Sudan University of Science and Technology
College of Graduate Study

Development and Validation of Chromatographic Methods for the Analysis of 4-Methylimidazole and Taurine in Carbonated Beverages

التطوير والتحقق من طرق الكروماتوغرافيا لتحليل 4-ميثيل إيمادزول و تورين في المشروبات الغازية

A thesis submitted in fulfillment of the requirements for the degree of Master of Science in chemistry

By

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December, 2018

الآية

قال تعالى: ﴿ وَمَا أَرْسَلْنَا مِنْ قَبْلِكَ إِلَّا رِجَالًا نُوحِي إِلَيْهِمْ فَاسْأَلُوا أَهْلَ الذِّكْرِ إِنْ كُنْتُمْ لَا تَعْلَمُونَ ﴾

صدق الله العظيم

سورة النحل الآية {43}

Dedication

To my beloved parents,

To my sisters and brothers,

To my friends

Acknowledgments

My full praise and thanks to our God for his guidance and gave me the strength to complete my study.

I would like to express my deepest grateful to my supervisor, Professor Abdalla Ahmed Elbashir for his encouragement, support, deep discussion and scientific guidance throughout the period of my study. I am deeply appreciate to him for arranging of the necessary funding.

My thankfulness to my co. supervisor Dr. Mohammed Elmukhtar Abd elaziz for his great help.

I gratefully acknowledge the financial assistance provided by Arab-German Young Academy of Sciences and Humanities (AGYA) and Ministry of Higher Education & Scientific Research, Commission of scientific research and Innovation for research funding.

I would like to thank Central Laboratory, Ministry of Higher Education & Scientific Research for the facilitations (workplace and equipment).

My deeply thanks to my sister Dr. Mei Musa Ali Omar for her unlimited support, guidance, and follow up the study step by step.

To my family, I thank my father, mother, sisters and brothers for their continuous praying, supporting and encouragement.

Abstract

This study described the optimization, validation, and application of anlaytical methods for determination of two hazardous compounds, namely: 4(5)-methylimidazole (4-MEI) and taurine that are usually found in carbonated drinks. 4-MEI was derivatized with 4-chloro-7 nitrobenzo-2-oxa-1,3- diazole (NBD-Cl) reagent and then determined using UV-Vis spectrophotometry and high performance liquid chromatography with photodiode array detecor (HPLC-PDA). The main parameters affecting the derivatization reaction between 4-MEI and NBD-Cl were optimized and gave excellent linearity with correlation coefficient (r^2 = 0.9998 and 0.9999) in the range of (1.0-50)mgL^{-1} and (0.5-50) mg L^{-1} for UV-Vis spectrophotometry and HPLC-PDA, respectively. The limit of detection (LOD) was found to be 0.183 mg L^{-1} and 0.152 mg L^{-1}, and limit of quantification (LOQ), 0.550 mg L^{-1} and 0.457 mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis respectively. The precision or relative standard deviation (RSD%) of the absorbance for intra-day and inter-day were 2.598 and 3.987 %, respectively. Good percent recoveries in range (91.8–106)% and (94.3-108)% were obtained for UV-Vis spectrophotometry and HPLC-PDA analysis, respectively. The developed methods were successfully applied for determination of 4-MEI in some soft drinks available in local markets i.e. Coca cola, Pepsi, Cola light, Pepsi diet, Veno and Vimto.

Two derivatzation methods using NBD-Cl and *o*-phthalaldehyde-sodium sulfite (OPA-Na_2SO_3) reagents were optimized and used for determination of taurine in energy drinks. The taurine-NBD derivative was determined using HPLC-PDA and HPLC coupled fluorescence detection (HPLC-FLD). In both validated methods, the derivatization product was separated on Inertsil ODS-3 analytical column with acetonitrile and 0.1% trichloroacetic acid (30:70, v:v) as mobile phase. The eluted derivative was detected at 472 nm by HPLC-PDA for absorption and at 472 nm for excitation and at 530 nm for emission by HPLC-

FLD. Good linearity was achieved for taurine (r^2 = 0.9998 and 0.9993) in the range of 5.0-50 mg L^{-1} and 5.0-50 µg L^{-1}, the LOD was 0.296 and 0.616 $\times 10^{-3}$ mg L^{-1}, the precision for peak area was 0.78% and 0.61% for HPLC-PDA and HPLC-FLD, respectively. Recovery of taurine, however, ranged from 92% to 103.3% for both.

The methods of determination of taurine after derivatization with OPA-Na_2SO_3 was optimized and validated. The taurine- OPA-Na_2SO_3 was measured using HPLC-PDA and UV-Vis spectrophotometry. The method was linear in the range of 0.5-20 mg L^{-1} and 0.5-15 mg L^{-1} , with correlation coefficient (r^2) of 0.9998 and 0.9996, LOD of 0.109 mg L^{-1} and 0.141 mg L^{-1} , intarday precision of 1.816% and 1.278%, interday precision of 2.858% and 2.236% , for HPLC-PDA and spectrophotometry, respectively. Recovery of taurine, however, ranged from 90% to 105% for both. These validated derivatization methods were successfully applied for determination of taurine in energy drink samples namely: Red bull, Tornado, Kratingdeang, Bison and Tiger.

المستخلص

تهدف هذه الدراسة لإيجاد طريقة ذات كفاءة عالية للتحليل الكمي للمشروبات الغازية لإثنين من أهم المركبات الخطرة هما 4-ميثيل إيمادزول والتوارين. تم إشتقاق 4-ميثيل ايمادزول بكاشف 4-كلورو-4-نايتروبنزو-2-اوكسا-3,1-داي ازول وتم تعيينه بالمضواء الطيفي للأشعة فوق البنفسجية والمرئية و جهاز كروماتوغرافيا السائل عالي الاداء المقترن بمكشاف (PDA). تمت متابعة ودراسة العوامل الرئيسية المؤثرة على تفاعل الإشتقاق تم الحصول على علاقة خطية ممتازة بمعامل إرتباط يساوي0.9998 و 0.9999 خلال مدى تركيز 1.0-50 و0.5-50 ملجم/لتر والحد الأدنى للكشف والحد الأدنى للقياس الكمي يساوي 0.183-0.152 و0.55-0.457 ملجم/لتر، الدقة والإنحراف المعياري لقيم الإمتصاص 2.598 %و3.9879% و نسبة إسترجاع جيدة في المدى 91.8-106 % و 94.3-108% للمضوائية الطيفية للأشعة فوق البنفسجية و كروماتوغرافيا السائل عالي الاداء المقترن بمكشاف (PDA) على التوالي .

تم تطبيق الطريقة المطورة المقترحة بنجاح لتقديرال 4-ميثيل إيمادزول الموجود في بعض المشروبات الغازية التجارية المتواجدة في المتاجر المحلية مثل البيبسي ,الكوكاكولا,الكوكا كولا لايت,الفيمتو,الفينو وبيبسي الريجيم.

تم عمل مشتقات من التوارين وال 4-كلورو-4-نايتروبنزو-2-اوكسا-3,1-داي ازول والاورثوفثالدهيد وكبريتيد الصوديوم وتم ضبط الطريقة وتطبيقها لتقدير التوارين الموجود في مشروبات الطاقة باستخدام كروماتوغرافيا السائل عالية الاداء المقترن بمكشاف (PDA) و مكشاف الفلورة. في كلتا الطريقتين تم فصل الناتج المشتق بواسطة عمود الانرتسيل(3- اوكتا ديسيل سيلان) وإستخدام طور متحرك من الاسيتونيتريل و0.1% ثلاثي كلورو حمض الخليك (70:30 حجم:حجم) تم تقدير المشتق الخارج من العمود بمكشاف (PDA) عند طول موجي 472 نانوميتر وايضا بمكشاف الفلورة عند طول موجي 472 نانوميتر للإثارة وطول موجي 530نانوميتر للإنبعاث.

تم الحصول على علاقة خطية جيدة بقيمة معامل إرتباط يساوي 0.9998 و 0.9993 في مدى تركيز0.5-50 ملجم/ لتر و5.0-50 ميكروجرام/لتر والحد الأدنى للكشف 0.296 و 0.616 *10^{-3} مليجرام/لتر والدقة لمساحة الاشارة كانت 0.78%و 0.61% لمكشافي (PDA) و الفلورة على التوالي. أما إسترجاع التوارين كان في المدى 92-103.3% لكليهما.

تم تقدير التوارين المشتق بالاورثوفثالدهيد وكبريتيد الصوديوم باستخدام جهاز كروماتوغرافيا السائل عالي الاداء المقترن بمكاشف (PDA) و جهاز المضواء الطيفي للأشعة فوق البنفسجية والمرئية وجد ان الطريقة خطية في مدى تركيز يساوي0.5-20ملجم/لتر و0.5-15ملجم/ لتر بمعامل إرتباط يساوي 0.9998 و0.9996 و حد كشف 0.109 ملجم/لتر و0.141 ملجم/لتر ودقة 1.816-2.858% و 1.278-2.236% لتقنية كروماتوغرافيا السائل عالية الاداء المقترن بمكشاف (PDA) و المضوائية الطيفية للأشعة فوق البنفسجية والمرئية على التوالي.أما إسترجاع التوارين كان 90-105% في كلتيهما. هذه الطريقة تم تطبيقها بنجاح لتقدير التوارين في مشروبات الطاقة الموجودة في المحلات التجارية مثل الريدبول,تورنيدو,بيسون,تايقر وكراتينغدينغ.

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List of Abbreviations

Carbonated soft drink.....	CSD
Dimethyl dicarbonate.....	DMDC
4(5)- Methylimidazole.....	4-MEI
(5)-(1,2,3,4 Tetrahydroxy butyl-imidazole.....	THI
Chromosome aberration.....	CA
Food Additives Organization.....	FAO
World Health Organization.....	WHO
European Union.....	EU
Central Nervouse System.....	CNS
High Performance Liquid Chromatograohy	HPLC
Mass Spectrometer.....	MS
Gas Chromatography	GC
Ultraviolet.....	UV
Magnetic Molecule Inprinted Polymer.....	MMIP
Photodiod array Detecter.....	PDA
Limit of Detection.....	LOD
Limit of Quantification.....	LOQ
Diod arrary Detecter.....	DAD
Solid Phase Extraction.....	SPE
Time of Flight.....	TOF
Electron Spray Ionization.....	ESI
Atmospheric Pressure Ionizatio.....	API

Multi Reaction Monitoring.....	MRM
Selected Ion Monitoring.....	SIM
Electro Spray Ionization.....	ESI
Bis-2-ethylhexyl phosohate.....	BEHPA
Electron Impact.....	EI
Nuclear Magnetic Resonance.....	NMR
0-Phthalaldehyde.....	OPA
2,4-Dinitrofluorobenzene.....	DNFB
2-[2-(Dibenzocarbazol)-ethoxy]ethyl chloroformate.....	DBCEC
4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride.....	DMS-Cl
Electrochemical Detection.....	ED
4-Chloro-4-nitrobenz-2-oxa-1,3-diazole.....	NBD-Cl
Evaporative Light Scattering.....	ELS
4-Fluoro-7-nitrobenzofurazan.....	NBDF
Octadecyl Silan.....	ODS
International Standardization Organization.....	ISO
International Conference Harmonisation.....	ICE
Fluorescence Detecter.....	FLD

List of Publications

Omer, M., Omar, M., Thiel, A., & Elbashir, A. (2018). High Performance Liquid Chromatographic Methods for Analysis of Taurine in Energy Drinks after Pre-column Derivatization. *Eurasian Journal of Analytical Chemistry, 13, 5.*

Omer,M., Omar, M., Almokhtar, M., Thiel, A., & Elbashir, A., (2019). Liquid Chromatographic and Spectrophotometric Determination of Taurine in Energy Drinks Based on O-Phthalaldehyde-Sulfite Derivatization. *Journal of Food Chemistry and Nanotechnology, 5(1) ,1-7.*

Chapter one

Introduction

1. Introduction

1.1. Carbonated beverages

Carbonated drinks are the second most consumed beverages in the world, which are made by incorporating carbon dioxide into a liquid. The origin of soft drinks lies in the development of fruit-flavored drinks. In 1767, Englishman Joseph Priestley first discovered a method of infusing water with carbon dioxide to make carbonated water (Fahim, 2015). These drinks are first appeared in the market as a mixture of lemon juice and water, sweetened with honey (Fahim, 2015).

Carbonated beverages, which include sodas, sparkling waters and beers, have unique properties. The principal properties of all common types of carbonated beverages are carbonation, acidity and high levels of sugar or artificial sweeteners. Carbonation, which produces the characteristic effervescence and bubbling associated with these drinks, is the result of dissolving gaseous CO_2 in a liquid under pressure (Johnson et al., 2010). Temperature and pressure influence the rate at which dissolved carbon dioxide converts into gas and is released, thus producing bubbles when a beverage container is opened (Cuomo et al., 2009). Acidity is a common chemical property of many carbonated beverages such as Colas, sodas and beers. In part, the acidic nature of sodas (pH 2.30-3.39) is derived from conversion of dissolved CO_2 to HCO_3) and H^+ by interaction with H_2O as well as additives such as citric and phosphoric acid. These acids are added to improve the organoleptic properties of a carbonated beverage such as taste which are important for their consumption (Johnso et al., 2010, Ratnayake & Ekanayake, 2010). Sugars and artificial sweeteners are added to many carbonated beverages, such as sodas, to impact a sweet taste, while beverages like beer have other forms of carbohydrate used in the fermentation process. Sugars and other carbohydrates add calories and increase the osmolality of the beverage. Artificial sweeteners provide a sugary taste without increasing the caloric content of the beverage. Other additives such as caffeine, taurine, alcohol, etc. may also be present, depending on the type of

beverage (Johnson et al., 2010). The effects of carbonated beverages on the human body have been the centre of much attention in the last decade, specifically, the consequences of carbonation, acid load and high carbohydrate consumption. Carbonated beverages are already notorious for their adverse effects on teeth, bone, liver and heart vessels. For more than a century, carbonated drink industry has been under political issues. A series of legislative acts were introduced in the congress to monitor and take actions against carbonated drinks and their ingredients which were proven harmful from time to time (Fahim, 2015). Carbonated drinks are considered to be associated with the development of cardiovascular disease, diabetes (Palmer et al., 2013) mellitus, dental, bone problems and obesity (Swinburn et al., 2004), all of which are strongly linked with kidney health. There is also the associated risk of formation of kidney stones (Passman et al., 2009). The most common types of beverages are the soft drinks and energy drinks (Fahim, 2015).

1.1.1. Soft drinks

Commonly, a soft drink refers to almost any drink that does not contain alcohol (Ashurst, 2016). The drinking of carbonated soft drinks (CSD) is as old as human history, and the serving of drinks for profit is as old as the concept of profit itself. In most cultures over the centuries, these have been accepted as an essential part of everyday life. CSD are consumed mainly for refreshment. Refreshment is very important to people of all ages at work and play. Sugar sweetened soft drink contains energy giving carbohydrate, which is a refreshing addition to a balanced diet (Godwill et al., 2015). These can also be psychologically refreshing during stress. Soft drinks contribute to the healthy and enjoyable diet. Soft drinks in addition to water also meet the fluid requirement. Besides water, body needs other nutrients for growth, energy and good health. Selected beverages can provide this vital combination of protein, carbohydrate, fat, vitamins, minerals and water. Hence, soft drinks provide part of the total daily intake of liquid and energy. The second area of nutritional

significance is the ability of soft drinks to promote rapid uptake of salts and water by body. The consumers choose the soft drinks that best suit their lifestyle, tastes, nutritional needs and physiological constraints (Ashurst, 2016).

1.1.1.1 Ingredients

The common components of the soft drinks are water, sugar and sweetener, fruit juices, acitity regulators and carbon dioxide, preservatives and flavorings and coloring agents.

(i)Water

Water is the main component of the soft drinks, mainly they contain approximately 90 percent water, while diet soft drinks may contain up to 99% water. Drinking water includes trace amounts of various ions which alter its taste. Water for soft drinks should fulfill the physical, chemical, and microbiological criteria for drinking water according to World Health Organization (WHO) standards (Kregiel, 2015).

(ii) Sugar and sweetener

Soft drinks usually contain between 1% and 12% sugar (w/w). Sucrose, glucose, or fructose, in various forms, is used as natural carbohydrate sweeteners (Fitch & Keim, 2012). The most common natural sweeteners provide glucose, the primary source of energy. Sucrose (saccharose) is a disaccharide composed of glucose and fructose molecules. This sugar can preserve and enhance the flavor of a drink and gives a satisfying sensation. The overconsumption of sugars can cause negative health effects, such as obesity, diabetes mellitus, or nonalcoholic fatty liver disease (Stratford & James, 2003).

(iii) Fruit juices

Fruits and fruit juices are added to soft drinks as a rich source of various nutrients and bioactive compounds, such as fiber, sugars, organic acids,

phosphates, minerals, and vitamins, as well as colors, flavors, and antioxidants (Stratford & James, 2003).

(iv) Acitity regulators and carbon dioxide

The carbonation of soft drinks varies from 1.5 to 5.0 g L^{-1}. Carbon dioxide is supplied to soft drinks manufacturers either in solid form (as dry ice) or in liquid form maintained under high pressure in heavy steel containers. This process makes the drink more acidic, which serves to sharpen the flavor and taste (Taylor, 2006). It also helps preserve soft drinks for longer time (Korzeniewskaet al., 2005). Acidity regulators are used in soft drinks to improve their taste by balancing the sweetness. Human saliva is almost neutral (pH approximately 6.8), and when our taste receptors interact with acids in food or drink this sensation is perceived as sourness (Chaudhari & Roper, 2010). Acids also play an important role in the natural preservation of soft drinks (Glevitzky et al., 2009).

(v) Preservatives

Chemical preservatives are used to improve the microbiological stability of soft drinks. The types of chemical preservatives that can be used depend on the chemical and physical properties of both the preservative and the beverage. The pH of the product, the presence of vitamins, the packaging, and the conditions of storage will determine what types of preservative, if any, should be used to prevent microbial growth. Sorbates (E 200-203), benzoates (E 210-213), and dimethyl dicarbonate (DMDC) (E 242) are permitted in ready-to-drink beverages in Europe. Sorbates are very effective preservatives against bacteria, yeasts, and molds. The antimicrobial effectiveness of sorbates depends on the physical and chemical properties of the beverage. Sorbates and benzoates are often used in combination, especially in highly acidic drinks (Kregiel, 2015). Sorbic acid affects yeast growth by inhibiting the uptake of amino acids and the function of sulfhydryl enzymes, while benzoic acid destroys the internal proton

level of microbial cells (Batteyet al., 2002). Other ingredients Various hydrocolloids, such as guar and locust gum, pectin, and xanthan, are used as stabilizers and thickeners, especially in diet drinks (to improve mouth feel) (Sengar & Sharma, 2014).

(vi) Flavorings and coloring

The use of colorings in soft drinks serves several important functions: (i) making the product more aesthetically appealing (ii) helping to correct for natural variations in color or for changes during processing or storage (iii) contributing to maintaining the qualities by which the drink is recognized. There are three basic categories of colorings: natural colors, artificial colors, and caramels. Natural colorings can be extracted from plants, fruits, and vegetables and comprise two main categories: (i) yellow to orange carotenoids, extracted from plants (ii) bright red to purple anthocyanins obtained commercially from a range of fruits and vegetables. Natural colorings are also added to soft drinks for their antioxidant properties (Gruenwald, 2009).

(vii) Caramel colors

Caramel colors have been used for a long time and in a wide variety of food products so that consumers tend to think of them as a single substance, but in reality they are a family of similar materials with slightly different properties (Sengar & Sharma, 2014). Caramel colors are brown powders or viscous liquids that are used to impart yellow to dark brown. This color additive can be prepared from various sources of carbohydrates such as glucose, corn, molasses, wheat, and tapioca hydrolysates by controlled heat treatment in the presence of certain reactants. Different reactants such as acids, alkalis, salts, ammonium salts and sulfites can be added depending on the type of caramel color (Kasim, 2010). Each type of caramel color has specific functional properties that ensure compatibility with a product and eliminate undesirable effects, such as haze, flocculation, and separation (Sengar & Sharma, 2014).

The most importance properties when dealing with caramel color are stability and compatibility with food which depend on types of reactant used and technical conditions such as time, temperature, moisture content and pressure. Since caramel color is a result of a complex chemical composition, the complete characterization of caramel is a difficult task (Delgado & Paredes, 2002). The stability of a caramel depends on the charge carried by reactants in a caramel. If reactants in a type of caramel are all negatively charged, then the color is stable. In contrast, if the reactants consist of negatively and positively charge molecules, then the caramel color will coagulate and this result in unstable caramel color (Kamuf et al., 2003). The compatibility of a caramel color can be defined as the absence of flocculation, precipitation and haze formation (Pintea, 2007). This again relates with the stability of caramel color. A stable colloidal system will prevent precipitation or coagulation which usually happens between oppositely charged molecules. A caramel color is said to be compatible when the particles of the caramel color have the same charge as the colloidal particles of the product to be colored. For example, a soft drink contains negatively charged colloidal particles, and therefore, a negative caramel color should always be used. Caramel color has a significance value as food additives that act as a provider food coloring and pleasant odor to foods. The importance of caramel color lies on its valuable functional properties. Therefore, caramel color is widely used in the beverages industry to gives color to drinks (Kasim, 2010).

(viii) Classes of caramel

Caramel color is a versatile food colorant. It is produced to have a broad spectrum of target consumers. Therefore, it is classified into four classes of caramel according to types of reactant and its color intensity

(i) Caramel Class I (plain or spirit caramel):

Prepared by heating carbohydrates with or without acids or alkalis; ammonium or sulfite compounds are used.

(ii) Caramel Class II (caustic sulfite caramel):

Prepared by heating carbohydrates with or without acids or alkalis in the presence of sulfite compounds; no ammonium compounds are used.

