ABSTRACT

SUMMERS, CAROLINE RUEDA. Modulation of Inflammatory Responses by Green and Black Tea in LPS-induced RAW 264.7 Cells. (Under the direction of Dr. Gabriel Keith Harris).

Recently, the World Health Organization estimated that chronic diseases are responsible for 46% of disease occurrence and 59% of all deaths worldwide. Growing evidence suggests that increasing chronic disease incidence is dependent upon several factors, including inadequate consumption of antioxidant-rich fruits and vegetables and chronic inflammation. Many researchers have previously investigated potential health benefits associated with consuming plant-based foods, and the ability of these foods to prevent or suppress the pathologies linked to chronic diseases. Specifically, plant-based bioactive compounds called polyphenols have been widely studied, and show promise as a therapeutic agent against chronic diseases.

Tea is produced from the leaves of the *Camellia sinensis* plant and, aside from water, is the most popular beverage in the world. Green tea, black tea, and oolong tea are the three major types of commercially produced tea. Black tea comprises over 75% of global tea production and consumption, followed by green tea at approximately 20%, and oolong tea at less than 2%. For centuries, tea has been known for its health effects. It has historically been used as a medicine in China and Japan. Tea polyphenols are particularly thought to have chemopreventive and cardioprotective effects due to their antioxidant and anti-inflammatory properties. Previous *in vitro* and animal studies have analyzed the anti-inflammatory activity of polyphenols, and the specific mechanisms by which these compounds suppress inflammation. But, to our knowledge, few studies have investigated tea's anti-inflammatory

capacity as a whole food, or compared the potential anti-inflammatory effects of different tea types.

Therefore, the objectives of the following study were to evaluate total phenol content in green tea (GT) and black tea (BT); to assess viability of cells exposed to GT, BT, and LPS; and to measure the modulation of inflammatory responses in lipopolysaccharide (LPS)-induced RAW 264.7 mouse macrophages by GT and BT. We predicted that GT and BT would differentially modulate inflammation because of differences in composition.

First, the total phenolic content in commercially produced GT and BT was measured using the Folin-Ciocalteu method. Freshly prepared GT had a significantly higher phenolic content than freshly prepared BT (1317.1 $\pm$ 6.0 GAE, 918.9 $\pm$ 10.7 GAE). Stored at -80° C, the phenolic content of GT and BT significantly increased at one month (1770.0 $\pm$ 35.2 GAE, 1124.0 $\pm$ 19.1 GAE) and two months post-preparation (1587.2 $\pm$ 21.5 GAE, 1003.2 $\pm$ 8.6 GAE), then decreased at 3 months post-preparation (1407.8 $\pm$ 13.4 GAE, 941.8 $\pm$ 0.5 GAE) ($p < 0.05$).

The second goal of our study was to assess viability in RAW 264.7 cells treated with GT, BT, and LPS. Viability was colorimetrically determined by the Trypan Blue assay and by measured absorbance values from the MTT assay. The GT, BT, and LPS treatments did not produce a cytotoxic effect, and cell viability values for each treatment were not significantly different ($p < 0.05$).

The third objective was to measure prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2), two major inflammatory mediators, in tea-treated, LPS-induced RAW 264.7 cells. We analyzed the inflammatory potency of LPS from two E. coli serotypes, 0111:B4 and 055:B5, and found that the latter serotype more significantly stimulated PGE_2 production.

When applied, GT and BT exhibited anti-inflammatory activity by similarly suppressing PGE_2. Western blotting analysis revealed that only GT applied at the highest concentration (5%) significantly inhibited COX-2 expression relative to the positive control ($p < 0.05$). Further studies are needed to elucidate the exact mechanisms by which tea exerts anti-inflammatory activity on these inflammatory mediators.

Modulation of Inflammatory Responses by Green and Black Tea in LPS-induced RAW 264.7 Cells

by
Caroline Rueda Summers

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APPROVED BY:

_____	_____
Dr. Gabriel Keith Harris Committee Chair	Dr. Jonathan C. Allen
_____	
Dr. Brenda P. Alston-Mills	

DEDICATION

I dedicate this work to my Mom and Dad. Thank you for all the sacrifices you made for me, your unconditional love and support, and for always encouraging me to pursue my dreams.

BIOGRAPHY

Caroline Rueda Summers was born on July 21, 1984 in Raleigh, North Carolina. She is the only child of Dr. Leopoldo (Pollie) Rueda and Mrs. Belen Rueda. Caroline completed her elementary and middle school education at Our Lady of Lourdes School, then attended Cardinal Gibbons High School, both in Raleigh. In the fall of 2002, she began her undergraduate studies at North Carolina State University, majoring in Biology. In addition to her coursework, Caroline was actively involved with the Pre-Health Club and the EMS and Fire Interest Organization, and volunteered as an Emergency Medical Technician at Cary Area EMS. She also participated in the College of Agriculture and Life Sciences Honors Program, and conducted research under the direction of Dr. Jonathan Allen and Gary Matsey from the Department of Food Science. In Spring 2006, Caroline graduated magna cum laude with a B.S. in Biological Sciences with a Nutrition Concentration.

Caroline's undergraduate research experience piqued her interest in nutrition, health, and research, so she decided to stay at NC State to work on a Master's degree in Nutrition and minor in Physiology. As a graduate student, Caroline worked both as a teaching and research assistant, was actively involved in the Food Science Club, and continued to volunteer with Cary Area EMS. She also completed the Instructional Technology Assistant Program through Distance Education and Learning Technology Applications. Caroline used the skills gained from this program to assist in the development of an internet-based undergraduate dairy course, and to enhance online introductory food science and nutrition

courses. Upon completion of her Master's program, Caroline will attend the Edward Via Virginia College of Osteopathic Medicine to pursue her dream of becoming a physician.

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TABLE OF CONTENTS

LIST OF TABLES x
LIST OF FIGURES xi
LIST OF ABBREVIATIONS AND NOMENCLATURE xiv

CHAPTER 1: LITERATURE REVIEW 1

1.1 Introduction 2
1.2 Tea Production and Consumption 4
1.3 Tea Compounds of Interest 6
1.4 Absorption, Bioavailability, and Metabolism of Tea Polyphenols 15
1.5 Reported Health Effects of Tea 19
1.5.1 Antioxidant Activity of Tea 20
1.5.2 Polyphenol Structure-Function Relationships 21
1.5.3 Inflammation and the Incidence of Chronic Disease 24
1.5.3.1 Anti-Inflammatory Effects of Tea on Arachidonic Acid-Dependent Pathways 25
1.5.3.2 Cyclooxygenase (COX) Pathway 26
1.5.3.3 Lipoxygenase (LOX) Pathway 28
1.5.4.1 Anti-Inflammatory Effects of Tea on Arachidonic Acid-Independent Pathways 30
1.5.4.2 Inducible Nitric Oxide Synthase (iNOS) 31
1.5.4.3 Nuclear Factor kappa B (NF-κB) 32
1.5.5 Methods of Analyzing Anti-Inflammatory Activity 35
1.5.5.1 Cell Viability Techniques 36
1.5.5.2 Prostaglandin E_2 (PGE_2) Enzyme-Linked Immunosorbent Assay (ELISA) 37
1.5.5.3 Protein Determination and Western Blotting (COX-2 Detection) 38
1.6 Clinical and Epidemiological Studies on Tea Consumption and Chronic Disease 39
1.6.1 Tea Consumption and Cancer 40
1.6.2 Tea Consumption and Cardiovascular Diseases 41
1.7 Recent Research on Tea Consumption and Human Disease 43
1.7.1 Tea Consumption and Diabetes Mellitus 44
1.7.2 Tea Consumption and Other Health Conditions 47
1.8 Summary and Conclusions 48
1.9 REFERENCES 50

CHAPTER 2: PREPARATION AND CHARACTERIZATION OF GREEN TEA AND BLACK TEA 62

2.1 ABSTRACT 63
2.2 INTRODUCTION 65
2.3 MATERIALS AND METHODS 67
2.3.1 Preparation of Green Tea and Black Tea 67

2.3.2 Preparation of Total Phenol Assay Reagents....68
2.3.3 Preparation of Standard Curve....68
2.3.4 Preparation of Samples....70
2.3.5 Statistical Analysis....71
2.4 RESULTS....71
2.5 DISCUSSION....73
2.6 CONCLUSIONS....76
2.7 REFERENCES....77

CHAPTER 3: CELL VIABILITY ANALYSIS, PGE_2 QUANTITATION, AND COX-2 DETECTION IN LPS-INDUCED RAW 264.7 CELLS TREATED WITH GREEN TEA AND BLACK TEA....79

3.1 ABSTRACT....80
3.2 INTRODUCTION....82
3.3 MATERIALS AND METHODS....86
3.3.1 Cell Culture and Cell Viability....86
3.3.1.1 Passaging, Plating, and Counting RAW 264.7 Cells....88
3.3.1.2 Cell Treatment and Harvesting....93
3.3.1.3 Cell Viability (MTT) Assay....96
3.3.2 PGE_2 ELISA....97
3.3.3 Protein Determination and Western Blotting (COX-2 Detection)....98
3.3.3.1 BCA Assay....98
3.3.3.2 Western Blotting....99
3.3.4 Experimental Design and Statistical Analysis....101
3.4 RESULTS....102
3.5 DISCUSSION....110
3.5.1 LPS....110
3.5.2 Cell Viability....111
3.5.3 PGE_2 ELISA....114
3.5.4 Western Blotting....114
3.6 CONCLUSIONS....115
3.7 REFERENCES....116

CHAPTER 4: SUMMARY AND SUGGESTIONS FOR FUTURE WORK....118

APPENDIX A. HPLC/MS ANALYSIS OF MULTIPLE INFLAMMATORY MARKERS IN LPS-INDUCED RAW 264.7 MACROPHAGES....121

A.1 INTRODUCTION....122
A.2 MATERIALS AND METHODS....123
A.2.1 Harvesting LPS-stimulated RAW 264.7 cells Treated with Green and Black Tea....123
A.2.2 Standard and Sample Isolation....125

A.2.3 Part I: Reverse-phase Liquid Chromatography and Mass Spectrometry.....125
A.2.3.2 Part II: Reverse-phase Liquid Chromatography and Mass Spectrometry.....126
A.2.4 Preliminary Experiments with RAW 264.7 Cells + LPS/PMA.....126
A.3 RESULTS.....127
A.4 DISCUSSION AND CONCLUSIONS.....173
A.5 REFERENCES.....174

LIST OF TABLES

CHAPTER 1: LITERATURE REVIEW

Table 1.1. Precursors of Major Theaflavins in Black Tea 12
Table 1.2. Role of Leukotrienes in Human Diseases 29

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

Figure 1.1. Methods of Tea Production 6
Figure 1.2. Structures of Major Polyphenols in Foods 7
Figure 1.3. Basic Structure of a Flavonoid 8
Figure 1.4. Catechins: Major Flavonoids in Green Tea 9
Figure 1.5. Tea Theaflavins and Thearubigins 11
Figure 1.6. Oolong Tea Flavonoids: Oolonghomobisflavans 12
Figure 1.7. Major Tea Flavonols 13
Figure 1.8. Flavonoid Content in Green Tea and Black Tea 14
Figure 1.9. Metabolites of Epicatechin and Quercetin 18
Figure 1.10. Structure of the Flavonol Quercetin and Highlighted Features of Flavonoid Antioxidant Activity 22
Figure 1.11. The Arachidonic Acid-Dependent Pathway 26
Figure 1.12. NF-κB Activation 34
Figure 1.13. Schematic of Cayman Chemical ACE™ Competitive PGE_2 ELISA 38

CHAPTER 2: PREPARATION AND CHARACTERIZATION OF GREEN TEA AND BLACK TEA

Figure 2.1. Gallic Acid Standards and Spectrophotometer 70
Figure 2.2. Effect of -80° C Storage on Phenol Content in GT and BT vs. Phenol Content in Freshly Prepared GT and BT 72
Figure 2.3. Effect of Incubator Conditions, RAW 264.7 cells, Media, LPS, GT, and BT on Total Phenol Content 73

CHAPTER 3: CELL VIABILITY ANALYSIS, PGE_2 QUANTITATION, AND COX-2 DETECTION IN LPS-INDUCED RAW 264.7 CELLS TREATED WITH GREEN TEA AND BLACK TEA

Figure 3.1. Metabolism of PGE_2 84
Figure 3.2. Laminar Flow Hood and Cell Culture Set-Up 87
Figure 3.3. Cell Culture Flasks 89
Figure 3.4. Cell Counter and Hemocytometer with Trypan Blue and Cell Suspension 91
Figure 3.5. Pipetting Media from 12-well Plates During Cell Harvest 95
Figure 3.6. MTT Assay: 96-well Plate Set-Up 96
Figure 3.7. PGE_2 ELISA: 96-well Plate Set-Up 97
Figure 3.8. BCA Assay: 96-well Plate Set-Up 99
Figure 3.9. Western Blotting: Protein Transfer to PVDF Membrane 101
Figure 3.10. Cell Viability (MTT) Assay 102

Figure 3.11a. Mean PGE_2 Concentrations in RAW 264.7 Cells Treated with Varying Levels of LPS from E. coli 0111:B4 104
Figure 3.11b. Mean PGE_2 Concentrations in RAW 264.7 Cells Treated with Varying Levels of LPS from E. coli 055:B5 105
Figure 3.12a. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 2 μg/ml of LPS from E. coli 0111:B4 106
Figure 3.12b. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 2 μg/ml of LPS from E. coli 055:B5 107
Figure 3.13. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 10 μg/ml of LPS from E. coli 055:B5 108
Figure 3.14. COX-2 Detection on Western Blot 109

APPENDIX A. HPLC/MS ANALYSIS OF MULTIPLE INFLAMMATORY MARKERS IN LPS-INDUCED RAW 264.7 MACROPHAGES

Figure A.1 Harvesting Cells for HPLC/MS Analysis 124
Figure A.2 Chromatogram for Standards Run on Finnigan LCQ Duo 128
Figure A.3 Chromatrogram for Media Sample "BT 1% + LPS" 129
Figure A.4a. Standard Curve for PGI_2 Standard 130
Figure A.4b. Chromatogram for PGI_2 Standard 131
Figure A.4c. Chromatogram for Negative Control Cell Lysate 132
Figure A.4d. Chromatogram for Positive Control Cell Lysate 133
Figure A.4e. Chromatogram for "GT 5% + LPS" Cell Lysate 134
Figure A.4f. Chromatogram for "BT 5% + LPS" Cell Lysate 135
Figure A.4g. Chromatogram for Negative Control Media 136
Figure A.4h. Chromatogram for Positive Control Media 137
Figure A.4i. Chromatogram for "GT 5% + LPS" Media 138
Figure A.4j. Chromatogram for "BT 5% + LPS" Media 139
Figure A.5a. Standard Curve for $PGF_{2\alpha}$ Standard 140
Figure A.5b. Chromatogram for $PGF_{2\alpha}$ Standard 141
Figure A.5c. Chromatogram for Negative Control Cell Lysate 142
Figure A.5d. Chromatogram for Positive Control Cell Lysate 143
Figure A.5e. Chromatogram for "GT 5% + LPS" Cell Lysate 144
Figure A.5f. Chromatogram for "BT 5% + LPS" Cell Lysate 145
Figure A.5g. Chromatogram for Negative Control Media 146
Figure A.5h. Chromatogram for Positive Control Media 147
Figure A.5i. Chromatogram for "GT 5% + LPS" Media 148
Figure A.5j. Chromatogram for "BT 5% + LPS" Media 149
Figure A.6a. Standard Curve for PGE_2 Standard 150
Figure A.6b. Chromatogram for PGE_2 Standard 151
Figure A.6c. Chromatogram for Negative Control Cell Lysate 152
Figure A.6d. Chromatogram for Positive Control Cell Lysate 153
Figure A.6e. Chromatogram for "GT 5% + LPS" Cell Lysate 154

Figure A.6f. Chromatogram for "BT 5% + LPS" Cell Lysate.....155
Figure A.6g. Chromatogram for Negative Control Media.....156
Figure A.6h. Chromatogram for Positive Control Media.....157
Figure A.6i. Chromatogram for "GT 5% + LPS" Media.....158
Figure A.6j. Chromatogram for "BT 5% + LPS" Media.....159
Figure A.7a. Standard Curve for LTB_4 Standard.....160
Figure A.7b. Chromatogram for LTB_4 Standard.....161
Figure A.7c. Chromatogram for Negative Control Cell Lysate.....162
Figure A.7d. Chromatogram for Positive Control Cell Lysate.....163
Figure A.7e. Chromatogram for "GT 5% + LPS" Cell Lysate.....164
Figure A.7f. Chromatogram for "BT 5% + LPS" Cell Lysate.....165
Figure A.7g. Chromatogram for Negative Control Media.....166
Figure A.7h. Chromatogram for Positive Control Media.....167
Figure A.7i. Chromatogram for "GT 5% + LPS" Media.....168
Figure A.7j. Chromatogram for "BT 5% + LPS" Media.....169
Figure A.8a. Chromatogram for Standards Run on Finnigan LCQ Duo.....171
Figure A.8b. Chromatogram for Cell Lysate + LPS/PMA.....172

LIST OF ABBREVIATIONS AND NOMENCLATURE

AA (arachidonic acid)
AMU (atomic mass units)
BCA (bicinchoninic acid)
BT (black tea)
CHD (coronary heart disease)
COMT (catechol-*O*-methyltransferase)
COPD (chronic obstructive pulmonary disease)
COX (cyclooxygenase)
COX-1 (cyclooxygenase 1)
COX-2 (cyclooxygenase 2)
COX-3 (cyclooxygenase 3)
DMEM (Dulbecco's Modified Eagle Medium)
EC (epicatechin)
ECG (epicatechin-3-gallate)
EGC (epigallocatechin)
EGCG (epigallocatechin-3-gallate)
ERK (extracellular signal regulated protein kinase)
FBS (fetal bovine serum)
GT (green tea)
HETE (hydroxyeicosatetraenoic acid)
HpETE (hydroperoxyeicosatetraenoic acid)
HPLC (high performance liquid chromatography)
IFNγ (interferon gamma)
IL (interleukin)
iNOS (inducible nitric oxide synthase)
LC (liquid chromatography)
LOX (lipoxygenase)
LPS (lipopolysaccharide)
LT (leukotriene)
LTB_4 (leukotriene B_4)
LTC_4 (leukotriene C_4)
LTD_4 (leukotriene D_4)
LTE_4 (leukotriene E_4)
MAPK (mitogen-activated protein kinase)
M-CSF (macrophage colony-stimulating factor)
MS (mass spectrometry)
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium))
NF-κB (nuclear factor kappa B)
NSAID (non-steroidal anti-inflammatory drug)
NO (nitric oxide)

PG (prostaglandin)
PGE_2 (prostaglandin E_2)
$PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$)
PGH_2 (prostaglandin H synthase)
PGI_2 (prostacyclin I_2)
PLA_2 (phospholipase A_2)
PMA (phorbol 12-myristate 13-acetate)
SULT (phenolsulfotransferases)
TLR4 (Toll-like receptor 4)
TNF-α (tumor necrosis factor alpha)
TPA (12-*O*-tetradecanoyl-phorbol-13-acetate)
TXA_2 (thromboxane A_2)
UGT (UDP-glucuronosyltransferases)
XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate)

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Over the past century, the causes for global morbidity and mortality have primarily shifted from infectious diseases to the emergence of chronic diseases, such as cancer, cardiovascular disease, obesity, type II diabetes, and neurodegenerative diseases. The World Health Organization recently estimated that chronic diseases are responsible for 46% of disease occurrence and 59% of deaths worldwide. Each year, approximately 35 million people die from chronic diseases. Growing evidence suggests that the increasing incidence of chronic disease can be attributed to a number of factors, including stress, lifestyle choices (e.g. smoking, alcohol intake), lack of physical activity, excessive intake of high-calorie foods rich in fat, sugar, and starch, as well as inadequate consumption of antioxidant fruits and vegetables (Bengmark 2004).

The growing emergence of chronic diseases has influenced trends in nutrition research, which have shifted from addressing nutrient deficiencies towards the prevention of chronic disease. Many researchers have investigated the potential health benefits associated with consuming plant-based foods, especially their ability to prevent the incidence of or suppress the pathologies linked to chronic diseases (Biesalski 2007). More specifically, plant-based compounds known as polyphenols have been widely studied and show much promise as a therapeutic aid against chronic diseases (Santangelo et al 2007).

Tea is a significant source of dietary polyphenols and has been extensively analyzed for its potential health benefits. Historically, tea has been consumed as a beverage and used as a medicine. Today, tea is the most popular beverage in the world, aside from water. Black tea and green tea comprise over 95% of world tea production and consumption.

Modern examinations of tea and its potential as a chemopreventive, cardioprotective, and neurostimulatory agent began approximately 50 years ago. In recent decades, tea studies have extended to investigating its effects on diabetes mellitus, obesity, dental caries, osteoporosis, bone mineral density, mood, and cognitive function (Arab & Blumberg 2008).

Research on teas as a source of phytochemicals has focused on analyzing specific tea polyphenols, such as epigallocatechin-3-gallate (EGCG) or theaflavins, which have demonstrated notable antioxidant and anti-inflammatory effects. While there have been observed positive health effects elicited by these individual components, tea is typically consumed as a whole food in beverage form. The American Heart Association recently recommended that antioxidants be consumed in the form of whole foods rather than as purified, individual compounds (Kris-Etherton et al 2004). Other naturally-occurring tea constituents, such as caffeine, may work synergistically with tea polyphenols to elicit more powerful antioxidant and anti-inflammatory activity. Additionally, tea is widely accessible and consumed by over two-thirds of the global population (McKay & Blumberg 2002). Therefore, validating the use of tea as a therapeutic whole food for the prevention or attenuation of chronic disease would be of benefit to tea drinkers worldwide.

It is not only important to investigate the potential health effects of tea. The differences in health effects between the two most widely consumed tea types, green tea and black tea, should also be evaluated. These two teas have dramatically different chemical compositions resulting from the varying degrees of enzymatic oxidation each tea undergoes during processing. The resulting compounds formed in each tea, and the amounts at which they are present, play prominent roles in the potential of each tea to function as an effective

anti-inflammatory agent. To our knowledge, little research has been done to compare the anti-inflammatory effects of green tea and black tea as a result of differences in their chemical composition.

Therefore, the objectives of the following study are to measure the total phenol content in each tea, to determine the modulation of inflammatory responses in lipopolysaccharide (LPS)-induced RAW 264.7 cells by green tea and black tea, and to compare the anti-inflammatory effects of green tea versus black tea by measuring specific inflammatory mediators. We will attempt to answer the following questions:

- Does green tea and/or black tea have an anti-inflammatory effect?
- Is one tea a better anti-inflammatory agent than the other tea?
- Is the total phenol content of each tea related to its effectiveness as an anti-inflammatory agent?

We hypothesize that green tea and black tea will suppress inflammation, and differentially alter markers of inflammation as a result of their unique chemical compositions. We predict that the tea with a higher phenolic content will exhibit a more powerful anti-inflammatory effect.

1.2 Tea Production and Consumption

Tea is produced from the leaves of the *Camellia sinensis* plant and was initially discovered in China almost 50 centuries ago. Tea is now grown in over 30 tropical and sub-tropical countries worldwide. Approximately three billion kilograms of tea are produced and consumed annually (Khan et al 2008). Aside from water, tea is the most widely consumed

beverage in the world (McKay & Blumberg 2002). Over two-thirds of the world's population drinks tea, and the per capita global consumption is 120 ml of brewed tea per day (Khan et al 2008).

There are over 300 different kinds of tea produced from the leaves of *Camellia sinensis*, which are divided into three general types: green tea, black tea, and oolong tea (Sang et al 2004). Black tea comprises 76%-78% of worldwide tea production and consumption, while 20%-22% is green tea, and less than 2% is oolong tea (McKay & Blumberg 2002). Black tea is most popular in North America, Europe (Satoh et al 2005), and India (Sang et al 2004), whereas green tea is a major beverage in Asian countries, such as China and Japan. Oolong tea is mainly consumed in China and Taiwan (Satoh et al 2005).

The chemical composition of green, black, and oolong tea vary because of differences in the production and processing, i.e. the level of drying and fermentation, of each tea. Fermentation is a term used by the tea industry that refers to the natural oxidation process by which tea leaves are physically and chemically converted by the leaf enzyme polyphenol oxidase. The enzymatic processes that occur during tea manufacturing are responsible for the development of the colors and flavors unique to each type of tea (Balentine & Pateau-Robinson 2000).

There are several steps involved in making tea. First, the leaves are picked from the *Camellia sinensis* plant. To produce black and oolong tea, the leaves are withered to allow the process of enzymatic oxidation to occur. Then, the leaves are bruised by shaking or rolling to speed up oxidation. Next, the leaves are left in a dark room to ferment or oxidize. During this process, enzymes from the leaves, including polyphenol oxidase, react with

tannins and catechins within the leaves and convert the polyphenols to theaflavins and thearubigins. Black tea is fully fermented, while oolong tea is partially fermented. For green tea, the leaves are withered then steamed to inactivate the enzymes and prevent the leaves from oxidizing. In other words, green tea is not fermented, and as much as 90% of the polyphenols are preserved in the leaves (Khan et al 2008) (Figure 1.1).

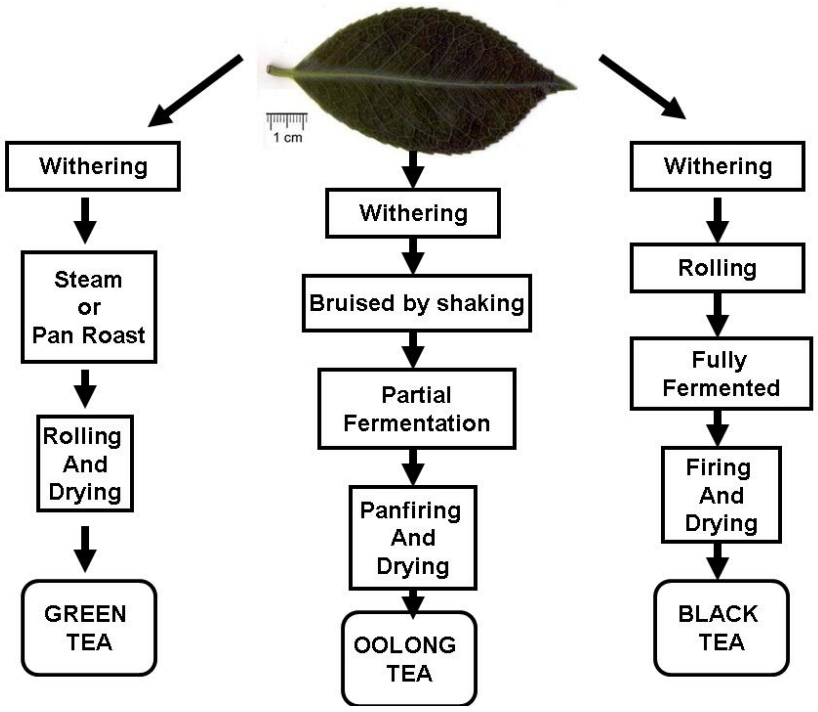


Figure 1.1. Methods of Tea Production (Khan et al 2008)

1.3 Tea Compounds of Interest

Polyphenols are a structurally diverse class of secondary metabolites that are ubiquitous in plants. To date, over 4000 polyphenolic compounds have been identified (McKay & Blumberg 2002, Kim et al 2004, Cheynier 2005). They are structurally

characterized as having one or more benzene rings with two or more hydroxal groups attached to the benzene ring. Polyphenols can be classified into four major groups: flavonoids, stilbenes, lignans, and phenolic acids (Figure 1.2). Within plants, polyphenols are primarily involved in protecting the plant against insects, microbes, fungi, and ultraviolet radiation. Polyphenols also act as antioxidants during photosynthesis (Biesalski 2007).

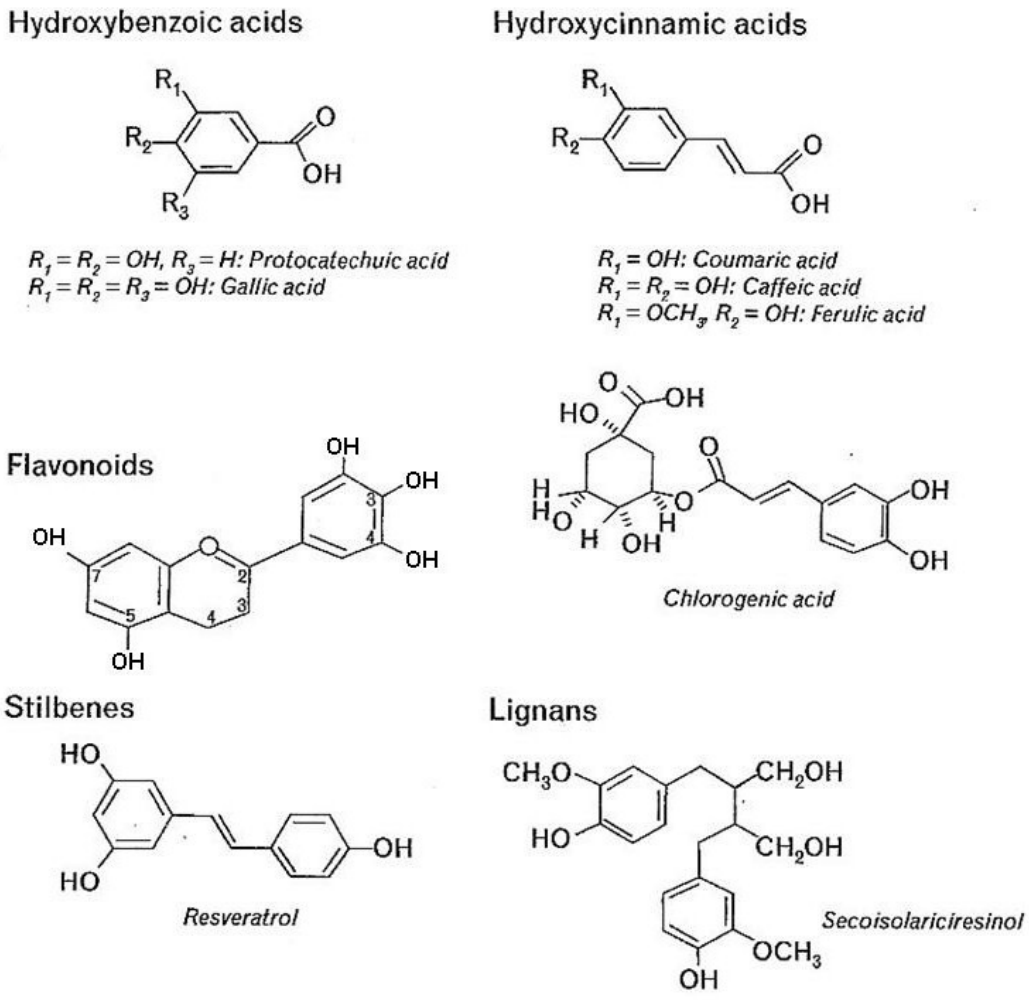


Figure 1.2. Structures of Major Polyphenols in Foods (Biesalski 2007)

Tea is a rich source of polyphenols, especially flavonoids. Flavonoids have a basic structure that includes three 6-carbon rings: an aromatic A-ring bound to a heterocyclic C-ring that is fused to an aromatic B-ring via a single carbon-carbon bond. Flavonoids vary based on the conjugation between the A- and B-rings, and by their hydroxyl, methoxyl, and glycosidic side groups (Babu & Liu 2009) (Figure 1.3). The primary flavonoids found in green tea are part of the flavanol subclass (Rice-Evans et al 1997) and include catechins such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG) (McKay & Blumberg 2002, Higdon & Frei 2003) (Figure 1.4). Green tea flavonoids are mostly catechin monomers (60-80%) (Higdon & Frei 2003). A tea's relative catechin content depends upon the geographical location in which the teas were grown, the drying process used on the leaves, and the growing conditions (McKay & Blumberg 2002).

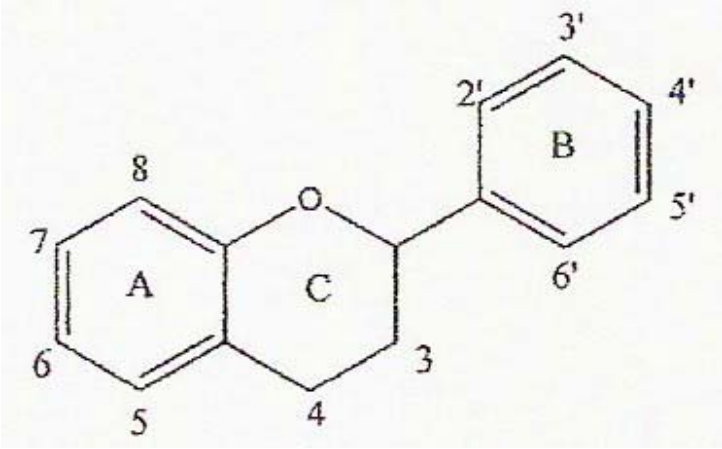


Figure 1.3. Basic Structure of a Flavonoid (Wang et al 2000)

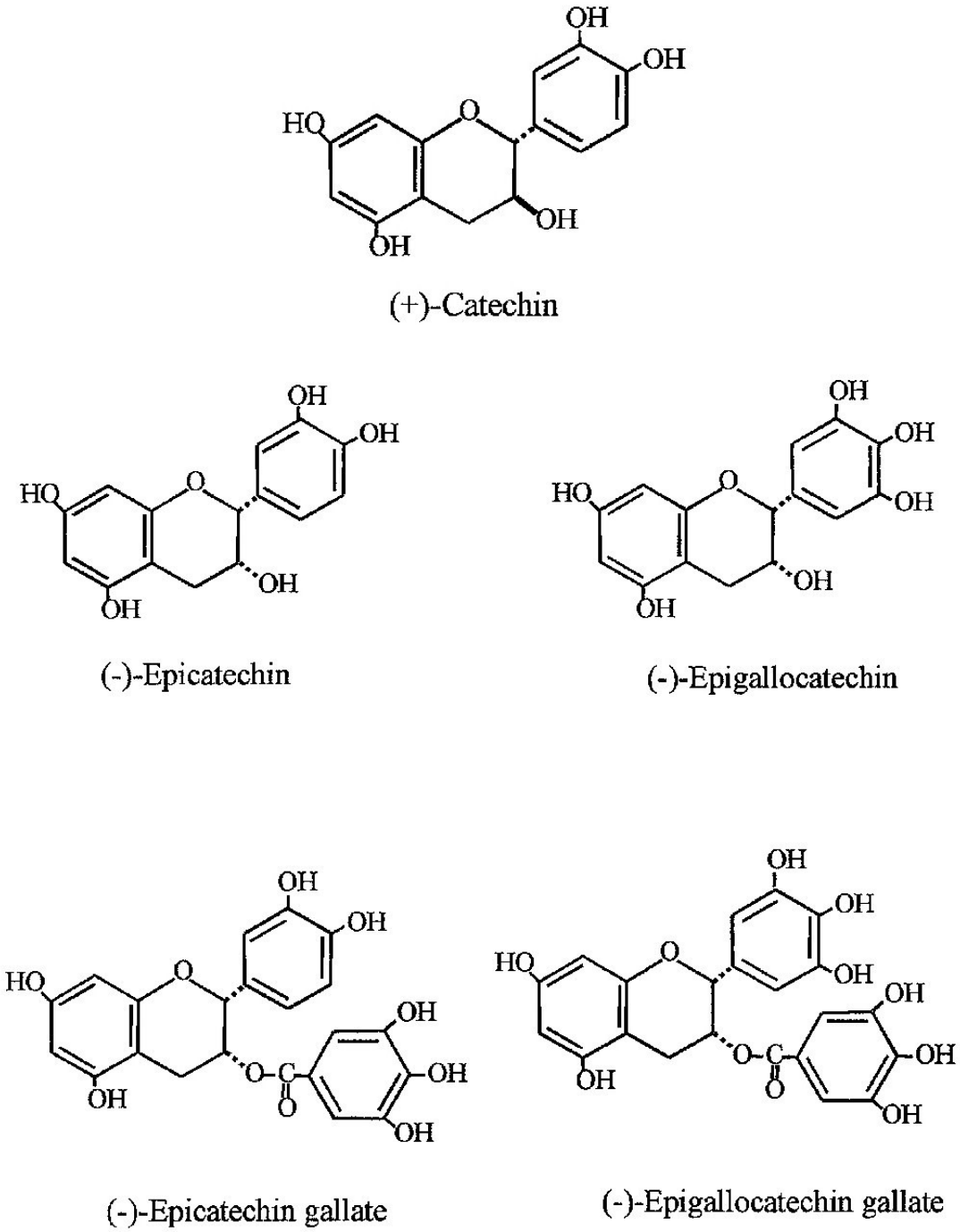


Figure 1.4. Catechins: Major Flavonoids in Green Tea (Higdon & Frei 2003)

The predominant flavonoids in black tea include polymerized catechins such as theaflavins and thearubigins (McKay & Blumberg 2002). Thearubigins are a diverse group of compounds that make up more than 70% of the total flavonoids found in black tea, while theaflavins comprise approximately 10% of black tea flavonoids (Lakenbrink et al 2000). The four major theaflavins are theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate, and theaflavin 3,3'-digallate (Figure 1.5). These compounds are formed from various catechin precursors during the enzymatic oxidation that occurs during tea processing (Table 1.1) (Hilal & Engelhardt 2007). Theaflavin derivatives, such as theaflavic acids and theaflavates, and theaflavin stereoisomers have also been identified in black tea (Sang et al 2004). Thearubigins, on the other hand, are poorly characterized chemically (Yang et al 2000). Since the oxidation period of oolong tea is shorter than that for black tea, oolong tea contains more catechins and less theaflavins and thearubigins than black tea (Balentine et al 1997). Oolong tea also includes unique flavanols, such as oolonghomobisflavans, as a result of undergoing semi-oxidation (Figure 1.6) (Nakai et al 2005). Flavonols, another flavonoid subclass, are present in green, oolong, and black tea in similar quantities because they are less affected by processing. Glycosides of the flavonols, such as kaempferol, quercetin, and myricetin, are also found in tea (Balentine & Paetau-Robinson 2000) (Figure 1.7). Figure 1.8 shows the relative flavonoid content in green tea and black tea (Higdon & Frei 2003).

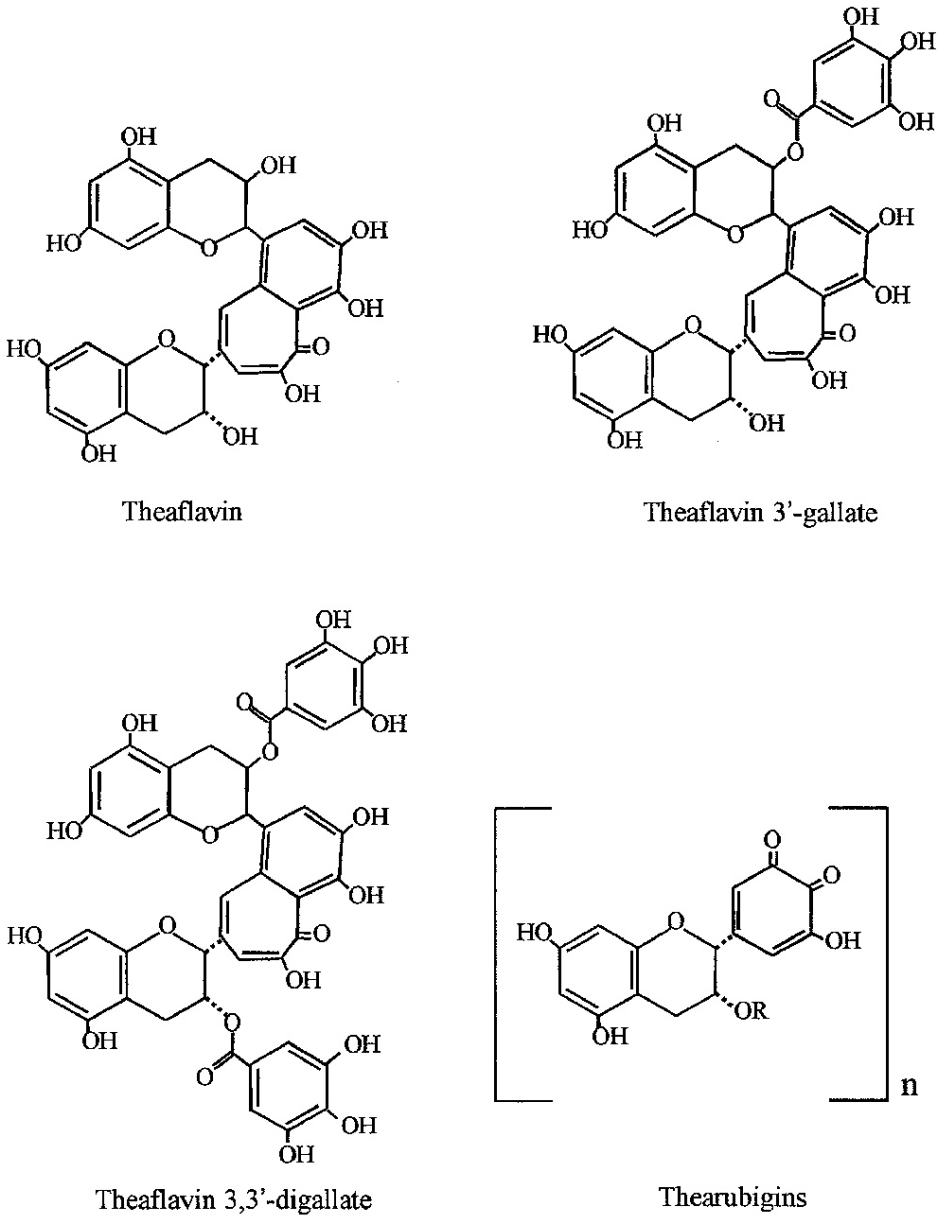


Figure 1.5. Tea Theaflavins and Thearubigins (Higdon & Frei 2003)

Table 1.1. Precursors of Major Theaflavins in Black Tea (Hilal & Engelhardt 2007)

Precursor A	Precursor B	Product
Epicatechin	Epigallocatechin	Theaflavin
Epicatechin	Epigallocatechin gallate	Theaflavin-3 gallate
Epicatechin gallate	Epigallocatechin	Theaflavin-3' gallate
Epicatechin gallate	Epigallocatechin gallate	Theaflavin-3,3' gallate

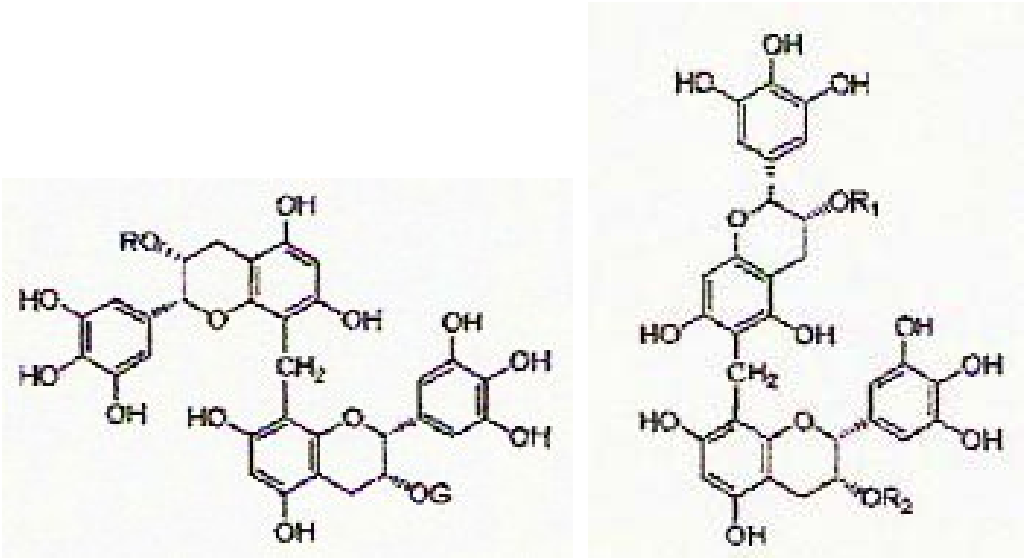


Figure 1.6. Oolong Tea Flavonoids: Oolonghomobisflavans (Nakai et al 2005)

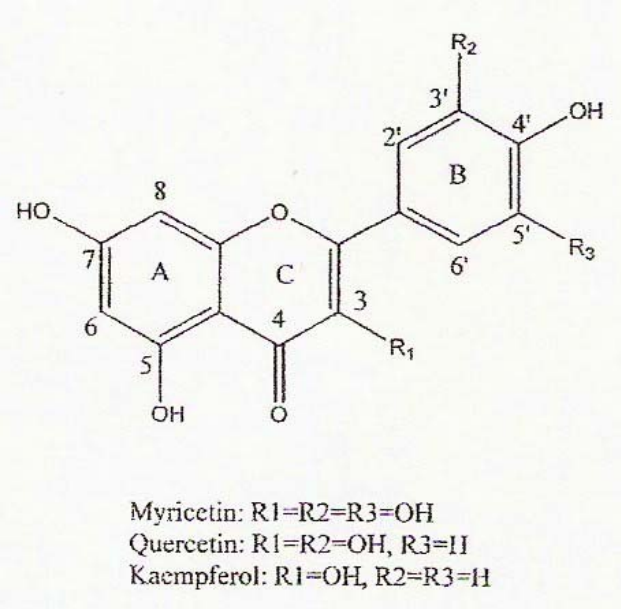
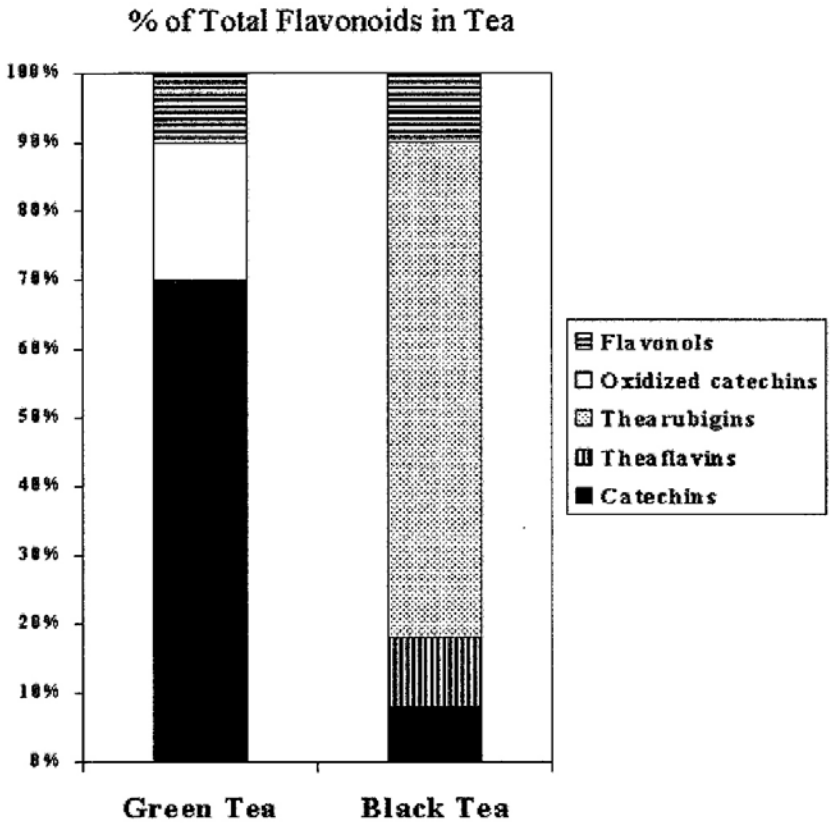


Figure 1.7. Major Tea Flavonols (Wang et al 2000)



Flavonols	7-9%	7-9%
Oxidized catechins	20-30%	
Thearubigins		63-74%
Theaflavins		5-12%
Catechins	60-80%	6-24%

Figure 1.8. Flavonoid Content in Green Tea and Black Tea (Higdon & Frei 2003)

For a tea beverage, the flavonoid concentration is dependent upon the type of tea and its method of preparation, i.e. amount of tea used, brewing time, and temperature. Brewed hot tea contains the highest amount of flavonoids at 541-692 μg/ml (Hakim et al 2000).

