Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
Department of Chemistry

Development of an Analytical Method for Morphinanes Determination in Poppy Seeds – Parametric Study and Limits of Applicability

Master's Thesis

Author: Aswathy Vinod
Study Program: Sustainable Agriculture and Food Security

Thesis Supervisor: doc. Ing. Petr Kačer, Ph.D.

Declaration

I hereby declare that I have authored this master's thesis carrying the name "Development of an Analytical Method for Morphinanes Determination in Poppy Seeds – Parametric Study and Limits of Applicability" independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the master's thesis, I further state that I have not infringed the copyrights of third parties in connection with its creation.

In Prague on 14th April 2023 _____

Aswathy Vinod

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Development of an Analytical Method for Morphinanes Determination in Poppy Seeds – Parametric Study and Limits of Applicability

Summary

The thesis aims to develop and validate an analytical method to determine the quantity of the various alkaloids present in poppy seeds collected from the opium poppy (*Papaver somniferum L.*). Despite the ban on poppy seeds in several countries due to major concerns owing to the trace presence of opium alkaloids, they are still widely used in the food and pharmaceutical industries. Therefore, it is imperative to understand the level of contamination of opium alkaloids (Morphine, Codeine, Thebaine, Noscapine and Papaverine) and develop methods to analyse the levels of alkaloids in poppy seeds while finding various techniques to reduce these levels to ensure adherence to European Commission Regulation (EC) No 1881/2006.

The thesis focuses on conducting experimental studies on various samples of poppy seeds by combining High-Performance Liquid Chromatography with detection by Mass Spectrometry. The study is also devoted to experimenting with varying parameters of temperature, UV-irradiation, treatment with hydrogen peroxide, exposure to microwave and pH value of the environment to see the effects on the content of the contaminant of alkaloids present on the surface of the poppy seeds.

The values of alkaloids present in some of the varieties of poppy seeds did not comply with EC No 1881/2006. It was observed that there was no significant difference in levels of alkaloids extracted from the whole and ground samples of poppy seeds. Furthermore, the pH value can significantly influence the concentration of morphinans extracted from the surface of the poppy seeds. Treating the poppy seeds at temperatures above 220°C substantially reduces the concentration of opium alkaloids. While treating the samples under UV-C irradiation, there was a slight reduction in the alkaloid content. Treating poppy seeds with H_2O_2 vapours was found to be more effective than liquid H_2O_2 in reducing the concentration of opium alkaloids.

Keywords: *Papaver somniferum*, Morphine, Alkaloids, Morphinans, HPLC-MS, Codeine, Thebaine, Noscapine, Papaverine

Contents

1. Introduction

Poppy seeds are obtained from the plant *Papaver somniferum L*. are classified as an oilseed (Carlin et al., 2020). Primarily harvested for its seeds, it has multi-faceted use across the food and pharmaceutical industries. Though the origin of the poppy remains vague, it is believed to have originated in the Middle Eastern and Mediterranean regions with the cultivation dating back to the Neolithic age. In 1806, morphine was isolated from the dried latex of opium poppy for the first time and used during the First World War as a pain suppressant. The history of the poppy seed also indicates its cultivation as an oilseed at the beginning of the 19th century.

Poppy seeds have a pleasant nutty taste and aroma and are used prominently in baked goods and as a spice, thickener, and fat replacer. Poppy seeds are good sources of minerals, carbohydrates, essential amino acids, and fatty acids. The poppy plant is utilized in its raw form to produce narcotics including morphine, codeine, heroin, and oxycodone which are employed therapeutically to treat pain and induce anaesthesia. The continuous use of narcotics can lead to physical and psychological dependence. While the edible poppy seed oil is extracted from the mechanical pressing of *Papaver somniferum*. The oil is a rich source of tocopherols including Vitamin E and is used primarily for manufacturing paints, varnishes, and soaps and occasionally as a cooking oil.