(iii) Caramel Color III (ammonia caramel):

Prepared by heating carbohydrates with or without acids or alkalis in the presence of ammonium compounds; no sulfite compounds are used.

(iv) Caramel Color IV (sulfite-ammonia caramel):

Prepared by heating carbohydrates with or without acids or alkalines in the presence of both sulfite and ammonium compounds (Kasim, 2010).

(ix) Caramelization process

It is a process of sugar turning brown when heat is applied. During this process, water is being removed from the sugar (Kamuf et al., 2003). The caramelization process can be conducted in open or closed vessels. The mixture obtained is cooled and filtered, and then the pH and specific gravity are adjusted by the addition of acids, alkalis, or water (Pintea, 2007). Caramelization causes important changes in foods, not only in color but also in flavor. As no enzymes are involved in the caramelization process, it is a non-enzymatic browning reaction. During a caramelization reaction, the sugars initially undergo dehydration and then condensation or polymerization into complex molecules of varying molecular weights. Lightly colored, pleasant-tasting caramel flavors are produced during the initial stages, but as the reaction continues higher molecular weight color bodies are produced, and the flavor characteristics become more bitter (Kamuf et al., 2003, Benhura et al., 1999) The Mechanism of caramelization comprises the reactions involved in the thermal decomposition of carbohydrates, in particular reducing mono- and disaccharides. Various stages of the caramelization reaction may be discerned (Kroh, 1994). The first step in most caramelization reactions involves internal reorganizations with the carbohydrates which are known as enolizations. The second step comprises the formation of the anhydro form of the carbohydrate

via the elimination of a water molecule. This is followed by variety of chemical reactions which depend strongly on the precise sugar composition, sample conditions like pH and the temperature. In these intermediate stages, most of the volatile compounds associated with caramel flavor are formed.

Heat treatment of foods is desirable because it helps develop color, flavor, aroma; however, despite these benefits, applying heat to foods also produces some undesirable compounds like polycyclic aromatic hydrocarbon (PAHs), furan, aromatic amines, 4(5)-methylimidazole (4-MEI), 5-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) and acrylamide, all of which can cause adverse health effects. Some of these compounds have even been categorized as cancer causing agents (UT, 2018).

1.1.1.2. 4(5)-Methylimidazole (4-MEI)

4-MEI (Figure 1.1) is a heterocyclic compound with two nitrogen atoms derived from imidazole. It is widely used in the pharmaceuticals, chemical, and agricultural industries. 4-MEI is widely present in a variety of foods including roasted meats, coffee, wine, carbonated beverages, and soy sauce (Yamaguchi & Masuda, 2011). Moreover, 4-MEI is the main imidazole in the class III and IV caramel colorings (Buckee & Bailey, 1978) and was also found in ammoniated forage and hay (Karangwa et al., 1990). Concentrations of 4-MEI in caramel colors can vary depending upon the manufacturing process, and the types of carbohydrates and nitrogen-containing compounds used (Müller & Jork, 1993).

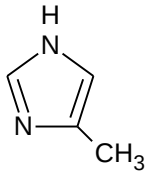

Figure 1.1. Chemical structure of 4-MEI molecule

1.1.1.2.1. Mechanism of 4-MEI formation in soft drinks

Formation of 4-MEI during Maillard reaction from glucose and ammonia was first reported in the early 1960 (Komoto, 1962). 4-MEI forms in thermally processed foods because of the reaction of carbohydrates and amino acids naturally found in foods (Figure 1.2). As an illustration, carbohydrates degrade upon heat treatment to dicarbonyl and alkyl ketones (Hodge, 1967). Therefore, 4-MEI forms from the reaction of dicarbonyl and ammonia. Moreover, 4-MEI can actually be added to foods when caramel color III or IV are added.

The formation of 4-MEI in Maillard reaction model systems consisting of D-glucose/NH_3, L-rhamnose/NH_3, methylglyoxal/NH_3 and methylglyoxal/ formaldehyde/NH_3 was investigated. 4-MEI was formed at levels ranging from 0.49 to 0.71 mg mL^{-1} in the D-glucose/NH_3 model system. The formation of 4-MEI was slightly higher in the L-rhamnose/NH_3 system (0.91 mg mL^{-1}) than in the D-glucose/NH_3 system under the conditions used in the present study. A methylglyoxal/NH_3 system produced significantly higher levels of 4-MEI (5.70 mg mL^{-1}), suggesting that methylglyoxal is an important precursor of 4-MEI. Ammonolysis of methylglyoxal, which is one of the glucose degradation products, was proposed to form formamide, which subsequently reacted with 2-aminopropanal (R-aminocarbonyl intermediate) formed from methylglyoxal to give 4-MEI. The levels of 4-MEI found in commercial cola soft drinks range from 0.30 μg mL^{-1} to 0.36 μg mL^{-1} (Moon & Shibamoto, 2010).

1.1.1.2.2. Physical and chemicals properties

At room temperature, 4-MEI is a light yellow solid crystal with a relative molecular mass of 82.11, boiling point of 263°C, and a flash point of 157°C (Palmer et al., 2013). Its melting point is between 46 - 48°C, and its vapor pressure is 0.007 mm Hg at 25°C. It is soluble in water and most polar organic solvents like ethanol and chloroform. 4-MEI ($C_4H_6N_2$) is a polar basic heterocyclic organic compound. Its basic nature relates to the ability of the nitrogen in the pyridine ring to accept a proton, and the electron-releasing

property of the methyl group tends to increase the electron density around the pyridine nitrogen (Hofmann & Schieberle, 1995). The imidazole ring in 4-MEI is amphoteric and can function as an acid or base. The acidic site is on nitrogen atom number one while the basic site is on nitrogen numbers three. Different basic pKa values for 4-MEI have been reported in the literatures. (Klejdus, Moravcová, Lojková, Vacek, & Kubáň, 2006) reported a pKa value of 7.52. A value of 7.6 was reported by (Klejdus, Moravcová, & Kubáň, 2003). (Petruci, Pereira, & Cardoso, 2013) and (Moretton, Crétier, Nigay, & Rocca, 2011) noted a value for pKa of 7.7 for 4-MEI. The low vapor pressure of 4-MEI (0.007 mm Hg at 25°C) dictates it should not exist in the atmosphere pKa of 4-MEI.

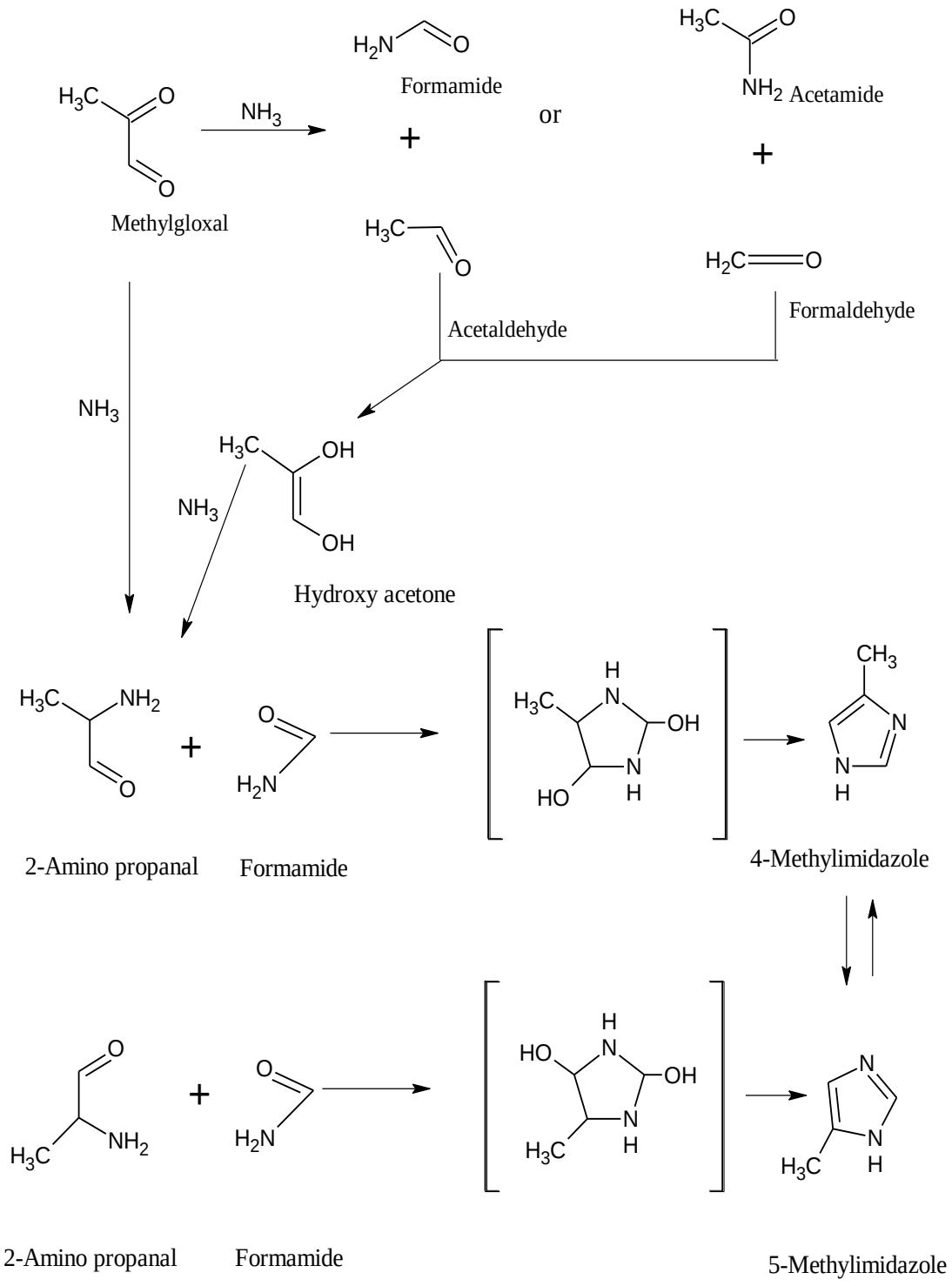

Figure 1.2. Formation mechanism of 4(5)-methylimidazole from methylglyoxal (Wieczorek, Przygoński, & Jeleń, 2018)

1.1.1.2.3. 4-MEI production and uses

An alkylimidazole, 4-MEI, was synthesized from the reaction of ammonia with either methyl alkyl ketones or α-dicarbonyl compounds (Debus, 1858): This method has a low yield, but it is suitable for creating C-substituted imidazole (Moon & Shibamoto, 2010). Commercially, 4-MEI is produced by cyclocondensation of aldehyde and ammonia with methylglyoxal (Chan et al., 2008).

4-MEI is widely used as a raw or intermediate material in pharmaceuticals, photographic chemicals, agricultural chemicals, dyes and pigments, cleaning products, and rubber products (Klejdus et al., 2006).

1.1.1.2.4. Factors affecting 4-MEI formation

The are many factors that affecting in 4-MEI formation such as:-

(i) Time and temperature

Differece in 4-MEI levels may be due to the applied temperature. In a D-glucose ammonium hydroxide Maillard model system, the temperature of heating time had significant effects on of 4-MEI formation when temperatures of 70,100, and 120°C were applied for 3, 6, or 12 h. In contrast, increasing the temperature by another 50°C increased level of 4-MEI by 2.3% (Moon & Shibamoto, 2010).

(ii) Moisture

No data are available in the literature on the effects of water content on 4-MEI formation. In the Maillard reaction the rate of formation depends on how much free water is available. In foods with high a_w, the Maillard reaction is slow, possibly because the reactant is diluted. Moreover, in low a_w foods, mobility of the reactants is limited. Therefore, the products of the Maillard reaction are low

even though the reactants concentration is higher. The best outcomes occur at intermediate a_w values (Wolfrom & Rooney, 1953).

(iii) Reactant concentrations

As more primary reactants like carbohydrates and compounds that contain nitrogen are present, more 4-MEI is formed (Lee et al., 2013).

(iv) Sugar types

Sugar type and sugar degradation rate directly effects formation of 4-MEI. In the Maillard model systems of D-glucose/ammonia and L-rhamnose/ammonia, the level of 4-MEI was slightly higher in the L-rhamnose/ammonia model (0.91mg mL^{-1}) than the D-glucose/ammonia model (0.71 mg mL^{-1}), L-rhamnose degrades more readily to yield precursors like formaldehyde, acetaldehyde, glyoxal, methylglyoxal than D-glucose (Martins et al., 2000).

(v) PH

The rate and the tendency of browning increases in the Maillard reaction as pH increases (Coca et al., 2004). Increasing the pH increases the reactivity of amino acids due to acid base equilibrium (Bostan & Boyacioğlu, 1997). In addition, pH plays an important role in formation of the end products types of Maillard reaction. Enolization of the Amadori product which is an intermediate compound in Maillard reaction at pH 7 or below produces hydroxymethylfurfural (HMF) (Martins et al., 2000).

1.1.1.2.5. Toxicity of 4-MEI

The toxicity of 4-MEI has been reported in many International agencies including the National Toxicology Program and other citations. In 2007, (MacKenzie et al., 1992) reported “clear evidence of carcinogenic activity” of 4-MEI in animal studies, based on increased incidences of alveolar/bronchiolar neoplasms (MacKenzie et al., 1992). The National Toxicology Program (MacKenzie et al., 1992) conducted a two year feeding cancer bioassay of 4-

MEI in mice and rats. In F344/N rat's ambiguous evidence of carcinogenic activity in female rats based on incidences of mononuclear cell leukemia and no evidence of carcinogenic activity in male rats were observed (Chan et al., 2008). Chromosome aberration (CA) assay is one of methods to measure cytotoxicity and genotoxicity . In 2015, Tazehkand & Topaktas, reported that 4-MEI might not anti-genotoxic and protective effects in bone marrow cells of Swiss Albino Mice, because 4-MEI could not reduce the chromosomal aberrations induced by EMS. In 2016, Tazehkand et al reported that 4-MEI induced structural CAs at all concentrations for 12 h and at highest concentration for 24 h treatment periods and decreased the 4-MEI at highest concentration for 12 h and at all concentrations for 24 h in bone marrow cells of Swiss Albino mice. This result showed that 4-MEI has cytotoxic and genotoxic effect in bone marrow cells of Swiss Albino mice (Tazehkand, et al., 2016). At another research from same researchers 4-MEI has cytotoxic effect on colon cancer cell lines and has necrotic effect on rat liver cells (Tazehkand et al., 2016, Tazehkand et al., 2017). In 2017, Norizadeh, observed that female and male rats given caramel color IV (110 mg 4MEI/kg body weight) in drinking water at concentration of 10 g kg^{-1} for 2 years had significantly lower body weights, but not observed any histopathological changes (Norizadeh, 2017). In another study in 1992 MacKenzie, reported that 4-MEI at lower concentrations ($\leq$ 100 μg mL^{-1}) has no significant effect on the biological characteristics, including proliferation, apoptosis, migration and genes of hematogenesis expression. Therefore, relatively low concentration of 4-MEI in foods and beverages may have no toxic effect on BMSCs (MacKenzie, et al., 1992). Therefore, it can be concluded that 4-MEI might pose a potential risk for humans. However, it must be investigated in other test systems for genotoxic and cytotoxic effects.

1.1.1.2.6. Level s of 4-MEI in foods

Most human exposure to 4-MEI is through ingestion. 4-MEI can be found in foods either as a result of interaction between amino acids and reducing sugar in the presence of heat and moisture or by adding caramel colors that contain 4-MEI produced by either the ammonia or ammonia-sulfite process. Some research has reported different levels of 4-MEI in food matrices, while in liquid coffee, as expected, because the solid coffee diluted, the highest level was 0.77 mg kg^{-1} (Klejdus et al., 2006).

At the 1969 meeting of the Joint Food Additives Organization (FAO)/ World Heaith Organization (WHO) Expert Committee On Food Additives was reported the levels of 4-ME1 in various types of caramel color was 200 mg kg^{-1} for ammonia caramel colors (Wilks et al., 1977).

The Codex Alimentarius of the World Health Organization (WHO) and the European Union (EU) have established a maximum of 250 mg kg^{-1} for 4-MEI, for caramels class III and IV. Until now, no limit levels have been established for the presence of 4-MEI in foodstuffs (Cunha et al., 2011).

1.1.1.2.7. Stratigies for 4-MEI reduction in foods

Total elimination of 4-MEI from processed food is difficult; however, reducing its levels in caramel III color and IV by investigating new manufacturing strategies is achievable.The γ-irradiation has recently become an alternative processing technology to preserve food with minimum interruption of its nutritional and sensory properties (Farkas, 2006). Besides the traditional purpose of using ɣ-irradiation, signi&icant reduction of pathogenic bacteria for sanitary purposes, new perspectives of γ-irradiation have been reported, that is, reduction of chemical toxicants such as N-nitrosamines in sausage (Ahn et al., 2002) biogenic amines in fermented soybean paste (Kim et al., 2006), allergenicity of foods γ-irradiation was applied to Caramel Color III to reduce the level of 4-MEI without color changes. The level of 4-MEI and color were analyzed on all caramel coloring. The concentration of 4-MEI in the non-

irradiated caramel coloring was 714.9 mg kg^{-1}. The level 4-MEI in caramel coloring was significantly reduced from ɣ-irradiated caramel coloring, the amount of 4-MEI in caramel color was at 658 mg kg^{-1}, which equates 7.9% reduce on from non-irradiated sample. No color change was observed on irradiated and non-irradiated caramel coloring . Therefore, γ-irradia on could be used as an alterna ve processing method to improve the quality of caramel coloring by reducing 4-MEI content without its color change (kim.H et al., 2006).

1.1.2.Energy drinks

Drinks containing ingredients with stimulant properties are called energy drinks) (Wolfrom & Rooney, 1953). Energy drinks are type of non-alcoholic functional beverage that increase alertness and enhance the psycho-physiological responses in human (Rai et al., 2016). Energy drinks are one of the fastest growing soft drink markets around the world. They have been recently consumed in different societies, especially by younger consumers. Energy drinks first appeared in Europe and Asia in the 1960s but did not become popular until the most widely known brand, Red Bull, was released in Austria in 1987; hitting the US. market in 1997. By 2006, there were over 500 brands of energy drinks around the world The main ingredients of energy drinks are caffeine, taurine, vitamins, carbohydrates, and other ingredients such as tyrosine, citicoline and guarana (Gliszczyńska & Rybicka, 2015, Pennay & Lubman, 2012). Vitamins are included for its essentiality in the normal biological functions as co-enzymes, caffeine for its well-known stimulant effect over the central nervous system (CNS) and taurine for being involved in a number of crucial physiological processes including modulation of calcium flux and neuronal excitability, osmoregulation, detoxification and membrane stabilization. Several investigations have been published indicating the effect of this kind of beverages over the CNS, showing significant improvements in mental performance (reaction time, concentration and memory) and reduction

in sleepiness and sleep-related driving incidents. Nevertheless, the consumption of these beverages is not recommended to persons with heart diseases, blood pressure problems, in pregnancy (Sharaf El Din & Wahba, 2015).

1.1.2.1. Taurine

Taurine (2-aminoethanesulfonic acid) (Figure 1.3) plays a very important role in several essential biological processes. This free sulfur containing β - amino acid is not incorporated into protein, and is the most abundant free amino acid in the heart, retina, skeletal muscle, leukocytes and brain (Sharaf El Din & Wahba, 2015), and distributes widely in biological fluids and tissues of many mammalians (Schaffer et al., 2010). Taurine is one of the most common free amino acid naturally present in the diet such as meat, seafood and milk. Taurine is thought to play an extensive role in numerous physiological processes (Triebel et al., 2007, Todorova & Pencheva, 2015). It is the main component of many energy drinks as a tonic medicine (Sawabe et al., 2008).

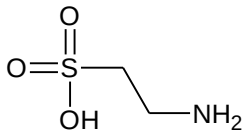

Figure 1.3. Chemical structure of taurine

The Physical and chemical properties of taurine, Taurine molecule ($C_2H_7SO_2N$) is a white crystals with a relative molecular mass of 125.15, odorless, melting point of 300°C, soluble in water and in absolute alcohol. Most amino acids have a L- or D-configuration, which means the molecule when put into a solution will rotate light either to the left or to the right. Taurine, like the amino acid glycine does not polarize light and consequently it does not have an L- or D-configuration. Taurine is an organic weak acid with dissociation constant pK 54.96 which it remains stable in acids and bases (Mou

et al., 2002). It occurs in the body as a free molecule and is never incorporated into muscle proteins (Löpez et al., 1981). The various physiological functions of taurine are explained with amino terminal group in the structure and sulfonic acid group moiety (Matinuzzi et al., 2012).

The Occurrence of taurine in nature, it is generally absent or present in traces in the bacterial and plant kingdoms. In many animals, including mammals, it is one of the most abundant of the low-molecular-weight organic constituents. Taurine is found in greater concentrations in all animal products. Meat, poultry, eggs, dairy products, and fish are good sources of taurine (Allen & Garrett, 1971).

1.1.2.1.2. Metabolism of taurine in mammalian tissues

Taurine was considered at one time to be only an end product of metabolism, it is now evident that this amino acid has several metabolic functions. Taurine is conjugated with the bile acids: cholic, chenodeoxycholic, and deoxycholic acids. Free bile acids and those conjugated with taurine facilitate the intestinal absorption of fat by contributing to lipolysis, micelle formation, and re-esterification of fatty acids within themucosal cells of the intestine (Jacobsen, 1963). When an excess of taurine was added to a rat liver homogenate, the conjugation of cholic acid with taurine increased (Bergstrom & Gloor, 1954).

1.1.2.1.3. Biological roles

(i) Osmorequlation and cellular tonicity:

Tauirne is essential for growth and survival of mammalian cells as well as fetus development, development of the newborn and during childhood (Kim et al., 2006), and plays an important role as an organic osmolyte in cell volume control in mammalian cells and a change in the cellular taurine content is an indication of a shift in the cell volume (Lambert, 2004). Taurine, an osmolyte,

helps to regulate osmolarity without causing additional perturbations of cellular tonicity (Trachtman et al., 1992).

