DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHIC METHOD FOR DETERMINATION OF MENTHOL IN PRESENCE OF OTHER ACTIVE INGREDIENTS IN COLD-COUGH SYRUPS

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DECLARATION

This thesis is my original work and has not been presented elsewhere for examination.

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DEDICATION

This thesis is dedicated to my late grandmother Febe Mugwang'a.

To my wife Lucy Lasoi, my son Willy Lemaiyan and my daughter Ella Alusa for their motivation and understanding especially during my absence for a prolonged period of time.

To all people who have contributed to humanity through growth of knowledge. We are able to do so much today because you showed us the way.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

µg/mol	Micrograms per mole
µm	Micrometre
AMB	Ambroxol
APIs	Active Pharmaceutical Ingredients
BP	British Pharmacopoeia
BRO	Bromhexine
CAM	Camphor
CHF	Chloroform
CHL	Chlorpheniramine
cm	Centimetre
CNS	Central Nervous System
CV	Coefficient of Variation
DARU	Drug Analysis and Research Unit
FID	Flame Ionization Detector
FTIR	Fourier Transform Infra-Red
g/mol	Grams per mole
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GMP	Good Manufacturing Practices
GUA	Guaifenesin
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization

IR	Infra-Red
IUPAC	International Union of Pure and Applied Chemistry
Kes	Kenya shillings
kg	kilogram
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Metre
MEN	Menthol
MET	Methanol
mg	Milligram
mg/L	Milligram per Litre
mL/min	Millilitre per minute
mm	Millimeter
MS	Mass spectrometry
nm	Nanometre
PDMS	Polydimethylsiloxane
PMS	Post market surveillance
PPB	Pharmacy and Poisons Board
RF	Response factor
RP	Reversed Phase
RSD	Relative Standard Deviation
S/N	Signal to Noise ratio

SAL	Salbutamol
URT	Upper Respiratory Tract
URTIs	Upper Respiratory Tract Infections
USA	United States of America
USP	United States Pharmacopeia
UV	Ultra Violet
WHO	World Health Organization

ABSTRACT

Introduction

The common cold is the most common infection of the upper respiratory tract. Management of the common cold is directed at mitigating clinical symptoms and cold-cough syrups are often prescribed. Menthol (MEN) is one of the common constituents of these syrups. Quality checks on cold-cough syrups normally target the major active pharmaceutical ingredients (APIs) without regard to MEN content in these cold-cough syrups. The quality of these medications is not given the same scrutiny as that directed towards other drugs.

Several methods have been developed, validated and are useful for analysis of cold-cough syrups in the Kenyan market but none of these methods is useful in the determination of MEN since they are based on high performance liquid chromatography (HPLC) with ultra violet (UV) detector.

Objectives

The main objective of the study was to develop and validate a gas chromatography (GC) method for assay of total MEN in cold-cough syrups in presence of other APIs. This method was then used in conjunction with another validated method to determine MEN and other APIs in commercially available cold-cough syrups in Nairobi county.

Methods

During method development, parameters such as type of column, detector and carrier gas were fixed from the onset. The column used was a ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol. Nitrogen was used as carrier gas as

well as make up gas and detection was done using a flame ionization detector. Preliminary analysis was run using values adopted from *Ravi et al*, 2016 as the starting point. Method development involved investigating the effect of varying column temperature, solvent type and carrier gas velocity on separation in order to obtain optimum conditions.

Validation of the developed method was based on International Conference on Harmonization (ICH) guidelines. The method was validated with respect to accuracy, specificity, precision, sensitivity, linearity of detector response, robustness and range of analyte concentration.

The validated method was used in conjunction with a previously developed and validated high performance liquid chromatography (HPLC) method for determination of menthol as well as other APIs under study.

Results and discussion

In this study, a simple, rapid, robust, accurate and reliable GC method was developed and validated for the determination of MEN in cold-cough syrups that may also contain ambroxol (AMB), chlorpheniramine (CHL), guaifenesin (GUA), bromhexine (BRO) and salbutamol (SAL).

From method development, optimized chromatographic conditions obtained were: A ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol held under temperature program as 110 ℃ (2 min), ramp 10 ℃/min to 190 ℃ (2 min). Injector port temperature was maintained at 240 ℃ with injection volume of 1.0 µl split in the ratio of 50:1. Carrier gas was nitrogen at 1.0mL/min which also served as make up gas (30 mL/min) in the flame ionization detector (260 ℃). Other detector gases were hydrogen (30 mL/

min) and industrial air (300 mL/ min) and the diluent for samples and standards was HPLC grade chloroform.

The accuracy of the method was tested based on recovery studies with 97.56 to 102.97 % recovery reported. The method was demonstrated to be precise through repeatability studies with a coefficient of variation (CV) of 0.55 as well as intermediate precision (of CV 0.32). The method was linear over a range of 0.042 to 0.169 mg/mL corresponding to 40 to 160 % of the working concentration with a coefficient of determination (R^2) of 0.9986. The limit of detection and limit of quantitation for menthol were 0.0063 mg/mL and 0.019 mg/mL, respectively. The method was robust with regard to small changes in column temperature, injector port temperature and carrier gas velocity.

From community pharmacies in Nairobi county, a total of 21 cold-cough syrups were sampled. In the determination of MEN content, 10 samples (47.6 %) complied with assay specifications of 90.0 to 110.0 % of the label claim for finished products according to the 2016 United States Pharmacopeia (USP). All samples that did not comply with these specifications recorded lower assay values. For completeness of analysis, other APIs of interest were assayed. From this assay, 20 samples (95.2 %) complied with assay specifications. In one sample, SAL and MEN content did not comply with USP 2016 assay specifications. Therefore, overall, 47.6 % of samples complied with USP 2016 assay specifications for all APIs with the highest failure rate attributable to MEN.

Conclusion and recommendation

A gas chromatographic method was developed for the determination of menthol in cold-cough syrups in Kenya. This method was used together with a validated HPLC method to assay cold-cough syrups that may also contain AMB, BRO, CHL, GUA and SAL.

Evaluation of menthol content in cold-cough syrups using the developed and validated method is crucial so as to ensure that only quality products are in the market for optimal therapeutic outcomes. The validated method can be used in assay of menthol as one of the APIs during pre-registration analysis as well as post market surveillance to curb substandard and counterfeit cold-cough syrups containing menthol.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Common cold and cough

The common cold is the most prevalent infectious disease in many parts of the world [1, 2] and it presents as a diverse group of conditions that are classified based on the affected region of the upper respiratory tract (URT). Conditions such as pharyngitis, rhinitis, nasopharyngitis, tonsilitis account for the largest proportion of upper respiratory tract infections (URTIs) that are generally referred to as common cold and are associated with cough [3, 4]. Although the term URTIs is generally used in clinical practice, it is misleading because it incorrectly suggests absence of symptoms from the lower respiratory tract [5].

1.1.1 Etiology, transmission and clinical course

The common cold is mainly caused by viral agents [6] with rhinovirus being the most common cause and accounting for half of the cases [7] with other causes being coronavirus, adenovirus, respiratory syncytial virus, influenza virus, parainfluenza virus, coxackie virus, echo virus, and bocavirus [6]. Bacterial agents are rarely responsible for initial infection but may lead to complication of the primary infection [7].

Transmission of common cold occurs via direct or indirect means with the most common means being droplet inhalation from infected persons and direct contact from hands to the nose or eyes [5]. Following infection, there is an incubation period of two to three days followed by symptoms of nasal congestion, throat irritation, sneezing and headache [5]. These are the most common early signs and are followed by myalgia, anorexia and rhinorrhea that is initially watery

and profuse but becomes purulent over time [5]. The severity and duration of symptoms vary with causative agent and age of patient. The common cold is an acute self-limiting infection that lasts 7-10 days and rarely presents serious sequelae [2, 5].

1.1.2 Epidemiology and impact

The common cold is more prevalent in children and prevalence rates decrease with age [6]. In the United States of America (USA), research indicates that an adult is infected with two to three episodes of the common cold annually [4]. A high frequency of episodes is observed in stressed patients as well as those that have inadequate sleep. In Uganda, the prevalence rate is estimated to be 37 % in children aged below two years [5]. In Kenya, there is no available data on overall prevalence rates. In a cohort study conducted in Kilifi in 2002 among children below five months, 40 % of the cohort was found to suffer from URTI caused by respiratory syncytial virus [8]. Therefore, the overall prevalence rate could be higher. By being highly contagious, URTIs transmission among children is particularly higher due to poor hygiene and overcrowding in schools [4].

Although the common cold is not fatal, it is debilitating and lowers productivity. The impact of the common cold is evaluated in terms of cost of medical care, number of hospital visits and days lost from work or school. In Canada, it was estimated that 40 % of days lost from work are due to the common cold while in the USA, the economic impact due to medical care and loss of productive days was estimated to be $ 42 billion annually in 1997 [9, 10].

1.1.3 Complications of common cold

In susceptible groups such as infants and people with immunosuppressive conditions, secondary infections may arise from the inflammatory response in primary infection [11]. In sinusitis,

inflammation of paranasal sinuses leads to fluid accumulation which encourages proliferation of normal flora. Sinusitis presents with cough, purulent rhinorrhea lasting more than 7 days, high fever and facial pain [9].

Inflammation may also be localized in the mucous membranes of the throat and cause bacterial pharyngitis. Clinical presentation of pharyngitis may involve nasal discharge in which case it is termed as nasopharyngitis. Complications of streptococcal pharyngitis can also occur in tissues and organs distant from site of primary infection. Children infected with *Streptococcus pyogenes* can develop rheumatic heart disease and acute glomerulonephritis [2].

Otitis media is another common complication in children and it has been reported to cause loss of hearing [7, 12]. The common cold may also trigger an asthmatic attack in susceptible individuals or exacerbate an existing disease. It is estimated that half of exacerbations of asthma in children are due to the common cold [5].

1.1.4 Diagnosis, treatment and management of common cold

Diagnosis of common cold is made on clinical presentation because laboratory testing is either unavailable or not practical in most settings [5]. These symptoms are not unique to common cold and will be present in other conditions that affect the respiratory tract therefore other respiratory conditions need to be investigated as well [5]. Definitive diagnosis can be made using polymerase chain reaction for influenza, parainfluenza and respiratory syncytial virus although clinical utility of this test is limited by cost [13].

Treatment of common cold is directed at minimizing severity of symptoms and therefore treatment with a single remedy may not be effective [5, 7, 14]. Analgesics and antipyretics are used to relieve headache and fever while nasal congestion is relieved by antihistamines and

topical drugs such as oxymetazoline [14]. Expectorants are used when cough is accompanied by production of sputum while antitussives are used to suppress dry cough [15]. Essential oils such as eucalyptus oil are used to improve breathing due to their clearing effect on nasal sinuses [16]. Menthol is used clinically as an antitussive to suppress dry irritating cough as well as symptomatic relief of nasal congestion, impaired breathing and irritation of the airways [17-19].

1.2 Components of cold-cough medicines

Management of the common cold and the associated cough utilizes drugs from different classes. Although there are products containing single ingredients, most cold-cough medicines are marketed as combinations of multiple active pharmaceutical ingredients (APIs) in different proportions. The APIs incorporated in cold-cough products are classified pharmacologically as decongestants, antitussives, analgesics, antipyretics, essential oils, antihistamines, expectorants or mucolytics [20].

1.2.1 Decongestants

Classic decongestants are α-adrenoceptor agonists and act by causing vasoconstriction to reduce inflammation and edema of nasal mucosa. Cold-cough syrups containing ephedrine and pseudoephedrine act systemically while those containing oxymetazoline (Figure 1.1) are topical decongestants.

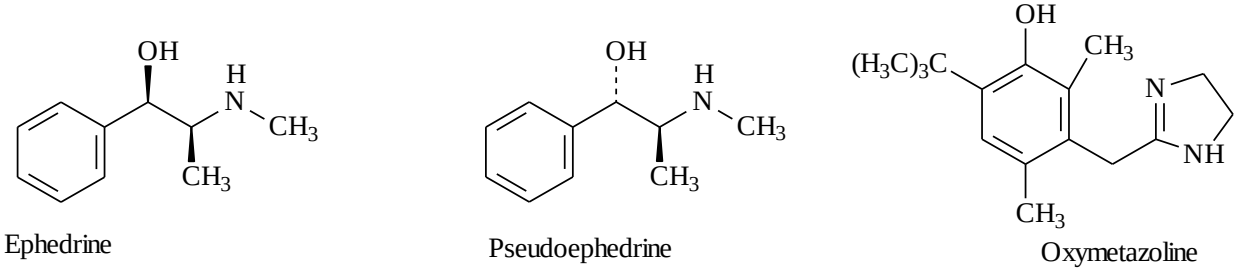
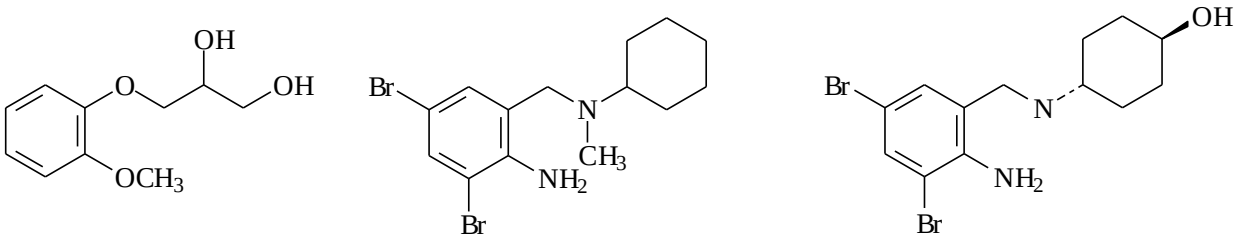


Figure 1.1: Chemical structures of selected decongestants

1.2.2 Expectorants and mucolytics

Expectorants are used in cough syrups to promote motility of cilia which helps to expel mucus in productive coughs. Mucolytics act by changing the structure of mucus such as breaking disulfide bonds to reduce viscosity of mucus and thus promote expectoration. Guaifenesin and ammonium chloride are common expectorants in cough syrups while bromhexine, sodium citrate and ambroxol are commonly used mucolytics (Figure 1.2).

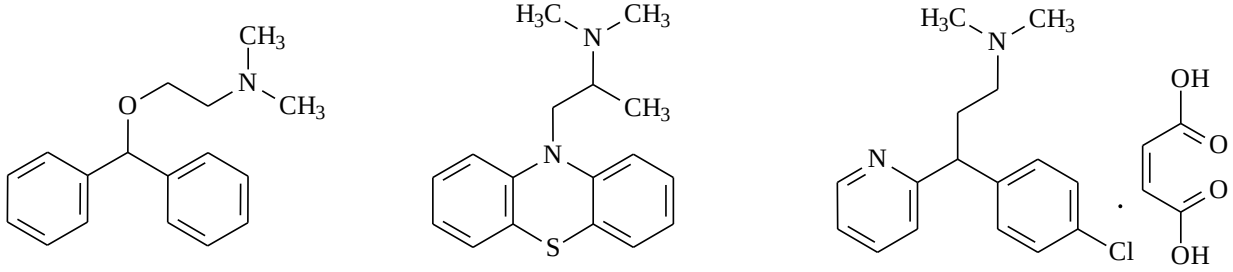


Guaifenesin Bromhexine Ambroxol

Figure 1.2: Chemical structures of selected expectorant and mucolytics

1.2.3 Antihistamines

Antihistamines used in cough preparations are drugs that act by antagonizing the activity of histamine at histamine 1 (H_1) receptors [22]. Activity of histamine on these receptors stimulates contraction of smooth muscles in the gut, uterus and bronchi. Histamine also causes relaxation of smooth muscles in capillaries which causes increased capillary permeability and edema [22- 24]. This can cause swelling of nasal passages and result in nasal congestion. Antihistamines are incorporated in cold-cough syrups due to their pharmacological activity of drying up mucosa and reducing secretions [25]. Antihistamines found in cold-cough syrups belong to diverse chemical groups some of which are related as shown in Figure 1.3.

