNA adduct formation when trout embryos were co-injected with both chlorophyll derivative and tritium labeled aflatoxin B₁ (Dashwood et al., 1998a). However, water-soluble copper chlorophyllin derivatives provided better protection against adduct formation suggesting that better distribution of water-soluble derivatives improves efficacy. Higashi-Okai et al. (1998) further demonstrated that magnesium-free chlorophyll derivatives pheophytin a and b, significantly suppressed tumor promotion and progression of skin tumorgenesis in a BALB/c mouse skin model.

These data from the described *in vitro* and animal studies have prompted an increased interest in potential usefulness of chlorophyll as an anticarcinogen. Currently, two investigations examining the benefits of commercial grade chlorophyllin are being supported by the NIH-NCI. These studies are designed to investigate the modulation of multi-organ carcinogenesis in rats and rainbow trout as well as identification of intermediate biomarkers to evaluate the chemopreventive of chlorophyll (Bailey, 1998; Dashwood, 1998b). Positive outcomes from these studies and the minimal toxicity
associated with chlorophyll, has prompted the NIH-NIEHS to support the first human clinical intervention with chlorophyllin. Qidong, People’s Republic of China, was selected to investigate the potential protective effects of dietary copper chlorophyllin for a population at risk for development of liver cancer from chronically high dietary exposure to aflatoxin (Kensler, 1998). Ninety participants consumed three supplements containing 100 mg chlorophyllin daily during a four month trial. Periodic serum and urine samples were collected for measurement of aflatoxin-albumin adduct formation and chlorophyllin content. Initial results from these studies has already provided valuable information regarding the presence copper chlorophyllin derivatives in human sera (Egner et al., 1999; 2000). Since large amounts of natural chlorophyll are consumed daily, it is imperative that similar investigations extend to these abundant dietary components.

1.3.4 Potential Mechanisms of Chlorophyll Bioactivity.

Elucidation of the underlying mechanism by which chlorophyll derivatives exhort their protective effects has been the focus of numerous studies. Two primary hypotheses regarding the potential mode of action have garnered much attention. These are the molecular complexation of planar mutagens and the trapping of potential genotoxic agents such as reactive oxygen species (Sarkar et al., 1994; Odin, 1997).
Molecular complexation of dietary carcinogen. Sequestration of dietary mutagens and carcinogens by interceptor molecules is a concept that applies to numerous compounds including porphyrins (Hartman & Shakel, 1990). In vitro complex formation between chlorophyllin and hydrophobic polycyclic planar mutagens was first reported by Arimoto et al. (1993). Arimoto-Kobayashi et al. (1997) further illustrated the binding of planar mutagens with either two or three fused rings to a chlorophyllin-chitosan insoluble complex. Hayatsu et al. (1999) demonstrated effective binding of heterocyclic amines by solid supported porphyrins including chlorophyllin-chitosan and hemin-agarose.

Complex formation between aflatoxin B1 and major porphyrins such as chlorophyllin, and zinc protoporphyrin IX was investigated using double beam spectrophotometric and fluorescence titration studies. Scatchard analysis demonstrated that binding strength was influenced by both presence and nature of central metal atom with metal-free derivatives being less effective in an interceptor role than either zinc or copper porphyrins (Breinholt et al., 1995b). It was subsequently determined that formation of chlorophyllin-aflatoxin complex contributed to a reduction in systemic aflatoxin bioavailability and thus relative mutagenicity/carcinogenicity (Breinholt et al., 1999; Hayashi et al., 1999). Purified chlorophyll derivatives are not required for the observed effect since co-administration of the unicellular green algae *Chlorella* with polychlorinated dibenzo-*p*-dioxin and dibenzofuran retarded gastrointestinal absorption and accelerated excretion of these industrial contaminants in Wistar rats (Morita et al., 1999).
The nature of mutagen-porphyrin complex has often been described as a reversible planer binding of overlapping ring systems (Dashwood et al., 1998a), but it is clearly dependent on the nature of both porphyrin and mutagen. In the case of chlorophyllin complexation may involve both electrostatic attraction between the porphyrin carboxyl groups with charged moieties such as exocyclic amines (Dashwood et al., 1996) and van der Walls interactions (Tachino et al., 1994; Breinholt et al., 1995b; Dashwood et al., 1998a). For specific mutagens such as aflatoxin B1, complex formation can be highly favorable (-16 to -21 kcal/mol) (Dashwood et al., 1998a).

**Antioxidant potential.** Chlorophyll derivatives are normally thought of as prooxidants due to their participation in photooxidation of fats and oils (Endo et al., 1984; Usuki et al., 1984). However, chlorophylls and pheophytins were protective against the autoxidation of vegetable oils in the dark (Endo et al., 1985a; Gutiérrez-Rosales et al., 1992). Endo et al. (1985b) suggested that chlorophyll derivatives may be acting as electron donors as evidenced by their ability to reduce free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH). Other studies have focused on select natural chlorophyll derivatives including pyropheophytin a by use of a ferric thiocyanate method (Cahyana et al., 1992). Both Cahyana et al. (1993) and Hoshina et al. (1998) demonstrated a clear structural relationship within porphyrins in inhibition of lipid hydroperoxide formation from ferric thiocyanate and ferric nitrilotriacetate respectively. Chlorophyll a was shown to act synergistically with vitamin E by quenching tocopherol radicals thereby enhancing observed antioxidant effects of vitamin E (Le Tutour et al., 1996; 1998).
Commercial grade sodium copper chlorophyllin also has antioxidant activity. A concentration dependent inhibition of lipid peroxidation was noted in rat liver microsomes (Sato et al., 1983). Intraperitoneally and intravenously administered chlorophyllin protected mitochondria and microsome lipid peroxidation and carbon tetrachloride induced injury in rat livers (Sato et al., 1984; 1985). In a subsequent study Sato et al. (1986) identified Cu(II)isochlorin e₄ as a major antioxidative component of sodium copper chlorophyllin by studying its effects on lipid peroxidation. The ability of various metallo-chlorophyllin compounds to quench superoxide was investigated by Suzuki et al. (1995). Nickel, copper and magnesium derivatives suppressed the oxidation of linoleic acid by superoxide anion, whereas, the iron derivatives enhanced oxidation. These data demonstrate that both the presence and nature of the central metal atom is important when considering the activities of chlorophyll derivatives.

**Other modes of action.** While antioxidant and antimutagenic potential of chlorophyll derivatives have received considerable investigation, a number of other potentially beneficial effects have been reported. The ability of chlorophyll derivatives to interact strongly with DNA (Neault & Tajmir-Riahi, 1998; Neault & Tajmir-Riahi, 1999) has raised the potential of minimizing chromosomal damage or influencing DNA repair (Madrigal-Bujaidar et al., 1997; Keshave et al., 1998). The ability of chlorophyllin to protect DNA against ionizing radiation has been demonstrated (Kumar et al., 1999). Smith et al. (1998) reported the enzyme independent inhibition of dibenzo[a,l]pyrene
DNA adduct formation by chlorophyllin in a microsomal in vitro assay system. Chlorophyllin was also found to be extremely effective at inhibiting 2-amino-3-methylimidazo[4,5-f]quinoline carcinogenicity (Dashwood, 1992; Guo and Dashwood, 1994).

Consideration has also been given to the possibility that chlorophyll modulates xenobiotic detoxification systems. Singh et al. (1996a, 1996b) demonstrated significant elevation of glutathione S-transferase activity after both topical and gavage administration of chlorophyllin to murine and suckling neonate mice while levels of cytochrome P450 and b5 remained unchanged. Non-specific inhibition of cytochrome P450 by chlorophyllin has also been observed in human liver microsomes (Yun et al., 1995). Chlorophyllin minimized bioactivation of pre-carcinogens and accelerated excretion of ultimate carcinogens by depression of Phase I cytochrome activity and stimulation of Phase II conjugation pathways.
1.4 Digestive and Absorptive Aspects of Chlorophyll Derivatives.

The digestive process can be divided into four components, the oral, gastric, small intestinal, and colonic phases (Davenport, 1978). Although all phases are important, it appears that the latter three are particularly relevant to chlorophyll digestion and absorption. Chlorophylls, with the exception of certain specific derivatives, are lipid soluble molecules. Digestion and absorption of these molecules are therefore likely to resemble that of dietary lipids, fat-soluble vitamins, heme and lipophilic xenobiotics.

The gastric phase involves shearing and acidification of the ingested material which aids in the formation of a crude emulsion that passes into the duodenum. Emulsifying actions of bile salts and pancreatic secretions facilitate the formation of mixed micelles, which aid in solubilization of lipophilic compounds. Micelles then passively transfer their contents into enterocytes (Porter & Charman, 1997). Unabsorbed/nonmicellarized compounds are subsequently passed into the colon for microbial digestion and/or excretion.

1.4.1 Chlorophyll digestion and absorption in mammals.

With the large amount of chlorophyll consumed via fruits and green vegetables, humans can achieve significant daily exposure. Early investigations provided initial data regarding chlorophyll digestion by identifying decomposition products of both natural and commercial grade derivatives in urine and feces. In general, magnesium-free derivatives were isolated. Their formation was attributed to gastric acidity. Pyroporphyrins, most likely pyropheophytins, were identified as a predominant product in human feces (Fisher & Hendschel, 1933; Brugsch & Sheard, 1938). Baxter, (1968)
found little evidence of chlorophyll metabolism by colonic microflora in several humans fed C14 labeled pheophytin and spinach. Decomposition that occurs during digestion is of great importance because some of the resulting metabolites may potentiate chlorophyll mediated antimutagenesis and chemoprevention (Breinholt et al, 1995a). While chlorophyll derivatives have been isolated in human feces, the contributions of specific digestive processes and colonic microbial activity to the outcome are unknown.

Evidence of chlorophyll absorption and transport in animals exists. Henderson and Long, (1941) orally administered chlorophyll and SCC to rats and discovered derivatives dispersed throughout the liver, lymph nodes, and spleen. More recently, Park & Surh (1996) showed rapid distribution of SCC in the heart, liver, skin, kidneys and lungs of ICR mice after administration of 15mg/kg body weight by gavage. Serine conjugates of chlorophyll and bacteriochlorophyll were found to distribute among tissues and rapidly clear (t ½ = 20 hours) from C57B1 mice following intraperitoneal injection (Rosenbach-Belkin et al., 1996). More recent investigations with SCC as part of a clinical trial in Qidong, People’s Republic of China, has provided characterization of a SCC metabolite in serum of human subjects consuming 100 mg pills daily. Individual steady state levels were found to vary between 0.5-6.0 μg/mL of serum (Egner et al., 1999). Subsequent work demonstrated the presence of a copper chlorin ethyl ester in the plasma of patients consuming 300 mg of SCC daily (Egner et al., 2000). However, natural chlorophyll derivatives in human tissues and body fluids have not been identified. Chlorophyll derivatives which are not absorbed may play an important role in human health by their capability to bind planar dietary mutagens and carcinogens, thereby reducing overall mutagenicity by suppressing bioavailability (Dashwood et al., 1991).
1.4.2 *In vitro* techniques & cell culture modeling of human digestion & absorption.

Absorption and bioavailability of micronutrients and phytochemicals by humans are preferentially investigated *in vivo*. However, time constraints and cost associated with this type of work has prompted a number of researchers to develop rapid and validated *in vitro* techniques for estimating bioavailability. In most cases these *in vitro* models rely on a series of predetermined physiological parameters, including meal size, duration of each digestive phase, pH, gastric and intestinal secretions, that are based on knowledge gained from *in vivo* studies in humans (Larsson et al., 1997). Techniques of this kind have been applied successfully for bioavailability assessment of calcium (Gangloff et al., 1996), cholesterol (Foud et al., 1991), iron and phosphorus (Glahn et al., 1999; Miller et al., 1981; Larsson et al., 1997), vitamin B₆ (Ekanayake & Nelson, 1986), and more recently, natural carotenoid pigments (Garrett et al., 1999a; Garrett et al., 2000). The later methods are of specific interest since they measure the degree to which carotenoid molecules partition into mixed micelles. Since micellarization is a prerequisite for absorption of lipophilic compounds, it may be used as a reliable estimate of bioavailability.

Likewise, the Caco-2 cell culture system is becoming a widely accepted model for studying compound bioavailability and absorption. This cell culture approach allows for systematic investigation of the physiochemical nature of absorption and bioavailability for a wide array of compounds. Caco-2 are human epithelial cells isolated from a human colo-rectal adenocarcinoma (Pinto et al., 1983). These cells spontaneously differentiate at confluence and exhibit enterocyte-like traits, including the polarized distribution of numerous nutrient transport systems, plasma membrane enzymes and hormone receptors,
as well as inducible phase I and II xenobiotic metabolizing systems (Hidalgo & Li, 1996; Gan & Thakker., 1997). The main advantages of using a Caco-2 cellular model include, its human origen, generation of valuable information at the cellular level regarding, uptake metabolism and transport of select compounds, and its use as a rapid screening tool for future studies reducing costly human or animal trials for all compounds of interest (Gan & Thakker, 1997).

While conclusions about bioavailability using these cellular models must be considered in the total context of a reductionist approach, a significant amount of work has demonstrated their efficacy as an effective predictor of human absorption into the intestinal tract (Artursson et al., 1996). Bailey et al. (1996) discusses the advantages of a Caco-2 cell system for absorption studies in drug discovery situations where long, expensive clinical trials often require initial data for effective planning. Gangolff et al. (1996) described a successful method for assessment of iron bioavailability using a combined in vitro digestion and Caco-2 cell culture system. Steensma et al. (1999) successfully studied bioavailability of the soy estrogens genistein and daidzein and their glycosides by Caco-2 intestinal epithelial cells. Studies by Lennernas et al. (1996) compared both active and passive drug transport in Caco-2 and human jejunum in vivo. A high correlation existed for passively transported drugs that are rapidly moved across Caco-2 monolayers. Both active and slower passive diffusion mechanisms (as found with hydrophilic xenobiotics) were depressed in Caco-2 cells compared to the human jejunum. However, the experimental conditions may not have been appropriate for the investigation of these transport routes.
The outcomes of these and many other experiments have demonstrated that Caco-2 monolayers provide accurate predictors of transport and have paved the way for study of a number of potentially bioactive dietary constituents. Using a Caco-2 model, Manna et al. (2000) evaluated both the passive transport mechanism and metabolism of hydroxytyrosol, a phenolic antioxidant present in olive oil. Tranchant et al. (1997) identified a carrier mediated transport system for α-linoleic acid in Caco-2 which supports the presence of a carrier mediated transport system for fatty acid in the human intestine. The investigation of the transepithelial transport of flavone by Caco-2 cultures revealed a rapid diffusional transport system with substantial cellular accumulation. Accumulation of lipophilic carotenoid derivatives from mixed micelles have also been investigated in cultures of Caco-2 cells (Garrett et al., 1999a; Garrett et al., 1999b; Garrett et al., 2000).
1.5 Experimental Aims

Our *long-term goal* is to establish a role for both natural and commercially derived chlorophyll derivatives in modulation of disease risk and outcome. The *objectives* of this investigation are to increase basic knowledge of chlorophyll digestion, absorption and modulation of their chemopreventive activities. To accomplish these goals, the following two specific aims were outlined:

1. *To characterize the digestion and absorption of chlorophyll derivatives from typical fruits, vegetables and supplements, including identification of major degradation products formed during the digestive process.*

2. *To assess the chemopreventive potential of specific chlorophyll derivatives formed during human digestion by measurement of antioxidant and antimutagenic potential of lipophilic and water soluble derivatives.*

*In vitro* digestion coupled with cellular uptake models were employed to characterize complex degradation and derivatization processes occurring to natural chlorophylls and to study key factors affecting their absorption in humans. Predominant derivatives were identified utilizing state of the art HPLC technology and subsequently assayed for potential antimutagenic and antioxidant properties. Results from this research provides basic information critical to the understanding of chlorophyll’s digestion, uptake, metabolism, and potential distribution in humans, enabling better definition of endpoints and experimental approaches for future studies involving dietary interventions toward modulation of disease outcomes.
Chapter 2

Assessment of degradation and intestinal cell uptake of chlorophyll derivatives from spinach puree using an \textit{in vitro} digestion and Caco-2 human cell model

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2.1 Abstract

While numerous studies have demonstrated the health benefits of chlorophyll derivatives, information regarding the digestion, absorption and metabolism of these phytochemicals is quite limited. To better understand the digestion of these pigments, green vegetables, including fresh spinach puree (FSP), heat and acid spinach puree (HASP), and ZnCl$_2$ treated spinach puree (ZnSP) were subjected to an *in vitro* digestion method which simulates both gastric and small intestinal phases of the process. Native chlorophylls were converted to Mg-free pheophytin derivatives during digestion. Conversely, Zn-pheophytins were completely stable during the digestive process. Transfer of lipophilic chlorophyll derivatives, as well as the carotenoids lutein and $\beta$-carotene, into the aqueous micellar fraction from the food matrix was quantified. Micellarization of total chlorophyll derivatives differed significantly ($p < 0.05$) for FSP (37.6%), HASP (17.2%), and ZnSP (8.7%). Micellarization of chlorophyll $a$ derivatives was determined to be significantly more efficient than chlorophyll $b$ derivatives in FSP and HASP ($p < 0.01$), but not in ZnSP ($p > 0.05$). Intestinal cell uptake of micellarized pigments was investigated using HTB-37 (parent) and clonal TC7 lines of human Caco-2 cells. Medium containing the pigment-enriched fraction generated during digestion was added to the apical surface of fully differentiated monolayers for four hours. Pigments were then extracted from cells and analyzed by $C_{18}$ HPLC with photodiode array detection. Both Caco-2 HTB-37 and TC7 clone cells accumulated 20 – 40% and 5 – 10% of micellarized carotenoid and chlorophyll derivatives respectively. These results are the first to demonstrate uptake of chlorophyll derivatives by human intestinal cells and support the potential importance of chlorophylls as health promoting phytochemicals.
2.2 Introduction

The health benefits associated with diets high in fruits and vegetables are well established (Wattenberg, 1990; Willet, 1994; Michaud et al., 1999; Slattery et al., 2000). The rapid accumulation of data in recent years has strengthened the association between chemoprevention and consumption of foods rich in phytochemicals, including natural plant pigments (Hayatsu et al., 1988; Giovannucci et al., 1995). Chlorophylls are the most abundant plant pigment in nature, compromising as much as 1% of the dry weight of certain plant tissues (Sheer, 1991). Sodium copper chlorophyllin, a commercial grade water-soluble mixture of copper-chlorophyll derivatives, exhibits potent antimutagenic and antioxidant activities (Chemomorsky & Segelman, 1988; Odin, 1997; Negishi et al., 1997; Dashwood et al., 1998). Various studies have also focused on the natural chlorophylls prominent in the human diet. Lai et al. (1980) showed a relationship between antimutagenic activity and chlorophyll content for a number of common vegetable extracts. More recently, in vitro and animal studies have shown that chlorophyll derivatives including chlorophyll a, pheophytin a, and pheophorbide a are potential chemopreventive agents (Nakamura et al., 1996a; Nakamura et al., 1996b; Negishi et al., 1997; Harttig and Bailey, 1998; Dashwood et al., 1998). Likewise, pheophytin, pyropheophytin and pheophorbide derivatives common to canned green vegetables were shown to have potent tumoricidal effects in vitro (Chernomorsky et al., 1999).

