Growth of lettuce with different content of inorganic nitrate as a feeding strategy for placebo-controlled nutritional interventions to test the effects of inorganic nitrate on human health

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Abstract

The chemical composition of vegetables is dependent on several growing conditions. This effect relates to phytochemical compounds including secondary metabolites and other bioactive non-nutritional compounds.

This study aimed to use different nitrogen fertilizer regimes to produce vegetables with so large differences in nitrate content that they can be used as treatment and placebo to study the effect of nitrate on human health. Green leafy vegetables, such as lettuce or rocket, are rich in inorganic nitrate (NO_3) and an increased consumption has been associated with beneficial effects on blood pressure (BP). The objective of this study was to compare whether two lettuce materials with controlled high and low NO_3 content may have different effects on BP in healthy subjects.

Firstly a gas chromatography mass spectrometry (GC-MS) method using pentafluorobenzyl bromide and chemical ionisation to determine nitrate and nitrite levels was modified to optimise the analytical process. This method has been used to measure samples of lettuce as well as samples of human plasma, urine and saliva obtained from this study and several other projects.

In this study, two sets of lettuce were produced as a model vegetable with high and low nitrogen fertiliser in controlled growing conditions to manipulate nitrate concentration of the vegetable as a tool for investigating the effect of nitrate content of food on human health. This was tested by determining the correlations between nitrate intake and blood pressure (BP), by measuring the short effect on systolic and diastolic BP and nitrate and nitrite contents in blood, urine and saliva samples. The novelty of this study is that the placebo and the treatment (lettuce with low and high nitrate content, respectively) have similar appearance despite being very different in nitrate content, making it possible to blind the subjects to the treatment and control placebo effects of vegetable consumption. Using 26 and 154ppm ammonium nitrate in the fertigation solution, lettuce was produced with a high (~530 mg nitrate/50g FW portion) or low (~3 mg/50g) nitrate content. However not all confounding factors could be controlled, e.g. the low nitrate lettuce produced a high amount of phenolic acids which was 69.5 mg/50g FW and high nitrate lettuce produced a much lower amount which10.5mg/50g FW ($P<0.001$). So while the nitrate content can be manipulated substantially via fertilizer treatments, the low-nitrate lettuce also differs from the high-nitrate lettuce in the content of other non-nutrient phytochemicals.

An intervention trial was carried out with twenty healthy young volunteers (12 females and 8 males) in a randomised, double-blind placebo controlled cross-over design, with two 24-hour

intervention phases separated by a 3-week washout period to avoid carry-over effects. Blood pressure was recorded by 24-hour ambulatory blood pressure monitoring (ABPM), every 30 mins during day-time and every 60 min at night-time.

Consumption of high nitrate lettuce significantly increased plasma NO_3 (3 hrs post-ingestion: 395±133 μM; $P<0.001$), salivary NO_3 (3 hrs post-ingestion: 7362±4666 μM; $P<0.001$) and urinary NO_3 (3 hrs post-ingestion: 2287±1233μM; $P<0.001$) concentrations whereas non-significant changes were measured in the low nitrate lettuce group. High nitrate lettuce significantly reduced systolic BP (-2.80±4.43 mmHg and -6.85±4.91 mmHg after 3 and 6 hrs, respectively; $P=0.003$) and diastolic BP (-2.25±2.34mmHg and Δ3.85±3.01 mmHg after 3h and 6 hrs, respectively; $P=0.002$) compared to low nitrate lettuce. No significant difference was observed between high nitrate lettuce and low nitrate lettuce for TEAC ($P = 0.32$), FRAP ($P = 0.26$), cGMP ($P = 0.19$) and plasma concentrations of phenolic compounds. The results showed that the amount of urinary nitrate excreted during a 24-hour period increased from 104mg with low nitrate lettuce to 391mg ($P<0.001$) with the high nitrate lettuce.

In conclusion, ingestion of high nitrate lettuce significantly increases plasma, saliva and urinary nitrate and lowers systolic and diastolic BP compared to low nitrate lettuce. The development of NO_3-enriched and NO_3-depleted vegetable products with similar organoleptic characteristics could provide a unique opportunity to conduct double-blind nutritional interventions and advance knowledge on the role of dietary nitrate on human health.

Declaration

I declare the work presented in this thesis is my own and has not been submitted anywhere for any other degree or qualification. Any reference to this work should be acknowledged and permission should be required from the author prior to the publication of any quotation from this work.

Publications arising from this work

The resulted from chapter 2 has been published in conference proceeding below:

1-Qadir, O., Teh, J., Siervo, M., Seal, C. and Brandt, K. (2013) 'Method using gas chromatography mass spectrometry (GC-MS) for analysis of nitrate and nitrite in vegetables', NUTRIHORT: Nutrient management, innovative techniques and nutrient legislation in intensive horticulture for an improved water quality. Ghent, Belgium: Institute for Agricultural and Fisheries Research.

2-O. Qadir, M. Siervo, C.J. Seal and K. Brandt. (2016) 'Recovery of urinary nitrate from ingestion of high-nitrate lettuce in healthy young subjects', Summer Conference: New technology in nutrition research and practice. University College Dublin (UCD), Ireland. The Nutrition Society.

Unpublished work

1- Manipulation of contents of nitrate, phenolic acids, chlorophylls and carotenoids in lettuce (*Lactuca sativa* L.) via contrasting responses to nitrogen fertilizer when grown in a controlled environment. ***In preparation.***

2- Effects of lettuce with different inorganic nitrate content on blood pressure and pharmacokinetic profile of nitrate and phenolic compounds in healthy subjects: A randomized, cross-over, double-blind clinical trial. ***In preparation.***

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List of abbreviations

A	Ampere
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ADI	Acceptable daily intake
ANOVA	Analysis of variance
AUC	Area under the curve
BL	Butterhead lettuce
bpm	Beats per minute
cGMP	Cyclic guanosine monophosphate
CHD	Chronic heart disease
CI	Confidence Intervals
DBP	Diastolic blood pressure
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
e.g	*exempli gratia* (for example)
et al.	*et alii* (and others)
eV	electron volt
FRAP	Ferric reducing ability of plasma
FW	Fresh weight
g	Gram
GCMS	Gas chromatography mass spectrometry
h	Hour / Hours
ha	Hectare
HPLC	High performance liquid chromatography
Kcal	Kilo calorie
Kg	Kilogram

kPa	Kilopascal
L	Litre
m	Meter
m/z	Mass to charge ratio
m^2	Square meter
METs	Metabolic equivalent of tasks
mg	Milligram
min	Minute / Minutes
ml	Millilitre
mM	Millimolar
mm	millimeter
mmHg	Millimeter of mercury
N	Nitrogen
n	Number of replicates
NICI	Negative-ion chemical ionization
nm	Nanometre
NO_2^-	Nitrite
NO_3^-	Nitrate
OL	Oakleaf lettuce
PFB-Br	2,3,4,5,6-Pentafluorobenzyl bromide
ppm	Part per million
RDA	Recommended dietary allowance
rpm	Round per minute
SBP	Systolic blood pressure
SCF	Scientific committee on food

SD	Standard deviation
sec	Second
SEM	Standard error of the mean
TEAC	Trolox equivalent antioxidant capacity
TP	Total phenolic
TRAP	Telomeric repeat amplification protocol
UV-VIS	Ultraviolet–visible
v/v	Volume to volume
Δ	Change in the value
µl	Microliter
µM	Micromolar
µmol	Micromole
°C	Degree centigrade (Degrees Celsius)
µ	Micron
µA	Microampere

Chapter 1. Introduction

1.1 General Introduction

Plant food contains numerous of bioactive compounds and health promoting properties, which are related to human health. Epidemiological studies widely recommends that consumption of a diet rich in vegetables and fruits has positive implications for human health (Cartea *et al.*, 2011), whereas vegetables and fruits contain a variety of health promoting actions such as indispensable minerals, vitamins, antioxidant and fibre as probiotics. A collection of investigations around the topic of plants, looking at the effect of plant secondary metabolites including anticancer agents and phenolic antioxidant (Brandt *et al.*, 2004; Nguyen and Niemeyer, 2008) also nitrate may have a beneficial effect on human health (Coles and Clifton, 2012). Phytochemical compounds have a widely beneficial for human health, for example, the phenolic acids. However, the growing conditions have also effect on chemical compositions of fruits and vegetables (Young *et al.*, 2005). Also, the on-going researches still looking for the investigation of a few compounds as benefit health from vegetables; for examples, reduce the cardiovascular risk disease by sulfoxides and/or flavonoids from Alliums (Griffiths *et al.*, 2002).

There are numerous of growing conditions have direct effects on chemical substances of plants, fruits and vegetables which are nutritional elements, including proteins, soluble carbohydrate and vitamins. It has been demonstrated in several recent studies that the accumulation of phytochemicals may be affected naturally by plant's conditions (Sari and Torronen, 2000; Ann *et al.*, 2002; Howard *et al.*, 2002). It has been stated that the effect of soil type is smaller than temperature and fertilisation on sensory quality and chemical composition in plants (Simon *et al.*, 1982; Hogstad *et al.*, 1997; Rosenfeld and Samuelsen, 1998).

Most of the epidemiological studies on plant nutrition and human health concern about the organic and conventional farming foods, which includes the effect of soil and fertilisation on chemical composition mainly secondary metabolites on human health. In last decades, there was some debate about the effects of bioactive compounds in plants which have an important role in defences and growing in plants. The secondary metabolites also have several benefits to prevent diseases and protect human health. In particular, polyphenol compounds have several benefits, including reduced risk of cancer and heart diseases (Brandt and Mølgaard, 2001; Zhao *et al.*, 2006; Mitchell *et al.*, 2007; Soltoft *et al.*, 2010). Epidemiology, it has been strongly

recommended the diet containing 400g fresh vegetables and fruits (Bazzano, 2006) as a nutritional goal for health promotion and the prevention of chronic diseases (Wang *et al.*, 2014).

The role of plant secondary metabolites for human health has been reviewed due to the investigation of their protective possible and health-promoting properties that range from the prompting of anti-oxidative mechanisms to reducing the risk of cancer and cardiovascular disease (Schreiner *et al.*, 2012). However, a little acknowledge is available between food components and health, particularly the understanding of deficiency or oversupply of any nutrients. On the other hand, some compounds classified as harmful toxicants, such as nitrate and plant secondary metabolites, appear to may have beneficial effects on human health. Due to the fact we may be able to connect food compositions and human health by additional investigations on secondary metabolite compounds in foods and will expose to getting a good health for consumers (Brandt, 2008).

Nitrates are a naturally constituent of plants, water and generally in other foods; some studies have found benefits for human health. Nitrate is also used as a food additive, preservative and antimicrobial agent (Santamaria, 2006). Vegetables are the main source of dietary nitrate (Weitzberg and Lundberg, 2013) and contain a different concentration of nitrate depends on plant species, soil and nitrogen fertiliser (Yamasaki *et al.*, 2014). Nitrate and nitrite represent the final pathway products of nitric oxide (NO) metabolism in human (Friedberg *et al.*, 1997). Most quantitative methods have measured nitrate (NO_3) and nitrite (NO_2) in biological samples as markers of NO release due to the radical nature and short half-life of NO. Nitrate is the primary source of NO metabolite in the blood (Moshage *et al.*, 1995; Bories *et al.*, 1999).

The Acceptable Daily Intake (ADI) for nitrate was established by the former Scientific Committee on Food (SCF) and was reconfirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002. ADI for nitrate is 0-3.7 mg/kg body weight/day, or 222 mg nitrate per person (60 kg adult). The most recent review has been completed by the JECFA in 2002 and reconfirmed an ADI of 0-0.07 mg/kg body weight for nitrite (Alexander *et al.*, 2008).

The remainder of this chapter includes a review of the literature across a number of nitrate and nitrite analytical methods such as caloremetric, chemiluminescence, capillary electrophoresis, high performance liquid chromatography (HPLC), chromatography-mass spectrometry (GC-MS), in addition to growing condition of vegetables, lettuce compositions and benefits of vegetable consumption on human health. The review will begin with a history and principles

of nitrate and nitrite analysis in different types of foods, in addition to advantage and disadvantage of the methods. The effect of growing conditions on lettuce compositions which include nitrate content and phytochemicals, the factors that affect phytochemical levels in plant and the association between plant phytochemicals and human health. The review will then give an overview of the role of vegetable in reducing the risk of cardiovascular diseases such as blood pressure. Finally, the understanding of dietary nitrate in the human body, pharmacokinetics of inorganic nitrate with nitrate-nitrite-nitric oxide pathway will be discussed.

1.2 Nitrates and nitrites

Most of the typical food contains nitrates and nitrites. They occur naturally in fruit and vegetables, which are considered as an important part of a healthy food due to the powerful evidence of beneficial health effects against cancer (Bradbury *et al.*, 2014) and it has been reported that the consumption of high nitrate vegetables significantly reduced blood pressure (Ashworth *et al.*, 2015). The last ten years, the evidence of physiological effects of nitrate has been increasing, particularly on the cardiovascular system and the understanding of the 'nitrate-nitrite-NO pathway' (Lidder and Webb, 2013). Additionally, nitrate and nitrite have been observed as inactive by-products of NO metabolism through the NO synthase–dependent pathway (Jonvik *et al.*, 2016).

1.2.1 Metabolism of dietary nitrate in human body

The understanding of the metabolism of dietary nitrate is a requirement for the assessment of the beneficial effects of nitrate on human health (McKnight *et al.*, 1999). Numerous studies that evaluate the metabolism of NO and the formation of reactive nitrogen species in vivo depend on the measurement of nitrate, nitrite, and 3-nitrotyrosine in human biological fluids. It has been acknowledged that dietary nitrate could affect urinary and plasma nitrate concentrations, and there are a few studies on the pharmacokinetics of ingested nitrate, which is degraded to nitrite in saliva, and its effect on the formation of these biomarkers. When swallowed through the mouth to the acidic conditions (pH 1–2) of the stomach, Nitrite is converted into nitrous acid and other reactive nitrogen species. Hence, the absorbed nitrite is reached into the plasma through the proximal small intestine, at this stage, it mixes with endogenously biosynthesized nitrate (L'Hirondel, 2001) (See figure 1.1). Then, it is ten times concentrated, from the plasma into the saliva (Spiegelhalder *et al.*, 1976) and then re-secreted into the upper intestinal tract. Approximately 25 % of dietary nitrate is present in saliva circulation (Tannenbaum *et al.*, 1976; McKnight *et al.*, 1999). Moreover, anaerobic bacteria on the dorsal surface of the tongue reduce nitrate to nitrite under hypoxic conditions (McKnight *et al.*, 1997). In another study, it has been found that the increase of nitrite concentration in saliva was 20ppm/100mg nitrate ingested, which is showed by a direct relationship between the nitrite levels in saliva and the amounts of dietary nitrate intake (Spiegelhalder *et al.*, 1976).

In a model study to assess the role of gastrointestinal absorption and metabolism of dietary nitrate intake in rats, the recovery of urinary nitrate excretion was 54 %, which is reported that about 40–45% of ingested nitrate appears to be metabolised or denitrified by the reside colon bacteria rather than excreted via the urine (Schultz *et al.*, 1985). Other investigators also have shown that 60% of ingested nitrate was excreted as ^{15}[NO3] labelled nitrate with the urine following 48h of an oral dose 217 mg and approximately 0.1% found in the faeces. Some of the nitrates appeared 3% in urine and a tiny amount 0.2% found in faeces in the form of ammonia NH_3 or urea CH_4N_2O. The loss of 35% of ingested nitrate is unknown (Green *et al.*, 1981; Wagner *et al.*, 1983). Interestingly, it has been discovered that the level of nitrous oxide N_2O has significantly increased in breath air after ingested lettuce and vegetable juice, which is a product of the metabolism process (Mitsui and Kondo, 1999). Wagner *et al.* (1983) believed in that a part of this lost might go through gaseous products which are exhaled in the breath or appear in flatus. As well as, the detection of ammonia and urea in urine and faeces indicated that a part of this lost might also have been combined into the colonic flora and consumed as a source of nitrogen (L'Hirondel, 2001).

The ingestion of nitrate rich- diet leads to an increase in the salivary, plasma, and urinary nitrate concentrations. Urinary nitrate excretion has increased about four-fold during a study, and this excretion has previously been stated after dietary inorganic nitrate and nitrate-rich food (Bartholomew and Hill, 1984; Cortas and Wakid, 1991; Oldreive *et al.*, 2001). In a clinical study, the maximum urinary nitrate excretion appeared after 4 to 6h of ingesting of each high nitrate meal and back to the baseline value after 24h; the total urinary nitrate excretion was approximately 75% (Pannala *et al.*, 2003). The other amount of 25% of nitrate excretion have been degraded to nitrite in the saliva, is excreted via sweat, and approximately 1% is excreted in faeces or some digestive secretions by bacterial action. Similarly, a group of authors found less than 2% of dietary nitrate intake in the terminal ileum which is a part of the end of the small intestine (L'Hirondel, 2001). Meanwhile, it has been found that 65-70% of ingested nitrate was excreted in urine in the following 24h (Spiegelhalder *et al.*, 1976; Bartholomew and Hill, 1984). This result shows that the 24-hr urinary nitrate analyses could be useful to determine the nitrate excretion in clinical trials.

The levels of plasma nitrate are certainly not zero. Plasma nitrate concentration is between 0.25 and 2.7mg/L under fasting condition. It might reach approximately 4mg/L after each regular meal, and 10–25mg/L following high dietary nitrate intake (Wagner *et al.*, 1983; Colbers *et al.*, 1996).

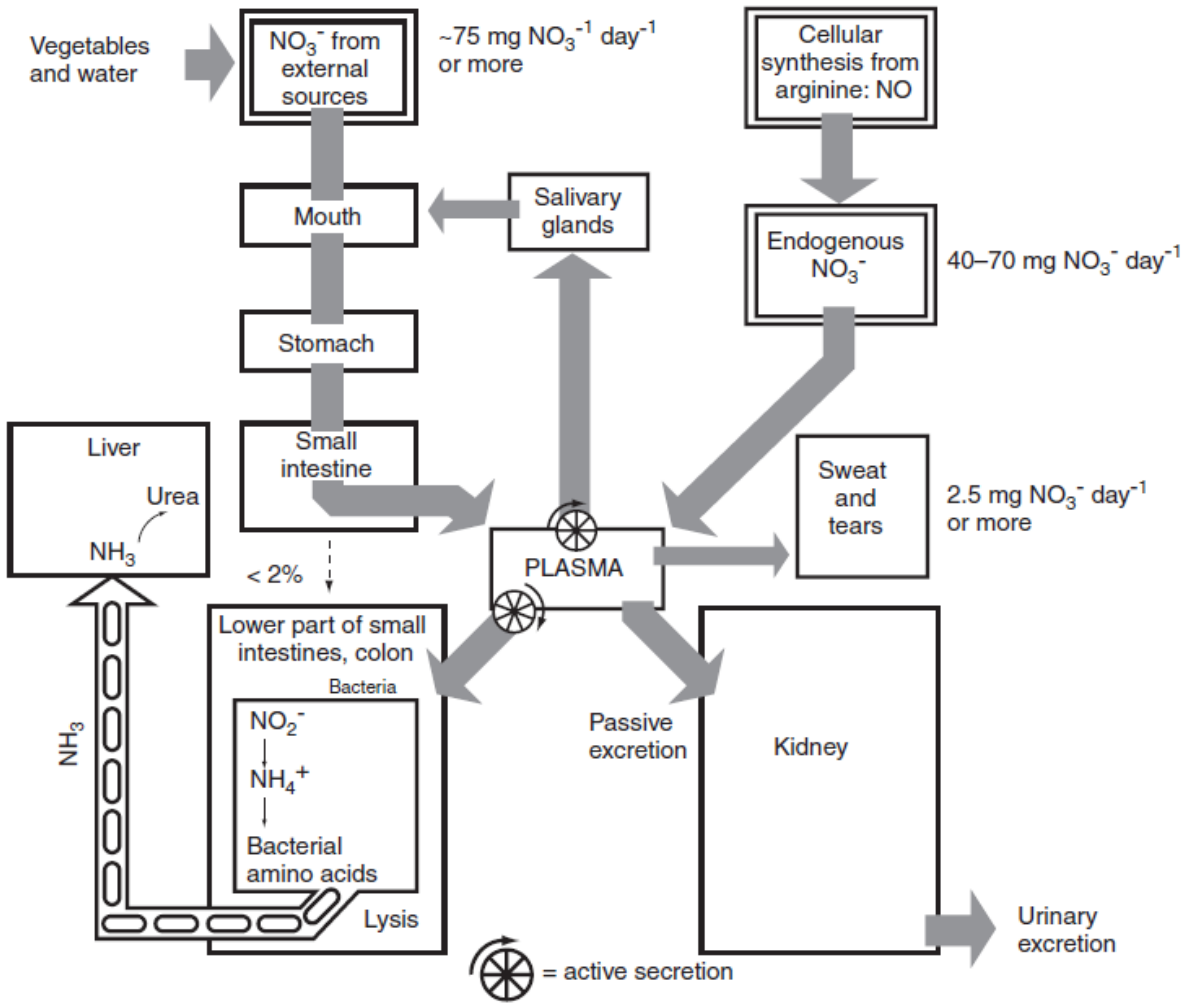


Figure 1.1. The metabolism of nitrate in human body

(L'Hirondel, 2001)

Ingested nitrate passes through the mouth and then the oesophagus before arriving in the stomach. It is then rapidly and almost immediately absorbed and passes from the upper small intestine to the bloodstream, where it mixes with endogenously synthesized nitrate (L'Hirondel, 2001)

1.2.1.1. Salivary nitrate, and its conversion to nitrite in the mouth

The human salivary glands can be divided into two separate exocrine groups. The major salivary glands are three paired including parotid, submandibular, and sublingual glands. The minor salivary glands are small glands which produced the mucosa of the upper aerodigestive tract. The function of the major salivary glands is to secrete saliva (Holsinger and Bui, 2007). Previous studies have discovered that amount of dietary nitrate intake has a linear relationship with the formation of salivary nitrite, which may have an effect on human health (Tannenbaum *et al.*, 1976; Eisenbrand *et al.*, 1980). In Chapter 3 of the book *"Nitrate and Man, Toxic, Harmless or Beneficial?"*, L'Hirondel (2001) provides us with some evidence that 25% of dietary nitrate intake is found in saliva secretions, and that 20% of such nitrate is converted to nitrite in the mouth, this means approximately 5% of nitrate stays in saliva in the form of salivary nitrite. In addition, dietary nitrate intake led to a rise in the pool of NO through the entero-salivary nitrate-nitrite-nitric oxide pathway which appears as a prospective regulator of physiological functions in the gastro-intestinal tract and the cardiovascular system (Lundberg and Weitzberg, 2009; Bryan and Loscalzo, 2011) (Figure 1.11).

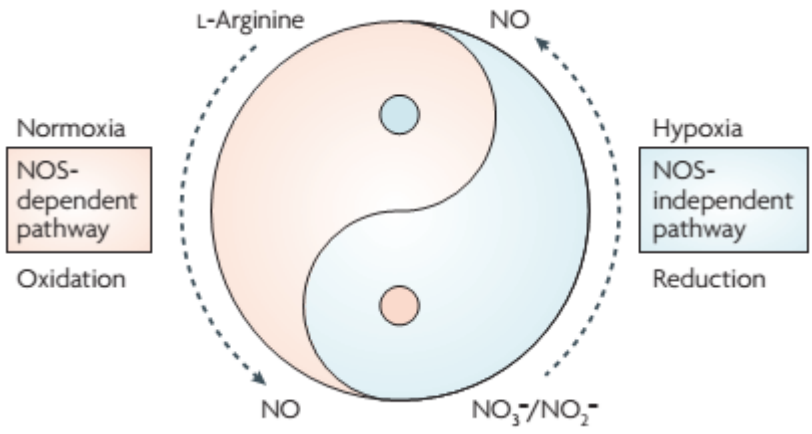


Figure 1.2 The nitrate–nitrite–NO pathway

Studies on human adults show that the salivary nitrate and nitrite, plasma nitrate and nitrite are increased from minutes to hours after nitrate loads (Lundberg and Govoni, 2004; Webb *et al.*, 2008; Gilchrist *et al.*, 2011; Hobbs *et al.*, 2013; Velmurugan *et al.*, 2016). The plasma nitrate level was raised rapidly within 30 min, and it reached maximum 90 min after ingestion of beetroot juice. In contrast, the appearance of plasma nitrite was considerably slower, peaking at 150 to 180 min (Lundberg and Govoni, 2004; Webb *et al.*, 2008). Meanwhile, the peak increase in plasma nitrate was reached at 210 min and in plasma nitrite at 190 min (Gilchrist *et*

al., 2011; Hobbs *et al.*, 2013). In saliva, the increase of salivary nitrite follows the amount of dietary nitrate intake. For instance, the ingestion of 30 and 60mg nitrate, increased salivary nitrite after 1 and 2h, respectively. A single subjects intake of 450 mg nitrate led to increased salivary nitrite of over 900 mg/litre at 2h (Tannenbaum *et al.*, 1976).

1.3 Analytical methods for the analysis of nitrate and nitrite

During the past 30 years, much more information has become available on the importance role of nitric oxide in human health. Nitrate and nitrite also are simple anions present mostly in green leafy vegetables (Hsu, 2009). A diet rich in fruit and vegetables reduces blood pressure and protects against cardiovascular disease. Recently, it was revealed that modest dietary supplementation with sodium nitrate reduces blood pressure in healthy volunteers (Larsen *et al.*, 2006). Nitric oxide has a short life with small diffusible radical, which causes difficulties in measuring it (Ricciardolo, 2003). Different analytical techniques are available to measure NO directly or indirectly. For instance, NO could be measured by bioassay (Gryglewski *et al.*, 1986), an oxyhemoglobin assay (Kelm *et al.*, 1988), chemiluminescence (Palmer *et al.*, 1987), high-performance liquid chromatography (HPLC) (Kelm *et al.*, 1999), the Griess reaction (Thomsen *et al.*, 1990), capillary electrophoresis (Kikura-Hanajiri *et al.*, 2002). NO is not stable and degrades rapidly in biological samples to the stable products nitrite and nitrate. Therefore, different assays have been developed to measure nitrate/nitrite concentrations as an indirect method to quantify NO formation (Berkels *et al.*, 2004). Because of the great variety in spatial expression and activity of NO in human tissues and vegetables, a high-resolution analysis of nitrite/nitrate concentrations in very small biological samples, is required. Therefore, more than 200 analytical methods have been conducted and developed as well as protocols assembled, and several applicable analytical parameters have been classified. Various of drawbacks and limitations in the use of all the methods has been reported which include the interference between the sample and reagent with a range of inaccuracy and insensitivity of some instruments (Moorcroft *et al.*, 2001).

A recent systematic literature review of nitrate and nitrite analysis concluded that the quantitative methods are based on the nitrate and nitrite reduction either by a metal catalyst in high performance liquid chromatography methods or by enzymatic reduction of nitrate ending spectrophotometrically after the Griess reaction (Bories *et al.*, 1999). The capillary electrophoresis (CE) which separates molecules by their charge and their hydrodynamic volume (Landers, 1995). In addition, gas chromatography-mass spectrometry methods include non-

derivatized and derivatized; various chemical substances have been used in derivatization procedures such as 1,3,5-trimethoxybenzene, mesitylene (Kage *et al.*, 2000), benzene and toluene for aromatic nitration and pentafluorobenzyl bromide (PFB-Br) for nucleophilic substitution (Tsikas *et al.*, 2010).

The demand for a rapid and accurate method for nitrate and nitrite analysis has increased in recent years. Thus, several attempts have been made to develop the methods, and this can continue to improve the accuracy in detection of nitrate and nitrate in environmental, biological fluids and food samples.

1.3.1 Colorimetric (Griess method)

The colorimetric methods are used for the determination of nitrate and nitrite. This method working based on the reaction of nitrite, sulphanilamide and N-(1-naphthyl)-ethylenediamine under acidic conditions to form a red azo dye. This forms basis for the nitrite determination in cured meats, cheese, dried milk and waste waters (Hill, 1996). The cadmium column has been used to develop the method to reduce nitrate in fruits and vegetables to nitrite (Pickston *et al.*, 1980). The advantages decrease the time require for colour development and less sensitive to pH variations. The disadvantages of this approach include interference of nitrate with chemical reagents, oxidising, and insufficient reduction of nitrate to nitrite (Norwitz and Keliher, 1985).

1.3.2 Chemiluminescence

The chemiluminescence method is one of the important analysis methods for detecting trace concentrations of nitrate and nitrite in aqueous samples. The chemiluminescence detector is highly sensitive to measure a trace amount of NO (Braman and Hendrix, 1989). The chemiluminescence instrument has been developed several times to increase the consistency and accuracy for NO detection, which has been produced by using different detectors such as Molybdenum (Mo-CLD), photolytic conversion (PC-CLD), luminol 3D (LMA3D) and NO_2-(LOPAP) (Kleffmann *et al.*, 2013). Mikuška and Večeřa (2002) reported that the flow injection analysis ultra-violet (FIA-UV) has been used to measure nitrate and the method is fast and highly sensitive, photolytic converter where nitrate is reduced to nitrite due to absorption of UV light at quartz capillaries, formed nitrite is rapidly determined as peroxynitrite by the chemiluminescent reaction with luminol. An UV photo-reductor consisting of a high pressure

mercury lamp and quartz capillaries provides an effective nitrate reduction however only deionized water is employed as a carrier medium.

The method is easy to use, and the photolytic conversion of nitrate induced by absorption of UV light seems to be a very promising technique for the reduction of nitrate to nitrite. Additionally, no toxic waste is produced. The photolytic reduction of nitrate needs low maintenance, which offers its usage for routine analysis of large series of samples or at various monitoring systems.

The drawbacks of this method is acidified cadmium reductor may cause interferences between copper or/and phosphate ions (Naidoo and Van Staden, 2001). In addition to, the photo-conversion of nitrate suffer from a low efficiency of nitrate reduction using distilled water as a carrier and a low-pressure mercury lamp as a light source (David *et al.*, 2000).

1.3.3 Capillary Electrophoresis

Capillary electrophoresis (CE) is another separation technique for different ions and micromolecules. The first used this method was for a clinical purpose, and it has been modified for analytical determinations in foods (Friedberg *et al.*, 1997) and many biological fluids because of low nitrite concentration in biological fluids (Hassid, 2004). The method needs a small volume of sample and buffer. The advantages of CE are a needs small sample and low buffer consumption, in comparison with HPLC. The equipment has autosampler, requires little maintenance and the technique is fast in simultaneous detection of different anions (Moorcroft *et al.*, 2001). This method has also been developed to increase the sensitivity by use capillary zone electrophoresis (Hill, 1996). The disadvantages of CE are sample needs several steps for of preparation and separation and, in addition to deproteinization technique for samples contain protein to avoid the interference of protein with the wall of the capillary and, it is more cost-effective for separation of anions than other techniques.

1.3.4 High performance liquid chromatography

Over the past few decades, the use of high performance liquid chromatography (HPLC) for nitrate and nitrite analysis has increased in environmental samples, plants and human fluids. The method has been developed for different analytical measurements due to sensitivity and accuracy of the instrument. The HPLC method on wide use based on the type of detectors and

the chemical properties of the sample. The principle of detections includes UV and VIS absorbance, chemiluminescence, fluorescence and electrochemistry (Jobgen *et al.*, 2007). A variety of HPLC methods has been applied to determine nitrate and nitrite. For instance, the recently developed method with pre-column derivatization with 2,3-diaminonaphthalene (DAN). The method shows a lack of interference, accuracy and consistency in measurement and can be applicable for biological fluids. On the other hand, the biological samples require dilution with double-distilled deionized water to minimize the effect of some compounds (NADPH, glutathione and dithiothreitol) existing in these samples on the derivatization of nitrite with DAN to yield 2,3-naphthotriazole (Li *et al.*, 2000).

1.3.5 Gas chromatography-mass spectrometry

Numerous gas chromatography-mass spectrometry (GC-MS) methods have been used to assess nitrate and nitrite in water and foodstuffs. [^{15}N] nitrate and [^{15}N] nitrite labelled as internal standards or isotope dilution techniques and derivatization with pentafluorobenzyl bromide (PFB-Br), benzene or toluene have been used in GC-MS methods to determine nitrate and nitrite in plasma, urine and water (Wennmalm *et al.*, 1993; Tsikas *et al.*, 1994; Johnson and Burleson, 1996). This method is mainly based on the nitration of aromatic compounds, the creation of a volatile derivative and extraction in an organic solvent and determination of the ionized molecules by using a selective detector (Tsikas *et al.*, 1994; Hill, 1996). Similarly, the GC-MS methods have been improved more than a few times to minimize the interference between the compounds and accuracy of the measure. According to Funazo *et al.* (1980) the GC-4BM method has been developed by derivatization and using ^{63}Ni electron capture detector, the sample was filtered and diluted with distilled water to a suitable concentration of nitrite (< 1ppm), to find a peak conversion reaction condition, the conversion yields of nitrite into ρ-bromochlorobenzene were examined by reaction of nitrite with ρ-bromoanillne and copper chloride ($CuCl_2$) in hydrochloric acid (HCl) medium and various of samples (foods, water and human saliva) has been analysed by this method.

Another GCMS preocedure has been prepared by using α-Bromo-2,3,4,5,6-pentafluorotoluene, the GC condition involves coiled stainless steel column (3.6 m x 2 mm I.D.) packed with 10% OV-210 on Chromosorb W HP (80-100 mesh). The injection port and detector temperatures were kept at 180 and 250°C respectively. The column was maintained isothermally at 150°C, the flow rate of the carrier gas (Nitrogen) was 50 ml/min. The method has been applied to

determine nitrite in a range of meat, water and saliva samples and the results were in good agreement with the literature (Wu *et al.*, 1984).

In the present study, the aim was to set up and modify a highly sensitive GC-MS method for quantitative determination of nitrite and nitrate in vegetables and human biological samples (plasma, saliva, and urine). The method is based on the conversion of nitrate and nitrite into α-nitro-pentafluorotoluenes using pentafluorobenzyl bromide (PFB-Br) as the alkylating agent, and isotope diulution with [^{15}N] labelled nitrate and nitrite as the internal standards.

1.4 Lettuce

Lettuce (*Lactuca sativa* L.) is one of the most popular annual crops of the Asteraceae family (Compositae) in the group of leafy vegetables and most diverse families of flowering plants (Romani *et al.*, 2002; Chon *et al.*, 2005; Sinha *et al.*, 2010). Lactuca means 'milk forming', sativa means 'common'. It has been used by Romans as vegetable salad however it is native to Egypt, where lettuce was integrated into their religious practices and arts by ancient Egyptians (Woys, 1997). It is the most widely consumed vegetable as fresh salads and the largest portion of filling in sandwiches and some forms also cooked (Křístková *et al.*, 2008). There is numerous type of lettuce, but the most common types are Iceberg, Crisphead, Romaine, Butterhead, Cos, and Leaf lettuce (Bassett, 1986).

The nutritional value of lettuce is different among lettuce types; lettuce is a dependable source of dietary fibres (USDA, 2016), vitamins such as vitamin A, K and C and a high concentration of β-carotene is found in darker green lettuces, such as Romaine (Sinha *et al.*, 2010). Lettuce rich in vitamin K and is involved in the synthesis of a number of proteins involved in blood clotting and bone metabolism (Damon *et al.*, 2005). There is a growing line of research highlighting the beneficial effects of vitamin K on cardiovascular diseases. The findings from the Rotterdam study suggest that t an adequate intake of menaquinone (vitamin K2) could be important for chronic heart disease (CHD) prevention (Geleijnse *et al.*, 2004). In another study, it has been stated that a high intake of vitamin K2 could protect against CHD (Gast *et al.*, 2009). In addition, it is also an excellent source of the essential minerals, folate and iron. However, lettuce is in very low calories with around 95% water content (Jaime and Fernando, 2008), but it also contains health beneficial bioactive compounds which help as an anti-inflammatory, cholesterol-lowering, and anti-diabetic activities. Leaf type lettuce and romaine are the most nutritious lettuces with folate content comparable to other rich leafy vegetable

sources. Red lettuce contains higher phenolic compounds than green lettuce. Baby green romaine lettuce is high in vitamin C (Kim *et al.*, 2016).

Table 1.1. Nutritional composition of different fresh lettuce (per 100 g edible portion)

Components	Butterhead	Romaine	Green leaf	Iceberg	Red leaf
Water (g)	95.63	94.61	94.98	95.64	95.63
Protein (g)	1.35	1.23	1.36	0.90	1.33
Total lipid (g)	0.22	0.30	0.15	0.14	0.22
Ash (g)	0.57	0.58	0.62	0.36	0.55
Total Carbohydrate (g)	2.23	3.29	2.87	2.97	2.26
Fibre, total dietary (g)	1.1	2.1	1.3	1.2	0.9
Energy (kcal)	13	17	15	14	16

Source: (USDA, 2016)

Table 1.2. Mineral element of different fresh lettuce (per 100 g edible portion)

Minerals	Butterhead	Romaine	Green leaf	Iceberg	Red leaf
Calcium, Ca (mg)	35	33	36	18	33
Iron, Fe (mg)	1.24	0.97	0.86	0.41	1.20
Magnesium, Mg (mg)	13	14	13	7	12
Phosphorus, P (mg)	33	30	29	20	28
Potassium, K (mg)	238	247	194	141	187
Sodium, Na (mg)	5	8	28	10	25
Zinc, Zn (mg)	0.20	0.23	0.18	0.15	0.20
Copper, Cu (mg)	0.016	0.048	0.029	0.025	0.028
Selenium, Se (µg)	0.6	0.4	0.6	0.1	0.5

Source: (USDA, 2016)

Table 1.3. Vitamin content of different fresh lettuce (per 100 g edible portion)

Vitamins	Butterhead	Romaine	Green leaf	Iceberg	Red leaf
Vitamin C (mg)	3.7	4.0	9.2	2.8	3.7
Thiamine (mg)	0.057	0.072	0.070	0.041	0.064
Riboflavin (mg)	0.062	0.067	0.08	0.025	0.077
Niacin (mg)	0.357	0.313	0.375	0.123	0.321
Pantothenic acid (mg)	0.15	0.142	0.134	0.091	0.144
Vitamin B6 (mg)	0.082	0.074	0.090	0.042	0.1
Folate (µg)	73	136	38	29	36
Choline (mg)	8.4	9.9	13.4	6.7	11.8
Betaine (mg)	0.1	0.1	0.2	0.1	0.2
Vitamin A (µg-RAE)	166	436	370	25	375
β-carotene (µg)	1987	5226	4443	299	4495

Lutein + zeaxanthin (µg)	1223	2312	1730	277	1724
Vitamin E (α-tocopherol) (mg)	0.18	0.13	0.29	0.18	0.15
γ-tocopherol (mg)	0.27	0.36	0.37	0.09	0.24
Vitamin K (phylloquinone) (µg)	102.3	102.5	126.3	24.1	140.3

Source: (USDA, 2016)

The data has been recorded by Economic Research Service (ERS) which is combined with data from the United Nations, Food and Agriculture Organization (FAO) shows that world production of lettuce and chicory was 23,733,803 metric tonnes in 2009. This production 54.16% came from China, 17.29% from the United States of America (USA), and 4.21% from Spain. China is the top producer, and Spain is the largest exporter of lettuce (USDA, 2011).

1.4.1 Effect of growing conditions on lettuce compositions

Lettuce is a plant of considerable agricultural and economic interest, but as a leafy vegetable, it accumulates large quantities of nitrate especially when grown in high nitrogen availability. The ability to accumulate nitrate in plants depends on various factors. The main factors are nitrogen supply, temperature regime, light intensity, soil, and water supply. Growth and nitrate accumulation in lettuce are affected by different growing conditions and declining nitrogen concentrations in nutrient solution (Maršić and Osvald, 2002; Pavlou *et al.*, 2007). Nitrate accumulation is a natural phenomenon resulting from the excess nitrate uptake and subsequent assimilation processes which may affect nitrate content in plant tissue (Maynard *et al.*, 1976). Thus, it would be difficult to compare high nitrate concentrations in plant tissues with high nitrogen applications without attempting to define the cause. Therefore, the role of environment factors on chemical compositions will be explained with some examples.

1.4.2.1 Nitrogen fertiliser

In 1997, European countries decided to formulate the maximum permissible nitrate content in lettuce. Consequently, a better understanding of the effects of environmental factors on nitrate accumulation in lettuce and producing lettuces with a low nitrate content, which is necessary for crop management leading to an acceptable production (Laurent *et al.*, 2000). Growing lettuce needs a high irrigation because of the content of water in lettuce (95%). The root of lettuce has a poor distribution and restricted rooting depth. These factors have an effect on nitrogen uptake. Therefore, lettuce is required a sufficient nitrogen content in the soil until the

harvest day, lack of nitrogen causes yellow leaves and reduction in growth (Althaus *et al.*, 2009). Thus, there is a positive relationship between the nitrate content and water content in lettuce (Laurent *et al.*, 2000). In contrast, numerous studies have shown that there is a negative correlation between nitrate concentration and carbohydrates in lettuce because the lettuce has a demand for more nitrate. This may cause an increase in photosynthetic activity through which nitrate acts as an osmoticum and is replaced by sugars (Millard, 1988; Behr and Wiebe, 1992). It has been discovered that the decline of nitrate in lettuce leaves is compensated for by Chloride (Cl^-). This means the nitrate ions have an important role in osmoregulation (Stienstra, 1986) and the nitrate content in lettuce has a positive correlation with water content, which could be the effect on the osmotic regulation of nitrate (Maynard *et al.*, 1976). Similarly, the decrease in nitrate content in lettuce was completely compensated for by an increase in organic acids and sugar to maintain the osmotic behaviour (Blom-Zandstra and Lampe, 1985). Moreover, a group of authors have investigated that the plants under high nitrogen supply have a higher water content (Radin and Boyer, 1982; Leigh and Johnston, 1985; Laurent *et al.*, 2000). On the other hand, several attempts have been made to reducing nitrate content in lettuce by continuous LED light 48 h pre-harvest stage (Wanlai *et al.*, 2013) or by nitrogen reduction in growing pattern hydroponics system with 0 g/L NO^3 (Croitoru *et al.*, 2015).

There are several sources of nitrate fertilisers, such as calcium nitrate $Ca(NO_3)_2$ and potassium nitrate (KNO_3) as a nitrogen source in growing vegetables. The use of nitrate NO_3 fertiliser led to accumulating of NO_3 in vegetable production (Santamaria *et al.*, 1998). High nitrogen supply in soil led to decrease the dry matter and increase chlorophyll content in lettuce (Fontes *et al.*, 1997). These studies clearly demonstrated that nitrogen fertiliser has an important effect on the composition of the plant.

1.4.2.1.1. Nitrate metabolism in plants

Nitrate is the main source of nitrogen for growing plants which is one of the most important components of in the nutrition solution. Plants can absorb nitrate through the plasma membrane of epidermal and cortical cells of the root. Nitrate is reduced to ammonium NH_4^+ by nitrate and nitrite reductases, and the organic nitrogen can be released from NH_4^+-N by ammonium assimilation to nitrogen compounds by donating nitrogen from the glutamine synthetase (GS)/glutamate synthase in (GOGAT) cycle (Forde, 2000).

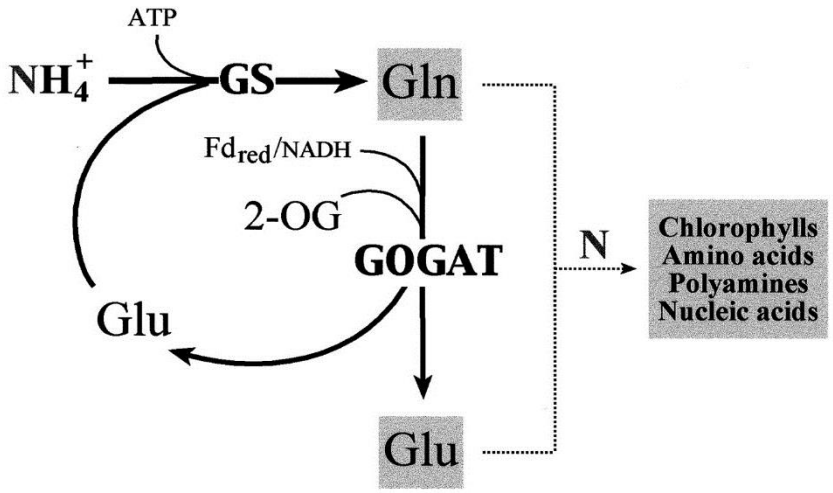


Figure 1.3. Assimilation of ammonium nitrate to nitrogen compound

"The amino acids glutamine and glutamate are the main N donors for the biosynthesis of nitrogen compounds. Net biosynthesis requires the supply of energy, reducing power and 2-oxoglutarate as the carbon skeleton." (Suárez *et al*., 2002).

1.4.2.2 Temperature

The production of quality head lettuce is very dependent on temperature regime. Lettuce is known as a cool ambient temperature plant (Jie and Kong, 1998). A high temperature produced a large amount of nitrate in lettuce leaves due to the nitrate translocation and assimilation processes (Maršić and Osvald, 2002). Lorenz and Wiebe (1980) have reported that increasing temperature led to increasing dry matter content in lettuce as well as enhancegrowth of leaf area. As a result, the associations between nitrate accumulations can be found with both high and low temperatures (Nightingale *et al.*, 1930; Kretschmer, 1958; Younis *et al.*, 1965) cited in (Maynard *et al.*, 1976). In some studies, the temperature has positive relationships with polyphenol and their antioxidant activities at relatively low temperatures led to high antioxidant and enzymatic status in lettuce plants. It has been reported by Van der Boon *et al.* (1990) that increasing the temperature of the nutrient solution and lowering the temperature of glasshouse air resulted in better growth as well as increased the nitrate concentration of the lettuce.

1.4.2.3 Light

The accumulation of nitrate in plants often occurs as a result of nitrate uptake over exceeding its metabolism under low light conditions. Therefore, different methods can be used to reduce nitrate in leaf. This system can supplies by various nitrogen concentration in harmony with a change of the light intensity to decrease accumulated nitrate in lettuce (Jernej *et al.*, 2004). It has been reported that the light intensity reductions are related to the increased nitrate contents in plant tissues (Maynard *et al.*, 1976). For example, Samuolienė *et al.* (2009) showed that increase light intensity reduced the nitrate content in lettuce grown under natural daylight (130 μmol. m-2. s-1) and increased to (200 μmol. m-2. s-1) six days before harvesting to for 24 h with the temperature regime at night was 12/16 °C, and at day time 16/18 °C. Given all that has been mentioned so far, it is clear that there is a strong correlation between light intensity and nitrate content in plants. Moreover, the association between light and nitrogen level are directly influencing the process of nitrate absorption, assimilation and translocation mechanism.

*"*Light factors were particularly significant at moderate N levels since, at low N levels, nitrate concentrations were relatively low, and at high N levels, concentrations were high. Light still exerted a marked effect at high N levels. Moreover, total N content over the experimental period was much greater at higher light levels suggesting that the reduced nitrate concentrations are indeed resulting from increased nitrate assimilation rates. Although growth was greatly accelerated at higher light intensity, the concentrations of mononitrate N were not reduced, indicating that the lower nitrate concentrations are not resulting from simple dilution effects" (Maynard *et al*., 1976).

1.5 Plant phytochemicals

Food provides the essential nutrients needed for survive life and also other bioactive compounds for health promotion and disease prevention (Liu, 2003). Although the original mechanisms of these effects have not been fully explained, the presence of secondary metabolites, commonly referred as phytochemicals, in fruits and vegetables might play major roles (Rodriguez-Casado, 2016). Epidemiological studies have revealed that increased fruit and vegetable consumption associated with a vital role in the prevention of chronic diseases (Temple, 2000; Bjelakovic *et al.*, 2012). Due to this fact, many epidemiological studies recommend consumers to eat a substantial amount of vegetables and fruits. Moreover, some researchers have investigated that consuming an enormous amount of vegetables and fruits, has been strongly associated with

reduced the risk cancers (Tanaka *et al.*, 2012; Jung *et al.*, 2013), and cardiovascular disease (Vasanthi *et al.*, 2012), diabetes, Alzheimer disease, and age-related functional decline (Willet, 1994). Vegetables contain a significant amount of bioactive compounds, such as phytochemicals, which provides health benefits and safety by decreasing chronic disease risks (Liu, 2003).

Phytochemicals are defined as bioactive non-nutritional secondary metabolite compounds in fruits and vegetables. Phytochemicals have been classified based on their chemical structure, function and source (Tiwari and Cummins, 2013). More than 5000 phytochemical structures have been recognised; however, the majority of them unknown function and require to more research investigate their beneficial effects on human health (Bruce and Lois, 1991; Liu, 2003). Many evidence shows that the majority of phytochemicals from fruits and vegetables exhibit antioxidant (Giampieri *et al.*, 2012), reduce platelet aggregation (El Haouari and A Rosado, 2011), blood pressure (Galleano *et al.*, 2010), and obesity (Calder *et al.*, 2011). Many reports have highlighted the free radical scavenging properties of phytochemical antioxidants such as polyphenols or carotenoids (Manach *et al.*, 2009).

The following sections will focus upon phenolic acids, flavonoids, carotenoids and chlorophylls which are abundant in vegetables and particularly in lettuce:

1.5.1 Phenolic compounds

Phenolic compounds are plant secondary metabolites synthesised mostly through the phenylproponaid pathway and are involved in defence of plants against attacking pathogens. Therefore, they are widely distributed in plant-derived foods significantly affecting their stability, colour, flavour, taste, nutritional and aesthetic value. In fact, plant secondary metabolites are important for most antioxidant properties (Pati *et al.*, 2006). It is believed that regular consumption of dietary antioxidants may reduce the risk of several diseases. Among natural antioxidants, plant polyphenols play a major role. Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities; the protective effects can be ascribed to their capacity to transfer electron free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce a-tocopherol radicals and inhibit oxidases (Heimler *et al.*, 2007). Recent studies have proved that tomato and lettuce are an interesting and cheap source of antioxidant phenolic compounds. These vegetables are the most popular vegetables in salads,

and their consumption is being increased due to they are considered as "healthy foods." (Alarcón-Flores *et al.*, 2013).

Phenolic compounds contain one or more aromatic rings with one or more hydroxyl groups. They present the largest group of molecules with a different function in plant growth (Vermerris and Nicholson, 2007). Their concertation is ranging around 1 to 3000mg/kg in foods (King and Young, 1999). Most of the phenolics are 'synthesized by phenylalanine ammonia lyase which catalyses the conversion of l-phenylalanine to trans-cinnamic acid' (Wen *et al.*, 2003). They are ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. The roles of phenolics in plants include defence against UV radiation and protecting the plant from pathogen disease and parasites as well as contributing to plants' colours. They commonly occur in food and beverages such as fruits, vegetables, legumes, cereals, chocolate, tea, coffee, beer, wine are and partially responsible for the organoleptic properties of plant foods (Dai and Mumper, 2010).

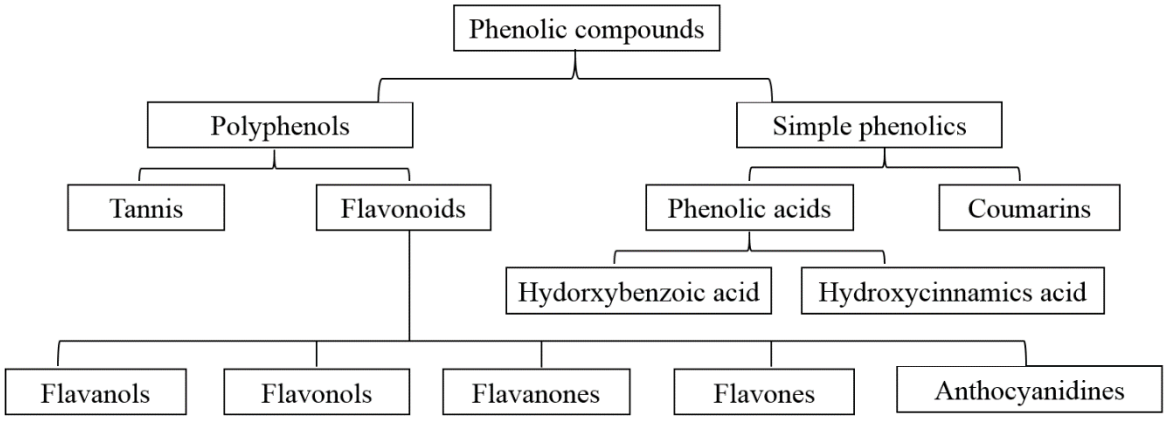


Figure 1.4. Chemical classification of phenolic compounds (Magnani *et al.*, 2014)

Previous studies have examined phenolic compounds and antioxidant activities in different varieties of lettuce (Llorach *et al.*, 2008). Numerous studies have shown that abundance of phenolic compounds in lettuce tissues could be affected by genetics, agronomical and environmental factors (DuPont *et al.*, 2000; Manach *et al.*, 2004). The antioxidant power of total phenolic acids has been investigated in lettuce; total phenolics accounted for more than 60% of the total antioxidant capacity. Dicaffeoyl tartaric acid (Chicoric acid) accounted for 55% (Nicolle *et al.*, 2004b). Among the major phenolic compounds are known to exert health-

promoting capacity, chlorogenic acid, caffeic acid, and chicoric acid play an important role (Olthof *et al.*, 2001).

Up to now, some studies have measured the phenol and polyphenol composition in different cultivars of lettuce. They have been identified into two main classes of products: caffeic acid derivatives and flavonols. In particular, 5-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, chicoric acid and two more caffeic acid derivatives, and the flavonoids quercetin 3- O-glucoside, quercetin 3-O-glucuronide, and quercetin 3-O-(6-O-malonylglucoside) (Romani *et al.*, 2002).

The amount of these antioxidant and micronutrients in lettuce leaves is susceptible to high variation in response to cultivars, growing conditions, environmental stress. For instance, exposure to high light intensity increased phenolic accumulation and antioxidant capacity (Oh *et al.*, 2009). Nitrogen availability appeared to reduce total polyphenols and antioxidant activity (Coria-Cayupán *et al.*, 2009).

1.5.1.1. Phenolic acids

Phenolic acids are non-flavonoid polyphenolic compounds which can be divided into two main groups, benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones (Tsao, 2010). A vast and growing body of literature have investigated that the correlation between a diet rich in fruits and vegetables with the health maintenance and protection of human health diseases. Current thought is the associations between high antioxidant content in foods with the prevention of oxidative damage in disease, for examples, stroke, cancer and coronary heart disease (Robbins, 2003). In lettuce, the ranges of phenolic acids were 8.4 to 12.9 and 27.8 mg/g dry weight in green and red oak leaf, respectively (Nicolle *et al.*, 2004b). In a comparison study of chemical composition and antioxidant capacity of lettuce, chlorogenic acid and caffeic derivates were found as major compounds among the free and bound phenolics, respectively in three different types of Romaine lettuce (regular, intermediate and low sizes) cultivated in greenhouse conditions (López *et al.*, 2014). Surprisingly, chlorogenic acid content ranges from 15.93 ± 1.59 to 22.24 ± 7.74 mg/100 g, and caffeic acid from 3.87±0.43 to 7.59±2.66 mg/100g were found in Italian head lettuce (Durazzo *et al.*, 2014).

The literature has highlighted the importance of phenolic acids such as chicoric acid in lettuce has enzyme inhibitory activities in lipid peroxidation (Mulabagal *et al.*, 2010). Some of the phenolic acids have antimicrobial properties against microorganisms such as the

hydroxycinnamic acids were bactericidal at pH 4.5 and bacteriostatic at higher pH as well as chlorogenic acid inhibited the growth of *Listeria monocytogenes* at pH 6.5 (Wen *et al.*, 2003). Also, some epidemiological studies, but not all, pointed out that chlorogenic acid might be involved in reducing the risk of colon cancer. A study found that the absorption of chlorogenic acid was 33 ± 17% and of caffeic acid 95 ± 4% in the human body following the a powder diet that was low in chlorogenic acid and quercetin, they found 33% of ingested chlorogenic acid and 95% of ingested caffeic acid were absorbed in the small intestine of humans, and they recovered 0.3% of chlorogenic acid and 11% of the ingested caffeic acid in urine. This result indicates that part of chlorogenic acid from foods enter into the blood circulation, but most reach the colon in human for some biological activities (Olthof *et al.*, 2001). It has been found that phenolic acids may implicate in inhibition process of activator protein-1 (AP-1) transcriptional activity in cell tumor proliferation. Additionally, the phenolic acids have a cell-mediated capacity to inhibit some of the processes involved in atherosclerosis in a plasma concentration range compatible with dietary intakes (Maggi-Capeyron *et al.*, 2001).

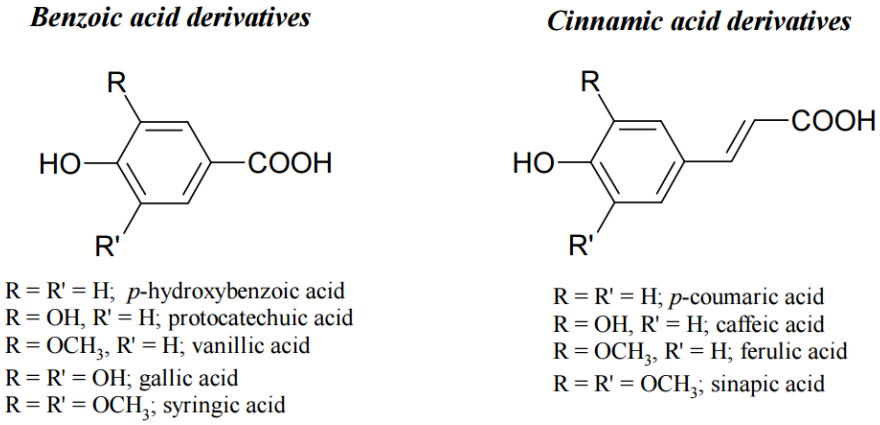
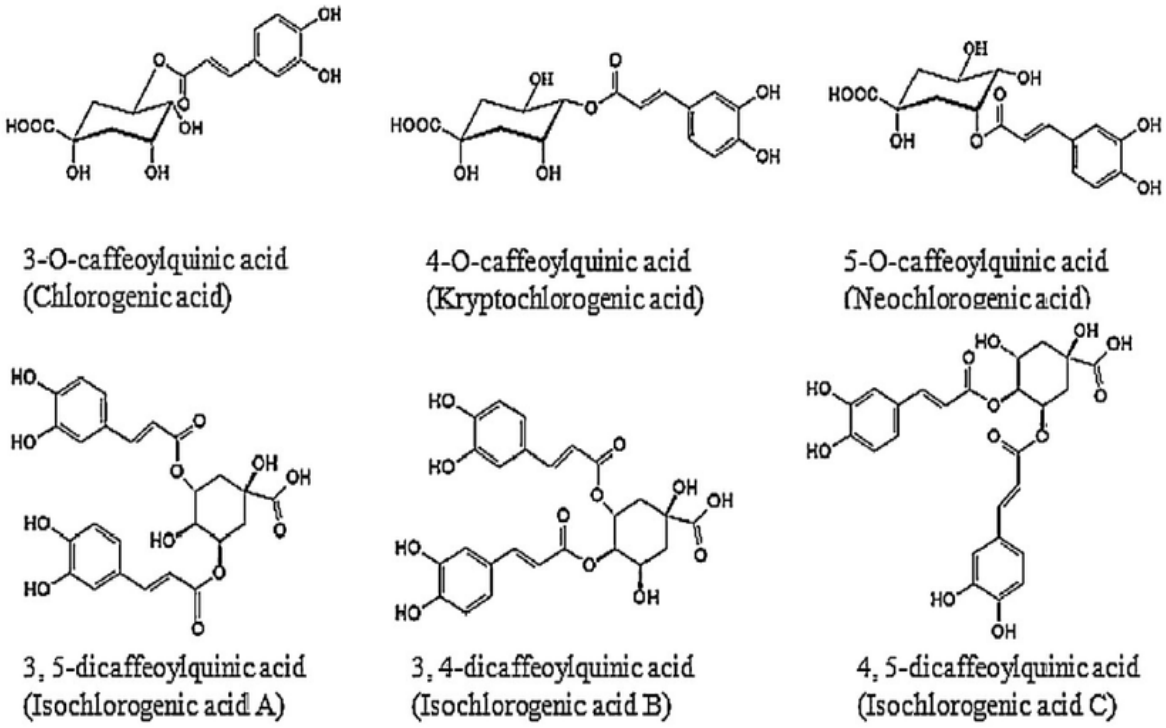


Figure 1.5. Chemical structure of Phenolic acids (Pereira *et al.*, 2009)



(Ma *et al.*, 2011)

Figure 1.6. Chemical structure of Caffeoylquinic acids in plants

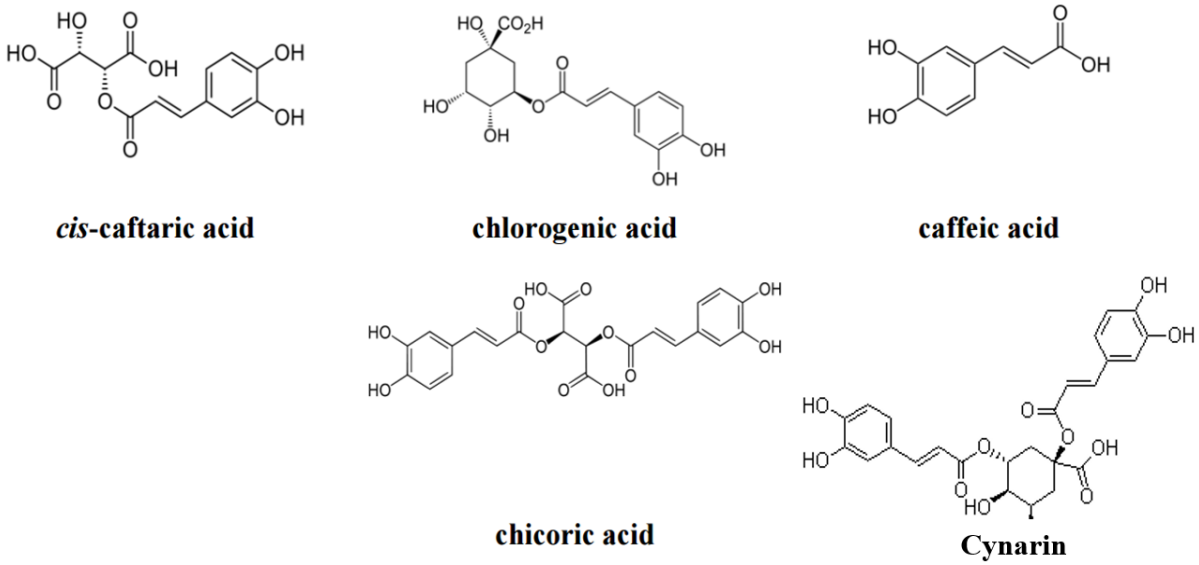


Figure 1.7. Chemical structure of some phenolic acids in plants

(Chen *et al.*, 2011)

1.5.1.2. Flavonoids

The flavonoids are secondary metabolism products, which can be divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central pyran ring. Almost 4000 flavonoids have been currently known in plants, and the list is continuously growing (D'Archivio *et al.*, 2007; Tsao, 2010). It has been reviewed by a group of the authors; only a few short-term intervention trials have been carried out to test the effect of flavanol-rich foods on lowering cardiovascular risks. Thus, while the literature regarding flavanols and vascular health is encouraging, more detailed and well-controlled clinical and investigational studies are required better to define the potential protective vascular effects of these compounds and their beneficial value in cardiovascular medication (Heiss *et al.*, 2010). Flavonoids are a group of polyphenolic that occurred as antioxidants properties to their inhibitory effects on reduced risk of cardiovascular disease is possibly associated with high intakes of dietary antioxidants (Hollman and Katan, 1999). Overall, flavonoids have so far been found to show a wide range of pharmacological properties, including antioxidative, anti-allergic, anti-inflammatory, antidiabetic, hepato and gastro protective, antiviral, and anti-neoplastic activities (Zand *et al.*, 2002; Ren *et al.*, 2003; Yao *et al.*, 2004).

Some dietary intervention trials have revealed the effects of flavonoids to enhance nitric oxide status and to improve endothelial function may be at least partly responsible for benefits on the risk of cardiovascular diseases. For example, the results of animal models to investigate the effects of tea and flavonoids derived from tea on blood pressure are inconsistent. Results of population studies suggest that long-term regular ingestion of tea may lower blood pressure (Riemersma *et al.*, 2001). A meta-analysis of one hundred thirty-three randomized controlled trials on the effects of flavonoids, flavonoid-rich foods on cardiovascular health demonstrated that chocolate increased d flow-mediated dilatation FMD after acute (3.99%; 95% CI: 2.86, 5.12; 6 studies) and chronic (1.45%; 0.62, 2.28; 2 studies) intake and reduced systolic (-5.88mm.Hg; -9.55, -2.21; 5 studies) and diastolic (-3.30mm.Hg; -5.77, -0.83; 4 studies) blood pressure (Hooper *et al.*, 2008).

The environmental factors are known to have effects on the content of polyphenol compounds. For example, the flavonol contents were higher in lettuce samples grown in the open air than the greenhouse. Moreover, the maximum amount of rutin and quercetin 2.4 and 7.5 mmol/g FW, respectively was found in *L. virosa* L. compared with *L. sativa* L., resistant *L. sativa* L. (Mariska), and *L. saligna* L. (Sedlářová *et al.*, 2011). Quercetin content varies from 0.99 ± 0.32

to 6.84 ± 1.53 mg/100g, and rutin content ranges from 17.51 ± 1.96 to 24.50 ± 2.08 mg/100 g in organic and conventional lettuce, respectively (Romani *et al.*, 2002; Durazzo *et al.*, 2014). Light also has an effect on the accumulation of flavonols by stimulating biosynthesis in in the outer and aerial tissues of leaves. It has been reviewed that the glycoside concentration in green lettuce and outer cabbage leaves is ten times higher than in the inner light coloured leaves (Herrmann, 1976; Manach *et al.*, 2004). Bilyk and Sapers (1985) measured both components of quercetin and kaempferol in 13 varieties of lettuce, 2-54 mg of quercetin/kg found in Leaf lettuce, while head lettuce varieties contained 1-28 mg quercetin /kg, more in the outer leaves than in the inner leaves. 0-2 mg of kaempferol/kg determined in lettuce samples.