The thesis aims to determine the content of alkaloids present in different varieties of poppy seeds. The thesis also verifies whether the varieties tested meet the permissible value of alkaloids as per the EC No 1881/2006. Subsequently, various experimental tools are used to determine the effects of physical and chemical factors to minimise the content of morphinans which can be applied by small-scale producers of poppy-based food products.

2. Scientific hypothesis and objectives of the thesis

The objective of the thesis is to develop an efficient and accurate analytical method using chromatographic separation and detection to identify the alkaloid contaminants in poppy seeds. The thesis is developed under the hypothesis that poppy seed surface is contaminated by morphine, codeine, thebaine, noscapine and papaverine, which can be separated, extracted, and analysed using a robust and efficient analytical method. The method needs to be validated to ensure its efficacy.

The thesis also hypothesis the influence of altering various physical-chemical parameters like temperature, UV-irradiation, use of hydrogen-peroxide, microwave, and pH (different acids) on the parametric value of the alkaloid contaminants on poppy seeds.

3. Literature Overview

3.1. Poppy

3.1.1. Taxonomic Hierarchy

Kingdom: *Plantae* (Plants)

Subkingdom: *Viridiplantae* (Higher Plants)

Superdivision: *Embryophyta*

Phylum: *Magnoliophyta* (Angiosperm)

Class: *Magnoliopsida* (Dicotyledonous)

Subclass: *Magnoliidae* (Flowering Plants)

Order: *Ranunculaes*

Family: *Papaveraceae* (Poppy Family)

Genus: *Papaver* (Poppy)

Species: *Papaver somniferum L.*

The poppy plant falls under the genus of *Papaver* which includes about 100 species with various concentrations of alkaloid compounds (phytochemicals) found in the latex of the plant. The plant family of *Papaveraceae* is known to produce isoquinoline alkaloids such as morphine, codeine, thebaine, noscapine, and papaverine. Isoquinoline alkaloids are heterogenous group biogenetically derived from L-phenylalanine and L-tyrosine and include an isoquinoline or a tetrahydroisoquinoline ring as a basic structural feature in their skeleton (Casciaro et al., 2020).

3.1.2. Poppy Plant

The poppy plant encompasses a variety of species falling under the *Papaveraceae* or the poppy family. Poppies are characterized by the presence of a milky sap (latex), brightly coloured petals and radially arranged seed stigmatic forming the tissue capsule which are the developed ovaries (fruit) (Hindawi et al., 2020). Though the defining features of poppy plants may vary across genera, the deeply cut leaves are an identifiable trait. Like the other plants in the order *Ranunculaes*, poppies are bisexual or perfect flowers due to the presence of both numerous stamens (male flower part) and solitary pistils (female flower part). Therefore, the poppy can reproduce through pollens and seed dispersion and can self-pollinate. The oily seeds are dispersed by the wind through pores from the top of the capsule.

Of the plant family *Papaveraceae* (common name poppy) the genus *Papaver* has two species containing morphine, codeine, thebaine, noscapine (also called Noscapine), and papaverine: *Papaver somniferum L.* and *Papaver setigerum D.C* (Garnock-Jones et al., 1990). The most significant species is the *Papaver somniferum laciniatum* (opium poppy) due to the comparatively higher concentrations of alkaloids by percent weight than in *Papaver setigerum D.C.* Another commonly cultivated species of the genus *Papaver* are *Papaver bracteatum* Lindl. (Iranian poppy or Persian poppy), *Papaver rhoeas linnaeus* (common poppy or corn poppy), *Papaver dubium* L., *Papaver pseudo-orientale* Medw., and *Papaver orientale* L. (Hadipour et al., 2020; Carlin et al., 2020).

The *Papaver bracteatum* is economically important due to its high content of thebaine which was historically utilized to produce codeine and non-narcotic papaverine. On the other hand, *Papaver rhoeas L.* is historically used for treating bronchial ailments such as cough, and sleep disorders and used as a pain suppressor. *Papaver orientale* L. are grown as ornamental flowers and are toxic upon ingestion.