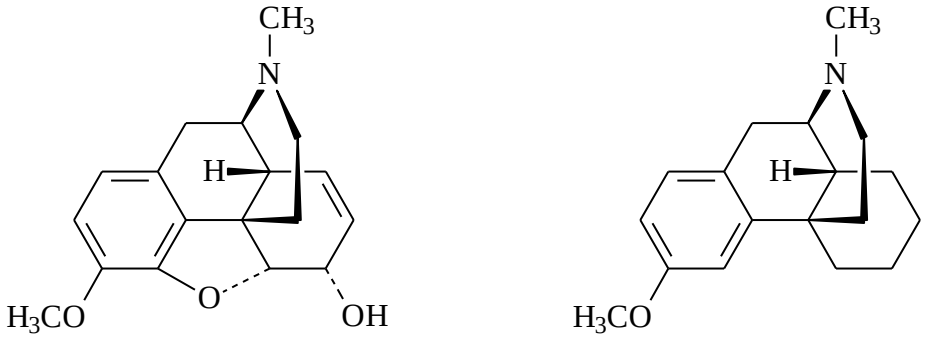


Diphenhydramine Promethazine Chorpheniramine maleate

Figure 1.3: Chemical structures of antihistamines common in cold-cough products

1.2.4 Antitussives

Antitussives are compounds that suppress cough by inhibiting the cough reflex in the central nervous system or by desensitizing receptors in the respiratory tract [26]. The most common centrally acting cough suppressants are opiates and their congeners (Figure 1.4).



Codeine Dextromethorphan

Figure 1.4: Chemical structures of selected antitussives

1.3 Menthol in cough-cold products

1.3.1 Pharmacology

In addition to the above discussed APIs, menthol is a common ingredient in a wide range of cold-cough medicines [27]. Biological activity due to menthol is attributed to stimulation of cold receptors and trigeminal afferents in the URT [18]. Besides antitussive and mucolytic properties,

menthol provides a cooling sensation in the nasal mucosa and increases breath-holding time which could promote deeper inhalation [18].

Despite the desirable pharmacological properties, menthol can induce adverse reactions such as dyspnoea, URT irritation, chest tightness and respiratory failure especially in children [18]. When incorporated in multi-component cold-cough syrups, menthol poses a high risk to children because children have a higher prevalence of URTIs than adults [12]. The lethal adult dose of menthol is estimated to be about 2g/day [28]. The World Health Organization (WHO) recommends a maximum daily intake of 0.2mg/kg body weight for menthol and toxicity is likely to occur at daily doses above 2mg/kg body weight [29].

1.3.2 Chemistry of menthol

Menthol is a cyclic monoterpene alcohol obtained either naturally or synthetically from various precursors. The racemic product is a mixture of equal parts of R and S enantiomers of cyclohexanol [5-methyl-2-(1-methylethyl)] with a molecular formula $C_{10}H_{20}O$ [30, 31].

Menthol crystals appear as white solids or shiny prisms with a peppermint odor and taste. It has a molecular weight of 156.3, melting point range of 41-44 ℃ and boiling point of 214.6 ℃. Menthol is highly lipid soluble, insoluble in water and very soluble in ethanol, methanol, chloroform and petroleum ether [30].

Menthol has three chiral centers, therefore has four pairs of enantiomers; (+) and (-) forms menthol, neomenthol, isomenthol and neoisomenthol as shown in Figure 1.5 [31, 32]. The (-) -form menthol is the most widely occurring natural isomer with a *1R, 3R, 4S* configuration which is the one generally referred to as menthol [33, 34].

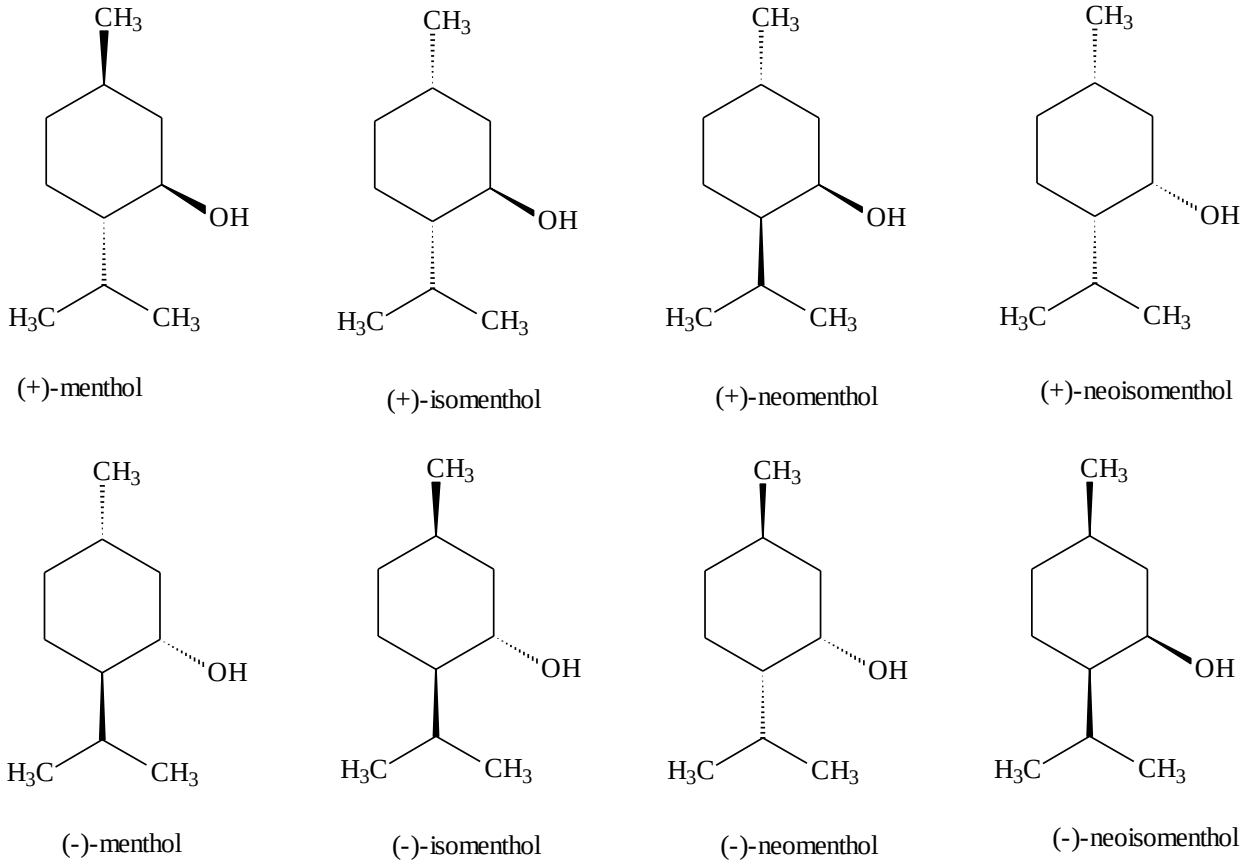
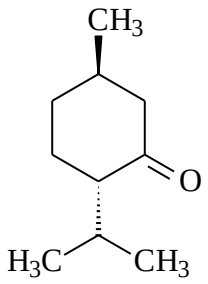


Figure 1.5: Chemical structures of the various stereoisomers of menthol

Like other saturated alcohols, menthol can undergo oxidation to produce menthone (Figure 1.6) which can occur as an impurity. The menthol enantiomeric mixture is identified by its specific optical rotation of -0.2° to +0.2° in 96 % ethanol. Thin layer chromatography can also be used for identification by comparing the retardation factor of the test solution with that of the menthol chemical reference substance [35].



l-menthone

Figure 1.6: Chemical structure of menthone

1.3.3 Production of menthol

Menthol is mainly obtained from natural plant sources such as from *Mentha piperita* and *Mentha arvensis* by steam distillation followed by crystallization. Biosynthesis of menthol as described by Croteau *et al 2005* shows the natural precursor as geranyl diphosphate [36]. In this biosynthesis, (-) - menthol (major constituent), (+)-isomenthol, (+)-neomenthol, (+)-neoisomenthol, menthone, menthyl acetate and cineole are produced [36, 37].

Various other pathways have been described for synthetic production of (-) - menthol. They include synthesis from (-)-β-pinene, δ-3-carene, thymol, (-)-piperitone, β-phellandrene, (+)-limonene, (+)-pulegone or (+)-citronellal as precursors [31, 38, 39].

The Japanese company Takasago utilizes β-pinene as the precursor which undergoes pyrolysis to form myrcene [31, 36, 38]. Diethylamine is then added in a base catalyzed reaction to form *N, N*-diethylgeranylamine. Isomerization of *N, N*-diethylgeranylamine produces an enamine of citronellal which then undergoes hydrolysis to form citronellal. Cyclization of citronellal

produces l-isopulegol which is hydrogenated to l-menthol. Highest biological activity is obtained with l-menthol. A simplified illustration of this synthesis is shown in Figure 1.7.

From the synthetic pathway shown in Figure 1.7, l-isopulegol and citronellal are the likely contaminants in synthetic menthol. Since the pathway is linear, starting materials are unlikely to cause contamination in the final product [40].

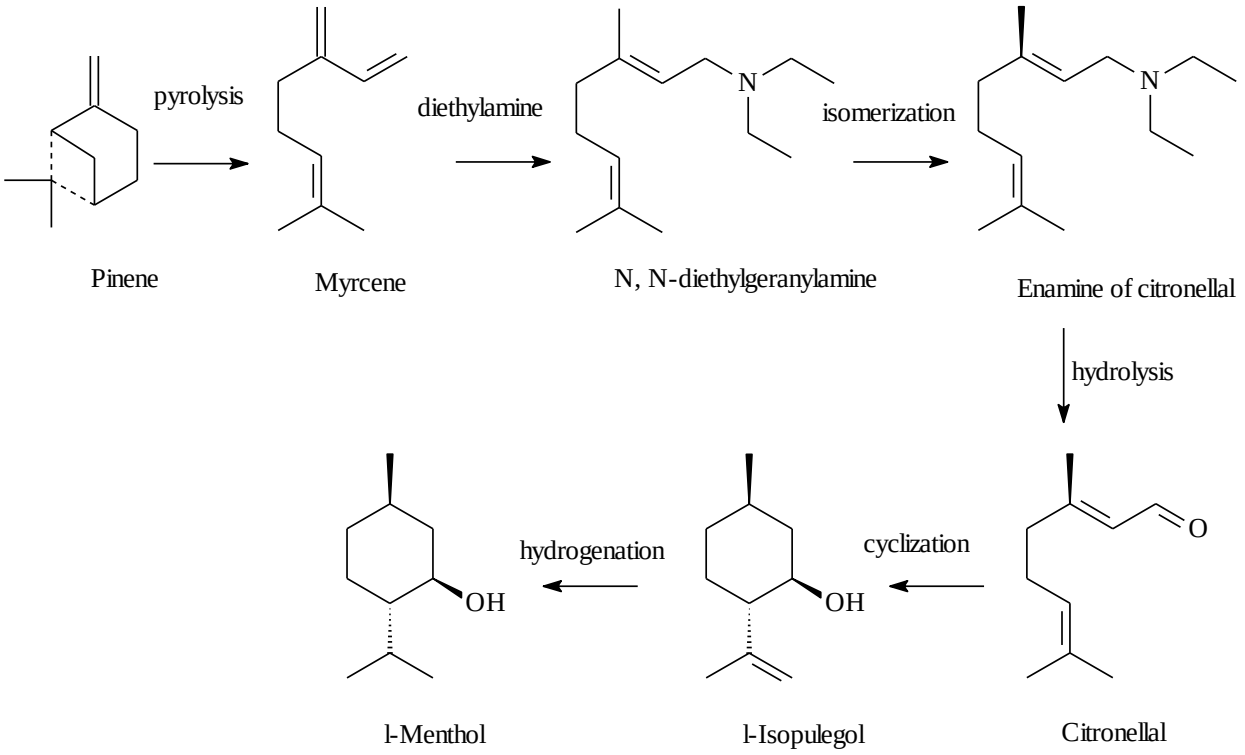


Figure 1.7: An example of a synthetic scheme of menthol

1.4 Choice of methods for quality control of menthol in cold-cough syrups

Typical multi-component cold-cough syrups in the market contain a combination of all or any of the following: 1 mg menthol (MEN), 2 mg chlorpheniramine (CHL), 100 mg guaifenesin (GUA), 2 mg salbutamol (SAL), 50 mg ambroxol (AMB) and 4 mg bromhexine (BRO) in every 5 mL [41]. Laboratory testing of such syrups is normally complicated by multiple additives other

than APIs. Such excipients include sweeteners, flavors, stabilizers, viscosity enhancers and coloring agents. Consequently, laboratory testing of cold-cough syrups requires a technique that separates the different compounds so as to enable the identification and quantitation.

Chromatographic methods possess such inherent separation capabilities and are suitable for analysis of compounds in mixtures. Since MEN is volatile, gas chromatography (GC) with a flame ionization detector (FID) was chosen for the analysis of menthol content in cold-cough syrups.

1.5 Assay methods for cold-cough syrups

1.5.1 Assay of non-volatile components

Several liquid chromatographic methods have been published for the determination of APIs in cold-cough syrups. Reverse phase high performance liquid chromatography (RP-HPLC) for the simultaneous analysis of paracetamol, phenylephrine and chlorpheniramine conducted on a waters Bondapak CN RP® column with UV detection at 265 nm has been reported. The method reported acceptable linearity with a correlation coefficient 0.9999 for all APIs under study [42].

There is reported another RP-HPLC method for simultaneous determination of dextromethorphan, guaifenesin and benzoate in cough syrups with a photodiode array detector where forced degradation of samples was studied to evaluate the suitability of the method for stability studies [43]. There is also a RP-HPLC method for simultaneous analysis of salbutamol/terbutaline, triprolidine, pseudoephedrine, guaifenesin, ambroxol, chlorpheniramine maleate and bromhexine. Separation was aided by an ion pairing reagent after ionization at pH 3.0 on a Phenomenex Gemini® column and UV detection at 254 nm [44].

Similarly, an electrophoretic method for simultaneous analysis of guaifenesin, salbutamol and dextromethorphan on a fused silica capillary column (43.5cm × 50µm) with ultra violet (UV) detection at 210 nm has been reported [45].