Instant tea contains less flavonoids (90-100 μg/ml). Ready-to-drink and iced tea contain even lower amounts (Arts et al 2000).

1.4 Absorption, Bioavailability, and Metabolism of Tea Polyphenols

Absorption and Bioavailability

Identifying the mechanisms of tea absorption and metabolism *in vivo* is essential for understanding how tea and its components function in physiological conditions. It is also important to consider the bioavailability of teas to determine if tea polyphenols are present in adequate amounts within the body to elicit health effects. Studies addressing these topics have generally focused on individual tea polyphenols, particularly catechins. These compounds, especially EGCG, generally have low bioavailability as a result of their relatively high molecular weights (> 500 atomic mass units, or amu) and a large number of hydrogen bond-donating groups. These characteristics contribute to polyphenols' large actual size and large apparent size (resulting from the formation of a large hydration shell), respectively (Sang et al 2006). In general, catechin levels in human plasma peak at 2-4 hours after ingestion (Higdon & Frei 2003). One study measured 0.6 μM EC, 0.7 μM EGCG, and 1.8 μM EGC in human plasma after 927 mg of catechins were administered. The highest reported levels of plasma catechins were 1 μM after regular, repeated green tea consumption of 8 cups per day (van het Hof et al 1999).

Despite the poor bioavailability of tea polyphenols, scientists have detected these compounds *in vivo*, and analyzed their absorption patterns and tissue distribution. Okushio and colleagues reported that, after orally administering tea catechins (EC, ECG, EGC, and

EGCG) to rats, these compounds were detectable in the portal vein. This indicates that tea catechins are, at least in part, absorbed in the intestines (Okushio et al 1996). In another study, rats were treated with 0.6% green tea polyphenols in drinking water over a 28-day period. After 14 days, plasma concentrations of EC and EGC were much higher than EGCG. Plasma catechin levels decreased over the following 14 days, suggesting an adaptive effect. Catechin concentrations, especially EGCG, were highest in the rat esophagus, intestine, and colon (sites in which tea catechins have direct contact), and lowest in the kidney, bladder, lung, and prostate (sites which rely on systemically bioavailable EGCG). When the analysis was performed in mice fed the same polyphenol preparation, EGCG levels were significantly higher in the plasma and lung. This implies that the absolute bioavailability of EGCG is higher in mice than rats, and that bioavailability of tea polyphenols is species specific (Kim et al 2000). In humans, Yang and others reported a maximum plasma catechin concentration 1.4-2.4 hours after oral ingestion of decaffeinated green tea (1.5, 3.0, and 4.5 g). After 8 hours, 90% of the EC and EGC were detected in the urine while EGCG was not detected. None of the catechins were measurable in the plasma after 24 hours. Just as in mice, the bioavailability of EGCG was lower than that of EGC in humans. Additionally, increased doses did not significantly raise plasma catechin levels (Yang et al 1998). This demonstrates a saturation effect, suggesting that catechins are absorbed from the intestines by passive diffusion (Sang et al 2006).

Black tea polyphenols are less widely studied because there is a lack of sufficient analytical methods to detect these compounds in plasma. Mulder et al identified theaflavins from black tea in human plasma and urine. After consumption of 700 mg of a mixture of

pure theaflavins (equivalent to approximately 30 cups of black tea), plasma and urine levels were only 1 ng/ml and 4.2 ng/ml, respectively at 2 hours post-consumption. Other theaflavin derivatives, including theaflavin mono- and di-gallates, were not detectable (Mulder et al 2001).

Metabolism

The relatively low absorption and bioavailability of tea polyphenols may be attributed to the rapid and extensive metabolic processes that occur following consumption of these compounds. Tea flavonoids, such as quercetin and epicatechin, are converted to glucuronide, *O*-methylated, and sulfate conjugates during absorption in the small intestine and again in the liver. Figure 1.9 shows examples of epicatechin and quercetin metabolites.

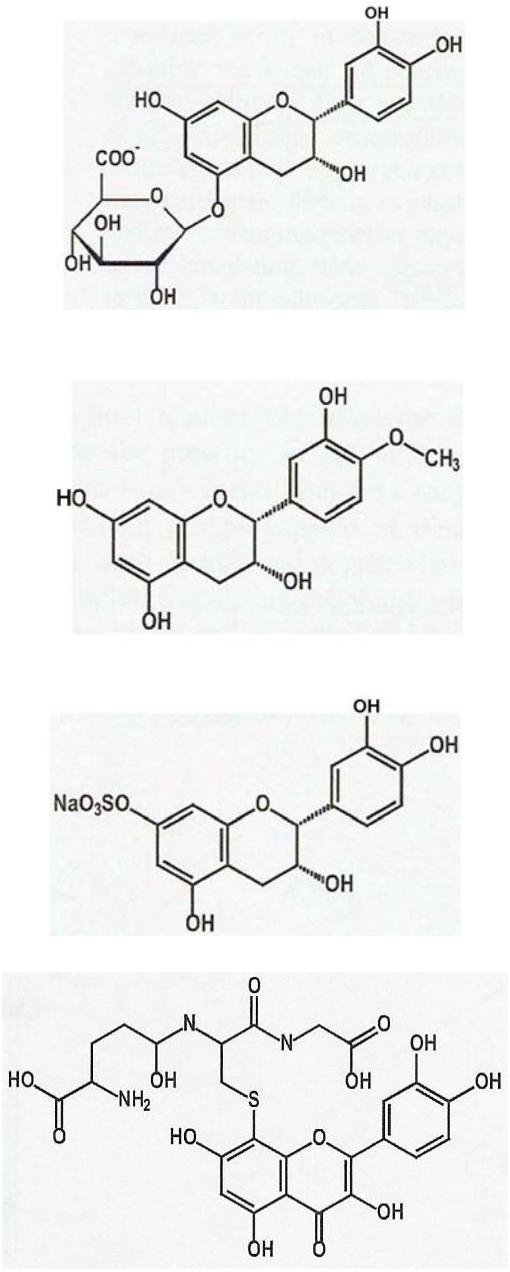


Figure 1.9. Metabolites of Epicatechin and Quercetin (Top to bottom: epicatechin-5-O-D-glucuronide, 3'-*O*-methyl epicatechin, epicatechin-7-sulfate, and 8-glutathionyl quercetin) (Williams et al 2004)

The enzymes responsible for polyphenol metabolism include UDP-glucuronosyltransferases (UGT), catechol-*O*-methyltransferase (COMT), and phenolsulfotransferases (SULT). Their functional sites include the small intestine (especially the jejunum), liver, and kidney (Higdon & Frei 2003). There is also evidence for further flavonoid metabolism in which flavonoids are broken down into phenolic acids by gut enzymes produced by colonic microflora. The resulting compounds may be absorbed and metabolized again in the liver (Williams et al 2004). Researchers have shown that tea catechins found in human plasma were present in their glucuronidated and O-methylated forms (Higdon & Frei 2003). Researchers should acknowledge the presence of these tea metabolites, especially when analyzing their potential biological activity *in vivo.* Failure to detect for tea metabolites may result in lower predicted concentrations of tea flavonoids in the body, and inaccurate estimations of relative bioavailability.

1.5 Reported Health Effects of Tea

The earliest known reports on the healthful properties of green tea date back to 2700 BC. In 1211 AD, the Japanese monk Eisai wrote a book entitled *Kissa-yojoki,* which loosely translates into "How to keep healthy drinking tea." Throughout history, documented reports have emerged on the use of tea in treating numerous ailments, including fever, headaches, and stomachaches (Benelli et al 2002).

In recent decades, scientific investigations have shown that polyphenols in foods, such as fruits, vegetables, and plant-based beverages, such as coffee, tea, and wine, may be linked to beneficial health effects. Studies have established that polyphenols exhibit

antioxidant and anti-inflammatory effects, and may be effective in treating the pathologies associated with an array of health conditions (Biesalski 2007), such as cancer, degenerative diseases, and acute and chronic inflammation (e.g. heart disease, rheumatoid arthritis, atopic dermatitis) (Kim et al 2004). Tea polyphenols, especially flavonoids, have ideal chemical structures that allow them to function as powerful antioxidants. These compounds also suppress inflammatory activity by interacting with metabolites in arachidonic acid (AA)-dependent and independent pathways. Because tea compounds have shown notable antioxidant and anti-inflammatory activity, there is much interest in using tea as a natural therapeutic agent to combat chronic health conditions.

1.5.1 Antioxidant Activity of Tea

Tea has experimentally demonstrated significant antioxidant activity, especially during conditions that simulate oxidative stress, inflammation, and the subsequent production of reactive oxygen species (ROS) (e.g. superoxide anion, hydroxyl free radicals, hydrogen peroxide, lipid peroxide) (Satoh et al 2005) and reactive nitrogen species, such as nitric oxide (NO). Normally, these compounds are synthesized to help the body's immune response in fighting pathogens (Sutherland et al 2006). However, an excess of free radicals produced from ROS and NOS in the body can cause cell damage, especially to proteins, lipids and DNA. This can lead to lipid peroxidation, apoptosis (Watanabe et al 1990), DNA strand disruption, and interruption of calcium ion metabolism (Halliwell & Cross 1994). Many human diseases and conditions, including heart disease, cancer, diabetes, Alzheimer's disease, and aging are strongly associated with oxidative tissue damage (Chen & Dou 2008).

Teas have the potential to be utilized as naturally occurring antioxidants for treating the symptoms associated with the development of disease. Teas contain flavonoids, such as EGCG and theaflavins, which have favorable chemical structures that enable tea to function as effective antioxidants. At adequate amounts in the body, tea flavonoids may quench free radicals, decrease oxidative stress, and prevent prolonged inflammation. Tea flavonoids also appear to exhibit more powerful antioxidant activity as compared to other antioxidant nutrients and plant-based flavonoids. On the other hand, tea flavonoids can display prooxidant activity when present at higher concentrations.

1.5.2 Polyphenol Structure-Function Relationships

Polyphenols, especially flavonoids, have ideal chemical structures for functioning as antioxidants and as free radical scavengers. Tea is rich in flavonoids, such as catechins in green tea, theaflavins in black tea, and flavonols (e.g. quercetin, myricetin, and kaempferol) in both teas. Figure 1.10 shows the structure of the flavonol quercetin, which possesses important features that define the antioxidant activity of flavonoids. These include: the *ortho*-3',4'-dihydroxy moiety in the B-ring (shaded blue) and the *meta* 5,7-dihydroxy set in the A-ring (red stars). The presence of the 2,3-double bond (shaded red) with the 4-keto group (shaded green) and the 3-hydroxyl moiety in the C-ring (purple circle) is also important in electron delocalization, especially when the *o*-dihydroxy group in the B-ring is present (shaded yellow) (Rice-Evans et al 1997, Williams et al 2004).

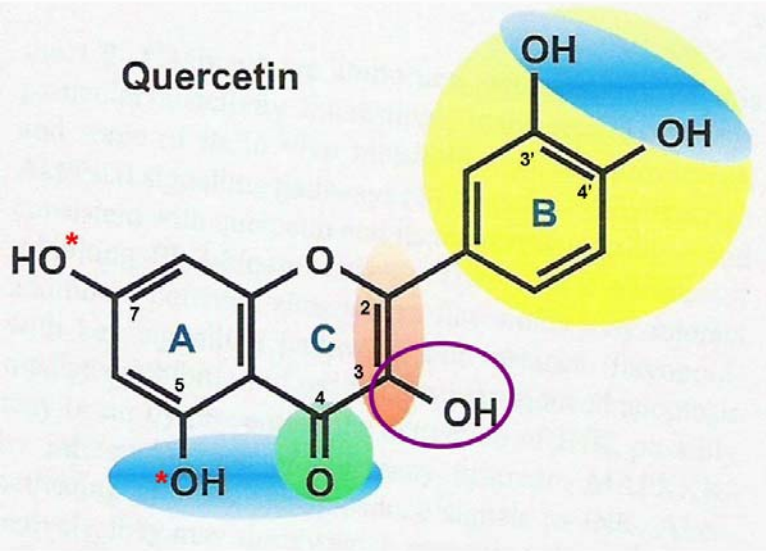


Figure 1.10. Structure of the Flavonol Quercetin and Highlighted Features of Flavonoid Antioxidant Activity (Williams et al 2004)

Flavonoids can act as free radical scavengers by directly quenching reactive oxygen species (ROS) and chelating metal ions such as iron (III) and copper (II). This leads to the inhibition of transition metal-catalyzed free radical formation. Metal chelation occurs when transition metal ions specifically bind to the phenolic groups on the *o*-3',4'-dihydroxy positions in the B-ring. Metal ions can also bind to 4-*keto*, 3-hydroxy or 4-*keto*, 5-hydroxy structures in the C-ring of flavanols (Rice-Evans et al 1997).

Reported health benefits of tea are generally attributed to the antioxidant properties of its polyphenolic compounds. A number of reports have also revealed that tea compounds are more effective than other antioxidants. ECG and theaflavin digallate have significantly higher total antioxidant activity (4.9 $\pm$ 0.02 mM and 6.2 $\pm$ 0.43 mM, respectively) than other antioxidants, including Vitamins C (1.0 $\pm$ 0.02 mM) and E (1.0 $\pm$ 0.03 mM), and other

flavonoids (antioxidant activity ranged from 0.8 $\pm$ 0.5 to 4.8 $\pm$ 0.19 mM) (Rice-Evans et al 1997). Additionally, Jovanavic and others showed that EGC and EGCG have the ability to function as better free radical scavengers than Vitamin E due to their lower reduction potentials. The researchers suggest that this property makes EGC and EGCG better electron donors than Vitamin E (Jovanavic et al 1996). Green tea catechins, especially EC and EGCG, have been shown to be 20 times more potent than ascorbic acid. The scientists observed that the antioxidant activity of these catechins increased as the number of *o*-dihydroxy groups increased (Vinson et al 1995).

Other studies have shown that tea constituents possess a higher antioxidant capacity than other plant-based flavonoids. Cao and colleagues found that green and black tea have a significantly higher antioxidant activity against peroxyl radicals compared to a number of vegetables, including garlic, Brussel sprouts, celery, kale, and spinach. However, the teas also demonstrated a pro-oxidant activity in the presence of Cu^{2+}. Cao and colleagues stated that the pro-oxidant activity of teas may not be significant *in vivo* since transition metals are largely suppressed (Cao et al 1996). In another study, the researchers found that catechin and epicatechin in particular were among the most potent antioxidants of the 24 plant-based flavonoids that were examined. The antioxidant activity of these compounds was mainly attributed to the 2,3 double bond in the C-ring (Rice-Evans et al 1995).

In contrast, some studies have reported an adverse effect from excessive concentrations of tea or its polyphenolic compounds. A 2002 study demonstrated that, at low concentrations (1-50 μM) *in vitro*, catechins function as antioxidants and as suppressors of apoptosis. However, reverse effects, including hydrogen peroxide-induced oxidative

damage, exaggerated oxidant activity, DNA damage, and cytotoxicity were observed at concentrations of 100-500 μM of EGC and EGCG (Szeto & Benzie 2002). Green tea extract (10-200 μg/ml) and EGCG applied at 20-200 μM to RAW 264.7 macrophages also produced the same effects. The green tea extract actually produced a more powerful pro-oxidant effect than hydrogen peroxide (Elbling et al 2005).

1.5.3 Inflammation and the Incidence of Chronic Disease

Inflammation is defined as the body's primary response to injury and is characterized by symptoms such as fever, swelling, redness, pain, and loss of function (Kim et al 2004). When an inflammatory response is triggered, the movement of serum proteins and leukocytes (e.g. macrophages, neutrophils, and eosinophils) from the blood to the extra-vascular tissue is activated. A large number of mediators, including eicosanoids, adhesion molecules, cytokines, and chemokines function during acute inflammation to regulate the recruitment of inflammatory cells and control vascular changes (Santangelo et al 2007). Typically, this complex process is self-limiting and is tightly regulated to prevent damage to the host. When the self-limiting capacity of the inflammatory response fails, a potentially deadly, chronic state of inflammation results. The incidence of many human conditions and diseases, such as cancer, obesity, type II diabetes, cardiovascular disease, aging, and neurodegenerative diseases is largely attributed to excessive inflammation (Bengmark 2004).

Inflammation can occur in the body via a number of cellular signaling pathways, including arachidonic acid (AA)-dependent and independent pathways. Researchers have demonstrated that tea and its compounds exhibit anti-inflammatory effects through tea and

tea polyphenol inhibition of cell signaling compounds within these pathways. In AA-dependent pathways, tea polyphenols appear to suppress major inflammatory mediators, including cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) enzymes. In AA-independent pathways, tea and its components promote the production of inhibitory proteins that prevent the expression of NF-κB, a key inflammatory regulator. The following section will discuss the specific teas and tea compounds that are responsible for anti-inflammatory activity, and the mechanisms by which these effects are achieved.

1.5.3.1 Anti-Inflammatory Effects of Tea on Arachidonic Acid-Dependent Pathways

Arachidonic acid (AA) is a hydrolysis product of cell membrane phospholipids that acts as a signaling molecule. AA metabolism is initiated by its release from membrane phospholipids by phospholipase A_2 (PLA_2) and leads to the production of eicosanoids through three major pathways (Figure 1.11). In the cyclooxygenase (COX) pathway, COX enzymes produce thromboxanes, prostaglandins, and prostacyclins. In the lipoxygenase (LOX) pathway, leukotrienes (LTs), hydroperoxyeicosatetraenoic acids (HpETES), and hydroxyeicosatetraenoic acids (HETEs) are formed. The third pathway involves the epoxygenase enzymes, which synthesize other HETEs and *cis*-epoxy-eicosatrienoic acids (EETs). These biologically active AA metabolites have a large array of functions in the body, including pro-inflammatory and anti-inflammatory effects (Boron & Boulpaep 2005, Yoon & Baek 2005).

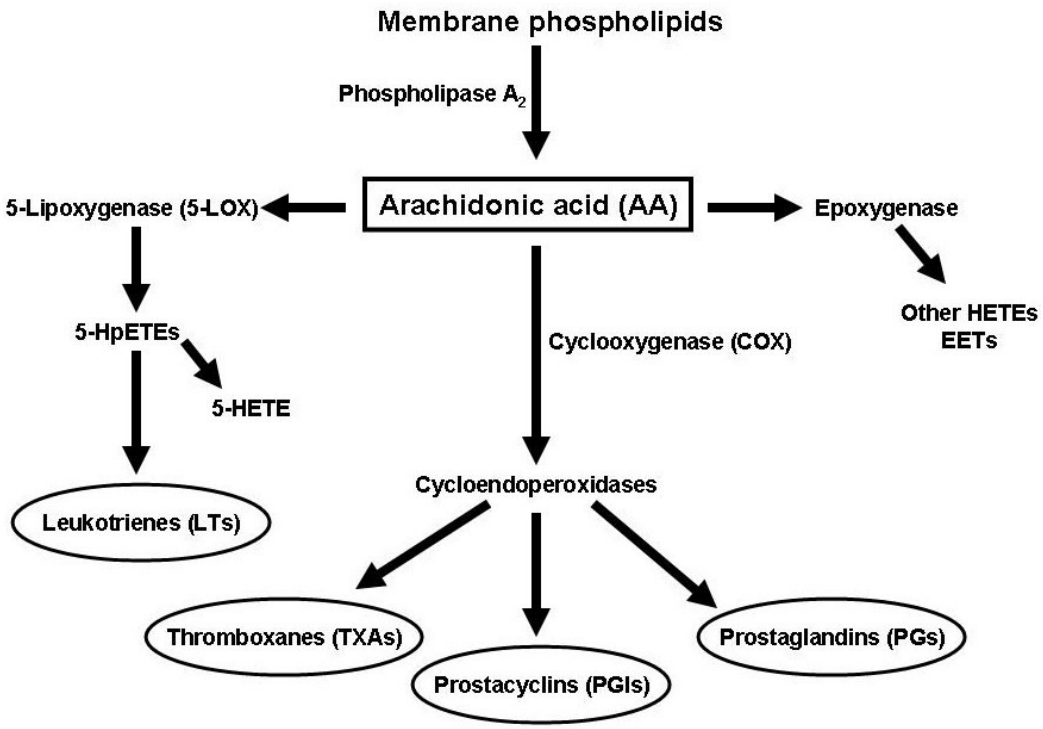


Figure 1.11. The Arachidonic Acid-Dependent Pathway

Tea polyphenols can target different components of the AA pathway (e.g. suppression of eicosanoid-producing enzymes, inhibition of AA metabolites). Since COX and LOX pathways are the major routes for the formation of inflammatory mediators, the following sections will focus on these particular pathways.

1.5.3.2 Cyclooxygenase (COX) Pathway

COX enzymes are isoforms of prostaglandin H synthase (PGH_2) that are involved in the conversion of AA into prostaglandins (PGs; i.e. PGE_2, $PGF_{2\alpha}$), thromboxane A_2 (TXA_2), and prostacyclin I_2 (PGI_2). Prostaglandins participate in the aggregation of platelets, airway

constriction, regulation of renal blood flow, and inflammation. TXA_2 is also involved with platelet aggregation, as well as coordinating platelet release and vasoconstriction of small blood vessels. PGI_2 is an anti-inflammatory AA metabolite that prevents the aggregation of platelets and vasodilates blood vessels (Boron & Boulpaep 2005).

There are two major types of COX, COX-1 and COX-2. COX-3, a third variant, also exists. COX-1 is constitutively expressed in many cells, while the induction of COX-2 is regulated by cytokines, mitogens, tumor promoters, and growth factors, and leads to the synthesis of prostaglandins. COX-2 is largely expressed in activated macrophages, mast cells, fibroblasts, and other cells that participate in the inflammatory response (Santangelo et al 2007). COX-1 and COX-2 both catalyze the rate-limiting step of prostaglandin production and are the targets of non-steroidal anti-inflammatory drugs, or NSAIDs. COX-3 is derived from the COX-1 gene and is sensitive to analgesic and antipyretic drugs, particularly acetaminophen (Chandrasekharan et al 2002). Studies have demonstrated that COX-2 is over-expressed in chronic inflammatory diseases such as arthritis (Sano et al 1992), and in almost every premalignant and malignant condition involving the colon (Kargman et al 1995), pancreas, liver, breast, lung, skin, bladder, stomach, esophagus, head, neck (Subbaramaiah & Dannenberg 2003), and prostate (Mohan & Epstein 2003).

COXs, particularly COX-2, are key enzymes that stimulate the inflammatory response via the production of prostaglandins. A number of studies have investigated the ability of tea polyphenols to suppress this enzyme. Kundu and colleagues demonstrated that COX-2 was down-regulated by EGCG in 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced human mammary cells (MCF-10A) and in mouse skin. The authors also found that

EGCG prevented catalytic activity of extracellular signal regulated protein kinase (ERK) and of p38 mitogen-activated protein kinase (MAPK), which are upstream enzymes that regulate the expression of COX-2 (Kundu et al 2003). EGCG and green tea catechins can also inhibit COX in LPS-induced macrophages (Gerhauser et al 2003). Other researchers have observed that, at doses of 30 μg/ml, black tea theaflavins and EGCG can significantly suppress COX in microsomes from tumors and from normal colon mucosa (Hong et al 2001). These studies show that tea flavonoids, especially EGCG, have notable anti-inflammatory function via its suppression of inflammatory COX enzymes.

1.5.3.3 Lipoxygenase (LOX) Pathway

Lipoxygenase (LOX) enzymes synthesize leukotrienes (LTs) from arachidonic acid (AA). There are 3 known isoforms of LOX. 5-LOX forms 5- hydroxyeicosatetraenoic acid (HETE) and many LTs, such as leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4), which are involved in a number of allergic disorders and inflammatory diseases. 12-LOX also participates in allergic and inflammatory conditions, and 15-LOX produces anti-inflammatory 15-HETE (Aggarwal & Shishodia 2006). Table 1.1 shows examples of several human diseases in which LTs may have an effect (Table 1.2) (Boron & Boulpaep 2005).

Table 1.2. Role of Leukotrienes in Human Diseases (Boron & Boulpaep 2005)

Disease	Evidence
Asthma	Bronchoconstriction from inhaled LTE_4; detection of LTC_4, LTD_4, and LTE_4 in serum and/or urine of asthma patients
Psoriasis	LTB_4 and LTE_2 found in fluids from psoriatic lesions
Adult respiratory distress syndrome	Elevated LTB_4 levels found in the plasma of ARDS patients
Allergic rhinitis	Elevated LTB_4 levels found in nasal fluids
Gout	LTB_4 in joint fluid
Rheumatoid arthritis	Elevated LTB_4 detected in serum and joint fluid
Inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis)	LTB_4 in GI fluids and LTE_4 in urine

Researchers have analyzed the ability of tea polyphenols to act as anti-inflammatory compounds by inhibiting LOX enzymes. Hong et al found that, at a concentration of 30 μg/ml, EGCG, ECG, and EGC from green tea and theaflavins from black tea reduced LOX-dependent activity in colon tumors (68.2% $\pm$ 3.1, 74.4% $\pm$ 0.8, 44.1% $\pm$ 3.3, and 29.8% $\pm$ 1.1

inhibition, respectively) (Hong et al 2001). Flavonols, such as quercetin, kaempferol, and myricetin, have also been shown to inhibit 5-LOX (Kim et al 2004). A 1984 publication indicated that quercetin and kaempferol were effective at stopping 12-LOX activity in mouse epidermis. The researchers stated that quercetin's ability to inhibit 12-LOX may be related to the presence of a C-2,3 double bond within its structure (Nakadate et al 1984). In another study, Kim et al determined that quercetin also strongly reduced 12- and 15-LOX activity in epidermal homogenate from guinea pigs (Kim et al 1998). These *in vitro, ex vivo,* and animal studies demonstrate that tea flavonoids suppress inflammation by their inhibitory action on LOX enzymes. The structural features of tea flavonoids, particularly the C-2,3 double bond in the C-ring, are closely related to their antioxidant and anti-inflammatory effects.

1.5.4.1 Anti-Inflammatory Effects of Tea on Arachidonic Acid-Independent Pathways

Numerous researchers have proven that tea polyphenols can alter arachidonic acid (AA) metabolism, primarily by inhibiting the eicosanoid-producing enzymes cyclooxygenase (COX) and lipoxygenase (LOX). In addition, tea polyphenols can also target components of AA-independent pathways, such as nitric oxide synthase (NOS) and the transcription factor nuclear factor kappa B (NF-κB) (Biesalski 2007). Like COX, LOX, and their inflammatory mediators, these two compounds also play key roles in regulating, initiating, and propagating inflammation.

1.5.4.2 Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide synthase (NOS) is responsible for producing nitric oxide (NO), a gaseous free radical, when L-citrulline is synthesized from L-arginine (Aggarwal & Shishodia 2006). There are three known isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The former two isoforms are constitutively expressed in the body and form nanomolar concentrations of NO in order to maintain normal cellular function (Kim et al 1999), including neurotransmission, vasodilation, and host defense (Mayer & Hemmens 1997). In contrast, iNOS is an inducible enzyme that is highly expressed in response to inflammatory stimuli, such as LPS, tumor necrosis factor alpha (TNF-α) (Ding et al 1988), and/or cytokines (Nathan 1992). When iNOS is induced, a micromolar amount of NO is made for a relatively long period of time. Although producing large amounts of NO by iNOS is important for host defense, excessive NO can react with superoxide anions to form peroxynitrite, a potent reactive nitrogen species, that can damage proteins, DNA, and lipids (Lin et al 2006). Thus, elevated NO production by iNOS has been implicated in the development of certain health conditions, including rheumatoid arthritis, septic and hemorrhagic shock, chronic infections (Nussler & Billiar 1993), diabetes, renal disease, cardiovascular disease (Lin et al 2006), and tumorigenesis (Aggarwal & Shishodia 2006).

There is significant interest among researchers in evaluating the potential anti-inflammatory effect of tea and its flavonoids on iNOS and NO since these compounds are inflammatory mediators. The synthesis of NO from iNOS can ultimately lead to the activation of COX-2, subsequent production of PGE_2, and enhance inflammation (Djoko et al 2007). Lin & Lin determined that EGCG, ECG, and gallic acid inhibited the expression of

iNOS and NO production in LPS-activated mouse peritoneal macrophages. The researchers reported that iNOS induction was blocked (especially by EGCG) by promoting the presence of IκB (an NF-κB inhibitory protein) in the cytosol and reducing iNOS mRNA expression by preventing NF-κB binding to the iNOS promoter (Lin & Lin 1997). In another study, both EGCG and quercetin had the same suppressive effect on iNOS and NO in LPS/interferon-gamma (IFNγ)-stimulated C6-astrocytes (Soliman & Mazzio 1998).

Tea as a whole food also appears to suppress iNOS activity. Lin et al demonstrated that, at a concentration of 500 μg/ml, the green tea inhibited iNOS enzyme activity and NO production more strongly than black tea (81.3%- 83.3% NO inhibition by green tea vs. 59.8%-66.4% NO inhibition by black tea). Based on these results, Lin and colleagues speculated that heavy fermentation of tea significantly decreased the suppression of NO by tea in LPS-stimulated RAW 264.7 cells (Lin et al 2006). Although the studies above have shown that some tea polyphenols can suppress iNOS induction and NO synthesis, the exact mechanisms by which this occurs is still not well understood (Kim et al 2004). These reports demonstrate the efficacy of tea and its constituent flavonoids to decrease inflammation by enhancing inhibitory protein production and preventing iNOS and NO synthesis.

1.5.4.3 Nuclear Factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is a transcription factor found in most cells and is critical in the regulation of the body's inflammatory response. Like iNOS and NO, NF-κB is also a component of arachidonic acid (AA)-independent pathways. In inactivated cells, NF-κB exists in the cytoplasm bound to a family of inhibitory proteins known as IκB. A variety

of cell stimuli, including ultraviolet (UV) light, endotoxins, free radicals, inflammatory cytokines (e.g. interleukin (IL)-1, TNF-α, etc), bacterial LPS, phorbol ester, or other cellular stressors, can cause rapid phosphorylation of the IκBα and IκBβ portions of the IκB protein complex by IκB kinase (Chen et al 1995). This leads to the ubiquitination (process of protein inactivation in which the protein ubiquitin binds to the protein and transports it to the proteosome for degradation) and degradation of IκB by proteosomes (large proteolytic enzyme complex) and NF-κB release. The liberated NF-κB translocates to the nucleus, binds to DNA, and activates transcription (Higdon & Frei 2003) of over 200 inflammatory and other genes (e.g. COX-2, iNOS, TNF) (Aggarwal & Shishodia 2006). Specifically, these activated genes include those that promote cellular transformation, proliferation, metastasis, and suppression of apoptosis (Biesalski 2007) (Figure 1.12).

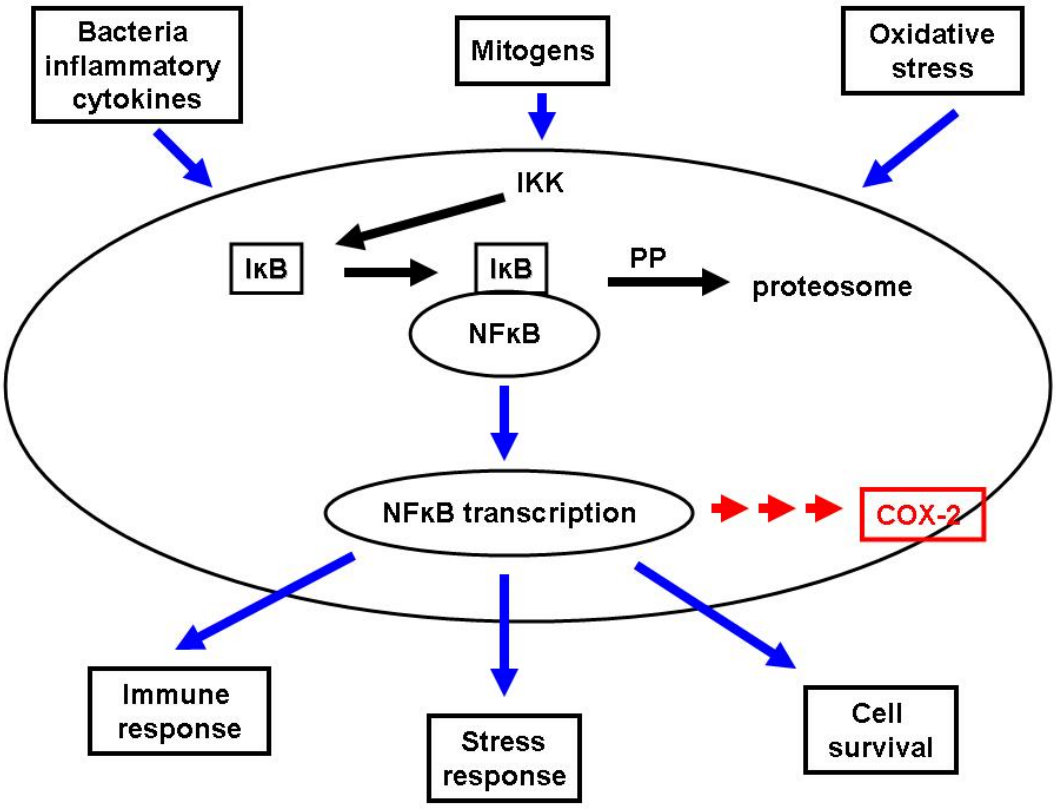


Figure 1.12. NF-κB Activation (Victor et al 2004)

Since NF-κB is redox-sensitive, its over-expression is more likely to occur with a poor antioxidant status, which will consequently produce an exaggerated stress response. The presence of antioxidants, such as tea polyphenols, can inhibit NF-κB activation. Green tea catechins and theaflavins from black tea have exhibited the ability to suppress NF-κB activation in various cultured cell lines (Higdon & Frei 2003). Pan and others investigated the effect of EGCG and black tea theaflavins on NF-κB activity in LPS-induced RAW 264.7 mouse macrophages. They found that these polyphenols, especially theaflavin-3,3'-digallate, inhibited IκB phosphorylation and IκK activity, preventing NF-κB from translocating to the

nucleus and binding to DNA (Pan et al 2000). Nomura et al observed that EGCG and theaflavins also inhibited IκK activity in a dose-dependent manner in TPA-treated mouse epidermal cells. These compounds blocked TPA-stimulated phosphorylation of IκBα (Nomura et al 2000). In cultured tumor cells, 1 to 10 μM of EGCG inhibited proteosome activity, causing an accumulation of the IκBα subunit in the cytoplasm and decreased activation of NF-κB. The researchers reported that the carbon within the ester bond of EGCG's structure was essential for proteasome inhibition (Nam et al 2001). Yang and colleagues also confirmed that EGCG was a potent inhibitor of IκK and of subsequent NF-κB activation in intestinal epithelial cells. The research group stated that the suppressive action of EGCG could be attributed to the presence of the gallate group within its structure (Yang et al 2001). Through the inhibition of NF-κB transcription, polyphenols in both black tea and green tea appear to prevent the production of hundreds of inflammatory metabolites formed when NF-κB is activated. Further investigation of the anti-inflammatory mechanisms of tea polyphenols on this key inflammatory mediator could lead to the development of plant-based treatments for systemic inflammation.

1.5.5 Methods of Analyzing Anti-Inflammatory Activity

A number of molecular biology techniques are commonly used to measure anti-inflammatory activity in cell models. Before these methods are performed, cell viability and potential cytotoxic effects must be evaluated using assays such as the Trypan Blue assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium (MTT) assay. Once viability of the cells and potential cytoxicity have been determined, anti-inflammatory activity can be

measured by analyzing major inflammatory markers, such as PGE_2 or COX-2. An enzyme-linked immunosorbent assay (ELISA) can be can be used to measure PGE_2. The BCA assay is done to determine total protein concentration for Western blotting. Finally, the presence of cyclooxygenase-2 (COX-2) can be detected with Western Blotting. ELISA and Western blotting provide evidence for potential anti-inflammatory activity by measuring the suppression of two specific inflammatory markers within the COX pathway.

1.5.5.1 Cell Viability Techniques

In order to maintain a successful mammalian cell culture, one must evaluate cell viability to determine cytotoxic or toxicological properties of compounds used experimentally to treat cells. There are a variety of methods used to evaluate cell viability, but two of the most commonly used techniques are the Trypan Blue (dye exclusion) assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium (MTT) assay. The Trypan Blue assay is a colorimetric analysis based on the selective permeability of viable cell membranes. Live cells typically present with a shiny appearance since their membranes do not absorb the dye. Dead cells, on the other hand, appear dark blue in color because their cell membranes are no longer selectively permeable. Thus, this assay is a rapid method of determining the number of live versus dead cells within a given volume of cell suspension (Jones & Senft 1985, Altman et al 1993).

The MTT assay is a colorimetric method that utilizes a tetrazolium salt to quantitatively detect mammalian cell survival, cytotoxicity, and proliferation. This assay identifies living cells only, and the resulting developed color is based on the extent to which

mitochondrial dehydrogenases in live cells can chemically reduce MTT to insoluble formazan salts (Mosmann 1983). The quantity of MTT-formazan product is verified spectrophotometrically after the formazan crystals have been dissolved in an appropriate solvent (Freshney 2005). A darker color is generally interpreted as a greater number of viable cells, while a lighter color indicates a lower number of live cells (Mosmann 1983). Our study utilizes these two techniques to establish potential cytotoxic effects of varying concentrations of green tea, black tea, and LPS on RAW 264.7 mouse macrophage cells.

1.5.5.2 Prostaglandin E_2 (PGE_2) Enzyme-Linked Immunosorbent Assay (ELISA) Experimental Detection of PGE_2

We measured the potential anti-inflammatory effects of tea by analyzing PGE_2 levels in tea-treated, LPS-induced RAW 264.7 cells using the Cayman Chemical Monoclonal PGE_2 Enzyme-Linked Immunosorbent Assay (ELISA). This kit measures PGE_2 via the competition between a PGE_2-acetylcholinesterase (AChE) conjugate, or PGE_2 Tracer and PGE_2 for a fixed quantity of PGE_2 Monoclonal Antibody. Because the concentration of PGE_2 is variable while the concentration of PGE_2 Tracer is constant, the amount of PGE_2 Tracer that can bind to the PGE_2 Monoclonal Antibody is inversely related to the PGE_2 concentration in each well. The bottom of each well is coated with goat polyclonal anti-mouse IgG, which is the compound that the antibody-PGE_2 group binds to. Once this reaction occurs, the plate is washed to eliminate unbound reagents. Then, Ellman's Reagent (which contains an AChE substrate) is added to each well and forms a yellow color that is strongly absorbed at 412 nm. The intensity of the yellow color is proportional to the amount

of PGE_2 Tracer attached to each well, and is inversely proportional to the quantity of unbound PGE_2 within each well (Figure 1.13):

$$\text{Absorbance} \propto [\text{Bound } PGE_2 \text{ Tracer}] \propto 1/[PGE_2]$$

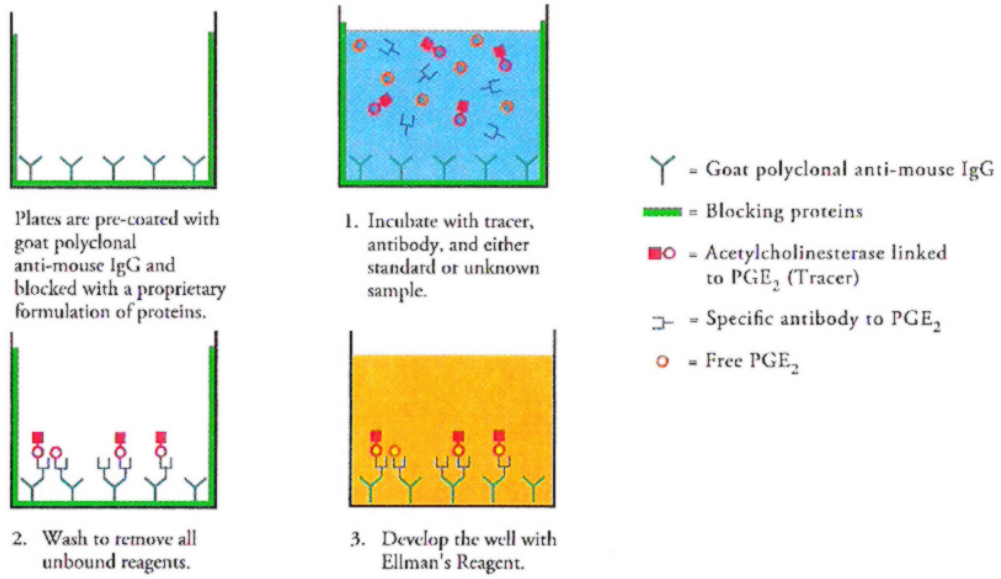


Figure 1.13. Schematic of Cayman Chemical ACE™ Competitive PGE_2 ELISA

1.5.5.3 Protein Determination and Western Blotting (COX-2 Detection)

Determination of Total Protein

Quantifying the amount of protein in a sample is necessary to evaluate the presence of a protein of interest (e.g. COX-2). This is also important for calculating equalized amounts of protein loaded into an electrophoresis gel. Total protein concentration can be colorimetrically identified and quantified by performing the bicinchoninic acid (BCA) assay. This method involves the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline solvent, and the

highly sensitive color formation that results from Cu^{+1} and the BCA reagent. The reaction product is formed by the chelation of two BCA molecules with one Cu^{+1}, which produces a purple color. The more intense the purple color, the higher the protein concentration in the sample (and vice versa). This mixture has a strong absorbance at 562 nm, and linearly increases with protein concentrations from 20-2,000 μg/ml (Smith et al 1985).

COX-2 Analysis

The presence of COX-2 in stimuli-induced cells can be detected by Western blotting. This is a molecular biology technique used to determine the presence of a protein of interest (i.e., COX-2). Generally, Western blotting involves electrophoresis of proteins on a gel, blotting them onto a membrane, probing the blot with a specific primary antibody, detecting the primary antibody with a compatible secondary antibody, and using visualization reagents to image the blot (Towbin et al 1979). COX-2 is a protein that, if present, produces a band at approximately 72 kD (Kundu et al 2001).

1.6 Clinical and Epidemiological Studies on Tea Consumption and Chronic Disease

A large number of studies have investigated the effects on teas and their flavonoids on various cellular signaling pathways. In addition to *in vitro* and *in vivo* research, scientists have observed positive health effects of tea via epidemiological and clinical studies. A majority of these projects have examined the possible relationship between tea compounds and different types of cancer or cardiovascular disease. While some studies show inverse

associations between tea consumption and the incidence of disease, the weight of evidence for the positive health effects of teas is still limited.

1.6.1 Tea Consumption and Cancer

In 2007, the American Cancer Society estimated that there were more than 12.3 million new cancer cases and 7.6 million cancer-related deaths worldwide (Chen & Dou 2008), making cancer largely responsible for chronic disease-related global mortality. A growing body of evidence from clinical and epidemiological studies has shown that consuming plant-based foods, such as tea, may be related to reducing cancer risk. Investigations performed in Asia, where green tea is frequently consumed in large amounts, have reported chemopreventive results. Nakachi and colleagues conducted a prospective 9-year study involving 8,552 Japanese adults. The researchers found that individuals who consumed 10 or more cups of green tea per day delayed the occurrence of cancer by 8.7 years in females and 3 years in males compared to those that consumed less than 3 cups daily (Nakachi et al 1997). In contrast, chemopreventive effects of tea are less frequent among Europeans who primarily consume black tea (Yang et al 2000).

The relationship between tea and the incidence of cancer also varies depending on the specific type of cancer. For example, studies performed in the United States, Italy, and the Netherlands have not shown a relationship between tea consumption and the incidence of breast cancer (McKay & Blumberg 2002). However, a Japanese study with 472 stage I and stage II breast cancer patients revealed an inverse correlation between green tea consumption and the rate of cancer recurrence after a 7-year period. The recurrence rate for patients

drinking 5 or more cups a day was 16.7% versus 24.3% for those that drank 4 or fewer cups a day (Nakachi et al 1998). For lung cancer, Mendilaharsu and colleagues found that consuming two or more cups of black tea each day was associated with a 66% decreased risk of lung cancer among 855 male smokers in Uruguay. The decreased risk was observed mostly in light smokers (Mendilaharsu et al 1998). In contrast, other studies have not shown a definitive relationship between black tea intake and lung cancer (Goldbohm et al 1996, Zheng et al 1998, Le Marchand et al 2000). For stomach cancer, a case-control study conducted in Japan on 22,834 individuals found that 7 or more cups of green tea daily was linked to a 31% decrease in the onset of stomach cancer. The researchers had no explanation for a site-specific mechanism in which green tea protects the stomach (Inoue et al 1998). In contrast, an 8-year prospective study of 26,311 Japanese adults at least 40 years old showed no correlation between tea intake and stomach cancer prevention (Tsubono et al 2001). While some studies show promise with the association between tea intake and decreased cancer risk, overall results are still mixed.

1.6.2 Tea Consumption and Cardiovascular Disease

Over the past few decades, studies have investigated the relationship between consuming tea and the incidence of cardiovascular disease. Sasazuki's group analyzed 512 patients aged 30 years and older who underwent coronary arteriography for the first time. They found that Japanese men who drank at least four cups of green tea daily showed a statistically significant inverse association with coronary atherosclerosis (Sasazuki et al 2000). The relationship between catechins and cardiovascular disease has been partly

attributed to the antioxidant activities of the catechins and preventing oxidation of low-density lipoprotein (LDL). Thus, the incidence of cardiovascular disease is reduced (Hertog et al 1993). In the Rotterdam Study, 1,836 men and 2,971 women with a mean age of 67.4 $\pm$ 7.8 years were followed for up to 7 years to detect for the incidence of myocardial infarction. The researchers revealed that individuals who drank at least 3 cups of black tea per day (>375 ml/day) had a lower risk of experiencing a myocardial infarction than those who did not drink tea at all. The inverse association between tea intake and myocardial infarction was stronger for fatal events compared to nonfatal events (Geleijnse et al 2002).