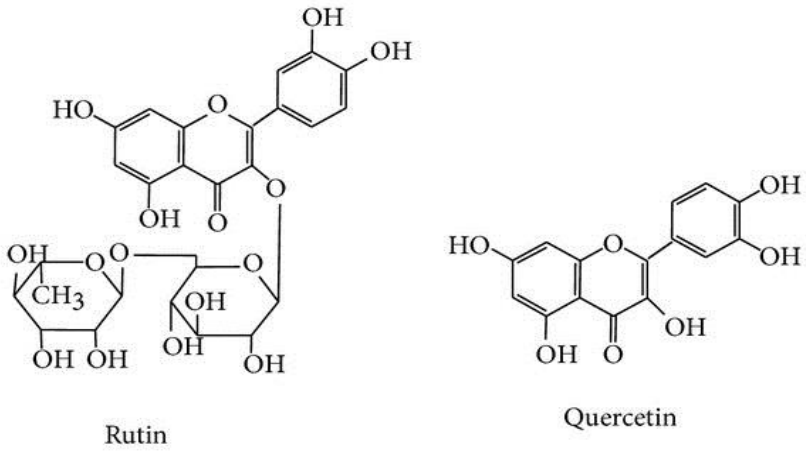
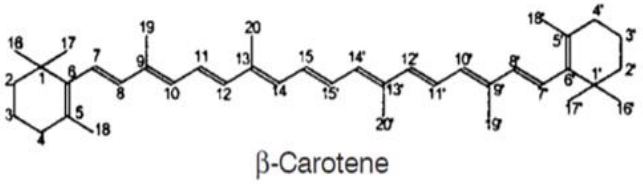


Figure 1.8. Chemical structure of Rutin and Quercetin in lettuce (Zhao *et al.*, 2014)

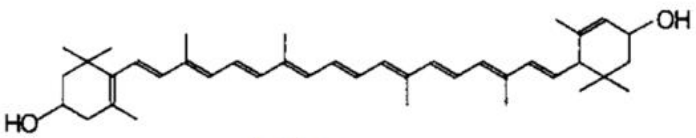
1.5.1.3. Carotenoids

Carotenoids are lipid-soluble pigments found in fruits and vegetables; some researchers believe that have the health benefits as an anti-cancer agent and eye benefits (Kopsell and Kopsell, 2006). Approximately 600 single carotenoids can be found in plant species (Bieri *et al.*, 1985). Nutritionally, lettuce is one of the good food sources of carotenoids (Britton *et al.*, 2009). The main carotenoid found was β-carotene followed by lutein, lactucaxanthin, violaxanthin and neoxanthin in Romaine lettuce (López *et al.*, 2014). In contrast, lutein found as the main carotenoids in other varieties of lettuce (Butterhead, Batavia, Oakleaf and Red oak leaf), together with another xanthophyll. (Nicolle *et al.*, 2004b). Lutein content was a significant difference in Italian head lettuce with 25.29 ± 6.90 and 9.30 ± 2.72 mg/100g in organic than conventional lettuce, respectively. In addition, β-carotene was highly significant in organic and conventional lettuce 53.56 and 27.71 mg/100g, respectively (Durazzo *et al.*, 2014).

Some epidemiological studies indicating that health benefits of dietary carotenoids is correlated with reducing the risk of a variety of chronic diseases such as cancers, cardiovascular disease, and age-related macular degeneration is associated with the high dietary intake of fruits and vegetables rich in carotenoids (Mayne, 1996). On the other hand, a meta-regression analysis shown β-carotene in doses higher than the RDA seem to significantly increase mortality (Bjelakovic *et al.*, 2013). Moreover, a randomized, double-blinded, placebo-controlled trial testing the effect of α-tocopherol and β-carotene supplementation performed to have no late effects on the incidence of lung cancer and other cancers in male smokers (Virtamo *et al.*, 2014).



β-Carotene



Lutein

Figure 1.9. Structure of some carotenoids in lettuce

(Rodriguez-Amaya, 2001)

1.5.1.4. Chlorophylls

Chlorophyll is required for photosynthesis and the uptake of radiant energy in plants, and it is considered a non-limiting compound unless the plant is exposed to severe nutritional deficiencies (Lopez-Cantarero *et al.*, 1994). The leaf chlorophyll content is significantly affected by concentration of nitrogen fertiliser in the plant (Konstantopoulou *et al.*, 2010). Natural chlorophyll derivatives can be defined as substituted tetrapyrroles with a centrally bound magnesium atom (Ferruzzi *et al.*, 2002). In lettuce, the chlorophyll a content is three times higher than chlorophyll b (Caldwell and Britz, 2006).

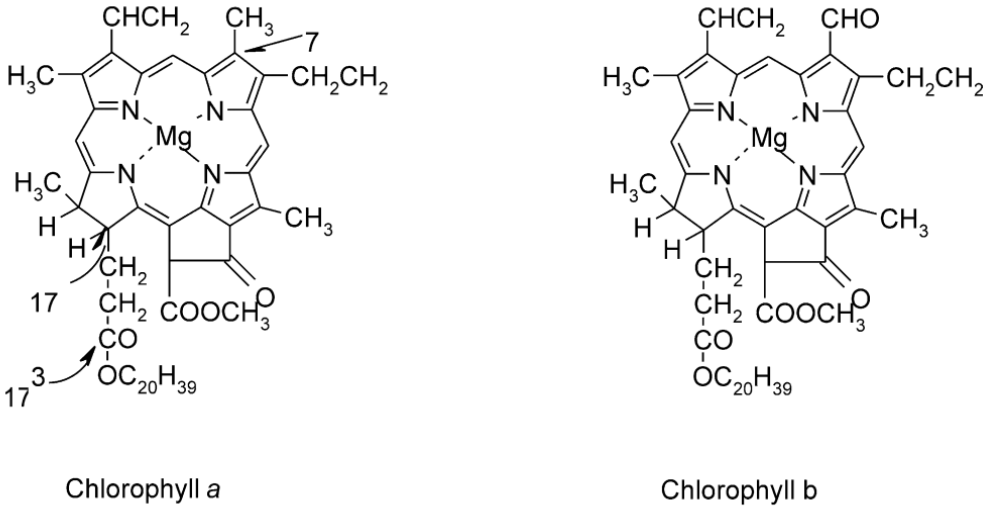


Figure 1.10 Structure of chlorophyll a and b in plants (Schoefs, 2002)

Epidemiological studies have investigated the association of foods rich in fruits and vegetables with prevention of chronic diseases such as cancer; this stimulated interest in plant phytochemicals as physiologically active nutritional components. Dietary chlorophylls as one of the phytochemical compounds that are potentially responsible for such associations (Tsuda *et al.*, 2004).

1.5.1.5. Polyphenols and cardiovascular system

It has been reviewed by Lundberg *et al.* (2015) that the polyphenols are directly related to activities of several enzymes which are connected to the NO system and may improve the bioactivity of NO. These agents inhibit the activity of vascular NADPH oxidases (NOXs), which are main creators of superoxide except some scavenging of reactive oxygen species (ROS) that might still occur. (Schewe *et al.*, 2008; Laurent *et al.*, 2012). Moreover, the ingestion of polyphenols (cocoa flavanols) can stimulate vascular function by increasing flow-mediated dilation (Schroeter *et al.*, 2006). The contribution of endothelial nitric oxide synthase (eNOS) was assessed by increasing level of urinary NO metabolite levels. A number of studies have confirmed the ingestion of diet rich in polyphenols causes lowering of blood pressure (Heiss *et al.*, 2010; Desideri *et al.*, 2012) because polyphenols stimulate endogenous nitric oxide (NO) formation through increased phosphorylation of (eNOS). These compounds also enhance the reduction of NO_2 to bioactive NO due to the production of reductive hydroxyl (OH) groups on the phenol ring (Lundberg *et al.*, 2015).

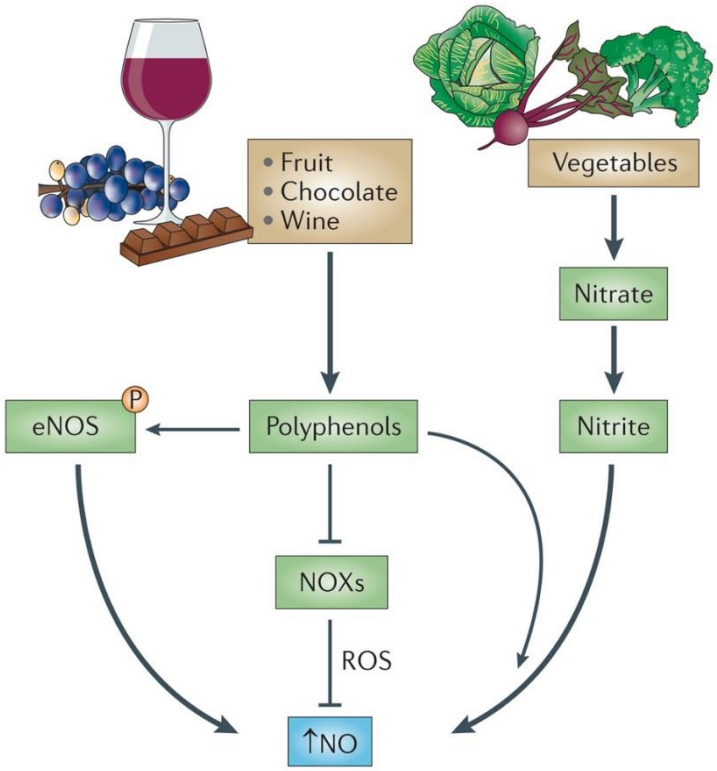


Figure 1.11. Polyphenols stimulate endogenous nitric oxide (NO) formation

(Lundberg *et al.*, 2015)

"Polyphenols stimulate endogenous nitric oxide (NO) formation through increased phosphorylation (indicated by P) of endothelial NO synthase (eNOS). These compounds also enhance the reduction of nitrite to bioactive NO owing to the presence of reductive hydroxyl (OH) groups on the phenol ring. By acting as scavengers of reactive oxygen species (ROS) and by inhibiting ROS-generating NADPH oxidases (NOXs), polyphenols may prevent these radicals from interacting with and destroying NO" (Lundberg *et al.*, 2015).

1.5.1.6. Factors that influence phytochemical levels in plants

Several studies have described the effect of environmental factors on amounts and types of phytochemicals in fruit and vegetables. Phytochemicals have been found in fruit and vegetables and are typically classified based on chemical structure, functional properties and their sources (Tiwari and Cummins, 2013). The levels of phytochemicals are different according to cultivar variation. On the other hand, environment conditions, growing locations, agronomic and harvest factors also significantly influence the level of phytochemicals in fruit and vegetables (Naczk and Shahidi, 2006; Padilla *et al.*, 2007; Singh *et al.*, 2007). Numerous environmental factors affect the contains of phenolic compounds in lettuce, in particular, fertiliser and light intensity (Kleinhenz *et al.*, 2003; Zhao *et al.*, 2007), and the high light intensity also has a positive effect on the accumulation of phytochemicals (Kopsell and Kopsell, 2008; Pérez-Balibrea *et al.*, 2008). In addition, the effect of organic and conventional farming system on phytochemicals has been studied. Veberic *et al.* (2005) have found higher concentrations of phenolic compounds in apple grown in organic production which could be a result of plant response to less fertiliser. The concentration of total phenolic was significantly different in organic compared to conventional in lettuce and collard (Young *et al.*, 2005). Dixon and Paiva (1995) have reviewed several effects on phytochemicals compounds in plants. They have mentioned that low nitrogen has significantly effect on the production of phytochemicals, in particular flavonoids and isoflavonoids. The increase of nitrogen fertiliser led to decrease phenolic compound levels (Bourn and Prescott, 2002). According to Lopez-Cantarero *et al.* (1994), the high nitrogen fertiliser led to increasing foliar concentrations of chlorophyll *a* and *b*, both individually and as total chlorophylls.

Table 1.4 The effect of different nitrogen levels on foliar concentrations of chlorophylls a and b (mg/100 g fresh weight)

Nitrogen levels (g/m^2)	Chlorophyll a	Chlorophyll b	Chlorophyll a+b
15	141 b	51 b	191 b
22.5	144 ab	52 b	195 b
30	148 a	55 a	206 a

Mean column separation according to Duncan's multiple range test (significant at level P=0.05)

Source: (Lopez- Cantarero et al., 1994)

A study of the effect of grwoing tempreture on antioxidant capacity in strawberry shows a significant decrease in phenolic acid, flavonols and anthocyanin content of strawberry cultivars with different night temperature from 12 to 22 °C and day tempreture kept at 25 °C (Wang and Zheng, 2001). In another study, a significant increase in phytochemical content was observed after mild environmental stress conditions, for example, the concentration of chlorogenic acid and chicoric acid was increased 7.5 and 4.5 fold, respectively when exposed to high light intensity (Oh *et al.*, 2009).

A search of systematic review has shown that only a few studies had been conducted on the factors that may contribute to increased phytochemical compound levels in organic systems (Zhao *et al.*, 2006). The head structure and size of lettuce could contribute to the differences found in nitrate concentrations, colour content, secondary metabolite compounds in lettuce, due to the sunlight penetration and therefore the photosynthesis affecting metabolites and the nitrogen assimilation (López *et al.*, 2014). From the last concept, the researchers consider investigating the effect each factor on phytochemical contents in vegetables in addition to their interaction. All together, these results provide additional information to improve the quality and health promoting benefits of plant foods by using controlled environmental factors with the consideration of their potentially negative effects on crop yield and growth.

1.6 The role of vegetables in human health

The primary sources of nitrate in our foods are leafy vegetables such as lettuce and spinach, root vegetables such as beetroot, and water (McKnight *et al.*, 1999). Nitrate in vegetables is the main source of dietary nitrate intake (Santamaria *et al.*, 1998). Recent intervention studies have investigated the role of vegetables in lowering blood pressure, although most of them were short term tests to investigate the effect of different vegetables (e.g., beetroot, lettuce, spinach…, etc.) on blood pressure. The vegetable is a natural source of dietary nitrate intake. Previous investigators have thought that the nitrate in drinking water poses a risk to human health,

including gastrointestinal cancer and methemoglobinaemia in infant (Fewtrell, 2004). In contrast, recent epidemiological studies have discovered that the nitrate might have a health benefit for human health to reduce the risk of harmful bacteria in the human intestine. Santamaria et al., 1998; Bartsch et al., 1988 and Slob et al., 1995) cited in (Du *et al.*, 2007). More recent researches show there is evidence that nitrate can undergo metabolic conversion to nitrite and nitric oxide and provide a useful protective function to prevent infection, protect our stomachs, improve exercise performance and prevent vascular disease (Gilchrist *et al.*, 2010). Therefore, numerous mechanisms have been presented to clarify the relation between diets with a high content of vegetables and fruits and cardiovascular diseases (Du *et al.*, 2007).

Table 1.5 Classification of vegetables according to nitrate content (mg/kg^{-1} FW).

Very low (<200)	Artichoke, Asparagus, Broad bean, Brussel sprouts, Aubergines, Garlic, Onion, Green bean, Melon, Mushroom, Peas, Peppers, Potato, Summer squash, Sweet potato, Tomato, Watermelon
Low (200–500)	Broccoli, Carrot, Cauliflower, Cucumber, Pumpkin, Chicory
Middle (500–1000)	Cabbage, Cima di rapa (broccoli rab), Dill, Radicchio, Savoy cabbage, Turnip
High (1000–2500)	Celeriac (celery root), Chinese cabbage, Endivie, Escarole, Fennel, Kohirabi, Leaf chicory, Leeks, Parsley
Very high (>2500)	Celery, Chervil, Water cress, Lamb's lettuce, Ordinary lettuce, Radishes, Red beetroot, Rocket, Spinach, Swiss chard

Source: (Santamaria, 2006)

1.6.1 Health benefits of lettuce

The health benefits of eating vegetables are associated with macronutrients, micronutrients and bioactive compounds (Coria-Cayupán *et al.*, 2009; Dai and Mumper, 2010). Lettuce is the greatest contributor to total vegetable intake (Sharma *et al.*, 2014) and it's one of the most important leafy vegetable commonly consumed fresh vegetable salads and a good nutritional source of vitamins antioxidant compounds. The antioxidant properties of lettuce have been attributed to their polyphenol contents (Altunkaya *et al.*, 2009). Quercetin, kaempferol, luteolin, apigenin and crysoeriol have been detected and identified in ten genotypes of lettuce belonging to *Lactuca sativa* (Heimler *et al.*, 2007). It has been also reviewed by Kim *et al.* (2016) that the lettuce contain anti-inflammatory agents which was related to phenolic compounds. Llorach *et al.* (2004) found that the antioxidant capacity (DPPH, ABTS, and FRAP assays) has been linearly correlated with phenolic content in lettuce. Serafini *et al.* (2002) pointed out that the ingestion of fresh lettuce leading to increase the antioxidant activities (TRAP) of plasma in healthy volunteers.

An in-vivo study has revealed that the beneficial effects of lettuce consumption on cardiovascular diseases. The results showed causes that the red-pigmented leafy lettuce-supplemented diet significantly reduced the level of total and LDL-cholesterol and triacylglycerol in the plasma of the mice. The effects could be explained by the amount of fibre in lettuce or could be synergistic effects with antioxidant compounds such as α-tocopherol, β-carotene, anthocyanins and phenolic compounds (Lee *et al.*, 2009). In another study, male rats fed on diet complemented with 20% red oak leaf lettuce led to decreased plasma low-density lipoprotein (LDL) to high-density lipoprotein (HDL), cholesterol ratio and liver cholesterol content. The authors identified the lettuce consumption causes lowering the absorption of cholesterol and increasing the bile acids in the faeces (Nicolle *et al.*, 2004a). Moreover, a high polyphenol content in lettuce could associate with antidiabetic and/or anti-inflammatory activities. (Cheng *et al.*, 2014) reported that mice on diets supplemented with 'Rutgers Scarlet' lettuce extracts (100 or 300mg/kg body weight), it has been observed that the glucose metabolism was improved. This is particularly because of phenolic compounds in lettuce have played a role in lowering blood glucose (Ong *et al.*, 2013). In a case control study showed the protective effects against lung cancer was observed for high consumption of lettuce (Odds ratio 0.6, 95% CI 0.3–1.2, p = 0.02), this could be due to its associated with carotenoids in lettuce (Brennan *et al.*, 2000).

Table 1.6 Nitrate concentration in different lettuce varieties.

Lettuce varieties	Nitrate concentration (mg/kg)
Butterhead lettuce	2026
Romaine lettuce (cos lettuce)	1015
Curled lettuce	1601
Iceberg lettuce	875
Lamb's lettuce	2104
Oakleaf lettuce	1534

Source: (Alexander *et al.*, 2008)

1.6.2 Nitric oxide and cardiovascular system

The role of nitric oxide (NO) is found in endothelium by US scientists in 1980 as a stimulator to vasodilate the blood vessels in the muscles (Furchgott and Zawadzki, 1980). This finding was an initiator to encourage researchers in the world to investigate what is the secret behind this unstable free radical molecule (NO). Later in 1988, other scientists found that the precursor of NO synthesis is L-arginine in vascular endothelial cells (Palmer *et al.*, 1988). The formation of NO following this formula:

L-arginine + NO synthase + Oxygen = NO + L-citrulline (Figure 1.12) (Wu *et al.*, 2013).

Nitric oxide is generated by endothelial cells and diffuses into smooth muscle causing vasodilation and then into the vessel lumen where most of the effective NO is rapidly deactivated by reduction or oxidation the oxygen from NO with haemoglobin to form nitrate. In consequence of this, diffusional barriers for NO around the erythrocyte and beside the endothelium in laminar passing blood decrease the inactivation reaction of NO by haemoglobin. Sufficient NO can escape for vasodilation and also react in plasma and tissues to form nitrite anions (NO_2^-) and NO-modified peptides and proteins (RX-NO) (Dejam *et al.*, 2004). Moreover, NO can contribute to vessel homoeostasis by preventing vascular smooth muscle tone and growth, platelet aggregation, and leukocyte adhesion to the endothelium (Fujiwara *et al.*, 2000).

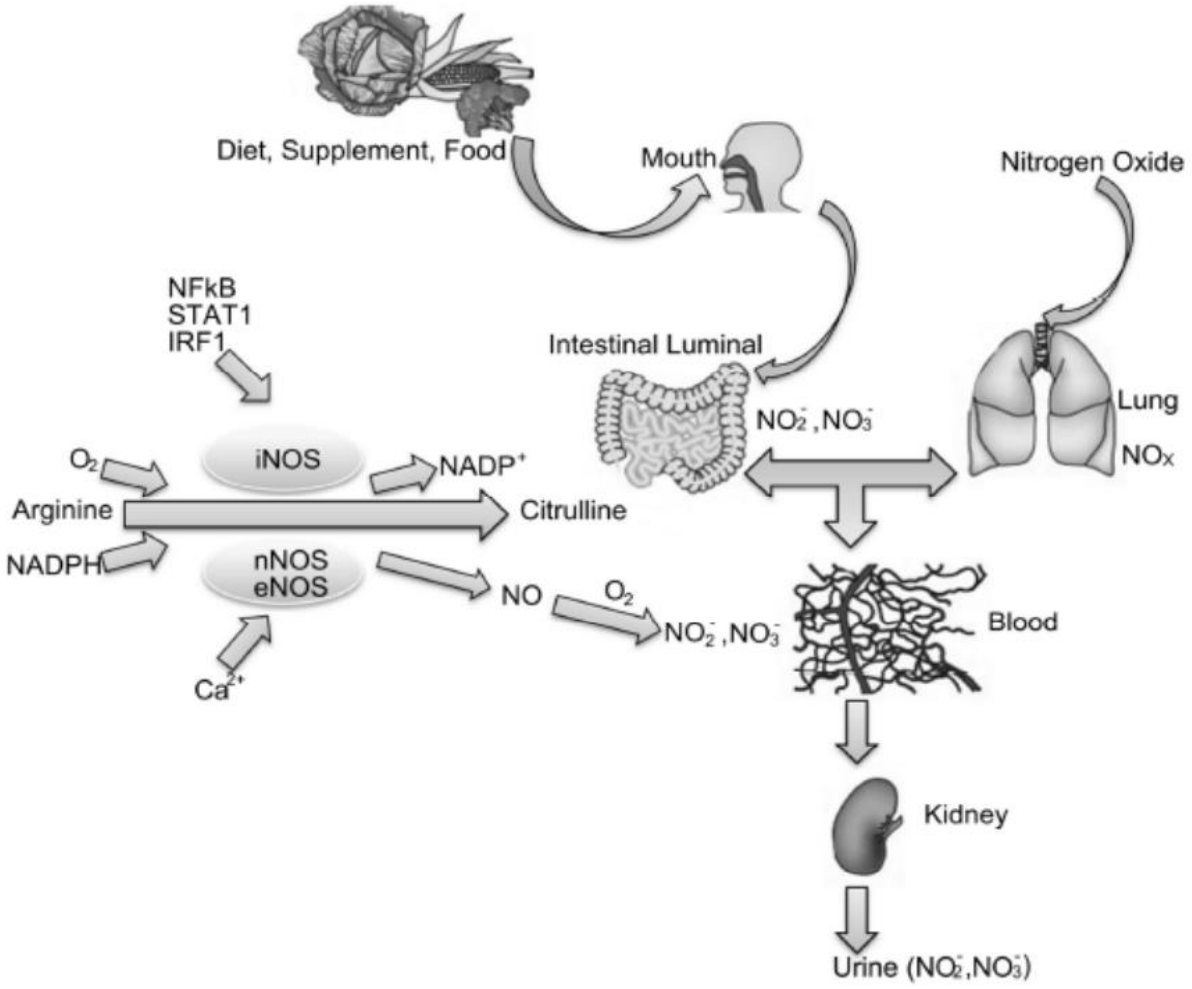


Figure 1.12. The nitric oxide synthesis from and the metabolism of NO3 and NO2 in human body.

1.6.2.1. Cyclic guanosine monophosphate (cGMP) and blood pressure

Relaxation of smooth muscle can result from activation of receptors coupled to adenylyl cyclase, generating cyclic AMP and guanylyl cyclase generating cyclic GMP (Pfeifer *et al.*, 1998). There are more than six isoforms of guanylyl cyclase. The work as receptor cyclases in transmembrane glycoproteins with an extracellular peptide receptor domain, a short transmembrane domain, and intracellular kinase and catalytic domains (Kuno *et al.*, 1986; Garbers, 1991). Soluble and particulate guanylyl cyclases and adenylyl cyclases have substantial homology in their catalytic domains. They may derive from a common ancestral gene. The activated sGC can synthesise cyclic AMP and cyclic GMP, the exchange of one

amino acid in adenylyl cyclase can transform it to an activated form of guanylyl cyclase (Murad, 2006). Soluble guanylate cyclase (sGC) is a precursor enzyme of the NO signalling pathway and rapidly attracting increasing attention as a therapeutic mark in cardiorespiratory disease, with several sGC agonists in clinical development. On binding of NO to a haem prosthetic group on sGC, the enzyme catalyses the synthesis of the second messenger cGMP, which produces relaxation in the vascular system and prevents smooth muscle proliferation, leukocyte recruitment, and platelet aggregation (Stasch *et al.*, 2011). Reduced NO and cGMP signalling has been linked to cardiovascular disease, including systemic arterial pressure and pulmonary hypertension, coronary artery disease, peripheral vascular disease, and atherosclerosis (Murad, 2006; Pacher *et al.*, 2007).

Recent efforts have focussed on identifying pharmacological agents that could target sGC-cGMP signalling directly. Activated sGC compounds can be divided into two groups based on their modes of action: sGC stimulators and sGC activators. Stimulators alert sGC to low levels of bioavailable NO by stabilising the nitrosyl haem complex. Thus, maintaining the enzyme in its active configuration which they can also produce sGC activity in the lack of NO (Stasch and Hobbs, 2009; Stasch *et al.*, 2011). cGMP signalling plays an important role in regulating hypertension and blood pressure by affecting the solute transport of several sodium channels in the proximal tubule, the thick ascending limb, and the collecting duct like the Na^+ /K^+ -ATPase, the type 3 Na^+/H^+ exchanger, the $Na^+/K^+/2Cl^-$ cotransport, and the epithelial sodium channel, respectively (Mergia and Stegbauer, 2016). In a study, it was shown for the first time that the stimulation of sGC is important for blood pressure regulation in the normotensive and hypertensive states in rats (Rothermund *et al.*, 2000). Altogether, several studies suggest that the role of NO-cGMP signalling in regulating blood pressure is well characterized in humans and animal models. NO is a key regulator of smooth muscle to a great part by controlling the sGC-mediated production of cGMP (Thoonen *et al.*, 2013). Thus, the above proposed roles of cGMP may suggest that an increase in plasma cGMP concentrations is associated with a lowering of blood pressure.

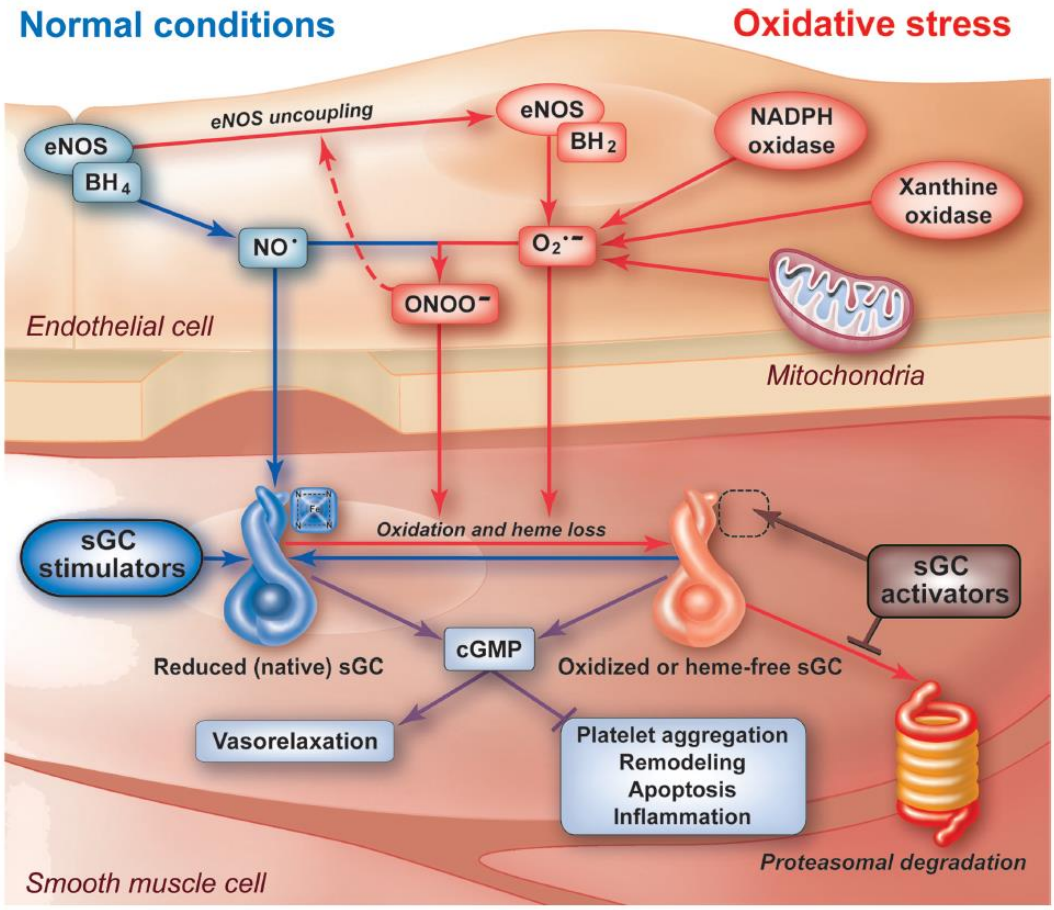


Figure 1.13. Soluble guanylate cyclase (sGC) stimulators and activators target 2 different redox states of sGC.

"The nitric oxide (NO)– sensitive reduced (ferrous) sGC and NO-insensitive oxidized (ferric) sGC, respectively. Stimulators of sGC stabilize the nitrosyl-heme complex of the reduced sGC and exhibit a strong synergism with NO. In various pathophysiological conditions (such as heart failure, pulmonary and systemic hypertension, atherosclerosis, and ischemia/ reperfusion injury), the sGC redox equilibrium can be shifted to the oxidized, ferric state by reactive oxygen species, and/or sGC can become heme deficient. Activators of sGC bind to the unoccupied hemebinding complex or displace the prosthetic heme of sGC and produce only an additive effect with NO. In certain cases, sGC activators also protect sGC from proteasomal degradation. BH4 indicates tetrahydrobiopterin; BH2, dihydrobiopterin; eNOS, endothelial nitric oxide synthase; O_2, superoxide; and $ONOO^-$, peroxynitrite" (Stasch *et al.*, 2011).

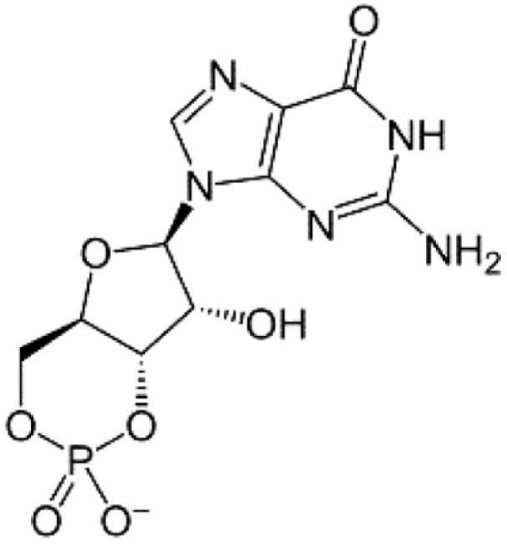


Figure 1.14. Chemical structure of cGMP

(Honorat *et al.*, 2013)

1.7 Aim and objectives

The previous literature review has focussed on the effect of vegetables, green leafy vegetables on cardiovascular disease particularly blood pressure, the nitrate content and other phytochemical compounds have the main role in reducing the risk of vascular diseases. Moreover, the evidence in regard to effect of dietary nitrate intake on blood pressure has been confirmed that the dietary nitrate foods have a positive effect in lowering blood pressure. There is a growing body of literature supporting that lowering blood pressure directly associated with the high consumption of green leafy vegetables rich-nitrate such as lettuce, rocket and spinach. In addition, the novelty of this study is that the placebo and the treatment (low nitrate lettuce vs. high nitrate lettuce, respectively) have similar appearance despite being very different in nitrate content, making it possible to blind the subjects to the treatment and control placebo effects of vegetable consumption. Therefore, this research aimed to produce lettuce with different nitrate contents by using different nitrogen fertilisers in controlled growing conditions.

The aim and objectives of this study can be divided into four main sections:

1- Modify the gas chromatography-mass spectrometry (GC-MS) method for analysis of nitrate and nitrite in vegetables by using the PFB-Br to develop an improved method for analysis of NO_3 and NO_2 in vegetables and biological fluids. The GC-MS method is one of the main requirements to analysis the plant materials and biological samples for the next steps of this project. Therefore, the strategy and priority were to develop a method.

2- Identify the effect of different nitrogen supply in controlled growing conditions on nitrate content and other secondary metabolites compounds such as phenolic acids, flavonoids, chlorophylls and carotenoids in lettuce. Additionally, to test the perception of the lettuce to develop blind interventions as a tool for investigating the effect of nitrate content on blood pressure.

3- It is necessary to acknowledge that the randomised, double-blind placebo-controlled crossover design in healthy young subjects will examine the pharmacokinetics of nitrate derived from a short-term high dietary nitrate intake in term of urinary nitrate excretion, salivary nitrate and nitrite concentrations, and plasma nitrate and nitrite concentration, and to assess the effect of nitrate circulation on blood pressure.

4- Investigate the two new produced lettuce materials with different nitrate contents (high nitrate content vs. low nitrate lettuce) will have different effects on systolic and diastolic blood pressure. On the other hand, the placebo has a substantial role in this type of studies to compare with the treatment and is powerful in human trials.

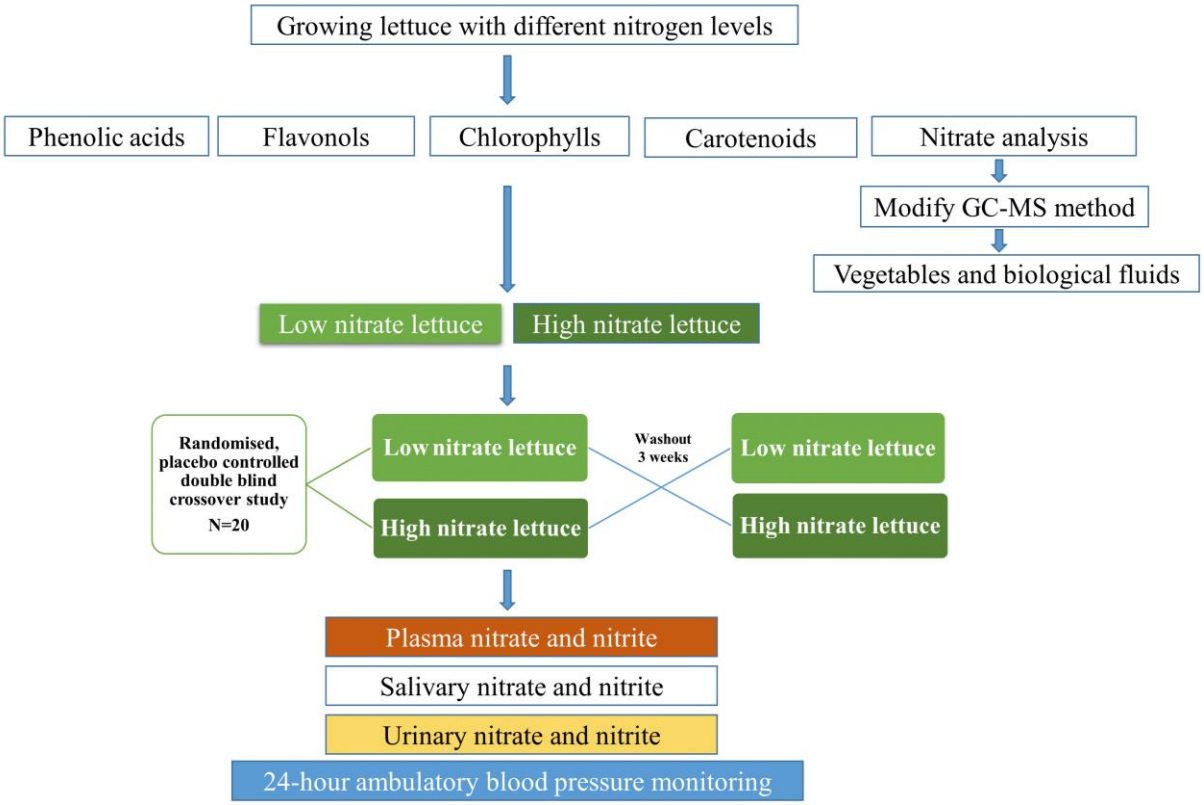


Figure 1.15. Work plan diagram

The diagram shows the work plan: starting with growing different varieties of lettuce by using different nitrogen levels and followed by the analysis of nitrate content and phytochemical compounds two produce to sets of lettuce (high nitrate lettuce and low nitrate lettuce) that can to be used in a human intervention trial.

Chapter 2. Method using gas chromatography mass spectrometry (GC-MS) for analysis of nitrate and nitrite in vegetables

2.1 Introduction

Vegetables are important constituents of a healthy diet. Adequate daily eating of vegetables can help reduce the major risk of diseases, including cardiovascular diseases, cancers, obesity and diabetes (Amine *et al.*, 2003). In addition, vegetables are also naturally rich in nitrate (NO_3) (Larsen *et al.*, 2014). Intervention studies have shown that short-term intake of inorganic NO_3 decreases blood pressure in healthy volunteers (Siervo *et al.*, 2013a). The main risk of NO_3 in vegetables is from its conversion to nitrite (NO_2), creating methaemoglobin by reacting with haemoglobin after ingestion, where the foetal haemoglobin in infants is particularly susceptible (Greer and Shannon, 2005). Therefore, the determination of the NO_3 and NO_2 levels in vegetables is important to evaluate their safety for consumers (Correia *et al.*, 2010). Analytical methods have an important role in NO_3 and NO_2 analysis of vegetable and human biological fluids, and there are several procedures available.

Capillary electrophoresis (CE) method can be applied to measure high amounts of NO_3 in vegetables. However, the samples must be diluted because of interfering oxalate in the same vegetables such as in spinach and tomato (Jimidar *et al.*, 1995). This method is working on separating the molecules by the charge and hydrodynamically injected volume (Landers, 1995). The advantages of this method are very fast, a small volume of sample which is suitable for clinical laboratories. However, the disadvantage CE was described in numerous studies that the protein has to remove the samples by filtration od deproteinization with acetonitrile because the capillary wall can absorb the protein in the samples which generate interactions and effects on the resolution on electrophoresis (Bories *et al.*, 1999)

Chemiluminescence is another method to measure nitric oxide in biological fluids by directe injection without any preparation for the samples and highly sensitive for low concentration of NO (Farell *et al.*, 1992). The chemiluminescence method has some drawbacks such as the interference of detection NO_2 and S-nitrosothiols (Marley *et al.*, 2000) and the instrument unable to detect the high NO_3 content in the samples precisely.

The most commonly used method is the colorimetric method based on the Griess reaction (Miranda *et al.*, 2001) the first established in 1879. This method is very popular and of low cost but it has a number of drawbacks. It is only accurate within a narrow concentration range 1–5

µM (Green *et al.,* 1982; Gillam *et al.,* 1993) and therefore difficult to use for measuring the very variable NO_3 content in vegetables. Also, the colour reaction is susceptible to interference from the dark colour in samples such as beetroot, red chard and cabbage. Additionally, a standard curve is needed in every test session because the changes in any conditions such as temperature affect the results. In addition, there is interference between NO_3 and NO_2. Several other methods were developed for analysis of NO_3 and NO_2 in human samples, but they require more modification to become suitable for analysis of vegetables.

High performance liquid chromatography (HPLC) reversed phase method commonly has been used for NO_3 and NO_2 analysis based on the use of tetrabutylammonium as an ion pair reagent with UV detector (205-220nm). The HPLC methods have been developed due to the interaction between the components with reagents and mobiles phases. Furthermore, the instrument is not very sensitive to detect the low concentration of NO_2 especially in urine, and plasma samples because of the highly UV absorptive which makes hiding the small peak of NO_2 (Zuo *et al.*, 2006); (Croitoru, 2012). However, the ion pair column has been used to remedy this problem instead of ion column to increase the selectivity of elutes.

GC-MS is widely engaged in quantitative analysis of trace compounds because of the accuracy and precisely in analytical measurements. It has a high sensitivity and selectivity for component detections especially the negative-ion chemical ionization (NICI). The sample and internal standard are in a stable condition because of GC-MS constant isotopes which make both in negative forms. This help us to measure NO directly in human biological fluids (Helmke and Duncan, 2007). Some GC-MS methods have been published for measuring NO_3 and NO_2 in foods and water (Massey, 1996). Among them, a GC/MS method using pentafluorobenzyl bromide (PFB-Br) derivatization and ^{15}N-labelled NO_2 and NO_3 as internal standards is very accurate and useful to investigate NO_2 and NO_3 metabolism and reactions in the human body (Tsikas, 2000). However, this method was developed for human fluids. This method measures the NO_3 and NO_2 contents simultaneously in the same sample based on isotope dilution. The isotope dilution means that the measured concentration values depend on the relative size of peaks with different molecular mass, not on the absolute size of the peaks. Thus, the method exceptionally robust about any external factor affecting peak size. Measurements are accurate across a range of more than three orders of magnitude, as long as the peaks are large enough for accurate measurement of their areas, and the volumes of sample and spiking solution are measured accurately.

The main aim of this study is to modify the PFB-Br GC/MS method to develop an improved method for analysis of NO_3 and NO_2 in vegetables and biological fluids. The method is based

on the conversion of nitrate and nitrite into α-nitro-pentafluorotoluene using pentafluorobenzyl (PFB) bromide as the alkylating agent and [^{15}N] labelled nitrate and nitrite as the internal standards.

2.2 Materials and methods

2.2.1 Sample preparation

Several vegetable samples (radish, tomato, red cabbage, red chard, mizuna, lettuce and beetroot) were used in this study; the source of vegetable samples were from the VegBP project; some of them have high NO_3 content, and others have low NO_3 content. All samples were prepared by homogenising approximately 14 to 16g of vegetables mixed with 15-25ml of de-ionized water using an Ultra TurraxT-25 for at least 1 minute and allowing the separation of residue and sample solution by centrifugation.

A high amount of plant materials per sample was used to obtain a high concentration of NO_3 and NO_2 in the extracts and to reduce random variation caused by variable concentrations in different parts of the sample (Anderson and Case, 1999). Direct homogenisation of fresh frozen samples prevented potential problems related to insufficient hydration of dry material or sequestration of NO_3 in intact plant cells.

Blood, saliva and urine samples were collected at baseline following 12 hours fasting in ten healthy young volunteers with an age range of 22-31 years and a body mass index (BMI) range of 20-24.6 kg/m2 who were recruited in the NU-Food research facilities for the intervention study.

2.2.2 Spiking solution preparation

The spiking solutions were prepared for each type of sample to ensure the optimum results. The NO_3 concentration needs to be considerably higher to ensure an accurate reading on the GCMS. To see an actual peak, the $^{15}NO_3$ and $^{15}NO_2$ concentrations should preferably be high enough to provide a signal of 200-1000 area units on the GC-MS (although areas down to approx. 50 can be detected, precision is reduced at these levels). Additionally, their concentrations should be at least 10% of the expected concentrations of the corresponding ^{14}N compounds. Two spiking solutions were prepared, one for high NO_3 vegetables and one for low, for the high NO_3 vegetables the average concentration is expected to be around 10mM NO_3, and the average NO_2 concentration in all vegetables is around 1mM.

A spiking solution has been made at a concentration of 5mM $^{15}NO_3$ and 0.05mM concentration of $^{15}NO_2$. All solutions and dilutions were prepared with pure water (Milli-Q).

2.2.3 Gas chromatography – mass spectrometry

The GCMS status was set as described by (Tsikas, 2000) with some modifications, aliquots (1 μL) were injected in the splitless mode using the following temperature program: column (15m*0.25mm OPTIMA-1701- 0.25μm) was held at 70 °C for 1 min, and then the temperature was increased to 280 °C at a rate of 30 °C/min. Helium (70 kPa for column carrier) and methane (200 Pa) were used as the carrier and the reagent gas, respectively, for negative-ion chemical ionization (NICI) in Shimadzu GCMS-QP2010 machine. Electron energy and electron current were set to 230 eV and 300 μA, respectively. Constant temperatures of 180, 280, and 200 °C were kept at the ion sources, interfaces, and injectors, respectively.

2.2.4 Derivatization Method

The flame ionization detection of GC-MS needs a conversion of hydrophilic NO_2^- and NO_3^- to volatile and an ionized formation to measure the NO in thermally stable derivatives before detection (Yang *et al.*, 2013). 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br) have been used as derivatization agent to generate the formation of PFB-NO_2 and PFB-NO_3. Additionally, several factors disturb the formation of PFB-NO_x in acetone medium such as temperature, pH, time and the amount of PFB-Br (Tsikas, 2000; Shigetoshi *et al.*, 2002; Wu *et al.*, 2013). Therefore, different time and temperatures were tested to maximise the formation of PFB-NO_x following the peak area of NO_3 and NO_2 by the ratio of m/z 47 to 46 for NO_2 and 63 to 62 for NO_3.

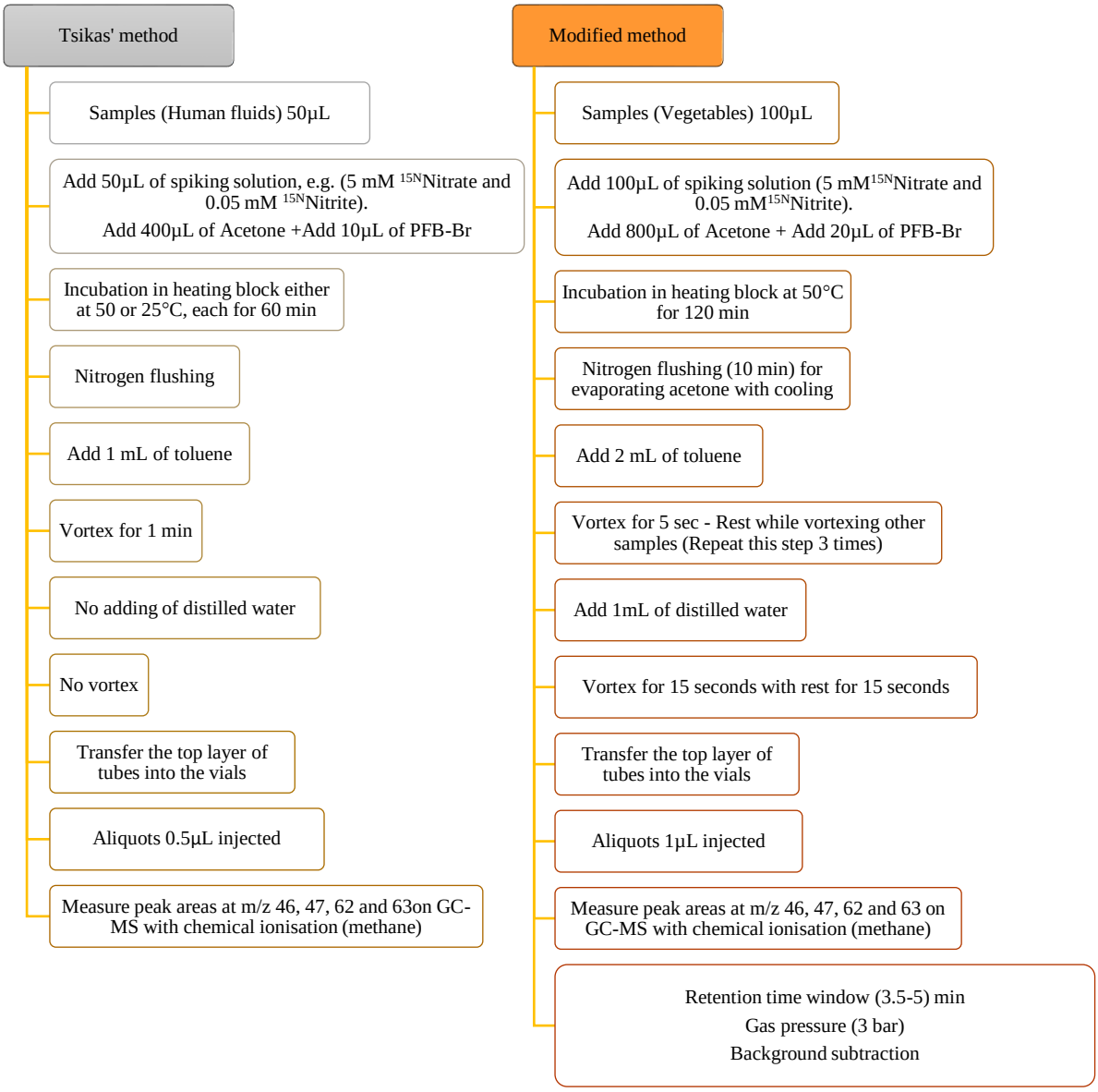


Figure 2.1. Sample preparation procedure for GC-MS Tsikas's and modified methods

For each of the following steps, two or more variants were tested to determine which version would give the best results with vegetables: gas pressure, derivatisation reaction time and temperature, duration of nitrogen flushing, time of vortex after adding toluene during sample preparation, and the amount of water added to separate the phases. In each case by analysing duplicate subsamples of 5-10 vegetable extracts with each method in one session. The volumes of sample and all reagents were doubled compared with the original method since sample volume is not a limiting factor for vegetable samples. Other variables such as column type, ionisation temperature, etc… were as described by (Tsikas, 2000).

The aim of each comparison was to maximise the peak area of NO_3 because this determines the sensitivity of the method (how low concentrations can be measured) and the precision (random variation).

2.2.5 Calculation and statistical analysis

The NO_3 and NO_2 contents were calculated by the following equations:

NO_3 content (mM) = 5*[^{14}N] NO_3 peak area/ [^{15}N] NO_3 peak area

NO_2 content (mM) = 0.05*[^{14}N] NO_2 peak area/ [^{15}N] NO_2 peak area

Statistical analysis was performed using the Minitab 16 software. The data were analysed using analysis of variance (ANOVA General Linear Model) and logarithmic transformation of the data. The results were presented as the back-transformed mean + SEM (standard error of the mean), and significance at ($P < 0.05$).

2.2.6 Reagent and Chemicals

Sodium [^{15}N]NO_3 (15N, 98 %+) and Sodium [^{15}N]NO_2 (15N, 98%+) from Cambridge Isotope Laboratories, Inc. (USA). 2,3,4,5,6-Pentafluorobenzyl bromide 99%from Sigma-Aldrich (Germany). Nitrogen compressed gas (oxygen free) and methane (low ethylene) from BOC gases (UK). Toluene (reagent grade) and acetone (general purpose grade) from Fisher Scientific (UK).

2.3 Results

2.3.1 Gas pressure

The pressure of the methane ionisation gas is important for the size of the peaks in the GC/MS chromatogram. Due to this, we adjusted the gas pressure through testing of different gas pressures from low (1 bar) to high (3.5 bar). The best result was obtained with 3 bars for both NO3 and NO2 (Figure 2.2).

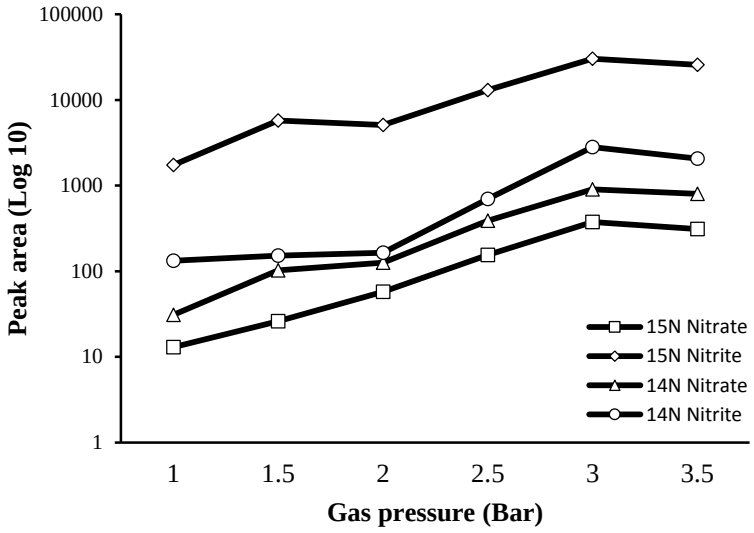


Figure 2.2. The effect of gas pressure on the sizes of nitrate and nitrite peaks of sample and the 15N standard in the GC/MS chromatograms.

Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

2.3.2 Retention time window

The retention time window interval where chemical ionisation takes place during the analysis was modified through comparing different retention time windows before the first peak (which comes at 3.6-3.8 minutes). There was no significant difference between the long retention time window (Long 2-5 minutes) and short retention time window (Short 3.5-5 minutes) ($P=0.181$), as shown in Figure 2.3.

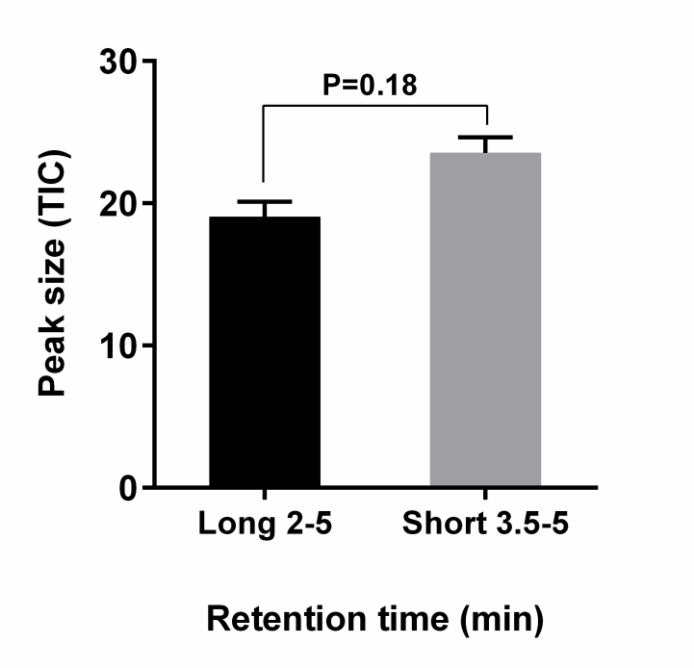


Figure 2.3. The difference between long and short retention time windows of GC-MS.

Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

MIC: Multi ion current.

2.3.3 Incubation

Reaction time has an effect on the production of derivatization reaction and stability of PFB-ONO_2^- and PFB-NO_2^- from nucleophilic substitution of bromide PFB-Br (Tsikas, 2005) during incubation on the heating block (Figure 2.4). Optimal times and temperatures of incubation on the heating block were determined in three different experiments through comparing different time periods and temperatures; [50°C/120min versus 50°C/90min]; [50°C/60min versus 50°C/90min]; and [50°C/90min versus 60°C/60min]. There was a highly significant difference in results for 50°C/120min versus 50°C/90min ($P<0.001$). On the other hand, there were no significant difference between 50°C/90min versus 60°C/60min ($P=0.187$) and 50°C/60min versus 50°C/90min ($P=0.302$), but here the peak areas of NO_2 were very small, so these conditions were not useful for our experiment (Figure 2.7). We found that the (50°C/120min) treatment is the best condition for the formation of PFB-NO_2^- (m/z 46) and in particular PFB-ONO_2^- (m/z 62) (Figure 2.5 and 2.6).

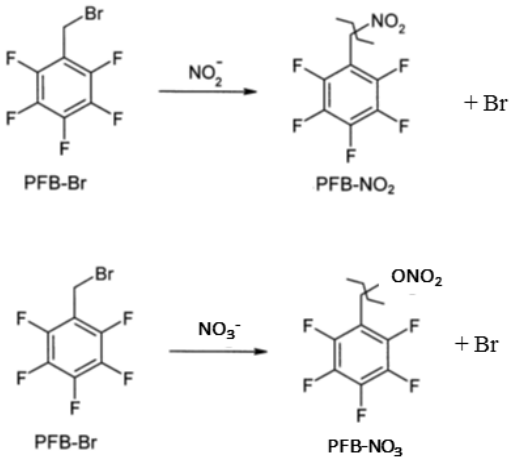


Figure 2.4. The production of derivatization reaction and stability of PFB-NO2- and PFB-ONO2- from PFB-Br during incubation on the heating block

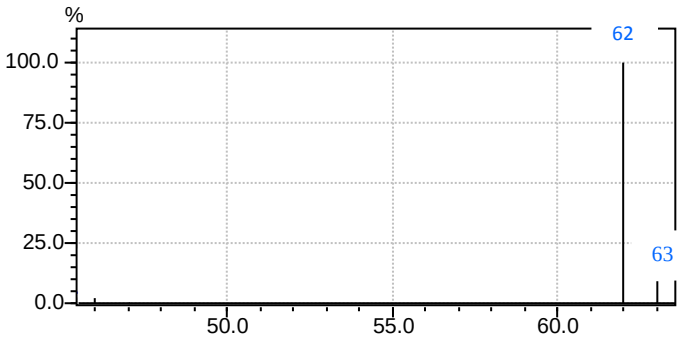


Figure 2.5. Chemical ionization mass spectrum of the reaction product of [15N]NO_3 with PFB-Br in aqueous acetone. Peak area ratio of m/z 62 to 63 for nitrate

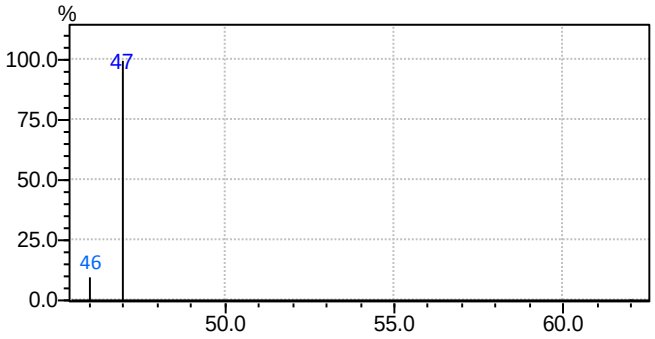


Figure 2.6. Chemical ionization mass spectrum of the reaction product of [15N]NO_2 with PFB-Br in aqueous acetone. Peak area ratio of m/z 46 to 47 for nitrite

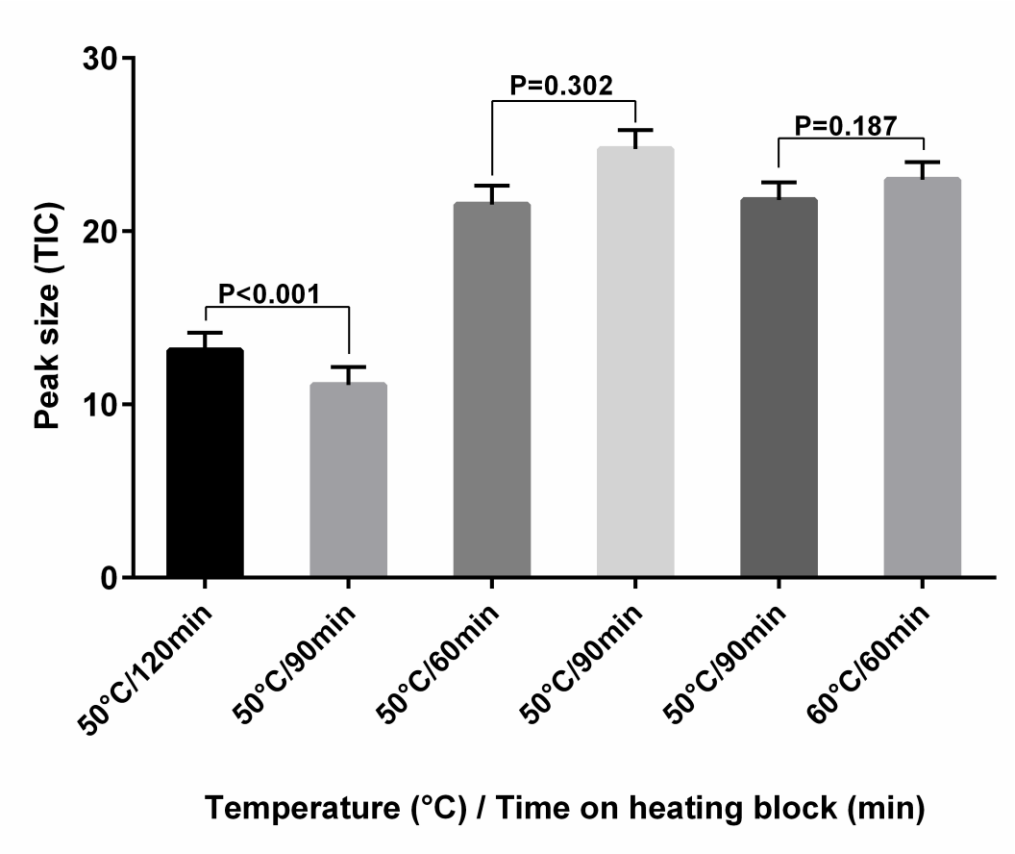


Figure 2.7. The effect of different time and temperature during the incubation step of sample preparation for GC-MS.

Optimal times and temperatures of incubation on the heating block were determined in three different experiments through comparing different time periods and temperatures; 50°C/120min versus 50°C/90min; 50°C/60min versus 50°C/90min; and 50°C/90min versus 60°C/60min. Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

2.3.4 Nitrogen flushing

Dry nitrogen flush was used to remove the water content in the samples and the acetone after the derivatisation reaction was complete. However, too much or too little flushing gave samples where it was hard to separate the phases afterwards. For this reason, we tried different periods of nitrogen flushing. Figure 2.8 shows that there was a highly significant difference between 10 and 20 minutes of nitrogen flushing ($P<0.001$); the best results were obtained with 10 minutes because if the mixture resides for a longer time it will get dry, and it may make other interfering organic compounds. No change was observed when comparing between 10 and 15 minutes of nitrogen flushing ($P=0.930$).

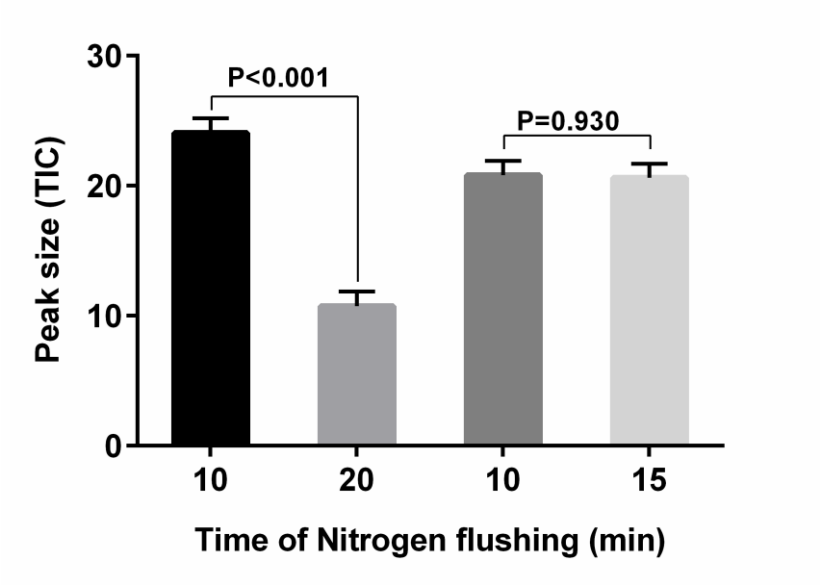


Figure 2.8. The effect of different time of nitrogen flushing on the size of peaks.

Optimal times of nitrogen flushing were determined in two different experiments through comparing different time periods; 10min versus 20 min and 10min versus 15min.Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

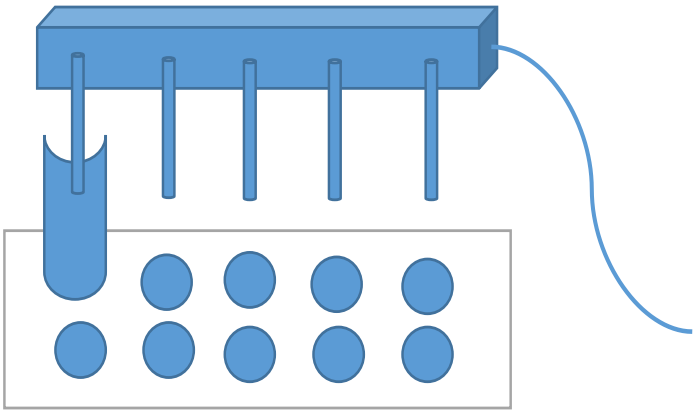


Figure 2.9. Nitrogen flushing equipment

2.3.5 Adding distilled water

Adding distilled water (DW) was introduced to make it easier to separate the organic phase from the extracted samples before transferring the top layer to vials, without interfering with the reaction with acetone. The observations of adding different amounts of DW for the first experiment (0.5 and 1 ml of DW) showed no significant differences (P=0.415). The results were the same for the second experiment (1.5 and 1 ml of DW) (P=0.558). Therefore, 1 ml of DW was chosen since it gave us a sufficient amount of supernatant phase after toluene evaporation (Figure 2.10).

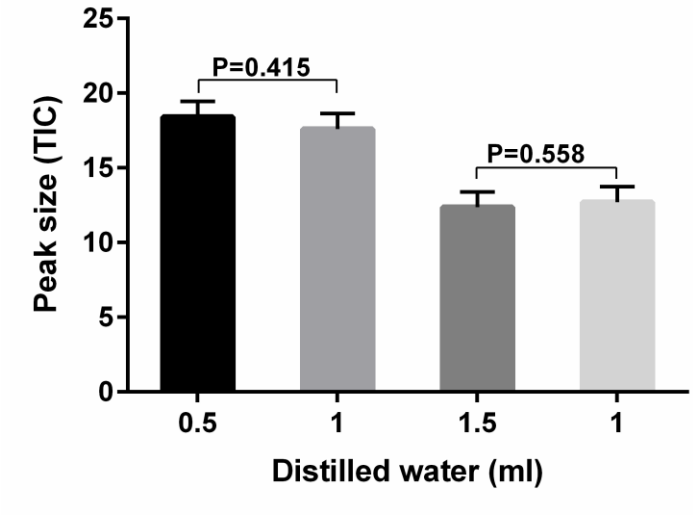


Figure 2.10. The effect of amount of distilled water added after adding toluene for extract samples.