Gas chromatography has been used in the simultaneous determination of bromhexine, chlorpheniramine, codeine, dextromethorphan, diphenhydramine, ephedrine/pseudoephedrine, guaifenesin and papaverine in cold-cough syrups. The analysis was done on glass column (5 ft. × 2 mm) packed with 3 % OV-25. Other chromatographic conditions were temperature programming from 170 ℃ to 260 ℃ at 10 ℃/min and nitrogen as carrier gas at 30 mL/min [46].

In addition to the above GC method, there is also a documented gas chromatographic method with mass spectrometry detection (GC-MS) for simultaneous analysis of guaifenesin, salbutamol and dextromethorphan in cough syrups. The method is based on a fused silica capillary column (30 m × 0.25 mm; 0.25 µm) coated with 5 % phenyl-95 % polydimethylsiloxane. In the method, sample preparation was done by liquid-liquid extraction using chloroform [45]. None of these methods was applicable in separation or quantitation of MEN in the products.

1.5.2 Assay methods for menthol in pharmaceuticals

1.5.2.1 Introduction

Currently, there is no monograph in official compendia for analysis of menthol in cold-cough syrups using GC. However, various studies have been conducted in the determination of menthol in cold-cough syrups as well as other formulations.

1.5.2.2 Methods for cold-cough preparations

The United States Pharmacopeia (USP) contains a GC-FID method for assay of menthol in lozenges. In this method, analysis is conducted on a fused silica capillary column coated with polyethylene glycol (PEG) and helium as a carrier gas. Unlike other methods reported in journals, chromatographic separation is run with column oven under isothermal conditions with anethole in hexane as the internal standard. From this method, identification of menthol is done by comparing the retention time of menthol peak in the sample chromatogram with that in the standard chromatogram [30].

There are relatively few reported specific studies on menthol content in cold-cough syrups. In one study, a GC analysis was conducted on Agilent DB-1 column (30m×0.32mm × 1.0µm) using nitrogen as carrier gas at 1.0 mL/min with FID. Other chromatographic conditions defined in the method include temperature programming of 100 ℃ for 2 min then ramped to 240 ℃ (hold for 25 min) at 10 ℃/min, injector port and detector maintained at 240 ℃ and 260 ℃, respectively and nitrogen as make up gas. Assay results obtained were precise (% RSD =1.8) with accuracy level of 95 to 105 % [47].

Mokhtar *et al.* published a GC-MS method for analysis of menthol together with cinnamaldehyde, carvone, cineole, limonene and thymol in cough syrups found in Egypt. Analysis was conducted on a Rtx-5 MS 30m × 0.25mm: 0.25µm column [48].

Other methods reported in the literature for analysis of menthol content in cough syrups include a RP-HPLC with refractive index detector. In this method, separation was achieved on a Inertsil ODS-3V (4.6mm ×250mm ×5µm) column using a mobile phase comprising water and methanol (30:70). Sample pre-treatment was not necessary. Forced degradation studies were also

conducted and reported 2-5 % degradation. However, none of the degradation products interfered with elution of the menthol peak [49].

1.6 Justification for the study and research problem

The quality of cold-cough medication is important in attaining appropriate healthcare outcomes. Poor quality cold-cough syrups may result in prolonged clinical signs, adverse drug reactions and wastage of resources [50]. Evaluation of the quality of pharmaceutical products in Kenya is the mandate of the Pharmacy and Poisons Board (PPB) and pharmaceutical manufacturers [51]. Currently, more attention is directed on the quality assessment of vital and essential drugs such as antimicrobials, antihelmintics and anti-malarials as seen in post market survey reports [51, 52]. In the period 2006-2010, 35 % of drugs analyzed at drug analysis and research unit (DARU) were antimicrobials. Cold-cough syrups accounted for less than 1 % in which none was analyzed for menthol content [53]. This suggests that less emphasis is placed on cold-cough products despite their widespread use and associated risks which are compounded by their ease of availability as over the counter drugs [54]. In addition, off-label use and self-medication further compounds risks associated with cough syrups [54].

In 2009, safety concerns led to some Kenyan hospitals withdrawing use of cold-cough syrups in children. Nonetheless, there is no national policy that guides the use of these medicines in children in Kenya. In the USA, the Food and Drug Administration (FDA) provides that cold-cough medicines should not be used in children less than two years with some studies limiting their use to over four years [55, 56].

The quality control of cold-cough syrups containing menthol is faced with challenges such as few reliable, simple and precise methods for their analysis. There is need for a simple, rapid,

reliable and precise method for the determination menthol in presence of other APIs such as AMB, CHL, GUA, BRO and SAL as well as the excipients found in commonly used cold-cough syrups.

Since menthol is volatile, gas chromatography with FID is a suitable method that would greatly reduce the cost of analysis. In addition, acquisition of carrier gas, oxidant and fuel used in the FID as well as their disposal is inexpensive compared to organic solvents used in HPLC. The validated method can be useful in conducting pre-registration analysis and routine analysis as well as post market surveillance to curb substandard and counterfeit menthol containing cough syrups.

1.7 Research questions

I. Can a suitable GC method be developed and validated for determination of menthol in cold-cough syrups found in the Kenyan market?

II. Is the developed and validated method applicable in determination of menthol content of cold-cough syrups used in Kenya.

1.8 Objectives

1.8.1 General objective

The main objective of this study was to develop and validate a gas chromatographic method for assay of menthol in cold-cough syrups.

1.8.2 Specific objectives

i. To develop and validate a gas chromatography method for quantitative analysis of menthol contained in cold-cough syrups.

ii. To assay menthol in presence of other active pharmaceutical ingredients in commercially available cold-cough syrups in Nairobi County.

CHAPTER TWO

METHOD DEVELOPMENT

2.1 Introduction

The need to develop new methods for qualitative and quantitative analysis of drugs is influenced by the continuous release of new formulations by pharmaceutical manufacturers. There are formulations and dosage forms for which there may not be a method in official compendia for the analysis of their APIs or existing methods are deficient [57, 58]. The development of new analytical methods relies on data obtained from existing methods with the aim of overcoming their deficiencies and improving on them [59, 60].

High Performance Liquid Chromatography (HPLC) currently dominates quantitative analyses in pharmaceutical industry. However, GC finds application in analysis of volatile but thermally stable compounds. Gas Chromatography has the same quantitative accuracy and precision as HPLC especially when used with an internal standard and a much greater efficiency than HPLC when used with capillary columns [61, 62].

In the present study, a GC method with FID was developed and validated for the assay of total menthol in cold-cough syrups in presence of other APIs such as, CHL, GUA, AMB, SAL and BRO. These other APIs were analyzed using an existing validated HPLC method [44].

2.2 Reagents ,chemicals, solvents and gases

Analytical grade sodium hexanesulfonate and ammonium acetate (Loba Chemie PVT Mumbai, India) were used as ion pairing reagent and buffer, respectively during HPLC analysis.

Standard substances used in GC method development were menthol (Sigma-Aldrich, St Louis, USA) and Camphor (May &Baker, Dagenham, England). In HPLC analysis, the following working reference standards were used: Ambroxol, guaifenesin, bromhexine, salbutamol and chlorpheniramine maleate which were kind donations from stock of standards used at DARU.

HPLC grade methanol (Sigma-Aldrich, St Louis, USA), HPLC grade acetonitrile (Scharlau chemie, sentmenat, Spain) and HPLC grade chloroform (Sigma-Aldrich, St Louis, USA) were used as solvents. Glacial acetic acid, and ethanol (Loba chemie, Mumbai, India) were analytical grade. Purified water was prepared in the laboratory using Aquatron-A 4000 water still (Cole-Parmer, Staffordshire, United Kingdom) which utilizes distillation followed by filtration through a 25 µm polypropylene filter.

Nitrogen, helium (99.99 %), hydrogen (99.9 %) and industrial air were obtained from BOC Gases (Nairobi, Kenya).

2.3 Instrumentation

2.3.1 Gas chromatographic system

A Shimadzu Gas Chromatograph 2010 plus system (Shimadzu Corporation, Kyoto, Japan) with an AOC-20s auto sampler, AOC-20i auto injector fitted with a FID and MS was used for gas chromatographic study. This chromatographic system was run using a GC-Solution software version 2.4 for data computation.

An injection volume of 1.0 µL was chosen as a compromise to ensure adequate resolution while preventing column overload. To provide comparable peak heights, concentration of reference working solutions used during method development was 0.48 mg/mL (CAM) and 0.40mg/mL (MEN), respectively.

2.3.2 High performance liquid chromatographic system

A 20AD Shimadzu Prominence liquid chromatograph was used for analysis. The system was equipped with an SIL-20A HT prominence autosampler and an SPD-20A prominence UV/VIS detector. In addition, the system incorporated a DGU-20A 5R prominence degassing unit and a CTO-10AS column oven heating block. The HPLC system was linked to a LC Solution software 1.22 for data acquisition and analysis. Mobile phase was degassed using a WiseClean ultrasonic set, model WUC-D06H, Daihan Scientific Co. Ltd, Korea.

2.3.3 Polarimeter

An ADP 220 Polarimeter, model 36-200, 36-20E, 30-20S (Bellingham and Stanley ltd, United Kingdom) was used to measure optical rotation of CAM and MEN standards.

2.3.4 pH meter

A Jenway pH meter 3510 serial no.42630 (Cole-Parmer, Staffordshire, United Kingdom) calibrated using standard buffer solutions at pH 4.0 and 7.0 was used to measure pH of buffer solutions.

2.4 Characterization of working standards

Working standards used in GC method development were characterized for identity according to the USP 2016 specifications involving specific optical rotation and trituration [30].

2.4.1 Specific optical rotation

Specific optical rotation for MEN and CAM was measured as shown in Table 2.1.

Table 2.1: Specific optical rotation of menthol and camphor

Working standard	**Observed optical rotation (°)**	**Literature optical rotation (°)** [30]
Menthol	+ 0.1	-2 to +2
Camphor	+ 41.7	-47 to +47

2.4.2 Trituration

When menthol was triturated with an equal weight of camphor, the mixture liquefied as specified in the USP 2016.

2.5 GC method development

2.5.1 Introduction

During GC method development, several factors were considered of that included: nature of the sample, mode of injection, column (type, thickness of stationary phase, length, and internal diameter), oven conditions, detector and carrier gas [58]. Some of these parameters were fixed from the onset of method development. Fixed chromatographic parameters were: column, type of detector and carrier gas.

2.5.2 Column selection

A Phenomenex Zebron ZB-WAXplus (60m ×0.25mm ×0.25µm) capillary column bonded with 100 % polyethylene glycol was used in the study. Capillary columns are reported to provide higher efficiency and shorter run time compared to packed columns. Bonding of stationary phase slows bleeding and allows the column to be rinsed when contaminated [61].

The choice of polyethylene glycol stationary phase was based on the principle of "like dissolves like" where "like" refers to the polarities of the analyte and stationary phase [61]. In the present

study, the analyte contains an alcoholic group which confers a polar character. Polarity of stationary phase should match that of sample components in order to obtain reasonable retention time. Under this condition, the sequence of elution is therefore determined by the boiling point of the sample components [63, 64].

Thickness of the stationary phase has an influence on retention time and capacity of the column. Analysis of highly volatile compounds requires columns with thick films since they retain analytes for a longer time [65]. However, for most analyses on a column with 0.25mm internal diameter, a 0.25µm film thickness is recommended [61].

2.5.3 Carrier gas selection

Several gases such as helium, nitrogen and hydrogen can be used as a carrier gas in GC. The choice of a particular carrier gas depends on the safety, cost, and availability. From the Golay curve, the same efficiency can be achieved with each of these three gases [64]. The difference between them lies in the average linear velocity that provides minimum plate height. Although this velocity is lower for nitrogen compared to helium and hydrogen, nitrogen was chosen because it is readily available, inexpensive and does not carry the risk of explosion [66].

2.5.4 Detector

The FID is the most widely used and generally applicable detector in analysis of organic samples. It has a high sensitivity, wide linear dynamic range and is rugged as changes in flow rate of make-up gas do not affect detector response [63, 65, 67]. Besides, detector gases such as hydrogen and synthetic air are inexpensive and readily available. Although FID is destructive to samples, no further characterization of the sample was necessary in the present study.

2.6 Optimization of GC conditions

2.6.1 Preliminary analysis

Preliminary analysis was first carried out by injecting methanol (diluent), menthol standard, camphor standard and a mixture of menthol and camphor standards using some of the chromatographic conditions as established by Kalgutkar *et al*, 2016 as the starting point [47]. Applicable chromatographic conditions adopted from this method were: nitrogen as carrier gas at 1.0 mL/min, oven temperature gradient as shown in the Table 2.2, injector inlet temperature of 240 ℃, flame ionization detector at 260 ℃, detector gases as hydrogen and industrial air at 30 and 300 mL/min, respectively and nitrogen as make up gas at 30 mL/ min. Methanol (MET) was used as a diluent for samples and standards.

Other parameters were fixed and used in conjunction with the above conditions. The fixed conditions were: a ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol and injection volume of 1.0 µL with split ratio of 50:1.

Table 2.2: GC temperature program

Time (minutes)	Temperature (℃)	Comment
0 to 2	100	Isothermal
2 to 16	100 to 240	Linear gradient
16 to 18	240	Isothermal

Under these conditions, there was adequate baseline resolution (Rs>1.5) between MET/CAM and CAM/MEN with retention times for MET (diluent), CAM and MEN as 4.7, 10.2 and 11.9 minutes, respectively, as shown in Figure 2.1 below and summarized in Table 2.3. However, these conditions resulted in a total run time of 18 minutes that is not suitable for a method

intended for routine analysis [68]. There was need to establish conditions that result in adequate resolution, capacity factors that lie between 1 and 5 as well as a total run time of 5-15 minutes [63, 65, 68, 69].

