FORENSIC ANALYSIS OF PEPPER SPRAYS BY CHROMATOGRAPHIC TECHNIQUE

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FORENSIC ANALYSIS OF PEPPER SPRAYS BY CHROMATOGRAPHIC TECHNIQUE

by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF EQUATIONS

Page

LIST OF SYMBOLS

°C	Degree Celcius
PPM	Parts Per Million
g	Gram
mL	Milliliter
µL	Microliter

LIST OF ABBREVIATIONS

GC-MS	Gas Chromatography-Mass Spectrometry
GC-FID	Gas Chromatography – Flame Ionisation Detector
PCA	Principal Component Analysis
DCM	Dichloromethane
OC	Oleoresin capsicum
N.Cap	Natural Capsaicin
MS	Mass spectrometry
M/z	Mass-to-charge ratio
SHU	Scoville Heat Unit
NMR	Nuclear Magnetic Resonance
IR	Infrared
UV	Ultraviolet
HPLC	High – Performance Liquid Chromatography
PAVA	Pelargonic Acid Vanillyl Amide
SAFE	Solvent-Assisted Flavour Evaporation
HCA	Hierarchical Cluster Analysis
DAD	Diode-Array Detection
SPME	Solid Phase Microextraction
HS	Headspace-Solid
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
CPOO	Chilli Pepper-Flavoured Olive Oil
LC-MS	Liquid Chromatography-Mass Spectrometry
PUFA	High Polyunsaturated Fatty Acid

SFA	Saturated Fatty Acid
TRM	Transient Receptor Potential
GCRP	Calcitonin Gene-Related
CNS	Central Nervous System
VR1	Vanilloid Type 1 Receptors
DARTTM	Direct Analysis in Real Time
AccuTOF	Accurate Time of Flight
LLOQ	Lower Limit of Quantification
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
SE	Solvent Extraction
ASTA	American Spice Trade Association
ECE	Empirical Cross Entropy
Ca^{2+}	Calcium ion
Na^{2+}	Sodium ion
USM	Universiti Sains Malaysia

ANALISIS FORENSIK BAGI PENYEMBUR LADA DENGAN TEKNIK KROMATOGRAFI

ABSTRAK

Penyembur lada telah mendapat populariti sebagai alat pertahanan diri disebabkan mudahnya pembuatan dan kebolehcapaian bahan-bahannya di rumah serta ketersediaannya di pasaran. Potensi penggunaan mereka dalam aktiviti jenayah memerlukan kaedah analisis forensik yang berkesan untuk mengenal pasti dan membezakan bahan kimia yang tersedia dengan tepat. Justeru, kajian ini bertujuan untuk menilai profil kimia bagi penyembur lada buatan sendiri dan penyembur lada komersial di Malaysia dengan gas kromatografi-spektrometri jisim (GC-MS). Dalam kajian ini, lima jenis penyembur lada buatan sendiri yang diperbuat daripada pelbagai jenis cili, iaitu cili merah dan hijau, cili burung merah dan hijau, dan habanero, serta empat jenis penyembur lada komersial yang berbeza telah disiasat. Kedua-dua sampel penyembur lada buatan sendiri dan sampel komersial telah menjalani langkah persediaan sampel untuk mengekstrak sebatian aktif daripada sampel dan dianalisis menggunakan GC-MS diikuti oleh analisis komponen utama (PCA). Daripada kajian ini, satu kaedah GC-MS telah dioptimumklan untuk menganalisis sampel penyembur lada. *Capsaicin* dan *dihidrocapcaicin* telah dijumpai hadir dalam kesemua sampel penyembur lada buatan sendiri. Puncak tambahan yang berkaitan dengan *homocapsaicin, homodihydrocapsaicin,* dan *nonivamide* juga telah diperhatikan dalam penyembur lada yang disediakan daripada cili burung hijau dan merah, serta habanero. Tambahan pula, penyembur lada yang dihasilkan daripada cili burung merah dan habanero menunjukkan puncak tambahan sepadan dengan *nordihydrocapsaicin*. Sebaliknya, penyembur lada komersial hanya mengandungi satu sebatian *capsaicinoid*

iaitu *nonivamide*. PCA membenarkan pembezaan penyembur lada buatan sendiri dan penyembur lada komersial dengan mengambil kira kandungan *capsaicinoid* sahaja. Pembezaan lanjutan, terutamanya antara sampel buatan sendiri boleh tercapai dengan menghuraikan 74 puncak boleh ditafsir daripada profil GC-MS. Secara kesimpulan, kandungan kimia penyembur lada, terutamanya penyembur lada buatan sendiri dan penyembur lada komersial boleh ditentukan melalui kaedah GC-MS. Hal ini boleh digunakan untuk menganalisis sebarang bahan bukti forensik yang disyaki mengandungi sisa-sisa semburan lada untuk mengaitkan kepada sumber yang berkemungkinan.