Associations between the reduced occurrence of cerebrovascular diseases and tea flavonoid intake, particularly catechins, have also been documented. Sato and colleagues performed a 4-year follow up study and found that the incidence of stroke and cerebral hemorrhage, as well as death from stroke, was more than twice as likely in individuals aged 60 years or older and drank less than 5 cups of green tea per day compared to those who consumed 5 or more cups daily. However, there was no relationship between drinking green tea and the incidence of hypertension (Sato et al 1989). In the Zutphen Study, 552 men between 50 and 69 years of age were followed for up to 15 years. The researchers found an inverse association between flavonoid consumption, particularly black tea, and the occurrence of stroke. Men who had a high flavonoid intake had a 73% decreased risk of experiencing a stroke (Keli et al 1996). Another Netherlands-based study reported an inverse correlation between catechin intake (primarily from black tea) and risk of ischemic heart disease in elderly Dutch men. However, this study did not find a relationship between catechin consumption and the incidence of stroke or stroke mortality (Arts et al 2001).

A number of studies have reported no significant decrease in chronic disease incidences as they relate to tea or tea flavonoid consumption. For a 12-year period between 1986 and 1998, Arts and colleagues followed 34,492 study participants to track the emergence of coronary heart disease, or CHD. They revealed an inverse association between (+)-catechin (C) and (-)-epicatechin (EC) intake and mortality caused by CHD. Apple and wine sources appeared to have the greatest correlation with a decreased CHD risk, but no association was found between tea catechins and the incidence of CHD (Arts et al 2001). Tabak and others reported that consuming solid fruit, not tea, was associated with a decreased incidence of chronic obstructive pulmonary disease (COPD) (Tabak et al 2001). Widlansky et al tested 66 human subjects who each drank 900 ml of black tea per day for an extended time, and determined that tea consumption only slightly increased plasma catechin levels. In addition, there was no observed relationship between improved endothelial function and changes in the levels of plasma catechins. Widlansky and his colleagues concluded that enhanced endothelial function and reduced cardiovascular disease risk after chronic tea consumption may actually be caused by noncatechin tea components, such as quercetin, kaempferol, or thearubigins (Widlansky et al 2005).

1.7 Recent Research on Tea Consumption and Human Disease

More recently, tea research includes analyzing the potential relationship between tea intake and reducing diabetes risk. Researchers have also reported possible associations between tea consumption and other health conditions, including stress, weight loss, and bone mineral density. *In vitro* and animal models have shown that tea may affect glucose

absorption and metabolism, decrease stress, promote weight loss, and increase bone mineral density. However, results are often conflicting among human studies.

1.7.1 Tea Consumption and Diabetes Mellitus

Diabetes mellitus is a prominent global health problem that affects almost 200 million people worldwide, or approximately 5% of the population (International Diabetes Federation 2005). Diabetes is a group of metabolic disorders that is characterized by glucose intolerance and hyperglycemia resulting from lack of insulin production, impaired insulin sensitivity, or both (Reaven 1995). In the United States and other developed countries, approximately 90-95% of individuals afflicted with diabetes have type 2 diabetes (International Diabetes Federation 2005). This type is often linked with obesity because obesity may lead to insulin insensitivity and hyperglycemia, which are characteristic of type 2 diabetes (Thorpe et al 2006, American Diabetes Association 2007). Lifestyle modifications, such as physical activity and dietary changes, may curb the symptoms associated with type 2 diabetes (Biesalski 2004). Changes in diet include higher intake of plant-based foods because their components, especially polyphenols, are linked to beneficial health effects (Willett 2006). Tea is a plant-based beverage containing polyphenols that may alter insulin signaling and the metabolism of glucose. Reports from *in vitro* and animal models have suggested a number of mechanisms by which tea and its constituents may affect glucose metabolism and diabetes. In rats, tea catechins suppress several enzymes that digest carbohydrates, including α-amylase, intestinal sucrase and α-glucosidase. This implies that glucose production may be slowed in the gut, lowering glucose and insulin levels (Kobayashi et al 2000, Shimizu et al

2000). Green, black, and oolong teas may also raise insulin sensitivity by enhancing insulin-stimulated glucose uptake in adipocytes. Tea polyphenols, such as epigallocatechin gallate (EGCG), epicatechin gallate (ECG), theaflavins, and tannins, may participate in improving insulin function (Anderson & Polansky 2002). Finally, green tea and EGCG may prevent liver, kidney, and pancreatic β-cell damage (Kao et al 2000, Crespy & Williamson 2004).

Several epidemiological studies have analyzed the possible association between tea intake and the risk for type 2 diabetes. Salazar-Martinez et al researched the correlation between tea consumption and diabetes in 84,276 American women from the Nurses' Health Study (aged 30-55 years) who were followed for 18 years; and 41,934 American men from the Health Professionals' Follow-Up Study (aged 40-75 years who were followed for 12 years. For both cohorts, there was no significant association between tea intake and risk for type 2 diabetes (Salazar-Martinez et al 2004). In a prospective cohort study in Japan, 17,413 Japanese adults aged 40-65 completed a 5 year follow-up questionnaire about self-reported, physician-diagnosed diabetes and tea consumption patterns. Researchers found that adults who drank more than 6 cups of green tea/day (1440 ml/day) had a 33% lower risk for diabetes. No correlation was found between diabetes risk and black or oolong teas (Iso et al 2006). In the United States, Song and colleagues examined the association between flavonoid intake and type 2 diabetes risk for a large cohort of women over 45 years old from the Women's Health Study. The researchers found no association between total flavonoid consumption or intake of flavonoid-rich foods and risk for type 2 diabetes. However, there was a relationship between women who consumed more than 4 cups of tea daily (960

ml/day) and diabetes risk (30% decrease) as compared to women who did not drink tea (Song et al 2005).

Human clinical interventions have also been done to determine the effects of tea on regulatory glucose molecules, but results are still conflicting. A recent study had young, healthy British men and women drink 75 g of glucose in either 250 ml of water (control), 250 ml of water with caffeine (caffeine content matched amount in black tea), or in 250 ml of water plus 1 g or 3 g of black tea. Fasting blood samples were collected, as well as at 30-minute time points up to 150 minutes post-treatment intake. The research group found that plasma glucose was lower at 120 minutes post-treatment consumption as compared to the control and caffeine treatments. The authors suggested that black tea may suppress glucose transport in the intestines and enhance insulin secretion from pancreatic β-cells (Bryans et al 2007). A study conducted in South Korea analyzed the effects of green tea intake on insulin resistance. South Korean men and women diagnosed with type-2 diabetes consumed either 900 ml of water with 9 g of green tea or water only for a 4 week period along with their normal diet. There were no significant changes found in fasting glucose levels or insulin concentrations (Ryu et al 2006). These observations spark debate as to whether biomarkers should be measured following tea consumption since tea polyphenols are quickly metabolized. Analyzing the effects of tea after an extended fasting period may yield inconsistent findings (Stote & Baer 2008).

1.7.2 Tea Consumption and Other Health Conditions

In addition to investigations of tea and chronic diseases, recent research on tea has examined the relationship between tea consumption and its effects on other health issues, such as weight loss, stress, and bone density. After a 4 month period of feeding mice green tea at 1% and 4% of their diets, the mice exhibited significantly lower food intake, body weight gain, and accumulation of fat tissue. Green tea has also been associated with a mild increase in thermogenesis, which leads to higher caloric expenditure. This phenomenon is explained by a possible synergistic effect between green tea's caffeine content (20-50 mg) in combination with its high catechin levels (Dulloo et al 1999, Dulloo et al 2000).

Green and black teas have been used for centuries in Asia as calming agents, and may be effective natural treatments for reducing stress and anxiety. The calming properties of teas are primarily result from the presence of L-theanine, an amino acid found in green tea and black tea. Tea polyphenols naturally convert theanine into catechins. Both green tea and black tea include approximately 20-40 mg of L-theanine. Theanine causes tranquilizing effects in the brain by facilitating the generation of alpha waves that help decrease anxiety, stress, and tension, causing a relaxed yet alert mental state. Theanine has also been shown to increase learning performance (in mice) and facilitate concentration (Talbott 2007, Huber 2003). During periods of stress, theanine can also lower cortisol levels (Cooper et al 2005).

While tea shows promise as a weight loss aid and calming agent, the associations between tea consumption and bone density are still unclear. A study from the United Kingdom involving 1256 women aged 65 to 76 found that tea drinkers had significantly greater mean bone mineral density measurements ($p < 0.05$). The researchers concluded that

tea consumption may help prevent osteoporosis in older women (Hegarty et al 2000). In contrast, an animal study reported that tannins in both green and black teas may reduce calcium and iron absorption. Black tea increased the rate of zinc absorption, while green tea decreased the rate (Zeyuan et al 1998). The conflicting results of these studies suggest that more work needs to be done to clearly define the effects of tea on bone density and the incidence of osteoporosis.

1.8 Summary and Conclusions

Many *in vitro, ex vivo,* and animal studies have provided ample evidence that tea polyphenols, particularly catechins and flavonoids, may prevent or reduce the incidence of a number of chronic diseases via their antioxidant properties, or by modulating various cellular signaling pathways. However, the evidence gathered from these studies has yet to extrapolate to human studies. The concentrations of tea and its compounds used in these models often far exceed (10-100 fold) the amounts of tea typically consumed by humans. The bioavailability of tea compounds, its metabolites, and their ability to elicit health effects at physiologically relevant levels is not yet understood. The experimental concentrations of teas may be higher than physiologically tolerable levels, which, if given to humans on a regular basis, could cause toxic effects over time. Additionally, cell culture conditions are drastically different from human physiological conditions, making it difficult to determine if the observed effects in culture will also occur in humans. The data from epidemiological and clinical studies are inconsistent as a result of many confounding factors (e.g. smoking, gender, alcohol intake, educational level, presence of caffeine and other non-polyphenol tea

compounds, etc). Many cohort and case-control studies use dietary assessment tools, such as food frequency questionnaires, that seldom differentiate between types of tea consumed, ask about its method of preparation, and define the amount per serving. Lack of or inaccurate reporting of tea preparation and consumption makes it extremely difficult to assess actual tea intake and the possible effects on health. Thus, a better understanding of the properties of tea, its polyphenolic compounds, and the specific mechanisms by which they exert their health effects in humans need to be established before recommendations for tea consumption can be implemented.

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CHAPTER 2: PREPARATION AND CHARACTERIZATION OF GREEN TEA AND BLACK TEA

2.1 ABSTRACT

There is growing interest in the antioxidant capacity of plant-based foods to combat excessive oxidative stress, chronic inflammation, and other pathologies linked to the development of chronic diseases. A variety of methods are available to measure antioxidant activity in plant-based foods. One of these ways is to evaluate phenolic content. The total phenol assay is a widely used colorimetric analysis of phenolic content in plants. This technique involves an oxidation/reduction reaction in which the phenols are oxidized by the reagent, causing a color change from yellow to blue. The Folin-Ciocalteu reagent is commonly utilized in this assay because it is very sensitive to reduction. Gallic acid is often used as the standard because it facilitates accurate and reproducible results. A number of studies have used this method to evaluate the differences in phenol content between different types of tea. Generally, green tea has a higher phenol content (and a greater reducing capacity) than black tea as a result of its lower state of oxidation or fermentation during processing.

The purpose of the following study was to compare the total phenolic content of green tea versus black tea. First, we developed a standardized method for preparing green tea and black tea in our laboratory. The total phenol assay was performed using the Folin-Ciocalteu method to determine the phenol content of each of the freshly prepared teas. Next, the assay was done using the media from lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages with or without the presence of green or black tea to establish if cell storage conditions and media components affected the total phenol content over a 24 hour period. Finally, a storage stability study was conducted on the prepared teas in order to observe

changes in the tea phenol concentration over time. We found that freshly prepared green tea has a significantly higher phenol content (1317.1 $\pm$ 6.0 GAE) than black tea (895.9 $\pm$ 10.7 GAE) ($p < 0.05$). The phenol content of both tea types increased significantly over the first 2 months of -80° C storage, but significantly decreased at 3 months post-preparation. Finally, the total phenol content between the experimental treatments over 24 hours were not significantly different ($p < 0.05$). This suggests that our experimental conditions are appropriate since they do not dramatically affect the stability of the phenol concentrations in the green tea and black tea.

2.2 INTRODUCTION

Measuring antioxidant activity of plants can be achieved by one of two accepted methods: (1) determining radical scavenging ability and (2) evaluating phenolic content. The total phenol assay, a colorimetric analysis using the Folin-Ciocalteu reagent, is a widely used technique for measuring phenolic compounds in plants because most plant-based antioxidants contain large quantities of polyphenols (Parejo et al 2002). The Folin-Ciocalteu reagent is very sensitive to reduction, mainly because of its relatively high molybdate content (Wu 1920). The basic mechanism of this assay is an oxidation/reduction reaction that involves a molybdotungstophosphoric heteropolyanion reagent:

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O$$

and

$$3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

When the phenols are oxidized by the reagent, the reagent undergoes a color change from yellow to blue. The λ_{max} for the resulting product is 765 nm (Prior et al 2005). Singleton and Rossi emphasized that using gallic acid as a standard would facilitate accurate and reproducible results (Singleton & Rossi 1965).

Studies have analyzed the differences in phenol content between different types of tea. Green tea generally appears to have a higher phenol content than black tea. Satoh and others found that the phenol content of green tea was 62.5 ± 0.3 Gallic Acid Equivalents (GAE)/200 μg water extract compared to black tea's phenol content at 50.2 ± 0.3 GAE/200 μg water extract (Satoh et al 2005). Lee and colleagues also found that green tea contained more phenolics than black tea (165 mg of GAE of green tea, 124 mg GAE of black tea) (Lee

et al 2003). Lin and others also confirmed that green tea has a higher phenol content (114.6 $\pm$ 5.4 mg GAE/g extract) compared to black tea (67.4 $\pm$ 3.9 mg GAE/g extract) (Lin et al 2006).

The differences in phenol content in green tea and black tea may be related to the differences in the chemical composition of each tea. Their chemical make-ups are dissimilar because each tea undergoes a unique method of processing. To produce green tea, the leaves of the *Camellia sinensis* plant are withered then steamed to prevent the leaves from oxidizing. Thus, green tea is not fermented, and up to 90% of the polyphenols are preserved in the leaves. On the other hand, leaves for making black tea are bruised and left in a dark room to fully oxidize. Some of the polyphenols in black tea are damaged as a result of the oxidation process (Khan et al 2008). Additionally, the theaflavins and thearubigins in black tea may interact with the tea leaves, causing a lower measured phenol content (Lakenbrink et al 2000).

The purpose of the following study was to compare differences in phenol content in freshly prepared green tea versus black tea, assess the stability of the polyphenols during exposure to experimental conditions, and monitor the changes in phenol content during extended storage. We also developed a standardized method for preparing green tea and black tea in our laboratory. The total phenol assay was performed using the Folin-Ciocalteu method to determine the phenol content of the freshly prepared green tea and black tea. The methods used were based on those conducted by Parejo and colleagues (2002). Next, the assay was performed using the media from LPS-stimulated RAW 264.7 macrophages with or without the presence of green or black tea to establish if cell storage conditions, media

components, and treatment compounds affected the total phenol content over a 24 hour period. Finally, a storage stability study was conducted on the prepared teas in order to observe changes in the tea phenol concentrations over time.

2.3 MATERIALS AND METHODS

Lipton® Green Tea and Black Tea were purchased from a local grocery store. Gallic acid, Folin-Ciocalteu phenol reagent, and anhydrous sodium carbonate were purchased from Sigma-Aldrich, Incorporated (St. Louis, MO). The ethyl alcohol (190 proof) was obtained from AAPER Alcohol and Chemical Company (Shelbyville, KY). The laminar flow hood used was a 1300 Series A2, Class II, Type A2 Biological Safety Cabinet bought from Thermo Scientific (Marietta, OH).

2.3.1 Preparation of Green Tea and Black Tea

The methods for tea preparation were similar to those performed by Lee et al (2003). First, 1.0 g of Lipton® Green Tea (GT) and 1.0 g of Lipton® Black Tea (BT) were measured. Next, two 250 ml beakers, each filled with 100 ml of deionized water, were covered with foil and brought to a boil. Once boiling, GT was placed into one beaker and BT was placed into the second beaker. Both beakers were immediately taken off the heat and the teas were steeped for 5 minutes. The beakers were transferred into the laminar flow hood and cooled for 1 minute. The tea liquid from each beaker were carefully pipetted into two separate 50 ml centrifuge tubes and centrifuged at 900 rpm (130 g) for 2 minutes. Finally, forty-eight 1 ml aliquots of each tea were measured and stored at -80° C.

2.3.2 Preparation of Total Phenol Assay Reagents

Sodium Carbonate Solution

First, 100 g of anhydrous sodium carbonate was weighed and transferred into a large beaker filled with 400 ml of deionized water. The beaker was slowly heated to boiling until the sodium carbonate was completely dissolved. Then, the beaker was removed from the hot/stir plate and allowed to cool to room temperature. The cooled solution was seeded with approximately 3 heaping tablespoons of anhydrous sodium carbonate. The beaker was covered with parafilm and left to sit for 24 hours. The following day, the solution was filtered through a Whattman® 4 filter, transferred to a 500 ml volumetric flask, and brought to volume with deionized water. The remaining solution was discarded after one month.

Phenol Stock Solution

Gallic acid was used as the standard. First, 0.25 g of dry gallic acid was measured into a 100 ml beaker. Next, 5 ml of ethyl alcohol (190 proof) was added to the beaker and gently swirled. Then, the beaker contents were transferred to a 50 ml volumetric flask, diluted to volume with deionized water, and mixed. The phenol stock solution was prepared and used the same day.

2.3.3 Preparation of Standard Curve

Eight disposable culture tubes were labeled as follows: 0, 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, and 2.0. The numbers on each tube corresponded to the amount (in ml) of added phenol stock solution. The total volume in each tube was brought up to 10 ml with deionized water

(i.e. 10 ml of deionized water was added to the tube labeled “0”, 9.9 ml of deionized water was added to the tube labeled “0.1”, and so forth).

Two separate sets of 8 disposable culture tubes were labeled in the following order: 0, 50, 100, 150, 250, 500, 750, and 1000. These values represented the gallic acid concentration (mg of gallic acid/L of solution) for each tube. Next, 0.1 ml of the diluted phenol stock solutions prepared above were pipetted into the appropriate tube (i.e. 0.1 ml of diluted phenol stock solution was pipetted from the tube labeled “2.0” into the tube labeled “1000”, 0.1 ml of diluted phenol stock solution was transferred from the “1.5” tube into the tube labeled “750”, and so forth). Then, 7.9 ml of deionized water was added to each tube and mixed. Folin-Ciocalteu phenol reagent (0.5 ml) was added to each of these tubes. After 30 seconds but before 8 minutes, 1.5 ml of sodium carbonate solution was added to each tube and gently vortexed. The tubes were covered and placed in the dark at room temperature for 2 hours. After the incubation period, absorbances were read at 765 nm using a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) (Figure 2.1). The total phenolic content was measured as gallic acid equivalents (GAE) of tea (mg of gallic acid/liter of solution) (Parejo et al 2002).

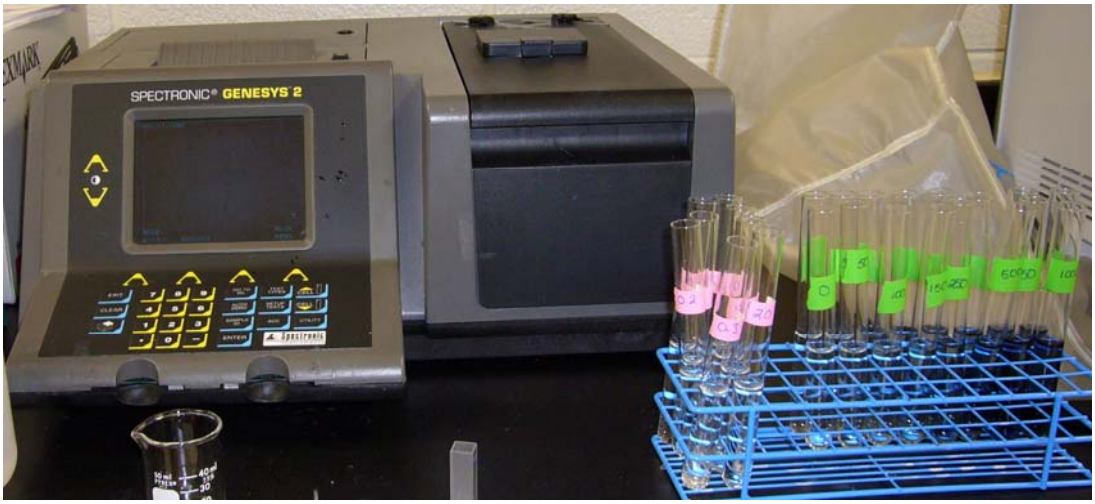

Figure 2.1. Gallic Acid Standards and Spectrophotometer

2.3.4 Preparation of Samples

First, 0.1 ml of tea or media sample was pipetted into a correspondingly labeled tube. Next, the total volume of each tube was brought to 8 ml by adding 7.9 ml of deionized water. The Folin-Ciocalteu phenol reagent (0.5 ml) was added to each sample tube and mixed. After 30 seconds but before 8 minutes, 1.5 ml of sodium carbonate solution was added to each tube and gently mixed. The sample tubes were covered and incubated in the dark at room temperature for 2 hours. The absorbance readings were taken at 765 nm using a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY). A standard curve based on gallic acid concentration was used to calculate total phenol content for the samples.

2.3.5 Statistical Analysis

For each experiment, all samples were run in triplicate. Statistical analysis was performed using the GLM procedure and Tukey's test on SAS® software (SAS Inc, Cary, NC). Data is expressed as the means ± standard error, and a probability (p) value < 0.05 is considered significant. On the figures below, treatments with the same letter indicate that the values were not statistically significant.

2.4 RESULTS

Figure 2.2 shows the effect of storage at -80° C on tea phenol content as compared to that of freshly prepared GT and BT. The phenol concentration of GT and BT stored at -80° C was also measured at 1 month, 2 months, and 3 months post-preparation. GT appeared to have a higher phenol content than black tea at all 4 time points. For both teas, the total phenol content increased at 1 month and 2 months post-preparation, and decreased slightly at 3 months post-preparation. The freshly prepared GT had an initial concentration of 1317.1 ± 6.0 GAE as compared to freshly prepared BT (895.9 ± 10.7 GAE). At one month post-preparation, the phenol concentration of GT increased significantly to 1684.7 ± 35.2 GAE, while BT concentration also significantly increased to 1088.0 ± 19.1 GAE ($p < 0.05$). At two months post-preparation, there was no significant change for either GT (1614.5 ± 21.5 GAE) or BT (1022.5 ± 8.6 GAE). Finally, GT and BT concentration were significantly lower at 3 months post-preparation (1439.8 ± 13.4 GAE, 942.7 ± 0.5 GAE, respectively).

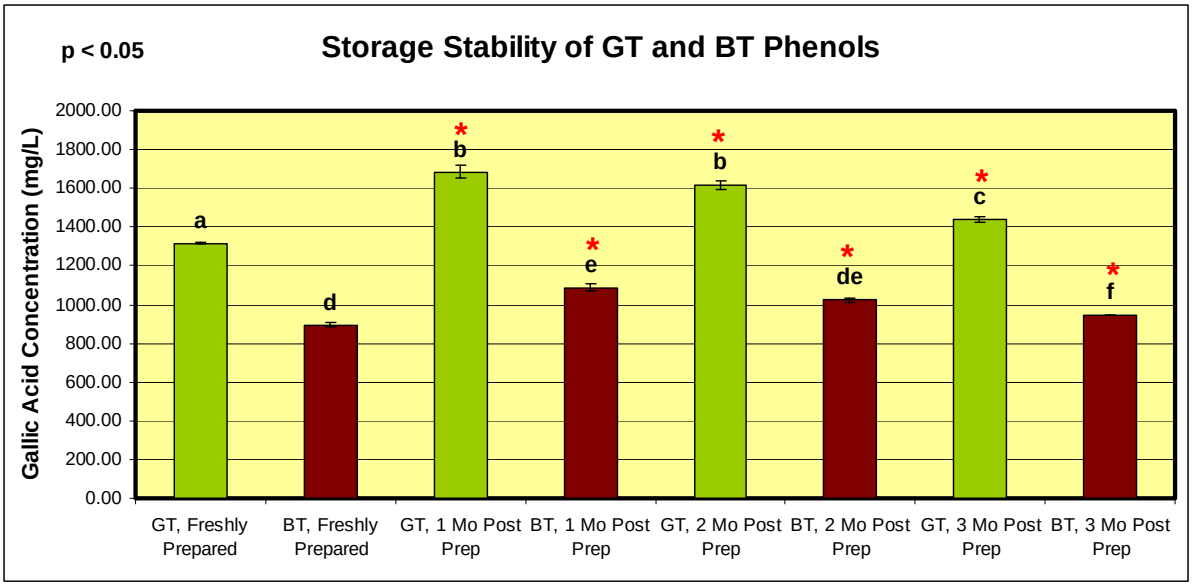

Figure 2.2. Effect of -80° C Storage on Phenol Content in GT and BT (Labeled with "*") vs. Phenol Content in Freshly Prepared GT and BT

Note: Treatments with same letter are not statistically different

Figure 2.3 represents the mean effect of incubation conditions (37° C, 5% CO_2), media composition, GT, BT, LPS, and of RAW 264.7 cells on the total phenol concentration at time 0, time 12 hours post-LPS application, and at 24 hours post-LPS application. There was a significant difference in mean GAE values between time 0 and time 12 and 24 hours post-LPS application, but no significant change between 12 and 24 hours post-LPS application. There was no significant difference in phenol content between the mean treatments ($p < 0.05$).

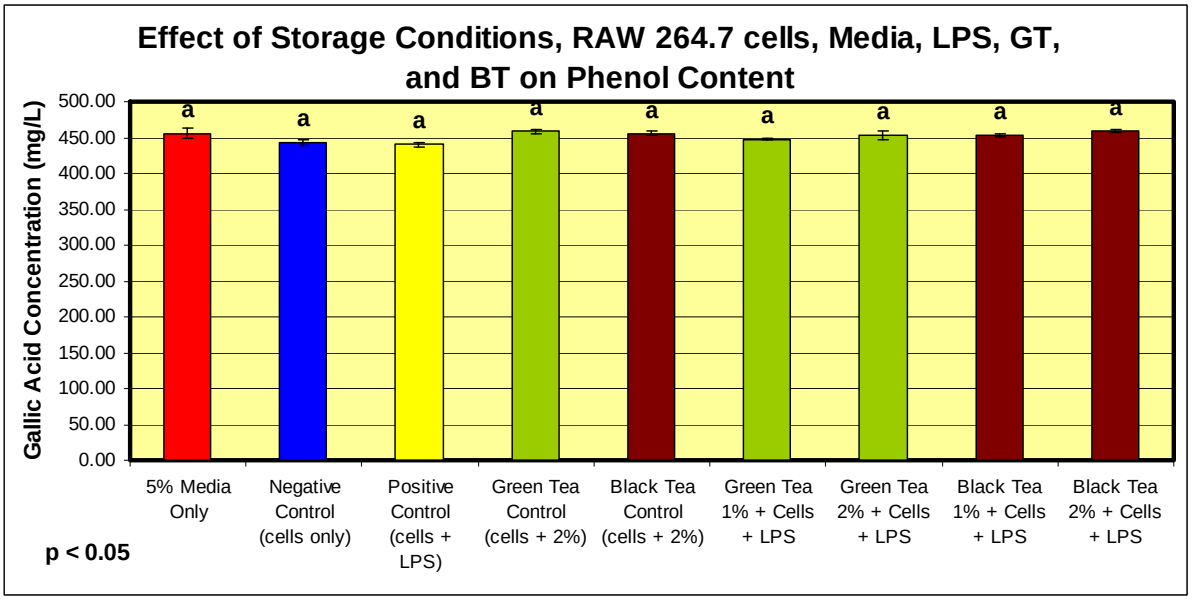

Figure 2.3. Effect of Incubator Conditions, RAW 264.7 cells, Media, LPS, GT, and BT on Total Phenol Content

Note: Treatments with same letter are not statistically different

2.5 DISCUSSION

The results above indicate that GT has a higher phenol concentration than BT. These differences in phenol content may be caused by differences in the teas' composition. In order to produce GT, leaves from the *Camellia sinensis* plant are steamed to inactivate enzymes within the leaf (e.g. polyphenol oxidase) that are responsible for converting leaf tannins and catechins to more oxidized polyphenols. Since GT is not oxidized or fermented, as much as 90% of polyphenols in the leaves are preserved. BT, on the other hand, is withered, bruised, and left to fully ferment and form more oxidized, complex polyphenols such as theaflavins and thearubigins (Khan et al 2008). GT has a higher phenol content than BT because GT is

less oxidized and has a greater capacity to reduce the Folin-Ciocalteu reagent. These results also imply that GT has a higher antioxidant capacity than BT.

Our method of tea preparation may have also affected the phenol concentration in the GT and BT used for this study. We prepared the teas according to manufacturer instructions, i.e. in a manner similar to the way teas would be prepared by a consumer. The polyphenol content in teas is highly dependent upon the amount of tea added, amount of water used, brewing time, and brewing temperature. For example, instant tea generally contains a lower amount of polyphenols as compared to hot tea and brewed iced tea, which have similar polyphenol concentrations (Hakim et al 2000). Although our teas were steeped for a relatively short time (5 minutes), past studies have demonstrated that brewing teas beyond 5 minutes and up to 10 minutes does not significantly alter phenol content (Liebert et al 1999, Lakenbrink et al 2000). In addition, brewing teas for an extended time causes precipitation of proline-rich proteins and mucopolysaccharides in saliva. This leads to a dry, astringent sensation in the mouth that decreases palatability of the tea (Dreosti 2000).

The changes in total phenol concentration over time may have been caused by a number of factors. As tea cools, caffeine and polyphenols interact and form a precipitated complex known as 'tea cream' (Hakim et al 2000). The earlier the cream develops, the higher the tea concentration (and possibly the total phenol content). The presence of theaflavins can also speed up the development of the cream (Jobstl et al 2005). Although all of the teas used for these experiments were prepared at the same time, variability in the rate of formation of tea cream in the cooled, freshly prepared tea aliquots could have affected the measured phenol content. Since the presence of tea cream was not considered, it is unknown

how significantly the measured phenol concentration was altered by the formation of this substance.

The temperature at which the teas were stored could have also significantly affected total phenol content. Tea polyphenols are susceptible to light exposure and temperature changes. Although the phenol content of each tea significantly increased at 1 month and 2 months post-preparation then decreased at 3 months post-preparation, the polyphenol concentration remained relatively stable in comparison to tea storage at a higher temperature. Preliminary experiments were conducted to determine the optimal temperature at which the teas were most likely to remain stable for an extended period of time. At -20° C, we found that the phenol concentration in both GT and BT were much lower (787.6 $\pm$ 37.8 GAE, 820.93 $\pm$ 9.3 GAE, respectively) than the values observed at -80° C storage. Based on these findings, we stored the teas at -80° C to maximize the stability of the tea polyphenols.

When the teas were exposed to experimental conditions over a 24 hour period, the changes in total phenol content were significant between time 0 and 12 and 24 hours post-LPS application, but not significant between 12 and 24 hours post-LPS application. The GAE values may have been altered by the presence of CO_2, which can act as a reducing agent on the Folin-Ciocalteu reagent. In addition, the sugar and protein fractions in media (e.g. glucose, fetal bovine serum), LPS, and various cell metabolites could have also affected the reduction of the Folin-Ciocalteu reagent and caused inflated absorbance readings (Prior et al 2002).

2.6 CONCLUSIONS

In summary, GT consistently had a higher phenol content than BT. This is mainly because GT is less oxidized during production, making it a more powerful reducing agent on the Folin-Ciocalteu reagent. This suggests that GT is a more effective antioxidant than BT. The differences between the teas' chemical composition may also explain the differences in their antioxidant capacity.

Although there were differences in phenol content between the teas, both teas were not significantly affected by the storage conditions used. When stored at -80° C, the teas remained relatively stable during the time they were utilized for experiments. Finally, total phenolic content was not significantly altered by experimental conditions and treatments used. This suggests that our experimental conditions are appropriate since they do not dramatically affect the stability of the phenol concentrations in the green tea and black tea.

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CHAPTER 3: CELL VIABILITY ANALYSIS, PGE_2 QUANTITATION, AND COX-2 DETECTION IN LPS-INDUCED RAW 264.7 CELLS TREATED WITH GREEN TEA AND BLACK TEA EXTRACTS

3.1 ABSTRACT

Lipopolysaccharide (LPS), a major structural and functional component of Gram-negative cell walls, is well-established as an effective stimulus for macrophage activation. Monocytes and macrophages possess a prominent role in the body's response to infection, inflammation, and associated innate and adaptive responses. Monocytes differentiate into macrophages via cytokine macrophage colony-stimulating factor (M-CSF). LPS binds to membrane protein CD14 and Toll-like receptor 4 (TLR4), which begins a signaling cascade and the subsequent secretion of pro-inflammatory cytokines (e.g. interleukins, tumor necrosis factor alpha) and secondary inflammatory mediators (e.g. nitric oxide, leukotrienes, prostaglandins). LPS produces inflammation in these cells in a manner similar to that observed in mammal and human macrophages in a relatively simplified form that allows inflammation to be analyzed.

The first objective of our study was to assess cell viability and potential cytotoxic effects of LPS, green tea, and black tea on RAW 264.7 mouse macrophage cells using the Trypan Blue assay and MTT assay. The Trypan Blue assay is a colorimetric analysis based on the ability of viable cell membranes to exclude the dye. The MTT assay is a colorimetric assay that measures live cells only by utilizing a tetrazolium salt to quantitatively detect mammalian cell survival, cytotoxicity, and proliferation. The teas, LPS, and experimental conditions were not shown to have a cytotoxic effect, and there were no significant differences between the treatments. Our cell viability results suggest that the treatments were added at appropriate concentrations to analyze the possible inflammatory effects of LPS and anti-inflammatory activity of the teas on the RAW 264.7 cells.

The second objective of this study was to measure PGE_2, a pro-inflammatory eicosanoid metabolized from arachidonic acid (AA), by performing an enzyme-linked immunosorbent assay (ELISA). First, the inflammatory potency of two LPS serotypes were analyzed and compared with this method. We found that LPS from E. coli 055:B5 significantly increased PGE_2 concentration as compared to LPS from E. coli 0111:B4. PGE_2 levels were also measured in tea-treated, LPS-induced RAW 264.7 cells. The results showed that PGE_2 formation was similarly suppressed by green tea (GT) and black tea (BT) relative to the positive control.

The final objective of this study was to compare the differential effects of GT and BT on COX-2, another inflammatory mediator in the AA-dependent pathway. The BCA Assay was used to measure total protein concentration, and Western blotting was performed to detect for the presence of COX-2. The GT and BT controls did not enhance COX-2 expression. GT applied at the highest concentration (5% of treatment well volume) was the only treatment that significantly inhibited COX-2 expression. Since the teas suppressed PGE_2, these findings suggest that the teas may modulate COX-2 activity rather than its expression, or affect a COX-2 metabolite rather than the protein itself.

3.2 INTRODUCTION

Many *in vitro* studies have utilized a wide array of cultured cell lines to investigate the potential effects of tea on inflammatory mediators. Our study used bacterial lipopolysaccharide (LPS) to induce inflammation in RAW 264.7 mouse macrophages. In general, monocytes and macrophages possess a prominent role in the body's response to infection, inflammation, and associated innate and adaptive responses. Monocytes differentiate into macrophages via cytokine macrophage colony-stimulating factor (M-CSF) (Murphy et al 2008). LPS, a major structural and functional component of Gram-negative cell walls, is well-established as an effective stimulus for macrophage activation. LPS is made up of 3 main components: the outer (O) polysaccharide, the core, and the lipid portion (lipid A). Lipid A is the component responsible for the development of septic shock (Victor et al 2004) and generalized inflammation (Rietschel et al 1994). LPS binds to membrane protein cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4) (Poltorak et al 1998), which begins a signaling cascade and the subsequent secretion of pro-inflammatory cytokines (e.g. interleukins (ILs), tumor necrosis factor alpha (TNF-α)) and secondary inflammatory mediators (e.g. nitric oxide (NO), leukotrienes (LTs), prostaglandins (PGs)). LPS produces inflammation in these cells in a manner similar to that observed in mammal and human macrophages in a relatively simplified form that allows inflammation to be analyzed (Moore et al 2000, Djoko et al 2007). In our study, we chose to measure prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2), two major inflammatory markers that are synthesized and expressed in LPS-induced RAW 264.7 cells.

Metabolism of PGE_2

In order to examine the pro-inflammatory or anti-inflammatory effects of our treatment compounds, a number of inflammatory markers can be experimentally measured. We chose to measure PGE_2, a pro-inflammatory eicosanoid that is a major product of arachidonic acid (AA) metabolism. When LPS activates macrophages by binding to CD-14 and TLR4 on the cell membrane, AA is released from the membrane via phospholipase A_2. This causes up-regulation of COX-2, which leads to the formation and release of PGE_2 into the extracellular space. PGE_2 is quickly converted into an inactive metabolite (13,14-dihydro-15-keto PGE_2) by the prostaglandin 15-dehydrogenase pathway (Granstrom et al 1980, Hamberg & Samuelsson 1971). *In vivo*, PGE_2 is typically present at 3-12 pg/ml in the plasma, and has a half-life of about 30 seconds in the circulatory system (Fitzpatrick et al 1980). Figure 3.1 represents the rapid metabolism of PGE_2.

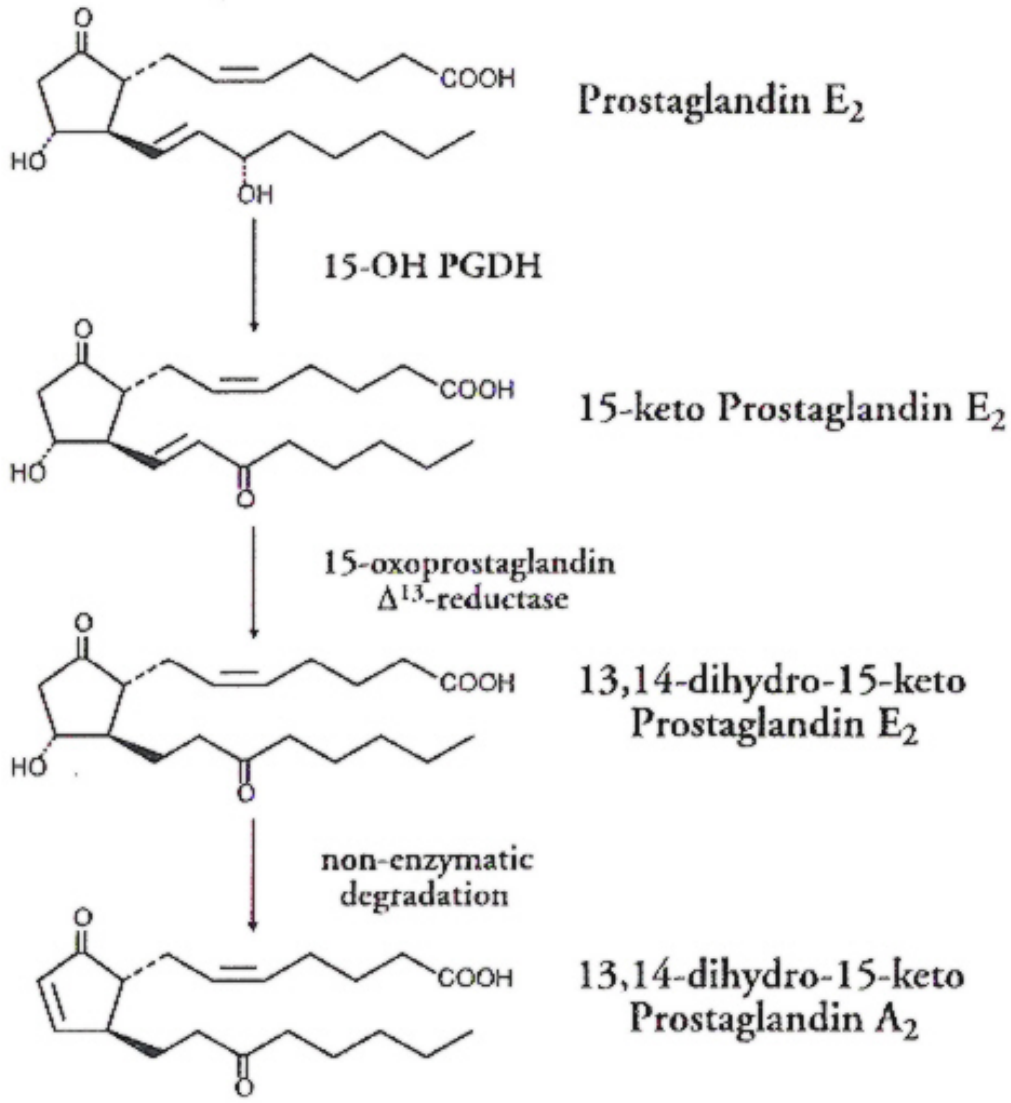

Figure 3.1. Metabolism of PGE_2

Metabolism of COX-2 and its role in inflammation

The PGE_2 ELISA is a relatively rapid screening assay that can establish the potential anti-inflammatory effect of green tea and black tea on LPS-induced RAW 264.7 cells. In addition to measuring PGE_2, we also investigated the effect of green tea and black tea on

COX-2, a precursor of PGE_2 in the AA-dependent pathway. Cyclooxygenase-2 (COX-2) is an isoform of prostaglandin H_2 synthase (PGH_2) that is responsible for converting AA released by membrane phospholipids into prostaglandins. These compounds mediate a number of cellular and physiologic processes, including acute inflammation (Yoon & Baek 2005). COX-2 is an important pro-inflammatory mediator that is largely expressed in activated macrophages, mast cells, fibroblasts, and other cells that participate in the inflammatory response (Santangelo et al 2007). In an *in vitro* system, COX-2 expression can be induced by a number of stimuli, such as phorbol esters, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), and LPS (Xie et al 1991). Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, indomethacin, and celecoxib, can reversibly or irreversibly inhibit COX-2 by competing with AA for binding to this enzyme's active site (Vane et al 1998). The suppression of COX-2 by green tea and/or black tea would probably lead to the inhibition of its metabolites, including PGE_2.

The objectives of the following study were to assess cell viability and potential cytotoxic effects of the treatment compounds using the Trypan Blue assay and MTT assay. The anti-inflammatory effects of green tea and black tea on LPS-induced RAW 264.7 macrophages were assessed by performing PGE_2 ELISA. The inflammatory capacity of two LPS serotypes were also analyzed with this method. Finally, the differential effects of green tea and black tea on COX-2 levels in LPS-stimulated mouse macrophage cells were observed by conducting Western Blot analysis. The BCA Assay was used to measure total protein concentration. We hypothesized that the treatment compounds used would not have a cytotoxic effect. We also predicted that the two LPS serotypes would have differential pro-

inflammatory effects on the cells because of structural variability between serotypes. Finally, we hypothesized that the teas would both suppress inflammation by decreasing PGE_2 levels and the expression of COX-2 in a dose-responsive manner.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture and Cell Viability

RAW 264.7 mouse macrophage cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Dulbecco's Modified Eagle Medium (DMEM), 100X Penicillin-Streptomycin-Glutamine, and Phosphate Buffered Saline (PBS) were purchased from Gibco®, Invitrogen™ (Grand Island, NY). Fetal bovine serum (FBS) was bought from Atlanta Biologicals (Lawrenceville, GA). LPS from E. coli 0111:B4, LPS from E. coli 055:B5, and Trypan Blue solution (0.4%) were purchased from Sigma-Aldrich, Incorporated (St. Louis, MO). M-PER® Mammalian Protein Extraction Reagent and HALT® Protease Inhibitor Cocktail Solution were obtained from Pierce (Rockford, IL). The MTT utilized was from Molecular Probes (Eugene, OR). The hydrochloric acid and isopropanol used were from Fisher Scientific (Fair Lawn, New Jersey) and were of analytical purity.

The laminar flow hood used was a 1300 Series A2, Class II, Type A2 Biological Safety Cabinet bought from Thermo Scientific (Marietta, OH) (Figure 3.2). The centrifuge was a IEC CL31 Multispeed centrifuge and was also purchased from Thermo Scientific. The incubator used was a Fisher Scientific Isotemp Laboratory CO_2 Incubator. Centrifuge tubes were from Corning Incorporated (Corning, NY). All cell culture supplies (serological pipets,

12-well plates, 75 cm^2 cell culture flasks) were purchased from Falcon® and BD Falcon™ (Becton Dickinson and Company, Franklin Lakes, NJ).

Figure 3.2. Laminar Flow Hood and Cell Culture Set-Up

Starting RAW 264.7 cells from freezer

Prior to use, RAW 264.7 cells were stored in liquid nitrogen. Before cells were thawed, 10 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin/glutamine ("10% media") were transferred into a 75 cm^2 cell culture flask and placed into the incubator at 37° C, 5% CO_2 for approximately 20 minutes before the cells were added. This allowed the media formulation to reach its normal pH of 7.0-7.4. Next, the frozen cell vial was gently thawed in a 37° C water bath for about 2 minutes. After thawing, the vial was removed from the water bath, patted dry with a paper towel, and sprayed with a 70% ethanol solution (700

ml ethyl alcohol, 300 ml deionized water). Next, the vial was placed under the laminar flow hood and its contents were transferred into a 15 ml centrifuge tube containing 9 ml of 10% media. The tube was spun for 7 minutes at 900 rpm (130 g). The supernatant was carefully removed from the cell pellet. Then, 7.5 ml of 10% media was removed from the incubated 75 cm^2 cell culture flask and added to the 15 ml centrifuge tube to resuspend the cell pellet. Finally, the tube contents were pipetted into the 75 cm^2 cell culture flask and incubated at 37° C and 5% CO_2 until cells were adherent and approximately 80% confluent (about 5-6 days).

3.3.1.1 Passaging, Plating, and Counting RAW 264.7 Cells

Passaging RAW 264.7 cells

After the cells had recovered from freezer storage, they were kept in 15 ml of DMEM containing 5% FBS and 1% penicillin/streptomycin/glutamine ("5% media"). The cells were maintained in six 75 cm^2 cell culture flasks (Figure 3.3) and passaged every 48 hours.

Figure 3.3. Cell Culture Flasks

When the cells were passaged, the old 5% media was removed from each flask and 5 ml of fresh 5% media was added to each flask. The cells were carefully scraped from the bottom of each flask, and the resulting cell suspensions were transferred into one flask. The cells were typically diluted 1 to 4:

(30 ml of old 5% media & scraped cells) * 0.75 = removed 22.5 ml old 5% media & cells)

Next, fresh 5% media was added to the remaining media and cell suspension to bring the total volume to 90 ml, or 15 ml of fresh 5% media and cells per flask:

(90 ml of total volume needed) – (7.5 ml of remaining 5% media and cells) = added 82.5 ml of fresh 5% media.

