Prevention of Iron-Polyphenol Complex Formation in Iron Fortified Tea

by

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This dissertation is submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

The fortification of black tea with iron has the potential to reduce the prevalence of iron deficiency in the developing world. Tea is an ideal vehicle for food fortification because it is centrally processed, is the most consumed beverage globally, aside from water, and is consumed in regular quantities by those of all socioeconomic strata in many regions. Unfortunately polyphenolic compounds present in tea, which are responsible for colour and flavour, form complexes with iron which reduce the bioavailability of both compounds and cause strong unattractive colour development. The objective of this study was to develop a technique for the fortification of tea with iron such that these complexes do not form. A spectrophotometric method was developed and validated for the quantification of iron-polyphenol complex formation. Iron-polyphenol complex formation was further investigated with a variety of iron sources, temperatures, and polyphenol concentrations using a gallic acid model system and tea extract. Analysis and modeling of iron-polyphenol complex formation prompted the investigation of predicted inhibitors, specifically reducing and chelating agents. Reducing agents were able to hinder iron complex formation at pH 5 (the pH of brewed tea) but not at pH 7 (an approximation of the small intestine’s pH). Chelating agents were successful at preventing iron-polyphenol complex formation at both pH 5 and pH 7. Due to its superior performance, disodium
ethylenediaminetetraacetate (EDTA) was optimized for use in black tea. In summary, a technique has been developed for producing iron fortified tea based on a comprehensive framework for understanding the formation and inhibition of iron-polyphenol complexes in the presence of black tea polyphenols. This tea maintains its visual acceptability while providing iron in a bioavailable form. A process for effective iron fortification based on our laboratory technique is ready for in-vivo evaluation of its effectiveness and pilot scale testing.
Acknowledgements

A very good friend of mine just recently asked me, “So, now that it’s all over, was it worth it?” This question is a bit premature, as I have not yet received my degree. Despite this, my answer is a very definite “Yes”, but not for the reason that I thought it would be. It was an odd childhood dream of mine to one day obtain a doctorate degree; I greatly admired those with high intelligence. Throughout the pursuit however, my perspective shifted. If a doctorate degree was solely a badge to indicate high intelligence, it would not have been worth it. Instead, the journey has made me grow, not only as a researcher, but as a person. I learned how to let go of an overwhelming ideal, sacrifice properly, and have faith that the outcome will be what is best. With God’s grace I found courage, resilience, and I am forever changed. For this, I have overwhelming gratitude to all of those involved.

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Table of Contents

ABSTRACT .............................................................................................................. II
ACKNOWLEDGEMENTS ....................................................................................... IV
TABLE OF CONTENTS .............................................................................................. VI
LIST OF TABLES ...................................................................................................... IX
LIST OF FIGURES ................................................................................................... XI
LIST OF APPENDICES .......................................................................................... XIV
LIST OF ABBREVIATIONS .................................................................................. XV

1 INTRODUCTION .................................................................................................. 1
1.1 Motivation ........................................................................................................ 1
1.2 Scope ............................................................................................................... 2
1.3 Outline and Objectives .................................................................................... 3

2 BACKGROUND .................................................................................................... 5
2.1 Potential Impact of Iron Fortified Tea ............................................................... 5
   2.1.1 Iron in the Human Body ......................................................................... 5
   2.1.2 Significance of Iron Deficiency .............................................................. 7
   2.1.3 Intervention Strategies ......................................................................... 9
   2.1.4 Tea as a Food Vehicle ........................................................................... 11
2.2 Factors for the Development of Iron Fortified Tea ............................................ 13
   2.2.1 Iron Source Selection ......................................................................... 13
   2.2.2 Iron Fortification Level ........................................................................ 14
   2.2.3 Black Tea Production and Chemistry .................................................... 15
   2.2.4 Iron Absorption in the Presence of Tea .................................................. 20
2.3 Iron-Polyphenol Chemistry .............................................................................. 21
   2.3.1 Formation of Iron-Phenol Coordination Complexes ............................. 21
   2.3.2 Electron Configuration and Colour of Coordination Complexes .......... 25
   2.3.3 Thermodynamics and Kinetics of Iron Coordination Complexes .......... 33
   2.3.4 Redox Reactions of Iron-Polyphenol Complexes .................................. 35
   2.3.5 Hydrolysis in Iron Coordination Complexes ......................................... 40
   2.3.6 Effect of pH Control on Metal Cations .................................................. 40
2.4 Overcoming Barriers Posed by Chelating Foods

2.4.1 Prevention of Iron-Polyphenol Complex Formation in Foods

2.4.2 Increasing the Bioavailability of Iron in Chelating Foods

2.5 Quantification Techniques

2.5.1 Polyphenol Quantification in Tea

2.5.2 Quantification of Iron-Polyphenol Complex

2.6 Opportunities for Scientific Advancement

3 MATERIALS AND METHODS

3.1 Materials

3.2 Tea and Model Solution Preparation Method

3.2.1 Tea Brewing

3.2.2 Gallic Acid and Tannic Acid Model Solutions

3.2.3 Tea Extract Preparation

3.2.4 Iron Solution Preparation

3.3 Analytical Methods

3.3.1 Measurement of pH

3.3.2 Phenolic Compound Concentration

3.3.3 Iron-Polyphenol Complex Quantification

3.3.4 Iron Content

4 IRON COMPLEXATION BY BLACK TEA POLYPHENOLS

4.1 Research Objective

4.2 Experimental Strategy

4.3 Results and Discussion

4.3.1 Tea Polyphenol Concentration and pH

4.3.2 Control of pH

4.3.3 Gallic Acid Calibration Curves

4.3.4 Comparison of Gallic Acid and Black Tea Polyphenols

4.3.5 Effect of Temperature and Concentration

4.4 Summary of Findings
5 REDUCING AGENTS ........................................................................................................ 87
5.1 Research Objective .................................................................................................. 87
5.2 Experimental Strategy ............................................................................................ 87
5.3 Results and Discussion ........................................................................................... 88
   5.3.1 Complex Formation of Ferrous versus Ferric Iron ........................................... 88
   5.3.2 Effect of Reducing Agents on Iron Complexation ........................................... 96
5.4 Summary of Findings ............................................................................................... 100

6 CHELATED IRON AND CHELATING AGENTS .......................................................... 101
6.1 Research Objective ................................................................................................. 101
6.2 Experimental Strategy ............................................................................................ 101
6.3 Results and Discussion ........................................................................................... 103
   6.3.1 Extent of Reaction with Chelated Iron Sources .................................................. 103
   6.3.2 Extent of Reaction with Chelating Agents ......................................................... 104
   6.3.3 Optimization of Disodium EDTA ................................................................. 107
6.4 Summary of Findings ............................................................................................... 109

7 IRON FORTIFIED TEA USING DISODIUM EDTA .................................................. 110
7.1 Research Objective ................................................................................................. 110
7.2 Experimental Strategy ............................................................................................ 110
7.3 Results and Discussion ........................................................................................... 111
   7.3.1 Iron-Polyphenol Complex Formation with Na₂EDTA .................................... 111
   7.3.2 Sequential pH Adjustment and Available Iron .............................................. 114
7.4 Summary of Findings ............................................................................................... 115

8 CONCLUSIONS ......................................................................................................... 116
8.1 Summary of Thesis Conclusions ............................................................................ 116
8.2 Recommendations .................................................................................................. 117
   8.2.1 Iron-Polyphenol Food Chemistry .................................................................... 118
   8.2.2 Development of Iron Fortified Tea ................................................................. 119

9 REFERENCES ............................................................................................................ 121

10 APPENDICES .......................................................................................................... 131
List of Tables

Table 2.1: Iron Losses in Adults..................................................................................................................6
Table 2.2: Appropriate Consumption of Iron ...........................................................................................7
Table 2.3: Common Iron Sources for Food Fortification ...........................................................................13
Table 2.4: Percentage of RDA and UL Expected for Adults Drinking Iron Fortified Tea ..............15
Table 2.5: Inhibition of Ferric Chloride Absorption in Rats from Tea and Tea Constituents .......21
Table 2.6: Molar Extinction Coefficients for Electronic Transitions of Iron ....................................31
Table 2.7: Equilibrium Values for Iron Chelators (adapted from .......................................................35
Table 2.8: Reported Stability of Iron-Phenolic Complexes ...................................................................36
Table 2.9: Initial Rates of Fe(II) Oxidation ............................................................................................38
Table 2.10: FC Reactivity of Phenols and Potentially Interfering Substances ..................................49

Table 3.1: Phenolic Compounds ............................................................................................................55
Table 3.2: Iron Compounds (Fortificants and Standard Curves) .............................................................55
Table 3.3: pH Stabilization and Adjustment ............................................................................................56
Table 3.4: Reducing and Chelating Agents .............................................................................................56
Table 3.5: Analytical Chemicals ..............................................................................................................57
Table 3.6: Experiments in Appendices 10.6 & 10.7 (Milk and Minerals) ............................................57
Table 3.7: Buffer Solution Preparation ..................................................................................................59
Table 3.8: Acid Digestion Sample Preparation .......................................................................................61

Table 4.1: Effect of Steeping Time on pH ..............................................................................................67
Table 4.2: Ferric Chloride versus Ferrous Sulphate Complex Formation in Gallic Acid ...............74
Table 4.3: Comparison of Iron Compounds in Gallic Acid and Tea Extract ......................................75
Table 4.4: Iron-Polyphenol Flow Cell Models .........................................................................................85

Table 5.1: Extent of Reaction at Initial Measurement for Ferric and Ferrous Iron ..........................89

Table 6.1: Stability of Iron in Tea Extract with Chelating Agents (pH 7) ........................................106
Table 6.2: Effect of Varying EDTA Concentration in Tea Extract with Added Iron ..................107
List of Figures

Figure 2.1: Estimates of Anemia Prevalence in Babies/Children (6–59 Months), 2011.................. 8
Figure 2.2: Estimates of Anemia Prevalence in Non-Pregnant Women Age 15 to 49, 2011......... 8
Figure 2.3: Estimates of Anemia Prevalence in Pregnant Women Age 15 to 49, 2011 ............. 9
Figure 2.4: Percentage of Total Tea Consumption by Country in 2013 ................................. 11
Figure 2.5: Global Black Tea Consumption, 2013 ................................................................... 12
Figure 2.6: Structures of Major Green Tea Polyphenols ......................................................... 17
Figure 2.7: Structures of Major Black Tea Polyphenols ......................................................... 18
Figure 2.8: Tea Processing Flow Chart ...................................................................................... 19
Figure 2.9: Common Phenolic Compounds in Food .................................................................. 20
Figure 2.10: Structures with Stabilized Oxygen Centers ......................................................... 22
Figure 2.11: Chemical Structures with Chelation Centers ....................................................... 23
Figure 2.12: Complex Formation of Fe(III) and Polyphenol (1:3 ratio) .................................... 24
Figure 2.13: σ Molecular Orbitals for an Octahedral Coordination Complex .......................... 27
Figure 2.14: Effects of π Bonding on Δo Using a d^5 Ion (Fe^{3+}) .......................................... 29
Figure 2.15: LMCT Transitions in an Octahedral Fe^{3+} Complex with π-Donor Ligands ........ 32
Figure 2.16: Iron Autoxidation ................................................................................................. 37
Figure 2.17: Reduction of Fe(III) in a Low pH Environment .................................................... 39
Figure 2.18: Isoelectric Forms of the Metal Quinone Chelate Ring .......................................... 39
Figure 2.19: pH Dependence of Fe(III) in the Presence of Various Ligands ......................... 39
Figure 2.20: Equilibrium between Ferric and Ferrous Chelate Forms ................................... 40
Figure 4.1: Increase of Polyphenol Concentration with Steeping Duration
Figure 4.2: Increase of Polyphenol Concentration with Tea Leaf Quantity
Figure 4.3: Dependence of pH on the Quantity of Tea Leaves
Figure 4.4: Spectrophotometric Scans of Brewed Tea
Figure 4.5: Spectrophotometric Scans of Iron Complex Formation in Gallic Acid at pH 5
Figure 4.6: Spectrophotometric Scans of Iron Complex Formation in Gallic Acid at pH 7
Figure 4.7: Spectrophotometric Scans of Iron Complex Formation in Tea Extract at pH 5
Figure 4.8: Spectrophotometric Scans of Iron Complex Formation in Tea Extract at pH 7
Figure 4.9: Polyphenol Calibration Curve Using Ferric Chloride at pH 5 (555 nm)
Figure 4.10: Polyphenol Calibration Curve Using Ferric Chloride at pH 7 (565 nm)
Figure 4.11: Iron-Polyphenol Complex Formation in the Flow Cell
Figure 4.12: Spectrophotometric Scans of 0.12 mM GAE Flow Cell Samples at pH 7
Figure 4.13: Models of Iron-Polyphenol Complex Formation at pH 5
Figure 4.14: Model of Iron-Polyphenol Complex Formation at pH 7

Figure 5.1: Absorbance Development of Ferrous Sulphate in Buffered Gallic Acid Solution
Figure 5.2: First Minute Readings of Ferrous Sulphate in Buffered Gallic Acid at pH 5
Figure 5.3: First Differences of Final 16 Absorbance Readings
Figure 5.4: Fitting Model Using Least Squares Regression
Figure 5.5: Model of Ferrous Sulphate in pH 5 Phthalate Buffered Gallic Acid
Figure 5.6: Effect of Reducing Agents on Iron-Polyphenol Complex Formation in Tea Extract
Figure 5.7: Spectrophotometric Scans of Ascorbate in Tea Extract at pH 7
Figure 5.8: Iron-Polyphenol Complex Development over Time with Reducing Agents (pH 5)
Figure 5.9: Spectrophotometric Scans of Iron in Tea Extract with Reducing Agents at pH 5

Figure 6.1: Extent of Reaction of Chelated Iron Compounds with Tea Extract (pH 7)
Figure 6.2: Average Scans of Chelated Iron Compounds with Tea Extract
Figure 6.3: Iron-Polyphenol Complex Formation in Tea Extract with Chelating Agents
Figure 6.4: Spectrophotometric Scans of Tea Extract with Chelating Agents (pH 7)
Figure 6.5: Chemical Structures of Chelating Agents
Figure 6.6: Absorbance of Brewed Tea Containing Ferrous Sulphate and Disodium EDTA
Figure 6.7: Tea Fortified Using Different Molar Ratios of Iron to Disodium EDTA
Figure 7.1: Iron-Polyphenol Complex Formation in Freshly Brewed Tea.......................... 112
Figure 7.2: Net Peak Absorbance of Freshly Brewed Iron Fortified Tea Solutions............... 113
Figure 7.3: Iron-Polyphenol Complex in Brewed Tea at pH 7, pH 6, and pH 5.................. 113
Figure 7.4: Retention of Soluble Iron in Tea Solutions after Sequential pH Adjustment........ 115

Figure 10.1: Sample Polyphenol Calibration Curve.......................................................... 131
Figure 10.2: Effect of Gallic Acid Concentration on Iron Complex Formation ................. 132
Figure 10.3: Spectrophotometric Scans of Ferrous Sulphate in Gallic Acid (pH 1 to pH 7) .... 134
Figure 10.4: Comparison of Ferric Sodium EDTA Sources in Tea Extract (pH 7) .............. 136
Figure 10.5: Calibration Curve Used in Flow-Cell Experiments ...................................... 137
Figure 10.6: Flow Cell Adding Tea Extract to Iron Solutions ............................................ 138
Figure 10.7: Flow Cell Adding Gallic Acid or Tea to Iron Solutions at 24°C ....................... 139
Figure 10.8: Iron-Polyphenol Complex Formation in the Presence of Minerals .................... 151
Figure 10.9: Retention of Soluble Iron in Tea with EDTA after Sequential pH Adjustment ... 153
List of Appendices

Appendix 10.1: Tea Parameter Measurement ......................................................... 131
  10.1.1 Polyphenol Calibration ............................................................................. 131
  10.1.2 Polyphenol Content of Dry Tea Leaves .................................................. 131
  10.1.3 Effect of Gallic Acid Concentration on Iron Complex Formation ............ 132
  10.1.4 Maximum Polyphenol Concentration in Brewed Tea ............................... 133

Appendix 10.2: pH Adjustment .............................................................................. 134
  10.2.1 Ferrous Sulphate in Gallic Acid Spectrophotometric Scans .................... 134
  10.2.2 Solution Preparation for Testing Buffer Solutions .................................. 134

Appendix 10.3: Comparison of Ferric Sodium EDTA Sources ......................... 136

Appendix 10.4: Additional Flow-Cell Calculations and Data .......................... 137
  10.4.1 Calibration Curves .................................................................................. 137
  10.4.2 Adding Tea Extract and Gallic Acid to Iron Solutions ............................. 137
  10.4.3 Temperature Estimation at the Flow-Cell ............................................. 139

Appendix 10.5: Use of EDTA in Food ................................................................. 141

Appendix 10.6: Effect of Milk on Iron Fortified Tea ........................................ 143
  10.6.1 Milk Testing Methods ........................................................................... 144
  10.6.2 Milk Results and Discussion .................................................................. 146
  10.6.3 Milk Conclusions and Recommendations ........................................... 148

Appendix 10.7: Interaction of Iron Fortified Tea with Minerals ....................... 150
  10.7.1 Mineral Testing Methods ....................................................................... 150
  10.7.2 Mineral Results and Discussion .............................................................. 151
  10.7.3 Mineral Conclusions and Recommendations ....................................... 151

Appendix 10.8: Soluble Iron Following Sequential pH Adjustment ............... 153

Appendix 10.9: Iron Fortified Tea Premix Preparation .................................... 154
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>ADI</td>
<td>Acceptable Daily Intake</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate Intake</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>Disodium EDTA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fe(III) or Fe³⁺</td>
<td>Ferric Iron</td>
</tr>
<tr>
<td>Fe(II) or Fe²⁺</td>
<td>Ferrous Iron</td>
</tr>
<tr>
<td>FF</td>
<td>Ferrous Fumarate</td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>Ferric Sodium EDTA</td>
</tr>
<tr>
<td>FS</td>
<td>Ferrous Sulphate</td>
</tr>
<tr>
<td>FC</td>
<td>Folin-Ciocalteu</td>
</tr>
<tr>
<td>JECFA</td>
<td>Joint FAO/WHO Expert Committee on Food Additives</td>
</tr>
<tr>
<td>LMCT</td>
<td>Ligand to Metal Charge Transfer</td>
</tr>
<tr>
<td>MLCT</td>
<td>Metal to Ligand Charge Transfer</td>
</tr>
<tr>
<td>FeCl₃/GAE</td>
<td>Ferric Chloride in Gallic Acid Equivalents (polyphenol concentration)</td>
</tr>
<tr>
<td>FS/GAE</td>
<td>Ferrous Sulphate in Gallic Acid Equivalents (polyphenol concentration)</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic Acid Equivalents (phenolic compound concentration)</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
</tr>
<tr>
<td>SHMP</td>
<td>Sodium Hexametaphosphate</td>
</tr>
<tr>
<td>TF</td>
<td>Theaflavins</td>
</tr>
<tr>
<td>TR</td>
<td>Thearubigins</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable Upper Intake Level</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultraviolet Light/Visual Light</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1 Introduction

1.1 Motivation

Micronutrient (i.e. vitamin and mineral) deficiencies directly affect more than 2 billion people worldwide and constitute the most severe health problem globally [1, 2]. Diets of micronutrient poor cereals and tubers are common in the developing world and are deficient in iron, iodine, folate, vitamin A, and zinc [3, 4]. Beneficial interventions include supplementation, food fortification, and advancements toward the consumption of diverse micronutrient rich foods (i.e. dietary diversification) [5]. Among these interventions, food fortification is often the most cost effective and easily implemented strategy.

The most common micronutrient deficiency is iron deficiency. It can lead to anemia which is characterized by a severely low concentration of erythrocytes and hemoglobin in the blood [3]. Anemia is a widespread condition affecting approximately 25% of the global population [6]. Insufficient intake of iron is responsible for the majority of iron deficiency and 50% of anemia globally [3]. Iron deficiency anemia is associated with impaired physical capacity, developmental delay, cognitive impairment, increased maternal mortality, premature delivery, low birth weight, and increased infant mortality [7]. Iron deficiency also lowers defense mechanisms of the body against infection and decreases the absorption of iodine and vitamin A [3]. Those most at risk are infants, children, adolescents, and women of childbearing age, especially those who are pregnant [4].

The food chosen for fortification (i.e. food vehicle or carrier) greatly affects the potential success of an intervention. Tea, the second most commonly consumed beverage worldwide (second only to water), is centrally processed, generally purchased, and has established distribution channels [8]. Black tea, in particular, accounts for the majority of tea produced, 65% in 2013 [9]. Research in several Indian states revealed that black tea is consumed by all age groups, consumption is not dependent on socioeconomic status, and it is consumed in both urban and rural areas [8]. Therefore, black tea is an ideal carrier to reach the largest populations in need.
1.2 Scope

Iron fortification of black tea is a logical intervention strategy to decrease iron deficiency caused by insufficient intake. However, once a suitable food vehicle is selected, other challenges pertaining to manufacturing, procurement by consumers, and consumption/absorption must be addressed. Manufacturing challenges include added complexity to manufacturing methods as well as the potential costs associated with new processing equipment and added processing steps. Consumers must be enticed to purchase the fortified tea; thus the product must be affordable and appealing in appearance. Furthermore, once brewed, the tea must have an acceptable colour and flavour. Finally, there must be a consistent and significant therapeutic benefit; iron content in the dry tea and in the brewed consumed tea must be consistent, and the consumed iron must be bioavailable.

Most of these challenges can be easily overcome. Iron can be attached to dried tea leaves or granules to form a premix. The premix can then be dry blended with unfortified dry tea in a manufacturing facility to produce fortified tea for distribution. When fortified tea is used to brew tea beverages, the attached iron will separate from the tea leaves, and dissolve or disperse through the beverage.

While the manufacturing strategy is clear, one key obstacle requires development to overcome and is the topic of this thesis. Tea contains high concentrations of polyphenolic compounds. Iron and polyphenols react to form non-bioavailable and intensely coloured complexes. The formation of iron-polyphenol complexes would hinder consumer acceptance, and reduce the efficacy of the iron fortification strategy. It is therefore critical to prevent iron-polyphenol complex formation in the brewed tea before consumption and when it reaches the site of iron absorption within the body. Consequently, the primary objective of this research is to develop a technique to prevent iron-polyphenol complex formation in iron fortified tea. Complications due to non-tea components (e.g. additives such as milk, varying mineral contents of water supplies, and interactions with other consumed foods) may also influence the appearance of brewed tea and iron bioavailability. However, these factors are outside of the scope of this research and will be addressed separately once a suitable strategy for the prevention of iron-polyphenol complex formation has been developed.
1.3 Outline and Objectives

This research is organized as a series of chapters beginning with the current introductory chapter which gives an overview of the motivation and research approach. In Chapter 2, background material is presented which further details these two aspects. The potential impact of iron fortified tea and factors that contribute to its development are further clarified. Then, current knowledge pertaining to the main hurdle, iron-polyphenol complex formation, is summarized. This is followed by a description of the current analytical methods used for the measurement of polyphenols and iron-polyphenol complexes. Finally, opportunities to advance scientific understanding while developing iron fortified tea are outlined.

In Chapter 3 materials used in experimentation are listed and methods are described. Chapters 4 through 7 present the principle investigations aimed at achieving the primary objective of preventing iron-polyphenol complex formation. These investigations explore the complexation of tea polyphenols with iron, the prevention of iron-polyphenol complex formation using a reducing agent approach or a chelation approach, and the use of disodium ethylenediaminetetraacetate (EDTA) in iron fortified black tea. Descriptions of Chapters 4-7 are presented in the following paragraphs.

The objective of the research presented in Chapter 4 was to quantify iron-polyphenol complex formation and gain a deeper understanding of how this reaction occurs in black tea. This objective was achieved through the development of an iron-polyphenol complex quantification method which allows for measurement at pH levels appropriate to iron fortified tea. Effects of iron source, polyphenol source, temperature, and polyphenol concentration were subsequently investigated. The complexation of a variety of iron sources by gallic acid and black tea polyphenols were compared and thermodynamic models were developed to explain the formation of iron-polyphenol complexes at pH 5 and pH 7.

Chapter 5 addresses the objective of determining the validity of using reducing agents to slow the formation of iron-polyphenol complexes in black tea. To accomplish this, experiments were conducted with reduced (i.e. ferrous) and oxidized (i.e. ferric) forms of iron in buffered gallic acid solutions followed by experimentation with reducing agents in solutions of black tea polyphenols. The reaction of ferrous sulphate in gallic acid solutions buffered to pH 5 was found
to be much slower than that of ferric iron. This reaction was modeled using first order kinetics, providing some insight into the need for iron oxidation to form iron-polyphenol complexes in tea. The reducing agents: sodium ascorbate, sodium metabisulphite, and glucose were then investigated for their ability to slow iron-polyphenol complex formation at pH 5 and pH 7 in solutions containing black tea extract.

In Chapter 6 the objective of the investigation was to **determine the ability of chelated iron sources and/or chelating agents to prevent the formation of iron-polyphenol complexes in tea**. Two chelated iron sources, ferric sodium EDTA and ferric trisglycinate, as well as one non-chelated source, ferrous D-gluconate, were selected to be compared to ferrous sulphate. Also, ferrous sulphate was tested in the presence of chelating agents. The chelating agents selected were sodium gluconate, sodium citrate, tartaric acid, disodium EDTA, glycine, and sodium hexametaphosphate (SHMP). Because disodium EDTA was the most effective, the amount required to prevent colour formation in dilute tea extract and in brewed tea was investigated.

In Chapter 7 an investigation of **the effectiveness of using disodium EDTA as an iron stabilizer in iron fortified black tea** is presented. Iron fortified tea was tested at pH 5 and pH 7 followed by further testing at pH levels between 5 and 7 and intervals of 0.2. To investigate potential iron bioavailability the quantities of total soluble iron and soluble iron-polyphenol complexes were measured in iron fortified tea following sequential pH adjustment simulating human digestion.

These chapters are followed by Chapter 8: Conclusions and Recommendations. Here it is concluded that a novel technique for fortifying tea with iron has been developed such that iron-polyphenol complex formation is prevented. Other conclusions with respect to iron fortified tea and advancing scientific knowledge are given as well as recommendations for further development in both of these domains.
2 Background

2.1 Potential Impact of Iron Fortified Tea

2.1.1 Iron in the Human Body

The primary source of information used for this section is the book “Inorganic Biochemistry of Iron Metabolism from Molecular Mechanisms to Clinical Consequences, 2nd Ed.” by Robert Crichton (John Wiley & Sons Ltd., 2001) [10]. This book presents a broad overview of the inorganic biochemistry of iron. It has been reviewed in the Journal of the American Chemical Society as a credible academic resource [11].

The iron content of the human body is usually between 40-50 mg Fe/kg of body weight with men typically possessing a higher concentration than women [10]. About 30 mg Fe/kg (60% to 75%) of the iron is found in hemoglobin, a protein in red blood cells (erythrocytes) that transports oxygen throughout the body [10]. Approximately 4 mg Fe/kg (8% to 10%) is found in myoglobin, an oxygen storage protein that resides in muscle cells [10]. Roughly 2 mg Fe/kg (4% to 5%) is found in other proteins throughout the body [10]. These proteins include cytochrome enzymes that act as electron carriers aiding in energy acquisition by aerobic respiration; enzymes that aid in the synthesis of steroid hormones and bile acids; enzymes that control some neurotransmitters; and enzymes that detoxify substances in the liver [4, 7]. Most of the remaining iron (generally approximately 10-12 mg Fe/kg for men and 5 mg/kg for women) is stored as ferritin and hemosiderin in the liver (with some storage in the spleen, bone marrow and muscle) [10]. These storage forms are mobilized for use in the body as transferrin protein [4].

Iron is absorbed by the body in the upper portion of the small intestine known as the duodenum [10]. Heme iron, which is acquired through meat consumption, and non-heme iron, which is acquired though vegetable and dairy sources, are absorbed by two separate pathways [10]. Heme iron, when ingested in globular protein forms (e.g. hemoglobin or myoglobin) is highly bioavailable and easily absorbed, generally between 20% to 30% [10]. The absorption is only thought to be hindered by calcium [4]. However, heme iron apart from globular proteins is not easily absorbed [10]. There are many factors that may enhance or inhibit non-heme iron absorption. Enhancers include meat, ascorbic acid, citric acid, malic acid, lactic acid, tartaric acid, and salts of these organic acids [10]. Inhibitors to non-heme iron absorption include polyphenols.
and phytates [10]. Polyphenols are found in high concentrations in tea and coffee as well as in many vegetables, legumes and condiments [10]. Phytates are found in cereals, nuts, and legumes [10]. Other non-heme iron absorption inhibitors are dietary fibre complexes, calcium, and certain proteins such as those from soy beans, nuts, or lupines [10].

Iron is lost from the body through sloughing off of cells (i.e. from skin, gastrointestinal tract, and urinary tract) and through the loss of blood [10]. In women additional iron loss occurs with menstruation and pregnancy [10]. Typical iron losses in adults are summarized in Table 2.1. Because of the greater amount of iron loss for women, their iron intake requirement is larger. Iron requirement is also increased due to growth during childhood. The recommended dietary allowances (RDAs) are summarized in Table 2.2.