Information regarding the digestion, absorption, and metabolism of chlorophylls and their derivatives by humans remains limited. Several early studies focused on the isolation of chlorophyll derivatives in feces of humans and animals assuming negligible
absorption (Baxter, 1968; Fisher & Hendschel, 1933; Brugsch & Sheard, 1938). Since natural chlorophylls are modified by heat and acid, they may be susceptible to degradation when subjected to the harsh digestive environment (Schwartz & von Elbe, 1983; Schwartz & Lorenzo, 1990; Gross, 1991; Ma & Dolphin, 1999). Select chlorophyll derivatives have been shown to exhibit different properties in in vitro tests (Chemomorsky et al., 1999, Ma & Dolphin, 1999). Therefore, a more complete understanding of the degree and nature of chlorophyll degradation through the gastrointestinal tract is important for the elucidation of the potential impact of this class of phytochemicals on human health.

The human intestinal cell line Caco-2 exhibits enterocyte like characteristics (Pinto et al., 1981), and its validity as a predictor of intestinal absorption in humans has been established for numerous lipophilic drugs (Bailey et al., 1996; Artursson et al., 1996). This model has also been utilized to investigate the uptake of nutrients and phytochemicals such as iron (Han et al., 1994; Glahn et al., 1999) and micellarized carotenoids (Garrett et al., 1999a; Garrett et al., 1999b). Recently, the TC7 clone of Caco-2 was found to express 15-15’dioxygenase activity (During et al., 1998). This cytoplasmic enzyme is responsible for cleavage of pro-vitamin A carotenoids, such as β-carotene into retinal, the precursor of vitamin A, or retinol (Krinsky et al., 1990).
The objectives of the present study were to investigate the modifications of natural chlorophylls during the gastric and small intestinal phases of digestion in vitro, and to characterize the uptake of micellarized chlorophyll and its degradation products by differentiated cultures of human intestinal Caco-2 cells. An understanding of these principles will expand our current understanding of the role these phytochemicals may play in human health.

2.3 Materials & Methods

Chemicals and Standards. Extraction and chromatography solvents, methanol, ethyl acetate, petroleum ether, and water were of certified HPLC and ACS grade (Fisher Chemical; Fair Lawn, NJ). Pure standards of chlorophyll a and b were obtained (Sigma-Aldrich; St. Louis, MO). Pheophytin a and b standards were synthesized from chlorophyll a and b by as described by Schwartz et al. (1981). Purity was confirmed by HPLC to be greater than 97%. Zn-pheophytin a and b were synthesized by as described by Schwartz (1984). Zn-pheophytins were extracted with petroleum ether, separated and isolated by HPLC to greater than 97% purity.

Sample Preparation. A spinach test meal was chosen for the study because of its high chlorophyll content. Spinach was purchased at a local market and homogenized in minimal deionized water to yield a puree. Lipid content of the homogenate was adjusted to 10% (wt/wt) by addition of corn oil, and the preparation was further homogenized to ensure even distribution of the oil and increase stability of the final emulsion.
Spinach puree was subjected to heat, acid, and ZnCl₂ treatments to induce formation of prominent dietary chlorophyll derivatives including pheophytins and Zn-pheophytins (Figure 2.1). Approximately 1000 g of spinach puree was adjusted to 10 % with corn oil and divided into aliquots of 300 g each. The first aliquot was frozen immediately at −80°C (fresh spinach puree referred to as FSP). The second aliquot was acidified to pH 3 by addition of glacial acetic acid and heated to 100°C for 20 min prior to freezing at −80°C (heat and acid spinach puree referred to as HASP). The final aliquot was heated for 20 min at 100°C with an excess of crystalline ZnCl₂, (ZnCl₂ treated spinach puree referred to as ZnSP).

**In Vitro Digestion.** Simulated digestions were performed as described by Garrett et al. (1999a). Briefly, representative aliquots of homogenized test meals were subjected to a two phase *in vitro* digestion process designed to mimic the *in vivo* process. The gastric phase included acidification of the sample to pH 2 with 100 mM HCl and treatment with porcine pepsin (3 mg/mL) with incubation at 37°C for 1 h in a shaking water bath. The intestinal phase was initiated by neutralization with NaHCO₃, addition of porcine pancreatin (0.4 mg/mL), lipase (0.2 mg/mL) and porcine bile extracts (2.4 mg/mL), adjustment of final pH to 7 with 1 N NaOH and incubation at 37°C for 2 h in a shaking water bath. Upon completion of the phases, aliquots of the digesta were centrifuged at 167,000g at 4°C for 35 min (Beckman model L7-65 Ultracentrifuge) to separate the aqueous micellar phase from the residual solid and oil. The aqueous fraction was filtered (0.2 micron pore size) to remove any contaminating non-micellarized aggregates. All procedures were performed in subdued light and samples were blanketed
with nitrogen to minimize oxidation. Aliquots of raw materials, digesta, and aqueous micellar fraction were collected and stored at –80°C until analysis.

**Cellular Uptake of Micellarized Chlorophyll Derivatives and Carotenoids.**

Studies for chlorophyll derivatives used the Caco-2 cellular model as described by Garrett et al. (1999a). The parent line of Caco-2 (HTB 37) was obtained from the American Type Cell Culture Collection (Rockville, Maryland), and the TC7 clone of Caco-2 was kindly provided by Dr. Monique Rousset, INERM, Cedex, France. Stocks were maintained as described by Han et al. (1994) with complete medium containing high glucose DMEM (Sigma, St. Louis; MO), 10.0% heat inactivated fetal bovine serum, nonessential amino acids (10 mL/L), L-glutamine (2.0 mmol/L), amphotericin B (0.5 mg/L), gentamicin (50 mg/L), HEPES (15 mmol/L), and sodium bicarbonate (44 mmol/L). Test cells were grown and differentiated in 12-well plastic dishes in a humidified atmosphere of air/CO₂ (95:5) at 37°C. All experiments used highly differentiated monolayers at passages 22-35 and 75-80 for HTB 37 and TC7, respectively, 11–14 days after reaching confluency. Prior to initiating the uptake experiment, monolayers were washed twice with 1 mL of Hank’s balanced salt solution. Monolayers were exposed to filtered aqueous fraction from the *in vitro* digestion diluted 1:3 with basal DMEM. Cultures were incubated for 4 h at 37°C. Test media was removed and monolayers were washed twice with ice-cold Hank’s balanced salts solution containing 5.0 mmol/L sodium taurocholate. Cells were collected in 1.0 mL of ice-cold phosphate buffered saline containing 10.0% ethanol and 45 μmol BHT and stored at –80°C.
**Raw Material Extraction.** Lipophilic carotenoids and chlorophyll derivatives were extracted from 5-20 g of raw material by homogenization in acetone with added CaCO₃ and subsequent vacuum filtration (repeated 3X). Filtrates were combined in a separatory funnel and mixed with 30 ml of petroleum ether. Deionized water was then added to partition the chlorophyll derivatives into the petroleum ether phase which was collected and diluted volumetrically to 50 ml. Aliquots were removed and dried under a stream of nitrogen. Dried samples were then dissolved in 2-4 mL of acetone in preparation for LC analysis.

**Digesta & Aqueous Micellar Fraction Extraction.** Carotenoids and chlorophyll derivatives were extracted from a 1-4.0 mL sample by addition of 4.0 mL acetone:petroleum ether (50:50) (0.1% BHT) and vortexed for 1 min. The sample was then centrifuged (2,000g) for 2 min to hasten phase separation. The petroleum ether layer was collected and saved. Extraction was repeated a total of three times and the combined petroleum ether fractions were dried under a stream of nitrogen, re-dissolved in acetone, and analyzed immediately.

**Caco-2 Cell Extractions.** Pigment extraction from Caco-2 cells was performed using a method modified from Peng & Peng (1992). Briefly, 200 µL of protease solution (100mg protease/10mL PBS) was added to each cell pellet. Following incubation at 37°C for 30 min, 0.5 mL of SDS-EtOH (0.1% BHT) solution was added and the sample vortexed for 1 min. Chlorophyll derivatives were extracted by addition of 0.5 mL of acetone:petroleum ether (1:2). The samples were vortexed and briefly centrifuged.
(2,000g) to hasten the phase separation. The petroleum ether layer was collected and saved. The extraction was repeated a total of three times and combined petroleum ether fractions were dried under a stream of nitrogen and analyzed immediately.

**Chromatography.** High performance liquid chromatography analysis of chlorophyll derivatives was performed by the method of Schwartz et al. (1981) with modification. A Waters 2690 Separations Module (Milford, MA) with a model 998 photodiode array detector was used for analysis. A Vydac 201TP54 analytical scale (4.6mm i.d. x 150mm) reversed phase column (Hesperia, CA) with a C18 stationary phase guard column was used.

Separations were achieved using a gradient elution with a binary mobile phase of methanol-water in reservoir A (75:25) and ethyl acetate in reservoir B. Initial conditions were set at 100% A with a linear gradient to 50:50 A:B over 10 min. The gradient was held for 10 min before following a 5.0 min linear gradient back to 100% A for a final chromatographic run time of 30 min. Detection and tentative identification of all chlorophyll derivatives was accomplished using in-line photodiode array data between 350 and 700 nm.
**Data Analysis.** The percent of lipophilic derivatives transferred from the raw material food matrix into the aqueous micellar fraction is defined as the micellarization percentage (%M). All data were analyzed using StatView 5.0 (SAS Institute; Cary, NC). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each pigment’s extent of micellarization and concentration in Caco-2 cells. Group differences were determined by analysis of variance using Fisher’s PLSD post-hoc test (α < 0.05).

**2.4 Results & Discussion**

**HPLC Analysis.** Representative LC chromatograms for FSP, HASP, and ZnSP are shown in Figure 2.2. Separation of six major chlorophyll derivatives is illustrated including predominant native chlorophyll a and b, pheophytin a and b, and Zn-pheophytin a and b. Further separation of corresponding allomers and epimers was also achieved (data not shown). The composition of the three starting materials was indicative of the method of preparative treatment. FSP contained chlorophylls a and b in a 2.5:1 ratio (Table 1) which is consistent with published literature values (Schwartz et al., 1981; Gross, 1991). In contrast, HASP contained the Mg free chlorophyll derivatives pheophytin a, a’, b, and b’. Pheophytin epimers represented approximately 15% of the total pheophytin (Table 1). Mg free pheophytin derivatives are known to be formed through commercial processing conditions (Schwartz et al., 1981). ZnSP contained Zn-pheophytin a, a’, b, b’, pheophytin b, and chlorophyll b. Residual chlorophyll b and
Pheophytin $b$ in this preparation are expected since the formation of Zn-pheophytin $b$ complexes is known to be poor (Jones et al., 1977). This has been attributed to both a lower concentration of the parent compound (pheophytin $b$) in the vegetable tissue, and the presence of the electron withdrawing formyl group lowering electron density that decreases its reactivity with metal ions (Schwartz & Lorenzo, 1990). The quantities of the major carotenoids, lutein and $\beta$-carotene, were not altered by the preparative treatments.

**Chlorophyll Degradation.** The impact of both gastric and small intestinal phases of digestion on dietary chlorophylls was examined. While the profile of chlorophyll derivatives in FSP and HASP raw material differed, distribution in the digesta of FSP and HASP were similar (Table 1). Approximately 75-77% of total chlorophyll pigments for both meals were pheophytin $a$ derivatives after digestion. Elevated levels of allomerized pheophytin $a$ and $b$ were present after digestion of FSP, but not HASP. Allomerized derivatives are hydroxy-chlorophylls formed through oxidation of parent chlorophylls with addition of oxygen to the isocyclic ring (Gross, 1991). It is unclear if the oxidation occurs prior to or after loss of the central magnesium atom. However, the low levels of allomerized pheophytin in HASP points to oxidation of chlorophyll in FSP prior to pheophytin formation during digestion.

The standard conditions for the gastric phase of *in vitro* digestion are incubation at 37°C in a shaking bath at pH 2 for 1 h. The sensitivity of chlorophyll derivatives to acidity is well established (Schwartz & Lorenzo, 1990). Numerous factors including age, physiological conditions, over the counter antacids and prescription H2-receptor
antagonists have been shown to affect human gastric pH (Hörter & Dressman, 1997; Graham et al., 1983; Furu & Straume, 1999). For this reason, the impact of elevated pH on the conversion efficiency of chlorophylls to their respective pheophytins in FSP was investigated. There was minimal loss of the central Mg from the chlorophyll porphyrin (6.0%) at pH 4 and the native chlorophylls were stable during incubation at pH 6 for 1 h (Figure 2.3). Therefore, both native chlorophylls and pheophytins may be present in the gastrointestinal tract of humans with elevated gastric pH. In addition, conversion of chlorophylls \( a \) and \( b \) to their respective pheophytins was complete after incubation at pH 2.0 for 0.5 h with greater than 95% of initial chlorophyll content from FSP recovered as pheophytin in the digesta (data not shown). The above data suggest that cleavage of the phytol tail to produce pheophorbides is unlikely to occur during the gastric and small intestinal phases of digestion. Thus, isolation of pheophorbides in human feces most likely resulted from subsequent metabolism by intestinal microflora (Sheer, 1991).

Regardless of initial food treatment, the aqueous micellar composition for both FSP and HASP samples was very similar with major components being pheophytin \( a \), \( a' \), \( b \), and \( b' \) (Table 1). The profile of the aqueous fraction provides insight about which lipophilic chlorophyll derivatives may be micellarized for uptake and possible transport by intestinal epithelial cells (Porter & Charman, 1997).

In contrast to the native chlorophylls from FSP, the Zn-pheophytins from the ZnSP were completely stable during \textit{in vitro} digestion (Table 1). The small amount of chlorophyll \( b \) (5.0%) found in the raw material was most likely degraded to pheophytin \( b \) through the simulated digestion. The absence of pheophytin \( b \) in the final aqueous micellar fraction may be explained by the poor association of the metal free derivatives
Micellarization of Chlorophyll Derivatives. The efficiency of transfer of the lipophilic chlorophyll derivatives from the food matrix to the aqueous fraction during the two phase digestion process is defined as percent micellarization (%M). The percentages of chlorophyll derivatives that were micellarized during digestion of the three spinach test meals are shown in Figure 2.4a. These data represent total chlorophyll, the sum of natural chlorophylls and their corresponding Mg free pheophytin derivatives previously described in Table 1. Micellarization of chlorophyll derivatives was significantly more efficient from FSP (37.6%) than either HASP (17.2%; \( p<0.01 \)) or ZnSP (8.7%; \( p<0.001 \)). Omission of bile extract from the standard digestion prevented transfer of chlorophyll derivatives to the aqueous fraction, confirming their association with lipid micelles (data not shown).

The decreased efficiency of micellarization of chlorophyll derivatives in digested HASP and ZnSP may be due to the effects of processing on the digestibility of the vegetable tissue. Components formed or released during thermal processing, acidification, or ZnCl\(_2\) treatment may impair digestion enzyme activities or alter micelle formation. For example, specific food components such as minerals and fiber have been
shown to interact with bile salts (Dongowski et al., 1996; Feroci et al., 1996). Excess zinc has been reported to limit activity of select pancreatic enzymes (Gomez-Ortiz et al., 1997). Chlorophyll derivatives have also been shown to hinder pancreatic enzyme catalytic capacity. This effect has been postulated to be a result of binding of the porphyrin backbone to proteolytic enzymes (Oda et al., 1971). Also, the central zinc atom of Zn-pheophytins maintains a coordination number of six. This allows the metal to participate in two interactions distinct from the four secondary valence bonds to the porphyrin backbone. It is possible that formation of coordinate covalent interactions between zinc near functional groups on the amino acid residues of enzymes may impair normal digestive events and lead to inefficient micellarization.

The relative efficiency of micellarization of chlorophyll \( \alpha \) and \( \beta \) derivatives (i.e., residual native chlorophylls and pheophytins for each class) was also compared for the three test meals (Figure 2.4b). Micellarization of chlorophyll \( \alpha \) derivatives was significantly greater than \( \beta \) derivatives for FSP and HASP \((p < 0.01)\), but not ZnSP \((p > 0.05)\). Although micellarization of chlorophyll \( \beta \) derivatives was significantly higher \((p < 0.05)\) for FSP than for HASP, the efficiency of micellarization remained only minimally affected by pre-digestion treatment ranging from 9.0% - 18%. Conversely, micellarization of chlorophyll \( \alpha \) derivatives was strongly affected by the pre-digestion treatment. Micellarization of chlorophyll \( \alpha \) derivatives was 40.0, 22.0, and 10.0% for FSP, HASP, and ZnSP, respectively. This profile closely matched the overall trend seen in Figure 2.4a, and demonstrates the fate of the more abundant chlorophyll \( \alpha \) derivatives contributes markedly to the profile of total chlorophyll micellarization.
The significant difference in micellarization between chlorophyll \( a \) and \( b \) derivatives may be explained by consideration of structural differences between each group of chlorophyll (\( a \) and \( b \)). The presence of a formyl group increases the hydrophilicity of chlorophyll \( b \) derivatives relative to chlorophyll \( a \) derivatives (Gross, 1991) evident by the more rapid elution of chlorophyll \( b \) derivatives on the reverse phase chromatography system employed in this study (Figure 2.2). The more hydrophobic chlorophyll \( a \) derivatives may be more efficiently micellarized because of more optimal solubility in the mixed micelle. This possibility is supported by our observations of the micellarization of the carotenoids lutein and \( \beta \)-carotene in the digested spinach samples. The efficiency of micellarization for lutein and \( \beta \)-carotene is depicted in figure 2.5. While micellarization of polar lutein was not significantly affected by type of treatment, there was a significant reduction in micellarization of apolar \( \beta \)-carotene in response to the digestion of HASP (\( p < 0.05 \)) and ZnSP (\( p < 0.001 \)).

**Caco-2 Cellular Uptake.** The uptake of chlorophyll derivatives by human intestinal cells was studied to validate the availability of micellarized chlorophyll derivatives. Studies were performed using fully differentiated cultures of both HTB-37 (parent) line and the TC7 clone of Caco-2 cells. Aqueous micellar fractions of each test meal were collected and diluted 4 fold with DMEM for presentation to monolayers. Cellular content was determined for spinach chlorophylls and carotenoids after 4 h. As with digesta and aqueous micelle fractions of both FSP and HASP, pheophytins \( a, a', b, \) and \( b' \) were the predominant chlorophyll derivatives in the Caco-2 cells. The total amount of chlorophyll \( a \) and \( b \) derivatives and carotenoids from FSP and HASP in both
HTB-37 and TC7 cell lines are shown in Figure 6a and 6b. Cells accumulated 3-30 pmol of chlorophyll $a$ and $b$ derivatives per mg cellular protein. Cells also accumulated between 10-30 pmol lutein per mg cell protein and 6-20 pmol β-carotene per mg cell protein. Cellular accumulation of chlorophyll derivatives represented 5-10% of the amount of micellarized chlorophyll $a$ and $b$ derivatives in the medium compared to 20-40% uptake of micellarized carotenoids. The extent of carotenoid uptake is similar to that previously reported by Garrett et al. (1999a). The lower pigment content of cells incubated with HASP test media resulted from depressed micellarization of pigments from HASP during simulated digestion (Figure 2.4a & b). These findings demonstrate that cellular content is proportional to micellar content and accentuate the importance of efficient micellarization for the maximization of cellular concentration of these lipophilic phytochemicals.