The solution was slowly mixed by pipetting up and down several times, and 15 ml was transferred to each cell culture flask. The flasks were returned to the incubator and stored at 37° C and 5% CO_2.

Trypan Blue Assay and Plating RAW 264.7 Cells

The Trypan Blue Assay was used to determine the live cell concentration, or number of cells per milliliter of media, in order to accurately transfer 500,000 cells/ml into 12-well plates. First, cells were scraped from each of the 6 cell culture flasks and transferred into 1 flask. Next, 10 µl of the cell suspension was pipetted into 90 µl of Trypan Blue and gently mixed. Approximately 30 µl of the Trypan Blue and cell suspension were pipetted into the hemocytometer chamber (Cascade Biologics™). The live cells and dead cells within each hemocytometer quadrant were counted (Figure 3.4), and the percent viability was calculated:

(Live Cells from Quadrant 1, 2, 3, and 4) + (Dead Cells from Quadrant 1, 2, 3, 4) = Total Cells

$$\text{Percent(\%) Viability} = \frac{\text{(Live Cells)}}{\text{(Dead Cells)}} \times 100$$

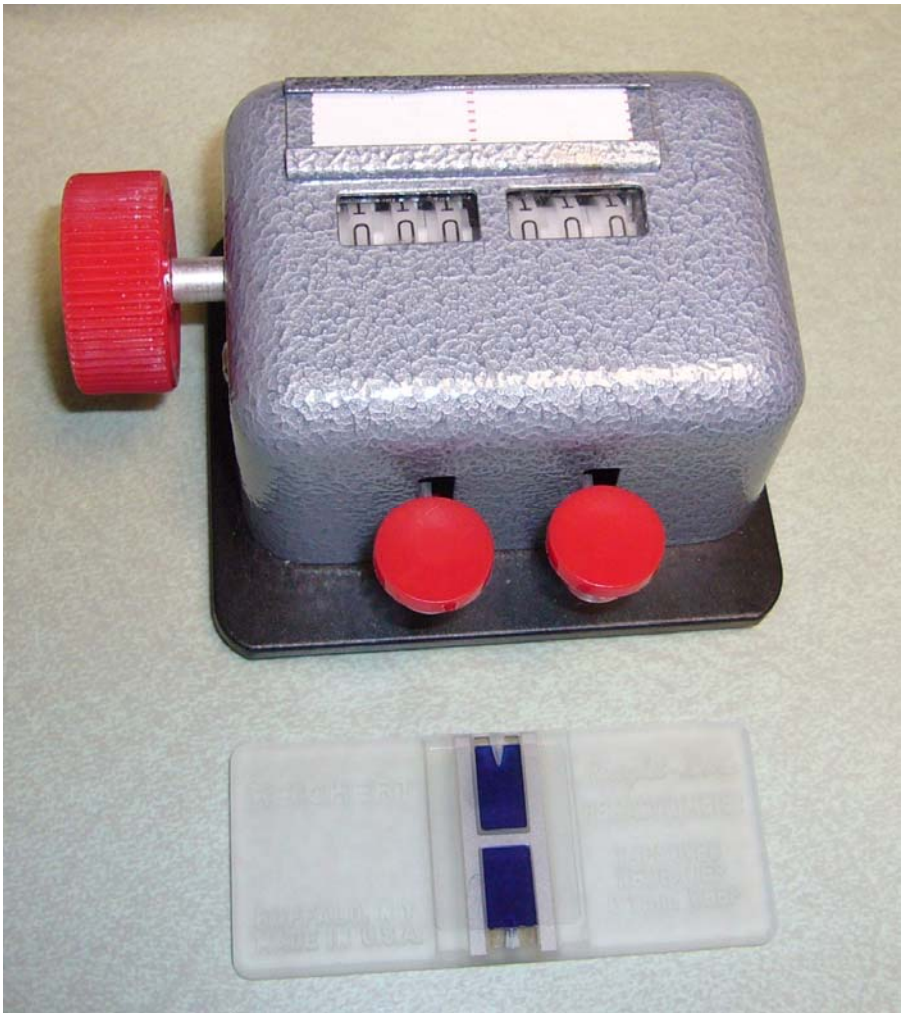

Figure 3.4. Cell Counter and Hemocytometer with Trypan Blue and Cell Suspension

Next, the live cell concentration was determined:

Live Cell Concentration (cells/ml) = (Total Live Cells) x (Dilution Factor) x 2500

$$= (\text{Total Live Cells}) \times \frac{(100\ \mu l\ \text{Trypan Blue} + \text{cell suspension})}{(10\ \mu l\ \text{of cell suspension})} \times 2500$$

= (Total Live Cells) x (Dilution Factor of 10) x 2500

Then, the dilution factor for the cell suspension was calculated in order to determine the volume of media needed to dilute the cells to a desired concentration of 500,000 cells/ml:

$$\text{Dilution Factor} = \frac{\text{(Live Cell Concentration)}}{\text{(500,000 cells/ml)}}$$

(6 cell culture flasks) x (5 ml of media and cells per flask) = 30 ml of cells and media

(30 ml of cells and media) x (Dilution Factor) = ___ ml of media needed to dilute cells to 500,000 cells/ml

Typically, 30 total wells were plated, with 1.5 ml of cells and media per well, and 10 wells filled in each 12-well plate. The proposed treatments were run in triplicate and are as follows:

1) Negative Control (cells and media only)
2) Positive Control (cells and LPS)
3) Green Tea (GT) Control (cells and 5% GT)
4) Black Tea (BT) Control (cells and 5% BT)
5) GT 1%, cells, and LPS
6) GT 2%, cells, and LPS
7) GT 5%, cells, and LPS
8) BT 1%, cells, and LPS
9) BT 2%, cells, and LPS
10) BT 5%, cells, and LPS

Next, the total amount of media needed for the 30 treatment wells and 6 cell culture flasks was calculated:

(30 treatment wells) x (1.5 ml of media and cells per well) = 45 ml of media and cells

(45 ml of media and cells) + [(15 ml of media and cells per flask) x (6 cell culture flasks)] = need 135 ml of media and cells ~ round to 140 ml of media and cells

Finally, calculations were performed to figure out the volume of scraped cells and media to be discarded. The volume of new 5% media that was to be added to dilute the remaining cell suspension was also determined:

(30 ml of media and cells)/ (___ ml media needed to dilute cells to 500,000 cells/ml) = (x ml)/(140 ml media and cells needed for 30 treatment wells and 6 flasks)

(30 ml of media and cells) – (x ml) = remove ___ ml of media and cells

(140 ml media and cells needed for wells and flasks) – (x ml) = add ___ ml of new 5% media

Once the final live concentration of the cell and media mixture was adjusted to 500,000 cells/ml, 1.5 ml of the mixture was pipetted into each of 30 treatment wells. The remaining cells and media were split into 15 ml for each cell culture flask. The 12-well plates and flasks were stored in the incubator at 37° C and 5% CO_2.

3.3.1.2 Cell Treatment and Harvesting

Preparation of LPS

Vials of LPS from E. coli 0111:B4 and of LPS from E. coli 055:B5 (1 mg/vial) were each diluted with 200 µl of DMEM in order to make 5 mg/ml LPS stocks (5 mg/ml is the solubility limit for LPS). The LPS stock aliquots were stored at -80° C.

Prior to treating the cells, LPS aliquots were diluted 1:100 (15 µl of 5 mg/ml LPS stock, 1485 µl DMEM) to make 0.05 mg/ml of LPS.

Treating RAW 264.7 cells with Green Tea, Black Tea, and LPS

The cells in 12-well plates were incubated for approximately 12 hours before achieving confluency. Once the cells were at least 80% confluent, the GT and BT were each applied to the tea treatment wells at 1% (15 µl) or 2% (30 µl) of the total volume in each well (1.5 ml) and incubated at 37° C, 5% CO_2 for 2 hours. After the incubation period, 64 µl of

0.05 mg/ml LPS stock was added at a final concentration of 2 μg/ml to the LPS treatment wells, and the 12-well plates were incubated for 12 hours at 37° C and 5% CO_2.

We later determined that a higher concentration of LPS was needed to induce a significant inflammatory response in the cells, and that LPS from E. coli 055:B5 was a better inflammatory stimulus. In addition, we added higher GT and BT treatments (5% of total treatment well volume, or 75 μl of tea extract) to determine if the teas could suppress inflammation to a greater extent (as compared to the 1% and 2% tea treatments). After incubating the cells with the tea treatments for 2 hours, LPS from E. coli 055:B5 (5 mg/ml stock) was diluted 1:10 to achieve a 0.5 mg/ml stock. The diluted stock (32 μl) was applied to the LPS treatment wells at a final concentration of 10 μg/ml, and the 12-well plates were incubated for 12 hours at 37° C and 5% CO_2.

Harvesting RAW 264.7 cells

First, 30 microcentrifuge tubes (1.5 ml) were labeled with treatment number 1-30, "C" (cell lysate samples for Western blotting and COX-2 analysis), the harvest date, and "CRS" (my initials). Another set of 30 microcentrifuge tubes were labeled with treatment number 1-30, "M" (PGE_2 ELISA media samples), harvest date, and "CRS". A third set of 30 microcentrifuge tubes were labeled with treatment number 1-30, "BCA" (samples for BCA assay), harvest date, and "CRS". An M-PER/Protease Inhibitor Cocktail solution was also prepared (3.2 ml of M-PER, 32 μl of Protease Inhibitor Cocktail).

The tea- and LPS-treated cells were incubated for 12 hours at 37° C and 5% CO_2 prior to harvesting. First, 1.2 ml of media was transferred from each well into the

correspondingly labeled tube and stored at -80° C (Figure 3.5). Next, the remaining media was carefully suctioned out of each well and discarded. Each well was rinsed 3 times with cold PBS, and the adhered RAW 264.7 cells were gently scraped from the bottom of each well with cell lifters. The cells were lysed by adding 100 µl of the M-PER/Protease Inhibitor Cocktail solution Finally, 60 µl of the cell lysate mixture was pipetted from each well into the microcentrifuge tubes labeled "C", while the remainder (40 µl) was transferred from each well into the microcentrifuge tubes labeled "BCA". All microcentrifuge tubes were stored in freezer boxes at -80° C.

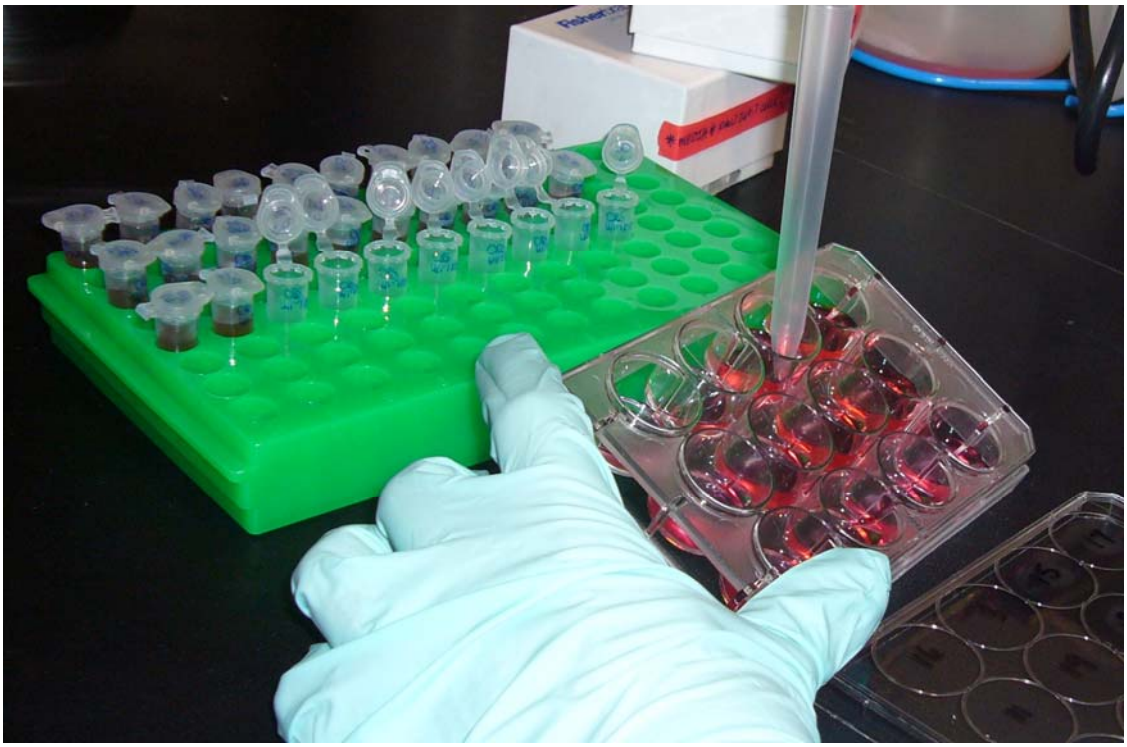

Figure 3.5. Pipetting Media from 12-well Plates During Cell Harvest

3.3.1.3 Cell Viability (MTT) Assay

The methods used are based on those developed by Mosmann (1983). First, 7.8 mg of MTT was mixed with 1 ml of deionized water. Then, 16 µl of the MTT stock was added to each of the treatment wells and incubated for 2 hours at 37° C and 5% CO_2 to allow formazan crystals to develop. After the incubation time, the media was slowly removed from each well to avoid disrupting the formed crystals. Next, 0.5 ml of acidified isopropanol (1.66 ml of hydrochloric acid in 50 ml of isopropanol) was added to each well to dissolve the crystals. Then, 200 µl was pipetted from each well and into a 96-well plate (Figure 3.6). The absorbances of the solutions were measured at 620 nm using a Multiskan® EX plate reader (Thermo Electron Corporation, Vantaa, Finland).

Figure 3.6. MTT Assay: 96-well Plate Set-Up

3.3.2 PGE_2 ELISA

The Prostaglandin E_2 Monoclonal EIA Kit was purchased from Cayman Chemical Company (Ann Arbor, Michigan). The 96-well plate reader is a Multiskan® EX (Thermo Electron Corporation, Vantaa, Finland).

Media samples were analyzed for PGE_2 by using a 96-well Prostaglandin E_2 Monoclonal EIA Kit (Cayman Chemical Company, Ann Arbor, Michigan) by following manufacturer instructions (Figure 3.7).

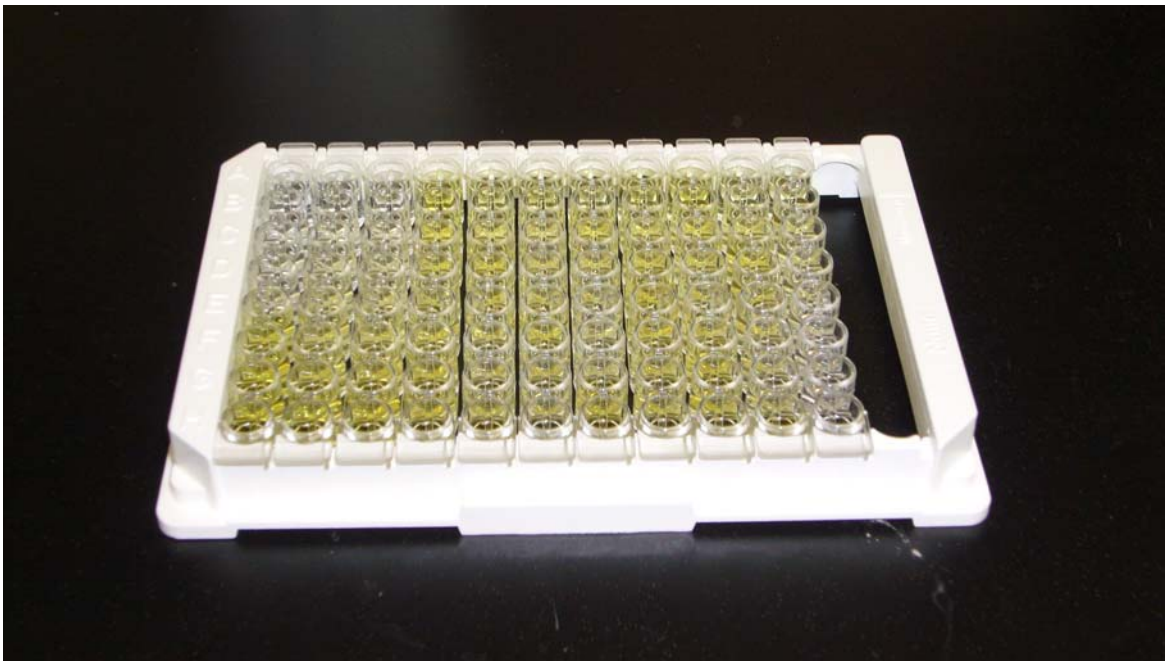

Figure 3.7. PGE_2 ELISA: 96-well Plate Set-Up

3.3.3 Protein Determination and Western Blotting (COX-2 Detection)

3.3.3.1 BCA Assay

The BCA Assay was performed using the Pierce® BCA Protein Assay Kit, and was purchased from Thermo Scientific (Rockford, IL).

Total protein content in the cell lysates was determined by following instructions provided in the Pierce® BCA Protein Assay Kit. Bovine serum albumin (BSA) was used as the protein standard, and the M-PER/Protease Inhibitor Cocktail Solution was used as the diluent for standard preparation. The standard or sample was added (25 µl/well), then 200 µl of the BCA Working Reagent was pipetted into each well. The 96-well plate was incubated at 37° C for 30 minutes. Once cooled, the absorbances were read spectrophotometrically at 512 nm. The samples were typically diluted 1:4 with the M-PER/Protease Inhibitor Cocktail solution (Figure 3.8).

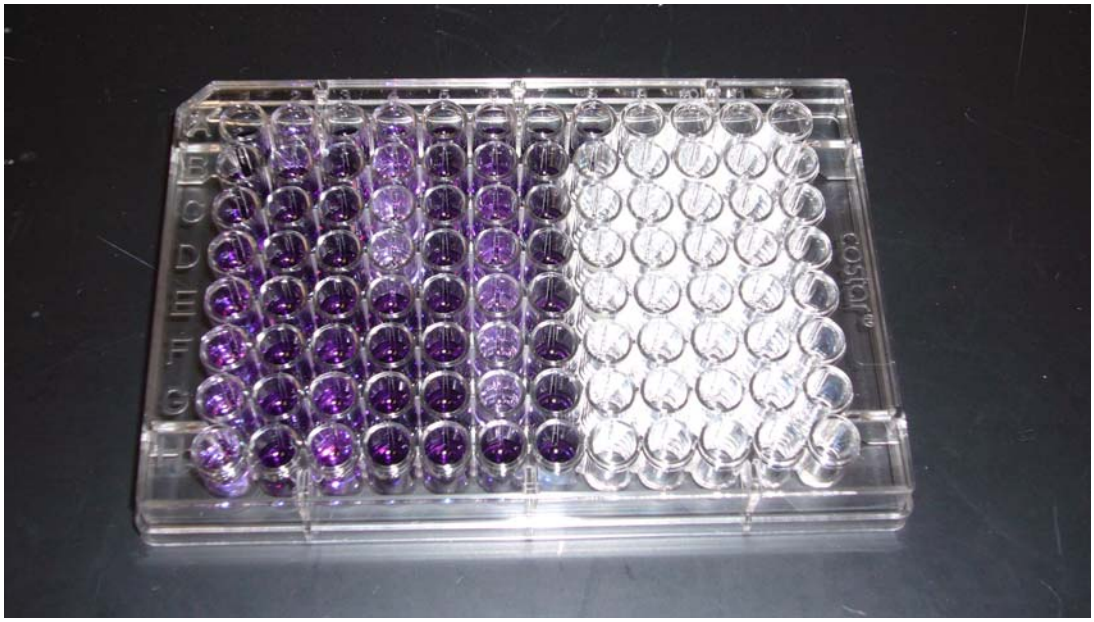

Figure 3.8. BCA Assay: 96-well Plate Set-Up

3.3.3.2 Western Blotting

Trizma base, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), bromophenol blue, glycerol, and sodium chloride were obtained from Sigma-Aldrich, Incorporated (St. Louis, MO). Glycine, sodium carbonate and sodium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ). Novex® Tris-Glycine 12% gels (1.0 mm, 12 well), PVDF membranes, filter paper, sponge pads, BenchMark™ Pre-Stained Protein Ladder, X-Cell SureLock™ gel box, and X-Cell II™ blot module were bought from Invitrogen™ (Grand Island, NY). The primary antibody (COX-2 (murine) Polyclonal Antibody – Affinity-Purified) was bought from Cayman Chemical Company (Ann Arbor, MI), and the secondary antibody (Anti-rabbit, IgG, AP-linked) was purchased from Cell Signaling Technology, Inc.

The CDP Buffer (25X) and CDP* Reagent were obtained from New England BioLabs, Inc. The methanol and hydrochloric acid used were of analytical purity.

The methods used for this procedure were based on a modification performed by Harris et al (2006). Proteins (80 μg/well) were separated on a 12% Tris-Glycine gel for 2 hours at 125 V, then transferred to PVDF membranes at 25 V for 3.5 hours (Figure 3.9). The membranes were blotted with primary antibody (COX-2 (murine) Polyclonal Antibody – Affinity-Purified, 1:100 dilution) overnight at 4° C and in secondary antibody (Anti-rabbit, IgG, AP-linked, 1:1000 dilution) for 1.5 hours at room temperature. CDP Buffer (diluted to 1X) and CDP* (1:200 dilution) were used to visualize the protein bands, and a densitometry program (UN-SCAN-IT, Silk Scientific, Inc., Orem, Utah) was used to quantify the protein.

Figure 3.9. Western Blotting: Protein Transfer to PVDF Membrane

3.3.4 Experimental Design and Statistical Analysis

A randomized complete block design was used for plating and treating the cells, and experiments were performed by blocking on day. Each treatment was run in triplicate. All experiments were run between cell passages #25 and #33 post-liquid nitrogen storage. Statistical analysis was performed using the GLM procedure and Tukey's test on SAS® software (SAS Inc, Cary, NC). Data is expressed as the means $\pm$ standard error, and a probability (p) value < 0.05 is considered significant. On the figures below, treatments with the same letter indicate that the values were not statistically significant.

3.4 RESULTS

Figure 3.10 shows the cell viability for each treatment relative to the negative control (cells and media only; 100%). The positive control (cells, media, and LPS), tea controls (cells, GT or BT at 5% of treatment well volume, and media), and tea + LPS treatments (cells, GT or BT at 1%, 2%, or 5% of treatment well volume, media, and LPS) did not appear to significantly affect cell viability. The "BT control" and "BT 5% + LPS" treatments had the highest values at 105.1 ± 8.0% and 107.8 ± 8.2%, respectively. The "GT 2% + LPS" treatment also had a mean value slightly above that of the negative control (100.3 ± 3.7%). These three values suggest that the presence of GT and BT at 5% and/or the LPS slightly enhanced cell viability.

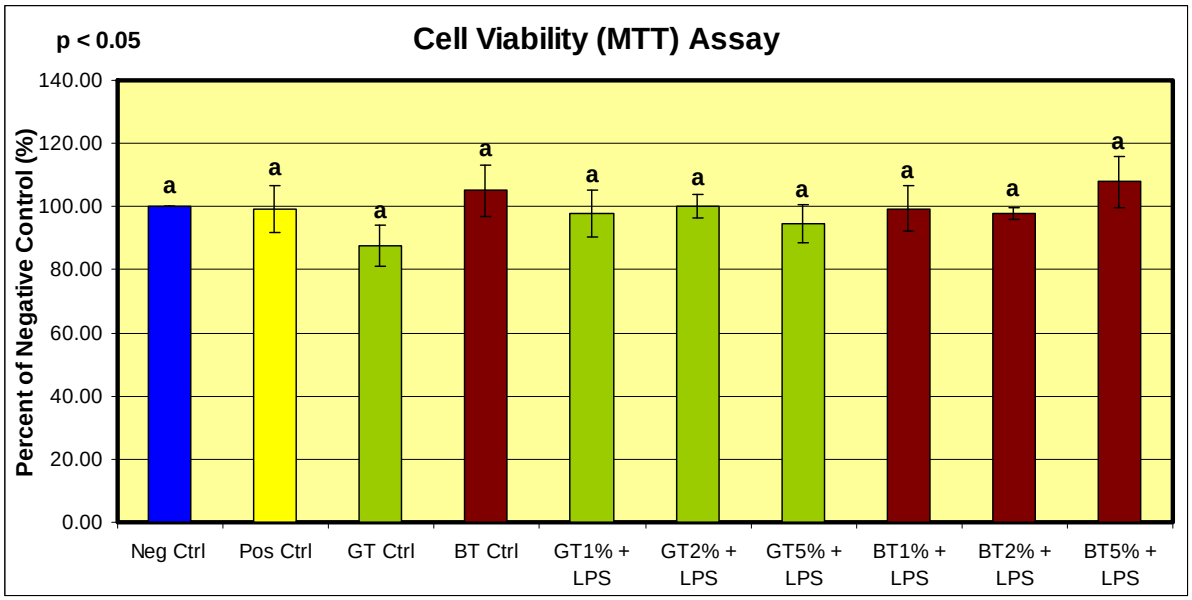

Figure 3.10. Cell Viability (MTT) Assay

Note: Treatments with same letter are not statistically different

Figure 3.11a and 3.11b show the mean PGE_2 concentrations for cells treated with 4 different concentrations of LPS from E. coli 0111:B4 and from E. coli 055:B5, respectively at 12, 16, and 24 hours post-LPS application. In Figure 3.11a, the PGE_2 levels were statistically different between all 3 times (indicated by the red stars on the X-axis labels). The concentration of PGE_2 significantly increased in a dose-responsive manner with each amount of LPS added up to 0.01 mg/ml ($p < 0.05$). PGE_2 levels were lowest for the negative control (14.47 ± 0.9 pg/ml), and highest at 0.01 mg/ml (159.7 ± 27.7 pg/ml). At 0.1 mg/ml, the PGE_2 concentration decreased (101.2 ± 17.3 pg/ml), and was statistically significant from the amount of PGE_2 from all other treatments except from that of 0.005 mg/ml of added LPS (90.0 ± 12.9 pg/ml). In Figure 3.11b, the values at 24 hours were significantly different from those at 12 hours and 16 hours post-LPS application. The concentration of PGE_2 also significantly increased in a dose-responsive manner with LPS up to 0.01 mg/ml. However, the PGE_2 levels in cells treated with 0.01 mg/ml LPS (106.2 ± 8.6 pg/ml) and 0.1 mg/ml (109.2 ± 10.5 pg/ml) were not statistically significant ($p < 0.05$).

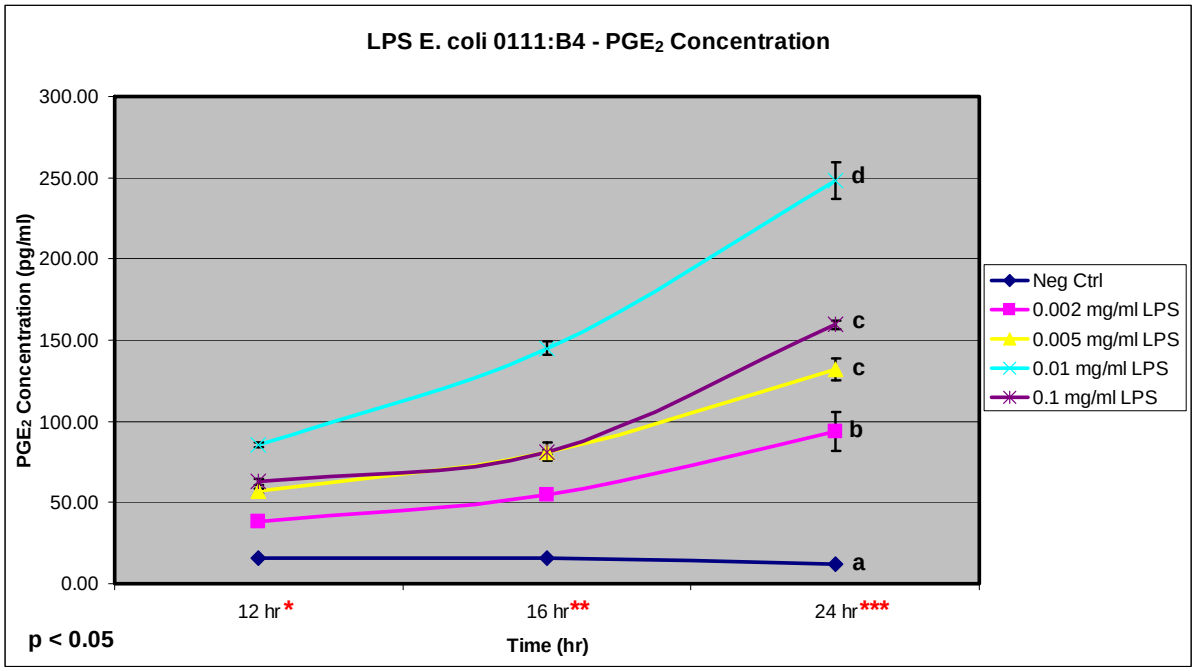

Figure 3.11a. Mean PGE_2 Concentrations in RAW 264.7 Cells Treated with Varying Levels of LPS from E. coli 0111:B4 (Different number of superscript "*" by X-axis time values indicate that mean PGE_2 concentrations for that time are statistically different from mean PGE_2 concentrations for other times)

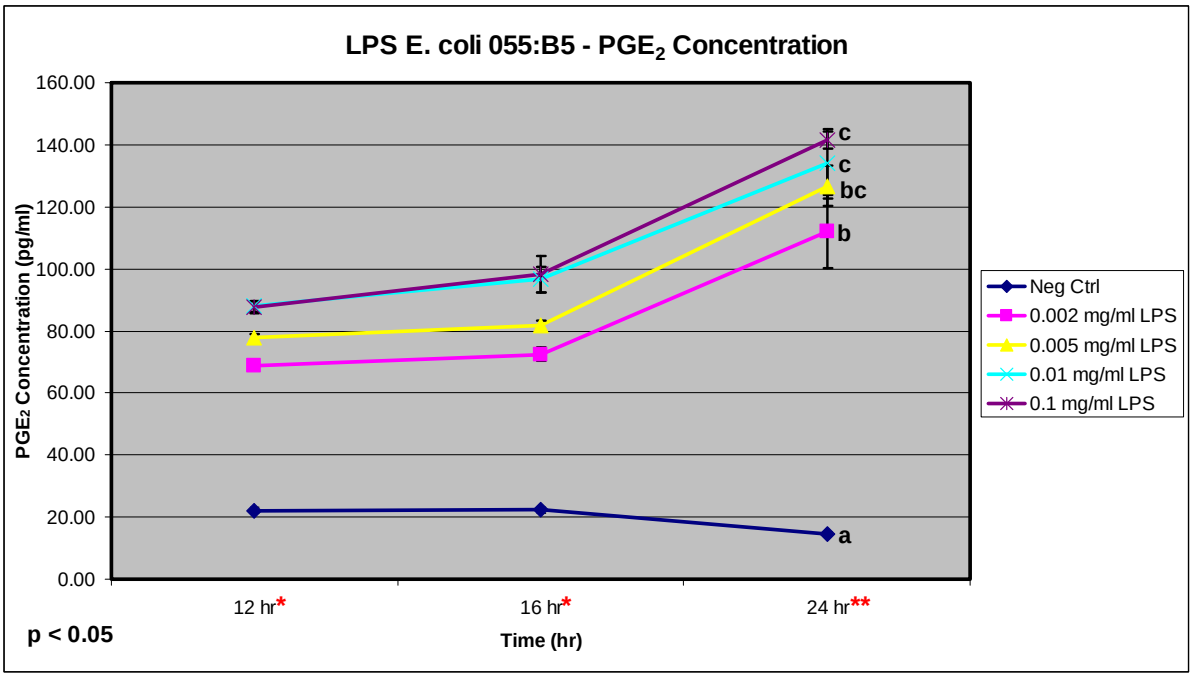

Figure 3.11b. Mean PGE_2 Concentrations in RAW 264.7 Cells Treated with Varying Levels of LPS from E. coli 055:B5 (Different number of superscript "*" by X-axis time values indicate that mean PGE_2 concentrations for that time are statistically different from mean PGE_2 concentrations for other times)

Figures 3.12a and 3.12b represent the relative PGE_2 concentration (expressed as a percent of the positive control) in cells treated with LPS from E. coli 0111:B4 and 055:B5, respectively, in the presence or absence of GT or BT. For each serotype, there are no statistically significant differences between any of the treatments ($p < 0.05$). However, for cells treated with LPS from E. coli 055:B5, there is a decreasing trend in PGE_2 levels in the "GT + LPS" and "BT + LPS" treatments (as tea concentrations increase, PGE_2 levels are decreasing) . At "GT 1% + LPS", the relative PGE_2 concentration was 83.3 $\pm$ 2.3%, as

compared to that for "GT 2% + LPS" (57.6 ± 11.2%). The same decreasing trend occurred from "BT 1% + LPS" (79.0 ± 4.8%) to "BT 2% + LPS" (67.5 ± 5.0%) (Figure 3.12b).

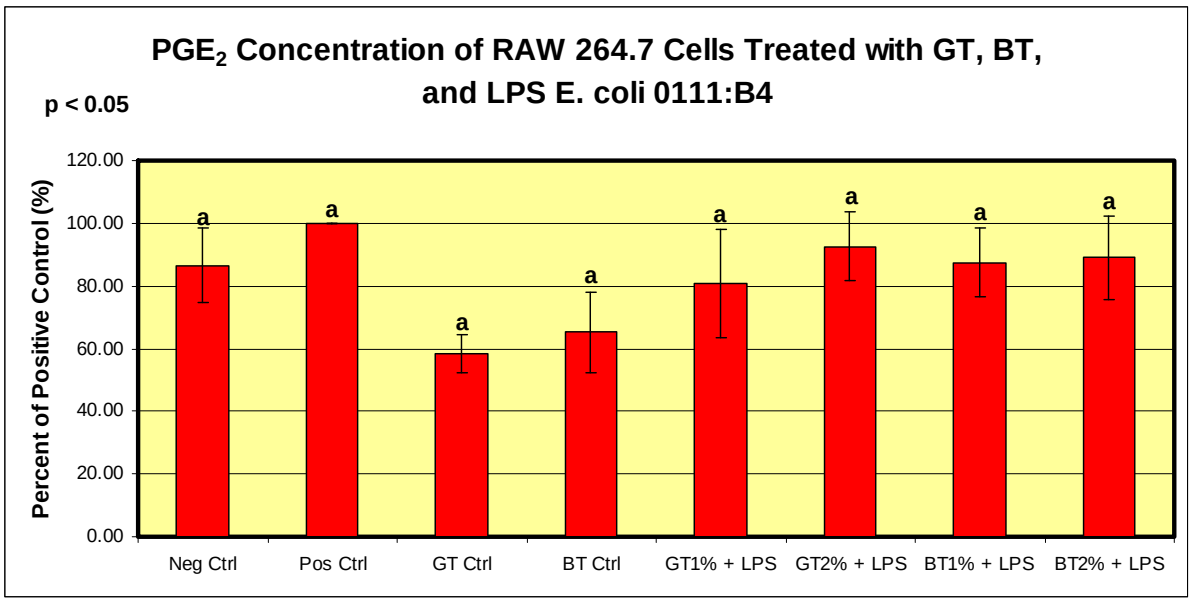

Figure 3.12a. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 2 µg/ml of LPS from E. coli 0111:B4

Note: Treatments with same letter are not statistically different

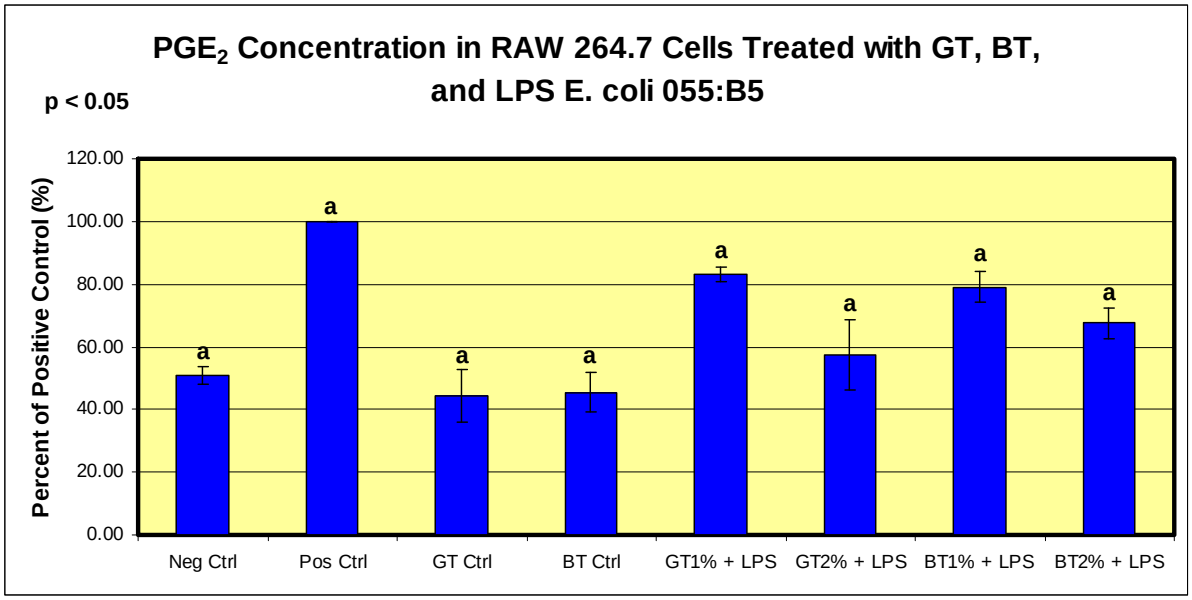

Figure 3.12b. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 2 μg/ml of LPS from E. coli 055:B5

Note: Treatments with same letter are not statistically different

Figure 3.13 shows the PGE_2 concentrations (relative to the positive control) in cells treated with GT, BT, and LPS from E. coli 055:B5 (10 μg/ml). There was statistical significance between the positive control and the tea treatments. There was no statistical difference between the negative control (37.2 $\pm$ 1.1%), GT control (36.6 $\pm$ 0.9%), and BT control (38.8 $\pm$ 1.3%). The PGE_2 values for "GT 1% + LPS" (62.3 $\pm$ 1.5%), "GT 2% + LPS" (56.8 $\pm$ 2.4%), "BT 1% + LPS" (65.6 $\pm$ 1.9%), and "BT 2% + LPS" (60.0 $\pm$ 1.3%) were also not statistically significant. The "GT 5% + LPS" and "BT 5% + LPS" treatments had even lower relative PGE_2 concentrations that were not statistically significant from one another (42.6 $\pm$ 1.1%, 51.0 $\pm$ 1.0%, respectively) ($p < 0.05$). However, there is a decreasing trend in

PGE_2 levels with increasing tea concentrations, suggesting that the teas may inhibit PGE_2 production in a dose-dependent manner.

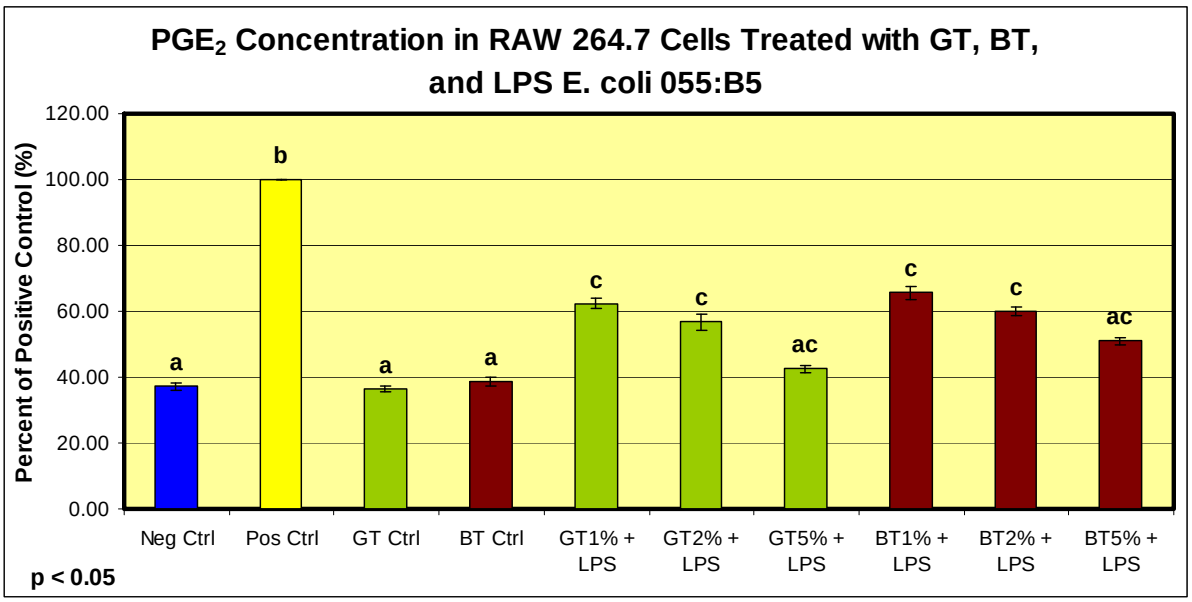

Figure 3.13. Relative PGE_2 Concentration of RAW 264.7 Cells Treated with GT, BT, and 10 µg/ml of LPS from E. coli 055:B5

Note: Treatments with same letter are not statistically different

Figure 3.14 represents a typical Western blot and the expression of COX-2 in RAW 264.7 cells treated with GT, BT, and LPS from E. coli 055:B5 (10 µg/ml) relative to the positive control. The negative control (16.9 $\pm$ 0.9%), GT control (12.0 $\pm$ 1.9%), and BT control (12.3 $\pm$ 4.1%) were not statistically significant ($p < 0.05$). This suggests that the teas alone did not promote COX-2 production. Only GT applied at the highest concentration

(5%) significantly inhibited COX-2 expression (84.7 ± 5.0%) relative to the positive control (100%).

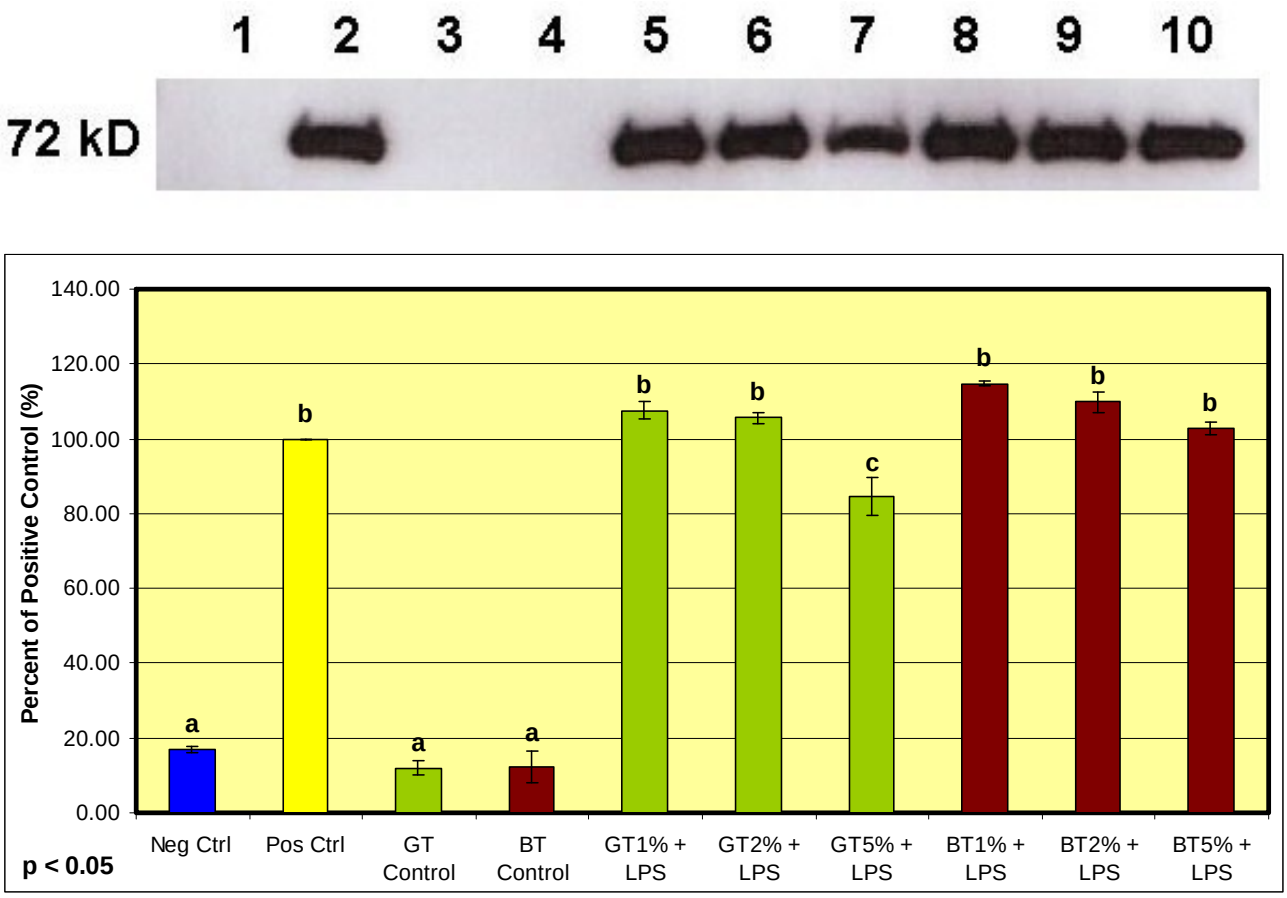

Figure 3.14. COX-2 Detection on Western Blot (*numbers above Western blot correspond to the treatments listed on the graph)

Note: Treatments with same letter are not statistically different

3.5 DISCUSSION

3.5.1 LPS

There is significant variability in LPS preparations, even when prepared from the same bacterial culture. We found that, depending on the LPS serotype, there are notable differences in macrophage activation. The O-specific portion of bacterial LPS is made up of a polymer of oligosaccharides with a repeating structure that varies widely in its number and structural components within and among serotypes. Additionally, structural differences exist in the outer core region of LPS. Thus, the serological specificity of LPS is affected by its extensive structural diversity. This extensive variability makes it difficult for commercial manufacturers to predict endotoxin content in LPS, even in products made from the same serotype (Rietschel et al 1994).

Since our goal was to effectively stimulate RAW 264.7 macrophages, we investigated differences in the inflammatory potency of LPS from two serotypes, E. coli 0111:B4 and 055:B5. Initially, we exclusively utilized the former, which was previously used by Harris et al (2006) in the same cell model to detect for the same inflammatory markers. However, for our experiment, we found that the latter was a more potent pro-inflammatory agent. LPS from E. coli 0111:B4 did not cause a significant increase in PGE_2 concentration as compared to LPS from E. coli 055:B5. At a lower final concentration (2 μg/ml), neither LPS serotype induced significant PGE_2 production in the cells. However, when LPS from E. coli 055:B5 was used at 10 μg/ml, a significant rise in PGE_2 concentration was observed in the positive control relative to the other treatments.