FORENSIC ANALYSIS OF HOMEMADE PEPPER SPRAY BY CHROMATOGRAPHIC TECHNIQUE

ABSTRACT

Pepper sprays have gained popularity as personal defence tools due to their ease of production and accessibility of ingredients at home, as well as readily availability in the market. Their potential use in criminal activities necessitates effective forensic analysis methods to accurately identify and differentiate the chemical constituents present. Therefore, this study was aimed to evaluate the chemical profiles of homemade and commercially available pepper sprays in Malaysia by gas chromatography-mass spectrometry (GC-MS). In this study, five homemade pepper sprays made up of different species of chilli pepper, namely red and green chilli, red and green bird's eye, and habanero pepper, as well as four commercial pepper sprays were investigated. Both homemade and commercial pepper spray samples underwent sample preparation step to extract the active compounds from the samples and analysed using gas GC-MS followed by principal component analysis (PCA). From the study, a GC-MS method was optimised for the analysis of pepper spray samples. Capsaicin and dihydrocapsaicin were found to be present in all homemade pepper spray samples. Additional peaks corresponding to homocapsaicin, homodihydrocapsaicin, and nonivamide were also observed in green and red bird's eye-, as well as habanero-prepared pepper spray solutions. Moreover, pepper spray solutions produced from red bird's eye and habanero exhibited extra peak corresponding to nordihydrocapsaicin. In contrast, the commercial pepper spray only contains one capsaicinoid compound, namely nonivamide. The PCA allowed for the

differentiation of homemade and commercially available pepper sprays by considering only the capsaicinoid contents. Further discrimination, especially among the homemade samples could be achieved by decomposing the 74 interpretable peaks from the GC-MS profiles. To conclude, the chemical composition of pepper sprays, especially the handmade and commercially available pepper sprays were determined through the GC-MS method. It would be used to analyse any forensic evidence suspected to have contained pepper spray residues to link to the possible sources.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Pepper spray contains an inflammatory compound called capsaicin which can cause burning, pain, and tears, especially when it encounters a person's eyes (Smith, 2023). Cases that were related to misuse of pepper spray had been reported, attracting the attention of forensic communities. In a case written by the New Straits Times (Mokhtar, 2018), eight policemen and five firemen were pepper sprayed by an American woman who refused to move out of her rented condominium near Mont Kiara. Another case related to pepper spray was reported by The Star (Timbuong & Zack, 2017) where a cosplayer dressed in full military tactical gear was arrested for allegedly spraying corrosive liquid at a security guard and a member of the public.

Although with limited statistics, criminal cases involving pepper spray occasionally happened as in the reports by local mass media from time to time. Hence, it is important to study such forensic evidence and its significance in assisting the forensic investigation. This study was conducted to differentiate the profiles of pepper sprays, both homemade and commercially available in Malaysia using analytical method, namely the gas chromatography-mass spectrometry (GC-MS).

1.2 Introduction

1.2.1 Pepper Spray

Oleoresin capsicum (OC) spray, popularly known as pepper spray, is a self-defence weapon that can severely paralyse victims by stinging and inflaming their eyes, noses, and respiratory systems when it is used. The self-defence sprays in portable, pressurised containers with nozzles contain unpleasant substances are sometimes referred to as "pepper sprays" and "tear gases". Law enforcement authorities, security staff, and others looking for personal protection frequently use them or standby for any unfortunate events. In most instances, these pepper sprays are available to these people through proper channels; however, homemade pepper sprays have also raised concerns about public safety. Video and procedure on making homemade pepper sprays are available online, and they can be made easily at home without any complicated procedure. In view of this, regardless of homemade or commercially available pepper sprays, they carry the potential of abuse or misuse (Borusiewicz et al., 2021).

In many countries, these devices can be purchased legally and without authorisation. Pepper spray should only be used for self-defence, but criminals frequently use them to attack and render their victims helpless (Borusiewicz et al., 2021). Examples of commercially available are demonstrated in Figure 1.1. In Malaysia, it is not against the law to carry pepper spray for personal protection since there are no specific regulations prohibiting its use for self-defence. However, the official guidelines on the police website state that obtaining a permit under the Arms

Act 1990 is required if you intend to import, sell, or possess pepper spray (The Sun Daily, 2018).

Figure 1.1 Examples of pepper spray in the bottle

Source: (Cain, 2020)

The most common form of pepper spray is in canisters, however, their availability can also tiny enough to be carried or hidden in jewellery like rings (Princy, 2023) as shown in Figure 1.2. Additionally, there are other varieties of pepper sprays, such as gel, foam, and foggers (Princy, 2023) as shown in Figure 1.3.

Figure 1.2 Examples of pepper spray in the form of rings

Source: (Princy, 2023)

Figure 1.3 Other varieties of pepper sprays, such as gel, foam, and foggers

Source: (Princy, 2023)

1.2.2 Chilli Pepper

The chilli pepper, scientifically referred to as Capsicum annuum L., is a perennial plant belonging to the Solanaceae family. Native to Central and South America, the Capsicum genus comprises around 25 to 30 different species. Chilli pepper fruits exhibit a diverse array of flavours, colours, shapes, and sizes, spanning from intensely spicy to mildly or pleasantly tangy. Figure 1.4 displays a selection of the chilli pepper varieties available. Notably, *Capsicum annuum* stands out as the most encountered species within the Capsicum genus (Idrees et al., 2019).