Different volumes of distilled water were tested to separate the organic phase; 0.5ml versus 1mL and 1 mL versus 1.5mL. Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

2.3.6 Vortex after adding toluene

Vortex mixing for samples is important to complete the chemical reaction among chemical material and different phases during sample preparation, e.g. after adding toluene or other materials. There was a highly significant difference between vortexing for 5 seconds and without vortex (P=0.026); the peaks of NO_2 were particularly small without vortexing. On the other hand, there was no significant difference for vortexing for 60 sec and 15 sec (P=0.475) and vortexing for 60 sec and 30 sec (P=0.253). The difference in the effect of interval vortexing for 5 seconds and resting between vortex periods repeating three times was highly significant compared with vortexing for 60 seconds (P=0.019). This method of mixing was better than other methods tested (Figure 2.11).

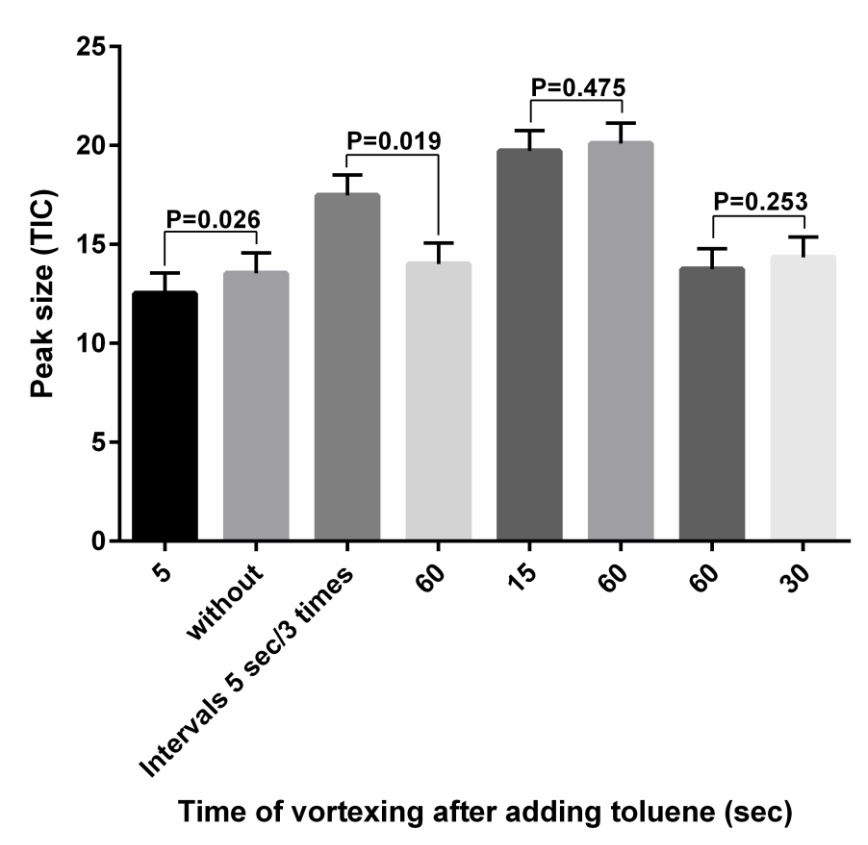


Figure 2.11. The effects of time of vortexing samples after adding toluene on the size of peaks.

Different times of vortexing were tested to complete the chemical reaction; 5sec versus without vortexing, intervals 5sec / 3 times versus 60sec, 15sec versus 60 sec and 30 sec versus 60 sec. Values are presented as the back-transformed mean + SEM, and significance at ($P < 0.05$).

2.3.7 Examples of measurement of contents of NO_3 and NO_2

In Table (2.1) and (2.2) shows a few examples of measurements by the GC/MS modified method for different types of vegetables. The NO_3 and NO_2 content of samples were calculated from the ratios of the peak areas with known concentrations of $[^{15}N]NO_3$ and $[^{15}N]NO_2$. The mass spectrometric detection was obtained in selected-ion monitoring (SIM). Since the commercially available $[^{15}N]NO_3$ contains a small amount of $[^{14}N]NO_3$, a blank sample can be used to determine this background content and subtract it before calculating the concentration in the sample. This is important particularly in vegetables that have a low content of NO_3 such as tomato. Table (2.3) shows some examples of urinary, salivary and plasma nitrate and nitrite results in samples were collected from ten healthy subjects to check the variability of the modified method for nitrate and nitrite concentration in biological fluids.

Table 2.1. Examples of recorded data including peak areas (SIM) of NO_3 and NO_2 in the GC/MS chromatograms in different types of vegetables*

Vegetables	Weight (g)	Total volume (ml)	15Nitrate (5mM)	15Nitrite (0.05mM)	14Nitrate	14Nitrite
Tomato	15.06 ± 0.03	50	614 ± 75	1484 ± 276	45 ± 5	237 ± 142
Lettuce	20.03 ± 0.23	40	6913 ± 940	4218 ± 347	46429 ± 11353	1528 ± 228
Radish	15.25 ± 0.00	50	8313 ± 1804	10124 ± 3259	16744 ± 1311	3985 ± 2110
Rocket	15.49 ± 0.07	50	16792 ± 4133	16562 ± 3264	114901 ± 25457	6772 ± 1431
Red Cabbage	15.11 ± 0.03	50	5125 ± 63	4871 ± 109	20890 ± 715	1285 ± 55
Mizuna	15.11 ± 0.00	50	5549 ± 882	4975 ± 110	20854 ± 1297	1176 ± 398
Red Chard	15.64 ± 0.00	40	1336 ± 850	2168 ± 954	5764 ± 1948	454 ± 281

Table 2.2. Examples of calculations of the amount of nitrate content in samples and vegetables**

Vegetables	In Samples				In vegetables			
	Nitrate content (mM)	Nitrite content (mM)	Nitrate content (mg/kg)	Nitrite content (mg/kg)	Nitrate content (mM)	Nitrite content (mM)	Nitrate content (mg/kg)	Nitrite content (mg/kg)
Tomato	0.37 ± 0.08	0.008 ± 0.004	22.96 ± 4.95	0.35 ± 0.17	1.23 ± 0.27	0.03 ± 0.01	76.23 ± 16.44	1.18 ± 0.56
Lettuce	33.26 ± 3.53	0.018 ± 0.002	2062.43 ± 218.65	0.83 ± 0.09	66.37 ± 6.30	0.04 ± 0.00	4115.24 ± 390.82	1.66 ± 0.16
Radish	10.26 ± 1.32	0.022 ± 0.016	636.09 ± 81.58	1.02 ± 0.73	33.64 ± 4.31	0.07 ± 0.05	2085.71 ± 267.50	3.35 ± 2.38
Rocket	34.69 ± 4.96	0.020 ± 0.001	2151.00 ± 307.80	0.94 ± 0.03	112.02 ± 16.45	0.07 ± 0.00	6945.29 ± 1020.20	3.03 ± 0.09
Red Cabbage	20.38 ± 0.45	0.013 ± 0.001	1263.46 ± 27.92	0.61 ± 0.04	67.42 ± 1.57	0.04 ± 0.00	4180.21 ± 97.08	2.01 ± 0.13
Mizuna	18.98 ± 1.81	0.012 ± 0.004	1176.51 ± 112.45	0.54 ± 0.18	62.79 ± 6.00	0.04 ± 0.01	3893.24 ± 372.11	1.80 ± 0.58
Red Chard	20.92 ± 2.63	0.010 ± 0.002	1297.08 ± 162.89	0.46 ± 0.09	53.49 ± 6.72	0.026 ± 0.005	3316.47 ± 416.48	1.18 ± 0.23

*Values are the mean ± SD of three replicates.

**Calculated using data from Table 1

Table 2.3 Examples of nitrates and nitrites were measured in some biological fluids[a]

Subjects[b]	Urine		Saliva		Plasma	
	Urinary nitrate (µM)	**Urinary nitrite (µM)**	**Salivary nitrate (µM)**	**Salivary nitrite (µM)**	**Plasma nitrate (µM)**	**Plasma nitrite (µM)**
S01	224.51 ± 0.77	2.87 ± 0.95	691.05 ± 74.63	60.82 ± 5.26	85.30 ± 1.75	9.11 ± 1.36
S02	335.78 ± 17.59	3.16 ± 0.45	449.01 ± 56.35	46.28 ± 5.26	75.75 ± 2.17	8.05 ± 0.90
S03	282.51 ± 10.92	4.09 ± 0.18	389.45 ± 4.00	72.16 ± 3.63	75.43 ± 8.76	6.52 ± 0.06
S04	345.36 ± 24.11	2.62 ± 0.39	360.58 ± 21.95	70.34 ± 3.46	85.42 ± 8.73	6.70 ± 0.08
S05	611.69 ± 38.75	3.25 ± 1.99	834.60 ± 145.53	74.02 ± 17.53	90.26 ± 14.56	8.28 ± 2.48
S06	209.74 ± 2.28	2.74 ± 0.21	1241.88 ± 208.02	119.66 ± 24.72	93.74 ± 5.40	8.85 ± 1.68
S07	248.92 ± 12.21	3.73 ± 0.13	528.73 ± 4.17	119.94 ± 2.06	90.96 ± 4.49	6.44 ± 0.14
S08	752.44 ± 20.63	2.67 ± 0.62	395.54 ± 13.99	41.51 ± 9.47	92.29 ± 4.44	5.90 ± 0.13
S09	539.17 ± 10.49	3.65 ± 1.41	481.26 ± 49.26	51.57 ± 4.17	75.72 ± 2.66	5.87 ± 0.05
S10	240.54 ± 5.78	4.03 ± 1.18	953.89 ± 195.31	150.72 ± 26.65	69.21 ± 2.09	5.83 ± 0.64
Mean ± SD[c]	379.07 ± 14.35	3.28 ± 0.75	632.60 ± 77.32	80.70 ± 10.22	83.41 ± 5.51	7.15 ± 0.75
SEMs	10.15	0.53	54.67	7.29	3.89	0.53

[a] Values are the mean ± SD of two replicates.

[b] Urine, saliva and plasma samples were collected at baseline from 10 participants after 12 hours fasting, on the experimental day of the human intervention study (more details in chapter 4).

[c] Mean ± SD of ten subjects.

2.4 Discussion

Accurate analytical methods are very important to detect the contents of chemical substances in vegetables and fruit. There is particular interest in NO_3 and NO_2, due to their possible benefits and harm on human health. The GC-MS method is one of the main requirements to analysis the plant materials and biological samples for the next steps of this project. Therefore, the strategy and priority were for modified this method. The aim of this research is to modify the method of measurement of NO_3 and NO_2 by GC-MS to be used in vegetables and human fluids, due to analytical difficulties of NO_3 and NO_2 analysis in biological samples (Yang *et al.*, 2013). Many procedures are available to measure NO_3 and NO_2. Some examples are capillary electrophoresis, spectrophotometry using the Griess reaction, various HPLC methods and different types of isotope dilution GC-MS: with electron ionisation by nitration of mesitylene, and with chemical ionisation by derivatisation with pentafluorobenzyl bromide. However, some of these methods are mostly used in research on human health, to measure NO_3 and NO_2 in human biological fluids such as plasma or urine. Methods developed for analysis of vegetable samples often give variable results, for example, due to interference from coloured compounds in the plant material. Therefore, the GC-MS modified method using pentafluorobenzyl bromide, and chemical ionisation was improved for the analysis of NO_3 and NO_2 in vegetable and human biological samples (urine, saliva and plasma). The purpose of using the bromide atom is mainly the single leaving group of PFB-Br (Tsikas, 2016) and replace it with NO. Experiments were carried out to maximise peak area, in particular for NO_3. Factors that were tested were gas pressure, derivatisation reaction time and temperature, duration of nitrogen flushing, time of vortex after adding toluene during sample preparation, and the amount of water added to separate the phases. Different samples were used between the runs for each factors. The modified method was used to analyse contents of NO_3 and NO_2 in samples from a range of different vegetables and biological samples to check the variability and this method has been used in several human trails. The spiking solution with different concentration of 5mM $^{15}NO_3$ and 0.05mM $^{15}NO_2$ has been used to the vegetable and biological samples to check the concentrations. All the measurements were accomplished with accuracy and precision during the method modification to obtain the optimal peak of nitrate and nitrite.

The results of vegetable samples were matching to NO_3 and NO_2 content in some vegetables (Siciliano *et al.*, 1975; Schuddeboom, 1993) which indicate the accuracy and precision of the new method. The results were corresponding to a control group in previous literature (Webb *et al.*, 2008; Liu *et al.*, 2013). The nitrite concentrations in saliva was significantly lower than in

plasma and urine because of the conversion of nitrate to salivary nitrite because approximately 20% of ingested nitrate is converted to nitrite by commensal Gram-negative bacteria in the mouth, resulting in increases nitrite in saliva (Hobbs *et al.*, 2012). However, the result of plasma nitrite was too low, but it was greatly improved than some previous GC-MS methods which they found less than 1µM (Wennmalm *et al.*, 1990; Wennmalm *et al.*, 1992) and 1.9 µM (Tsikas *et al.*, 1994) nitrite in plasma. In the whole investigation, the small standard error values of urinary nitrate 379.07 ± 14.35 µM; salivary nitrate 632.60 ± 77.32µM and plasma nitrate 83.41 ± 5.51 µM indicate the accuracy and precision of the modified method.

Quantification of nitrite and nitrate was conducted in the NICI mode by selected ion monitoring (SIM) of the ions m/z 46, for nitrite and nitrate-derived nitrite, and m/z 47, for [^{15}N]nitrite and [^{15}N]nitrate-derived [^{15}N]nitrite (Tsikas *et al.*, 1999). Quantification of nitrate as PFB-ONO2 was performed in the NICI mode by SIM of the ions m/z 62 for nitrate and m/z 63 for [^{15}N]nitrate (Tsikas *et al.*, 1998). PFB-Br with nitrate and nitrite produced two separate forms of PFB-NO_2 and PFB-NO_3 in aqueous acetone, this has been shown to depend on reaction mixture pH, reaction temperature and time, and amount of PFB-Br (Wu *et al.*, 1983; Wu *et al.*, 1984). The effect of reaction time and temperature on the formation of PFB-NO_2 and of PFBNO_3 at a great excess of PFB-Br (20µL) by derivatizing a mixture containing unlabelled and ^{15}N-labeled compounds and analysing by GC/MS. Consequently, the maximum formation of PFB-NO_2 and PFBNO_3 were achieved in acetone as water-miscible after 120 min when heated at 50 °C. We assumed that there was a lack of interference based on the column (Optima-1701) which provide a better separation for the PFB-NO2 and PFB-ONO2 (Wu *et al.*, 1984; Tsikas, 2000), another reason of lack of interference can be explained by the observations that the extent of PFB-ONO2 formation and its ionization to m/z 46 is very low relative to that of PFBNO2 (Figure 2.12).

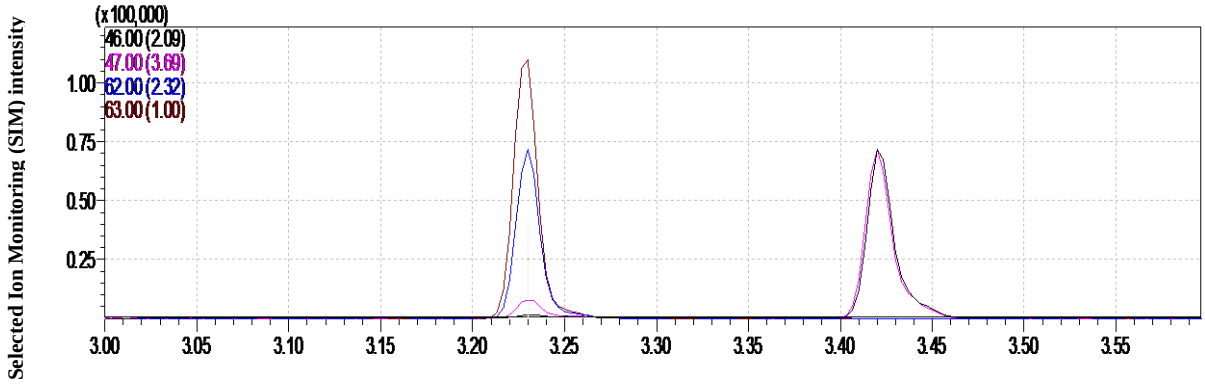


Figure 2.12. A partial GC-MS chromatograms of nitrate and nitrite as PFB derivatives of nitrate (SIM of m/z 62 and m/z 63) and nitrite (SIM of m/z 46 and m/z 47) in the NICI mode.

In conclusion, the modified GC-MS method is a very accurate and relatively rapid method for the determination of high and low contents of NO_3 and NO_2 in various vegetables and biological samples. Due to its large dynamic range, there is no need for extensive dilution of samples, irrespective of initial NO_3 content. There was no interference with other compounds, which can occur when using the Griess or Chemiluminescence methods. However, the method has a limited capacity (96 samples per 24 hours), the equipment is very expensive, and sample preparation takes a long time, and needs some additional equipment for heating and flushing.

Chapter 3. Contents of nitrate, phenolic acids and carotenoids show contrasting responses to nitrogen fertilizer in lettuce grown in a controlled environment.

3.1 Introduction

Plant growing conditions involve a broad range of factors that affect chemical compositions of vegetables. One of these is the use of fertilizers, which may influence the nutrient composition of plants, including secondary metabolites. For example, the different availability of nitrogen resulting from organic and conventional crop management practices has been suggested as a key determinant of the differences in the content of plant secondary metabolites between organic and conventional vegetables and fruit (Brandt *et al.*, 2011).

Nitrate exists naturally in plants, mainly in vegetables. Nitrate is taken up from fertilizers as a key plant nutrient, and excess may be stored in plant tissues (Santamaria, 2006); apart from its important role in plants, nitrate and its metabolite nitrite also have effects on human health. The high amount of nitrate can be found in leaves, while hardly any nitrate is found in seeds and mature fruits. According to European Commission Regulation (EC) No.563/2002 the maximum level of nitrate in leafy vegetables were recorded in spinach (3000mg NO_3/kg FW) and lettuce (4500mg NO_3/kg FW lettuce grown under cove). The main purpose of this EC regulation was to minimize the risk of nitrate in these vegetables because some previous research focused mainly on potential harmful effects of nitrate on human health (Santamaria, 2006). In contrast, recent studies have focused on benefits, e.g. that a diet rich in leafy vegetables has a positive effect on the incidence of high blood pressure, myocardial infarction and stroke (Habermeyer *et al.*, 2015).

Previous studies of lettuce show that the increase nitrogen fertilization tends to reduce of total phenolic content while it increases the contents of nitrate, carotenoids and chlorophylls (Coria-Cayupán *et al.*, 2009; Stagnari *et al.*, 2015). However the growing conditions, such as light intensities, temperature and nitrogen fertilizer, all have an effect on nitrate accumulation in lettuce; there can also be interactions between the genotype and environment with the nitrate content in lettuce (Stagnari *et al.*, 2015).

Due to this, to investigate the effect of lettuce consumption on human health, it is necessary to understand how specific clearly defined growth conditions can be used to obtain reproducible variances in the composition of this vegetable. The results will be useful to design a human

intervention trial to assess the bioavailability of nitrate from lettuce and corresponding effects of lettuce with high or low nitrate content on blood pressure.

The aim of this work was therefore to identify the effect of nitrogen fertilization regimes on concentrations of nitrate and other potentially health relevant compounds such as phenolic acids, flavonoids, chlorophylls and carotenoids under otherwise identical completely controlled growing conditions.

3.2 Material and Methods

3.2.1 Growth condition and plant materials

This work was conducted in Agriculture Building, Newcastle University. Oakleaf lettuce (*Lactuca sativa* L. var. *crispa*) cv Sansula and Butterhead lettuce (*Lactuca sativa* L. var. *capitata*) cv Egery were sown in 11x10x10 cm square pots in a growth cabinet (Plant Growth/Environmental Chambers - MLR-351, SANYO) with five shelves. Pots contained 1 litre peat moss with structure (fine 0-10mm) (White soda peat, KLASMAN code 822 with 33.3g $CaCO_3$ l^{-1}, three seeds were sown and reduced to 1 plant per pot after germination. Seeds were irrigated with deionized water at 20°C for three days. For plant growth, the temperature for Oakleaf lettuce was 22°C with 150µmol m^{-2} for 12hrs/day in light period and 18°C in dark period, for Butterhead lettuce it was 27°C with 150µmol m^{-2} for 12hrs/day, 23°C/150µmol m^{-2} for 2hrs/day in light period and 23°C 10hrs/day in the dark period. During plant growth, the location of shelves and plants were changed every ten days to even out any differences in irradiance among shelves and positions on a shelf. The mean humidity was 64%, recorded by a humidity meter (Ebro LEBI-20TH, Klipspringer, UK). Plants were irrigated by providing 50ml nutrient solution per pot every day for the first four weeks and then increased to 100ml per day for the last three weeks until one day before harvesting.

3.2.2 Nutrient solution

The nutrient solution was designed based on Hoagland's nutrient solution, to allow the nitrogen concentrations to differ at five levels 26, 39, 51, 103 and 154 ppm, corresponding to 7.21, 10.81, 14.41, 28.82, and 43.24 kg/ha while keeping all other nutrients constant. The source of macronutrients nitrogen, phosphorus and pottasium (NPK) were changed such as ammonium nitrate (NH_4NO_3), potassium hydroxide (KOH) and Doff super phosphate - Ready-to-Use

(phosphorous pentoxide P_2O_5) instead of ammonium phosphate $(NH_4)_2PO_4$), potassium nitrate (KNO_3) and calcium nitrate ($Ca(NO_3)_2$) respectively, (calcium was provided from the $CaCO_3$), see Table 3.1.

Each treatment included 6 plants, which were grown for seven weeks. After weighing the harvested above-ground material, half of the fresh plants were used for nitrate analysis and the second hlaf was frozen at -20°C and freeze-dried in a VirTis GPFD 24DX48 (SP Scientific) freeze-drier. The freeze-dried material was weighed and broken into to a coarse powder, which was stored at -20°C up to 6 months used for analysis.

Table 3.1 Modified Hoagland's nutrition solutions, providing five levels of nitrogen for lettuce.

Nitrogen levels	Chemical	Stock solution g L^{-1}		Stock solution mL L^{-1} of final solution	Element	Final solution
Nitrogen source						**ppm**
N1	NH_4NO_3	14.706		**5mL L^{-1}**	N	**26**
N2	NH_4NO_3	14.706		**7.5mL L^{-1}**		**39**
N3	NH_4NO_3	14.706		**10mL L^{-1}**		**51**
N4	NH_4NO_3	14.706		**20mL L^{-1}**		**103**
N5	NH_4NO_3	14.706		**30mL L^{-1}**		**154**
Macronutrients						**ppm**
For all nitrogen levels	$Ca(H_2PO_4)_2$ + 2 $CaSO_4$	15		10mL L^{-1}	P	11.4
	KOH	20		10mL L^{-1}	K	139
Micronutrients						**ppm**
For all nitrogen levels	KCl	3.728	In 1 litre mixed stock solution (storable)	1mL L^{-1}	Cl	1.77
	H_3BO_3	1.516			B	0.27
	$MnSO_4 .H_2O$	0.338			Mn	0.11
	$ZnSO_4. 7H_2O$	0.575			Zn	0.131
	$CuSO_4. 5H_2O$	0.125			Cu	0.032
	H_2MoO_4 (85% MoO_3)	0.081			Mo	0.05
	Fe-EDTA[3]	6.922	Prepared fresh	1mL L^{-1}	Fe	1.12

3.2.3 Analytical methods

3.2.3.1 Nitrate analyses

14 to 16g of lettuce was mixed with 15-25ml of de-ionized water in a 50 ml polypropylene centrifuge tube using an Ultra Turrax T-25 homogeniser for at least 1 minute, followed by separation of residue and sample solution by centrifugation (2000rpm, 10min). The nitrate concentration was analysed by gas chromatography-mass spectrometry (GC–MS) as described by (Qadir *et al.*, 2013).

3.2.3.2 Extraction of samples for phenolic acid analysis

The method was described by Alarcón-Flores *et al.* (2013) with some modifications. Lettuce samples were kept in plastic bags and stored at -20°C for 48 hours and then transferred into the freeze-dryer. 150mg of lyophilized sample were weighed into a 15mL polypropylene centrifuge tube, and ten mL of methanol: water (80:20, v/v) were added. The mixture was agitated for 30min with a shaker, and all samples were centrifuged for ten mins at 4000rpm. After that, 1.5ml of supernatant were transferred into an HPLC vial.

3.2.3.3 Phenolic acid compositions and flavonoids analysis

The HPLC column was a Kinetex EVO Reverse phase (C18, 100A, 250×4.6mm, 5µ), and the column oven was set at 25°C. The injection volume was 20µL, and the HPLC; system was equipped with a Shimadzu 2 LC-10AD pump, SiL-10A system Autosampler, SPD-M 10A photodiode array UV-VIS detector set to collect all data from 200 to 600 nm, and a CTO-10AD column oven (Shimadzu Corporation. Kyoto, Japan). The mobile phase was 0.1% v/v trifluoroacetic acid in ultra-pure water (solvent A) and 0.1% v/v trifluoroacetic acid in HPLC-grade acetonitrile (solvent B) with a flow rate of 1ml/minute. The solvent gradient (A:B) was 0 min (100: 0), 5 mins (100:0), 15 mins (83:17), 17 mins (83:17), 22 mins (75:25), 30 mins (65:35), 35mins (50:50), 40 mins (0:100), 50mins (0:100), 55mins (100:0) and 65 mins (100:0).

3.2.3.4 Quantification of phenolic acids

The identification and quantification of phenolic acids were based on the retention times and absorption spectra of authentic standards. The diode-array detector was set at 320nm for quantification of phenolic acids and 354nm for flavonols. The concentrations of unknown phenolic acids were expressed as chlorogenic acid equivalents for the total of all the phenolic acids, and the unknown flavonols derivatives were quantified as rutin equivalents and combined whole HPLC peaks of unknown flavonols. Table 3.2 shows the different standards were tested for the identification.

Table 3.2. List of standards used to compare and identify phenolic compounds.

All standards were prepared as stock by concentration 0.1 mg /mL in 70% Methanol; the standards were stored in dark in refrigerant

Standards	Other name	CAS No	Sourced
3-Caffeoylquinic acid	Chlorogenic acid	327-97-9	Chemstrong scientific Co Ltd
5 -Caffeoylquinic acid	Neochlorogenic acid	906-33-2	Biopurify Phytochemicals Ltd
1,3-Dicaffeoylquinic acid	Cynarin, Cynarine	19870-46-3	Biopurify Phytochemicals Ltd
3,5-Dicaffeoylquinic acid	Isochlorogenic acid A	2450-53-5	Biopurify Phytochemicals Ltd
3,4-Dicaffeoylquinic acid	Isochlorogenic acid B	14534-61-3	Biopurify Phytochemicals Ltd
4,5-Dicaffeoylquinic acid	Isochlorogenic acid C	32451-88-0	Biopurify Phytochemicals Ltd
Dicaffeoyltartaric acid	Cichoric acid	70831-56-0	Biopurify Phytochemicals Ltd
Caffeoyltartaric acid	Caftraric acid	67879-58-7	Biopurify Phytochemicals Ltd
Rutin	Quercetin-3-rutinoside	250249-75-3	Sigma-Aldrich Company Ltd

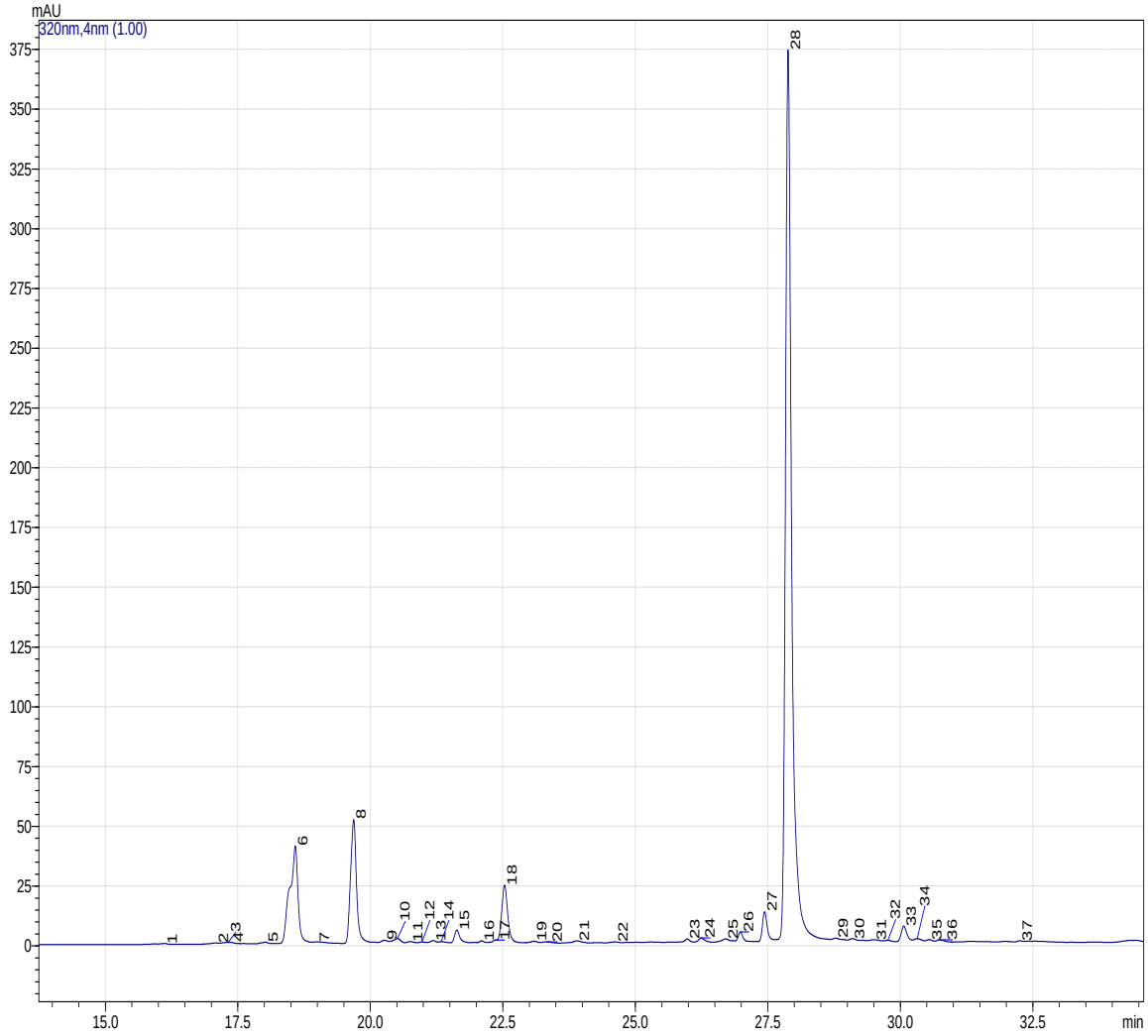


Figure 3.1. HPLC Chromatogram of phenolic compounds in lettuce

Peaks identified by comparison with authentic standards: 6 Caftaric acid (Caffeoyltartaric acid), 7 Neochlorogenic acid (5 -Caffeoylquinic acid), 8 Chlorogenic acid (3-Caffeoylquinic acid), 9 Cynarine (1,3-Dicaffeoylquinic acid), 17 Isochlorogenic acid B (3,4-dicaffeoylquinic acid), 18 Isochlorogenic acid A (3,5-dicaffeoylquinic acid), 26 Rutin (Quercetin-3-rutinoside), 28 Cichoric acid (Dicaffeoyltartaric acid), 29 Isochlorogenic acid C (4,5-Dicaffeoylquinic acid), 20, 21, 27, 30, 31 and 32 Unknown Phenolic acids, 23 and 24 unknown Quercetin derivatives.

3.2.3.5 Extraction of samples for determination of chlorophylls and carotenoids

The method as described by Rashed (2009) with some modifications. 70mg of the freeze-dried sample was put in a 10ml screw glass tubes with 3ml of ethyl acetate. Samples were covered with aluminium foil to keep them dark. Samples were vortexed for a few minutes and left in the fridge overnight. The samples were centrifuged for 10 minutes at 4000 rpm and the supernatant transferred to an HPLC vial.

3.2.3.6 Chlorophylls and Carotenoids analyses

The analysis was carried out on the same HPLC and column as the phenolic acids. The HPLC oven was set at 40°C with the flow at 1ml/min, and 20 µL samples were injected. The solvents of mobile phase were pure water (A), methanol (B), and ethyl acetate (C). The solvent gradient (A:B:C) was: 0 min (50:50:0), 6 min (50:50:0), 11 min (30:70:0), 30 min (15:85:0), 35 min (0:100:0), 38 min (0:90:10), 56 min (0:60:40), 62 min (0:0:100), 64 min (0:0:100), 70 min (0:100:0), followed by re-equilibration as 75 mins (50:50:0), 95 (50:50:0).

3.2.3.7 Identification and quantification of carotenoids and chlorophylls

Retention times and peak areas of carotenoids and chlorophylls were measured by comparing to authentic standards. The diode-array detector was set at 450nm for quantification of carotenoids and chlorophylls.

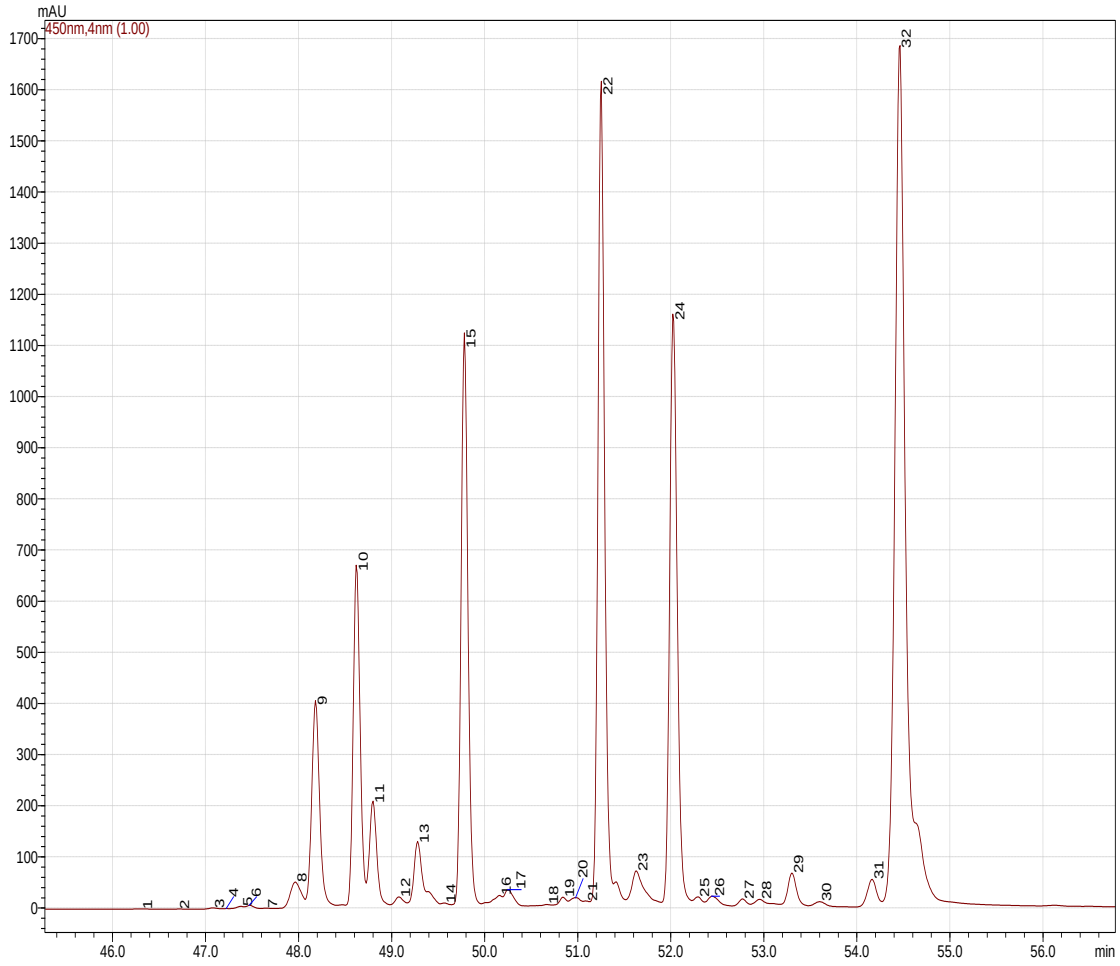


Figure 3.2. HPLC Chromatogram of carotenoids and chlorophylls in lettuce

Chromatographic profile acquired by HPLC: 10 lutein, 22 Chlorophyll b, 23 Cis-Chlorophyll, 24 Chlorophyll a, 32 β-carotene; 8, 9, 11, 12, 13, 14, 15, 16 and 17 Unknown Carotenoids.

3.3 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the main effect of nitrogen levels on nitrate contents and each phytochemical compound. Statistical significance was determined at the $P < 0.05$ level and significantly different values distinguished using Tukey's test. All statistical analyses were completed using Minitab, version 17 Statistical software.

3.4 Results

3.4.1 Study 1: Oakleaf lettuce

3.4.1.1. Effects of nitrogen levels on nitrate concentration

There was a positive relationship between the responses of Oakleaf lettuce (OL) for increasing the nitrogen fertiliser concentration with the nitrate content. The only statistical significant difference of nitrate content was observed between 51 compared to 103 and 154ppm N (P=0.002) (Figure 3.3).

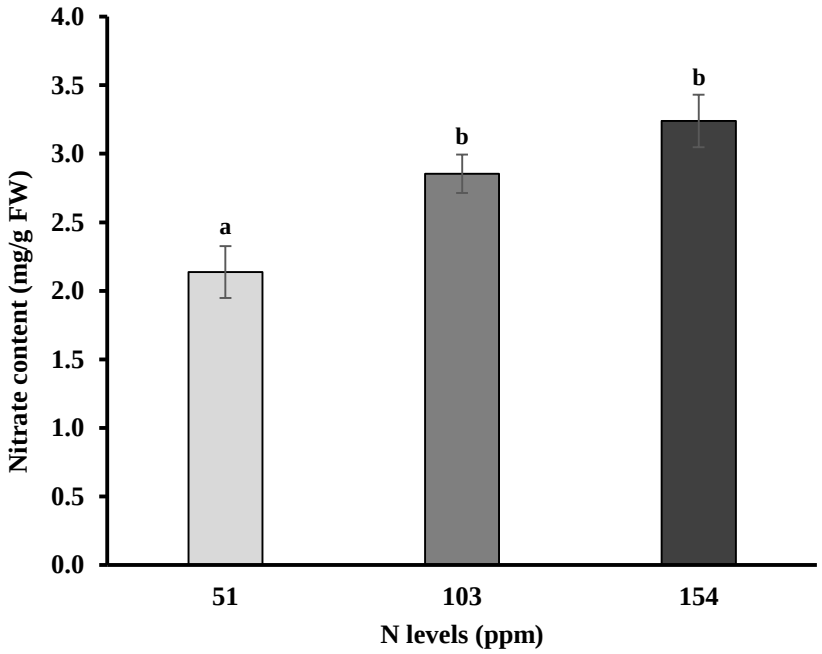


Figure 3.3. Effect of nitrogen levels on nitrate content in Oakleaf lettuce (OL) grown at 22/18°C.

Values are the mean ± SEM of six replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$.

3.4.1.2. Effects of nitrogen supply on plant biomass and dry matter

The size of lettuce was reduced with high nitrogen supply, probably due to increasing the stress in the plants. The results of figure 3.4 showed a noticeable decrease in fresh weight of OL of the treatments received from low to high nitrogen concentration. The fresh weight of OL plants significantly declined from 39.52 to 28.42g when nitrogen contents in the nutrient solutions increased from 51 to 154ppm ($P<0.009$). There was also no significant difference in plant biomass between 103 and 154ppm N in OL.

The nitrogen concentration in the nutrient solution was slightly decreased the percentage of dry matter in Oak leaf lettuce. The dry matter content was significantly decreased 7.25 to 5.86% in OL ($P=0.042$) when nitrogen supply was increased from 51 to 154ppm (Figure 3.5).

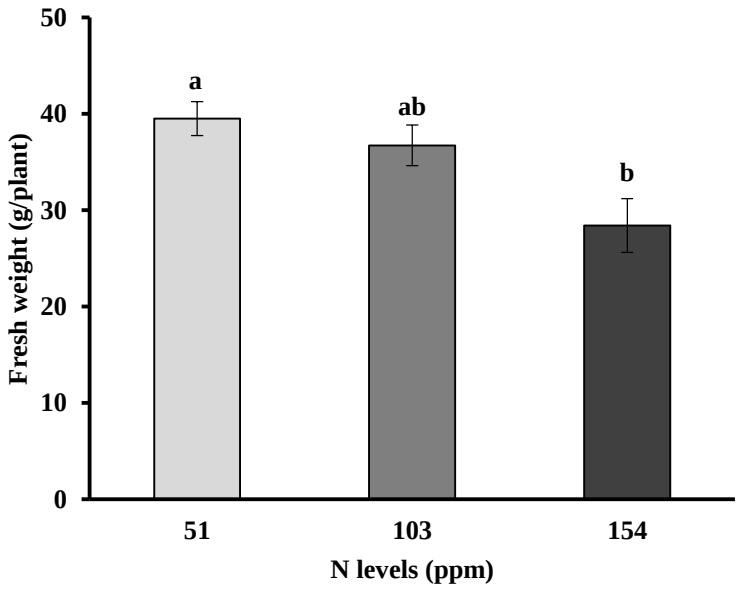


Figure 3.4. Effect of nitrogen levels on plant biomass in Oakleaf lettuce (OL).

Values are the mean ± SEM of six replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$.

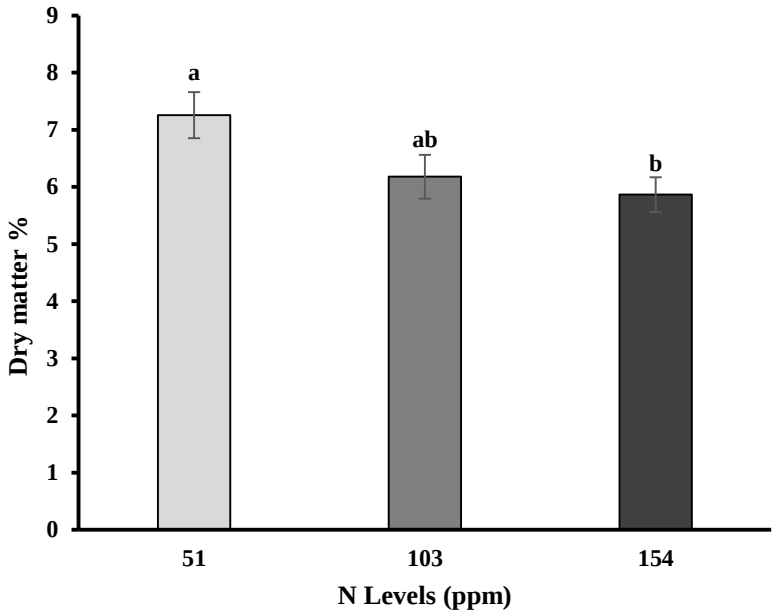


Figure 3.5. Effect of nitrogen levels on the percentage of dry matter in Oakleaf lettuce (OL).

Values are the mean ± SEM of six replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$.

3.4.1.3. Effects of nitrogen supply on chlorophyll contents

Figure 3.6 shows the chlorophyll profile contents of a, b and total chlorophylls in OL. For all treatments, the concentration of chlorophyll a was always higher than b. The significant difference were only observed between 51 and 154ppm N of chlorophyll a (P=0.006), chlorophyll b (P=0.007), and for the total chlorophylls (P=0.002). The values of chlorophyll b and a reached 2.47 and 9.58 mg/g DW after increasing the nitrogen concentration to 154ppm, respectively.

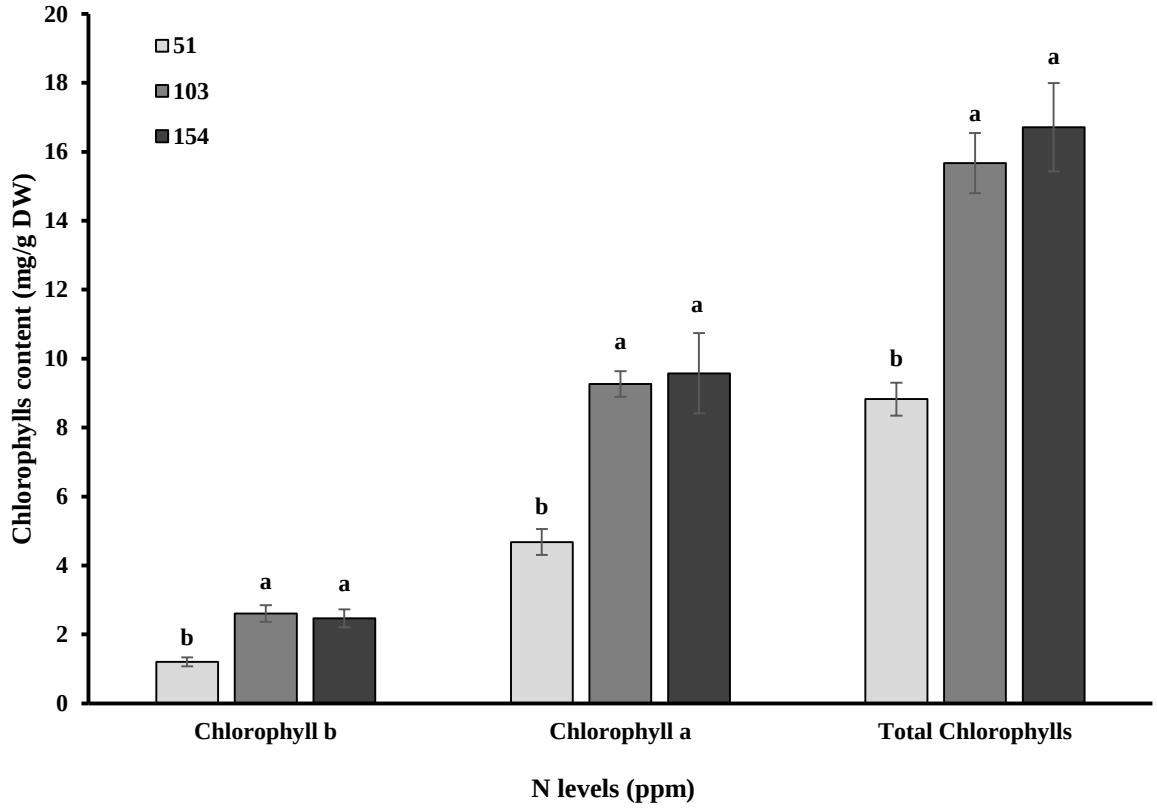


Figure 3.6. Effect of nitrogen levels on chlorophyll content in Oakleaf lettuce (OL).

Values are the mean ± SEM of three replicates, each replicate consisting of three plants. Bars with different letters indicate significant differences at $P < 0.05$ among the different N-levels.

3.4.1.4. Effect of nitrogen supply on carotenoid contents in lettuce

Data presented in Figure 3.7 shows that the high nitrogen treatments were likely to increase carotenoid contents. However, the effect of nitrogen was not consistent for all treatments on lutein, β-carotene and total carotenoids from 51 to 154ppm in OL. In addition, a significant difference was observed for total carotenoids between 51 and 103ppm N in OL (P=0.01).

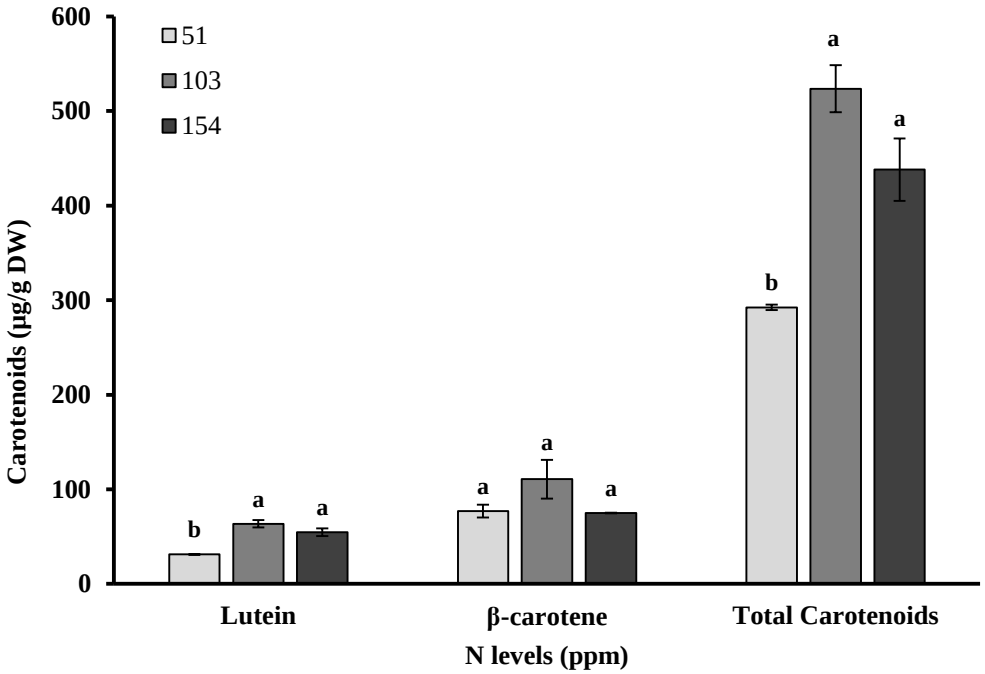


Figure 3.7. Effect of nitrogen levels on carotenoids contents in Oakleaf lettuce (OL).

Values are the mean ± SEM of three replicates, each replicate consisting of three plants. Bars with different letters indicate significant differences at $P < 0.05$ among the different N levels.

3.4.1.5. The effect of nitrogen on phenolic phytochemical compounds

The experimental data of the effect on different nitrogen supplies on phenolic acids and some flavonols of OL are listed in Table 3.3. The individual standard deviation was obtained from 3 replicate consisting of three different plants. There were no significant effects of the different nitrogen applications on Cichoric acid, Caftaric acid, Neochlorogenic acid, Chlorogenic acid, Isochlorogenic acid B acid and Rutin in OL. Only significant effect was obtained on Isochlorogenic acid A for 51ppm N compared to 103 and 154ppm N (P=0.010). No significant difference was observed of the effect of N supplies on total flavonols in OL (P=0.458).

Table 3.3. Phenolic compounds in Oakleaf lettuce (OL)[a]

Compounds	**Nitrogen levels (ppm)**			
(µg/g DW)	**51**	**103**	**154**	***P-value***
Cichoric acid	1520 ± 150	625 ± 160	540 ± 325	0.144
Caftaric acid	629 ± 357	332 ± 81	318 ± 179	0.063
Neochlorogenic acid	88 ± 78	103 ± 28	136 ± 73	0.428
Chlorogenic acid	31.9 ± 14.6	20.1 ± 8.6	20.7 ± 9.1	0.155
Isochlorogenic acid B acid	14.2 ± 11.3	11.4 ± 5.0	16.8 ± 10.5	0.385
Isochlorogenic acid A	11.6 ± 5.2 **a**	6.3 ± 1.3 **b**	5.6 ± 1.4 **b**	0.010
Rutin	101 ± 75	104 ± 28	169 ± 87	0.200
Unknown flavonols	111.8 ± 126.2	57.6 ± 17.3	83.6 ± 51.6	0.512
Total flavonols	212.9 ± 151.0	161.7 ± 42.8	250.5 ± 136.8	0.458

[a] Values are the mean ± SD of three replicates, each with 3 plants. In a row, different letters indicate significant differences at ($P < 0.05$) among nitrogen levels.

3.4.2 Study 2: Butterhead lettuce

3.4.2.1. Effects of nitrogen levels on nitrate concentration

The response of Butterhead lettuce (OL) for increasing the nitrogen concentration significantly increased the nitrate content in BL from 0.06 to 10.8mg/g FW, from 26 to 154ppm N in the nutrient solution, respectively (Figure 3.8).

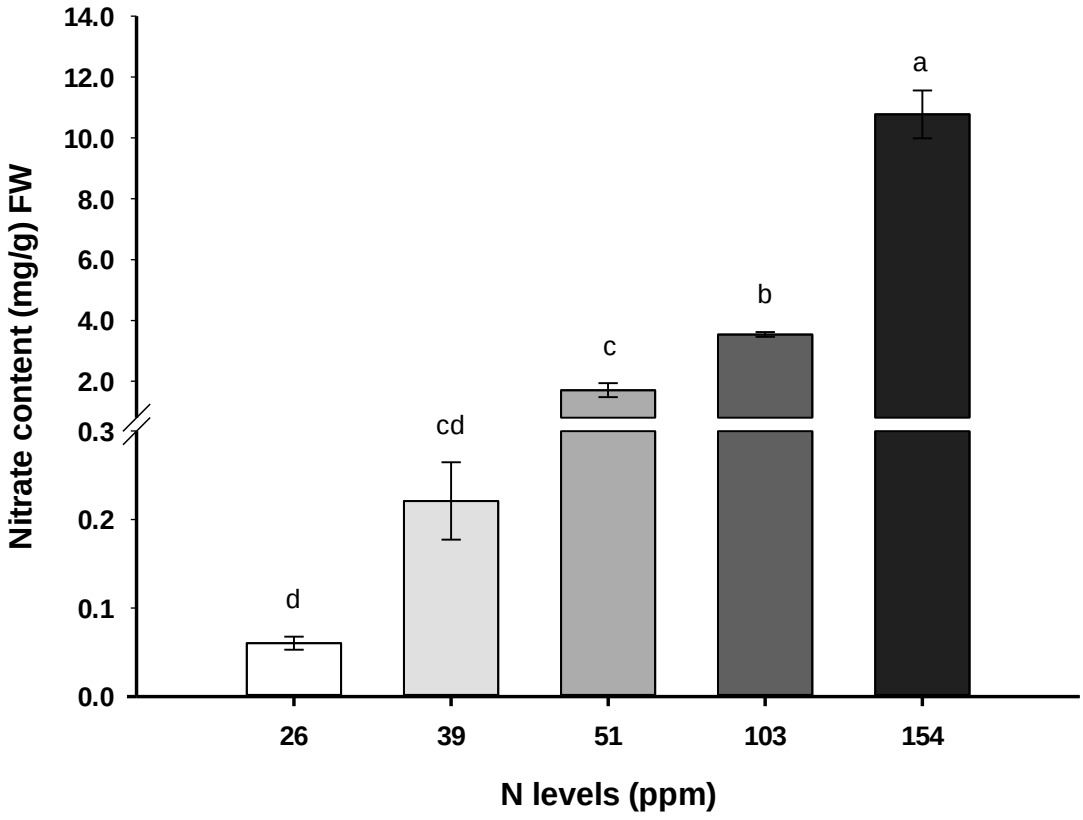


Figure 3.8. Effect of nitrogen levels on nitrate content in Butterhead lettuce (BL) grown at 27/23°C.

Values are the mean ± SEM of six replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$.

3.4.2.2. Effects of nitrogen supply on plant biomass and dry matter

The size of lettuce was reduced with high nitrogen supply, probably due to increasing the stress in the plants. The results showed noticeable decrease in fresh weight (plant biomass) of BL leaves of the treatments received from low to high nitrogen concentration. The fresh weight of BL plants significantly declined from 108.5 to 47.6g when nitrogen contents in the nutrient solutions increased from 26 to 154ppm ($P<0.001$).

The relationship between nitrate concentration in the nutrient solution and the dry matter was linear for BL. The dry matter content was significantly decreased from 8.90 to 4.55% and 9.67 to 2.17 g/plant in BL ($P=0.002$ and $P<0.001$), respectively, when nitrogen supply was increased from 26 to 154ppm (Figure 3.9).

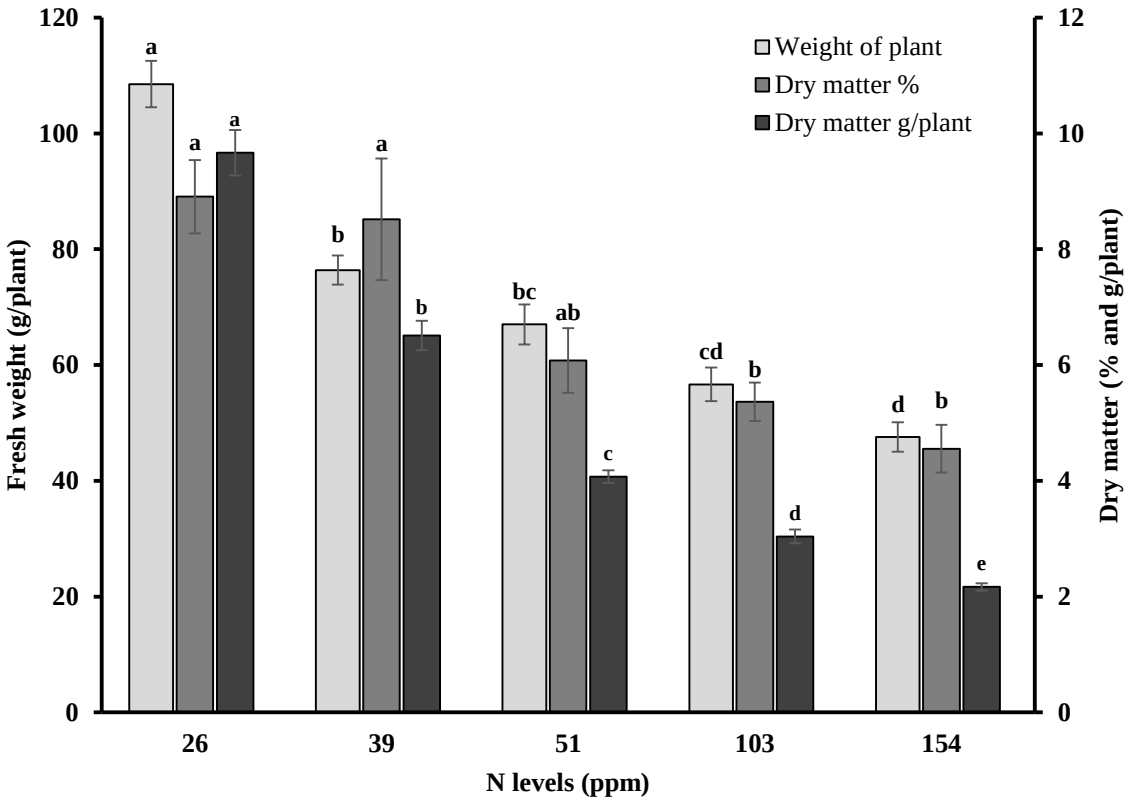


Figure 3.9. Effect of nitrogen levels on fresh weight (g/plant) and dry matter (% and g/plant) in Butterhead lettuce (BL).

Values are the mean ± SEM of six replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$.

3.4.2.3. Effects of nitrogen supply on chlorophyll contents

Figure 3.10 shows the chlorophyll profile contents of a, b and total chlorophylls in BL and OL. As expected, a gradual increase of chlorophyll contents was observed from 26 to 154ppm nitrogen concentration in BL. For all treatments, the concentration of chlorophyll a was always higher than b in both varieties of lettuce. Concerning the chlorophyll b significant differences (P=0.005), chlorophyll a (P=0.008), and for the total chlorophylls (P=0.006) were observed among the N application rates. A one-way ANOVA - Tukey test showed that there was a significant difference between 26 and 154ppm for total chlorophylls in BL. The total chlorophylls were approximately three times higher in BL than OL.

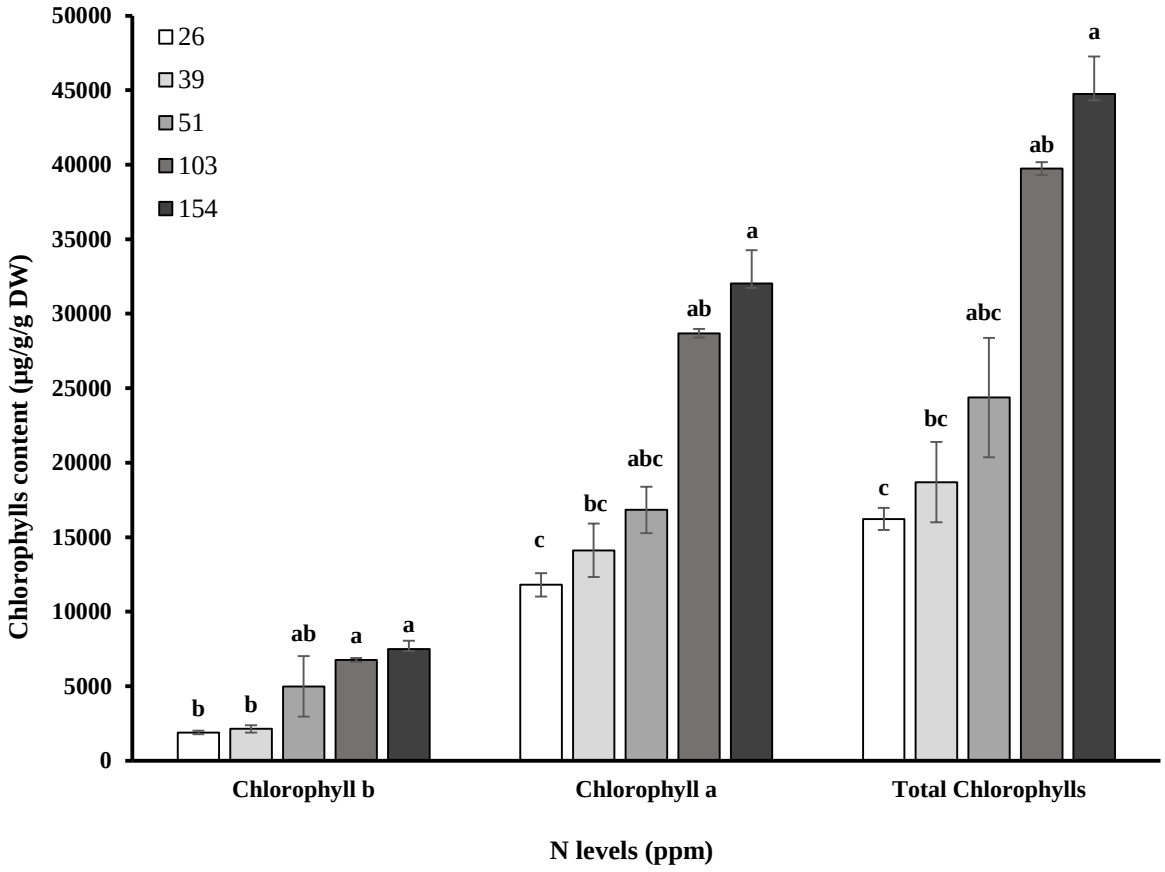


Figure 3.10. Effect of nitrogen levels on chlorophyll content in Butterhead lettuce (BL).

Values are the mean ± SEM of three replicates, each replicate consisting of three plants. Bars with different letters indicate significant differences at $P < 0.05$ among the different N-levels.

3.4.2.4. Effect of nitrogen supply on carotenoid contents in lettuce

Data presented in Figure 3.11 shows that the high nitrogen treatments were likely to increase carotenoid contents. However, the effect of nitrogen was not consistent for all treatments on lutein, β-carotene and total carotenoids from 26 to 154ppm in BL. There was a highly significant difference between 26 and 154ppm for β-carotene (P=0.014) and total carotenoids (P=0.014) in BL. Overall, the total carotenoid content was seven times higher in BL than OL.

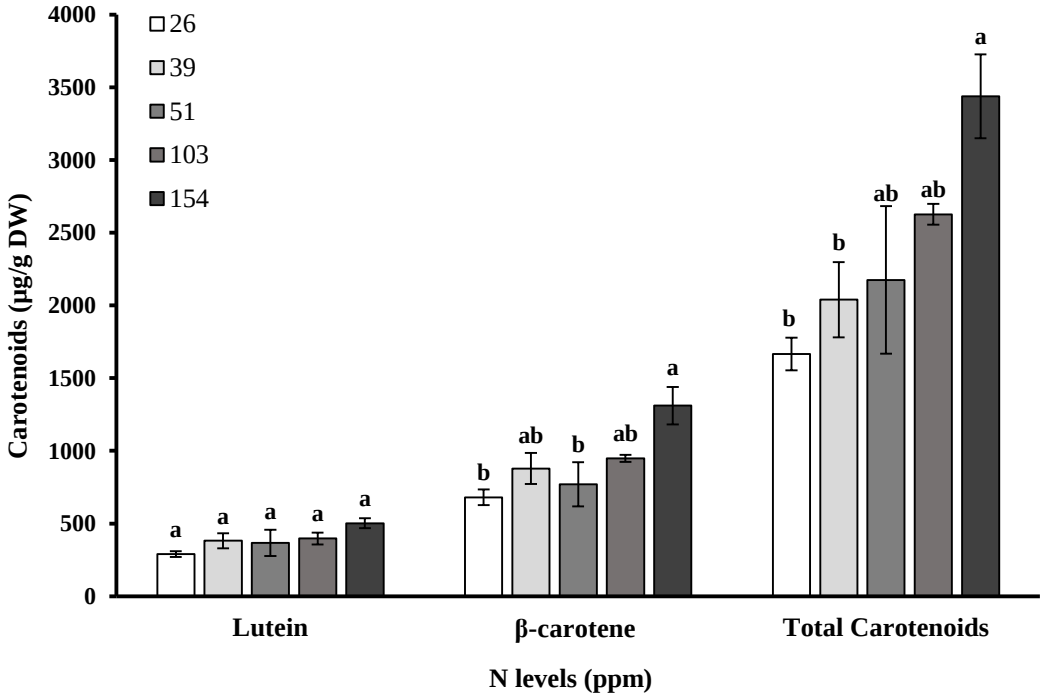


Figure 3.11. Effect of nitrogen levels on carotenoids contents in Butterhead lettuce (BL).

Values are the mean ± SEM of three replicates, each replicate consisting of three plants. Bars with different letters indicate significant differences at $P < 0.05$ among the different N levels.