Apart from the alkaloids, the poppy plant is a rich source of phenolic compounds, such as anthocyanins, flavanols, and the characteristic indole derivatives nudicaulines, and essential oil volatiles, which altogether are responsible for its pharmacological activities (Hindawi et al., 2020; Carlin et al., 2020).

3.1.3. Opium Poppy

The *Papaver somniferum L.* is a plant species not only known for its brightly coloured flowers and nutty-flavoured seeds used in the food industry, but for the presence of powerful opium alkaloids, namely morphine, codeine, papaverine, noscapine and thebaine. The opium poppy is one of the oldest medicinal plants and the milky latex originating in its seed capsule, is the source of morphinans. The mature and ripe kidney-shaped beans are non-narcotic in nature and therefore the plant is timely harvested according to the purpose of cultivation. The high content of alkaloids in the plants can either be utilized in the pharmaceutical industry or treated to minimize the concentration of morphinans and used in the food industry.

The blue-coloured poppy seeds are synonymous with a low concentration of alkaloids and can be used as a source of raw material in the food industry or to produce oil for culinary, cosmetic, or technical applications (Svoboda et al., 2020). The blue-coloured poppy is one of the most sought-after varieties in the Czech market.

The existence of various phytochemicals not limited to alkaloids, makes the plant diverse and versatile. The existence of the opium poppy is a double-edged sword due to its

benefits in various industries and the use of the alkaloids obtained for illegal opium production. Many opioid drugs such as heroin which are highly addictive narcotic drugs are manufactured from the natural phytochemicals released by the opium poppy. The heroine is derived from morphine which is the most abundant alkaloid present in the poppy plant. One of the major factors contributing to the production of illicit drugs from opium alkaloids is the illegal cultivation of the opium poppy globally. For instance, in most marginalised regions of Mexico, farmers have depended for survival on the illegal cultivation of opium poppy for the US market (Smith et al., 2019).

3.1.3.1. History

The opium poppy is considered one of the oldest cultivated plants tracing back to the Neolithic ages, making it several centuries old. The earliest recordings suggest it was used as a pain reliever and was considered a cure-all for many ailments back in ancient times. The initial set of radiocarbon dates directly from the opium poppy remains of eleven Neolithic sites (5900–3500 cal BCE) in the central and western Mediterranean, north-western temperate Europe, and the western Alps (Salavert et al., 2020). Throughout the course of ancient history, scholars believe that there have been mentions of the poppy plant, its flower, and the capsule in ancient manuscripts and tablets. Many prehistoric artifacts refer to poppy and its uses, including Greek Minoan culture, Sumerian clay tablet (about 2100 BC), and juglets imitating poppy capsules found in Cyprus and Egypt (Poul R. Kruse et al., 2014).

The opium poppy is believed to have originated in Sumer, a region in ancient Mesopotamia (modern-day Iraq and Kuwait), in the Mediterranean region around 5000 BC (Aragón -Poce et al., 2002). The existence of the opium poppy has many historical ties, it is known to be linked to the eighteenth-century Silk Road, opium like many other products was traded. The historical significance of opium can be linked to the current ban on opium products in China. The infamous Opium War (Anglo-Chinese war) was waged between China and Britain between 1840-42 has been narrated by Waley (Gray, 1960) highlighting the struggles, the disturbance in the Chinese Empire and the accounts of smuggling Indian opium into China by the British.

3.1.4. Poppy in Commercial Industries

Poppy is a versatile ingredient used in many industries. The opium poppy is used in the food industry due to its gustatory property, flavour profile, nutritional benefits, and its ability to be used as a thickening agent. Furthermore, the substantial quantities of morphine,

codeine, thebaine, papaverine, and noscapine found in the latex of the poppy plant can be used to derive opiates for medicines as well as street drugs. The cold-pressed oil is used as raw material to manufacture varnishes and paints due to its high content of fatty acids and in the cosmetic industry.