Two-way analysis of variance showed a significant interaction ($p < 0.05$) between cell line and test meal for lutein only. A main effect of test meal was seen for lutein, chlorophyll $a$ and chlorophyll $b$ derivatives with mean cellular content of these pigments consistently higher after exposure to diluted micellar fraction from digested FSP for the reasons described above. The failure to detect Zn-pheophytins in Caco-2 cells is attributed to the low degree of micellarization of these chlorophyll derivatives from the digested ZnSP. Assuming that the monolayers accumulated 5-10% Zn-pheophytins as observed with pheophytin derivatives from aqueous micelle fraction of both FSP and HASP, the employed methodology lacked sufficient sensitivity to confidently detect and identify these analytes.
Relative accumulation of chlorophyll derivatives was similar for both HTB-37 and TC7 Caco-2 cells ($p > 0.05$). In contrast, cellular accumulation of β-carotene and lutein by the TC7 clonal line was significantly higher than by HTB-37 cells ($p < 0.05$). This initial observation was surprising since 15-15′ dioxygenase activity has been reported in the TC7 clone (During et al., 1998), but is not present in the HTB-37 line of Caco-2. While the metabolism of spinach carotenoids by these cells was not investigated, the greater accumulation might represent more efficient uptake or retention of carotenoids by this clonal line.

The lower cellular accumulation efficiency of chlorophyll derivatives compared to carotenoids may be a result of specific physiochemical properties of chlorophyll. Parameters such as hydrophobicity, ionization, and molecular size have been strongly related with differential intestinal absorption (Chan & Stewart, 1996). In models systems such as rat intestinal perfusion, rat intestinal rings and Caco-2 cells, an increase in molecular weight has been correlated with lower intestinal permeability (Chan & Stewart, 1996). Chlorophyll content in spinach can be as high as 1500 ppm on a wet weight basis compared to 200 ppm for carotenoids (Gross, 1991). Therefore, it is likely that enterocytes can accumulate significant amounts of chlorophyll derivatives after ingestion of meals containing green vegetables. The possibility of subsequent cellular metabolism and transport of these compounds merits investigation.

In conclusion, pheophytins and their epimers were micellarized from spinach test meals subjected to \textit{in vitro} digestion. Although Zn-pheophytins were stable during simulated gastric and small intestinal phases of digestion, native chlorophylls degraded rapidly to pheophytins in response to the high acidity of the gastric phase. These results
indicate that it is possible for multiple chlorophyll derivatives to coexist within mixed micelles. The presence of chlorophyll derivatives in the micelle fraction after digestion and accumulation by Caco-2 enterocyte like cells suggests that these compounds or their metabolites may be absorbed and transported to peripheral tissues. Furthermore, the absence of chlorophyll $a$ and $b$ in the digesta and aqueous micellar fractions when the pH of the gastric phase of digestion was low (pH 2) indicates that pheophytins are abundant and likely represents the biologically relevant forms of these natural pigments in most humans.

2.5 Abbreviations

HPLC, high performance liquid chromatography; FSP, fresh Spinach Puree; HASP, heat and acid treated spinach puree; ZnSP, ZnCl$_2$ treated spinach puree; DMEM, Dulbecco’s modified eagles medium; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PBS, phosphate buffered saline; BHT, butylated hydroxytoluene.

2.6 Acknowledgments

The authors thank Dale Butterfoss for her technical assistance with in vitro sample digestion.
2.7 References


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Table 2.1. Relative amounts of chlorophylls and pheophytins in Raw Material (RM), digesta (Dig), and aqueous micellar fraction (Aqu) for Fresh Spinach Puree (FSP), Heat & Acid Spinach Puree (HASP), ZnCl₂ Spinach Puree (ZnSP). Raw materials were subjected to the standard digestion procedures described in Materials & Methods. Data are presented as percent of total and represent an average of 5 independent measurements. CHL = chlorophyll; PHE = pheophytin; PHE' = pheophytin epimer; PHE (ox) = allomerized pheophytin; ZnPHE = Zn-pheophytin.
Figure 2.1. Structure of three main chlorophyll derivatives from Fresh Spinach Puree (FSP), Heat & Acid Spinach Puree (HASP) and ZnCl$_2$ Spinach Puree (ZnSP).

For $a$ derivatives $R = \text{CH}_3$; $b$ derivatives $R = \text{CHO}$.
Figure 2.2. Representative HPLC chromatograms of chlorophyll and carotenoid derivatives present in (a) Fresh Spinach Puree (FSP), (b) Heat & Acid Spinach Puree (HASP), and (c) ZnCl₂ Spinach Puree (ZnSP). The preparation of these spinach based meals is described in Materials & Methods. Peak identifications: 1 = lutein; 2 = Chlorophyll b; 3 = Chlorophyll a; 4 = Pheophytin b; 5 = β-carotene; 6 = Pheophytin a; 7 = Zn-Pheophytin b; 8 = Zn-Pheophytin a.
Figure 2.3. Influence of gastric pH on conversion of chlorophyll to pheophytin during the gastric phase of digestion. Raw material composition prior to digestion is indicated as FSP. Gastric pH was adjusted to experimental conditions with 1.0 N HCl prior to addition of pepsin. After incubation at 37°C for 1 h in shaking water bath, samples were neutralized (pH 7) by addition of 1.0 M NaHCO₃ followed by titration with 1.0 N NaOH. Analysis was performed as described in Materials & Methods. Values represent an average of four independent gastric digestions.
Figure 2.4. Micellarization of chlorophyll derivatives from test meals (a) Percent micellarization of total chlorophyll (CHL = sum of residual chlorophylls and pheophytins) from FSP, HASP, and ZnSP spinach test meals. (b) Comparison of CHL a and b derivatives from digested FSP, HASP, and ZnSP test meals. Data represent mean +/- SEM for 5 independent digestions. The presence of different letters over the error bars indicate that micellarization of both total chlorophyll and chlorophyll a and b differed significantly between test meals (p < 0.05) with the exception of chlorophyll b derivatives between HASP and ZnSP where the difference was determined to be statistically insignificant (p > 0.05). The presence of the asterisk (*) above the bars indicates those test meals in which micellarization of chlorophyll a and b derivatives differed significantly (p < 0.05); FSP (p < 0.001), HASP (p < 0.05); ZnSP (p > 0.05).
Figure 2.5. Comparison of lutein and β-carotene micellarization from digested FSP, HASP and ZnSP test meals. Data represent mean +/- SEM for 5-9 independent digestions for each test meal. The presence of different letters over the error bars indicate that mean β-carotene micellarization differed significantly (p < 0.05) between test meals. Differences in mean micellarization of lutein between test meals were determined to be statistically insignificant (p > 0.05). The presence of the asterisk (*) above the bars indicates those test meals in which micellarization of lutein and β-carotene derivatives differed significantly (p < 0.05); HASP (p < 0.05), ZnSP (p < 0.001), FSP (p > 0.05).
Figure 2.6. Caco-2 cellular content (pmol/mg protein) of micellarized chlorophyll and carotenoid pigments generated from the digestion of (a) Fresh Spinach Puree (FSP); (b) Heat & Acid Spinach Puree (HASP). Data represent mean quantity of pigment present per mg cell protein after 4 hour incubation in medium containing aliquots of aqueous (micellar) fraction of the digestate. CHL = sum of residual native chlorophylls and pheophytins. Data represents mean +/- SEM for 3-4 independent measurements. The presence of different letters indicates mean cellular accumulation of each pigment differed significantly ($p < 0.05$) within both HTB-37 parent and TC7 clonal lines of Caco-2. The presence of the asterisk (*) above the bars for β-carotene and lutein in panel a indicate that the uptake of these carotenoids by HTB-37 parent line and TC7 clonal lines of Caco-2 differed significantly ($p < 0.05$).
Chapter 3

Thermal degradation of commercial grade sodium copper chlorophyllin.

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Key words: Sodium copper chlorophyllin, Cu(II)chlorin e₄, thermal degradation, UV-Vis spectrophotometry, HPLC.
3.1 Abstract

Sodium copper chlorophyllin (SCC), a water-soluble commercial derivative of chlorophyll, has gained importance as a food colorant and dietary supplement with apparent chemopreventive activities. The thermal stability of SCC was studied to assess the potential application of this chlorophyll derivative for use in thermally processed foods and supplements. Thermal degradation of an aqueous 500 ppm SCC solution was monitored between 25 and 100°C by the loss of absorbance at 627 nm. Decomposition was also followed by reverse phase C18 HPLC with photodiode array detection in order to monitor the loss of Cu(II)Chlorin e4, the major component of commercial grade SCC. Rate of thermal degradation of SCC was found to follow first-order reaction kinetics. HPLC analysis confirmed the ultraviolet and visible (UV-Vis) absorbance data and also demonstrated loss of the major SCC component, Cu(II)Chlorin e4, at a rate faster than overall SCC. Activation energy (Ea) was estimated using the Arrhenius equation and found to be 13.77 ± 0.70 and 16.70 ± 2.06 Kcal/mol for the thermal degradation of SCC and Cu(II)Chlorin e4 respectively. The observed temperature sensitivity of SCC was determined to be similar to natural chlorophyll and raises the possibility of color deterioration when used in food products where mild to severe thermal treatment is applied. Furthermore, the implication of rapid loss of Cu(II)Chlorin e4, a potential bioactive component of SCC, upon heating may result in alteration of potential dietary benefits.
3.2 Introduction

Sodium copper chlorophyllin (SCC), the bright green colorant derived from natural chlorophyll, has been marketed in the United States as a common dietary food supplement, in both liquid and powdered form, with functions ranging from internal deodorizer to natural wound healer and general promoter of kidney health (Kephart, 1955). This mixture of water soluble copper chlorophyll derivatives has gained interest as a potential chemopreventive agent (Odin, 1997). SCC has been found to have antimutagenic activity against a variety of known mutagens including aflatoxin B₁, benzo[a]pyrene, heterocyclic amines, 3-amino-1-methyl-5H-pyrido-[4,3-β]indole and 2-amino-3-methylimidazo[4,5-f]quinolino (Harttig & Bailey, 1998; Dashwood et al., 1991; Breinholt et al., 1995a). SCC has also been shown to have potential antioxidative properties because of its highly delocalized electron system with the ability to act as a radical scavenger (Sato et al., 1986). Together, these studies have prompted considerable interest in SCC for its possible use in prevention of chronic disease.

Commercial grade SCC is a mixture of numerous chlorin type compounds derived from natural chlorophyll (Chernomorsky et al., 1997; Dashwood, 1997). Observed benefits most likely arise from action stemming from this mix of metallo-porphyrins. Two main components have been identified in a complex and variable mixture, (Figure 3.1) Cu(II)Chlorin e₄ and Cu(II)Chlorin e₆ (Inoue et al., 1994; Chernomorsky et al., 1997). Numerous studies have demonstrated the thermal instability of natural chlorophylls and have characterized their major degradation products including metal free pheophytin and pyropheophytins (Mackinney and West, 1940; Schwartz et al., 1983; Schwartz & Lorenzo, 1990). Canjura et al. (1991) studied the degradation kinetics of
chlorophylls and chlorophyllides during thermal processing demonstrating first order reaction kinetics with activation energies between 15.0 to 22.8 Kcal/mol respectively. The structural similarity of SCC to natural chlorophylls make this commercial preparation potentially labile to thermal treatments encountered during typical food processing conditions. However, only a few studies have addressed the potential sensitivity of SCC. Pentillä et al. (1996) investigated photobleaching of chlorophyllin demonstrating a biphasic aerobic degradation. Salin et al. (1999) further reported that photobleaching of SCC is temperature dependent and proceeds by a multistep pseudo first-order process with a potential oxidative component.

The purpose of this study is to gain a better understanding of the temperature sensitivity of commercial grade SCC. Utilizing an aqueous model system, degradation kinetics of crude SCC solutions were assessed by both Ultraviolet and Visible (UV-Vis) spectrophotometry and high performance liquid chromatography (HPLC). Results of these experiments will aid in assessing the potential usefulness of this mixture of copper chlorophyll derivatives for food grade applications involving thermal processing.

### 3.3 Materials & Methods

**Chemicals & Standards.** Commercial grade sodium copper chlorophyllin was purchased form Sigma-Aldrich (St. Louis, Mo). Purity of commercial SCC was calculated to be 47.8% based on 4.5% copper content, specified by the manufacturer, with respect to the main component, Cu(II)-chlorin e₄. These findings agree with
previously published values for commercial grade SCC (Chernomorsky et al., 1997). SCC concentrations for experiments were adjusted based on this level of purity. Authentic standards of Cu(II)-chlorin e₄ were purchased from Frontier Scientific (Logan, Utah), and determined to be >95% pure by HPLC analysis.

**Assessment of SCC Thermal Stability.** All experiments and manipulations were conducted under subdued light in order to minimize photooxidative degradation of SCC. Approximately 250 mg of commercial grade SCC was solubilized in deionized water to a final volume of 500 mL yielding a 500 ppm working solution. Two mL aliquots of the SCC working solution were placed into 2-mL cryogenic vials and tightly sealed. Thermal treatments were applied using a shaking water bath (Thelco Precision Scientific; Chicago, IL) set between 25 and 100°C. Sample vials were exposed to thermal treatment by submersion into the water bath and collected at predetermined time intervals over 120 min. Analysis was performed by monitoring SCC’s electronic absorption spectrum with a Hewlett Packard model 8453 UV-Vis spectrophotometer (Avondale, PA). Spectral data was collected between 300nm and 700nm. SCC degradation was assayed as a loss of absorbance at 627nm, corresponding to both the observed and published red absorption maxima of SCC (Chernomorsky et al., 1997). This method has been previously determined to be in close agreement to data calculated from elemental analysis (Chernomorsky, 1994b).
Assessment of SCC Degradation by Liquid Chromatography. High performance liquid chromatography was used to monitor the degradation of Cu(II)Chlorin e₄, the main component of SCC, and for monitoring the formation of distinct thermal degradation products of SCC. A Hewlett Packard model 1100 (Santa Clara, CA) equipped with a model 1100 diode array detector was used. A Beckman Ultrasphere C₁₈ analytical scale (4.6mm i.d. x 150mm) reversed phase column with a C₁₈ stationary phase guard column was also used. Gradient elution parameters determined for separation of chlorophyllin derivatives were based on a binary mobile phase of methanol-water-acetic acid in reservoir A (75:24.5:0.5) and ethyl acetate in reservoir B. Initial conditions were set at 100% A with a linear gradient to 50:50 A:B over 20 min, followed by a 5.0 min linear gradient back to 100% A for a final chromatographic run time of 25 min. Details of this method including chromatographic output can be seen in Appendix B: Collected Analytical Methods.

3.4 Results & Discussion

Thermal degradation of SCC was investigated at temperatures between 25 and 100°C by following the electronic absorption spectra between 350 and 700nm. Reduction in absorbance at 627 nm was chosen to monitor degradation of copper chlorophyll complexes as loss of metal ions from chlorophyll derivatives has been shown to significantly reduce absorbitivity in the red region (Sheer, 1991). Degradation of total SCC and Cu(II)Chlorin e₄ was found to be temperature dependent following first-order
reaction kinetics as depicted by linear relationship in the thermal degradation curves (Figure 3.2). Reaction rate constants were calculated based on apparent first-order models. Activation energy was estimated within the range of 25° to 100°C from the slopes of Arrhenius plots (Figure 3.3) using the Arrhenius equation.

Experimental rate constants and calculated activation energies for both crude SCC and Cu(II)chlorin e₄ are depicted in Table 3.1. Cu(II)chlorin e₄ was determined to degrade at a rate between three and ten times faster than that of crude SCC. The apparent slower decay of crude SCC may be due to residual absorbance from the decomposition products of the main chlorin components including Cu(II)Chlorin e₄. Electronic absorption spectra of fully degraded SCC maintained a depressed absorbance in the Soret region (406 nm) while the absorbance in the Q region was almost completely lost (data not shown). Degradation of the porphyrin structure would result in significant depression of Soret absorbance consistent with previously published results (Salin et al., 1999). Loss of absorbance in the Q region is similar to the spectral shifts associated with chlorophyll to pheophytin transformation (pheophytinization) wherein the central magnesium atom is replaced by two hydrogens (Sheer, 1991). This results in an olive brown discoloration encountered during thermal processing of green vegetables (Schwartz & Lorenzo, 1990).

Accumulation of specific SCC thermal degradation products was not observed using the chromatographic conditions employed in this study. However, formation of unique compounds should not be discounted since the observed alteration of SCC absorption spectra and the evident olive brown discoloration, supports the possibility that degradative components of SCC may include copper free porphyrins or cleavage products similar to those encountered during degradation of natural chlorophylls (Heaton 82
Further treatment most likely results in a complete deterioration of the tetrapyrrole structure and formation of colorless porphyrin breakdown products including organic acids (Sheer, 1991; Salin et al., 1999).

Activation energies determined from the Arrhenius plots were 13.77 ± .77 and 16.77 ± 2.06 Kcal/mol for crude SCC and Cu(II)Chlorin e₄ respectively (Table 3.1; Figure 3.3). These values are similar to those previously reported for natural chlorophyll derivatives (Macinney & Joslyn, 1941; Canjura et al., 1991; Schwartz & Lorenzo, 1990), and are indicative of only a minor temperature sensitivity for these copper chlorophyll derivatives. Other degradative food reactions have activation energies similar to those observed for SCC. Specifically, oxidation of lipids occurs with activation energies between 10 and 30 kcal/mol (Fennema, 1996). Bleaching of SCC in the dark was previously shown to be highly aerobic and linked to active oxygen intermediates including peroxides (Pentillä et al., 1996). Salin et al. (1999) reported a pseudo-first order, temperature dependent rate of SCC photobleaching, and concluded that both oxidative and photochemical components were responsible for overall SCC degradation. As oxygen was not excluded from the experimental condition prior to thermal treatments, it is possible that an oxidative component contributed to thermal degradation of SCC in our system.

The crude nature of commercial grade SCC results in the presence of non-chlorin material which has not been fully characterized. With the high batch to batch variability often encountered with these commercial preparations, non-chlorin components may comprise as much as 40-50% of total SCC (Inoue et al., 1994; Chernomorsky, 1997).
The extent to which these components contribute to SCC degradation is unclear and remains to be investigated.

3.5 Conclusions

In the United States, SCC is marketed as a liquid and powdered dietary supplement, both of which are subjected to a certain degree of thermal processing. Thermal treatment of aqueous SCC results in temperature and time dependent first-order degradation of copper chlorin components. Cu(II)chlorin $e_4$, a major functional component comprising greater than 85% of total SCC, was found to degrade between three and ten times faster than that of crude SCC. Loss of major SCC components such as Cu(II)chlorin $e_4$ through typical food processing and preparation must be considered as it may impact both color and potential functional properties of this commercial grade chlorophyll preparation.

3.6 Abbreviations

HPLC, high performance liquid chromatography; SCC, sodium copper chlorophyllin; $E_a$, activation energy; UV-Vis, ultraviolet and visible.