Although the measured PGE_2 values were significantly higher at 16 hours and 24 hours post-LPS application, we chose to measure the effects of the teas on PGE_2 levels at 12 hours of incubation. Optimal cell morphology and confluency was observed at this time. In order to obtain reproducible results, it is essential to maintain cells in the same media formulation and conduct cell passages and experiments at the same time of day (Freshney 2005). When RAW cells are healthy and grown in the conditions specified, they typically have a regular, double teardrop shape with clearly defined edges. When RAW cells are induced by LPS, they transform into amorphous, distended shapes with long, thin extensions that protrude from the body of the cell. After 12 hours of incubation in LPS and tea treatments, the cells were confluent and present at adequate concentrations for experimental analysis. Beyond 12 hours, the cells became very crowded in the wells, appeared rounded, and began to clump and stack. These changes in morphology and growth can alter the cells' response to treatment compounds and yield erroneous results.

3.5.2 Cell Viability

The Trypan Blue assay is a rapid method of determining cell viability and concentration. However, there are a number of limitations to using Trypan Blue dye. Researchers have observed that the dye may overestimate cell viability (Smith & Smith 1989), produce inaccurate results after adherent cells are scraped (Tennant 1964), and be blocked from uptake from both live and dead cells grown in cell culture media containing serum proteins (Black and Berenbaum 1964). Also, cells should be counted within 3-5

minutes after adding the dye because the number of stained cells increases after the dye is added (Jones & Senft 1985).

The MTT assay is a quick and commonly used technique for establishing cytotoxic effects of compounds used to treat cells. Because none of the values were significantly different from the negative control, the concentrations of the teas and LPS used did not appear to have a cytotoxic effect. In contrast, the highest level of BT added alone and in combination with LPS seemed to increase cell viability slightly above the positive control. Although these values are not statistically significant, high concentrations of BT and LPS may have caused a stress response in the cells adequate enough to stimulate cell growth and increased metabolism.

Just as with Trypan Blue, using MTT also has disadvantages. First of all, the proper incubation time of the MTT with the cells is difficult to determine. Generally, 3-4 hours of incubation time is suggested (Mosmann 1983, Freshney 2005). However, the treatment(s) used may interfere with the ability of the cells to reduce the MTT dye such that the incubation period may need to be adjusted. Furthermore, it is challenging to avoid pipetting and disrupting the formazan crystals when the cell culture media is removed.

DMEM was the cell culture media used in this study. Phenol red is included in DMEM as a colorimetric pH indicator – as pH decreases, this compound changes from red to orange to yellow. As RAW 264.7 cells proliferate and metabolize, the phenol red makes it relatively easy to visually determine if the cells need to be passaged due to increased growth and/or metabolism. Despite careful efforts to remove all media from MTT-treated wells, trace amounts of media and the applied tea treatments may have remained in the well with

the crystals. The phenol red, FBS (which is added to the media), and the green and black tea may all add background noise and affect absorbance readings. This can lead to variable results within treatment replicates. Therefore, using cell culture media without phenol red or FBS in future studies may reduce erroneous absorbances.

There are other cell viability methods that may be more accurate and versatile. Simultaneous fluorometric staining with fluorescein diacetate and propidium iodide is similar to the Trypan Blue assay. As with Trypan Blue, the dyes can be applied within a few minutes. The fluorescent dyes remain stable after cell uptake, and cell viability estimates can be achieved up to 1 week after dye application. This latter staining technique may be more convenient when a large number of samples need to be counted (Jones & Senft 1985).

The XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay has similar sensitivity as compared to the MTT assay. The former is also a colorimetric assay that measures cell proliferation. Like MTT, XTT is also a tetrazolium salt that is cleaved by dehydrogenases in viable cells. In contrast to the MTT assay, the resulting colored XTT formazan product is water soluble. This eliminates the need to solubilize the formazan product (as in the MTT assay), and shortens the amount of time and sample handling needed to perform cell viability measurements (Roehm et al 1991). Therefore, the use of XTT rather than MTT should be considered for future cell viability studies.

3.5.3 PGE_2 ELISA

GT and BT significantly and similarly suppressed PGE_2 levels. These results show that the teas can function as anti-inflammatory agents by inhibiting the enzyme that produces PGE_2. The teas may also suppress the formation or activity of upstream metabolite(s) in the COX pathway of the arachidonic acid-dependent pathway. The mechanism by which each of these teas decreased PGE_2 is unknown. Future studies should explore the possibility that, because of differences in composition, each type of tea has different anti-inflammatory mechanisms.

3.5.4 Western Blotting

Detection of COX-2 in our tea-treated, LPS-induced RAW 264.7 cells demonstrated that only GT 5% significantly inhibited COX-2 relative to the positive control. However, the values for the GT and BT controls were not statistically different from the negative control, which implies that the teas in the absence of LPS are not inflammatory stimulants. Our results show that the teas may not suppress inflammation by acting directly on COX-2 synthesis. The teas may have alternate mechanisms of inhibiting inflammation, such as altering COX-2 activity, rather than blocking its expression. Western blots simply detect the presence of a protein of interest, not the activity of that protein. COX-2 was produced as a result of LPS stimulation, but the COX-2 present may not necessarily be active. Future work should measure the activity of COX-2 in the presence of teas to determine if there is an effect. In addition, other metabolites in the COX pathway, such as PGE_2 synthase, should be analyzed to isolate the specific targets by which the teas modulate inflammation.

3.6 CONCLUSIONS

Based on our results, the tea and LPS treatments did not significantly affect cell viability. GT and BT both exhibited anti-inflammatory activity by similarly suppressing PGE_2 in LPS-induced RAW 264.7 mouse macrophages. However, except for GT 5%, the teas did not significantly inhibit the production of COX-2. This does not necessarily mean that the teas cannot inhibit inflammation in the COX pathway; rather, the teas may interact with a COX-2 metabolite or interfere with COX-2 activity. Future research should further investigate the specific mechanisms by which green tea and black tea alter the inflammatory response.

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CHAPTER 4: SUMMARY AND SUGGESTIONS FOR FUTURE WORK

Tea is one of the most popular beverages in the world, with green tea and black tea comprising over 95% of the world's tea production and consumption. Tea is widely accessible and is consumed by over two-thirds of the world's population. For centuries, tea has been known for its health effects. Modern investigations have demonstrated the antioxidant and anti-inflammatory effects of tea and tea polyphenols. Some studies have even shown teas' superior antioxidant and anti-inflammatory function as compared to other nutrients and plant-based foods. While the mechanisms of teas' health effects are not completely understood, the chemical structures of tea polyphenols play a key role in tea's ability to effectively modulate oxidative stress and chronic inflammation. This is achieved by the interaction of tea polyphenols with inflammatory metabolites in a number of pathways that regulate inflammation.

The weight of evidence shows that teas have the capacity to suppress or inhibit inflammation in cell culture and animal models. According to our results, green tea and black tea did exhibit anti-inflammatory activity in LPS-induced RAW 264.7 mouse macrophages by similarly suppressing PGE_2. However, only green tea applied at the highest treatment concentration (5% of treatment well volume) significantly decreased COX-2 expression. These results suggest that the teas may affect the activity of the enzyme rather than its expression, or interact with a different inflammatory marker within this pathway. Further research needs to be conducted to understand the exact mechanisms by which green tea and black tea suppress inflammation, and the specific compounds that they inhibit.

It is well-established that teas suppress pro-oxidant activity and inflammation in *in vitro* and animal studies. Currently, there is no consensus among human studies that supports

a strong association between tea intake and the prevention of chronic disease. Many epidemiological studies have often lacked precise documentation of the amounts of tea consumed, the specific type of tea being analyzed, and how subjects prepare tea. In order to uncover the true effect of teas on human diseases, the development of more accurate reporting and addressing these confounding factors is needed.

Additionally, future studies should include human studies that analyze the efficacy of teas in inhibiting inflammation. These include translational studies, i.e. analyzing inflammatory markers in human blood samples from clinical trial participants who have consumed controlled amounts of tea. These types of studies will allow for a better understanding of teas' effects in humans and their potential to effectively decrease the incidence of inflammation and the symptoms associated with the emergence of chronic diseases. Determining the health effects of teas in humans may lead to the development of recommendations for tea consumption in order to maintain health and as a preventive measure against the incidence of chronic disease.

APPENDIX A. HPLC/MS ANALYSIS OF MULTIPLE INFLAMMATORY MARKERS IN LPS-INDUCED RAW 264.7 MACROPHAGES

A.1 INTRODUCTION

When an inflammatory response is induced within a cell or tissue, a large number of mediators, such as eicosanoids, are synthesized, released, and cause a broad range of pro-inflammatory and anti-inflammatory effects (Santangelo et al 2007). Eicosanoids are 20 carbon-containing regulatory molecules that are products of arachidonic acid (AA). AA is released from membrane phospholipids by phospholipase A_2 (PLA_2) (Horton et al 2002). The free AA is converted into hundreds of bioactive compounds by a number of enzymes via three main pathways: cyclooxygenase, lipoxygenase, and cytochrome P450. Examples of eicosanoids include prostaglandins (PGs), prostacyclins, and thromboxane A_2 (TXA_2). Despite the presence of these distinct pathways, some eicosanoids can be produced by more than one enzyme. In addition, some enzymes can make more than one eicosanoid. Therefore, the production of eicosanoids is a complex, integrated web of intermingled pathways and associated metabolites. In order to fully understand a cell or tissue's overall response to inflammatory stimuli and eicosanoid interaction with each other and their target sites, multiple markers of inflammation must be simultaneously analyzed (Deems et al 2007).

For decades, enzyme-linked immunosorbent assays (ELISAs) have been used to quantify one eicosanoid at a time. Nonetheless, very few commercial antibodies have been made specifically for detecting eicosanoids. Many ELISA kits tend to be inefficient since only one compound can be detected for each kit (Deems et al 2007). There is also much variability between treatment replicates and kits within the same lot, suggesting a lack of standardization and quality control in manufacturing ELISA kits. In recent years, ELISA kits

for multiple inflammatory markers have been developed. However, these kits are even more expensive and less accurate than those that detect for only one compound.

The goals of the following experiment were to develop a method of accurately identifying and quantifying multiple eicosanoids and inflammatory mediators by using HPLC/MS. Our lab provided LPS-induced RAW 264.7 cells treated with varying amounts of green tea and black tea, and prepared the samples for analysis. Dr. Lisa Dean from the USDA, ARS, Market Quality and Handling Research Unit (Raleigh, NC), and Dr. Tad Dean from Microbac Laboratories Southern Testing Division (Wilson, NC) performed high performance liquid chromatography (HPLC)/mass spectrometry (MS) analysis on a set of standards and samples in an effort to detect for specified inflammatory markers.

A.2 MATERIALS AND METHODS

Standards (8-*iso* prostaglandin $F_{2\alpha}$, prostaglandin E_2, prostaglandin I_2, and leukotriene B_4) were purchased from Cayman Chemical Company (Ann Arbor, Michigan). All chemicals were of analytical purity.

A.2.1 Harvesting LPS-stimulated RAW 264.7 cells Treated with Green Tea and Black Tea

RAW 264.7 cells were plated at 10^5 cells/ml into 12-well plates at 1.5 ml/well. Once the cells had achieved 80% confluency, cells were treated with 1%, 2%, or 5% green tea (GT) or black tea (BT) extracts, and incubated at 37° C and 5% CO_2 for 2 hours. Next, LPS from E. coli 055:B5 (5 mg/ml stock) was added to the LPS treatment wells at a final concentration of 10 μg/ml and incubated at 37° C and 5% CO_2 for 12 hours.

Upon harvest, 1.5 ml of media was pipetted out of each well and into correspondingly labeled microcentrifuge tubes. Ethanol (75 µl or 5% of total volume) was added to the media in each tube and mixed. Then, each well was rinsed 3 times with cold phosphate-buffered saline (PBS). Next, methanol (250 µl) was added to each well, and adhered cells were scraped using cell lifters. PBS (500 µl) was pipetted into each well, and the entire contents from each well (~750 µl) were transferred into correspondingly labeled microcentrifuge tubes. All media and cell lysate samples were stored at -80° C. Media and cell lysate triplicates of the negative control (cells only), positive control (cells and LPS), GT 5% + cells + LPS, and BT 5% + cells + LPS (24 total samples) were given to Dr. Dean for HPLC/MS analysis. Figure A.1 shows how harvesting is performed.

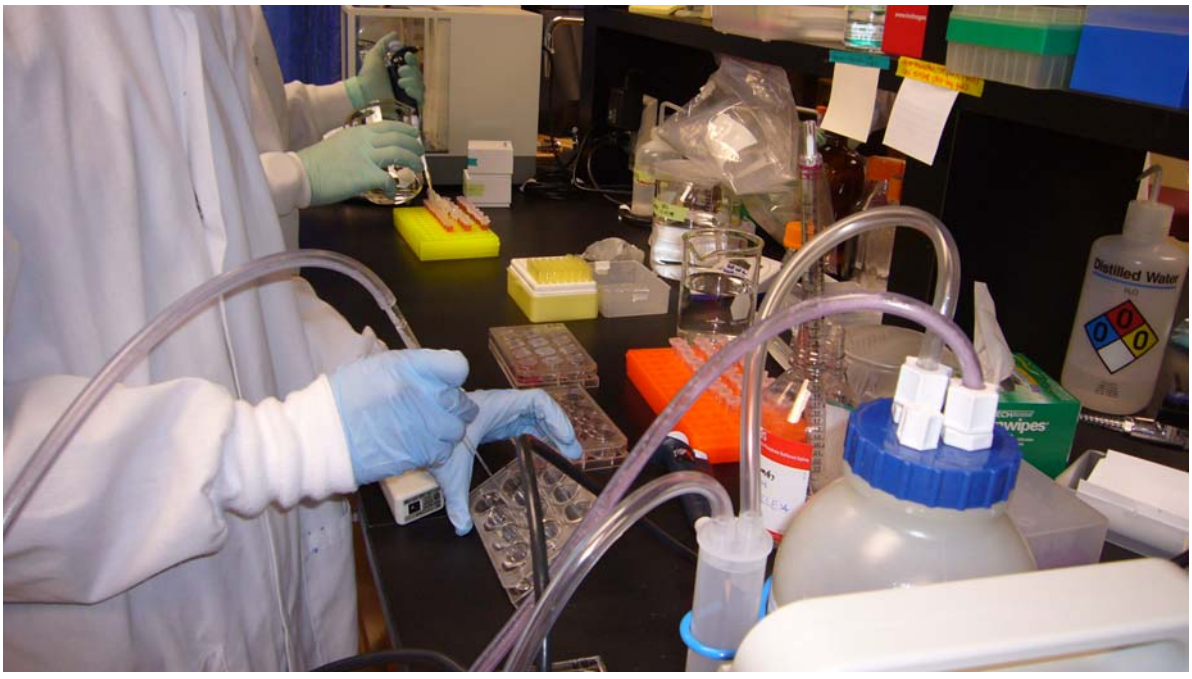

Figure A.1. Harvesting Cells for HPLC/MS Analysis

A.2.2 Standard and Sample Isolation

Standard and sample extraction was achieved using Strata® X SPE columns (Phenomenex, Torrance, CA). First, the columns were washed with methanol (2 ml), then 2 ml of water. Next, the sample or standard was applied, and the columns were rinsed with 10% methanol (1 ml), then eluted with 1 ml of methanol. The resulting eluant was dried under a vacuum and re-dissolved in 0.1 ml of mobile phase A (63% water, 37% acetonitrile, 0.2% formic acid) for LC-MS/MS analysis.

A.2.3 Part 1: Reverse-phase Liquid Chromatrography and Mass Spectrometry

Initial HPLC analysis was performed on a Thermo Quest Surveyor (Thermo Scientific, Marietta, OH). The analysis of the standards and samples were performed by LC-MS/MS. The standards and prepared samples were separated on an Accutis RP-Amide column (3.9 mm x 150 mm, 5 μm) at a flow rate of 200 μl/min. Standards and samples were injected by an auto-sampler kept a constant temperature of 10° C to prevent sample degradation prior to and during analysis. The column was equilibrated in mobile phase A. The standards and samples were dissolved in mobile phase A, injected using a 20 μl injection loop, and eluted from 0 to 100% mobile phase B (50% isopropyl alcohol, 50% acetonitrile, 0.2% formic acid) between 0 to 10 minutes; mobile phase A was increased to 100 while mobile phase B was dropped to 0% between 10 to 11 minutes; and mobile phase A was sustained at 100% until 15 minutes.

MS was run on a Finnigan LCQ DUO (Thermo Scientific, Marietta, OH). The IonTrap parameters are as follows: capillary temperature = 250° C, sheath flow rate (N_2) =

60 Abu, auxillary flow rate (He) = 10 Abu, and voltage = 4.55 kV. For all standards, 40 V collision energy was used.

A.2.3.2 Part II: Reverse-phase Liquid Chromatrography and Mass Spectrometry

A second method of standard and sample detection was performed that enabled higher sensitivity and a lower detection limit (5 ng). HPLC analysis was done using a Shimadzu LC10AT (Shimadzu Scientific Instruments, Columbia, MD). Samples were harvested and isolated as previously discussed. The standards and samples were separated on a Phenomenex Luna 3 u, C18 column (2.0 x 150 mm, 3 µm) at a flow rate of 200 µl/min. Standards and samples were loaded using a Shimadzu SIL10AD. The standards and samples were dissolved in mobile phase A (70% water, 30% acetonitrile, 0.2% formic acid) and increased from 0 to 100% mobile phase B (50% isopropanol, 50% acetonitrile) in 10 minutes.

A 4000 QTRAP hybrid, triple-quadropole, linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) was used for MS analysis. The QTRAP parameters are as follows: CUR = 10 psi, TEM = 600°, GS1 = 70 psi, GS2 = 50 psi, ihe = ON, IS = -4500 V, CAD = HIGH, and CXP = -50 V. For all standards, -30 V collision energy was used.

A.2.4 Preliminary Experiments with RAW 264.7 Cells + LPS/PMA

Our laboratory also performed preliminary experiments in which we treated RAW 264.7 cells with LPS from E. coli 0111:B4 and phorbol 12-myristate 13-acetate (PMA) in an attempt to further exacerbate the inflammatory response in the cells. The cell harvest

methods and procedure for HPLC/MS analysis of these samples is the same as those discussed in sections A.2.1 and A.2.3.

A.3 RESULTS

Figure A.2 shows the chromatogram for the standards (see section A.2.3). The top peak is LTB_4, followed by PGE_2, 8-iso $PGF_{2\alpha}$, and PGI_2. Figure A.3 is a representative chromatogram for the "BT 1% + LPS" media samples. The chromatogram shows that the eicosanoids of interest could not be detected in the sample. These samples were analyzed according to "Part I: Reverse-Phase Liquid Chromatography and Mass Spectrometry".

Figures A.4a, 5a, 6a, and 7a are representative standard curves for the PGI_2, $PGF_{2\alpha}$, PGE_2, and LTB_4 standards, respectively. Figures A.4b-4j, 5b-5j, 6b-6j, and 7b-7j are chromatograms of standards, cell lysate samples, and media samples. The standards and samples were analyzed according to "Part II: Reverse-Phase Liquid Chromatography and Mass Spectrometry". The standards were each detected and quantified. However, the cell lysate and media samples did not reach the equipment detection limit of 5 ng and could not be detected or quantified. The majority of the samples had relatively low intensities and differing retention times relative to the standard peaks.

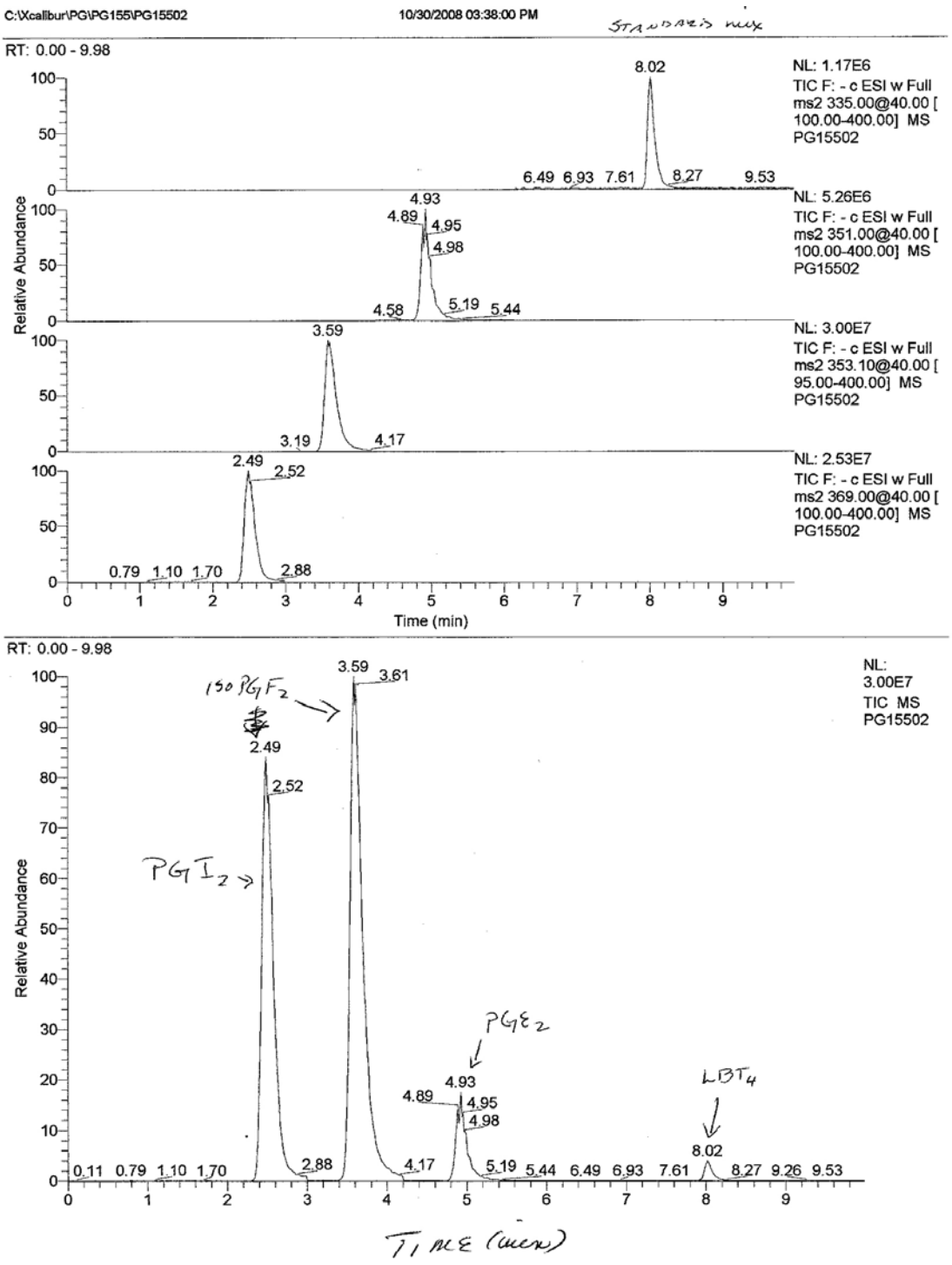

Figure A.2. Chromatogram for Standards Run on Finnigan LCQ Duo

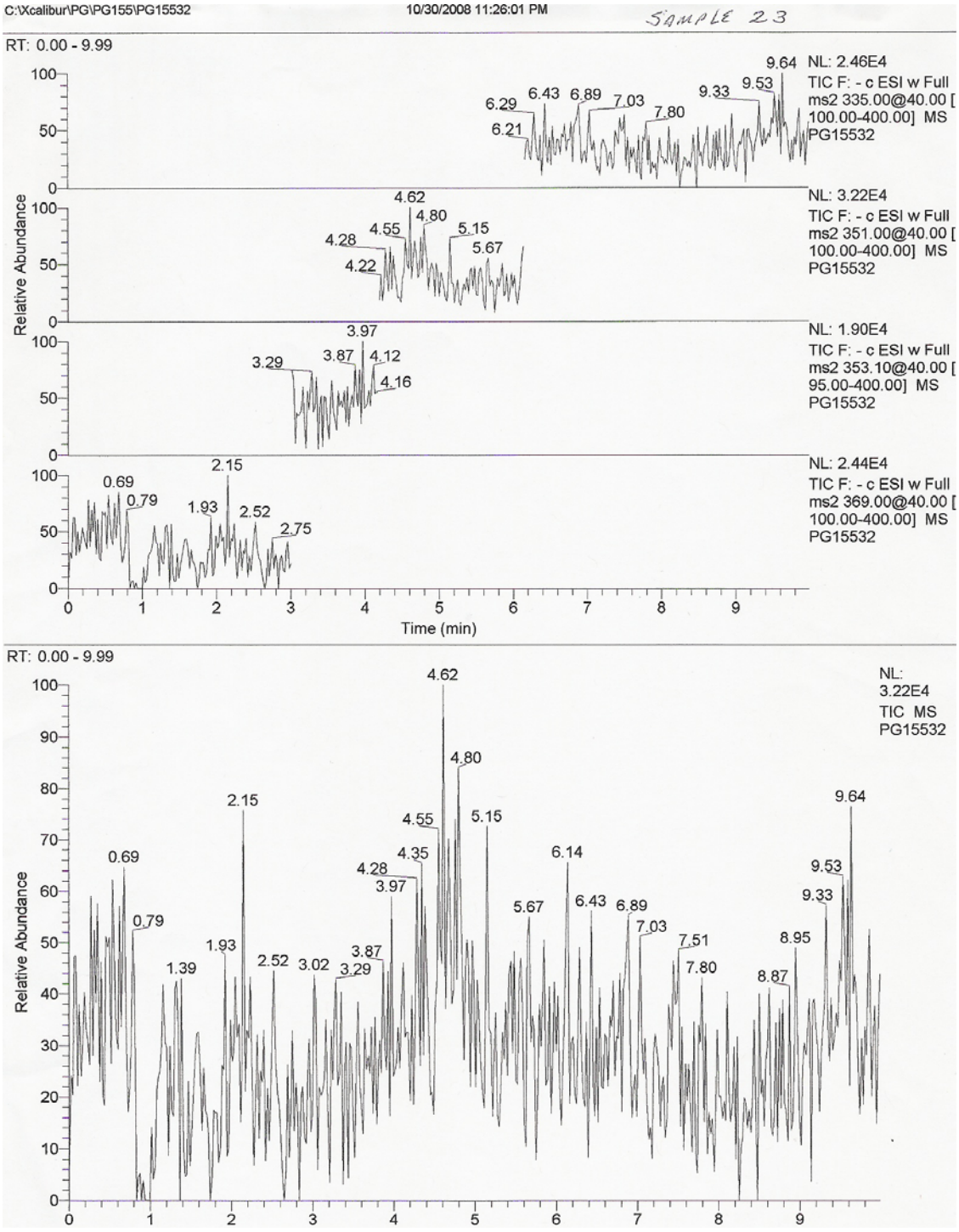

Figure A.3. Chromatrogram for Media Sample "BT 1% + LPS"