(ii) Brain aging:

Taurine is found in high concentrations in the brain, though levels decline with age (El Idrissi, 2008). Researchers showed that the learning ability of older rats was impaired, impairment correlated to the reduction in taurine levels (Dawson et al., 1999). In cerebrospinal fluid of patients with Parkinson's disease, levels of a few amino acids including taurine are lowered (Engelborghs et al., 2003). Taurine is reported to promote release of dopamine, a neurotransmitter lacking in Parkinson‘s disease, from the neuronal pool. Alzheimer's disease, is due to in part, an increase in the generation of nitric oxide (Togo et al., 2004). The supplementation of taurine, along with magnesium inhibits nitric oxide production (Kim.C et al., 2006).

(iii) Heart health:

Taurine makes up nearly 50% of the free amino acids in the heart cells (Huxtable et al., 1980). Taurine level is depleted in the failing heart (Azuma & Schaffer, 1993). Taurine‘s electrophysiological actions in cardiac cells are brought about by modulation of ion channels (Satoh, 1998). Calcium homeostasis is critical to stable myocardial contractile function. Changes in the intracellular taurine pool modulate calcium transport and taurine exerts a cardioprotective action (Punna et al., 1994). Taurine has an essential function in ensuring stable calcium levels, which thereby promotes proper contractile function of the heart tissue. Likewise, potassium is also an important ion in heart cells. Taurine directly modulates the potassium ion current by increasing the current's action potential duration (Satoh, 1998).

(iv) Effect on skeletal muscle

Taurine is necessary for normal skeletal muscle functioning. This was shown by a study, using mice with a genetic taurine deficiency (Ito et al., 2008). They

had a nearly complete depletion of skeletal and cardiac muscle taurine levels. These mice had a reduction of more than 80% of exercise capacity compared to control mice (Geny et al., 2006).

1.1.2.1.4. Taurine carcinogenicity

In cancerous conditions, taurine is a potent cytoprotective agent and immune enhancer (Redmond et al., 1998). Taurine has been shown to be depleted in people taking chemotherapy (Desai et al., 1992), When added to the cancer therapy program, taurine acts to reduce endothelial cell death and actually increases the tumor cytotoxicity. The calcium homeostatic mechanism of taurine was found to be the critical feature in these anti-cancer functions (Finnegan et al., 1998).

1.1.2.1.5. Daily intake of taurine

The mean daily intake of taurine from diet was estimated to vary between 40 to 400 mg/day (McConn, 2012). Some energy drinks contain high level of synthetic taurine (4000 mg L^{-1}), hence the daily intake of taurine would be 2000 mg/day from consumption of 0.5 L/day of these drinks (Todorova & Pencheva, 2015). This is five times greater than the highest estimated intake of 400 mg/day from naturally occurring taurine in omnivore diets. High doses of taurine greater than 2g per day may cause unintended side effects ranging from high blood pressure to strokes, induction of psoriasis and seizures to heart disease (González et al., 2012, Matinuzzi et al., 2012).

1.2 Analytical methods for 4-MEI and taurine determination

1.2.1 Analytical methods for determination of 4-MEI

The 4-MEI compounds are difficult to analyze due to their polar nature, lack of chromophore and low molecular weight. From literature survey there are many techniques have been developed to determine 4-MEI such as high performance liquid chromatography-mass spectrophotometeric methods (HPLC-MS) and

gas chromatography-mass spectrophotometeric methods (GC-MS) (Moretton et al., 2011). The main advantage of the recent HPLC–MS methods is the possibility to analyze 4-MEI without derivatization (Lojkova et al., 2006, Wolfrom & Rooney, 1953). Notwithstanding the high selectivity achieved by this technique, the methods include a previous tedious solid-phase extraction or supercritical fluid extraction (Fernandes & Ferreira, 1997).

1.2.1.1. HPLC based methods for determination of 4-MEI

Several HPLC methods have been developed for the determination of 4-MEI in various matrices based on using ultraviolet (UV), MS and MS/MS as detection techniques.

Feng et al., 2017 were established new method for the determination of the 4-MEI in soy sauce based on magnetic molecularly imprinted polymer (MMIP) solid-phase extraction coupled with HPLC photo diodarray detecter (PDA). A Sino-Chrom ODS-AP column (5 μm, 230 mm ×4.6 mm) was used. For the analytical chromatography, isocratic elution with methanol and KH_2PO_4 (0.05 M) in a ratio of 12:88 (v/v) was performed. The detection wavelength and column temperature were set at 233 nm and 28 ºC, respectively. The method was linear in the concentration range of 5.7 to 1148 μg L^{-1} with a correlation coefficient 0.9977. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.71 and 5.64 μg L^{-1}, respectively. The recoveries in the range of (97.33-104.57 %) and the precision as RSD was in the range of 0.158% to 2.38% are obtained.

Content of 4-MEI in commercial coffee brands was determined using HPLC with UV DAD and MS detectors. Solid phase extraction (SPE) cartridges were used for solid phase extraction. Sample analysis was performed under gradient elution on a Zorbax Eclipse XDB C-18 (4.6 x 150 mm, 5 μm) analytical column using mobile phase consisting of 5.0 mM NH_4OH (phase A) and acetonitrile (ACN, phase B) with following steps: 0 – 9 min: 2 - 20% ACN, 10-

13 min: 20–2% ACN, 13–16 min: 2% ACN). Column temperature was set at 25 °C. Signal was monitored using UV detector at 215 nm MS spectra were recorded with an Agilent 6224 Accurate-Mass Time of Flight (TOF) mass spectrometer using an electron spray ionization (ESI) in a positive mode. The MS spectra were recorded from 30 to 500 *m/z* and the chromatogram for 4-MEI determination was extracted for *m/z* 83.06.The satisfied linearity in the concentration range of 5–100 ng mL^{-1} was achieved with correlation coefficient of 0.9980. The LOD of 2.5 ng mL^{-1} and LOQ of 8.4 ng mL^{-1} were calculated (Dieu et al., 2015).

Quick HPLC-MS/MS method was used for the separation of 4-MEI in caramel color and processed foods. HPLC separation was performed on a Luna C18 (2×100 mm, 3 μm) column-thermostatted at 35°C using mobile phase A (5 mM NH_4HCO_3 in high-purity water; pH 9) and mobile phase B (5 mM NH_4HCO_3 in methanol; pH 9). Mass spectrometer was used with atmospheric pressre ionization (API) Qtrap and the multiple reaction monitoring (MRM) mode was monitored for quantitative analysis. The method was linear between 5-1,000 μg kg^{-1} in the measuring solutions with correlation coefficient 0.999. The LOD and LOQ were found to be 3.0 μg kg^{-1} and 5.0 μg kg^{-1} , respectively in the measuring solutions. The recovery rate of 4-MEI spiked in sauce and curry was 102.3% and 96.4% (Kim .T. R et al., 2013).

A method for the determination of 4-MEI was developed using solid phase extraction (SPE) and HPLC/MS. An RP chromatographic column MetaChem Polaris C-18A (150 mm× 4.6 mm, 3.5 μm) was employed as analytical column. The mobile phase was acentonitrile (solvent A) and 5.0 mM ammonia in water (solvent B). A linear gradient that follows at 0 min, 98% B; at 10 min 80% B; at 15 min 80% B; and at 20 min 98% B was used for the separation. The flow rate was 0.4 mL/min and the temperature of the column oven was set at 40 °C. The single quadruple mass spectrometer equipped with ESI interface operated in the positive mode The spectra were recorded from *m/z* 50 to 250 for

full scan mode and the data for the selective ion montoring (SIM) mode were acquired at *m/z* 83/56. The calibration curve was linear in the concentration ranges 1.0−70 ng mL^{-1} with correlation coefficient > 0.9993. The LOD was estimated at 0.1 ng mL^{-1} while the LOQ was 0.4 ng mL^{-1}. The inter-day accuracy for the standard solution was found to be 101.0%. The precision of the inter-day analysis, expressed as RSD%, was calculated as 2.6%. The recovery was 98%. The method was used to determine 4-MEI in beverages, coffee, caramel colors and other samples (Klejdus et al., 2006).

HPLC-MS method has been developed to determine 4-MEI. Among three sorbents tested, best peak shape of 4MeI was achieved on A reversed-phase chromatographic column MetaChem Polaris C18-A (Polaris 2) with a guard cartridge Meta Guard (4.6mm i.d., Inertsil ODS—3.5m particle size,MetaChem Technologies, Torrance, CA, USA) was employed. The mobile phase was acetonitrile (solvent A) and 5.0 mM ammonium hydroxide in Milli-Q water (solvent B). A linear gradient that follows at 0.0 min, 2:98 B; at 10 min, 20:80 B; at 15 min, 15:75 B; and 20 min, 2:98 B was used for the separation. The flow rate was 0.4 mL min^{-1} and the temperature of column oven was set at 40 °C. The column effluent was monitored with diode array detector at 215 nm for 4-MEI. The sensitivity was compared to the parameters achieved by the electrospray ionization mass spectrometric (ESI/MS) detection in the presence of 5.0 M ammonium hydroxide using selected ion-monitoring (SIM) mode. The LOD was 0.147 ng using UV detection and 1.0 pg using ESI/MS detection. The method could be applied for a fast and sensitive determination of 4-MEI different biological materials as well as in Class III Caramels (Klejdus et al., 2003).

4-MEI was determined in cigrette smoke by HPLC with UV-Vis detector after derivatization with NBD-Cl. Then the derivative was separated on LiChrosorb Si 60 column (200 mm x 4.6 mm) using mobile phase containing methylene

chloride-butanol (94:6) % . The detector was set at 360 nm. This method is very selective since no clean-up procedure is necessary. The levels of 4-MEI and imidazole in cigarette smoke condensate vary between 100 and 500 mg L-1 (Moore et al., 1984).

1.2.1.2. GC-MS based methods for determination of 4-MEI

Sensitive GC-MS methods after derivatization have been published for MEI determination (Fernandes & Ferreira, 1997, Casal et al., 2002, Lojkova et al., 2006).

Cunha et al., 2011 have been reported fast and robust GC-MS method for 4-MEI determination in soft drinks that dark beer based on ion-pair extraction with bis-2-ethylhexylphosphate (BEHPA) and acylation as derivatization process using isobutylchloroformate. The GC separation was conducted with a DB-5ms column (15.0 m ×0.25 mm I.D. × 0.25 mm film thickness. The mass selective detector was set at electron impact (EI) ionization mode. The performance of the method was evaluated in terms of linearity ($r > 0.998$); recovery (90–101%, 3 levels); and precision (3–8%, 3 levels, $n = 6$). Limits of detection and quantification in the matrices studied were 0.60 mg L^{-1} and 2.2 mg L^{-1}, respectively. The optimized method was applied to a wide variety of soft drinks brand name and generic colas, uncarbonated flavor and energy drinks) and dark beers. Overall, soft drinks presented higher amounts of 4-MEI (ranging from 37 to 613 μg L^{-1}) than those found in the dark beers (ranging from 3 to 424 μg L^{-1}), with colas presenting the highest levels. When the different colas analyzed were compared, the 4-MEI levels in generic colas were generally higher than those in brand-name colas. 4-MEI was found in only one of eight energy drinks studied. The main drawback of GC–MS methods with isobutylchloroformate derivatization is the degradation of the columns following injection of chloroformic extracts containing excess of isobutylchloroformate (Casal et al., 2002).

The identification of imidazole derivatives in cigarette smoke was performed by GC-MS on a fused-silica capillary column coated with Carbowax 20.0 m. A fraction, enriched imidazoles, was prepared from the smoke condensate by liquid chromatography on Sephadex LH 20 prior to the GC-MS analysis. Ten alkylated imidazoles were identified in cigarette smoke, 4-MEI and imidazole being the most abundant (Moore et al., 1984).

An improved method for isolation and quantification of 4-MEI in caramel color has been presented. The method consists of a methylene chloride extraction of a semidry mixture of the sample and Celite 545, followed by concentration and GC analysis. The GC analysis is carried out using a base-modified column packing of 7.5% Carbowax (20M) and potassium hydroxide (KOH) 2% . With this packing, the 4-MEI peak is symmetric, and the need for derivatization prior to analysis is avoided. Quantification is done using 2-methylimidazole (2-MEI) as an internal standard. Standard addition recovery experiments and reproducibility studies involving two analysts indicate that the method is acceptably accurate and has a precision of 2-3% relative standard deviation. The minimum level of 4-MEI detectable by this method is estimated to be 0.2 pg g^{-1} of caramel color (Wilks et al., 1977).

1.2.2. Analytical methods for determination of taurine

Many analytical methods have been described for the measurement of the amount of taurine in food such as spectrophotometry (Dranganv, 2014), spectrofluorometric (Sharaf El Din & Wahba, 2015), HPLC (Sawabe et al., 2008), capillary electrophoresis (Vochyánová et al., 2014) and nuclear magnetic resonance spectroscopy (^{1}H-NMR) (Hohmann, 2014). The most common methods for taurine determination are UV-Vis spectrophotometry and HPLC with different detection systems. Due to the lack of chromophoric group in taurine molecule, most of the analytical methods of its analysis involve derivatization to enhance its detectability using ultraviolet (UV), visible or fluorometric detection.

(i) Derivatization agents for taurine

Several derivatizing agents have been used for the determination of taurine such as *o*-phthalaldehyde (OPA) (Kanjana, 2017, Mehdinia et al., 2017)., 4-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) (Ghandforoush et al., 2009, Mohamed et al., 2017), 2,4-dinitrofluorobenzene (DNFB) (McConn, 2012), 2,4-dinitrofluorobenzene (DNFB) (Rai et al., 2016), 2-[2-(dibenzocarbazol)-ethoxy]ethyl chloroformate (DBCEC) (X. Chen, You, Suo, & Fan, 2014), ninhydrine (Dranganv, 2014), Hantzsch reagent, tetracyanoethylene (Sharaf El Din & Wahba, 2015), 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl) (Inoue et al., 2003), phenol and sodium hypochlorite (Muangthai & Klongnganchui, 2015), dansyl chloride, 9-Fluorenylmethyl chloroformate (Saleh et al., 2007), and fluorescamine (McMahon et al., 1996). However most of these derivatizing agents have some drawbacks such as time-consuming (Vochyánová et al., 2014). DNFB method either applied tedious time consuming procedures or offered low sensitivity values (McConn, 2012). 4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride and fluorescamine methods required complicated pretreatment procedure (Inoue et al., 2003). Reaction between taurine and dansyl chloride requires high temperatures and the presence of salts can have detrimental effects on the reaction yield (Stocchi et al., 1994). Fluorenylmethyl chloroformate forms stable derivative with taurine, but hydrolysis products of the reagent interfere unless removed prior to analysis (Saleh et al., 2007).

(ii) *o*-Phthalaldehyde (OPA) derivatizing agent

OPA (Figure 1.4) is a common cheap reagent used for derivation of free amino acids including taurine followed by HPLC analysis (Mehdinia et al., 2017). OPA reacts rapidly with primary amine and amino acids in presence of reducing agent alkylthiol (mercaptoethanol) or sulfite group to form isoindole ring (Maldonado & Maeyama, 2013, Michail et al., 2011). The compound 1-

alkylthio-N-alkylisoindole produced from the reaction of amino acids and OPA-alkylthiol reagent is unstable due to further reaction with excess OPA in the derivatization matrix. Sodium sulfite is used in combination with OPA as a derivatization agent in order to improve amino acid derivatives stability and derivatization occurs instantaneously (Jacobs, 1987). HPLC with fluorescence and electrochemical detection (ED) are the most frequent methods for determination of taurine after pre-column derivatization with OPA (Kanjana et al., 2009, Ghandforoush et al., 2009). The OPA fluorometric method has low to nanomolar's detection limit (Liang et al., 2016).

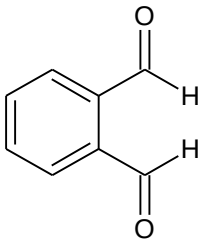

Figure 1.4. Chemical structure of OPA

(iii) 4-Chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) derivatizing agent

NBD-Cl (Figure 1.5) as an electroactive halide reagent (Mohamed et al., 2017), is a highly sensitive chromogenic and fluorogenic reagent used in many investigations. It was used as a coloring agent for determination of imidazole (Saleh et al., 2007), and amines and amino acids produce a highly fluorescent NBD-product. This approach for labeling amines and amino acids has become a standard method for analysis of very low levels of amines and has widespread analytical applications. A large number of studies have been carried out with these fluorogenic dyes to develop methods for quantification of amines and amino acids in pharmaceutical and biological samples by labeling amine functional groups (Aboul-Enein et al., 2011).

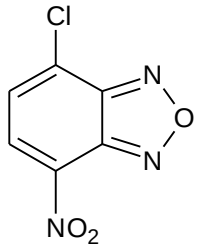

Figure 1.5. Chemical structure of NBD-Cl

1.2.2.2 Spectrophotometric and spectrofluorometric methods for determination.of taurine

Spectrophotometry is probably the most convenient analytical technique for routine analysis of taurine because of its inherent simplicity, low cost and wide availability in most quality control laboratories (Mohamed et al., 2017). A literature review revealed spectrophotometric determination of taurine, using phenol/sodium hypochlorite) as reagent. The LOD and LOQ are 6.27 μM and 189.99 μM, respectively (Muangthai & Klongnganchui, 2015).

Spectrophotometric method based on reaction between the taurine and NBD-Cl at alkaline medium has been developed. The formed yellow adduct, exhibiting maximum absorption (λ_{max}) at 474 nm. Under the optimized reaction conditions, the method was linear in the concentration range of 10-50 μg mL^{-1} with a correlation coefficient 0.9993. The LOD and LOQ were found to be 1.69 and 5.15 mg mL^{-1}, respectively. The recoveries in the range of (92.4-102.4 %) and the precision as RSD was in the range of (0.14 – 1.59%) are obtained. The method was successfully applied for determination of taurine in energy drinks. The content of taurine measured compared to the labeled amount in three commercial energy drinks containing taurine was found to be (93.9 –101.8%) (Mohamed et al., 2017).

UV-spectrophotometry method has been developed and validated for taurine determination after derivatization with ninhydrin. The product of reaction is a

color compound and it has absorption max at 570 nm in 70 vol. % ethanol matrix. The correlation coefficients about 1–0.99692 (Draganov et al., 2014).

Sharaf El Din & Wahba, 2015 have been developed and validated two sensitive, selective, economic and validated spectrofluorimetric methods for the determination of taurine in energy drinks and spiked human urine. Method I is based on fluorimetric determination of the amino acid through its reaction with Hantzsch reagent to form a highly fluorescent product measured at 490 nm after excitation at 419 nm. Method II is based on the reaction of taurine with tetracyanoethylene yielding a fluorescent charge transfer complex, which wasmeasured at λex/em of (360 nm/450 nm). The proposed methods were subjected to detailed validation procedures, and were statistically compared with the reference method, where the results obtained were in good agreement. Method I was further applied to determine taurine in energy drinks and spiked human urine giving promising results. The linear ranges of methods I and II were 0.15–1.5 μg mL^{-1} and 0.2–2.0 μg mL^{-1}, respectively. LOD and LOQ for methods I and II were 0.12 μg $mL^{-1}$and 0.15 μg mL^{-1}, respectively (Sharaf El Din & Wahba, 2015).

1.2.2.3. HPLC methods for determination of taurine

HPLC with different detection systems such as UV-Vis, electrochemical, evaporative light scattering (ELS), fluorescence refractive index detection and mass spectrometry (Triebel et al., 2007, Chen et al., 2006, wang et al., 2011) has been reported for taurine determination. The most frequent used detector for taurine detection is UV-Vis detector (Sawabe et al., 2008).

Taurine in human plamsa has been determined by a reversed-phase HPLC system after derivatization using fluorescamine. The derivatization product was separated on a Bondclone C-18 analytical column (300 mm x 3.9 mm) and the mobile phase was tetrahydrofuran-acetonitrile phosphate buffer (pH 3.5)

(4:24:72, v/v/v). The taurine derivative was detected by measuring the UV absorbance of 385 nm. The correlation coefficient of the regression line was 0.9998 and the LOQ was 5.0 μg mL^{-1}. The inter- and intra-assay calibration curves were 5.56% and 3.87%, respectively. The relative recovery of taurine from plasma was found to be 89.7% (McMahon et al., 1996).

The determination of taurine in some energy drinks using HPLC after derivatization with 2,4-dinitrofluorobenzene (DNFB) has been reported. A reversed phase column (Phenomenex Kinetex XB-C18 100 Å) with a 2.1 μm particle size and column dimensions of 4.6 mm x 50 mm was used as analytical column. An isocratic elution of 80:20 (v/v) of the phosphate buffer/acetonitrile was used. The detection wavelength for the taurine derivative was 360 nm. The correlation coefficient of the regression line was 0.9893 (McConn, 2012).

A rapid and simple method to determine taurine in energy drinks by pre-column HPLC using 4-fluoro-7-nitrobenzofurazan (NBD-F) has been developed. The reaction of taurin with NBD-F finished in 10 min at 60°C the derivative was measured on a UV-Vis detector (470 nm) by HPLC using conventional octadecylsilan (ODS) column. Amixture of sodium hydrogenphosphate-citric acid buffer solution pH 5.4 containing 10 mM tetra butylammonium bromide and acetonitril (70:30)% was used as a mobile phase. The recoveries were in the range 98.2-99.9%. The precision as standard division was in the rang 0.3–0.5% , the linearty as a coffecient of correlattion value was 0.999 and the specification was confirmed by addition of taurine to three commercial energy drinks. The content of taurine measured compared to the labeled amount in commercial energy drinks in the range 92.9–105.1% (Sawabe et al., 2008).