3.7 Acknowledgments

The authors would like to thank Dr. Minhthy Nguyen and Ni Luh Puspitasari-Nienaber for their assistance throughout the study and in analysis of data.
3.8 References


Figure 3.1. Structures of major chlorophyllin derivatives present in commercial grade SCC. Cu(II)Chlorin $e_4$ was identified as the major component (>85%) in SCC preparations utilized in this study.
Figure 3.2. First order thermal degradation of crude SCC (Top panel) and Cu(II)Chlorin e₄ (Bottom panel) in aqueous solution. Samples were transferred into 2 mL vials and exposed to thermal treatments by submersion into the appropriate water bath temperature. Each point for crude SCC represent the mean ± SEM for triplicate spectrophotometric measurements. Cu(II)chlorin e₄ was determined by HPLC as described in Material & Methods and each point represents an average of two independent measurements.
<table>
<thead>
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<th>Temperature ($^{0}\text{C}$)</th>
<th>UV-Vis $k$ (min$^{-1}$)</th>
<th>HPLC $k$ (min$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>25</td>
<td>$3.20 \times 10^{-4}$</td>
<td>$9.72 \times 10^{-4}$</td>
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<td>$1.49 \times 10^{-2}$</td>
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<td>$6.60 \times 10^{-3}$</td>
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<td></td>
</tr>
<tr>
<td>100</td>
<td>$2.94 \times 10^{-2}$</td>
<td>$2.81 \times 10^{-1}$</td>
</tr>
</tbody>
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**$E_a = 13.77 \pm 0.77$ kcal/mol 16.70 ± 2.06 kcal/mol**

Table 3.1: Reaction rate constants and calculated activation energy for commercial grade SCC degradation. Values obtained from the slopes of Arrhenius plots (Figure 3.3).
Figure 3.3. Arrenhius plots for crude SCC (•) and Cu(II)Chlorin e₄ (O).
Chapter 4

Sodium copper chlorophyllin: In vitro digestive stability and accumulation by Caco-2 human intestinal cells

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Key Words: Sodium copper chlorophyllin, in vitro digestion, Caco-2 cells, cellular accumulation.
4.1 Abstract

Sodium copper chlorophyllin (SCC), a mixture of water-soluble chlorophyll derivatives, is used as both a food colorant and a common dietary supplement. While the potential antimutagenic and antioxidant properties of this commercial preparation have been demonstrated, limited information is available on its digestion and absorption by humans. Stability of SCC was examined during simulated gastric and small intestinal digestion. Three preparations were subjected to in vitro digestion: SCC in water, SCC in water +10% corn oil, and SCC in applesauce. SCC components from raw material preparations and digestive fractions were analyzed by C\textsubscript{18} HPLC with photodiode array detection. Cu(II)chlorin e\textsubscript{4}, the major chlorin component of SCC, was relatively stable during simulated digestion. In contrast, greater than 90% of Cu(II)chlorin e\textsubscript{6} was degraded to undetermined products during digestion. Recovery of Cu(II)chlorin e\textsubscript{6} after digestion was increased by incorporation of SCC into applesauce, suggesting alteration of inclusion matrix as a possible means to stabilize labile SCC components. Accumulation of SCC derivatives was investigated using differentiated cultures of the TC7 clone of the Caco-2 human intestinal cell line. Cellular accumulation from media containing 0.5 to 60 ppm SCC was linear with intracellular content ranging between 0.2 and 29.6 μg total SCC per mg cellular protein. Uptake of SCC by Caco-2 cells was significantly (p < 0.01) lower in cultures incubated at 4°C compared to 37°C. Stability of Cu(II)chlorin e\textsubscript{4} during in vitro digestion and effective accumulation by Caco-2 enterocyte-like cells support the likelihood that this SCC component or its metabolites are absorbed from the human intestine.
4.2 Introduction

Increased fruit and vegetable consumption has been associated with a decreased risk in the development of chronic diseases such as cancer and cardiovascular disorders (Hayatsu et al., 1988; Steinmetz & Potter, 1996; Su et al., 1999). This has resulted in intense efforts to identify physiologically active compounds in plant foods (Hayatsu et al., 1988; Giovannucci et al., 1995; Martinez & Giovannucci, 1997). Chlorophyll is the most ubiquitous of all natural pigments and functions as the primary photosynthetic pigment of all green plants (Sheer, 1991). Sodium copper chlorophyllin (SCC) is a bright green mixture commercially derived from natural chlorophyll that is being used increasingly as both a food supplement and colorant. Commercial grade SCC is prepared from a crude chlorophyll extract by reaction with methanolic sodium hydroxide followed by a replacement of the central magnesium atom with a heavy metal such as copper (Kephart, 1955). The final mixture is composed of numerous chlorin compounds derived from natural chlorophyll and includes two main components referred to as Cu(II)chlorin e₄ and Cu(II)chlorin e₆ (Dashwood, 1997; Chernomorsky et al., 1997).

A wide range of health benefits has been reported for SCC. In addition to the anti-inflammatory, deodorizing, and erythropoietic activities reported almost 50 years ago (Kephart, 1955), SCC also exhibits potent antimutagenic activity against a variety of known dietary and environmental mutagens such as aflatoxins, benzo[a]pyrene, and heterocyclic amines (Harttig & Bailey, 1998; Dashwood et al., 1991; Breinholt et al., 1995a; Breinholt et al., 1995b). Cu(II)chlorin e₄ and Cu(II)chlorin e₆ also have been identified as potent antioxidants in the commercial preparations of SCC that minimize lipid peroxidation (Sato et al., 1983 and 1986).
In contrast to numerous reports about the apparent health promoting benefits of SCC, there is minimal information about the absorption and metabolism of these compounds in humans. The instability of natural chlorophylls to acid, heat, and light is well established (Schwartz & Lorenzo, 1990). We recently reported that native Mg-chlorophylls are completely converted to metal free pheophytin derivatives during digestion \textit{in vitro}. Lipophilic pheophytin derivatives that were micellarized during the small intestinal phase of \textit{in vitro} digestion process were subsequently accumulated by the Caco-2 human intestinal cell line (Ferruzzi et al., 2001). The recent identification of Cu-chlorin e, and a Cu-chlorin ethyl ester in serum of human subjects ingesting SCC daily provides the first evidence that SCC can be absorbed by humans (Egner et al., 2000). However, neither the digestive stability nor the efficiency of SCC absorption in humans have been investigated.

The primary objectives of the current investigation were to evaluate the stability of SCC during \textit{in vitro} digestion, and to examine SCC uptake from the luminal compartment using monolayers of the Caco-2 cell line. This human intestinal cell line exhibits enterocyte-like characteristics (Pinto et al., 1981), and has been validated as a general predictor of intestinal absorption in humans for numerous lipophilic drugs (Bailey et al., 1996; Artursson et al., 1996). This model also has been successfully utilized to investigate the uptake of nutrients such as iron (Han et al., 1994; Glahn et al., 1999) and phytochemicals, including micellarized carotenoids (Garrett et al., 1999a; Garrett et al., 2000) and natural chlorophylls (Ferruzzi et al., 2001).
4.3 Materials & Methods

Chemicals and Standards. Extraction and chromatography solvents, methanol, ethyl acetate, diethyl ether, and water were of certified HPLC and ACS grade (Fisher Chemical, Fair Lawn, NJ). Phosphate buffered saline was prepared in HPLC grade water and acidified to pH 3 by titration with 5.0 N HCl. Commercial grade sodium copper chlorophyllin was purchased from Sigma-Aldrich (St. Louis, MO). Purity of commercial SCC was calculated to be 47.8% based on 4.5% copper content as specified by the manufacturer with respect to the main component Cu(II)chlorin e₄ (MW = 644.3). These findings agree with previously published values for commercial grade SCC (Chernomorsky et al., 1997). Final experimental SCC concentrations were adjusted in accordance with the level of purity. Authentic standards of Cu(II)chlorin e₄, Cu(II)chlorin e₆, and Cu(II)pheophorbide a were purchased from Frontier Scientific (Logan, UT) and determined to be >95% pure by HPLC analysis.

Assessment of Digestive Stability. Stability of SCC to human digestion was assessed using an in vitro protocol as described originally by Garrett et al. (1999a). Three test preparations were prepared from an aqueous stock solution of SCC. The first preparation simply contained aqueous SCC (W). The second preparation consisted of a homogenized mixture of SCC and 10% lipid (wt/wt) as corn oil in water (WCO). The third preparation contained SCC homogenized in an applesauce matrix with 10% corn oil (AS). Final SCC concentrations ranged from 50 to 250 µg/mL for all experimental preparations. Representative aliquots of each preparation were subjected to a two-phase
in vitro digestion protocol designed to mimic the in vivo process. The gastric phase included acidification of the sample to pH 2 with 100 mM HCl, and exposed to porcine pepsin (3 mg/mL) with incubation at 37°C for 1 h in a shaking water bath (95 rpm). The small intestinal phase was initiated by neutralization of the gastric phase with NaHCO₃, addition of porcine pancreatin (0.4 mg/mL), lipase (0.2 mg/mL) and bile extract (2.4 mg/ml), and adjustment of final pH to 7.0 with 1 N NaOH prior to incubation at 37°C in a shaking water bath (95 rpm). After 2 h, aliquots of the digesta were centrifuged at 167,000g at 4°C for 35 min (Beckman model L7-65 Ultracentrifuge) to separate the aqueous micellar phase from residual solids and oil. Isolated aqueous fractions were filtered (0.2 micron pore size) to remove contaminating aggregates. All procedures were performed in subdued light, and samples were blanketed with nitrogen to minimize oxidation. Aliquots of raw material, digesta, and aqueous fraction were collected and stored at -80°C until analysis.

Accumulation of SCC by Intestinal Cells. Caco-2 human intestinal cells were used to characterize the accumulation of SCC. The TC7 clone of Caco-2 was kindly provided by Dr. Monique Roussel, INERM, Cedex, France. Stocks and test cells were maintained as described by Han et al. (1994) with complete medium containing high glucose Dulbecco Modified Eagle Media (DMEM) (Sigma-Aldrich; St. Louis, MO), 10.0% heat inactivated fetal bovine serum, nonessential amino acids (10 mL/L), L-glutamine (2.0 mmol/L), amphotericin B (0.5 mg/L), gentamicin (5.0 mg/L), HEPES (15 mmol/L) and sodium bicarbonate (44 mmol/L), in a humidified atmosphere of air/CO₂ (95:5) at 37°C. Test cells (passages 75-80) were grown and differentiated in 6-well
plastic dishes. Highly differentiated monolayers were used for experiments 11–14 days after reaching confluency. Prior to initiating experiments, monolayers were washed twice with 1 mL of basal DMEM. Six test media were prepared by solubilizing SCC in basal DMEM at concentrations of 0.5, 1.5, 6.0, 15.0, 25.0, and 60.0 ppm. Monolayers were incubated with 2 mL of test media for 15 min to 4 h at either 4 or 37°C. Test media were removed at indicated times and monolayers were washed twice with ice-cold phosphate buffered saline (PBS). Cells were collected in 1.0 mL of ice-cold PBS (pH 7.4), centrifuged (500g for 5 min), and the pellet was blanketed with N₂ and stored at -80°C until analysis.

Preliminary characterization of the transepithelial transport of SCC was accomplished utilizing differentiated cultures of Caco-2 cells grown on Transwell® inserts (Corning; Corning, NY). Intact monolayers were incubated with high phenol red DMEM (500 μM phenol red) containing 30 ppm SCC. After 4 h, inserts were washed with phenol-free DMEM before transfer to new wells. Fresh DMEM medium with and without 500 μM phenol red was added to the insert and the well respectively. Both apical and basolateral media were collected at 4 and 20 h post loading and saved. Monolayers were washed twice with ice-cold phosphate buffered saline (PBS) and cells were collected either after the four hour exposure to SCC, or 4h and 20h post-loading times. Monolayer integrity was assessed by monitoring the transfer of the phenol red from the apical to the basolateral compartment. Only monolayers with less than 0.01%/h permeability were utilized for transport studies.
Extraction of SCC. Components of SCC were extracted from 1 to 4 mL of the digesta and the aqueous fraction from the digestion by homogenization with an equal volume of acidified PBS (pH=3.0). Reduction of the pH ensures protonation of SCC derivatives facilitating their extraction into organic solvent. SCC components were then extracted by addition of 4.0 mL diethyl ether (0.1% BHT) and vortexed for 1 min. Samples were then centrifuged (2,000g) for 2 min to hasten phase separation for collection of the diethyl ether layer. Extraction was repeated a total of three times, and the combined diethyl ether fractions were dried under a stream of nitrogen, re-dissolved in methanol, and immediately analyzed as described.

Extraction of SCC from Caco-2 cells was performed by addition of 200 μL of protease solution (10 mg protease per mL PBS) to resuspended cell pellet and incubation at 37°C. After 30 min, 0.5 mL of acidified PBS was added and the sample vortexed for 1 min. SCC components were extracted into 0.5 mL of diethyl ether (0.1% BHT). Samples were vortexed and briefly centrifuged (2,000g) to separate the phases. The diethyl ether layer was collected and extractions were repeated a total of three times. Combined fractions of diethyl ether were dried under a stream of nitrogen and analyzed immediately.

SCC Analysis. High performance liquid chromatography (HPLC) of chlorophyllin derivatives was performed using a Hewlett Packard model 1100 HPLC system equipped with a model 1100 diode array detector (Santa Clara, CA). A Vydac 201TP54 analytical scale (4.6 mm i.d. x 250 mm) C18 reversed phase column (Hesperia, CA) with a C18 stationary phase guard column was used. Separations were achieved by
gradient elution with a binary mobile phase of methanol-water-acetic acid in reservoir A (75:24.5:0.5) and ethyl acetate in reservoir B. Initial conditions were set at 100% A with a linear gradient to 50:50 A:B over 20 min followed by a 5.0 min linear gradient back to 100% A for a final chromatographic run time of 25 min. Detection and tentative identification of major SCC components was accomplished by co-chromatography with authentic standards, and comparison of electronic absorption spectra obtained from in-line diode array detection with previously published electronic absorption spectra of major SCC derivatives (Inoue et al., 1994).

**Data Analysis.** The quantity of SCC derivatives recovered in digesta and aqueous fractions relative to that in the raw material is defined as percent recovery. All data were analyzed using StatView 5.0 (SAS Institute, Cary, NC). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each SCC derivative's percent recovery and Caco-2 intracellular content. Group differences were determined by analysis of variance using Fisher's PLSD post-hoc test ($\alpha < 0.05$).

4.4 Results & Discussion

**HPLC Analysis.** A representative HPLC chromatogram of commercial grade SCC is shown in Figure 4.2. This material contained numerous copper chlorophyllin complexes. Four derivatives were tentatively identified based on co-chromatography with authentic standards and comparison of electronic absorption spectra to published
values (Inoue et al., 1994). As shown in Table 4.1, Cu(II)chlorin e₄ (81%) was the major component followed by Cu(II)chlorin e₆ (10%), Cu(II)rhodin g₇ (3%), and Cu(II)pheophorbide a (1%). These results are comparable to previously published reports with expected differences arising from lot to lot variability (Inoue et al, 1994; Chernomorsky et al., 1997). A number of other chlorin components were detected and observed to have similar spectral properties to the main derivatives. These likely represent isomers and/or minor degradation products of the parent compounds. The described chromatographic method facilitates rapid and selective separation of major components from commercial grade SCC.

**Stability of SCC to In Vitro Digestion.** Stability of SCC during simulated digestion was examined using an *in vitro* model that simulates both gastric and small intestinal phases of digestion. SCC was incorporated at 50 to 250 μg per g into three separate matrices designed to mimic typical consumption: applesauce (AS), water (W) and a water-corn oil emulsion (WCO). The chromatographic profile of chlorophyllin species in the test meal, digesta, and aqueous fraction was subsequently determined by HPLC. Chlorophyllin derivatives transferred from the starting raw material to the aqueous fraction of the digesta are considered to be available for uptake by the intestinal cells. SCC components from the raw materials were efficiently transferred (>60%) to the aqueous fraction for all preparations presumably due to the hydrophilicity of SCC.
Minimal differences were observed between W and WCO preparations, indicating that association of SCC with lipid-bile salt micelles is not required for transfer to the aqueous fraction as is required with natural chlorophyll derivatives (Ferruzzi et al., 2001). Therefore, ingestion of foods containing fat may not be required to optimize intestinal cell uptake and, by extension, bioavailability of these chlorophyll derivatives.

Quantitative and qualitative changes in the chlorin content of SCC were noted after digestion of the three preparations. Cu(II)chlorin \(e_4\), the main component of SCC, exhibited excellent stability with greater than 90% from the raw material present in the digesta fraction after \textit{in vitro} digestion. Recovery of Cu(II)chlorin \(e_4\) in the aqueous fraction was 70 to 80% for all preparations (Figure 4.3). The amounts of SCC in the aqueous fraction was consistently lower than in digesta. This may reflect binding of porphyrins to remnant solids from crude porcine pancreatin and bile extract, as well as residual pectins and fiber in applesauce, since a green precipitate was visible following centrifugation of the digesta fraction in all three preparations.

In contrast with the relative stability of Cu(II)chlorin \(e_4\), recovery of Cu(II)chlorin \(e_6\) after digestion of both W and WCO preparations was only 10% \((p < 0.001;\) Figure 4.3). Incorporation of SCC into applesauce significantly \((p < 0.05)\) increased recovery of Cu(II)chlorin \(e_6\) after digestion. The decline in Cu(II)chlorin \(e_6\) relative to Cu(II)chlorin \(e_4\) contributes to the qualitative changes in SCC profile observed post digestion, but does not greatly affect final SCC content because Cu(II)chlorin \(e_4\) is most abundant in this commercial SCC preparation.
Degradation of chlorophyll derivatives by reactive oxygen species including singlet oxygen has been demonstrated (Telfer et al., 1994). Penttilä et al. (1996) reported that chlorophyllin bleaching proceeds by a peroxidative process and can be retarded by addition of reducing agents such as ascorbate. Moreover, Salin et al. (1999) reported the selective loss of Cu(II)chlorin e₆ during photobleaching of crude SCC. We speculated that the preferential degradation of Cu(II)chlorin e₆ during the digestion occurred by oxidative processes. However, the addition of ascorbic acid to levels as high as 1 mg per g test meal failed to improve the recovery of Cu(II)chlorin e₆. Therefore, the greater stability of Cu(II)chlorin e₆ in applesauce requires consideration of other matrix effects including buffering capacity, light protection, and non-nutritive components such as phenolic antioxidants. The significance of these factors becomes evident when preparing supplementary products with SCC that are intended for human consumption.

The stability of phytochemicals during digestion is important for speculating about their potential health benefits. *In vitro* studies assessing functional activity often use purified natural compounds without consideration of digestive stability and bioavailability. We recently reported that natural chlorophylls are converted to metal-free pheophytins during simulated digestion, thereby generating a diverse array of metal-free chlorophyll derivatives available for uptake by human intestinal cells (Ferruzzi et al., 2001). Thus, the protective effects of natural chlorophyll *a* and *b* reported in the literature (Negishi et al., 1997; Harttig & Bailey, 1998) would more likely reflect the activities of their metal-free pheophytin derivatives rather than the native compounds. The stability of some water-soluble copper chlorophyll derivatives such as Cu(II)chlorin e₄ during simulated digestion indicates that these Cu(II) complexes are the predominant
chlorophyllin derivatives in the human intestinal lumen after consumption of SCC. It is interesting that Egner et al. (2000) identified both Cu(II)chlorin e₄ and a Cu(II)chlorin e₄ ethyl ester, but not Cu(II)chlorin e₆ in plasma of patients ingesting 300 mg of SCC daily for four months. Our results suggest that failure to detect Cu(II)chlorin e₆ in plasma may be due to selective degradation of this derivative in the GI tract.

Uptake and Accumulation of SCC by Intestinal Epithelial Cells. Cellular uptake and accumulation of SCC derivatives by enterocyte-like cells was studied using fully differentiated cultures of the TC7 clone of Caco-2 cells. SCC was solubilized in basal DMEM and serially diluted to concentrations ranging from 0.5 to 60 ppm for presentation to the apical surface of the monolayers. This range approximates concentrations in the lumen resulting from consumption of 1.0 to 100 mg SCC, assuming that the ingested softgel or liquid SCC supplement (60-100 mg SCC per serving) will be diluted by approximately 2L of gastric and intestinal fluids (Tortora and Evans, 1986). Cells were harvested after incubation for indicated times and SCC content was determined.