3.1.4.1. Food Industry

Papaver somniferum or the opium poppy has been a long-standing crop containing various phytochemicals, including alkaloids and bioactive phytochemicals. The poppy is cultivated for the food industry as the poppy seed and the cold-pressed oil obtained from the poppy have multifaceted uses. The gustatory properties of the poppy seeds make them irresistible to consumers. The flavour profile of the poppy seeds is nutty, with a characteristic earthiness. The oilseed can be ground into a paste and used to enhance the richness of dishes. Ground poppies are used as fat substitutes and thickeners in cuisines across the world.

When harvested correctly, the seeds rarely contain opium and therefore can be used in the food industry. With the high content of fatty acids, and tocopherols including vitamin E and phospholipids, it becomes a staple that increases the gustatory and nutritional value of the product. Since consumers demand functional foods with new ingredients and have developed a preference towards natural ingredients with bioactive compounds and minimally processed sustainable food, recently the seeds have attained great popularity (Melo et al., 2022).

The whole poppy seeds are incorporated into Indian, North American, European, and Middle Eastern cuisines. The seeds are ingredients in baked goods such as muffins, bagels, and cakes. There exist reports of the poppy seeds being steeped to produce poppy tea. The ground oilseed becomes a paste due to its high-fat content. The paste is used as fillings for pastries, a source of flavour in baked goods, and a thickener in dishes like curries. While poppy seed oil is more often commercially used, the high smoking point and flavour enable it to be used as salad dressings and occasionally for cooking.

3.1.4.2. Pharmaceutical Industry

Since ancient times opium poppy has been prescribed to patients as a pain reliever. The content alkaloids like morphine, codeine, papaverine, and thebaine are base ingredients from which drugs such as oxycodone and heroin are derived. The Latin name of the plant *Papaver somniferum* loosely translates to ‘sleep bringer’ which indicates why opium poppy is necessary for the pharmaceutical industry. The use of poppy seed tea (PST) for sleep has been linked to the development of Opioid Use Disorders (OUD) which has been a steadily

growing problem in the United States owing to the readily available PST for purchase (Hagan et al., 2021).

Despite the illicit use of the drugs obtained from poppy seeds, they are still useful in the pharmaceutical industry. Poppy seed is used to sometimes diagnose conditions like vesicoenteric fistula and colovesical fistula, the poppy seed test is proven to be cost-effective and accurate in most patients (Kwon et al., 2008). Though evidence remains insufficient, the analgesic properties of the poppy are believed to be able to treat respiratory conditions like asthma and bowel disorders like constipation and diarrhoea. The pharmaceutical industries also use the opiates (morphine and codeine) found in the plant as base ingredients for painkillers.

3.1.5. Legislations

Organisations like the European Commission (EC), European Food Safety Authority (EFSA), Food and Drug Administration (FDA), Food and Agricultural Organisation (FAO) of the United Nations (UN), Centre for Food Safety and Applied Nutrition (CFSAN) aims to ensure food safety by introduces rules, laws and legislative policies. These policies can control the import and export markets, and production of food and establishes safety regulations and limits to ensure that all aspects of food production including animal welfare and agriculture are regulated. Just as the FDA is the food legislation organisation based in the United States of America, the EC implements various policies across the European Union.

The European Commission has established several regulations to make the production and consumption of foodstuffs safer. One such policy is the amendment of Regulation (EC) No 1881/2006 OF 19 December 2006, which sets maximum levels for certain food contaminants including opium alkaloids (morphine and codeine) in foodstuffs (Knutsen et al., 2018).

The EFSA in 2018 developed and updated a scientific opinion on the maximum level of alkaloids in poppy seeds with the Acute Reference Dose (ARfD) of 10 μg morphine/kg body weight (bw) and concluded that the concentration of codeine in the poppy seed samples should be taken into account by converting codeine to morphine equivalents, using a factor of 0,2 (Knutsen et al., 2018).