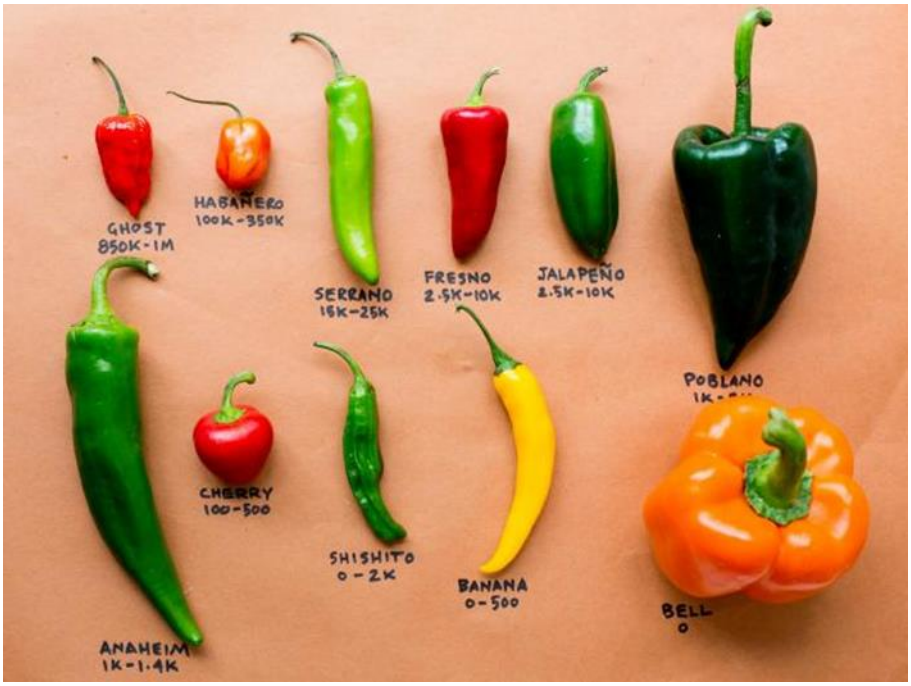

Figure 1.4 Types of chillies

Source: (Brickley, 2015)

Chilli pepper's fruit contains capsaicin and a variety of related chemicals, whose main chemical constituents include straight-chain alkyl vanillyl amides and homologous series branched, collectively known as capsaicinoids. Nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin are the minor capsaicinoids while capsaicin and 6,7-dihydrocapsaicin appear to be the major ones. Steroid alkaloids and glycosides are also present in certain plant. Seeds contain the steroidal glycosides capsicoside A through D and all furostanol (Idrees et al., 2019). Four novel steroidal glycosides, referred to as capsicosides A–D, were discovered in the roots and seeds of both Capsicum annuum var. conoides and Capsicum annuum var. fasciculatum. Additionally, proto degalactotigonin could also be isolated from these sources. Through thorough chemical and spectroscopic analysis, it was confirmed that the structures of capsicosides A–D are furostanol glycosides, which correspond to oligoglycosides of gitogenin and tigogenin (Yahara et al., 1994).

Research conducted by Trovato et al. (2022) demonstrated that the identification of distinct aroma compounds could serve to distinguish between various chilli pepper species, aiding in their detection and traceability within chilli pepper products. The study identified a collective of 269 volatile compounds present in the *Capsicum annuum, Capsicum chinense,* and *Capsicum baccatum species*. Notably, the *Capsicum annuum* species also displayed a notable prevalence of acids and ketones, whereas the *Capsicum chinense* and *Capsicum baccatum* species were recognized for their higher levels of esters and aldehydes, respectively.

1.2.3 Gas Chromatography-Mass Spectroscopy (GC-MS)

Chromatography is a separation process depending on the differential distribution of sample components between moving and stationary phases. It covers all types of chromatography, including gas, liquid, and supercritical fluid chromatography, except for size exclusion chromatography. The use of chromatography was first reported by Ramsey (1905) and Tswett (1906); however, the process during the invention was tedious and used primarily for the separation of compounds derived from natural products and the preparation of small amounts of their components for subsequent investigation (Robertson, 1992). Figure 1.5 shows the schematic diagram of gas chromatography.