SCC uptake by Caco-2 cells was investigated at various times during exposure to medium containing 30 ppm SCC for as long as 1 h (Figure 4.4). The intracellular ratio of Cu(II)chlorin e₄ to e₆ was similar to that of the test medium (data not shown) indicating that uptake of the major SCC derivatives was proportional to their concentration in the media. Uptake of Cu(II)chlorin e₆ by human intestinal cells in vitro further highlights the impact of digestive instability of this chlorin derivative. Cellular content of total SCC, i.e., Cu(II)chlorin e₄ + e₆ increased linearly with length of exposure at 37°C. After 1 h,
cells contained 10.3 μg total SCC per mg cellular protein. Cellular uptake decreased by approximately 75% \( (p < 0.01) \) in cultures incubated at 4°C (Figure 4.4). The accumulation of total SCC also was decreased approximately 70% \( (p < 0.001) \) when cultures were exposed to medium containing 1.5, 15, and 30 ppm SCC at 4°C for 4 h (data not shown). These data suggest that SCC derivatives are transported into enterocytes by a facilitated process. Receptor mediated transport of porphyrins has been reported. A heme binding protein was partially purified from the duodenum of iron-repleted rats (Smith, 1983), and transferrin mediated internalization of heme and other porphyrins was observed in murine erythroleukemia cells (Stout, 1992). Additional studies of SCC transport across the intestinal epithelium are merited, especially in light of recent isolation of SCC metabolites in human sera (Egner et al., 2000).

Intracellular content of SCC was proportional to the amounts in medium with cell levels ranging from 0.1 to 28 μg total SCC per mg cellular protein, after incubation in medium containing 0.5 to 60.0 ppm SCC at 37°C for 4h (Figure 4.5). Therefore, cellular accumulation of SCC was not saturated over the 120 fold range of SCC in the medium. The quantity in the cells represented 45 to 60% of SCC added to the test medium.

Stability of SCC test media was investigated in a cell-free tissue culture environment. Media containing 30 ppm SCC test media was added to cell free wells and incubated at 37°C for 4h in a humidified atmosphere of air/CO₂ (95:5). Recovery of total SCC was 70% with 30% losses of both Cu(II)chlorin e₄ and e₆ (data not shown). There was no indication that SCC adhered to the walls of the cell culture vessel. Thus, the loss of SCC most likely resulted from oxidation in culture medium.
A preliminary investigation of SCC retention by Caco-2 cells was undertaken in order to observe potential metabolism and transepithelial transport of these chlorophyllin derivatives. Differentiated cultures of Caco-2 cells were grown on Transwell® inserts and incubated with high phenol red DMEM containing 30 ppm SCC. Caco-2 monolayers accumulated 6.31 ± 1.7 µg SCC per g cellular protein after 4h. Following removal of exogenous SCC, inserts were washed with phenol free DMEM and transferred to new wells before adding 1.5 and 2.5 mL of fresh phenol free high phenol red DMEM and phenol free cDMEM to the apical and basolateral compartments, respectively. After incubation for 4h, (i.e. 8h from initial loading), only 13% (0.80 ± 0.26 µg SCC per g cellular protein) of the previously accumulated SCC was present in Caco-2 cells (Figure 4.6). Intracellular SCC was not detectable at 20h post-loading. Approximately 1% of the initially accumulated SCC were recovered in the apical and basolateral compartment, respectively. The transport of some SCC across the intestinal epithelia agrees with the presence of low concentration of several SCC components in human plasma after consumption of supplemental SCC (Egner et al., 1999; Egner et al., 2000). More detailed studies are required to explain the apparently low efficiency of transfer across the basolateral membrane. These preliminary data suggest that efficient uptake of SCC by human intestinal cells is accompanied by poor retention of these compounds indicating potential transport, metabolism, and/or oxidative degradation such as that noted in cell free media. Caco-2 cells express heme oxygenase and biliverdin reductase activities (Cable et al., 1993). Therefore, the possibility that some portion of intracellular SCC is being metabolized merits further investigation.
4.5 Conclusions

In conclusion, commercial grade SCC is composed of two prominent derivatives, Cu(II)chlorin e₄ and Cu(II)chlorin e₆. When subjected to simulated digestion, Cu(II)chlorin e₄ was relatively stable with greater than 70% recovery in the aqueous fraction. Conversely, the majority of Cu(II)chlorin e₆ in the preparation was lost during *in vitro* digestion. Incorporation of SCC into an applesauce matrix diminished the loss of Cu(II)chlorin e₆, suggesting that an inclusion matrix can be considered for stabilizing labile SCC components following ingestion. Caco-2 human intestinal cells readily accumulated SCC when the extracellular concentration was between 0.5 and 60 ppm. Non-saturable but temperature dependent accumulation of SCC in Caco-2 suggest the possibility of both passive and active transport through the intestinal epithelia. The stability of Cu(II)chlorin e₄ during *in vitro* digestion, and its efficient uptake by Caco-2 enterocyte-like cells, suggests that this SCC derivative can be absorbed and transported to peripheral tissues.

4.6 Abbreviations

SCC, sodium copper chlorophyllin; CuCe₄, Cu(II)chlorin e₄; CuCe₆, Cu(II)chlorin e₆; HPLC, high-performance liquid chromatography; AS, applesauce preparation; WCO, water lipid preparation; W, Water no lipid preparation; DMEM, Dulbecco’s modified eagles medium; PBS, phosphate buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-thanesulfonic acid; BHT, butylated hydroxytoluene.
4.7 References


Figure 4.1. Structure of main sodium copper chlorophyllin derivatives for Cu(II)chlorin e₄:
R = CH₃; Cu(II)rhodin g₇: R = CHO.
Figure 4.2. HPLC separation of predominate derivatives of commercial grade sodium copper chlorophyllin. Peak identifications: 1 = Cu(II)rhodin g; 2 = Cu(II)chlorin e₅; 3 = Cu(II)chlorin e₆; 4 = Cu(II) pheophorbide a. Ultraviolet and visible absorption spectra were collected online by diode array detection between 350 and 700nm.
Table 4.1 Absorption Maxima of main SCC components obtained by online photodiode array detection from a C18-HPLC separation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Observed $\lambda_{\text{max}}$ (nm)</th>
<th>Published $\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soret band</td>
<td>Q band</td>
</tr>
<tr>
<td>Cu(II) Pheophorbide a</td>
<td>402,424</td>
<td>654</td>
</tr>
<tr>
<td>Cu(II) chlorin e₆</td>
<td>408</td>
<td>634</td>
</tr>
<tr>
<td>Cu(II) chlorin e₄</td>
<td>404</td>
<td>627</td>
</tr>
<tr>
<td>Cu(II) rhodin g₇</td>
<td>438</td>
<td>623</td>
</tr>
</tbody>
</table>

*a Inoue et al., (1994)*
Figure 4.3. Recovery of major SCC derivatives in (■) digesta and (□) aqueous fractions from *in vitro* digestion of AS (panel A); W (panel B); WCO (panel C). Data represent the percentage of SCC present in digesta and aqueous fraction of digesta relative to starting raw material, which is arbitrarily set at 100%. Digestions and analyses were performed as described in Materials & Methods. Values represent means ± SEM for 4 independent digestions. Different letters above error bars indicate that recovery of Cu(II)chlorin e₆ was significantly lower than Cu(II)chlorin e₄ in AS (p < 0.05), WCO (p < 0.001), and W (p < 0.001). Preparations for which pigment recoveries differed significantly (p < 0.05) from the original raw material are indicated by an asterisk (*).
Figure 4.4. Uptake of SCC by Caco-2 human intestinal cells. Differentiated monolayers of Caco-2 cells were incubated in medium containing 30 ppm SCC. Medium was removed and monolayers washed with ice cold PBS at indicated times, and the quantity of SCC in cells was determined as described in Material & Methods. Means ± SEM for 3-5 independent measurements are reported. The presence of different letters indicate significant \((p < 0.01)\) differences for cellular uptake at 37 and 4°C after incubation for 15, 30, and 60 min. Cellular content increased \((p < 0.01)\) linearly with time at both temperatures as indicated by the different superscripts with the letters.
Figure 4.5. Accumulation of SCC by Caco-2 cells is proportional to medium concentration of SCC. Data represent means ± SEM for 3-5 independent measurements of total SCC present per mg cell protein after 4 h incubation in basal DMEM containing between 0.5 and 60ppm SCC.
Figure 4.6. Retention of SCC by Caco-2 cells. Monolayers were grown on Transwell® inserts and loaded with SCC by incubating cultures with apical medium containing 30 ppm SCC for 4 h. Cellular SCC was measured and replicate monolayers were utilized to examine retention. Medium was then removed and the monolayers was extensively washed to remove extracellular SCC before addition of fresh medium with and without 500 μM phenol red to the apical and basolateral compartments, respectively. SCC cellular content was determined 4 and 20 h post loading. Data represent mean ± SEM for 3 independent measurements of total SCC per mg cell protein remaining after incubation for 4 and 20 h in high phenol red DMEM. The presence of different letters indicate significant (p < 0.001) differences between cellular SCC content after incubation for 4 and 20 h post loading.
Chapter 5

Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays

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5.1 Abstract

In vitro antioxidant and antimutagenic activity of both natural and commercially derived dietary chlorophyll derivatives prevalent in foods and dietary supplements was assessed in order to better understand the potential protective effects. Antioxidant activity was determined by the ability of each compound to scavenge the long-lived free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS"). Antimutagenic activity of chlorophyll derivatives was assayed with a modified microscreen bacterial reverse mutagenicity assay using Salmonella typhimurium TA100 and benzo[a]pyrene as the tester strain and mutagen respectively. Derivatives of chlorophyll a were found to be more effective radical quenchers than those of chlorophyll b. Furthermore, metal free derivatives such as chlorins, pheophytins, and pyropheophytins exhibited significantly lower antiradical capacity than metallo-derivatives such as Mg-chlorophylls ($p < 0.05$), Zn-pheophytins ($p < 0.01$), Zn-pyropheophytins ($p < 0.05$), Cu-pheophytin a ($p < 0.01$), and Cu-chlorophyllins ($p < 0.001$). Both metal free and metallo-chlorophyll derivatives demonstrated similar dose-dependent inhibitory activity against benzo[a]pyrene induced mutagenesis. These results demonstrate that dietary chlorophyll derivatives prevalent in both fresh and processed foods and dietary supplements have antioxidant and antimutagenic activities.
5.2 Introduction

Rapid accumulation of data in recent years has strengthened the association between prevention of chronic diseases such as cancer and cardiovascular disorders and consumption of fruits and vegetables (Hayatsu et al., 1988; Giovannucci et al., 1995; Steinmetz & Potter, 1996; Slattery et al., 2000). The bioactivity of phytochemical constituents common to these foods has been the subject of a number of investigations (Chernomorsky & Segelman 1988; Mayne, 1996; Lu & Foo, 2000; Jiménez-Escrig et al., 2000). Chlorophylls are the most abundant phytochemical pigments in higher plants. Since Lai et al. (1980) showed a relationship between antimutagenic activity and chlorophyll content for a number of common vegetable extracts, numerous derivatives have been identified as potential antimutagens. Endo et al. (1985a; 1985b) first suggested that chlorophyll derivatives may be chain-breaking antioxidants by acting as effective electron donors. Sato et al. (1986) subsequently identified Cu-isochlorin e₄ as a major antioxidative component of commercial grade sodium copper chlorophyllin. More recently, Cahyana et al. (1993) and Hoshina et al. (1998) demonstrated a clear structural relationship within related porphyrins for the inhibition of lipid hydroperoxide formation from ferric thiocyanate and ferric nitrilotriacetate, respectively.

All natural chlorophyll derivatives can be described as substituted tetrapyrroles with a centrally bound magnesium atom (Sheer, 1991). While chlorophyll a and b derivatives predominate in higher plants, numerous alterations occurring naturally, during food processing, and in response to gastrointestinal digestion may dramatically increase the spectrum of dietary chlorophyll derivatives present in the intestinal lumen of humans consuming green vegetables (Schwartz & Lorenzo, 1990; Ma & Dolphin, 1999; Ferruzzi
et al., 2001). Basic structural modifications may include the formation of metal-free 
pheophorbides, pheophytins, and pyropheophytins, as well as metallo-chlorophyll 
derivatives including zinc and copper complexes. Water soluble chlorophyllin 
preparations produced from alkali hydrolysis of the native pigments are finding increased 
use as food colorants and dietary supplements (Kephart, 1955). Dietary derivatives other 
than naturally abundant Mg-chlorophylls have since been separately identified as 
potential chemopreventative agents. These include Cu-chlorophyllins, pheophorbides, 
pheophytins, and pyropheophytins (Waters et al., 1996; Nakamura et al., 1996a; 
Nakamura et al., 1996b; Negishi et al., 1997; Harttig & Bailey, 1998; Dashwood et al., 
1998; Chernomorsky et al., 1999). However the extent to which chlorophyll 
derivatization modulates potential bioactivity has not been systematically investigated.

The purpose of this study was to compare the antioxidant and antimutagenic 
activity of prominent chlorophyll derivatives prevalent in the human diet in order to 
assess the effect of processing or digestive alterations on the bioactivity of chlorophyll. 
Fifteen chlorophyll derivatives (Figure 5.1) were chosen based on their abundance in 
fresh, processed foods and dietary supplements. Derivatives were assayed for their 
ability to scavenge two long lived free radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH') 
and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS'), as well as to inhibit 
benzo[a]pyrene (B[a]P) induced mutagenesis in Salmonella typhimurium TA100. 
Information from these studies will further address the possible role of these dietary 
phytochemicals in modulating disease risk and outcomes.
5.3 Materials & Methods

**Chemicals & Standards.** All solvents including methanol, petroleum ether, diethyl ether, and water were of HPLC and ACS grade. Antioxidant assay reagents including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox®) and manganese dioxide were obtained from Sigma-Aldrich (St. Louis, MO). Benzo[a]pyrene (B[a]P), the test mutagen for the bacterial reverse mutagenicity assay, was purchased from Moltox (Boone, NC).

Commercial grade sodium copper chlorophyllin (SCC), as well as pure standards of chlorophyll a and b, were obtained from Sigma-Aldrich (St. Louis, MO). Standards of chlorin e₄, chlorin e₆, pheophorbide a, and copper complexes of each were purchased from Frontier Scientific (Logan, UT). Pheophytin and pyropheophytin a and b were synthesized from corresponding chlorophylls as described by Schwartz (1984). Zinc and copper complexes of pheophytins and pyropheophytins were synthesized as described by Schwartz (1984). Each chlorophyll derivative was checked for purity by HPLC and further purified when necessary to >95% by semi-preparative high performance liquid chromatography as described previously (Ferruzzi et al., 2001).

**Assessment of Trolox Equivalent Antioxidant Capacity (TEAC).** The ability of each chlorophyll derivative to scavenge the stable DPPH⁺ and long lived ABTS⁺ radical was determined. A 50 µmol L⁻¹ DPPH⁺ stock solution was prepared daily in methanol. The ABTS⁺ radical was prepared fresh daily using the method of Miller et al.
(1996) with modification. Briefly, 5.0 g of magnesium dioxide was added to a 5.0 mmol L$^{-1}$ stock solution of ABTS in methanol. The slurry was placed in a sonicating water bath for 5 min after which the solution was filtered first through a Whatman no. 5 filter paper before a 0.2 μm PTFE syringe filter. ABTS$^+$ stock was diluted with methanol to a final absorbance of 0.70 at 734nm after which it was allowed to equilibrate for two hours at room temperature prior to use.

Individual chlorophyll derivatives were dissolved in methanol to a final concentration of 50-100 μmol L$^{-1}$ as determined by preliminary experiments with SCC. A 100 μL aliquot of each chlorophyll solution or methanol blank was added to either 1.9 mL of DPPH$^-$ stock solution or 0.9 mL of ABTS$^+$ in an 1 mL glass vial. Solutions were briefly vortexed to ensure proper mixing. After 10.0 min absorbance was quantified spectrophotometrically (Hewlett Packard Ultraviolet and Visible Spectrophotometer model 8453; Avondale, PA) at 515nm and 734nm for DPPH$^-$ and ABTS$^+$, respectively. The decrease in absorbance after 10.0 min was corrected with that of a methanol control and used to calculate antioxidant capacity based on a calibration curve prepared with standard solutions of 0-200 μmol L$^{-1}$ Trolox® for both DPPH$^-$ and ABTS$^+$ assays.

**Bacterial Reverse Mutation Assay.** The modified microscreen method of Diehl et al. (2000) was utilized with *Salmonella typhimurium* TA100 (Moltox; Boone, NC) as the tester strain. This method is based from the traditional plate incorporation assay of Maron & Ames (1983), modified to utilize six-well cell-culture dishes, thereby minimizing the quantity of test compound required. Six-well plates pre-poured with 5mL
of Vogel-Bonner minimal glucose agar per well and top agar (2% agar) were purchased from Moltox (Boone, NC). Bacterial stock cultures were inoculated into 10mL of Oxoid #2 nutrient broth and incubated at 35 ± 2°C for approximately 10 h until an optical density of 0.8 was achieved. Mammalian microsomal activation system (S-9 mix) was prepared by diluting Aroclor 1254-induced rat liver microsomes (Moltox; Boone, NC) into a glucose-6-phosphate-NADP solution at 4% (v/v). B[α]P stock solution (100 μg/mL) was prepared in sterile dimethyl sulfoxide (DMSO). Individual chlorophyll derivatives were dissolved in DMSO, filter (0.2μm PTFE) sterilized, and diluted to concentrations of 40-400 μmol L⁻¹ for incorporation into the mutagenicity assay.

Microscreen assays were plated by incorporation of 25 μL of B[α]P, 100 μL of S-9 mix, 25 μL of bacterial culture, and 25 μL test compound into a 2 mL vial. Samples were preincubated for 20 min at 37°C, combined with 500 μL of molten top agar, mixed and poured onto surface of well. Upon hardening of the top agar, plates were incubated for 48 hr at 37°C after which plates were counted for his⁺ revertant colonies. Spontaneous mutations were determined by plating controls containing DMSO in place of B[α]P and chlorophyll derivatives. Antimutagenic activity of each chlorophyll derivative was determined by the ability of each derivative to reduce the number of his⁺ revertants caused by 10nmol B[α]P per well (86 ± 5) corrected for spontaneous background revertants.
Data Analysis. All data was analyzed using StatView 5.0 (SAS Institute; Cary, NC). Trolox® equivalent antioxidant capacity (TEAC) was determined for each chlorophyll derivative based on the calculated regression coefficients from the appropriate Trolox® calibration curve. Results were expressed on a molar basis as mM Trolox® equivalent to activity 1 mM of chlorophyll derivative. The reduction of total his+ revertants relative to B[a]P controls, corrected for spontaneous mutations, is defined as Percent Inhibition (%I). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each chlorophyll derivative’s TEAC and %I. Group differences were determined by analysis of variance using Fisher’s PLSD post-hoc test ($\alpha < 0.05$).