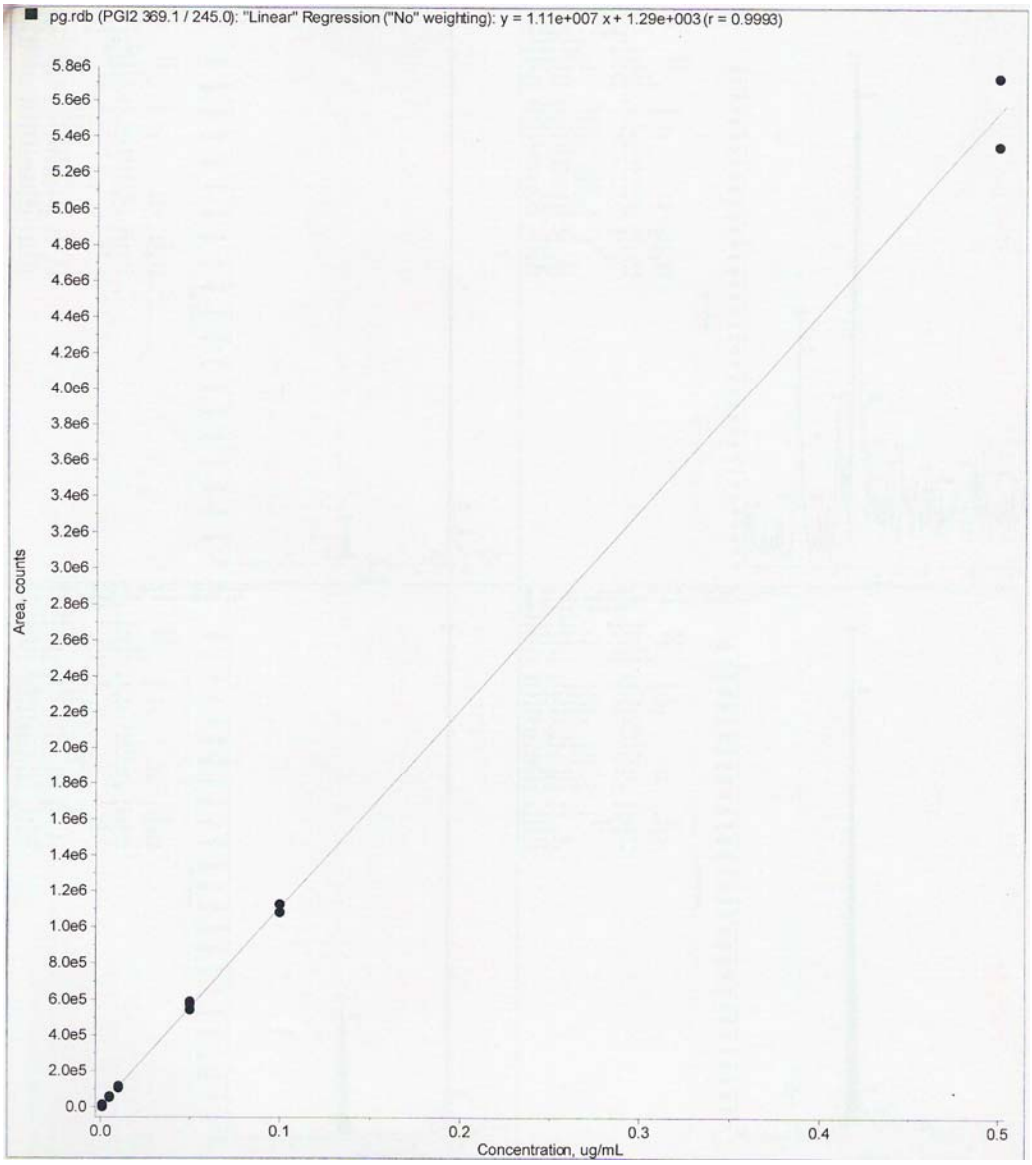

Figure A.4a. Standard Curve for PGI_2 Standard

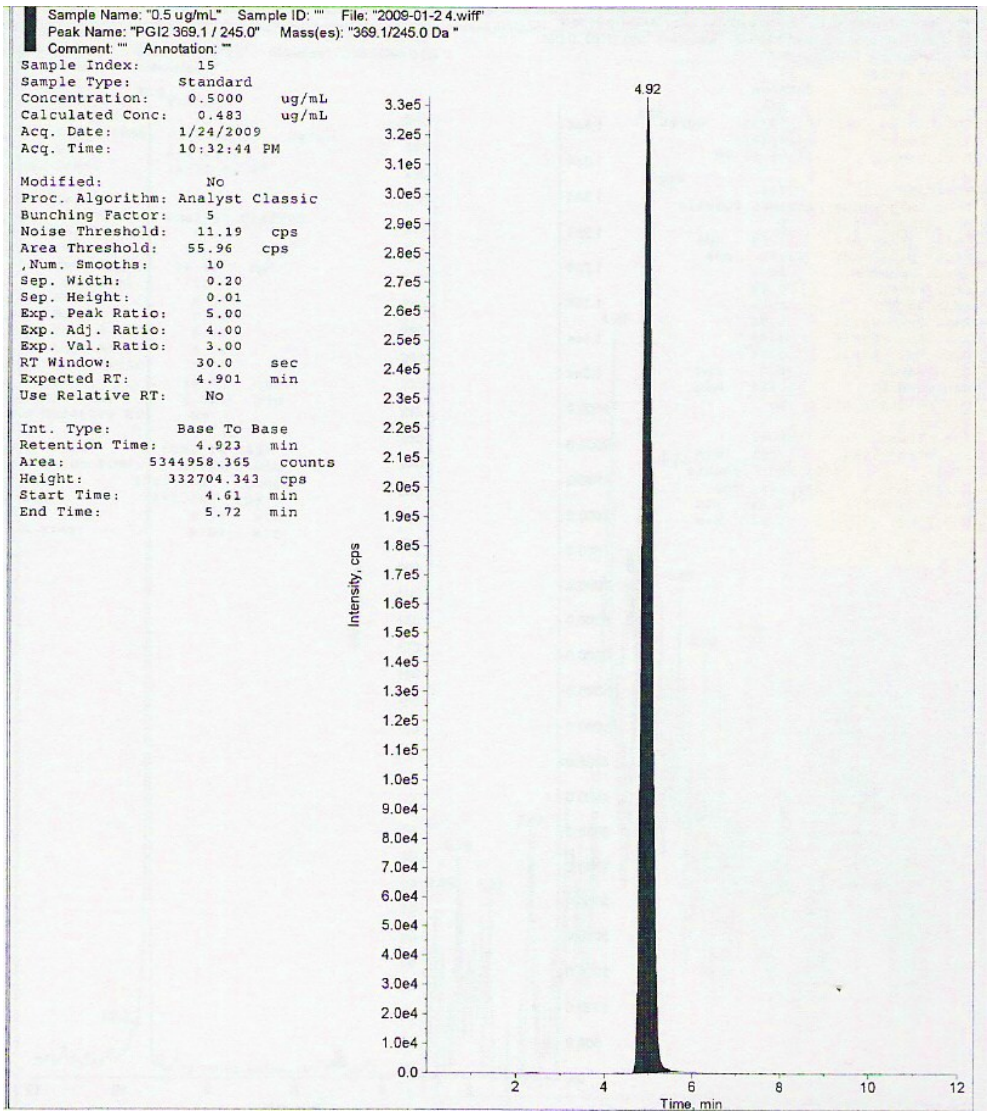

Figure A.4b. Chromatogram for PGI_2 Standard

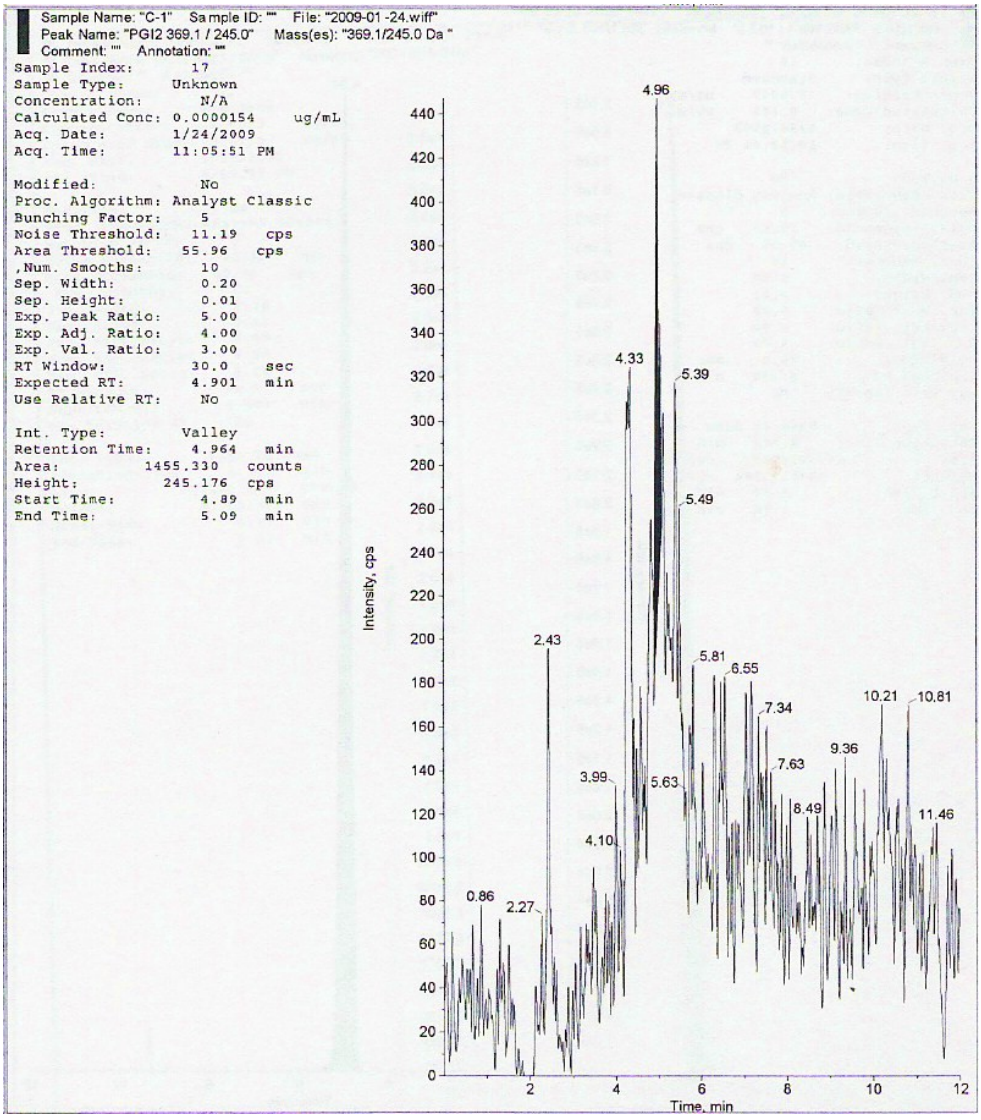

Figure A.4c. Chromatogram for Negative Control Cell Lysate

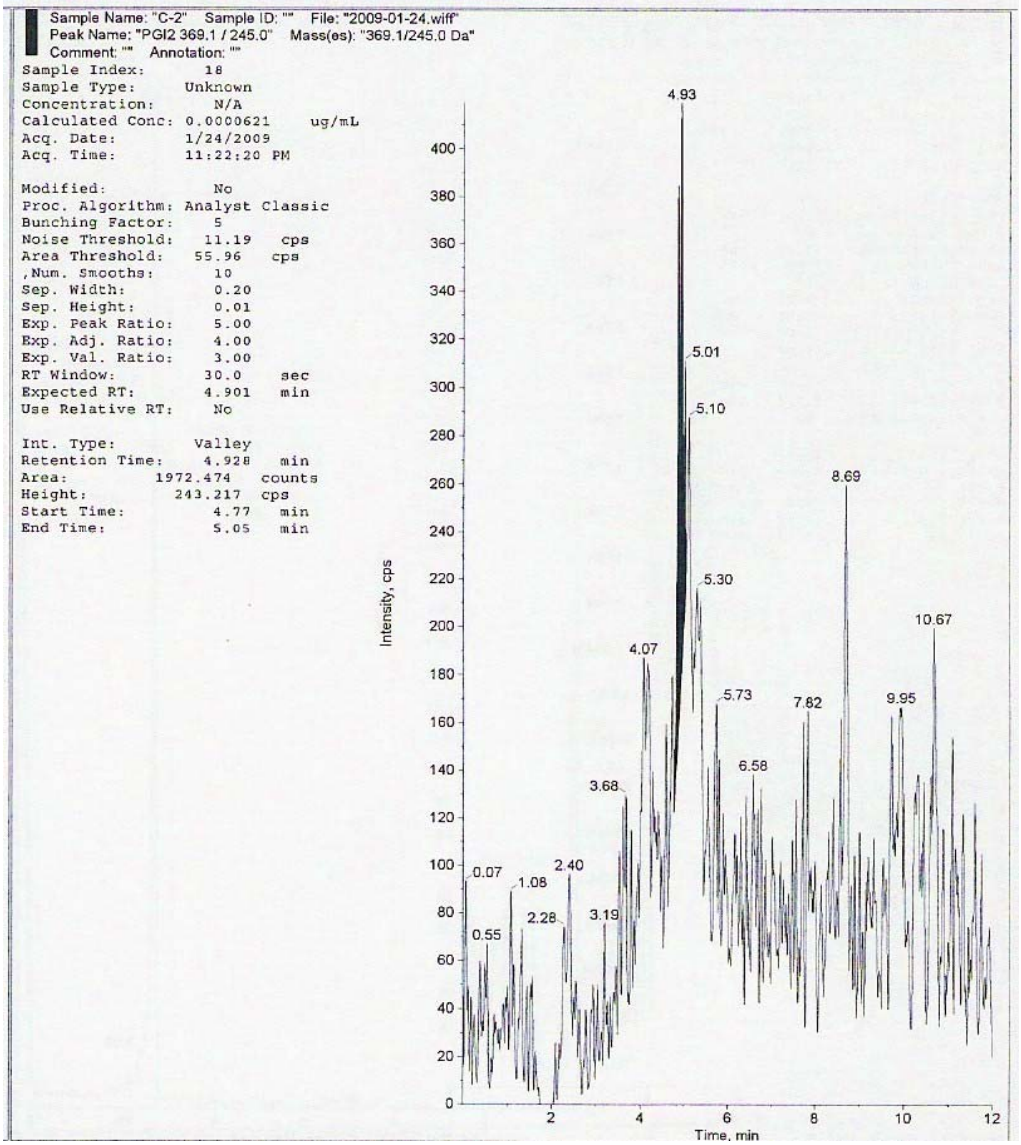

Figure A.4d. Chromatogram for Positive Control Cell Lysate

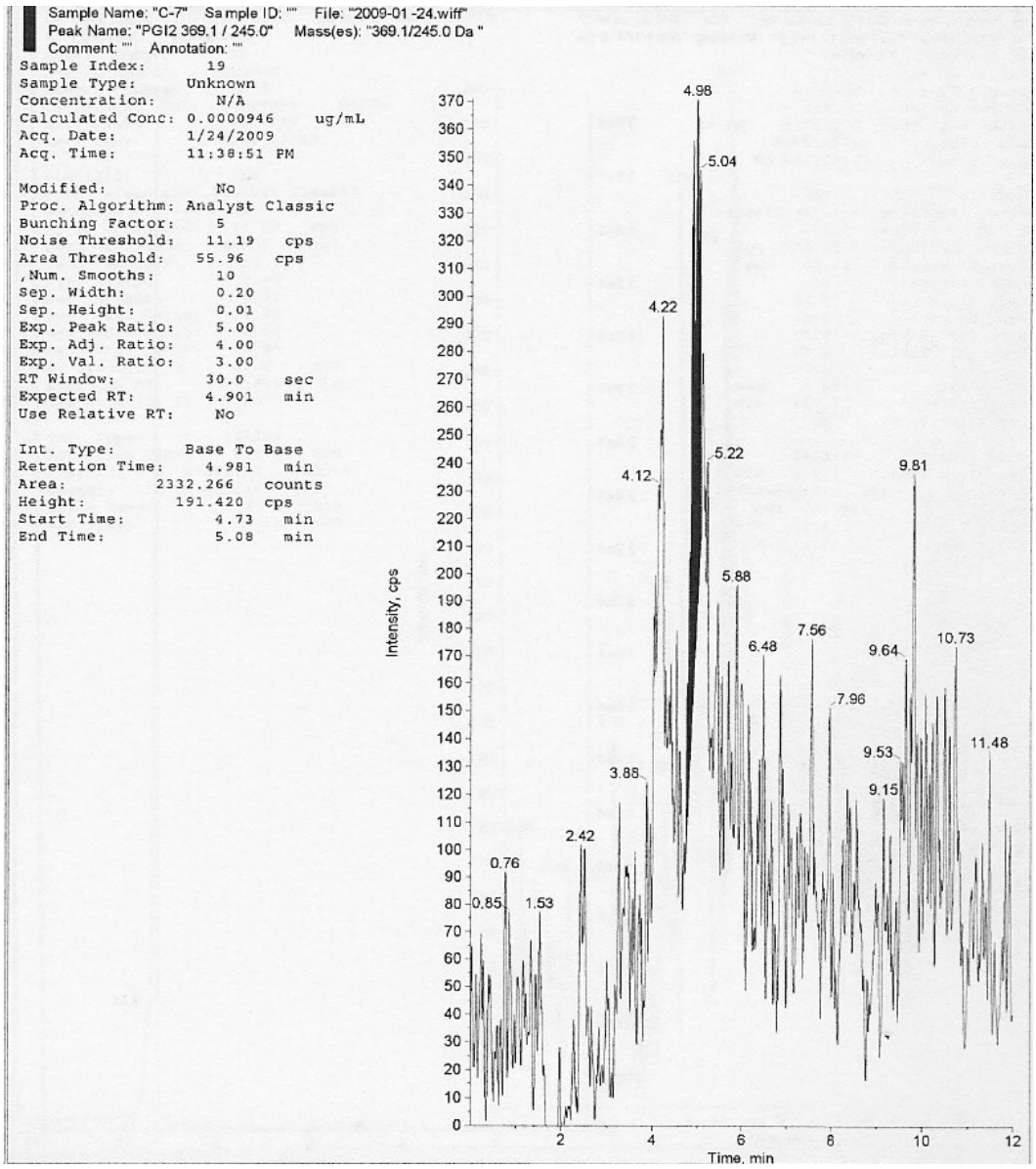

Figure A.4e. Chromatogram for "GT 5% + LPS" Cell Lysate

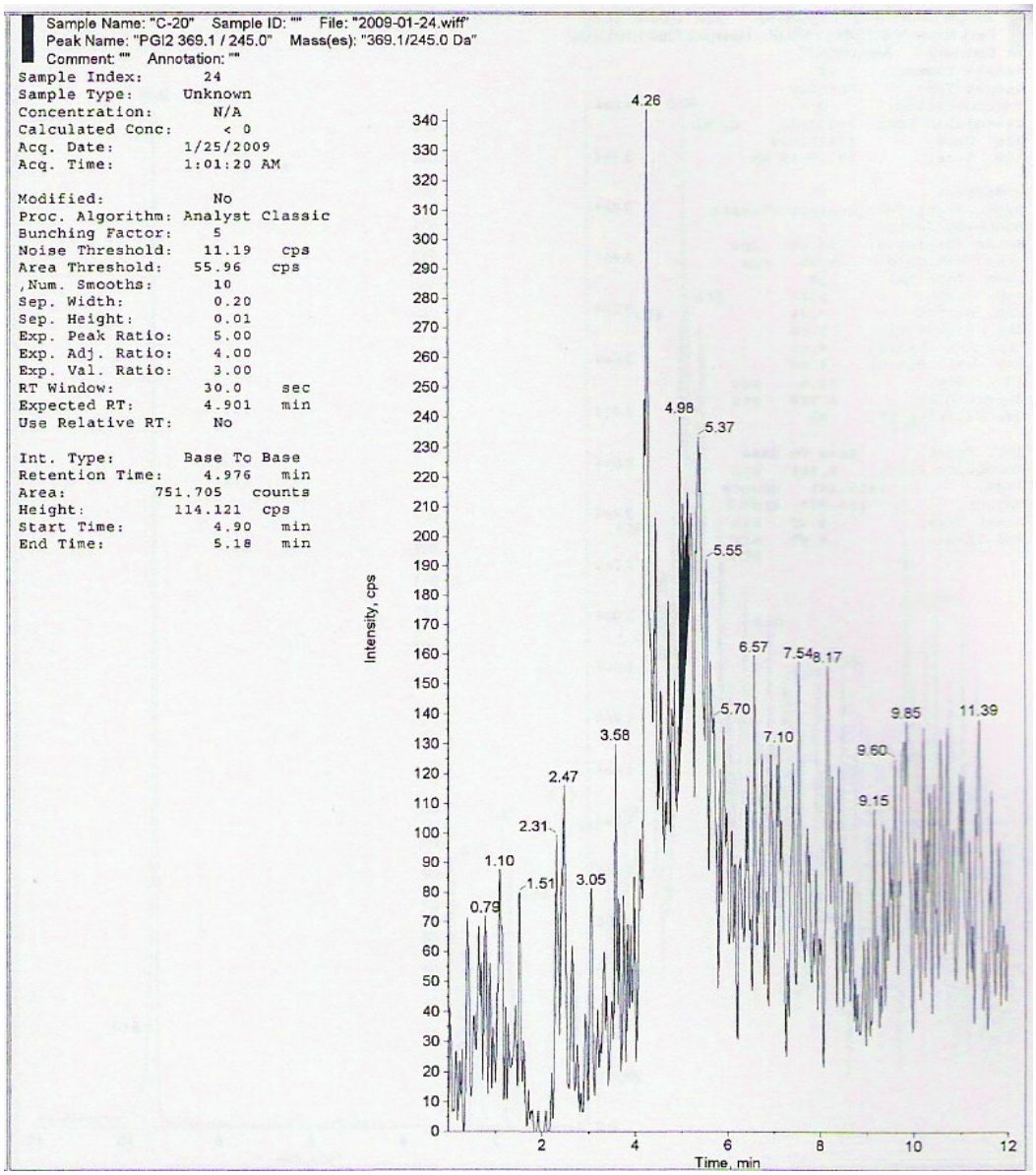

Figure A.4f. Chromatogram for "BT 5% + LPS" Cell Lysate

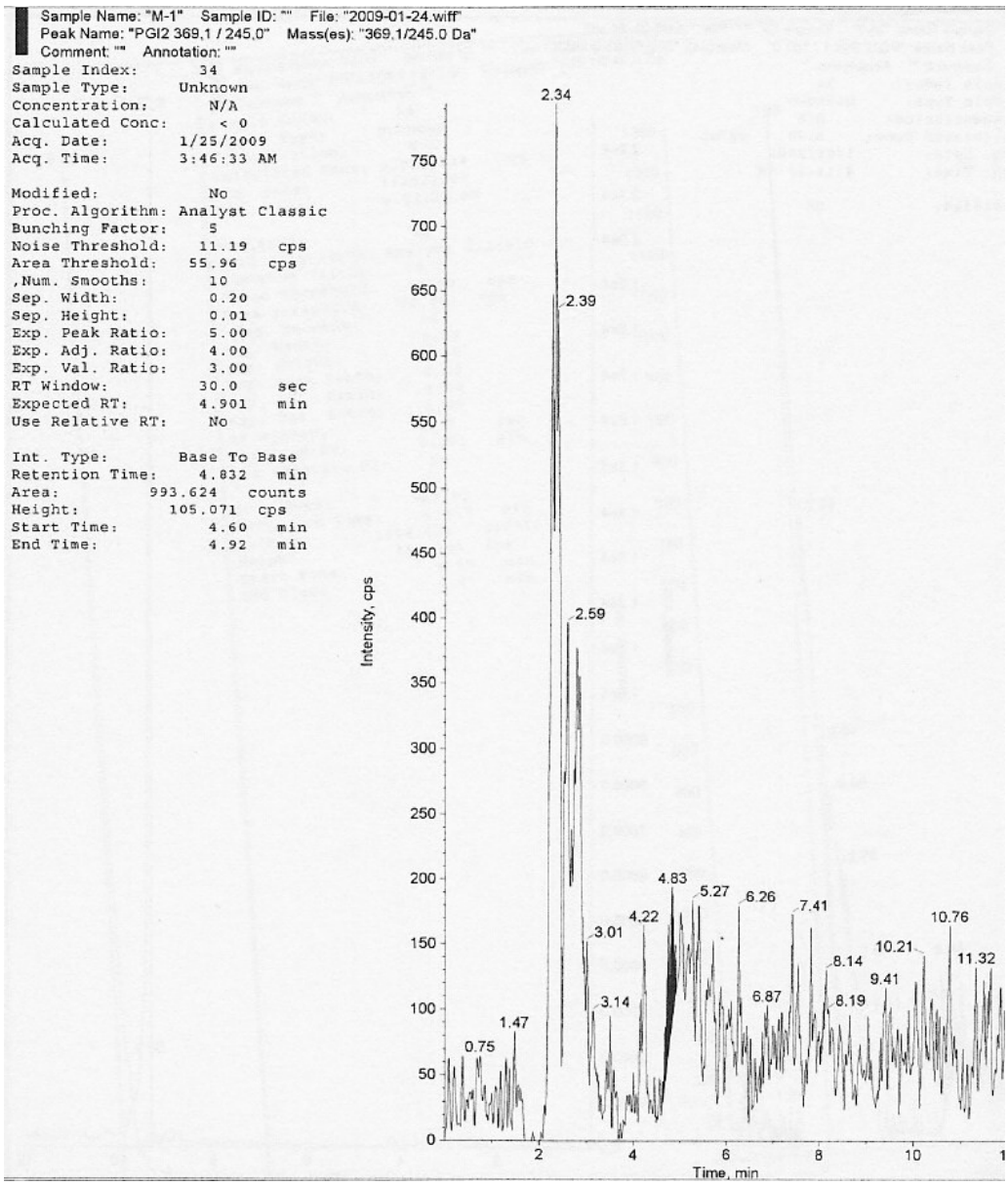

Figure A.4g. Chromatogram for Negative Control Media

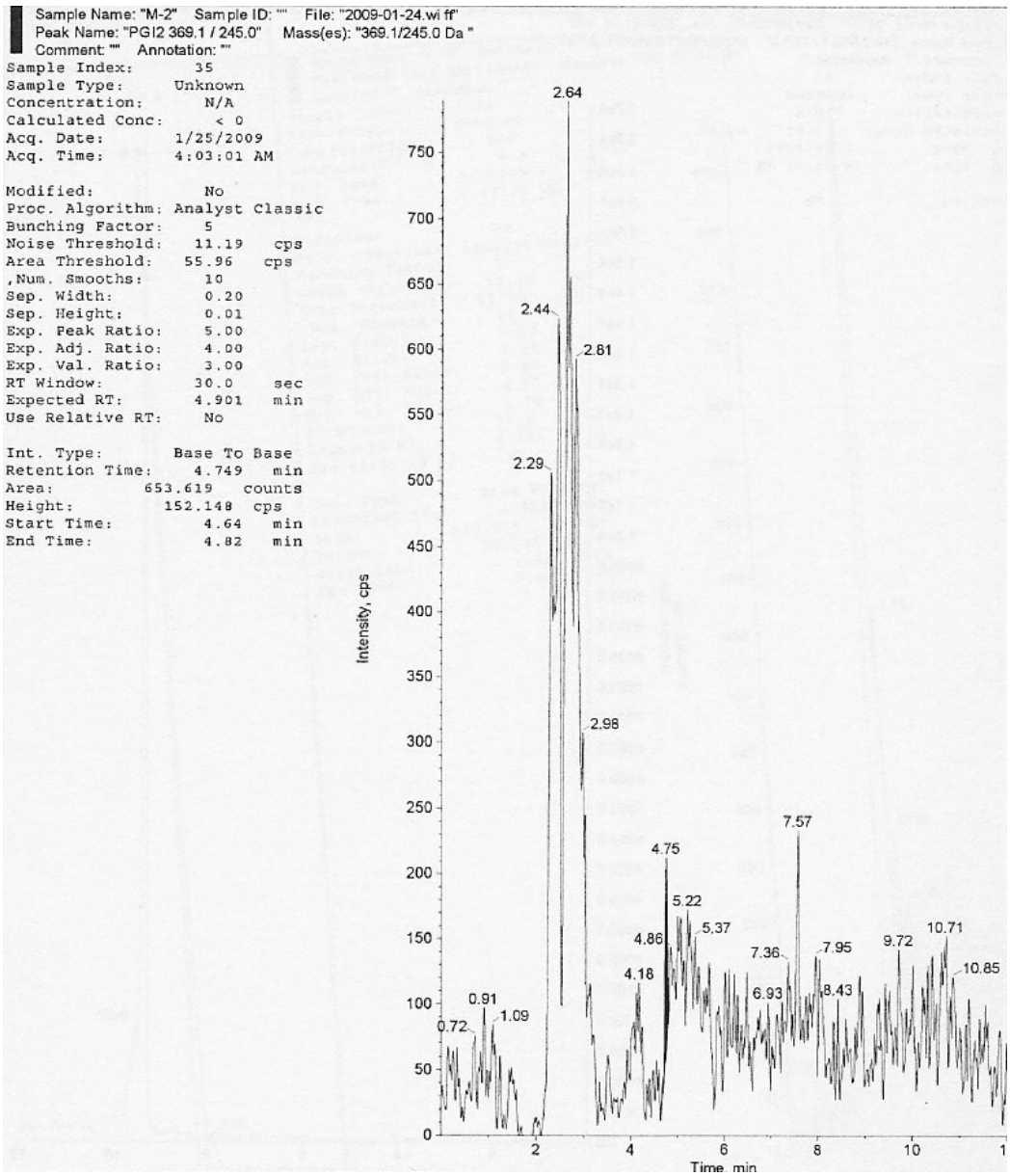

Figure A.4h. Chromatogram for Positive Control Media

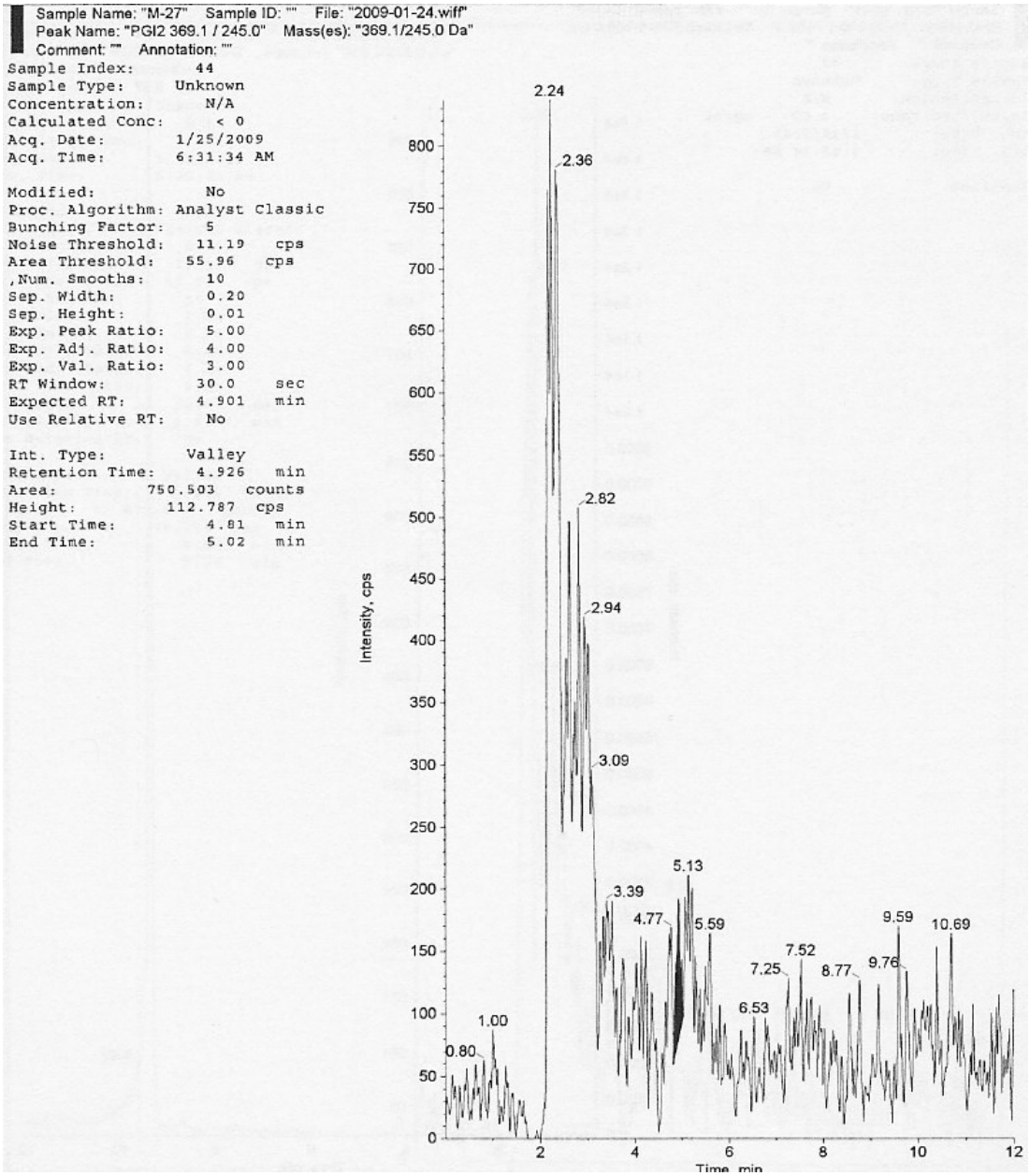

Figure A.4i. Chromatogram for "GT 5% + LPS" Media

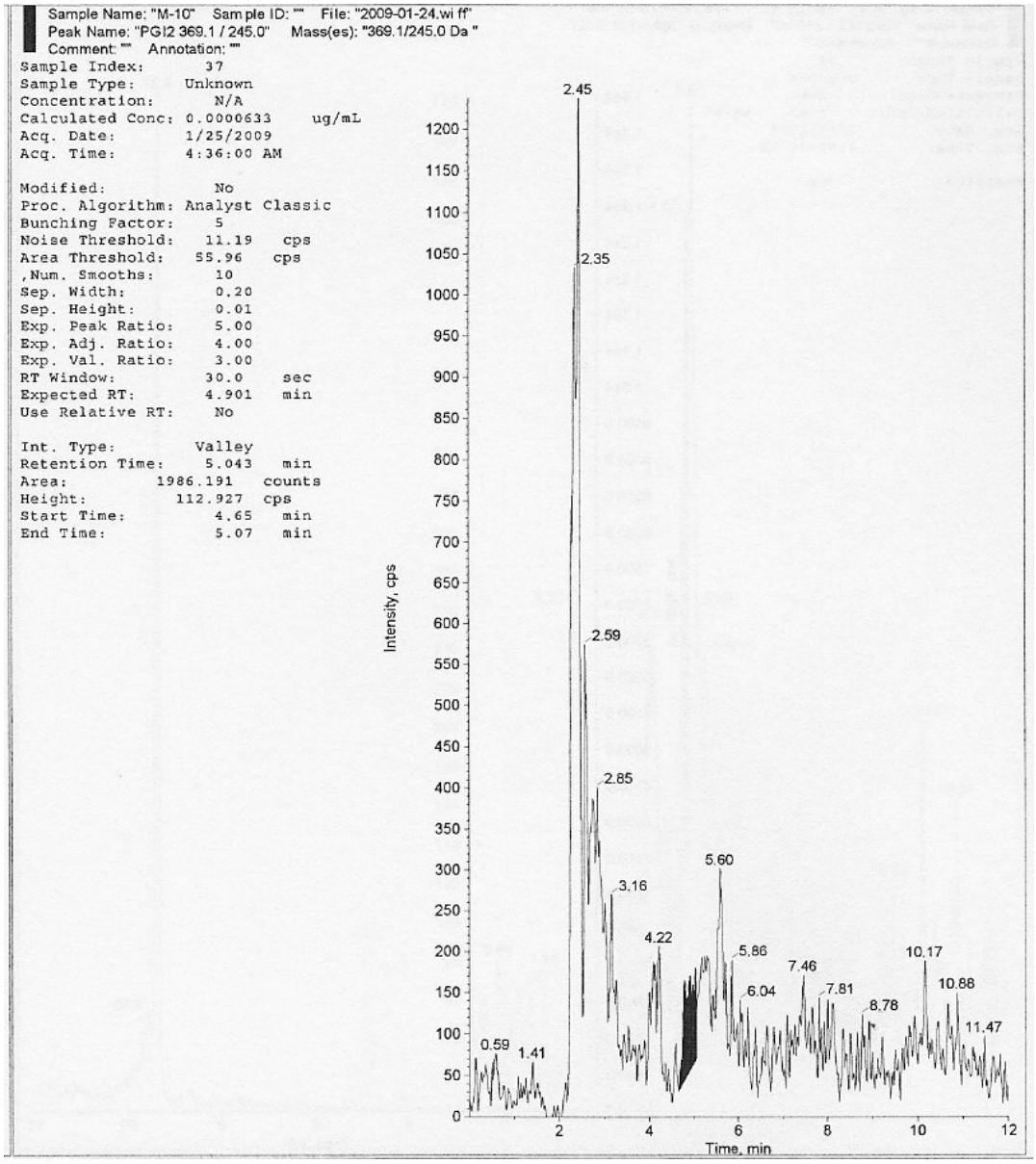

Figure A.4j. Chromatogram for "BT 5% + LPS" Media

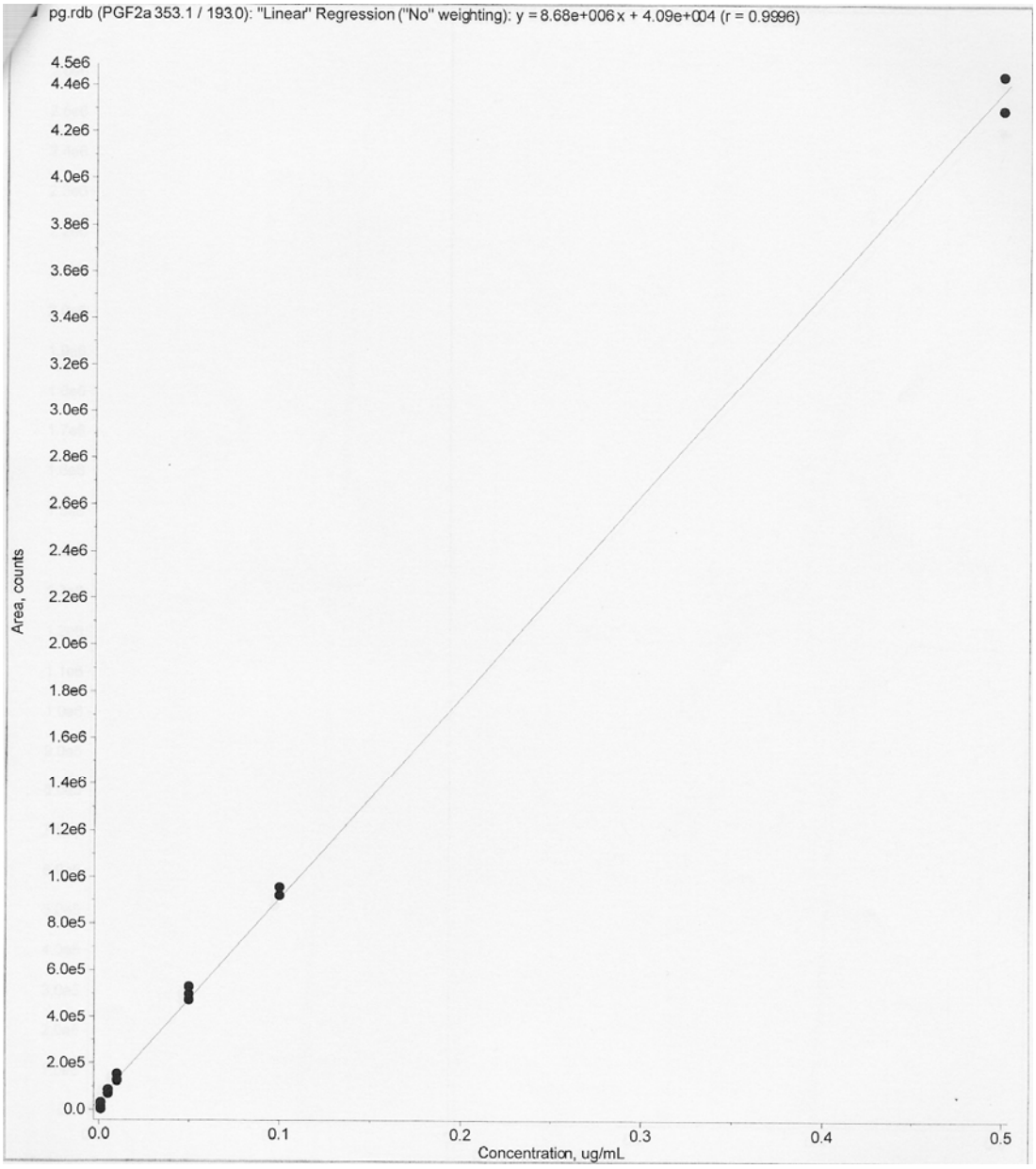

Figure A.5a. Standard Curve for $PGF_{2\alpha}$ Standard

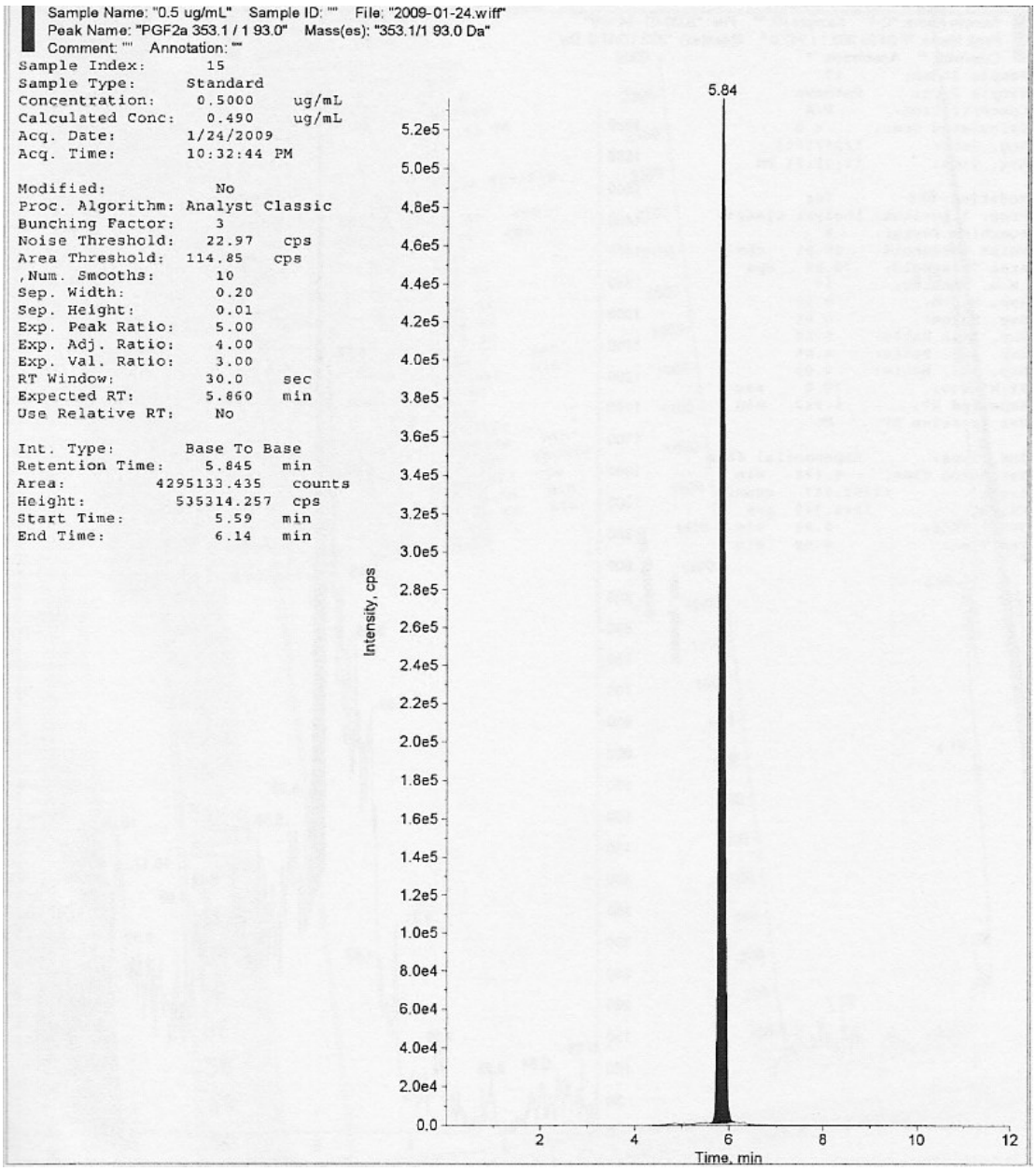

Figure A.5b. Chromatogram for $PGF_{2\alpha}$ Standard

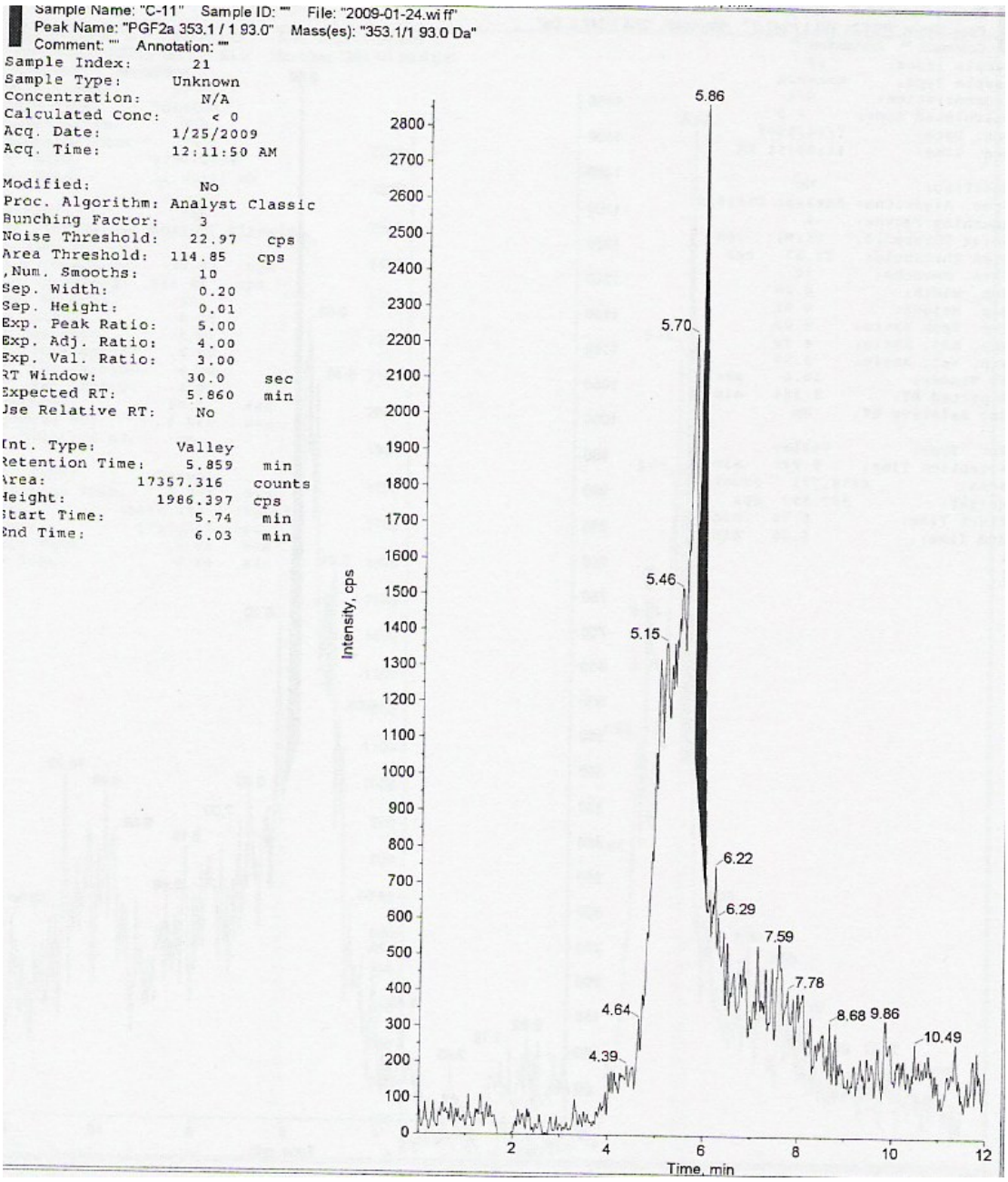

Figure A.5c. Chromatogram for Negative Control Cell Lysate

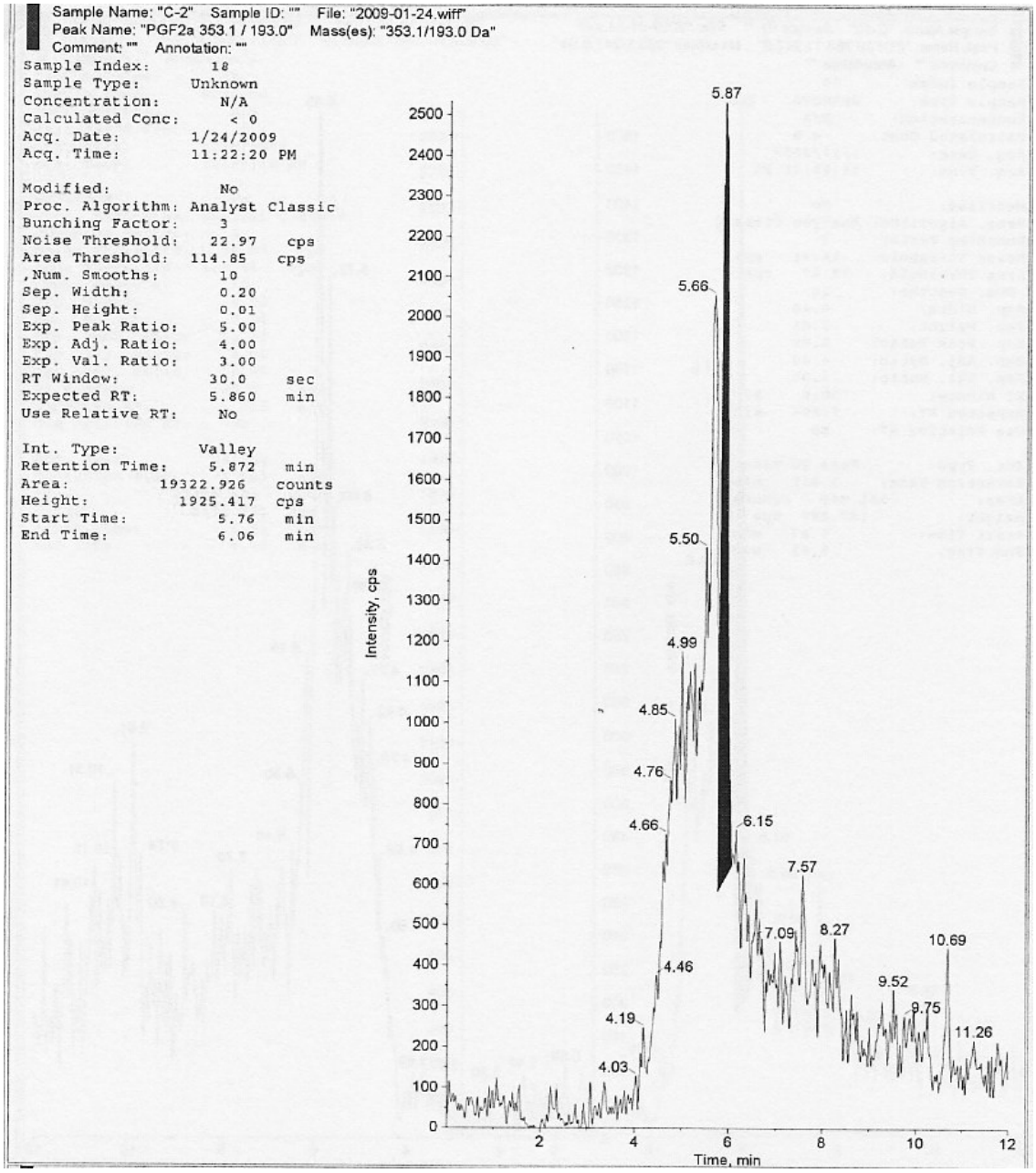

Figure A.5d. Chromatogram for Positive Control Cell Lysate

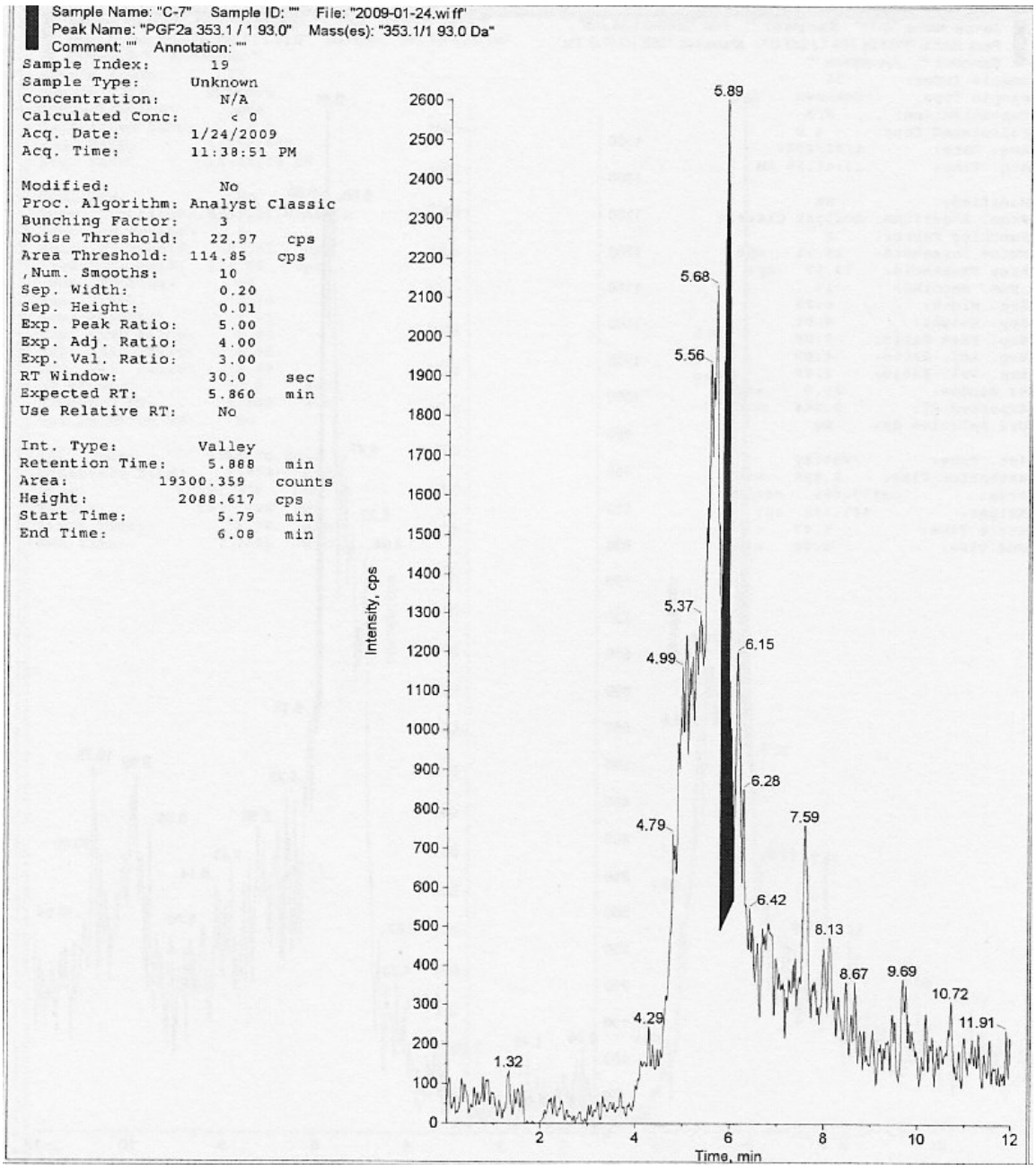

Figure A.5e. Chromatogram for "GT 5% + LPS" Cell Lysate

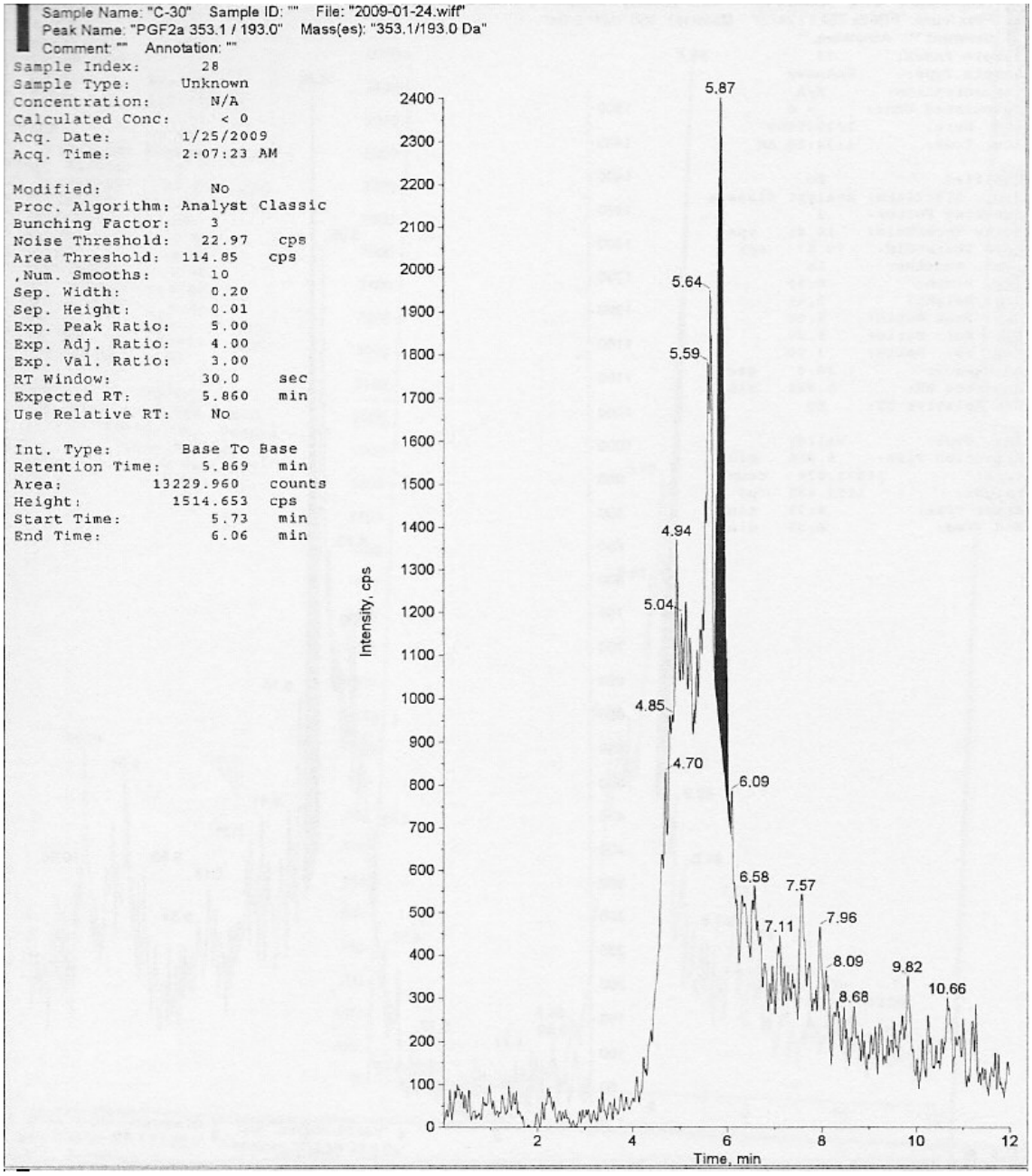

Figure A.5f. Chromatogram for "BT 5% + LPS" Cell Lysate

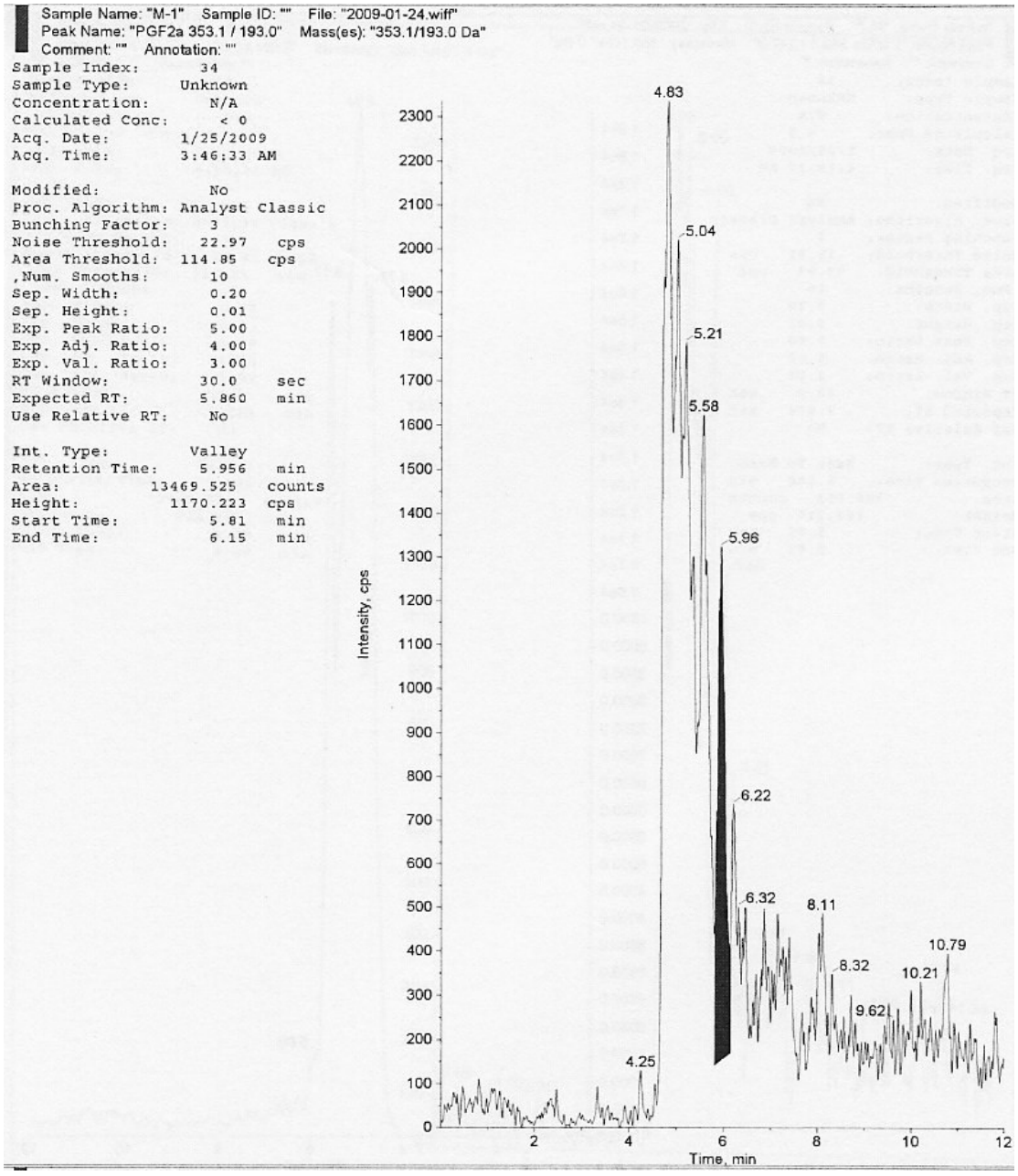

Figure A.5g. Chromatogram for Negative Control Media

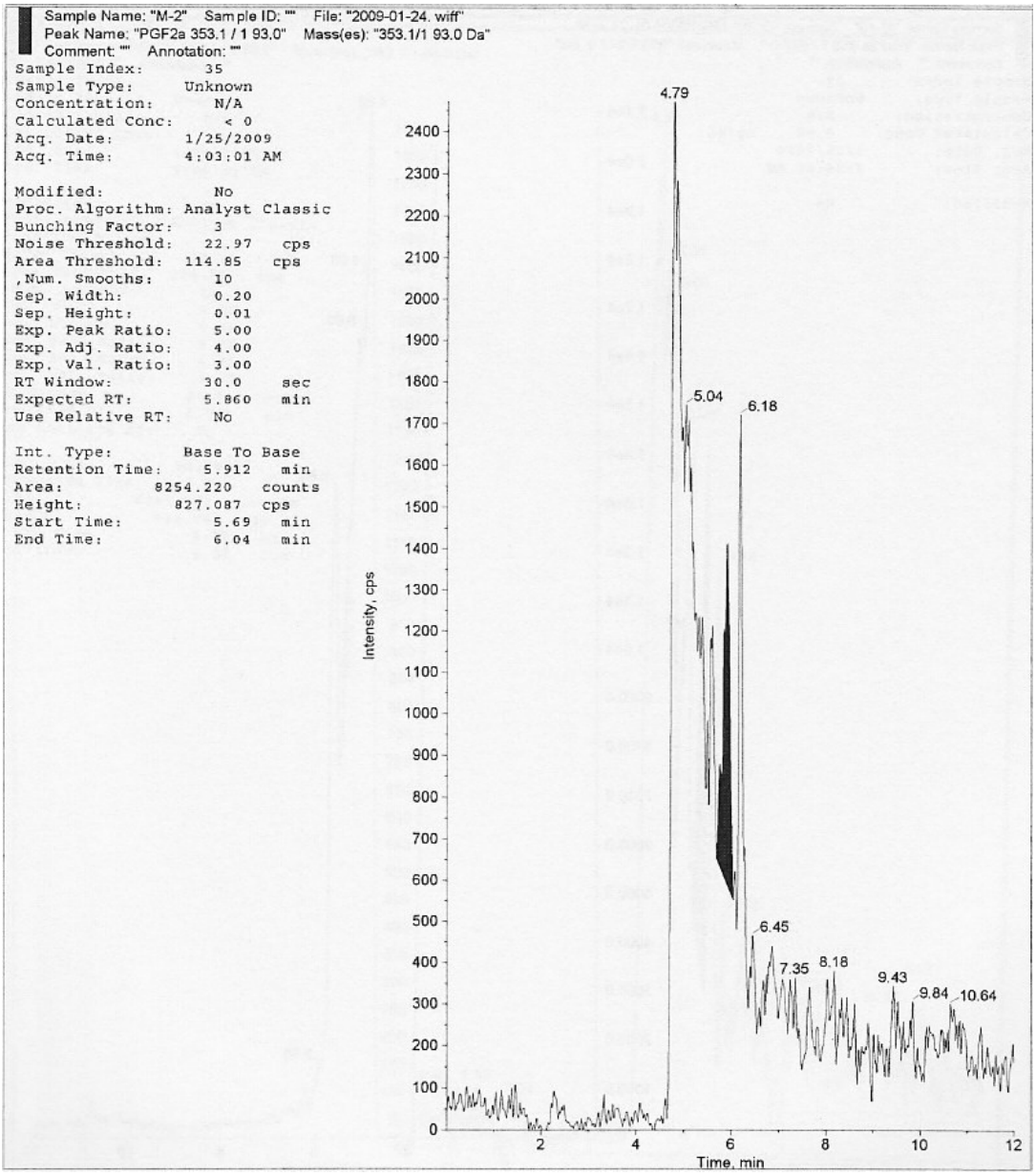

Figure A.5h. Chromatogram for Positive Control Media

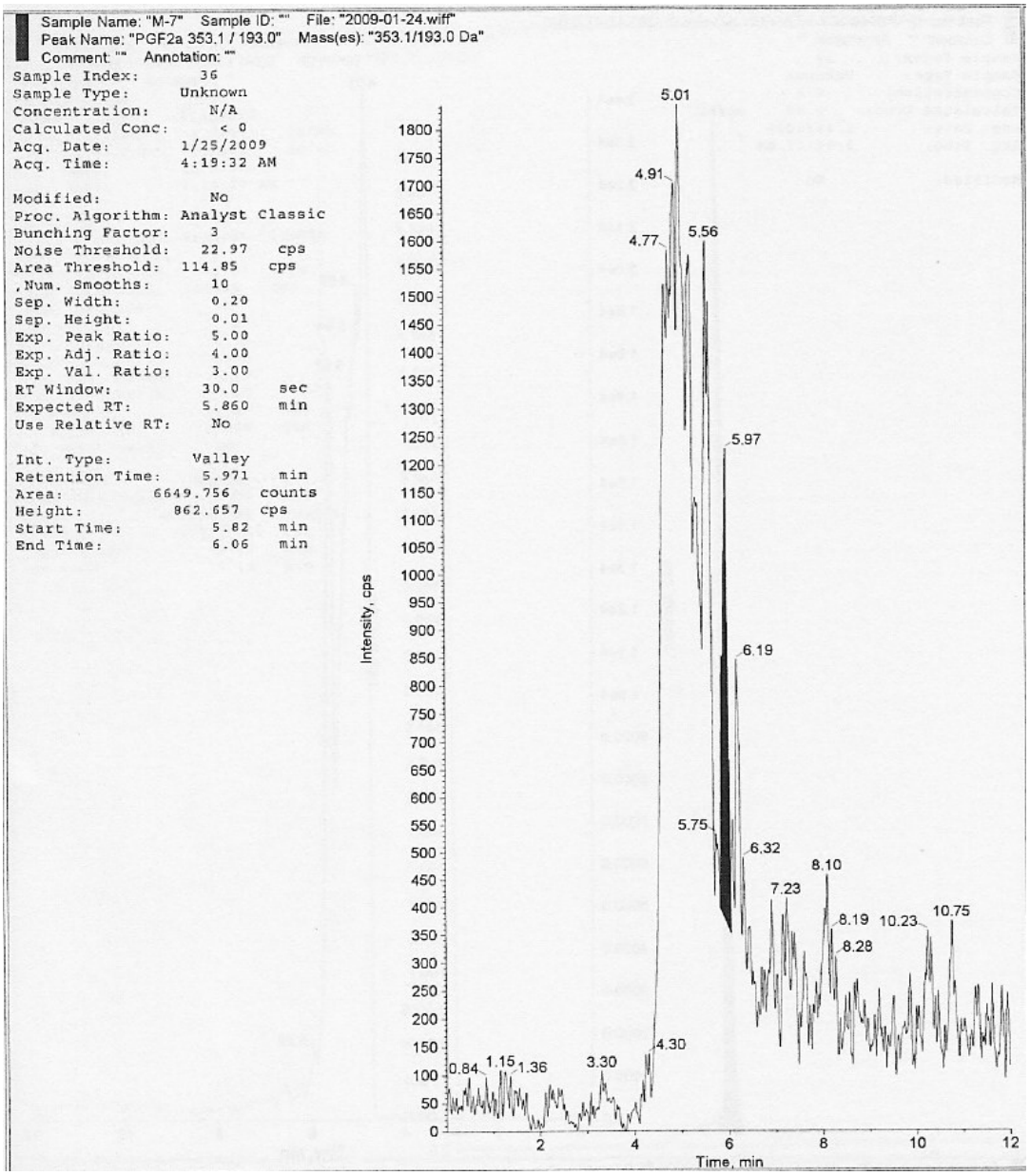

Figure A.5i. Chromatogram for "GT 5% + LPS" Media

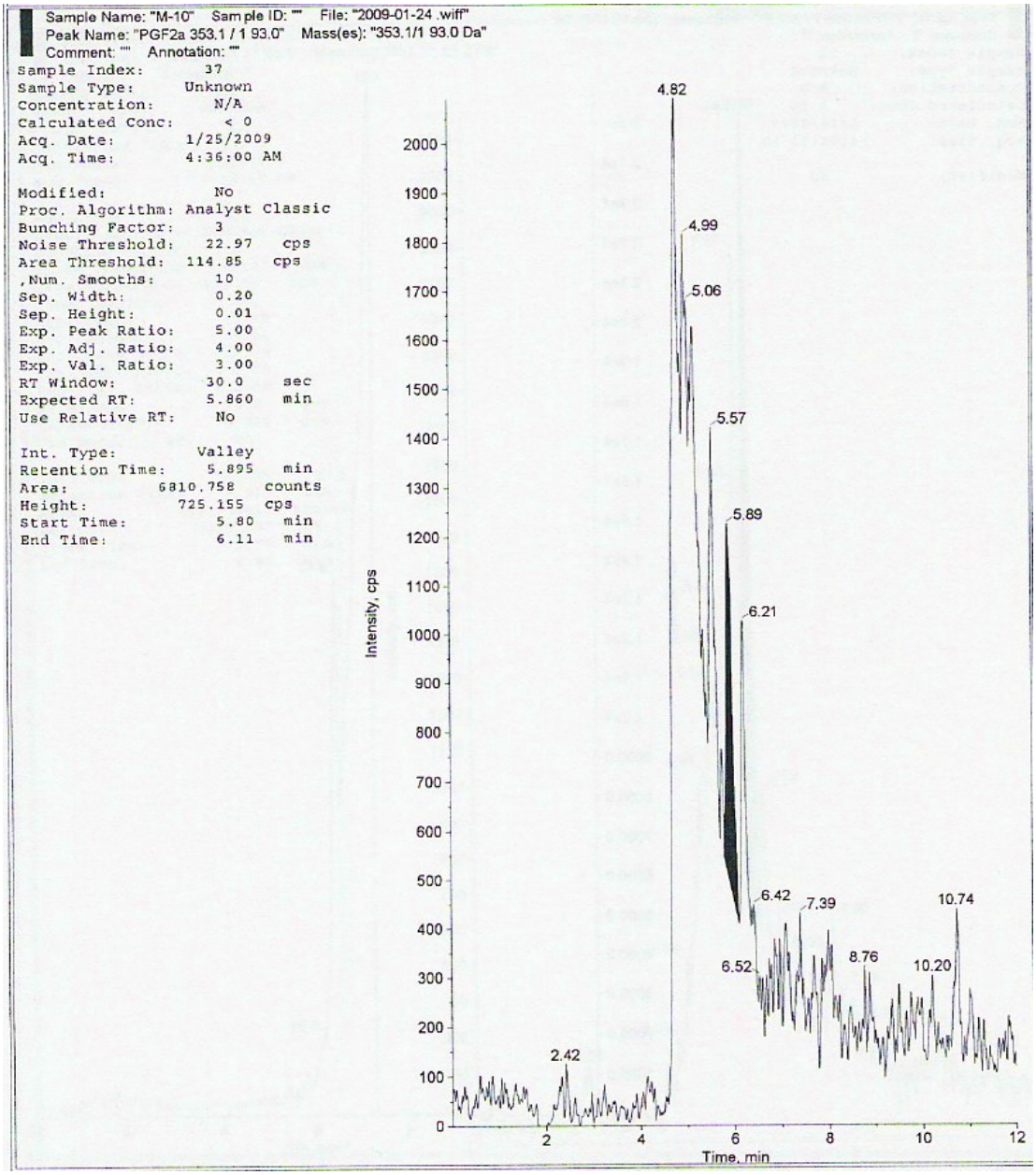

Figure A.5j. Chromatogram for "BT 5% + LPS" Media