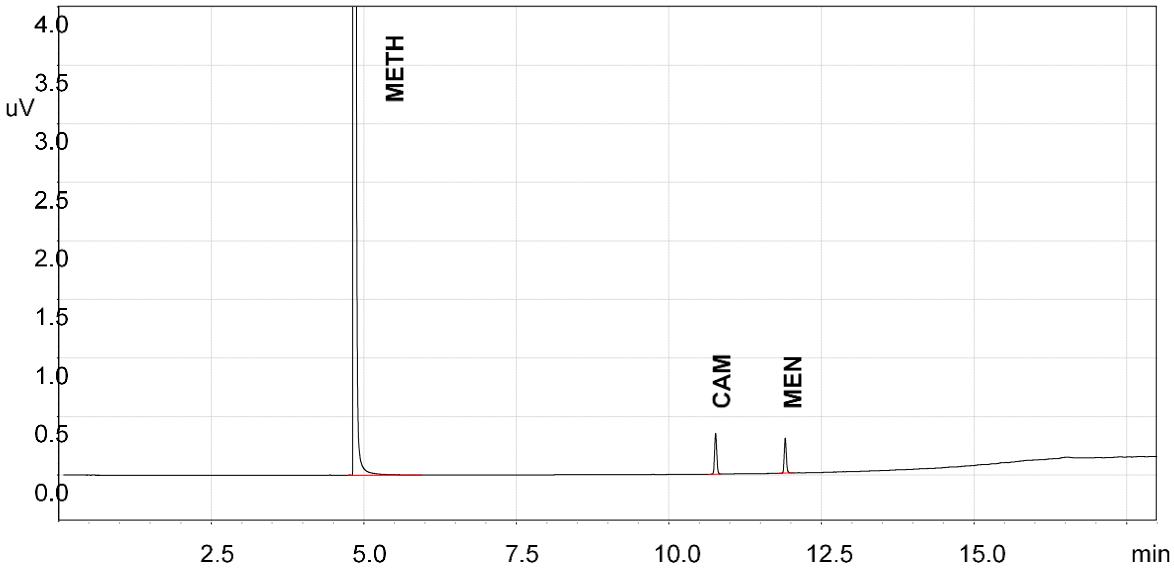


Figure 2.1: Typical standards gas chromatogram from preliminary analysis. Methanol (MET), camphor (CAM) and menthol (MEN). Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol. Oven temperature 100 ℃(2 min), ramp 10 ℃/min to 240 ℃

Table 2.3: Peak parameters for camphor and menthol

Final column temperature (℃)	Compound	Retention time (min)	Capacity factor	Resolution
240.0	Camphor	10.21	0.95	31.77
	Menthol	11.90	1.27	3.12

Column:ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 100 ℃(2 min), ramp 10 ℃/min to 240 ℃

2.6.2 Effect of column temperature on separation

In gas chromatography, adequate resolution with desirable run time is achieved with a column temperature that approximates the boiling point of the analyte [58, 63]. During temperature programming, resolution of earlier eluting peaks can be achieved by either lowering the initial column temperature or increasing the initial isothermal (hold) period [58]. From preliminary analysis, the earliest eluting peak was MET followed by CAM. There was adequate resolution of CAM peak from MET and therefore it was not necessary to lower initial column temperature or increase the isothermal period. It was noted that methanol eluted at 120 °C and therefore to reduce the overall run time, the initial temperature was raised from 100 °C to 110 °C.

The last peak (MEN) eluted at 190 °C which therefore formed the basis for investigating the effect of final column temperature at 190, 210, 220 and 230 °C on separation. Resolution and capacity factors for CAM and MET decreased with increase in final column temperature from 190 to 240 °C (Table 2.4). Resolution between CAM and MEN was highest (5.23) at 190 °C and lowest (3.12) at 240 °C although still adequate. The decrease in resolution with increase in temperature could be a result of reduced interaction with stationary phase since vapor pressure of analytes also increase with increase in temperature [64]. The effect of final column temperature on capacity factors of camphor and menthol is illustrated in Figure 2.2 and a final column temperature of 190 °C was hence considered optimum.

Table 2.4: Effect of column temperature on peak parameters of camphor and menthol

Temperature (X ℃)	Compound	Retention time (min)	Capacity factor(K′)	Resolution
190	Camphor	10.8	1.45	47.97
	Menthol	11.9	1.70	5.23
210	Camphor	10.8	1.29	42.04
	Menthol	11.9	1.53	5.19
220	Camphor	11.0	1.25	33.94
	Menthol	11.7	1.49	4.86
230	Camphor	10.4	1.11	33.54
	Menthol	11.6	1.46	3.83
240	Camphor	10.2	0.95	31.77
240	Menthol	11.9	1.27	3.12

Oven temperature 110 ℃ (2 min), ramp 10 ℃/min to X ℃ where X-final column temperature.

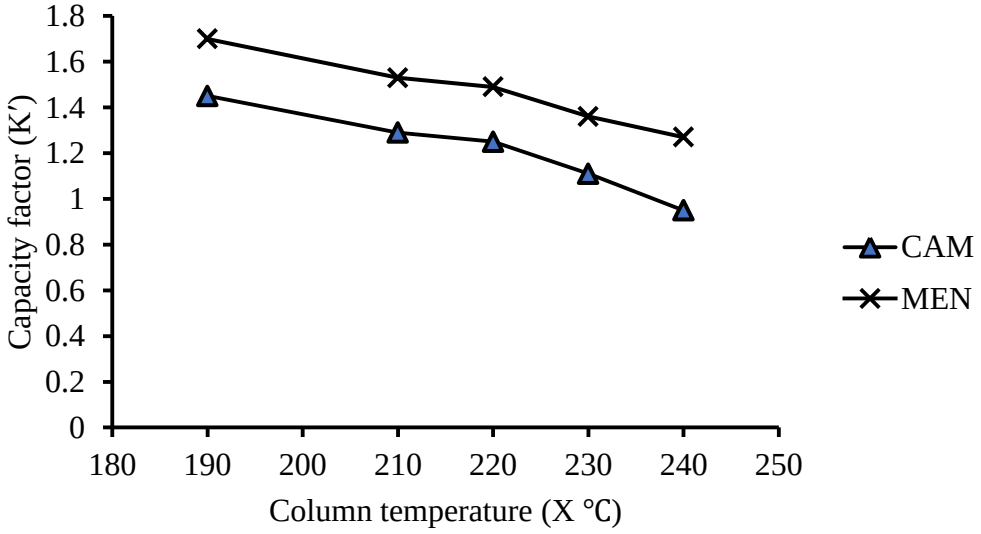


Figure 2.2: Effect of final column temperature on capacity factors of CAM and MEN. Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol. Oven temperature 110 ℃ (2 min), ramp 10 ℃/min to X ℃. CAM-camphor, MEN-menthol, X-final column temperature

2.6.3 Effect of changing solvent on separation

Trial runs were conducted on a cough syrup sample spiked with CAM to check for interference from the formulation matrix and a representative chromatogram is shown in Figure 2.3. There were several other peaks besides those attributable to MET, CAM and MEN. In addition, sample solutions retained their original color which upon cooling could solidify and clog the capillary column.

Menthol is very soluble in chloroform (CHF) and it has been used as a diluent in previous studies on cough syrups [45]. By using chloroform as a diluent, menthol peak was identified by comparing with the retention time of the standard solution. The same sample was then spiked with CAM and diluted in CHF whereby all coloring agents were removed leaving the analyte in the organic colorless solution. There were no interfering peaks in the elution of menthol and camphor as shown in Figure 2.4.

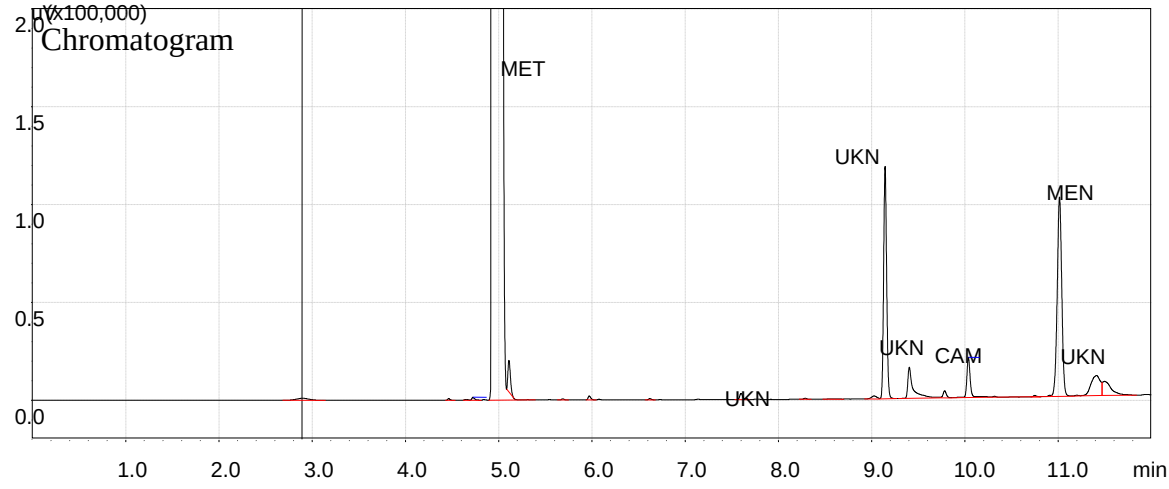


Figure 2.3: Typical sample in methanol gas chromatogram. Methanol (MET), menthol (MEN) and three unknown compounds (UKN). Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol. Oven temperature 110 ℃ (2 min), ramp 10 ℃/min to 190 ℃

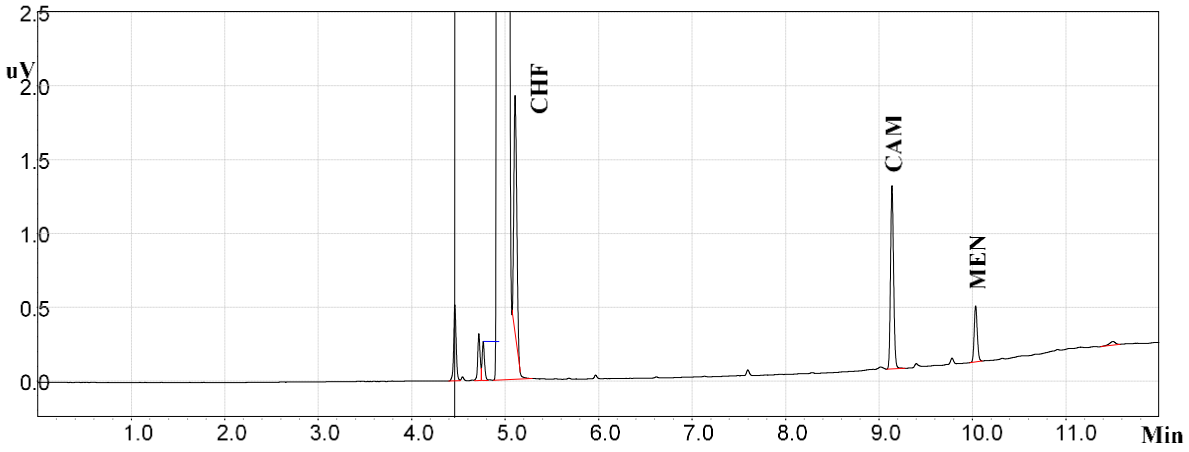


Figure 2.4: Typical sample in chloroform gas chromatogram. Chloroform (CHF), camphor (CAM) and menthol (MEN). Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110℃ (2 min), ramp 10℃/min to 190℃

2.6.4 Effect of carrier gas velocity on separation

The effects of carrier gas velocity on retention time, asymmetry factor, capacity factor and resolution of camphor and menthol were further investigated at 0.5, 1.5 and 2.0 mL/min (Table 2.5). Figure 2.5 illustrates the effects of carrier gas velocity on capacity factors for camphor and menthol.

At 0.5 mL/min, the retention time and capacity factor for CAM and MEN were increased, resolution between CAM and CHF as well as CAM and MEN was improved (Rs > 6.9) while asymmetry factors for CAM and MEN were 0.99 and 1.03, respectively. The disadvantage of working at 0.5 mL/min was the long run time (26 minutes).

At 1.5 mL/min, retention times and capacity factors for CAM and MEN reduced with CAM eluting at 7.8 minutes. Although baseline separation was achieved, resolution was reduced as compared to that obtained at 1.0 mL/min.

At 2.0 mL/min, capacity factors for CAM and MEN were less than 1 although the two peaks were well resolved (Rs >1.5). Consequently, 1.0 mL/min was chosen as the optimum carrier gas velocity because it gave the shortest analysis time with adequate resolution.

Table 2.5: Effect of carrier gas velocity on peak parameters of camphor and menthol

Carrier gas velocity (mL/min)	Compound	Retention time (min)	Asymmetry factor	Capacity factor	Resolution
0.50	CAM	18.82	0.99	1.21	48.87
	MEN	20.20	1.03	1.59	6.92
1.00	CAM	9.13	1.13	1.17	44.07
	MEN	10.02	1.02	1.42	5.79
1.5	CAM	7.83	1.57	1.03	36.89
	MEN	9.42	1.66	1.13	3.12
2.0	CAM	7.26	1.29	0.54	36.28
	MEN	9.22	1.11	0.96	4.19

Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110 ℃ (2 min), ramp 10 ℃/min to 190 ℃ CAM-camphor, MEN-menthol

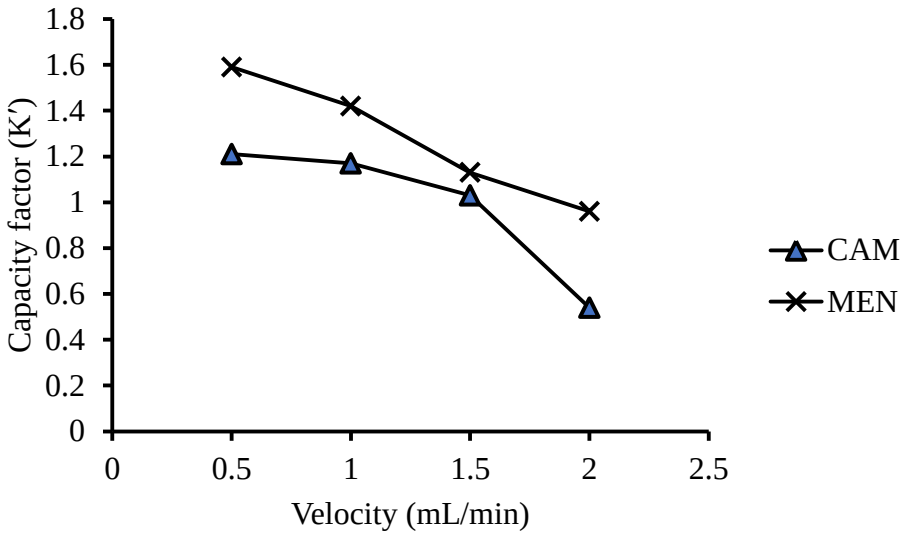


Figure 2.5: Effect of carrier gas velocity on capacity factors of camphor and menthol. Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110 ℃ (2 min), ramp 10 ℃/min to 190 ℃. CAM-camphor, MEN-menthol

2.6.5 Optimized chromatographic conditions

The initial and final isothermal periods remained 2 minutes and from trial analyses, the following conditions resulted in adequate resolution with lowest possible temperature, time and were therefore considered as optimum conditions : A ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol, column temperature program as 110 ℃ (2 min), ramp 10 ℃/min to 190 ℃ (2 min), injector port temperature at 240 ℃ into which injection volume of 1.0 µl is delivered, split in the ratio of 50:1, carrier gas as nitrogen at 1.0mL/min which is also used as make up gas (30 mL/min) in the flame ionization detector (260 ℃). Other detector gases were hydrogen (30 mL/ min) and industrial air (300mL/ min). The diluent for samples and standards was HPLC grade chloroform. A representative chromatogram for standard analytes obtained under these conditions is shown in figure 2.6.