5.4 Results & Discussion

Calibration and Optimization of Radical Scavenging Assay. DPPH• and ABTS•- assays are based on transfer of electrons from a suitable donor molecule to the corresponding radical resulting in a loss of color measurable at 515 and 734 nm respectively. Both assays have been routinely utilized in assessment of antioxidant capacity for crude fruit and vegetable extracts (Fogliano et al., 1999; Sanchez-Moreno et al., 1999; Lu & Foo, 2000; de Ancos et al., 2000), carotenoids (Miller et al., 1996; Jiménez-Escrig et al., 2000), and phenolics (Liebert et al., 1999).
Results of dose-dependent Trolox® calibration curves for both the stable DPPH and long lived ABTS⁺ radicals were analyzed by linear regression, and describe the relationship through the experimental concentration range by the following equations:

\[
\begin{align*}
\text{ABTS}^+ & \quad \Delta A_{734} = 2748.20 \text{ (mM Trolox®)} - 0.005 \quad R^2 = 0.9963 \quad (1) \\
\text{DPPH} & \quad \Delta A_{515} = 1291.70 \text{ (mM Trolox®)} + 0.005 \quad R^2 = 0.9998 \quad (2)
\end{align*}
\]

In order to optimize the assay for assessment of chlorophyll derivatives, concentration-dependence of experimental TEAC values was determined. Commercial grade SCC was chosen for preliminary investigation because this mixture of chlorin derivatives has previously demonstrated measurable antioxidant activity (Sato et al., 1983; 1984; 1985; 1986). A stock solution of 800 μmolL⁻¹ was serially diluted in 3-fold increments. Experimental TEAC values for SCC dilutions ranged from 0.5 to 1.3 mmolL⁻¹ for DPPH and 1.4 to 2.6 mmolL⁻¹ for ABTS⁺ assays with higher SCC concentrations resulting in reduced TEAC values (Figure 2). At the highest concentrations of SCC (800 μmolL⁻¹), TEAC values could not be determined with the ABTS⁺ assays since quenching was beyond the range of the Trolox calibration curves. The observed concentration dependence may be a result of minor background interference originating from SCC’s strong electronic absorption spectra. While not maintaining a maximum absorbance at assay wavelengths of 515 and 734 nm, highly concentrated chlorophyll solutions potentially introduce minor absorbance contributions (≤ 0.05 Abs) at the assay wavelengths, which manifest as an apparent reduction in
quenching efficacy. Conversely, minimal quenching at lower SCC concentrations resulted in only small reductions in absorbance at experimental wavelengths resulting in an increase in assay variability. For these reasons, a concentration of 100μM was employed for subsequent radical quenching experiments since this concentration provided both minimal background absorbance and excellent assay reproducibility.

**TEAC of Dietary Chlorophyll and Chlorophyllin Derivatives.** Dietary chlorophylls consumed primarily from fresh and processed green vegetable sources provide a variety of natural derivatives, including native Mg-chlorophylls, metal-free pheophytins, pyropheophytins, and in some cases zinc and copper complexes (Schwartz & Lorenzo, 1990; Ma & Dolphin, 1999; Ferruzzi et al., 2001). Standard solutions of 9 chlorophyll and 6 chlorophyllin derivatives were screened for antioxidant activity (Figure 5.1). TEACs for prominent dietary chlorophyll derivatives as determined from DPPH and ABTS$^-$ radical assays are shown in Tables 5.1 and 5.2, respectively.

Antioxidant capacity of native chlorophyll $a$ was significantly $(p < 0.01)$ higher than chlorophyll $b$ maintaining approximately three-fold higher TEAC in both DPPH$^-$ and ABTS$^-$ assays. Metal-free derivatives such as pheophytin and pyropheophytin had significantly $(p < 0.01)$ lower TEACs than native chlorophyll $a$, pointing to a potential loss of antioxidant activity as native chlorophylls are degraded/derivatized through food processing and human digestion. Previous studies with natural chlorophylls have yielded similar results. Hoshina et al. (1998) showed that chlorophyll $b$ had approximately 46% activity of chlorophyll $a$ in a ferric nitrilotriacetate induced singlet oxygen lipid peroxidation assay. Also, metal-free derivatives such as pheophytins and
pyropheophytins displayed only 17% of the activity of chlorophyll \(a\). Endo et al. (1985b) showed that while the basic porphyrin structure is crucial for free radical reduction, magnesium chelation strengthened apparent antioxidant activity of chlorophylls.

Modulation of antioxidant activity by metal chelation was further investigated with zinc and copper derivatives of pheophytins and pyropheophytin. A significant \(p < 0.001\) increase in TEACs was observed for metallo-chlorophyll derivatives compared to their metal free counterparts (Table 5.1 & 5.2). Experimental antioxidant activities of zinc and copper pheophytin were comparable to and in some instances greater than that of the parent magnesium chelate, chlorophyll \(a\) (Figure 5.3). Alteration of porphyrin electronic state by inclusion of a central metal atom is readily observed by the significant changes in the electronic absorption spectra of chlorophyll derivative (Sheer, 1991). Metal chelation would result in concentration of electron density toward the chelated metal and away from the porphyrin backbone of the chlorophyll molecule, resulting in an increased ability to donate electrons from the conjugated porphyrin system. The nature and extent to which this capacity is altered will greatly depend on the nature of the chelated metal. Suzuki et al. (1995) determined that while nickel, copper, and magnesium porphyrin derivatives suppressed superoxide mediated oxidation of linoleic acid, the iron derivatives enhanced the oxidation.

Degradation of natural chlorophylls to pheophytins and pyropheophytins during typical food processing and human digestive conditions is well documented. However, zinc and copper chlorophyll demonstrate excellent stability during both food processing and simulated human digestion (Schwartz and Lorenzo, 1990; Ferruzzi et al., 2001). Metallo-chlorophyll complexes are readily formed during vegetable tissue through
thermal processing in the presence of copper and zinc salts, resulting in a re-greening effect that has been commercialized in the VeriGreen® process (Jones et al., 1977; Segner et al., 1984). Total amount of zinc complexes of pheophytin and pyropheophytin approaches 35% of the total chlorophyll derivatives in typical VeriGreen® products (von Elbe et al., 1986). Addition of heavy metal salts for the purpose of re-greening metal-free pheophytins and pyropheophytins formed during food processing may have the secondary effect of modulating antioxidant capacity of the abundant chlorophyll fraction present in green vegetables. Derivation of improved strategies for more efficient in situ synthesis of metallo-chlorophyll derivatives in the diverse array of thermally processed green vegetable products merits attention in light of potential health benefits that would accompany any improvement in quality/sensory attributes by the re-greening process.

Commercial grade sodium copper chlorophyllin is derived from a crude natural chlorophyll extract by reaction with methanolic sodium hydroxide, followed by a replacement of the central magnesium atom with copper (Kephart, 1955). The final mixture is composed of numerous chlorin type compounds derived from natural chlorophyll, including two main components Cu(II)chlorin $e_4$ and Cu(II)chlorin $e_6$ (Inoue et al., 1994; Dashwood, 1997; Chernomorsky et al., 1997). Commercial SCC was found to have significantly ($p < 0.01$) higher antioxidant capacity than all natural chlorophyll derivatives in both DPPH• and ABTS•+ assays. As with the natural chlorophylls, metal-free chlorin derivatives maintained significantly ($p < 0.01$) lower activity compared to copper chelated chlorin derivatives. Specifically, chlorin $e_4$, chlorin $e_6$, and pheophorbide $a$ experienced a 3- to 5- fold increase in experimental TEAC values upon copper chelation (Table 5.1 & 5.2).
Antioxidative activities of commercial SCC preparations have been demonstrated previously. Sato et al. (1980) demonstrated the ability of crude SCC to reduce DPPH· with similar efficiency to α-tocopherol. Disodium Cu(II)isochorin e₄ and trisodium Cu(II)chlorin e₆ were identified as two main antioxidative components of commercial SCC preparations contributing approximately 92% of the observed activity (Sato et al., 1986). These results are consistent with our current findings regarding the contribution of copper chlorins to overall antioxidant capacity of SCC. While Cu(II)pheophorbide a maintained significant antioxidant capacity, it remains only a minor constituent in this SCC preparation and therefore most likely will not contribute significantly to the overall antioxidant activity of this commercial SCC preparation. The elevated experimental TEAC of Cu(II)chlorin e₄ and e₆, combined with the prominence of these two derivatives in commercial grade SCC, supports the notion of that these derivatives are the main antioxidative constituents of SCC.

**Antimutagenic Activity of Dietary Chlorophyll Derivatives.** Six dietary chlorophyll derivatives were further screened for their ability to inhibit the mutagenicity of B[a]P in a Salmonella typhimurium TA100 microscreen assay. Chlorophyll a and b, pheophytin a and b, Zn-pheophytin a, and Cu-pheophytin a were dissolved in sterile DMSO at concentrations between 40 and 400 μM, resulting in 1.0 to 10.0 nmol pigment per test well. Assays were conducted with 10 nmol of B[a]P per well providing 86 ± 5 his⁺ revertants per well corrected for spontaneous background revertants. Commercial grade SCC was utilized as an antimutagenic control in light of the numerous reports
highlighting the potent antimutagenic activity of this commercial preparation (Ong et al., 1986; Arimoto et al., 1995; Waters et al., 1996; Chernomorsky, 1997; Surh, 1998).

All chlorophyll derivatives exhibited strong dose-dependent antimutagenic activity (Figure 5.4). SCC was found to inhibit a maximum of $54 \pm 5\%$ of $B[a]P$ induced mutagenicity at its highest experimental concentration of $400 \mu M$ SCC (10 nmol pigment per well). These results are similar to those reported by Arimoto et al. (1995) who found that a 1:1 ratio of pigment:mutagen resulted in approximately 50% inhibition. Inhibition by all chlorophyll pigments did not differ significantly from each other or SCC ($p > 0.05$), ranging from 48 to 62 % at highest pigment concentrations (400 $\mu M$). Previous reports have highlighted antimutagenic behavior of chlorophyll $a$ and pheophytin $a$ (Okai et al., 1996; Yoshikawa et al., 1996; Chernomorsky et al., 1999). However, few have addressed derivatives of chlorophyll $b$ and lipophilic metallo-chlorophyll complexes such as Zn- and Cu-pheophytins. These results suggest that dietary chlorophyll derivatives of both natural and food processing origin maintain similar activity, and are as effective as commercial grade SCC in inhibiting $B[a]P$ induced mutagenesis in Salmonella typhymurium TA100. Furthermore, total conversion of native chlorophylls to metal-free pheophytin derivatives during digestion (Ferruzzi et al., 2001) should not impact the antimutagenic nature of plant derived chlorophylls in the gastrointestinal tract.

Hydrophobic mutagen trapping via complex formation between porphyrins and hydrophobic polycyclic planar mutagens, such as $B[a]P$, have been postulated as a potential mode of antimutagenic action (Arimoto et al., 1993). In this manner, chlorophylls are thought to reduce mutagen bioavailability and, by extension, relative mutagenicity/carcinogenicity (Breinholt et al., 1999). The presence of a transition metal
in the tetrapyrrole macrocycle has been postulated to alter potential antimutagenic activity (Odin, 1997). However, similar activity of all chlorophyll derivatives assayed in this investigation may indicate only minor alteration of the porphyrin:mutagen complex. The intact nature of the macrocycle has been shown to be the essential component in effective complex formation and antimutagenic behavior (Arimoto et al., 1995; Odin, 1997). While the specific nature of this inhibition merits further investigation, these data further support a role for the tetrapyrrole macrocycle, and not the presence of a central metal atom as a crucial component of chlorophyll antimutagenic activity.

5.5 Conclusions

Antioxidant and antimutagenic activity of prominent dietary chlorophyll derivatives were assayed in vitro. Both natural and commercially derived chlorophyll derivatives were chosen based on their prevalence in foods and dietary supplements. Antioxidant activity of natural chlorophylls was found to be significantly lower than commercial grade chlorophyllins. Metallo-chlorophyll derivatives, exhibited significantly higher antioxidant capacity than metal-free derivatives indicating that the nature of chlorophyll derivatives present in fresh and processed fruits and vegetables is important when considering antioxidant capacity of these foods. Conversely, metal-free and metallo-chlorophyll derivatives demonstrated equal dose-dependent inhibitory activity against B[α]P induced mutagenesis. These results further demonstrate the potential bioactivity of dietary chlorophyll derivatives and support a role for these phytochemicals in human health and disease prevention.
5.6 Abbreviations

DPPH', 1,1-diphenyl-2-picrylhydrazyl; ABTS', 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate); Trolox™, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; SCC, sodium copper chlorophyllin; B[a]P, benzo[a]pyrene; HPLC, high performance liquid chromatography.

5.7 Acknowledgments

The authors thank Ni Luh Puspitasari-Nienaber and Igor Milosevic for their assistance with derivative synthesis, purification and TEAC assay development. The kind assistance of Julie Jenkins with the antimutagenicity studies is greatly appreciated.
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Determination of copper (II) chlorophyllin by reversed-phase high-performance liquid

Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-

formation of zinc and copper complexes of chlorophyll derivatives in vegetable tissue by


chlorophyllin on lipid peroxidation. V. Effect on peroxidative damage of rat liver

on lipid peroxidation. VI. Effect on its administration on mitochondrial and microsomal

on lipid peroxidation. VIII. Its effects on carbon tetrachloride-induced liver injury in rats.

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Antioxidant activity of chlorophyll derivatives, oxygen stress relievers, to superoxide

<table>
<thead>
<tr>
<th>Chlorophyll Derivative</th>
<th>TEAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyropheophytin α</td>
<td>0.02 ± 0.01 a</td>
</tr>
<tr>
<td>Pheophytin α</td>
<td>0.04 ± 0.02 a</td>
</tr>
<tr>
<td>Pheophytin β</td>
<td>0.05 ± 0.02 ab</td>
</tr>
<tr>
<td>Chlorophyll β</td>
<td>0.06 ± 0.03 b</td>
</tr>
<tr>
<td>Zn-Pheophytin β</td>
<td>0.13 ± 0.03 c</td>
</tr>
<tr>
<td>Chlorophyll α</td>
<td>0.19 ± 0.02 d</td>
</tr>
<tr>
<td>Pheophorbide α</td>
<td>0.21 ± 0.02 d</td>
</tr>
<tr>
<td>Chlorin e₈</td>
<td>0.26 ± 0.01 d</td>
</tr>
<tr>
<td>Zn-Pyropheophytin α</td>
<td>0.44 ± 0.04 e</td>
</tr>
<tr>
<td>Zn-Pheophytin α</td>
<td>0.51 ± 0.04 f</td>
</tr>
<tr>
<td>Chlorin e₉</td>
<td>0.60 ± 0.01 g</td>
</tr>
<tr>
<td>Cu-Chlorin e₇</td>
<td>0.81 ± 0.02 h</td>
</tr>
<tr>
<td>Cu-Pheophorbide α</td>
<td>0.98 ± 0.09 i</td>
</tr>
<tr>
<td>Cu-Pheophytin α</td>
<td>0.99 ± 0.03 i</td>
</tr>
<tr>
<td>Crude SCC</td>
<td>1.04 ± 0.11 i</td>
</tr>
<tr>
<td>Cu-Chlorin e₅</td>
<td>2.88 ± 0.06 k</td>
</tr>
</tbody>
</table>

Table 5.1. Trolox equivalent antioxidant cap-acity (TEAC) for 15 prominent chlorophyll derivatives and crude SCC as determined by a DPPH radical quenching assays. TEAC data represent means ± SEM for 5 independent measurements. Presence of different letters indicate values which differed significantly as determined by ANOVA with Fisher’s PLSD post-hoc test (α < 0.05).
<table>
<thead>
<tr>
<th>Chlorophyll Derivative</th>
<th>TEAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pheophytin a</td>
<td>0.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pheophytin b</td>
<td>0.08 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyropheophytin a</td>
<td>0.16 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.23 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn-Pheophytin b</td>
<td>0.29 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn-Pheophytin a</td>
<td>0.43 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pheophorbide a</td>
<td>0.45 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorin e&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.53 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu-Pheophytin a</td>
<td>0.58 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorin e&lt;sub&gt;6&lt;/sub&gt;</td>
<td>0.64 ± 0.03&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn-Pyropheophytin a</td>
<td>0.67 ± 0.05&lt;sup&gt;a,h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.73 ± 0.05&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude SCC</td>
<td>1.25 ± 0.28&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu-Chlorin e&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.35 ± 0.14&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu-Chlorin e&lt;sub&gt;6&lt;/sub&gt;</td>
<td>2.25 ± 0.13&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cu-Pheophorbide a</td>
<td>2.40 ± 0.13&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 5.2. Trolox equivalent antioxidant capacity (TEAC) for 15 prominent chlorophyll derivatives and crude SCC as determined by a ABTS<sup>•</sup> radical quenching assays. TEAC data represent means ± SEM for 5 independent measurements. Presence of different letters indicate values which differed significantly as determined by ANOVA with Fisher’s PLSD post hoc test (α < 0.05).
Figure 5.1. Structure of 15 prominent dietary chlorophyll and chlorophyllin derivatives assayed for potential antioxidant activity. Pheophytin, pyropheophytin and corresponding Zn and Cu chelates were synthesized as described in Materials & Methods. SCC derivatives including, pheophorbide a, chlorin e₄ and e₅ and their corresponding Cu chelates were purchased from Frontier Scientific (Logan, UT). Purity of all derivatives was determined to be greater than 95%.
Figure 5.2. Concentration dependence of DPPH (●) and ABTS⁺ (○) quenching by SCC. Assays were conducted as described in Materials & Methods using methanolic solutions between 10 to 800 μmol L⁻¹ SCC. Data represent the means ± SEM of 5 independent measurements. The presence of different letters indicate significant (p < 0.01) differences between TEAC values for different SCC concentrations determined by ABTS⁺ (above) and DPPH⁻ (below) assays.
Figure 5.3. The effect of central metal chelation on experimental TEAC values for derivatives of pheophytin a. Data represent mean ± SEM for 5 independent measurements. The presence of different letters over the error bars indicate that TEAC values for pheophytin a were significantly lower than Mg-pheophytin a ($p < 0.001$), Zn-pheophytin a ($p < 0.001$), Cu-pheophytin a ($p < 0.001$) in the DPPH’ and ABTS‘ assays.
Figure 5.4. Inhibition of benzo[a]pyrene induced mutagenesis in *Salmonella typhimurium* TA100 by chlorophylls (panel A); pheophytins (panel B); and Zn- and Cu-pheophytin *a* (panel C). Microscreen assays were conducted as described in Materials & Methods utilizing 10nmol B[a]P resulting in 86 ± 5 his" revertants per well corrected for spontaneous background revertants. SCC was utilized as a antimutagenic control.
Chlorophylls belong to a larger class of phytochemical plant pigments that have been implicated in observational studies associating fruit and vegetable consumption to decreased disease risk. Investigations highlighting specific derivatives (chlorophylls, pheophorbides, pheophytins, and chlorins), preparations (natural vegetable extracts and SCC), and effects (antioxidant, antimutagenic, ant-inflammatory) have been reported in the literature. However, most fail to address important considerations such as the impact of food processing, digestion, absorption, and bioactivity of the parent compounds as modulated through each step. Examination of chlorophyll degradation through the digestive process was accomplished in order characterize the activity and absorption potential of prominent derivatives present in the gastrointestinal tract.

Fresh spinach puree, Heat+Acid treated spinach puree, Heat+ZnCl treated spinach puree, and commercial grade sodium copper chlorophyllin (SCC) were digested by a two step \textit{in vitro} process. Pheophytins were identified as predominant pigments micellarized from each spinach puree regardless of preparative treatment. Large amounts of Zn-pheophytins were noted in the micellar fraction of the ZnCl treated puree. SCC derivatives readily associated with the aqueous fraction, presumably due to their high aqueous solubility. Major SCC component Cu(II)chlroin e$_4$ underwent minimal degradation compared to Cu(II)chlroin e$_6$ which was found to be extremely sensitive to simulated digestion.
Cellular uptake studies were performed using both HTB-37 (parent) line and TC7 clones of Caco-2 cells. Both Caco-2 HTB-37 and TC7 clone, accumulated between 5.0-10.0% of micellarized chlorophyll derivatives from all spinach purees. SCC derivatives were readily accumulated by Caco-2 cells when the extracellular concentration was between 0.5 and 60ppm. Observed temperature dependent cellular accumulation of SCC in Caco-2 allows for the possibility of both passive and active transport for this class of chlorophyll derivative through the intestinal epithelia. The presence of chlorophyll derivatives such as pheophytins and SCC in both the micellar fraction and Caco-2 human intestinal cells suggests that these compounds can be absorbed through the digestive tract. Furthermore, the absence of chlorophyll \( a \) and \( b \) in the aqueous micellar fraction points to metal-free pheophytins formed during food processing and digestion as the biologically relevant forms of these pigments.