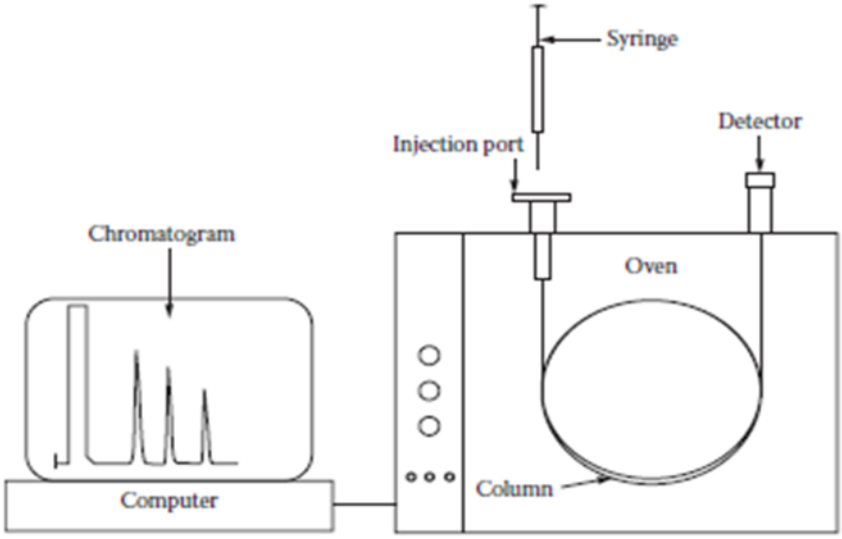

Figure 1.5 Schematic diagram of gas chromatography

Source: (Carlin & Dean, 2013)

Mass spectrometry (MS) is an analytical tool for determining a sample's mass-to-charge ratio (m/z) of one or more molecules of one or more molecules in a sample. These data are frequently used to determine the molecular weight of the sample components. Mass spectrometers can be typically used to identify unknown chemicals through the molecular weight and mass spectra, to quantify the known compounds, and to analyse molecular structure and chemical characteristics of a target compound (Rockwood et al., 2018). Figure 1.6 illustrates the schematic diagram of mass spectroscopy.

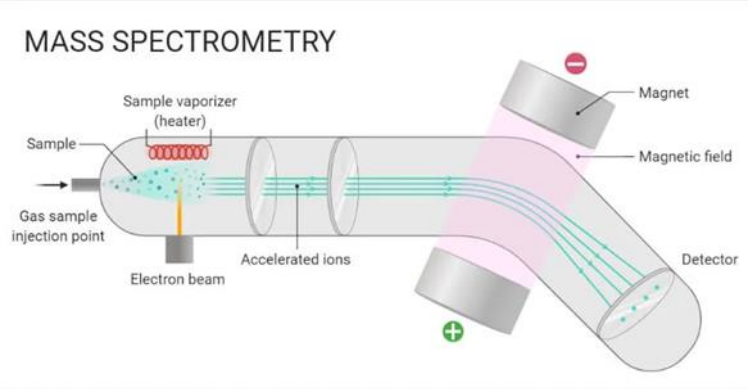

Figure 1.6 Mass spectroscopy

Source: (Rockwood et al., 2018)

1.3 Problem Statement

According to Sentul police commander ACP R. Munusamy, carrying pepper spray for personal protection is not prohibited in Malaysia. There are no regulations that specifically restrict the use of pepper spray for personal safety. It is vital to understand, however, that if the pepper spray is mishandled or utilised inappropriately, legal action may be taken (The Sun Daily, 2018).

Homemade pepper spray can be simply manufactured using many recipes and instructions on the internet. However, because they are neither regulated nor standardised, the composition and effectiveness of homemade pepper sprays can vary greatly. A specific study has not been done in Malaysia to describe homemade pepper sprays created with Malaysian peppers. This indicates that little is known about the precise chemical makeup or efficacy of such handmade sprays.

Forensic investigation in the context of pepper spray may entail investigating its chemical composition, pinpointing the active chemicals that produce its effects, and examining the physical and chemical characteristics of the pepper spray. The

compositional profiles of pepper spray sold commercially available online are also unknown, indicating that a forensic study would be required to comprehend the chemical content of pepper spray. This investigation would involve comparing several pepper sprays that are readily accessible on the market to identify their similarities, differences, and probable compositional variances.

Forensic investigation of the substance may be crucial in legal situations involving the use, abuse, or occurrences related to pepper spray, forensic investigation of the substance may be crucial. If necessary, it could help law enforcement and judicial authorities identify the source or origin of a certain pepper spray sample by offering useful information. Given this, the profile of homemade pepper and commercially available pepper spray is worth investigating.

1.4 Aim and Objectives

This study was aimed to evaluate the chemical profiles of homemade and commercially available pepper sprays in Malaysia by chromatographic technique. To achieve the aim of this study, the objectives were set as follows:

I. To optimise a GC-MS method for the determination of pepper spray profiles.
II. To determine the chemical signature attributes in both homemade and commercially available pepper sprays in the marketplace.
III. To distinguish the profiles of homemade and commercially available pepper sprays through statistical approach.

1.5 Significance of Study

The chemical composition of a pepper spray can indeed reveal crucial forensic evidence about its origin, whether it was homemade or commercially available. Assessing the chemical composition of a sample of pepper spray and identifying any unique markers or characteristics associated with various sources and production processes, can also provide the investigative lead on any cases involving the uses of pepper sprays.