Many countries such as Afghanistan, the Czech Republic, Myanmar, Turkey etc have regulations to ensure fair trade, cultivation, and production of poppy seeds. While countries such as China, Saudi Arabia and the United Arab Emirates despite Arab cuisine use the poppy as a spice and to prepare bread, Singapore and Taiwan have prohibited the *Papaver*

somniferum species of poppy due to the presence of alkaloids in poppy seeds caused by the contamination because of the improper harvesting techniques. Though during food processing the morphine content is considerably reduced (up to 90%). The possibility of false-positive opiate drug tests after poppy food ingestion exists and therefore, lawmakers in several countries continue to declare the cultivation, distribution and use of poppy illegal (Lachenmeier et al., 2010).

3.2. Poppy Seed Cultivation and Harvest

Poppy plants is categorised and cultivated according to the purpose of production. Poppy plants that are grown for the food industry must contain low levels of morphinans to meet the regulatory standards of production and distribution. The other variety comprises of poppy plant with high levels of opium alkaloids in other parts of the plant (straw and capsules) which is cultivated for pharmaceutical use.

3.2.1. Poppy Seed Cultivation

The opium poppy best thrives in warm and dry, temperate climates with well drained and fertile soil with neutral pH. It is grown in regions of Turkey, Asia (Mediterranean regions), South America, and some regions of Europe. The highest producers of poppy are Turkey, the Czech Republic and Spain, while regions of India and China cultivate it as an annual crop. There is a significant amount of illicit opium cultivated around the world, including countries such as Afghanistan, Myanmar and other Southeast Asian countries. One of the biggest risks attributed to poppy cultivation is its sensitivity to geographical variations which can affect the yield.

The poppy plant has 6 stages of development during its cultivation. When developed it grows five to eight pods with a height of 60 to 120 cm. The latex is distributed throughout the crop but found in the highest concentration in the capsules (fruit). The germination occurs at higher temperatures, following the sowing and takes around five to ten days. The opium poppy can thrive in cooler temperatures as well. The cultivation period is 4-5 months. The method and frequency of irrigation plays a huge role in the growth and yield of the poppy plant, this is demonstrated by Chung (Chung, 1987) who theorizes that for maximum yield, one irrigation of 50 mm should be applied at the 50% hook stage, at 50% flowering, at the end of flowering and 2 weeks after the end of flowering.

Good cultivation practices involve preventing diseases and pest control which can significantly affect the food quality and the concentration of morphinans released by the poppy crop. Pests damage can result in penetration of the plant that release milky sap which contaminates the seeds.

For poppy seeds cultivated for pharmaceutical industries, they are collected right after flowering when the capsules release maximum alkaloid rich latex.

3.2.2. Poppy Seed Harvest and Storage

Once the petals shed, the crop is allowed to dry for nearly a month and harvest either mechanically or manually. The seeds are obtained from the harvested capsules and dried. The harvest time heavily influences the content of alkaloids present in the plant.

Poppy that is harvested as raw material for the food industry thrives well in low humidity as the water holding capacity can severely impact the dry matter content of the seed. To prevent losses in the case of unfavourable weather conditions, the poppy can be harvested for its straws and capsules to extract opium alkaloids instead.

Proper cleaning post harvesting is necessary to minimise the levels of alkaloid contamination in the edible variety of poppy. Environmental moisture content needs to be controlled when storing the poppy seeds.

3.3. Alkaloids in Poppy Seeds

3.3.1. Opium Alkaloids

Papaver somniferum is characterised by the presence of opium alkaloids present in the latex of the plant. The alkaloids present in opium poppy are morphine, codeine, papaverine, thebaine and noscapine.

Of the opium alkaloids found on poppy seeds, morphine, and codeine are the most pharmacologically active and have been detected in biological matrices collected in workplace and roadside drug testing resulting in positive opiate results (Carlin et al., 2020).