Table 2.1: Iron Losses in Adults [10]

<table>
<thead>
<tr>
<th>Modes of Iron Loss</th>
<th>Average Iron Loss per Day (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrointestinal tract exfoliation of mucosal cells and loss of blood</td>
<td>0.67</td>
</tr>
<tr>
<td>Skin and urinary tract exfoliation</td>
<td>0.33</td>
</tr>
<tr>
<td>Menstruation (averaged over the month)</td>
<td>2</td>
</tr>
<tr>
<td>Pregnancy (averaged over pregnancy duration)</td>
<td>2.7 (may reach as high as 6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group of Adults</th>
<th>Total Iron Loss per Day (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>1</td>
</tr>
<tr>
<td>Post-menopausal women</td>
<td>1</td>
</tr>
<tr>
<td>Pre-menopausal non-pregnant women</td>
<td>3</td>
</tr>
<tr>
<td>Pregnant women (averaged over pregnancy duration)</td>
<td>3.7 (may reach as high as 7)*</td>
</tr>
</tbody>
</table>

*Iron loss reported is due to pregnancy in addition to other causes
Table 2.2: Appropriate Consumption of Iron* (adapted from [12], Health Canada)

<table>
<thead>
<tr>
<th>Age</th>
<th>RDA (mg/day)</th>
<th>Tolerable Upper Intake Level (UL) (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6 months</td>
<td>0.27**</td>
<td>40</td>
</tr>
<tr>
<td>7-12 months</td>
<td>6.9</td>
<td>40</td>
</tr>
<tr>
<td>Child</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3 years</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>4-8 years</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>9-13 years</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>Man</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18 years</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>19-50 years</td>
<td>8</td>
<td>45</td>
</tr>
<tr>
<td>Woman</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Pregnant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18 years</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>19-50 years</td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 years</td>
<td>27</td>
<td>45</td>
</tr>
<tr>
<td>Lactating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤18 years</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>18-50 years</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>Senior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51+ years</td>
<td>8</td>
<td>45</td>
</tr>
</tbody>
</table>

*Iron requirement is 1.8 times larger for vegetarians due to lower iron bioavailability in the diet

**Indicates adequate intake instead of RDA

2.1.2 Significance of Iron Deficiency

Iron deficiency is the most common and widespread micronutrient deficiency in the world. Insufficient intake of iron resulting in iron deficiency is responsible for 50% of anemia cases globally. Anemia is a medical condition characterized by a severely low amount of erythrocytes or hemoglobin in the blood [3]. Anemia affects approximately 25% of the population [6]. Different portions of the population are affected differently. Globally anemia affects 47% of preschool-age children (3-5 years old), 25% of school aged children (6-12 years old), 42% of pregnant women, 30% of non-pregnant women, 13% of men, and 24% of the elderly [6]. The prevalence of anemia globally is shown in Figures 2.1, 2.2, and 2.3 for babies/children, non-pregnant women of childbearing age, and pregnant women respectively. As shown in the pictorial representations, anemia has a global presence, but, is much more prevalent in developing countries.
Figure 2.1: Estimates of Anemia Prevalence in Babies/Children (6–59 Months), 2011 [13]

Figure 2.2: Estimates of Anemia Prevalence in Non-Pregnant Women Age 15 to 49, 2011 [13]
Iron deficiency anemia impacts health in a variety of ways. It is associated with impaired physical capacity, developmental delay, cognitive impairment, increased maternal mortality, premature delivery, low child birth weight, and increased infant mortality [7]. Iron deficiency anemia results in the death of 136,000 women and children as well as 600,000 stillbirths or neonatal deaths annually [5]. Furthermore, one fifth of global maternal mortality is due to iron deficiency anemia [5]. Iron deficiency decreases physical capacity due to reduced oxidative metabolism in muscles [4]. This leads to a decreased ability to work which causes economic losses of as much as 1.9% of the GDP for Bangladesh and losses of over 1% of the GDP for India, Malawi, and Oman [14]. Iron deficiency also increases the likelihood of infection because iron is required for the production and action of immune T lymphocytes [4]. Furthermore, iron deficiency leads to a decreased absorption of iodine and vitamin A leading to further deficiencies [3]. The most vulnerable groups include infants, children, adolescents, and pre-menopausal women, especially those who are pregnant, due to growth and greater iron loss [4].

2.1.3 Intervention Strategies

There are three main strategies to combat iron deficiency: dietary diversification, supplementation, and food fortification. However each of these strategies has inherent obstacles. With reference to documentation published by the World Health Organization (WHO) and the
Micronutrient Initiative (MI), these difficulties together with some successes are outlined below [3, 5].

Dietary diversification involves helping the target population to eat a variety of nutrient dense foods and thus acquiring all required nutrients. Although this is the preferred method to ensure adequate overall nutrition, it is very difficult to implement. It requires that the target population be educated on nutrition and change the foods they consume. This change is difficult because many factors contribute to food consumption decisions such as preference, culture, religious beliefs, and affordability of higher quality foods. Implementation also requires financial and natural resources for producing or otherwise acquiring the foods. Because of these challenges, these programs are the most difficult to implement especially as the sole source of intervention [3].

Iron supplementation and fortification strategies have been implemented successfully in developing countries. Supplementation strategies include giving tablets or powders that contain iron to high risk groups such as pregnant women and babies [5]. Numerous foods have been fortified with iron including salt, sugar, chocolate/cocoa, wheat flour, maize flour, breakfast cereals, fish sauce, soy sauce, milk (powdered and fluid), soft drinks, and cereal based complementary foods used for weaning infants [3]. These programs have been successful and have produced a 20% reduction in maternal mortality in the developing countries which have adopted them [5]. However, there is still a vast portion of the population for whom supplements and fortified foods have not yet reached.

Although very useful, supplementation and fortification programs have challenges and limitations. Supplementation programs often suffer from poor compliance [3]. This is largely due to the cost of the supplements, inadequate nutrition education, and difficulty in obtaining the supplements due to distance and time constraints [3]. Food fortification strategies are often the most cost effective and most likely to reach those in need [3]. However, these programs require effective distribution channels for the fortified food, resources for food processing, financial resources so that the target population may afford the fortified food, and the presence of foods that are eaten in regular amounts which can be selected for fortification [3]. As this strategy does not require behavioural change in the target population, it is the most easily implemented. The
success of a fortified food lies in the identification of the food vehicle (or food carrier) that will contain the added nutrient (i.e. iron) and the development of a low cost fortification method.

2.1.4 Tea as a Food Vehicle

The concept of tea fortification is not novel. Tea was first suggested in 1943 as a carrier for vitamin A in Pakistan, India, and Tanzania [8]. Vitamin A fortified tea was developed such that it retained 90% of the vitamin A after 6 months of storage at 37°C [8]. However, iron fortified tea had not yet been developed.

Tea is an excellent choice as a micronutrient carrier because it is the most commonly consumed beverage globally aside from water, has established central processing facilities, is generally purchased, and has established distribution channels [8]. The vast majority of global tea consumption, 83.1%, occurs in developing countries (Figure 2.4) [9]. Therefore tea is an excellent option to reach those in developing countries.

Figure 2.4: Percentage of Total Tea Consumption by Country in 2013 (data from the FAO [9])
Black tea is the most produced variety of tea [9]. In 2013, 65% of total tea production was devoted to black tea [9]. Figure 2.5 shows black tea consumption by country or region as of 2013. As the figure demonstrates, the Far East is the greatest black tea consuming region, consuming 52.5%, and the Near East is second, consuming 22.4% [9]. Also, by country, India is by far the largest consumer with 32.2% of total black tea consumption [9]. Furthermore, research in several Indian states showed that black tea is consumed by all age groups, consumption is not dependent on socioeconomic status, and it is consumed in both urban and rural areas [8]. Therefore, black tea is a logical choice for iron fortification in India and likely other Eastern countries.

Figure 2.5: Global Black Tea Consumption, 2013 (data from the FAO [9])

Black tea was chosen to be the food vehicle in this study because it is the most produced variety of tea globally and will easily reach those in India as well as other Eastern countries. However, the challenges posed by iron fortification are very similar for all varieties of tea. Therefore the technology developed for black tea is expected to be easily applicable to other tea varieties. This includes the second most produced tea, green tea, which comprised 32% of total tea production in 2013 [9].
2.2 Factors for the Development of Iron Fortified Tea

2.2.1 Iron Source Selection

There are four types of iron used for fortification: ferrous iron salts, ferric iron salts, elemental iron, and chelated iron. Although their colour depends on the specific iron compound, there are certain trends that can be seen when it comes to solubility, bioavailability, and cost. Typically, the least expensive iron sources are elemental iron followed by iron salts then chelated iron sources [3]. Solubility and bioavailability are often linked. Ferrous iron salts and chelated iron are more soluble and more bioavailable than ferric iron salts and elemental iron [3]. Table 2.3 outlines a comparison of different categories of iron sources relative to ferrous sulphate. Despite having seemingly high bioavailabilities, soluble iron salts are susceptible to reactions with polyphenols and phytates forming complexes which are not absorbable by the body. There is a need to test whether the more expensive chelated iron sources sufficiently increase iron bioavailability in the presence of polyphenols.

Table 2.3: Common Iron Sources for Food Fortification [3]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Bioavailability (%)</th>
<th>Relative cost (per mg iron) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous Iron Salts: water soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ferrous gluconate</td>
<td>89</td>
<td>670</td>
</tr>
<tr>
<td>Ferrous Iron Salts: poorly water soluble but soluble in dilute acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrous fumarate</td>
<td>100</td>
<td>220</td>
</tr>
<tr>
<td>Ferrous succinate</td>
<td>92</td>
<td>970</td>
</tr>
<tr>
<td>Ferric Iron Salts: water insoluble, poorly soluble in dilute acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric orthophosphate</td>
<td>25-32</td>
<td>400</td>
</tr>
<tr>
<td>Ferric pyrophosphate</td>
<td>21-74</td>
<td>470</td>
</tr>
<tr>
<td>Elemental Iron: water insoluble, poorly soluble in dilute acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-reduced</td>
<td>13-148*</td>
<td>50</td>
</tr>
<tr>
<td>Atomized</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>CO-reduced</td>
<td>12-32</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Electrolytic</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>5-20</td>
<td>220</td>
</tr>
<tr>
<td>Chelated Iron: water soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrous bisglycinate</td>
<td>&gt;100</td>
<td>1.760</td>
</tr>
<tr>
<td>Sodium iron EDTA</td>
<td>&gt;100</td>
<td>1.670</td>
</tr>
</tbody>
</table>

*Experimental data is only for a greatly reduced particle size
In the development of an iron fortified food targeted for use in developing countries, cost should be kept low and bioavailability kept acceptably high. In general, adding overages of iron can increase the amount of absorbed iron. However, this comes at the detriment of the fortified product (e.g. cloudiness in brewed tea) and adds extra cost. In fortifying tea with iron specifically, the bioavailability consideration is further complicated by the presence of polyphenols. To develop an effective iron fortified tea at a low cost, iron-polyphenol complex formation must be stifled.

2.2.2 Iron Fortification Level

When determining the iron fortification level, per capita tea consumption was considered. It was assumed that tea was brewed using 1% w/v tea leaves, in accordance with typical instructions [15-17]. The literature indicated that India, the country with the largest consumption of black tea, had a per capita consumption of 2 cups of tea daily. This was somewhat confusing, as in North America a cup is a food measure equal to 237 mL, while the actual consumption was closer to 213 mL/day or approximately 90% of a cup (in 2013) [9, 18]. However, per capita tea consumption was as much as 821 mL/day or more than 3 cups in Turkey [9, 18]. These data points are a bit skewed as adults are likely to drink more than children. For a balanced but conservative estimate, tea consumption was assumed to be 500 mL/day (or approximately 2 cups) per adult. The fortification level recommended in order to have impact and avoid overconsumption is 20% to 40% of the RDA [19]. Since pregnant women are the main target group, tea containing 30% of the RDA for a pregnant women or 8.1 mg of iron per 500 mL is expected to be suitable. This is approximately 4.1 mg of iron per 250 mL (or 3.8 mg per cup). For ease of experimentation, solutions were made on a molar basis using a 0.3 mM iron solution as a close approximation, which is 4.2 mg per 250 mL (or 4.0 mg of iron per cup). Using the latter 4 mg per cup with an expected consumption of 2 cups per day, the percentage of recommended dietary allowance (RDA) and tolerable upper intake level (UL) were calculated. The results can be seen in Table 2.4.
Table 2.4: Percentage of RDA and UL Expected for Adults Drinking Iron Fortified Tea [12]

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Percentage of RDA (2 cups of tea)</th>
<th>Percentage of Tolerable Upper Intake Level (2 cups of tea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18</td>
<td>73%</td>
<td>18%</td>
</tr>
<tr>
<td>19-50</td>
<td>100%</td>
<td>18%</td>
</tr>
<tr>
<td>Woman</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Pregnant</td>
<td>14-18</td>
<td>53%</td>
</tr>
<tr>
<td>Pregnant</td>
<td>19-50</td>
<td>44%</td>
</tr>
<tr>
<td>Lactating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>30%</td>
<td>18%</td>
</tr>
<tr>
<td>≤18</td>
<td>80%</td>
<td>18%</td>
</tr>
<tr>
<td>18-50</td>
<td>89%</td>
<td>18%</td>
</tr>
<tr>
<td>Senior</td>
<td>51+</td>
<td>100%</td>
</tr>
</tbody>
</table>

At this proposed fortification level no segment of the adult population would receive more than 100% of the RDA. For all adults only 18% of the upper limit is added. However, if in practice adding 20% of the RDA for pregnant women is more advantageous, the modification required to iron fortified tea will be a simple adjustment.

2.2.3 Black Tea Production and Chemistry

2.2.3.1 Tea Plants

The most popular teas, omitting herbal infusions, are black, green, and oolong. All three of these teas are produced from the cultivated plant species *Camellia sinensis* [20-22]. There are traditionally three varieties of tea plant (i.e. China, Assam, and Cambod) [20]. These varieties are distinguished based on leaf characteristics (i.e. size, shape, and pose), as well as growth habit [20]. However, cross-breeding has made these varieties quite similar to one another [20]. The currently available tea hybrid plants are referred to by the traditional names based on similarity to the traditional breeds. The China variety is small-leaved and predominantly cultivated in China and Japan for the production of green tea [20]. The large-leaved Assam variety is cultivated in India, Sri Lanka, Africa and Argentina for the production of black tea [20].

2.2.3.2 Tea Polyphenols

Tea shoots have high levels of polyphenols and caffeine. The largest group of polyphenolic compounds in green leaves are catechins (flavanols) [20]. Young tea shoots are 20% to 35% phenolic compounds and 2% to 5% caffeine, on a dry weight basis [22]. Catechins are water-soluble pigments present in the vacuole of the plant leaf cells [20]. They are the major reducing agents of the leaves [20]. During the production of black tea, the green leaves are fermented [20]. The term fermentation is misleading as this usually refers to an anaerobic process involving
microorganisms. However, the term used here refers to enzymatic reactions that require oxygen. During this fermentation oxidation and polymerization of the catechins occurs [20]. The three main types of processed commercial tea are differentiated based on the degree of fermentation: unfermented green tea, semi-fermented oolong tea, and fully fermented black tea [22].

During fermentation the tea catechins (including simple catechin (SC), epicatechin (EC), gallocatechin (GC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG)) are polymerized by the endogenous polyphenol oxidase enzyme into theaflavins and thearubigins [20, 21]. Compared to unfermented green tea, the catechin content of black tea is reduced by approximately 85% [20]. Theaflavins (TF) constitute 0.3 to 1.8% of black tea on a dry weight basis [20, 22]. The TF may be any of the following: simple theaflavin-3 (STF); theaflavin-3-gallate (TF-3-G); theaflavin-3’-gallate (TF-3’-G); theaflavin-3, 3’-digallate (TF-3, 3’-G); digallate equivalent of theaflavins (DGETF); isotheaflavin; and theaflavic acids [20]. These are the yellow/orange/red compounds that give colour, briskness, and strength to the tea [20, 21]. Thearubigins (TR) are red/brown in colour and make up 10-20% of the dry weight of black tea [20-22]. They are the pigments formed from the oxidative degradation of theaflavins [20]. The structures of major green tea polyphenols and black tea polyphenols may be seen in Figures 2.6 and 2.7 respectively. The majority of antioxidants found in tea are polyphenolic compounds. The approximate mean dry weight percentages of antioxidant components in solid black tea extracts are: catechins (i.e. flavanols) (10%-12%), theaflavins (3%-6%), thearubigins (12%-18%), phenolic acids (10%-12%), flavonols (6%-8%), and methylxanthines (8%-11%) (e.g. caffeine) [20].
Figure 2.6: Structures of Major Green Tea Polyphenols [20]
Figure 2.7: Structures of Major Black Tea Polyphenols [20]
2.2.3.3 Processing of Black Tea

Black tea may be produced using a batch method referred to as “orthodox” or a continuous process referred to as “CTC” (crush, tear, and curl) [20, 21]. The process for making black tea consists of withering, crushing/rolling, fermentation, drying, and sorting (Figure 2.8 is a flow chart displaying the process) [20-22]. Withering causes changes to take place such as moisture reduction, an increase in cell membrane permeability, a decrease of leaf rigidity, and an increase in aroma forming compounds [20, 21]. During the crushing/rolling process the leaves are broken into small pieces and the internal cell constituents, namely polyphenol oxidase and polyphenols, are mixed [20, 21]. Prior to this stage the polyphenol oxidase is located in the cytosol of the tea leaf cells while the polyphenols are located in the vacuoles [20]. During fermentation the polyphenols in the leaves are oxidized with the aid of polyphenol oxidase [20, 21]. This process is controlled to ensure the correct ratio of TF to TR (1:10 to 1:12) and to maximize the theaflavin amount (to above 0.8%) [20]. Also during fermentation, free gallic acid and catechin are formed from other catechin fractions (EGCG, ECG, and EGC) by oxidative degallation [20]. This is followed by drying where the moisture is removed and the fermentation is arrested [21]. Moisture is reduced to approximately 3% which enables the tea to have high shelf stability [20].

![Figure 2.8: Tea Processing Flow Chart](image-url)
2.2.4 Iron Absorption in the Presence of Tea

A three-membered flavan ring system is typical of most polyphenols in food (see Figure 2.9) [23]. Polyphenolic compounds, mainly flavonoids, are abundant in tea. The major polyphenolic compounds in tea are catechins and, in black tea, polymerized catechins [20].

![Common Phenolic Compounds in Food](image)

R groups may be H, OH, OCH₃, galloyl esters, or carbonate groups.

Figure 2.9: Common Phenolic Compounds in Food [23]

Catechol, gallol, and polyphenolic compounds containing these groups are effective metal chelators [23]. Polyphenols in tea are known to form chelation complexes that inhibit iron absorption. Interactions within the gut lumen depend on the type of polyphenols present and their degree of protonation, and thus pH. At a pH of less than 4, such as in the adult stomach, most dietary iron is released from its matrix. In the duodenum the pH increases allowing iron to form chelation complexes with polyphenols or quinones (which the polyphenols may have been converted into). In vitro models of digestion (pH adjustment followed by enzymatic digestion) indicate that tea decreases iron absorption by ~30%. This is more pronounced if there is a high stomach pH, such as in infants where the pH is above 4. The effect increases with the dose of tea and is observed when iron is given as a solute in the tea solution or in a meal consumed with tea [20].

Inhibition of iron absorption occurs in proportion to the amount of gallol groups present [20]. Other components of tea may also affect these interactions but this is not fully understood [20].
In a study by Disler et al. (1975) it was concluded that the reduction of iron absorption was caused solely by the tannins in black tea [24]. Data from this study shows a decrease in iron absorption in rats that consumed tea, tannin extract, and tannic acid [24]. A reduction was not seen from tea in which the tannins had been removed or after the consumption of a caffeine solution (Table 2.5) [24].

<table>
<thead>
<tr>
<th>Possible inhibitor</th>
<th>Absorption of iron: mean% ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea Control</td>
<td>21.6 ± 7.3*</td>
</tr>
<tr>
<td>Tea</td>
<td>42.4 ± 8.4</td>
</tr>
<tr>
<td>Tea tannin solution</td>
<td>13.8 ± 5.6*</td>
</tr>
<tr>
<td>Tannin-free tea Control</td>
<td>16.1 ± 10.3*</td>
</tr>
<tr>
<td>Tea</td>
<td>49.6 ± 7.5</td>
</tr>
<tr>
<td>Tannic acid solution Control</td>
<td>53.2 ± 11.2</td>
</tr>
<tr>
<td>Tea</td>
<td>21.7 ± 8.7*</td>
</tr>
<tr>
<td>Tannic acid solution Control</td>
<td>24.1 ± 2.6*</td>
</tr>
<tr>
<td>Caffeine Control</td>
<td>40.9 ± 7.3</td>
</tr>
<tr>
<td>Caffeine Control</td>
<td>56.4 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>61.7 ± 12.6</td>
</tr>
</tbody>
</table>

*significantly different from control, P < 0.001 (rest P > 0.05)

The mechanism of this malabsorption was partially described by Eun-Young Kim et al. (2008) who found that cells of the intestinal lining, enterocytes, absorb iron in the presence of polyphenols but do not allow for the basolateral exit of the iron-polyphenol complex [25]. Simply, iron is absorbed by the intestinal wall but is not allowed to pass through it into the blood stream [25]. This inhibition of iron absorption has been shown to contribute to iron deficiency in at-risk populations [20].

### 2.3 Iron-Polyphenol Chemistry

#### 2.3.1 Formation of Iron-Phenol Coordination Complexes

Deprotonated phenols form oxygen centers with a high charge density (i.e. are hard ligands) that are stabilized by the mesomeric effect (see Figure 2.10a) [26]. These oxygen centers may form coordinate covalent bonds with metal cations resulting in coordination complexes [26]. A coordinate covalent bond is a type of covalent bond in which both electrons in the bonding pair originate from one species. Deprotonated polyphenol ligands behave as hard Lewis bases and have high binding stabilities when bound to hard Lewis acids such as ferric iron (Fe(III)) [23]. A
Lewis base donates an electron-pair to a Lewis acid to form a Lewis adduct [23]. The typical pH range of polyphenol deprotonation is 9-10 but in the presence of certain cations, such as Fe(III), this may be lowered to 5-8 [26]. Aliphatic alcohols do not form these bonds because there are no resonance structures that stabilize a deprotonated oxygen center [26]. However, pyrones can bind metal cations as well because there is partial delocalization of the lone pairs of electrons associated with the heteroatom (the oxygen atom in the ring structure) (see Figure 2.10b) [26]. This delocalization is more prominent in non-fused rings than fused ones [26].

![Figure 2.10: Structures with Stabilized Oxygen Centers; (A) phenol, (B) pyrone (modified from [26])](image)

For chelation to take place, as in iron-polyphenol complex formation, ligands must have a minimum denticity of 2 [26]. Polyphenolic compounds are often bidentate, meaning they can form two coordinate covalent bonds with a metal cation [26]. A polyphenol and a pyrone may act as a bidentate ligand if they have oxygen centers in the ortho position (see Figure 2.11a and Figure 2.11b respectively) [26]. The peri positions of two fused rings may also provide a chelation center (see Figure 2.11c) [26]. However, if two chelation centers share an oxygen center, only two oxygen centers will form a chelation complex with a metal cation [26]. Also, the affinity of chelating sites to metal cations is lower on attached rings than single rings [26]. For example, the affinity of the molecule in Figure 2.11d is lower than the molecule in Figure 2.11b [26].
Figure 2.11: Chemical Structures with Chelation Centers; (A) catechol, (B) pyromeconic acid (3-hydroxy-4-pyrene), (C) 1,8-dihydroxynaphthalene, (D) 3-hydroxychromone (modified from [26])

A phenoxide group (i.e. a deprotonated phenol) is a hard ligand, having high charge density, and thus interacts preferentially with cations of high charge density (e.g. iron(III), iron(II), copper(II), zinc(II), aluminum(III), gallium(III)) [26]. Iron(III) is preferred to iron(II) due to the electronegativity of the oxygen electron donor (O') of phenols [26, 27]. This is in contrast to chelating agents that use nitrogen electron donors (N) which are softer ligands [26, 27]. Phenoxide groups generally do not interact with alkali or alkaline earth metal cations (e.g. sodium, potassium, and calcium) although there may be some weak interaction with magnesium [26].

Bidentate polyphenol ligands can form 1:1, 1:2, and 1:3, iron:polyphenol complexes [26]. The amounts of each type of complex in a solution depend on the concentration of the metal, the concentration of the ligand, the pH of the solution, and the structure of the polyphenols [23, 26]. Due to deprotonation there is an increasing amount of polyphenol per complex as pH increases [26]. In the 1:1 and 1:2 iron:polyphenol complexes, iron also coordinates water molecules (4 in the 1:1 complex and 2 in the 1:2 complex) [26]. The reaction of Fe(III) with a polyphenol may be seen in Figure 2.12. Ferrous iron (Fe(II)) and Fe(III) both prefer octahedral geometry when binding to polyphenols [23]. Some polyphenols possess multiple metal-binding sites and thus are capable of oligomerization and polymerization [26].
There are a few complicating factors when dealing with black tea polyphenols and even gallic acid when investigating potential iron fortification strategies. Firstly, certain polyphenols contain other groups that are expected to bind iron such as the pyrone groups in flavonols [23]. Solid black tea extracts are composed of 6-8% flavonols [20]. Secondly, polymerized polyphenols may have multiple chelation centers which may bind to multiple iron atoms, increasing the likelihood of polymerization and precipitation. Thirdly, some iron sources possess a counter ion that may coordinate iron. However, the effect of a counter ion is likely to be less prominent in tea due to the relative concentrations of iron to polyphenol (0.3 mM iron and approximately 6 mM GAE polyphenols) [28]. Finally, some acids and bases that may be used to maintain pH may also coordinate iron. However, if strong acids and bases are used, less of them will be added thus minimizing this effect.

The ratio of iron:polyphenol that will be present upon complexation may be anticipated from the pH of the solution. At slightly acidic pH (5-6.5) each iron is typically bound to 2 catecholate or 2 to 3 gallate ligands, forming complexes which are blue-purple in colour [23]. At more acidic pH (less than 4) polyphenols bind iron in a 1:1 ratio forming complexes which are blue-green in colour [23]. At pH 8 and above, the 1:3 iron:polyphenol complexes dominate which are red in colour [23, 29]. Using black tea tannins, Disler et al. measured that the iron:tannin ratio in the dominant complexes were 1:1 at pH 2.0, 1:2 at pH 5.5, and 1:3 at pH 8 [24].
2.3.2 Electron Configuration and Colour of Coordination Complexes

Charge transfer between orbitals of metal character and orbitals of ligand character causes vivid colour formation in some coordination compounds [30]. To gain a more complete understanding of how this applies to iron-polyphenol complexes it is necessary to first review the electronic structure of iron and iron coordination complexes. The term “iron-phenol” will be used to refer to both iron-phenol and iron-polyphenol complexes. Iron has an electronic structure of $1s^22s^22p^63s^23p^64s^23d^6$ or [Ar]$4s^23d^6$ [30]. The most common ions of iron are ferrous (Fe$^{2+}$, [Ar]$3d^6$) and ferric (Fe$^{3+}$, [Ar]$3d^5$) [30]. Also importantly, oxygen has an electronic structure of $1s^22s^22p^4$ or [He]$2s^22p^4$ [30]. If one electron of an oxygen atom is being used to bond to another atom (such as to carbon in phenol) this leaves a lone pair of electrons to form a coordination bond with iron and a single extra electron in the 2p orbitals. If two electrons are being used to bond to other atoms (such as to two hydrogens in water) this leaves only a lone pair of electrons to form a coordination bond with iron.

Molecular orbital theory explains how orbitals of atoms may become bonding orbitals when their symmetries and relative energies permit it [30]. When atomic orbitals interact they join to produce a bonding molecular orbital and an antibonding molecular orbital [30]. The bonding orbital is lower in energy than the atomic orbitals and the antibonding orbital is higher in energy than the atomic orbitals [30]. Commonly an antibonding orbital is denoted with an asterisk. If an atomic orbital does not interact it forms a nonbonding molecular orbital [30]. The types of molecular orbitals important to iron coordination chemistry are σ and π orbitals. The σ orbitals are symmetric to rotation about the line connecting the nuclei of the bonding atoms (bond axis) while π orbitals change in sign with 180° rotation about the bond axis [30]. Orbitals may be gerade, symmetric to inversion (e.g. d orbitals or the molecular orbital $\sigma_g$), or ungerade, antisymmetric to inversion (e.g. p orbitals). In the cases of iron-phenols and iron aquo ions the $3d$, $4s$, and $4p$ orbitals from iron interact with the $2p$ orbitals from the oxygen present in the phenol or water molecules.

Upon the formation of a coordination compound, the $3d$ orbitals of the free iron ion split into different energy levels [30]. This is because the geometry of the interacting orbitals prohibits some of the $3d$ iron orbitals from forming σ bonds with the $2p$ oxygen orbitals. In the case of complex formation with only σ bonding (e.g. iron aquo ions), the $3d$ orbitals that do not form σ bonds form nonbonding orbitals at the same energy level as uncoordinated iron [30]. The splitting of the $3d$ orbitals...
orbitals of iron is dependent on the coordination geometry of the complex [29]. Because iron-phenol complexes and iron aquo ions have octahedral coordination geometry, this geometry will be the one explained further [23, 29].

Figure 2.13 denotes the molecular orbitals for an octahedral transition metal coordination complex with only σ interaction. Presented on the left are the atomic orbitals from the metal, on the right are the ligand orbitals, and in between are the molecular orbitals. The “A1, E, T1, and T2” indicate symmetry information for the atomic orbitals while “a1, e, t1, and t2” indicate the same information for the molecular orbitals [30]. The symmetry information is not crucial to the understanding of colour formation but the labels are used to refer to specific orbitals. In an octahedral complex the $d_{x^2-y^2}$ and the $d_{z^2}$ iron orbitals are oriented directly toward ligand lone pairs of electrons thus forming $\sigma$ and $\sigma^*$ orbitals ($e_g$) [30]. The other 3d orbitals ($d_{xy}, d_{xz},$ and $d_{yz}$) are oriented between ligand lone pairs such that they do not interact with them, thus form nonbonding orbitals ($t_{2g}$) [30]. The 4s and 4p orbitals from the iron are used to form the remainder of the $\sigma$ molecular orbitals [30]. All of the $\sigma$ bonding orbitals will be occupied by electrons from the ligands and are defined as having ligand character [30]. The $t_{2g}$ orbitals and in some circumstances the antibonding $e_g$ orbital ($e_g^*$ or $\sigma^*$) will be occupied with electrons from the metal, thus are of metal character [30].

There are two possible distributions of metal electrons between the $t_{2g}$ and $e_g^*$ energy levels referred to as low-spin and high-spin [30]. A low-spin electron distribution is one in which the electrons form pairs (of opposite spin) in the lower energy orbitals before occupying those of higher energy [30]. A high-spin distribution is one in which the electrons occupy all orbitals (of both energy levels) before pairing [30]. In determining the electron distribution both the total pairing energy ($\Pi$) and the difference in energy between the split metal d orbital electrons (i.e. between $t_{2g}$ and $e_g^*$ molecular orbitals), denoted “$\Delta_o$”, must be taken into account [30]. In general, if $\Delta_o$ is large, ligand orbitals are interacting strongly with the metal orbitals, thus there is a “strong ligand field” and low spin electron configurations are favoured. In contrast if $\Delta_o$ is small, there is a “weak ligand field” and high spin electron configurations are favoured [30]. Both iron aquo ions and iron-phenols form high-spin complexes [29, 30]. Therefore the high-spin configuration will be considered further.
Coordination compounds of iron and phenols also have π interaction due to the oxo ligand having a spare electron in the p orbital [31, 32]. In contrast, water forms purely σ bonds in an iron coordination complex [29]. This π interaction causes bonding molecular orbitals to form from the d_{xy}, d_{xz}, and d_{yz} iron orbitals in conjunction with the interacting ligand orbitals, forming bonding t_{2g} and antibonding t_{2g}^* molecular orbitals [30].
Two types of $\pi$ interactions may occur: ligands may accept electrons from the metal ($\pi$-acceptor ligands) or they may donate electrons ($\pi$-donor ligands) [30]. Ligands with unoccupied orbitals of higher energy than the transition metal d orbitals may accept electrons from the occupied metal d orbitals [30]. When this occurs, $\Delta_o$, the energy difference between the split metal d electrons, is increased [30]. This is because the $\pi$ bonding molecular orbitals of metal character are the bonding $t_{2g}$ orbitals [30]. The $\sigma$-only nonbonding $t_{2g}$ orbitals are thus lowered in energy becoming bonding $t_{2g}$ orbitals while the $\sigma$ antibonding $e_g$ orbitals ($e_g^*$, also of metal character) are unaffected [30]. The other type of $\pi$ interactions can occur when ligands with occupied p orbitals become $\pi$-donors [30]. When $\pi$-donor interaction occurs, the resulting $\Delta_o$ decreases because the $\pi$ molecular orbitals of metal character are $t_{2g}^*$ which have higher energy than the $\sigma$-only $t_{2g}$ nonbonding orbitals [30].