Commercially available pepper sprays are often made up of capsaicinoids, the active components extracted from chilli peppers, as well as other substances such as solvents, propellants, and additives. To ensure safety, efficacy, and uniformity, these commercial formulas can be standardised and controlled through the examination of respective profiles. On the other hand, the chemical content of homemade pepper sprays might vary greatly. Individuals making their pepper spray may utilise a variety of chilli peppers, different extraction methods, and additional substances that are not frequently seen in commercial sprays. These variables may affect the overall effectiveness and use of the homemade pepper spray. Therefore, a policy shall be established to control the production of homemade pepper sprays as not induce harm to the public if there is any misuse.

Forensic analysis using GC-MS can assist in establishing a link between the suspect and the pepper spray used in the crime by comparing the pepper composition retrieved from samples belonging to the suspect and from the crime scene. Forensic scientists can also determine whether an unknown sample matches a commercially accessible product or exhibits features compatible with homemade manufacturing by

comparing its composition to the recognised commercial formulas or reference standards. Furthermore, knowing where a pepper spray came from can help investigators pinpoint its origin, either attributing it to a specific manufacturer or identifying the method by which it was obtained.

Through this study, the forensic investigators would get a better understanding of the varied composition between the two types of pepper sprays, namely handmade and commercially available pepper spray when it is recovered from the crime scene. This information could be essential when formulating sample plans to gather any pepper spray evidence and proposing the appropriate extraction and analytical procedures.

CHAPTER 2

LITERATURE REVIEW

2.1 Species of Chilli pepper

Chilli pepper (*Capsicum annuum L.*) is a Solanaceae family perennial herb. Perennial herb is a type of plant that persists through multiple seasons, with the majority flowering on a yearly basis once they are established (Jekka, 2023). *Capsicum annuum* is called by different names around the world, depending on the type and location. In English, it is commonly referred to as chillies, long chillies, or red chillies. *Surkhmirch* is the Urdu word for it in Pakistan. It is known as *lalmirca* in India, particularly in Hindi, and as *fulfil-e-Ahmar* in Arabic. In other language such as Danish, Dutch, and English known as *chilli peber, chilli peper* and chilli pepper respectively. Capsicum annuum is the most prevalent Capsicum. Only five species are farmed and domesticated *(Capsicum frutescens, Capsicum pubescens, Capsicum annuum L., Capsicum chinense Jacq., and Capsicum baccatum L.)* (Idrees et al., 2019).

Chilli pepper plants have uncomplicated, alternately arranged leaves. Within the family, the defining features include flowers that are either solitary or grouped, consisting of five fused sepals and petals. There are five stamens and a superior ovary, positioned above the attachment point of the other flower components. The ovary is formed from two fused carpels, housing ovules, and it is slightly slanted within the flower on a basal tissue disk. The style, found at the ovary's upper end, is simple and carries a stigma with two lobes that receive pollen. These flowers are typically easily noticeable and attract insects. The resulting fruit is commonly a berry or capsule (Britanicca, 2023).

Depending on the species, the plant can grow up to 2 to 4 feet tall. The leaves are smooth, simple, whole, globous, hairless, and flat, and vary in shape from ovate to elongate with different species. Flowers are often solitary and creamy white, with straw-coloured seeds. All Capsicum species are perennials when grown in unfavourable (semitropical or tropical) climes. Chilli peppers are also grown for their ornamental value, owing to their brilliant, shining fruits that come in a variety of colours (Idrees et al., 2019).

Chilli pepper holds the distinction of being the most extensively utilized spice and condiment globally, highly valued for its spiciness and its ability to impart distinctive flavours to a wide range of cuisines worldwide. Initially utilized primarily for seasoning and medicinal purposes, its applications have expanded to include fresh and processed vegetables, spice production, dried forms, use as a food dye, cultivation as an ornamental plant, and the creation of extracts for diverse industries like pharmaceuticals and cosmetics. In Eritrea, pepper plays a crucial role as an ingredient in the preparation of nearly all Eritrean dishes. The average weekly household consumption of dried pepper in Eritrea is approximately 140 grams. Typically, it's consumed in powdered form derived from dried pods known as 'berbere,' which not only adds flavour but also acts as a food dye. These dried red pods are also central to the creation of 'shiro' powder, a popular sauce in Eritrean cuisine. Moreover, the green pods are enjoyed both raw as a salad or appetizer and cooked alongside various ingredients (Saleh et al., 2018).

It is believed that growing conditions, genetics, geographical origin, the presence of similar chemotypes, and variations in the nutritional quality of plants are all related to the essential oil concentration of chilli peppers. It was reported that trans-

b-ocimene, linalool, 2-methoxy-3-isobutylpyrazine, limonene, hex-cis-3-enol, and methyl salicylate are the main chemical constituents of chilli pepper essential oil. Chemically and morphologically, chilli peppers are very varied. The flavours, fragrances, and medical applications are influenced by the plant's origin, source, and growing environment (Idrees et al., 2019).