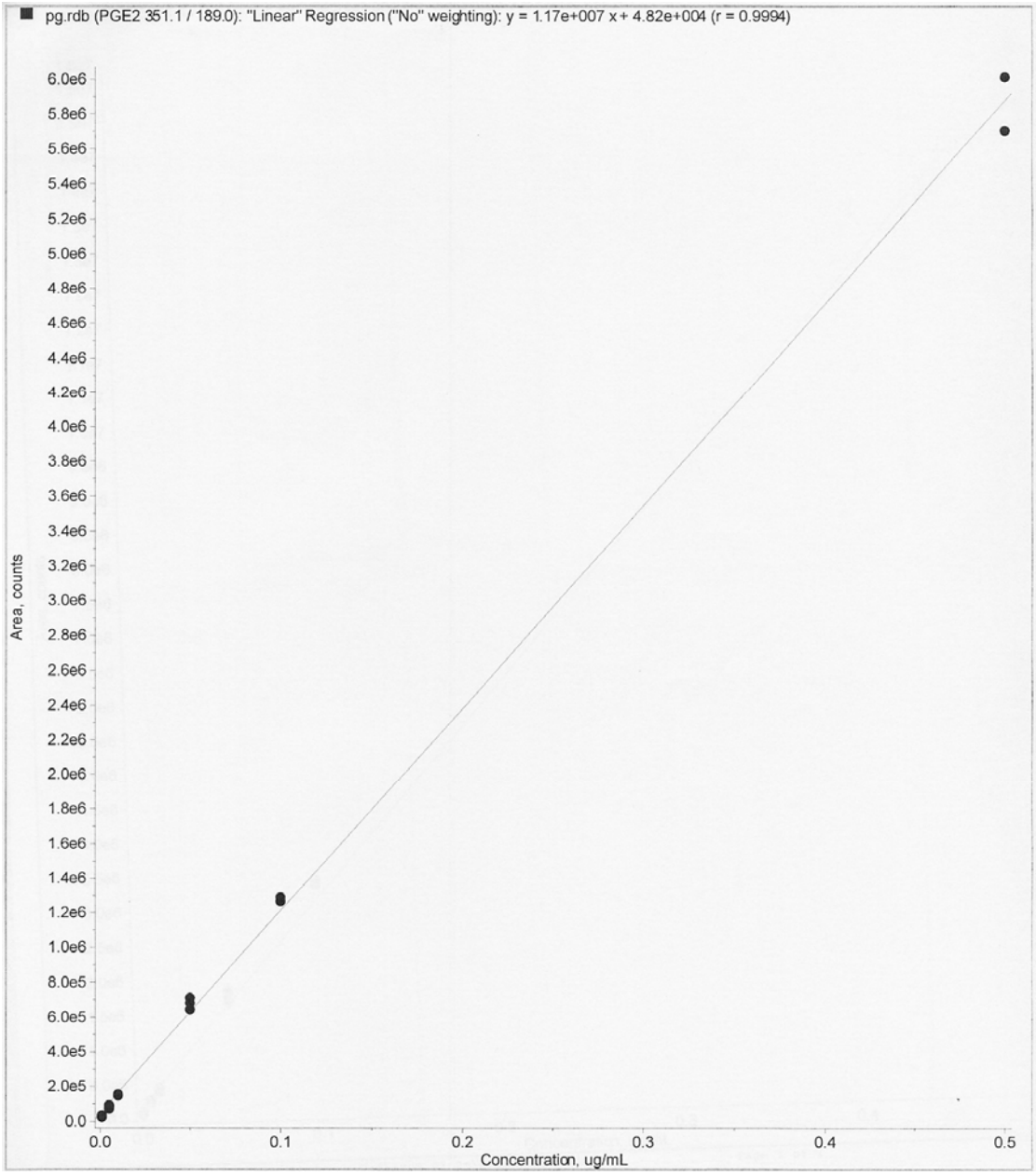

Figure A.6a. Standard Curve for PGE_2 Standard

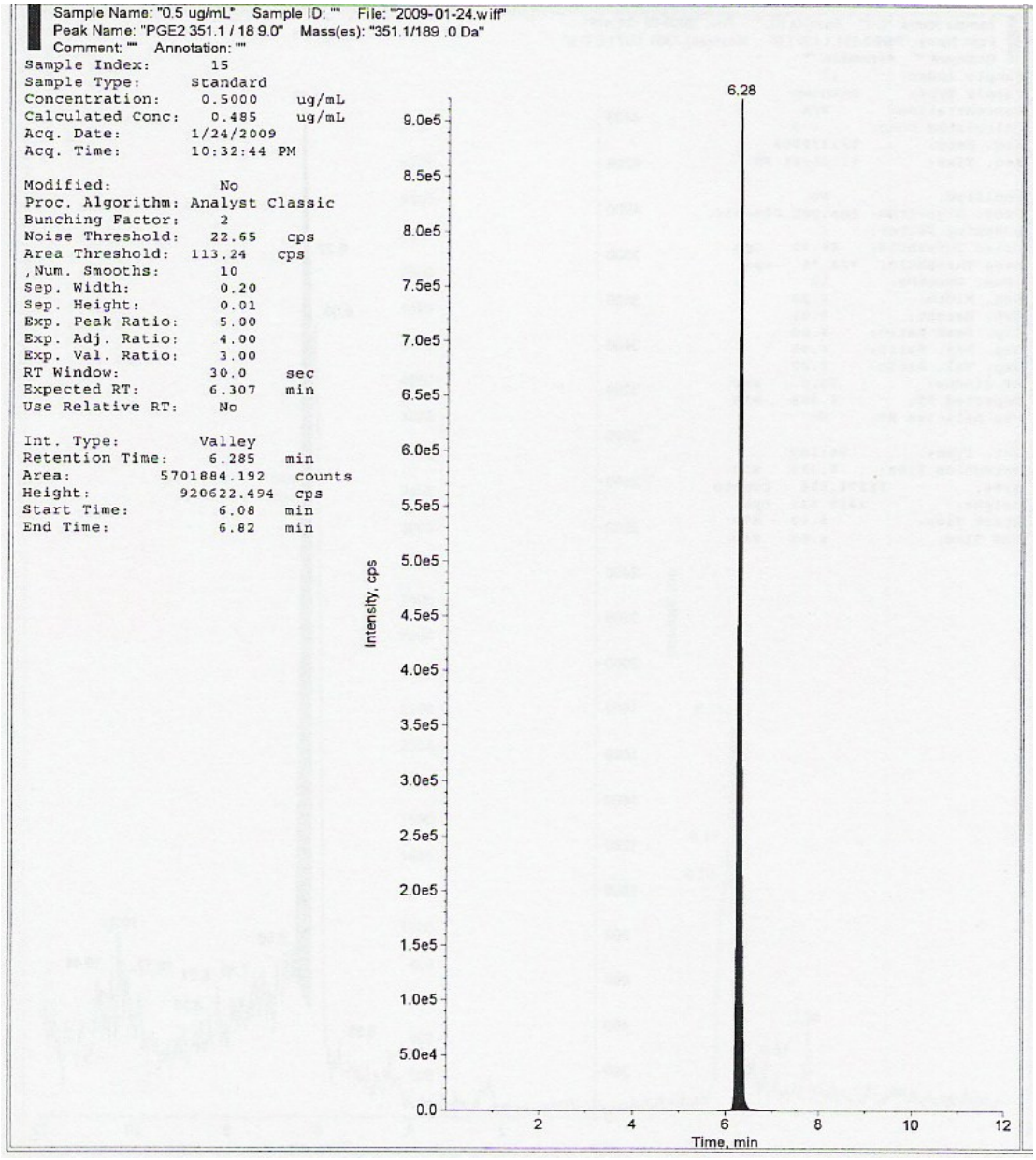

Figure A.6b. Chromatogram for PGE_2 Standard

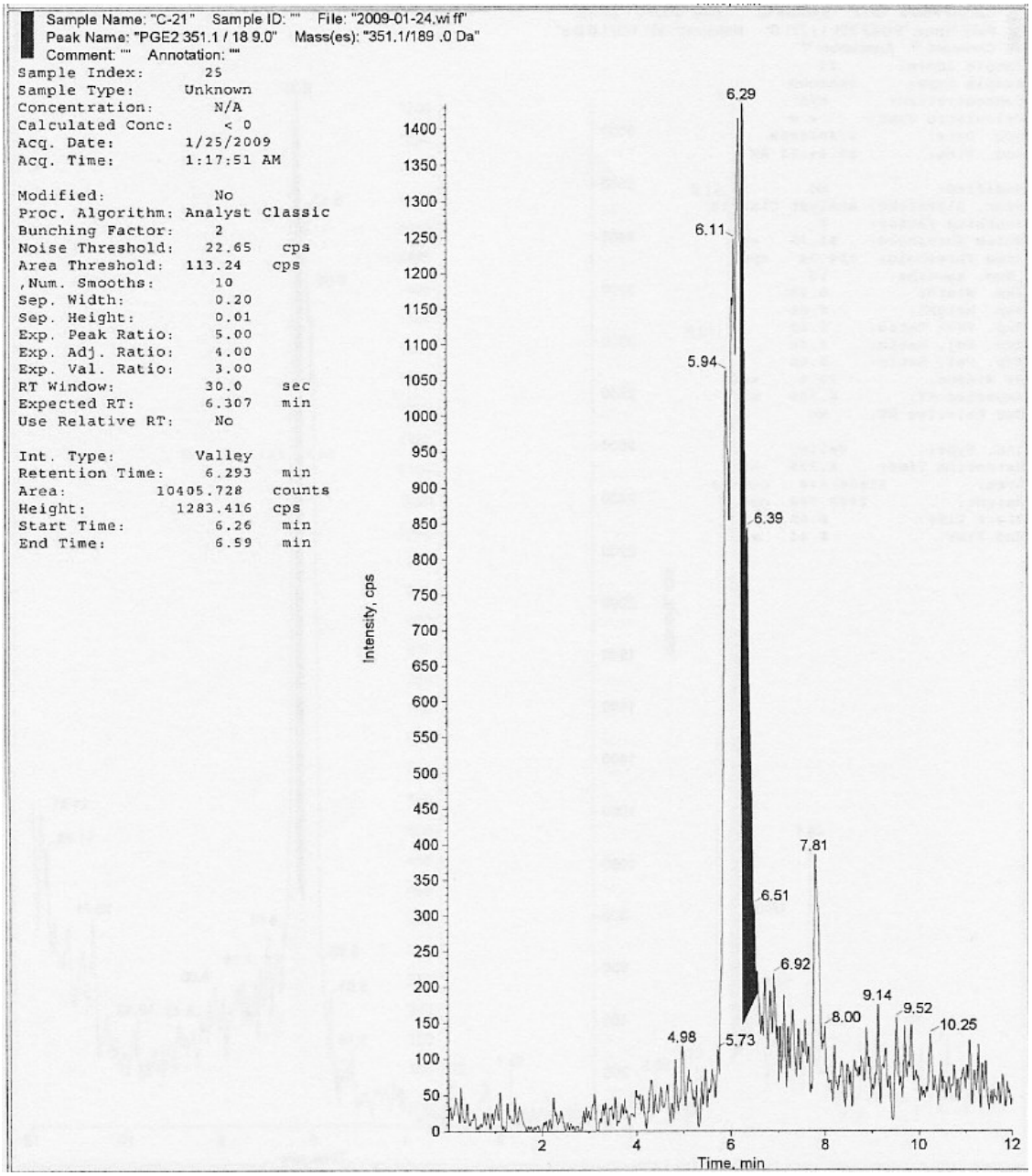

Figure A.6c. Chromatogram for Negative Control Cell Lysate

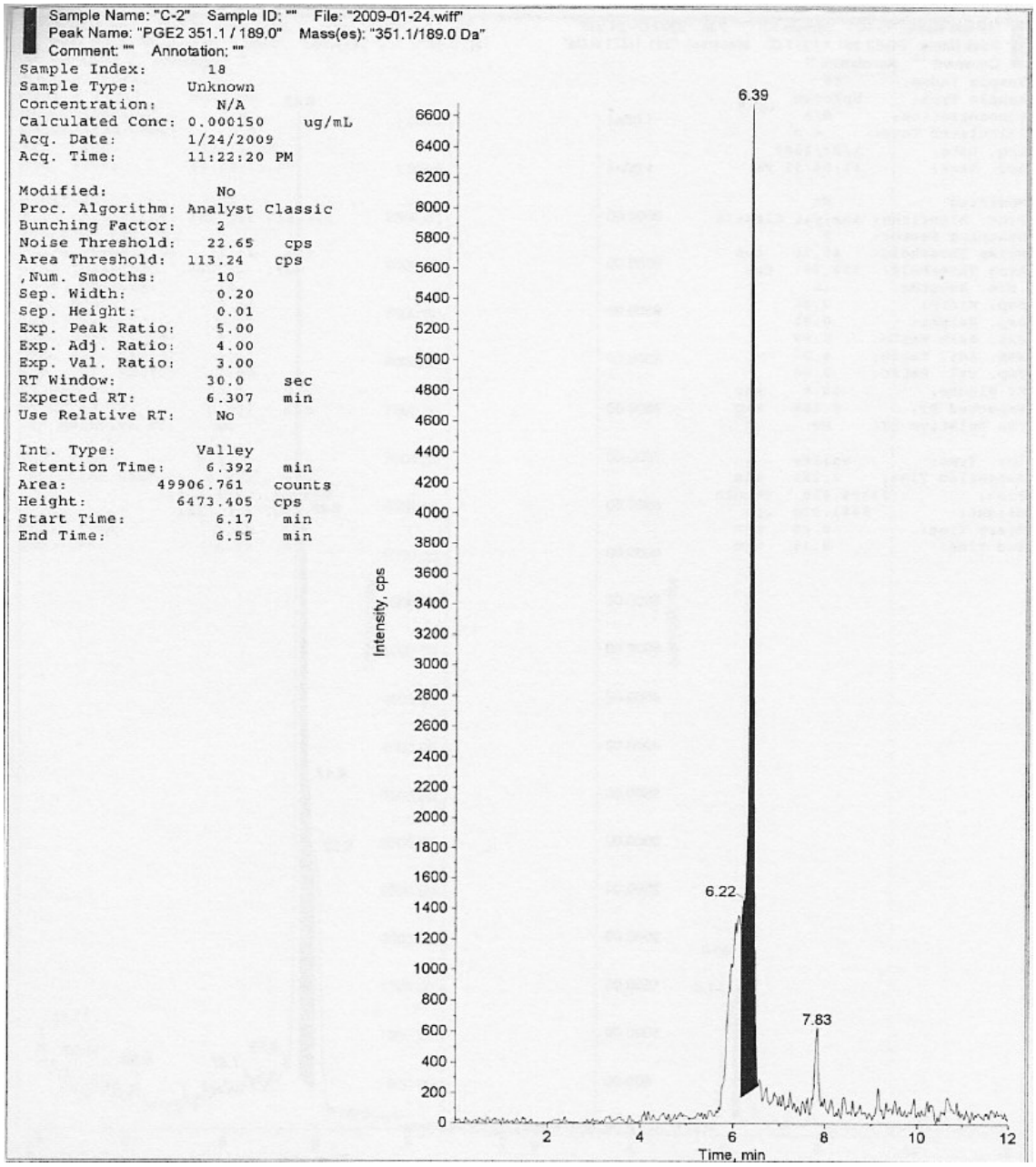

Figure A.6d. Chromatogram for Positive Control Cell Lysate

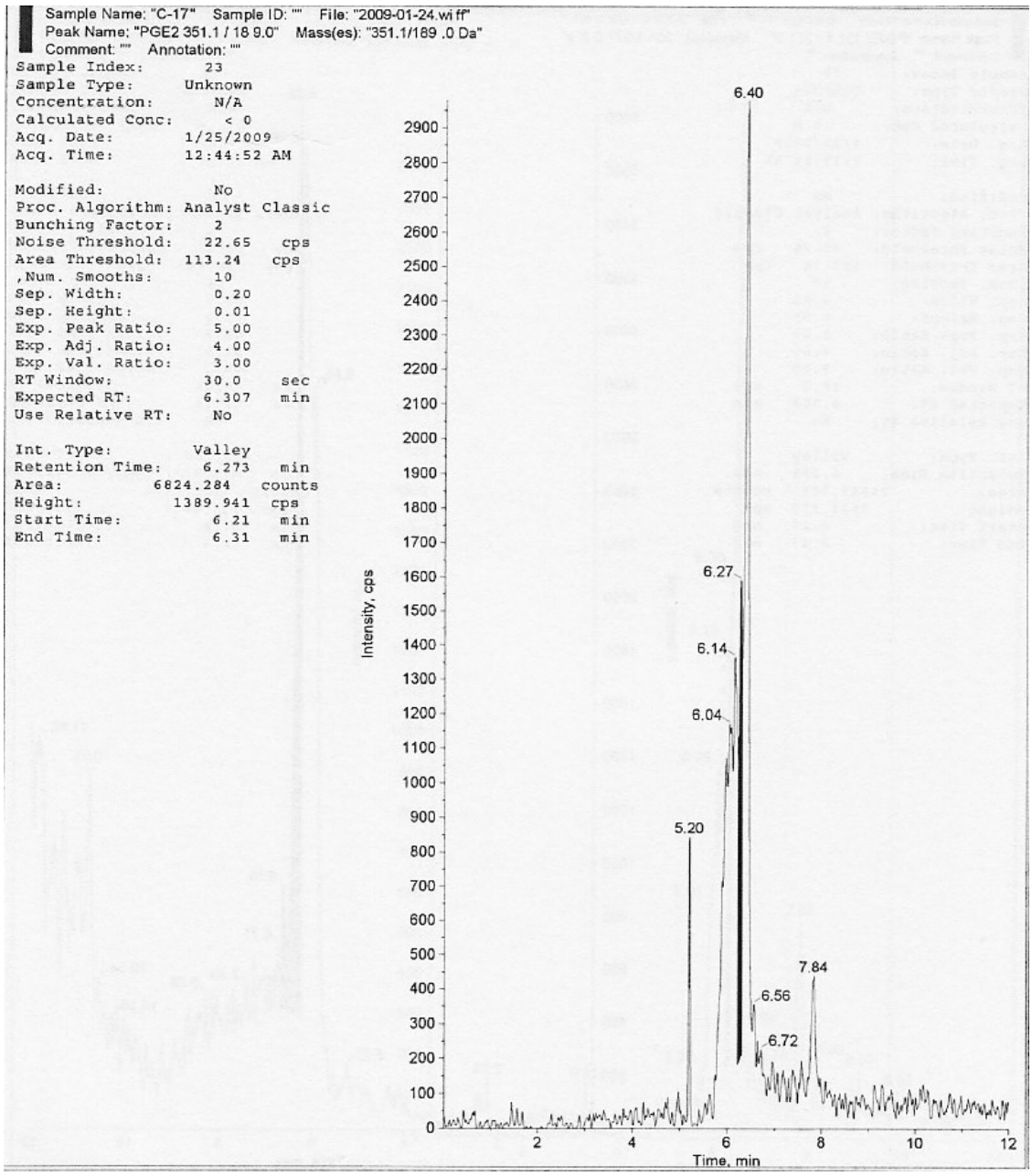

Figure A.6e. Chromatogram for "GT 5% + LPS" Cell Lysate

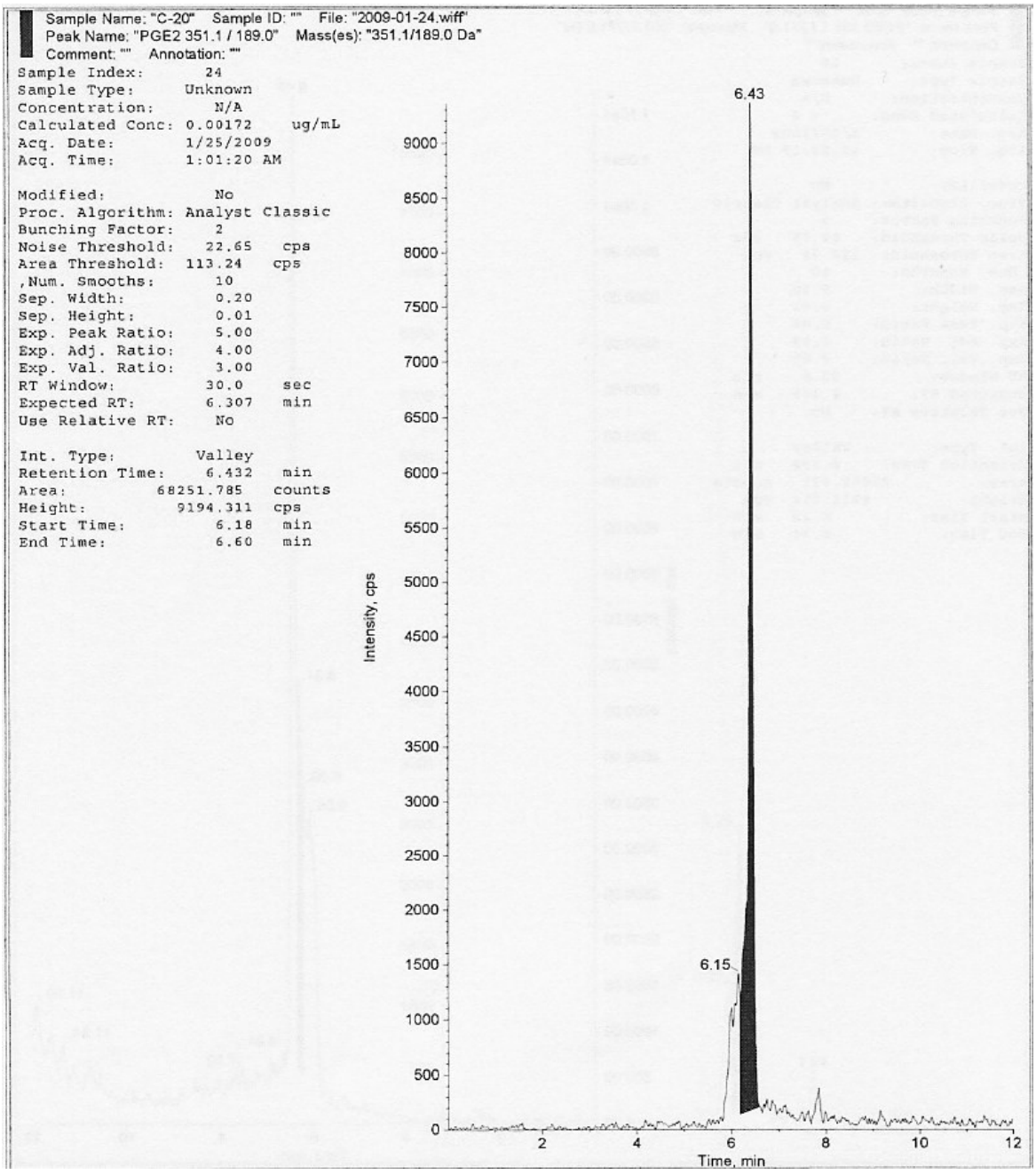

Figure A.6f. Chromatogram for "BT 5% + LPS" Cell Lysate

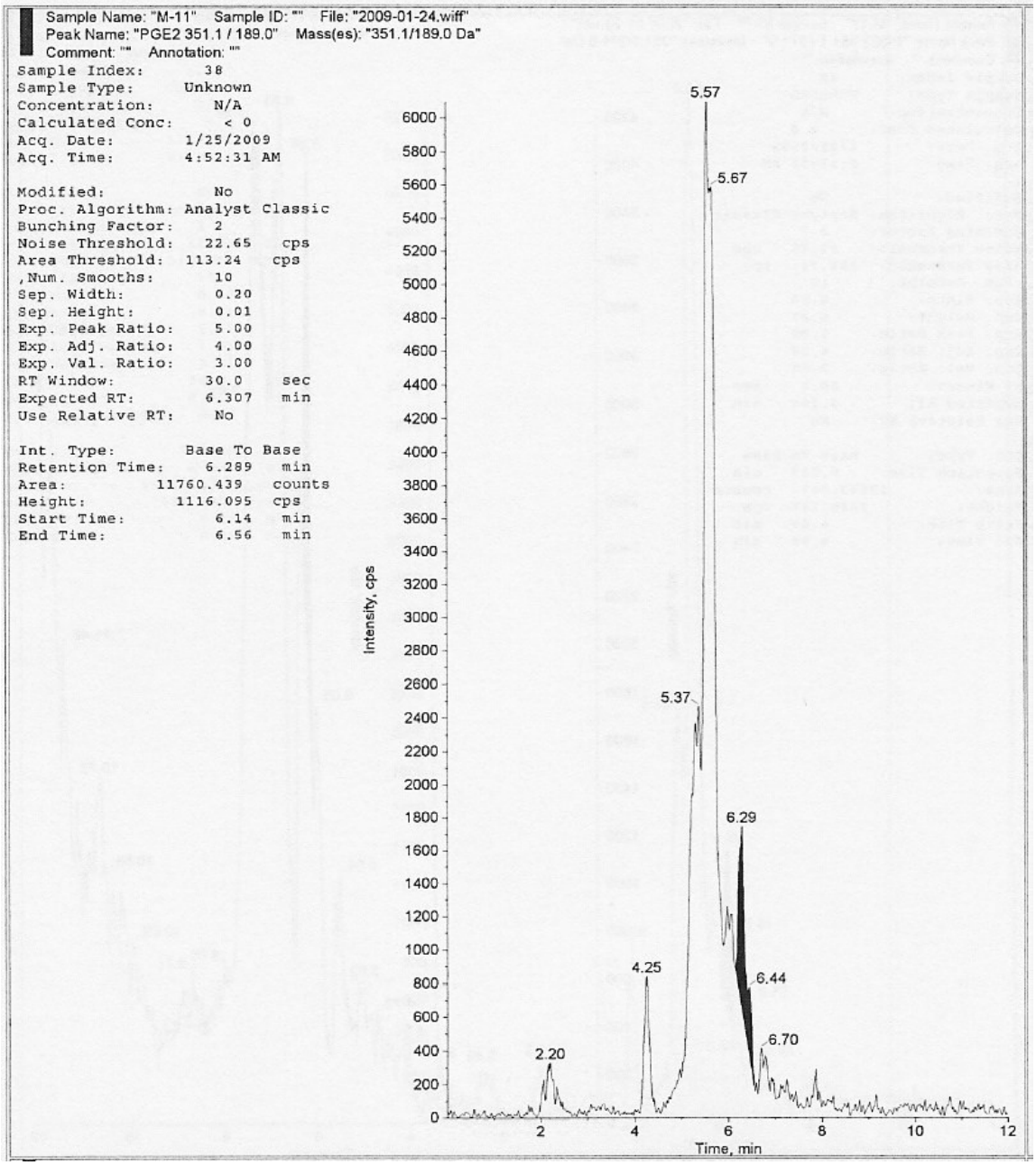

Figure A.6g. Chromatogram for Negative Control Media

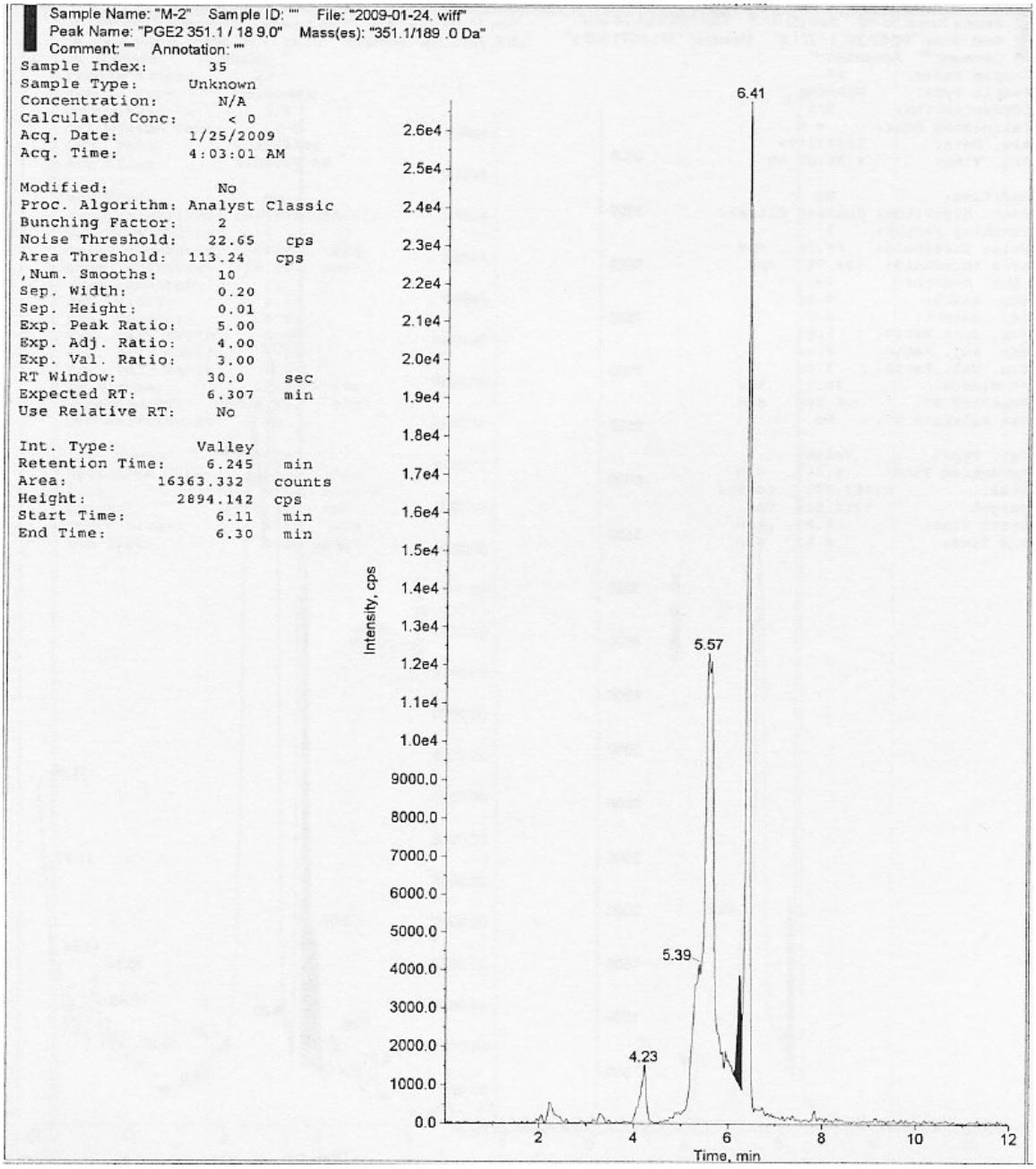

Figure A.6h. Chromatogram for Positive Control Media

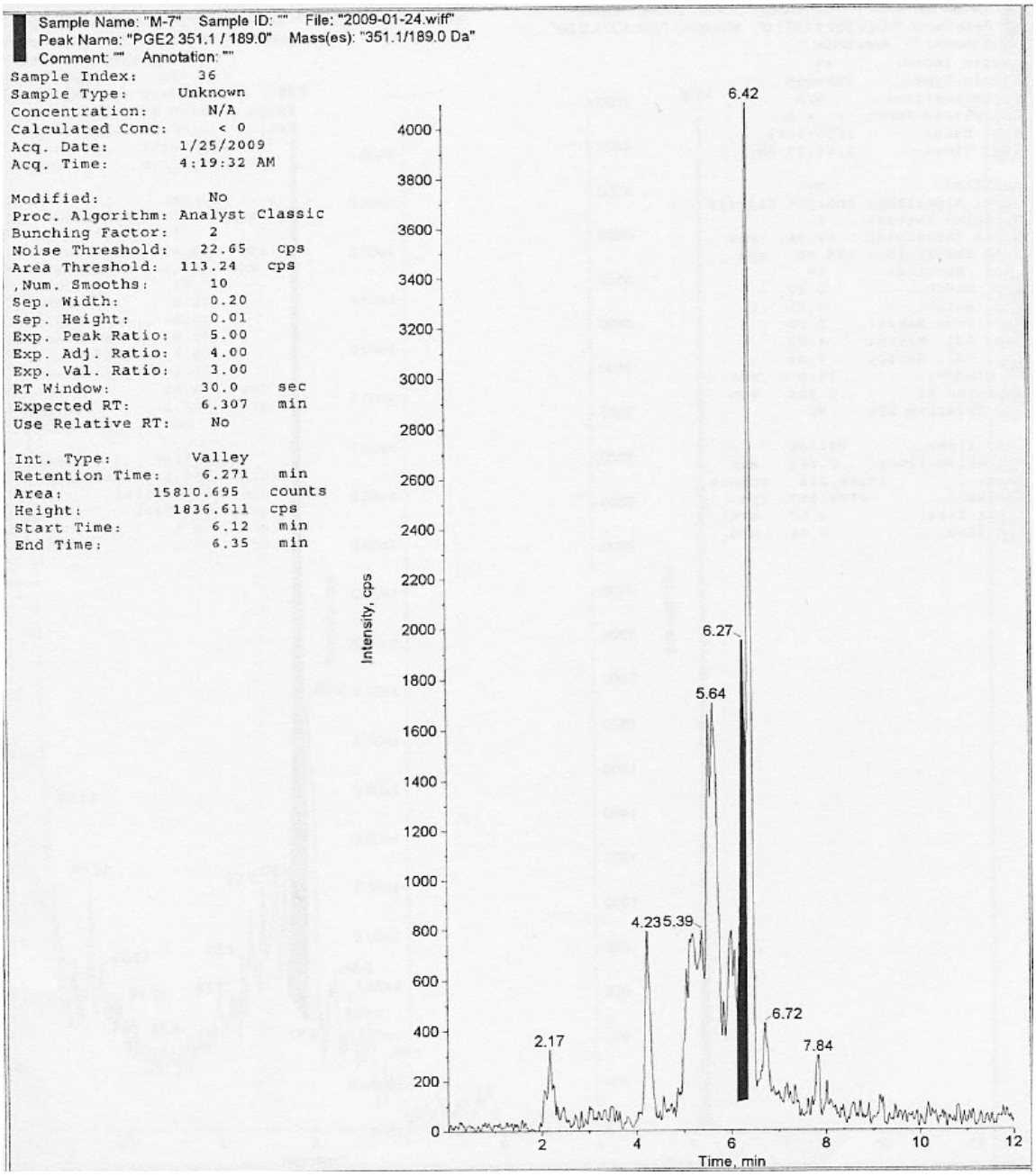

Figure A.6i. Chromatogram for "GT 5% + LPS" Media

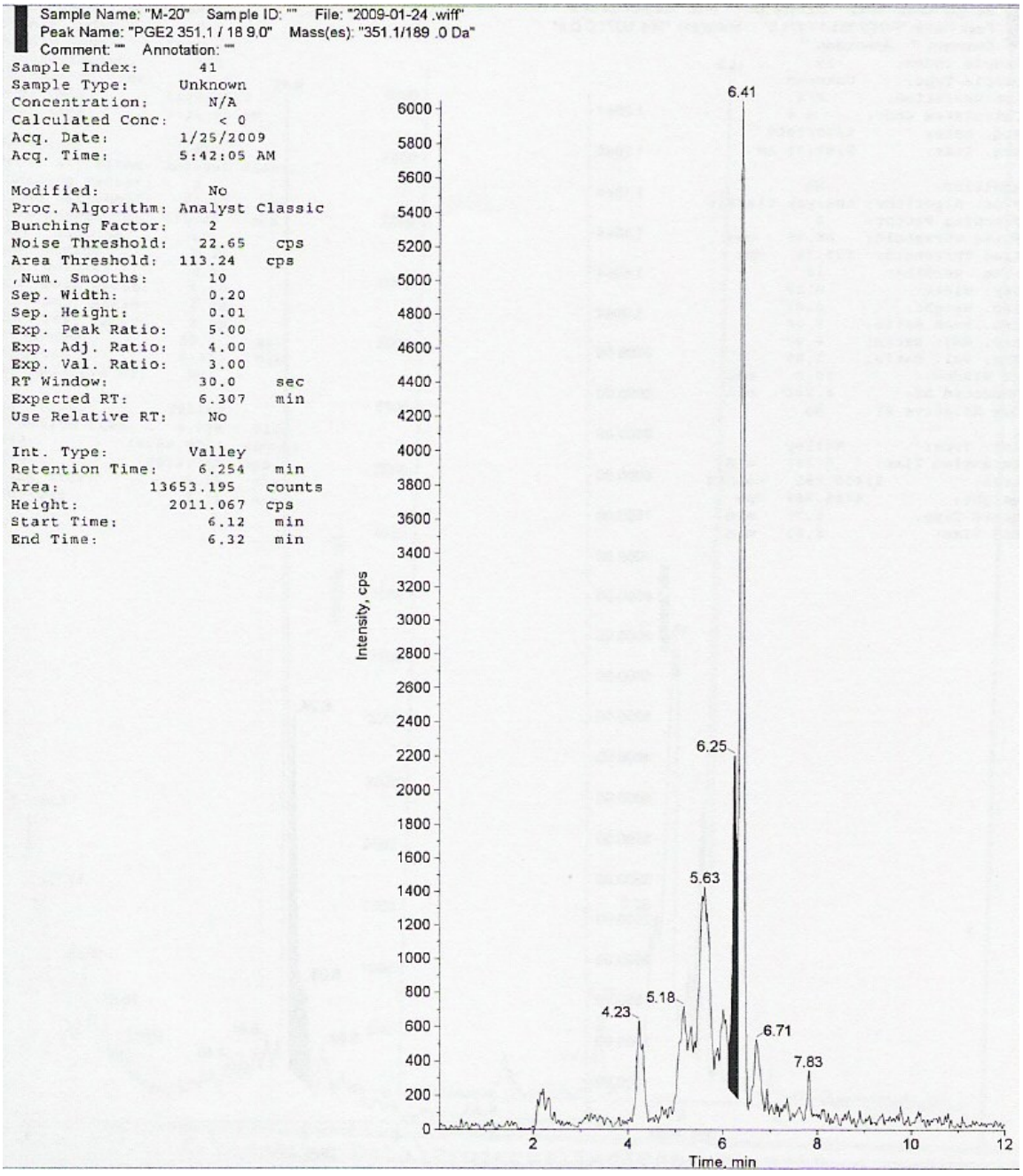

Figure A.6j. Chromatogram for "BT 5% + LPS" Media

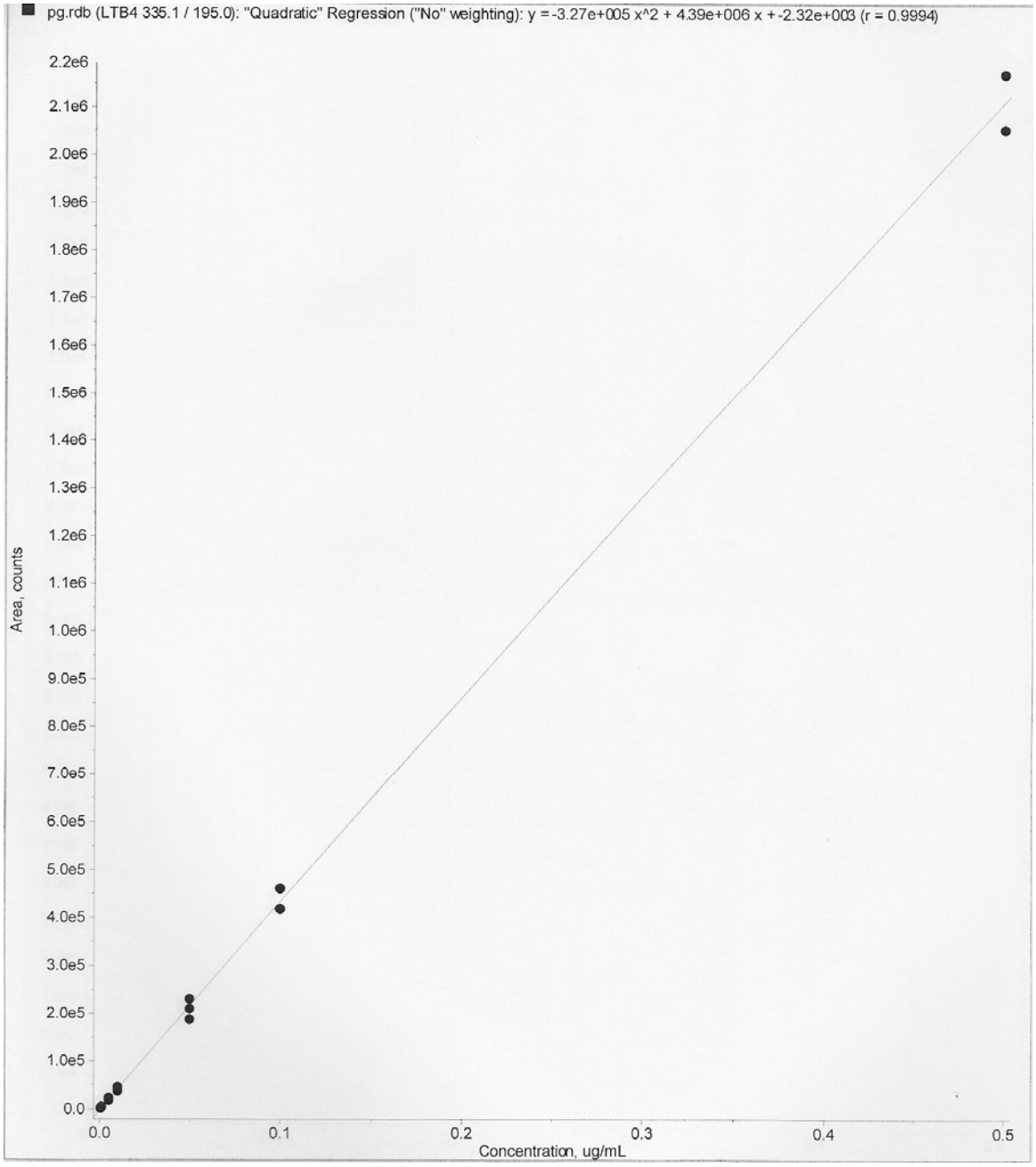

Figure A.7a. Standard Curve for LTB_4 Standard

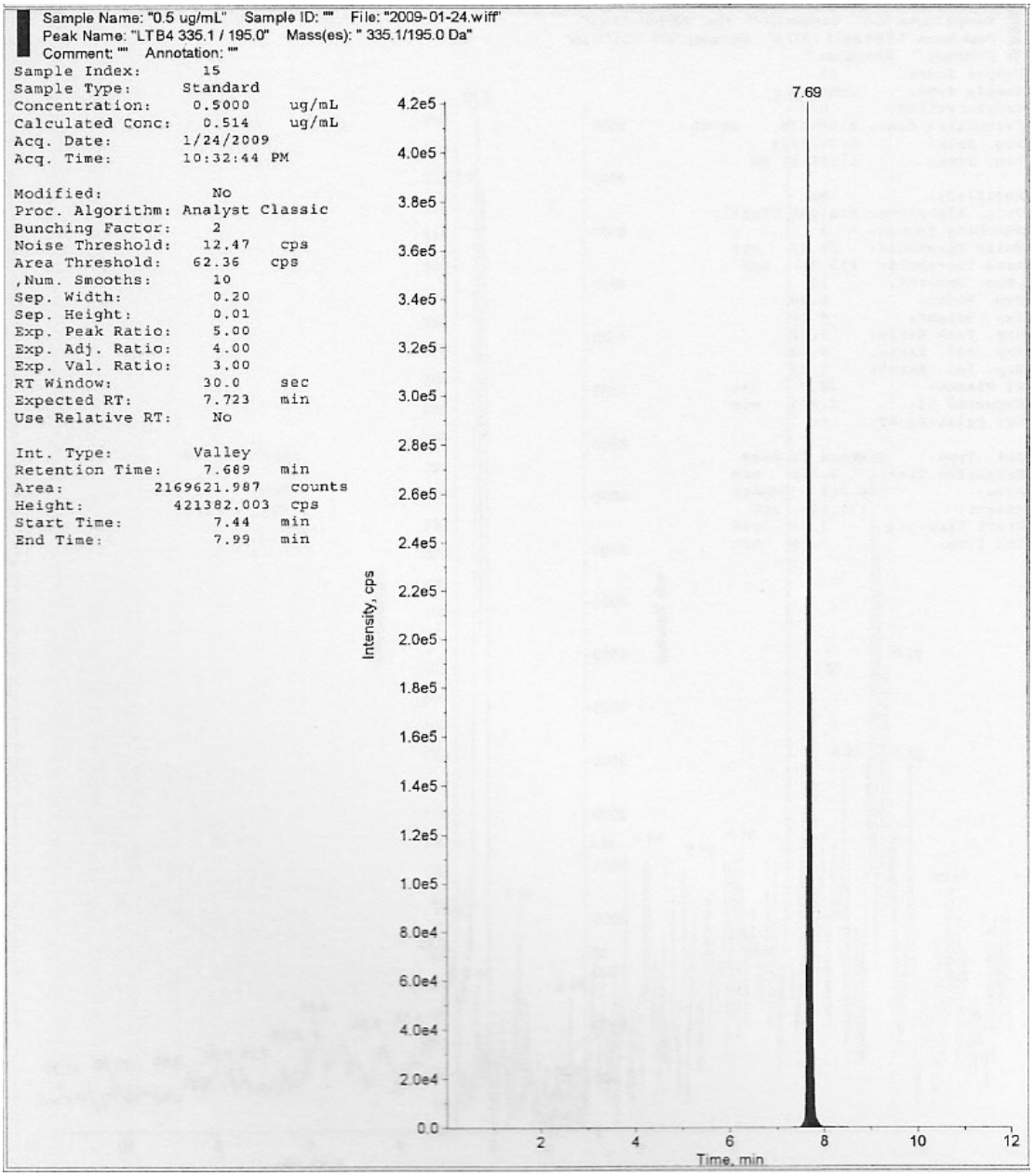

Figure A.7b. Chromatogram for LTB_4 Standard

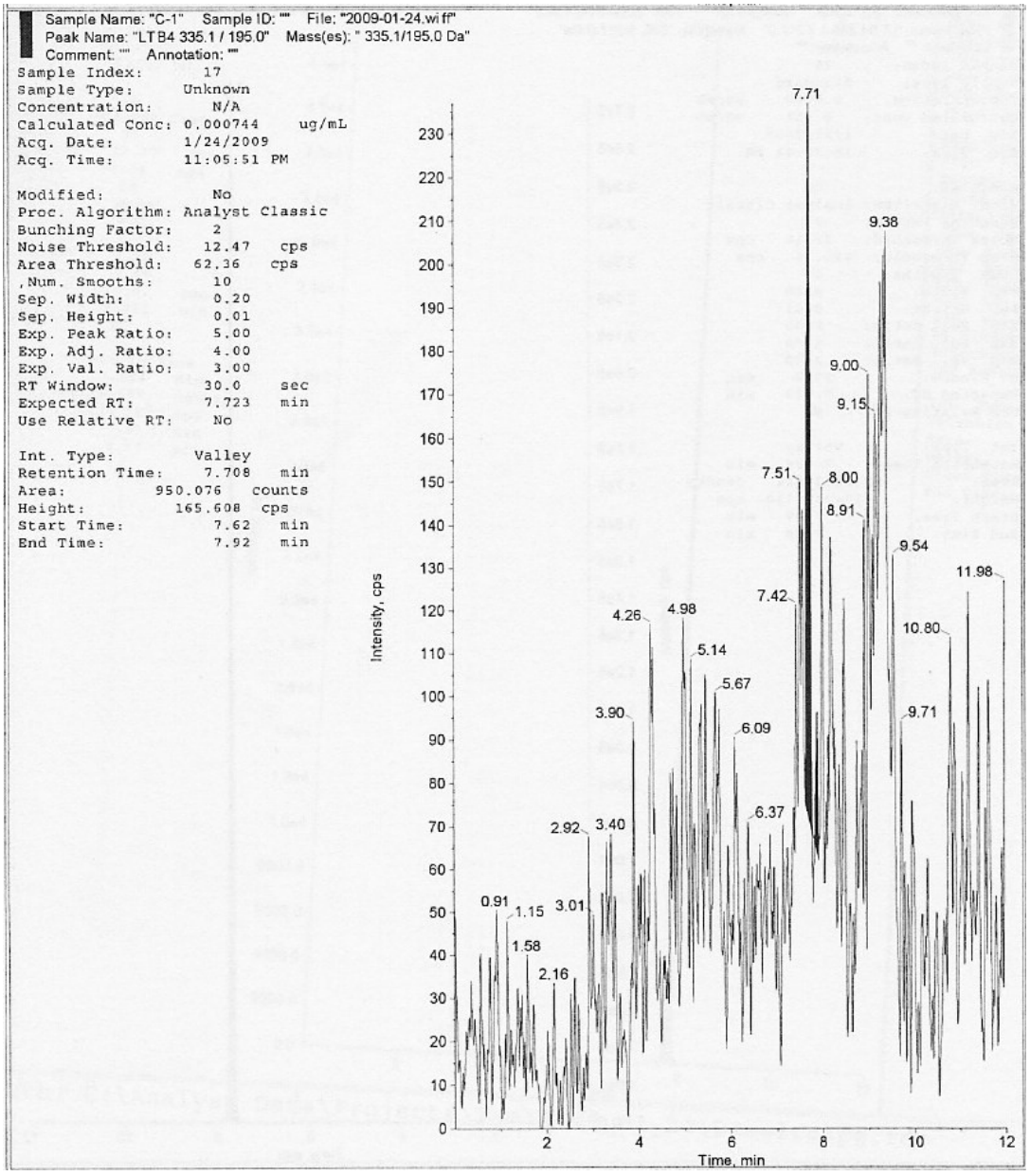

Figure A.7c. Chromatogram for Negative Control Cell Lysate

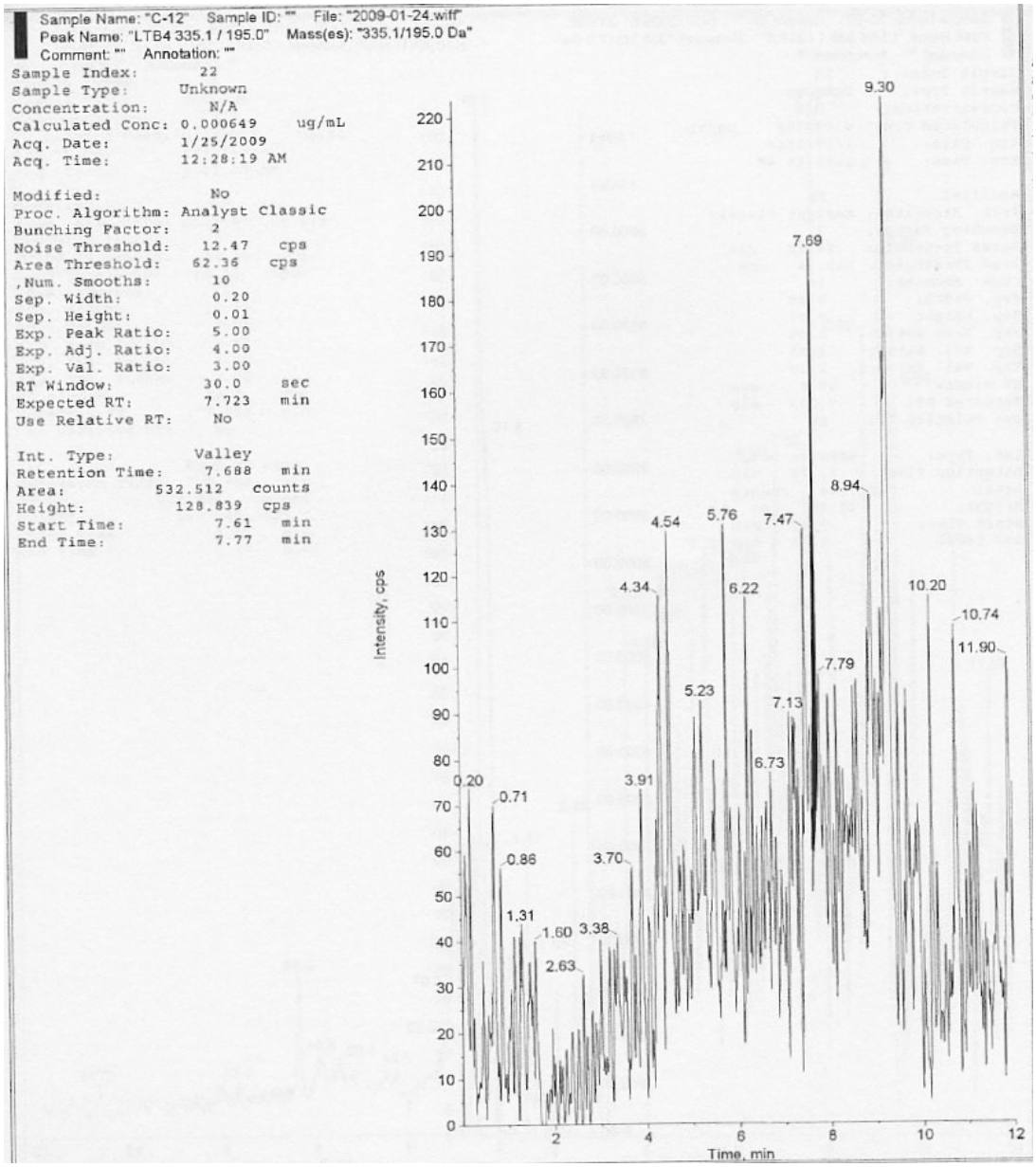

Figure A.7d. Chromatogram for Positive Control Cell Lysate

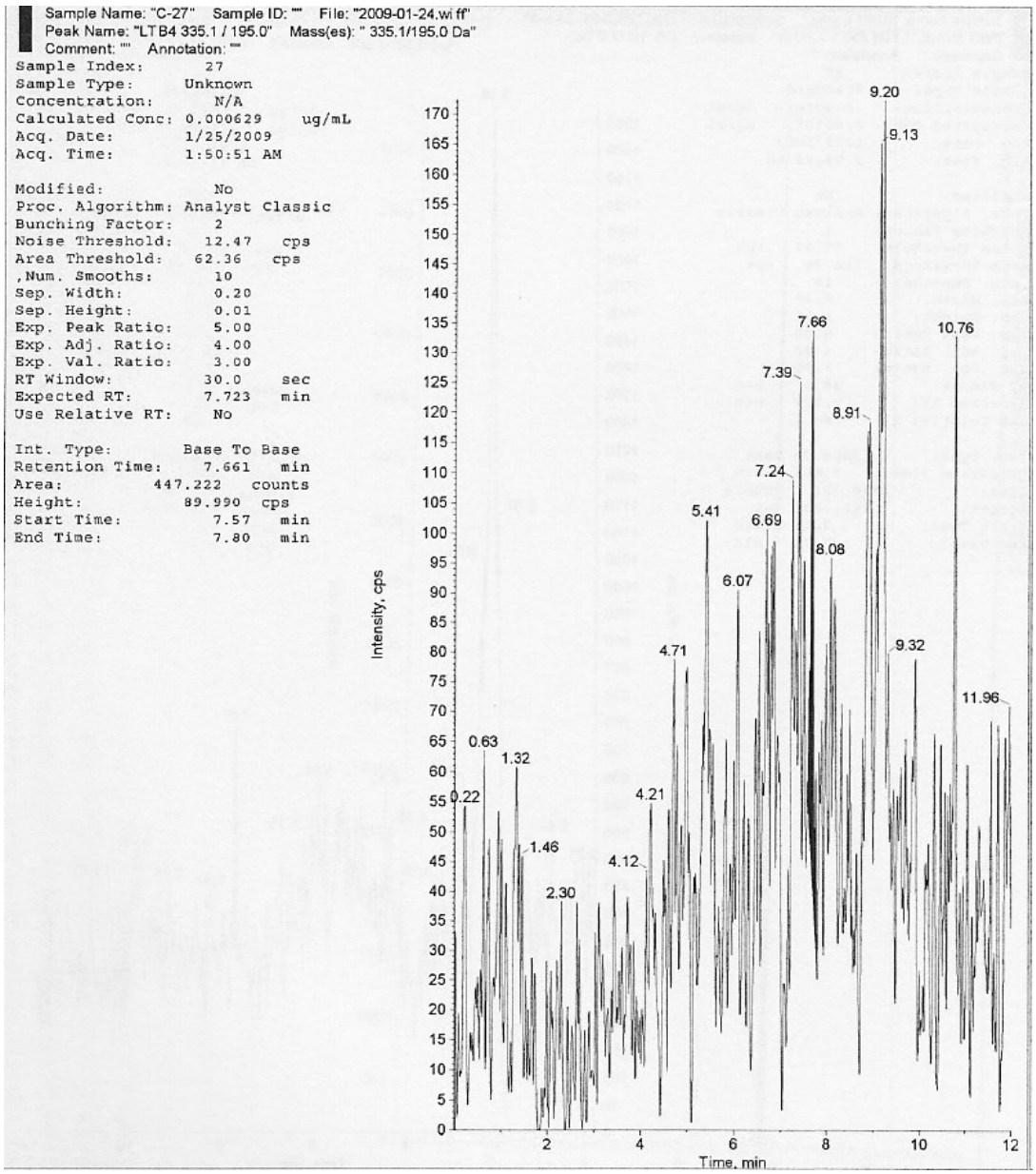

Figure A.7e. Chromatogram for "GT 5% + LPS" Cell Lysate

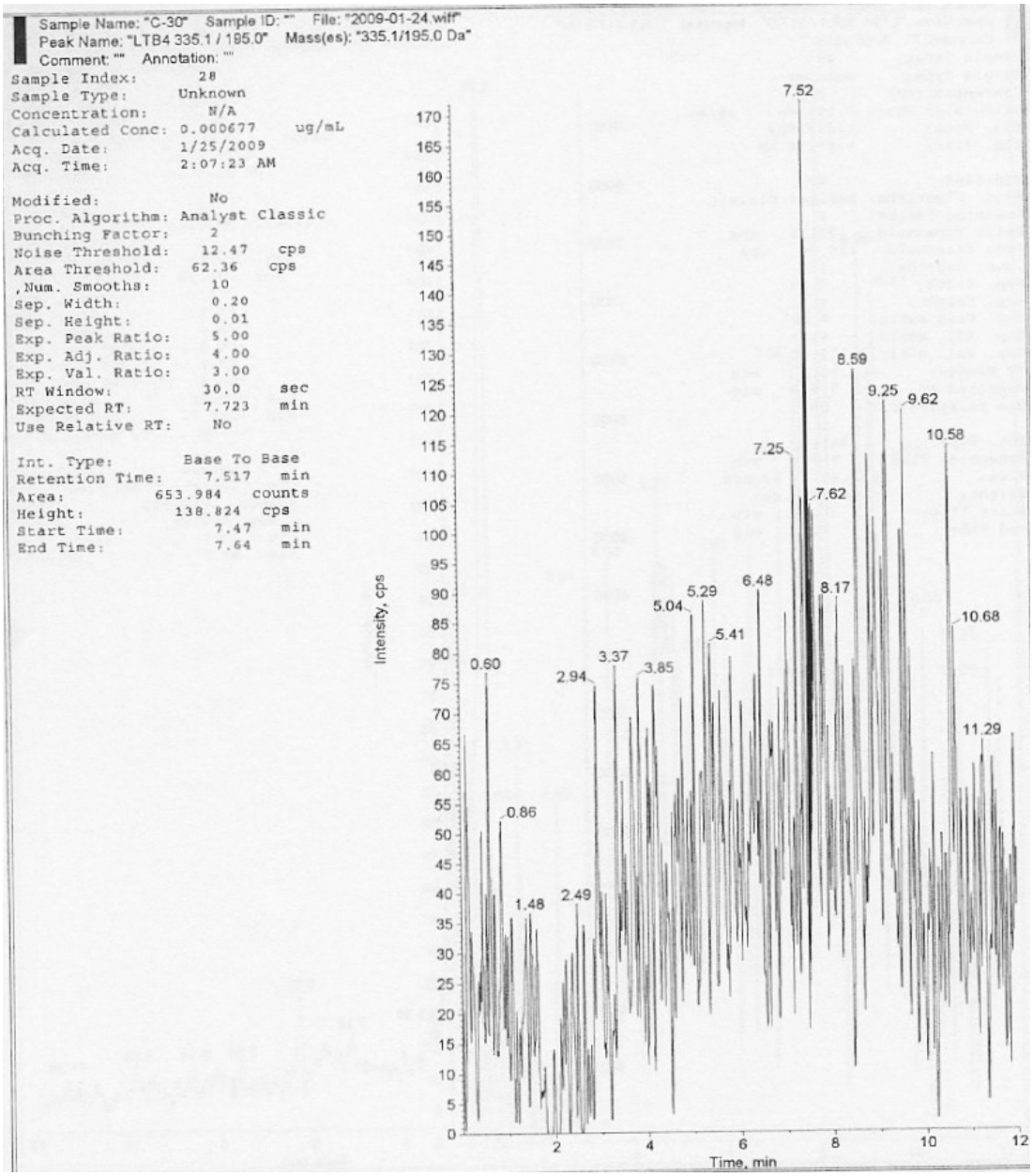

Figure A.7f. Chromatogram for "BT 5% + LPS" Cell Lysate

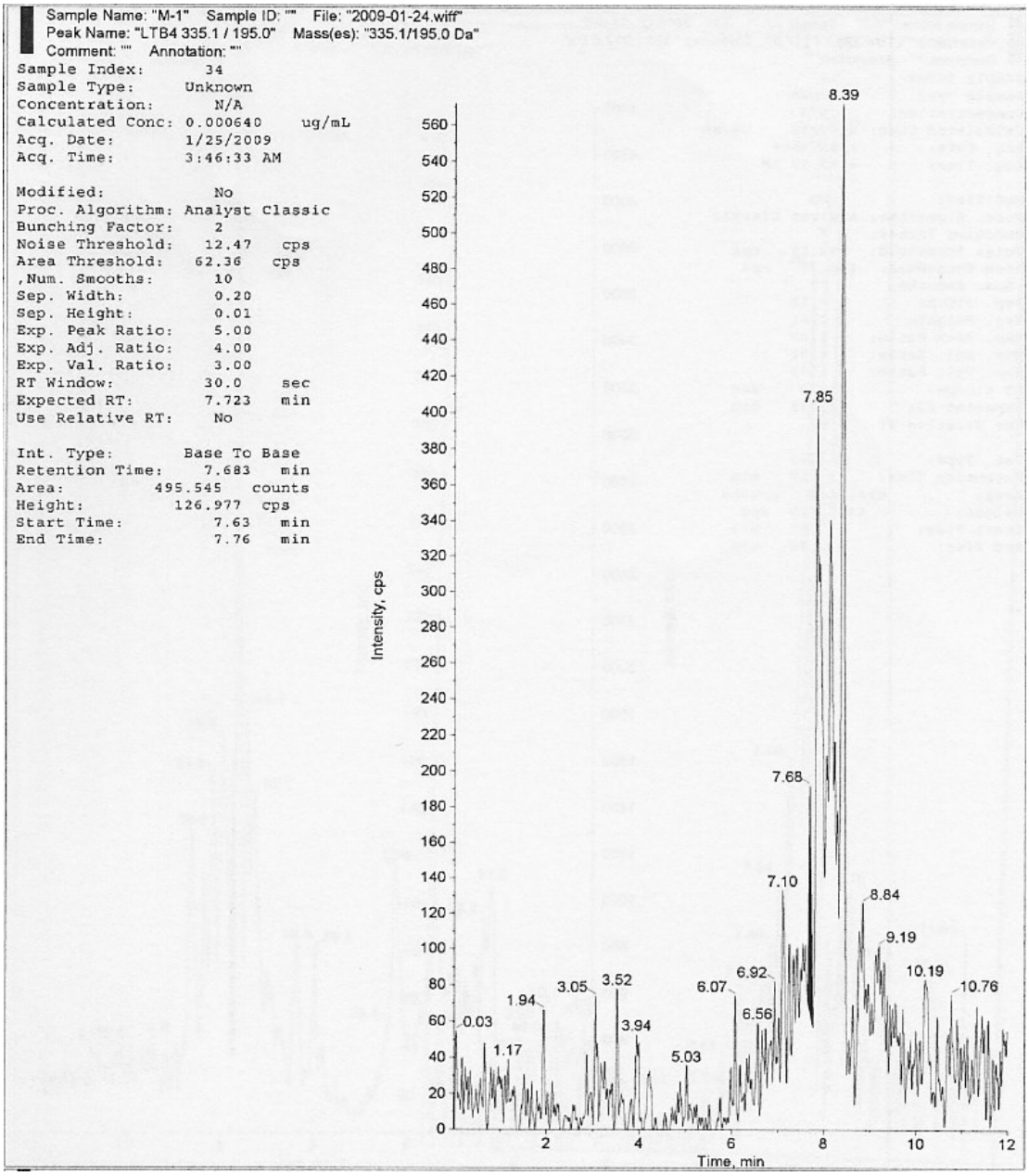

Figure A.7g. Chromatogram for Negative Control Media

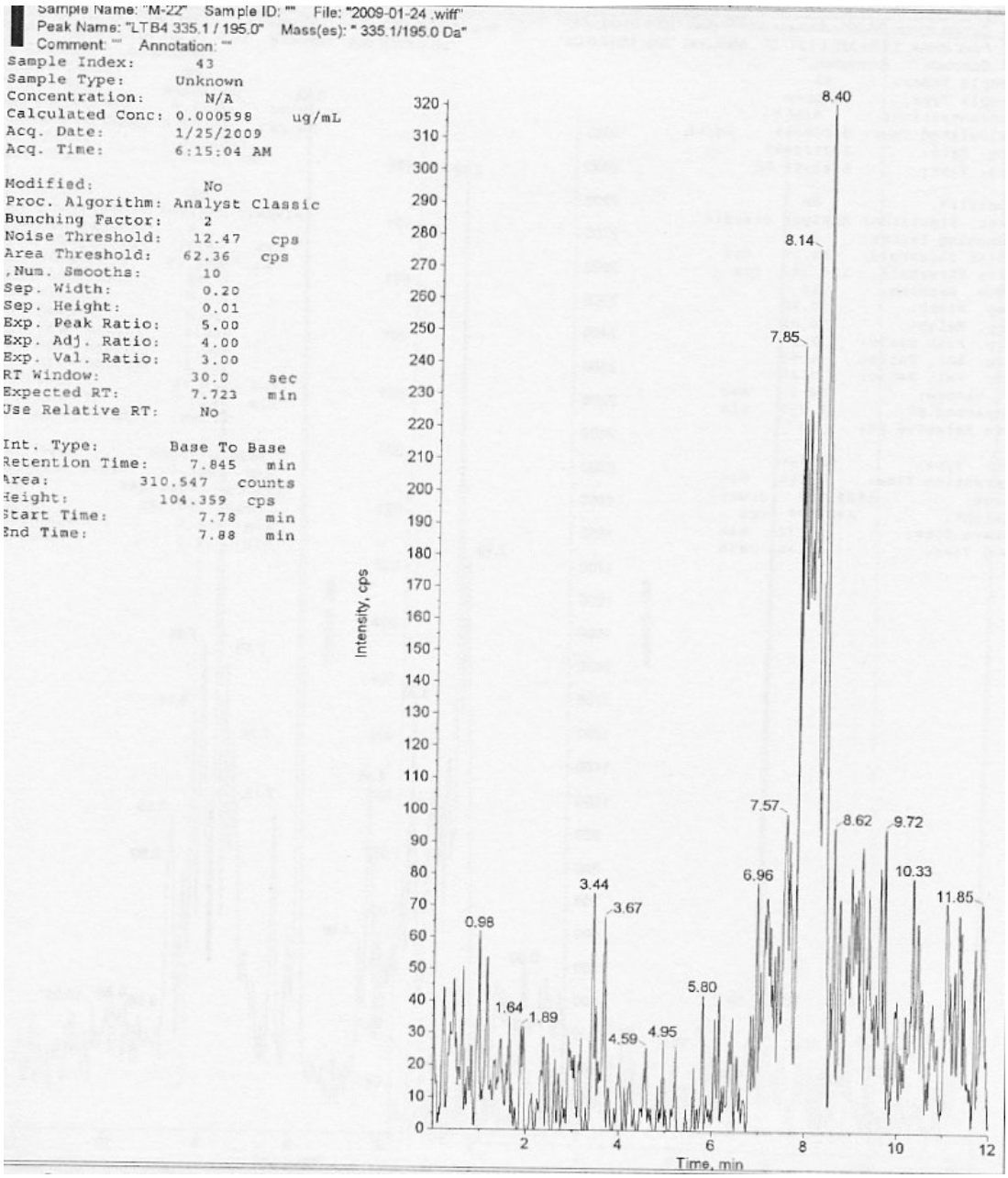

Figure A.7h. Chromatogram for Positive Control Media

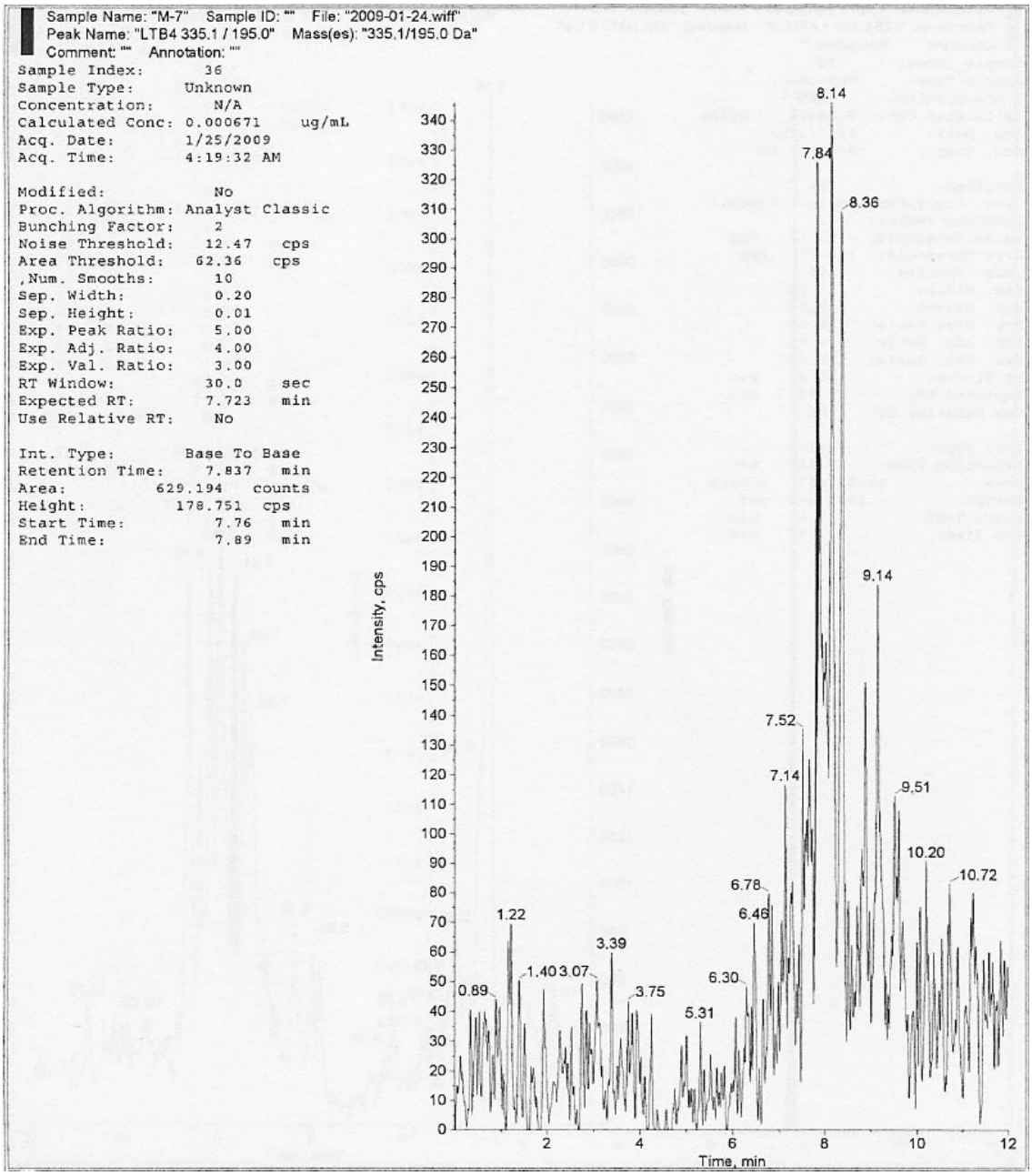

Figure A.7i. Chromatogram for "GT 5% + LPS" Media

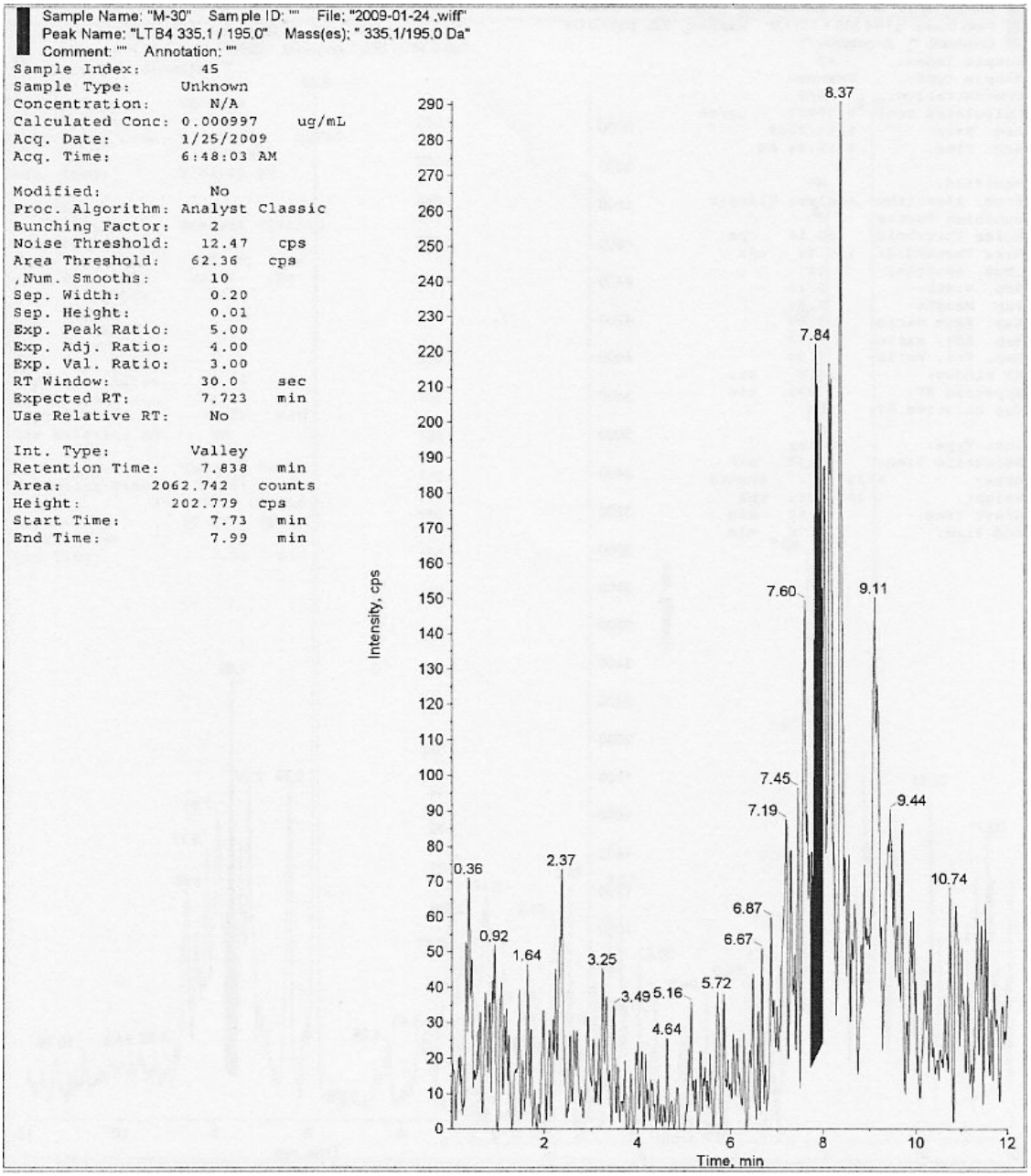

Figure A.7j. Chromatogram for "BT 5% + LPS" Media