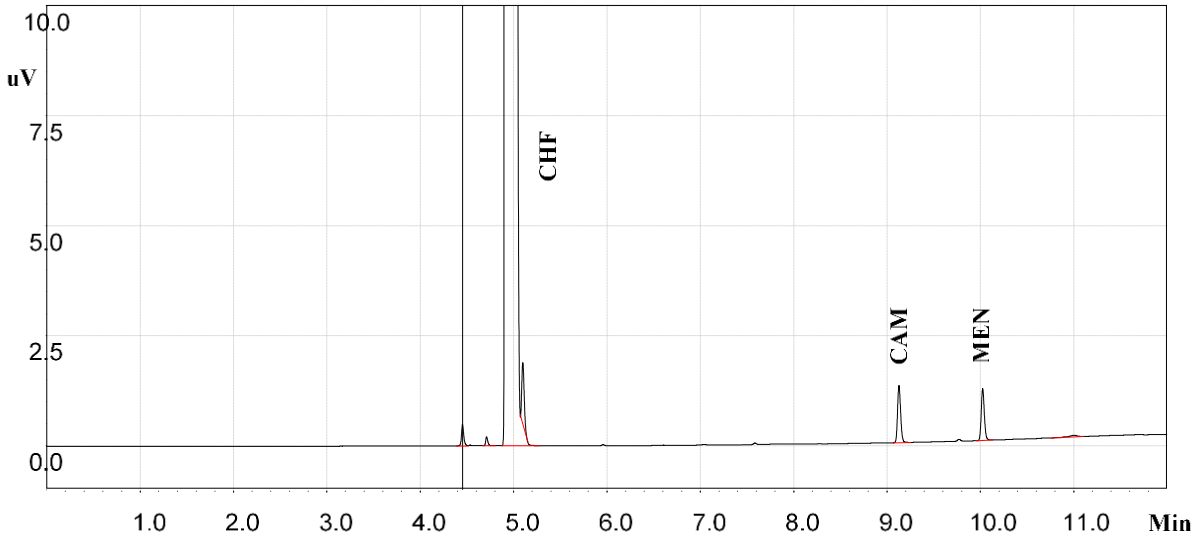


Figure 2.6: Typical gas chromatogram for Chloroform (CHF), camphor (CAM) and menthol (MEN).at optimized conditions. Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100 % polyethylene glycol. Oven temperature 110 ℃(2 min), ramp 10 ℃/ to 190 ℃(2min)

CHAPTER THREE

ANALYTICAL METHOD VALIDATION

3.1 Introduction

Validation of an analytical method is the process of establishing the suitability of the method for intended analytical use by way of laboratory studies [70]. Validation therefore provides assurance that the method will provide consistent results that correctly reflect the quality attributes being tested [60, 71]. Typical characteristics used in method validation are accuracy, specificity, precision, sensitivity, linearity of detector response, robustness and range of analyte concentration.

In the present study, validation was carried out using reference working solutions of menthol and camphor each at concentration of 0.1mg/mL (100 %) which is comparable with that used in the assay of menthol in lozenges [30].

3.2 Accuracy

The accuracy of the GC test results was determined by spiking three blank commercial cold-cough syrup sample with menthol and camphor standards. Each working standard solution was prepared so as to contain a constant amount (0.049 mg/mL) of CAM with MEN at 0.08, 0.1 and 1.2 mg/mL corresponding to the three concentration levels of 80, 100 and 120 % menthol. Triplicate determinations were made at each of these concentration levels and then percent recovery of the added menthol standard was calculated using equation 3.1 and summarized in Table 3.1.

$$\% \text{ recovery } = \frac{\text{RFa}}{\text{RFb}} \times 100. \quad \text{Equation 3.1}$$

Where, RFa and RFb are response factors from spiked placebo and a directly injected menthol standard solution respectively. The placebo was a sample syrup containing AMB, BRO, CHL, GUA and SAL but not MEN.

Table 3.1: Percent recoveries of menthol from a spiked placebo

Concentration level (%)	Sample	Average response factor	% recovery
80	Standard	4.38	
	Placebo	4.51	102.97
100	Standard	4.86	
	Placebo	4.73	98.32
120	Standard	6.12	
	Placebo	6.15	100.49

Additionally, the accuracy was also determined from commercial samples using standard addition method at 80,100 and 120% of menthol concentration level. Assay for MEN was conducted on a commercial cold-cough syrup and the peak area response factor (RF) noted. Samples were then spiked with a constant amount of CAM and menthol at a concentration corresponds to 80, 100 and 120% level and then calculating RF. Percent recovery was calculated from equation 3.2 below and the results summarized in Table 3.2.

$$\% \text{ recovery} = \frac{\text{RF ss} - \text{RF s}}{\text{RF std}} \times 100. \text{ Equation 3.2}$$

Where, RFss is the average response factor for spiked sample, RFs is the average response factor for unspiked sample and RF std is the average response factor for standard solution.

Table 3.2: Percent recoveries of menthol from standard addition to a cough syrup

Concentration level (%)	Average Response factor for sample (not spiked)	Average Response factor for sample (spiked)	Average Response factor for standard.	% recovery
80	0.522	1.423	0.919	98.04
100	0.524	1.406	0.904	97.56
120	0.493	1.386	0.876	101.94

The recovery of menthol from spiked placebo ranged from 98.32 to 102.97 % while that from standard addition to sample solutions ranged from 97.56 to 101.94 %. The percent recovery was adequate since it fell within 97 to 103% [71]. These values indicate that the method had adequate accuracy and was suitable for quantitative determination of menthol in cold-cough syrups.

3.3 Specificity

The International Conference on Harmonization (ICH) guidelines define specificity of an analytical method as the ability of the method to assess an analyte in the sample with complete discrimination from other components that may be present [70]. Other authorities such as International Union of Pure and Applied Chemistry (IUPAC) use selectivity to relay the same meaning and reserve specificity for analytical procedures that produce a response for a single analyte only [72]. Experiments to determine specificity/selectivity are done to distinguish the response of target compounds from responses of impurities, sample matrix and other APIs that may be present and this is assessed using resolution and asymmetry factor [70].

Specificity of the developed method was investigated by analyzing standard solution containing CAM and MEN and then determining resolution, asymmetry factors and peak purity. Resolution

between CAM/CHF and CAM/MEN was > 1.5, asymmetry factors for CAM and MEN peaks were < 2.0 while GC-MS analysis of the MEN peak gave a purity index of 0.98. Specificity was also assessed using a sample syrup solution in chloroform (blank) containing AMB, CHL, GFN, SAL and BRO. There was no peak eluting at the same retention time as that observed for menthol and camphor as shown in Figure 3.1.

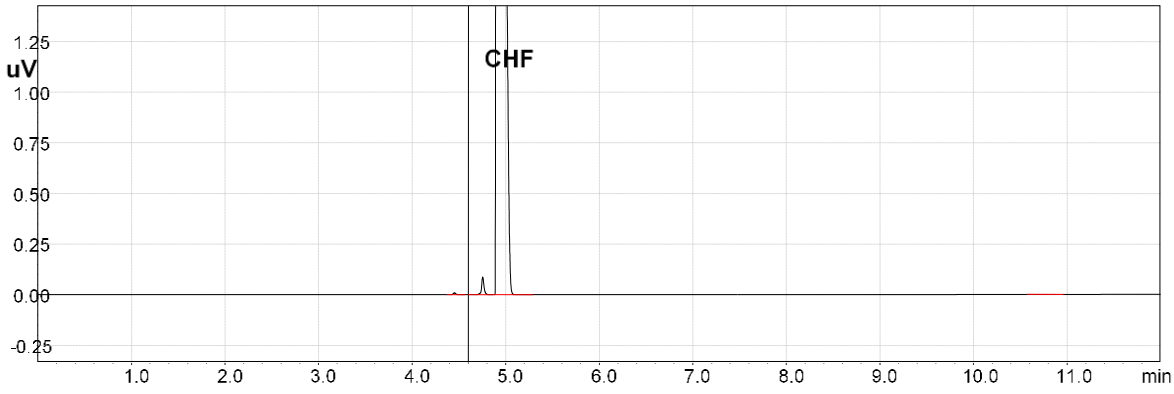


Figure 3.1: Typical gas chromatogram of a blank sample syrup. Chloroform (CHF). Column: ZB-WAXplus 60m ×0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110℃(2 min), ramp 10℃/min to 190℃(2 min)

3.4 Sensitivity

The capability of the method to discriminate small differences in concentration depends on the degree of detector response per unit concentration. In validation of an analytical method, assessment is made on the method and ancillary instruments included in the method. Detector noise is important as it determines the minimum concentration at which the signal from an eluted solute can be discerned unambiguously from the noise [61].

Sensitivity of the method was assessed quantitatively through determination of the limit of detection (LOD) and limit of quantitation (LOQ).

3.4.1 Limit of detection

The LOD is the lowest concentration of an analyte that can be detected but not necessarily quantified as an exact value under specified experimental conditions [70].

The limit of detection can be determined in various ways. Since a standard substance was available, signal to noise ratio (S/N) was first evaluated [73]. Working solutions of the standard were prepared by serial dilution of a stock standard solution (2.06 mg/mL) and then injecting the solutions into the chromatograph in triplicate. The signal to noise ratio at each concentration was then determined from peak height with reference to a blank injection of the diluent. The ICH stipulates an acceptance criterion for LOD as the lowest concentration that produces a definitive peak whose signal to noise ratio (S/N) lies between 2:1 and 3:1 [70]. From analysis of S/N, LOD was determined to be 0.0059 mg/mL.

3.4.2 Limit of quantitation

Limit of quantitation refers to the lowest amount of an analyte in a sample that can be determined quantitatively with suitable precision and accuracy. The degree of precision considered acceptable in determining LOQ from peak areas of replicate injections (n = 6) is a relative standard deviation (RSD) of approximately 10 % at S/N 10:1 [60]. From the S/N, LOQ was determined to be 0.023 mg/mL. [71,73-75].

3.5 Linearity of detector response

Within a given range of concentration, it is important for an analytical method to produce a detector response that is directly proportional to the concentration of the respective analyte. In

GC, the peak areas should be directly proportional to analyte concentration over such a specified concentration range (linear dynamic range). This is the linearity of detector response and it is important in establishing the accuracy of the analysis [72]. It is recommended that linearity should be evaluated using a minimum of five concentrations that span 80 % to 120 % of 0.1 mg/mL which is the expected working concentration [70]. The acceptance criteria for linearity determination is a coefficient of determination (R^2) ≥ 0.99 for all the concentrations used [30, 60].

In this study, a stock solution of menthol was prepared from which various dilutions (working solutions) were made. Linearity was then determined by making injections from working solutions corresponding 40, 60, 80, 120, 140 and 160 % of 0.1 mg/mL (100 %). Each working solution was analyzed in triplicate and data obtained from response factors of menthol shown in Table 3.3.

Table 3.3: Various concentrations of menthol with respective CV of RF

Concentration level (%)	Actual concentration (mg/mL)	CV of response factors
160	0.169	1.6
140	0.148	1.3
120	0.127	0.5
100	0.106	0.3
80	0.084	1.0
60	0.063	0.8
40	0.042	1.1

This data was further subjected to linear regression analysis with exact concentration of menthol (x-axis) being plotted versus response factor (y-axis). Values for coefficient of determination, y-intercept, slope of the regression line and residual sum of squares are summarized in Table 3.4.

Table 3.4: Linear regression analysis for menthol

Drug	Slope of regression curve	y-intercept	Coefficient of determination (R^2)	Residual sum of squares
Menthol	218,187	-626.43	0.9986	835131.8

The method was found to be linear in the range 0.042 to 0.169 mg/mL with $R^2 = 0.9986$. Since the expected working concentration for analysis was 0.1mg/mL, the lower level for linearity determination (40 %) was 0.042 mg/mL which is slightly higher than 0.023 mg/mL for LOQ.

3.6 Precision

Analytical experiments often involve multiple determination of a particular quality attribute from the same homogeneous sample. Precision is the degree of closeness of individual test results obtained from repeated analysis of the same homogeneous sample. Precision is evaluated with respect to repeatability, intermediate precision and reproducibility.

Repeatability expresses precision under the same operating conditions over a short interval of time. It is also called intra-assay precision. Intermediate precision expresses variation within laboratories and is meant to verify that in the same laboratory, the method produces consistent results. Findings from precision experiments are expressed as a coefficient of variation (CV) of responses from replicate determinations which should be < 3 [70].

In the present study, CV of response factors of nine replicate determinations made from 80, 100 and 120 % concentration on the same day was used as a measure of repeatability [70]. Intermediate precision was assessed from CV of response factors obtained from three determinations daily for a three-day interval. Analyses for intermediate precision were done from 100 % concentration level of menthol and the results are summarized in Table 3.5. Tests to determine reproducibility were beyond the scope of this study and therefore were not done.

Table 3.5: Precision results for menthol

Concentration level (%)	**Coefficient of Variation of Response Factors**	
	Repeatability (n =3)	**Intermediate precision (n = 9)**
120	0.34	
100	0.32	1.03
80	0.99	

The CV from response factors from repeatability (<1) and intermediate precision (<3.0) indicate that the method has acceptable precision.

3.7 Robustness

Robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate changes in operational factors. The purpose of making deliberate changes is to reflect normal variations expected when the method is applied routinely. Therefore, robustness provides an indication of reliability of a method in normal application [60].

Temperature has a significant influence on separation of analytes especially on selectivity between critical peak pairs. In general, optimum resolution is associated with minimal temperature at the expense of elution time [61].

Carrier gas velocity affects resolution and efficiency of a chromatographic system [76, 77]. To determine robustness of the developed method, the effect of small changes on carrier gas velocity (< 1 %) [76] and oven temperature as well as injector port temperature on peak area and retention time of menthol and camphor was investigated. The results were summarized (Table 3.6) with each factor, the level varied and the corresponding CV in retention time and response factor indicated.

Table 3.6: Effect of column, injector port temperature and carrier gas velocity on peak parameters

Parameter varied	Compound	CV of retention time	CV of response factor
Oven temperature (°C) (109.5, 110.0, 110.5)	CAM	0.39	-
	MEN	0.41	-
	MEN/CAM	-	1.27
Injector port temperature (°C) (239.5, 240.0, 240.5)	CAM	0.0082	-
	MEN	0.0049	-
	MEN/CAM	-	0.33
Carrier gas velocity (mL/min) (0.98, 1.0, 1.02)	CAM	0.73	-
	MEN	0.65	-
	MEN/CAM	-	0.37

Data obtained from robustness studies showed that oven temperature had the greatest effect on peak areas and subsequently response factors (CV =1.27) as compared to injector port

temperature (CV =0.33) and mobile phase flow rate (CV =0.37). Therefore, it is necessary to take precautions by ensuring that the method is set with the correct temperature program and that the gas chromatograph oven is well sealed to ensure no entry of air. Proper functioning of the fan was ensured so that the oven temperature adjusted appropriately to the temperature program [64].

Mobile phase flow rate had appreciable effect on retention times for CAM and MEN (CV =0.73 and 0.65) as compared to initial oven temperature and injector port temperature. This is consistent with literature reports [62,64]. It was necessary to ensure that carrier gas supply system was free from leakages and that pressure regulators were well calibrated.

Injector port temperature demonstrated negligible effect on both peak areas and retention times. However, higher injector port temperature can lead to sample decomposition which would affect peak areas and subsequently response factors, therefore a CV of <3 would be admissible [62, 71].