3.4.2.5. The effect of nitrogen on phenolic phytochemical compounds

Table 3.4 shows the list of phenolic acids and some flavonols were in BL. The highest amount of Cichoric acid (Dicaffeoyltartaric acid) 10186 ± 906µg/g DW was found in BL. However, there was a significant effect of nitrogen on Cichoric acid for 26ppm N compared to 39, 51, 103 and 154ppm N (P=0.002) and the effect of nitrogen was almost similar for Caftaric acid (Caffeoyltartaric acid) (P=0.001). There were no significant effects of the different nitrogen applications on Chlorogenic acid (3-Caffeoylquinic acid) (P=0.114), Neochlorogenic acid (5-Caffeoylquinic acid) (P=0.144), and Cynarine (1,3-Dicaffeoylquinic acid) (P=0.461) because the samll sample size of 3 replicate was too small in the current exepeiment. Therefore, the outcome of power and sample size for one way ANOVA was calcaulated after the experiment were 10 samples to achieve the significant differences.

The concentrations of Isochlorogenic acid A (3,5-dicaffeoylquinic acid) and Isochlorogenic acid C (4,5-Dicaffeoylquinic acid) were not significantly different among the nitrogen levels, but Isochlorogenic acid B (3,4-dicaffeoylquinic acid) was significantly different for 26ppm N compared to 39, 103 and 154ppm N (P=0.018). In addition, some unknown phenolic acids found in BL and their concentration were highly significantly different for 26ppm N compared with 154ppmN, but no significant differences were found between the other nitrogen levels. Overall, these results indicate that increasing nitrogen supply led to decreases of phenolic phytochemical compounds.

It is apparent from Table 3.4 that some flavonol compounds were found in BL. A one-way ANOVA revealed that there was a significant difference between 26ppm N and other nitrogen concentration 39, 51, 103 and 154ppm N on the concentration of Rutin (Quercetin-3-O-rutinoside) and some unkown flavonols derivatives (P<0.001). Furthermore, the total flavonols showed a significant difference of the concentration at 26ppm N compared to that at other nitrogen levels.

Data from this table can be compared with the previous data in Table 3.3 which shows that the two varieties appeared to have different concentrations of each compound. The single most striking observation to emerge from this data comparison was that the concentration of Rutin in Oakleaf was higher than in Butterhead lettuce, even though most other phenolic compounds were highest in Butterhead.

Table 3.4. Phenolic compounds in Butterhead lettuce (BL)[a]

Compounds	Nitrogen levels (ppm in nutrient solution)					
	26	**39**	**51**	**103**	**154**	***P-value***
Cichoric acid (µg/g DW)	10200 ± 900 **a**	4400 ± 2300 **b**	5300 ± 2700 **b**	2500 ± 700 **b**	2700 ± 1300 **b**	0.002
Caftaric acid (µg/g DW)	1530 ± 60 **a**	1180 ± 270 **a**	960 ± 540 **ab**	330 ± 80 **b**	310 ± 60 **b**	0.001
Chlorogenic acid (µg/g DW)	1600 ± 500	960 ± 1100	390 ± 80	590 ± 50	740 ± 320	0.114
Neochlorogenic acid (µg/g DW)	31.8 ± 7.8	16.0 ± 8.9	19.2 ± 14.9	13.2 ± 3.2	13.7 ± 6.0	0.144
Cynarin (µg/g DW)	14.0 ± 1.5	9.8 ± 3.2	10.6 ± 5.8	13.4 ± 2.4	11.5 ± 1.6	0.461
Isochlorogenic acid A (µg/g DW)	113 ± 31	96 ± 88	74 ± 64	59 ± 4	65 ± 25	0.532
Isochlorogenic acid B (µg/g DW)	23.7 ± 4.3 **a**	6.9 ± 3.0 **b**	11.4 ± 8.0 **ab**	4.2 ± 3.1 **b**	7.2 ± 7.5 **b**	0.018
Isochlorogenic acid C (µg/g DW)	2.85 ± 0.71	2.78 ± 1.51	5.13 ± 2.39	5.13 ± 0.96	5.59 ± 5.48	0.500
Unknown phenolic acids (µg/g DW)	41.52 ± 3.91 **ab**	72.24 ± 36.98 **a**	25.04 ± 4.69 **ab**	15.29 ± 912 **b**	20.97 ± 9.16 **b**	0.025
Total phenolic acids (µg/g DW)	13826 ± 1483 **a**	6891 ± 3403 **b**	6902 ± 3389 **b**	3474 ± 832 **b**	3280 ± 1733 **b**	0.002
Rutin (µg/g DW)	1100 ± 30 **a**	350 ± 210 **b**	250 ± 110 **b**	140 ± 20 **b**	160 ± 70 **b**	0.000
Unknown flavonols (µg rutin equivalents/g DW)	390 ± 80 **a**	178 ± 61 **b**	75 ± 28 **b**	50 ± 20 **b**	53 ± 12 **b**	0.000
Total flavonols (µg rutin equivalents/g DW)	1490 ± 92 **a**	528 ± 274 **b**	325 ± 93 **b**	195 ± 11 **b**	212 ± 77 **b**	0.000
Total phenolic compounds (µg/g DW)	15320 ± 1521 **a**	7417 ± 3676 **b**	7167 ± 3420 **b**	3750 ± 839 **b**	4192 ±1800 **b**	0.001

[a] Values are the mean ± SD of three replicates, each with 3 plants. In a row, different letters indicate significant differences at ($P < 0.05$) among nitrogen levels.

Figure 3.12 compares the results obtained from the total phenolic acids (TPA) in BL and OL. There is a clear trend of decreasing TPA with increasing nitrogen levels. From this data, we can see that OL resulted in a lower value of total phenolic acids compared to BL. Total phenolic acid values were 13826, 6891, 6902, 3474 and 3280 µg/g DW at 26, 39, 51, 103 and 154ppm N supplies in BL, respectively. Morover, there was also a significant difference between the results of TPA for 26ppm N and other nitrogen levels (P=0.002). However, the TPA values were 2441, 1137 and 1068 µg/g DW at 51, 103 and 154ppm N supplies in OL, with no significant differences between the N levels on TPA contents in OL (p=0.126).

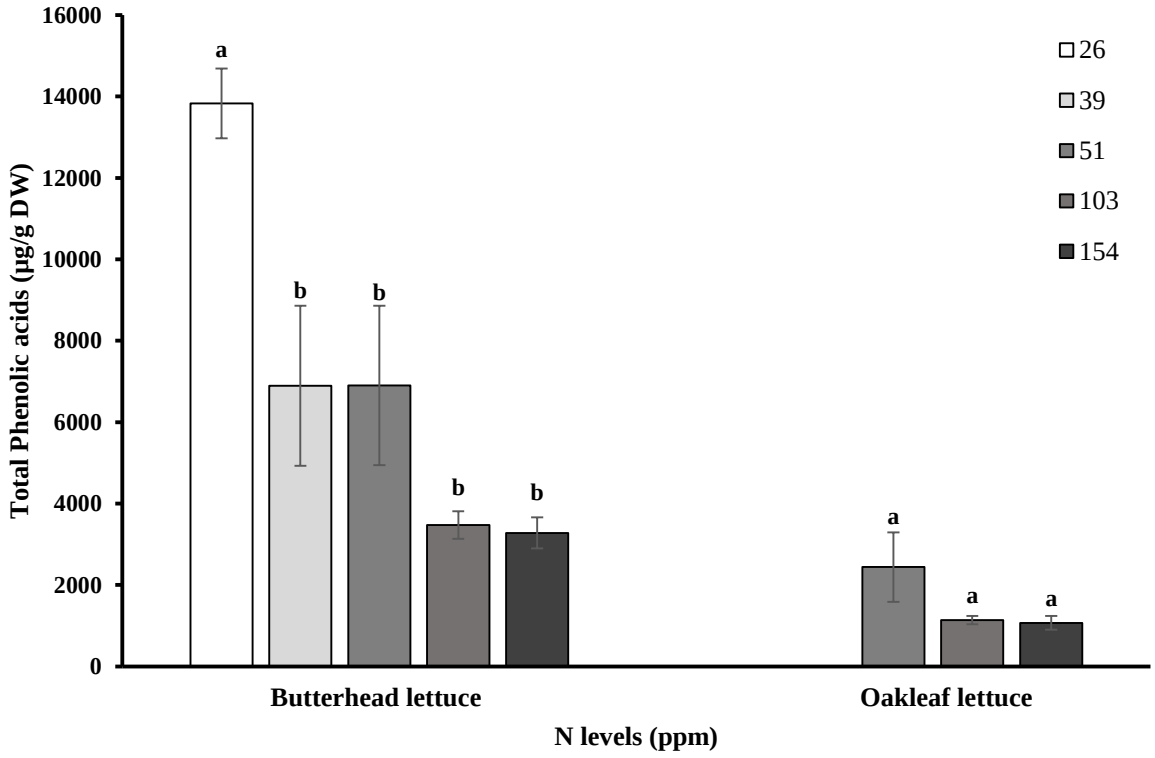


Figure 3.12. Effect of nitrogen levels on total phenolic acids in Butterhead (BL) and Oakleaf (OL) lettuces.

Values are the mean ± SEM of three replicates, each replicate consisting of six plants. Bars with different letters indicate significant differences at $P < 0.05$ among the different N levels within a variety.

3.5 Discussion

The present study was designed to determine the effect of nitrogen fertilisation on concentrations of nitrate and other potentially health relevant phytochemical compounds such as phenolic acids, flavonoids, chlorophylls and carotenoids in controlled growing conditions. In addition, it aimed to reduce nitrate accumulation in lettuce to develop a treatment that could be used as a placebo control tool together with a high nitrate lettuce in an intervention trial study. Nitrogen is a necessary nutrient constituent for all plants (Wang and Li, 2004). Fertigation did increase the nitrate content in both verities/temperature combinations of lettuce (BL and OL), as was also found by (Chen *et al.*, 2004). The results showed that there were significant differences of nitrate content in BL among the five N supply levels and in OL among three N levels. The highest nitrate concentration was found in BL at the higher nitrogen supply (154ppm N). It has been shown previously that plant have the ability to accumulate nitrate, if nitrate is supplied in excess of existing demands (Clarkson, 1986; Millard, 1988). Increasing N application as NO_3 fertiliser increased the nitrate content in white cabbage, green cabbage, spinach and rape (Wang and Li, 2004; Turan and Sevimli, 2005) and in lettuce (Liu *et al.*, 2014).

However, the growth chamber temperature was different between the two varieties of lettuce, 22/18°C for OL and 27/23°C for BL, but it is not the only factor effects on nitrate accumulation because the process of absorption, translocation, and assimilation are all affected (Maynard *et al.*, 1976). At 14/6 °C the nitrate content was significantly higher than at 6/6°C in Butterhead lettuce grown in a growth chamber (Behr and Wiebe, 1992).

The results showed that dry matter content and fresh weight decreased with increased inorganic nitrogen supply from 26 to 154ppm of N as ammonium nitrate. The cause of these decreases could not be, explained from other factors known to affect nitrate accumulation, such as light intensity and temperature in the growth chambers which contained the lettuce plants (Breimer, 1982), since these were kept identical for all plants. The inverse relationship between nitrate and dry matter is associated with inefficient nitrate assimilation (Santamaria *et al.*, 1999). However the effect of N supply on the plant biomass was in line with a recent study carried out by (Croitoru *et al.*, 2015) to reduce nitrate accumulation in lettuce, so it seems to be a general phenomenon that using an excess of inorganic nitrogen negatively influences the biomass of lettuce.

Recent studies show that vegetables provide approximately 40–92% of the average daily intake of nitrate by a human (Liu *et al.*, 2014). The largest amount can be obtained from leafy

vegetables. Therefore, lettuce as the main vegetable in salad and sandwich fillings is responsible for much of the population's intake of nitrate.

Nitrate supply shows a very important role, not only in nitrate accumulation but also in the contents of phytochemical compounds. The results show that the chlorophyll contents response was highly dependent on the nitrate supply with the highest amount achieved at 154ppm N of BL. In this project, carotenoid and chlorophyll contents were obtained by use of HPLC analysis to better understanding the effect of different N supplies on these pigments. The increase of nitrate uptake increases the chlorophyll contents in BL and OL. The results of chlorophyll contents of BL are in agreement with recent findings (Fontes *et al.*, 1997) which showed that chlorophyll reached 11.02mg/g DW and 6,361 mg/kg DW nitrate in lettuce (Brasil 202 cultivar). These results confirm that there was a strong linear relationship between the total chlorophyll (R^2=0.967) and N concentrations in BL and likewise the relationship was not similar in OL (R^2=0.846) (see Appendix of chapter 3). Moreover, the concentration of chlorophyll a was almost three times higher than b in BL and OL (Ferruzzi *et al.*, 2002; Ferruzzi and Blakeslee, 2007). These findings may help us to use the chlorophyll contents as an indicator to detect the N status and foreseeing crop N requirements is plants. It has been observed by Mitchell *et al.* (1991) that nitrogen fertilizer enhances chlorophyll contents in lettuce leaves. On the other hand, differences in chlorophyll content among the N application rates could be attributed to the fact that the leaves are more exposed to different light intensity between the shelves inside the growth chamber. Although one would expect different in growth chamber temperatures which were 22/18°C for OL and 27/23°C for BL. A portable chlorophyll meter is currently available to measure green color intensity in plant leaves, a feature directly related to leaf chlorophyll and N contents (Takebe and Yoneyama, 1989; Lopez-Cantarero *et al.*, 1994). The plant used nitrate as a nutrient source of growing by assimilation of N to chlorophylls, amino acids, polyamines and nucleic acids (Forde, 2000) and the nitrate which is in excess of accumulates in plant cells.

Regarding phytochemical contents, phenolic compounds are produced through the phenylpropanoid pathway by environmental stresses which activate the plant's defence systems (Kim *et al.*, 2006). Concerning carotenoid contents, no significant difference was observed in lutein content among the N supplies in BL (P=0.159), whereas for β-carotene, a significant difference (P=0.014) between 26 and 154ppm N was recorded for BL harvested after seven weeks. These results are in accordance with previous literatures (Mitchell *et al.*, 1991; Mozafar, 1993; Cardoso *et al.*, 2009). There were also significant effects of N supplies on β-carotene in OL. The chlorophyll and carotenoid quantities are identified to determine the plant stress level

(Cruz *et al.*, 2012). It has been pointed out by Kim *et al.* (2008) that the total carotenoid content in Romaine lettuce increases when plants are under nutritional stress. In consequent, the increasing carotenoids might have increased antioxidant capacity against photo-bleaching of chlorophyll (Bode *et al.*, 2009). The amounts of chlorophyll contents in lettuce grown at 26ppm N concentration are in agreement with the literature (Agüero *et al.*, 2008). The results of β-carotene and total carotenoids are in agreement with other research, Kopsell *et al.* (2007), which concluded that the linear increases in lutein/zeaxanthin and β-carotene were observed with increasing N concentrations in parsley grown in nutrient solution culture.

With regard to phenolic acids, the present study found that the relationship between the phenolic acid concentrations was inverse with increases of N concentration. There are different environmental factors affecting the phenolic acids in plants. In addition, different phenolic compounds were found in different varieties of lettuce. The most significant difference was observed in the effect of 26ppm N on Cichoric acid compared with 39, 51, 103 and 154ppm N supply. For Caftaric acid, no significant effect was observed in lettuce produced at 26 and 39ppm N, but the effect of N supplies was revealed at 51ppm N. It is obvious that the phenolic acids declined when the N application rates increased (Li *et al.*, 2008). The results of phenolic acids BL are in agreement with those obtained by (Hanafy Ahmed *et al.*, 2002) that the soluble phenols concentrations were decreased with increased nitrogen fertiliser in the leaves of rocket plants. Mreover, it has been pointed out that nitrate accumulation was inversely related to accumulation of sugars and organic acids in lettuce which they are play a role in osmotice adjustments (Blom-Zandstra and Lampe, 1985; Blom-Zandstra *et al.*, 1988; Behr and Wiebe, 1992). The availability of sugars might affect the need for nitrate as an osmoticum (Barlow, 1981). This confirms the osmotic regulatory function of nitrate in replacement of organic acids and sugars (Santamaria *et al.*, 1999).

On the other hand, the OL at lower temperature had lower contents of phenolic acids than BL. The findings of the current study do not fully support the previous research. For example, contents of flavonoids and caffeic acid derivatives very low in Iceberg and Butterhead lettuce. In contrast, Oakleaf pointed out that the synthesis and phenolic compound accumulations are essential characteristics of secondary metabolism and several of the biosynthetic reactions leading to the major phenolic classes of plants (Cole, 1984). The results of flavonols concentration are in line with the findings demonstrated by (Hohl *et al.*, 2001) that quercetin concentrations were detected between 19.4 and 152.1 µg/g DW in inner leaves and from 417.3 to 2482.7 µg/g DW in outer leaves of BL. Stewart *et al.* (2001) showed that low N led to

increased quercetin content and total flavonols in tomatoes. Researchers have not studied the effect of different nitrogen application rates on phytochemicals in much detail.

The study of using different N levels for BL confirmed differences in the nitrate content as well as in their phytochemical content under controlled growing conditions (identical temperature and light). Despite the temperature combinations are contributed in growing the plants, these results limit the use of nitrogen fertilizer as an index to predict vegetable nitrate and phytochemical contents.

The aim of using different nitrogen application rates on two varieties of lettuce was to produce two sets of lettuce materials with controlled low and high nitrate content as a model, to investigate the short-term effects on blood pressure. However, the results showed that such treatment and placebo would be different in contents of both nitrate and phytochemicals. Still, it can be used for studies to investigate whether low and high nitrate vegetables produced with different phytochemical contents have the same or different effects and bioavailability as nitrate-rich juices. This project may provide a new perspective for quality management of vegetable contents, especially in leafy vegetable production under controlled growing condition.

In the next chapter, the findings of the intervention trial will be presented the impact of such low and high nitrate lettuces on blood pressure and pharmacokinetics of nitrate in health young subjects.

The effect of nitrogen fertilizers on plant materials could be summarized as follows:

1- The effect of N on nitrate content in lettuce strongly depends on the use of N supplies and temperature.

2- The most consistent effects of increasing nitrogen fertilizers on lettuce materials with this light and temperature ranges are:

- The decrease in fresh biomass and dry matter.
- Increase in the accumulation of plant pigments, especially total chlorophylls, β-carotene, total carotenoids in leaf tissue of lettuce.
- The decrease in phenolic acids and flavonols derivatives, particularly in Butterhead lettuce at 27/23°C.

Chapter 4. Effect of lettuce with different inorganic nitrate content on blood pressure and pharmacokinetic profile of nitrate in healthy young volunteers

4.1 Introduction

Recently, the interest in the effect of inorganic nitrate has been increased as a possibility to control blood pressure in humans. Several placebo-controlled studies have shown clinically relevant effects in short-term studies using beetroot juice or other nitrate-containing liquids compared with similar drinks with a low nitrate content, (Webb *et al.*, 2008; Bailey *et al.*, 2009; Lansley *et al.*, 2011; Coles and Clifton, 2012). Inorganic nitrate and beetroot juice supplementation were associated with a significant reduction in systolic BP (Siervo *et al.*, 2013b). These results suggest that the nitrate derived from leafy vegetables may also cause short-term lowering of blood pressure particularly systolic blood pressure (SBP), but placebo-controlled studies have not been possible, due to the lack of reliably different products with a similar appearance. It is known that dietary inorganic nitrate from vegetables is metabolised in vivo to form nitrite in the mouth and then bioactive nitric oxide in the stomach (Govoni *et al.*, 2008). Most of the nitrite in saliva results from the reduction of nitrate by a variety of micro-organisms in the oral cavity. Intake of nitrate-rich lettuce increased the nitric oxide values in expelled air fourfold compared with fasting conditions, because of the high amounts of nitrate in lettuce being reduced to nitrite by bacterial enzymes in the saliva. It is also supported by the finding that chewed lettuce mixed with HCl in vitro formed much more NO than saliva alone with HCl. An alternative explanation may be that agents with reducing capacity in the lettuce might enhance NO_2 formation from NO_3 in the saliva (Lundberg *et al.*, 1994).

Regarding the placebo, the evidence has been obtained from studies using pharmaceuticals as placebo for examples, potassium chloride (Kapil *et al.*, 2010), drinking water (Webb *et al.*, 2008) or apple juice supplementation (Coles and Clifton, 2012), which has a suitable placebo for the design of double-blind clinical trials. The design of food-based nutritional interventions have been concerned by the fact that an appropriate and ethical use of placebo treatment is required carefully more monitored than balanced by substantial benefits for the entire community (Weber, 2008). Therefore, it is not possible to meet the criteria for proper double-blind, randomised placebo-controlled intervention trials. In addition, the biological effects of processed products such as beetroot juice or solutions with a pharmacological status may be different from that of fresh vegetables, e.g. due to the conversion of nitrate to nitrite in the mouth during mouth chewing. Due to this, it could be expected that the fresh vegetable to be

chewed may have a different (greater) effect than liquid (juice), which is quickly swallowed without chewing.

Nitrate is naturally found in plants, particularly in vegetables. Nitrate is used as fertilizer (Santamaria, 2006); it plays an important role as a growing factor in plants and also has an effect on human health. High amounts of nitrate (250mg/100g fresh weight) could be found in beetroots and leafy vegetables such as spinach, rocket and lettuce. The growing conditions such as light intensities, temperature and nitrogen fertilizer have an effect on nitrate accumulation in lettuce; also the genotype interacts with the environment (Reinink, 1993). Due to this, lettuce purchased from retail stores have a highly variable nitrate content and is not suitable for a controlled intervention study.

In recent studies, it is suggested that vegetables rich in nitrate content might benefit health by reducing blood pressure. However, the placebo has a major role in this type of studies to compare with the treatment (Preston *et al.*, 2000), and is particularly important in human trials on blood pressure, since blood pressure can easily be affected by the expectations of participants on lowering blood pressure (Bienenfeld *et al.*, 1996). Therefore, this study was designed to investigate if two new produced lettuce materials with controlled high and low nitrate content, respectively, may have different effects on blood pressure. The new element of this study is that the placebo and the treatment (lettuce with low and high nitrate content) are sufficiently similar in appearance to make blinding feasible, in particular for the study subjects. If successful, the results of this and future studies can determine whether nitrate from vegetables may have the same or higher effects and bioavailability than nitrate-rich juices.

4.2 Materials and Methods

4.2.1 Ethical approval

The trial procedures were approved by the Ethics Committee of Faculty of Science, Agriculture and Engineering at Newcastle University (15-QAD-15). All participants were informed of the trial process prior to the study and provided with an information sheet of the trial, and they signed an informed consent form before participation. The trial was registered on ClinicalTrials.gov, with identifier NCT02701959.

4.2.2 Participants

Twenty healthy young volunteers (12 females and 8 males) participated in a randomised, double-blind placebo-controlled cross-over design with two 24-hour intervention phases separated by a 3-weeks washout period to reduce carry-over effects.

The inclusion criteria were: non-smoking, male and female aged 18-35 years with a body mass index (BMI) in the range of 20 to 25 kg/m^2.

The exclusion criteria were (reason of exclusion):

- Current participation in other clinical investigations.
- Mouthwash users.
- Vegetarianism (likely to have very high nitrate intake)
- Dislike to lettuce consumption or inability to comply with the study diet (lack of compliance)
- Use of antihypertensive or cholesterol lowering medication.
- History of any major illness such as cancer; or cholesterol lowering medication; history of cardiovascular or peripheral vascular disease;
- History of any major illness such as cancer; a psychiatric illness; recent history of asthma, renal, liver or gastrointestinal disease.
- Use of antibiotics within previous 2 months; current or recent (within previous 6 months) significant weight loss or gain (>6% of body weight); woman who were pregnant, lactating or wishing to become pregnant during the study.

- Previous diagnosis of type 1 or type-2 diabetes treated with insulin (modification of regulation of intermediate metabolism).
- Major surgical operations are interfering with the study outcomes (systemic effects on study outcomes).
- Alcohol intake >21 units/week for men and >14 units/week women
- Non English speakers or volunteers are requiring translators or interpreters (since these services are not available for this study).

International Physical Activity Questionnaire (IPAQ) was used for assessment of physical activity level (IPAQ, 2005) and dietary intake was assessed using a Food Frequency Questionnaire (FFQ) (EPIC, 2014) and a Nitrate Screening Questionnaire (See Appedices of Chapter 4). All measurements were performed at baseline of each session of high or low nitrate lettuce with the exception of dietary intake assessment using FFQ, which was done at baseline only.

4.2.3 Sample size

The sample size calculation was performed using Minitab 17 Statistical software. Calculations are based on power and samples size - 1-Sample t. A randomized controlled cross-over study was performed by (Liu *et al.*, 2013) compared a meal of Spinach (220 mg of nitrate derived from spinach) compared with low nitrate meal (100 ml rice milk) in health young adults (Age 38–69 years). The sample size calculation was 17 with a power of 80% and SD of 7; this corresponds to an estimation of sample size when the difference of -5.2mmHg at 150min, and the sample size was 24 when the difference SBP was -4.2mmHg at 210min. This reference has been used for this calculation because no other published study was found that was more similar to this project. Based on assessing similar values from different studies we will recruit a total sample size of 24 participants to be on the safe side.

4.2.4 Intervention

Participants received either 50g of high nitrate lettuce (~530 mg nitrate per portion) or low nitrate lettuce (~3 mg nitrate per portion) in the morning in two separate sessions, separated by at least 3 weeks washout period. The trial was double-blind, the lettuce labelled with a code number for each participant and reordered by a staff member at the nu-food research facility, neither the researchers nor the participants know which is which until the study ends. The list of participants and their code numbers is kept secret until the end of the trial.

Table 4.1 The phytochemical profile of high and low nitrate lettuces*

Components	High nitrate lettuce	Low nitrate lettuce
Dry matter %	~4.6	~8.9
Nitrate content (mg/50g FW)	~530	~3
Total phenolic acids (mg chlorogenic acid equivalents/50g FW)	~10.5	~69.5
Total Chlorophylls (mg/50g FW)	~116	~57
Total Carotenoids (mg/50g FW)	~8.9	~5.8
Total flavonols (mg rutin equivalents/50g FW)	~0.5	~6.5

***for more details refer to chapter 3**

Urine, saliva and blood samples were collected just before and during 24h following the consumption, to assess the pharmacokinetics and bioavailability and compliance to the interventions (details in the diagram of human trial, Figure 4.1).

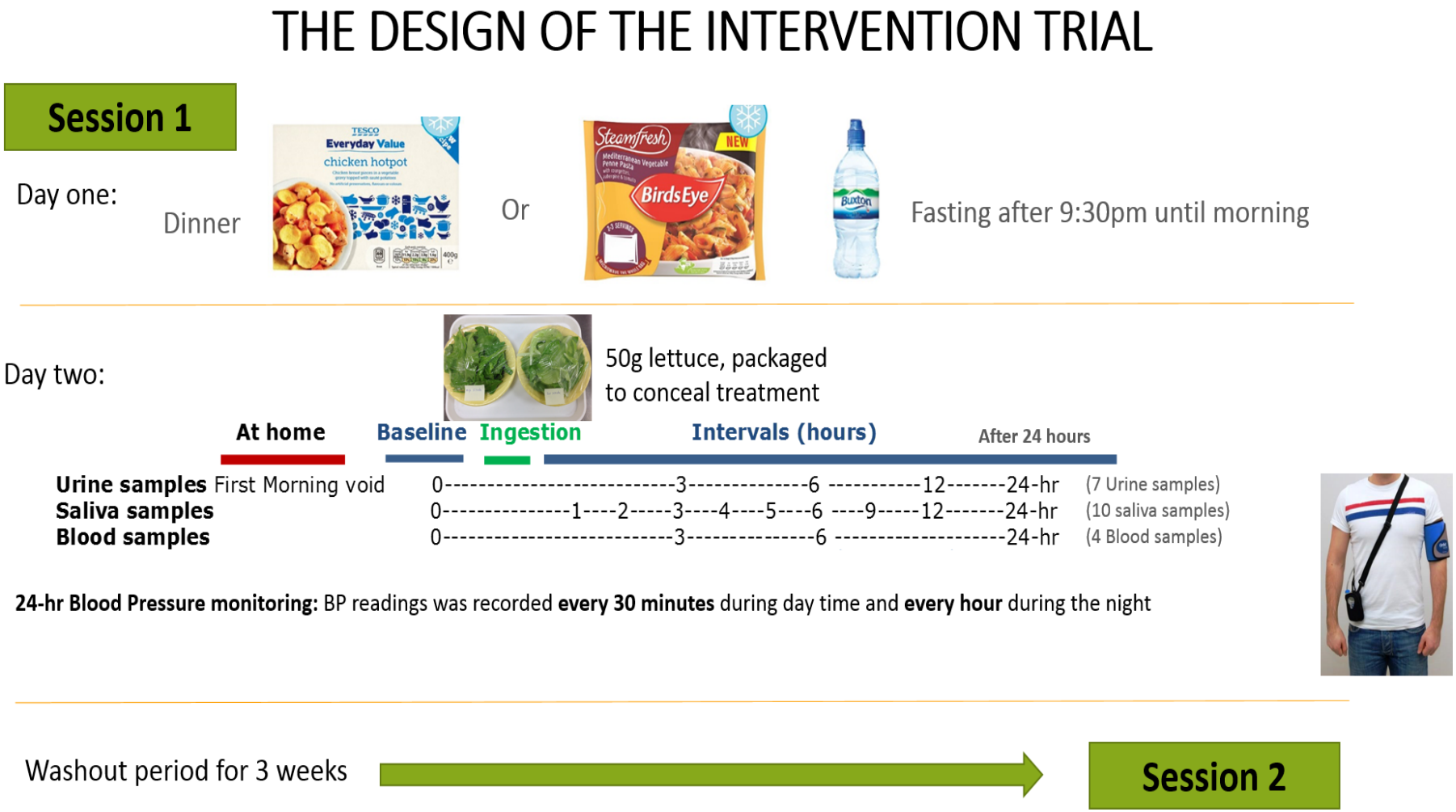


Figure 4.1 the design of the intervention trail

4.2.5 Anthropometry

Body weight, height and waist circumference were measured according to standardised protocols using a body composition analyser TANITA BC420 MA with an electronic scale. Body mass index (BMI) was calculated as weight (kg) / height squared (m^2), fat mass (%) and waist circumference (cm) by measuring tape. Procedures were safe, not invasive and induced minimal discomfort for the participants. The duration of the measurement was approximately 3-5 minutes.

4.2.6 Urine analysis

One urine sample (First Morning Void) was collected by the participants at home before arriving for the experimental day, one sample was collected at baseline (just before providing the lettuce) and further urine samples (all that the volunteer can produce) were collected at set times after the supplementation (at 3 hours and 6 hours) at NU-Food research facility. Subsequent collections (at 12 and 24 hours after supplementation) took place at home in appropriate plastic containers, and the samples were delivered to the researcher at the final visit 24hr after the consumption. After measuring the volume of each sample, subsamples were stored in -20 ºC until analysis for nitrate and nitrite by GC-MS.

4.2.7 Saliva analysis

A small saliva sample (~2ml) was collected in disposable plastic containers at baseline and then every hour for 6 hours at NU-Food research facility and immediately transferred to -20 ºC; additional subsequent collections after 9 and 12 hour at home (kept in the freezer at home) with the final sample taken after 24 hr. Samples were analysed for nitrate and nitrite by GC-MS.

4.2.8 Plasma analysis

Venous blood samples were collected in lithium-heparin containing tubes (~6ml) at baseline and then after 3 and 6 hours following the consumption and after 24 hours, sample were

centrifuged within 30 min at 3000 rpm for 10 min at 4 ºC and plasma was immediately transferred to -80 ºC. Plasma samples were analysed for nitrate and nitrite by GC-MS as well as for Cyclic GMP, TEAC and FRAP.

4.2.9 Dietary restrictions and non-intervention foods

Participants were provided with 2 portions of a standard meal, to consume the evening before and the evening after the lettuce intervention, the Chicken Hotpot - ASDA (372g) for dinner and Mediterranean Vegetable Penne Pasta - Birds Eye (350g) for lunch, the nitrate content was measured as 55.26 and 61.07 mg/meal, respectively. They were provided with drinking water, a low-nitrate mineral water (nitrate <0.1mg/L) from Buxton Mineral Water Company Limited to consume the evening before and during the study day for 24 hours. Also they were asked to follow a diet for 24 hr prior to the study that excluded a list of specific foods containing high nitrate (rocket, spinach, other leafy vegetables, radish and beetroot), cured meat, cured seafood and cured fish, mature cheese. Finally, they were asked to fast for at least 12 hours before the lettuce intervention (from 20.00 in the evening to 8.00 in the morning) and to arrive fasting on the morning of the study.

4.2.10 24-hr Ambulatory Blood Pressure Monitoring (ABPM)

Participants were fitted with an automated portable device to measure BP over a 24-hr period. ABPM measurements were automatically taken every 30 min (between 0900 to 2200h) at day time and every 60 min (between 2200 to 0900h) at night time to minimise the potential impact on sleep quality, an authenticated device approved by the British Hypertension Society was used to monitor 24-hr systolic and diastolic Blood Pressure (Mobil-O-Graph NG, I.E.M. GmbH). Participants were instructed on how to operate the device safely and advised to continue their normal activity during the monitoring period. All of the valid BP recordings were analysed to obtain an average 24-hour systolic and diastolic blood pressure (SBP and DBP).

4.2.11 Nitrate Analysis

Nitrate concentration in all plasma, saliva and urine samples were analysed by gas chromatography-mass spectrometry (GC–MS) as described by Qadir et al. (2013) and in Chapter 2.

4.2.12 TEAC and FRAP assay

Plasma TEAC and FRAP (Nagah and Seal, 2005) was measured using an ABX Pentra 400 (Horiba Medical, Northampton, UK).

4.2.13 Performing cyclic GMP assay

Cyclic GMP in plasma was determined by using an immunoassay ELISA kit per the manufacturer's instructions (catalog#ADI-900-013, Enzo Life Sciences, Inc., USA). Samples were non-acetylated. Assays were run in duplicate. The concentration of cyclic GMP is expressed as picomoles per millilitre.

4.2.13.1. Principle of the assay

The kit used a polyclonal antibody to cGMP to bind, in a competitive manner, the cGMP in the standard or sample which has cGMP covalently attached to it. After a simultaneous incubation at room temperature, the excess reagents were washed away, and the substrate was added. After a short incubation time, the enzyme reaction stopped, and the yellow colours generated were read on a microplate reader at 405nm. The intensity of the bound yellow colour was inversely proportional to the concentration of cGMP in either standards or samples.

4.2.13.2. Reagent Preparation

cGMP Standard: 5000pmol/ml cGMP standard solution were brought to the room temperature (18 – 25ºC). Six 12 x 75 mm glass tubes #1 to #6 were labelled. 900µL standard diluent (assay buffer) was pipetted into tube #1 and 800µL of standard diluent into tubes #2-6. 100µL of the

5000pmol/mL standard were added to tube #1 and were vortexed thoroughly. 200µL of tube #1 were added to tube #2 and were vortexed thoroughly. These processes were continued for tubes #3 through #6.

Wash Buffer: The wash buffer was prepared by diluting 5ml of the supplied concentrate with 95ml of deionized water.

Table 4.2. Dilution table for making standards 1-6

Standards	Assay Buffer 2 (µL)	Volume added (stock and tubes 1-5) (µL)	cGMP concentrations (pmol/ml)
1	900	100 of stock solution	500
2	800	200 of Std. 1	100
3	800	200 of Std. 2	20
4	800	200 of Std. 3	4
5	800	200 of Std. 4	0.8
6	800	200 of Std. 5	0.16

4.2.13.3. Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

1. 100µL of standard diluent (assay buffer) was pipetted into the NSB and the Bo (0pmol/mL standard) wells.
2. 100µL of standards were pipetted into the appropriate wells.
3. 100µL of samples were pipetted into the appropriate wells.
4. 50µL of standard diluent were pipetted into the NSB wells.
5. 50µL of blue conjugate were pipetted into each well, except the TA and Blank wells.
6. 50µL of yellow Antibody were pipetted into each well, except the Blank, TA and NSB wells.
7. The plate were covered and incubated at room temperature for 2 hours on a plate shaker at ~500rpm.
8. After the incubation period, the contents were emptied and washed by adding 400 µL of wash solution to every well. This procedure was repeated for 2 times for a total of 3 washes.

9. After the final wash, the wells were aspirated and the plate was tapped firmly on a lint free paper towel to remove any remaining wash buffer.
10. 5µL of blue conjugate was added to the TA wells.
11. 200µL of the pNpp Substrate solution was added to every well and then incubated at room temperature for 1 hour without shaking.
12. 50µL of stop solution was added to every well. This stopped the reaction, and the plate were read immediately.
13. The optical density in each well was determined using a microplate reader set to 405 nm.

Non-Acetylated Well I.D.: Acetylated Well I.D.:	Blank A1, B1 A1, B1	TA C1, D1 C1, D1	NSB E1, F1 E1, F1	Zero Std. G1, H1 G1, H1	Stds. A2 - D3 A2 - B3	Samples E3 - H12 C3 - H12
Assay Buffer 2	---	---	150 µL	100 µL	---	---
Std. and/or Sample	---	---	---	---	100 µL	100 µL
Conjugate	---	---	50 µL	50 µL	50 µL	50 µL
Antibody	---	---	---	50 µL	50 µL	50 µL
Incub. 2 hours @ RT, shaking	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Asp. & Wash 3 x 200µL	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Conjugate	---	5 µL	---	---	---	---
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incub. 1 hour @ RT	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Figure 4.2. Assay protocol flow chart

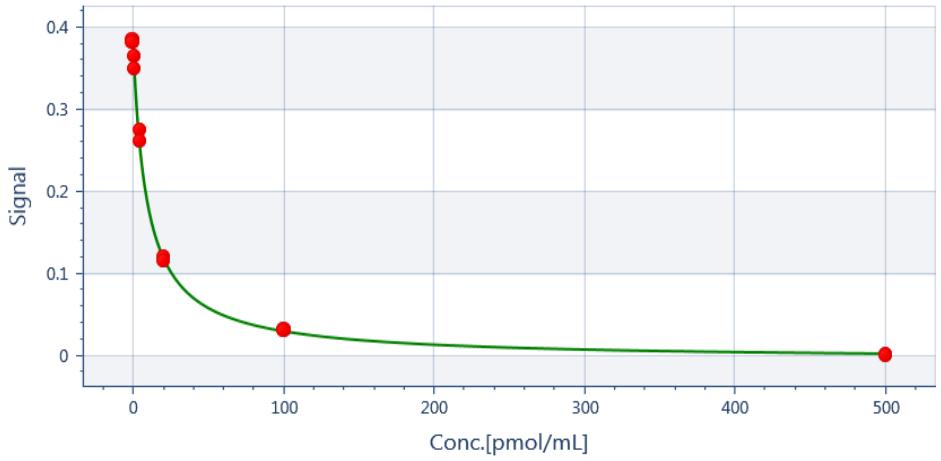


Figure 4.3. The standard curve to calculate cGMP concentrations.

y = 0.393122 + ((-0.00497801 - 0.393122) / (1 + (x/9.03917)^-0.96937))

$R^2 = 1$

4.2.14 Phenolic acids Analysis in plasma samples

4.2.14.1. Chemicals and reagents

All solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-caffeoylquinic acid and ferulic acid were also purchased from Sigma-Aldrich. Caffeic acid-3′-β-D-glucuronide, ferulic acid-4′-O-β-glucuronide, ferulic acid-4′-O-sulfate, isoferulic acid-3′-O-β-D-glucuronide, dihydrocaffeic acid-3′-O-sulfate, dihydroferulic acid-4′-O-sulfate, and dihydroisoferulic acid-d3-3′-O-sulfate were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Feruloylglycine was kindly provided by Prof. Alan Crozier (Department of Nutrition, University of California Davis, USA).

4.2.14.2. Extraction of phenolic metabolites

Plasma samples were extracted using the micro solid-phase extraction (SPE) protocol recently reported by (Feliciano *et al*., 2016), with minor modifications. Briefly, plasma samples were thawed and centrifuged at 20,000g for 10 min at 4 °C. Supernatants (350 µL) were mixed with phosphoric acid 4% (1/1, v/v), for protein precipitation, and spiked with 5 µL of the internal standard dihydroisoferulic acid-d3-3′-O-sulfate stock (28 µM). Each plasma sample (600 µL) was loaded on an OASIS HLB µElution plate (30 µm), washed with 200 µL of ultrapure water and 200 µL of acetic acid 0.2%, and eluted with 60 µL of methanol. The eluted sample was transferred into MS vials before ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) analysis. Each sample was extracted in duplicate.

4.2.14.3. UHPLC-QqQ-MS/MS analysis

Plasma samples were analysed using an UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI; Thermo Fisher Scientific Inc.).

For UHPLC, mobile phase A was acetonitrile containing 0.2% formic acid and mobile phase B was 0.2% formic acid in water. Separations were performed with a Kinetex EVO C18 (100 x 2.1 mm) column, 2.6 µm particle size (Phenomenex, Macclesfield, UK). The gradient started with 5% A, isocratic conditions were maintained for 0.5 min, and reached 95% A after 6.5 min, followed by 1 min at 95% A and then 5 min at 5% A to re-equilibrate the column. The flow

rate was set at 0.4 mL/min, the injection volume was 5 μL, and the column was thermostated at 40 °C.

The MS worked in negative ionization mode with capillary temperature at 270 °C, while the source was at 300 °C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high purity argon gas was used for collision-induced dissociation (CID). Characteristic MS conditions (S-lens RF amplitude voltage and collision energy) were optimized for each compound. The applied method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in the "selected reaction monitoring" (SRM) mode. Two molecular transitions were used to qualify and quantify the phenolic metabolites (Table 2). Data processing was performed using Xcalibur software from Thermo Scientific.

Table 4.3. Retention times and optimized SRM conditions for identification and quantification of caffeoylquinic acid-derived metabolites

No.	Compound	RT (min)	Parent ion (*m/z*)	S-lens	Quantifier		Qualifier	
					Product ion (*m/z*)	CE (V)	Product ion (*m/z*)	CE (V)
1	5-caffeoylquinic acid	3.42	353	83	191	20	135	36
2	Caffeic acid-3′-β-D-glucuronide	3.33	355	98	179	21	135	37
3	Ferulic acid	4.94	193	71	134	19	178	18
4	Ferulic acid-4′-*O*-β-glucuronide	3.18	369	93	193	18	134	39
5	Ferulic acid-4′-*O*-sulfate	4.36	273	92	193	18	178	28
6	Feruloylglycine	3.96	250	79	206	14	134	22
7	Isoferulic acid-3′-*O*-β-D-glucuronide	3.90	369	93	193	18	178	32
8	Dihydrocaffeic acid-3′-*O*-sulfate	3.89	261	96	181	20	137	25
9	Dihydroferulic acid-4′-*O*-sulfate	4.15	275	75	195	31	136	17

CE: collision energy

4.2.15 Statistical analysis

Twenty participants completed the present study using a cross-over design. Data were expressed as mean ± 95% CI. The area under the curve (AUC) was calculated as accumulative measure of change in systolic and diastolic BP (AUC={bp_1+bp_2/2*(t_2-t_1)}+…) for the periods between 0-3 , 3-6, 6-12 and 12-24 hrs periods after ingestion of the meal. A paired t-test for independent samples was used to compare differences between baseline characteristics of the participants taken before the start of each intervention. Effects were analysed by ANOVA - General Linear Model. Significance shown for comparisons between high nitrate lettuce vs low nitrate lettuce of for Multiple Pairwise Bonferroni test at each time points. Repeated-measure ANOVA was used to compare the effects of high nitrate lettuce and low nitrate lettuce on systolic and diastolic BP, TEAC, FRAP, caffeoylquinic acid-derived metabolites and cGMP; time and treatment were set as fixed factors and subjects nested as a within-subject factor. To assess individual responses, Bonferroni post-hoc analyses were performed to compare between high nitrate lettuce and low nitrate lettuce at different time points. Paired t-test was used to compare the AUC of ΔSBP and ΔDBP. The AUC was calculated as an average for ΔSBP and ΔDBP from the baseline. The period between 0 and 3 represents the post dose time, 3 and 6 h represents the time participants spent in the NU-Food research facilities, 6 and 12 h represents the remain active period at home, and 12 and 24 h represents passive period. Statistical significance was set at the 0.05 level for all cases.

4.3 Results

4.3.1 Participant's baseline characteristics

Twenty four healthy young participants were recruited, but only twenty participants completed the study and were included in the analysis (12 females and 8 males) with an age range of 22-31 years and a body mass index (BMI) range of 20-24.6 kg/m^2 who were recruited in the NU-Food research facilities, Newcastle University from 25th February 2015 until 04th August 2015. Four participants withdrew from the study after the recruitment due to time restrictions. The baseline characteristics for each session are reported in Table 4.3. Body weight did not change, but and physical activity level was slightly higher in low nitrate lettuce participants.

Table 4.4. Baseline characteristics of the participants before consumption of lettuce*

Characteristics	High nitrate lettuce		Low nitrate lettuce		
	Mean	SD	Mean	SD	P-value
Age (years)	24.95	2.91			
Height (cm)	1.68	0.08			
Body weight (kg)	62.32	7.67	62.34	7.46	0.99
Body mass index (kg/m^2)	22.04	1.63	22.03	1.55	0.98
Waist circumference (cm)	79.90	5.68	79.95	4.99	0.97
Fat mass (%)	20.53	6.40	20.42	6.40	0.95
Heart rate (bpm)	67.43	10.53	69.26	10.96	0.59
Systolic BP (mmHg)	111.85	11.60	112.60	10.33	0.82
Diastolic BP (mmHg)	72.28	8.40	71.95	7.90	0.89
Energy intake (Kcal/day)	2608	1027	2608	1027	1.00
Physical activity (METs)	3410	2004	3501	2369	0.89
Total NO_3 intake (mg/day)	27.54	3.89	27.20	3.74	0.58

*Mean value with their standard deviations (n= 20), 12 females and 8 males, A paired t-test for independent samples was used to compare between the sessions differences of baseline measurements.

4.3.2 Plasma nitrate and nitrite concentration in participants

The value of baseline plasma NO_3 concentration was similar in participants when they received low and high nitrate lettuce (P=0.994). Ingestion of high nitrate lettuce resulted in an increase in the plasma NO_3 concentration by nearly 5-fold, from 86 ± 33μM to 395 ± 133μM (Mean ± SD), 3h post ingestion (P<0.001). In contrast, there was no significant change (P=1.00) after ingestion of low nitrate lettuce, levels of plasma NO_3 were 91 ± 35μM and 87 ± 42μM at baseline and 3h after ingestion of low nitrate lettuce, respectively. The difference in plasma NO_3 was still significant (P<0.001) 6 h post high nitrate ingestion, where the plasma NO_3 concentration was lowered to 4-fold (315 ± 95μM), and it returned to basal levels by 24h. There was also no significant difference in change between baseline 0h and 24h for high and low nitrate lettuce, P=0.250 and P=0.200, respectively (Figure 4.4).

The plasma nitrite concentrations did not show any statistically significant differences between any treatments or time points (Figure 4.5).

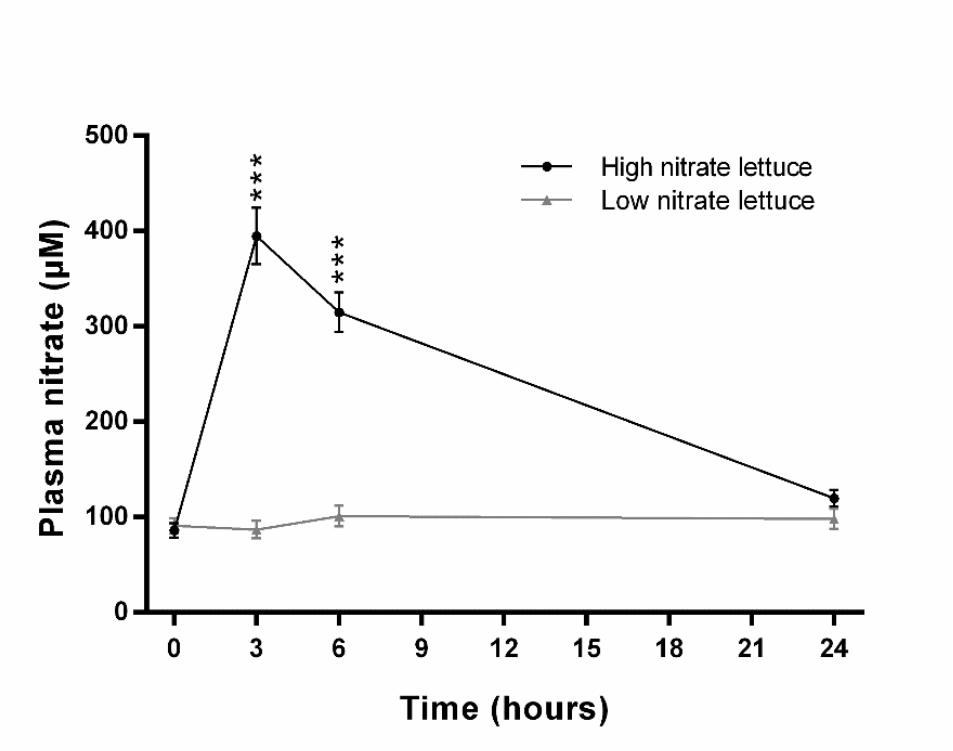


Figure 4.4 The effect of high and low nitrate lettuce on the plasma nitrate concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of (P<0.05) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *P < 0.05; **P < 0.01; ***P < 0.001.

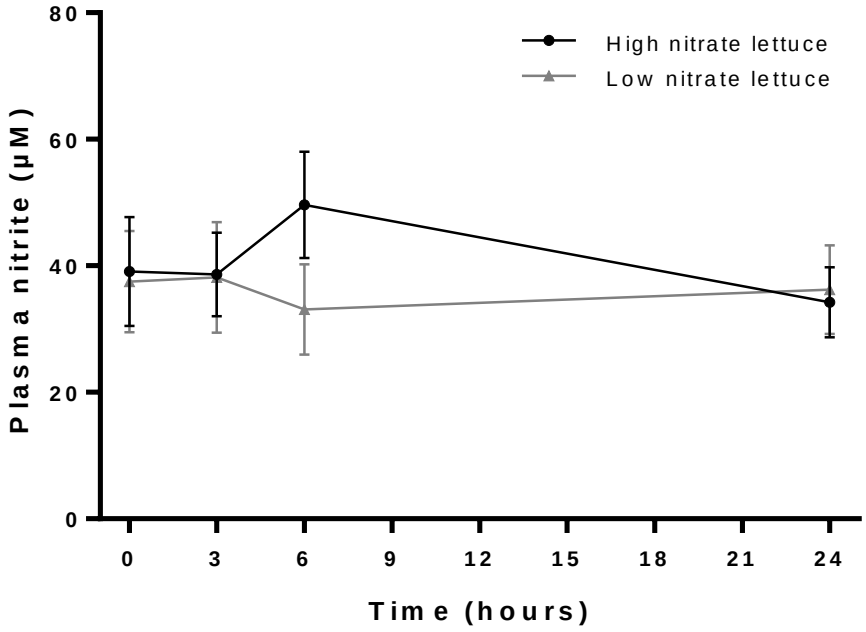


Figure 4.5 The effect of high and low nitrate lettuce on the plasma nitrite concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.3.3 Salivary nitrate and nitrite

Salivary nitrate and nitrite concentrations were significantly increased following high nitrate lettuce ingestion relative to low nitrate lettuce. In the case of a low nitrate lettuce, the basal salivary nitrate and nitrite concentrations did not vary significantly after ingestion for up to 24h with concentrations ranging from 455 to 871μM and, 131 to 173μM, respectively. Basal salivary nitrate and nitrite did a very significant increase for up to 6h post high nitrate lettuce with concentrations ranging from 337 to 3557 μM and 163 to 971μM, respectively. Moreover, salivary nitrate concentration was increased dramatically within 1h from 337 to 5801μM ($P<0.001$) and peaked at 7362μM ($P<0.001$) by 2h after the high nitrate lettuce consumption. However, the salivary nitrate concentrations decreased after 3 to 6h post ingestion, but there were still highly significant differences ($P<0.001$) of high nitrate lettuce intake comparative to low nitrate lettuce. Moreover, the salivary nitrate concentration gradually returned to baseline by declined to 2155 and 1649μM at 9 and 12 h, respectively, and then reached to 879μM after 24h post consumption (Figure 4.6).

Because about 20-30% of ingestion nitrate secrets into the saliva where it reduces into nitrite by oral bacteria, saliva was considered for the amount of salivary nitrate and nitrite. At baseline, there was no significant difference between the salivary nitrite concentration of the high nitrate lettuce group and the low nitrate lettuce group. Salivary nitrite concentration raise from 163 to 1003μM at 1 hour and peaked to 1719μM ($P<0.001$) by 3h following the high nitrate lettuce intake, indicating a delay compared with the time course for the nitrate. The highly significant difference in salivary nitrite concentration remained up to 12 hours after ingestion of high nitrate lettuce and returned to 211μM after 24h which was not different ($P=1.00$) from the 173μM in the low nitrate lettuce treatment (Figure 4.7).

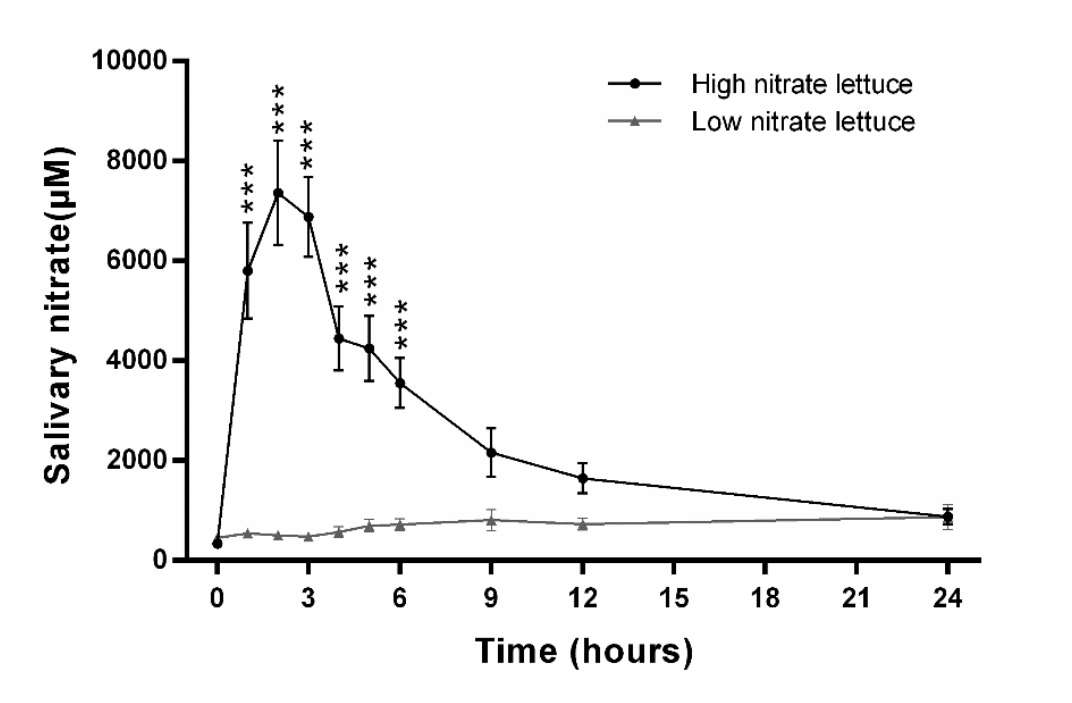


Figure 4.6 The effect of high and low nitrate lettuce on the salivary nitrate concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of (P<0.05) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *P < 0.05; **P < 0.01; ***P < 0.001.

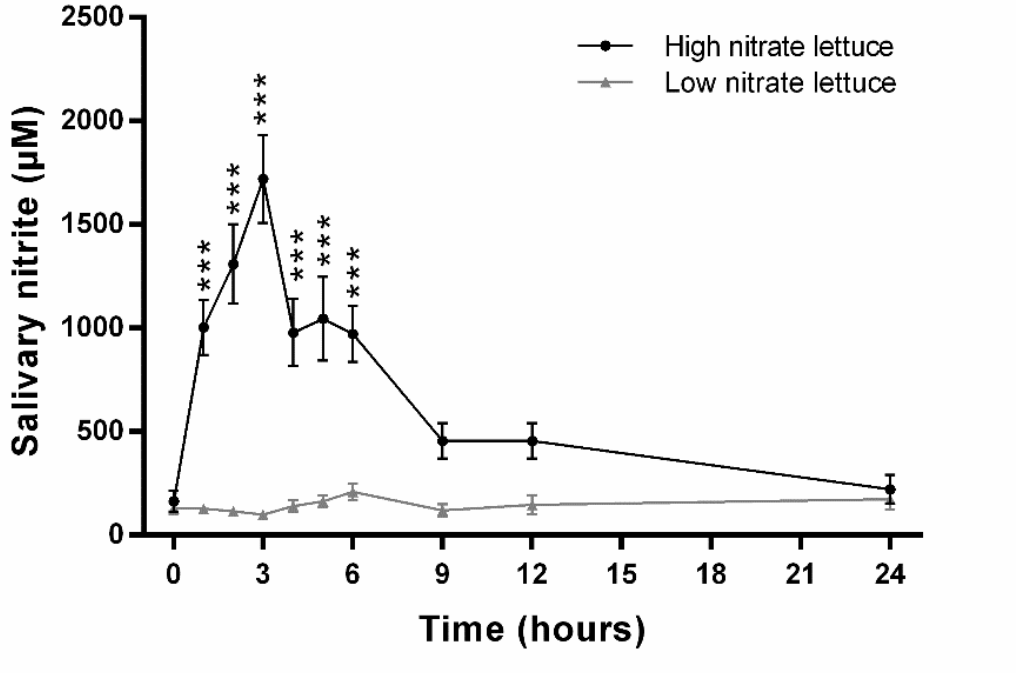


Figure 4.7 The effect of high and low nitrate lettuce on the salivary nitrite concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of (P<0.05) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *P < 0.05; **P < 0.01; ***P < 0.001.

4.3.4 Urinary nitrate and nitrite concentrations

There was no significant difference in the baseline urine samples that were collected at home (First Morning Void, FMV) and upon arrival to the NU-Food research facility. In the case of low nitrate lettuce, the mean urinary nitrate concentration decreased 3h after ingestion compared with baseline value (P=0.399) and then slightly increased after 6h (P=0.543) and remained with no significant difference for up to 24h (P=0.196). In contrast, the high nitrate lettuce ingestion led to a highly significant increase in the basal urinary nitrate concentration, from 656±362 to 2287±1233µM in the urine collected from 0 to 3h post ingestion (P<0.001). However, there was wide variation in levels between individuals. The average peaked at 2586µM (P<0.001) in the urine collected from at 6h post ingestion, and then there was also a significant difference of urinary nitrate concentration in the urine collected from 6 to 12h (P=0.003) and 12 to 24h (Figure 4.8). There were no significant differences in the urinary nitrite concentrations after the ingestion of high nitrate lettuce compared with baseline values and low nitrate lettuces at different time points (Figure 4.9).

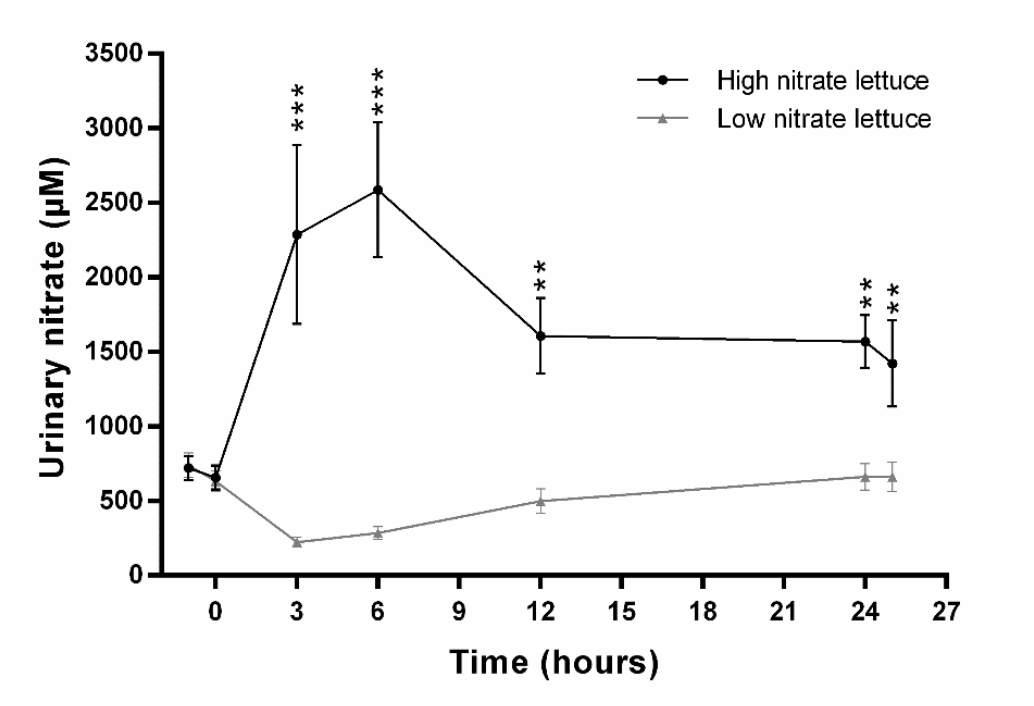


Figure 4.8 The effect of high and low nitrate lettuce on the urinary nitrate concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of (P<0.05) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *P < 0.05; **P < 0.01; ***P < 0.001.

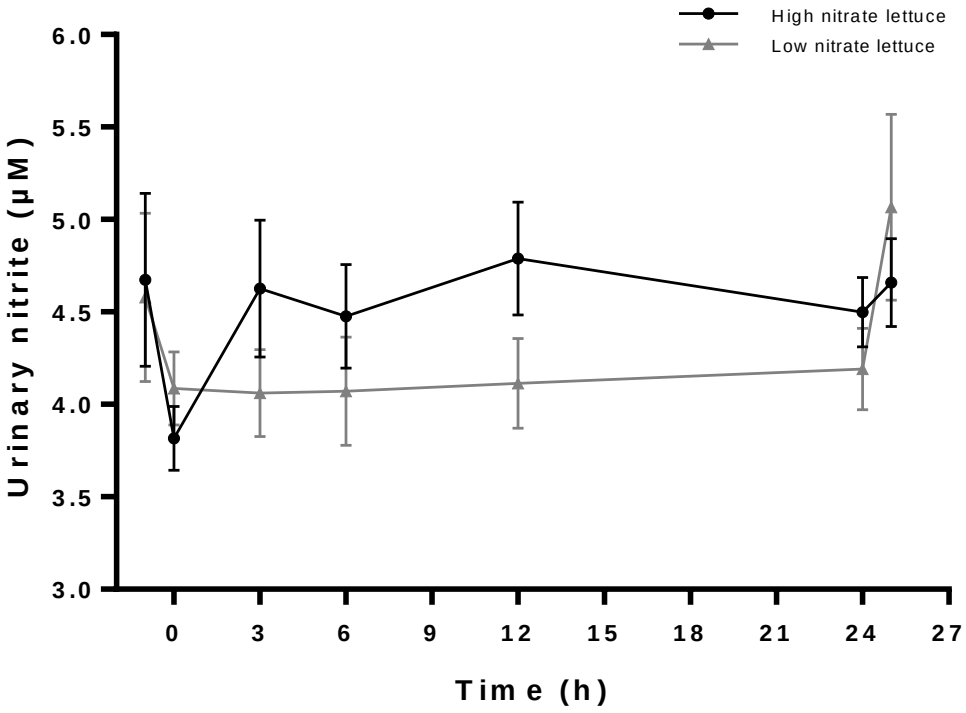


Figure 4.9 The effect of high and low nitrate lettuce on the urinary nitrite concentrations.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.3.5 Pharmacokinetic profile of urinary nitrate excretion

The high nitrate lettuce consumption led to an elevation in total urinary nitrate concentration. In the case of low nitrate lettuce, there was no significant difference between the mean of total urinary nitrate concentration in 24h urine volume relative to baseline (0h) for up to 24h post ingestion, ranging from 292 to 390μM. There was also no significant difference in urinary NO_3 concentrations in urine collected at home (FMV) and baseline urine sample upon arrival to the NU-Food research facilities. There was a significant difference in the total urinary nitrate concentration for the urine volumes collected at 3h (P=0.004) after high nitrate lettuce intake relative to baseline and low nitrate lettuce. Then, the total urinary nitrate concentration was a significant difference in collected urine volumes between 3 to 6h (P=0.003), and then gradually reduced after 6 to 12h (P=0.275), and 12 to 24h (P=0.616). There was also no significant change of total urinary nitrate 24h post high and low nitrate lettuce consumptions (Figure 4.10). Additional information of total urine volumes over 24 h of participants after ingestion of high and low nitrate lettuce were provided in Table 4.4.

The mean of accumulative urinary nitrate excretion of high nitrate ingestion gradually increased from 27mg at baseline to 126mg between at 3h but statistically there was no significant difference relative to baseline, and low nitrate lettuce intakes due to wide variation in levels among individuals, from 126 to 227mg at 6h (P=0.003), from 227 to 300mg at 12h (P<0.001), from 300 to 368mg at 24h (P<0.001), and the total urinary nitrate excretion was 391mg after 24h (P<0.001) of high nitrate lettuce consumption. The amount of cumulative urinary nitrate excreted was calculated based on the urine volumes that were collected at separate times for each individual subject (Figure 4.9). In the case of low nitrate lettuce, while no significant change in concentrations were observed for up to 24h post ingestion relative to baseline mean, the accumulative urinary nitrate excretion was gradually increased from 23mg at baseline to 104mg, presumably because of the endogenous formation of nitrate. Overall, the results show that it took more than 12 hours after the consumption of high nitrate lettuce until the excretion rate returned to baseline and that the amount of nitrate excreted through urine during more than 24 hours increased from 104mg with low-nitrate lettuce to 391mg (P=0.015) with the high-nitrate lettuce. This 287mg increase in urinary excretion (Figure 4.11) corresponded to 54% of the 527mg additional dietary intake of nitrate provided from the high nitrate lettuce treatment.