Most chilli peppers belong to the five different species in the Capsicum genus. While each existing pepper variety can be categorised into one of these five species, there are 25 known species in the Capsicum genus, most of them being non-domesticated in the New World, with their varieties spanning from the southern United States through Central and northern South America (Morris & Taylor, 2017).

Many well-known varieties like cayenne, jalapeño, serrano, and Thai chillies are derived from *Capsicum annuum*. The extremely hot peppers, such as habanero, Carolina reaper, and ghost chilli (bhut jolokia), are varieties of *C. chinense.* Tabasco comes from *C. frutescens*. These peppers are enjoyed fresh or dried and are utilized to create chilli powder, add flavour to dishes like barbecue and hot curry, and spice up various sauces (Britanicca, 2023).

2.1.1 Capsicum Annum

Native to southern North America, the Caribbean, and northern South America, Capsicum annuum is a member of the plant genus Capsicum. This species is the most widespread and widely grown. The species includes chilli peppers in a wide range of sizes and forms, including sweet bell peppers and various nightshade-related chilli

pepper cultivars like jalapenos, New Mexico chiles, and cayenne peppers (Swamy, 2023).

Its herb or small shrub can reach heights of 0.3 to 1.2 meters and a width of 15 to 30 cm. It has roughly oval-shaped, smooth-margined, glossy leaves that can grow up to 7.5 cm long. Some varieties have dark purple or black foliage. Flowers are 4-5 petalled, star- or bell-shaped, and can be white, green, or purple in hue. Its fruit is a genuine berry and comes in various sizes, colours, flavours, and sweetness. The fruit can be green, red, yellow, orange, or even black, depending on the variety, and many of them change colour as they ripen (Swamy, 2023). Figure 2.1 illustrates an example of chilli pepper in *Capsicum annum* species.

Figure 2.1 Example of Capsicum annum species

Source: (Britanicca, 2023)

2.1.2 Capsicum Chinense Jacq.

The immense morphological plasticity of the domesticated forms of *C. chinense* is seen in the many fruit sizes, colours, and shapes, which are typically quite spicy and aromatic. There are also some varieties with sweet fruits, such as the popular "pimenta de bico" in Brazil's Minas Gerais state(Carvalho et al., 2014).

The peppers from this species known as "habanero," "pimenta-de-cheiro," "murupi," "pimenta-de-bico" (also known as "biquinho," "pimentade- bode," and "cumari-do-Pará") are the more popular varieties. In Roraima State, Brazil, both indigenous and non-indigenous people choose the morphological type "murupi" to make sauces and "jequitaia" (pepper powder). When ripe, murupi fruits are elongated, have a rough surface, and are coloured yellow or red. They are weighed at less than 4.5 gram each and have a distinctive aroma and strong pungency that can reach levels of over 220,000 SHU (Scoville Heat Unit). This species is popular throughout the tropical area and is frequently used in Caribbean cuisine to add flavour and pungency(Carvalho et al., 2014). Figure 2.2 illustrates an example of *Capsicum chinense Jacq* species.

Figure 2.2 Example of *Capsicum chinense Jacq* species

Source: (Britanicca, 2023)

2.1.3 Capsicum Frustescens

The general characteristics of *C. frutescens* include small, conical, upright, spicy fruits with thin fruit walls that are often red when ripe and lack the calyx annular constriction between the calyx and pedicel. However, populations of C. frutescens

have previously shown differences in the location, size, shape, and colour of fruits; as a result, they are regarded as domesticated. The most prevalent example, which was long thought to be the only domesticated type, is the well-known "Tabasco pepper" grown in the United States(Carvalho et al., 2014).

Baral & Bosland, (2004) reported the existence of the accessions of *C. frutescens* showing big, pendant, and persistent fruits, suggesting the existence of other domesticated forms, besides cultivated “Tabasco”. Figure 2.3 illustrates an example of Capsicum frutescens species.

Figure 2.3 Example of *Capsicum frutescens* species

Source: (Carvalho et al., 2014)

2.1.4 Capsicum Baccatum

In contrast to the other domesticated chilli pepper species, which are widely cultivated across the Americas, baccatum peppers are mostly only popular in South American nations like Peru and Brazil. These peppers stand out among variations of other species because of their distinct citrus or fruity flavour and attractively aromatic aroma. Every baccatum pepper plant has cream or yellow dimples on its flower pedals. Most peppers of baccatum origin have the prefix "aji" at the beginning of their names,

such as the aji amarillo and aji omnicolour (Swamy, 2023). Figure 2.4 illustrates an example of *Capsicum baccatum* species.

Figure 2.4 Examples of *Capsicum baccatum* species

Source: (Geek, 2023)

2.1.5 Capsicum Pubescens

Pubescens pepper plants, the most recognisable pepper species, with dark purple blooms, big, black seeds, and short, hairy stems and leaves. With origins in Peru before the Inca Empire, this pepper was perhaps the first to be domesticated. Like the manzano and locoto peppers, the fruits often have thick flesh and are enormous in size. It is originated so near the equator, but due to its long history of cultivation at high altitudes in the Andes Mountains, it is the pepper plant that can tolerant the frigid environmental conditions (Swamy, 2023). Figure 2.5 illustrates an example of *Capsicum pubescens* species.