1.3. Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the

requirements for the intended analytical application. The international standardization organization (ISO) defined validation as “confirmation by examination and provision of objective evidences that the particular requirements of a specified intended use are fulfill (Magnusson, 2014).

There are many reasons for the need to validate analytical procedures. Among them are regulatory requirements, good science, and quality control requirements. Of course, we want to apply good science to demonstrate that the analytical method used had demonstrated accuracy, sensitivity, specificity, and reproducibility. Finally management of the quality control unit would definitely want to ensure that the analytical methods that the department uses to release its products are properly validated for its intended use so the product will be safe for human use. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications (Guideline, 2005).

1.3.2. Parameters for method validation:

1.3.2.1. Accuracy (Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (Ravichandran et al., 2010).

The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentrations (e.g., a control sample or certified reference material) and comparing the measured value with the true

value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume.

After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the recovery factor determined for different concentrations can be used to correct the final results.

Accuracy is usually reported as percent by the assay (using the proposed analytical procedure) of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. The range for the accuracy limit should be within the linear range.

The accuracy of a method may vary across the range of possible assay values and therefore must be determined at several different fortification levels. The accuracy should cover at least three concentrations (80, 100 and 120%) in the expected range. Accuracy may also be determined by comparing test results with those obtained using another validated test method. The accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated determination for each concentration) (Ravichandran et al., 2010).

1.3.2.2. Precision

When a sample is analyzed several times, the individual results are rarely the same instead, the results are randomly scattered. Precision is a measure of this variability. The closer the agreement between individual analyses, the more

precise the results. Precision is a measure of the spread of data about a central value and may be expressed as the range, the standard deviation, or the variance. Precision is commonly divided into two categories: repeatability and reproducibility.

Repeatability is the precision obtained when all measurements are made by the same analyst during a single period of laboratory work, using the same solutions and equipment. Reproducibility, on the other hand, is the precision obtained under any other set of conditions, including that between analysts, or between laboratory sessions for a single analyst. Since reproducibility includes additional sources of variability, the reproducibility of an analysis can be no better than its repeatability (Harvey, 2000).

1.3.2.3. Linearity

Linearity of an analytical procedure is defined as the ability (within a given range) to obtain test results of variable data (e.g., absorbance directly proportional to the concentration (amount of analyte) in the sample. Quantitation of the analyte depends on it obeying Beer's law for the spectroscopic method over a concentration range. Therefore, the working sample concentration and samples tested for accuracy should be in the linear range

There are two general approaches for determining the linearity of the method. The first approach is to weigh different amounts of standard directly to prepare linearity solutions at different concentrations. However, it is not suitable to prepare solution at very low concentration, as the weighing error will be relatively high. Another approach is to prepare a stock solution of high concentration. Linearity is then demonstrated directly by dilution of the standard stock solution. This is more popular and the recommended approach. Linearity is best evaluated by visual inspection of a plot of the signals as a function of analyte concentration. Subsequently, the variable data

are generally used to calculate a regression line by the least - squares method. At least five concentration levels should be used. Under normal circumstances, linearity is acceptable with a coefficient of determination (r^2) of $\geq$ 0.997. The slope, residual sum of squares, and y intercept should also be reported as required by international conference harmonization (ICH) (Belajová**,** 2006).

1.3.2.4. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure (Ravichandran et al., 2010).

1.3.2.5. Limit of detection

The limit of detection (LOD) of an analytical procedure is the lowest amount of an analyte in a sample that can be detected. It is a limit that specifies whether or not an analyte is above or below certain value. The LOD of detection of instrumental procedures is carried out by determining the signal-tonoise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-to noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold.

Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (SDa) which may be related to LOD and the slope of the calibration curve, b, by LOD = 3.3 SDa / b. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analysed at the time to validate the level (Harvey, 2000). .

1.3.2.6. Limit of quantification

Limit of quantification (LOQ) is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy and precision under the stated operational conditions of the method. Like LOD, LOQ is expressed as concentration, with the precision and accuracy of the measurement also reported. The standard deviation multiplied by a factor usually (10) (LOQ =10 Sa/b) provides an estimate of the limit of quantitation. In many cases, the limit of quantitation is approximately twice the limit of detection (Harvey, 2000).

1.3.2.7. Selectivity and Specificity

The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The United State Pharmacopeia (USP) monograph (Morrison et al, 1992) defines the selectivity of an analytical method as its ability to measure

accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix (Convention, 2010).

1.3.2.8. Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, temperature and determines the effect on the results of the method. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used**.**

1.4. Objectives:

The main aim of this thesis was to develop different spectrophotometric and chromatographic methods for analysis of 4-MEI and taurine in manufactured after derivatization using different reagents. The specific objectives of the research were to:

- To develop, optimize and validate simple UV-Vis spectrophotometric and HPLC-PDA methods after derivatization with NBD-Cl for determination of 4-MEI in Sudanese soft drinks containing carmel.
- To validate a reliable, sensitive, simple and low cost high performance HPLC methods with PDA and fluorescence detectors using NBD-Cl as derivatization reagents for routine analysis of taurine in energy drinks.

- To optimize and validate UV-Vis spectrophotometric method for taurine determination after derivatization with *o*-phthalaldehyde and sodium sulfite **(**OPA-Na_2SO_3).
- To optimize and validate HPLC-PDA method for taurine determination after derivatization with OPA-Na_2SO_3.
- To determine the levels of 4-MEI and taurine in carbonated beverages available in the local market.
- To establish a basic research infra-structure for the monitoring of chemical contaminants of food in the country.

Chapter Two

Materials and Methods

2. Materials and methods

2.1. Analytical Methods for determination of 4-MEI using NBD-Cl

2.1.1. UV-Vis spectrophotometry analysis

All ultraviolet-visible spectrophotometric measurements were carried out using a double beam V-530 (JASCO, Japan), the instrument is provided with 1.0 cm quartz cells.

2.1.2. High Performance Liquid Chromatography (HPLC) Analysis

The separation of 4-MEI derivative was carried out on Waters HPLC with PDA detector (Waters, Milford, MA) consisted of a Waters alliance 2695 gradient separations module equipped with auto sampler and column oven, a Waters 2996 Photodiode array detector. All data were processed using Empower software (Waters, Milford, MA). The chromatographic separation was done using an Inertsil ODS-3 (4.6 mm× 250 mm) and a Waters 2996 Photodiode array detector (PDA). The separation was performed at room temperature .the mobile phase consisting of acetonitrile: (0.1%) trichloro acetic acid (30:70, v:v) with the flow rate set at 1.3 mL min^{-1} and injection volume 20 µL. The wavelengths were set at 450 nm.

2.1.3. Chemicals

4(5)-Methylimidazole (98%) and 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride AR. was obtained from CDH (New Delhi, India). Potassium hydroxide pellets was from Lab Tech Chemical (India). Boric acid was purchased from VWR International (Leuven, Belgium). Methanol was supplied by Chem. Lab NV (Belgium). Water was purified with Daihan Lab Tech (Kyonggi, Korea). Acetonitrile HPLC grade was purchased from Duksan (Korea). Trichloroacetic acid was from (Scharlau, European Union).

2.1.4. Preparation of standards and solutions for 4-MEI analysis

2.1.4.1. Preparation of 4-MEI stock solution

Stock solution of 4-MEI (500 mg L^{-1}) was prepared by dissolving 0.025 g in distilled water in a 50.0 mL volumetric flask and made up to the mark with distilled water It was appropriately diluted to prepare intermediate standard for spectrophotometer and HPLC-PDA detection.

2.1.4.2. Preparation of borate buffer for 4-MEI derivatization

The borate buffer (0.005 M) was prepared by dissolved 0.31 g boric acid and 0.29 g of sodium chloride in 80 mL of distilled water and the pH adjusted to 7.0 with 1.0 M potassium hydroxide then the volume was completed to 100 mL with distilled water.

2.1.4.3. Preparation of NBD-Cl solution for 4-MEI derivatization

NBD-Cl solution (0.2%) was prepared by dissolving 0.05 g of the reagent in methanol, then transferred to 25.0 mL volumetric flask and the volume was completed to the mark with methanol. This solution was stable for one week when kept in refrigerator in amber container bottle**.**

2.1.5. Procedures used for 4-MEI analysis

2.1.5.1. Preparation of soft drink samples for 4-MEI determination

A total of six bottled carbonated beverage samples namely Coca cola, Pepsi cola, Cola light, Pepsi diet, Veno and Vimto were purchased from local supermarkets. All samples were stored in refrigerator until opened for analysis. Aliquots of 20.0 mL of each sample was poured into a 50.0 mL beaker and degassed for 15.0 min in a Bandelin Sonorex Ultrasonic bath (Bandelin electronic GmbH &co. KG, Berlin) . A 10.0 mL aliquot of each degassed sample was then pipetted 100 mL beaker and adjusted to pH 7.0 then diluted with 50.0 mL distilled water until the concentration of 4-MEI was 200 mg L^{-1}

for HPLC-PDA based on the labeled amount. Then 0.5 mL of the samples were subjected to derivatization.

2.1.5.2. Pre-column derivatization of 4-MEI with NBD-Cl

Different amounts of (0.1-1.0 mL) were transferred from 500 mg L^{-1} for HPLC detection into a series of 10.0 mL volumetric flasks. Aliquots of 0.5 mL of each diluted sample were transferred to 10.0 mL volumetric flask the volumes of standards and samples were adjusted to about 1.0 mL with distilled water, following addition of 1.0 mL of borate buffer (pH 7.0) and 1.0 mL of 0.2% NBD-Cl. The mixture was heated in thermostatically controlled water bath (WERKE GmbH, Co. KG, Germany) at 90°C for 45.0 min in the dark. The reaction was terminated by cooling in ice water. Then the volume was brought to 10.0 mL with distilled water. The mixture was filtered through 0.45μm cellulose acetate syringe filter from thermo scientific (Mexico) into HPLC auto sampler vial for analysis. Finally, the obtained derivative was analyzed either by measuring its absorbance at 450 nm against reagent blank using spectrophotometer or separation and detection using HPLC-PDA.

2.2. Analytical methods for determination of taurine after derivatization with NBD-Cl

2.2.1. HPLC-PDA and HPLC-FLD Instrumentations

The separation of taurine derivative was carried out on Waters HPLC with PDA detector (Waters, Milford, MA) and Shimadzu HPLC with fluorescence detector (HPLC-FLD) (Shimadzu Corporation, Kyoto, Japan). The HPLC-PDA consisted of a Waters alliance 2695 gradient separations module equipped with auto sampler and column oven, a Waters 2996 Photodiode array detector. All data were processed using Empower software (Waters, Milford, MA). The HPLC-FLD equipped with Prominence LC-20 AD pump, DGU- 20 A3R degassing unit, Prominence SIL-20A autosampler, Prominence CTO-20 A column oven, CBM-20A Communications bus module and RF-20 A

fluorescence detector. All data were processed using LC solution software (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was done using an Inertsil ODS-3 (250 mm× 4.6 mm, 5 μm) column (GL Sciences Inc., Japan). The separation was performed at ambient temperature. The mobile phase consisting of acetonitrile and 0.1% trichloroacetic acid (30:70, v:v) with flow rate of 1.0 mL min^{-1} and injection volume of 20 μL. The wavelength of PDA detector was 470 nm and the excitation and emission wavelengths of fluorescence detector were 470 nm and 530 nm, respectively

2.2.2. Chemicals for taurine analysis

Taurine purity (≥99 %) and NBD-Cl purity 98% were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was obtained from CDH (New Delhi, India). Potassium hydroxide pellets was from Lab Tech Chemical (India). Boric acid was purchased from VWR International (Leuven, Belgium). Methanol (99.8%) was supplied by chem-lab NV (Belgium). Water was purified with Daihan Lab Tech (Kyonggi, Korea). Acetonitrile HPLC grade was purchased from Duksan (Korea). Trichloroacetic acid was purchase from Scharlau (European Union). Hydrochloric acid (37%) was purchased from Sham Laboratory (Addra, Syria).

2.2.3. Preparation of standards and solutions for taurine analysis

2.2.3.1. Preparation of taurine stock solution

Stock solution of taurine of concentration of 1000 mg L^{-1} was prepared by dissolving 0.05 g taurine in distilled water in a 50 mL volumetric flask and made up to the mark with distilled water. It was appropriately diluted to prepare intermediate standard solutions at concentrations of 500 mg L^{-1} and 0.5 mg L^{-1} for HPLC-PDA and HPLC-FLD, respectively**.**

2.2.3.2. Preparation of borate buffer for taurine derivatization with NBD-Cl

The borate buffer (5.0 mM) was prepared by dissolving 0.31 g boric acid and 0.29 g of sodium chloride in 80 mL of distilled water and the pH adjusted to 10 with 1.0 M potassium hydroxide then the volume was completed to 100 mL with distilled water.

2.2.3.3. Preparation of derivatization reagent

NBD-Cl solution (0.05%) was prepared by dissolving 0.0125 g of the reagent in methanol then transferred to a 25 mL volumetric flask and the volume completed to the mark with methanol. This solution was stable for one week when kept in refrigerator in amber container bottle.

2.2.4. Procedures used for taurine derivatization with NBD-Cl

2.2.4.1. Energy drink samples preparation A total of five samples of energy drinks namely Red Bull, Tornado, Kratingdeang, Bison and Tiger were purchased from local supermarkets in Khartoum, Sudan. All samples were stored at refrigerator until opened for analysis and the content of taurine was measured compared to the labeled amount in the samples.

An aliquot of 20 mL of each sample was poured into a 100 mL beaker and degassed for 30 min in a Bandelin Sonorex ultrasonic bath (Berlin, Germany). Then the pH was adjusted to 7.0 using potassium hydroxide (1.0 M). The samples were diluted with distilled water until the concentrations of taurine were 200 mg L^{-1} and 0.2 mg L^{-1} for HPLC-PDA and HPLC-FL, respectively based on the labeled amount. Then 0.5 mL of the samples were subjected to derivatization.

2.2.4.2. Pre-column derivatization with NBD-Cl of taurine standard and taurine in energy drink samples

The derivatization procedure was done according to previously reported method by (Mohamed et al, 2017), with some modifications. Varied volumes (0.1-1.0 mL) were transferred from intermediate standard solutions (500 mg L^{-1} and 0.5 mg L^{-1}) for HPLC-PDA and HPLC-FL detection, respectively into a series of 10 mL volumetric flasks. An aliquots of 0.5 mL of each diluted sample were transferred to 10 mL volumetric flasks. Then the volumes of standards and samples were adjusted to about 1.0 mL with distilled water. Following addition of 1.0 mL of borate buffer (pH 10) and 1.0 mL of 0.05% NBD-Cl solution. The mixture was heated in5 thermostatically controlled water bath (WERKE GmbH, Co. KG, Germany) at 70 °C for 35 min in the dark. The reaction was terminated by cooling in ice water. Then 0.2 mL of HCl was added to adjust the pH to 3.5 to prevent the analytical column from damage. Then the volume was brought to 10 mL with distilled water. The mixture was filtered through a 0.45 μm cellulose acetate syringe filter from Thermo Scientific (Mexico) into a HPLC auto sampler vial for analysis.

2.3. Analytical methods for determination of taurine using *o*-phthalaldehyde (OPA)

2.3.1. UV-Vis spectrophotometry analysis of taurine using OPA

All ultraviolet-visible spectrophotometric measurements were carried out using a double beam V-530 (JASCO, Japan), The instrument is provided with 1-cm quartz cells.

2.3.2. HPLC-PDA analysis of taurine using OPA

The chromatographic separation of taurine derivative was carried out on Shimadzu HPLC coupled with PDA detector (Shimadzu Corporation, Kyoto, Japan). The instrument equipped with Prominence LC-20AD pump, DGU-20A3R degassing unit, Prominence SIL-20A autosampler, CBM-20A system controller and Prominence SPD-M20A photodiode array detector. An Inertsil ODS-3 (250 mm× 4.6 mmi.d., 5 μm) (GL Sciences Inc., Japan) was used as

analytical column. The separation was performed at ambient temperature. The mobile phase consisted of acetonitrile and 0.1% trichloroacetic acid (30:70, v/v) with flow rate of 0.8 mL min^{-1}. Aliquots of 20 µL of the samples and standards were injected into the chromatographic system using the auto-sampler. The wavelengths of PDA detector were set in range of 210-400 nm and the quantitative analysis was performed at 298 nm.

2.3.3. Chemicals for taurine analysis using OPA derivatization

Taurine (purity ≥99 %) and *o*-Phthalaldehyde (OPA) (purity 98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride (99.9%) was obtained from CDH (New Delhi, India). Potassium hydroxide pellets (85%) was from Lab Tech Chemical (India). Boric acid (85%) was purchased from VWR International (Leuven, Belgium). Methanol (99.8%) was supplied by Chem-lab NV (Belgium). Water was purified with Daihan Lab Tech (Kyonggi, Korea). Acetonitrile HPLC grade (99.9%) was purchased from Duksan (Korea). Trichloroacetic acid (80%) was from Scharlau (European Union). Hydrochloric acid (37%) was purchased from Sham Laboratory (Addra, Syria). Sodium sulfite (99%) was from BDH chemicals (United Kingdom).

2.3.4. Preparation of standards and stock solutions for taurine analysis using OPA derivatization

2.3.4.1. Taurine standard stock solution

Stock solution of taurine of concentration 1000 mg L^{-1} was prepared in distilled water and stored at 4 ºC. It was appropriately diluted with distilled water to prepare intermediate standard solution (50 mg L^{-1}).

2.3.4.2. Preparation of borate buffer for taurine derivatization with OPA

The borate buffer (0.1 M) was prepared by dissolving 0.618 g boric acid and 0.584 g of sodium chloride in 70 mL of distilled water then pH adjusted to 9.5 with 1.0 M potassium hydroxide then the volume was brought up to 100 mL with distilled water.

2.3.4.3. Preparation of OPA derivatization reagent

Stock solution of OPA was prepared by dissolving 0.156 g of the reagent in methanol then transferred to a 25 mL volumetric flask and the volume was completed to the mark with methanol. This solution was stable for 3 days when kept in refrigerator in the dark.

Stock solution of OPA was prepared by dissolving 0.156 g of the reagent in 5.0 mL methanol then transferred to a 25 mL volumetric flask and the solution was made up to volume with methanol. This solution was stable for 3 days when kept in refrigerator in the dark.

Stock solution of sodium sulfite (Na_2SO_3) (0.25M) was prepared by dissolving 1.575 g in distilled water in a 100 mL volumetric flask and made up to the mark with distilled water.

Derivatization reagent working solution was prepared by mixing 0.6 mL of OPA stock solution and 0.3 mL of sodium sulfite stock solution then complete the volume to 5.0 mL with borate buffer (0.1 M). The reagent was prepared daily and kept in amber bottle.

2.3.5. Procedures for taurine analysis using OPA reagent

2.3.5.1. Optimized derivatization method

Aliquots of taurine intermediate standard solution (50 mg L^{-1}) over the volume ranges 0.10-1.6 mL and 0.10-2.0 mL were transferred to a set of 5.0 mL

volumetric flasks and the volume were adjusted to 1.6 mL and 2.0 mL with distilled water for UV-Vis spectrophotometer and HPLC-PDA analysis respectively. After addition of 0.4 mL OPA-Na_2SO_3 intermediate solution, the reaction is allowed to proceed for 5.0 min in dark. For HPLC analysis, the pH of the mixtures was adjusted to 3.0 by addition 0.1 mL of HCl (0.5 M) to prevent the analytical column from damage. Afterwards, the solution is made up to volume with distilled water. Finally, the obtained derivative was analyzed by measuring its absorbance at 323 nm either using spectrophotometric determination against reagent blank or by chromatographic separation coupled with PDA detection.

2.3.5.2. Determination of taurine in energy drinks Samples

Approximately 50 mL of each energy drink sample was poured into a 100 mL beaker and degassed by sonicating for 30 min in a Bandelin Sonorex ultrasonic bath (Berlin, Germany). Then, the pH of each degassed sample was adjusted to 7.0 with potassium hydroxide (1.0 M). The samples were diluted with distilled water until the concentration of taurine was 40 mg L^{-1} for all energy drink samples based on the labeled amount. Then 0.5 mL of the samples were subjected to derivatization as described in section 2.3.6.1.

2.4. Method validation

2.4.1. Percentage recovery calculation

The average percent recovery was calculated from equation:

$$\text{Percent Recovery} = [(C_{obs} - \hat{C}_{native})/ C_{spike}] \text{ X } 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.1)$$

Where C_{obs} is the mean of the result from the replicate analyses of spiked sample, $\hat{C}_{native}$ is the observed concentration of the analyte in the unspiked sample, and C_{spike} is the nominal concentration of analyte in spiked sample (Abas, Takruni, Abdullah, & Tahir, 2002).

2.4.2. Precision

The precision of the system was evaluated by estimating the percent relative standard deviation (%RSD) of multiple successive determinations for standards. %RSD was calculated with the equation:

$$\%RSD = [([\Sigma (x_i - x_{avg})2/ n-1]1/2)/ x_{avg}] \text{ X } 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.2)$$

where x_i corresponds to the analyte concentration measured in each standard, n is the number of standards, and x_{avg} is the mean value of the standards (Keith, 2005).

2.4.3. Accuracy

The accuracy was calculated with the equation (Harvey, 2000).

$$\% \text{ Error (percent relative error)} = \frac{(\text{obtained result} - \text{expected result}) \times 100}{\text{expected result}} \quad (2.3)$$

2.4.4. Limit of detection and limit of quantification

Limit of detection (LOD) was calculated from the following equation (Harvey, 2000):

$$LOD = 3.3 \times (s/S) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.4)$$

Limit of quantification was calculated with the equation:

$$LOQ = 10 \times (s/S) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.5)$$

where s is the standard deviation of the intercept and S is the slope of the curve.