3.8 Stability of working solutions

The stability of working solutions was monitored over 72 hours under different storage and handling conditions in order to determine suitable conditions for sample handling during analysis. Solutions containing known concentration of menthol and camphor were designated A, B and C and handled as follows:

Solution A stored at room temperature in a clear glass.

Solution B stored at room temperature in amber colored glass.

Solution C stored in the refrigerator (2-8 ℃) in a clear glass.

Triplicate injections of each solution were made at 0, 24, 48 and 72 hours and mean response factors computed relative to a freshly prepared solution from the first day. Results were

summarized in a table showing each solution, response factor, % response factor relative to freshly injected solution from 0 hours.

Table 3.7: Results for stability studies

Sample	Time in hours	Average response factor	% Relative response factor
A	0	4.82	100
	24	4.86	100.8
	48	4.87	101
	72	4.85	100.6
B	0	4.82	100
	24	4.79	99.4
	48	4.72	97.9
	72	4.70	97.5
C	0	4.82	100
	24	4.73	98.1
	48	4.76	98.8
	72	4.42	91.7

Working standard solutions stored at ambient temperature in a clear glass showed minimal change in % response factor over the entire 72-hour test period. Whereas chloroform is known to decompose at room temperature in absence of air, the test period was within the 2-week recommended period for use of chloroform when taken from original container [26].

Working standard solutions stored at ambient temperature but protected from light showed a slight change in % response factor (2.5) over the entire test period while the working standard solutions stored in the refrigerator (2-8 ℃) showed the highest change (8.3 %) in response factor.

Findings from stability studies implied that standard working solutions could be used within 72 hours from time of preparation if stored at room temperature. These studies were not conducted on samples and therefore the most practical precaution to be taken during analysis with this GC method would be to use freshly prepared solutions.

CHAPTER FOUR

ANALYSIS OF COMMERCIAL SAMPLES

4.1 Introduction

The validated GC method was used to analyze the content of total menthol in cold-cough syrups that also contained CHL, GUA, SAL, AMB and BRO. In addition, determination of APIs other than menthol was done using a previously validated HPLC method [44].

4.2 Sample acquisition

Samples of commercial products were purchased from retail pharmacies within Nairobi County using a simple stratified random sampling method. The drug register maintained at PPB as at December 2015 was used as the sampling frame. All available cough syrups containing menthol and any other API under study were sampled from at least two batches and were collected according to the r-sampling plan [78].

A total of twenty-one samples were collected for analysis. One sample had four batches, two samples had three batches, seven samples had two batches while the remaining eleven samples had one batch each. From samples collected, 33.3 % were manufactured locally while 66.7% were imported from India. This corroborates with a December 2008 report on analysis of healthcare industry which found out that majority of drugs in the Kenyan market are imports [79]. Samples were coded and details pertaining to their country of origin, date of manufacture, expiry date, batch number and label claim (mg/5mL) recorded as shown in Table 4.1.

Table 4.1: Particulars of samples analyzed

Sample code	Country of origin	Batch number	Manufacturing date	Expiry date	Composition (mg/5mL)
CON	Kenya	CON67	09/2015	08/2018	Ammonium chloride 60 mg
		CON21	03/2016	02/2019	Chlorpheniramine maleate 2mg
					Menthol 0.25 mg
TSPP	India	TSPP63	04/2016	03/2019	Bromhexine 4mg
		TSPP53	08/2015	07/2018	Salbutamol 1 mg
		TSPP63	05/2016	04/2019	Menthol 0.75 mg
		TSPP63	07/2016	06/2019	
ASC	India	ASC90	06/2016	05/2018	Salbutamol 1mg
					Bromhexine 2 mg
					Guaifenesin 50 mg
					Menthol 0.5 mg
ONA	India	ONA08	03/2016	02/2018	Salbutamol 1mg
					Bromhexine 2 mg
					Guaifenesin 50 mg
					Menthol 0.5 mg
SAL	India	SAL94	05/2015	04/2018	Salbutamol 1mg
		SAL37	11/2015	10/2018	Bromhexine 2 mg
					Guaifenesin 50 mg
					Menthol 0.5 mg
BROM	Kenya	BROM81	03/2016	02/2019	Salbutamol 1mg
		BROM31	03/2015	02/2018	Bromhexine 2 mg
		BROM83	03/2017	02/2020	Guaifenesin 50 mg
					Menthol 0.5 mg
COF	India	COF02	11/2015	10/2018	Chlorpheniramine maleate 4 mg
		COF01	04/2016	03/2019	

Sample code	Country of origin	Batch number	Manufacturing date	Expiry date	Composition (mg/5mL)
					Menthol 1.5 mg
TUSP	India	TUSP63	04/2016	03/2018	Chlorpheniramine maleate 4 mg
		TUSPH63	08/2016	07/2018	
		TUSPJ63	10/2016	09/2018	Menthol 1.5 mg
ZDX	India	ZX92	09/2015	08/2018	Bromhexine 8 mg
		ZX91	09/2015	08/2018	Menthol 5 mg
TRIC	Kenya	TRIC1T	06/2016	05/2019	Chlorpheniramine maleate 2 mg
		TRIC7T	07/2016	06/2019	
					Menthol 0.25 mg
BROX	India	BROX34	08/2015	07/2018	Ambroxol 15 mg
					Guaifenesin 50 mg
					Chlorpheniramine maleate 2 mg
					Menthol 1 mg
BZN	India	BZN86	11/2015	10/2017	Ambroxol 30 mg
					Salbutamol 2 mg
					Guaifenesin 50 mg
					Racementhol 1 mg
BZX	India	BZX26	02/2016	01/2019	Bromhexine 4 mg
					Guaifenesin 50 mg
					Menthol 2.5 mg
DAC-M	Kenya	DAC-M66	12/2015	11/2018	Salbutamol 2 mg
					Bromhexine 4 mg
					Menthol 1 mg
LUN	Kenya	LUN76	04/2017	03/2020	Chlorpheniramine 2 mg
					Menthol 0.25 mg

Sample code	Country of origin	Batch number	Manufacturing date	Expiry date	Composition (mg/5mL)
DEL-C	Kenya	DEL-C47	11/2016	10/2019	Guaifenesin 100 mg Menthol 0.75 mg
CAD	India	CAD39 CAD75	09/2016 03/2016	08/2020 02/2020	Chlorpheniramine maleate 2 mg Guaifenesin 80 mg Menthol 1.0 mg
CADP	India	CADP20 CADP01	10/2015 01/2016	09/2019 12/2019	Chlorpheniramine maleate 2 mg Guaifenesin 80 mg Menthol 1.0 mg
BEN-A	India	BEN-ABE BENA5BE	06/2016 05/2017	05/2019 04/2020	Menthol 1.5 mg Ammonium chloride 60 mg
DEL-P	Kenya	DELC02 DELC89	10/2016 05/2016	09/2019 04/2019	Chlorpheniramine maleate 2 mg Menthol 0.75 mg
ASC-D	India	ASCD25	05/2016	04/2018	Chlorpheniramine maleate 2 mg Menthol 1.5 mg

4.3 Analysis of samples

4.3.1 Gas chromatographic analysis of menthol

4.3.1.1 Sample preparation

When determining sample volume for analysis, consideration was given to the volume that would result in concentration of menthol that lies within the linear range of the detector while producing relatively large peak areas. Large peak areas are preferred because they minimize

errors associated with measurement. Besides, when less sample volume is used, sample differentiation may result in the analyte being lost in the split injector [62].

During analysis, 10 to 20 mL (depending on label claim) of the sample syrup was measured into the 50mL volumetric flask and diluted to volume with chloroform. The mixture was then shaken and sonicated for 5 minutes. The aqueous layer was removed and the organic layer made to volume with chloroform. This solution was then filtered through a 125mm Whatman's filter paper and stored in a stoppered container.

4.3.1.1 Assay for menthol

In the present study, quantitative determination of menthol involved preparation of stock standard solutions of CAM and MEN from which appropriate dilutions were made. A calibration solution was made by pipetting 1.2 mL of a 2.06 mg/mL CAM and 1.0 mL of a 2.06 mg/mL MEN into a 50-mL volumetric flask and diluted to volume with chloroform. The resulting working standard solution contained 0.041 mg/mL (MEN) and 0.049mg/mL (CAM) and was labeled as solution 1.

Sample solution for analysis was then prepared by measuring 1.2 mL of a 2.06mg/mL CAM stock standard solution into a 50-mL volumetric flask to which 10 to 20 mL (depending on label claim) of the sample syrup added and diluted to volume with chloroform. The mixture was shaken and sonicated for 5 minutes. The aqueous layer was then removed and the organic layer made to volume with chloroform. This solution was then filtered through a 125mm Whatman's filter paper and stored in a stoppered container. The resulting solution was labeled as solution 2.

System suitability test was conducted before analyzing samples whereby a standard solution of CAM and MEN was injected six times and CV of peak area response factors, retention time and

resolution (<2.0) determined which was obtained as <2.0 and therefore the system was considered suitable [44].

Standard solutions (solution 1) were run in duplicate while sample solutions (solution2) were prepared in quadruplet for each batch and then respective peak areas for CAM and MEN recorded. Response factors for solution 1 (Rf1) and solution 2 (Rf2) were then calculated using equation 4.1 and 4.2 respectively [62].

$$\text{Rf1} = \frac{\text{area of menthol peak in calibration solution}}{\text{area of camphor peak in calibration solution}} \quad \text{Equation 4.1}$$

$$\text{Rf2} = \frac{\text{area of menthol peak in sample solution}}{\text{area of camphor peak in sample solution}} \quad \text{Equation 4.2}$$

Amount of menthol in the syrup was then calculated as follows:

$$\frac{\text{R f2}}{\text{Rf1}} \times \text{C1} \left(\frac{\text{mg}}{\text{mL}}\right) \times \text{Vs} \times \text{P}. \quad \text{Equation 4.3.}$$

Where, C1 is the concentration of menthol in standard solution 1, Vs is the volume of sample syrup taken for analysis and P is the potency of menthol standard

The amount of menthol expected in the analytical sample was calculated from the label claim. Results from the twenty-one samples analyzed showed that the range of menthol content was 26.3 to 107.8 % f the label claim. It was however noted that in the sample that resulted in 26.3 % of the label claim, menthol was declared to be included as a flavor. In the assay, ten samples complied with assay specifications of 90.0 to 110.0 % label claim for finished products

according to the USP 2016 [30]. All samples that complied with these assay specifications were manufactured by local industries. This is an indicator of improvement in good manufacturing practices, good distribution practices as well as adequate supervision from the PPB. Results for GC analysis of menthol were summarized in Table 4.2.

4.3.2 HPLC analysis of non-volatile APIs.

Analysis of non-volatile APIs was done according to the method developed and validated by Njaria *et al,* 2016 [44]. Samples were prepared by taking 5.0 mL of the cold-cough syrup and diluting to 50 mL in a volumetric flask. The diluent used was a mixture of acetonitrile and water in the ratio 2:3. The sample solution was then filtered through a 154 mm filter paper and 20 µL of the filtrate injected into the liquid chromatograph [44]. System suitability test was conducted before analyzing samples whereby a standard solution was injected six times and CV of peak areas, retention time and resolution (<2.0) determined.

In calculating content, the average peak area of standard solutions was normalized to produce concentration per unit area and then compared to average sample peak areas obtained from sample analysis [44]. Based on USP 39 specification for assay of finished products, AMB, CHL, GUA and BRO in all samples that were analyzed complied with assay limits of 90.0 to 110.0 % label claim. The ranges for percent label claim were: 97.2 to 100.6 for AMB, 91.7 to 101.3 for CHL, 90.9 to 105.2 for GUA and 91.2 to 108.8 for BRO. Twenty samples containing SAL complied with USP 39 specifications for assay of finished products with the highest result being 99.3 % label claim. One sample had 86.1 % of label claim for SAL. From HPLC analysis results, there was insignificant inter-batch variability which could be as a result of adherence to good

manufacturing practices. Results for HPLC analysis of cold-cough syrups recorded as percent label claim were shown in Table 4.2.

4.4 Determination of pH

The pH of each cough syrup was determined at room temperature using a Jenway pH meter calibrated with two buffer solutions at pH 4.0 and 7.0. The pH for the cough syrups ranged from 3.4 to 5.1 as shown in Table 4.2. All the twenty-one samples recorded pH values below 5.5 which is considered critical for enamel dissolution [80-82]. Therefore, all the cough syrups analyzed in this study carry the potential for dental erosion [81,83].

Table 4.2: Analysis report for samples

Sample code	Batch no.	pH	Percent label claim					
			MEN	AMB	CHL	GUA	BROM	SALB
ASC	ASC90	3.6	79.9 (0.9)	-	-	101.7 (0.3)	96.4 (1.2)	94.8 (1.6)
ONA	ONA08	4.5	77.9 (1.1)	-	-	104.9 (0.4)	108.8 (0.9)	86.1 (1.8)
BZX	BZX26	3.4	39.8 (0.6)	-	-	105.2 (0.3)	107.9 (1.0)	-
TSPP	TSPP63	4.2	58.0 (0.8)	-	-	-	100.4 (1.1)	98.6 (0.4)
	TSPPH63	4.3	43.9 (0.2)	-	-	-	98.2 (0.7)	99.3 (1.2)
	TSPPE63	4.2	68.3 (0.1)	-	-	-	98.5 (0.5)	97.8 (0.8)
	TSPPG63	4.2	57.5 (0.3)	-	-	-	96.9 (0.6)	93.4 (0.1)

Sample code	Batch no.	pH	Percent label claim					
			MEN	**AMB**	**CHL**	**GUA**	**BROM**	**SALB**
SAL	SAL94	4.8	79.5	-	-	97.6	100.4	96.3
			(0.4)			(1.5)	(0.7)	(0.8)
	SAL37	4.9	100.8	-	-	98.4	99.6	97.9
			(0.9)			(0.7)	(1.8)	(1.1)
CON	CON67	5.1	43.1	-	98.7	-	-	-
			(0.6)		(0.4)			
	CON21	5.1	34.4	-	98.3	-	-	-
			(0.2)		(0.5)			
	CON13	5.1	36.1		96.2	-	-	-
			(1.6)		(0.8)			
TUSP	TUSPD63	4.2	56.1	-	91.7	-	-	-
			(0.9)		(0.3)			
	TUSPH63	4.2	56.5	-	96.8	-	-	-
			(1.5)		(0.1)			
	TUSPJ63	4.3	59.2	-	93.6		-	-
			(0.8)		(0.6)			
COF	COF02	4.2	26.3	-	99.7	-	-	-
			(0.8)		(0.2)			
	COF01	4.2	26.4	-	101.3	-	-	-
			(1.1)		(0.6)			
BROM	BROM81	3.9	93.7	-	-	93.1	98.0	97.6
			(0.7)			(0.2)	(0.9)	(0.2)
	BROM31	3.7	90.9	-	-	90.9	95.3	99.1