Figure 2.14 shows the difference in the $t_{2g}$ energy levels and $\Delta_o$ for $\pi$-acceptor ligands, $\sigma$-donor only ligands, and $\pi$-donor ligands [30].

The size of $\Delta_o$ is an indication of the strength of the ligand field. When ligands are ordered from strongest to weakest field strength (i.e. the spectrochemical series) they are ordered from $\pi$-acceptor ligands (large $\Delta_o$, strong field, low spin), to $\sigma$-donor only ligands, to $\pi$-donor ligands (small $\Delta_o$, weak field, high spin) [30]. Figure 2.14 also demonstrates the possible electron configurations of each type of coordination complex using a $d^5$ transition metal ion as an example since Fe$^{3+}$ is the most relevant species to this thesis. The oxygen in phenol contains an electron that is not bound to carbon nor forms $\sigma$ bonds with iron, i.e. it has an occupied p orbital. This allows phenols to act as $\pi$-donors and form high-spin iron-phenol complexes [31, 32].
Movement of electrons from one energy level to another may occur with the absorption of specific wavelengths of electromagnetic radiation [30]. The wavelength required is related to the difference in energy between the orbitals the electrons move between [30]. Intensities of absorption bands are governed by selection rules:
1. Laporte selection rule [30]
Transitions between states of the same parity are forbidden. Parity refers to gerade (symmetrical to inversion, e.g. d orbitals) or ungerade (unsymmetrical to inversion, e.g. p orbitals) [30]. Therefore, d-d transitions are not allowed but p-d transitions are allowed, such as from ligand to metal [30].

2. Spin selection rule [30]
Transitions between states of different spin multiplicities are forbidden. Spin multiplicity is calculated as $2S + 1$ where $S$ is the total spin angular momentum quantum number which is the largest possible value of the sum of the quantum spin numbers ($+\frac{1}{2}$ or $-\frac{1}{2}$ for each electron) [30]. More simply, the total spin must remain constant before and after the transition, or, no electrons may change their spin during the transition.

These rules are not strict because there are a few mechanisms for which they may be relaxed [30]. However, intensity of the bands is dependent on how well these selection criteria are adhered to [30]. An example of how these rules may be relaxed is that transition metal complexes undergo vibrations that may temporarily change the symmetry [30]. Octahedral complexes vibrate allowing the center of symmetry to temporarily be lost and in this way d-d transitions result [30]. However, in the case of Fe$^{3+}$ high spin complexes, all d-orbitals are filled with one electron and therefore movement of electrons will necessitate a change in spin multiplicity, which is also forbidden. There is no visible d-d transition associated with high-spin octahedral iron complexes [29]. Ferrous iron (Fe$^{2+}$) has only one spin-allowed band in the near-IR range (~1000 nm) [29]. Thus iron aquo complexes and iron-phenol complexes do not have visibly coloured d-d bands.

Charge transfer transitions can occur between the iron d orbitals and ligand p orbitals [30]. This transfer can occur from metal to ligand (MLCT) or from ligand to metal (LMCT) [30]. These transitions are fully allowed and therefore have high intensity [30]. When they occur the effects of other electronic transitions are generally no longer observable due to their relatively lower intensities [30]. The absorbance of an electronic transition is related by the Beer-Lambert law to the concentration of the complex and to the molar extinction coefficient, $\varepsilon$, which represents the
intrinsic intensity of the absorption [29, 30]. Table 2.6 lists some molar extinction coefficients for different types of electronic transitions for iron.

Table 2.6: Molar Extinction Coefficients for Electronic Transitions of Iron (modified from [29])

<table>
<thead>
<tr>
<th>Type of band</th>
<th>Molar Extinction Coefficient ε (M⁻¹ cm⁻¹)</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge transfer (LMCT or MLCT), fully allowed</td>
<td>2,000–10,000</td>
<td>Fe³⁺–phenol</td>
</tr>
<tr>
<td>d–d, Octahedral, spin-allowed</td>
<td>1–10</td>
<td>Octahedral Fe²⁺ (high spin)</td>
</tr>
<tr>
<td>d–d, Octahedral, spin-forbidden</td>
<td>0.01–0.1</td>
<td>Octahedral Fe³⁺ (high spin)</td>
</tr>
</tbody>
</table>

Iron-phenol complexes are ligand to metal charge transfer complexes (LMCT) [23]. For a complex to be MLCT it must contain π-acceptor ligands such that the electrons may move from metal d-orbitals to higher energy orbitals of ligand character [30]. LMCT bands are often at visible wavelengths when ferric iron (Fe³⁺) is coordinated to electron-rich ligands (i.e. π-donors) [29]. Ligand to metal charge transfer transitions result in a formal reduction of the metal (i.e. Fe³⁺→Fe²⁺) [30]. The energies of these transitions (i.e. frequencies of light required) may be conceptualized on the basis of redox reactions [29]. The more easily reducible the metal ion (e.g. Fe³⁺ is more easily reduced than Fe²⁺) and the more reducing the ligand (e.g. phenol is a better reducing agent than water), the lower the energy required for charge transition [29]. Ferric iron-phenol complex has a band in the visible region and is coloured while the charge transfer bands of ferrous iron-phenol are of higher frequency and the complex is not coloured [29]. In accordance with this, iron aquo complexes are also colourless. Figure 2.15 is a diagram illustrating the possible LMCT transitions that may occur in ferric iron coordination complexes.
Figure 2.15: LMCT Transitions in an Octahedral Fe$^{3+}$ Complex with $\pi$-Donor Ligands (modified from [33])

Only one LMCT band resides in the visible spectrum for ferric iron-polyphenol complexes [23]. The LMCT transition with the smallest energy difference is likely to cause the absorbance in the visible spectrum. The wavelength of the visible absorption band for each complex ratio (1:1, 1:2, 1:3 (iron:polyphenol)) is different. At a 1:1 iron:polyphenol ratio the complex is blue-green and absorbs at about 670 nm; at a 1:2 ratio the complex is blue-purple and absorbs at 542-586 nm; and at a 1:3 ratio the complex is red and absorbs at 490-520 nm [23]. This decrease in wavelength signifies an increase in the energy differences between the orbitals of ligand character and metal character which determine the visible LMCT absorbance band. The increase in energy difference with increased polyphenol is reasonable because the lowest energy charge transfer occurs between $t_{2g}$ and $t_{2g}^*$. In the iron aquo ion $t_{2g}$ is comprised of nonbonding orbitals because there is no $\pi$ interaction. As the amount of polyphenol increases the amount of $\pi$ interaction increases. This forms and further separates the energy levels of the bonding $t_{2g}$ orbitals and antibonding $t_{2g}^*$ orbitals. Another factor is distortion caused by the molecule having a combination of different ligands (water and polyphenol).
Tea is a very complicated system and there are many factors that may complicate the measurement of iron-polyphenol complex formation through the observation of LMCT absorbance bands. Measurement may be complicated by interference, change in molecule symmetry (distortion), several orbital energies, and differences in degrees of orbital overlap. Some specific examples are given below:

- Coloured polyphenols due to extended conjugations [23]
- Other possible ligands (e.g. OH⁻, pH dependant)
- Pyrone groups interacting with iron to form intensely coloured compounds [34, 35]
- A variety of different polyphenols present in tea forming iron complexes

2.3.3 Thermodynamics and Kinetics of Iron Coordination Complexes

Bonding strengths are indicated by stability constants. In the case of coordination complexes these are generally equilibrium constants measured in aqueous solution [30]. Often water molecules are omitted from equilibrium constant expressions for simplicity [30]. As a result, large stability constants indicate that bonding with the incoming ligand is much more favourable than bonding with water [30]. Temperature dependence of equilibrium constants can be used to calculate enthalpies and entropies of reaction but because of complicating factors they are often most valuable for viewing relationships among similar complexes (e.g. different ligands reacting with the same metal) [30].

For polydentate ligands, once one site binds to a metal it becomes more likely that the others will also bind [30]. This is seen as an increase in the stability constant (and decrease in ΔG) indicating a more favourable reaction. This tendency is referred to as the chelate effect. It is most prominent when the total chelation ring size is 5 or 6 atoms [30]. This is because small chelation rings become strained and larger ones may require awkward bending in order to bind [30]. An increase in entropy is used to explain the chelate effect and can be simply observed from the increase in the total number of molecules when iron binds to a polydentate ligand [30].

Example:

\[ \text{Fe(H}_2\text{O)}_6 + \text{polyphenol} \rightarrow \text{Fe(polyphenol)(H}_2\text{O)}_4 + 2 \text{H}_2\text{O} \]

\text{Equation (Eq.) 2.1}

2 molecules \rightarrow 3 molecules
Complexation equilibria in aqueous solutions usually involve metal (M), ligand (L), and protons (H⁺) [29]. Each complex (M_iL_jH_k) is characterized by stability constant (i.e. an equilibrium constant, β_{ijk}) [29].

\[ iM + jL + kH^+ \leftrightarrow M_iL_jH_k \]  
Eq. 2.2

\[ \beta_{ijk} = \frac{[M_iL_jH_k]}{[M]^i[L]^j[H]^k} \]  
Eq. 2.3

For a formal stability constant, such as the one defined in the equation above, “L” refers to a fully deprotonated form of the ligand [29]. To take pH dependence into account, effective binding constants have been defined such as [29]:

\[ K_{eff} = \frac{\Sigma_k [MLH_k]}{[M] \Sigma_i [H_iL]} \]  
Eq. 2.4

The summation term in the numerator accounts for protonated complexes (k>0) and for hydrolyzed complexes (k<0) [29]. The summation term in the denominator accounts for the degree of protonation of the uncomplexed ligand [29]. But this effective binding constant is written specifically for 1:1 metal:ligand complexes and if the system contains different metal:ligand stoichiometries this method becomes cumbersome [29].

A common alternative is to calculate the concentration of free metal ions in standard solutions with specified pH, total ligand concentration, and metal concentration [29]. The concentration of free metal ions is expressed as pM (-log[M]) [29]. A smaller concentration of free metal ion produces a larger pM, which denotes a more effective iron-complexing agent [29]. This method takes into account the effects of ligand protonation, chelate protonation/hydrolysis, and the formation of a mixture of complexes with differing metal:ligand stoichiometry [29]. An example of some of these pM values is given in Table 2.7 in conjunction with overall binding coefficients. This data indicates that for polyphenols, such as catechol, it is likely that no significant free ferric iron will remain in solution at equilibrium and that polyphenols do not bind strongly to ferrous iron.
Table 2.7: Equilibrium Values for Iron Chelators (adapted from [29])

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ion</th>
<th>log $\beta_{110}$</th>
<th>log $\beta_{120}$</th>
<th>log $\beta_{130}$</th>
<th>pM</th>
<th>% Fe Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,10-phenanthroline</td>
<td>Fe(II)</td>
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<td>11.15</td>
<td>21</td>
<td>11.53</td>
<td>100</td>
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<tr>
<td></td>
<td>Fe(III)</td>
<td>6.5</td>
<td>11.4</td>
<td>14.1</td>
<td>7.67</td>
<td>98*</td>
</tr>
<tr>
<td>oxalic acid</td>
<td>Fe(II)</td>
<td>3.05</td>
<td>5.15</td>
<td>-</td>
<td>6.01</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>7.53</td>
<td>13.64</td>
<td>18.49</td>
<td>11.54</td>
<td>100*</td>
</tr>
<tr>
<td>iminodiacetic acid</td>
<td>Fe(II)</td>
<td>5.8</td>
<td>-</td>
<td>-</td>
<td>6.03</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>10.72</td>
<td>-</td>
<td>20.12</td>
<td>12.04</td>
<td>100*</td>
</tr>
<tr>
<td>catechol</td>
<td>Fe(II)</td>
<td>7.95</td>
<td>13.5</td>
<td>-</td>
<td>6.00</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>20</td>
<td>34.7</td>
<td>43.8</td>
<td>15.5</td>
<td>100*</td>
</tr>
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<td>citric acid</td>
<td>Fe(II)</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>6.09</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>11.4</td>
<td>18.2</td>
<td>-</td>
<td>16.73</td>
<td>100*</td>
</tr>
<tr>
<td>nitrilotriacetic acid</td>
<td>Fe(II)</td>
<td>8.33</td>
<td>12.8</td>
<td>-</td>
<td>7.07</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>15.9</td>
<td>23.97</td>
<td>-</td>
<td>18.05</td>
<td>100*</td>
</tr>
<tr>
<td>EDTA</td>
<td>Fe(II)</td>
<td>14.27</td>
<td>-</td>
<td>-</td>
<td>12.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>25.1</td>
<td>-</td>
<td>-</td>
<td>23.52</td>
<td>100*</td>
</tr>
<tr>
<td>DTPA**</td>
<td>Fe(II)</td>
<td>16.4</td>
<td>-</td>
<td>-</td>
<td>13.25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fe(III)</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>28.24</td>
<td>100*</td>
</tr>
</tbody>
</table>

Values for 0.1 M ionic strength, 25°C, neutral pH, 10^-6 M Fe
*Calculated from pM
**DTPA = diethylenetriaminepentaacetic acid

In general high-spin octahedral $d^6$ (Fe$^{2+}$) and $d^5$ (Fe$^{3+}$) compounds undergo rapid substitution reactions (or are labile) [30]. Commonly reaction half-lives of one minute or less are said to be labile [30]. Both high-spin aquo ions (Fe$^{2+}$ and Fe$^{3+}$) are labile [29]. The water-exchange reactions for Fe$^{2+}$ and Fe$^{3+}$ follow $I_d$ (interchange by dissociation) and $I_a$ (interchange by association) mechanisms, respectively [29]. Polydentate complexes are thermodynamically more stable but also this chelate effect may be observed kinetically [30]. Substitution for a chelated ligand is generally slower than for similar monodentate ligands [30]. This can reduce reactions rates by a factor of 20 to 10^5 [30].

2.3.4 Redox Reactions of Iron-Polyphenol Complexes

Ferrous iron (Fe(II)) oxidation usually occurs slowly in the presence of oxygen. However, when bound to polyphenol ligands at neutral or basic pH, the reduction potential is lowered and the rate of iron oxidation is increased [23]. This may be rationalized through electrostatic repulsion which favours the removal of an electron from Fe(II) and stabilizes ferric iron (Fe(III)) [26]. Another explanation may be derived from the fact that coordination complexes are also acid-base adducts [30]. Deprotonated polyphenol ligands, which are hard Lewis bases, bind more strongly to Fe(III), which is a hard Lewis acid, than to Fe(II), which is a borderline Lewis acid [23, 26].
The tendency towards iron oxidation can be observed by comparing the total stability, $\beta$, of the ferrous and ferric iron complexes. This is defined as the product of the equilibrium constants. The equilibrium constants $K_1$, $K_2$, and $K_3$ represent 1, 2, or 3 polyphenols binding to an iron ion, respectively. The total stability is often presented as “log $\beta$” which is the sum of the log of the equilibrium constants. This is defined below:

$$\log \beta = \Sigma \log K_n$$

Eq. 2.5

Where,

$\log \beta$ = total stability
$K$ = equilibrium constant
$n$ = number of species

Catechol and gallic acid equilibrium constants together with total stabilities are presented in Table 2.8. As can be seen, the total stability of ferric complexes is higher than that of ferrous complexes [23]. Furthermore, the total stabilities for ferric complexes are large, indicating that only negligible iron remains as an aquo complex.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fe$^{n+}$, $n$=</th>
<th>log $K_1$</th>
<th>log $K_2$</th>
<th>log $K_3$</th>
<th>log $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>catechol</td>
<td>2</td>
<td>8.2*</td>
<td>5.55</td>
<td>–</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20.01</td>
<td>14.69</td>
<td>9.06</td>
<td>43.76</td>
</tr>
<tr>
<td>gallic acid</td>
<td>2</td>
<td>7.0</td>
<td>–</td>
<td>–</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12.6*</td>
<td>8.5*</td>
<td>4.7*</td>
<td>25.8*</td>
</tr>
</tbody>
</table>

*average of two studies

Therefore, the autoxidation that occurs at neutral pH is characterized by the rapid oxidization of Fe(II)-polyphenol complexes into Fe(III)-polyphenol complexes [23]. A pictorial representation of this reaction may be seen in Figure 2.16.
Figure 2.16: Iron Autoxidation (modified from [23])

The iron oxidation rate varies for different polyphenol complexes. For example, gallate complexes oxidize iron faster than catecholate complexes [23]. The rate of Fe(II) autoxidation is also dependent on the counterion of the ferrous salt [23]. Other ligands also cause autoxidation, such as hydroxide, pyrophosphate, citrate, phosphate, chloride, sulphate and perchlorate [23, 26]. The half-life of Fe(II) (0.4 mM) at pH 7.0 in the presence of phosphate (1.2 mM) is 210 seconds and in the presence of citrate (1.5 mM) is 60 seconds [26]. Similar rates are expected to occur in the presence of tea polyphenols [26].

Perron et al. (2010) measured ferrous iron oxidation rates in the presence of polyphenols at pH 6 by observing the LMCT absorbance bands [36]. The results are summarized in Table 2.9. Polyphenols with gallol groups generally had faster initial rates than those with only catechol groups [36]. The assumed general reaction rate equation was [36]:

\[
    \text{rate} = k_{\text{obs}}[\text{polyphenol}]^a[\text{Fe}^{2+}]^b[\text{O}_2]^c[\text{H}^+]^n
\]

Eq. 2.6

For the catechol and gallol compounds tested (EC, EGCG, MEPCA, MEGA, GA), the reaction rates were first order with respect to the ligand concentration and Fe(II) concentration [36]. That is, the reaction rates increased in proportion to increases in polyphenol concentration or Fe(II) concentration [36]. The complexes MEPCA and MEGA were selected to represent catechol and gallol containing polyphenols. These were used to investigate the dependence of the reaction on O₂, which was found to be linear [36]. As expected, the rate of iron oxidation was found to decrease with a decreased pH [36]. Therefore the rate expression, for when pH was held constant, was concluded to be:

\[
    \text{rate} = k_{\text{obs}}[\text{polyphenol}][\text{Fe}^{2+}][\text{O}_2]
\]

Eq. 2.7
In an acidic environment Fe(III) is reduced to Fe(II) when bound to catecholate or gallate ligands [23]. This is because these ligands are susceptible to oxidation [26]. During this process the polyphenol is oxidized to a semiquinone [23]. At low pH, the ligand is protonated and is therefore a neutral ligand. Fe(II) is stabilized relative to Fe(III) by neutral unsaturated ligands [23]. Once the semiquinone form of the polyphenol is generated, it can reduce another equivalent of Fe(III) while oxidizing from a semiquinone to a quinone [23]. Studies of this phenomenon were done at very low pH, which may be relevant to tea fortification due to low stomach pH [23]. At higher pH conditions, the formation of bis- and tris-polyphenol complexes with iron (two and three polyphenol ligands coordinated to a single iron, respectively) inhibit the ferric iron reduction process [23]. The semiquinone and quinone are also able to coordinate cations but generally with a reduced affinity [26]. A diagram of the reaction can be seen in Figure 2.17. In the case of theaflavins at low pH, quinone forms develop (theabenazoquinone and theanephthoquinone) and oxydative polymerization occurs [37]. The formation of these products occurs within 30 seconds at pH 3 (Fe(III) = 2 mM, theaflavin = 0.2 mM) [37].
The redox state of iron coordinated to polyphenol ligands is dependent on the pH of the solution [26]. Iron can be cycled between Fe(II) and Fe(III) by manipulation of pH [26]. There are three isoelectric forms of the metal quinone chelate ring (Figure 2.18) [26]. At low pH, Fe(II) is more prominent and at pH 6 and above it appears to be non-existent (see Figure 2.19). The proportion of Fe(II) to Fe(III) is dependent on the pH, the phenolic compound used, and other ligands present [26]. However, under many conditions, equilibrium occurs between the two forms (Figure 2.20) [26]. The Fe(II)-semiquinone complexes are green in colour and may be mistaken for Fe(III)-mono(polyphenol) complexes at low pH [23].
2.3.5 Hydrolysis in Iron Coordination Complexes

Fe(III) aquo complexes have a tendency towards deprotonation of the coordinated water molecules to form yellow hydroxo complexes [29]. Fe(III) begins to hydrolyze slightly below pH 2 while Fe(II) does not begin to hydrolyze until a pH of approximately 8.5 [29]. Fe(III) progresses through complexes of 1:1 to 1:4 Fe:OH ratio from pH 2 to pH 10 (i.e. Fe(OH)$^+$, Fe(OH)$_2$, Fe(OH)$_3^-$, Fe(OH)$_4^{2-}$) [29]. At neutral pH, insoluble Fe(OH)$_3$ is the prominent species ($K_{sp} = 10^{-20}$ M) [29]. If an iron source is highly insoluble it may not react with polyphenols. However, at low pH, ferric iron is likely to take a soluble form (e.g. in the stomach) and proceed to form complexes with polyphenols or quinones in the duodenum where pH is increased. As discussed in Section 2.3.4, the iron will likely be reduced to its ferrous form in the presence of polyphenols at low pH while the polyphenols are oxidized into quinones.

When Fe(III) is complexed with a multidentate ligand hydroxylation may still occur [29]. The tendency of the chelate to hydrolyze is inversely related to the stability of the chelate [29]. However, even the relatively stable ferric-EDTA complex exists at neutral pH as a mixture of Fe(EDTA)$^-$, Fe(EDTA)(OH)$^{2-}$, and (Fe-EDTA)$_2$O$^{4-}$ [29]. Possible hydrolyzed species of iron-polyphenol complex may cause some change in ligand field energy and thus change in colour (shift in absorbance band) in relation to the pH of the solution.

2.3.6 Effect of pH Control on Metal Cations

Buffering compounds are well known to interfere with cations, such as iron, in solution. Common buffering compounds such as various citrate, acetate, and phosphate salts are listed as sequestrants in the Codex Alimentarius [38]. Specialized biological buffer solutions have been developed to lessen the interaction with cations, including HEPES, Tris, MES, and MOPS. However, studies of these buffer solutions still show interference with iron [39, 40]. Research with these buffer
solutions is generally done at pH levels representative of biological systems, generally between pH 6 and pH 8. Investigations have been done in the presence of iron comparing phosphate to these specialized buffer solutions. In a paper by Tadolini (1987), iron autoxidation in MOPS and HEPES buffer solutions were compared to phosphate [41]. It was found that MOPS and HEPES better represented an unbuffered system that was brought to the intended pH with only sodium hydroxide (NaOH) [41]. Andjelkovic et al. (2006) investigated phosphate, HEPES, and Tris buffer solutions when studying the iron-chelation properties of various phenolic acids [39]. It was found that phosphate and HEPES interfered considerably while Tris only slightly interfered [39]. Citrate and acetate have not been studied with direct comparisons to other buffering agents.

Adjustment of pH is necessary in order to determine if iron-polyphenol complex will form in brewed tea and at site of iron absorption in the body. Since tea polyphenols are strong chelating agents, interactions between a buffer solution and iron may be minimal. However, this must be investigated and a decision must be made on whether to use buffer solutions or resort to the use of strong acids and bases for pH adjustment during experimentation.

2.4 Overcoming Barriers Posed by Chelating Foods

2.4.1 Prevention of Iron-Polyphenol Complex Formation in Foods

There have not been many studies conducted on the prevention of iron-polyphenol complex formation in foods. The most comprehensive study regarding this topic was conducted by Mellican et al. (2003) and will be discussed at length in this section [42]. Mellican et al. studied iron-phenolic compound complex formation for the purpose of preventing off-colour development in food. The study covers iron interaction with simple phenolic compounds followed by a deeper investigation of monomeric polyphenols. The impacts of various environmental factors were tested on monomeric polyphenols and modeled. These models were then compared to various food and beverage products with added iron.

The LMCT absorbance bands were observed for iron complex formation with the following simple phenolic compounds: phenol, catechol, resorcinol, and hydroquinone. Both ferrous sulphate and ferric sulphate were used as iron sources. Iron-phenol complex was only found to occur in solutions of catechol because it was the only phenolic compound containing ortho-hydroxyl groups. Ferrous sulphate reacted more slowly than ferric sulphate. The absorbance of
the ferrous sulphate solutions increased over a 24 hour period whereas ferric sulphate solutions were fully developed in less than 5 minutes. The extent of the reaction for ferric sulphate was ten times greater than ferrous sulphate but this may have been caused by the duration of the experiment.

Monomeric polyphenols (i.e. catechol, gallic acid, catechin, caffeic acid, and chlorogenic acid) were tested under different conditions expected to affect iron-polyphenol complex formation. These conditions included variation of the following:

- **iron oxidation state**: ferrous iron, ferric iron
- **presence of oxygen**: with, without
- **reducing agents**: none, ascorbic acid, sodium bisulphite, hydroxylamine
- **chelating agents**: none, EDTA, sodium hexametaphosphate (SHMP)
- **temperature**: 0°C, 23°C, 61°C, 100°C
- **pH**: 2, 4, 6, 8
- **redox potential** (varied by using a variety of iron compounds): ferrous sulphate, ferrous lactate, ferrous gluconate, ferrous bisglycinate, ferric sulfate, ferric citrate, ferric EDTA

An interesting finding was that temperature has an inverse effect on absorbance, and thus iron-polyphenol complex formation. An explanation given for this is that the concentration of dissolved oxygen in the water is lower at higher temperatures. Since this oxygen is required to oxidize any reduced iron to form the coloured iron-polyphenol complex, less of it was formed.

Linear regression models were generated which can predict colour development (based on absorbance) as a function of the various conditions. Only one of these models was presented in detail but all binding polyphenols were reported to have behaved similarly. Although the model presented had a fairly low coefficient of regression, 0.67, it is useful in ranking the relative significance of the various conditions. In order of decreasing significance for influencing iron-polyphenol complex formation the factors are ordered as: presence of reducing agents (model coefficient = -0.348), presence of chelating agents (-0.333), pH (0.262), the presence of oxygen (0.131), the oxidation state of iron (-0.049), and the redox potential of the iron source (0.001). The effect of temperature was found to be insignificant for the model.
As expected, reducing agents, chelating agents, and the use of ferrous iron (over ferric iron) decreased iron-polyphenol complex formation. Also as expected, an increase in pH or oxygen increased iron-polyphenol complex formation. However, Mellican et al. were surprised that lower redox potentials for ferrous iron sources lead to greater iron-polyphenol complex formation whereas the opposite was true for ferric iron sources. This was surprising because iron must be in its oxidized form in the coloured complex. Therefore, the expectation was that a lower redox potential would be beneficial for both types of iron. However, dissociation of ferrous iron sources will lower the redox potential of a solution while dissociation of ferric iron sources will increase the redox potential of a solution. Therefore, it is likely that redox potential was an indication of the tendency of the iron source to dissociate in solution. This would then indicate that higher dissociation leads to greater iron-polyphenol complex formation.

A more in depth investigation was done on the reducing agents, iron sources, and chelating agents with all of the monomeric polyphenols mentioned previously. The results were similar for all of them so specific substances were selected to demonstrate the results. Of the reducing agents, ascorbic acid and sodium bisulphite were able to eliminate colour formation in the presence of ferric sulphate. Hydroxylamine was also effective but some colour formation occurred. The iron sources tested may be ordered from most to least complex formation as follows: ferrous bisglycinate (maximum absorbance with gallic acid ~2), ferrous gluconate (~0.8), ferrous lactate (~0.3), ferric citrate (no clear peak absorbance, ~0.2), and ferric EDTA (no clear peak absorbance, ~0). As for the chelating agents, SHMP completely prevented colour development in the presence of ferric sulphate and thus iron-polyphenol complex formation. EDTA was less effective, forming a dark green to yellow solution. These results are difficult to interpret because the pH of the solutions, the concentration of the reducing agents, and the concentration of the chelating agents were not reported. It was stated, however, that the reducing agents and chelating agents were added in excess.

Mellican et al. also tested a variety of foods for colour development using the Hunter Lab scale to determine the usefulness of the linear regression models. The foods tested include black tea, green tea, coffee, hot chocolate, and banana baby cereal. The amount of colour development in each food was found to be directly related to the amount of complex formation in the monomeric polyphenol model systems. This was done using various iron sources (ferric sulphate, ferrous
bisglycinate, ferrous lactate, ferrous gluconate, ferric citrate, and ferric sodium EDTA) of which ferric sodium EDTA showed markedly less colour formation than the others. However, pH was not controlled which limits the relevance of these results. The foods were also tested for colour development with ferric sulphate in each of the following situations: with the addition of ascorbic acid (a reducing agent), the addition of EDTA (a chelating agent), the reduction of pH to 2, the removal of oxygen, and the reduction of the temperature to 0°C [42]. Ascorbic acid, EDTA, reduced pH, and removal of oxygen all reduced or eliminated coloured complex formation. The reduced temperature increased iron(III)-polyphenol complex formation as it did with monomeric polyphenols. The amount of iron added was 30% RDI (recommended daily intake) per serving. Unfortunately the age, sex, or pregnancy status criteria used to select the RDI was not specified. Also, pH was not reported although it is a very important factor in iron-polyphenol complex formation. The pH of a black tea sample was only reported when ferric sulphate alone was added to black tea. This lowered the pH from 5.01 to 4.03. EDTA prevented colour formation but the amount necessary was not measured since both reducing and chelating agents were added in excess. Although the model served as a good predictor of colour formation and indicated effective methods of reducing iron-polyphenol complex formation (i.e. the addition of reducing or chelating agents) the results were not quantitatively useful. Furthermore, pH levels relevant to iron absorption from tea were not measured.

As seen from Mellican et al. (2003), there are quite a few strategies for reducing iron-polyphenol complex formation in tea. However, many are not feasible if both colour formation and iron bioavailability are to be conserved. These include reducing oxygen content, reducing pH, removing polyphenols, and adding an insoluble or microencapsulated iron source. When being consumed, the tea beverage will be open to the air thus allowing for oxygen exchange. A reduction of pH would result in a change of flavour and appearance. Furthermore, once the tea is consumed, the body will regulate the pH so pH adjustment of the food will likely not alter iron bioavailability. Removing the polyphenols is not possible because these compounds impart the desired colour and flavour to tea. Iron sources with low solubilities may not react with polyphenols. However, they generally have low bioavailabilities, requiring a greater amount be added. This would increase cost and likely impact the acceptability of the tea. Also, care must be taken so that the iron remains suspended in the tea solution. Microencapsulation of iron would be difficult as the iron must remain encapsulated in hot water, must remain suspended, must stay intact in the stomach, and
finally must be released in the duodenum. Even so, it is still likely to form non-bioavailable complexes before being absorbed. Therefore, to develop a visually acceptable and bioavailable iron-fortified tea, iron source, reducing agents, and chelating agents should be investigated.