Chapter Three

Results and Discussions

3. Results and discussions

3.1. Results of determination of 4-MEI

3.1.1. Absorption Spectrum of the reaction of 4-MEI with NBD-Cl

The absorption spectrum of 4-MEI was recorded against water, it was found that 4-MEI exhibits a maximum absorption peak (λ_{max}) at 227 nm. The reaction between 4-MEI and NBD-Cl was performed, and the absorption spectrum of the product was recorded against reagent blank. It was found that the brown colored product is exhibiting (λ_{max}) at 450 nm (Figure 3.1), and the (λ_{max}) of NBD-Cl was 342 nm.

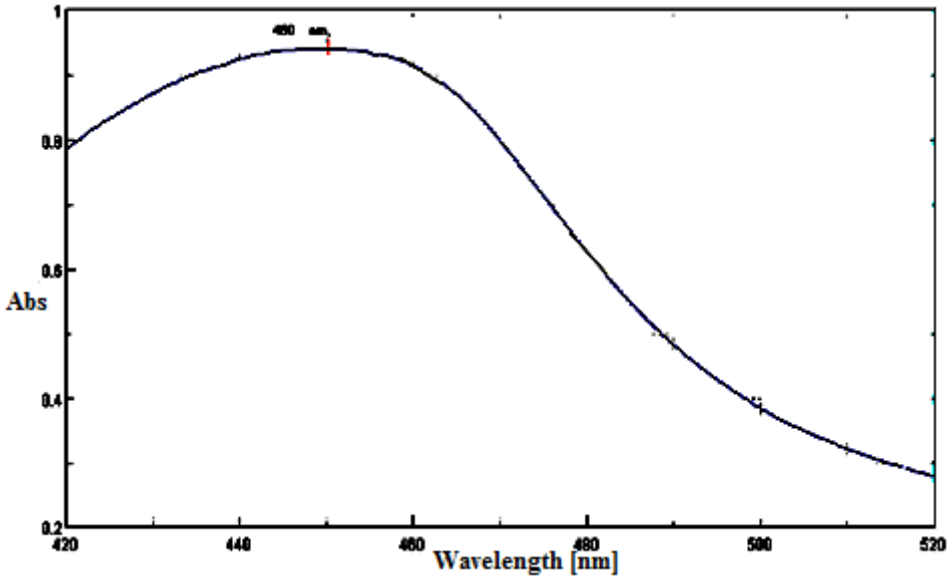

Figure 3.1. UV-Vis spectra of 4-MEI and NBD-Cl derivative against reagent blank.

3.1.2. Optimization of derivatization conditions between 4-MEI and NBD-Cl

Experiments were conducted to investigate the optimal reaction conditions using UV-Vis spectrophotometer. The main parameters affectingthe

derivatization reaction between 4-MEI and NBD-Cl such as pH, concentration of NBD-Cl, buffer volume, dilution solvent, job's method, time and temperature of the reaction were studied.

3.1.2.1. Effect of pH on derivatization process

The influence of buffer pH on the absorbance of derivatization product was investigated in the range of 6.0-8.2. It was observed that, the absorbance of the solution increases up to pH 7.0 and then decrease as shown in Figure 3.2. This means at pH 7.0 the degree of the nucleophilic substitution reaction is reach its maxima. The decrease in the absorbance of solution at pH higher than 7.0, this may be due to the increase of hydroxide ion holds back the nucleophilic substitution reaction between 4-MEI and NBD-Cl. In order to keep the high sensitivity for the determination of 4-MEI, pH 7.0 was selected for optimal experimental conditions.

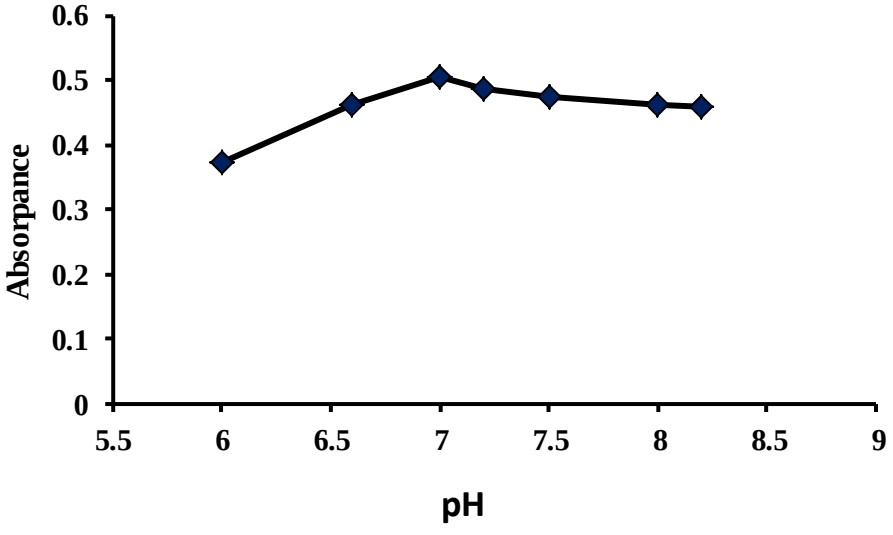

Figure 3.2. Effect of the PH on the reaction of 4-MEI with NBD-Cl.

3.1.2.2. Effect of NBD-Cl concentration on 4-MEI derivatization product

The effect of NBD-Cl concentration was studied over the range (0.05–0.5)% (w/v). Increasing the concentration of NBD-Cl results in more products up to an amount of 0.2%, after which the absorbance is decreased (Figure 3.3). Therefore, a concentration of 0.2% NBD-Cl was considered optimum.

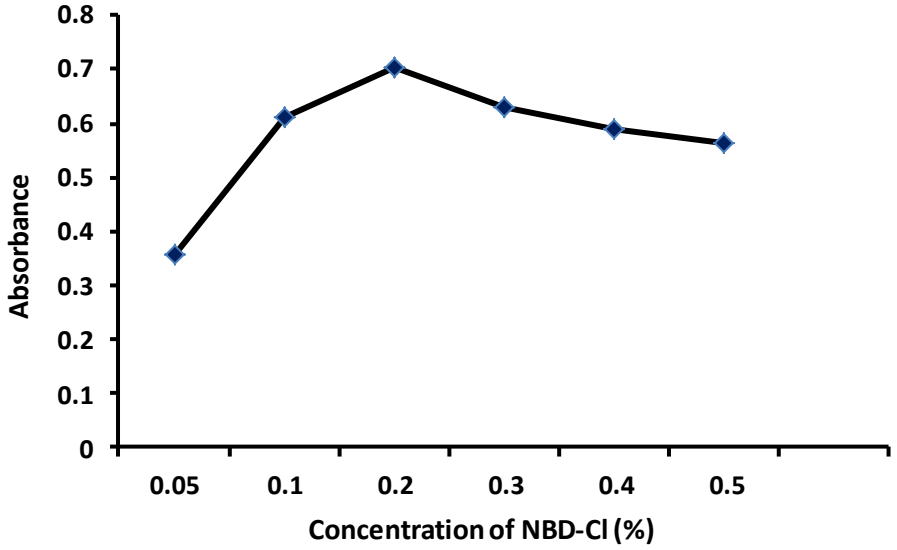

Figure 3.3. Effect of the concentration of NBD-Cl on the reaction 4-MEI with NBD-Cl

3.1.2.3. Effect of temperature and time on derivatization of 4-MEI

The optimum temperature and time for derivatization were investigated. The effect of temperature on derivatization reaction was examined by varying temperature from 50 to 99 °C. The results (Figure 3.4) indicated that the highest absorbance was observed at 90 °C. Therefore the temperature of 90 °C was selected as optimum derivatization temperature.

The effect of time in derivatization process was studied in range from 15 to 60 min. It can see from Figure 3.5, the peak area of 4-MEI derivative increased

with increasing reaction time up to 45 min and longer reaction time did not affect the reaction. Hence reaction time of 45 min was chosen as optimal time.

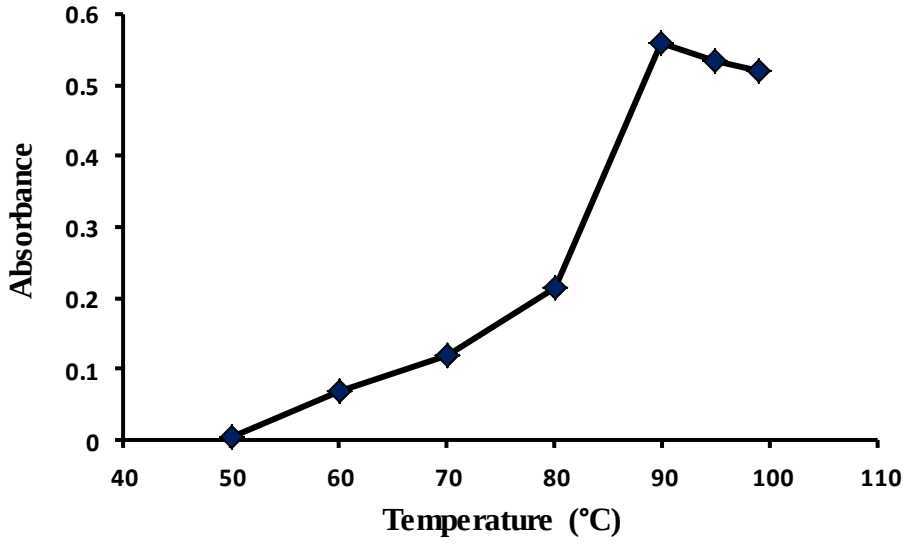

Figure 3.4. Effect of the temperature on the reaction of 4-MEI with NBD-Cl.

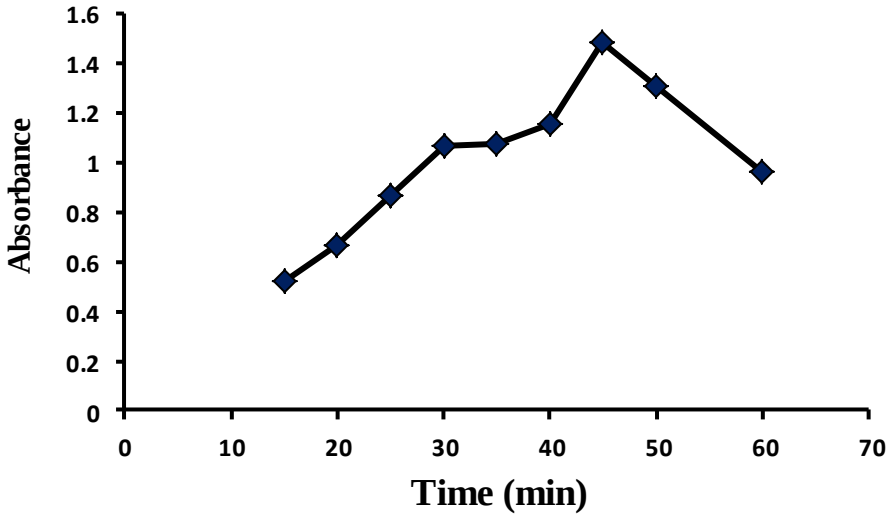

Figure 3.5. Effect of the time on the reaction of 4-MEI with NBD-Cl.

3.1.2.4. Effect of dilution solvent on 4-MEI derivatization with NBD-Cl

Acetonitrile, ethanol, methanol, acetone and water were investigated as reaction diluting solvents for derivatization procedure. The results indicated that water gave the best results as assessed by the detector responses (Figure 3.6). Acetonitrile and methanol gave the lowest response under the proposed conditions. In general, water was used as diluting solvent throughout this study because of the solubility of 4-MEI in water.

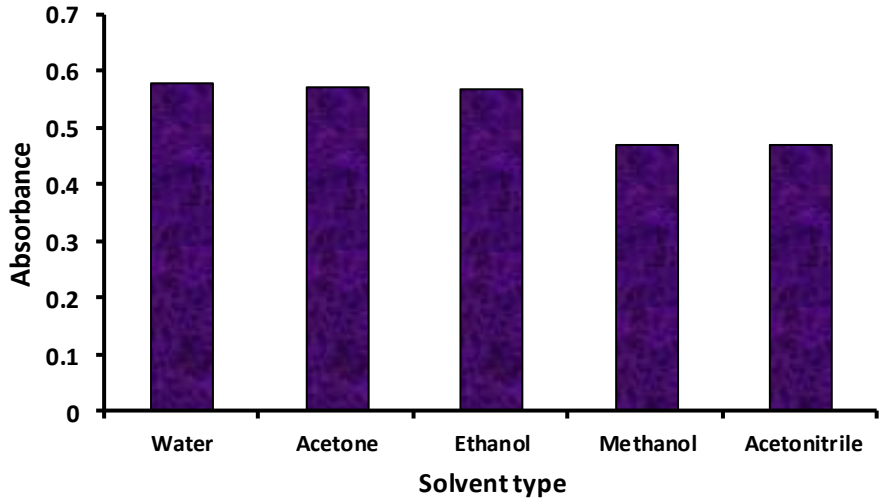

Figure 3.6. Effect of the diluting solvents on the reaction of 4-MEI with NBD-Cl.

3.1.2.5. Effect of amount of borate buffer in 4-MEI derivatization

The effect of amount of borate buffer solution on the absorbance of product was studied in range of 0.25-2.0 mL. It was found that the absorbance of product enhances rapidly with the rise of amount of buffer solution, and becomes maxima when the amount of buffer solution is 1.0 mL (Figure 3.7). Therefore, the amount of 1.0 mL buffer solution was selected to ensure the highest absorbance of product.

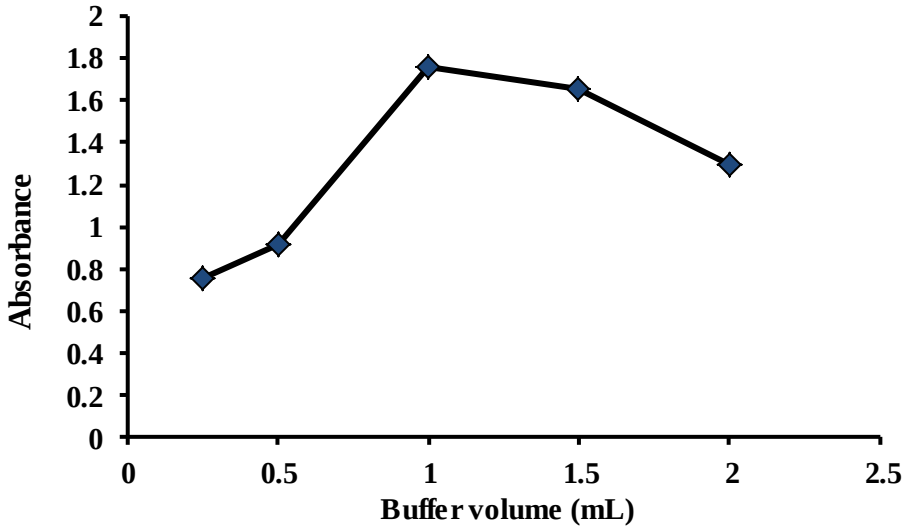

Figure 3.7. Effect of the buffer volume on the reaction of 4-MEI with NBD-Cl.

3.1.2.6. Job's Method

The molar ratio of NBD-Cl and the 4-MEI in the reaction mixture was studied according to Job's method of continuous variation. Utilizing equimolar solution of 4-MEI and NBD-Cl, the reaction stoichiometry was found to be a good approximation 1:1 ratio (4-MEI/reagent) as shown in Figure 3.8.

According to optimization studies, the optimized conditions used for further studies were found as borate buffer pH 7.0, NBD-Cl concentration of 0.2%, reaction temperature 90 ºC, reaction time 45 min, olume of the buffer 1.0 mL, and reaction stoichiometry 1:1 ratio (4-MEI/reagent).

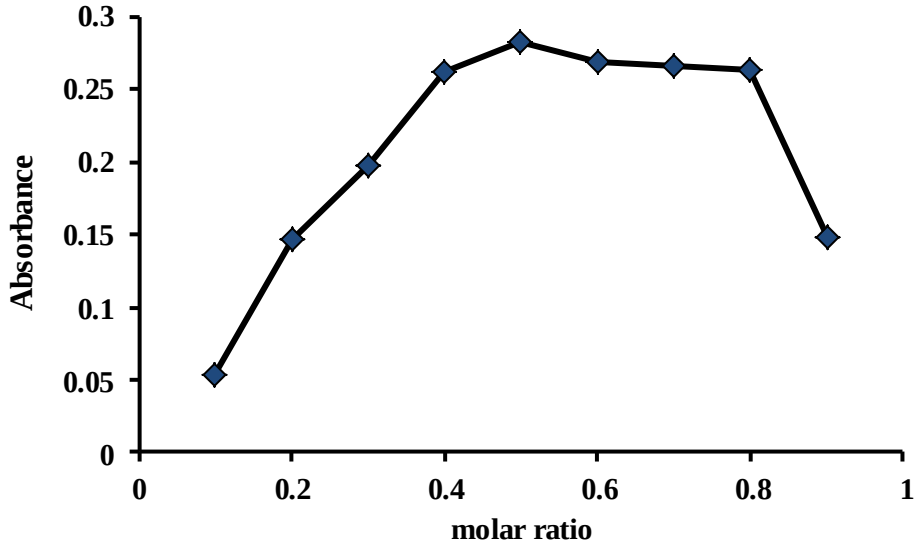

Figure 3.8. The continuous variation plot for the stoichiometry of the reaction of 4-MEI with NBD-Cl.

3.1.3. Linearity and Limit of detection and quantification

For the linearity measurement for the 4-MEI-NBD derivative, six standard solutions containing 4-MEI were prepared over the range from 1.0 to 50 mg L^{-1} and 0.5 to 50 mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis, respectively. Then, the calibration curves were constructed by plotting absorbance (spectrophotometer) as in Figure 3.9 or peak area (HPLC-PDA) as in Figure 3.10 against the concentration of 4-MEI. Good linearities were obtained with correlation coefficients (r^2) of 0.9992, 0.9997 for UV-Vis spectrophotometer and HPLC-PDA respectively as shown in Table (3.1).

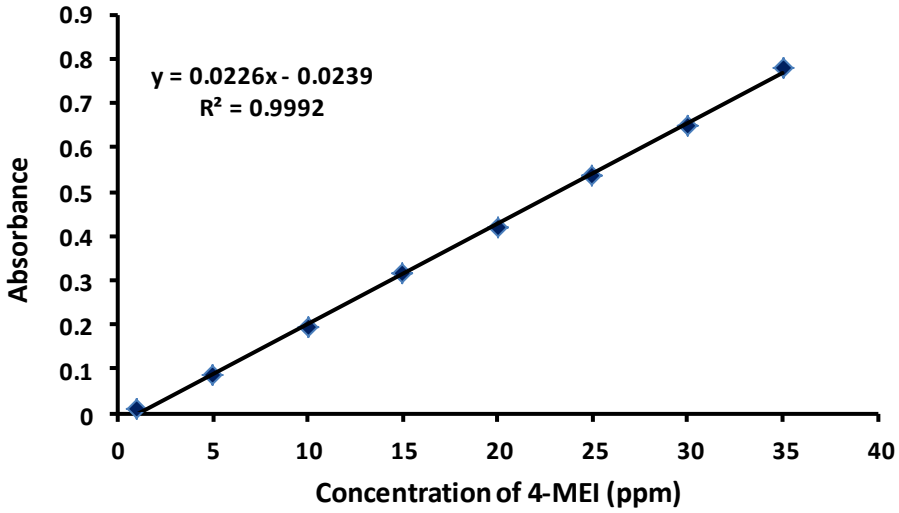

Figure 3.9. Calibration curve of derivatization product of 4-MEI-NBD using UV-Vis spectrophotometer. Under optimized condition.

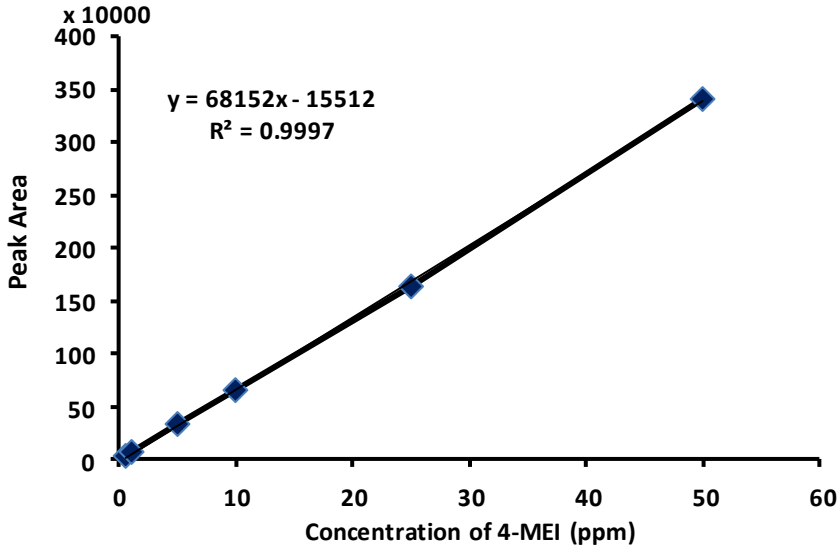

Figure 3.10. Calibration curve of derivatization product of 4-MEI-NBD using HPLC-PDA. Under optimized condition.

The LODs and LOQs of these methods were calculated from the calibration curve data. They were determined according the following formula: LOD= 3.3× SDa/b, and LOQ= 10× SDa/b, where: SDa is the standard deviation of intercept, b is the slope. LOD and LOQ were found to be 0.183, 0.152 mg L^{-1} and 0.550,

0.457 mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis respectively (Table 3.1).