Sample code	Batch no.	pH	Percent label claim					
			MEN	**AMB**	**CHL**	**GUA**	**BROM**	**SALB**
			(1.4)			(0.1)	(0.7)	(0.3)
	BROM83	3.9	97.8	-	-	96.1	95.7	98.0
			(0.4)			(0.6)	(0.3)	(0.8)
ZDX	ZDX92	3.8	37.7	-	-	-	93.6	-
			(0.7)	-	-	-	(0.1)	-
	ZDX91	3.8	36.0	-	-	-	91.2	-
			(1.4)				(0.9)	
TRIC	TRIC1T	4.8	50.7	-	96.9	-	-	-
			(0.3)		(0.8)			
	TRIC7T	4.8	66.6 (0.6)	-	100.4 (0.2)	-	-	-
BROX	BROX34	3.5	54.1	100.6	94.1	95.6	-	-
			(1.8)	(0.1)	(1.3)	(0.7)		
BZN	BZN86	3.6	107.3	97.2	-	95.8	-	97.3
			(0.2)	(0.5)		(0.9)		(0.1)
DAC-M	DAC-M66	4.9	103.5	-	-	-	99.3	98.2
			(0.5)				(0.3)	(0.7)
LUN	LUN47	4.7	99.3	-	98.1	-	-	-
			(1.5)		(0.1)			
DEL-C	DEL-C47	4.8	92.5	-	-	-	-	-
			(1.7)					
CAD	CAD39	4.4	105.8	-	96.4	97.3	-	-
			(0.1)		(0.9)	(0.4)		
	CAD75	4.4	104.3	-	99.2	96.8	-	-
			(0.6)		(0.7)	(0.3)		

Sample code	Batch no.	pH	Percent label claim					
			MEN	**AMB**	**CHL**	**GUA**	**BROM**	**SALB**
CADP	CADP20	4.4	74.4 (0.5)	-	97.6 (0.8)	99.1 (0.1)	-	-
	CADP01	4.5	84.7 (0.7)	-	97.3 (0.5)	98.9 (0.5)	-	-
BEN-A	BEN-ABE	4.3	103.4 (0.7)	-	-	-	-	-
	BENA0BE	4.3	107.8 (1.3)	-	-	-	-	-
DEL-P	DEL-P02	4.8	98.0 (1.6)	-	96.8 (0.4)	-	-	-
	DEL-P89	4.8	95.2 (0.9)	-	94.2 (0.7)	-	-	-
ASC-D	ASCD25	3.9	94.6 (0.5)	-	100.5 (0.3)	-	-	-

Values in parentheses is the RSD from replicate determination (n=3)

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General discussion

A simple, reliable and inexpensive GC method was developed for the analysis of menthol in cold-cough syrups that may also contain AMB, CHL, GUA, BRO and SAL in various combinations. The validated method was used together with a validated HPLC method to completely determine all APIs in nine out of twenty-one samples analyzed.

The method utilizes nitrogen as a carrier gas which is inexpensive thereby making it readily applicable. In addition, the choice of FID imparts versatility to the method since it can be used in detection of most hydrocarbons provided no further characterization is required.

During method validation, it was necessary to determine LOD and LOQ. The LOQ provides an indicator of the lowest concentration of an analyte in a sample that can be reliably quantitated. This provides a measure of applicability of the method in quantification of low level analytes such as in toxicological studies. Small variations in initial oven temperature, injector port temperature and mobile phase flow rate did not affect the method.

5.2 Conclusion

A gas chromatographic method was developed for the determination of menthol in cold-cough syrups in the Kenyan market. Compared to other published methods, this GC method is inexpensive with a shorter run time This method was used together with a validated high-performance liquid chromatography method to fully assay MEN in presence of other APIs such

as AMB, GUA, CHL, BRO and SAL which are common ingredients in cold-cough syrups in Kenya.

5.3 Recommendations

Chloroform was used as a diluent for samples and standards. However, the use of chloroform is associated with potential health risks and therefore, more studies should be conducted to evaluate the suitability of alternative solvents. Additionally, collaborative studies to evaluate ruggedness of the method should be conducted. This allows the method to be applicable in different analytical laboratories with suitable accuracy and precision.

This method can be used in pharmaceutical quality control laboratories to carry out pre-registration analyses in which case PPB can include menthol to be analyzed as part of the active pharmaceutical ingredients. This method can also be used in routine analyses such as post market surveillance to curb substandard and counterfeit cold-cough syrups containing menthol.

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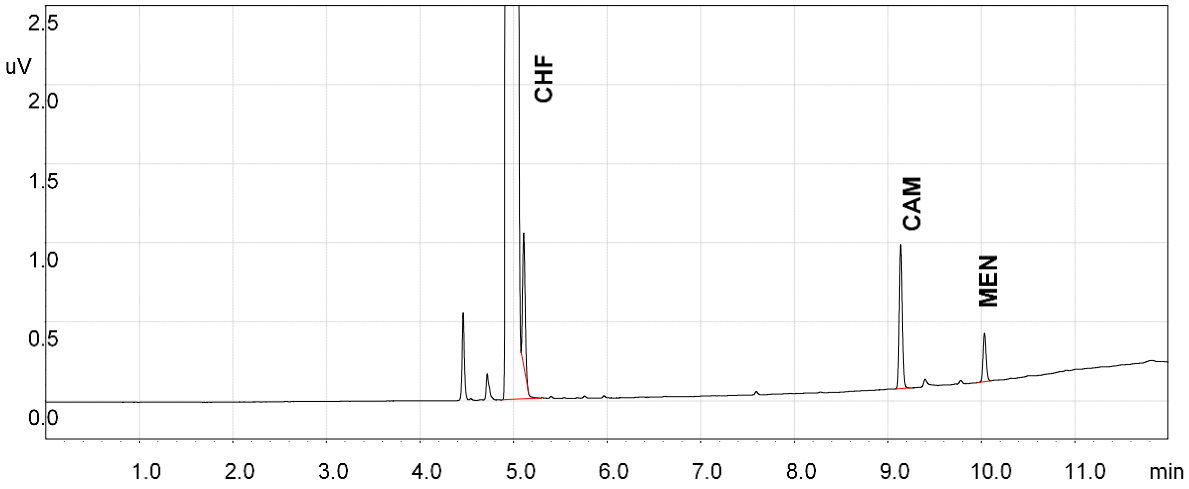
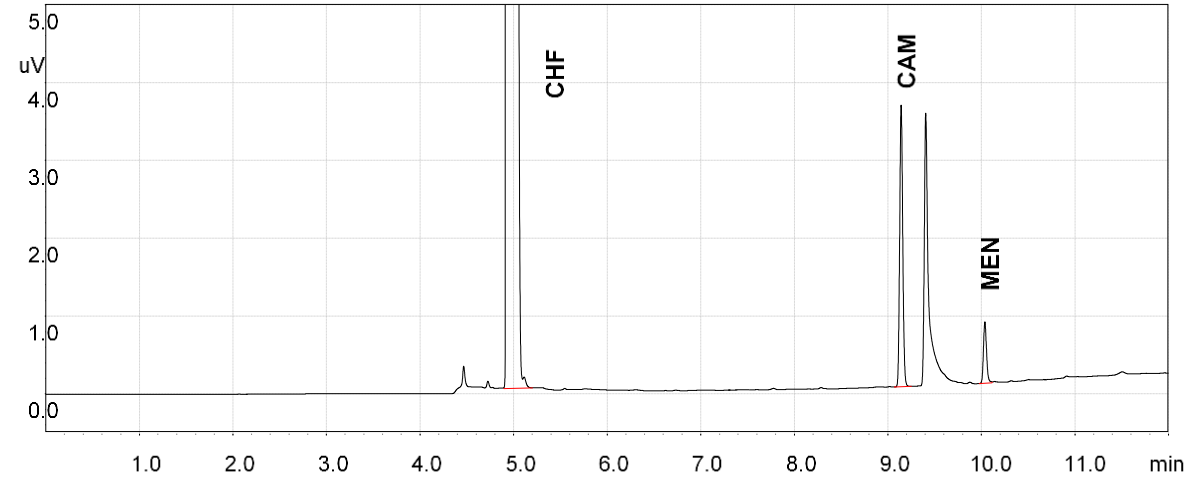
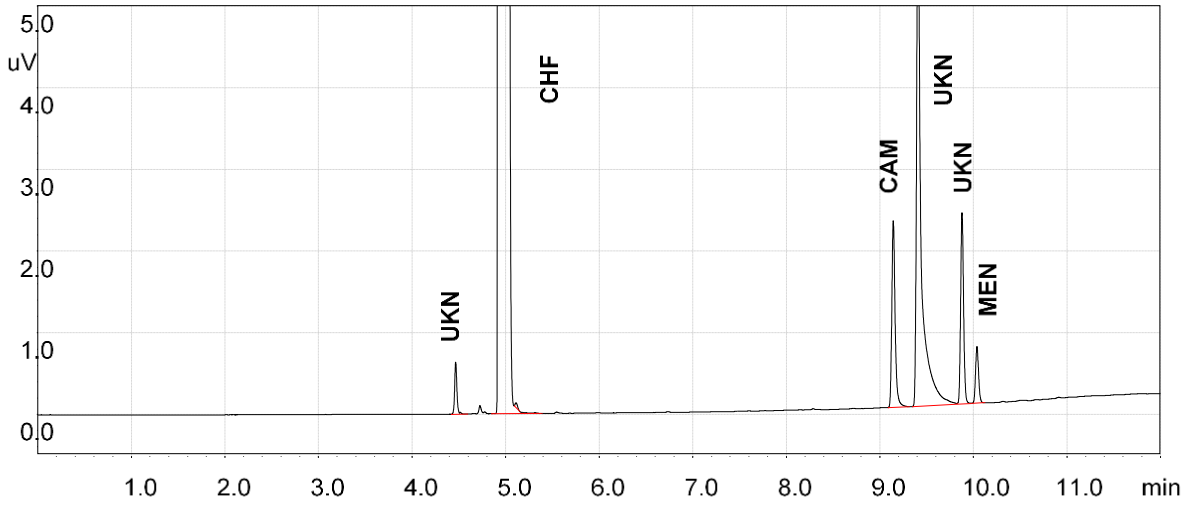
APPENDICES



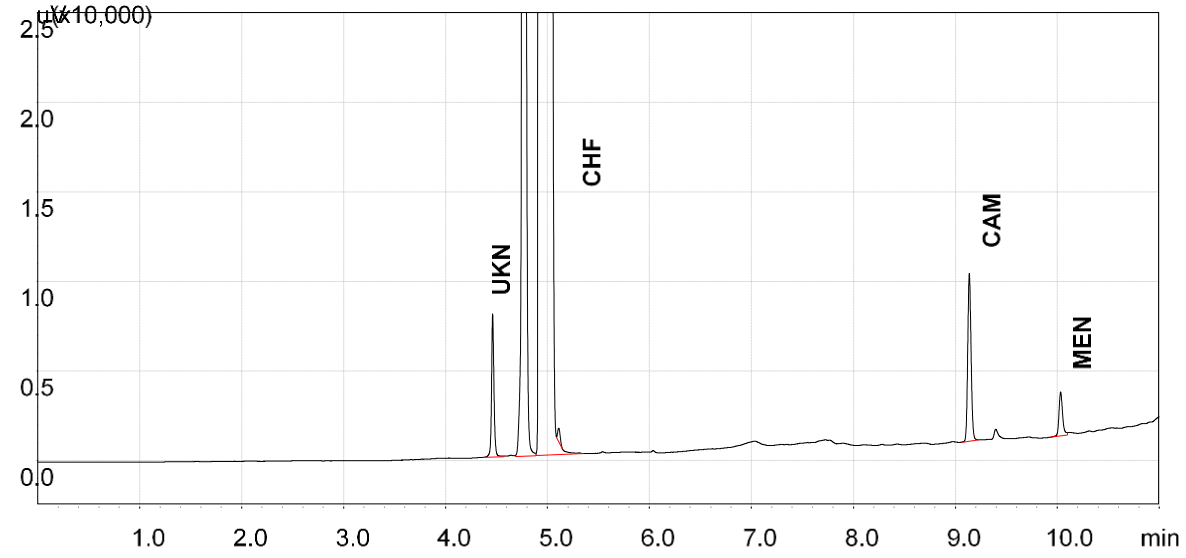
Appendix 1: A representative gas chromatogram for BEN-A sample



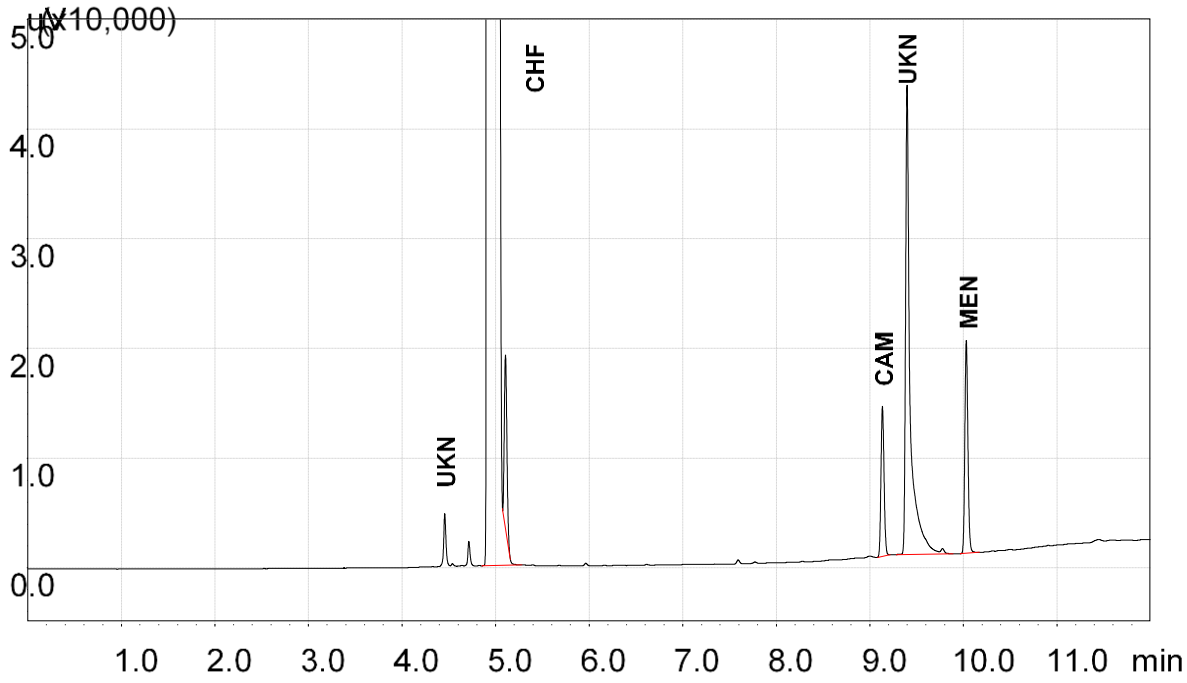
Appendix 2: BRX representative sample gas chromatogram