Tamilmani et al. (2016) investigated the effect of ascorbic acid (a reducing agent) and EDTA (a chelating agent) on the iron binding ability of pure phenolic compounds, coffee, and tea [43]. They concluded that ascorbic acid is the most effective [43]. However, the pH in this study was not reported despite samples being prepared in an acetate buffer. One reason that ascorbic acid may be more effective is that it may lower the pH, lessening the iron-polyphenol interaction. The typical pH used for iron-polyphenol quantification in the method used by Tamilmani et al. is 5.1. They used the “two wavelength method,” see Section 2.4.2 for further detail. If the pH of the solutions was maintained at 5.1, iron-polyphenol complex formation was still not measured at all relevant pH levels for brewed tea and digestion. Furthermore, more research was needed because the amount of iron added (0.7 mM) was high for iron fortified tea. Also, the samples tested were of freeze-dried tea extract dissolved in an acetate solution which may not accurately represent brewed tea [43].

For the application of iron-fortified tea, the prevention of iron-polyphenol complex formation at pH levels applicable to brewed tea and iron absorption needed to be investigated. The most promising strategies outlined by literature are the use of reducing agents (e.g. ascorbic acid), chelated iron sources (e.g. ferric sodium EDTA), or chelating agents (e.g. disodium EDTA). It is likely that strategies for improving iron bioavailability will also result in visually acceptable brewed tea. However, strategies such as microencapsulation may be revisited if required.

2.4.2 Increasing the Bioavailability of Iron in Chelating Foods

In studies where iron absorption is reliably increased in the presence of polyphenols (in vitro with Caco-2 cells and in vivo) ascorbic acid or EDTA (as an additive or as part of the iron source) are used [39, 43-46]. Similar results are found in the presence of phytate, another inhibiting chelating agent present in food [3]. Some studies have claimed that vitamin A or β-carotene prevent the inhibiting effect of polyphenols and phytates on iron absorption [47, 48]. However, these results are controversial. Walczyk et al. (2003) could not confirm the results and suggested that the
increase in iron absorption may be an effect of increasing vitamin A intake in people that are vitamin A deficient [49]. Deficiency of vitamin A is known to lead to anemia [49].

The majority of studies related to the bioavailability of iron in food focus on foods that contain phytate. It is likely that prevention of iron-phytate complexes is similar to iron-polyphenol complexes and that the principles are transferable. The most studied chelated iron sources are sodium iron EDTA and ferrous bisglycinate [3]. It was found that both of these iron sources are absorbed into the body at 2-3 times greater amounts than ferrous sulphate in high phytate foods [3]. Ferric trisglycinate, a closely related compound, was found to not be well absorbed from maize (a high phytate food) [3]. These compounds are costly. When compared to ferrous sulphate, they are greater than 15 times more expensive [3]. Consequently, another chelation strategy has been employed using less expensive iron sources (e.g. ferrous sulphate) plus a chelating agent (e.g. disodium EDTA) and allowing the chelating agent to complex the iron within the food [3].

There is a reasonable amount of research pertaining to increased iron absorption in high phytate foods when EDTA (as ferric sodium or disodium EDTA) is added. MacPhail et al. (1994) found that the optimal molar ratio of EDTA (added as disodium EDTA) to iron (added as ferrous sulphate) in a rice-based meal was between 1:0.25 to 1:1 iron:EDTA when 3 mg of iron were present in each 200 g portion of boiled rice [50]. Evaluated against samples devoid of disodium EDTA, there was an increase in iron absorption of 7.5%, 9.7%, and 5% for molar ratios of 1:0.25, 1:0.5, and 1:1 iron:EDTA respectively [50]. Hurrell et al. (2000) fortified cereal foods with ferric sodium EDTA or ferrous sulphate with disodium EDTA [51]. In infant cereals and bread rolls fortified with ferric sodium EDTA there was a 1.9-3.9 fold increase in iron absorption over ferrous sulphate for each product [51]. Furthermore, when disodium EDTA was added up to a 1:1 molar ratio iron:EDTA (with ferrous sulphate as the iron source) in wheat cereal, iron absorption increased from 1% to 5.7% and in wheat-soybean cereal iron absorption increased from 0.7% to 2.9% [51]. Nayak et al. (2003) found that the in vitro bioavailability of iron from wheat flour fortified with ferrous sulphate and EDTA (60 mg iron/kg Indian bread, chapathi) at a 1:1 iron:EDTA ratio doubled the iron absorption compared to fortification with ferrous sulphate alone [52]. Therefore, in previous research, ratios of 1:0.25 to 1:1 iron:EDTA have been used to successfully increase iron absorption from iron chelating foods.
2.5 Quantification Techniques

2.5.1 Polyphenol Quantification in Tea

Accurate methods for the quantification of thearubigins, thus black tea polyphenol content in its entirety, have not been developed [53, 54]. These high molecular weight (1-40 kDa) polyphenolic fermentation products have not been fully characterized [22, 53]. Because of these compounds, quantification of total polyphenolic compounds in black tea is not done with the use of chromatography (e.g. high-pressure liquid chromatography (HLPC) and solid phase microextraction gas chromatography (SPME-GC)) or capillary electrophoresis; although these methods have been used when quantifying certain specific polyphenols in tea [55].

Thearubigins were first observed in 1959 but were only described by the term “thearubigins” since 1962 [20]. From chromatographic analysis (HPLC) of black tea a Gaussian-shaped hump was observed, termed the “thearubigin hump” [20]. For decades no suitable methods were found to resolve it [20]. However in 2010, progress was made when ultra-high resolution mass spectrometry ESI-FT-ICR MS (electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry) was used [20]. It was found that thearubigins are comprised of several thousand compounds (10,000 molecular ions were resolved in a single direct infusion ESI-FT-ICR MS experiment) [20]. Also, 1500 molecular formulae have been assigned using the data, which accounts for over 90% of the total ion intensity [20]. Most recently, Kelebek (2016) used LC-DAD–ESI-MS/MS (liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry) to identify and quantify 35 phenolic compounds in Turkish black tea [56]. Even if all thearubigins were characterized, it would be cumbersome to use a chromatographic method for quantification. This is in part because there are many compounds, but also, the way in which polyphenols interact with iron is subject to their individual structures, mainly their active iron-chelating groups.

Alternative spectrophotometric methods have been developed to measure total polyphenol content. The most popular is the Folin and Ciocalteu method especially in the tea industry (ISO 14502-1) [55, 57]. The Folin-Ciocalteu (FC) reagent is a yellow acidic solution which contains polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids [57]. The molybdenum ion begins in a +6 valance state which is not coloured [57]. It may be reduced to a +5 valence state which is a blue colour [57]. But, it may also be further reduced to a +4 valence
state which is not coloured [57]. Phenols become deprotonated at high pH and ionize [57]. These ionized phenols may reduce the molybdenum allowing for the coloured +5 valence state complex to form [57]. At pH 9-10, which is accomplished by the addition of a saturated sodium carbonate solution, 50% of phenol groups are expected to be ionized [57]. A standard compound, usually gallic acid, is used and the total phenol content is measured in “gallic acid equivalents” (GAE) [57]. It would seem that this method measures the number of hydroxyl phenolic groups (hydroxyl groups attached to an aromatic ring). However, the structures of different phenolic substances as well as other substances in complex samples can interfere. Therefore, the results of the FC method more accurately measure the reducing power of the sample at basic pH levels. Table 2.10 outlines the reactivity of different phenolic compounds and potential interfering complexes.

Some polyphenols have hydroxyl phenolic groups that do not react in the FC assay [57]. Gallic acid is an example, with 3 hydroxyl phenol groups but only 2 able to react due to a lack of deprotonation [57, 58]. Other factors that lower expected values for FC are molecules containing additional groups which raise the phenolic pKa such that deprotonation does not occur or generate a masking steric effect [57]. Some molecules can react more than expected such as flavonols. The enolic c ring (pyrone group) of a flavonol participates in the reaction and causes an increase in absorption compared to what would be expected [58]. Reducing agents, in general, will affect the results of the FC assay. However, one such substance, ascorbic acid, may be distinguished from phenols as it will cause the reduction to occur in acidic conditions as well as basic ones [59]. Therefore, the difference between the absorbance readings of the two conditions may be used to determine the amount of “total phenols” [59]. Interferences from non-phenol substances may be additive, inhibitory or enhancing (augmenting) [58]. Although there are many compounds that interfere with this method, none of them occur in tea. Caffeine has been proven to not interfere with the FC assay [60].
Table 2.10: FC Reactivity of Phenols and Potentially Interfering Substances [58]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Free phenolic hydroxyls</th>
<th>Reacting groups</th>
<th>Molar absorbance (÷ 1000)</th>
<th>Molar absorptivity per reactive group</th>
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</thead>
<tbody>
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<td>1</td>
<td>12.7</td>
<td>12.7</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>1</td>
<td>1</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Vanillin</td>
<td></td>
<td></td>
<td>14.9</td>
<td>14.9</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td></td>
<td>1+</td>
<td>19.2</td>
<td>19.2</td>
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<tr>
<td>Tyrosine</td>
<td></td>
<td>2</td>
<td>21.8</td>
<td>10.9</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td></td>
<td>1+</td>
<td>19.2</td>
<td>19.2</td>
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<tr>
<td>o-Aminophenol</td>
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<td>2</td>
<td>21.8</td>
<td>10.9</td>
</tr>
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<td>Sinapic acid</td>
<td></td>
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<td>33.3</td>
<td>16.6</td>
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<td>p-Hydroquinone</td>
<td></td>
<td>2</td>
<td>12.8</td>
<td>12.8</td>
</tr>
<tr>
<td>Resorcinol</td>
<td></td>
<td>2</td>
<td>19.8</td>
<td>19.8</td>
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<td>Catechol</td>
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<td>11.2</td>
</tr>
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<td>Dihydroxyphenylalanine (DOPA)</td>
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<td>28.9</td>
<td>14.4</td>
</tr>
<tr>
<td>4-Methylesculetin</td>
<td></td>
<td></td>
<td>31.1</td>
<td>15.6</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td></td>
<td>3</td>
<td>13.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td></td>
<td>2</td>
<td>24.8</td>
<td>12.4</td>
</tr>
<tr>
<td>Gallic acid</td>
<td></td>
<td></td>
<td>25.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Kaempferol</td>
<td></td>
<td>3</td>
<td>29.6</td>
<td>14.8</td>
</tr>
<tr>
<td>Malvin</td>
<td></td>
<td></td>
<td>40.5</td>
<td>20.2</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td></td>
<td></td>
<td>34.3</td>
<td>11.5</td>
</tr>
<tr>
<td>Quercitrin</td>
<td></td>
<td></td>
<td>44.8</td>
<td>14.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td></td>
<td>48.3</td>
<td>16.1</td>
</tr>
<tr>
<td>D-Fructose</td>
<td></td>
<td></td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>4-Hydroxycoumarin</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Flavone</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Flavanone</td>
<td>0</td>
<td>0</td>
<td>1.9</td>
<td>N/A</td>
</tr>
<tr>
<td>3-Hydroxyflavone</td>
<td>0</td>
<td>0</td>
<td>3.5</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>1</td>
<td></td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>p-Methylaniline</td>
<td></td>
<td>1</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>1</td>
<td>1</td>
<td>17.1</td>
<td>17.1</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1+</td>
<td></td>
<td>17.5</td>
<td>17.5</td>
</tr>
<tr>
<td>o-Diaminobenzene</td>
<td>2</td>
<td></td>
<td>21.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

There are two main concerns when using the FC method to quantify “total polyphenols” in black tea. Especially when relating this information to iron-polyphenol complex formation. The first is that this method does not accurately measure the amount of iron chelating polyphenol groups. The second is the variety of different polyphenol complexes which potentially react with the FC reagent differently (e.g. flavonol). Despite these limitations, this method yields useful quantitative
information for comparing tea samples and allows for consistent concentration control. Also, with the assumption that the relative amount of iron-chelating polyphenols is consistent, correlation of “total polyphenols” and iron-polyphenol complex formation will allow for prediction of reduced bioavailability and colour formation based on the most widely used method of polyphenol quantification (FC).

2.5.2 Quantification of Iron-Polyphenol Complex

Spectrophotometry, measuring LMCT absorbance bands of iron-polyphenol complexes, is the most prominent method for investigating iron-polyphenol complex formation [24, 31, 36, 39, 42, 43, 61-64]. Electrospray mass spectrometry (ESI-MS) has been used to aid in the characterization of simple iron-polyphenols such as complexes with iron(III)-catechin and iron(III)-β-glucogallin (a compound structurally related to tannins) [31, 65, 66]. Mass spectrometric methods are not viable for quantifying iron-polyphenol complexes in black tea. To start with, no mass spectrometric methods have been developed for determination of total polyphenol content. Secondly, the structures of the thearubigins have not yet been fully elucidated. Another method that has been used is a ferrozine assay in which Fe$^{2+}$ is measured [67]. Since the coloured iron-polyphenol complex contains Fe$^{3+}$, under certain circumstances the amount of Fe$^{2+}$ may be used to measure the amount of unbound iron. However, this method has been called into scrutiny because ferrozine will compete with the polyphenols to bind ferrous iron [36].

In many studies LMCT bands are used to observe iron-polyphenol complex formation and the results are left in terms of absorbance instead of converting to molar or mass quantities [42, 24, 36]. In others with simple polyphenols either a binding ratio is assumed with reference to literature or other methods (e.g. Job’s method) are used to determine the binding ratio [39, 61]. This ratio is then used to interpret absorbance data [39, 61]. In complex samples, qualitative analysis has been conducted for over a century using an LMCT absorbance assay termed the “ferric chloride test for phenols” [68]. This assay is still in use [68]. More recently there have been attempts to use LMCT absorbance data as a quantitative measure. In 1991, Brune et al. developed a spectrophotometric assay in which iron-binding phenolic compounds in food may be quantified [63]. This assay involves extraction with a solution of 50% v/v dimethylformamide and 50% v/v 0.1M acetate buffer, the addition of a ferric ammonium sulphate (FAS) reagent, and measurement of LMCT bands in solutions at pH 5.1 [63]. The resulting colour is due to the formation of Fe(III)-
gallol (blue colour) and Fe(III)-catechol (green colour) complexes which have distinguishable absorbance maxima [63]. Food blank absorbances are subtracted from samples containing the FAS reagent yielding Fe(III)-catechol measurements in catechol-equivalents and Fe(III)-gallol in tannic acid-equivalents [63]. This assay became known as the “two wavelength method” and is used for the measurement of iron-polyphenol complexes in tea [43, 64].

Although useful, there are some weaknesses in the two wavelength method:

- Iron-polyphenol complex formation may only be measured at pH 5.1.
  To obtain useful data relevant to the in-vivo behaviour of the iron-polyphenol complex, its concentration must be measured both at the pH of tea, to deter colour formation, and at the pH in which iron is absorbed into the body.

- Measurement is required at two wavelengths
  Most non-bioavailable iron-polyphenol complexes in tea form with gallol moieties [69]. Therefore, there may be little need for the measurement of two wavelengths. This is especially likely in iron fortified tea where the iron added will be at a much lower concentration than the polyphenol. Also, polyphenols with gallol groups form coloured complexes more quickly than those with catechol groups [36]. The spectrum of tea polyphenols with added iron may be investigated to determine whether two absorption peaks form.

- The use of a buffer solution
  Buffering agents are known to interfere with iron-polyphenol complex formation. The effect of buffer solutions may be compared to the use of a strong acid and base.

- The use of ferric ammonium sulphate
  A different iron source may be more advantageous than the use of ferric ammonium sulphate. Ferric chloride may be beneficial because it is already used in the popular qualitative polyphenol assay. Ferrous sulphate may be useful since it is the most used iron source to evaluate food fortification strategies.

- The use of “tannic acid equivalents” to measure iron-gallol complex
  Brune et al. found that the standard curve was non-linear for gallic acid [63]. However, gallic acid is more soluble than tannic acid and is the standard used in the FC method to determine polyphenol content. Therefore, it would be useful to
determine if gallic acid may be used as a standard for the quantification of iron-polyphenol content. This would consequently allow “total phenolic content” and “iron-polyphenol complex content” to be easily comparable.

2.6 Opportunities for Scientific Advancement

The development of iron fortified tea will potentially prevent a large number of deaths and decrease a substantial amount of suffering caused by iron deficiency in the developing world. However, this project is not only a humanitarian effort but also a scientific one. Among the many factors that need to be considered for the development of iron fortified tea, the most substantial obstacle is the presence of polyphenols in tea and their interaction with iron. Review of the literature revealed several domains in which scientific advancement was logical in the pursuit of developing a technique to prevent iron-polyphenol complex formation in tea. The domains which required further inquiry as well as specific consequential goals, which are congruent with the overall objective of this project, are summarized in the following list:

1. Iron-polyphenol complex quantification

There was no published iron-polyphenol quantification method that allowed measurement at different pH levels. The method developed by Brune et al. (1991) was the only method where LMCT bands were converted into molar or mass equivalence quantities [63].

Consequential goal:
- To develop a new quantification method that may be used at a variety of pH levels based on the ideas of Brune et al. (1991) and improvements suggested in Section 2.5.2.

2. Understanding the factors influencing iron-polyphenol complex formation

Mellican et al. (2003) conducted the only study of how various factors influence iron-polyphenol complex formation in iron fortified foods [42]. Due to shortcomings mentioned in Section 2.4.1 more research was needed on factors including pH (5-7), iron source, temperature, and polyphenol concentration.
Consequential goal:
- To investigate the effects of pH (5-7), iron source, temperature, and polyphenol concentration on iron-polyphenol formation in tea.

3. Predictive models for the extent of iron-polyphenol complex formation
Simple polyphenols have been characterized by stability constants (Section 2.3.3 and Section 2.3.4) which are the product of equilibrium constants for the expected species. These serve as predictive models for the quantity of iron-polyphenol complex formation. Mellican et al. (2003) developed predictive models for colour development due to iron-polyphenol complex formation (Section 2.4.1). These models were produced using monomeric polyphenols and applied to predict colour development in foods [42]. The models were useful qualitatively but were lacking quantitative rigor as demonstrated by the low reported coefficient of regression (0.67) [42]. Predictive models have not been developed in complex food systems.

Consequential goals:
- To develop predictive models for iron-polyphenol complex formation based on total phenolic content in tea, a complex food system.
- To compare these models with current knowledge on the formation of simple iron-polyphenol complexes.

4. Kinetics of ferrous iron-polyphenol reaction
Mellican et al. (2003) found that ferrous iron reacted more slowly than ferric iron [42]. This important finding needed to be confirmed. Also, there was no kinetics model used to describe this phenomenon.

Consequential goals:
- To confirm the decrease in reaction rate due to the use of ferrous iron.
- To determine a suitable kinetics model for ferrous iron in a polyphenolic solution.
- To interpret the predicted reaction chemistry with the use of a well-fitting kinetics model.
5. Utility of reducing agents, chelated iron sources, and chelating agents

From literature the use of reducing agents, chelated iron sources, and chelating agents seemed to be promising strategies to prevent iron-polyphenol complex formation and improve iron bioavailability. However, bioavailability studies do not measure iron-polyphenol complex formation directly and studies that measure iron-polyphenol complex formation either do not quantify it and/or do not measure it at relevant pH levels.

Consequential goals:
- To investigate the utility of reducing agents, chelated iron sources, and chelating agents to lessen iron-polyphenol complex formation in tea at relevant pH levels.
- To compare varieties of these substances to determine factors that contribute to the prevention of iron-polyphenol complex formation.

6. EDTA as a chelating agent to reduce iron-polyphenol complex formation

There have been many studies done on the use of EDTA in chelating foods to improve bioavailability. Less studies have been done on the prevention of iron-polyphenol complex formation. In high phytate foods the optimal iron:EDTA ratios were found to be between 1:0.25 and 1:1 for bioavailability improvement (Section 2.5.2). This ratio was not known for foods containing high levels of polyphenol.

Consequential goal:
- To determine a suitable ratio of iron:EDTA for use in tea, a food with high polyphenol content.
3 Materials and Methods

3.1 Materials

The following tables present the materials used for this research.

Table 3.1: Phenolic Compounds

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Material</th>
<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea and tea extract solutions</td>
<td>Black tea leaves</td>
<td>The Metropolitan Tea Company Ltd.</td>
<td>Toronto, Ontario, Canada</td>
<td>Grown in Behora Assam, Golaghat, India. Dry FBOP (flowery broken orange pekoe) tea leaves.</td>
</tr>
<tr>
<td></td>
<td>N,N-dimethylformamide</td>
<td>Caledon Laboratory Chemicals</td>
<td>Caledon, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Simple model compound</td>
<td>Gallic acid</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Tannic acid</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
</tbody>
</table>

Table 3.2: Iron Compounds (Fortificants and Standard Curves)

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Material</th>
<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade and/or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous iron salts</td>
<td>Ferrous sulphate, heptahydrate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Ferrous fumarate</td>
<td>Dr. Paul Lohmann GmbH KG</td>
<td>Emmerthal, Lower Saxony, Germany</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Ferric iron salts</td>
<td>Ferric chloride, hexahydrate</td>
<td>VWR</td>
<td>Radnor, Pennsylvania, U.S.A.</td>
<td>BDH brand, reagent grade</td>
</tr>
<tr>
<td></td>
<td>Ferric nitrate, hexahydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Chelated iron</td>
<td>Ferric trisglycinate (&quot;Iron Taste Free&quot;)</td>
<td>Albion Minerals</td>
<td>Layton, Utah, U.S.A.</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Ferrous D-gluconate dihydrate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Ferric sodium EDTA</td>
<td>Bio Basic Canada Inc.</td>
<td>Markham, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fisher Scientific</td>
<td>Toronto, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Ferric pyrophosphate</td>
<td>Dr. Paul Lohmann GmbH KG</td>
<td>Emmerthal, Lower Saxony, Germany</td>
<td>Reagent grade</td>
</tr>
</tbody>
</table>
### Table 3.3: pH Stabilization and Adjustment

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade and/or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Potassium phosphate monobasic (potassium dihydrogen phosphate)</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Potassium hydrogen phthalate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>Caledon Laboratory Chemicals</td>
<td>Caledon, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>1N hydrochloric acid (HCl)</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>5N sulphuric acid (H₂SO₄)</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
</tbody>
</table>

### Table 3.4: Reducing and Chelating Agents

<table>
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<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade and/or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium EDTA dihydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Purity &gt; 99.5%</td>
</tr>
<tr>
<td>Sodium acetate anhydrous</td>
<td>EMD Millipore</td>
<td>Etobicoke, Ontario, Canada</td>
<td>ACS grade</td>
</tr>
<tr>
<td>Glycine</td>
<td>Fisher Scientific</td>
<td>Ottawa, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Sodium hexametaphosphate</td>
<td>EMD Millipore</td>
<td>Etobicoke, Ontario, Canada</td>
<td>ACS grade</td>
</tr>
<tr>
<td>Sodium citrate tribasic dehydrate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Purity &gt; 99.0%</td>
</tr>
<tr>
<td>Sodium pyrophosphate tetrabasic</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>ACS grade</td>
</tr>
<tr>
<td>Sodium D-gluconate dihydrate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Purity 98%, ACS grade</td>
</tr>
<tr>
<td>Sodium phosphate monobasic</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Purity &gt; 99.0%</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Purity &gt; 99.5%</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Purity &gt; 99%</td>
</tr>
<tr>
<td>Dextrose</td>
<td>ACP Chemicals Inc.</td>
<td>Toronto, Ontario, Canada</td>
<td>ACS grade</td>
</tr>
<tr>
<td>Sodium ascorbate</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>ACS grade</td>
</tr>
</tbody>
</table>
Table 3.5: Analytical Chemicals

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Material</th>
<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade and/or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis of phenolic compounds</td>
<td>Folin and Ciocalteu’s phenol reagent</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td></td>
<td>Sodium carbonate</td>
<td>Fisher Scientific</td>
<td>Toronto, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Iron analysis using atomic absorption spectroscopy</td>
<td>Iron standard for atomic absorption spectroscopy (AAS)</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>Fluka brand, analytical grade</td>
</tr>
<tr>
<td>Sample digestion</td>
<td>Sulphuric acid, concentrated</td>
<td>Sigma-Aldrich Canada Co.</td>
<td>Oakville, Ontario, Canada</td>
<td>ACS reagent ~ 70.4 wt%</td>
</tr>
<tr>
<td></td>
<td>Nitric acid, concentrated</td>
<td>Caledon Laboratory Chemicals</td>
<td>Caledon, Ontario, Canada</td>
<td>Reagent grade ~ 96.0 wt%</td>
</tr>
</tbody>
</table>

Table 3.6: Experiments in Appendices 10.6 & 10.7 (Milk and Minerals)

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Supplier Location</th>
<th>Grade and/or Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>Neilson/Beatrice</td>
<td>Local grocery store, Toronto, Ontario, Canada</td>
<td>Food grade</td>
</tr>
<tr>
<td>Casein</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Analytical grade</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>VWR</td>
<td>Radnor, Pennsylvania, U.S.A.</td>
<td>BDH brand, reagent grade</td>
</tr>
<tr>
<td>Calcium chloride, dihydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Copper(II) chloride, dihydrate</td>
<td>EMD Millipore</td>
<td>Etobicoke, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Manganese sulphate, monohydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Magnesium chloride, hexahydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Zinc sulphate, heptahydrate</td>
<td>BioShop Canada Inc.</td>
<td>Burlington, Ontario, Canada</td>
<td>Reagent grade</td>
</tr>
</tbody>
</table>
3.2 Tea and Model Solution Preparation Method

3.2.1 Tea Brewing
Reverse osmosis (RO) purified water was boiled in a Betty Crocker electric kettle. Then the hot water was added to tea leaves in an Erlenmeyer flask such that there was 1 g of tea leaves for every 100 g of water (1% wtea_leaves/wwater). This mixture was thus approximately 1% w/v (gtea_leaves/mLwater). To demonstrate this, assuming that the water was 100°C (i.e. at the lowest possible density, 0.9584 g/mL):

$$1 \text{ g} / (100 \text{ g} / (0.9584 \text{ g/mL})) = 0.96\% \text{ w/v}. $$

As the water cools it becomes denser, thus solution became closer to 1% w/v. The mixture was gently stirred and allowed to steep for 5 minutes. After 5 minutes the mixture was gently stirred again and poured through a strainer to separate the solution from the tea leaves. There was a slight loss of water due to evaporation of ~1-2 g during steeping.

This procedure was followed unless otherwise stated. This method of tea preparation is in accordance with recommendations from a variety of loose leaf black tea manufacturers (1% to 1.3% w/v followed by steeping for 3-5 minutes) [15-17].

3.2.2 Gallic Acid and Tannic Acid Model Solutions
The gallic acid and tannic acid solutions were prepared at the same concentration as the measured polyphenols concentrations in tea (1 g/L). Tannic acid is composed of molecules containing ten gallic acid groups. Therefore, the molar concentrations of the tannic acid solutions were one tenth of the molar concentrations of the gallic acid solutions. Concentrated solutions of gallic acid and tannic acid were prepared using RO water (10 g/L and 100 g/L respectively). These were diluted to the target concentration, generally 1 g/L, with RO water or buffered solutions.

The pH levels investigated were pH 1 (stomach pH), pH 3, pH 5 (tea beverage pH), and pH 7 (approximation of small intestine pH). The pH was maintained using either buffered solutions or strong acid and/or base solutions (i.e. HCl, H2SO4, and/or NaOH). The buffered solutions were prepared with RO water containing the buffering agent and adjusted to the target pH with HCl or NaOH. The buffer solutions were prepared as outlined in Table 3.7.
Table 3.7: Buffer Solution Preparation

<table>
<thead>
<tr>
<th>Solution Target pH</th>
<th>Solution Prepared</th>
<th>Chemical for pH Adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>potassium chloride</td>
<td>HCl</td>
</tr>
<tr>
<td>3</td>
<td>potassium hydrogen phthalate</td>
<td>HCl</td>
</tr>
<tr>
<td>5</td>
<td>potassium hydrogen phthalate</td>
<td>NaOH</td>
</tr>
<tr>
<td>7</td>
<td>potassium phosphate monobasic</td>
<td>NaOH</td>
</tr>
</tbody>
</table>

3.2.3 Tea Extract Preparation

Tea extract was prepared such that it was a more concentrated solution than brewed tea. This allowed it to be diluted to the required concentrations with added iron and pH adjustment. Another benefit of the extract is the ability to be stored at sub-zero temperatures without freezing, thus allowing it to remain stable for a prolonged period of time. The tea extract concentration was determined by the method outlined in Section 3.3.3. The most concentrated extract contained 20 gGAE/L (g of gallic acid equivalents/L). The extract was prepared using a solution of 50% N,N-dimethylformamide and 50% RO water (v/v) with black tea leaves as described by Turkmen et al. (2006) [70]. Tea extract was diluted to the concentration of tea (1 gGAE/L) or to one tenth of that concentration (0.1 gGAE/L) during experiments as needed.

3.2.4 Iron Solution Preparation

All iron solutions were prepared in RO water. Hydrochloric acid was used in cases where solubility was an issue (5-10 mL of 1N HCl in 100 mL of concentrated iron solution). They were prepared at concentrations of 12.5-30.0 mM allowing for dilution to 0.3 mM which is the target fortification concentration for iron fortified tea.

3.3 Analytical Methods

3.3.1 Measurement of pH

A VWR Scientific Model 8000 pH meter was used to measure the pH of solutions.

3.3.2 Phenolic Compound Concentration

The method used for polyphenol measurement was based on the method developed by Singleton and Rossi in 1965 [57]. In short, a calibration curve was prepared using gallic acid diluted to 0.001-0.013 g/L. Gallic acid solutions were treated with Folin & Ciocalteu’s reagent, mixed, and allowed to react for 5 minutes. A saturated solution of sodium carbonate was then added and the
solutions were agitated. This was followed by the addition of RO water to dilute the solutions to the proper concentrations. The solutions were allowed to rest for 30 minutes. Then the absorbance was measured at 765 nm using a Cary 50 UV-Vis spectrophotometer. A blank was also prepared that did not contain gallic acid. Tea and tea extract were measured similarly. The concentration of phenolic compounds was reported in terms of gallic acid equivalents (GAE). A sample calibration curve can be found in Appendix 10.1.1.

3.3.3 Iron-Polyphenol Complex Quantification

A Cary 50 UV-Vis spectrophotometer was used to measure the absorbance of solutions by scanning the spectrum from 300 nm to 800 nm. Net absorbance was calculated by subtracting the absorbance of a pH adjusted blank (i.e. gallic acid, tea extract, or brewed tea) from the sample absorbance (i.e. polyphenol solution containing iron). The maximum net absorbance peak was calibrated using gallic acid calibration curves. These were prepared using gallic acid at a concentration 1 g/L (to model tea) and iron concentrations of 0 to 0.5 mM, since 0.3 mM is the target concentration for tea fortification. The net absorbance peak occurred between 550 and 570 nm unless otherwise stated.