Figure 2.5 Example of *Capsicum pubescens* species

Source: (Britanicca, 2023)

2.2 Components in Chilli Pepper

Carotenoid pigments, such as cucurbitaxanthin A, zeaxanthin, capsorubin, lutein epoxide, zeaxanthin, capsanthin, β-carotene, anteraxanthin, violaxanthin, β-cryptoxanthin, and neoxanthin are abundant in chilli peppers (Yanti & Warsi, 2021). The calorie value of chilli peppers is lower, and they do not have any cholesterol or fat in it. Chilli pepper is a good source of various vitamins, including vitamin E, vitamin C, vitamin A, and vitamin B complex, as well as minerals including calcium, iron, potassium, thiamine, folate, manganese, molybdenum, and luteolin, as well as flavonoids, quercetin, and polyphenols (Idrees et al., 2019).

Phenolics, flavonoids, carotenoids, and alkaloids are all present in chilli pepper. Amongst, a class of alkaloids found in chilli pepper is known as capsaicinoids. Alanine, scopoletin, chlorogenic acid, caffeic acid, linalool, amyrin, camphor, carvone, citric acid, linoleic acid, oleic, cinnamic acid, piperine, as well as vitamins B1, C, B3, and E are among the other phytochemicals found in the plant. Compared to oranges, chilli pepper provides seven times more vitamin C. Strong antioxidants that aid in destroying free radicals such as the vitamins A and C as well as the beta-

carotenoids can also be found in chilli (Idrees et al., 2019). According to Yanti & Warsi, (2021) additional bioactive compound such as peptides (e.g., defensin, thionin-like peptide), phytol, fatty acids (e.g. myristic acid, methyl stearic, methyl linoleic), and flavonoids (quercetin, luteolin, rutin) are also made up the compositions of chilli pepper.

Chilli peppers also contain magnesium, sodium, phosphorus, sulfur, and selenium. In the fruit of Capsicum species, there are low levels of volatile or essential oils, which range from 0.1% to 2.6% in paprika. Additionally, the oil extracted under atmospheric pressure contains other compounds in higher concentrations. These compounds include benzaldehyde, nona-trans, non-1-en-4-one, nontrans-2-en-4-one, and trans-2 and 5-dien-4-one (Idrees et al., 2019)

2-isobutyll-3-methoxy pyrazine found in chilli peppers gives them their flavour. Aroma can be contributed by substances like trans-2, 4-dienal, nona-trans, cis-2, 6-dienal, and decatrans. Fixed oil made from seeds contains roughly 60% triglycerides, including linoleic acid and other unsaturated fatty acids. There are several fatty acids to be found in the composition, including linoleic acid, tricosanoic acid, linolenic acid, palmitic acid, behenic acid, stearic acid, arachidic acid, and lignoceric acid(Idrees et al., 2019).

It was noted that bioactive compound refers to a substance with biological effects, influencing one or more metabolic processes to enhance overall health. Capsanthin imparts the red colour to chilli peppers and possesses potent antioxidant properties. Violaxanthin is also a predominant carotenoid antioxidant in yellow chilli peppers, constituting a significant portion (37–68%) of total carotenoid content. Lutein, found

most abundantly in green (young) chilli peppers, is reported to decreases as the peppers mature. Increased lutein consumption can be linked to better eye health. Sinapic acid, also known as sinapinic acid, can offer diverse potential health benefits due to its antioxidant properties. On the other hand, ferulic acid is an antioxidant with potential to protect against chronic ailments. Notably, the antioxidant content in mature (red) chilli peppers is considerably higher than that in immature (green) ones (Arnarson, 2023).

Chilli peppers may boast a wealth of vitamins, minerals, and antioxidants. However, due to their consumption in small quantities, their contribution to daily nutrient intake is modest. They abundant in vitamin C, vital for immune function and wound healing. Vitamin B6 supports the energy metabolism, while vitamin K1 is crucial for blood clotting, bone health, and kidney function. Potassium, an essential dietary mineral, serves diverse roles and adequate intake may lower the risk of heart disease. Apart from that copper, a vital trace element, is integral to sturdy bones and the health of neurons (Arnarson, 2023).

2.3 Capsaicinoids

The term “capsaicinoids” describes a group of pungent chemical analogues found in chilli peppers (*Capsicum annum and C. frutescens)* (Reilly et al., 2001). The phenolic alkaloids known as capsaicinoids, which include the compounds capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin, are unique to the genus Capsicum (Antonious, 2018).