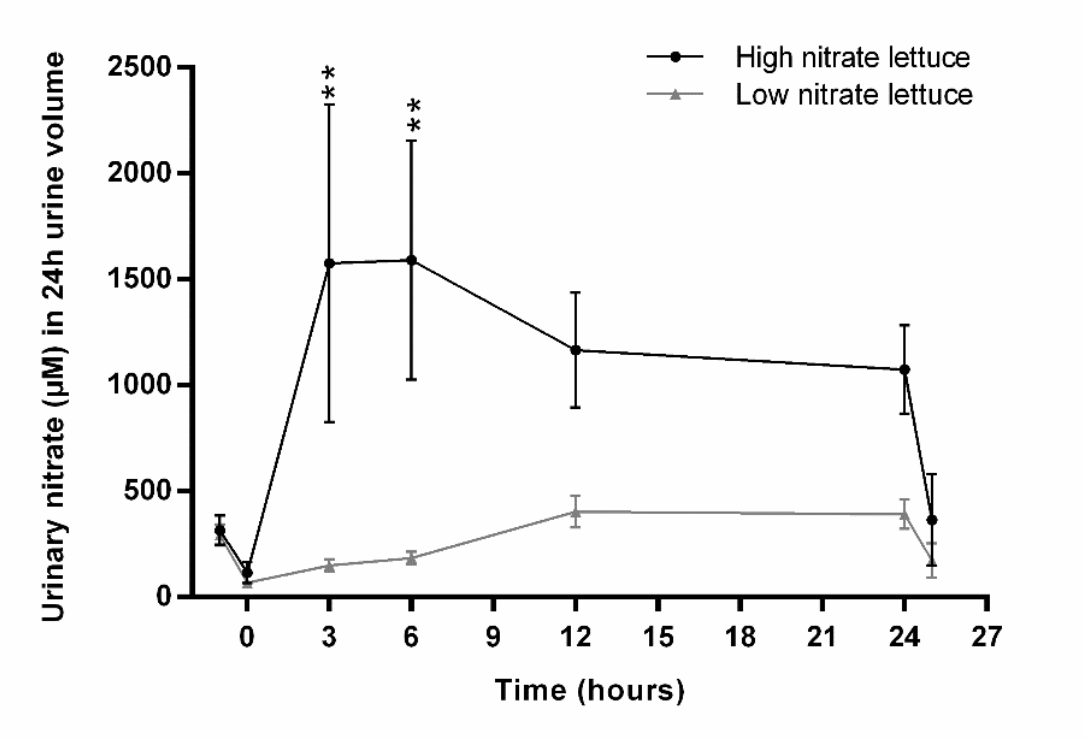


Figure 4.10 Total urinary nitrate concentration at separate time of 24h urine volume.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4.5. Total urine volumes over 24 h of participants after ingestion of high and low nitrate lettuce

	Total urine volume (ml)	
Time (h)	**Low nitrate lettuce**	**High nitrate lettuce**
FMV	377±188	392±219
Baseline	98±76	149±182
0-3	695±473	599±482
3-6	709±360	540±301
6-12	760±429	716±459
12-24	614±362	682±337
After 24	193±243	155±174
Total	3444±1466	3233±1685

*Mean value with their standard deviations (n= 20)

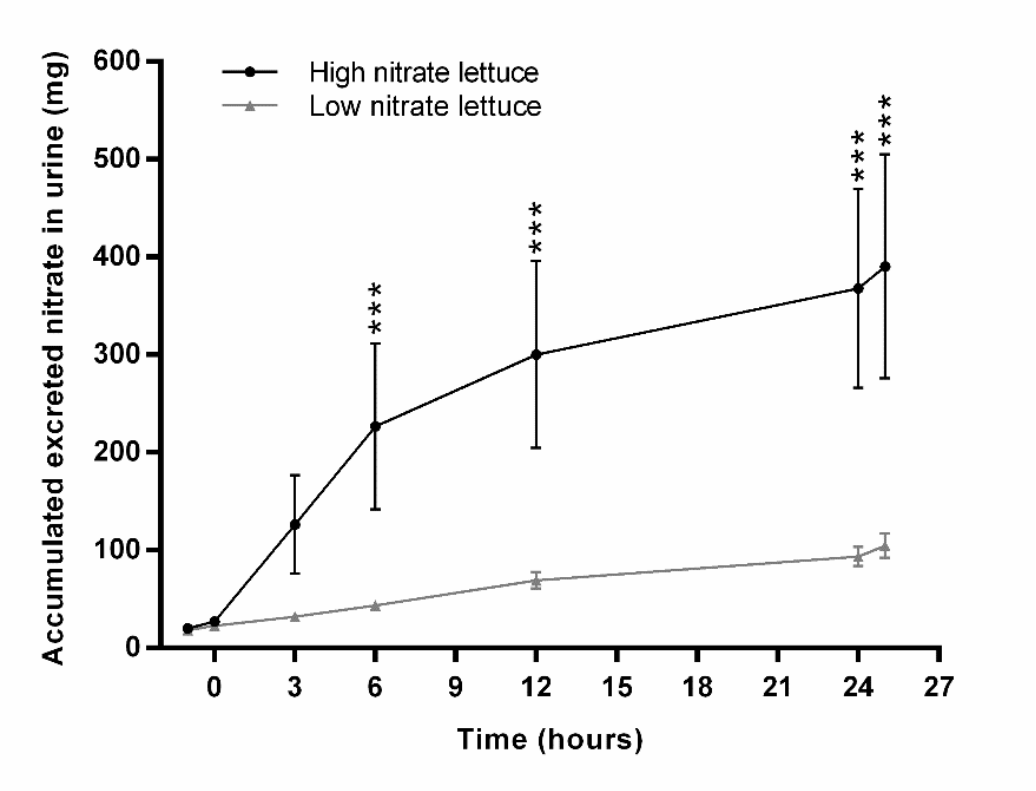


Figure 4.11 Pharmacokinetic cumulative nitrate excretion profile after the ingestion of high and low nitrate lettuce.

Data expressed as mean ± 95% CI. Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.3.6 24-hour Ambulatory Blood Pressure Monitoring (24-hr ABPM)

A repeated measure ANOVA of 24-ABMP showed that high nitrate lettuce compared with low nitrate lettuce resulted in an overall lower postprandial SBP (P=0.003) and DBP (P=0.002). Relative to baseline, high nitrate lettuce began to reduce the SBP and DBP between 3 to 7h after ingestion and the reduction peaked at 5h. The correlation of change in postprandial SBP and DBP with high nitrate lettuce was r^2 =71.91% and r^2 =78.71%, respectively.

The difference in SBP was 6.93 ± 12.33mmHg and 4.10 ± 10.74mmHg after 3h and 6h following high nitrate ingestion, respectively, when compared with baseline SBP, and the difference in SBP was 3.38 ± 10.63mmHg and 10.20 ± 16.88mmHg after 3h and 6h following low nitrate ingestion, respectively, when compared with baseline SBP. Regarding DBP, The difference in mean of DBP was 1.27 ± 4.80mmHg and 5.25 ± 8.44mmHg after 3h and 6h following high nitrate ingestion, respectively, when compared with baseline DBP, and the difference in DBP was 4.60 ± 7.78mmHg and 3.7 ± 9.05mmHg after 3h and 6h following low nitrate ingestion, respectively, when compared with baseline DBP. The change of SBP in the current study is corresponding with the resulted obtained from the power and samples size calculation. On the other hand, the difference in SBP was 2.80 ± 4.43mmHg and -6.85 ± -4.91mmHg after 3h and 6h, respectively, following high nitrate ingestion when compared with low nitrate lettuce, and the difference in mean of DBP was -2.25 ± 2.34mmHg and 3.85 ± 3.01mmHg after 3h and 6h, respectively, following high nitrate ingestion when compared with low nitrate lettuce (Figure 4.12). No significant difference was observed between high and low nitrate lettuce treatments in mean heart rate (P=0.18) and peripheral pulse pressure (P=0.629) over the 24h post ingestion. In addition, there was also a significant difference in the average daytime of systolic blood pressure (SBP) following high nitrate lettuce of 116.10± 4.47mmHg compared to low nitrate lettuce with 118.77±3.57mmHg (P=0.013). There was also a significant difference in average value for SBP during the entire recording period following high nitrate lettuce of 114.67±4.80mmHg and low nitrate lettuce with 116.56±5.39mmHg (P=0.047). There were statistically significant differences between the high and nitrate lettuces in post-dose (3h) SBP (P=0.038), and no significant change found in post-dose DBP (P=0.729). Additional information of heart rate, mean arterial pressure and pulse pressure were provided in Table 4.4.

However, the AUC for ΔSBP and ΔDBP from 0 to 3h following high nitrate lettuce ingesting was lower compared with the low nitrate lettuce, but the paired t-test revealed no significant difference in the AUC of ΔSBP and ΔDBP. There was only a significant difference in the AUC of ΔSBP and ΔDBP from 3 to 6h. There were also no significant difference in the time of 6 to

12h and 12-24 h (Figure 4.13: A and B).

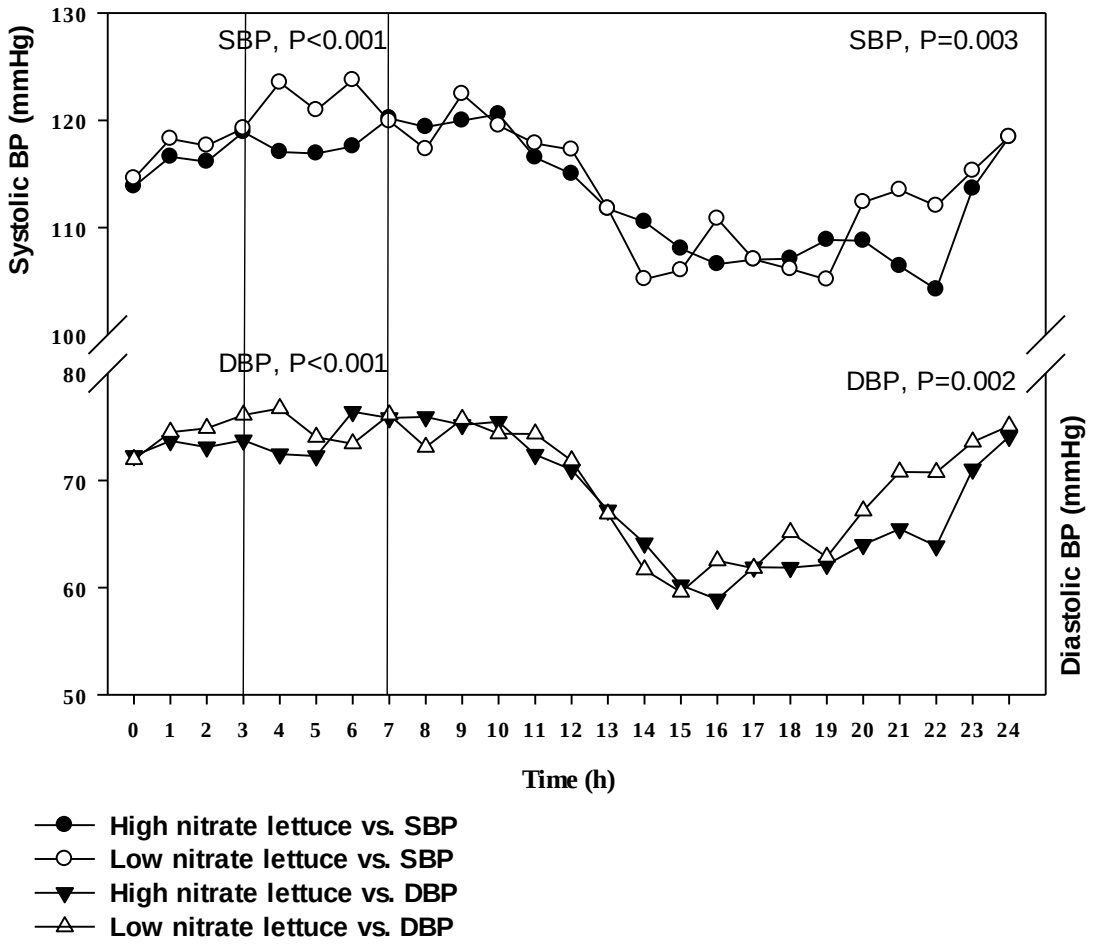


Figure 4.12 The effect of high and low nitrate lettuces on 24-hr ABPM systolic and diastolic blood pressure.

Effects were analysed by General Linear Model - Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20), H = high nitrate lettuce and L= low nitrate lettuce ($P<0.05$)

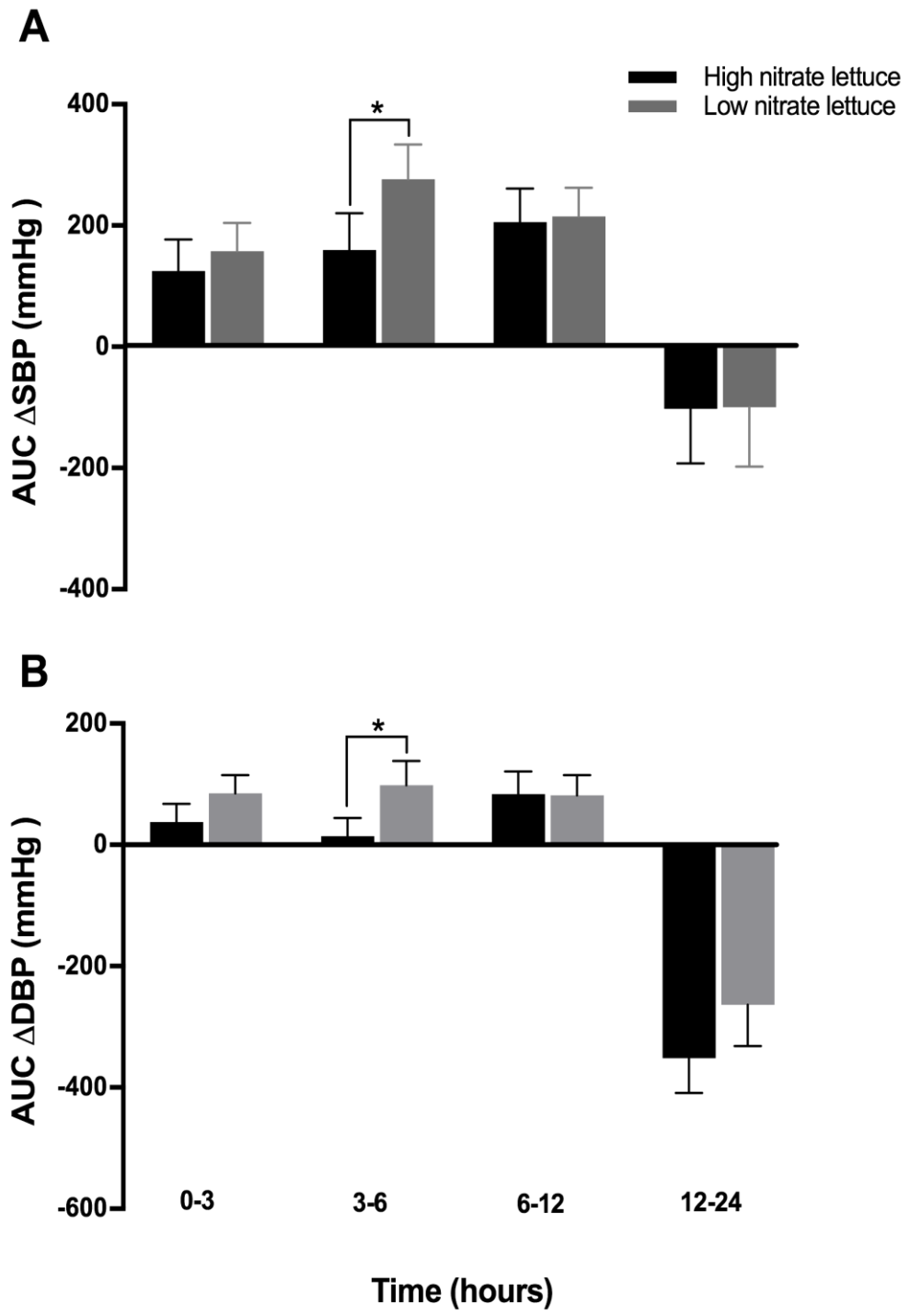


Figure 4.13. Mean ± SEM of change in AUC of ΔSBP (A) and ΔDBP (B) curve from 0 to 3h, 3 to 6h, 6 to 12h and 12 to 24 h following high and low nitrate lettuce ingestions.

Effects were analysed by paired t test in SPSS. Significant shown for comparisons between high nitrate lettuce vs low nitrate lettuce of ($P<0.05$). *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4.6 Systolic blood pressure, diastolic blood pressure, heart rate, mean arterial pressure and pulse pressure of participants during the active and passive periods in the 24-h following ingestion of high and low nitrate lettuce

Measurement	High nitrate lettuce	Low nitrate lettuce	P-value
Average daily value			
SBP (mmHg)	114.67±4.80	116.56±5.39	0.035
DBP (mmHg)	70.87±5.14	71.98±4.90	0.107
Heart rate (bpm)	69.86±5.72	71.02±4.41	0.348
Mean arterial pressure (mmHg)	90.95±4.87	92.40±4.98	0.022
Pulse pressure (mmHg)	43.79±2.27	44.56±2.43	0.368
Average day - active period (awake)			
SBP (mmHg)	116.10±4.47	118.77±3.57	0.000
DBP (mmHg)	72.95±3.58	74.20±2.32	0.000
Heart rate (bpm)	72.25±4.34	73.02±2.55	0.011
Mean arterial pressure (mmHg)	92.73±3.85	94.61±2.66	0.000
Pulse pressure (mmHg)	43.14±2.20	44.57±2.63	0.430
Average night - passive period (asleep)			
SBP (mmHg)	111.23±3.83	111.18±5.37	0.967
DBP (mmHg)	65.86±4.97	66.63±5.43	0.326
Heart rate (bpm)	64.08±4.43	66.17±4.24	0.159
Mean arterial pressure (mmHg)	86.63±4.43	87.07±5.32	0.615
Pulse pressure (mmHg)	45.37±1.63	44.54±1.96	0.447
Post-Dose Mean (3 hours)			
SBP (mmHg)	114.67±2.82	118.14±3.23	0.062
DBP (mmHg)	73.33±1.52	73.96±1.60	0.622
Heart rate (bpm)	71.44±2.62	72.43±2.30	0.563
Mean arterial pressure (mmHg)	92.21±1.84	94.15±2.25	0.103
Pulse pressure (mmHg)	41.24±2.42	44.18±1.91	0.100
BP DIP			
SBP (mmHg)	-4.87	-7.60	0.080
DBP (mmHg)	-7.09	-7.57	0.683
Heart rate (bpm)	-8.17	-6.85	0.277
Mean arterial pressure (mmHg)	-6.10	-7.55	0.219
Pulse pressure (mmHg)	2.23	-0.03	0.083
BP DIP %			
SBP (mmHg)	-4.19	-6.40	0.083
DBP (mmHg)	-9.72	-10.20	0.744
Heart rate (bpm)	-11.31	-9.38	0.22
Mean arterial pressure (mmHg)	-6.58	-7.98	0.221
Pulse pressure (mmHg)	5.41	0.45	0.095
Max Value			
SBP (mmHg)	122.38	125.28	0.472
DBP (mmHg)	77.80	78.70	0.242
Heart rate (bpm)	84.51	77.94	0.433
Mean arterial pressure (mmHg)	97.20	100.03	0.203

Pulse pressure (mmHg)	47.83	51.65	0.374
Min Value			
SBP (mmHg)	104.25	105.15	0.28
DBP (mmHg)	58.90	59.58	0.957
Heart rate (bpm)	58.13	59.71	0.204
Mean arterial pressure (mmHg)	80.70	81.03	0.362
Pulse pressure (mmHg)	38.45	40.35	0.611

Values are means ± SD

Pulse pressure refers to SBP minus DBP.

Mean arterial pressure refers to MAP =1/3 (SBP – DBP) + DBP

BP DIP refers to active period minus passive periods.

4.3.7 TEAC and FRAP

There was a significant difference in TEAC at 6h following high and low nitrate lettuces consumptions (P=0.03) (Figure 4.14). There was no significant difference in FRAP post high and low nitrate lettuce ingestions. However, the FRAP values were decreased after high and nitrate lettuce ingestions (Figure 4.15). The repeated measure ANOVA revealed no significant difference was observed between high and low nitrate lettuces for TEAC (P=0.325) and FRAP (P=0.268).

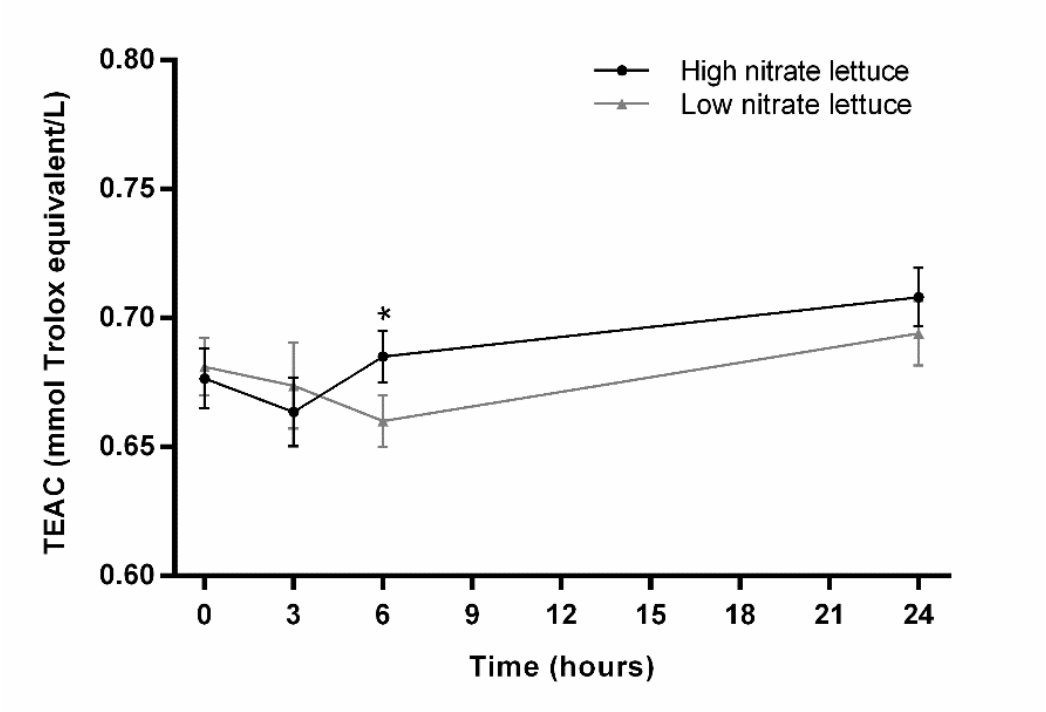


Figure 4.14 The effect of high and low nitrate lettuce on TEAC.

Data expressed as mean ± 95% CI. Effects were analysed by General linear Model – Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of (P<0.05) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *P < 0.05; **P < 0.01; ***P < 0.001.

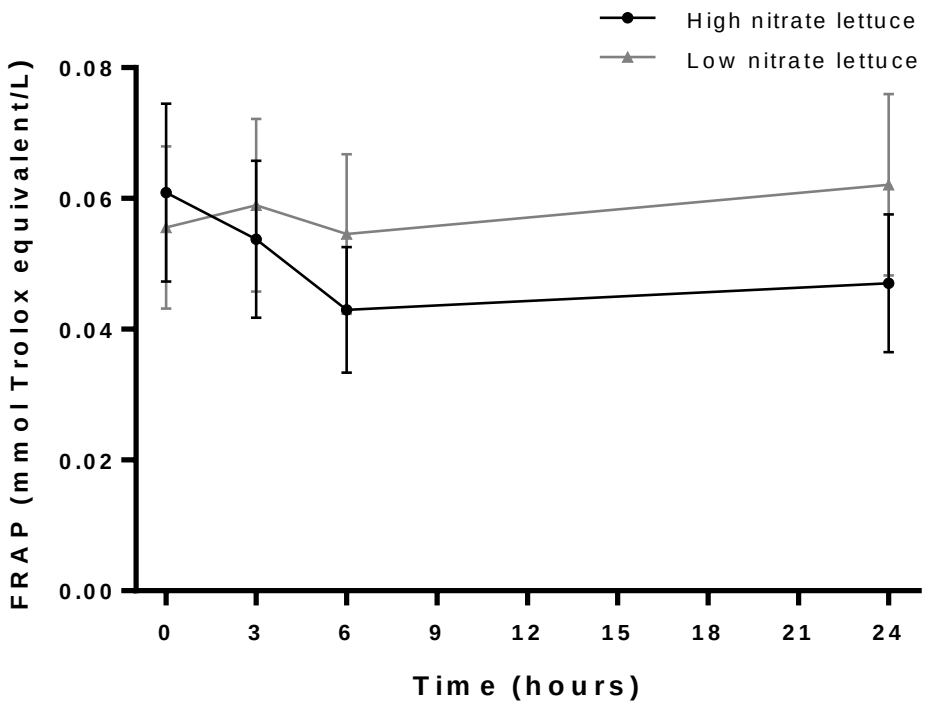


Figure 4.15 The effect of high and low nitrate lettuce on FRAP.

Data expressed as mean ± 95% CI. Effects were analysed by General linear Model – Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.3.8 Cyclic guanosine monophosphate (cGMP)

Figure 4.16 shows mean of plasma cGMP following high and low nitrate ingestions. The cGMP plasma was almost increased after 6h of high nitrate lettuce ingestion, but statistically, no significant difference was observed relative to the baseline value, and the same effects remained up to 24h. Surprisingly, there was no significant difference in the effect of high and low nitrate lettuce on cGMP at different time points relative to baseline values for up to 24h. In addition, the mean of AUC of high nitrate lettuce was higher than low nitrate lettuce (see appendices of chapter 4).

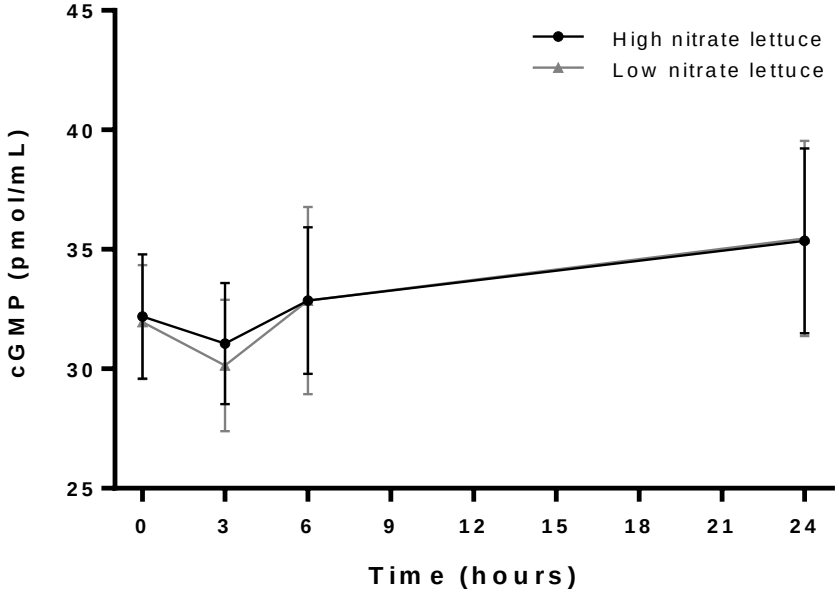


Figure 4.16 The effect of high and low nitrate lettuce on cGMP.

Data expressed as mean ± 95% CI. Effects were analysed by General linear Model – Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce (n=20) vs low nitrate lettuce (n=20) of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

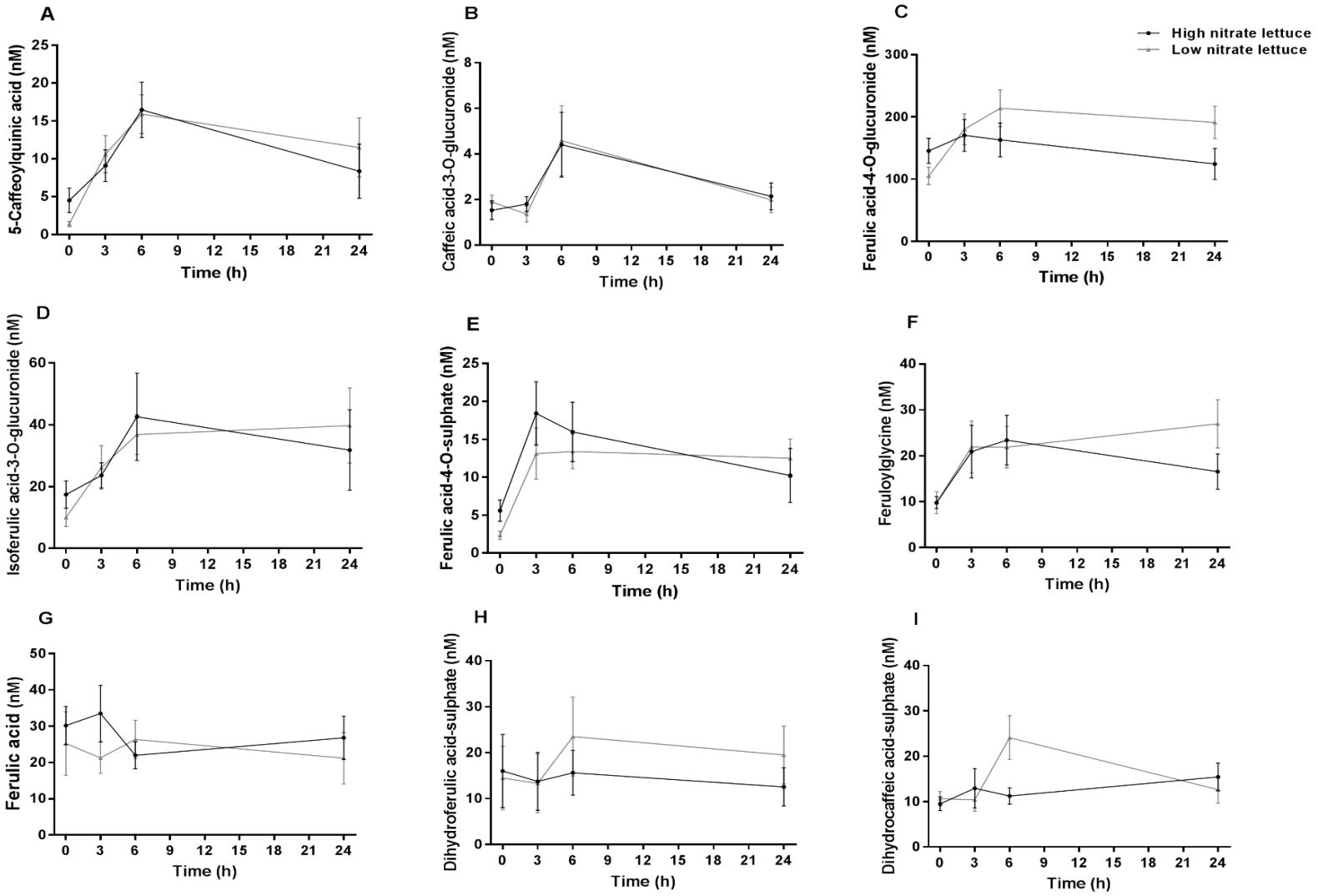


Figure 4.17. (A, B, C, D, E, F, G, H and I), the effect of high and low nitrate lettuce on caffeoylquinic acid-derived metabolites.

Data expressed as mean ± 95% CI. Effects were analysed by General linear Model – Repeated Measure ANOVA in Minitab. Significant shown for comparisons between high nitrate lettuce vs low nitrate lettuce of ($P<0.05$) for Pairwise Bonferroni test. *, **, *** Different from low nitrate lettuce: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.4 Discussion

This is the first study to use lettuce with high and low nitrate contents as the treatment and placebo with the same appearance in an acute human intervention trial. Recently, the attention to the effects of green leafy vegetable consumption on vascular health has been increased due to their high dietary nitrate content (Liu *et al.*, 2013; Jovanovski *et al.*, 2015). As a model to assess this concept, two sets of lettuces were produced, and the change in SBP and DBP measured after ingestions in healthy young subjects. The results demonstrated that high nitrate lettuce containing ~530mg of nitrate, increased plasma nitrate, salivary nitrate and nitrite and urinary nitrate. These findings indicate that dietary nitrate may enhance an increase of the NO circulation through the entero-salivary circulation via NO_3-NO_2-NO pathway (Govoni *et al.*, 2008; Bondonno *et al.*, 2014). The level of nitrate contained in the high nitrate lettuce was 8.5mg/kg body weight vs. 0.04mg/kg body weight in the low nitrate lettuce. Miller *et al.* (2012) reported that a similar amount of nitrate intake (527mg, corresponding to 6.5mg/kg body weight/day) in a high nitrate beetroot juice supplement was used to assess the effect of plasma nitrate and nitrite in older adults.

Plasma NO_3 increased by nearly 5-fold 3h post ingestion of high nitrate lettuce ($P<0.001$), and then the effects was reduced to 4-fold after 6h relative to baseline values ($P<0.001$). The plasma nitrate levels and changes following the high nitrate lettuce in accordance with the results reported by (Webb *et al.*, 2008). Dietary nitrate returned to a baseline of plasma and saliva nitrate and nitrite within 24h (van Velzen *et al.*, 2008; Bondonno *et al.*, 2015a). This result is in agreement with the data obtained by (Miller *et al.*, 2012) that plasma nitrate peaked at 2h after the consumption of the high-nitrate beetroot juice. The results of plasma nitrate concentration peaked 395 ± 133μM (Mean ± SD) after 3h of ingestion high nitrate lettuce ($P<0.001$), which is almost in line with the results in recent research paper by Jonvik *et al.* (2016) a semi-randomized, crossover study observed the peak plasma nitrate concentrations were similar (NaNO3: 583 ± 29μmol/L, beetroot juice: 597 ± 23μmol/L, rocket salad beverage: 584 ± 24μmol/L and spinach beverage: 584 ± 23μmol/L) after 2h an ingestion of almost 800mg dietary nitrate provided in 4 different sources. Surprisingly, in the present study, there was no significant difference in the plasma nitrite concentrations after consumption of the high and low nitrate lettuces. It was expected that the high nitrate lettuce would cause a significant rise in plasma nitrite compared with the low nitrate lettuce, this is because of the instability of nitrite after blood collection if the samples are not processed immediately, then nitrite can be quickly oxidized to nitrate (Wang *et al.*, 2013).

These results clearly show that high nitrate lettuce can lead to substantial increases within 1h in the salivary nitrate and nitrite concentration, peaking at 2 and 3h, respectively. These results are in agreement with those obtained by (Tannenbaum *et al.*, 1976), they found the same change and raise in salivary nitrite and nitrite in 14 individuals after consumption 200ml of celery juice which contained 240mg $NaNO_3$. It has been reported that after nitrate absorption in stomach and the upper gastro-intestinal tract ~25% of ingested nitrate enter the entero-salivary circulation through the plasma into the saliva where it is reduced to nitrite by facultative anaerobic bacteria on the surface of the tongue (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994; Lundberg and Govoni, 2004; Webb *et al.*, 2008; Hobbs *et al.*, 2012). This finding confirmed that there is a linear relationship between salivary nitrite and dietary nitrate intake (Spiegelhalder *et al.*, 1976). On the other hand, an amount 20-25% of nitrate is absorbed quickly and is secreted in body fluids such as saliva and sweat following ingestion of high nitrate lettuce (Ruddell *et al.*, 1976; Hobbs *et al.*, 2012).

The results show that it took more than 12h after the consumption of high nitrate lettuce until the excretion rate returned to baseline and that the amount of nitrate excreted through urine during more than 24h increased from 104mg with low nitrate lettuce to 391mg (P=0.015) with the high nitrate lettuce. This 287mg increase in urinary excretion corresponded to 54% of the 527mg additional dietary intake of nitrate. The amount of nitrate excreted in urine 24h after low nitrate lettuce ingestion in a good agreement with the results obtained by (Green *et al.*, 1981) under the condition of low nitrate ingestion. In another study on the effect of dietary nitrate and nitrite on urinary nitrate excretion, the percent recovery of nitrate and nitrite as urine nitrate could be calculated during the three feeding periods. This was 57% (nitrate), 59% (nitrite), and 52% (nitrate + nitrite) (Granger *et al.*, 1991). In a clinical study, the recovery of urinary nitrate excretion was approximately 75% after 4 to 6h of ingesting of high nitrate meal (Pannala *et al.*, 2003).

These findings confirm that dietary nitrate is highly bioavailable. These results are in accord with findings reported by (Schultz *et al.*, 1985) which is reported that 40–45% of nitrate intake appears to be metabolised in the body or denitrified by the enteric colon bacteria rather than excreted via the urine in rats, the other half disappears through mammalian cell processes. Although, these results of presents were in accordance with a study published by (Bartholomew and Hill, 1984) where they found that 65-70% of a challenge dose of nitrate was excreted in the urine of 7 volunteers after oral doses of 25, 50, 100 and 170mg potassium nitrate (KNO_3) in distilled water, which was given 72h after the volunteers were put on nitrate free diets. Moreover, other researchers also stated that 60% of ingested nitrate was excreted as ^{15}[NO3]

labelled nitrate with the urine following 48h of an oral dose 217mg and approximately 0.1% found in the faeces (Green *et al.,* 1981). Some of the nitrates appeared 3% in urine and a tiny amount 0.2% found in faeces in the form of ammonia NH_3 or urea CH_4N_2O. The loss of 35% of ingested nitrate is unidentified (Wagner *et al.,* 1983). However additional research is required to determine whether dietary nitrate may suppress the endogenous synthesis of nitrate via L-arginine and NO, or if the remaining 240mg nitrate from the lettuce was lost via other routes, e.g. excreted in faeces, via sweat, exhaled via the air etc. (L'Hirondel, 2001), absorbed from the colon or metabolized to nitrogen or protein by the gut bacteria (Bartholomew and Hill, 1984). In future investigations, it might be possible to determine the 24h urinary nitrate excretion from a different source of dietary nitrate intake and use it as an indicator to assess the bioavailability of nitrate in the circulation system and achieve a further understanding of the pharmacokinetic properties of nitrate.

The present study investigated the effects of high nitrate lettuce as inorganic nitrate source on blood pressure in healthy subjects. It has been demonstrated by (Gilchrist *et al.,* 2010) that eating a large amount of healthy vegetable-rich diet have many positive effects on human health, which may prove to be useful in preventing and treating a wide range of diseases such as those with high blood pressure and atherosclerosis in future studies. However, there is still need to evidence in future studies whether it is useful in preventing high blood pressure. Therefore, any attempt on this aspect will help us to determine the mechanism of the effect of vegetables with high nitrate content on human health. The results demonstrated that high nitrate lettuce containing ~530mg nitrate linked to lower postprandial SBP and DBP compared with the low nitrate lettuce placebo. The AUC of ΔSBP reductions was of greatest size for the period 3-6hr post ingestion, corresponding to the period with the highest salivary nitrate and nitrite concentrations and so presumably also the largest increase in NO-generation inside blood vessels. The results thus supported the hypothesis of this study that the vegetable-derived nitrate intake caused the beneficial effects on lowering BP. However, the phenolic acid concentrations were different in the high and low nitrate lettuces but the results of caffeoylquinic acid-derived metabolites in plasma shows no significant differences (Figure 4.17). Therefore, we can assume that the effect on lowering BP comes from the nitrate content in the lettuce. In addition, some notice has been recorded that some of the participants reported about a slightly unpleasant taste of low nitrate lettuce, this might be because of the high phenolic acid contents.

Some authors claim that high nitrate lettuce can be used as a superfood for athletic performance to reduce the oxygen cost of low-intensity exercise (Bailey *et al.,* 2009; Vanhatalo *et al.,* 2010). In comparison, the ADI for nitrate is 3.7mg/kg body weight/day which established by WHO

(Alexander *et al.*, 2008), which is much less than what was used in the present study and those other studies.

On the other hand, one unanticipated finding was that no significant difference was observed for cGMP in the response of high and low nitrate lettuces. We have also believed that changes in cGMP could be associated with the phenolic acids and flavonoids of low nitrate lettuce, which may contribute to increasing the cGMP and lowering BP. Also, it has been mentioned in a review paper that polyphenolic compounds derived from fruit and vegetable have been shown as a stimulator in endothelium-dependent vasodilation effects to be mediated by the NO–cGMP pathway (Morton *et al.*, 2000).

A limitation of the study is that the postprandial blood samples were only taken every three hours, it would have been better to take them every hour as for the saliva samples. Thus, it is possible that plasma nitrate was affected by high nitrate lettuce or low nitrate lettuce ingestions in between the sampling times and may have occurred in association with variations in BP. However, the saliva samples could be used to monitor the combination between the nitrate circulation and BP.

In summary, this trial showed that lettuce produced with different fertilizer treatments could be sufficiently different to be used in a double-blind study to measure how much dietary nitrate intake could affect blood pressure in health young adults. Furthermore, the trial was well designed to detect a lowering of blood pressure between the high and low nitrate lettuces in males and females by using 24-hour AMBP. In addition, patterns of urinary nitrate excretion indicated that adherence of the participants to the lettuce consumptions was excellent. It is also important to highlight that the lowering of blood pressure was observed in healthy participants, so the results are relevant for improvements of athletic performance (short term), but not necessarily for participants with hypertension, whose blood pressure regulation does not function normally (Siervo *et al.*, 2015). Therefore, we suggest assessing the effect of high and low nitrate lettuces using the same trial design in at risk populations such as older individuals or hypertension patients to compare the outcomes, since this could show whether the difference between older and younger subjects is caused by differences in uptake of nitrate from food into blood and saliva, or in the body's response to (similarly) elevated nitrate and nitrite contents in blood and saliva.

Chapter 5. General discussion

5.1 Nitrate and nitrite analysis

Numerous of analytical methods have been reported to determining nitrate and nitrite in biological fluids, including spectrometry (Giovannoni *et al.*, 1997), ion chromatography (Everett *et al.*, 1995), liquid chromatography (El Menyawi *et al.*, 1998), GC-MS (Kage *et al.*, 2000) and capillary electrophoresis (Friedberg *et al.*, 1997). Many studies have shown that parallel comparison of nitrite and nitrate quantification by different methods produces diverging and difference values, mostly due to methodological difficulties (Tsikas *et al.*, 1997; Becker *et al.*, 2000). The amounts of nitric oxide (NO) and its metabolites appear to have an important effect on human health and disease development and it is important to monitor NO synthesis in order to correlate changes in NO levels with disease (Bories *et al.*, 1999). Due to its short half-life of less than 0.1 sec (Kelm and Schrader, 1990), most quantitative methods have measured nitrate and nitrite in biological samples as markers of NO release. In the presence of oxygenated haemoglobin, oxygen and superoxide, circulating NO is rapidly converted to both nitrite and nitrate (Helmke and Duncan, 2007). Therefore, the first aim of this thesis was to adapt the PFB-Br – GC-MS isotope dilution method to the analysis of nitrate and nitrite in vegetables and human biological fluids. GC-MS is considered the most accurate technique for the quantitative determination of nitrite and nitrate in biological samples (Romitelli *et al.*, 2007). Plasma nitrate and nitrite can be analysed by gas chromatography, the derivatization of nitrate and nitrite followed by the GC-MS analysis is an excellent procedure for the identification and determination of these compounds (Kage *et al.*, 2000). The method was based on the conversion of nitrate to nitrite into α-nitro-pentafluorotoluene using pentafluorobenzyl (PFB) bromide as the alkylating agent and [^{15}N] labelled nitrate and nitrite as the internal standards with the samples. Up to now, this is the only derivatization reaction that allows for the simultaneous analysis of nitrite and nitrate by GC–MS (Tsikas, 2000).

Chapter two shows the details of the modified GC-MS method with improvements in sample preparation which includes the size of the sample (100µL), increasing the volume of spiking solutions, acetone, PFB-Br (Qadir *et al.*, 2013). Also, effects of the time and temperature of derivatization reaction on formation and stability of PFB-NO_3 and PFB-NO_2, dry nitrogen flushing to remove the water content in the samples and acetone after the derivatization, in addition to some improvements in the GC-MS method file which include the sampler port, GC and MS acquisitions. The importance of this modified GC-MS method to this project was to achieve a reliable method to determine nitrate content in produced lettuce with different

nitrogen applications. In chapter three of this thesis, it is described how this and other methods were used to measure the effects of different concentrations of nitrogen fertilisers (26, 39, 51, 103 and 154ppm N) on nitrate content in two varieties of lettuce (Butterhead and Oakleaf). Additionally, biological fluids (urine, saliva and plasma) samples from the intervention trial study (Chapter four) were also successfully analysed by the modified method (Tannenbaum *et al.*, 1976; Webb *et al.*, 2008).

The methods have produced diverging values of nitrite and nitrate in particular in biological samples (Table 2.3). The relatively great differences regarding nitrite and nitrate levels in the circulation of healthy humans can only in part be explained by differences in dietary nitrate intake (Tsikas, 2005).

5.2 The association between nitrogen fertiliser and phytochemicals in vegetables

Nitrogen fertiliser is directly associated with the accumulation of nitrate in plants. Nitrate contents generally change in green leafy vegetables in line with the change in nitrogen concentration of fertiliser applications, in addition to other factors such as temperature, light and water supply (Pavlou *et al.*, 2007). The use of different N was especially to determine the effect of N applications on nitrate content and phytochemical compounds in growth chamber lettuce as a model to be used in a further study. As mentioned in chapter three, the purpose of testing different N concentration with two varieties of lettuce (BL and OL) was to produce two sets of lettuce containing high and low nitrate as treatment and placebo, respectively, to examine the short-term effects on blood pressure, in particular, systolic BP.

BL and OL were supplied with different N fertiliser concentrations and obtained different nitrate levels (Figure 3.3 and 3.4). N significantly increased plant nitrate content, the effect being extra marked at the highest rate of fertiliser application (154ppm). The significant increase of nitrate in BL with increased nitrogen supplies is in good agreement with previous reports (Hansen, 1978; Greenwood and Hunt, 1986; Hähndel and Wehrmann, 1986).

The plant biomass and dry matter of the lettuces were significantly decreased by the high nitrate concentrations. These findings are in accord with recent studies by (Sørensen *et al.*, 1994) who demonstrated that the dry matter content decreased in crisphead lettuce with increased nitrogen supply of 50, 100, 150 and 200 kg N per ha, these N levels conform with those in the current study; 7.21, 10.81, 14.41, 28.82, and 43.24 kg N per ha. However it contrasted to the findings by (Hansen, 1978), who found that the fresh weight of Butterhead lettuce was gradually

increased to 105, 134, 145, 176 and 147 g/plant with increasing N supplies of 0, 50, 100, 200 and 400 kg N per ha 54 days after planting with 14h day length in a field experiment. This discrepancy may be due to the low level of light in the growth chamber, which limited photosynthesis, causing the nitrate to accumulate in the plant rather than being consumed for additional growth as in the field (Santamaria *et al.*, 2001).

The total chlorophyll content was increased in BL and OL with increased N supplies. These results are in line with those of previous studies by (Fontes *et al.*, 1997) who reported that chlorophyll level was directly related to leaf N concentration and may help as a useful index for N status in lettuce and hence could be an assistance in predicting crop N supplies. It was outlined in chapter three that the increased N led to increases in carotenoid pigments, especially β-carotene. A possible explanation for these increases might be that there is a correlation between the accumulations of chlorophyll pigment and carotenoids (Mou, 2005). Moreover, vegetables are main sources of carotenoids in the human nutrition, β-carotene and lutein have been related to reduced risks of cancer and chronic disease (Chenard *et al.*, 2005). If confirmed, this may increase the nutritional value of lettuce, a question that could be investigated in the same way as the present investigation of nitrate.

Increasing the level of nitrogen fertilisation as ammonium nitrate decreased both the phenolic acids and flavonoids, especially this decline was marked for cichoric and caftaric acid, rutin and total flavonols in BL. This result has not previously been described, however a possible explanation for these results may be a lack of carbon in plant tissues which is associated with increased N concentration, the plant used most of the carbon in structural growth, and it is believed that the decreased carbon led to less soluble organic compounds in the plant (Hanafy Ahmed *et al.*, 2002). In this respect, nitrate accumulation was negatively related to the accumulation of soluble carbohydrates in the cell sap (Seginer *et al.*, 1997). There is a negative correlation between nitrate concentration and carbohydrates in lettuce because the lettuce has a demand for more nitrate. This may cause an increase in photosynthetic activity through which nitrate acts as an osmoticum and is replaced by sugars (Millard, 1988; Behr and Wiebe, 1992). The outcome of this idea is the decreases in phenolic acids are caused by the decreases in sugar content. The findings demonstrated by (Reinink and Biom-Zandstra, 1989) confirm that the increase in nitrate accumulations led to reduced organic acids in lettuce, additionally the difference in osmolarity could lead to differences in nitrate accumulation. However, more research on this topic needs to be undertaken before the association between nitrogen

fertilisation and phenolic compounds are more clearly understood. It would be possible to develop and standardised the phenolic concentrations of high and low nitrate lettuce by increasing sulphur application for the low nitrate lettuce (Li *et al.*, 2008).

In general, increasing the concentration of nitrogen fertilisation decreased both fresh biomass and DM of lettuce, increased chlorophyll content, β-carotene and other carotenoids in leaf tissue of lettuce and decreased phenolic acids and flavonoids, however, this decrease was accompanied by increasing nitrate concentration as it was intended. So the low-nitrate plants are not a perfect placebo for the high-nitrate treatment. However there are no reports of short-term effects of dietary carotenoids on blood pressure, and the increased amount of phenolic compounds in the low-nitrate lettuce only correspond to a cup of green tea (Kodama *et al.*, 2010), which would not be expected to cause a measurable change in blood pressure.

5.3 Effect of vegetable consumptions on the association between dietary nitrate intake and blood pressure

The study presented here is the first comprehensive study investigating the effect of a green leafy vegetable on BP, salivary nitrate and nitrite, plasma nitrate and nitrite, urinary nitrate and nitrite, and urinary nitrate excretion. The current work demonstrated in this thesis has a number of key strengths. The use of 24-h ABPM provides a robust and trustworthy method of measuring BP and allows direct comparisons with the changes in saliva composition during the post-prandial period. This is also the first study exploring the effect of dietary nitrate on BP using the treatment and placebo (high nitrate lettuce vs. low nitrate lettuce) with the same appearance.

As mentioned in the literature review, the role of vegetable intake in protection against cardiovascular diseases may be mediated by the nitric oxide, which has a role in endothelium tissue and diffuses into muscle causing vasodilation in blood vessels. Previously the protective effects of rich-nitrate vegetables have been examined against lowering BP in young and old individuals. There is a rapidly growing literature on consumption of beetroot juice and rich-nitrate foods due to the effect of nitrate present in this food on increasing the bioavailability of NO (de Oliveira *et al.*, 2016), which indicate that the dietary nitrate intake could be responsible for the effect on vascular health mainly in BP. These consequences suggest that alerting the public to increase dietary nitrate intake, which requires high consumption of green leafy vegetables and beetroot rich in inorganic nitrate, could be an important method to reduce the risk of cardiovascular disease in healthy subjects (Ghosh *et al.*, 2013; Gilchrist *et al.*, 2013;

Jajja *et al.*, 2014). However, the effect on lowering BP seems to be most pronounced in young people with a well-functioning vascular epithelium, since a meta-analysis study showed that there was no significant difference of the main effect of inorganic NO_3 on 24-hr ABPM outcomes in older subjects (55 years and older) (Siervo *et al.*, 2015). Due to this, young people were chosen for the present study.

On the other hand, some researchers also concerned about the potential risk of dietary nitrate, nitrite and nitrosamine and gastric cancer and this has been investigated by several studies. A recent meta-analysis study was conducted on relevant articles, the high nitrates intake was associated with a weak but statistically significant reduced risk of gastric cancer. Whereas increased consumption of nitrites and N-nitrosodimethylamine appeared to be risk factors for cancer. Further studies will requires to confirm these conclusions (Song *et al.*, 2015).

In recent years, numerous clinical intervention trials have been conducted aiming to specify the association between the dietary nitrate intake as a source of NO-mediated vasodilation through the NO_3-NO_2-NO pathway, decreasing BP in healthy young subjects (Bondonno *et al.*, 2015b). Hence, the physiologic target of NO is soluble guanylate cyclase, and NO activates guanylate cyclase, causing increased cGMP levels. Even though in the present study the difference between treatments was not significant, it is important that cGMP mediate NO-dependent relaxation of vascular smooth muscle cells and resulting in vasodilation in the blood vessels by increasing in cGMP production (Napoli and Ignarro, 2001; Napoli *et al.*, 2006; Napoli and Ignarro, 2009).

Moreover, ecological and epidemiological evidence suggest that diets rich in fruit and vegetables are directly associated with reducing the risk of cardiovascular disease in human. Predominantly, the consumption of nitrate-rich green leafy vegetables, such as lettuce, rocket and spinach could have the beneficial effects in raising plasma concentrations of phytochemicals and lowering blood pressure (John *et al.*, 2002). Furthermore, the Dietary Approaches to Stop Hypertension (DASH) diet is directly associated with a reduced risk of cardiovascular disease by lowering BP (Appel *et al.*, 1997); in particular, it may be the high nitrate vegetables that are linked to blood pressure reductions (Hord *et al.*, 2009).

This is the first trial to demonstrate that compared with the low nitrate lettuce, the high nitrate lettuce led to increased plasma NO_3, salivary NO_3 and NO_2, urinary NO_3 and that the time periods with the highest salivary NO_3 and NO_2 coincided with the greatest relative lowering of

blood pressure in healthy young adults. These findings confirm that the rich-nitrate green leafy vegetables could reduce blood pressure and that this effect was caused by the nitrate (or, hypothetically and much less likely, the chlorophyll or carotenoids), not by lettuce consumption as such. The increases in plasma nitrate, salivary nitrate and nitrite, and urinary nitrate after high nitrate lettuce contained (~530mg derived NO_3) demonstrated increased NO_3 metabolism through the entero-salivary NO_3-NO_2-NO pathway and the increase of molecules with the potential to be converted to NO (Bondonno *et al.*, 2015b). In addition, the patterns of urinary nitrate excretion indicated that the adherence of the participants to the lettuce consumptions was excellent.

The randomised, double-blind, crossover study was designed to investigate the effect of inorganic nitrate derived from high and low nitrate lettuce on 24-h ABPM. This trial was conducted in twenty healthy young participants. The repeated measure ANOVA outcomes show that there was a significant difference between the treatment (high nitrate lettuce) and placebo (low nitrate lettuce) in SBP (P=0.003) and DBP (P=0.002). No significant change was observed in mean heart rate and peripheral pulse pressure over the 24h post ingestion between high and low nitrate lettuces. The kinetics of nitrate was also monitored through a collection of biological samples (blood, saliva and urine) to measure the level of nitrate and nitrite after the lettuce ingestion for 24h.

The mechanisms of lowering blood pressure have not been fully clarified. As mentioned in chapter one, the literature states that the NO_3 can be converted to nitrite by bacteria on the tongue, and that this conversion is essential for these benefits. Meanwhile, the enterosalivary NO_3 reduction prevents the increase in plasma NO_2 level and blocks the decrease in blood pressure following high dietary nitrate intake (Webb *et al.*, 2008; Liu *et al.*, 2013). Our data confirmed this by showing that ingestion of high nitrate lettuce resulted in significant increases in salivary levels of both NO_3 and NO_2, indicating a substantial increase in NO_3 reduction to NO_2.

With respect to urinary nitrate excretion, on average the increased excretion of nitrate found in the urine corresponded to 54% of the increased intake from high nitrate lettuce. However, the amount of urinary nitrate excretions throughout the study were not similar for all the subjects. A limited number of articles exists assessing urinary nitrate excretion after the consumption of dietary nitrate foods in human adults. In fact, no such data are available for nitrate balance studies in healthy young adults, comparing the amount of urinary nitrate excreted after the

ingestion of high dietary nitrate vs. low dietary nitrate. However the results from the present study correspond well with other comparable trials: After consuming free nitrate diet for 72h, approximately 65-70% of nitrate is excreted following an oral dose of potassium nitrate (Bartholomew and Hill, 1984). Similarly, in a comparison study, the mean of urinary nitrate recovery were 66.7, 70.8 and 74.1% following the consumption of 200g ham (55 mg NO_3), nitrate in water (aqueous solution) (62 mg NO_3) and 20g lettuce (36 mg NO_3), respectively. Similarly, the recovery of urinary nitrate ranged from 61-78% following the consumption of lettuce in thirteen subjects. (Packer *et al.*, 1989).

Very little was found in the literature on the effect of inorganic nitrate on 24hr ABPM in healthy young volunteers. Therefore, the outcomes of this trial are most relevant for research assessing the effect of high intake of rich-nitrate vegetables to improve exercise performance and oxygen cost during exercise (Larsen *et al.*, 2007; Lidder and Webb, 2013; Wylie *et al.*, 2013). For instance, the consumption of 500ml beetroot juice contained 750mg NO_3 increased plasma nitrate and nitrite. This elevation associated with an increase in exercise performance which includes claudication onset time (COT), peak walking time (PWT) and a reduction in fractional O_2 extraction at the working tissues during cardiopulmonary exercise compared to placebo (Kenjale *et al.*, 2011). Another example shows dietary nitrate supplementation positively decreasing oxygen cost without increasing plasma lactate during submaximal work; this was achieved through a diet rich in vegetables (Larsen *et al.*, 2007). Other suggestions arising from this study are whether the changes in BP would have been different after long-term exposure to nitrate-rich green leafy vegetables and whether administration of a similar lettuce to hypertensive patients would have a significant result in lowering BP as for the normotensive subjects recruited in this trial.

5.4 Conclusion and future research

5.4.1 Conclusion

The present study was designed to determine the effect of different nitrogen application on nitrate and phytochemical compounds in lettuce in controlled growing conditions. Additionally, to manipulate nitrate concentration of the vegetables as a tool for investigating the effect of nitrate content of food on vascular health. In this context, two sets of lettuces were produced with different nitrate contents (high nitrate content vs. low nitrate lettuce);

- to test if they would have correspondingly different effects on blood pressure, as short-term effects on systolic and diastolic blood pressure;
- to examine the kinetics of nitrate in circulation system from a short-term high dietary nitrate intake in healthy young subjects in terms of urinary nitrate excretion, salivary nitrate and nitrite concentrations and plasma nitrate and nitrite concentration.

In summary, the original contribution to knowledge achieved from this work in this thesis are:

- The modified GC-MS method has been used successfully for nitrate and nitrite analysis in vegetables and biological samples and achieved excellent accuracy in results, which has been used in several studies.
- The study of plant growth has confirmed the effects of increasing nitrogen fertiliser on nitrate content and phytochemical compounds in lettuce. The nitrate content in lettuce affected by the excess in use of nitrogen supplies; decrease in fresh biomass and dry matter; increase in accumulation of plant pigments, especially total chlorophylls, β-carotene and total carotenoids in leaf tissue of lettuce; and decrease in total phenolic acids and total flavonols particularly in Butterhead lettuce.
- Altogether, the principle to use fertiliser to produce two sets of a food plant with different composition and effects on human health could have substantial potential effects for research and public health nutrition.

- The application in a clinical trial showed how to use lettuces in double-blind settings; measured as effects on BP and nitrate metabolism in healthy young subjects. Hence, increased plasma nitrate, salivary nitrate and nitrite, urinary nitrate were achieved together with lowering blood pressure. The results of urinary nitrate excretion indicate that 24h urinary nitrate analyses can be used in nitrate balance studies.

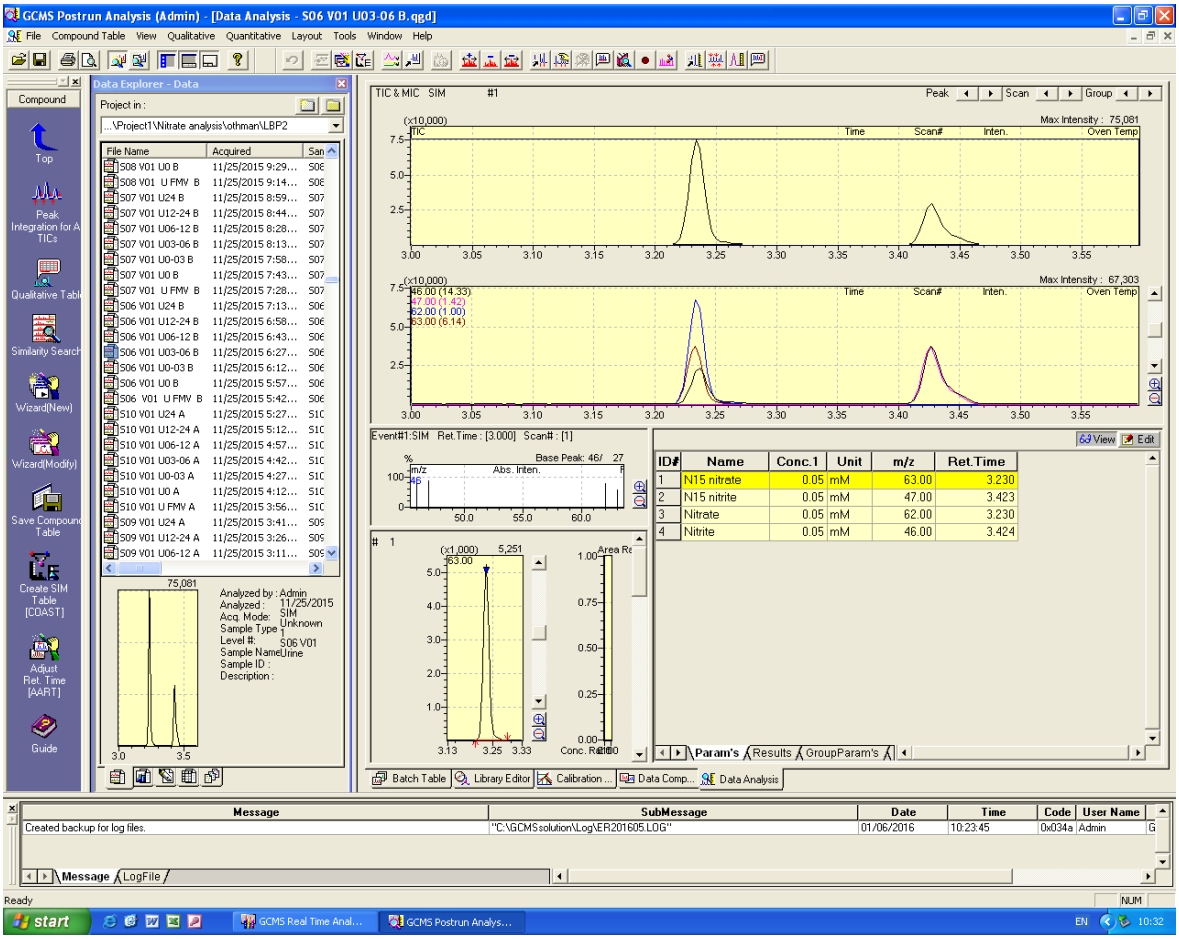
5.4.2 Recommendations for further research

This study has shown that increasing nitrogen fertiliser represents a viable strategy to modify nitrate content and phytochemicals in lettuce. However, the phytochemical contents of low nitrate lettuce were not similar to high nitrate lettuce. Therefore, future studies are required, for example, to confirm the effect of different concentrations of nitrogen fertilisers under conditions that eliminate differences in phenolic compounds in lettuce or to use nitrogen and other nutrients to change other phytochemical contents. Further research could also be conducted in multiple varieties of lettuce as a popular vegetable associated with lowering BP, for example, to enhance athletic performance.

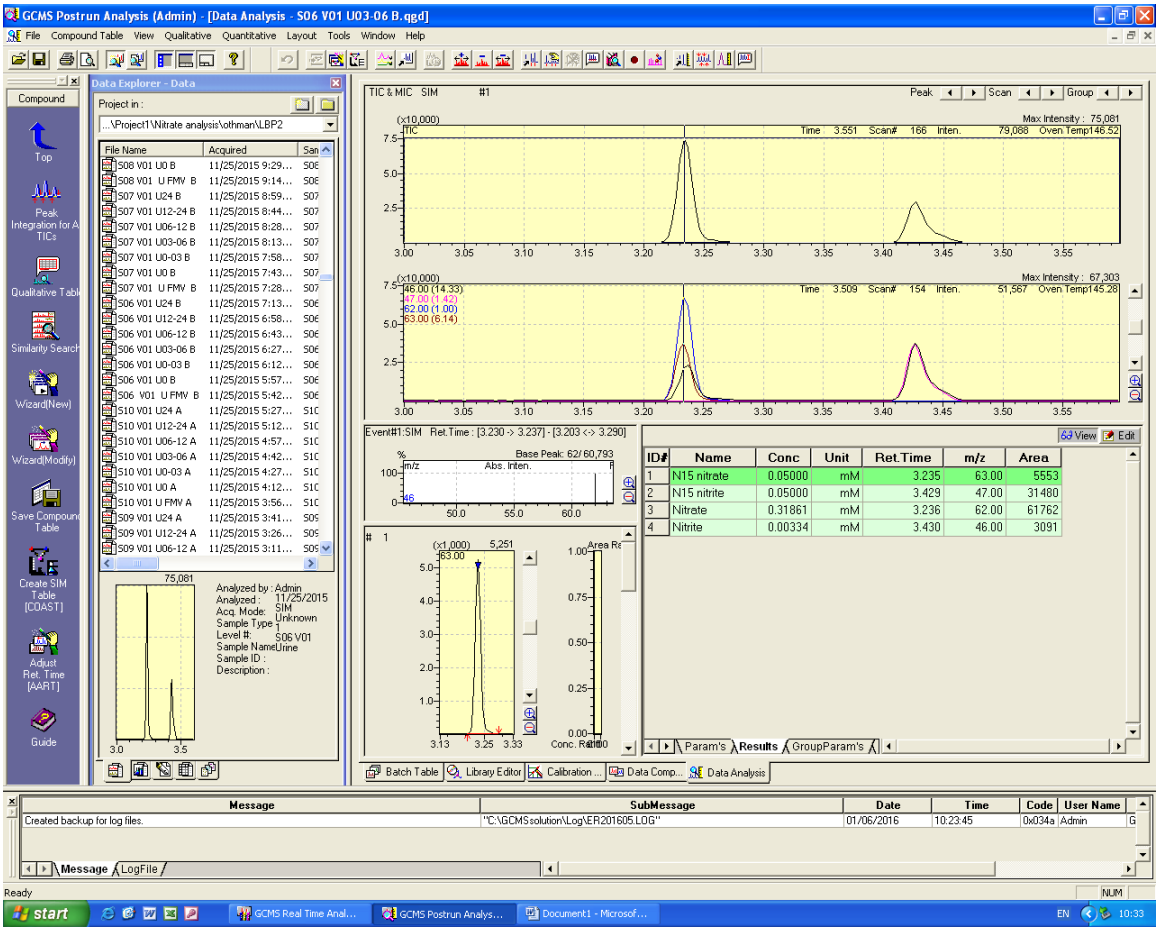
A limitation of the trial study is that we have used one portion of lettuce 50g and following the effect on pharmacokinetic of nitrate for 24h. Thus, future studies need to establish if longer-term regular intake of high nitrate lettuce can continue to lower blood pressure in healthy young subjects vs. older patients with hypertension as well as determine if it reduces the risk for cardiovascular diseases. Now that the principle of the method has been demonstrated in this short-term study, a longer-term randomised controlled trial could provide more definitive evidence.