Table (3.1) Equation for external calibration curve, regression coefficient, limit of detection (LOD) and limit of (LOQ) for 4-MEI derivative with NBD-Cl using UV-Vis spectrophotometer and HPLC-PDA

Parameter	Spectrophotometer	HPLC-PDA
Concentration range (mg L^{-1})	1.0-50	0.5-50
Equation	Y= 0.0226x - 0.0239	Y= 68152x – 15512
Regression coefficient (r^2)	0.9992	0.9997
LOD (mg L^{-1})	0.183	0.152
LOQ (mg L^{-1})	0.550	0.457

3.1.4. Precision of UV-Vis spectrophotometer method

The precision of this method was estimated by intra-day (repeatability) and inter-day (reproducibility). The intra-day repeatability was evaluated by analyzing five replicates of 4-MEI standard derivative (10 mg L^{-1}) over one day. The inter-day reproducibility was determined by analyzing twelve replicates of the 4-MEI standard derivative (10 mg L^{-1}) over three days. The precision is presented as the relative standard deviation. The RSD% of the absorbance for intra-day and inter-day were 2.598 and 3.987 %, respectively, which indicate that the proposed method were adequately precise (Table 3.2).

Table (3.2) Intraday and interday precisions for the determination of 4-MEI derivative by UV-VIS spectrophotometer

Parameter	**UV-Vis spectrophotometer**	
	SD	RSD%
Intraday precision (n=5)	0.004	2.598
Interday precision (n=12)	0.007	3.987

3.1.5. Accuracy for UV-Vis spectrophotometer and HPLC

The accuracy of these methods was determined by recovery test. The five soft drink samples (Pepsi, Pepsi diet, coca cola, cola light, Veno and Vimto) containing known amount of 4-MEI were spiked with 4-MEI standard at concentration levels of (5.0, 20) mg L^{-1} and (5.0, 12.5) mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis respectively . Good percent recoveries in range (91.8–106%) and (94.3-108%) were obtained for UV-Vis spectrophotometer and HPLC-PDA analysis Table (3.3) and (3.4), respectively.

Table (3.3) Percentage recovery (n = 3) of 4-MEI in soft drink samples using UV-Vis spectrophotometer

Sample	**UV-Vis spectrophotometer**			
	Sample content (mg L^{-1})	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery% ± SD (n=3)
Pepsi	11	5	16.04	101 ± 0.004
	11	20	30.93	99.7 ± 0.002
Pepsi diet	11	5	15.82	96.4 ± 0.001
	11	20	30.30	96.5 ± 0.006
Coca Cola	7	10	16.18	91.8 ± 0.010
	7	20	26.58	97.9 ± 0.017
Cola light	13	5	18.31	106 ± 0.019
	13	20	32.40	97.0 ± 0.006
Veno	40	5	45.10	102 ± 0.007
	40	20	58.86	99.3 ± 0.010
Vimto	16	5	21.22	104 ± 0.001
	16	20	35.87	99.3 ± 0.001

Table (3.4) Percentage recovery (n = 3) of 4-MEI in soft drink samples using HPLC-PDA.

Sample	HPLC-PDA			
	Sample content (mg L^{-1})	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery% ± SD (n=3)
Pepsi	30	5	34.77	99.2 ± 0.50
	30	12.5	42.35	99.5 ± 0.58
Pepsi diet	30	5	34.07	96.9 ± 0.42
	30	12.5	43.77	104 ± 1.01
Coca Cola	16	5	21.31	101 ± 0.24
	16	12.5	27.77	95.4 ± 1.05
Cola light	35	5	40.37	101 ± 0.77
	35	12.5	47.99	101 ± 0.52
Veno	20	5	26.77	108 ± 0.95
	20	12.5	33.58	105 ± 0.65
Vimto	20	5	25.07	100 ± 0.07
	20	12.5	31.37	94.3 ± 0.19

3.1.6. Robustness for derivatization of 4-MEI with NBD-Cl determined by UV-Vis spectrophotometer

Robustness was assessed by testing the susceptibility of measurements to deliberate variation of the analytical conditions. one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that variation in the NBD-Cl concentration of 0.2 ± 0.02 % (w/v) and optimal experimental conditions of temperature (90 ± 2°C), time (45 ± 2min) and pH (7.0 ± 0.2), did not significantly affect the procedures and recovery values were 98-99 % and the SD values did not exceed 0.0099 (Table 3.5).

Table (3.5) Robustness values for derivatization 4-MEI with NBD-Cl measured by spectrophotometer

Parameter	Value	Recovery (% ± SD)
pH	6.8	99.5 ± 0.001
	7.2	98.9 ± 0.009
NBD-Cl concentration (w/w)%	0.18	96.2 ± 0.028
	0.22	99.1 ± 0.016
Reaction time (min)	43	98.58 ± 0.033
	47	99.90 ± 0.003

(*n*=3)

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3.1.7. Application of the method

The validated methods were assessed by analyzing a total of six soft drinks contain 4-MEI (Pepsi, Pepsi diet, Coca cola, Cola light, Vimto, Veno) using UV-Vis spectrophotometer and HPLC-PDA. Figure 3.11 shows the UV-Vis Spectra of MEI-NBD derivative present in Cola light sample. Symmetrical peaks with reasonable retention time (7.551 and 7.534 min) were obtained for 4-MEI standard (5.0 mg L^{-1}) and 4-MEI in Pepsi diet sample after derivatization with NBD-Cl as shown in Figure 3.12 and Figure 3.13, respectively.

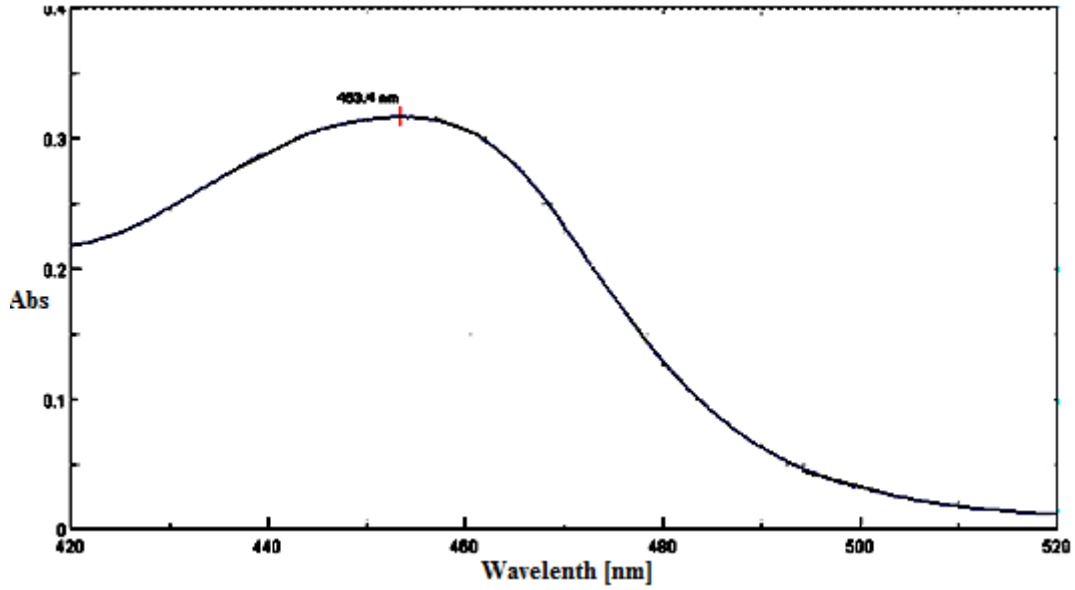

Figure 3.11. UV-Vis Spectraum of MEI-NBD derivative (8 mg L^{-1}) present in Cola light sample

Table (3.6) Concentration of 4-MEI in soft drink samples obtained by UV-Vis spectrophotometer and HPLC-PDA

Sample	**Spectrophotometer**	**HPLC-PDA**
	Conc. (mg L^{-1} ± SD)	**Conc. (mg L^{-1} ± SD)**
Pepsi	56.51 ± 0.0004	59.84 ± 0.465
Pepsi diet	56.87 ± 0.013	63.50 ± 0.420
Coca cola	73.22 ± 0.050	66.71 ± 0.381
Cola light	65.63 ± 0.019	70.7 ± 0.588
Vimto	84 ± 0.014	80.67 ± 0.306
Veno	90.4 ± 0.004	87.12 ± 0.393

From Table (3.6) it can be noted that the 4-MEI content of all analyzed soft drinks samples is between 56.51 mg L^{-1} and 90.4 mg L^{-1}. The highest concentration of 4-MEI was obtained in Veno sample and the lowest concentration in Pepsi sample. A good correlation is observed between the 4-MEI content quantified with the two methods. The HPLC-PDA method is very selective as shown in Figure 3.12, only one peak was appear in the chromatogram of 4-MEI standard derivatized with NBD-Cl.

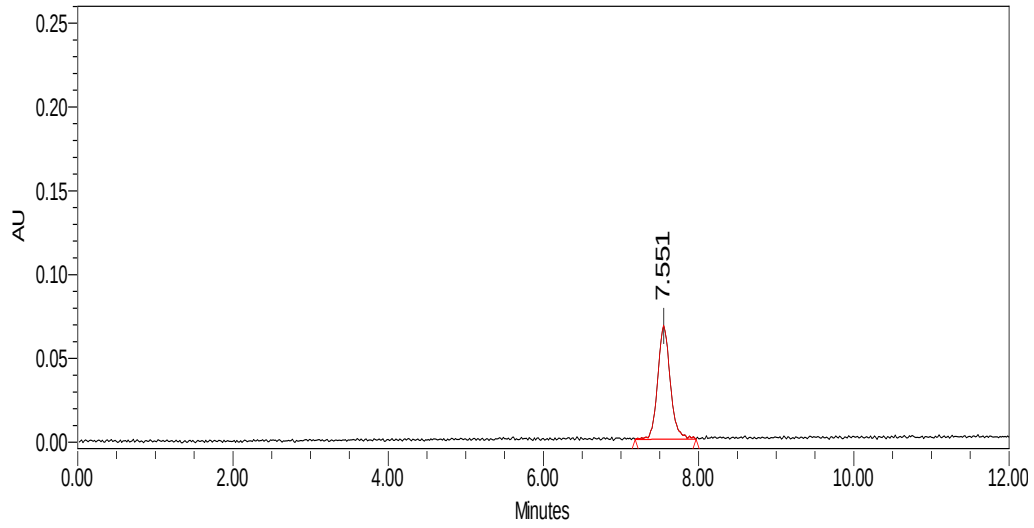

Figure 3.12. Chromatogram of 4-MEI standard. (5.0 mgL^{-1}) derivatized with NBD-Cl analyzed by HPLC-PDA.

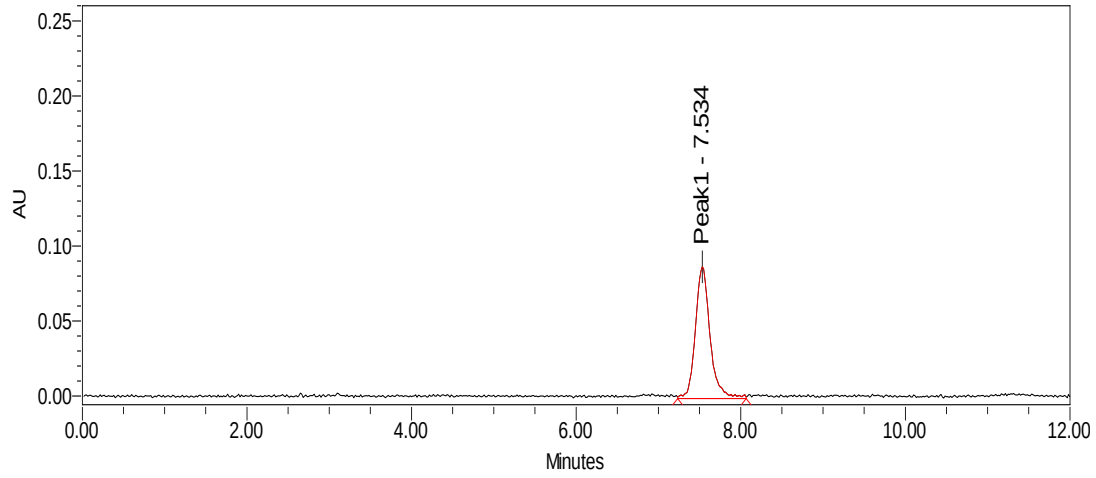

Figure (3.13). Chromatogram of 4-MEI (5 mg L^{-1}) in Pepsi diet sample derivatized with NBD-Cl analyzed by HPLC-PDA.

3.2. Result of determination taurine using NBD-Cl

Herein, taurine was derivatized with NBD-Cl, then the product was measured using HPLC-PDA and HPLC-FLD.

3.2.1. Validation of the methods

To ensure that this method for determination of taurine after derivatization with NBD-Cl was applicable to real samples, several basic analytical parameters were evaluated, including linearity, LOD, LOQ, precision (intra-day and inter-day repeatability) (RSD%) and accuracy (recovery). All these parameters were determined for both HPLC-PDA and HPLC-FLD analysis.

3.2.1.1. Linearity

The calibration curves were constructed by plotting taurine derivatives peak areas against concentrations of taurine. For HPLC-PDA measurement linearity Figure 3.14 was studied in the concentration range of 5.0-50 mg L^{-1}. As shown in Table (3.7) good linearity was obtained for taurine derivative with correlation coefficient (r^2) of 0.9993 (*n*=6). The concentration range of taurine that was used linearity study for HPLC-FLD analysis was 5.0-50 μg L^{-1} (Figure 3.15). The results show a good linear relationship with coefficient of 0.9998 (*n*=6) Table (3.7).

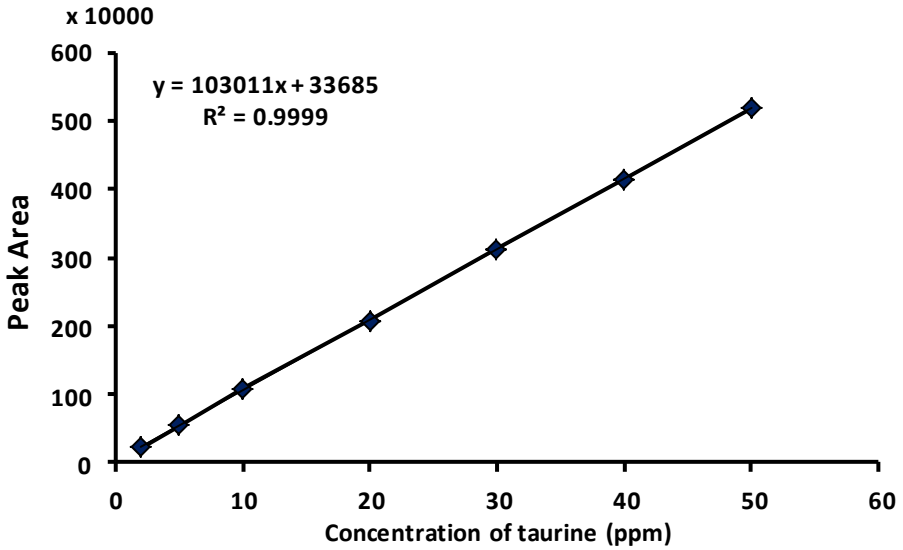

Figure 3.14. Calibration curve of the of taurine derivatized with NBD-Cl under optimized condition and measured with HPLC-PDA

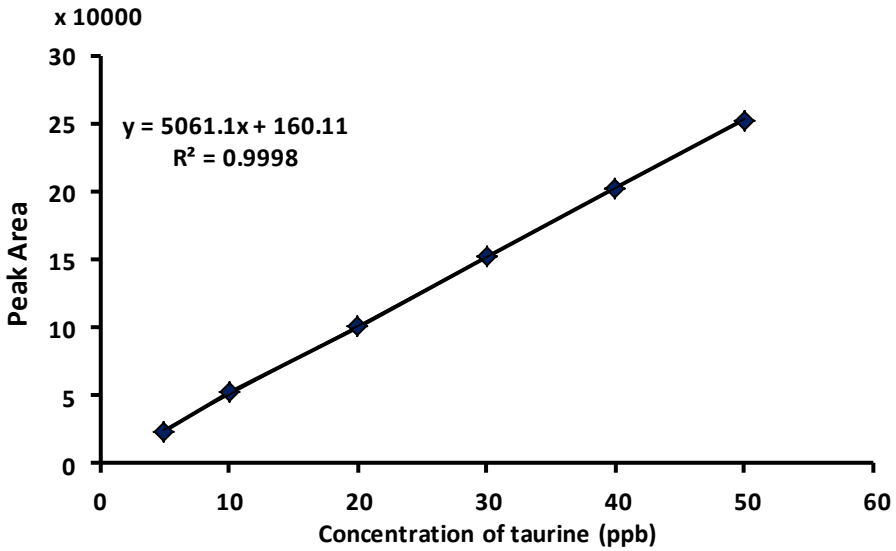

Figure 3.15. Calibration curve of the of taurine derivatized with NBD-Cl under optimized condition using HPLC-FLD.

3.2.1.2. Limit of detection and limit of quantification

The LOD and LOQ were calculated using the external standard calibration curve. The LOD was established using LOD = 3.3×(s/S) and the LOQ = 10×(s/S), where s is the standard deviation of the intercept and S is the slope of the curve. The LOD and LOQ of the methods were estimated to be 0.295, 0.888 mg L^{-1} and 0.615, 1.847 μg L^{-1} for HPLC-PDA and HPLC-FLD, respectively as shown in Table (3.7). It was found that HPLC-FLD is more sensitive 480 times than HPLC-PDA analysis. Hence this derivatization method with HPLC-FLD analysis can use for determination of taurine at trace level.

Table (3.7) Equations for external calibration curves, regression coefficient, LOD) and LOQ for taurine derivative with NBD-Cl using HPLC with PDA and FLD detectors.

Parameter	**HPLC-PDA**	**HPLC-FLD**
Concentration range (mg L^{-1})	5-50	0.005-0.05
Equation	Y= 103011x + 33685	Y= 5061.1x + 160.11
Regression coefficient (r^2)	0.9998	0.9993
LOD (mg L^{-1})	0.296	0.616 $\times 10^{-3}$
LOQ (mg L^{-1})	0.888	1.847 $\times 10^{-3}$

3.2.1.3. Precision for HPLD-PDA and HPLC-FLD analysis

The precision of these methods were tested by intra-day repeatability and inter-day reproducibility as RSD%. The intra-day repeatability was studied by performing six successive injections of 10 mg L^{-1} and 10 μg L^{-1} taurine derivative for HPLC-PDA and HPLC-FLD, respectively. The inter-day reproducibility was determined by nine consecutive injections of the same concentration of intra-day repeatability. As summarized in (Table 3.8) the RSD of the peak area and retention time for intra-day repeatability and inter-day reproducibility were less than 0.78, 1.34% and 0.25, 0.71%, respectively, which indicate that the precision of these methods are satisfactory

Table (3.8). Intra-day and inter-day precisions for the determination of taurine derivative with HPLC-PDA and HPLC-FLD

Parameter	**HPLC-PDA**		**HPLC-FLD**	
	Peak area	**Retention time (min)**	**Peak area**	**Retention time (min)**
Intraday precision (RSD%) (n=6)	0.78	0.25	0.61	0.13
Interday precision (RSD%) (n=9)	1.34	0.71	1.13	0.52

3.2.1.4. Accuracy (Recovery) for HPLD-PDA and HPLC-FLD analysis

For the recovery study, all energy drink samples (Red Bull, Tornado, Kratingdeang, Bison and Tiger) with known taurine concentration were spiked with taurine at levels (5.0, 20 and 40 mg L^{-1}) and (10, 15 and 20 μg L^{-1}) for HPLC-PDA and HPLC-FLD analysis, respectively. Good percentage recoveries (92–103.3%), RSD (0.145-2.53%) were obtained for both HPLC-PDA and HPLC-FLD as demonstrated in Table (3.9) and Table (3.10), respectively.

3.2.1.5. Application of the optimized method

The validated methods were assessed by analyzing a total of five energy drinks contain taurine (i.e., Red bull, Tornado, Kratingdeang, Bison and Tiger). Figure (3.16) (A-D) presents the chromatograms of taurine standard derivative (10 mg L^{-1}), Tornado sample (10 mg L^{-1}) and taurine standard derivative (30 μg L^{-1}), Tornado sample (30 μg L^{-1}), determined by HPLC-PDA and HPLC-FLD, respectively. The obtained concentrations of analyzed energy drinks were found to be not significantly different from the concentration values in the labels. The percentage recovery was less than (106%), this indicate the high accuracy of the two proposed methods (HPLC-PDA and HPLC-FLD) for determination of taurine in energy drinks. The concentrations of taurine in energy drink samples that obtained by the two proposed methods were statistically compared with each other using T and F-tests (Table 3.11). The results obtained showed there was no significant difference between these values at the 95% confidence level this indicated similar accuracy and precision in the two methods.

Table (3.9) Percentages recovery (n = 3) of taurine in energy drink samples by HPLC-PDA.

Sample	HPLC-PDA			
	Sample content (mg L^{-1})	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery% ± SD (n=3)
Tornado	5.0	5.0	10.21	99.7 ± 0.05
	5.0	20.0	25.74	102.2 ± 0.65
	5.0	40.0	45.33	100.2 ± 0.41
Krating-daeng	5.0	5.0	10.20	102.4 ± 0.13
	5.0	20.0	25.26	100.9 ± 0.43
	5.0	40.0	44.68	99 ± 0.15
Bison	10.0	5.0	15.14	99.9 ± 0.25
	10.0	20.0	30.17	100.1 ± 0.59
	10.0	40.0	49.30	97.7 ± 0.80
Tiger	10.0	5.0	15.0	100.1 ± 0.27
	10.0	20.0	29.94	99.7 ± 0.30
	10.0	40.0	49.21	97.8 ± 0.71
Red Bull	10.0	5.0	15.03	103.3 ± 0.14
	10.0	20.0	30.27	102 ± 0.20
	10.0	40.0	49.6	99.2 ± 0.49

Table (3.10) Percentage recovery (n = 3) of taurine in energy drink samples by HPLC-FLD.