An acyl chain with 10 to 11 carbon atoms and a vanillamide moiety (4-hydroxy-3-methoxybenzylamide) make up the distinctive chemical structure of capsaicin (or its

variants). Pain is caused by capsaicinoids that activate the vanilloid receptor. This receptor functions as a molecular integrator of inputs that could be harmful (e.g., low pH and high temperature). Studies on the structure and action of capsaicin and other related compounds have shown that pungency strictly requires the vanillyl ring and an acyl chain of 8–12 carbon atoms. Due to variations in natural capsaicinoids to stimulate membrane depolarisation by binding to the vanillamide receptor, the pungency of these compounds varies (Reilly et al., 2001).

The active component in chilli peppers, capsaicin, is also the hot pepper's most common irritating molecule and what gives people a burning feeling (Antonious, 2018). Cold water does not dissolve capsaicin; however, alcohol, acetone, ether, and other comparable solvents do. One of the most noxious substances known may be tasted at 1 in 15 to 17 million dilutions. The vanillyl group, the acid-amide connection, and the alkyl sidechain are the three distinguishing characteristics of capsaicin molecule. Any one of these changes will lessen pungency (Pickersgill, 2003).

Despite having the same pungency as capsaicin, nonivamide, or "synthetic" capsaicin, has not been positively identified as a natural substance (Reilly et al., 2001). Several researchers concluded that the presence of nonivamide in oleoresin capsicum was a sign that the sample contain adulteration (Cordell & Araujo, 1993). In 1919, Nelson achieved the first synthesis of nonivamide, and subsequently, he and other scientists explored various alkyl vanillylamides for their spiciness. When a diluted alcoholic solution of this compound matched the pungency of capsaicin, only nonivamide was determined by a taste-testing panel to possess an equivalent level of spiciness as capsaicin. As a result of this study, nonivamide came to be known as "synthetic capsaicin" (Constant et al., 1996).

According to a study by Constant and Cordell (1996), they used complexation chromatography to separate the co-eluting substances to distinguish between natural and synthesised capsaicinoids. Nonivamide was extracted from capsicum oleoresin using the same mobile-phase method. Purified samples of the substance were subjected to 1H- and 13C-Nuclear Magnetic Resonance (NMR) (tests, and they were contrasted with a real sample of synthetic nonivamide. The pure natural nonivamide's chemical shifts were identical to the genuine sample of synthetic nonivamide. Apart from that, nonivamide isolated from capsicum oleoresin had spectra that matched those of the real material in the mass spectrometer (MS), infrared (IR), and ultraviolet (UV). According to high-performance liquid chromatography (HPLC) analysis, nonivamide represented 0.25% of the total capsaicinoids. It could be inferred that nonivamide is a natural product and a part of capsicum oleoresin as a result (Constant et al., 1996).

Raw materials originated from chilli, particularly the capsaicinoids could be identified using GC-MS. The primary capsaicinoids present in the pepper spray solution could also be quantified and used for the differentiation of various pepper spray solutions. Nonivamide could be separated using GC-MS. It was noted the nonivamide shares the same chemical structure as pelargonic acid vanillyl amide, known as PAVA, and is made artificially. Thus, PAVA is not a natural extract (Junior et al., 2019).

The most abundant and potent analogues in peppers (and consequently pepper extracts) are capsaicin and dihydrocapsaicin (Reilly et al., 2001). Dihydrocapsaicin, which constitutes up to 22% of all capsaicinoids, has a pungent flavour that is like that of capsaicin. Of the naturally occurring capsaicinoids in spicy peppers, capsaicin (N-vanillyl-8-methyl-6-nonenamide) and dihydrocapsaicin made up roughly 80–90% of

the mixture. Approximately 7% of the mixture of capsaicinoids is nordihydrocapsaicin. Homocapsaicin, which makes up 1% of all capsaicinoids and has half the pungency of capsaicin, is more potent. About 1% of all capsaicinoids are homodihydrocapsaicin, and it has about half the pungency of capsaicin (Antonious, 2018). Nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin are also present, but generally contribute little to the total capsaicinoid concentration and pungency of the pepper (Reilly et al., 2001). Commonly capsaicin and dihydrocapsaicin ratios range from 1:1 to 2:1. Each capsaicinoid has a vanilloid group, which is an aromatic ring with a hydroxyl and a methyl group, as well as a lengthy hydrocarbon chain and a polar amide group attached (Antonious, 2018). Table 2.1 shows the percentage of capsaicinoids commonly present in the chilli pepper.

Table 2.1 Percentage of capsaicinoids present in the chilli pepper.

Capsaicinoids	**Percentage of capsaicinoids present in the chilli pepper**
Capsaicin	80–90%
Dihydrocapsaicin	≈22%
Nordihydrocapsaicin	≈7%
Homocapsaicin	≈1%
Homodihydrocapsaicin	≈1%

The concentration of capsaicinoids in a pepper varies depending on the type of pepper, the growing environment, and the time of harvest and ranges from 0.1 to 2.0% (dry weight). Variations in the relative proportions of capsaicinoid analogues also occur in response to the parameters in addition to variances in the overall capsaicinoid content (Reilly et al., 2001).