To test iron-polyphenol complex formation at various temperatures, a flow-cell system was used. A stirred solution at a specified temperature in an Erlenmeyer flask situated on a stirring hotplate was pumped with a peristaltic pump into a flow-cell within the spectrophotometer. The absorbance of the solution was scanned similarly to the aforementioned solutions. The pump used was from a Micon CH4 concentrator.

3.3.4 Iron Content

The iron content of solutions was measured using atomic absorption spectroscopy (AAS) using the Perkin Elmer AAnalyst 100 AAS system. Calibration curves were made using solutions of 1 ppm, 5 ppm, 10 ppm, and 20 ppm iron standard for AAS. Samples were digested with stock concentration sulphuric acid and nitric acid prior to iron measurement. The digestion was considered complete when the solutions became clear and colourless. The digestion was performed at the boiling point of the solutions. Table 3.8 outlines the preparation of the samples.
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Quantity</th>
<th>Sulphuric Acid</th>
<th>Nitric Acid</th>
<th>Final Volume</th>
<th>Expected Maximum Iron Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix</td>
<td>0.5 g</td>
<td>25 mL</td>
<td>2 mL</td>
<td>250 mL</td>
<td>20 ppm</td>
</tr>
<tr>
<td>Tea (fortified or unfortified)</td>
<td>0.5 g</td>
<td>25 mL</td>
<td>2 mL</td>
<td>250 mL</td>
<td>4 ppm</td>
</tr>
<tr>
<td>Tea beverage (fortified or unfortified)</td>
<td>30 mL</td>
<td>10 mL</td>
<td>1 mL</td>
<td>100 mL</td>
<td>5 ppm</td>
</tr>
</tbody>
</table>
4.1 Research Objective

The objective of the study presented in Chapter 4 was to quantify iron-polyphenol complex formation and gain a deeper understanding of how this reaction occurs in black tea. This task was divided into four sub-objectives. The first sub-objective was to gain a deeper understanding of the polyphenol concentration and pH of black tea. Investigation of how these parameters depend on brewing duration and quantity of tea leaves used is important to understanding the potential variability. The second sub-objective was to investigate the use of some common buffer solutions for pH control. Buffer solutions are known to interfere, to a degree, with iron chelation. However, their ability to maintain a constant pH solution can be useful for simplifying experimental procedures. This allows for more precise repeatability. Also, they allow for precise pH maintenance throughout the duration of a reaction, which is critical to kinetics studies. The third sub-objective was to develop a spectrophotometric method for measuring iron-polyphenol complex formation that is tailored to tea. This was needed as there were no analytical methods to quantify iron-polyphenol complex at pH levels critical to the development of iron fortified tea. The fourth sub-objective was to use this analytical method to study the effects of iron source, temperature, and polyphenol concentration on iron-polyphenol complex formation. Overall, a better understanding of iron-polyphenol complex formation in black tea was gained and better hypotheses could be formulated for strategies to prevent iron-polyphenol complex formation.

4.2 Experimental Strategy

For the first sub-objective, the polyphenol content of the tea specimen used as well as the polyphenol concentration and pH of brewed tea were analyzed. Tea polyphenol content and pH were measured as functions of the amount of tea leaves used during brewing and brewing duration. Quantities of tea leaves and steeping durations were chosen to span a minimum 4 fold range above and below the typical tea brewing recommendations of 1% w/v tea leaves and 5 minute steeping time. This was done to encompass cultural differences and individual preferences as well as to observe trends. Furthermore, brewed tea was prepared from the same sample of tea leaves before and after two years of storage at room temperature in the original packaging.
material. This was done to determine if there was a significant change in polyphenol concentration over the course of the experimental period.

To achieve the second sub-objective, the control of pH through the use of buffer solutions was compared to manual adjustment using a strong acid (HCl) and base (NaOH). The control of pH is important as iron-polyphenol reactions are pH sensitive. Also, in the context of iron-fortified tea, the pH of the tea beverage and pH at which iron absorption occurs in the body should be taken into account. Control of pH is most easily achieved with buffer solutions. Two simple and inexpensive buffer solutions were chosen: phthalate for pH 5 and phosphate for pH 7. Phosphate was selected due to a large volume of research surrounding its use and its known ability to interfere with chelation (see Section 2.3.6). No literature report was found on the use of phthalate buffer solutions in the presence of cations. Therefore, this was an unknown to be investigated. Although it is well known that buffering agents can interfere with metal chelation, the extent of this interference was investigated in both gallic acid and tea. Gallic acid and tea polyphenols are strong chelating agents. When present at the high concentrations representative of tea, the interactions between buffering agents and iron were expected to be minimal.

The third sub-objective of developing a spectrophotometric method for the quantification of iron-polyphenol complex was attained by adapting the two wavelength method developed by Brune et al. (1991). Explanations of potential areas for improvement for the two wavelength method are summarized in Section 2.5.2. An analytical method was developed that can quantify iron at pH 5 (tea pH) to pH 7 (a pH representative of the small intestine). The requirement for two wavelengths was examined by observing spectrophotometric scans of tea polyphenol solutions with added iron. A strong acid and base were used instead of a buffer solution. As iron standards, ferric chloride and ferrous sulphate were investigated. Ferric chloride is advantageous because it has been used in qualitative polyphenol analysis for more than a century. Also, since it is in the ferric form, it does not have to undergo oxidation to form coloured iron-polyphenol complexes. Ferrous sulphate is advantageous because it is the most commonly used iron source in food fortification research. The use of gallic acid as a standard or reference compound was explored. It is advantageous over tannic acid, which was used by Brune et al. (1991), because Folin and Ciocalteu polyphenol measurements are done in “gallic acid equivalents” (GAE) [63].
For the fourth sub-objective, the developed analytical method was used to study the effects of iron source, temperature, and polyphenol concentration on iron-polyphenol complex formation. Tea extract and gallic acid were tested for iron complex formation with ferrous sulphate, ferrous fumarate, and ferric sodium EDTA (i.e. two common iron salts and one chelated iron source). All selected iron sources have high bioavailabilities in non-chelating foods. Also, to elucidate more iron-polyphenol chemistry, a flow cell within a spectrophotometer was used to measure the effect of temperature and polyphenol concentration on the extent of iron-polyphenol complex formation (for method see Section 3.3.3). Equilibrium models were developed for pH 5 and pH 7 which relate “total polyphenols” to iron-polyphenol complex formation.

4.3 Results and Discussion

4.3.1 Tea Polyphenol Concentration and pH
The amount of polyphenols in the tea leaves used in this study was found to be 212 ± 7 mgGAE/g tea leaves. This is 21.2% ± 0.7% gGAE/g tea leaves (Appendix 10.1.2). In a study by Turkmen et al. (2006) where a similar method was used, black tea samples from Ankara, Turkey were found to only have 131.9 ± 0.59 mgGAE/g tea leaves [70]. In another study black tea samples from Malaysia were found to have between 6.06% ± 0.54% and 8.49% ± 0.80% gGAE/g tea leaves [71]. Also, black tea from Argentina was found to have between 8.42% ± 0.55% and 17.62% ± 0.42% gGAE/g tea leaves [71]. Therefore, the tea sample used in this study had a comparatively high concentration of polyphenols.

Both the polyphenol concentration and pH of the tea beverage are expected to affect iron-polyphenol complex formation. As such, it is essential to understand how these parameters are affected by brewing time and amount of tea leaves used. Although a 3-5 minute steeping time is common for many, tea may be brewed for much longer durations due to cultural differences and personal preference. Polyphenol extractions were performed for different durations of steeping using 1% \( w_{\text{tea leaves}}/w_{\text{water}} \) (an approximation of 1% w/v). This was done initially and after a 2 year storage period to confirm stability. The tea was stored at ambient conditions in the original packaging material. In the initial test only one replicate was taken but three were taken in the subsequent test. Figure 4.1 shows that the quantity of phenolic compounds in brewed tea did not substantially change over two years. Also, the increase in polyphenol concentration with time slows greatly after 10 minutes of steeping. This equilibration occurs at a concentration of
approximately 1 gGAE/L. Despite a lower concentration occurring at the typical 5 minute steeping time, to err on the side of stronger tea, a concentration of 1 gGAE/L was selected to model tea. This concentration is in accordance with the study from Rechner et al. (2002) in which the polyphenol content of seven brands of UK market black tea, once brewed, were measured to contain an average of 262 mgGAE/230mL or 1.14 gGAE/L (a range of 0.95-1.25 gGAE/L) [28].

![Figure 4.1: Increase of Polyphenol Concentration with Steeping Duration](image)

The impact of the amount of tea leaves for a defined brewing duration of 5 minutes was investigated as well. The time points were the same as in the former experiments, with one replicate taken for the initial measurements and three replicates taken for the ones at 2 years. The results can be seen in Figure 4.2. There is a pronounced decrease in the amount of polyphenols in the tea solution over this time period. However, when 1% \( w_{\text{tea leaves}}/w_{\text{water}} \) is used, this difference is negligible. It is only 0.13 ± 0.12 gGAE/L, where the standard deviation was calculated given the assumption that the relative standard deviation of the initial measurement would be equivalent to the relative standard deviation of the measurements taken after 2 years of storage. There is a linear relationship between the polyphenol concentration and amount of tea leaves. Only a small portion of water remains unabsorbed by the tea leaves when 4% \( w_{\text{tea leaves}}/w_{\text{water}} \) is used. Therefore, using this amount is impractical.
There is a large amount of polyphenols in black tea compared to the target amount of iron. Black tea contains about 1 gGAE/L of polyphenols which is approximately 6 mM GAE whereas the target concentration of iron is only 0.3 mM. Therefore, polyphenol is present at a molar concentration 20 times greater than iron. Due to this overage of polyphenols, it was expected that within the reasonable bounds of half to quadruple the expected concentration of polyphenols, iron-polyphenol complex formation would not be greatly influenced by polyphenol concentration. Evidence in favour of this expectation was collected in preliminary studies where gallic acid solutions (0.5-4 g/L), adjusted to pH 7, were spectrophotometrically scanned after the addition of 0.3 mM ferric chloride. Gallic acid was used as a model compound for tea polyphenols because precipitation occurs when iron is added to tea polyphenols at concentrations representative of brewed tea. As expected, the amount of iron-polyphenol complex formation was not greatly affected (Appendix 10.1.3). Therefore, it is reasonable that 1 gGAE/L is representative of all practical tea polyphenol concentrations.
Similarly to polyphenol analysis, the pH of brewed tea solutions was measured at a variety of steeping durations and quantities of tea leaves. Reported in Table 4.1 is the effect of steeping time on pH taken after 2 years of storage. This data is in agreement measurements taken before storage. The pH was only subtly affected by steeping duration beyond 1 minute when tea leaves were added at 1% \( \text{w}_\text{tea leaves}/\text{w}_\text{water} \). The average pH at all steeping times (1, 2, 5, 10, and 30 minutes) was \( 5.18 \pm 0.06 \). The quantity of tea leaves had an effect on the pH, which can be seen in Figure 4.3. Similar to polyphenol concentration measurements, only single measurements were made at the initial time point but after 2 years of storage three replicates were taken. The pH of the tea ranged from 5.5 to 4.9 with tea leaf amounts of 0.2% to 4% \( \text{w}_\text{tea leaves}/\text{w}_\text{water} \). From this data a pH of 5 was selected as a reasonable pH to represent tea.

Table 4.1: Effect of Steeping Time on pH

<table>
<thead>
<tr>
<th>Steeping Time (minutes)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.21 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>5.24 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>5.21 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>5.14 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>5.12 ± 0.01</td>
</tr>
</tbody>
</table>

Figure 4.3: Dependence of pH on the Quantity of Tea Leaves
More measurements were taken to better understand the tea system. It was found that the solubility of black tea polyphenols in boiling water is very high and would not be a limiting factor for the maximum practical polyphenol levels found in brewed tea (Appendix 10.1.4). Also, absorbance scans of black tea with concentrations of 0.5%-2% \(\text{w}_{\text{tea leaves}}/\text{w}_{\text{water}}\) and steeped for 5 minutes can be found in Figure 4.4. When less than or equal to 1% \(\text{w}_{\text{tea leaves}}/\text{w}_{\text{water}}\) is used, the absorbance of tea is low, below 0.62, within the region typically associated with iron-polyphenol complex formation in tea (500-600 nm). Therefore, accurate measurements of the coloured iron-polyphenol complex may be taken. A blank tea that does not contain iron may be used to correct for the background absorbance caused by tea. At 2% \(\text{w}_{\text{tea leaves}}/\text{w}_{\text{water}}\) precipitation begins to occur upon cooling, thus a vertical shift in the absorbance scan.

![Spectrophotometric Scans of Brewed Tea](image)

To summarize, characteristics of tea leaves and brewed tea were investigated. The tea source used in this study has a high polyphenol concentration rendering it a good source for testing iron-polyphenol complex formation. Brewed tea, prepared using the recommended brewing guidelines, is approximately pH 5 with a polyphenol concentration of 1 gGAE/L. An increase in the amount of tea leaves used has a linear effect on the polyphenol concentration and only a slight effect on pH. Polyphenol concentration in brewed tea, using 1% \(\text{w}_{\text{tea leaves}}/\text{w}_{\text{water}}\), did not markedly change during the two year period in which the majority of the experiments were completed.
4.3.2 Control of pH

Iron-polyphenol complex formation is known to be affected by pH level. Therefore, the pH must be controlled. Preliminary experiments of ferrous sulphate in buffered gallic acid solutions demonstrated that at pH 3 and pH 1 no complex formation occurred in 1 g/L gallic acid with 0.1-0.5 mM ferrous sulphate (Appendix 10.2.1). Complex formation did occur at pH 5 and pH 7.

Brune et al. (1991) made use of an acetate buffer in the development of an iron-polyphenol quantification method [63]. However, it is well recognized that buffering compounds can interfere with cations, such as iron, in solution. In this study the use of buffer solutions was investigated. The buffer solutions selected were phosphate for pH 7 and phthalate for pH 5. These were compared to using NaOH and HCl for pH adjustment. In preliminary testing, to maintain adequate pH with the addition of ferrous sulphate and gallic acid or tea extract, buffer solutions needed to be at a concentration of 0.3 M. Solutions containing buffering agents, 0.3 mM ferrous sulphate, and 1 g/L gallic acid or 1 gGAE/L tea extract, were prepared and left at room temperature for three hours. The solutions were then scanned using a spectrophotometer. Scans of polyphenol solutions that did not contain added iron were subtracted from scans of samples with added iron in order to account for the absorbance caused solely by the polyphenols. For a more in depth description of the reaction solution preparation see Appendix 10.2.2.

In gallic acid (1 g/L, equivalent to tea polyphenol content) the phthalate buffered solutions (pH 5) and phosphate buffered solutions (pH 7) decreased iron complex formation in a predictable manner. At both pH levels the amount of iron-gallate complex formation was lower when buffering agents were used. This can be seen by the height of their respective peaks in Figures 4.5 and 4.6. This effect is likely due to the phthalate and phosphate binding to a portion of the iron. Otherwise, it may be explained by an increased ionic strength when the buffering agents are used. An increase in ionic strength could decrease the ability of gallic acid to deprotonate and thus bind to iron.

The changes in the peak maxima are interesting because at pH 5 the curve with no buffer solution is to the left of the one with buffer solution and at pH 7 the opposite is true. The most likely reason for this is that the complexes that form with gallic acid also incorporate a component from the buffer solution (or hydroxide in the absence of buffer solution) which changes their electronic
structure such that the LMCT absorbance band is different. This shift would be dependent on the composition of the solution, thus different buffering agents would cause different shifts.

Figure 4.5: Spectrophotometric Scans of Iron Complex Formation in Gallic Acid at pH 5

Figure 4.6: Spectrophotometric Scans of Iron Complex Formation in Gallic Acid at pH 7

Using tea extract at a concentration of 1 gGAE/L resulted in precipitate formation. Consequently, tea extract at the lower concentration of 0.1 gGAE/L was used to determine the effect of buffer solutions. Figures 4.7 and 4.8 present the results of iron complex formation in tea extract at pH 5 and pH 7 respectively. Interestingly, when using tea extract at pH 5, the net absorbance increased with the use of phthalate buffer. This is likely due to a slight salting out effect caused by the 0.3
M phthalate buffer. At pH 7 with the phosphate buffer there was no difference in the absorbance at ~575nm which is the maximum absorbance wavelength of the non-buffered solutions. However, at both pH 5 and pH 7, the shape of the curves dramatically changed between the two conditions. It is suspected that this is due to the buffer solutions interacting and altering the spectral effects of the polyphenols. Alternatively, a variety of iron-polyphenol-buffer complexes may have formed which have different LMCT absorbance bands.

![Graph](image-url)

**Figure 4.7**: Spectrophotometric Scans of Iron Complex Formation in Tea Extract at pH 5

![Graph](image-url)

**Figure 4.8**: Spectrophotometric Scans of Iron Complex Formation in Tea Extract at pH 7
In summary, phthalate and phosphate buffers should not be used to investigate iron interactions with phenolic compounds in tea or model tea systems. For gallic acid the buffering agents lowered the amount of iron-polyphenol complex formation, likely due to coordinating iron. When used with tea polyphenols the shape of the absorbance scan changed and the defined peak was lost. Also, some salting out of polyphenols occurred at pH 5. Therefore, pH adjustment should be done manually using a strong acid or base that does not strongly bind iron, such as HCl or NaOH.

4.3.3 Gallic Acid Calibration Curves

Inhibition of iron absorption in tea occurs in proportion to the concentration of gallol groups [20]. Also, spectrophotometric scans of black tea polyphenols with iron showed only a single absorbance peak. This peak corresponds to gallol complexes because the peak absorption occurs at ~578 nm as opposed to catechol complexes which absorb at ~680 nm [63]. Due to this, quantification of iron-polyphenol complex in black tea can be done using a single wavelength. It is logical then, for calibration curves to be prepared in gallic acid, a simple well studied phenolic acid known to form coloured complexes with iron similarly to tea polyphenols. It is also the preferred reference substance used in the Folin and Ciocalteu phenol assay [57].

Calibration curves were prepared in gallic acid (1g/L, the concentration of tea) to quantify iron-polyphenol complex formation. In preliminary testing, tannic acid was also used because it had been employed in the iron-polyphenol quantification method developed by Brune et al. (1991) [63]. Unfortunately, precipitates form when tannic acid is used at 1 gGAE/L rendering it unsuitable for spectrophotometry at this concentration.

Ferric chloride was the iron source chosen to create these calibration curves because it has been traditionally used in a colorimetric assay to test for the presence of phenolic compounds, termed the “ferric chloride test” [72]. Also, the use of a ferric iron source increases the probability of a rapid reaction rate because iron oxidation is not required. Figures 4.9 and 4.10 show the ferric chloride calibration curves for pH 5 (8 replicates) and pH 7 (10 replicates) respectively. From the calibration curves it can be seen that there is a linear relationship within the iron concentration range needed for analysis of iron fortified tea (0.3 mM).
The results from ferric chloride were compared to those of ferrous sulphate, the iron source most often used in fortification studies. Table 4.2 shows the absorbance readings of ferrous sulphate compared to ferric chloride when tested at 0.5 mM. This concentration was selected because it is the largest in the calibration curve. Therefore, it would yield the most pronounced difference. At both pH 5 and pH 7 no significant difference was found in the amount of coloured iron complex formed (p>0.09).
To summarize, ferric chloride in gallic acid calibration curves were created for quantifying iron-polyphenol complex formation at pH 5 and pH 7. The amount of complex formation can be converted by these calibration curves into “ferric chloride in gallic acid equivalents,” or FeCl₃/GAE.

### 4.3.4 Comparison of Gallic Acid and Black Tea Polyphenols

Three potential iron sources (ferrous sulphate (FS), ferrous fumarate (FF), and ferric sodium EDTA (FeNaEDTA)) at a concentration of 0.3 mM were compared based on the amount of coloured iron complex formation with gallic acid and tea extract at both pH 5 and pH 7. Ferrous sulphate and ferrous fumarate were chosen due to their low cost and high bioavailability (in non-chelating foods) and ferric sodium EDTA was selected for its high bioavailability in chelating foods such as those containing phytates and polyphenols. Variation was found when using different sources of ferric sodium EDTA. A comparison of these different sources can be found in Appendix 10.3. Ferric sodium EDTA from Sigma-Aldrich was used in these experiments. When tea extract was tested at the typical polyphenol concentration of tea (1 gGAE/L) using ferrous sulphate or ferrous fumarate, precipitation of the iron-polyphenol complexes occurred rendering the samples unmeasurable. This likely occurred due to polymerization of the polyphenols. Due to this, tea extract at the reduced concentration of 0.1 gGAE/L was tested as well. Some peak shifting occurred for the different iron sources as well as between gallic acid and tea extract. The results are summarized in Table 4.3. Three replicates were measured at each condition and the error expressed is standard deviation.
Table 4.3: Comparison of Iron Compounds in Gallic Acid and Tea Extract

<table>
<thead>
<tr>
<th>Target pH</th>
<th>Phenolic Substance</th>
<th>Iron Source</th>
<th>Actual pH</th>
<th>Wavelength of Peak Absorbance</th>
<th>Iron Complex (mM FeCl₃/GAE)</th>
<th>Iron Complex (% FS in GA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Gallic acid (1 g/L)</td>
<td>FS</td>
<td>4.96 ± 0.00</td>
<td>550</td>
<td>0.323 ± 0.006</td>
<td>100 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>4.98 ± 0.02</td>
<td>550</td>
<td>0.334 ± 0.007</td>
<td>103 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeNaEDTA</td>
<td>4.91 ± 0.01</td>
<td>no peak *(570)</td>
<td>0.016 ± 0.001</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Tea extract (1 gGAE/L)</td>
<td>FeNaEDTA</td>
<td>5.00 ± 0.01</td>
<td>no peak *(570)</td>
<td>0.01 ± 0.02</td>
<td>4 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FS</td>
<td>5.01 ± 0.03</td>
<td>570</td>
<td>0.232 ± 0.003</td>
<td>72 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>5.11 ± 0.05</td>
<td>570</td>
<td>0.210 ± 0.004</td>
<td>65 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeNaEDTA</td>
<td>5.02 ± 0.03</td>
<td>no peak *(570)</td>
<td>0.010 ± 0.001</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Tea extract (0.1 gGAE/L)</td>
<td>FS</td>
<td>6.92 ± 0.00</td>
<td>565</td>
<td>0.318 ± 0.005</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>6.87 ± 0.05</td>
<td>560</td>
<td>0.350 ± 0.006</td>
<td>110 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeNaEDTA</td>
<td>6.95 ± 0.02</td>
<td>**485 *(555)</td>
<td>0.019 ± 0.002</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Tea extract (1 gGAE/L)</td>
<td>FeNaEDTA</td>
<td>7.03 ± 0.02</td>
<td>540</td>
<td>0.22 ± 0.03</td>
<td>70 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FS</td>
<td>6.87 ± 0.02</td>
<td>565</td>
<td>0.269 ± 0.001</td>
<td>85 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>6.86 ± 0.01</td>
<td>565</td>
<td>0.209 ± 0.008</td>
<td>66 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeNaEDTA</td>
<td>6.94 ± 0.02</td>
<td>555</td>
<td>0.010 ± 0.002</td>
<td>3.0 ± 0.7</td>
</tr>
</tbody>
</table>

*wavelength used for measurement (same as that of other FeNaEDTA sample at equivalent pH; when there were two, wavelength closest to that of calibration curve was selected)

**unlikely to be typical iron-polyphenol complex, however it would be equivalent to 0.043 ± 0.002 mM FeCl₃/GAE and 13.6% ± 0.7% of FS in GA at pH 7

Comparisons may be made between gallic acid and tea polyphenols. At pH 5, when a chelated iron source was used, complex formation was similar for gallic acid and tea extract. However, at pH 7, complex formation was greater in tea extract than in gallic acid. This is due to a difference in the structures of tea polyphenols and gallic acid. It seems that at pH 7 tea polyphenols have more iron binding groups per GAE (as measured by the Folin and Ciocalteu method) than gallic acid. Also, precipitation occurred when the non-chelated iron sources were added to 1 gGAE/L tea extract but not to 1 g/L gallic acid. This was likely due to polymerization brought about by the multiple binding groups present on the oxidized long-chain tea polyphenols. Consequently, a comparison could not be made for non-chelated iron sources. As expected, there was less complex formation in the dilute tea polyphenol solutions (0.1 gGAE/L) when compared to 1 g/L gallic acid.
Differences may also be seen between different iron sources. Ferrous sulphate and ferrous fumarate generally perform equivalently poorly in gallic acid. In 0.1 gGAE/L tea extract, ferrous fumarate formed less complex than ferrous sulphate. At pH 5, ferrous fumarate formed about 10% less complex than ferrous sulphate and at pH 7 it formed about 20% less. This is surprising but is likely due to fumarate forming coordination complexes with iron and thus protecting it from this lower concentration of polyphenols. It is not due to the difference in solubility because ferrous fumarate was dissolved in HCl immediately before use.

Ferric sodium EDTA formed much less complex than the other two varieties of iron. In solutions of gallic acid and ferric sodium EDTA, only 5%-6% of the complex formed with respect to the amount that formed for ferrous sulphate in gallic acid at the same pH level. For 0.1 gGAE/L tea extract, only 3% of the complex formed. In tea extract at the expected concentration of tea (1 gGAE/L) there was also a decrease. However, it was much more pronounced at pH 5, where 4% of the complex formed, than pH 7, where 70% of the complex formed. This decrease in complex formation is due to ferric sodium EDTA being a chelated iron source. Therefore, EDTA binds strongly to iron protecting it from forming complexes with other molecules. At pH 7 there is more deprotonation of the phenolic compounds, which drives iron-polyphenol complex formation. This may be used to explain the large difference in complex formation between pH 5 and pH 7 in tea extract (1 gGAE/L) with ferric sodium EDTA. Furthermore, since the iron-EDTA complex is bioavailable, iron chelation may be a useful strategy in developing iron fortified tea.

This novel analytical method allows for comparison of iron-polyphenol complex formation between simple polyphenolic compounds (i.e. gallic acid) and tea polyphenols (a complex system of polyphenols) at pH levels relevant to iron fortified tea. It is also advantageous as it allows for prediction of the efficacy of different iron sources to prevent off-colour and allow for iron absorption into the body. Chelated iron sources such as ferric sodium EDTA form less iron-polyphenol complex in tea than simple iron salts. However, they may not be enough to prevent malabsorption because a significant amount of iron-polyphenol complex forms at pH 7 when tea extract is at a polyphenol concentration of 1 gGAE/L.
4.3.5 Effect of Temperature and Concentration

Binding between iron and polyphenols is expected to be affected by many factors including the type of polyphenols, pH, concentrations, and temperature. At the target iron concentration for fortified tea (0.3 mM) iron-polyphenol binding was measured at various polyphenol concentrations and temperatures in solutions at pH 5 and pH 7. The pH of these solutions was adjusted using sulphuric acid and sodium hydroxide. Sulphuric acid was selected because sulphate was already present due to the selection of ferrous sulphate as the iron source. It was chosen over hydrochloric acid for ease of use as hydrochloric acid is more volatile. Iron in sulphate complexes is highly susceptible to chelation, thus the extra sulphate added by way of sulphuric acid was not expected to dampen the iron-polyphenol reaction [42]. Complex formation was measured by means of a flow cell and spectrophotometer. Absorbance scans were performed for all solutions and the net peak absorbances were at 560 nm. The net peak absorbances were calibrated using a ferrous sulphate in gallic acid (1 gGAE/L) calibration curve. Due to this, the units for iron-polyphenol complex concentration are “ferrous sulphate in gallic acid equivalents” (FS/GAE). Further explanation may be found in Appendix 10.4.1. Ferrous sulphate was selected as it is the typical iron source used for comparative research in food fortification. It was found to react within a few minutes under the conditions of this experiment. Also, it was shown to react to the same extent as ferric chloride, Section 4.3.3.

In the initial trials polyphenols were increasingly added to iron solutions which were held at a specific temperature and pH level. This is the typical procedure for producing binding isotherms. The polyphenols are regarded as the ligands because they are in excess in the target application. Also, in the field of coordination chemistry the non-metal species is generally conceptualized as the ligand. However, the reaction proceeded to a lesser extent than expected due to the formation of an inactive form of iron prior to the addition of polyphenols. This inactive form is likely insoluble ferric hydroxide which forms more quickly at pH 7 than pH 5. More detail of this is given in Appendix 10.4.2.

To gain further insight into the iron-polyphenol reaction, the aforementioned approach was modified such that solutions of tea extract at various concentrations were prepared at pH 5 and pH 7 before adding iron. Once iron was added, these solutions were heated to a range of temperatures while being tested for iron-polyphenol complex formation. The previously
mentioned complications with iron did not seem to occur when this procedure was used. Also, since more iron-polyphenol complex formation occurs at lower temperatures, there will not be a tendency toward increased iron-polyphenol complex formation upon heating for potential inactive iron side products to impede the reaction [42]. The ratios of iron:polyphenol were approximately 2.5:1, 1:1, 1:2, 1:3 on a molar basis with 0.3 mM of iron remaining constant. These ratios were chosen due to the potential formation of 1:3 iron:polyphenol complexes. Also, nonlinearity due to lesser iron-polyphenol complex formation (i.e. a plateau effect) was observed at the 1:3 iron:polyphenol ratio so testing higher polyphenol concentrations would not be useful. This ratio of 1:3 iron:polyphenol is quite different from the ratio in iron fortified tea, i.e. 1:20. More specifically, the concentration of polyphenols in this experiment ranged from 0.12 mM GAE to 0.93 mM GAE whereas brewed black tea has a concentration of approximately 6 mM GAE. However, having less polyphenol is useful for understanding iron-polyphenol complex formation during the initial stages of tea brewing. One of the common methods for tea brewing in some areas of the world, such as India, is to add tea leaves to water (or milk) followed by heating. These lower concentrations are also useful to the understanding of what occurs in digestion because the tea will be diluted.

In Figure 4.11 it can be seen that there is a linear relationship between the polyphenol concentration and the amount of complex formed for the first three points. This is followed by a plateau at higher concentrations. These points are averages of iron-polyphenol complex quantity formed at 4 different temperatures: 30°C, 40°C, 50°C, and 60°C. More detail regarding the estimation of the temperatures at the flow cell can be found in Appendix 10.4.3. Averages were taken across the temperatures because there were no substantial trends seen in forming iron-polyphenol complexes. This can be readily observed in Figure 4.11 by the presence of small error bars which represent standard deviation. Mellican et al. (2003) found greater complex formation at lower temperatures, though, temperature was deemed an insignificant factor for the prediction of iron-polyphenol complex formation [42]. Also, a larger range of temperatures was used in the experiments of Mellican et al. (2003). That is, from 0°C to room temperature for experiments using foods and 0°C to 100°C for experiments using monomeric polyphenols [42]. Therefore, the concentration of dissolved oxygen, a factor claimed to be lowering the extent of the reaction, would have been more greatly affected [42]. There is one notable abnormality in the data observed at pH 7 and 0.12 mM GAE (Figure 4.12). There is a considerable decrease in absorbance between
40°C and 50°C. However, this is not due to a change in iron-polyphenol complex formation because peak height was not greatly affected. Instead, there is a shift of the entire spectrophotometric scan. The amount of complex formation at pH 5 and pH 7 was similar for concentrations of polyphenols up to 0.62 mM GAE. At higher values more complex was formed at pH 7 than at pH 5. This was expected because deprotonation of polyphenols is more likely to occur at pH 7.