Sample	HPLC-FLD			
	Sample content (µg L^{-1})	Added amount (µg L^{-1})	Found (µg L^{-1})	Recovery% ± SD (n=3)
Tornado	10.0	10	20.13	98.2 ± 0.13
	10.0	15	25.05	98.2 ± 0.13
	10.0	20	30.06	98.9 ± 0.01
Krating-daeng	10.0	10	19.87	96.6 ± 0.08
	10.0	15	25.12	99.4 ± 0.20
	10.0	20	29.8	98.4 ± 0.06
Bison	10.0	10	19.99	99.9 ± 0.06
	10.0	15	25.05	98.2 ± 0.13
	10.0	20	30.06	98.9 ± 0.01
Tiger	10.0	10	20.08	91.9 ± 0.09
	10.0	15	25.11	94.7 ± 0.09
	10.0	20	30.22	97 ±0.05
Red Bull	10.0	10	20.11	99.9 ± 0.15
	10.0	15	25.11	100 ± 0.19
	10.0	20	29.93	99 ±0.07

Table (3.11) Comparison between measured taurine contents in this study and labeled amount of taurine in energy drink samples and values of T and F tests

Sample	**Labeled amount in (mg L^{-1})**	**HPLC-PDA**		**HPLC-FLD**		**T**	**F**
		Conc. mg L^{-1} ± SD[a]	Recovery (%)	Conc. mg L^{-1} ± SD	Recovery (%)		
Tornado	100	106 ± 2.12	106	103 ± 0.63	103	1.92	11.31
Kratingdaeng	4000	4005± 3.54	100.1	4002 ± 5.66	100	0.63	0.39
Bison	4000	4008 ± 2.83	100.3	3990 ±7.07	99.7	3.34	0.16
Tiger	3200	3199± 2.12	99.9	3202 ± 1.41	100	1.67	2.26
Red Bull	4000	3996 ± 4.24	99.9	3992 ± 2.83	99.8	1.11	2.24

[a] n=2

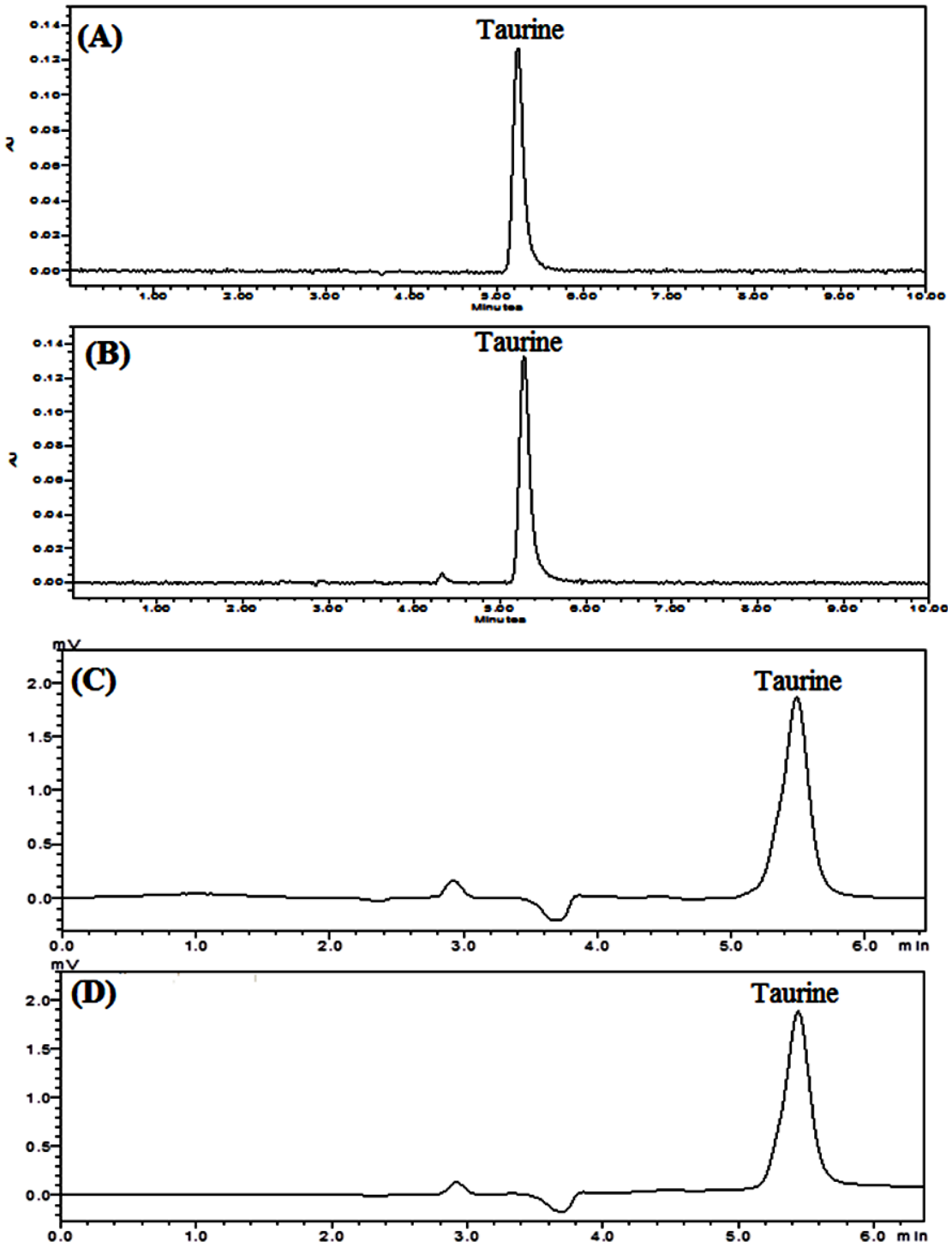

Figure 3.16. Chromatograms of taurine derivative (A) taurine standard. derivative (10 mg L^{-1}) by HPLC-PDA, (B) taurine derivative in Tornado sample (10 mg L^{-1}) by HPLC-PDA (C) taurine standard. derivative (10 µg L^{-1}) by HPLC-FLD, (D) taurine derivative in Tornado sample (10 µg L^{-1}) by HPLC-FLD.

3.2.1.6. Analytical performance comparison of present work with other reported studies

Comparison of the LODs or LOQs obtained with the present work of pre-column derivatization of taurine with NBD-Cl followed by HPLC-PDA and HPLC-FLD detection with the LODs achieved with other high performance liquid chromatographic methods using different derivatization procedures are shown in Table (3.12). Compared with the previously reported HPLC methods for the determination of taurine, the mobile phase used in this proposed method (acetonitrile: trichloroacetic acid) is simpler than the majority of mobile phases used in reported HPLC methods. Most of the mobile phases used in previous HPLC methods for taurine analysis are containing buffer solutions or ion pair reagents which are needed long time to wash them out the analytical column . The sensitivies of the validated methods are higher than the results obtained using HPLC-UV/VIS pre-column derivative with fluorescamine (McMahon, O'Kennedy, & Kelly, 1996), HPLC-PDA direct analysis (Begum, 2014) and direct HPLC with evaporative light scattering detector (ELSD) analysis (Chirita, Dascalu, Gavrila, & Elfakir, 2010). The LOD of current HPLC-PDA is comparable with previous HPLC-UV/VIS pre-column derivative with O-phtha-aldehyde/ 2-mercaptoethanol (Ferreira et al., 1997).

Table (3.12) Comparison of HPLC conditions, LOD and LOQ of present work with other HPLC methods for taurine analysis published in literature

Chromatographic method	**Mobile phase**	**Column**	**Derivtization agent**	**Detection method**	**LOD & LOQ (mg L^{-1})**	**Ref**
HPLC-UV/VIS pre-column derivative	Tetrahydrofuran-acetonitrile-phosphate buffer (15 mM, pH 3.5) (4:24:72)%	Bondclone C-18 (300 x 3.9 mm, l0 µm)	Fluorescamine	Fluorescence	LOQ: 5.0	[18]
HPLC-UV/VIS with pre-column derivative	Disodium hydrogenphosphate-citric acid (PH 5.4) containing 10mM tetrabutylammonium bromide: acetonitrile (70:30)%	ODS, (150 x 4.6 mm, 5µm)	4-fluoro-7-nitrobenzofurazan (NBDF)	UV-Visible	LOQ: 0.50	[7]
HPLC-UV/VIS pre-column derivative	phosphate buffer (0.05M, pH: 5.3):Methanol (60:40)%	C18 (S10 ODS_2)	O-phtha]aldehyde/ 2-mercaptoethanol	UV-VIS	LOD: 0.3	[24]
HPLC/ESI-MS Direct analysis	Gradient between (A) 5 mM heptafluorobutyric acid and (B) methanol	Spherigel- C18 silica 5µm	-	MS	LOD: 0.01	[11]
HPLC-PDA Direct analysis	orthophosphoric acid :acetonitrile (60:40)%	Inertsil ODS (250 x 4.6 mm, 5 µm)	-	PDA	LOD: 2.76	[22]
HPLC- ELSD Direct analysis	Methanol: water (70:30)%	Astec apHeraTM NH_2 polymer (150 x 2.1 mm ,5µm)	-	ELSD	LOD: 2.0	[23]
HPLC-PDA pre-column derivative	Acetonitrile : 0.1% trichloroacetic acid (30:70)%	Inetrsil ODS-3 (250× 4.6 mm, 5 µm)	NBD-Cl	PDA	LOD: 0.296 LOQ:0.888	Present work
HPLC-FLD pre-column derivative	Acetonitrile : 0.1% trichloroacetic acid (30:70)%	Inertsil ODS-3 (250 × 4.6 mm, 5 µm)	NBD-Cl	Fluorescence	LOD: 0.62×10^{-3} LOQ:1.85×10^{-3}	Present work

3.3. Result of determination taurine using *o*-phthalaldehyde (OPA)

3.3.1. Derivatization reaction and absorption spectra

The reaction between amino acids and OPA in presence of thiol compounds. The most common thiols used with OPA for taurine derivatization are 2-mercapto-propionic acid (Ghandforoush et al., 2009) and 2-mercapto-ethanol (Kanjana, 2017) which have unpleasant stench, toxic effects and produced unstable product (Maldonado & Maeyama, 2013, Belajovà, 2006). Therefore, in this work odorless sodium sulfite was used instead of alkylthiol to react OPA and taurine under experimental conditions and produce relatively stable N-alkyl 1-isoidonle sulfonate derivative.

Figure (3.17) shows overlapped absorption spectra of taurine in water, OPA in methanol and derivative taurine-OPA-sulfite. The taurine and OPA exhibit maximum absorption peak at 201 nm and 251 nm, respectively, while the derivatization product absorbed at 227 and 323 nm. In spite of the absorption of derivatization product is higher in 227 nm, the wavelength 323 nm was used for all spectrophotometric measurements to enhance the selectivity.

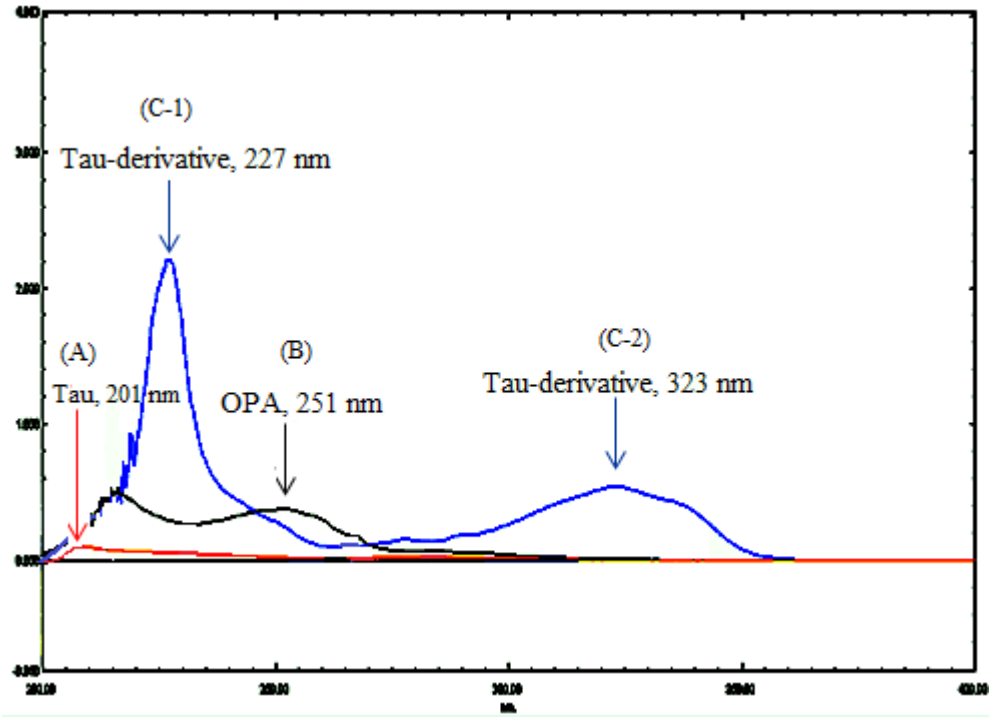

Figure 3.17. Absorption spectrum of (A) taurine (8.0 mg mL-1) against water. (B) Absorption spectrum of OPA (50 mg mL^{-1}) against methanol. (C1,2) Absorption spectrum of reaction product of taurine with OPA-Sulfite against reagent blank.

3.3.2. Optimization of derivatization conditions between taurine and OPA-Na_2SO_3

Experiments were conducted to investigate the optimal reaction conditions using UV-Vis spectrophotometer. The main parameters affecting on the derivatization reaction between taurine and OPA-Na_2SO_3 such as pH, concentration of OPA and Na_2SO_3, dilution solvent and time of the reaction were studied.

3.3.2.1. Effect of pH on derivatization of taurine with OPA-Na_2SO_3

The derivatization reaction took place under basic conditions (M.salah et al., 2007). Therefore, the effect of pH on the absorbance of derivatization product was investigated in range of (8.0-11) using 0.1 M borate buffer. The results show that the absorbance of taurine derivative increases with increasing the pH of borate buffer up to pH 9.5 and then decrease as shown in Figure 3.18. This result in an agreement with that reported by (Monge & Fornaguera, 2009) for

determination of taurine after pre-column derivatization with OPA. Thus pH of 9.5 was selected for derivatization reaction. However, most of published works for derivatization of amino acids with OPA has been done with buffer at pH 10 or 10.4 (Ghandforoush et al., 2009, Mehdinia et al., 2017, Monge & Fornaguera, 2009).

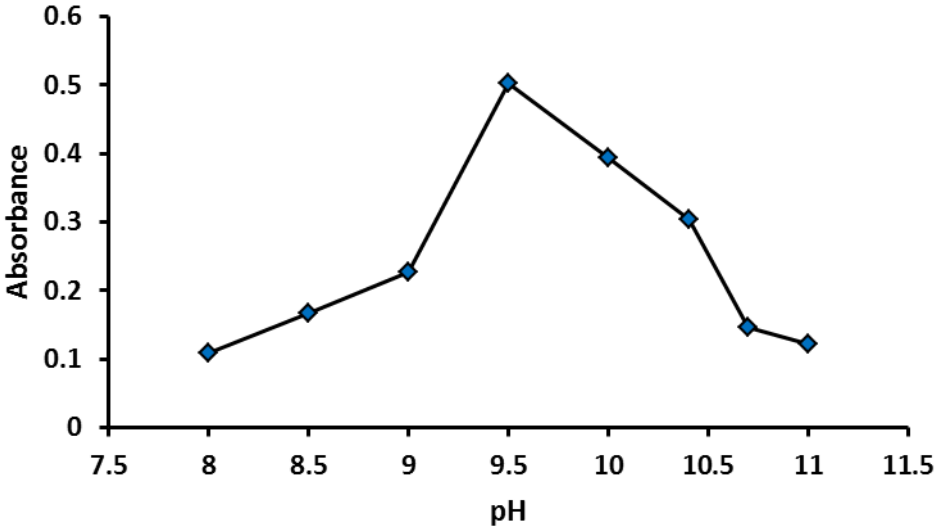

Figure 3.18. Effect of the PH on the reaction of taurine with OPA Na_2SO_3.

3.3.2.2. Effect of OPA and Na_2SO_3 concentration on derivatization of taurine

The effect of OPA concentration was studied over the range (5–150) mg L^{-1} in the final solution. From Figure 3.19, it was observed that, increasing the concentration of OPA increase the reaction yield up to an amount of 60 mg L^{-1} and then leveled off. Therefore a concentration of 60 mg L^{-1} was considered optimum.

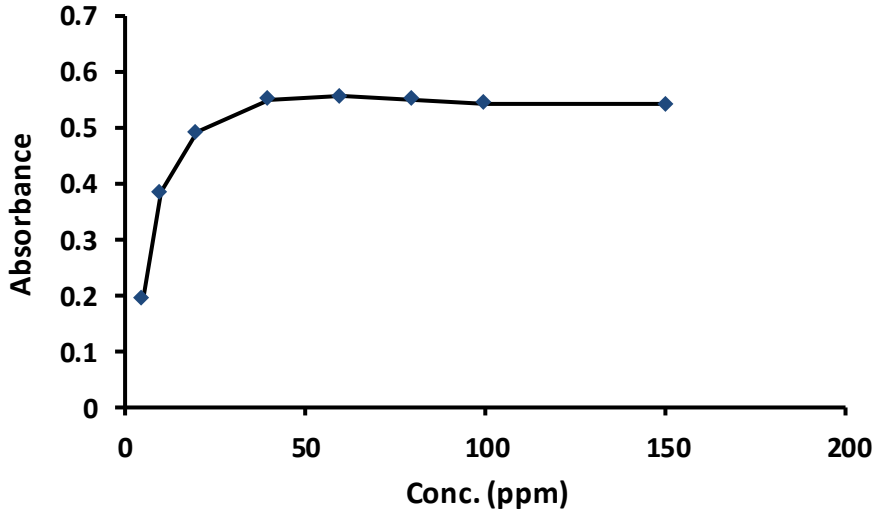

Figure 3.19. Effect of the concentration of OPA on the reaction taurine with OPA-Na_2SO_3.

Also the influence of Na_2SO_3 concentration was investigated over the range (50-353 mg L^{-1}). It was observed that, the response of taurine derivative increase with the rise of concentration of Na_2SO_3 solution, and becomes maxima at concentration of 202 mg L^{-1} (Figure 3.20). Therefore, the concentration of 202 mg L^{-1} was chosen to ensure the highest absorbance of product.

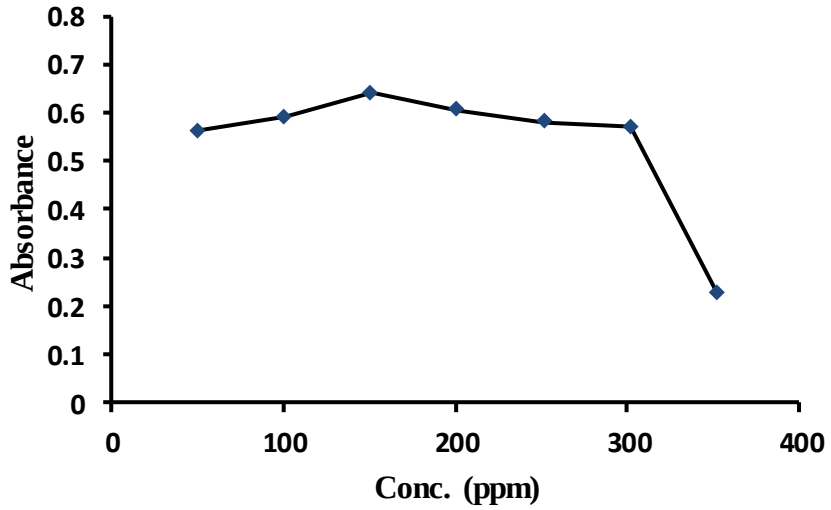

Figure 3.20. Effect of the concentration of Na_2SO_3 on the reaction taurine with OPA-Na_2SO_3.

3.3.2.3. Effect of time on derivatization of taurine with OPA-Na_2SO_3

By following the reaction for various periods (0.5–10 min), it was found that the reaction was completed in 5.0 min and then the response is slightly declined with prolonged reaction time (Figure 3.22). This may be due to instability of OPA derivatives at room temperature over a long time. So the reaction time was set to 5.0 min for the further experiments.

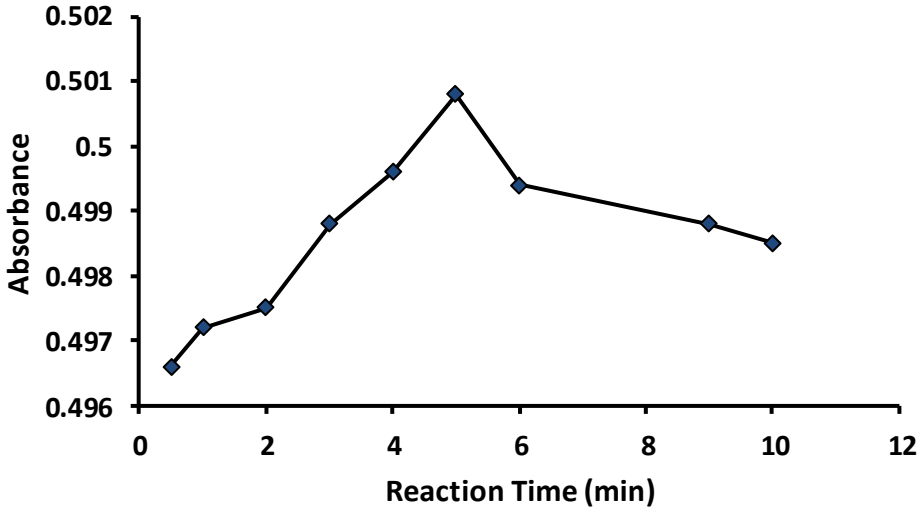

Figure 3.21. Effect of the reaction time on the reaction of taurine with OPA-Na_2SO_3.