Predominant dietary chlorophyll derivatives identified through \textit{in vitro} studies were assayed for potential antioxidant activity with two radical scavenging assays, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS\(^{+}\)). Trolox equivalent antioxidant capacity (TEAC) for all derivatives ranged from 0.02 to 2.9 mmol/L and 0.02 to 2.4 mmol/L for DPPH and ABTS\(^{+}\) assays respectively. Antioxidant activity of natural chlorophylls was found to be significantly lower than commercial grade chlorophyllin derivatives. In general, metallo-chlorophyll derivatives exhibited significantly higher antioxidant capacity than metal-free derivatives, indicating that the nature of chlorophyll derivatives present in fresh and processed fruits and vegetables is important when considering antioxidant capacity of these foods.

Dietary chlorophyll derivatives were also screened for their ability to inhibit the mutagenicity of B[a]P in a \textit{Salmonella typhimurium} TA100 microscreen assay. Both metal free and metallo-chlorophyll derivatives demonstrated equal dose dependent
inhibitory activity against benzo[a]pyrene induced mutagenesis. These results further demonstrate the potential bioactivity of dietary chlorophyll derivatives and support a potential role for these phytochemicals in human health and disease prevention.

Results of this work will facilitate the design of future chemopreventive research on chlorophyll, since specific derivatives can be targeted and completed studies which have investigated biologically relevant derivatives can be validated. Future studies must move beyond reductionist \textit{in vitro} and cell culture models to address \textit{in vivo} absorption and distribution of chlorophyll derivatives. Furthermore, the true physiological implications of chlorophyll absorption into human intestinal epithelia cells must be addressed. The highly conserved nature of the porphyrin backbone places chlorophylls in the unique position of structural homology with numerous biologically relevant human porphyrins including heme and cytochromes. How specific dietary chlorophylls may interplay with the biochemistry of these compounds may provide further insight into biological activity of these plant pigments. Also, the development of improved \textit{in situ} synthesis strategies for biologically relevant chlorophyll derivatives could potentially add a secondary functional benefit to value-added processed fruit and vegetable products. Information gained from these studies continues to support the association between consumption of chlorophyll rich foods and risk or outcomes of specific diseases.
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APPENDIX A:
Micellarization of chlorophyll derivatives from commercially available green vegetables products.
Table A1. Average pigment content of green vegetable test meals. Amount given in nmol pigment per 100 g test meal. Abbreviations: CHL a = Total chlorophyll a derivatives; CHL b = Total chlorophyll b derivatives. Test meals were prepared by complete homogenization of vegetable tissue with minimal water. Lipid content was adjusted to 10% (wt/wt) by the addition of corn oil. Pigment content was determined by complete acetone extraction followed C18 HPLC analysis with diode array detection as described in Appendix B.
<table>
<thead>
<tr>
<th>Sample</th>
<th>CHL a</th>
<th>CHL b</th>
<th>Total CHL</th>
<th>β-carotene</th>
<th>Lutein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Spinach</td>
<td>56.98 ± 3.27</td>
<td>20.74 ± 2.72</td>
<td>47.30 ± 2.96</td>
<td>35.78 ± 5.61</td>
<td>22.07 ± 2.12</td>
</tr>
<tr>
<td>Canned Spinach</td>
<td>27.60 ± 3.64</td>
<td>25.80 ± 0.74</td>
<td>25.85 ± 0.17</td>
<td>30.61 ± 1.90</td>
<td>35.97 ± 1.44</td>
</tr>
<tr>
<td>Fresh Broccoli</td>
<td>34.49 ± 1.90</td>
<td>27.80 ± 0.82</td>
<td>32.84 ± 1.45</td>
<td>31.82 ± 3.36</td>
<td>61.78 ± 2.20</td>
</tr>
<tr>
<td>Frozen Peas</td>
<td>42.83 ± 3.17</td>
<td>38.05 ± 2.45</td>
<td>41.72 ± 2.98</td>
<td>28.16 ± 4.54</td>
<td>87.20 ± 6.70</td>
</tr>
<tr>
<td>Canned Peas</td>
<td>28.47 ± 0.72</td>
<td>24.71 ± 1.42</td>
<td>27.76 ± 0.86</td>
<td>21.76 ± 2.52</td>
<td>61.11 ± 0.48</td>
</tr>
<tr>
<td>Fresh Green Beans</td>
<td>33.29 ± 1.53</td>
<td>27.32 ± 1.83</td>
<td>32.13 ± 1.58</td>
<td>N/D</td>
<td>87.76 ± 4.96</td>
</tr>
<tr>
<td>Canned Green Beans</td>
<td>22.33 ± 2.04</td>
<td>15.60 ± 1.91</td>
<td>21.18 ± 2.02</td>
<td>41.09 ± 1.29</td>
<td>65.21 ± 5.30</td>
</tr>
<tr>
<td>Veri-Green Beans</td>
<td>36.60 ± 1.83</td>
<td>33.21 ± 1.44</td>
<td>34.10 ± 0.68</td>
<td>36.22 ± 1.95</td>
<td>57.19 ± 1.45</td>
</tr>
</tbody>
</table>

Table A2. Pigment micellarization efficiency (%M) from commercially available green vegetable products. Abbreviations: CHL a = Total chlorophyll a derivatives; CHL b = Total chlorophyll b derivatives. Test meals were digested as described in Chapter 2. Pigment content was determined for each digestive fraction by complete acetone extraction followed by C18 HPLC analysis with diode array detection as described in Appendix B. Micellarization efficiency is expressed as percent micellarization ± SEM.
Figure A1. Average micellarization efficiency of pigments from 8 commercially available green vegetable preparations. CHL a = Total chlorophyll a derivatives; CHL b = Total chlorophyll b derivatives. Digestions and analyses were performed as described in Chapter 2: Materials & Methods. Values represent means ± SEM of 32 independent digestions.
APPENDIX B:
Collected Analytical Methods
Extraction of Chlorophyll Derivatives from Green Vegetable Tissue

Compound(s) of interest: All major natural chlorophyll derivatives.

Materials Required:
- Acetone
- Petroleum Ether
- Sodium Bicarbonate
- 5% Aqueous Sodium Sulfate
- Crystalline Sodium Sulfate
- Blender or Homogenizer
- #42 and #1 Filter paper
- Funnel
- Vacuum Flask
- Separatory funnel
- 50mL volumetric flask
- Pasteur pipettes

Method:
1. Weigh out ~5-10g of chopped vegetable tissue in a 100 mL beaker.
2. Add ~5g of sodium bicarbonate to neutralize organic acid release.
3. Add 50 mL of acetone.
4. Homogenize at 10,000 rpm for 30 seconds.
5. Vacuum filter through #1 and #42 filter paper into vacuum flask.
6. Quantitatively collect filtrate and return to beaker.
7. Add 25 mL of acetone and homogenize.
8. Vacuum filter through #1 and #42 into vacuum flask.
9. Repeat until no residual color remains in plant tissue filtrate (3X minimum).
10. Quantitatively transfer acetone extract into 250mL separatory funnel.
11. Add 35mL of petroleum ether to acetone and mix.
12. Wash with ~10mL of 5% aqueous sodium sulfate followed by excess distilled water.
13. Drain excess aqueous layer and collect petroleum ether.
14. Dry petroleum ether through crystalline sodium sulfate and dilute in 50 mL volumetric flask.
15. Dry aliquots of extracts, redissolve in 100% acetone for immediate analysis.
**Extraction of Chlorophyll Derivatives From Caco-2 Cells**

Compound(s) of interest: All major natural chlorophyll derivatives.

**Materials Required:**

- Acetone
- Phosphate buffered saline (pH = 7.4)
- Protease solution
  - 100 mg protease (Sigma-Aldrich; St. Louis, MO) into 10 mL PBS.
- SDS-EtOH BHT solution
  - 1 mL of 20% aqueous SDS into 19 mL of ethanol (0.1% BHT)
- Acetone:Petroleum Ether:BHT solution
  - 1 part Acetone into 2 parts petroleum ether (0.1% BHT)
- Pasteur pipettes
- 2 mL microcentrifuge tubes
- Microcentrifuge

**Method:**

1. Thaw samples at room temperature.
2. To each cell pellet, add 200 µL of protease solution and digest at 37°C for 30 minutes.
3. Add 500 µL of SDS-EtOH-BHT solution and vortex for 30 seconds.
4. Add 500 µL of acetone:petroleum ether (0.1% BHT).
5. Vortex 60 seconds.
6. Centrifuge @ 13,000 x g for 1 minute.
7. Remove petroleum ether layer into 11 mL glass vial.
8. Repeat extraction for total of 3X.
9. Combine petroleum ether layers and dry under nitrogen.
10. Resolubilize in 100% acetone for immediate analysis.
Extraction of Chlorophyllin Derivatives from Food Preparations

Compound(s) of interest: Derivatives of commercial grade sodium copper chlorophyllin.

Materials Required:

- Methanol
- Phosphate buffered saline (pH = 7.4)
- Acidified phosphate buffered saline (pH = 3.0)
  
  *Standard PBS acidified with 1N HCl to pH 3*
- Diethyl ether
- BHT solution
  
  *1 part Acetone into 2 parts petroleum ether (0.1% BHT)*
- Crystalline Sodium Sulfate
- Pasteur pipettes
- 50 mL centrifuge tubes
- 100 mL volumetric flask
- Blender or Homogenizer
- Centrifuge

Method:

1. Weigh out ~0.5-1g of material into 50 mL centrifuge tube.
2. Add 5 mL acidified PBS.
3. Homogenize for 30 seconds at 10,000 rpm.
4. Add 25 mL of diethyl ether (0.1% BHT).
5. Vortex for 60 seconds.
6. Centrifuge at 2000 x g for 5 minutes.
7. Quantitatively remove diethyl ether layer.
8. Repeat extract for a total of 3X.
10. Dry combined diethyl ether through crystalline sodium sulfate and dilute in 100 mL volumetric flask.
11. Dry aliquots of extract, redissolve in 100% methanol for immediate analysis.
Extraction of Chlorophyllin Derivatives from Caco-2 Cells

Compound(s) of interest: Derivatives of commercial grade sodium copper chlorophyllin.

Materials Required:

- Methanol
- Phosphate buffered saline (pH = 7.4)
- Acidified phosphate buffered saline (pH = 3.0)
  
  *Standard PBS acidified with 1N HCL to pH 3*
- Protease solution
  
  *100 mg protease (Sigma-Aldrich) into 10 mL PBS.*
- SDS-EtOH BHT solution
  
  *1 mL of 20% aqueous SDS into 19 mL of ethanol (0.1% BHT)*
- Diethyl ether:BHT solution
  
  *1 part Acetone into 2 parts petroleum ether (0.1% BHT)*
- Pasteur pipettes
- 2 mL microcentrifuge tubes
- Microcentrifuge

Method:

1. Thaw samples at room temperature.
2. To each cell pellet, add 200 μL of protease solution and digest at 37°C for 30 minutes.
3. Add 500 μL of SDS-EtOH-BHT solution and vortex for 30 seconds.
4. Add 500 μL of diethyl ether (0.1%BHT).
5. Vortex 60 seconds.
6. Centrifuge @ 13,000 x g for 1 minute.
7. Remove diethyl ether layer into 11 mL glass vial.
8. Repeat extraction for total of 3X.
9. Combine petroleum ether layers and dry under nitrogen.
10. Redissolve in 100% methanol for immediate analysis.
Synthesis of Pheophytin Derivatives from Chlorophyll

Materials Required:

Chlorophyll a and b (Sigma-Aldrich)
0.1N HCl
HPLC grade Acetone
HPLC grade Petroleum Ether
11 mL glass vials

Method:

1. Dissolve 1 mg chlorophyll in 4 mL of acetone.
2. Split into 4 x 1 mL aliquots in 11 mL vials.
3. To each vial add ~ 200 μL of 0.1N HCl.
   Solution should visibly and rapidly (no longer than 1 min) turn from green to olive brown indicating a complete conversion. Chlorophyll b derivatives may require more time but no more than 5 min in order to avoid epimerization and allomerization.
4. After complete conversion extract pheophytins into 4 mL of petroleum ether for each vial.
5. Retain aliquot for HPLC purity check.
6. Dry each vial under stream of nitrogen.
7. Rap in foil and store frozen in desiccant.
Synthesis of Zinc and Copper Pheophytin Derivatives from Chlorophyll

Materials Required:

Chlorophyll a and b (Sigma-Aldrich)
0.1N HCl
HPLC grade Acetone
HPLC grade Petroleum Ether
ZnCl₂
CuCl₂
11mL glass vials

Method:

1. Synthesize corresponding pheophytin from appropriate chlorophyll derivative as described above.
2. To each vial add excess metal salt and mix thoroughly. Solution should visibly and rapidly (no longer than 1 min) turn from olive brown to blue green indicating a complete conversion. Pheophytin b and both copper derivatives may require more time but no more than 5 min in order to avoid epimerization and allomerization.
3. After complete conversion extract Zn- or Cu-pheophytins into 4 mL of petroleum ether for each vial.
4. Retain aliquot for purity determination by HPLC.
5. Dry each vial under stream of nitrogen.
6. Wrap in foil and store frozen in desiccant.
Chromatographic Separation of Chlorophyll by RP-C18 Method

Compound(s) of interest: Lutein, beta-carotene, chlorophyll a and b, pheophytin a and b, pyropheophytin a and b.

Detector: Photodiode Array

Wavelengths monitored: 350nm & 700nm  Maximum wavelength: 450nm & 661nm

Column (type, dimensions): Vydac 201TP54 (4.6 x 250 mm)

Guard Column: C18 at your discretion

Injection Volume: 5-100 μL  Injection Solvent: Acetone or Methanol

Flow Rate: 1.0 mL/min

Column Temperature: Ambient  Sample Temperature: Ambient

Solvents:
Solvent A: 98% MeOH: 2% 1M Ammonium Acetate
Solvent B: 100% HPLC Water
Solvent C: 100% Ethyl Acetate

Isocratic  Gradient

Timetable:

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<tr>
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<th>%A</th>
<th>%B</th>
<th>%C</th>
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<td>75.0</td>
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<td>Linear</td>
</tr>
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Comments:
Dry extracts should be dissolved in 100% HPLC and ACS grade acetone. The volume of acetone is dependent on the original concentration, volume of the dried extract, and on the desired concentration range for the analysis. Highly concentrated samples such as spinach may need to be diluted to insure adherence to the linear range as prescribed by the response curves for each chlorophyll derivative. Pass extract through a 2μm filter using a disposable 5.0mL syringe.
Sample chromatogram:

Typical HPLC chromatogram of four major chlorophyll derivatives. Peak identifications: 1 = Chlorophyll b, 2 = Chlorophyll b', 3 = Chlorophyll a, 4 = Chlorophyll a', 5 = Pheophytin b, 6 = Pheophytin b', 7 = Pheophytin a, 8 = Pheophytin a'.
**Chromatographic Separation of Copper Chlorophyllin by RP-C18 Method.**

Compound(s) of interest: Cu(II)chlorin e₄, Cu(II)chlorin e₆, Cu(II)pheophorbide a, Cu(II)rhodin g₇.

Detector: Photodiode Array

Wavelengths monitored: 350nm & 700nm  Maximum wavelength: 409nm & 627nm

Column (type, dimensions): Beckman Ultrasphere 5µm (4.6 x 150 mm)

Guard Column: C18 at your discretion

Injection Volume: 5-100 µL  Injection Solvent: Water or Methanol

Flow Rate: 1.0 mL/min

Column Temperature: Ambient  Sample Temperature: Ambient

Solvents:
- Solvent A: 99.5% MeOH: 0.5% Glacial Acetic Acid
- Solvent B: 100% HPLC Water
- Solvent C: 100% Ethyl Acetate

Isocratic ___  Gradient ___

Timetable:

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Comments:

Mobile phase pH should be 3.5 +/- .2. Adjust with Glacial acetic accordingly. Dry extracts should be dissolved in 100% HPLC and ACS grade methanol. Volume of methanol is dependent on original concentration, volume of the dried extract, and on the desired concentration range for the analysis. Pass extract through a 2µm filter using a disposable 5.0mL syringe. Detection and tentative identification of major SCC components is accomplished by co-chromatography with authentic standards and comparison of electronic absorption spectra obtained from in-line diode array detection with previously published electronic absorption spectra of SCC derivatives.
HPLC separation of predominate derivatives of commercial grade sodium copper chlorophyllin. Peak identifications: 1 = Cu(II)rhodin g₇; 2 = Cu(II)chlorin e₆; 3 = Cu(II)chlorin e₄; 4 = Cu(II) pheophorbide a.
**Preparative Separation of Chlorophyll a and b by RP-C30 HPLC**

Compound(s) of interest: Chlorophyll a and b.

**Detector:** UV-Visible

Wavelengths monitored: 654 nm

**Column (type, dimensions):** YMC C30 S-5 200A (20 x 250 mm)

**Guard Column:** C18 at your discretion

**Injection Volume:** 1-3.0 mL  
**Injection Solvent:** Acetone

**Flow Rate:** 5.0 mL/min

**Column Temperature:** Ambient  
**Sample Temperature:** Ambient

**Solvants:**
- Solvent A: 90% MeOH: 5% Ethyl Acetate: 5% Water
- Solvent B: 50% MeOH: 50% Ethyl Acetate

**Isocratic_____ Gradient___ x**

**Timetable:**

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<th>%B</th>
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**Comments:**

Purification should be done on high concentration samples such as spinach extracts. Dry extracts should be dissolved in 100% HPLC and ACS grade acetone. Injection solutions should be highly concentrated to ensure maximum collection. Pass extract through a 2μm filter using a disposable 5.0mL syringe. Begin collection half way up the slope of the desired peak and terminate half way down. This will ensure maximum purity while allowing for significant collection. Final purity checks should be completed by analytical scale methods. Extracts should be dried under nitrogen gas and stored frozen and desiccated. Samples should be utilized within 1 month.
Sample chromatogram:

Preparative HPLC chromatogram of Chlorophyll b and Chlorophyll a.
Preparative Separation of Pheophytin a and b by RP-C30 HPLC

Compound(s) of interest: Pheophytin a and b,

**Detector:** UV-Visible

Wavelengths monitored: 654 nm

**Column (type, dimensions):** YMC C30 S-5 200A (20 x 250 mm)

**Guard Column:** C18 at your discretion

**Injection Volume:** 1-3.0 mL

**Injection Solvent:** Acetone

**Flow Rate:** 5.0 mL/min

**Column Temperature:** Ambient

**Sample Temperature:** Ambient

**Solvents:**

Solvent A: 90% MeOH: 5% Ethyl Acetate: 5% Water

Solvent B: 50% MeOH: 50% Ethyl Acetate

Isocratic _____ Gradient x

**Timetable:**

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**Comments:**

Purification should be done on high concentration samples such as spinach extracts. Dry extracts should be dissolved in 100% HPLC and ACS grade acetone. Injection solutions should be highly concentrated to ensure maximum collection. Pass extract through a 2μm filter using a disposable 5.0mL syringe. Begin collection half way up the slope of the desired peak and terminate half way down. This will ensure maximum purity while allowing for significant collection. Final purity checks should be completed by analytical scale methods. Extracts should be dried under nitrogen gas and stored frozen and desiccated. Samples should be utilized within 1 month.
Sample chromatogram:

Preparative HPLC chromatogram of Pheophytin b and Pheophytin a from processed spinach.
APPENDIX C
Published Abstracts
CHLOROPHYLL DEGRADATION PRODUCTS ARE MICELLARIZED FROM SPINACH PUREE BY AN IN VITRO DIGESTION PROCESS.