Figure 4.11: Iron-Polyphenol Complex Formation in the Flow Cell
The expected iron-polyphenol complex for both pH 5 and pH 7 has an iron:polyphenol ratio of 1:2, since complexes formed were blue-purple in colour and had maximum wavelengths between 542-586 nm [23]. Also, pH levels of 5-7 are generally dominated by complexes of a 1:2 iron:polyphenol ratio [23]. A 1:1 ratio dominates below pH 4 whereas a 1:3 ratio dominates above pH 8 [23]. Because only one absorbance peak was present, it is assumed that the other ratios of iron:polyphenol (i.e. 1:1 and 1:3) were not present. Therefore, if a species of 1:1 iron:polyphenol was formed, it must have been converted into a 1:2 complex or dissociated. This 1:1 complex is assumed to be a labile transient product. In the literature equilibrium expressions are generally used to describe coordination complex formation, as described in Section 2.3.3. The expected reactions are as follows:

\[
Fe + GA \leftrightarrow FeGA \quad \text{…Eq. 4.1}
\]

\[
FeGA + GA \leftrightarrow Fe(GA)_2 \quad \text{…Eq. 4.2}
\]

\[
Fe = \text{iron}
\]

\[
GA = \text{polyphenol}
\]

\[
FeGA = 1:1 \text{ iron-polyphenol complex}
\]

\[
Fe(GA)_2 = 1:2 \text{ iron-polyphenol complex}
\]
At equilibrium:

\[ K_{D1} = \frac{[Fe][GA]}{[FeGA]} \quad \ldots \text{Eq. 4.3} \]

\[ K_{D2} = \frac{[FeGA][GA]}{[Fe(GA)_2]} \quad \ldots \text{Eq. 4.4} \]

\( K_{D1} \) = disassociation constant 1

\( K_{D2} \) = disassociation constant 2

\([Fe]\) = concentration of iron at equilibrium

\([GA]\) = concentration of polyphenols at equilibrium

\([FeGA]\) = concentration of 1:1 iron-polyphenol complex at equilibrium

\([Fe(GA)_2]\) = concentration of 1:2 iron-polyphenol complex at equilibrium

This may be expressed in terms of total dissociation constant:

\[ K_{DT} = K_{D1}K_{D2} = \frac{[Fe][GA]^2}{[Fe(GA)_2]} \quad \ldots \text{Eq. 4.5} \]

\( K_{DT} \) = total disassociation constant

Because only the 1:2 iron:polyphenol complex was observed:

\([Fe] = [Fe]_o - [Fe(GA)_2] \quad \ldots \text{Eq. 4.6} \)

\([Fe]_o \) = initial concentration of iron

\([GA] = [GA]_o - 2[Fe(GA)_2] \quad \ldots \text{Eq. 4.7} \)

\([GA]_o \) = initial concentration of polyphenol
Combining equations 4.5, 4.6, and 4.7:

$$K_{DT} = \frac{([Fe]_o - [Fe(GA)_2])([GA]_o - 2[Fe(GA)_2])^2}{[Fe(GA)_2]}$$

...Eq. 4.8

This may be rearranged to be in terms of initial polyphenol concentration.

$$[GA]_o = \left( \frac{K_{DT}}{\frac{[Fe]_o}{[Fe(GA)_2]} - 1} \right)^{1/2} + 2[Fe(GA)_2]$$

...Eq. 4.9

The value of the total dissociation constant was estimated by fitting the model to the 4 data points shown in Figure 4.11, for each pH level, using least squares regression (i.e. minimizing the sum of the squared residuals).

$$S = \sum_{i=1}^{4} ([Fe(GA)_2]_i - [Fe(GA)_{2i}])^2$$

...Eq. 4.10

$S = \text{sum of the squared residuals}$

$[Fe(GA)_2]_i = \text{experimental concentration of complex at a given } [GA]_o$

$[Fe(GA)_{2i}]_i = \text{model predicted concentration of complex at a given } [GA]_o$

A few implicit assumptions are made when this model is applied to iron-polyphenol complex formation. One of these is that the phenolic content measured by the Folin & Ciocalteu method represents the amount of polyphenols present with the ability to bind iron. A second is that the ability of all polyphenols in tea to bind iron is equal. A third is that iron-polyphenol complex concentration measured as FS/GAE accurately represents the molar quantity of these complexes. The final important assumption is that all iron and polyphenols remain in a reactive form.
These models are plotted with experimental data in Figures 4.13 (pH 5) and 4.14 (pH 7). At pH 5 the model appeared to over predict complex formation at higher polyphenol concentrations. The first hypothesis tested was that less complex forms because less of the polyphenols are deprotonated. This can be addressed by altering GA\textsubscript{0} such that a certain fraction of it is unreactive. Unexpectedly, this was unsuccessful at improving the fit of the model. The pH 5 data appear to plateau below the maximum of 0.3 mM (total iron concentration). To account for this, the model was adjusted such that a factor (x) was added in front of Fe\textsubscript{0} term. This adjustment greatly improved the fit of the model. This adjustment would support the hypothesis that a specific fraction of iron became unreactive. However, this hypothesis seems unlikely. Consequently, I suggest further investigation of iron-polyphenol complex formation at pH 5.

Figure 4.13: Models of Iron-Polyphenol Complex Formation at pH 5
The formula for the adjusted model is as follows,

\[
[GA]_0 = \left( \frac{K_{DT}}{x[Fe]_0} - 1 \right)^{1/2} + 2 [Fe(GA)_2]
\]

...Eq. 4.11

\( x \) = fraction of total iron that is able to form iron-polyphenol complex

This model may be used to predict the amount of iron-polyphenol complex at equilibrium given the total phenolic concentration (as measured by the Folin & Ciocalteu method). Parameters include a total dissociation constant (\( K_{DT} \)) as well as a pH dependant factor (\( x \)). These parameters as well as calculated total binding constants, and coefficients of determination (\( R^2 \)) are presented in Table 4.4. The pH 5 adjusted model predicts that 84% of the iron remains active with the ability to form iron-polyphenol complex. This is in contrast to pH 7 where 100% of the iron remains available. The total binding constants are large which is in agreement with literature that suggested that the reaction nears completion.
Table 4.4: Iron-Polyphenol Flow Cell Models

<table>
<thead>
<tr>
<th>Description</th>
<th>x</th>
<th>$K_D$ (mM$^2$)</th>
<th>Total binding constant (M$^{-2}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5 model</td>
<td>1</td>
<td>0.015</td>
<td>6.7*10$^7$</td>
<td>0.956</td>
</tr>
<tr>
<td>pH 5 adjusted model</td>
<td>0.84</td>
<td>0.0047</td>
<td>2.1*10$^8$</td>
<td>0.998</td>
</tr>
<tr>
<td>pH 7 model</td>
<td>1</td>
<td>0.011</td>
<td>9.1*10$^7$</td>
<td>0.998</td>
</tr>
</tbody>
</table>

The novel analytical method for quantifying iron-polyphenol complex formation was applied to determining the effect of temperature and polyphenol concentration at pH 5 and pH 7. The effect of temperature on the extent of reaction between 30°C and 60°C was negligible. Models with excellent correlation to the observed data were produced which predict the amount of iron-polyphenol complex formation based on the polyphenol content of the tea solution.

4.4 Summary of Findings

Iron-polyphenol complex formation was quantified and a deeper understanding of the formation of this complex in tea was developed. The variation in polyphenol concentration and pH of black tea based on steeping time and quantity of tea leaves was investigated. The tea source used in this study is comparatively high in polyphenol content and was stable throughout the study. A suitable pH and polyphenol concentration for the representation of brewed tea were found to be pH 5 and 1 gGAE/L. Phosphate and phthalate buffer solutions were found to interfere with iron-polyphenol complex formation. Because other buffer solutions are known to act similarly, sodium hydroxide and hydrochloric acid were selected to be used for pH adjustment. A novel spectrophotometric iron-polyphenol quantification method tailored to black tea was developed. This method is improved in comparison to that of Brune et al. (1991) in the following ways:

- It allows for quantification at pH 5-7.
- Sodium hydroxide and hydrochloric acid are used for pH adjustment eliminating the potential interference of a buffer solution.
- Only one absorbance wavelength is required for quantification. This is specific to black tea.
- Gallic acid is used instead of tannic acid in calibration curves. This makes the results more comparable to “total phenolic content” which is measured in “gallic acid equivalents”.
- Ferric chloride or ferrous sulphate is used in calibration curves allowing for comparison to current literature on iron binding polyphenols and iron fortification.
This method was used to study the effects of iron source, temperature, and polyphenol concentration on iron-polyphenol complex formation. The chelated iron source formed less iron-polyphenol complex than the iron salts. Also, it formed more complex with tea polyphenols than gallic acid at pH 7. Temperature did not substantially affect the extent of iron-polyphenol complex formation. Equilibrium models relating “total phenolic content” to iron-polyphenol complex formation were produced with excellent fit to experimental data. The total binding constant derived from these models is large which confirms that the reaction moves to completion.

Through the development of a deeper understanding of the interaction between iron and tea polyphenols, Chapter 4 presents strides forward in the development of a technique to prevent iron-polyphenol complex formation in iron fortified tea. Because temperature did not notably alter iron-polyphenol complex formation, it did not need to be studied further in the initial development of the technique. A suitable pH and polyphenol concentration to model tea were determined and an analytical method for the quantification of iron-polyphenol complex formation was developed. The following two Chapters present the application of this novel analytical method to the investigation of strategies for the reduction of iron-polyphenol complex formation in model tea solutions (i.e. gallic acid or tea extract).
5 Reducing Agents

5.1 Research Objective

In the food industry sacrificial antioxidants, which become oxidized and thus preserve the more useful reduced form of other components, are used. Such reducing agents may be useful in iron fortified tea because ferrous iron is first oxidized during the formation of stable coloured iron-polyphenol complexes [67]. It has been shown that reducing agents, especially ascorbic acid, increase iron absorption in general [73]. Therefore, the objective of the investigation presented in this chapter was to determine the efficacy of employing reducing agents to slow the formation of iron-polyphenol complexes in black tea for the purposes of preserving iron bioavailability and colour acceptability.

5.2 Experimental Strategy

The effect of ferric and ferrous iron addition to model tea solutions was compared at pH 5 (the pH of brewed tea) and pH 7 (a pH to approximate the small intestine). The behaviour of tea was modeled by gallic acid in buffered solutions. This was done to eliminate confounding factors due to the complexity of black tea such as polymerization issues upon iron addition. Buffer solutions were used despite not being ideal, as shown in Section 4.3.2, because the reaction is pH dependant and periodic adjustment of pH impacts the kinetics. The kinetics of iron-gallate complex formation was modeled mathematically. This provided some insight into the need for iron oxidation to form iron-polyphenol complexes in tea. Then reducing agents were selected and added to solutions containing black tea extract at a concentration of 0.1 gGAE/L (one tenth the concentration of tea). This polyphenol solution was selected to represent the complexity of black tea polyphenols but avoid polymerization which occurs upon iron addition at higher polyphenol concentrations. The pH levels of these solutions were adjusted using sodium hydroxide and hydrochloric acid.

The reducing agents were selected by consulting the Codex Alimentarius for antioxidant additives. The list was narrowed to those that are water soluble. Most antioxidant additives remaining were ascorbates or sulphites [38]. One of each was selected such that they were not in an acid form, to prevent lowering the pH of the tea. As a result, sodium ascorbate and sodium metabisulphite were selected. It should be noted that in literature ascorbic acid is the most widely
sighted reducing agent for improving iron bioavailability. Some sugars are referred to as reducing sugars because they can act as reducing agents. Dextrose, or D-glucose, was selected for this reason. Dextrose is also an excellent water soluble adhesive. Therefore, it may also be useful in tea fortification by attaching iron to tea leaves during processing. In the remainder of this document dextrose is referred to simply as glucose.

The reducing agents were added to solutions containing black tea extract and the pH was adjusted to that of the tea beverage (pH 5) or of the small intestine (pH 7) using sodium hydroxide and hydrochloric acid. The reducing agents were added to the solutions at 5% w/v. This relatively large quantity was chosen to determine efficacy but is impractical for use in iron fortified tea. For perspective, the amount of tea leaves used is only ~1% w/v. In molar concentrations the quantities of reducing agents were 277 mM, 252 mM, and 263 mM for glucose, sodium ascorbate, and sodium metabisulphite respectively. Therefore, all solutions tested contained more than 800 times the molar the concentration of iron added (0.3 mM). These solutions were also tested for complex formation at time zero, one hour, and two hours to determine their stability.

5.3 Results and Discussion
5.3.1 Complex Formation of Ferrous versus Ferric Iron
Various iron compounds (at 0.3 mM, the target fortification level) were tested in gallic acid (1 gGAE/L, to simulate tea) at pH 5 and pH 7 using buffered solutions (0.1 M). This included ferrous iron sources (ferrous sulphate and ferrous fumarate) and ferric iron sources (ferric chloride, ferric sodium EDTA, and ferric nitrate). In Section 4.3.2 it was mentioned that a 0.3 M buffer was necessary. However, this concentration was needed to maintain adequate pH upon the initial addition of iron and gallic acid or tea extract, not to maintain the pH throughout the reaction. The lower concentration was used here in order to cause less impact on the reaction but still maintain the target pH level. It was found that ferric iron reacted very quickly, achieving close to its maximum absorbance at both pH 5 and pH 7 before the first measurement was taken, whereas ferrous iron sources took longer at pH 5. For demonstration purposes, the initial absorbance, expressed as a percentage of final absorbance (after 4 hours of reaction time), for samples of ferrous sulphate and ferric chloride are given in Table 5.1.
Table 5.1: Extent of Reaction at Initial Measurement for Ferric and Ferrous Iron

<table>
<thead>
<tr>
<th>Iron Source</th>
<th>Initial Peak Absorbance as Percentage of Final Peak Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>8%</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>88%</td>
</tr>
</tbody>
</table>

The absorbance of a gallic acid solution (1 g/L) containing ferrous sulphate (0.3 mM) at pH 5 and was traced over time (see Figure 5.1). Absorbance was measured at 560 nm over the duration of 4 hours (i.e. 240 minutes). The spectrophotometric program was:

- 0-1 minute → reading every 0.1 seconds
- 1-15 minutes → reading every second
- 15-30 minutes → reading every 2 minutes
- 30-90 minutes → reading every 5 minutes
- 90-240 minutes → reading every 10 minutes

![Absorbance Development of Ferrous Sulphate in Buffered Gallic Acid Solution](image)

In order to analyze the kinetics at pH 5 the measured reading times were shifted forward by a calculated value of 2.386 minutes. This shift takes into account the time delay between sample preparation and the first reading. The time shift required was calculated by fitting a linear equation to the data points collected during the first minute. The graph was extrapolated to the x-intercept, i.e. the point at which absorbance = 0. It was calculated that $t = -2.386$ minutes at the x-intercept.
so all of the data was shifted forward by that amount. Figure 5.2 shows the linear plot used to determine the time shift.

![Figure 5.2: First Minute Readings of Ferrous Sulphate in Buffered Gallic Acid at pH 5](image)

The reaction of ferrous iron with a phenolic binding group such as gallol (the group present in gallic acid (GA)) can be summarized by four reaction steps: the deprotonation of polyphenols, polyphenol binding of the first iron, oxidation of the 1:1 iron-polyphenol complex, and finally binding of another polyphenol to create a 1:2 iron-polyphenol complex [23]. These reactions may be summarized as follows:

\[
GA(H_2) \rightarrow GA^{2^-} + 2H^+ \quad \text{...Eq. 5.1}
\]

\[
GA^{2^-} + Fe^{2+} \rightarrow [Fe(II)GA] \quad \text{...Eq. 5.2}
\]

\[
[GAFe(II)] + O_2 \rightarrow [Fe(III)GA]^+ + O_2^{2^-} \quad \text{...Eq. 5.3}
\]

\[
[Fe(III)GA]^+ + GA^{2^-} \rightarrow [Fe(III)GA_2]^- \quad \text{...Eq. 5.4}
\]

The hypothesis of a rate limiting first order reaction step would be supported if a first order kinetics model is able to fit the experimental data. The rate limiting reaction would not be reaction 5.1 because this step is also required for ferric iron, which reacted quickly. Reaction 5.4 also could not be rate limiting because the [Fe(III)GA]^+ complex was not observed.
Equation 5.2 may be treated as pseudo first order because the amount of gallic acid in solution is much larger than the amount of ferrous iron (6 mM versus 0.3 mM). Also, since the solutions are exposed to air, oxygen concentration is assumed to not be a limiting factor. With these assumptions the potential rate limiting reactions may be simplified to:

Step 1: \( \text{Fe}^{2+} \rightarrow \text{[Fe(II)GA]} \) \hspace{1cm} \text{OR} \hspace{1cm} \text{A} \rightarrow \text{B} \hspace{1cm} \text{...Eq. 5.5}

Step 2: \( \text{[Fe(II)GA]} \rightarrow \text{[Fe(III)GA]}^+ \) \hspace{1cm} \text{OR} \hspace{1cm} \text{B} \rightarrow \text{C} \hspace{1cm} \text{...Eq. 5.6}

In developing a kinetics model for ferric gallate complex formation in solutions of ferrous sulphate and gallic acid some assumptions were made. The reaction was assumed to move to completion such that the final concentration of the product at \( t = \infty \) would be 0.3 mM. This is a reasonable assumption because the overall binding coefficients for gallic acid and catechol (a related polyphenol) are very large (\( 10^{25.8} \) and \( 10^{43.76} \) respectively) [23].

Therefore,

\[ [A_0] = [C_{t=\infty}] \hspace{1cm} \text{...Eq. 5.7} \]

Where “\([A_0]\)” is the concentration of \( \text{Fe}^{2+} \) at time zero and “\([C_{t=\infty}]\)” is the concentration of product at infinite time. The coloured product is represented as “\( C \)” despite this letter being used in equation 5.6 to represent an intermediate product. This is appropriate due to the assumption that this intermediate product is quickly converted into the final coloured product. The quantity of the product is proportional to the absorbance, i.e. follows the Beer-Lambert law [29, 30]. Therefore, the ratio of the absorbance to the absorbance at \( t = \infty \) is assumed to be equal to the ratio of the amount of complex formation to the amount of complex formation at \( t = \infty \).

So,

\[ [C] = [A_0] \left( \frac{\text{Abs}}{\text{Abs}_{t=\infty}} \right) \hspace{1cm} \text{...Eq. 5.8} \]

Here, “\([C]\)” is the concentration of product at a given time \( (t) \). Also, “\( \text{Abs} \)” represents the peak absorbance at a given time \( (t) \) and “\( \text{Abs}_{t=\infty} \)” represents the maximum peak absorbance (at \( t = \infty \)). \( \text{Abs}_{t=\infty} \) was estimated by plotting the first differences of the absorbance values for the last 16 data points against time and extrapolating the exponential trend (Figure 5.3). These last 16 data points
were captured at 10 minute intervals. To approximate the absorbance at $t = \infty$ the time point of 3000 minutes was selected because no significant changes in absorbance were observed near this time point. The absorbance at $t = \infty$ was predicted to be 1.036.

![Graph showing first differences of absorbance readings with the equation $y = 0.1348e^{0.018x}$ and $R^2 = 0.9539$.]

Figure 5.3: First Differences of Final 16 Absorbance Readings

A first order model may represent one of three reaction paradigms. Each of these includes a first order rate limiting reaction step. For Case 1, the iron binding reaction is rate limiting. For Case 2, the iron oxidation reaction is rate limiting. For Case 3, the iron binding reaction is in rapid equilibrium followed by a rate limiting iron oxidation reaction. Each of these cases may be represented by the same first order rate equation.

If Case 1 is assumed:

Step 1: $A \rightarrow B$ slow
Step 2: $B \rightarrow C$ fast

$$Rate = \frac{d[C]}{dt} = -\frac{d[A]}{dt} = k_1 [A]$$

...Eq. 5.10

$$Rate = k_1 ([A_0] - [C])$$

...Eq. 5.11

Where “$k_1$” is the rate constant and “$A_0$” is the amount of Fe$^{2+}$ at time zero.
This maybe assumed because [B] will be very small.

If Case 2 is assumed:
Step 1: A \rightarrow B \quad \text{fast}
Step 2: B \rightarrow C \quad \text{slow}

\[
Rate = \frac{d[C]}{dt} = -\frac{d[B]}{dt} = k_1 [B]
\]

...Eq. 5.12

\[
Rate = k_1 ([A_0] - [C])
\]

...Eq. 5.11

This may be assumed because [A] will be very small.

If Case 3 is assumed:
A \leftrightarrow B, such that K = [B]/[A], were K is the equilibrium constant. \quad \ldots \text{Eq. 5.13}
B \rightarrow C, rate = k_2 [B] \quad \ldots \text{Eq. 5.14}

Also,

\[
[A] = [A_0] - [B] - [C]
\]

...Eq. 5.15

So,

\[
[A] = [A_0] - K[A] - [C]
\]

...Eq. 5.16

\[
[A] = ([A_0] - [C])/(1 + K)
\]

...Eq. 5.17

\[
Rate = \frac{d[C]}{dt} = -\frac{d[B]}{dt} = -\frac{d[A]}{dt}
\]

...Eq. 5.18

\[
Rate = k_2[B] = k_2K[A]
\]

...Eq. 5.19

\[
Rate = k_2 \frac{K}{1 + K}([A_0] - [C])
\]

...Eq. 5.20
This final equation is the same as for the other two cases except that instead of just having the reaction constant “k₁” it has become “k₂ * K/(1+K)”. So, if k₁ is defined as follows:

\[ k_1 = k_2 \left( \frac{K}{1 + K} \right) \]  

...Eq. 5.21

Then,

\[ Rate = k_1 ([A_o] - [C]) \]  

...Eq. 5.11

Therefore, it is established that the three cases are indistinguishable by way of a first order kinetics model.

Least squares regression was used to fit a first order reaction model to the experimental data. The first order reaction model expression was derived as follows:

\[ \frac{d[C]}{dt} = k_1 ([A_o] - [C]) \]  

...Eq. 5.22

\[ \int_0^C \frac{d[C]}{([A_o] - [C])} = \int_0^t k_1 dt \]  

...Eq. 5.23

\[-\ln([A_o] - [C]) + \ln[A_o] = k_1 t\]  

...Eq. 5.24

\[ \ln \left( [A_o] - \left( [A_o] \left( \frac{Abs}{Abs_{t=\infty}} \right) \right) \right) - \ln[A_o] = -k_1 t \]  

...Eq. 5.25

\[ \ln \left( 1 - \left( \frac{Abs}{Abs_{t=\infty}} \right) \right) = -k_1 t \]  

...Eq. 5.26
1 − \left( \frac{\text{Abs}}{\text{Abs}_{\text{t=\infty}}} \right) = e^{-k_1t} \quad \text{…Eq. 5.27}

1 − \left( \frac{\text{Abs}}{1.036} \right) = e^{-k_1t} \quad \text{…Eq. 5.28}

Skewing of the model due to frequent initial sampling was avoided by using only evenly spaced data points at 10 minute intervals. Figure 5.4 depicts the model with the data employed for least squares regression. From this, \( k_1 \) was determined to be 0.0258 min\(^{-1}\). Therefore, in terms of the concentration of iron-polyphenol complex, the first order model can be described by the following equations:

\[
[C] = [A_o](1 - e^{-k_1t})
\quad \text{…Eq. 6.29}
\]

Or,

\[
[Fe(III)GA_2]^- = 0.3 \left(1 - e^{-0.0258t}\right)
\quad \text{…Eq. 6.30}
\]

Figure 5.4: Fitting Model Using Least Squares Regression
In Figure 5.5 this model is plotted in terms of iron-gallate complex concentration. This was done assuming that an absorbance of 1.036 (Abs$\infty$) is equal to 0.3 mM. The experimental data is well described by the first order model as demonstrated by the high coefficient of determination.

![Graph showing iron-gallate complex concentration vs. time](image)

**Figure 5.5: Model of Ferrous Sulphate in pH 5 Phthalate Buffered Gallic Acid**

To summarize, the rate of the reaction of ferrous iron buffered gallic acid solutions at pH 5 is slower than at pH 7 and slower than ferric iron at either pH level. The experimental data from ferrous sulphate in gallic acid is well described by a first order kinetics model. This supports the hypothesis that there is a first order rate limiting reaction step. It is expected that either initial iron-polyphenol complex formation or oxidation holds precedence over the overall reaction rate. The reduced reaction rate for ferrous iron at pH 5 may be due to the lower affinity of gallic acid for ferrous iron or the need for ferrous iron to be oxidized in order to form the stable intensely coloured iron-gallate complex.

### 5.3.2 Effect of Reducing Agents on Iron Complexation

Oxidation of ferrous iron is required for the formation of coloured iron-polyphenol complexes. As shown in Section 5.3.1, rate of iron-polyphenol complex formation is slower when ferrous iron, as opposed to ferric iron, is added to buffered gallic acid solution at pH 5. Also, the rate of complex formation is slower at pH 5 than pH 7 for ferrous iron. As iron oxidation occurs more quickly at higher pH, this is further evidence that iron oxidation may slow complex formation. It
is therefore a reasonable hypothesis that reducing agents will further slow the formation of coloured iron-polyphenol complex. The addition of various reducing agents was tested to determine if the reaction rate could be slowed such that no detectible iron-polyphenol complex would form in the presence of tea polyphenols at pH levels critical to colour formation and iron bioavailability. This is an ambitious goal as a 10% to 20% loss of bioavailable iron to the formation of iron-polyphenol complex is still expected to suitably impact iron status.

Ferrous sulphate (0.3 mM) solutions were prepared in tea extract (0.1 gGAE/L) at pH 5 and pH 7. Each reducing agent (i.e. sodium ascorbate, glucose, or sodium metabisulphite) was added to these solutions at a concentration of 5% w/v. Iron-polyphenol complex formation at time zero can be seen in Figure 5.6. The samples were prepared in triplicate except for pH 5 glucose which represents the average of five replicates. In this experiment iron-polyphenol complex formation occurred rapidly with the addition of ferrous sulphate at pH 5. This indicates that the pH 5 phthalate buffer used in Section 5.3.1 was not a trivial factor, likely contributing to the reduction of reaction rate for ferrous iron at pH 5.

Reducing agents, aside from glucose, appeared to be successful at hindering iron-polyphenol complex formation at pH 5. Unfortunately, at pH 7 they were less effective. At pH 7, sodium ascorbate appeared to cause more iron-polyphenol complex formation than the control which had no added reducing agent. However, spectrophotometric scans of these solutions are oddly shaped,
see Figure 5.7. Likely, ascorbate is interacting with ferrous iron producing ferrous ascorbate, a purple pigment complex. Coloured ferrous ascorbate chelate forms at pH 7 but does not form at pH 5 [74]. From the absorbance scans it is impossible to distinguish the iron-polyphenol absorbance peak from that of ferrous ascorbate at 525 nm. The absorbance bands of both complexes are caused by charge-transfer [74].

Figure 5.7: Spectrophotometric Scans of Ascorbate in Tea Extract (pH 7)

Figure 5.8 displays iron-polyphenol complex formation in solutions that were observed at 0, 1, and 2 hours to determine stability. All data points are averages of three replicates except glucose at time zero which is an average of five. Glucose did not prevent the reaction and thus the full extent of the reaction was achieved immediately. Although glucose can act as a reducing agent, it is generally not used for that purpose, as it is thought to be a weak reducing agent, which was confirmed by these results. Sulphites, including metabisulphite, are used in food as antioxidants. Metabisulphite successfully prevented ~60% of iron-polyphenol complex formation even after two hours. At initial time points the variation in the amount of complex formation was quite large. However, after two hours this variation decreased. The samples that formed less complex initially reached the same levels as those that formed more. Ascorbate was the most successful as it prevented ~90% of iron-polyphenol complex formation over a 2 hour duration. This may be in part because, as a bidentate ligand, it can act as both a reducing agent and a weak sequestering agent.
Spectrophotometric scans of each of the pH 5 reducing agent solutions at several time points may be seen in Figure 5.9. Each line is an average of 3 replicates except glucose at 0 hours which is 5 replicates. These scans show that typical iron-polyphenol peaks formed for solutions containing glucose and metabisulphite. However, ascorbate solutions did not form these peaks. Therefore, it is likely that there was no iron-polyphenol complex formation.

Figure 5.9: Spectrophotometric Scans of Iron in Tea Extract with Reducing Agents at pH 5
Reducing agents alone are not likely to improve iron absorption from fortified tea. This is because they do not hinder iron-polyphenol complex formation at pH 7, an approximate pH of the small intestine. They may be useful to improve the colour of the tea beverage in the presence of iron or other foods with a pH at or below 5. Ascorbate was the most effective of the reducing agents tested. Unfortunately, due to confounding complex formation at pH 7, another analytical method, such as in-vivo iron absorption, is needed to verify that ascorbate will increase iron bioavailability in iron fortified tea.

5.4 Summary of Findings

The strategy of employing reducing agents to slow the formation of iron-polyphenol complexes in black tea was evaluated. Solutions of ferrous iron at pH 5 in buffered gallic acid formed iron:polyphenol complex slowly in comparison to ferrous iron at pH 7 and ferric iron. This is an indicator that reducing agents may be useful to slow iron-polyphenol complex formation. A first order kinetics model excellently fit the experimental data pertaining to ferrous iron at pH 5.

Unfortunately, none of the reducing agents tested, effectively slow iron-polyphenol complex formation at pH 7. However, further investigation of ascorbate would be useful because a coloured ascorbate complex confounded the results. There is clear evidence in the literature that ascorbic acid and ascorbate improve iron absorption in many foods. At pH 5, both metabisulphite and ascorbate successfully decrease the amount of iron-polyphenol complex formation. Since no absorbance peak was observed when sodium ascorbate was added to black tea extract at pH 5, likely iron-polyphenol complex formation is prevented by sodium ascorbate. This success may be due to the fact that ascorbate is not only a reducing agent but also a weak chelating agent.

Chapter 5 demonstrates that, for iron fortified tea, reducing agents insufficiently slow the formation of iron-polyphenol complexes. However, they may be used to improve the colour of the tea beverage in the presence of iron. As reducing agents are not able to fully inhibit iron-polyphenol complex formation, competing complexing by chelation was thus further investigated.
6 Chelated Iron and Chelating Agents

6.1 Research Objective

Chelation refers to the binding of a central atom, usually a metal ion, to a ligand at two or more binding sites. The binding of iron to polyphenols in tea is an example of chelation. The iron(III)-polyphenol chelate is coloured and is not absorbed by the body. A potential strategy for preventing iron-polyphenol complex formation in tea is to add chelating agents or chelated iron sources which compete with polyphenols to form (or maintain) bioavailable iron complexes. Competing chelating compounds had not yet been studied at concentrations and pH levels useful for iron fortified tea. Also, insight into the reasons behind the relative effectiveness of different chelating agents is useful for iron fortification endeavours in general. Therefore, the objective of the investigation presented in this chapter was to determine the ability of chelated iron sources and chelating agents to prevent the formation of iron-polyphenol complexes in tea.