Appendices

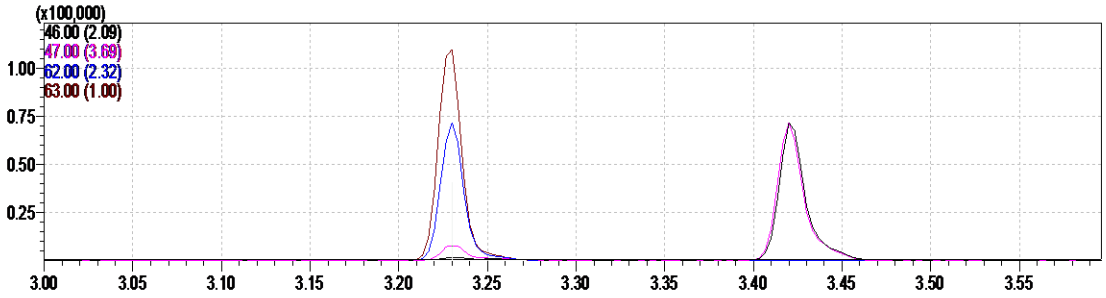
Appendices of chapter 2.



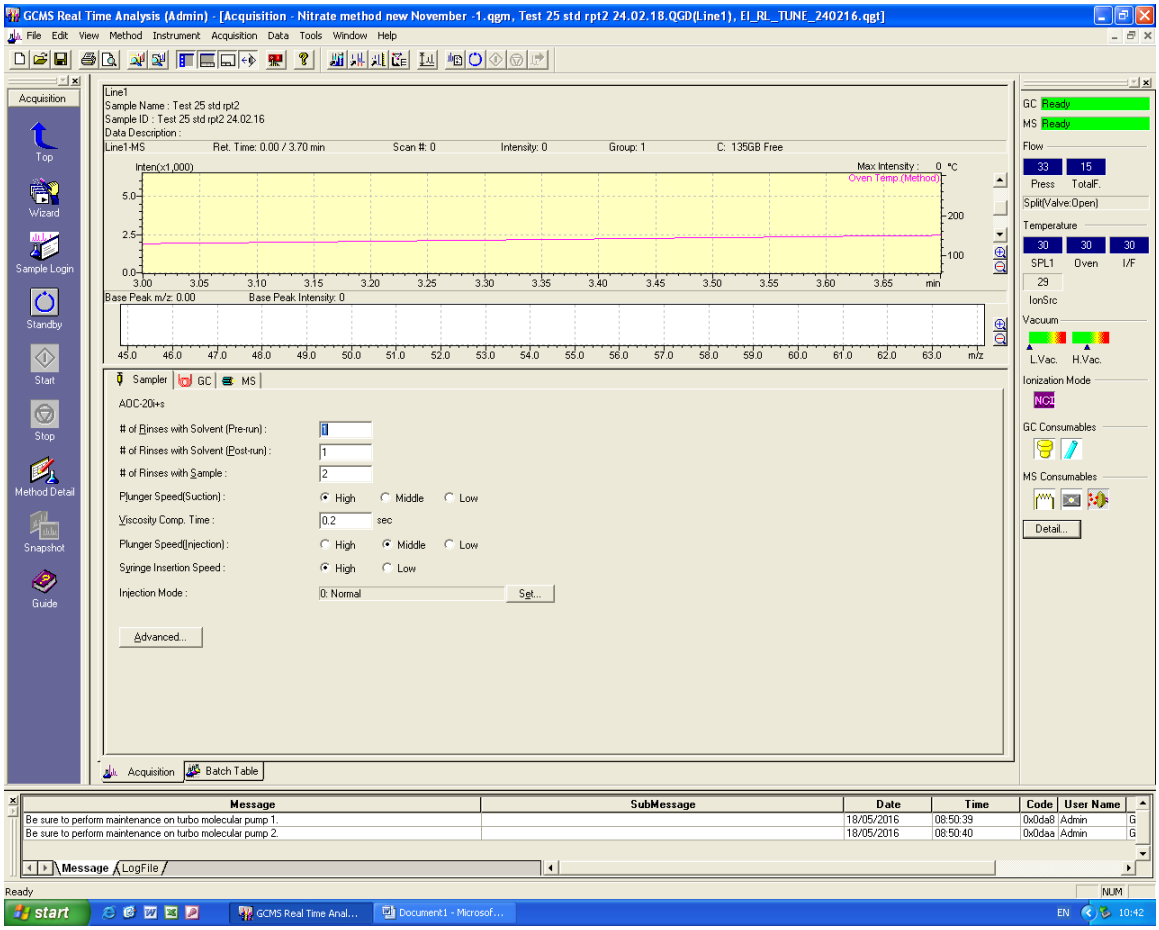
Appendix 1. Example of GC-MS chromatogram window and parameters of nitrate and nitrite



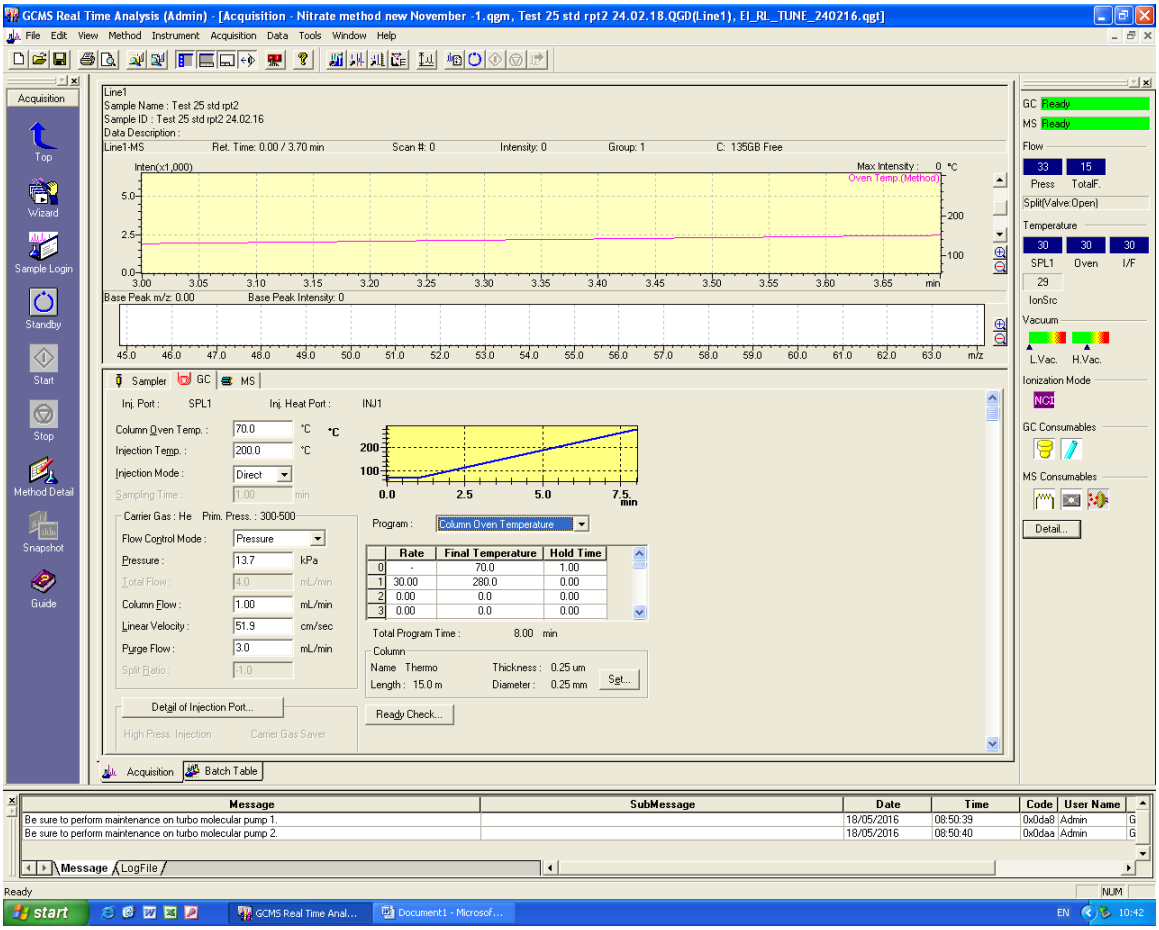
Appendix 2. GC-MS chromatogram window and results of nitrate and nitrite



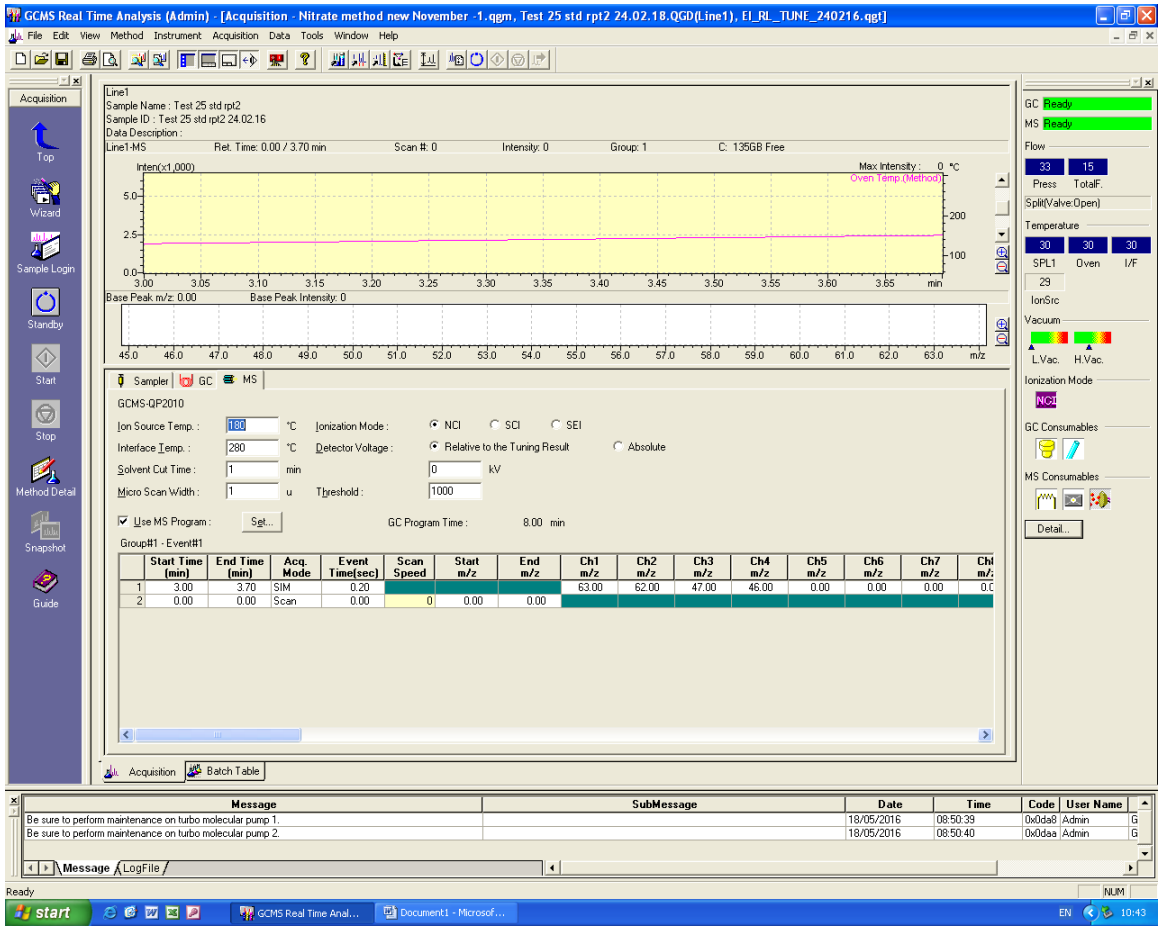
Appendix 3. Partial chromatogram from the simultaneous GC/MS analysis of NO_3 (SIM of m/z 62 and m/z 63) and NO_2 (SIM of m/z 46 and m/z 47) in the NICI mode in lettuce sample by the modified method.



Appendix 4. The GC-MS parameters of auto-sampler

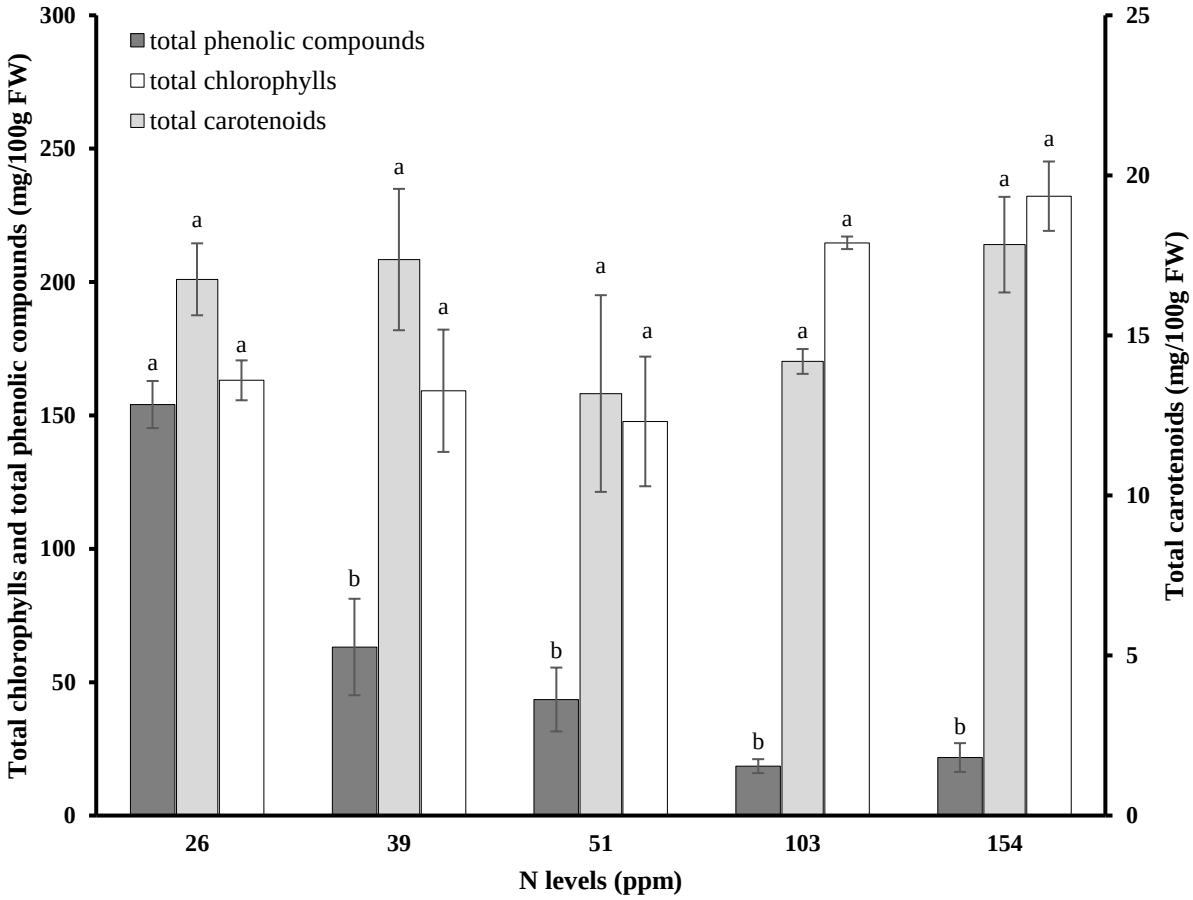


Appendix 5. The GC-MS parameters of gas chromatography tools



Appendix 6. The GC-MS parameters of mass spectrometry tools

Appendices of chapter 3.



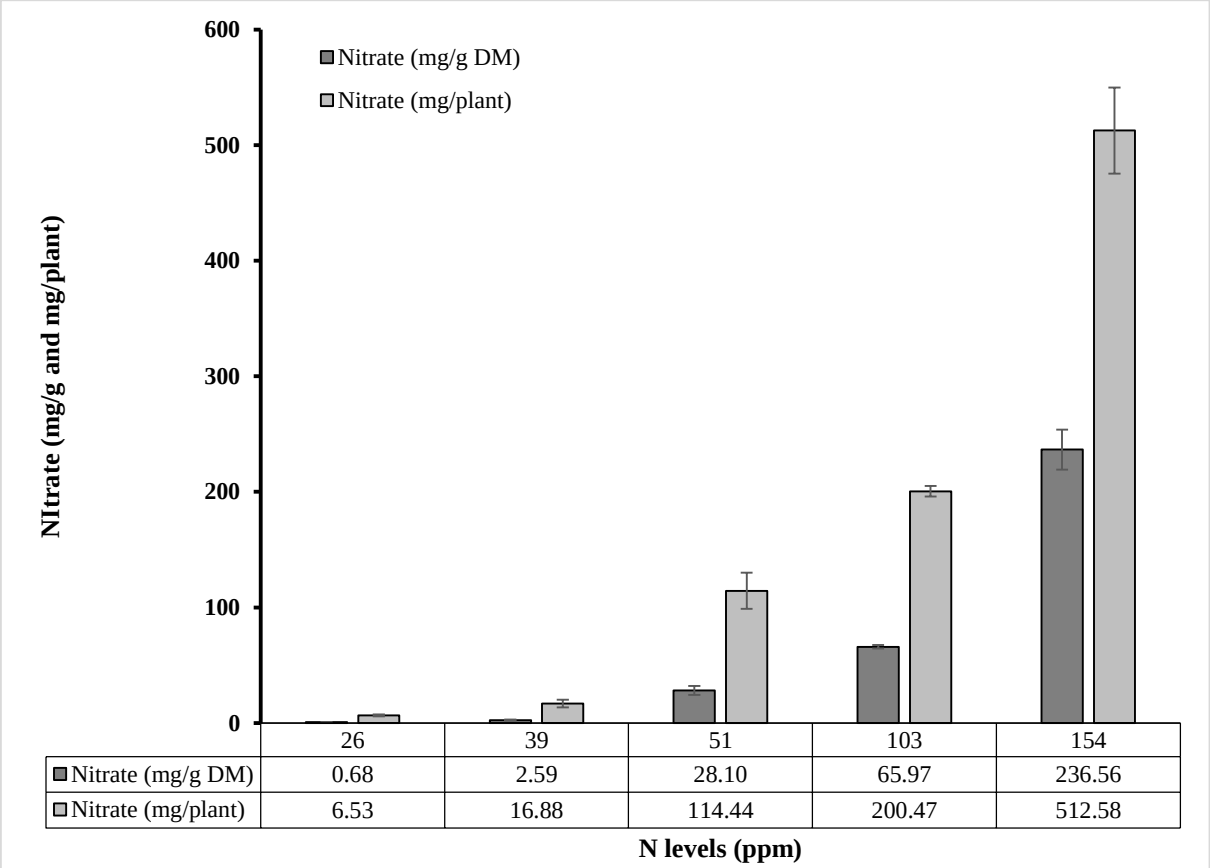
Appendix 7. Effect of nitrogen levels on total phenolic compounds, total chlorophylls and total carotenoids contents in Butterhead lettuce.

Values are the mean ± SEM of three plants for each N level (n=3). Bars with different letters indicate significant differences at $P < 0.05$ between different N levels.

Appendix 8. Carotenoids and chlorophylls in Butterhead lettuce [a]

Compounds	**Nitrogen levels (ppm in nutrient solution)**					
	26	**39**	**51**	**103**	**154**	***P-value***
β-carotene (µg/g DW)	680 ± 94 **b**	879 ± 184 **ab**	771 ± 263 **b**	948 ± 42 **ab**	1310 ± 223 **a**	0.014
Lutein (µg/g DW)	291 ± 34 **a**	382 ± 89 **a**	367 ± 155 **a**	397 ± 70 **a**	502 ± 59 **a**	0.159
Unknown carotenoids (µg β-carotene equivalents/g DW)	695 ± 119 **b**	779 ± 176 **b**	1037 ± 492 **ab**	1280 ± 51 **ab**	1626 ± 277 **a**	0.010
Total carotenoids (mg/g DW)	1.7 ± 0.2 **b**	2.0 ±0.4 **b**	2.2 ± 0.9 **ab**	2.6 ± 0.1 **ab**	3.4 ± 0.5 **a**	0.014
Chlorophyll b (mg/g DW)	1.9 ± 0.2 **b**	2.1 ± 0.4 **b**	5.0 ± 3.5 **ab**	6.8 ± 0.2 **a**	7.5 ± 1.0 **a**	0.005
Chlorophyll a (mg/g DW)	11.8 ± 1.4 **c**	14.1 ± 3.1 **bc**	16.8 ± 2.7 **abc**	2.9 ± 0.5 **ab**	3.2 ± 3.9 **a**	0.008
Total chlorophylls (mg/g DW)	16.2 ± 1.3 **c**	18.7 ± 4.7 **bc**	24.4 ± 17.4 **abc**	39.7 ± 0.8 **ab**	44.8 ± 4.3 **a**	0.006

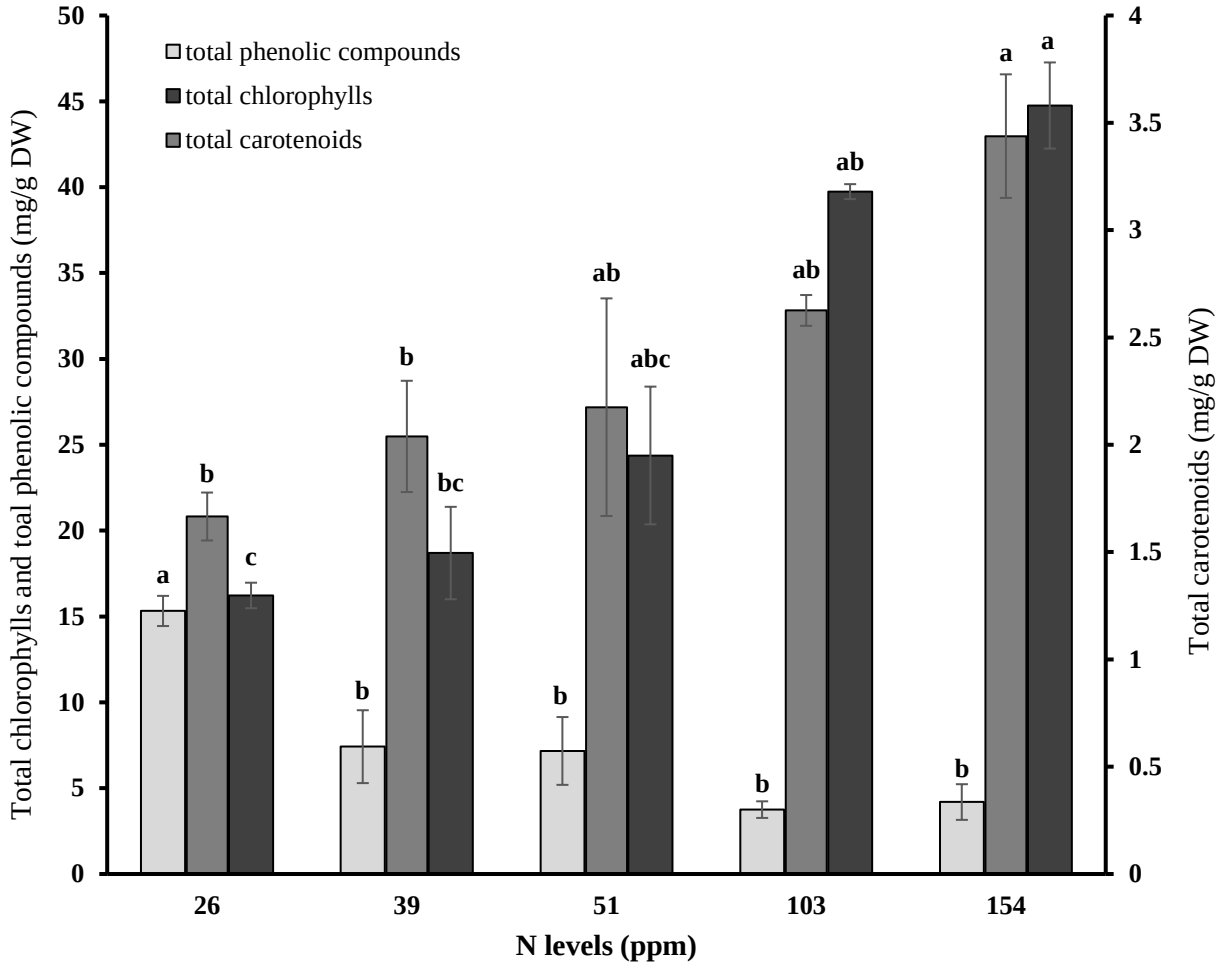
[a] Values are the mean ± SD of three plants for each N levels (n=3). In a row, different letters (a, b and c) indicate significant differences at $P < 0.05$ among nitrogen levels.



	26	39	51	103	154
Nitrate (mg/g DM)	0.68	2.59	28.10	65.97	236.56
Nitrate (mg/plant)	6.53	16.88	114.44	200.47	512.58

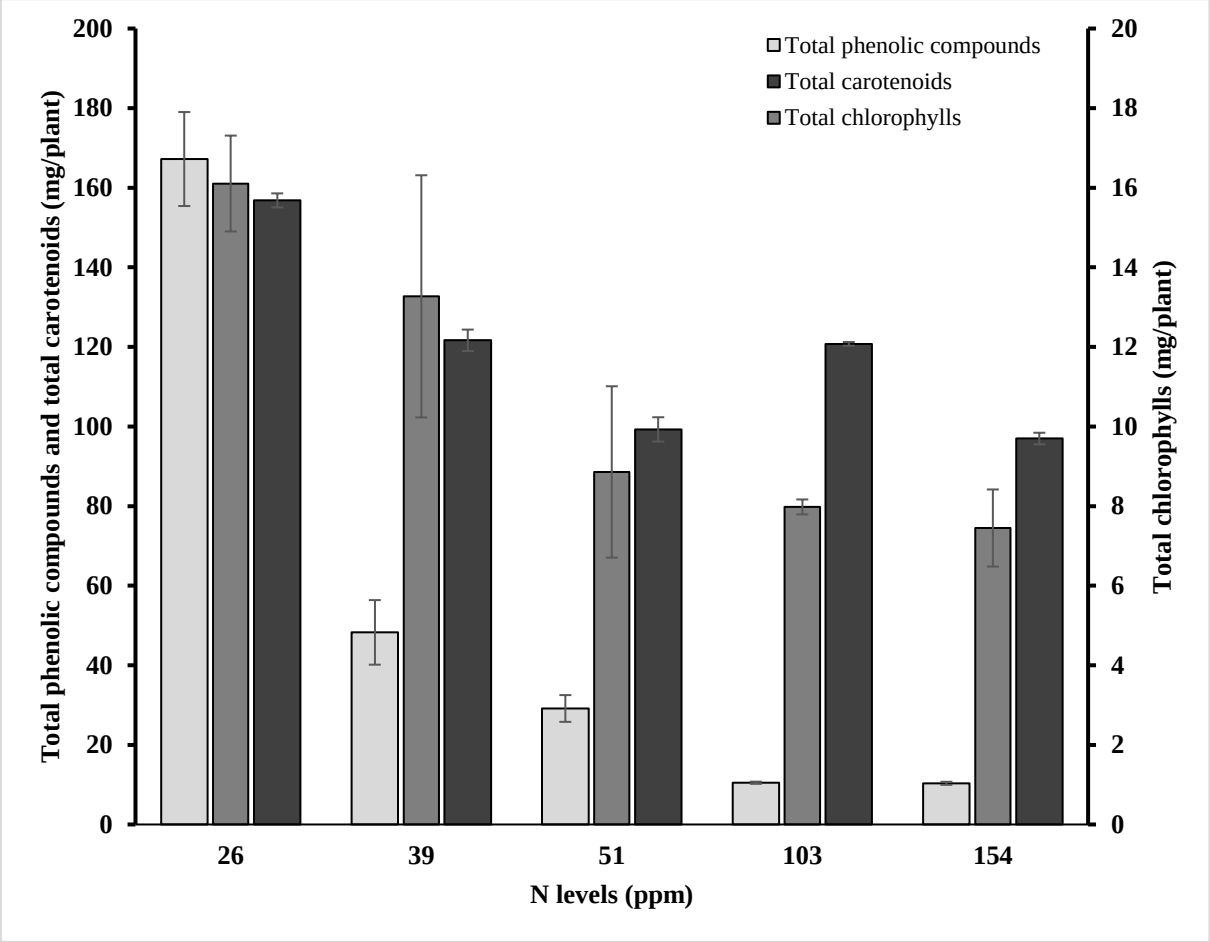
Appendix 9. Effect of nitrogen levels on nitrate content (mg/g dry matter and mg/plant) in Butterhead lettuce.

Values are the mean ± SEM of six plants for each N level (n=6).



Appendix 10. Effect of nitrogen levels on total phenolic compounds, total chlorophylls and total carotenoids contents (mg/g DW) in Butterhead lettuce.

Values are the mean ± SEM of three plants for each N level (n=3). Bars with different letters indicate significant differences at $P < 0.05$ between different N levels.

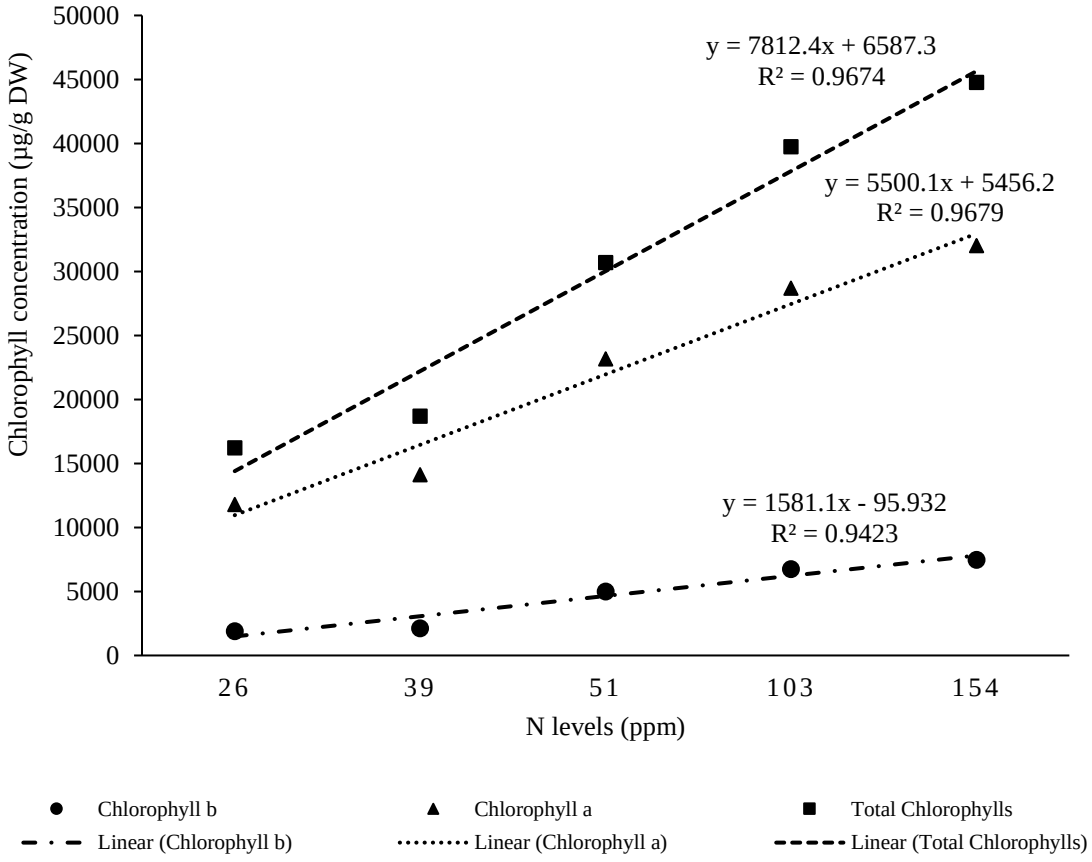


Appendix 11. Effect of nitrogen levels on total phenolic compounds, total chlorophylls and total carotenoids contents (mg/plant) in Butterhead lettuce.

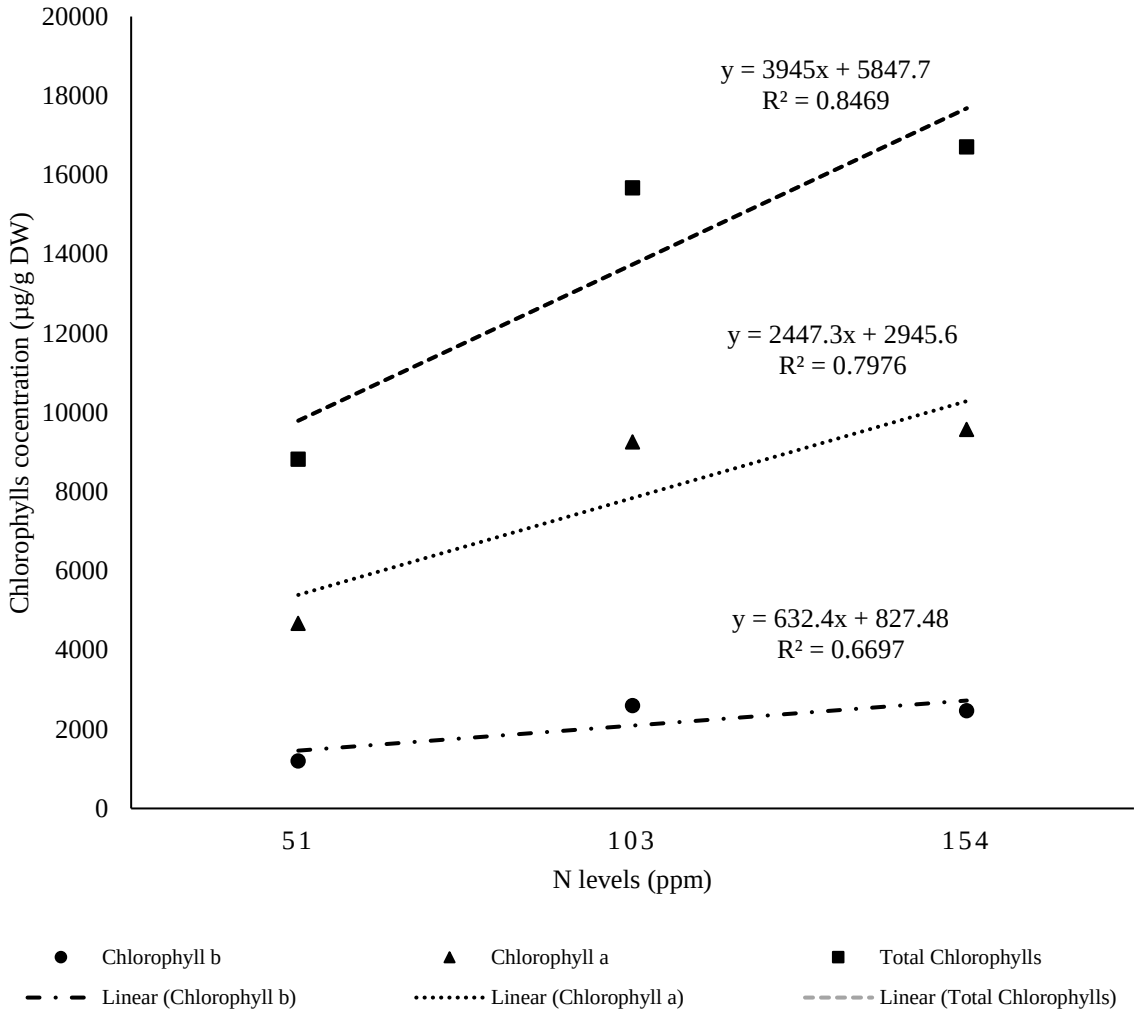
Values are the mean ± SEM of three plants for each N level (n=3).

Appendix 12. Oakleaf lettuce in growth chamber

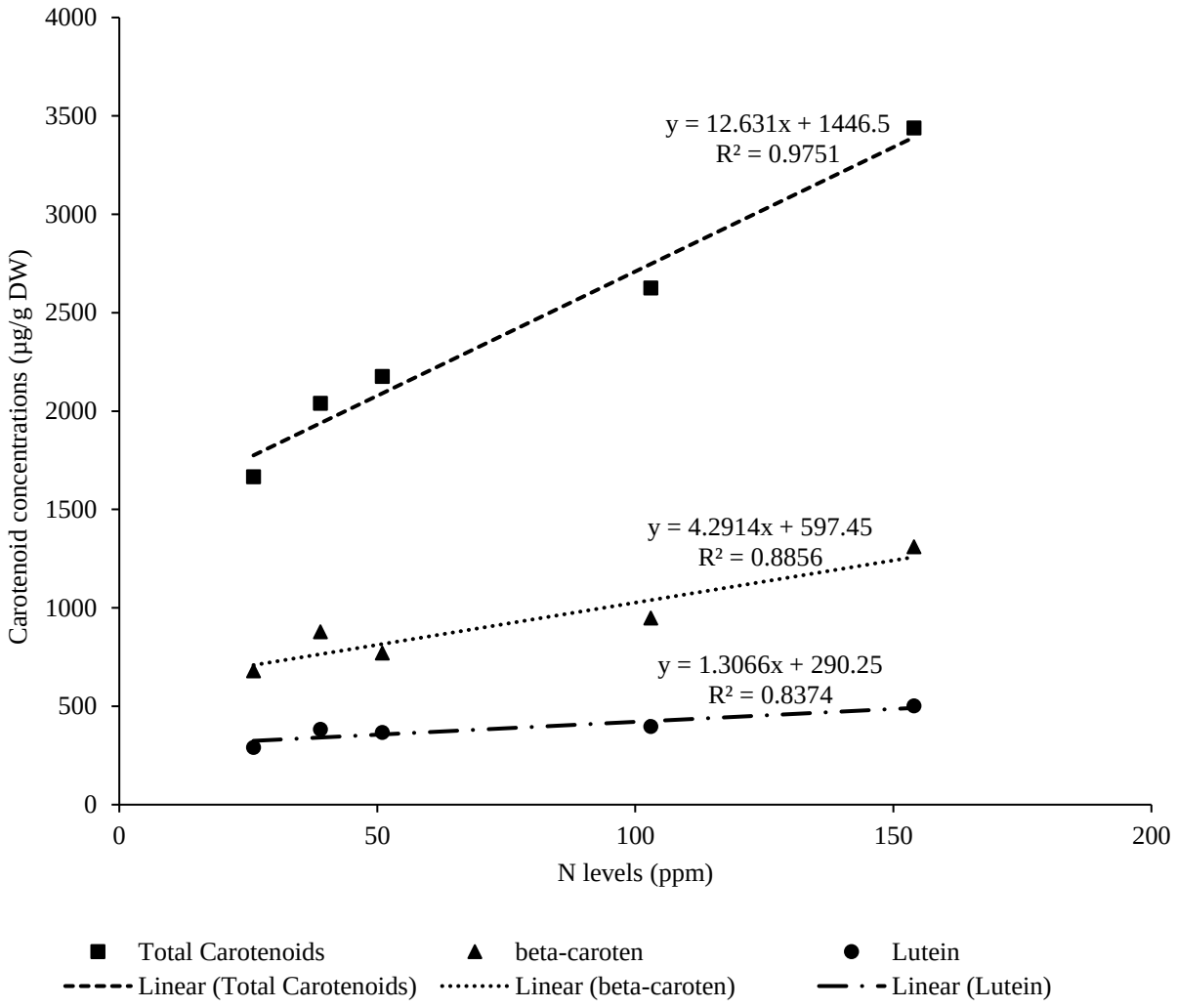
Appendix 13. Butterhead lettuce in growth chamber



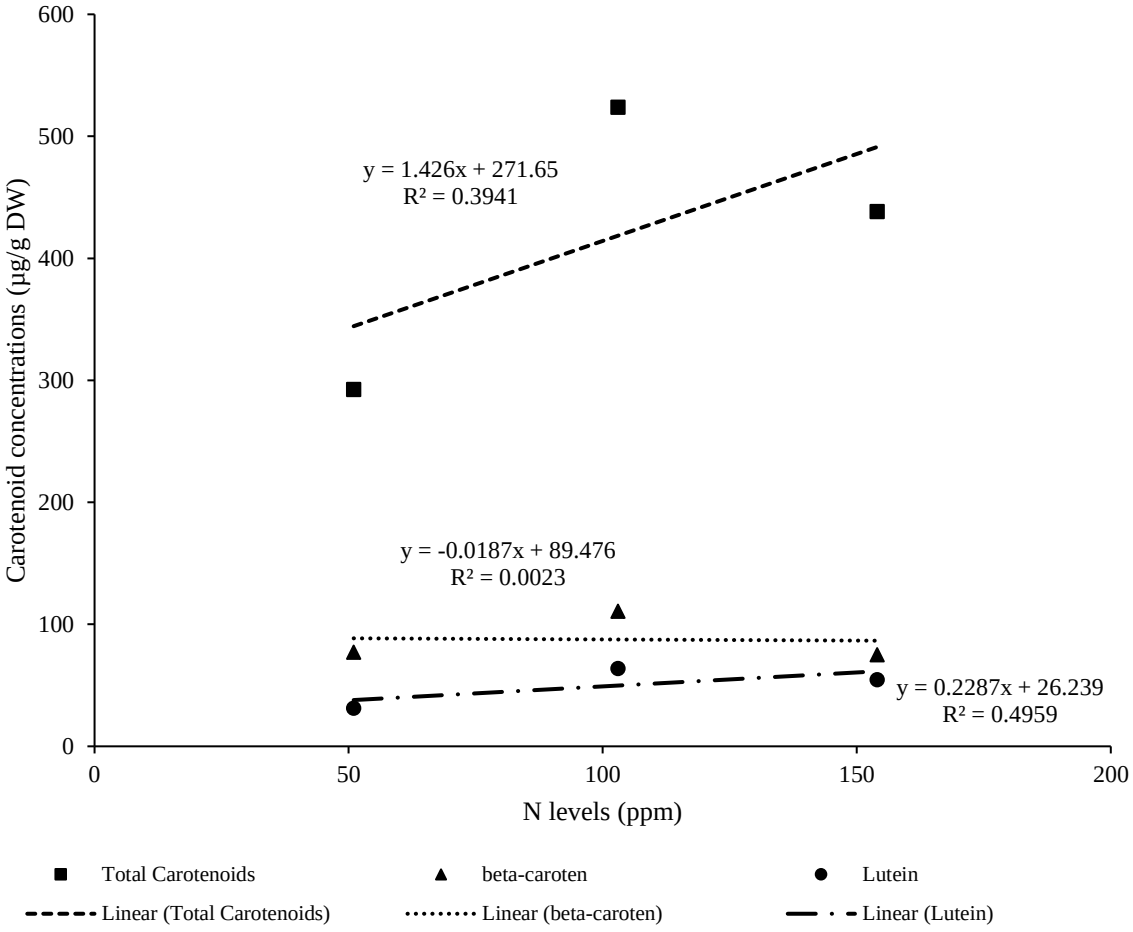
Appendix 14. The correlation between chlorophyll contents and nitrogen concentrations in Butterhead lettuce



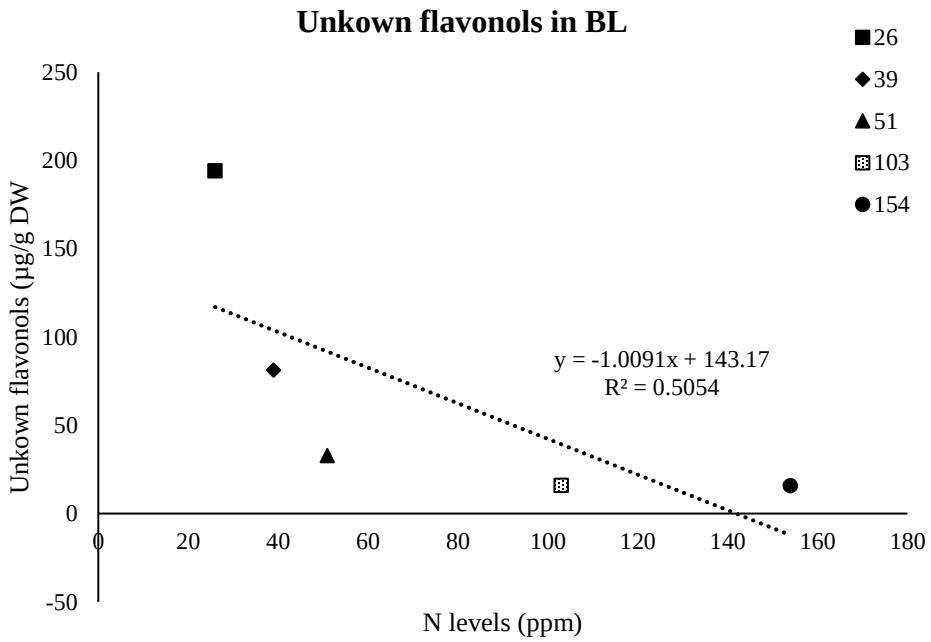
Appendix 15. The correlation between chlorophyll contents and nitrogen concentrations in Oakleaf lettuce



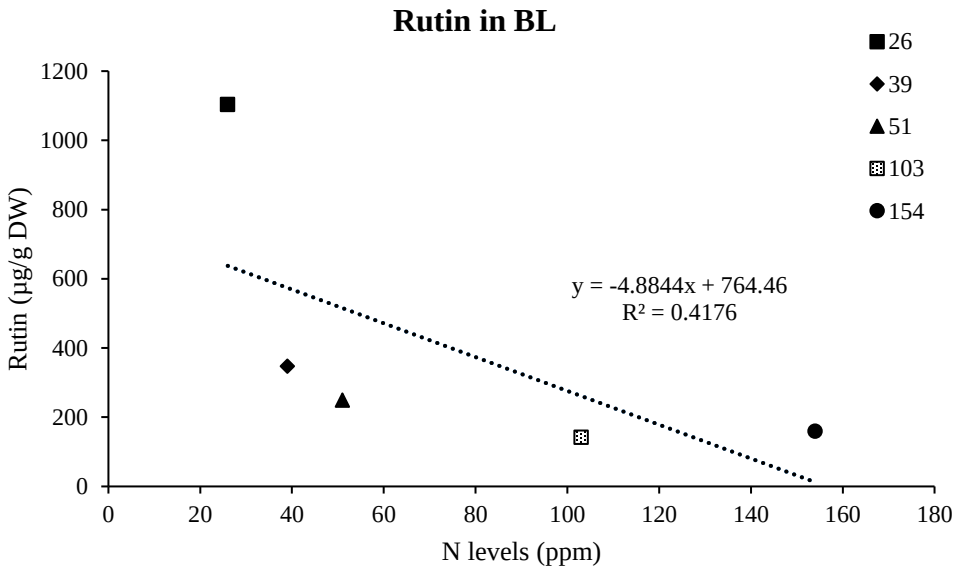
Appendix 16. The correlation between carotenoid contents and nitrogen concentrations in Butterhead lettuce



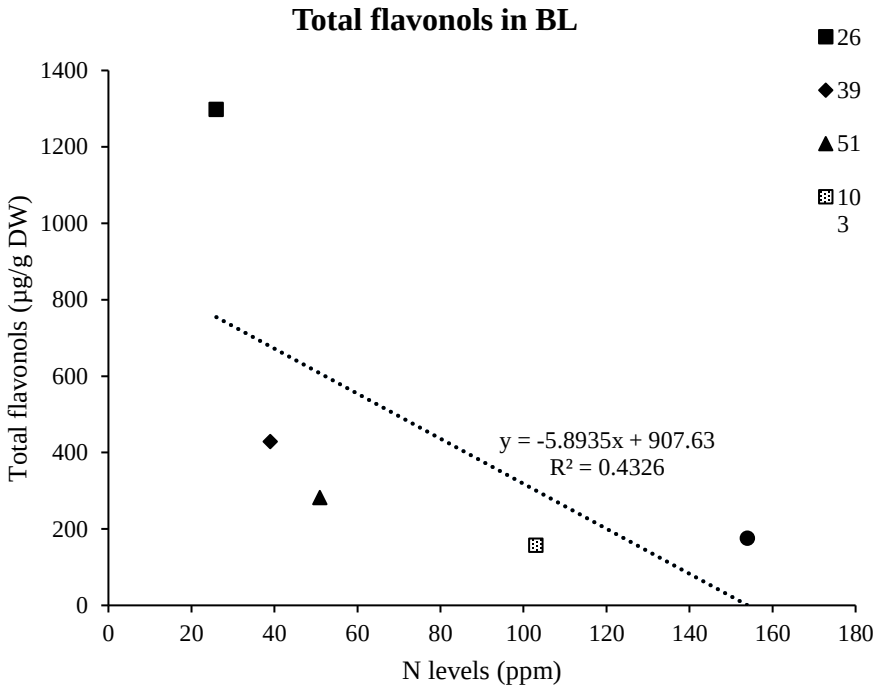
Appendix 17. The correlation between carotenoid contents and nitrogen concentrations in Oakleaf lettuce



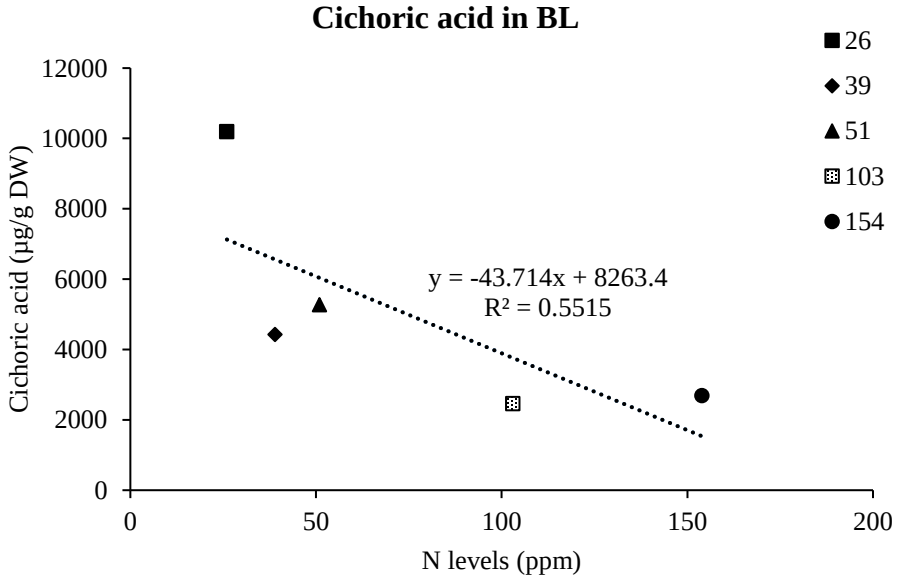
Appendix 18. The correlation between unknown flavonols and nitrogen concentrations in Butterhead lettuce



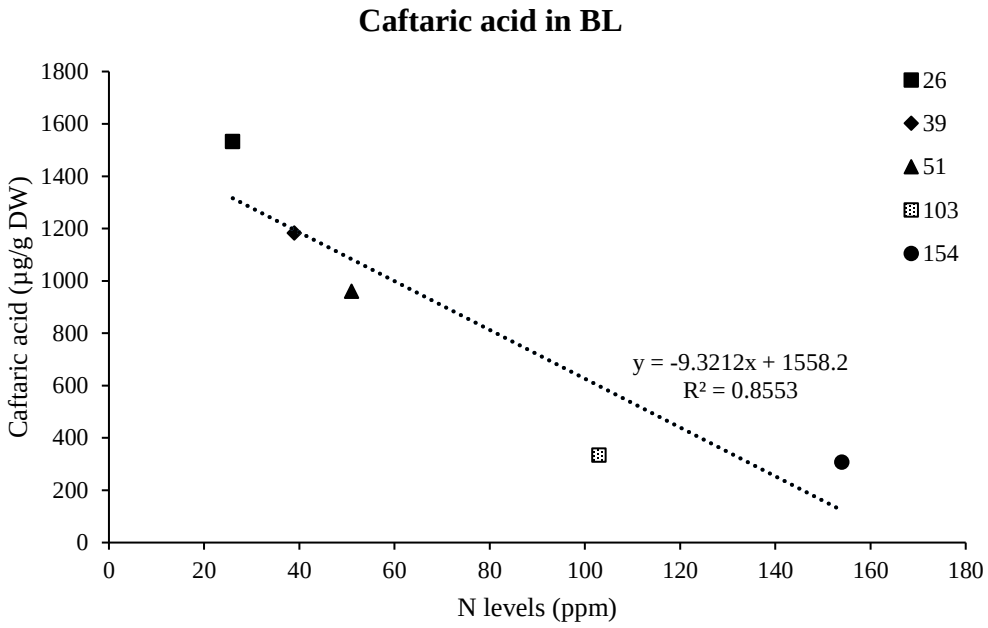
Appendix 19. The correlation between Rutin and nitrogen concentrations in Butterhead lettuce



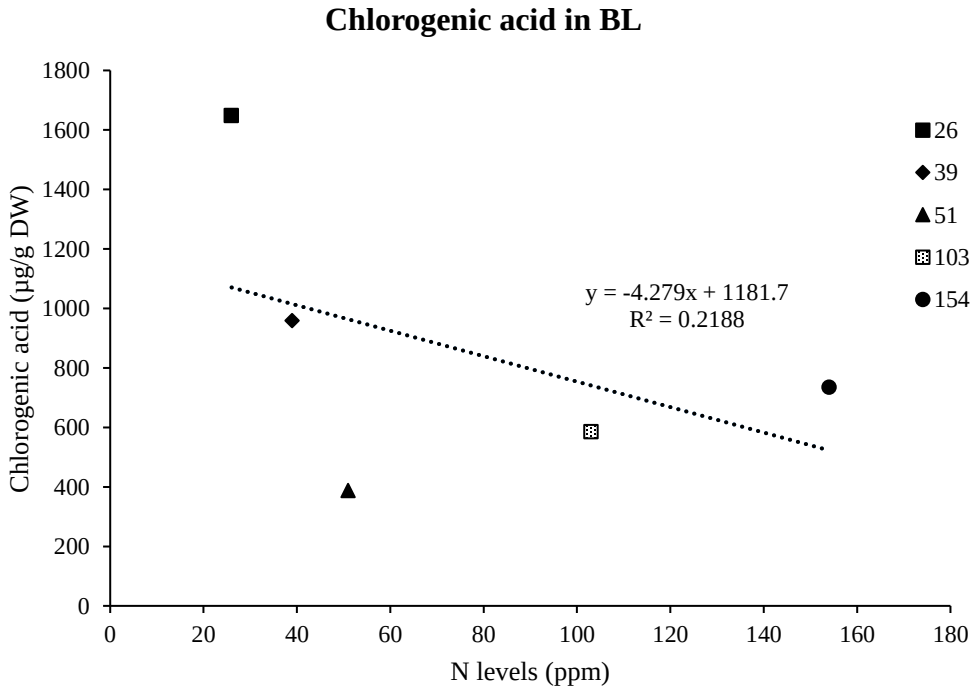
Appendix 20. The correlation between Total flavonols and nitrogen concentrations in Butterhead lettuce



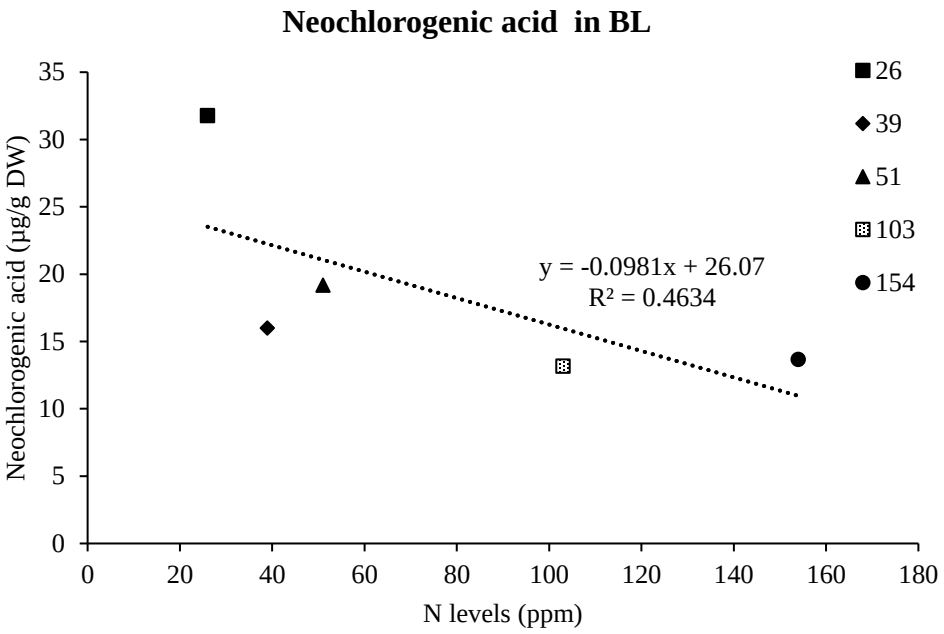
Appendix 21. The correlation between Cichoric acid and nitrogen concentrations in Butterhead lettuce



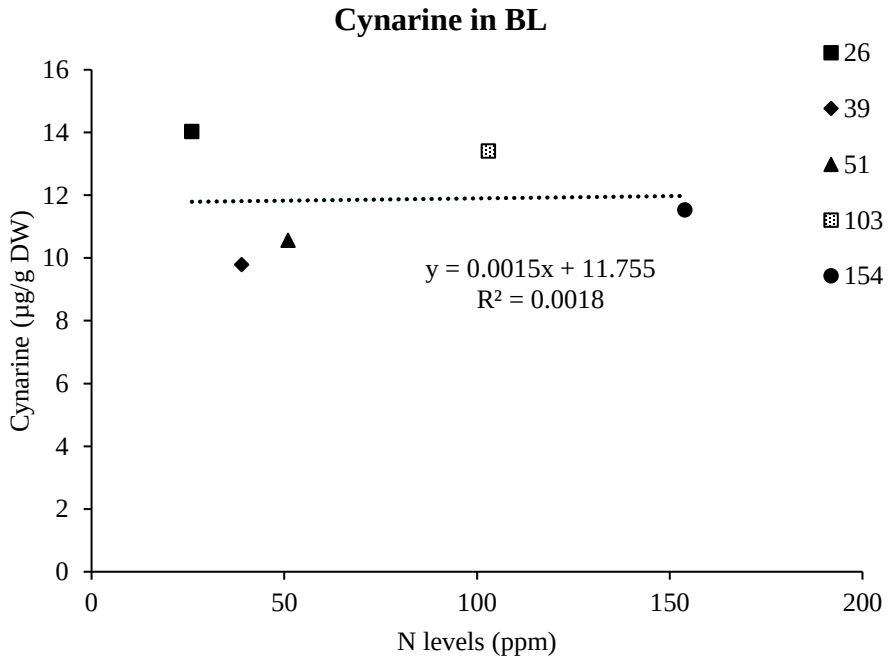
Appendix 22. The correlation between Caftaric acid and nitrogen concentrations in Butterhead lettuce



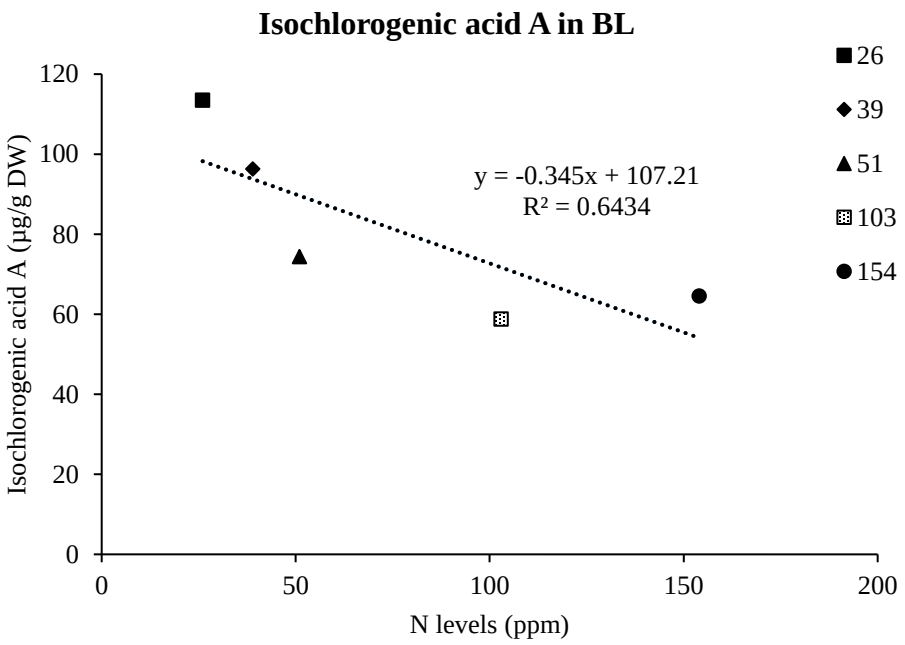
Appendix 23. The correlation between Chlorogenic acid and nitrogen concentrations in Butterhead lettuce



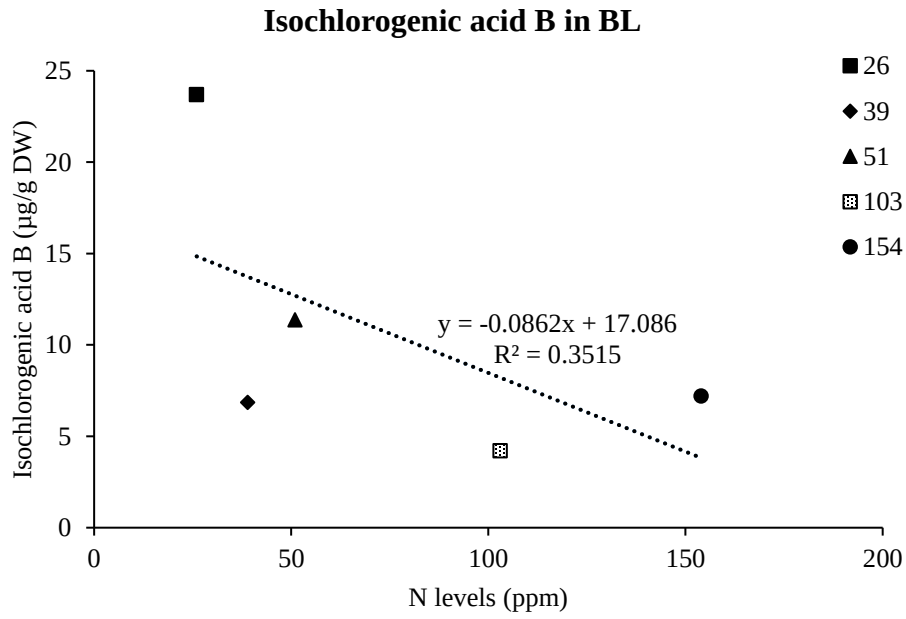
Appendix 24. The correlation between Neochloregenic acid and nitrogen concentrations in Butterhead lettuce



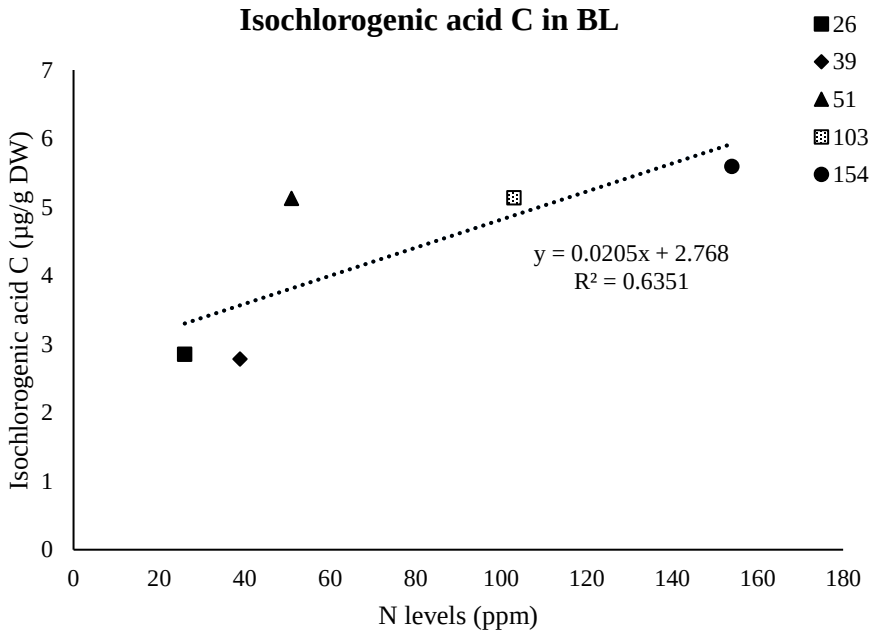
Appendix 25. The correlation between Cynarine and nitrogen concentrations in Butterhead lettuce



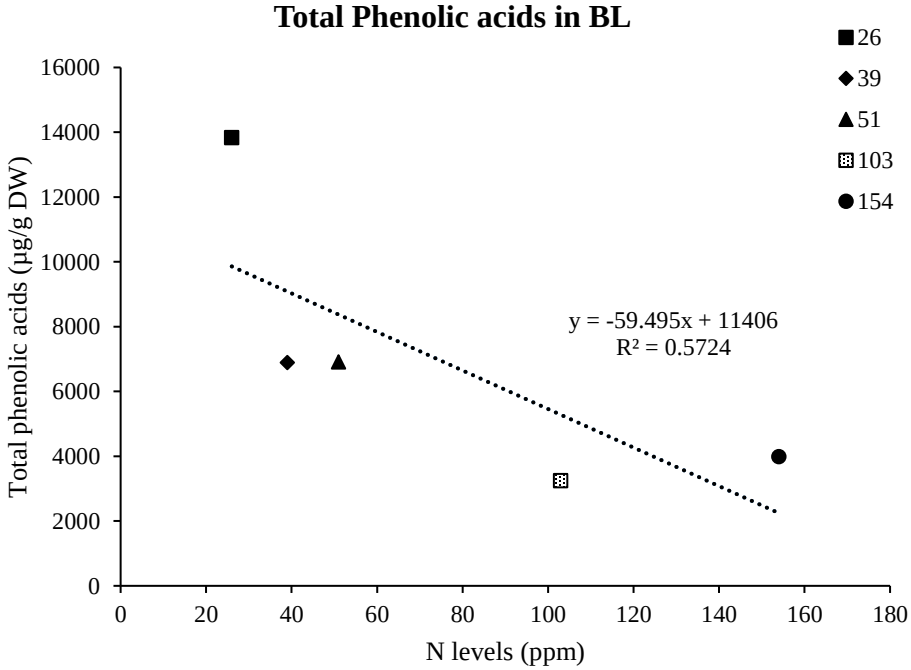
Appendix 26. The correlation between Isochloregenic acid A and nitrogen concentrations in Butterhead lettuce