M.G. Ferruzzi, D.A. Garrett*, M.L. Failla*, S.J. Schwartz. Dept. of Food Science & Technology, The Ohio State University, Columbus, OH 43210 and *The University of North Carolina, Greensboro, NC 27402.

Presented at the Institute of Food Technologist Annual Meeting 1999; Chicago, IL.

JUSTIFICATION: Numerous studies have shown that chlorophyll and Cu-chlorophyllin, a water-soluble chlorophyll derivative, possess antimutagenic and antigentoxic activities. However, little information about the absorption and metabolism of these compounds by humans is available. In vitro digestion has been used to assess the relative bioavailability of numerous minerals and nutrients from different food matrices.

OBJECTIVE: In order to establish a better understanding of chlorophyll absorption in humans, food samples rich in these pigments were subjected to an in vitro digestion to mimic the in vivo process.

METHODS: Fresh spinach puree, Heat+Acid treated spinach puree, Heat+ZnCl treated spinach puree, and commercial grade Cu-chlorophyllin were digested by a two step process. A gastric phase including acidification and pepsin treatment was followed by an intestinal phase which involved neutralization and treatment with pancreatin and bile extract. Samples were then centrifuged to separate the aqueous micellar phase from the solid non-digested fraction. Chlorophyll components that are transferred from the food to the aqueous phase are possibly bioavailable. The aqueous fraction was then analyzed by C18 HPLC with photodiode array detection.

RESULTS: Pheophytins and pyropheophytins were micellarized from each of the three spinach puree regardless of preparative treatment. Large amounts of Zn-pheophytins were noted in the micellar fraction of the ZnCl treated puree. Cu-chlorophyllin was readily micellarized without undergoing any and degradation.

SIGNIFICANCE: The presence of chlorophyll derivatives such as pheophytins and chlorophyllins in the micellar fraction suggests that these compounds can be absorbed through the digestive tract. Furthermore, the absence of chlorophyll a and b in the aqueous micellar fraction points to degradation products formed during digestion such as pheophytins and pyropheophytins as the biologically absorbable forms of these pigments.
Factors governing the higher ratio of lycopene cis-trans isomers in human biological fluids and tissues relative to those in the diet remain unclear. Using an in vitro digestive system, we monitored the extent of lycopene isomerization prior to absorption for nine different lycopene sources. The five varieties of fresh tomatoes that were studied included normal red, reduced total carotenoid, high beta-carotene, high delta-carotene and tangerine-type containing high level of cis lycopenes. Commercial lycopene sources such as tomato sauce, and guava baby food along with supplement pills and standards in applesauce were also subjected to digestion. All samples were normalized to contain 10% vegetable oil before digestion. C30 reversed phase HPLC analysis of micellarized carotenoids from digesta fractions revealed that lycopene is stable in both the gastric and intestinal phases of digestion. Likewise, the distribution of geometrical isomers for other tomato carotenoids including lycopene, beta-carotene, delta-carotene and gamma-carotene also remain the same. The results from this in vitro study indicate that the likelihood of cis isomers of lycopene and other carotenoids being incorporated into micelles is similar to their all-trans counterparts. This finding, along with our previous published report of lycopene's remarkable stability during food processing, suggests that the ratio of lycopene cis-trans isomers in foods is not altered during the digestive process. This information is useful in our current effort to investigate the bioavailability and tissue deposition pattern of lycopene geometrical isomers in humans.
THERMAL DEGRADATION OF SODIUM COPPER CHLOROPHYLLIN.

I. Milosevic, M.G. Ferruzzi, S.J. Schwartz. Dept. of Food Science & Technology, The Ohio State University, Columbus, OH 43210

Presented at the Institute of Food Technologist Annual Meeting 2000; Dallas, TX.

JUSTIFICATION: Sodium copper chlorophyllin (SCC), a water-soluble commercial derivative of chlorophyll, has gained importance as a legally accepted food colorant within Japan and the European Community, and as a common dietary supplement found in both liquid and powdered form. While many studies demonstrate the antimutagenic and antioxidant properties of this compound, very little information is available on the stability of SCC to food processing conditions, specifically thermal treatments.

OBJECTIVE: The stability of SCC was studied to assess the potential usefulness of this chlorophyll derivative as a commercial grade food colorant and dietary supplement for use in thermally processed foods.

METHODS: Aqueous solutions of SCC were prepared from a commercial grade standard. Thermal degradation at 25, 37, 50, 70, and 100°C of 500ppm of SCC solutions was monitored by a loss of UV-Vis absorbance at 627nm over a 60-minute period. Decomposition was also followed by reverse phase C18 HPLC with photodiode array detection in order to monitor the loss of specific SCC components and the formation of distinct degradation products.

RESULTS: The rate of thermal degradation of SCC was found to follow first order reaction kinetics. HPLC analysis confirmed the UV-Vis data and also demonstrated loss of the major SCC component, Cu-Chlorin e₄, at a faster rate than the overall SCC. Activation energy (Ea) was estimated using the Arrhenius equation and found to be 16.1 Kcal/mol for the thermal degradation of SCC and 16.2 Kcal/mol for Cu-Chlorin e₄ in SCC preparations.

SIGNIFICANCE: The observed temperature sensitivity of SCC raises the possibility of color deterioration when used in food products where mild to severe thermal treatment is necessary. Furthermore, the implication of rapid loss of Cu-Chlorin e₄, the major component of SCC, upon heating may result in a change in the potential dietary benefits.
SIMULTANEOUS DETECTION OF TOCOPHEROLs, CAROTENOIDS AND CHLOROPHYLLs IN VEGETABLE OILS BY DIRECT INJECTION ON RP-HPLC WITH COULOMETRIC ELECTROCHEMICAL ARRAY DETECTION.

N.L. Puspitasari-Nienaber, M.G. Ferruzzi, and S.J. Schwartz Dept. of Food Science & Technology, The Ohio State University, Columbus, OH 43210

Presented at the Institute of Food Technologist Annual Meeting 2000; Dallas, TX.

JUSTIFICATION: In addition to the fatty acid and triglyceride composition, edible oil purity criteria is often determined by the characteristic profile of minor constituents such as tocopherols, sterols, chlorophylls, and carotenoids. These compounds are isolated in the unsaponifiable fraction and analyzed separately by GC and/or HPLC using different detectors. Simultaneous detection of these compounds with electrochemical detection would allow for rapid and sensitive analysis by direct injection of diluted oil samples. Sample loss due to saponification could also be avoided.

OBJECTIVE: To demonstrate the application of direct injection on RP-HPLC with coulometric electrochemical array detection in the simultaneous detection of tocopherols, carotenoids and chlorophyll in vegetable oils.

METHODS: Vegetable oils were dissolved in appropriate organic solvents. Tocopherols, carotenoids and chlorophylls were separated by direct injection on a C30 reversed-phase HPLC column using a coulometric electrochemical array detector equipped with eight channels in series.

RESULTS: Effective separation of tocopherols (α, δ, γ tocopherols, α and δ tocotrienols), carotenes (α, β carotenes and their isomers), chlorophylls, and pheophytins was achieved. The characteristic hydrodynamic voltammo-grams (HDV) can be used in the identification of each compound. Detection limits for tocopherols, carotenes and chlorophylls were approximately 5-1000 fold better than the conventional UV-vis detection.

SIGNIFICANCE: This method can be applied for rapid and sensitive analysis in the study of oil quality and adulteration.
UPTAKE OF MICELLAR CHLOROPHYLL DERIVATIVES BY CACO-2 HUMAN INTESTINAL CELLS.


Presented at Experimental Biology Annual Meeting 2000; San Diego, CA.

While numerous studies have demonstrated the health benefits of chlorophyll derivatives, very little research has been done to ascertain the degree to which humans adsorb and metabolize these phytochemicals. To better understand the digestion of these pigments, fresh spinach puree (FSP), heat-acid treated spinach puree (HASP), and sodium copper chlorophyllin powder (SCC) were subjected to a two stage in vitro digestion method, which includes acidification and incubation with pepsin (gastric phase) followed by neutralization and incubation with pancreatin and bile extract (small intestinal phase. Transfer of the lipophyllic chlorophyll derivatives, as well as carotenoids lutein and β-carotene, into the aqueous micellar phase was subsequently quantified. Cellular uptake studies were performed using both HTB-37 (parent) line and TC7 clones of Caco-2 cells. Fully differentiated monolayers were washed and then incubated with DMEM media containing 25% chlorophyll rich aqueous micellar fraction from the in vitro digestion. Cells were then washed, extracted for pigments, and analyzed by C18 HPLC with photodiode array detection. Native chlorophylls degraded during the in vitro digestion process to the metal-free derivatives pheophytin and pyropheophytins. Conversely, SCC was found to be very stable during the digestive process. Both Caco-2 HTB-37 and TC7 clone accumulated between 5.0-10.0% of micellarized chlorophyll derivatives compared to 20-40% of micellarized carotenoids from all spinach purees. These results are the first to demonstrate uptake of chlorophyll derivatives by human intestinal cells and support the potential importance of chlorophylls as health promoting phytochemicals.
APPLICATION OF A C30 HPLC-EC METHOD TO ASSESS POST-PRANDIAL CAROTENOID ABSORPTION FROM VEGETABLES IN HUMANS.


Presented at Experimental Biology Annual Meeting 2000; San Diego, CA.

Conventional detection methods only afford the measurement of carotenoids in total plasma instead of newly absorbed carotenoids in the plasma chylomicron fraction. Our objective is to demonstrate the ability of an HPLC-EC method to accurately quantify low levels of dietary carotenoids and their isomers in the chylomicron fraction. Subjects consumed a representative salad (247 g) along with salad dressing (60 g). Blood samples (10mL) were drawn at 0, 3, 6 and 9 hours following the consumption of the test meals and chylomicron fraction isolated by ultracentrifugation. α-carotene, β-carotene and lycopene in various geometrical forms were extracted with hexane containing 0.1% w/v BHT, separated via a C30 gradient HPLC method and quantified by an eight-channel coulometric electrochemical array detector. Under the potential setting of 200 to 680 mV, all-trans carotenoids and several corresponding cis isomers were detected in all chylomicron samples at above the 1 fmol detection limit. The enhanced sensitivity of this method allows for the direct comparison of bioavailable carotenoids in plasma chylomicron fraction as a function of fat levels and post-injection time while facilitating the reduction in sample volume of each blood collection. We expect to implement this method to study key factors influencing the bioavailability of major carotenoids and formulate strategies to enhance their absorption from the diet. (Supported by the Procter & Gamble Co.)
Control of *in vivo* oxidative stress is thought to decrease the relative risk of specific disease states including cancer and cardiovascular disorders. Chlorophylls, the most abundant class of natural plant pigments, have demonstrated potent antimutagenic activity both *in vitro* and *in vivo* studies. While antioxidant capacity for select chlorophyll derivatives has been demonstrated, this potential activity remains largely under investigated. To better ascertain the antioxidant potential of chlorophylls, prominent dietary derivatives were assayed for their ability to scavenge two different long lived free radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS). Derivatives common to both fresh and processed foods were chosen including: Native chlorophylls, metal free pheophytins and pyropheophytins, Zn chelates of pheophytins and pyropheophytins and commercial grade chlorophyllins. Trolox equivalent antioxidant capacity (TEAC) was calculated for each derivative based on calibration curves for both radicals. Experimental TEAC values for all derivatives ranged from 0.01-1.0 mmol/L and 0.01-1.5 mmol/L for DPPH and ABTS assays respectively. Derivatives of chlorophyll a were found to be more effective radical quenchers than those of chlorophyll b. Furthermore, metal free derivatives such as pheophytins and pyropheophytins were found to have significantly lower antiradical capacity than those with centrally bound metals such as Mg-chlorophylls, Zn-pheophytins, Zn-pyropheophytins and Cu-chlorophyllins. These results demonstrate antioxidant activity of dietary chlorophyll derivatives prevalent in both fresh and processed foods.
DIGESTION, MICELLARIZATION AND INTESTINAL CELL UPTAKE OF TRANS AND CIS β-CAROTENE.

Univ. North Carolina Greensboro, NC 27402 and The Ohio State University, OH 43210.

Presented at Experimental Biology 2001 Annual Meeting; Orlando, Florida.

Insights regarding the basis for the difference among isomer ratios of carotenoids in foods, body fluids and tissues are limited. We used in vitro digestion and the Caco-2 human intestinal cell line to examine the transfer of β-carotene (BC) isomers from a meal to micelles with subsequent uptake into cells. Dunaliella salina (DS) (68% trans, 17% 9-cis, 6% 13-cis, 9% 15-cis BC) or water-soluble beadlets (WSB) (87% trans, 2% 9-cis, 9% 13-cis, 2% 15-cis BC) were added to a meal containing applesauce and 7.5% corn oil. Meals were homogenized and subjected to simulated gastric and small intestinal digestion. The micellar fraction was isolated and BC isomers in test meal, digestate, and micellar fraction were quantified by HPLC. Results show that BC isomers are stable during the digestion process. Efficiency (%) of micellarization was: 15-cis > 9-cis > 13-cis > trans (DS meal) and 13-cis > 15-cis > 9-cis > trans (WSB meal). Aqueous fractions were diluted 1:4 with basal DMEM medium and added to differentiated cultures of Caco-2 cells to evaluate isomeric profile following cellular uptake. Cells accumulated 18% (DS meal) and 10% (WSB meal) of BC from medium after 4-h. The intracellular profile of BC isomers (% trans > % cis) differed from that in micelles. Moreover, the intracellular ratio of trans to cis isomers of BC further increased when cells pre-loaded with BC were incubated overnight. Concentrations of micellarized isomers were not altered after incubation in cell-free dishes after 18-h. These data suggest that cis isomers of BC are preferentially micellarized during digestion and that cis isomers are either transferred across the brush border surface less efficiently than trans isomers and/or isomerized within the cell.
USE OF ELECTROCHEMICAL DETECTION TO QUANTIFY THE EFFECT
OF ADDED FAT ON INTESTINAL CAROTENOID ABSORPTION FROM
FRESH VEGETABLES IN HUMANS.

W.S. White, M.B. Brown, M.G. Ferruzzi, M.L. Nguyen, D.A. Cooper, A.L. Eldridge,
and S.J. Schwartz. Dept. of Food Science and Human Nutrition, Iowa State Univ., Ames,
IA 50011; Dept. of Food Science, Ohio State Univ., Columbus, OH 43210; The Procter
& Gamble Co., Cincinnati, OH 45224.

Presented at the Gordon Conference on Carotenoids 2001; Ventura, CA.

We previously showed that HPLC with high-sensitivity coulometric electrochemical
array detection (ECD) can be used to quantify the small amounts of carotenoids absorbed
from a single serving of fresh vegetables in humans (Ferruzzi et al., FASEB J 14:A485,
2000). The objective of this project was to apply the same technology to compare the
appearance of carotenoids in the plasma chylomicron fraction after subjects ingested
fresh vegetable salads with fat-free, reduced-fat, or full-fat salad dressings. The subjects
(n = 7) were healthy, normolipidemic men and women. Each salad consisted of 48 g
spinach, 48 g romaine lettuce, 66 g grated carrot, and 85 g cherry tomato. The salad
dressings were prepared from a commercial fat-free salad dressing mix with varying
amounts of added canola oil to provide either 0 g, 6 g, or 28 g of fat per 60-g serving.
The salads with the 3 salad dressings were ingested in random order and separated by a
washout period of ≥ 2 weeks. After a fasting blood sample was drawn, the subjects
consumed the test salad within 30 minutes. Blood samples were then drawn at hourly
intervals for 9 h. The plasma chylomicron fraction was isolated by cumulative rate
ultracentrifugation (Hu et al., Am J Clin Nutr 71:1170-80, 2000). The carotenoids were
extracted with hexane:acetone (3:1 by vol) and analyzed by HPLC–ECD (Ferruzzi et al.,
Anal Biochem 256:74-81, 1998). After ingestion of the salads with the fat-free salad
dressing, the appearance of carotenoids in the plasma chylomicron fraction was low but
detectable. After ingestion of the salads with the reduced-fat as compared with the full-
fat salad dressing, the areas under the curve (AUCs) for α-carotene, β-carotene, and
lycopene in the chylomicron fraction were decreased by 60.9 ± 24.9% (P = 0.05), 53.0 ±
23.5% (P < 0.07), and 76.3 ± 33.1% (P = 0.06), respectively. The use of HPLC–ECD to
measure the postprandial carotenoid response in plasma chylomicrons was shown to be a
sensitive and efficient approach to screen for dietary factors such as added fat that
enhance β-carotene bioavailability. (Supported by The Procter & Gamble Co.)
ANTIOXIDANT ACTIVITY OF CAROTENOID GEOMETRICAL ISOMERS.
V Böhmi, NL Puspitasari-Nienaberm, MG Ferruzzib, SJ Schwartzb
iaInstitute of Nutrition, Friedrich-Schiller University Jena, 07743 Jena, Germany; 
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BACKGROUND: Carotenoids and their potential as biological antioxidants are currently 
the focus of numerous investigations. Isomerization of carotenoids is often encountered 
in food processing under the influence of temperature and light. These may play a role in 
observed protective effects in light of recent reports highlighting a large percentage of 
carotenoid (Z)-isomers in biological fluids and tissues. This study investigated the 
antioxidant activity of prominent geometrical isomers of α-carotene, β-carotene, 
lycopene and zeaxanthin, using an in vitro assay.

MATERIALS AND METHODS: α-carotene, β-carotene, lycopene and zeaxanthin were 
isolated from food or supplements. All extracts were photoisomerized using iodine. The 
resulting equilibrium mixture of isomers were fractionated on a semi-preparative C30-
column and analyzed for purity by analytical C30-column HPLC. The antioxidant activity 
of all isomers collected was measured photometrically using the Trolox Equivalent 
Antioxidant Capacity (TEAC) assay.

RESULTS: Fractionations resulted in five α-carotene isomers ((E)-, 9(Z)-, 9'(Z)-, 13(Z), 
13'(Z)), three β-carotene isomers ((E)-, 9(Z)-, 13(Z)-), five lycopene isomers ((E)-, four 
unidentified (Z)-isomers) and three zeaxanthin isomers ((E)-, 9(Z)-, 13(Z)-). Their anti­
oxidant activity was measured using the TEAC assay with Trolox®, a watersoluble vita­
m E analogue, as a calibration standard. TEAC values of isomers ranged as follows: 
lycopene (Z)-isomer 1 = lycopene (Z)-isomer 2 = lycopene (Z)-isomer 3 > (E)-lycopene 
≈ lycopene (Z)-isomer 4 > (E)-α-carotene = 9'(Z)-α-carotene = 13'(Z)-α-carotene = 
(E)β-carotene = 9(Z)-β-carotene ≈ (E)-zeaxanthin = 13 (Z)-zeaxanthin = 9(Z)-α-carotene 
= 13(Z)-α-carotene = 13(Z)-β-carotene ≈ 9(Z)-zeaxanthin.

CONCLUSIONS: This study showed the in vitro antioxidant activity of different 
carotenoid geometrical isomers for the first time. One lycopene isomer (tentatively 
identified as the 5(Z)-), eluting shortly after (E)-lycopene, showed the same efficacy as 
the (E)-isomer. In contrast, the other three lycopene isomers had significantly higher (p < 
0.05) antioxidant capacity. On the other hand, the 9(Z)-zeaxanthin had a more than 80 % 
lower TEAC value compared to (E)-lycopene. With the results presented the in vivo 
relevance of (Z)-isomers of carotenoids can be considered.