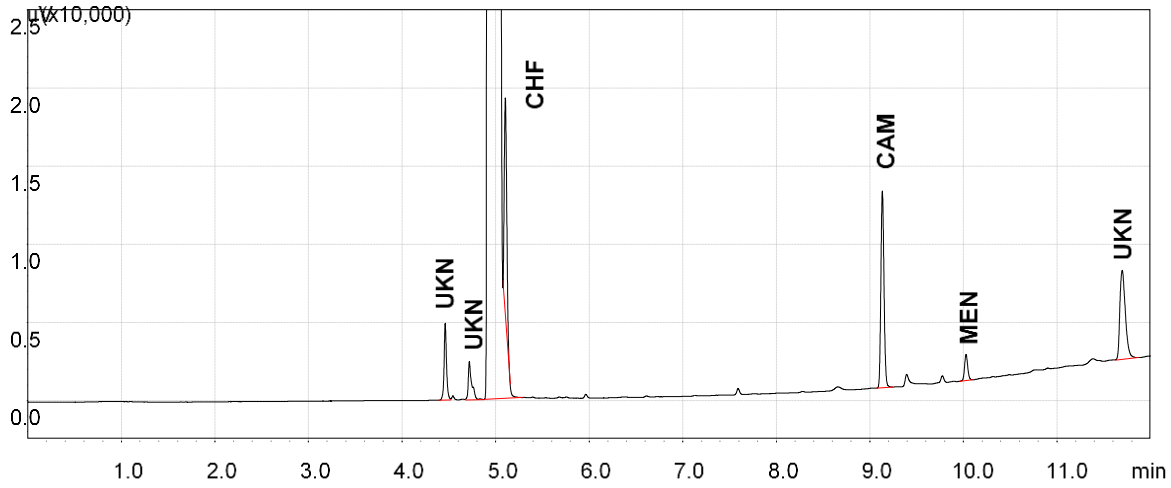
Appendix 3: BZN representative sample gas chromatogram



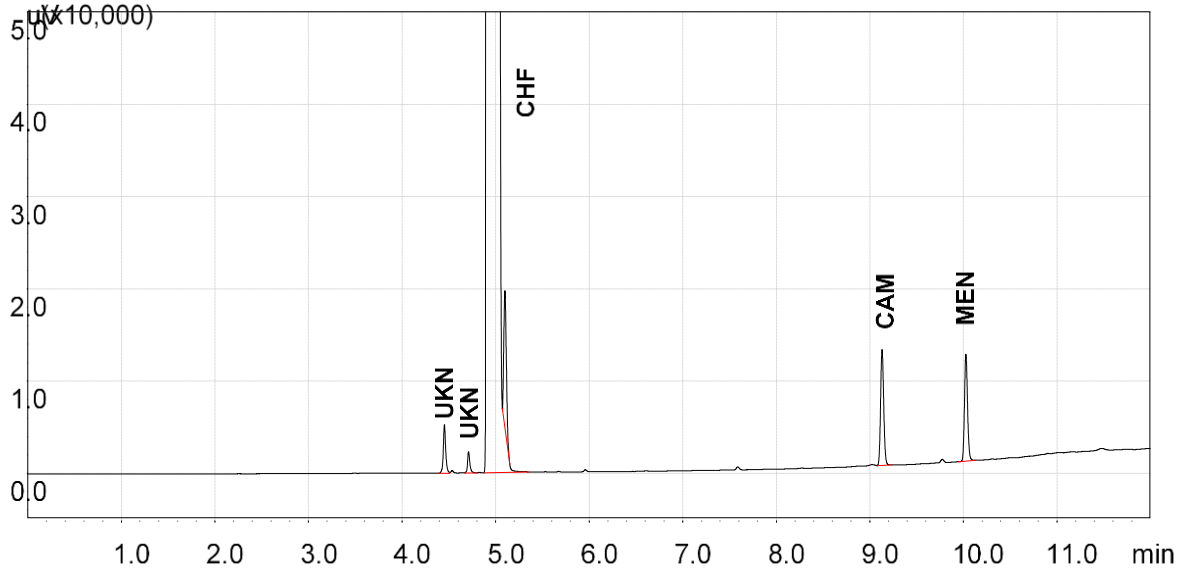
Appendix 4: DAC-E representative sample gas chromatogram



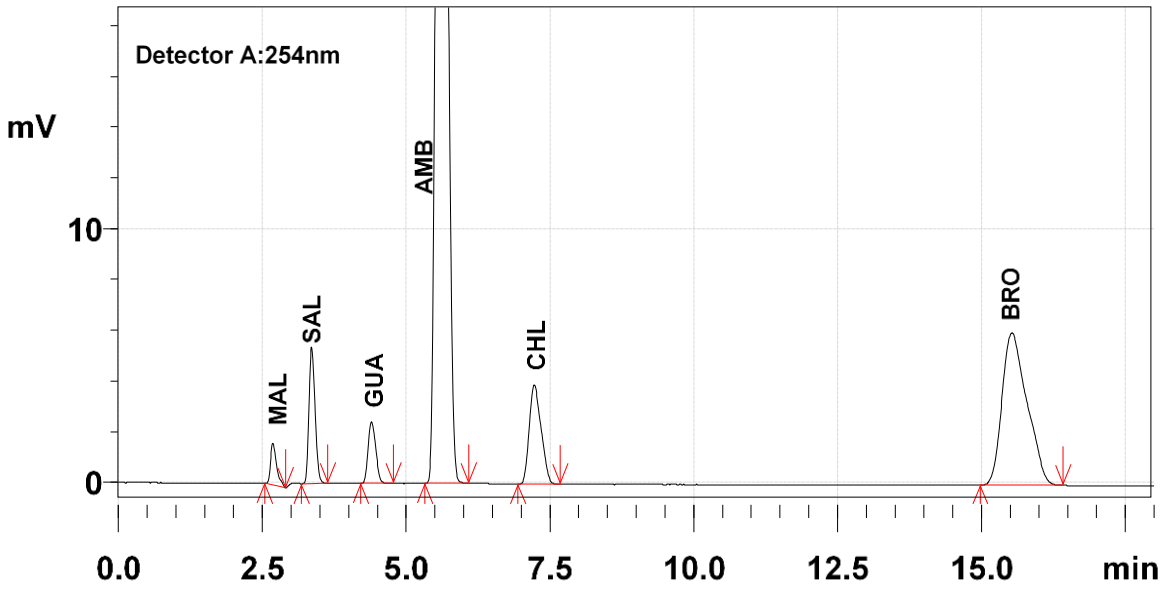
Appendix 5: ASC-D representative sample gas chromatogram



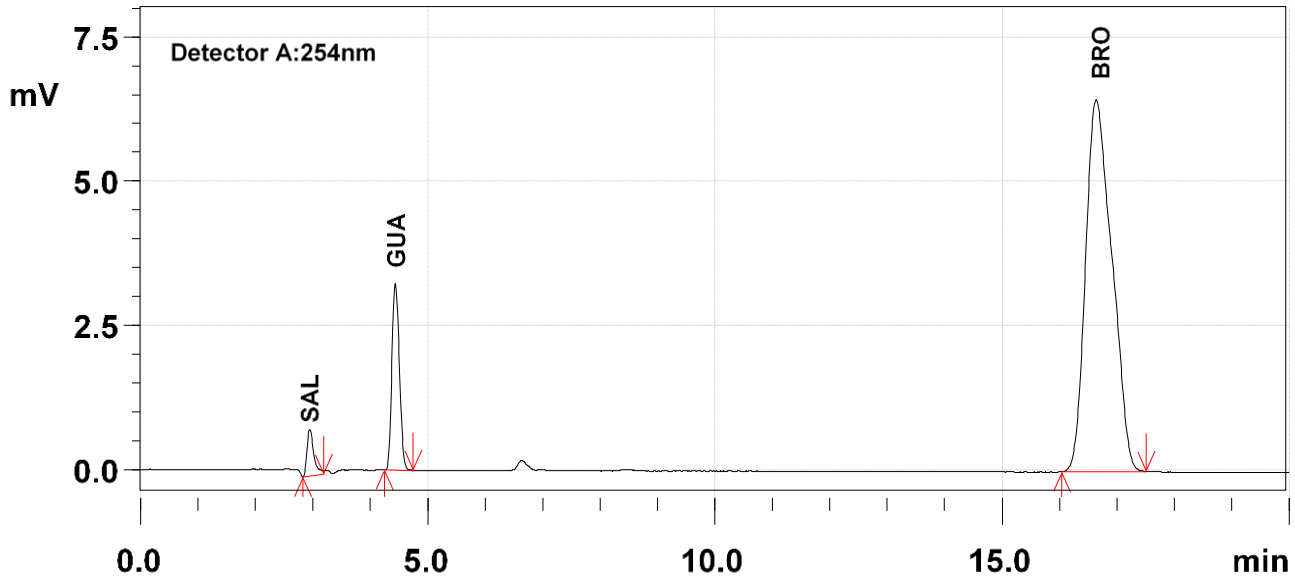
Appendix 6: SAL representative sample gas chromatogram



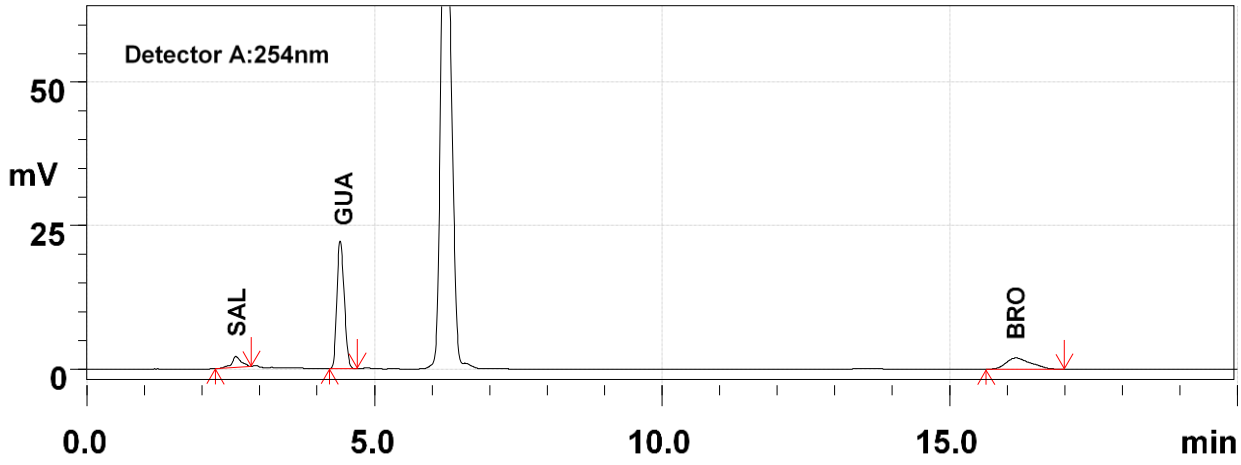
Appendix 7: Camphor and Menthol standards representative gas chromatogram



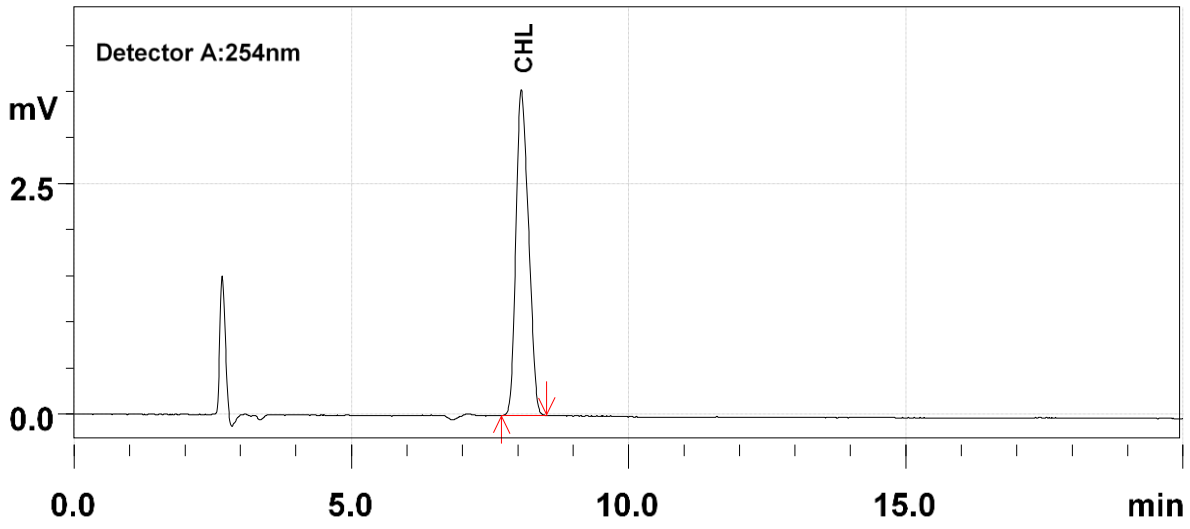
Appendix 8: HPLC standards representative liquid chromatogram. MAL-maleic acid, SAL-salbutamol, GUA-guaifenesin, AMB-ambroxol, CHL-chlorpheniramine, BRO-bromhexine



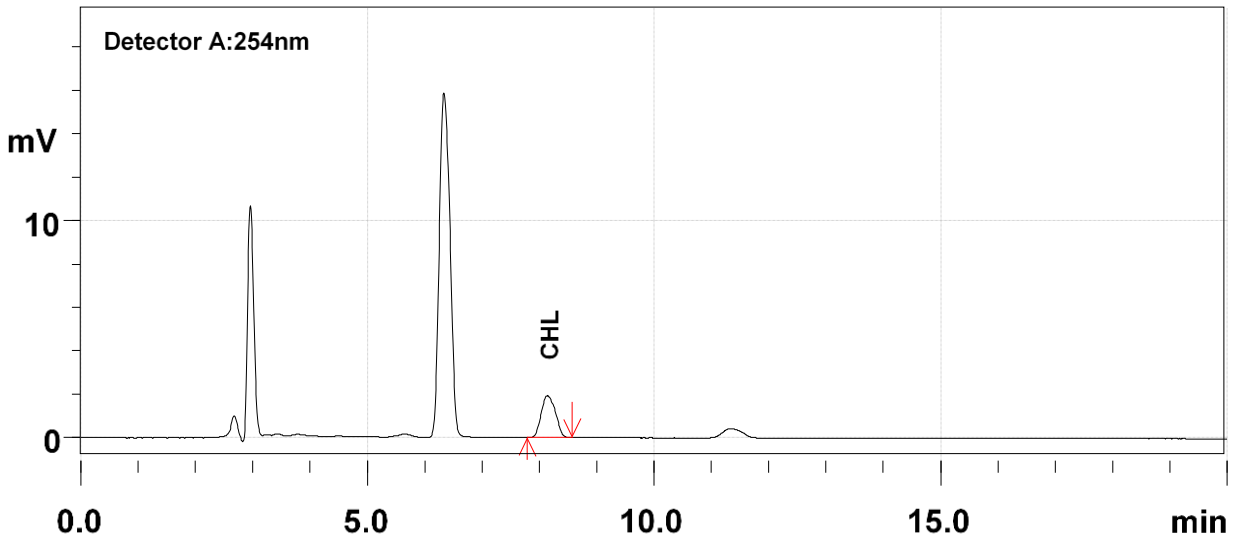
Appendix 9: SAL sample representative liquid chromatogram



Appendix 10: ASC sample representative liquid chromatogram



Appendix 11: DEL sample representative liquid chromatogram



Appendix 12: CON sample representative liquid chromatogram