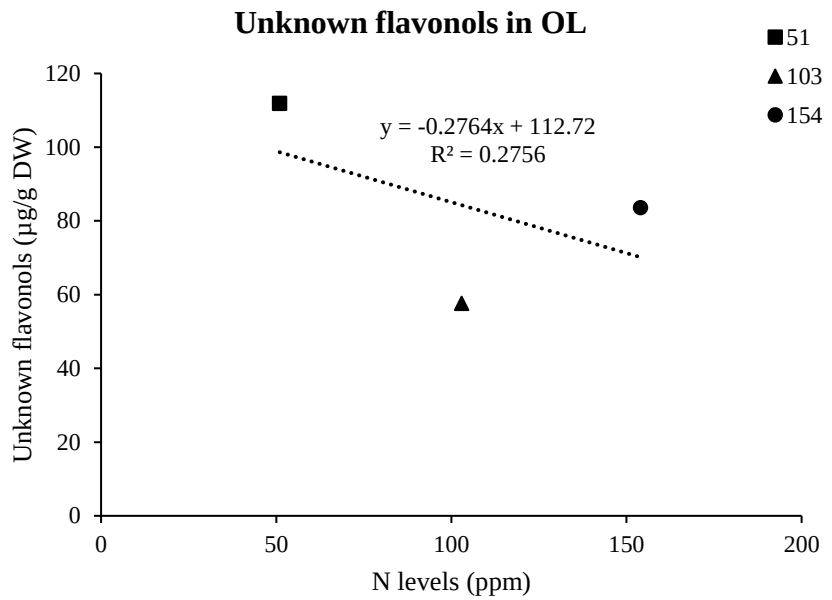
Appendix 27. The correlation between Isochloregenic acid B and nitrogen concentrations in Butterhead lettuce



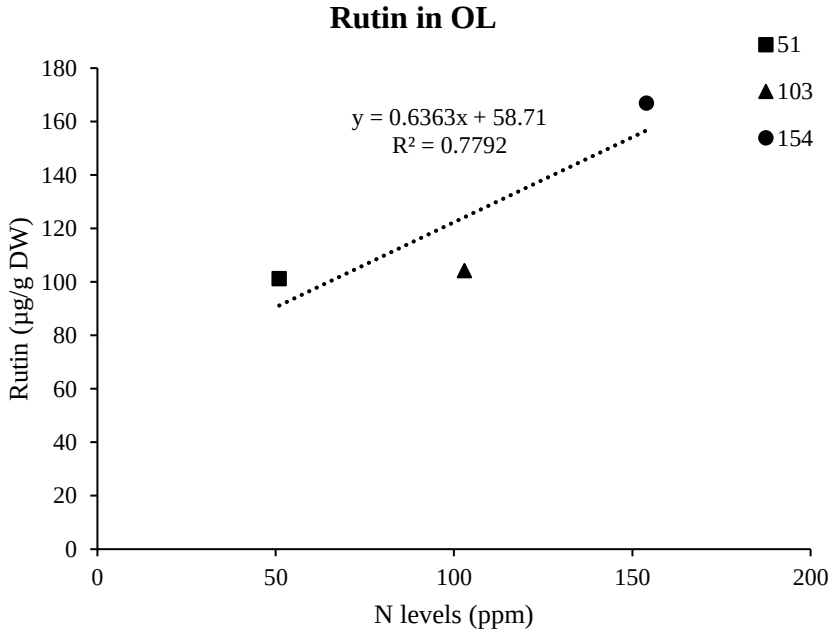
Appendix 28. The correlation between Isochloregenic acid C and nitrogen concentrations in Butterhead lettuce



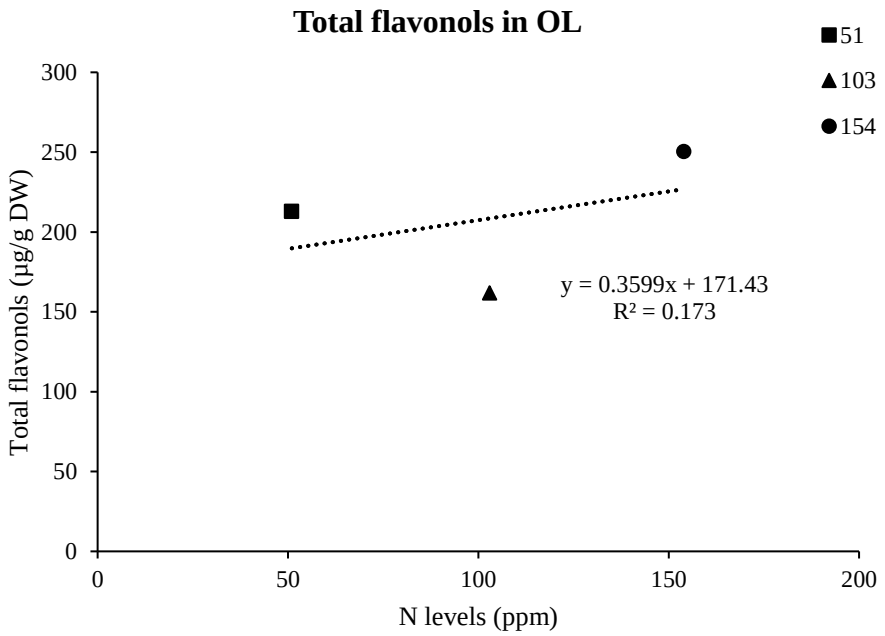
Appendix 29. The correlation between Total phenolic acids and nitrogen concentrations in Butterhead lettuce



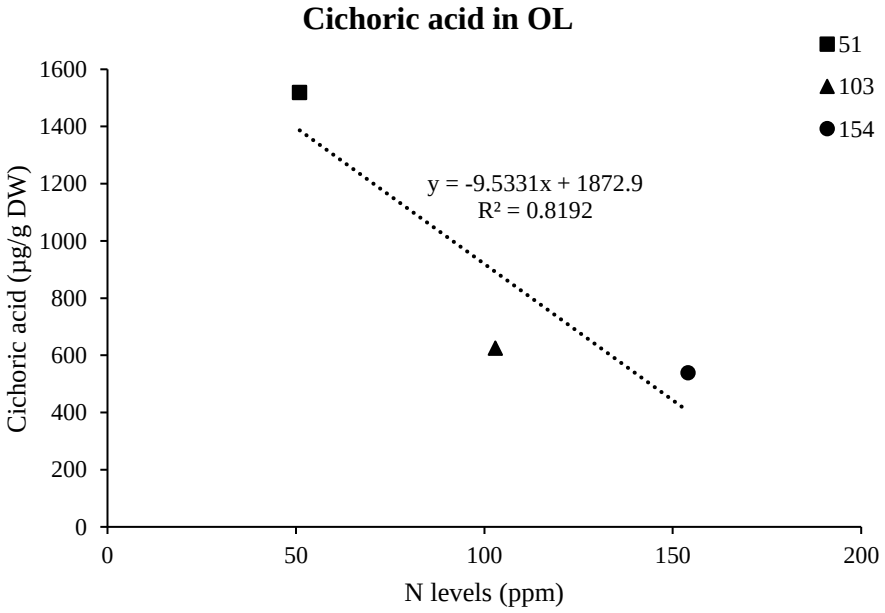
Appendix 30. The correlation between unknown flavonols and nitrogen concentrations in Oakleaf lettuce



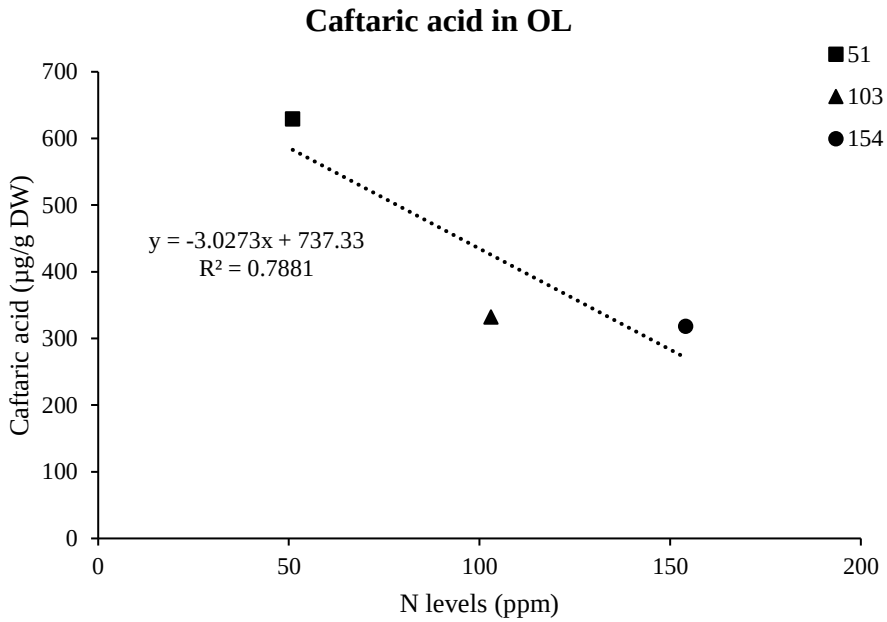
Appendix 31. The correlation between Rutin and nitrogen concentrations in Oakleaf lettuce



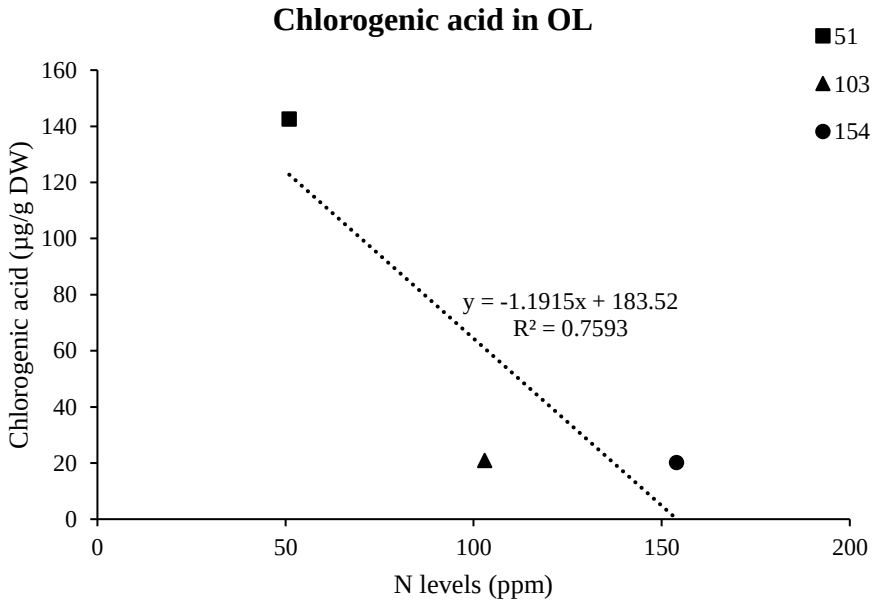
Appendix 32. The correlation between Total flavonols and nitrogen concentrations in Oakleaf lettuce



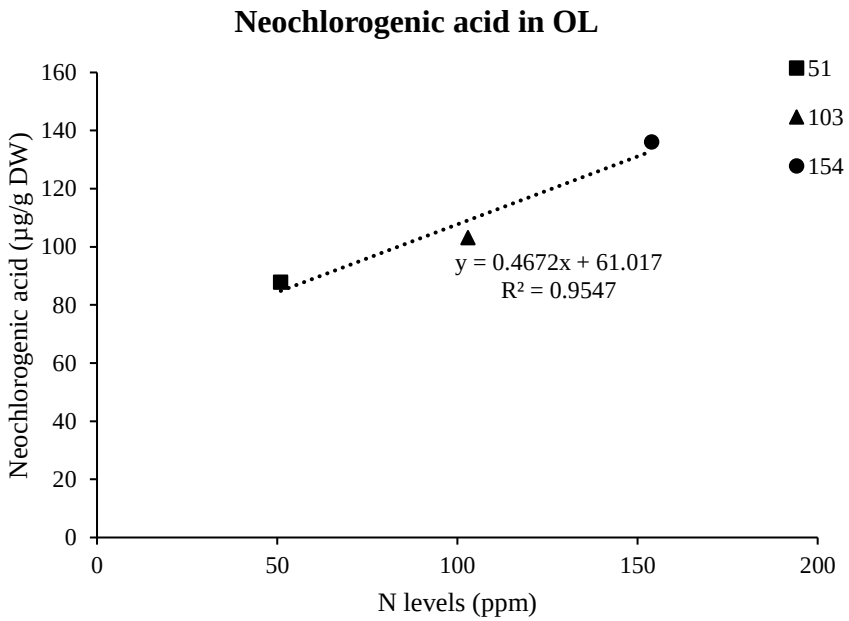
Appendix 33. The correlation between Cichoric acid and nitrogen concentrations in Oakleaf lettuce



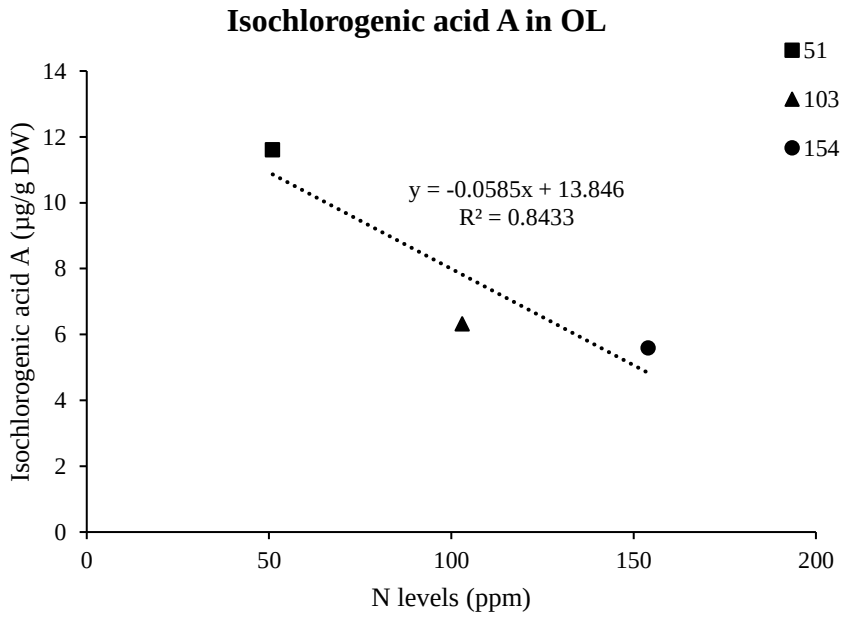
Appendix 34. The correlation between Caftaric acid and nitrogen concentrations in Oakleaf lettuce



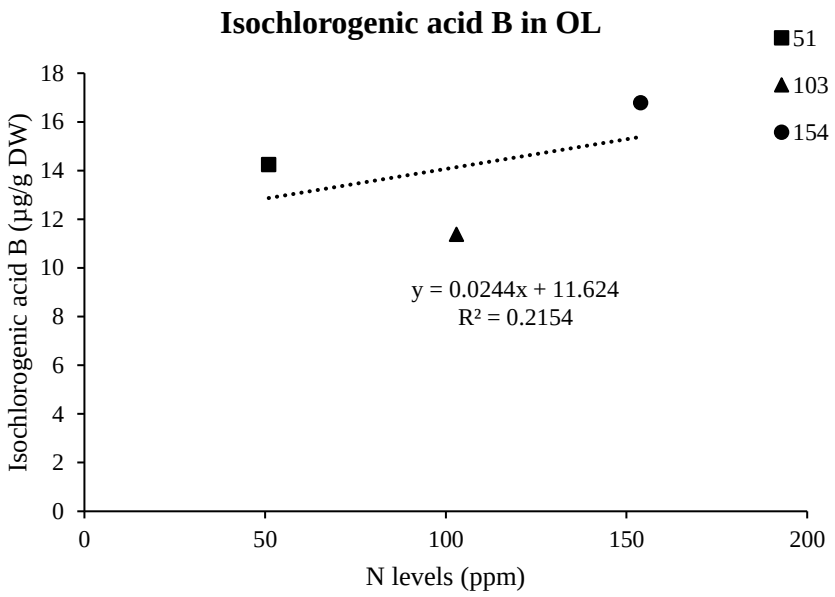
Appendix 35. The correlation between Chlorogenic acid and nitrogen concentrations in Oakleaf lettuce



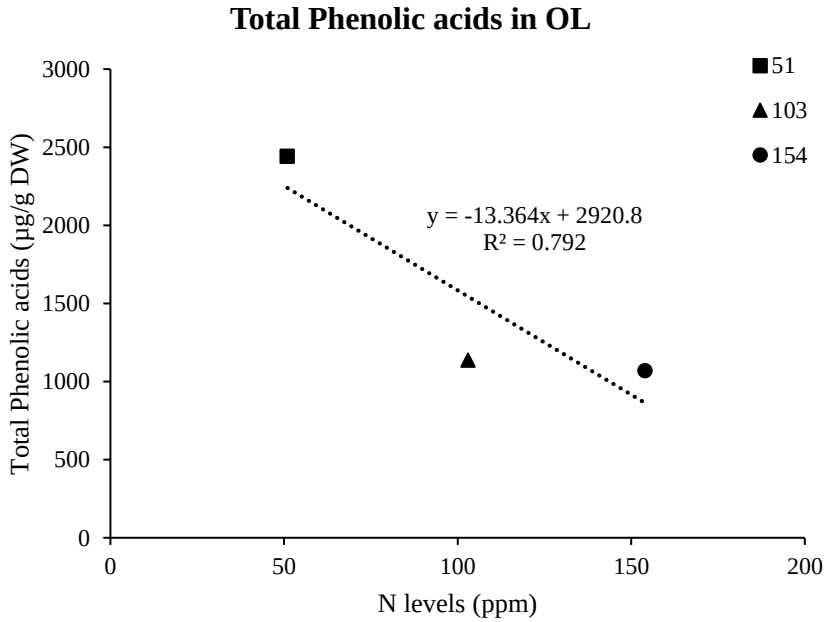
Appendix 36. The correlation between Neochlorogenic acid and nitrogen concentrations in Oakleaf lettuce



Appendix 37. The correlation between Isochlorogenic acid A and nitrogen concentrations in Oakleaf lettuce

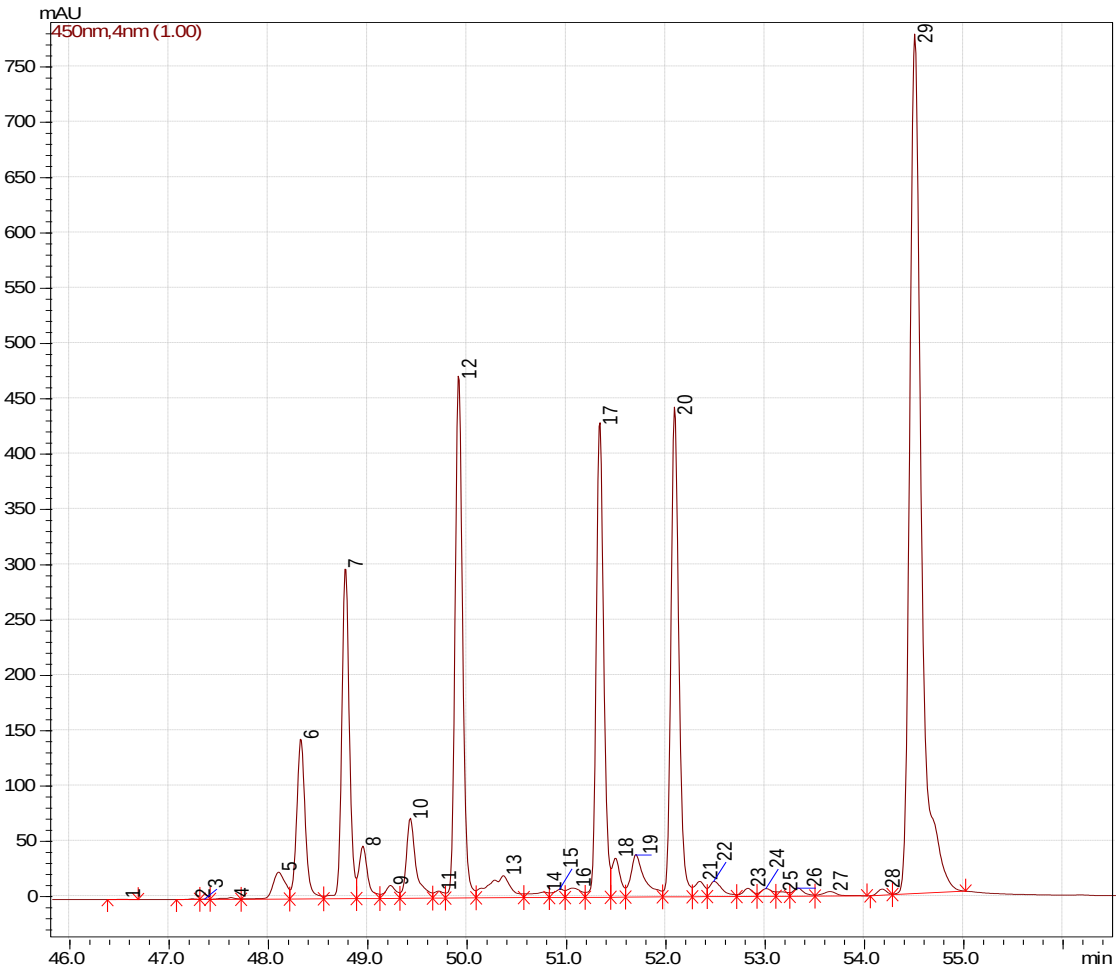


Appendix 38. The correlation between Isochlorogenic acid B and nitrogen concentrations in Oakleaf lettuce

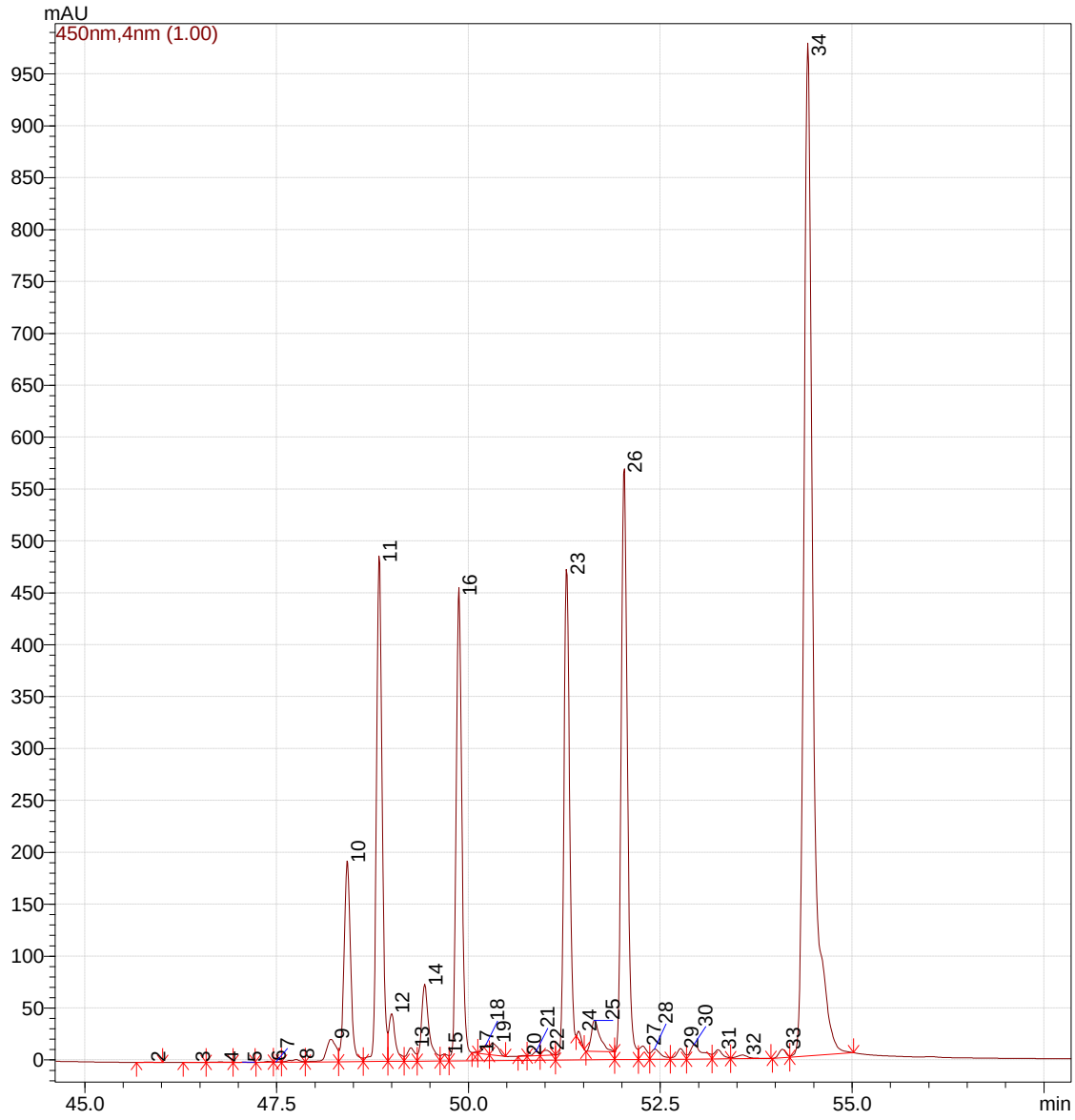


Appendix 39. The correlation between total phenolic acids and nitrogen concentrations in Oakleaf lettuce

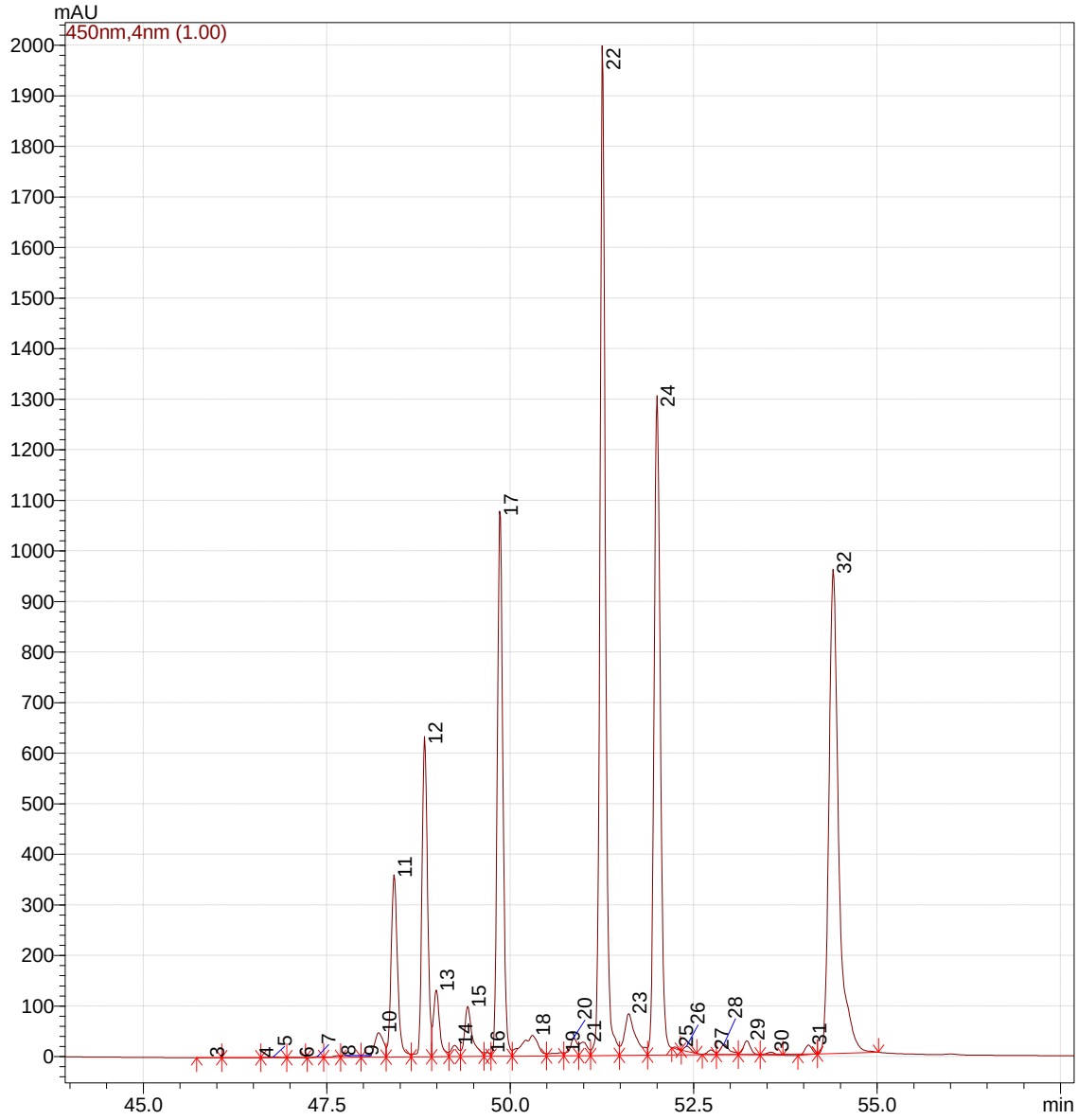
HPLC chromatograms of Chlorophylls a and b, Carotenoids in Lettuce Butterhead



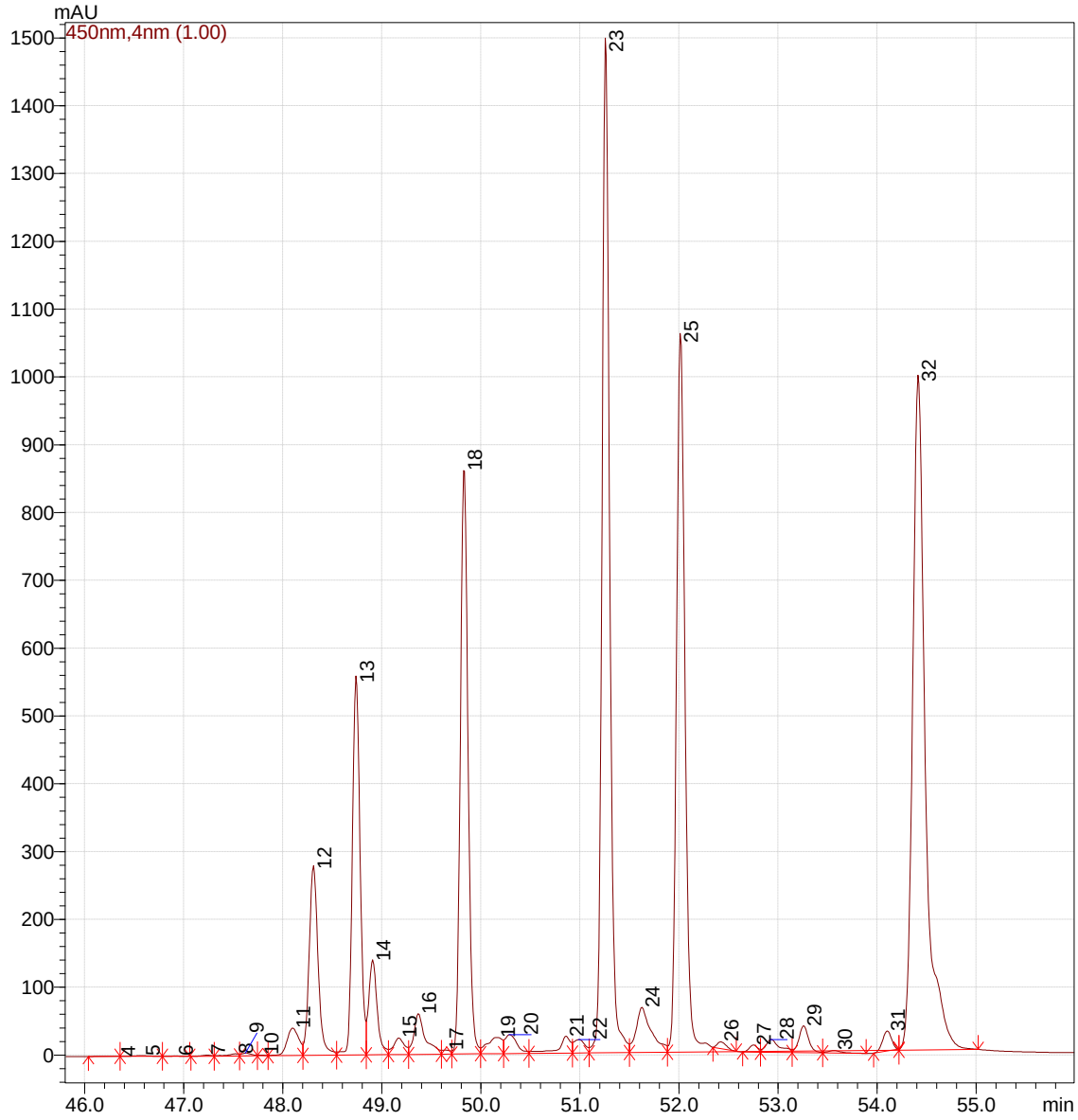
Appendix 40. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Butterhead lettuce at 26ppm N



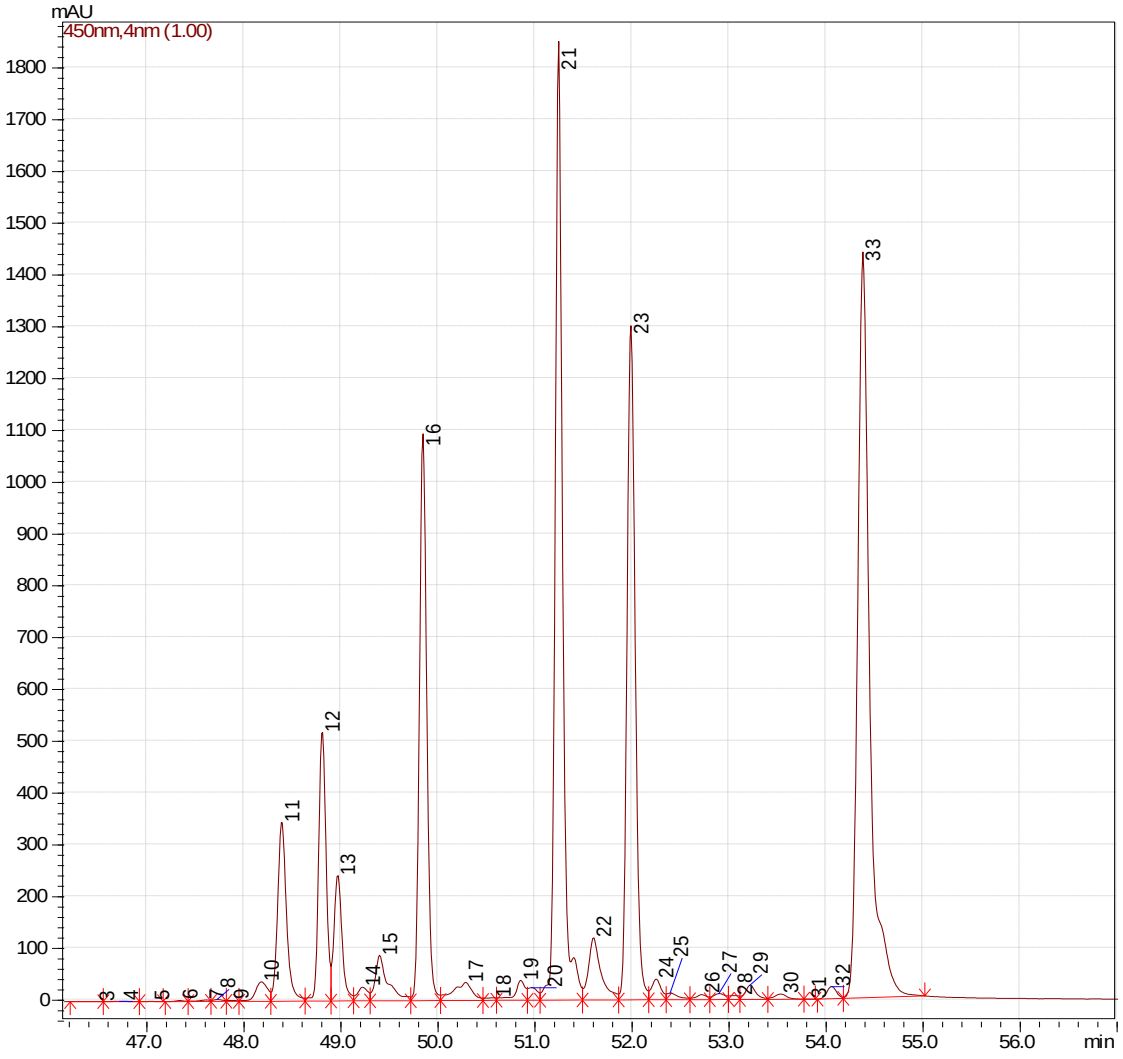
Appendix 41. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Butterhead lettuce at 39ppm N



Appendix 42. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Butterhead lettuce at 51ppm N

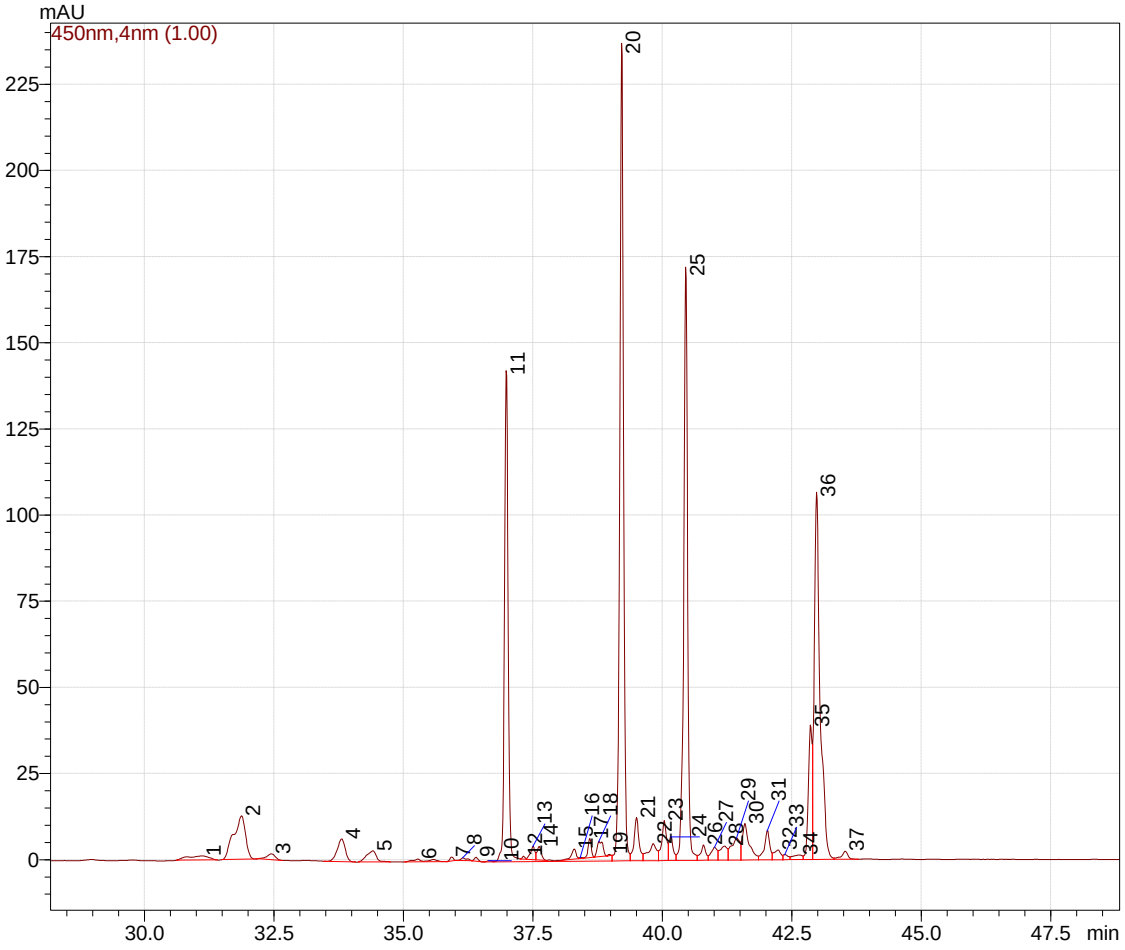


Appendix 43. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Butterhead lettuce at 103ppm N

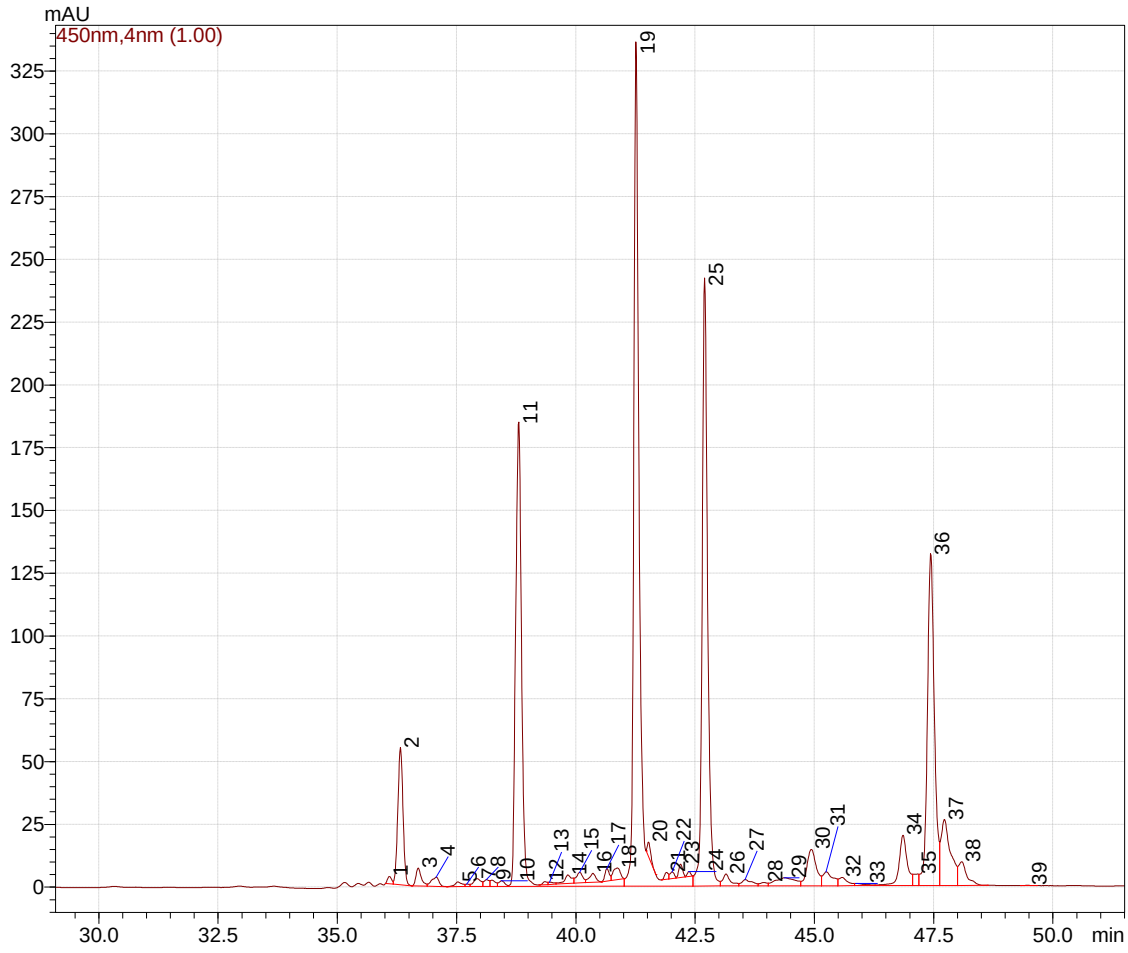


Appendix 44. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Butterhead lettuce at 154ppm N

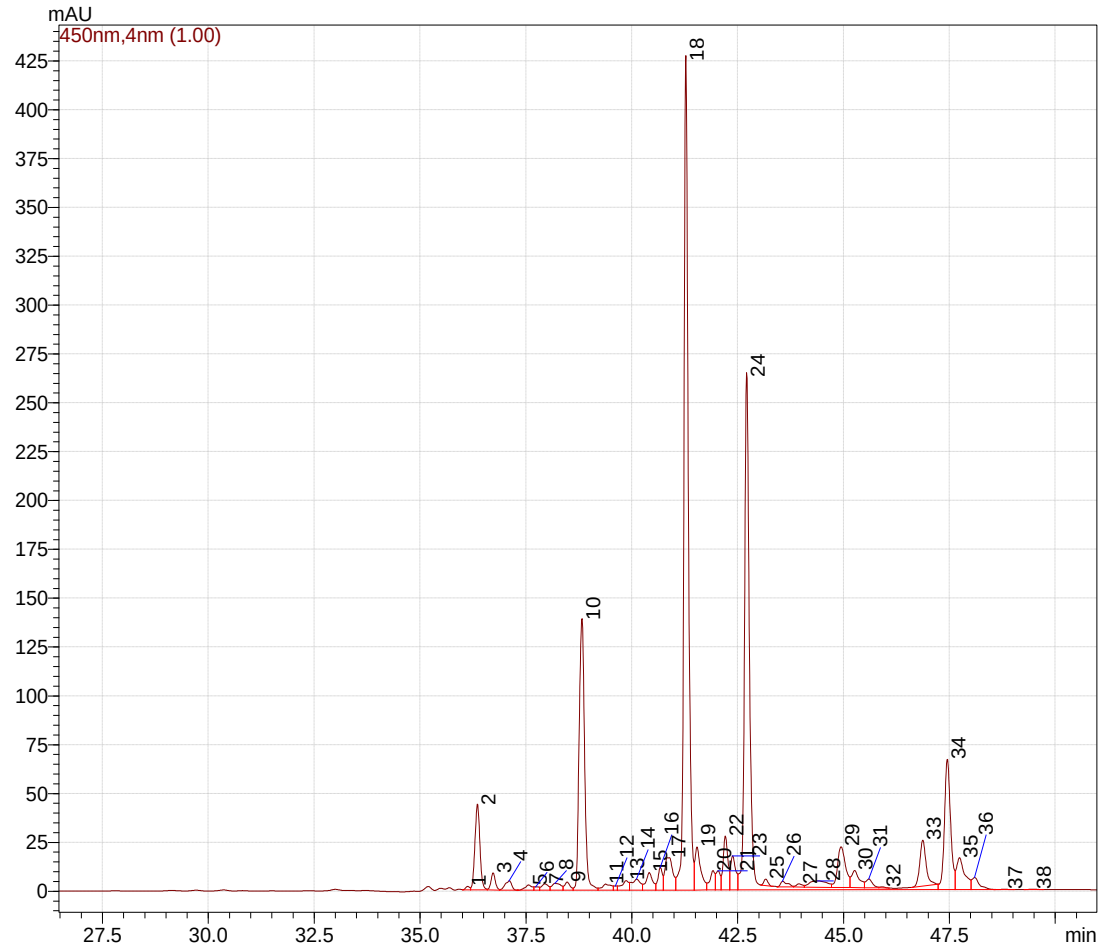
HPLC chromatogram of Chlorophylls a and b, Carotenoids in Lettuce Oakleaf



Appendix 45. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Oakleaf lettuce at 51ppm N

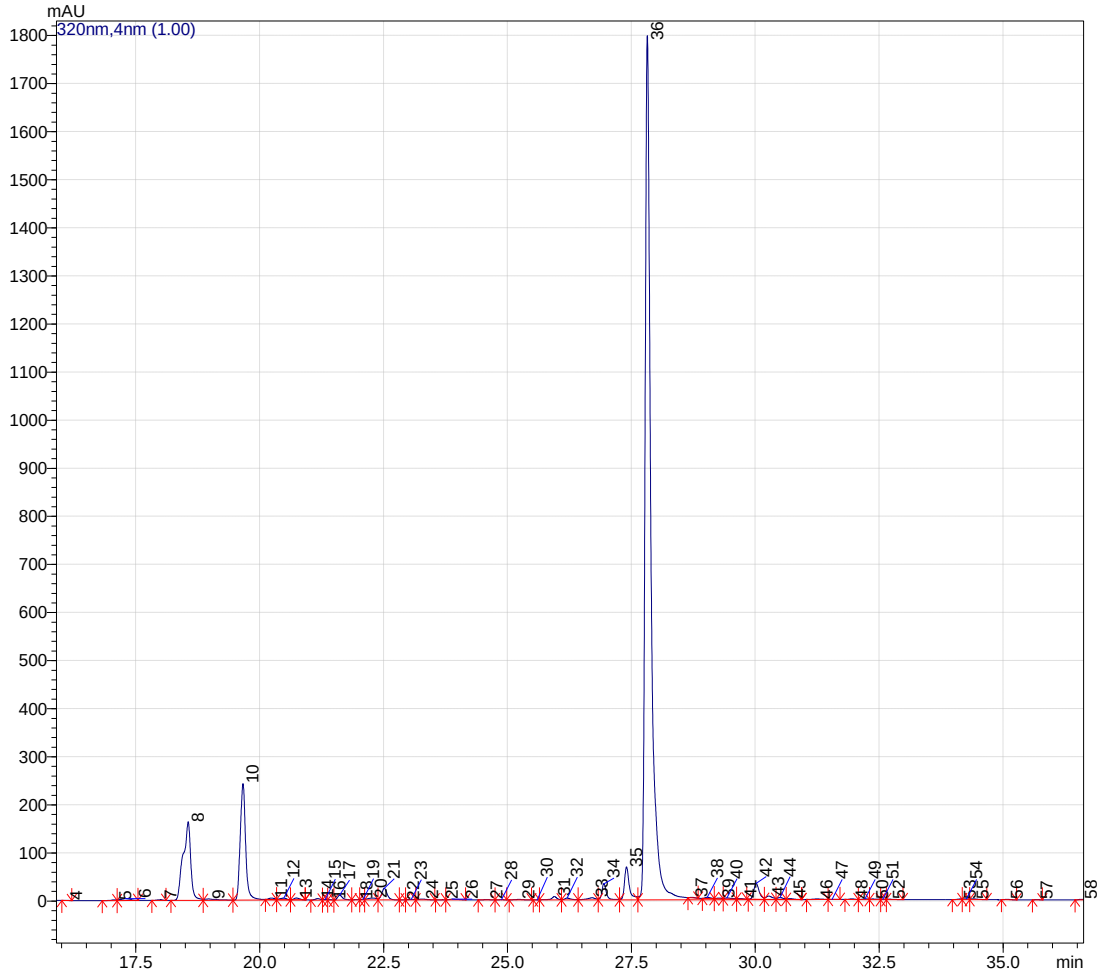


Appendix 46. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Oakleaf lettuce at 103ppm N

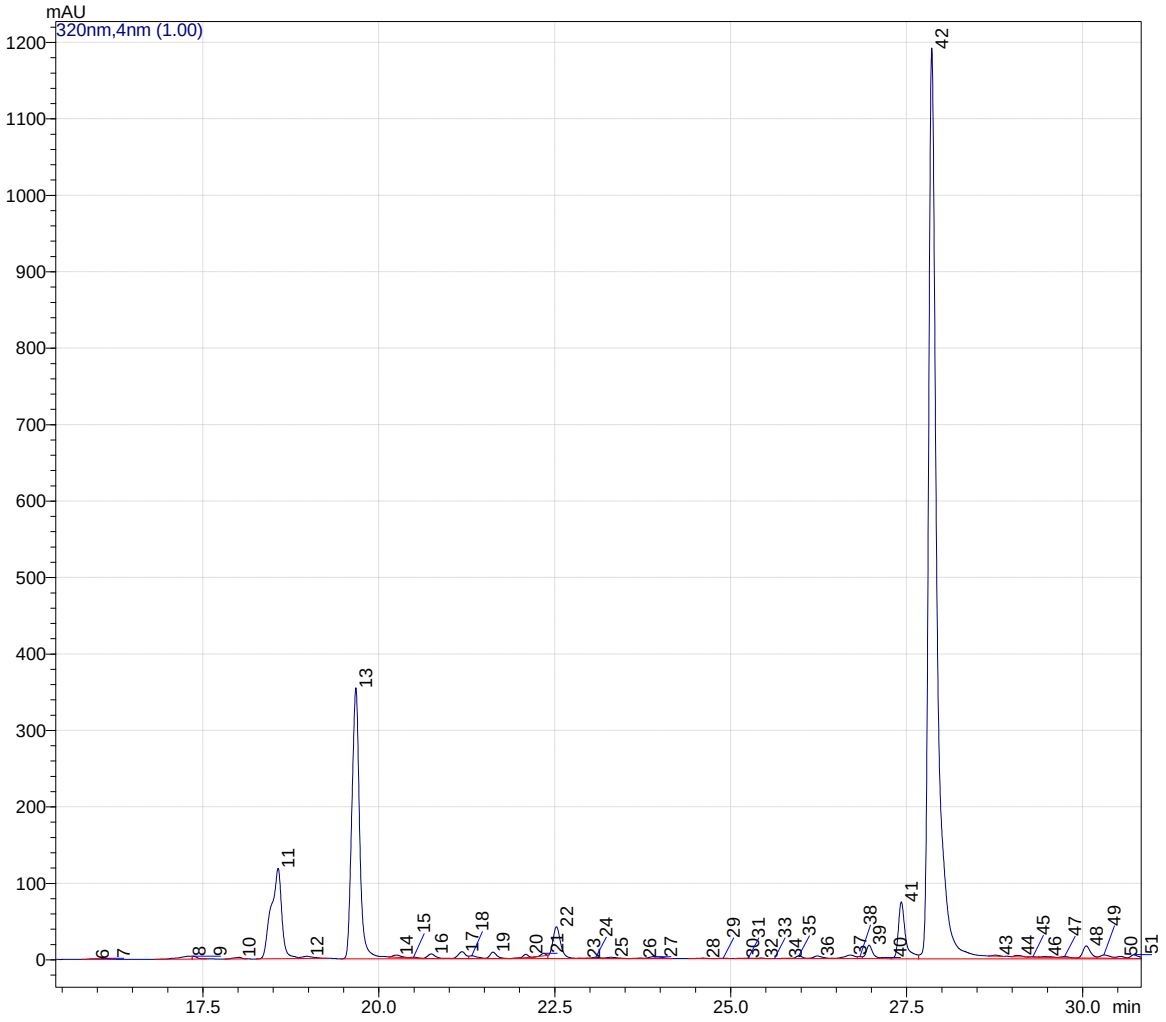


Appendix 47. HPLC Chromatogram of Chlorophylls a and b, Carotenoids in Oakleaf lettuce at 154ppm N

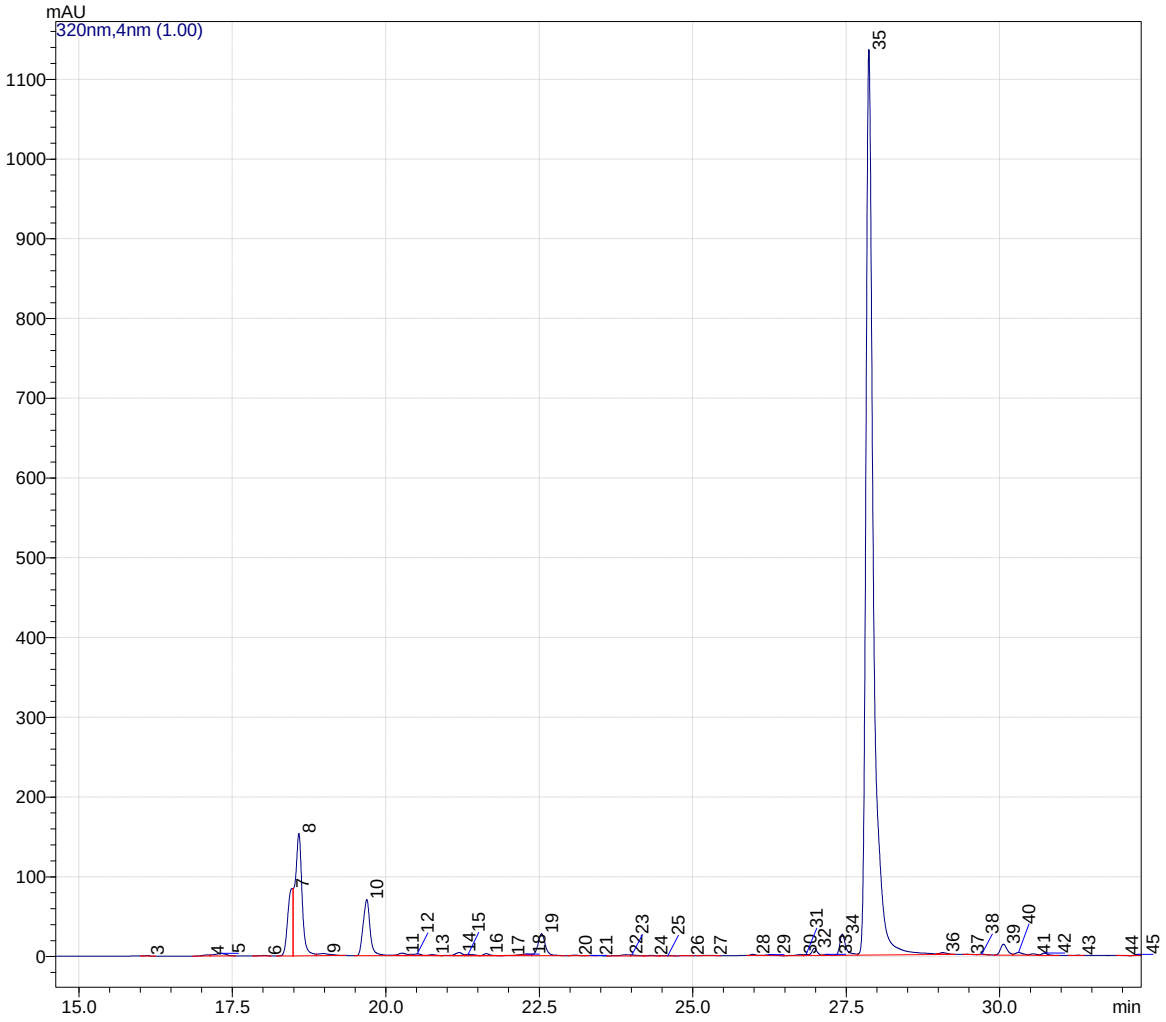
HPLC chromatogram of phenolic acids profile in Lettuce Butterhead



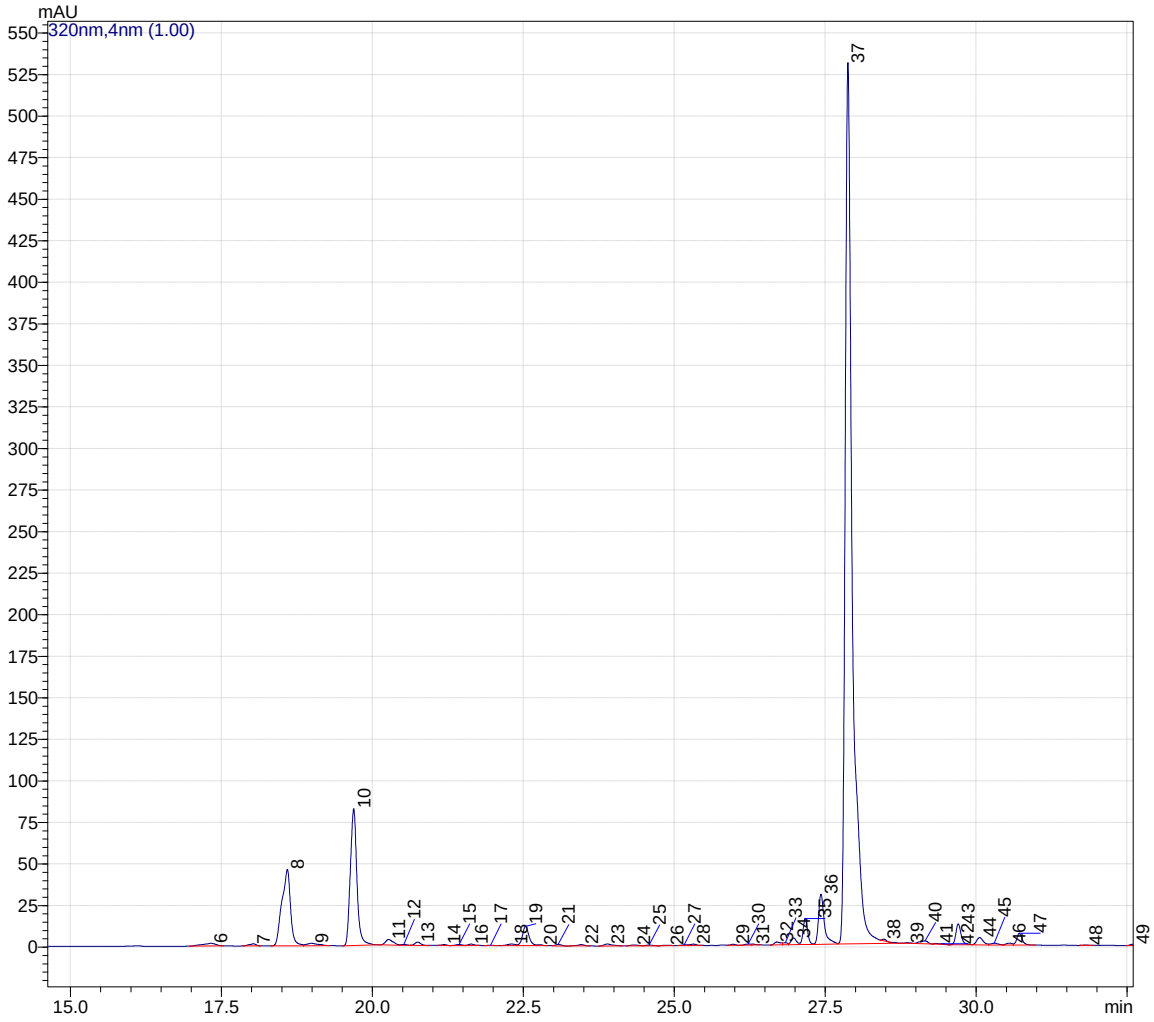
Appendix 48. HPLC Chromatogram of phenolic acids profile in Butterhead lettuce at 26ppm N



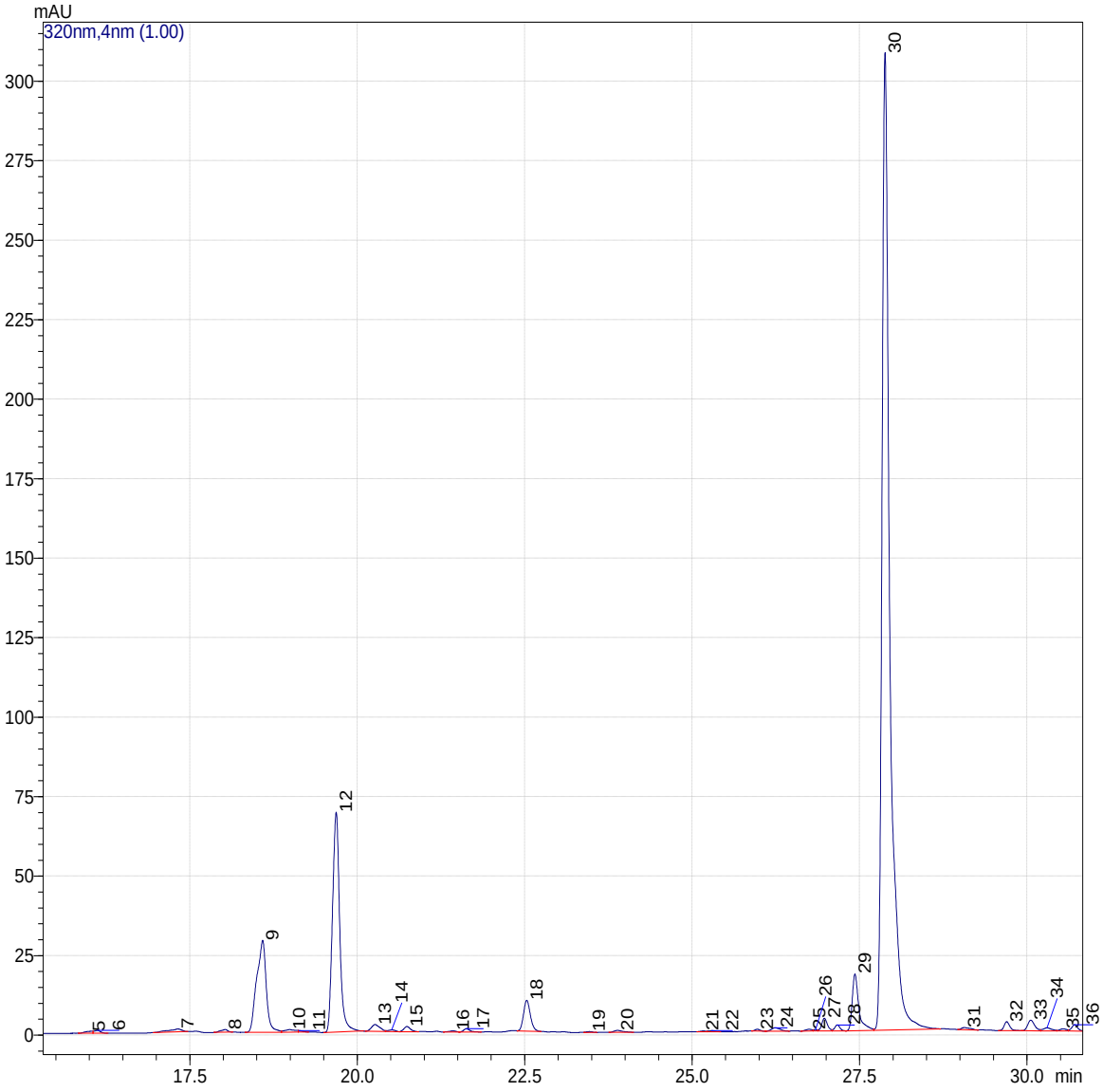
Appendix 49. HPLC Chromatogram of phenolic acids profile in Butterhead lettuce at 39ppm N