3.3.2.4. Effect of diluting solvent on derivatization of taurine with OPA-Na_2SO_3

Different solvents, such as acetonitrile, ethanol, methanol, acetone and water were tested as diluting solvents for derivatization product. The results in Figure 3.22 showed that water is the best solvent as the highest absorbance value was obtained.

According to optimization studies, the optimized conditions used for further studies were found as borate buffer pH 9.5, OPA concentration of 60 mg L^{-1}, concentration of Na_2SO_3 of 202 mg L^{-1}, reaction time of 5.0 min.

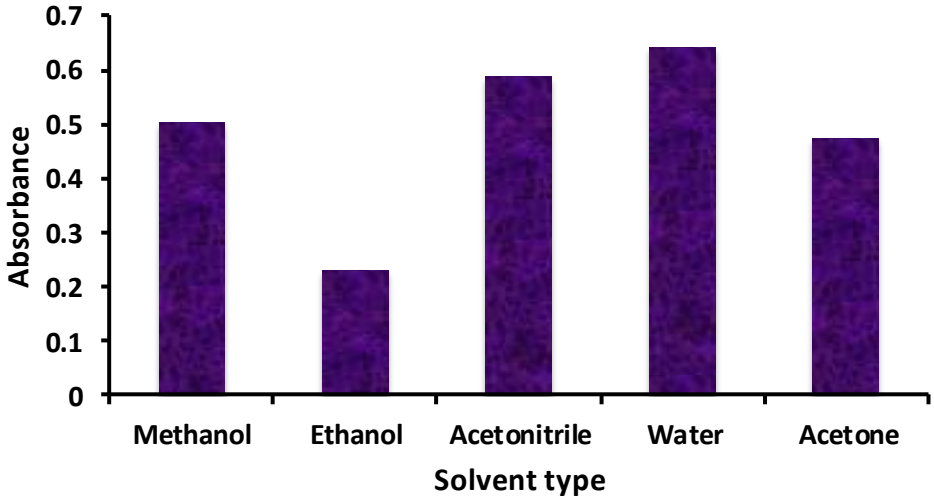

Figure 3.22. Effect of the diluting solvents on the reaction of taurine with OPA-Na_2SO_3.

3.3.3. Validation of derivatization method of taurine with OPA-Na_2SO_3

The current method was validated throughout linear range, LOD, LOQ, precision (repeatability) (RSD%) and accuracy (recovery).

3.3.3.1. Linearity and Limit of detection and quantification

For the linearity measurement for the taurine-OPA/Na_2SO_3 derivative, six standard solutions containing taurine were prepared over the range from 0.5 to16 mg L^{-1} and 0.5 to 20 mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis, respectively. Then the calibration curves were constructed by plotting absorbance (spectrophotometer) or peak area (HPLC-PDA) against the concentration of taurine as shown in Figure 3.23 and Figure 3.24, respectively. Good linearities were obtained with correlation coefficients (r^2) of 0.9996, 0.9998 for UV-Vis spectrophotometer and HPLC-PDA respectively as shown in Tables (3.13).

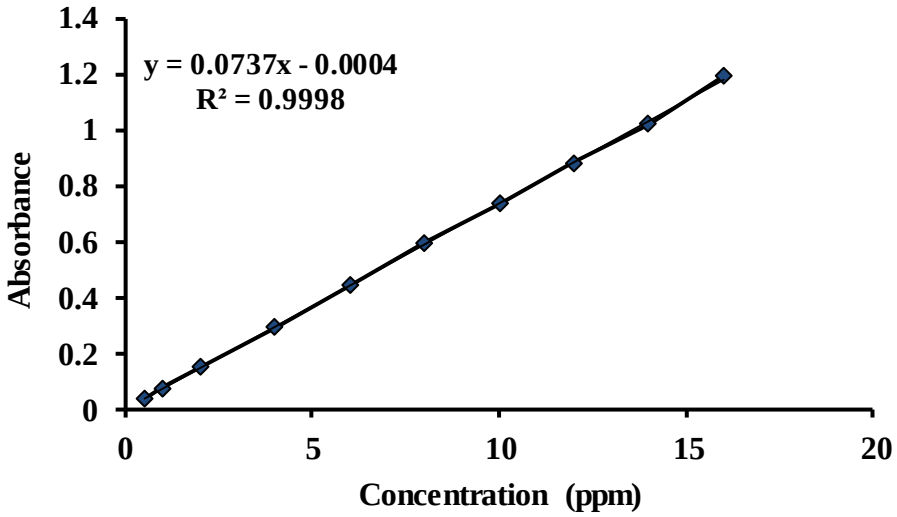

Figure 3.23. Calibration curve of the reaction of taurine with OPA-Na_2SO_3 by UV-Vis Spectrophotometer

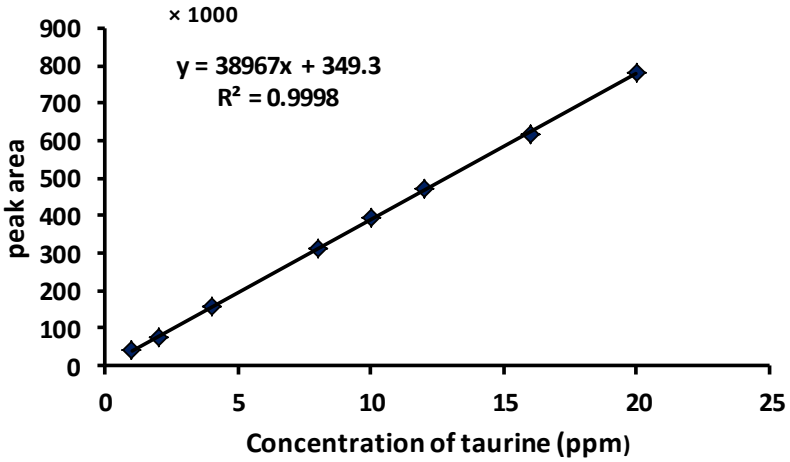

Figure 3.24. Calibration curve of the reaction of taurine with OPA-Na_2SO_3 by HPLC-PDA

The limit of detection (LOD) and limit of quantification (LOQ) of these methods were calculated from the calibration curve data. They were determined according the following formula: LOD= 3.3× SDa/b, and LOQ= 10× SDa/b, where: SDa is the standard deviation of intercept, b is the slope

(Omer, Omar, Thiel, & Elbashir, 2018). LOD and LOQ were found to be 0.141, 0.109 mg L^{-1} and 0.423, 0.328 mg L^{-1} for UV-Vis spectrophotometer and HPLC-PDA analysis respectively, Tables (3.13).

3.3.3.2.Precision

The precision of these methods was estimated by intra-day repeatability and inter-day reproducibility. The intra-day repeatability was evaluated by analyzing six replicates of taurine standard derivative (8.0 mg L^{-1}) over one day. The inter-day reproducibility was determined by analyzing twelve replicates of the taurine standard derivative (0.8 mg L^{-1}) over three days. The precision is presented as the percentage relative standard deviation (RSD%). The intra-day precision for UV-Vis spectrophotometer and HPLC-PDA were 1.278 % and 1.816%, respectively. The inter-day precision for UV-Vis spectrophotometer and HPLC-PDA were 2.236% and 2.858%, respectively, which indicate that the proposed methods were adequately precise Table (3.14). analysis respectively, which indicate that the proposed methods were adequately precise.

Table (3.13) Equations for external calibration curves for taurine derivative with OPA-Na_2SO_3 using UV-Vis Spectrophotometer and HPLC -PDA

Parameter	**UV-Vis Spectrophotometer**	**HPLC-PDA**
Concentration range (mg L^{-1})	0.5-15	0.5-20
Equation	Y= 0.073*x* - 0.0004	Y= 38967*x* + 349.3
Regression coefficient (r^2)	0.9996	0.9998
LOD (mg L^{-1})	0.141	0.109
LOQ (mg L^{-1})	0.423	0.328

Table (3.14) Intraday and interday precisions for the determination of taurine OPA-Na_2SO_3 derivative by UV-Vis spectrophotometer and HPLC-PDA

Parameter	**UV-Vis Spectrophotometer**	**HPLC-PDA**
Intraday precision (RSD%) (*n*=6)	1.278	1.816
Interday precision (RSD%) (*n*=12)	2.236	2.858

3.3.3.3 Accuracy (Recovery)

The accuracy of these methods was determined by recovery test. Aliquots of 1.0 mL of diluted energy drink samples containing taurine at concentration level of 20 mg L^{-1} were spiked with aliquots of 0.2, 0.5 and 1.0 mL taurine intermediate standard solution (50 mg L^{-1}). Then the derivatization for spiked samples was the same as described previously in section 2.6. The final concentrations of added amount of taurine standard were 2.0, 5.0 and 10 mg L^{-1}. Good percentage recoveries in range (93.3–105%) and (90.2-104%) were obtained for UV-Vis spectrophotometer Table (3.15) and HPLC-PDA analysis Table (3.16).

Table (3.15) Percentage recovery of taurine in energy drink samples by UV-Vis Spectrophotometer

Sample	UV-Vis spectrophotometer			
	Sample content (mg L^{-1})	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery% ± SD (*n*=3)
Tornado	4.0	2	5.972	98.6± 0.02
	4.0	5	9.072	101 ± 0.01
	4.0	10	13.94	99.4 ± 0.02
Krating-daeng	4.0	2	6.099	105 ± 0.01
	4.0	5	9.116	102 ± 0.01
	4.0	10	13.99	99.9 ± 0.06
Bison	4.0	2	6.130	106 ± 0.05
	4.0	5	8.970	99.4 ± 0.12
	4.0	10	13.33	93.3 ± 0.14
Tiger	4.0	2	5.994	99.7 ± 0.11
	4.0	5	9.143	103 ± 0.08
	4.0	10	13.84	98.4 ± 0.11
Red Bull	4.0	2	5.950	97.5 ± 0.08
	4.0	5	8.850	97.0± 0.16
	4.0	10	13.43	94.3± 0.12

(*n* = 3)

Table (3.16) Percentage recovery of taurine in energy drink samples by HPLC-PDA

Sample	HPLC-PDA			
	Sample content ($\mu g\ L^{-1}$)	Added amount ($\mu g\ L^{-1}$)	Found ($\mu g\ L^{-1}$)	Recovery% ± SD (*n*=3)
Tornado	4.0	2.0	5.760	96.0 ± 0.05
	4.0	5.0	8.527	90.5 ± 0.01
	4.0	10.0	13.621	96.2 ± 0.16
Krating-daeng	4.0	2.0	5.840	92.0 ± 0.03
	4.0	5.0	8.783	95.6 ± 0.09
	4.0	10.0	13.882	98.2 ± 0.12
Bison	4.0	2.0	5.804	90.2 ± 0.07
	4.0	5.0	9.197	104 ± 0.10
	4.0	10.0	13.525	96.6 ± 0.18
Tiger	4.0	2.0	5.841	95.2± 0.04
	4.0	5.0	8.866	97.3 ± 0.07
	4.0	10.0	13.932	99.3 ± 0.17
Red Bull	4.0	2.0	6.086	104 ± 0.06
	4.0	5.0	8.976	99.5 ± 0.09
	4.0	10.0	13.415	94.1 ± 0.16

(*n* = 3)

3.3.4. Applications of the methods of derivatization of taurine with OPA-Na_2SO_3

The proposed UV-Vis spectrophotometer and HPLC-PDA methods were applied to determine taurine content in some energy drink samples available in local markets namely (Red bull, Tornado, Kratingdeang, Bison and Tiger). Figure (3.26) (A-B) shows the chromatograms of taurine standard derivative (8.0 mg L^{-1}) and taurine derivative in Tiger energy drink sample taurine standard derivative (8.0 mg L^{-1}) and drivatized tiger energy drink sample (4.0 mg L^{-1}) spiked with taurine at concentration of 2.0 mg L^{-1} determined by HPLC-PDA. As seen in Figure (3.25), there no interference peak from other ingredients was observed, which could be attributed to the selectivity of the method. As indicated in Table (3.17), the obtained taurine concentrations of analyzed energy drinks were very close to the concentration values in the labels. The percentage was (94.75 – 102%) and (95.15-100.8%) that indicate

the high accuracy of the two proposed methods UV-Vis spectrophotometer and HPLC-PDA respectively, for the determination of the studied analyte.

Table (3.17) Comparison between measured taurine contents in this study and labeled amount of taurine in energy drink samples

Sample	**Labeled amount in (mg L^{-1})**	**HPLC-PDA**		**UV-Vis Spectrophotometer**	
		Conc. mg L^{-1} ± SD[a]	Recovery (%)	Conc. mg L^{-1} ± SD	Recovery (%)
Tornado	100	97 ± 0.03	97.0	102.9 ± 0.006	103
Kratingdaeng	4000	4035 ± 0.02	100.8	4022 ± 0.18	100.5
Bison	4000	3951 ± 0.04	98.7	3638 ± 0.07	102.1
Tiger	3200	3132 ± 0.02	97.8	3165 ± 0.11	98.9
Red Bull	4000	4207 ± 0.08	105	3908 ± 0.04	97.7

(*n*=2)

3.3.5. Comparison of present work with other reported studies

The sensitivity and chromatographic separation conditions for the current modified methods were compared with the previous published studies for determination of taurine after derivatization with OPA reagent. As indicated in Table (3.18) most of used techniques for taurine determination are based on HPLC with fluorescence detection (Liang et al., 2016, Monge & Fornaguera, 2009, Maldonado & Maeyama, 2013) which is not available in many laboratories. In present work used spectrophotometer and HPLC-PDA for detection taurine-OPA-sulfite derivative. For HPLC separation, simpler mobile phase has been used (0.1% trichloroacetic acid and acetonitrile) . In this study, simple mobile phase i.e (0.1% trichloroacetic acid and acetonitrile) has been used for separation of taurine-OPA-Na_2SO_3 derivative on HPLC system. Most of the other HPLC methods for taurine determination after derivatization used buffers such as a constituent of mobile phase (Ghandforoush et al., 2009, Kanjana , 2017, Ferreira et al., 1997, Mehdinia et al., 2017).

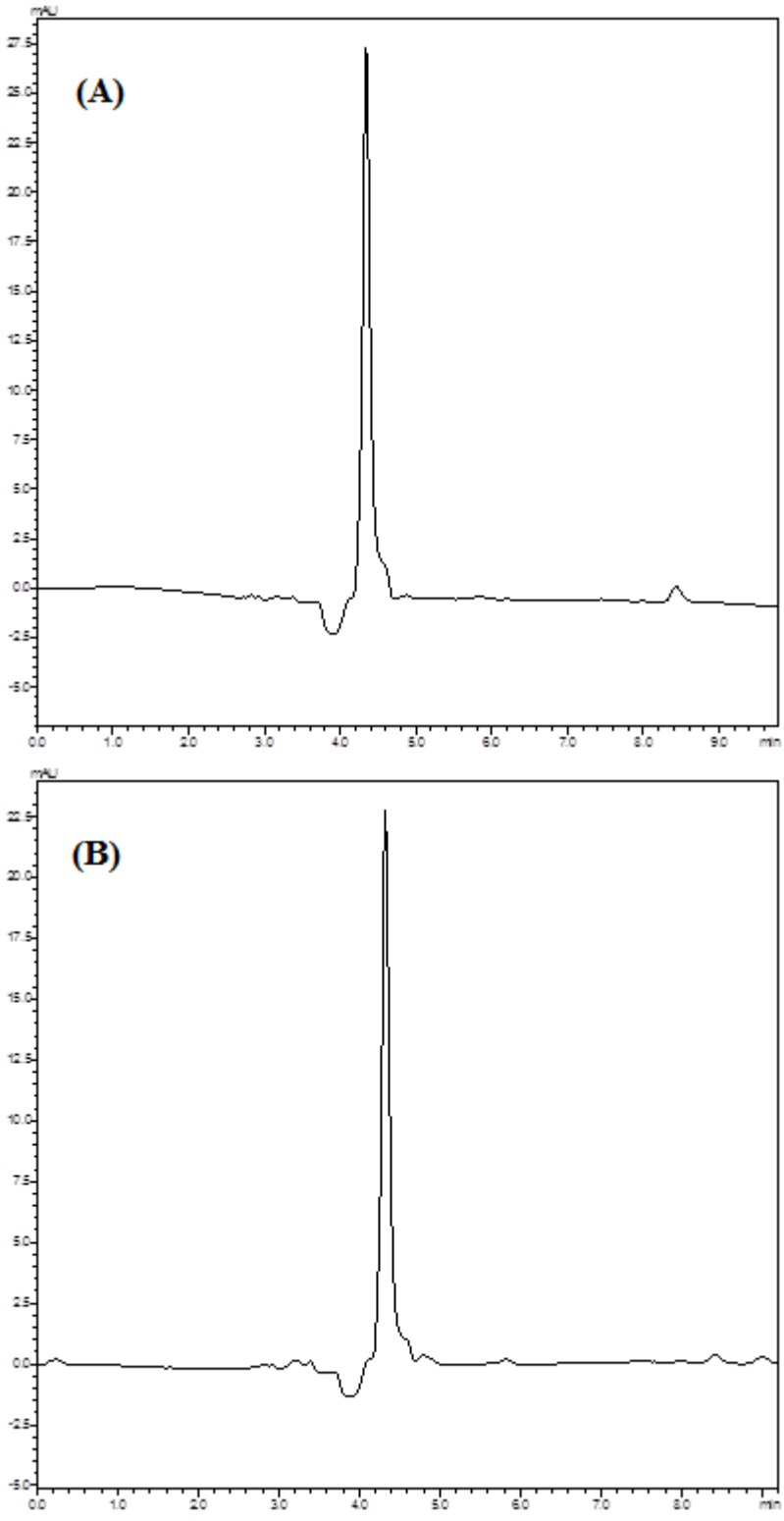

Figure 3.25. HPLC-PDA chromatograms of taurine derivative (A) taurine std. derivative (8.0 mg L^{-1}) (B) taurine derivative in Tiger sample(6.0 mg L^{-1}

The relatively higher LODs obtained by these methods is due to the use of UV-Vis and PDA as a detection techniques while the other methods used the fluorescence detector. Because of the high concentrations of taurine in energy drink samples, the LODs of the introduced methods were satisfactory for the energy drink samples.

Table (3.18). Comparison of conditions, LODs and LOQs of present study with those from literature.

Analytical method	**Mobile phase**	**Column**	**Derivtization agent**	**Detection method**	**LOD & LOQ (mg L^{-1})**	**Ref**
UV-Vis spectrophotometer	-	-	Na_2SO_3	UV-Vis	LOD: 0.141 LOQ: 0.423	Present study
HPLC-PDA	Acetonitrile : 0.1% trichloroacetic acid (30:70)%	Inertsil ODS-3 (250 × 4.6 mm i.d., 5 μm)	Na_2SO_3	PDA	LOD: 0.109 LOQ: 0.328	Present study
HPLC-FLD	Methanol: Acetonitrile: phosphate buffer 0.02M, pH 7.5) (8.0:17.5:74.5)%	Agilent C18 (150× 4.6 mm , 5 μm)	3-mercaptopropionic acid	Fluorescence	LOD: 0.03	Mehdinia et al., 2017
HPLC-FLD	Phosphate buffer (0.02 M, pH 4.8): Acetonitrile (65:35) %	Fortis (250 × 4.6 mm, 5 μm)	2-mercaptoethanol	Fluorescence	LOD: 0.001 LOQ: 0.005	Kanjanaklon gnganchu et al., 2016
HPLC-FLD	Disodium hydrogen phosphate (0.0125 M, pH = 7.2) : Acetonitrile (94:6) %	Genesis C18 (150 mm, 4 μ)	3-mercaptopropionic acid	Fluorescence	LOD: 0.4 pg mL^{-1} LOQ : 1.2	Ghandforous h-Sattari et al., 2008
HPLC-UV/Vis	Phosphate buffer (0.05M, pH: 5.3):Methanol (60:40)%	C18 (S10 ODS_2)	2-mercaptoethanol	UV-VIS	LOD: 0.3	Ferreira et al., 1997

4. Conclusion

In this work, two analytical methods have been developed for Determination of 4-MEI in soft drinks. These methods are based on derivatization of 4-MEI with NBD-Cl and then determined with UV-Vis Spectrophotometer and HPLC-PDA. The parameters that affecting on derivatization have been optimized. The developed methods have been validated throughout linear range, LOD, LOQ, precision (repeatability) (RSD%) and accuracy (recovery). The methods provide good performance. Then the developed methods have been applied for determination of 4-MEI in soft drinks samples collected from local markets i.e (Pepsi, Pepsi diet, Coca cola, Cola light, Veno and Vimto). The methods are suitable for routine quality monitoring as well as for research applications.

Also in this study, analytical methods have been validated for taurine determination in energy drinks. Firstly, analytical methods based on pre column derivatization with NBD-Cl and analysis by HPLC-PDA and HPLC-FLD have been validated. Secondly, analytical methods based on pre column derivatization with OPA-Na_2SO_3 and analysis by UV-Vis spectrophotometer and HPLC-PDA have been optimized and validated. In this study we found that using NBD-Cl and OPA-Na_2SO_3 as derivatization agents are compatible with taurine analysis in energy drink samples. The proposed methods show good analytical figures of merits and have been applied successively for determination of taurine in some energy drinks available in local markets i.e. Red bull, Tiger, Bison, Kratingdaeng and Tornado. The obtained concentrations of taurine measured these techniques are comparable and do not significantly differ for the taurine amount written in Label of the energy drinks by manufactures.

5. Future work

- ❖ Conformation the obtained results for 4-MEI in soft drinks by HPLC-MS
- ❖ Determination of other caramelization by-products in soft drinks such as 5-(1,2,3,4-tetrahydroxybutyl)- imidazole (THI).
- ❖ Development of simple analytical methods for routine determination of additives in carbonated drinks.
- ❖ Investigate the effect of storage conditions on the concentration of 4-MEI and taurine in carbonated drinks.

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