6.2 Experimental Strategy

As presented in Section 4.3.4, polyphenol solutions (1 g/L gallic acid, 1 gGAE/L tea extract, and 0.1 gGAE/L tea extract) were tested by adding three iron sources (ferrous sulphate, ferrous fumarate, and ferric sodium EDTA) at pH 5 and pH 7. Ferric sodium EDTA, a chelated iron source, prevented iron-polyphenol complex formation to a greater degree than the other iron sources. In this chapter further testing of chelated iron sources is presented. Experiments compared four iron sources: ferrous sulphate, ferric sodium EDTA, ferric trisglycinate, and ferrous D-gluconate. This was done in tea extract at one tenth the concentration of tea (0.1 gGAE/L) in order to prevent precipitate formation. Iron was added to a concentration of 0.3 mM and the solutions were adjusted to pH 7. This pH was selected because iron-polyphenol complex formation is more likely to occur at pH 7 (approximation of intestinal pH) than pH 5 (tea pH) due to the increased tendency for polyphenol deprotonation. Ferrous sulphate was selected as it is the simple iron salt typically used in food fortification. Ferric sodium EDTA was selected as it is the most widely reported chelated iron source in the literature and earlier testing indicated that it is a promising contender. An amino acid chelated iron source was selected from the two most reported, ferrous bisglycinate and ferric trisglycinate. Ferric trisglycinate was selected despite its poor performance in maize, a high phytate food [3]. This is because ferrous bisglycinate was shown to be relatively poor at preventing iron-polyphenol complex formation by Mellican et al.
(2003) [42]. Ferrous D-gluconate was also selected as it performed better than ferrous bisglycinate in the aforementioned study and is less expensive than chelated iron sources [3, 42]. Furthermore, gluconate salts are known chelating agents despite ferrous D-gluconate generally not being referred to as a chelated iron source [38].

Due to the relatively high cost of chelated iron sources, an alternative method was investigated. Chelating agents (also known as sequestrants) were added to tea polyphenols together with non-chelated iron sources. It was anticipated that the chelating agents would compete with the polyphenols in solution to bind iron. Six compounds were chosen that had the potential to function as sequestration agents. The Codex Alimentarius was consulted for food sequestrant additives [38]. Chelating agents chosen from the list include: sodium gluconate, sodium citrate, tartaric acid, and disodium EDTA. Two others were tested as well: glycine and sodium hexametaphosphate (SHMP). These are currently listed under food additives by the Food and Drug Administration (FDA) [75]. SHMP was selected because it has been shown to prevent iron-polyphenol complex formation through sequestration by Mellican et al. (2003) [42]. Glycine was selected because ferric trisglycinate and ferrous bisglycinate are chelated iron compounds used in food. These chelating agents were tested by adding them to tea extract (0.1 gGAE/L) at a concentration of 5% w/v in the presence of 0.3 mM ferrous sulphate at pH 7. The concentration of the chelating agents was chosen to be in excess, ranging from 82 mM to 666 mM.

Since disodium EDTA was the most successful chelating agent, the amount needed to prevent iron-polyphenol complex formation was investigated. This was first done in tea extract with 0.1 gGAE/L polyphenols at pH 5 and pH 7. Two iron sources were used, ferrous sulphate and ferrous fumarate. Ferrous fumarate was chosen because it has a colour which resembles black tea (reddish brown), it has greater stability compared to ferrous sulphate (often due to its lower solubility), it is relatively inexpensive, it has a high bioavailability (when not in chelating foods), and it has a bland flavour. This was followed by testing the amount of disodium EDTA required in freshly brewed tea.
6.3 Results and Discussion

6.3.1 Extent of Reaction with Chelated Iron Sources

Ferrous sulphate was compared to ferrous gluconate, ferric trisglycinate, and ferric sodium EDTA for preventing iron-polyphenol complex formation in tea extract at pH 7, see Figure 6.1. All compounds were tested in triplicate except for ferric sodium EDTA which was tested in quadruplicate. Ferrous gluconate was not a stable compound in the presence of black tea polyphenols and performed similarly to ferrous sulphate. This is likely because it is not truly a chelated iron source. Both ferric trisglycinate and, to a greater extent, ferric sodium EDTA prevented iron-polyphenol complex formation. However, from observing the spectrophotometric scans (Figure 6.2), it can be seen that there was an increase in the background absorbance but not peak height for ferric trisglycinate. Therefore, both ferric sodium EDTA and ferric trisglycinate prevented iron-polyphenol complex formation. However, ferric trisglycinate is not readily water soluble. In these experiments hydrochloric acid was used to aid in dissolution but this is not feasible in tea. As an alternative, a smaller particle size or more buoyant particles would allow iron to remain suspended in the beverage. Both ferric sodium EDTA and ferric trisglycinate are expensive. Accordingly, an alternative strategy of adding chelating agents to simple iron salts was tested.

![Graph showing extent of reaction of chelated iron compounds with tea extract (pH 7)](image)

Figure 6.1: Extent of Reaction of Chelated Iron Compounds with Tea Extract (pH 7)
6.3.2 Extent of Reaction with Chelating Agents

A control of 0.1 gGAE/L tea extract with 0.3 mM ferrous sulphate at pH 7 was compared to similar solutions containing the following chelating agents at 5% w/v: sodium gluconate, glycine, sodium citrate, tartaric acid, sodium hexametaphosphate (SHMP), and disodium EDTA. The amount of complex formation from each of these solutions, except sodium gluconate, can be seen in Figure 6.3. The sodium gluconate solutions became a dark red colour, likely from the formation of ferric sodium gluconate, confounding the results. Glycine performed poorly forming as much iron-polyphenol complex as the control. This was surprising as ferric trisglycinate had performed well, as seen in Section 6.3.1. For the production of the related chelated iron source ferrous bisglycinate, citric acid must be present [76]. Therefore, the addition of other substances may be required for glycine to complex iron in the presence of tea polyphenols. Sodium citrate and tartaric acid solutions produced about 60% less iron-polyphenol complex than the control. According to the spectrophotometric scans, Figure 6.4, these complexes could be iron-polyphenol. The peak in the tartaric acid solution is evident and there is a plateau in the citric acid solution scan at wavelengths that may indicate iron-polyphenol complex. The citric acid samples, however, are also likely producing other complexes which are causing the changed shape of the spectrophotometric scan. For the tartaric acid samples, there was a shift in the peak. This may indicate a change in the LMCT absorbance band caused by the formation of hybrid complexes. Furthermore, SHMP and disodium EDTA reduced iron-polyphenol complex formation by more
than 80% when compared to the control (Figure 6.3). Also, no prominent absorbance peak was observed from solutions containing either substance (Figure 6.4). Therefore, there was minimal, or conceivably no coloured iron-polyphenol complex formed in these solutions.

![Iron-Polyphenol Complex Formation in Tea Extract with Chelating Agents](image1.png)

(a) 6 replicates, (b) 3 replicates, (c) 5 replicates

Figure 6.3: Iron-Polyphenol Complex Formation in Tea Extract with Chelating Agents (pH 7)

![Spectrophotometric Scans of Tea Extract with Chelating Agents](image2.png)

Figure 6.4: Spectrophotometric Scans of Tea Extract with Chelating Agents (pH 7)

A pair of SHMP and the disodium EDTA solutions were tested over a period of two hours to ensure their stability. The results can be found in Table 6.1. For SHMP there may have been a
slight increase in complex formation over time. For disodium EDTA no notable increase was observed.

Table 6.1: Stability of Iron in Tea Extract with Chelating Agents (pH 7)

<table>
<thead>
<tr>
<th>Chelating Agent</th>
<th>Time (hours)</th>
<th>Complex Formation (mM FeCl$_3$/GAE)</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium hexametaphosphate (SHMP)</td>
<td>0</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.03</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.06</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>disodium EDTA</td>
<td>0</td>
<td>0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.03*</td>
<td>0.04</td>
<td>0.03*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.01*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*decrease from 1 hour to 2 hours is likely due to experimental error

The chemical structures of the chelating agents that were successful at reducing iron-polyphenol complex formation are shown in Figure 6.5. Tartaric acid and sodium citrate are bidentate and tridentate ligands respectively. Iron binds to their respective carboxylate groups when forming a chelated compound. The chelating agents that were more successful at preventing iron-polyphenol complex formation, SHMP and disodium EDTA, are hexadentate. In the case of SHMP, iron is able to bind to the six phosphate groups. In the case of disodium EDTA the four carboxylate groups as well as the two amine groups are able to bind to iron. These hexadentate ligands typically form more stable complexes than bidentate and tridentate ligands.

![tartaric acid](image)

![sodium citrate](image)

![sodium hexametaphosphate](image)

![disodium EDTA](image)

Figure 6.5: Chemical Structures of Chelating Agents [77]
SHMP, although useful in stabilizing iron, has not been shown to increase iron absorption. For example, Binata Nayak et al. (2003) compared SHMP to disodium EDTA and ascorbic acid in Indian bread [52]. In this high phytate wheat-based food SHMP did not benefit iron absorption whereas other two substances did [52]. This is likely because SHMP is sensitive to acid catalyzed hydrolysis, which is likely to occur in the stomach [78]. Therefore, of the chelating compounds tested, disodium EDTA is the most likely to increase iron bioavailability.

6.3.3 Optimization of Disodium EDTA

The amount of disodium EDTA that is needed to prevent iron-polyphenol complex formation was investigated. Solutions of tea extract (0.1 gGAE/L) adjusted to pH 5 and pH 7 were tested in the presence of disodium EDTA at iron:EDTA molar ratios of 1:0, 1:1, and 1:2. Two iron sources were tested at a concentration of 0.3 mM: ferrous sulphate (FS) and ferrous fumarate (FF). All samples were prepared in triplicate. The results may be found in Table 6.2. From these results it is clear that ferrous sulphate and ferrous fumarate react almost equivalently in the presence of disodium EDTA. Also, at an iron:EDTA ratio of 1:1 there was very little complex formation, which may only be slightly improved at a ratio of 1:2.

<table>
<thead>
<tr>
<th>Iron:EDTA</th>
<th>pH</th>
<th>Iron Compound</th>
<th>Iron-Polyphenol Complex Formation (mM FeCl₃/GAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>5</td>
<td>FS</td>
<td>0.232 ± 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.198 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>FS</td>
<td>0.267 ± 0.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.214 ± 0.059</td>
</tr>
<tr>
<td>1:1</td>
<td>5</td>
<td>FS</td>
<td>0.007 ± 0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.005 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>FS</td>
<td>0.033 ± 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.014 ± 0.004</td>
</tr>
<tr>
<td>1:2</td>
<td>5</td>
<td>FS</td>
<td>(-0.0007)* ± 0.0004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.010 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>FS</td>
<td>0.009 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF</td>
<td>0.016 ± 0.004</td>
</tr>
</tbody>
</table>

*Negative value may be due to slight difference in pH between the samples and the blanks

However, further testing in brewed tea (~1 gGAE/L) using ferrous sulphate indicated that an iron:EDTA ratio of 1:2 was required. To avoid precipitation, samples were not allowed to rest for more than 10 minutes prior to spectrophotometric analysis. Figure 6.6 shows the maximum net
absorbances of the three different ratios of iron to EDTA. The amount of complex was not calculated because pH was not controlled. Figure 6.7 may be referenced for a visual comparison of plain tea and iron fortified tea at three different iron:EDTA ratios. In this example the tea was fortified using ferrous fumarate but similar results were seen using ferrous sulphate. The blank tea solution appeared nearly identical to the fortified tea with a 1:2 iron:EDTA molar ratio.

(a) 3 replicates, (b) 4 replicates

Figure 6.6: Absorbance of Brewed Tea Containing Ferrous Sulphate and Disodium EDTA

Figure 6.7: Tea Fortified Using Different Molar Ratios of Iron to Disodium EDTA
6.4 Summary of Findings

The efficacy of using chelated iron sources and/or chelating agents to compete with tea polyphenols and prevent the iron-polyphenol complex formation was evaluated. Of chelated iron sources, both ferric trisglycinate and ferric sodium EDTA caused very little iron-polyphenol complex formation. The use of chelating agents was found to be an effective alternative to using chelated iron sources. Sodium citrate and tartaric acid prevented approximately 60% of iron-polyphenol complex formation in tea extract at pH 7. However, SHMP and disodium EDTA prevented complex formation by more than 80% due to their higher denticities. Because SHMP is prone to acid hydrolysis it does not aid iron absorption. Therefore, disodium EDTA is the best candidate for iron-fortified tea. In tea extract, at a concentration of 0.1 gGAE/L, the 1:1 molar ratio of iron:EDTA appeared to prevent iron-polyphenol complex formation to a similar degree as the 1:2 ratio. However, it was apparent in brewed tea that a 1:2 molar ratio was required to keep the visual appearance similar.

To summarize, Chapter 6 validates that iron chelation is an effective strategy for inhibiting iron-polyphenol complex formation. Disodium EDTA can prevent iron-polyphenol complex formation at both pH 5 and pH 7. Also, tea brewed with the addition of disodium EDTA at a 1:2 iron:EDTA molar ratio retains its original colour. In the following chapter a more thorough investigation of disodium EDTA in the context of iron fortified tea is presented.
7 Iron Fortified Tea Using Disodium EDTA

7.1 Research Objective

Disodium EDTA is an approved food additive [79]. In Chapter 6 disodium EDTA is shown to be a reasonable candidate for preventing iron-polyphenol complex formation in iron fortified tea. It was tested in tea extract at one tenth the concentration of tea with pH adjustment to 5 and 7 as well as in brewed tea with no pH adjustment. It was shown to prevent the reaction even at quite low concentrations (0.6 mM). At the 1:2 iron:EDTA molar ratio that was required in brewed tea, its addition level is well within U.S. FDA regulations [79] (see Appendix 10.5).

Further investigation was required to determine if the use of disodium EDTA is a viable strategy for the prevention of iron-polyphenol complex formation in iron fortified tea. This is partly because iron-polyphenol complex had not been quantified in freshly brewed tea at pH levels important for tea appearance and iron absorption. Also, during digestion there is a sequence of pH changes. Accordingly, iron fortified tea with added disodium EDTA was tested after sequential pH adjustment to mimic the gastrointestinal system. Therefore, the objective of the research presented in this chapter was to investigate the effectiveness of using disodium EDTA as an iron stabilizer in iron fortified black tea. The potential complications resulting from the consumption of other foods with black tea are outside the scope of this project. However, under my direction, two visiting students conducted preliminary experiments to examine the effect of milk and of added trace minerals on tea colour and bioavailability when fortified with iron (Appendix 10.6 and Appendix 10.7).

7.2 Experimental Strategy

It was found that in freshly brewed tea a 1:2 iron:EDTA molar ratio is required to inhibit iron-polyphenol complex formation when 0.3 mM of iron is present (Section 6.3.3.). However, the pH of the brewed tea was not controlled and changed from 4.98 ± 0.02 to 4.47 ± 0.05 in the presence of ferrous sulphate and disodium EDTA. Using a different iron source, such as ferrous fumarate, may limit this effect. For the purpose of experimental design, pH should be controlled. Also, the pH of the small intestine is important for iron absorption. Because of this, iron-polyphenol complex formation was observed in brewed tea fortified using a 1:2 molar ratio of iron:EDTA followed by pH adjustment to pH 5 and pH 7. To gain further insight, pH adjustments were also
done at intervals of 0.2 from pH 5 to pH 7. In addition, the effect of increased disodium EDTA was investigated at pH 7.

Another important factor for the digestion of tea is the variation of pH in the gastrointestinal tract. Brewed tea has a pH of 5. Once it is consumed and enters the stomach, the pH is lowered to 1. It then enters the small intestine where the pH is raised to approximately 7. After sequential pH adjustment of brewed tea with added ferrous sulphate, to mimic digestion, measurements of soluble iron and soluble iron-polyphenol complex were taken. The sequential pH adjustment consisted of an adjustment to pH 5 for 5 minutes, acidification to pH 1 for 2 hours, and a final adjustment to pH 7. This was done both in the presence and absence of disodium EDTA. Hydrochloric acid and sodium hydroxide were used to lower and raise the pH level. To separate the iron that remained in solution from precipitate, solutions were filtered using a 0.5 μm syringe filter. These experiments yielded a deeper understanding of the potential amount of bioavailable iron in fortified tea.

7.3 Results and Discussion
7.3.1 Iron-Polyphenol Complex Formation with Na₂EDTA
Two formulations of iron fortified tea were prepared. The first contained ferrous sulphate with disodium EDTA and the second contained ferric sodium EDTA with added disodium EDTA. These formulations were prepared using freshly brewed tea to contain a 1:2 iron:EDTA molar ratio (0.3 mM iron) and were adjusted to pH 5 or pH 7 (Figure 7.1). Three replicates were prepared of each formulation at each pH level. There was very little complex formation at pH 5 but quite significant complex formation at pH 7. This is in contrast to the findings in tea extract with approximately one tenth the polyphenol concentration of tea (Section 6.3.3). Therefore, it seems that the larger polyphenol concentration affected iron-polyphenol complex formation.
The pH of the small intestine reaches 7-8.5. However, iron absorption occurs in the duodenum, the first compartment of the small intestine. Here, the pH only approaches 6 [80]. In order to understand complex formation in iron fortified tea (with a 1:2 molar ratio of iron to EDTA) solutions were prepared at intervals of 0.2 from pH 5 to pH 7 (see Figure 7.2). These solutions were prepared with 0.3 mM ferrous sulphate. From pH 5 to pH 6, inclusive, no net absorbance was observed, thus no complex was formed. This was then quantified for pH 5, pH 6, and pH 7 (see Figure 7.3). From these results, fortified tea using a 1:2 molar ratio of iron to EDTA is expected to be bioavailable.
Additional disodium EDTA was added with the intent of decreasing the amount of iron-polyphenol complex formation to nearly zero at pH 7, see Figure 7.3. However, even at very high concentrations, such as 5% w/v, complex formation still occurred, although, at reduced levels. More precisely, this was 5 g of Na₂EDTA per 100 g of boiled water. Therefore, increased addition of disodium EDTA will further decrease iron-polyphenol complex formation at pH 7 but will not suppress it completely in brewed tea.
7.3.2 Sequential pH Adjustment and Available Iron

Sequential pH adjustment from pH 5 to pH 1 to pH 7 was used to mimic the gastrointestinal system. It was observed that when the pH was adjusted from pH 5 to pH 1 to pH 7, black tea polyphenols largely precipitated from the solution due to polymerizing at pH 1. They did not dissolve into solution once the pH was brought up to 7. When the pH of brewed tea containing iron was adjusted similarly, much of the iron was incorporated into the precipitate. Samples of brewed tea containing ferrous sulphate without added disodium EDTA and with added disodium EDTA (1:2 iron:EDTA molar ratio) were prepared in triplicate. Figure 7.4 shows the percent retention of soluble iron, as total soluble iron and soluble iron-polyphenol complex, after this sequential pH adjustment. In the case of tea with only ferrous sulphate, 33% ± 6% of the iron remained soluble and in a form other than iron-polyphenol complex, thus, is expected to be bioavailable. In fortified tea, with added disodium EDTA, 81% ± 18% remained soluble and unattached to polyphenol. Therefore, the fraction of iron expected to be bioavailable is higher in iron fortified tea containing a 1:2 molar ratio of iron to EDTA.

The binding of iron is very sensitive around pH 7 for tea containing disodium EDTA. This can be seen from the large standard deviation (shown as the error bar in Figure 7.4) of total soluble iron. It was also indicated by a drop from 100% retention of total soluble iron to 67% from pH 6.96 to 7.08 (Appendix 10.8). However, since the duodenum has a gradient of pH values only reaching approximately pH 6, it is expected that the bioavailable iron fraction will be even higher than the values indicated here (i.e. close to 100%). These results are supported by the observations that the iron-polyphenol complex does not form at pH 6 in brewed tea, as previously stated.
Summary of Findings

The effectiveness of disodium EDTA in preventing iron-polyphenol complex formation in iron fortified black tea has been evaluated. The formulation of iron fortified tea containing 0.3 mM iron and 0.6 mM disodium EDTA, brewed using 1% \( \frac{\text{wtea-leaves}}{\text{water}} \), was investigated. At pH 5 this formulation successfully suppressed iron-polyphenol complex formation. However, at pH 7, complex formation occurred to a high degree (~0.20-0.25 mM FeCl\(_3\)/GAE). Despite this, at pH 6, the highest pH of the duodenum, no complex formation occurred. Since the duodenum is the site of iron absorption into the body, the iron is likely to be bioavailable. Furthermore, this formulation was sequentially adjusted through expected pH levels of digestion. In comparison to tea that did not contain added EDTA, 47% ± 19% more of the total iron added remained in solution without being bound by polyphenols. These results were sensitive to pH in the vicinity of pH 7. Because the duodenum only approaches pH 6, it is expected that 100% of the iron will remain in solution in the duodenum and not be bound to tea polyphenols. Therefore, a technique to prevent iron-polyphenol complex formation in iron fortified tea so as to prevent colour formation and preserve iron bioavailability has been developed.
8 Conclusions

8.1 Summary of Thesis Conclusions

This research is an integral contribution to the development of iron fortified tea. The development of intensely coloured non-bioavailable iron-polyphenol complexes in tea is a barrier to consumer acceptance and effectiveness of iron fortified tea. The main objective of this research was to understand the formation of these complexes, and to develop strategies for their prevention. A novel technique has been successfully developed to prevent iron-polyphenol complex formation in iron fortified tea.

The results of this research, i.e. novel contributions, are summarized as follows:

1. **The addition of disodium EDTA is an effective strategy for overcoming the barriers posed by iron-polyphenol complex formation in black tea.**
   1.1. The optimized technique involves the addition of disodium EDTA at a molar ratio of 1:2 iron:EDTA.
   1.2. Disodium EDTA prevents iron-polyphenol complex formation at the pH of the brewed beverage and duodenum.
   1.3. Disodium EDTA competes with polyphenols to form a non-coloured bioavailable iron chelate complex.

2. **Iron chelation is an effective strategy for preventing iron-polyphenol complex formation.**
   2.1. Chelated iron sources are successful at limiting the formation of iron-polyphenol complexes.
   2.2. Chelating agents may be added with simple iron salts and decrease or prevent iron-polyphenol complex formation.

3. **Reducing agents are capable of slowing/decreasing iron-polyphenol complex formation at pH 5.**
   3.1. Sodium ascorbate was the most effective of the reducing agents tested.
3.2. Reducing agents alone do not readily prevent iron-polyphenol complex formation at pH 7 which may impact the bioavailability of iron if used as a sole strategy in iron fortified tea.

4. A novel analytical method has been developed for the quantification of iron-polyphenol complexes at pH levels relevant to brewed tea and iron absorption.
   4.1. A direct comparison may be made between “total phenolic content” (as measured by the Folin and Ciocalteu method) and iron-polyphenol complex concentration.
   4.2. Only one wavelength is required for the measurement of iron-polyphenol complex formation in gallic acid or black tea (pH 5-7).

5. Thermodynamic models that predict the extent of iron-polyphenol complex formation at equilibrium with black tea polyphenols were developed.
   5.1. The amount of iron-polyphenol complex formation is dependent on “total phenolic content” (GA₀), total iron concentration (Fe₀), and pH (represented as factor “x”).
   5.2. The equilibrium constants derived from these models support expectations from literature that the reaction moves to completion.

6. A kinetics model has been developed for ferrous iron in buffered gallic acid solution.
   6.1. A first order kinetics model fits experimental data very well.
   6.2. This model supports the hypothesis of a first order rate limiting reaction step. It is expected that this step is the first binding of iron to the polyphenol or oxidation.

8.2 Recommendations

This work has a concentrated focus on a particular application, i.e. on the development of iron fortified tea. Therefore, my recommendations are organized into two sections. The first is focused on suggestions solely for the advancement of scientific knowledge pertaining to iron-polyphenol food chemistry. The second is more focused on the application and how I suggest to further the economic development of iron fortified tea.
8.2.1 Iron-Polyphenol Food Chemistry

1. Interaction of buffer solutions with iron-polyphenol complex formation
It has been confirmed that buffer solutions can interfere with iron-polyphenol complex formation. A more comprehensive study of the effect of different buffer solutions on iron-polyphenol complex formation would be advantageous. It would be useful for kinetics studies where buffer solutions are required to counter the pH shift. Additionally, it would be interesting to determine if all polyphenols and iron sources are affected equally by the interference of buffering agents.

2. Thermodynamics of iron-polyphenol complex formation
Equilibrium models were developed at pH 5 and pH 7 that predict the amount of iron-polyphenol complex formation with tea polyphenols. The pH 5 model contains a pH dependent factor “x” which was presented based on the assumption that a certain fraction of iron becomes inactive. However, this hypothesis seems unsatisfactory and should be revisited. Experiments should be repeated in three ways: as they were done previously, with a few different iron sources, and with changes in iron concentration. If factor “x” is persistent then its dependence on pH should be investigated. Furthermore, the models should be expanded to take into account iron-polyphenol complex inhibitors such as chelating agents (e.g. EDTA) and reducing agents (e.g. ascorbic acid). Also, a variety of polyphenols should be tested. This will lead to a deeper understanding of the extent of iron-polyphenol complex formation and allow for prediction in high polyphenol containing foods.

3. Kinetics of iron-polyphenol complex formation
A first order kinetics model was able to describe iron-polyphenol complex formation of ferrous sulphate in a buffered gallic acid solution at pH 5. However, in other chapters it was shown that ferrous sulphate reacted quickly at pH 5. Therefore, the potential interference of the buffer solution on the rate of the reaction should be investigated. In order to build more robust kinetic models the effects of pH, concentration, and temperature should be investigated. Also, the kinetics of other simple monomeric polyphenols as well as complex systems such as tea should be tested and compared. Furthermore, these models may be expanded to include other factors, such as reducing agents, on the speed of the reaction. These recommendations would lead to a more complete understanding of the kinetics of iron-polyphenol complex formation which may be utilized in food applications.
8.2.2 Development of Iron Fortified Tea

The development of iron fortified tea will require investigation of alternative formulations, confirmation of bioavailability, and commercial scale implementation of the process developed here.

1. **Investigation of Alternative Formulations**

Ascorbate has been seen in literature to greatly increase iron absorption in highly chelating foods such as those with phytate. From this study it was shown to greatly reduce iron-polyphenol complex formation at pH 5, inhibiting colour formation. However, at pH 7, with the analytical techniques available to me, iron-polyphenol complexes were not distinguishable from ferrous ascorbate. Further research is required to determine the amount of sodium ascorbate that would be necessary to improve bioavailability. If the added cost is low, this approach has advantages over using disodium EDTA alone. Ascorbate is a vitamin, so it would add additional health benefits, and is listed by the FDA as generally recognized as safe (GRAS). Furthermore, investigation into potential synergistic effects of ascorbate and EDTA in preventing iron-polyphenol complex formation may lower both cost, ease of approval, and acceptance by consumers.

2. **Confirmation of Bioavailability**

For a more thorough understanding of the bioavailability of iron in fortified tea, Caco-2 cell permeability testing and in vivo testing should be conducted. Some of this work is proceeding, and results from Caco-2 cells and basic rat studies are positive. Further investigation into the effect of different black tea varieties, mineral content of brewing water, and common additives such as milk, is required. Preliminary studies conducted under my directions by visiting students on both milk and mineral addition may be found in Appendix 10.6 and Appendix 10.7.

3. **Implementation**

Currently, a method has been developed by my colleagues for adhering ferric sodium EDTA and disodium EDTA to tea leaves using a pan coating technique making use of hydroxypropyl methylcellulose (HPMC) as the adhering agent. These tea leaves serve as a premix that may be dry mixed with plain tea leaves to reach the target level of iron. The adhered iron and disodium EDTA detaches and dissolves in the tea beverage when water is added. A more detailed outline
of this production method can be found in Appendix 10.9. This process should be expanded to other iron sources and pilot tested.

Food fortification is only effective if the affected population accepts the fortified food. Initial tests in India and North America showed that the tea of iron fortified tea prepared by this technique is acceptable. However, many Indian consumers prepare tea in milk and in this form the colour of the beverage was not acceptable to the average rural consumer. Therefore, further research is required to improve consumer acceptability for implementation in these areas.

The further development of iron fortified tea is expected to generate an inexpensive but efficacious iron delivery system. Implementation of this fortified beverage is expected to markedly reduce the grave disparity caused by iron deficiency in the vast portions of the developing world where black tea is consumed ubiquitously. Furthermore, the technique developed for the prevention of iron-polyphenol complex formation is expected to be easily adaptable to other high polyphenol beverages. These include green tea and coffee which are often regularly consumed in areas where black tea is less common. Therefore, the results of this thesis have the potential to greatly reduce suffering and deaths caused by iron deficiency.
9 References


[56] H. Kelebek, "LC-DAD–ESI-MS/MS characterization of phenolic constituents in Turkish black tea: effect of infusion time and temperature," *Food Chemistry*, vol. 204, p. 227–238, 2016.


10 Appendices

Appendix 10.1: Tea Parameter Measurement

10.1.1 Polyphenol Calibration

Figure 10.1 shows a sample calibration curve used for the determination of the polyphenol content of tea.

![Sample Polyphenol Calibration Curve](image)

**Figure 10.1: Sample Polyphenol Calibration Curve**

10.1.2 Polyphenol Content of Dry Tea Leaves

Extraction procedures were performed similarly to the procedure developed by Turkmen et al. (2006) using a 50% N,N-dimethylformamide (DMF) aqueous solution as extraction solvent [70]. Briefly, polyphenols were extracted from ground tea leaves (1 g) with 15 mL of solvent for 1 hour on a horizontal shaker. The mixture was centrifuged at 2000 rpm for 10 min. The extract was collected and 15 mL of solvent was added to the centrifuge tube to extract again. This procedure was repeated 4 more times with the same ground tea leaves but with shaking durations of 2 hours for the following two extractions and 3 hours for the last two extractions. The five supernatants were collected in a 100 mL volumetric flask and completed to the correct volume with additional extraction solvent. Eight samples were made and these were then analyzed for polyphenol content, see Table 10.1.
Table 10.1: Dry Tea Leaves Polyphenol Extraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tea Weight (g)</th>
<th>Amount of Extract (mL)</th>
<th>mgGAE/g leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0127</td>
<td>100</td>
<td>222.12</td>
</tr>
<tr>
<td>2</td>
<td>1.0225</td>
<td>100</td>
<td>210.67</td>
</tr>
<tr>
<td>3</td>
<td>1.0010</td>
<td>100</td>
<td>212.04</td>
</tr>
<tr>
<td>4</td>
<td>1.0247</td>
<td>100</td>
<td>216.78</td>
</tr>
<tr>
<td>5</td>
<td>1.0013</td>
<td>100</td>
<td>212.59</td>
</tr>
<tr>
<td>6</td>
<td>1.0201</td>
<td>100</td>
<td>213.29</td>
</tr>
<tr>
<td>7</td>
<td>1.1002</td>
<td>103.8</td>
<td>198.96</td>
</tr>
<tr>
<td>8</td>
<td>1.0164</td>
<td>100</td>
<td>210.87</td>
</tr>
</tbody>
</table>

The amount of polyphenols in the tea leaves was found to be 212 ± 7 mgGAE/g tea leaves which is equivalent to 21.2 ± 0.7% w/w. This is reasonable as the major two types of polyphenols found in black tea (theaflavins and thearubigins) make up 10% to 22% of the dry weight of black tea [20]. Also, when tea was brewed for 5 minutes with 1% \text{w/} \text{water}, a 50% extraction of the polyphenols occurred, resulting in a solution of 1 gGAE/L of polyphenols.