Appendix 50. HPLC Chromatogram of phenolic acids profile in Butterhead lettuce at 51ppm N

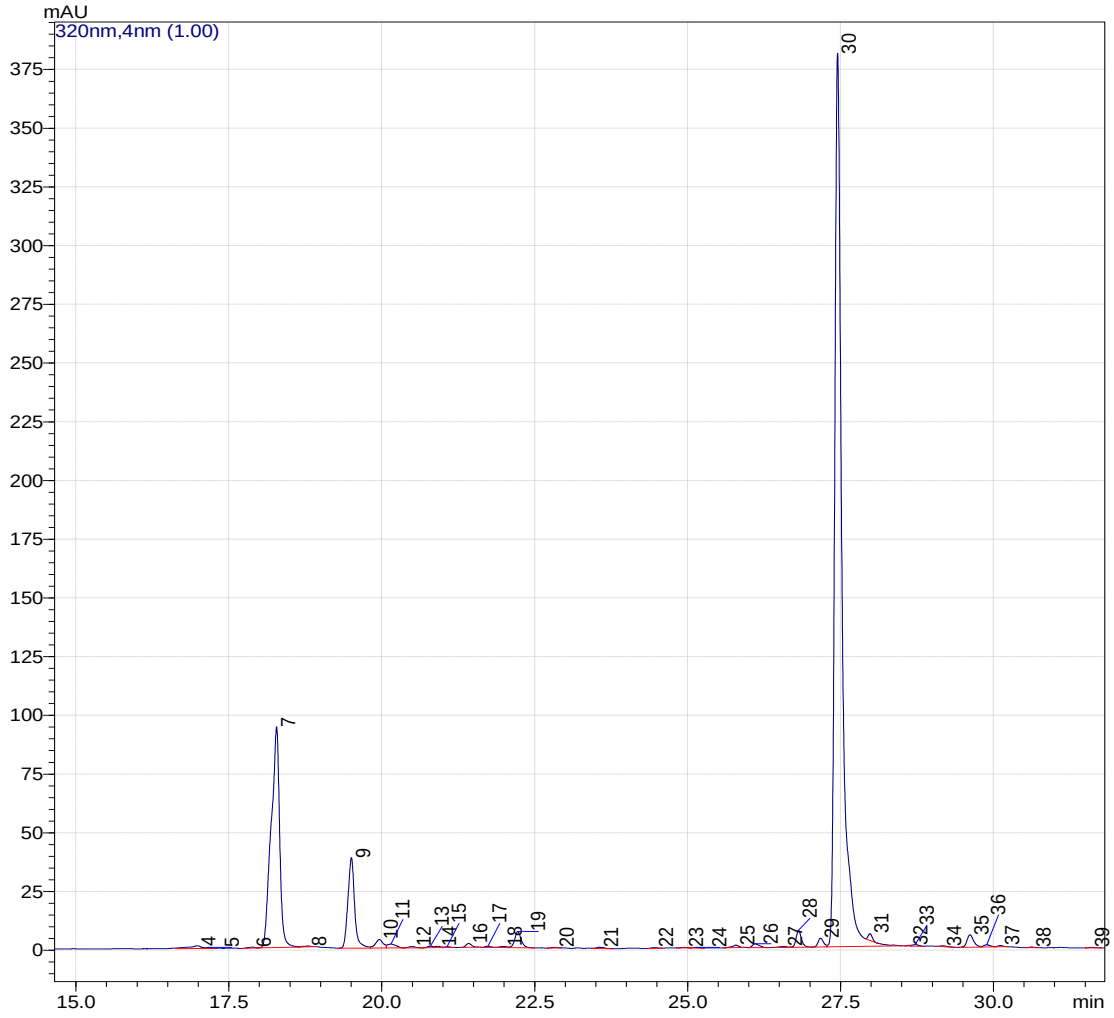


Appendix 51. HPLC Chromatogram of phenolic acids profile in Butterhead lettuce at 103ppm N

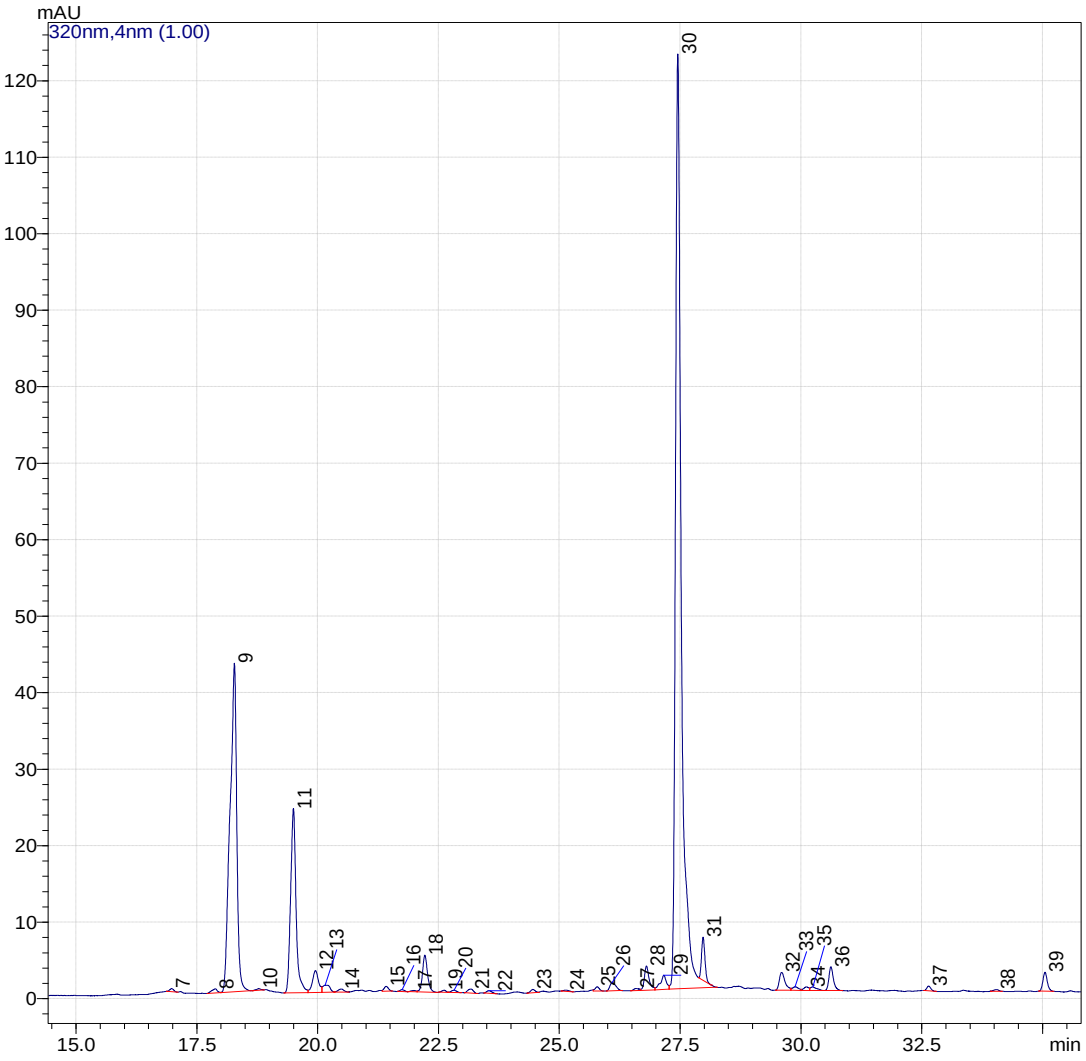


Appendix 52. HPLC Chromatogram of phenolic acids profile in Butterhead lettuce at 154ppm N

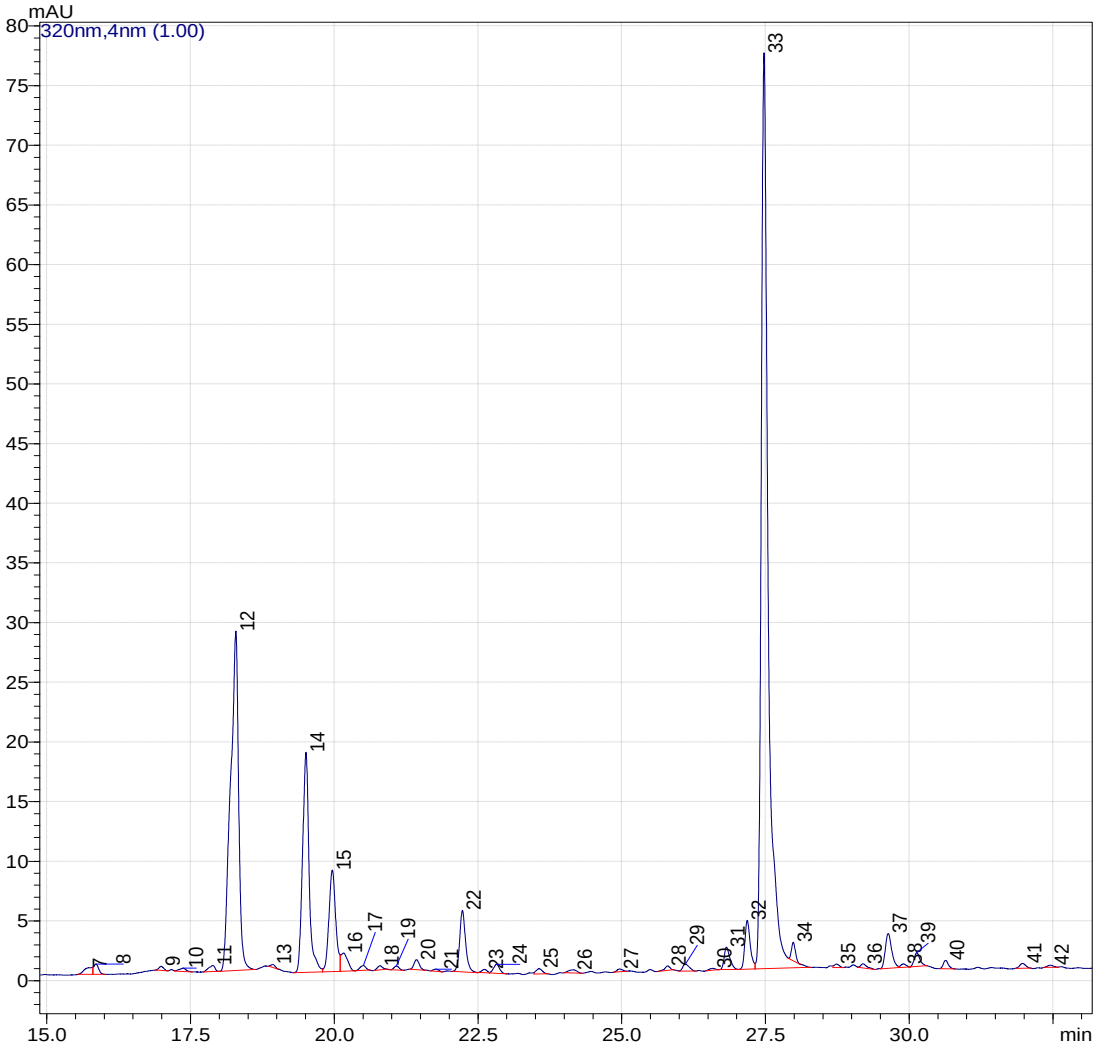
HPLC chromatogram of phenolic acids profile in Oakleaf lettuce



Appendix 53. HPLC Chromatogram of phenolic acids profile in Oakleaf lettuce at 51ppm N

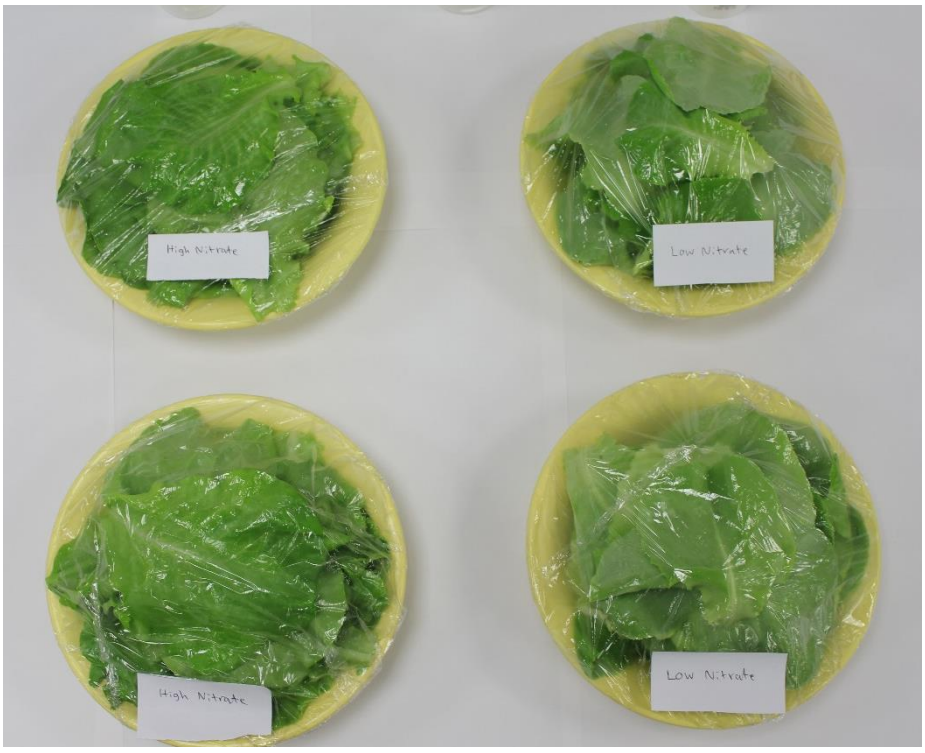


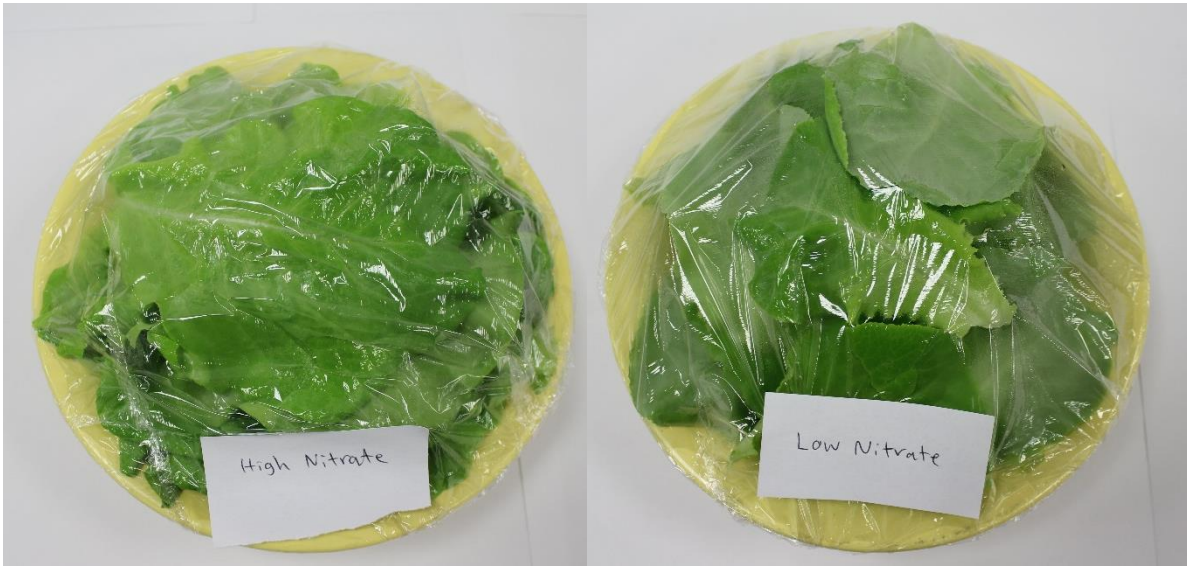
Appendix 54. HPLC Chromatogram of phenolic acids profile in Oakleaf lettuce at 103ppm N



Appendix 55. HPLC Chromatogram of phenolic acids profile in Oakleaf lettuce at 154ppm N

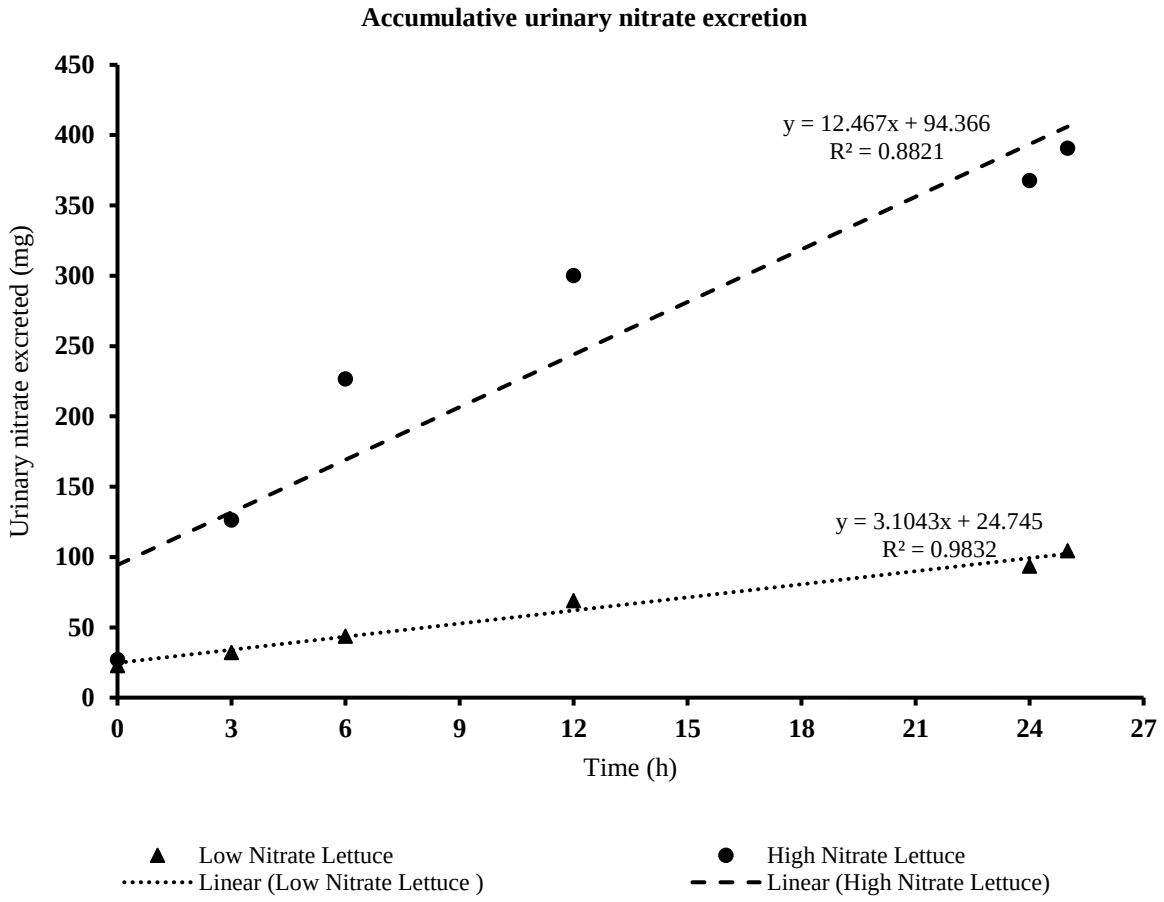
Appendices of chapter 4.



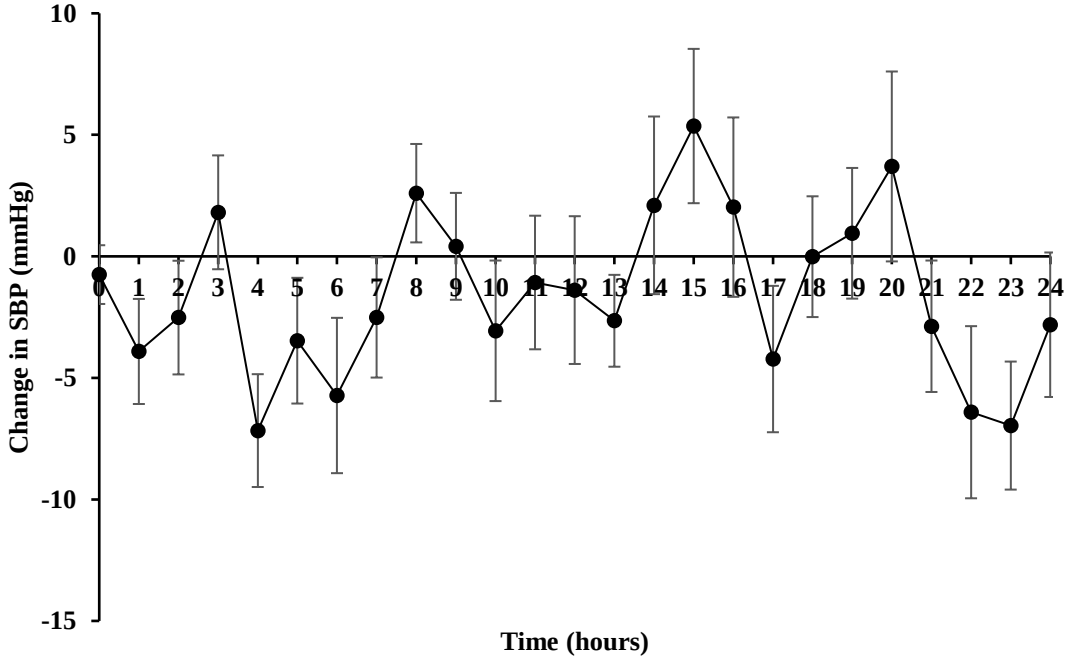


Appendix 56. Participants were received 50g-portions of lettuce (one with low and one with high nitrate content) on separate days

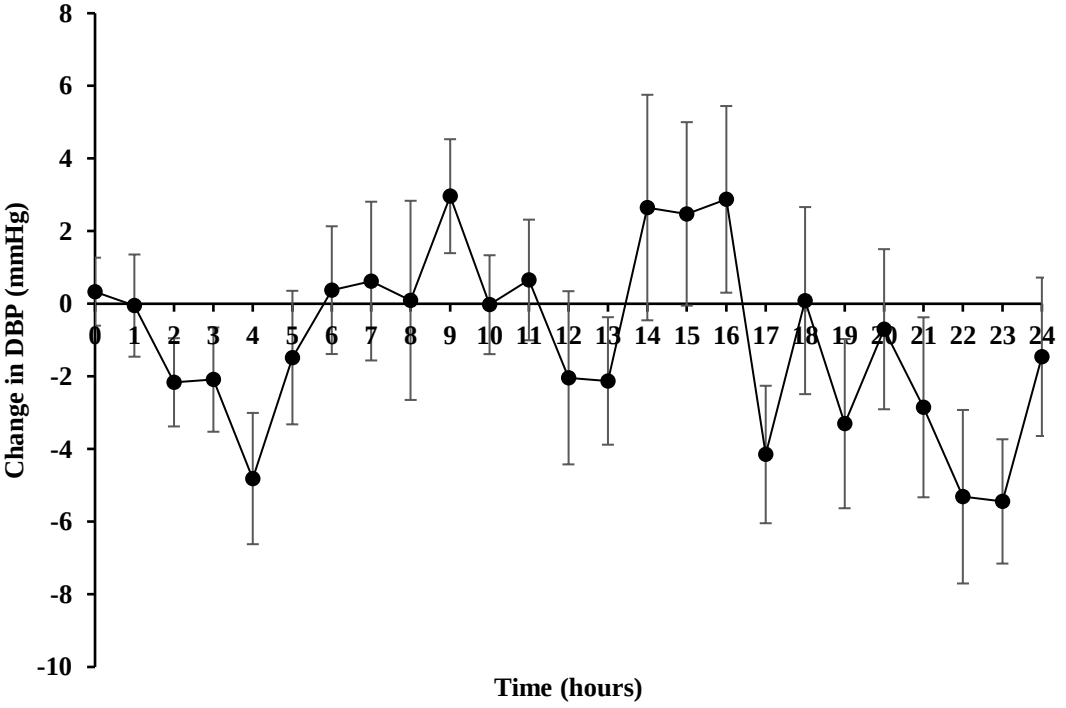
Appendix 57. 24-hr ambulatory blood pressure measurement, taking blood sample, anthropometry measurements.



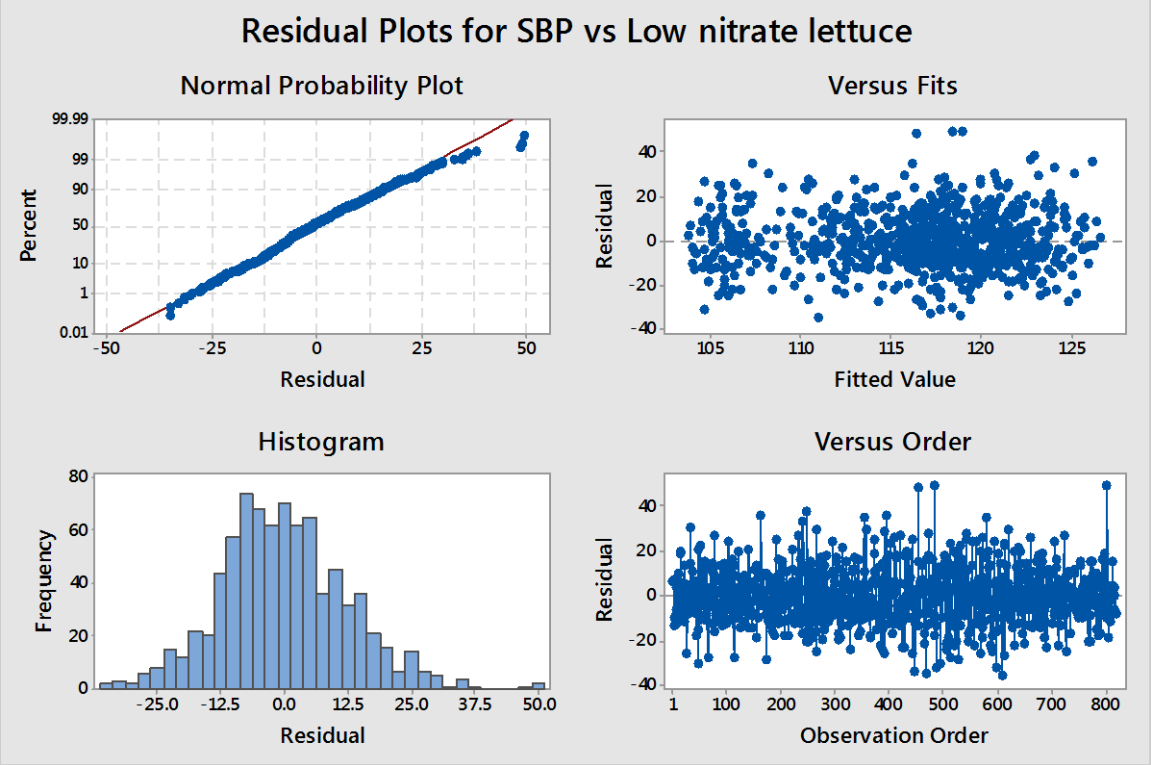
Appendix 58. The relationship between high and low nitrate lettuce intake and urinary nitrate excretion in healthy young subjects

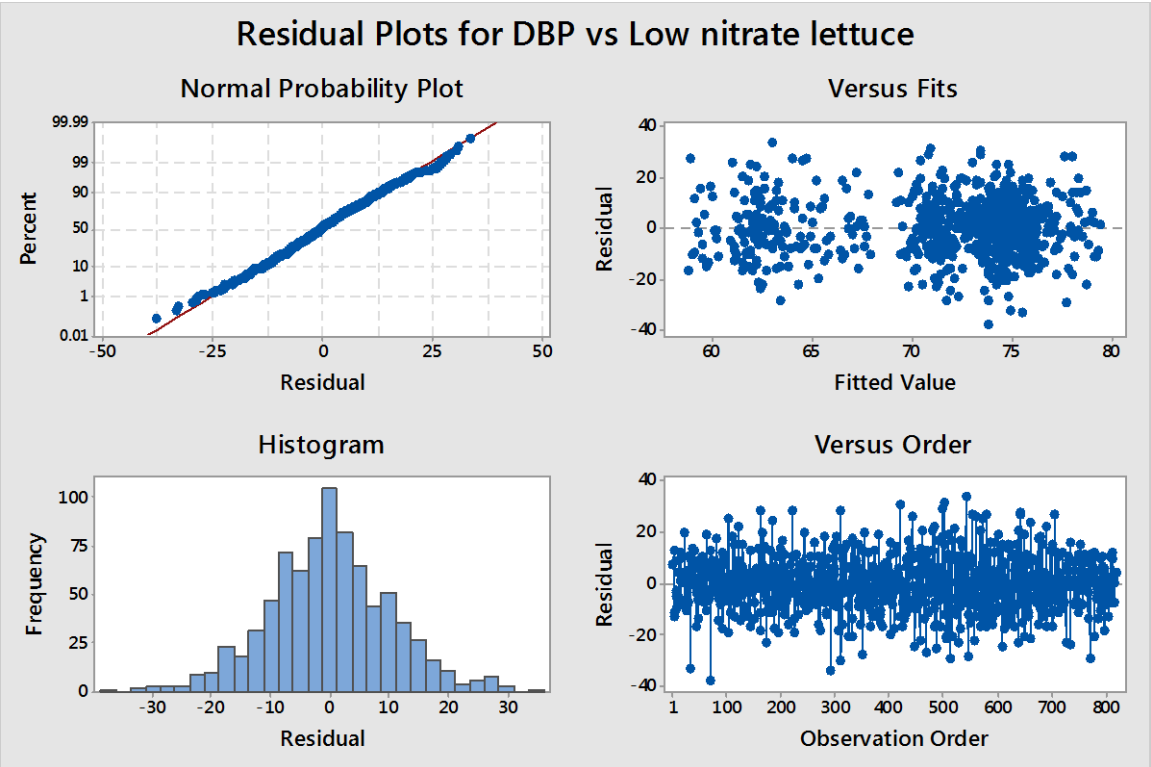


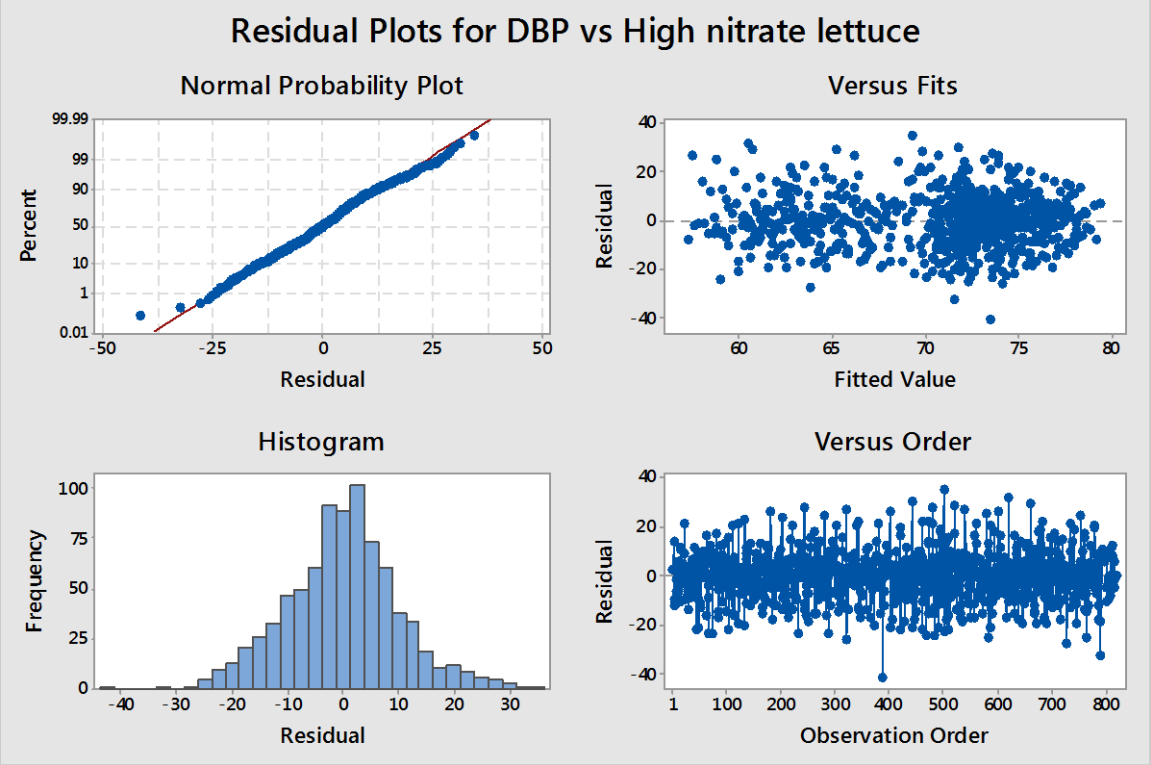
Appendix 59. Average of difference of SBP between high nitrate lettuce and low nitrate lettuce. Values are expressed as mean±SE

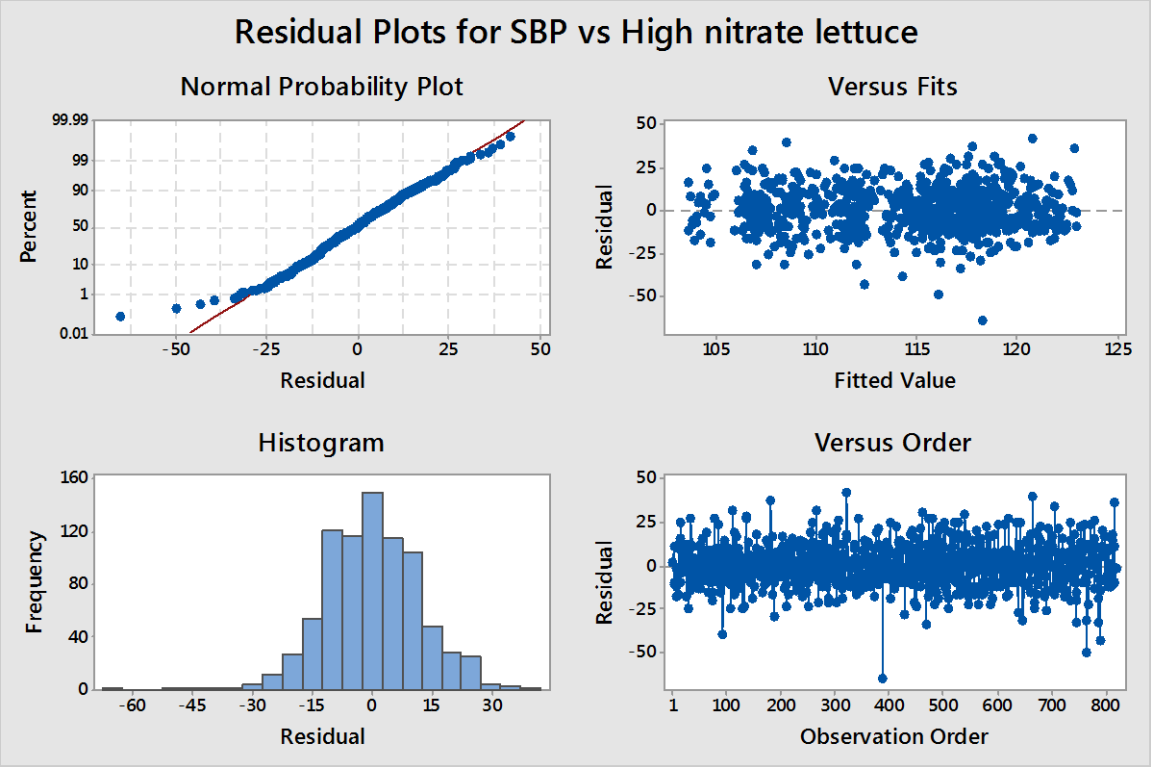


Appendix 60. Average of difference of DBP between high nitrate lettuce and low nitrate lettuce. Values are expressed as mean±SE

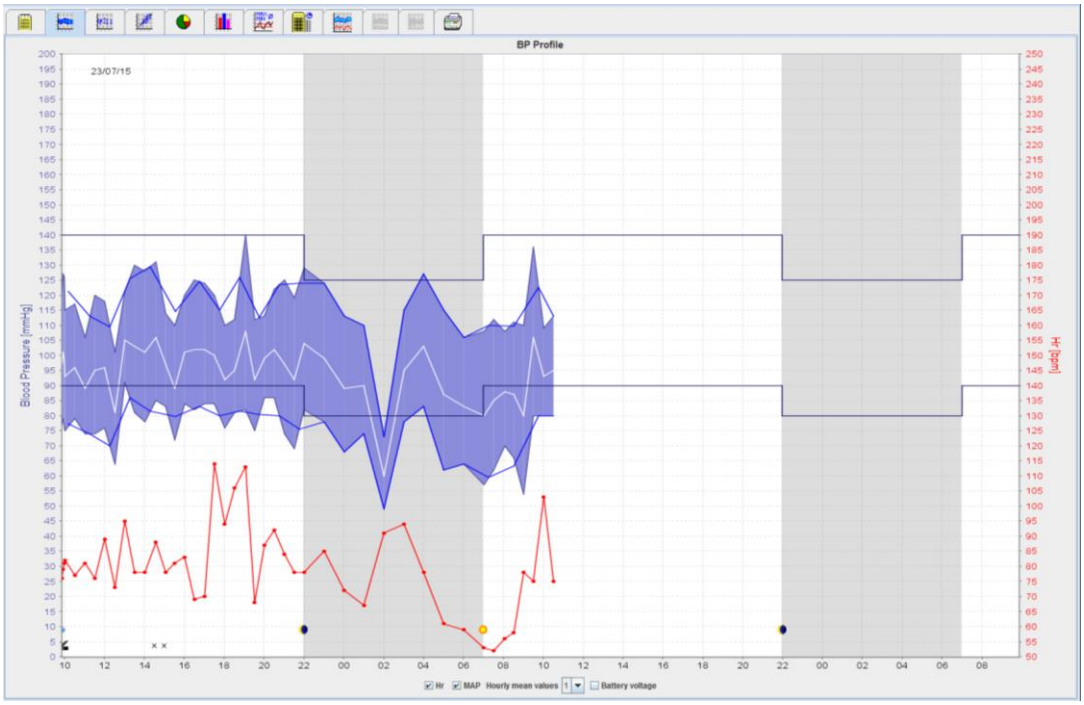

Residual Plots for SBP vs Low nitrate lettuce
Normal Probability Plot
Percent
Residual
Versus Fits
Residual
Fitted Value
Histogram
Frequency
Residual
Versus Order
Residual
Observation Order

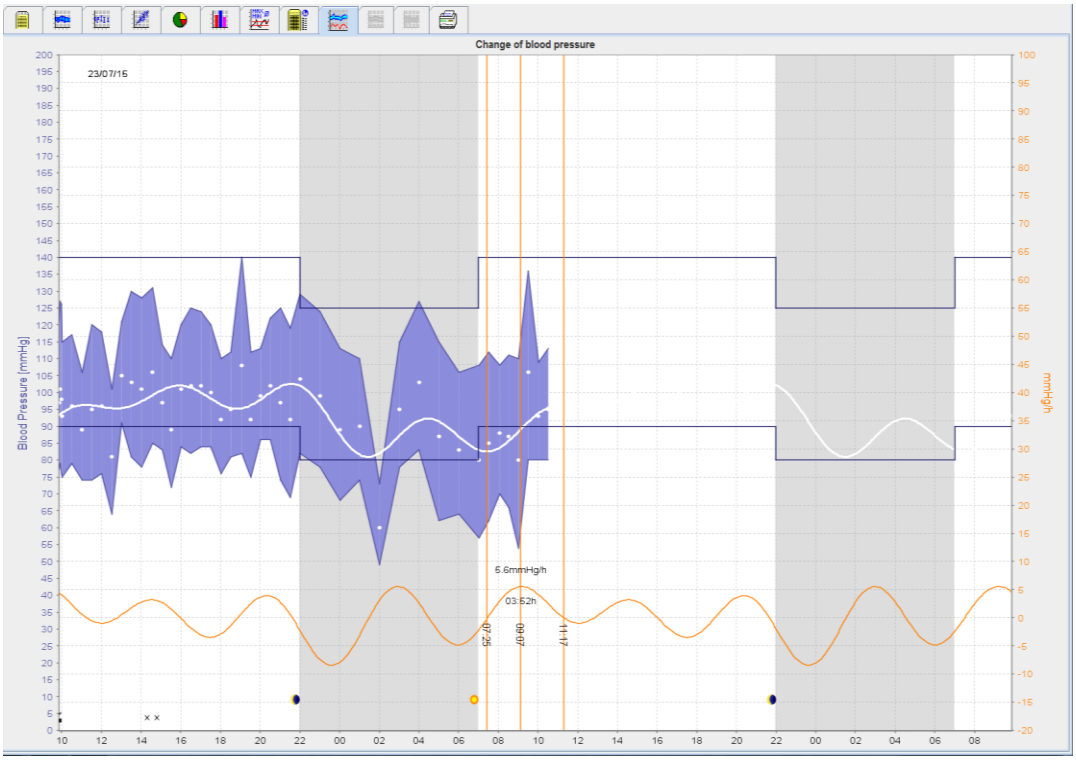

Residual Plots for DBP vs Low nitrate lettuce
Normal Probability Plot
Percent
Residual
Versus Fits
Residual
Fitted Value
Histogram
Frequency
Residual
Versus Order
Residual
Observation Order


Residual Plots for DBP vs High nitrate lettuce
Normal Probability Plot
Percent
Residual
Versus Fits
Residual
Fitted Value
Histogram
Frequency
Residual
Versus Order
Residual
Observation Order

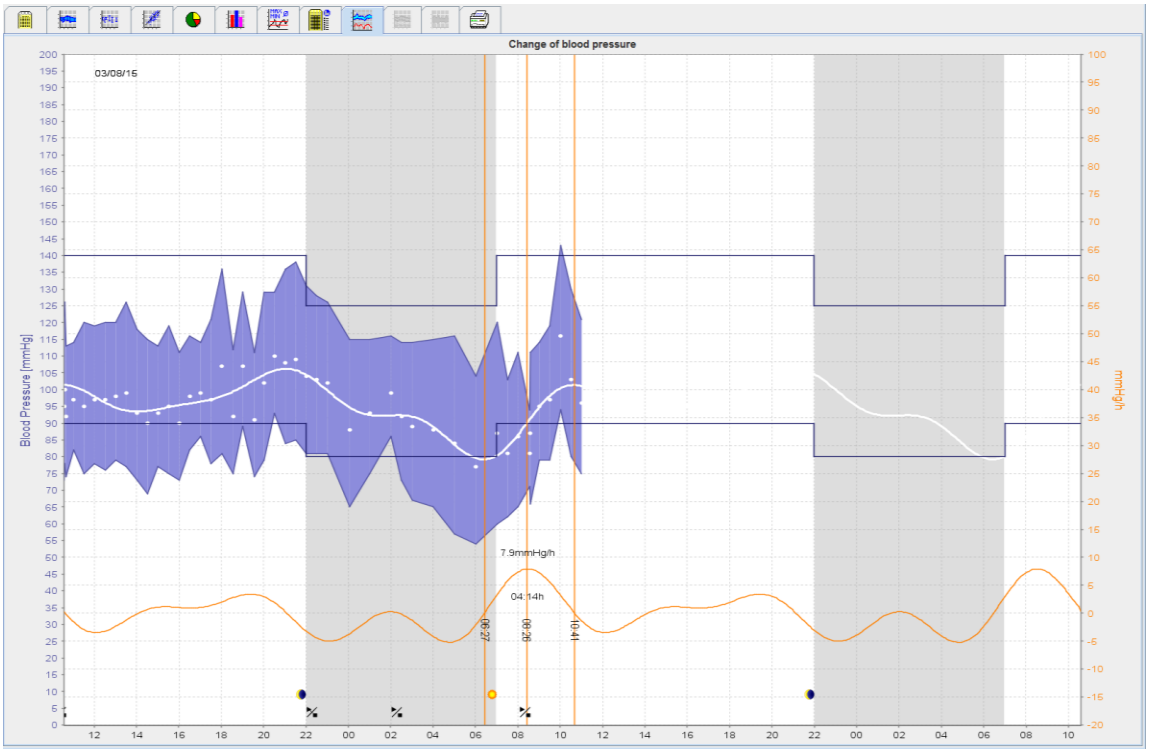

Residual Plots for SBP vs High nitrate lettuce
Normal Probability Plot
Percent
Residual
Versus Fits
Residual
Fitted Value
Histogram
Frequency
Residual
Versus Order
Residual
Observation Order

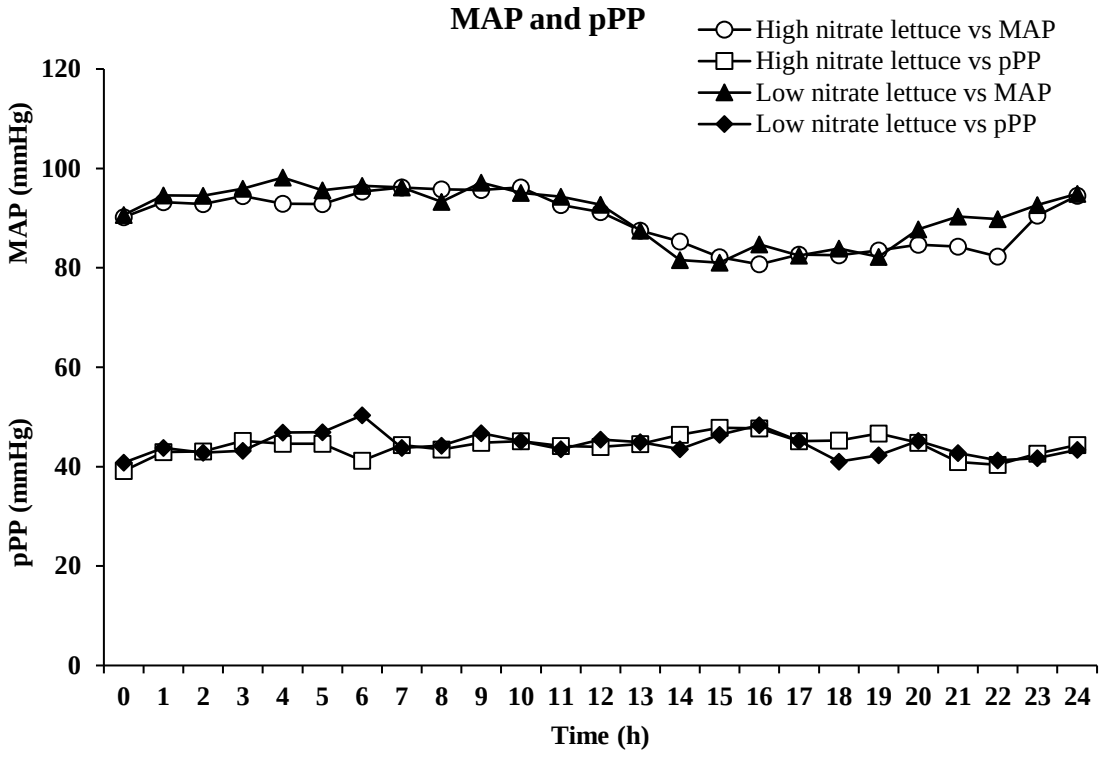
Example of 24hr- ABPM record of a subject received low nitrate lettuce



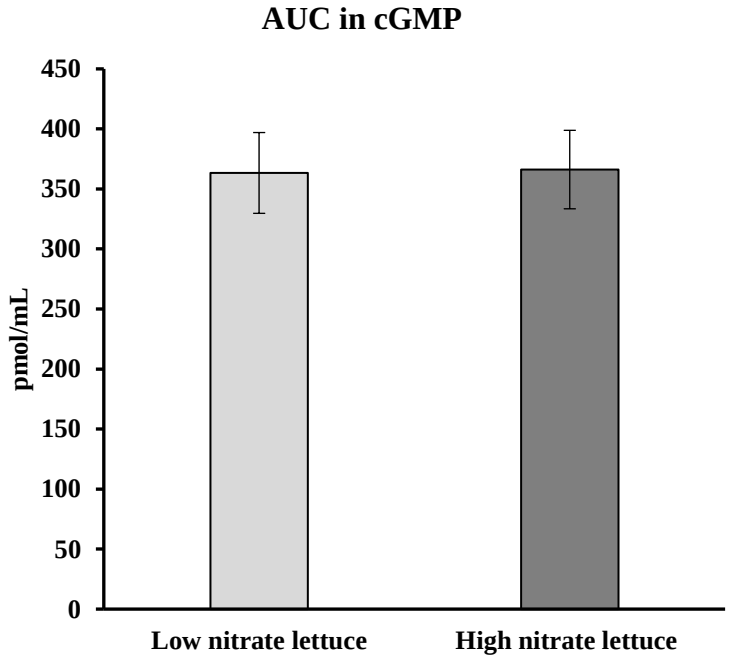


Example of 24hr- ABPM record of a subject received high nitrate lettuce





Appendix 61. Mean arterial pressure (MAP) and Peripheral Pulse Pressure (pPP) outcomes after ingestion low nitrate lettuce vs. high nitrate lettuce



Appendix 62. AUC of plasma cGMP after high and low nitrate intake

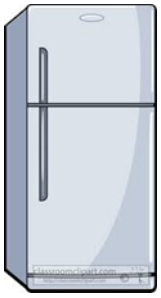
Instruction of urine collection for volunteers

Record the volume

Transfer 5 ml of the urine to the small tube

Ice gel

Zip-lock bag



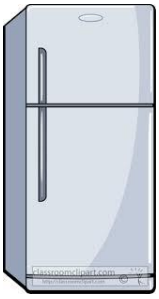
Bringing the urine containers back to NU-food

Transferring ~5ml of collected urine from the urine containers to the small tubes (7ml) & stored in -20 °C

Instruction of saliva sample collection at home by volunteers

1- Open the lid of 30ml universal tube.
2- You spit about (3ml) of your saliva in the tube and close the lid immediately.
3- Put the tube in zip-bag and put it in the freezer immediately.
4- When you come back after 24 hr please take the saliva samples with you.

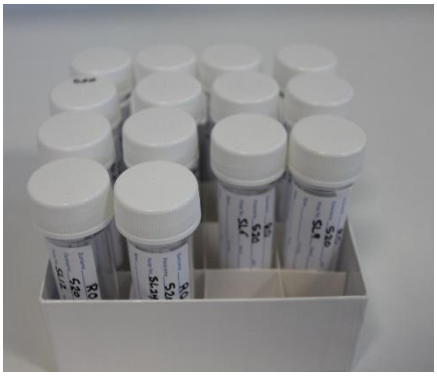
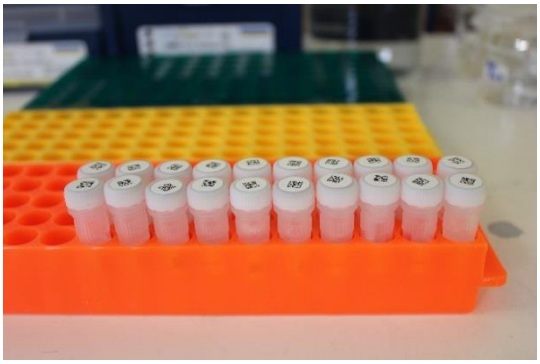


Zip-lock bag

Take the saliva samples after 24hr to NU-food

Saliva samples were transferred from the universal tubes to eppendorf tubes and stored in -20°C

Example of screening questionnaire

Lettuce and blood pressure control

Screening Questionnaire

Name…….

Address……

Age ……..

Phone Number: Work ……; **Home**……; **Mobile**…….

Email: ……..

Are you currently in any other research studies, or have you been in any in the last year?
……..…….....

Self-reported: **Weight: ……..…kg/lbs; Height ……..…….cm/ft inch;**

BMI …… (20-25 kg/m2) *(exclude if outside this range)*

Health:

Do you have any medical conditions or are you taking any medications currently?

YES □ NO □

(exclude if use anti high BP medications)

If YES please describe

……
……
……
……

Do you use mouthwash? **YES □ NO □**

(Exclude if yes)

Do you drink alcohol? **YES □ NO □**

If yes, how many units of alcohol would you consume in an average week?

(1 unit = 1 measure of spirits / 1 small glass of wine / 1 half pint of beer)

……

Are you vegetarian? **YES** □ **NO** □

(Alternative meal for the chicken hotpot meal)

Has your weight changed in the last month? **YES** □ **NO** □

If yes, how much weight have you gained or lost? …….……kg

Availability and Transport:

Would you have any difficulty in:

(i) Attending the research centre on 6 occasions? **YES** □ **NO** □

(ii) Change your diet for about 3 days and avoid certain type of food (mature cheese, green leafy vegetables (rocket, lettuce), high Nitrate containing-food, beetroot processed meat (bacon, cured meats)? **YES** □ **NO** □

(iii) Collecting 12 urine, 8 blood and 20 saliva samples, 24 hr Blood pressure monitor fitted? **YES** □ **NO** □

(iv) Fasting after an evening meal until approx. 9.30 the following morning? **YES** □ **NO** □

(Exclude if yes)

(v) Eating lettuce? **YES** □ **NO** □

(Exclude if yes)

(vi) Abstain from using mouthwash during the entire study? **YES** □ **NO** □

(Exclude if yes)

Do you have any preferred days to attend the unit? **YES** □ **NO** □

If yes, please give days…….

Volunteer database questionnaire sent..... **YES** □ **NO** □

_____

Name of Research Team member

(Please print)

_____

Signature

_____

Date Version 1, January, 2015

Example of Nitrate Screening Questionnaire

We are interested in finding out about the amount of nitrate containing food that people consume as part of their everyday lives. The questions will ask you about the types and frequency of food you consume in the **last 7 days**. Think about all the **foods you ate** in the **last 7 days**. Please answer each question or ask the investigators in this study if there are any questions that you are still unsure of.

Please tick on every line.

During the last 7 days how many times did you eat the following foods?

Food Type	**Frequency**	**Portion Size**
Green leafy vegetables (Broccoli, cabbage, spinach, lettuce, etc).	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
a. Eggplant, Courgette, Turnip, Pumpkin.	**Frequency** ☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	**Portion Size** ☐ Small ☐ Medium ☐ Large
b. Canned Products (Canned corn, canned peas, canned tomatoes, etc)	**Frequency** ☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	**Portion Size** ☐ Small ☐ Medium ☐ Large
c. Cured Meat and/or Bacons.	**Frequency** ☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	**Portion Size** ☐ Small ☐ Medium ☐ Large
d. Mature Cheese.	**Frequency** ☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	**Portion Size** ☐ Small ☐ Medium ☐ Large
e. Beet and Beetroot	**Frequency** ☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	**Portion Size** ☐ Small ☐ Medium ☐ Large

Please tick every line

During the last 7 days how many times did you eat the following foods?

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
1. Fresh Cheese				
• Brie	☐	☐	☐	☐
• Ricotta	☐	☐	☐	☐
• Cream Cheese	☐	☐	☐	☐
• Mozarella	☐	☐	☐	☐
• Others	☐	☐	☐	☐
2. Cereals				
• Potatoes	☐	☐	☐	☐
• Pasta	☐	☐	☐	☐
• Rice	☐	☐	☐	☐
• Breakfast Cereals	☐	☐	☐	☐
• Bread	☐	☐	☐	☐
• Savory Biscuits	☐	☐	☐	☐
3. Meat & Fish				
• Turkey	☐	☐	☐	☐
• Chicken	☐	☐	☐	☐
• Beef	☐	☐	☐	☐
• Pork	☐	☐	☐	☐
• Fish	☐	☐	☐	☐
• Other	☐	☐	☐	☐
4. Legumes				
• Bean	☐	☐	☐	☐
• Peas	☐	☐	☐	☐
• Chickpeas	☐	☐	☐	☐
• Lentils	☐	☐	☐	☐
5. Vegetables				
• Carrot	☐	☐	☐	☐
• Cucumber	☐	☐	☐	☐
• Pepper	☐	☐	☐	☐
• Tomato	☐	☐	☐	☐
• Mushroom	☐	☐	☐	☐
• Cauliflower	☐	☐	☐	☐

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
6. Fruits				
• Apples	☐	☐	☐	☐
• Banana	☐	☐	☐	☐
• Strawberries	☐	☐	☐	☐
• Peach	☐	☐	☐	☐
• Berries	☐	☐	☐	☐
• Others	☐	☐	☐	☐
7. Sweets				
• Chocolate	☐	☐	☐	☐
• Cakes	☐	☐	☐	☐
• Sweets	☐	☐	☐	☐
• Biscuits	☐	☐	☐	☐
• Ice Cream	☐	☐	☐	☐
8. Sauces				
• Ketchup	☐	☐	☐	☐
• Mayonnaise	☐	☐	☐	☐
• Others	☐	☐	☐	☐
9. Dairy Product				
• Milk	☐	☐	☐	☐
• Yogurt	☐	☐	☐	☐
10. Drink				
• Caffeinated	☐	☐	☐	☐
• Non – caffeinated	☐	☐	☐	☐
• Soft Drinks	☐	☐	☐	☐
• Fruit Juice	☐	☐	☐	☐

THANK YOU FOR TAKING YOUR TIME

TO COMPLETE THIS QUESTIONAIRE

International physical activity questionnaire

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Example of visit records of participants

Lettuce and Blood Pressure control

First Visit

Name: ……Code ID ……

Date: ……; Time:……;

Health Check-up:
……

……

Medications: ……
……

Fasting **YES ☐ NO ☐**
Explained aims of study? **YES ☐ NO ☐**

Eligibility Check

Date of birth (dd/mm/yyyy) ……/……/……

Age……years

Non- Smoker: YES ☐ NO☐

<u>If answered NO to any questions, subjects are not eligible and are excluded</u>

Signed informed consent **YES ☐ NO ☐** **Offered copy to volunteer** **YES ☐ NO☐**

Anthropometry

Height……m; Weight……kg; BMI…… kg/m²

***BMI 20-25 kg/m² YES ☐ NO☐; <u>*If BMI is less than 20kg/m² and more than 25 kg/m² then EXCLUDE</u>**

Eligible for study YES ☐ NO ☐

Code: ……; Randomization Order: ……

Body Composition

Waist Circumference……cm

Fat Mass (Kg)	
Fat Free Mass (Kg)	

Note: Remove socks, stockings and excessive outer wear for BIA measurement.

Blood Pressure Measurement (Baseline)

Lying supine position **YES ☐ NO ☐**

Subjects rested for at least 15 minutes **YES ☐ NO☐**

Cuff Size **REGULAR ☐ LARGE☐**

Blood sample

Patient Lying Supine ☐; Insertion of IV Butterfly needle; Right Arm ☐; Left Arm ☐;

Operator: ……

Notes……
……
……

Baseline blood sample LH (6ml) **YES ☐ NO ☐ TIME: ……**

Number of Blood Samples	**Milliliter (mL) Lithium Heparin**	**Blood Pressure Measurement**				
		Time 00:00	**Systolic (mmHg)**	**Diastolic (mmHg)**	**Heart Rate (BPM)**	**MEAN**
Baseline blood sample (0 hour)						
3 hours blood sample						
6 hours blood sample						
24 hours blood sample						

Notes

……

……

……

Blood Pressure Measurement (Baseline)

Cuff Size **REGULAR ☐ LARGE☐**
Arm **Right ☐; Left ☐;**
Starting time: ..... am;

Minutes	Systolic (mmHg)	Diastolic (mmHg)	Heart rate (BPM)	Mean Arterial Pressure
1				
1 Minute rest				
2				
1 Minute rest				
3				
1 Minute rest				
4				
1 Minute rest				
5				

**Three baseline blood pressure recordings will be taken*

Lettuce Intake: YES ☐ NO ☐ TIME: .....; CODE: .....;

Type of salad dressing: YES ☐ NO ☐

Salad Cream ☐ Reduced fat salad cream 60% ☐ Light salad cream 30% less fat ☐

Administered by: .....; Signature: .....;

Dinner before the Experimental day:

Chicken Hotpot □ Vegetarian Penne Pasta □

Breakfast

Nestlé Curiously Cinnamon ☐ Nestlé Cookie Crisp ☐ Nestlé Original Shreddies ☐

Nestlé Golden Nuggets ☐ Nestlé Nesquik ☐ Nestlé Cheerios ☐ Nestlé Coco Shreddies ☐

Subjects received light refreshments? YES ☐ NO ☐

Any concerns? .....

.....

Lunch

Chicken Hotpot □ Vegetarian Penne Pasta □

Dinner

Chicken Hotpot □ Vegetarian Penne Pasta □

Drinking water (Buxton Water)

Subjects received water? YES ☐ NO ☐

Any concerns? .....

.....

Urine samples

Number of Urine Samples	**Volume of urine samples (mL)**	**Time** **(00:00) minutes**	**Comments**
First Morning Void (FMD) **At home**			
0 Baseline urine sample			
0 - 3 hours urine sample			
3 - 6 hours urine sample			
6 - 12 hours urine sample **At home**			
12 -24 hours urine sample **At home**			
After 24 hours urine sample			

**Provide urine aid for female volunteers*

Note

.....
.....
.....
.....
.....

Saliva Samples

Number of saliva Samples	**Volume of saliva samples (mL)**	**Time** **(00:00) minutes**	**Comments**
0 Baseline saliva sample			
1 hour saliva sample			
2 hours saliva sample			
3 hours saliva sample			
4 hours saliva sample			
5 hours saliva sample			
6 hours saliva sample			
9 hours saliva sample **At home**			
12 hours saliva sample **At home**			
After 24 hours saliva sample			

Notes

.....
.....
.....

Intervention (washout period for 3 weeks)

Explained procedure for 2nd visit to volunteer YES ☐ NO ☐

Subject told to fast 12 hours before coming to 2nd visit YES ☐ NO ☐

Told subject we will call 2 days in advance to remind them of their 2nd visit

YES ☐ NO ☐

END of FIRST VISIT

Comments

……
……
……

***Update Clinical Research File of participant: YES ☐ NO ☐**

Signature: ……; Date:……

***Include a copy of the participants consent form: YES ☐ NO ☐**

Signature: ……; Date:……

Lettuce and Blood Pressure control

Second Visit

Code ID : ……; Date:……; Time:……

Health Check-up: ……

……

Medications:……

……

Fasting **YES** ☐ **NO** ☐

Explained aims of study? **YES** ☐ **NO** ☐

Body Composition

Waist Circumference ☐☐☐ . ☐ **cm**

BIA

Fat Mass (Kg)	
Fat Free Mass (Kg)	

Note : Remove socks, stockings and excessive outer wear for BIA measurement.

Insertion of Butterfly needle

Subject is happy to have the blood sample taken **YES ☐ NO ☐**
Subject understands we will take 4 samples in total **YES ☐ NO ☐**
Which arm is the butterfly needle used **RIGHT ☐ LEFT ☐**

Notes.....
.....
.....
.....

Blood Pressure Measurement (Baseline)

Lying supine position **YES ☐ NO ☐**
Subjects rested for at least 15 minutes **YES ☐ NO☐**
Cuff Size **REGULAR ☐ LARGE☐**

Baseline samples

Blood sample

Patient Lying Supine ☐; Insertion of IV Butterfly needle; Right Arm ☐; Left Arm ☐;
Operator: ……
Notes……
……
……

Baseline blood sample LH (6ml) YES ☐ NO ☐ TIME: ……

Number of Blood Samples	Milliliter (mL) Lithium Heparin	Blood Pressure Measurement				
		Time 00:00	Systolic (mmHg)	Diastolic (mmHg)	Heart Rate (BPM)	MEAN
Baseline blood sample (0 hour)						
3 hours blood sample						
6 hours blood sample						
24 hours blood sample						

Notes

……

……

Blood Pressure Measurement (Baseline)

Cuff Size **REGULAR □ LARGE□**
Arm **Right □; Left □;**
Starting time: ……. am;

Minutes	Systolic (mmHg)	Diastolic (mmHg)	Heart rate (BPM)	Mean Arterial Pressure
1				
1 Minute rest				
2				
1 Minute rest				
3				
1 Minute rest				
4				
1 Minute rest				
5				

**Three baseline blood pressure recordings will be taken*

Lettuce Intake: **YES □ NO □ TIME: ……; CODE: ……;**

Type of salad dressing: YES □ NO □

Salad Cream □ Reduced fat salad cream 60% □ Light salad cream 30% less fat □

Administered by: ……; Signature: ……;

Dinner before the Experimental day:

Chicken Hotpot □ Vegetarian Penne Pasta □

Breakfast

Nestlé Curiously Cinnamon □ Nestlé Cookie Crisp □ Nestlé Original Shreddies □ Nestlé Golden Nuggets □ Nestlé Nesquik □ Nestlé Cheerios □ Nestlé Coco Shreddies □

Subjects received light refreshments? YES □ NO □

Any concerns?

……

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Lunch

Chicken Hotpot □ Vegetarian Penne Pasta □

Dinner

Chicken Hotpot □ Vegetarian Penne Pasta □

Drinking water (Buxton Water)

Subjects received water? YES □ NO □

Any concerns?

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Urine samples

Number of Urine Samples	**Volume of urine samples (mL)**	**Time** **(00:00) minutes**	**Comments**
First Morning Void (FMD) **At home**			
0 Baseline urine sample			
0 - 3 hours urine sample			
3 - 6 hours urine sample			
6 - 12 hours urine sample **At home**			
12 -24 hours urine sample **At home**			
After 24 hours urine sample			

**Provide urine aid for female volunteers*

Notes

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Saliva Samples

Number of saliva Samples	**Volume of saliva samples (mL)**	**Time** **(00:00) minutes**	**Comments**
0 Baseline saliva sample			
1 hour saliva sample			
2 hours saliva sample			
3 hours saliva sample			
4 hours saliva sample			
5 hours saliva sample			
6 hours saliva sample			
9 hours saliva sample **At home**			
12 hours saliva sample **At home**			
After 24 hours saliva sample			

Notes……
……
……

Intervention Finished

Thank volunteer for their time and commitment **YES ☐ NO ☐**

Hand volunteer there Gift/Reimbursement **YES ☐ NO ☐**

END of STUDY VISIT

Comments

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***Update Clinical Research File of participant: YES ☐ NO ☐**

Signature: ……; Date:……

***Include a copy of the participants consent form: YES ☐ NO ☐**

Signature: ……; Date:……

Post Study
***Results letter sent to participant: YES ☐ NO ☐**

Date…… Signature……

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