Figures A.8a and A.8b represent chromatograms for the standards (see section A.2.3) and cell lysate samples containing LPS and PMA, respectively. Figure A.8b shows that PGE_2 levels met the detection limit of the equipment used, and that this eicosanoid was detected in the sample. These results imply that LPS and PMA can effectively stimulate RAW 264.7 cells to levels that can be measured using HPLC/MS.

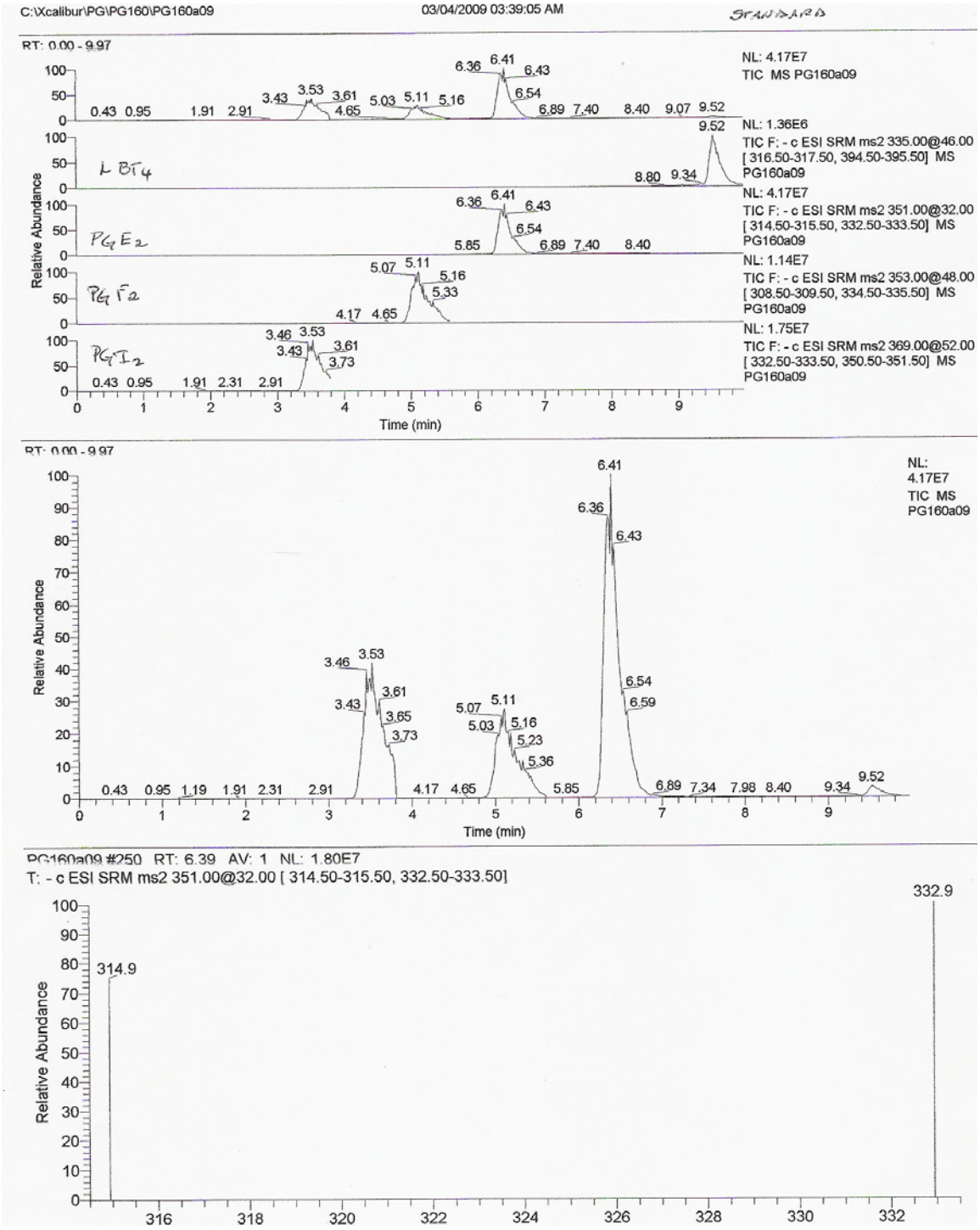

Figure A.8a. Chromatogram for Standards Run on Finnigan LCQ Duo

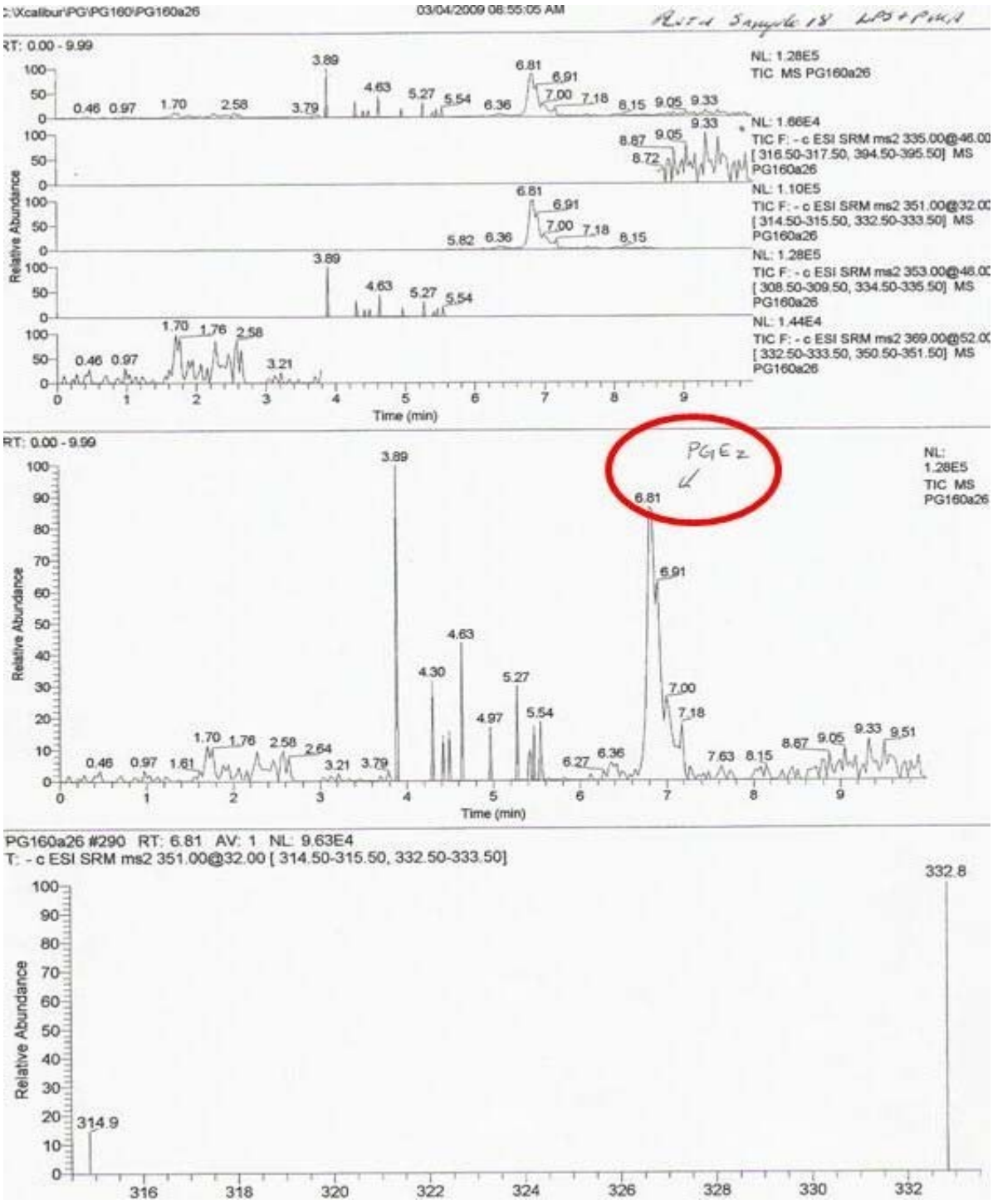

Figure A.8b. Chromatogram for Cell Lysate + LPS/PMA

A.4 DISCUSSION AND CONCLUSIONS

Based on our results, there were inadequate levels of the eicosanoids of interest in the cell lysates and media samples treated with LPS only. The analytes in the samples did not meet the detection limit of the equipment used, making it difficult to detect or quantify the eicosanoids from the samples. However, the ability of the equipment to detect and quantitate the standards shows promise. This indicates that the instruments used are highly sensitive and capable of identifying inflammatory markers. Preliminary PGE_2 ELISA results from our lab show that using LPS + phorbol 12-myristate 13-acetate (PMA, a potent tumor promoter) can increase PGE_2 levels in RAW cells by 15-fold (relative to the negative control). HPLC/MS analysis of cells treated with LPS and PMA did allow for PGE_2 detection within the sample. Future cell culture experiments should utilize PMA or other stimuli, such as TPA, with LPS to enhance the expression of multiple inflammatory mediators and approach the detection limit of the equipment used. This will enable accurate, simultaneous eicosanoid detection, and allow for a better understanding of the complex processes that occur during an inflammatory response.

A.5 REFERENCES

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