10.1.3 Effect of Gallic Acid Concentration on Iron Complex Formation

Solutions of gallic acid (0.5 to 4 g/L) and ferric chloride (0.3 mM), adjusted to pH 7 using sodium hydroxide, were scanned using spectrophotometry. The results can be seen below in Figure 10.2. There is no substantial change in the maximum absorbance of the solutions indicating that the amount of iron-polyphenol complex formation at all concentrations remains constant. Therefore, results taken at 1 gGAE/L will be representative of all practical tea polyphenol concentrations.

![Figure 10.2: Effect of Gallic Acid Concentration on Iron Complex Formation](image-url)
10.1.4 Maximum Polyphenol Concentration in Brewed Tea

Finding the upper limit of black tea polyphenols extractable by water was done to find the highest concentration of polyphenols that could be present in a tea beverage. Tea leaves were added to boiling reverse osmosis purified (RO) water such that no more could be added due to their swelled volume. It was allowed to steep for 10 minutes and the extract was analyzed for polyphenol content. It should be noted that, at temperatures slightly lower than boiling, the polyphenolic compounds precipitated out of solution. This was done with four replicates. The resulting solution contained $18.2 \pm 0.3 \text{ gGAE/L}$, which is approximately $2\% \text{ w/v}$. This is much larger than would be found in practice as only $\sim 1\% \text{ w/v}$ tea leaves are generally used to brew tea.
Appendix 10.2: pH Adjustment

10.2.1 Ferrous Sulphate in Gallic Acid Spectrophotometric Scans

Ferrous sulphate (0.1-0.5 mM) was allowed to react with gallic acid (1 g/L) in buffered solutions (0.1 M), see Figure 10.3. The compositions of the buffered solutions are outlined in Section 4.2.2. From pH 7 to pH 5 the peak absorbance increases as expected from literature. At pH 3 and lower no complex formation occurred.

Figure 10.3: Spectrophotometric Scans of Ferrous Sulphate in Gallic Acid (pH 1 to pH 7)

10.2.2 Solution Preparation for Testing Buffer Solutions

Phenolic Compound Solution Preparation

Gallic acid was dissolved in RO water at a concentration of 10 g/L. While dissolving, it was stirred vigorously at a slightly elevated temperature (approximately 35°C). Tea extract was diluted to 1 gGAE/L using RO water.

Solutions for pH Adjustment

**Sodium hydroxide:** Pellets were added to RO water then diluted to a concentration of 0.8 M.

**Buffer solution (pH 5):** 0.3 M potassium hydrogen phthalate was made in RO water and adjusted to pH 5 with sodium hydroxide.
Buffer solution (pH 7): 0.3 M monopotassium phosphate was made in RO water and adjusted to pH 7 with sodium hydroxide.

Ferrous Sulphate Solution
Ferrous sulphate heptahydrate was dissolved in RO water to make a 0.0125 M solution.

Buffered Sample Preparation
In a 50 mL volumetric flask, 5 mL of the polyphenol solution (gallic acid or tea extract) and 20 mL of buffer solution was added. To that 1.2 mL of the ferrous sulphate solution was added into the mixture. The pH of these solutions was measured to ensure they were at the levels needed. These solutions had a final concentration of 1 g/L gallic acid or 0.1 gGAE/L for tea extract and 0.3 mM iron.

Unbuffered Sample Preparation
The pH of the polyphenol solutions were adjusted using sodium hydroxide (NaOH), ferrous sulphate was added, and the pH was readjusted and monitored every hour. The pH 5 tea extract solution had large fluctuations in pH that were adjusted every hour. Generally, the pH of the solutions was kept between 5.0-5.2 or 7.0-7.2. NaOH was mostly used for pH adjustment however small amounts of HCl were also used to maintain the desired pH level. The final volume was the same as in the buffered samples.
Appendix 10.3: Comparison of Ferric Sodium EDTA Sources

Three sources of ferric sodium EDTA were compared. The first iron source was from Fisher Scientific, the second from Sigma-Aldrich, and the third from Bio Basic. The source from Bio Basic was called into question when the solubility was not as per the specification. It was observably different in colour than the other two sources as well. Absorbance scans were done of these three sources (at 0.3 mM) in 0.1 mM GAE tea extract (pH 7) to compare complex formation. Samples were produced in triplicate. Results can be seen in Figure 10.4. There is a large deviation between the amount of iron-polyphenol complex formed by the Bio Basic ferric sodium EDTA and the others. The Bio Basic source formed 10 times the amount of complex as the other two sources.

The Bio Basic source of ferric sodium EDTA was only used in preliminary studies on interactions of milk and additional minerals on iron fortified tea (Appendix 10.6 and Appendix 10.7 respectively). The main body of experimentation was not done using the Bio Basic ferric sodium EDTA source.
Appendix 10.4: Additional Flow-Cell Calculations and Data

10.4.1 Calibration Curves

Figure 10.5 displays the calibration curves used for the flow-cell experiments. To produce these curves ferrous sulphate was added to a 1 g/L gallic acid solution. This concentration of gallic acid was selected because it is an approximation of the phenolic compounds in tea. Also it is much higher than the concentration of iron added and thus will push the reaction to completion.

Figure 10.5: Calibration Curve Used in Flow-Cell Experiments

\[
\begin{align*}
\text{linear (pH 5 (550 nm))} \\
y &= 3.8475x + 0.018 \\
R^2 &= 0.9979
\end{align*}
\]

\[
\begin{align*}
\text{linear (pH 7 (565 nm))} \\
y &= 3.8623x + 0.0297 \\
R^2 &= 0.999
\end{align*}
\]

10.4.2 Adding Tea Extract and Gallic Acid to Iron Solutions

For the initial trials, solutions of ferrous sulphate were first made at pH 5 or pH 7 (adjusted using sodium hydroxide) and heated to the desired temperature. All solutions, except that at pH 5 and 24°C (room temperature), turned yellow/orange. Then, increasing amounts of tea polyphenol extract was added and the amount of complex formation was measured. In all solutions that turned yellow/orange very little complex formation was observed. This is likely due to the formation of ferric hydroxide which does not react to the same extent as ferrous sulphate under these conditions, likely due to its low solubility. Figure 10.6 shows iron-polyphenol complex formation versus polyphenol concentration for pH 5 solutions at 24°C, an average of the other pH 5 solutions (at 35°C, 50°C, 65°C), and an average of all of the pH 7 solutions (24°C, 35°C, 50°C, 65°C).
The experiment was repeated with gallic acid instead of tea. The results were similar confirming that this lack of binding is not due to the unique polyphenol structure of tea but to the form of the iron itself. Figure 10.7 shows the results of this experiment with both pH 5 and pH 7 solutions at 24°C. Of the possible forms that the iron may have become, ferric hydroxide (or mixture of ferric hydroxide and ferric oxide) is the most probable due to the yellow/orange fine precipitate formation. Tannins, polyphenolic compounds, are a common ingredient in rust converter, thus chelating and dissolving iron-oxide. However these products likely have much higher concentrations of polyphenols. Furthermore, because ferric hydroxide is not highly bioavailable its lack of reactivity in this context is irrelevant to the tea project.
The temperature at the flow cell was estimated by making a few simplifying assumptions. These include that the flow cell was midway along a tube, of even wall thickness and diameter, containing the solution in turbulent flow, with no heat gain (due to friction in the peristaltic pump for example), and that the tube was in direct contact with well mixed room temperature air ($T_\infty$). The temperatures of the solution moving into the tube and coming out of the tube were measured. Then the value of temperature (T) at any distance along the tube (x) may be solved as follows:

$$- \frac{dT}{dx} = k(T - T_\infty)$$

Eq. 10.1

$$\int_{T_{in}}^{T} \frac{dT}{T - T_\infty} = \int_{0}^{x} -k \, dx$$

Eq. 10.2

$$\ln \frac{T - T_\infty}{T_{in} - T_\infty} = -kx$$

Eq. 10.3
First the known out temperature was used to solve for “k” when \( x = L \) (the full length of the tube), then \( T \) was solved for when \( x = 0.5L \). Room temperature was measured to be \( 24.8 \pm 0.8 \). Table 10.2 outlines these calculations and results.

**Table 10.2: Estimated Temperatures at the Flow Cell**

<table>
<thead>
<tr>
<th>( \text{Average } T_{in} )</th>
<th>( \text{Standard Deviation} )</th>
<th>( \text{Average } T_{out} )</th>
<th>( \text{Standard Deviation} )</th>
<th>( \frac{T_{out} - T_{\infty}}{T_{in} - T_{\infty}} )</th>
<th>( k ) at ( x = L )</th>
<th>( T ) (˚C) at ( x = 0.5L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.4</td>
<td>0.2</td>
<td>30.2</td>
<td>0.5</td>
<td>0.973</td>
<td>0.0272/L</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>40.5</td>
<td>0.3</td>
<td>39.5</td>
<td>0.4</td>
<td>0.936</td>
<td>0.0666/L</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>50.5</td>
<td>0.5</td>
<td>48.2</td>
<td>0.4</td>
<td>0.911</td>
<td>0.0928/L</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>60.4</td>
<td>0.3</td>
<td>56.9</td>
<td>0.5</td>
<td>0.902</td>
<td>0.1037/L</td>
<td>59 ± 1</td>
</tr>
</tbody>
</table>

*Room temperature was measured to be \( 24.8 \pm 0.8 \)*

For simplicity, within this document the temperatures at the flow cell were indicated as 30˚C, 40˚C, 50˚C and 60˚C.
Appendix 10.5: Use of EDTA in Food

The formulation determined to be effective for the prevention of iron-polyphenol complex formation in brewed tea (with 0.3 mM (~16.75 mg/L) iron) contained 0.6 mM EDTA (a 1:2 molar ratio of iron:EDTA). This may be achieved using disodium EDTA (dihydrate) alone (~0.223 g/L) or in combination with ferric sodium EDTA (0.112 g/L Na$_2$EDTA·2H$_2$O, 0.110 g/L FeNaEDTA). Neither disodium EDTA nor ferric sodium EDTA is generally recognized as safe (GRAS) by the FDA. Disodium EDTA is classified as an additive permitted for direct addition to food for human consumption and is primarily used as a preservative or to promote colour retention. Both of these purposes are related to its ability to sequester iron [81]. Table 10.3 shows the foods it is permitted to be added to as well as the amount permitted. Also, for comparison, the amount is present in brewed iron fortified tea. Ferric sodium EDTA is only currently approved by the FDA as an indirect food additive, specifically for use in adhesives [81]. However, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated it as safe for use in supervised food fortification programs [82].

Table 10.3: Disodium EDTA Additive Permissions from the FDA (as of July 2016) [81]

<table>
<thead>
<tr>
<th>Food</th>
<th>Limitation (ppm Na$_2$EDTA·2H$_2$O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brewed Fortified Tea (under development)*</td>
<td>223</td>
</tr>
<tr>
<td>Aqueous multivitamin preparations</td>
<td>149</td>
</tr>
<tr>
<td>Canned black-eyed peas</td>
<td>144</td>
</tr>
<tr>
<td>Canned kidney beans</td>
<td>164</td>
</tr>
<tr>
<td>Canned strawberry pie filling</td>
<td>497</td>
</tr>
<tr>
<td>Cooked sausage</td>
<td>36 (used as a cure accelerator)</td>
</tr>
<tr>
<td>Dressings</td>
<td>75</td>
</tr>
<tr>
<td>French dressing</td>
<td>75</td>
</tr>
<tr>
<td>Frozen white potatoes including cut potatoes</td>
<td>99</td>
</tr>
<tr>
<td>Gefilte fish balls or patties in packing medium</td>
<td>50 (in total weight including packing)</td>
</tr>
<tr>
<td>Legumes (all cooked canned, other than black-eyed peas)</td>
<td>164</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>75</td>
</tr>
<tr>
<td>Ready-to-eat cereal products containing dried bananas</td>
<td>313 (in dried banana component)</td>
</tr>
<tr>
<td>Salad dressing</td>
<td>75</td>
</tr>
<tr>
<td>Sandwich spread</td>
<td>99</td>
</tr>
<tr>
<td>Sauces</td>
<td>75</td>
</tr>
</tbody>
</table>

*Not currently approved by the FDA
The acceptable daily intake (ADI) of EDTA on a disodium EDTA basis as prescribed by the JECFA is currently 2.5 mg/kg body weight per day (determined in 1973) [83]. If one considers the average adult body weight to be 62 kg this is 154.1 mg/day on a Na₂EDTA·2H₂O basis (or 155 mg/day on a CaNa₂EDTA basis). It is expected that people drink two cups of tea per day and would then receive 105.5 mg/day of EDTA (Na₂EDTA·2H₂O basis) which is under the ADI. There has also been impetus to update the ADI to a higher level especially for iron fortification purposes. In an article by Carel Wreesmann, he argues for an increase in the ADI to allow for the fortification of cereals (high phytate foods) with iron [79]. His argument hinges on more recent animal feed studies and use of EDTA in medicinal formulas having no ill effects [79].
Appendix 10.6: Effect of Milk on Iron Fortified Tea

One issue with iron in milk is that it can cause off-flavours and odours due to lipid peroxidation [84]. In developing a method to fortify milk with iron it was found that the use of iron(III) chelates reduced these effects, with ferric EDTA being the most effective [84]. Most attempts at fortifying milk with iron have used microencapsulation, either of the iron source alone or with acetic acid (an antioxidant) [85-88]. Because the iron in iron fortified tea is stabilized with a strong chelating agent (disodium EDTA) and tea polyphenols are antioxidants, lipid peroxidation is unlikely.

A second issue with milk is that it has an excess of iron absorption inhibiting substances [89]. One of which is calcium. It is thought that the mechanism of inhibition has to do with both iron and calcium competing for the same transporter in mucosal cells of the duodenum [90]. However, calcium consumed in black tea may also not be absorbed well due to binding to oxalate in the tea [91]. Therefore, it is less likely to compete with iron for the transporter. The main concern for calcium in iron fortified tea is whether it competes with iron to bind to EDTA and thus allows more iron to bind to polyphenols in the tea rendering them non-bioavailable. Further study into the effect of calcium on iron metabolism in tea is outside of the scope of this research as it concerns the biology of intestinal mucosal cells and not the chemical composition of the beverage. Milk proteins (casein and whey) were found to decrease iron bioavailability both in their enzyme-hydrolyzed or acid-hydrolyzed forms but also (to a lesser extent) as intact milk protein [92]. This is likely due to milk proteins chelating the iron.

A preliminary study was conducted under my direction by Mirium Chiarmasso, a visiting student from École des Mines de Saint-Étienne, France, regarding milk proteins, calcium, and iron in tea. Membrane separation was used to separate iron complexes formed by tea, milk constituents (calcium and proteins), and milk. This was attempted with ferrous sulphate, ferric sodium EDTA, and ferric sodium EDTA with added disodium EDTA (1:1 molar ratio).
10.6.1 Milk Testing Methods

Iron-Polyphenol Complex Content
The iron-phenol complex was measured using spectrophotometry with a Cary50 UV-Vis spectrophotometer. Absorbance data was calibrated using a gallic acid ferrous sulphate curve.

Iron Content
The iron content of solutions was measured using atomic absorption spectroscopy (AAS) using the AAAnalyst 100 AAS system. Calibration curves were made using samples of 1 ppm, 5 ppm, 10 ppm, and 20 ppm of iron standard for AAS. For samples containing ferric sodium EDTA, a calibration curve was made using ferric sodium EDTA.

Polyphenol Content
The method used for polyphenol measurement was based on the method for phenol measurement developed by Singleton and Rossi in 1965 [57]. In tea, the phenolic compounds are polymerized polyphenols. In short, a calibration curve was made using gallic acid diluted to 0.001-0.013 g/L. The gallic acid solutions were treated with Folin-Ciocalteu reagent, mixed and allowed to react for 5 minutes. Sodium bicarbonate was then added and the solutions were shaken. This was followed by the addition of 2 mL of water to dilute the gallic acid to the proper concentration. The solutions were allowed to sit for 30 minutes and their absorbance was measured at 765 nm along with a blank that did not contain gallic acid. Tea was measured similarly and the amount of phenol in the tea was reported as gallic acid equivalents (GAE).

Casein and Calcium Addition
The amount of calcium and casein protein in milk was taken into consideration when determining the amount to add to solutions. The quantity of calcium in milk is 1.1 g/L and the quantity of casein is approximately 25.6 g/L [93]. However, for the purposes of these experiments, 1.2 g/L of calcium was used and 16.2 g/L of casein was used. Calcium carbonate was the calcium source chosen for these experiments. Skim milk was used instead of milk containing fat to lower the complexity of the system. Also, milk was added such that the final solutions contained were 1/3rd milk by volume.
Membrane Separation

A 5 kDa membrane made of polyethersulphone (PES) can separate compounds containing polyphenols from tea with a rejection percentage of 93.7% [94]. Given the large molecular weight cut-off, the mode of separation is not size exclusion. Tea polyphenols, ferrous sulphate, ferric sodium EDTA, milk components, and skim milk were investigated to determine if useful separations could be made. Samples of permeate and retentate solutions were tested for iron and iron-polyphenol complex amount.

When working with membranes, mathematical models are used to analyze membrane efficiency. Firstly, concentration factor $C_f$ expresses how much liquid has passed through the membrane at a given time.

$$C_f = \frac{V_o}{V_r}$$

Where $V_o$ represents the initial feed volume and $V_r$ represents the retentate volume [95]. A specific value of concentration factor may be chosen so that other variables may be compared. An indicator for the efficiency of the separation is rejection (R), or the probability that a particle cannot pass through the membrane. If nothing passes through the membrane, then $R = 100\%$.

$$R = (1 - \frac{c_p}{c_r}) \times 100$$

Where $c_p$ is the solute concentration in the permeate and $c_r$ is the solute concentration in the retentate [95]. The solute concentration in the retentate can be more accurately measured through the concentration of the initial solution as follows:

$$(c_o)(v_o) = (c_p)(v_p) + (c_r)(v_r)$$

Where $c_o$ is the concentration of the original solution and $v_p$ is the volume of the permeate. This eliminates error due to loss on the membrane.
The concentration factor used in these experiments was 2 and the rejection of iron-polyphenol complexes as well as iron alone was measured.

10.6.2 Milk Results and Discussion

When tea was filtered alone, it resulted in a $91\% \pm 2\%$ rejection of polyphenols which is in line with the 93.7% seen in literature [94]. The results from all solutions tested with ferrous sulphate are presented in Table 10.4. Ferrous sulphate in water only had a $25\% \pm 3\%$ rejection. This is advantageous because the small rejection means an increase will likely be due to binding to tea polyphenols or milk constituents. The rejection of iron in the solution of ferrous sulphate in tea was equivalent to the rejection of iron-polyphenol complex. This was expected as almost all iron would be complexed by tea polyphenols. The rejections of iron and complex are significantly larger than that of ferrous sulphate in water. Therefore, iron-polyphenol complex formed in tea is mostly rejected. Calcium carbonate was also added to a ferrous sulphate and tea mixture. This was done because calcium in milk when added to tea may displace iron which is bound to polyphenols. However the addition of calcium did not affect the rejection and thus the same amount of iron remained bound to tea polyphenols. There was also a large rejection of ferrous sulphate in skim milk ($88\% \pm 2\%$) which is likely due to the binding of iron to milk proteins. The rejection of iron from the solution containing ferrous sulphate, skim milk, and tea was also high, as expected. Overall this qualitatively shows that ferrous sulphate binds to both milk proteins and tea polyphenols. The ratio of the types of compounds in tea was not elucidated because the skim milk clouded the spectrophotometric readings for iron-polyphenol complex and the membrane did not separate iron-protein complexes from iron-polyphenol complexes.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Average Iron Rejection (%)</th>
<th>Standard Deviation</th>
<th>Average Iron Polyphenol Complex Rejection (%)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulphate + water</td>
<td>25</td>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferrous sulphate + tea</td>
<td>81</td>
<td>15</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td>Ferrous sulphate + CaCO₃ + tea</td>
<td>79</td>
<td>4</td>
<td>81</td>
<td>4</td>
</tr>
<tr>
<td>Ferrous sulphate + casein protein + water</td>
<td>72</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferrous sulphate + casein protein + tea</td>
<td>93</td>
<td>4</td>
<td>92</td>
<td>2</td>
</tr>
<tr>
<td>Ferrous sulphate + skim milk</td>
<td>88</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferrous sulphate + tea + skim milk</td>
<td>90</td>
<td>3</td>
<td>N/A (milk clouded solution)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Ferric sodium EDTA was tested in much the same way as ferrous sulphate (see Table 10.5). Ferric sodium EDTA in water did not move through the membrane as easily as ferrous sulphate. It had a 73% ± 3% rejection as opposed to only a 25% ± 3% rejection of ferrous sulphate. However, in tea, iron moved through the membrane more easily indicating that complexes formed other than that of ferric sodium EDTA and iron-polyphenol complexes. This intermediate complex may have an iron binding component that causes an absorbance reading. Thus, a similar rejection was seen of iron and iron-polyphenol complexes in this situation. A much larger percentage was rejected when calcium was added for both iron and iron-polyphenol complex. This could be because the calcium pushes those intermediate complexes to form either ferric-EDTA or iron-polyphenol complexes. Ferric sodium EDTA in skim milk alone caused a low rejection and thus intermediate complexes may have formed with milk proteins. The combination of both milk and tea increase the rejection, possibly pushing the iron towards ferric-EDTA or iron-polyphenol complexes and away from intermediates that are passed through the membrane.
Table 10.5: Ferric Sodium EDTA Membrane Tests

<table>
<thead>
<tr>
<th>Solution</th>
<th>Average Iron Rejection (%)</th>
<th>Standard Deviation</th>
<th>Average Iron Polyphenol Complex Rejection (%)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric sodium EDTA + water</td>
<td>73</td>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferric sodium EDTA + tea</td>
<td>27</td>
<td>4</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>Ferric sodium EDTA + CaCO₃+tea</td>
<td>73</td>
<td>16</td>
<td>79</td>
<td>16</td>
</tr>
<tr>
<td>Ferric sodium EDTA + skim milk</td>
<td>18</td>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferric sodium EDTA + tea + skim milk</td>
<td>52</td>
<td>19</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Finally, tea made with ferric sodium EDTA and additional EDTA (to the molar ratio of 1:2 iron to EDTA) was tested (Table 10.6). This ratio was found to produce very low amounts of visible iron-polyphenol complex. The extra EDTA increased the amount of iron rejection from 27% ± 4% to 68% ± 7% which was predicted because more iron-EDTA complex is expected, and this complex is mostly rejected. The iron-polyphenol rejection in this increased EDTA tea is similar to ferrous sulphate in tea. This indicates that the tiny amount of complex present is likely bound to tea in the same form as when EDTA is not present. Furthermore, this increased EDTA tea had a similar iron rejection when skim milk was added as when EDTA was not increased. Likely intermediate complexes are forming between iron, milk proteins, and EDTA of which the ratio is not greatly affected by the increase in EDTA.

Table 10.6: Ferric Sodium EDTA with Added EDTA Membrane Tests

<table>
<thead>
<tr>
<th>Solution</th>
<th>Average Iron Rejection (%)</th>
<th>Standard Deviation</th>
<th>Average Iron Polyphenol Complex Rejection (%)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fortified tea</td>
<td>68</td>
<td>7</td>
<td>81</td>
<td>4</td>
</tr>
<tr>
<td>Fortified tea + skim milk</td>
<td>58</td>
<td>20</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

10.6.3 Milk Conclusions and Recommendations

The results of these tests give a qualitative understanding of the tea system with milk and added constituents. On the whole it appears that ferrous sulphate is greatly affected by tea and by milk whereas chelated iron in the form of ferric sodium EDTA is less affected, forming intermediate complexes. More intricate details of the mechanisms behind these observations are currently speculation and further investigation is required. The use of a model system with smaller phenolic
acids such as gallic acid may prove useful as the smaller acids would be more easily separated from milk proteins when using membrane filtration. Separation using a membrane in which size exclusion is the principle mode of separation may be useful if one is found such that iron-EDTA chelate may be separated from iron-polyphenol complexes. Also tests should be conducted at relevant pH levels. Furthermore, the impact of milk on bioavailability would be best tested using Caco-2 cells or in vivo to take into account the more complicated nature of mineral absorption. Also, the effect of milk on the colour of iron fortified tea must be addressed in future research.
Appendix 10.7: Interaction of Iron Fortified Tea with Minerals

Excess chelating agents may be used to fortify tea with iron. An example of a formulation that appears to halt the iron-polyphenol reaction is a 1:2 molar ratio of iron to EDTA. Additional minerals may bind to EDTA however, and allow iron to react with polyphenols. This scenario was investigated under my direction by Laura Godard, a visiting student from Agrosup Dijon, France. The tea used contained ferric sodium EDTA with additional disodium EDTA (1:2 iron:EDTA). Seven micronutrients were studied: Ca, Cu, K, Na, Mg, Mn and Zn by adding the Recommended Dietary Allowance (RDA) for pregnant women of each of these minerals to iron-fortified tea (Table 10.7). The values pertaining to pregnant women were chosen as they are known to be one of the main groups of people who suffer from iron deficiency.

Table 10.7: Recommended Dietary Allowance for Pregnant Women [12]

<table>
<thead>
<tr>
<th>Mineral (mg/day)</th>
<th>Calcium (mg/day)</th>
<th>Copper (µg/day)</th>
<th>Magnesium (mg/day)</th>
<th>Manganese (mg/day)</th>
<th>Zinc (mg/day)</th>
<th>Potassium (g/day)</th>
<th>Sodium (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/day)</td>
<td>1000</td>
<td>1000</td>
<td>360</td>
<td>2</td>
<td>11</td>
<td>4.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

10.7.1 Mineral Testing Methods

Iron-Polyphenol Complex Quantification

The iron-polyphenol complex was measured using spectrophotometry with a Cary50 spectrophotometer at 560 nm. The amount of iron-polyphenol complex was determined by reading the absorbance after 5, 20 and 60 minutes at 560 nm for each sample. Readings from blank samples without mineral addition were subtracted from those with it. Absorbance data was calibrated using a ferric chloride in gallic acid curve at pH 5.

Sample Preparation

Iron fortified tea (1:2 iron to EDTA molar ratio) was brewed by adding RO water to fortified tea leaves and allowing it to sit for 5 minutes at room temperature. The ratio of tea leaves to water was 1% \(\frac{w_{\text{tea leaves}}}{w_{\text{water}}}\). After steeping the solution was poured through a strainer separating the tea leaves from the liquid. Concentrated solutions of sodium chloride, potassium chloride, calcium chloride, manganese sulphate, magnesium chloride, copper (II) chloride, zinc chloride were added either individually or together to tea. These solutions were added until the RDA for pregnant women of each mineral was achieved.
**10.7.2 Mineral Results and Discussion**

The addition of each mineral appears to cause an increase in iron-polyphenol complex formation. Figure 10.8 shows the increase in iron-polyphenol complex formation due to the addition of each mineral. This increase is most pronounced for calcium and zinc; both exhibiting greater than 1/6th of the added iron becoming iron-polyphenol complex after one hour. When all minerals were added, this effect was even more pronounced, with over 2/3rds of the iron forming iron-polyphenol complex after one hour.

![Figure 10.8: Iron-Polyphenol Complex Formation in the Presence of Minerals](image)

Although the pH of brewed tea is approximately 5, pH was not measured or controlled for in these experiments. Change in the pH of the tea due to the addition of minerals would lead to very different results. The polyphenols in the tea are pH sensitive in that the colour changes from light yellow to brown when moving from acidic to basic conditions, which will affect spectrophotometric readings. Also, the ability of the polyphenols to bind iron (as well as other minerals) is also pH dependant.

**10.7.3 Mineral Conclusions and Recommendations**

In summary, minerals added to tea containing ferric sodium EDTA with added EDTA (with a 1:2 molar ratio of iron to EDTA) appeared to cause the formation of some iron-polyphenol complexes. However, pH was not measured or controlled. Similar experiments would be able to determine
the effects of each mineral on iron-polyphenol complex formation in brewed tea and in the duodenum if pH were controlled. Initial tests should add minerals in large amounts, such as a 1:2 molar ratio with iron added (iron:other mineral) to determine if they will compete for EDTA when in tea. Further, the levels of those that cause a significant change in iron-polyphenol complex formation by displacing iron in the EDTA complex may be characterized over a range of possible concentrations. These results will then be easily adaptable for the purposes of predicting colour formation in water with different mineral compositions as well as the amount of complex formation that may occur in the duodenum. Further testing for bioavailability using Caco-2 cells or in vivo with the presence of minerals may be useful because the mechanisms of absorption are not limited to iron-polyphenol complex formation but also include competition for transporters from cells in the intestinal lumen.
Appendix 10.8: Soluble Iron Following Sequential pH Adjustment

Solutions of tea fortified with the use of disodium EDTA were adjusted through pH levels applicable to digestion. When the final pH was brought to pH 7 the solubility of iron was greatly affected. From Figure 10.9 it may be seen that there was a drop from 100% retention of soluble iron to 67% from pH 6.96 to pH 7.08.

![Figure 10.9: Retention of Soluble Iron in Tea with EDTA after Sequential pH Adjustment](image)

Error bars are based on the standard deviation of triplicate AA measurements.
Appendix 10.9: Iron Fortified Tea Premix Preparation

The following method for iron fortified tea production was developed by researchers of the Food Engineering Group at the University of Toronto, specifically Bih-King Chen and Veronica Dueik. Materials used include: hydroxypropyl methylcellulose (HPMC), specifically METHOCEL™ E15 Premium LV; ferric sodium EDTA (Sigma-Aldrich); and disodium EDTA dihydrate (BioShop).

To prepare fortified tea, a solution of 2 wt% HPMC, 10 wt% ferric sodium EDTA, and 10 wt% disodium EDTA dihydrate is prepared then sprayed onto tea leaves in a pan coater. On 43 g of tea leaves, 40 g of solution is sprayed (addition of 93 wt%). They are then dried at room temperature (25°C) under high vacuum resulting in 50 g of treated tea leaves. These treated tea leaves contain 1 wt% iron once dried and serve as a pre-mix to dry blend with untreated tea leaves. This blending is completed using one part pre-mix to four parts tea leaves, however may be easily adjusted to reach target iron levels. This results in fortified tea that contains 2 mg of iron per gram of fortified tea leaves. In the brewed tea drink this would result in about 4.7 mg of iron per cup.