The latex released from the capsule of the poppy plant can be dried and is the major source of opium alkaloids, which can contaminate the poppy seeds due to improper harvesting techniques. Opium poppy is one of the most important crops for the pharmaceutical industry for the production of natural opiate alkaloids, mainly morphine, codeine and thebaine, that are extracted mainly from the crushed dried capsules emptied of seeds (Mahdavi-Damghani et al., 2010).

Opium alkaloids activate the mesocorticolimbic dopaminergic system, which project from the ventral tegmental area to the nucleus accumbens and medial prefrontal cortex, and dopamine is critically important in opioid consumption and sustaining. The reward effect resulting from the activation of the dopaminergic system leads to continued opioid consumption and occurs opioid dependence (Jafarova Demirkapu et al., 2021).

3.3.2. Morphine

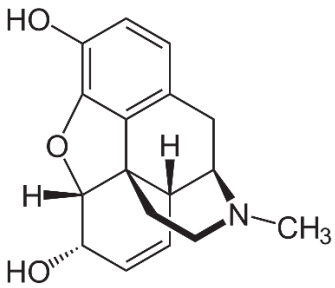


Figure 1 Chemical Structure of Morphine

Morphine is the most important poppy alkaloid and is mostly found in the highest concentration amongst all other opium alkaloids. Morphine is known for its analgesic properties and its ability to reduce pain threshold by about 50% via oral administration.

The chemical structure of morphine is that of a phenanthrene alkaloid (see Figure 1), which consists of five fused rings and two hydroxyl groups (phenolic and alcoholic) at C3 and C6. Morphine is a weak base that has an acid strength (pKa) of 7.9

Morphine, however, is greatly misused by illegal conversion into its diacetyl-derivative: heroin (Hubert G et al., 1986).

Thanks to the two -OH groups at C3 and C6, it is a substance relatively soluble in water and poorly soluble in fats. Morphine is available for therapeutic use as the hydrochloride, sulphate, and tartrate salts in a wide variety of uses (Davis et al. 2009).

Morphine is mainly metabolized in the liver, where it undergoes conjugation with glucoronic acid at the 3-hydroxyl group (Zentai et al. 2012). Metabolism also occurs in other organs, especially in the CNS (Davis et al. 2009). Morphine is excreted in the urine mainly as morphine-3-glucuronide. In addition to 3,6-diglucuronide, other minor metabolites including normorphine and 3-ether sulfate have been identified. Secondary conjugation also occurs at the 6-hydroxyl group to form the 6-glucuronide, which is pharmacologically active, and to a limited extent the 3,6-diglucuronide (Zentai et al. 2012). The metabolites morphine-3-glucuronide and morphine-6- glucuronide account for about 50–60% of the dose (Davis et al. 2009).

Morphine is commonly used in heart failure patients to treat shortness of breath and chest pain. However, studies have found that increased reactivity of blood platelets, reduced spontaneous myocardial reperfusion and larger infarct size occurred during its administration (Gaweda et al. 2020).

3.3.3. Codeine

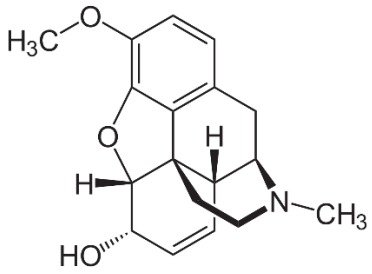


Figure 2 Chemical Structure of Codeine

Codeine is the only other bioactive morphinan other than morphine, found in the poppy plant. Codeine is highly soluble in alcohol, chloroform, and ether. In terms of toxicity, codeine is less toxic than morphine and produces similar effects to morphine. Therapeutically used to alleviate respiratory distress and used as a sedative.

Codeine, medically the most widely used opiate, is mostly derived from morphine, isolated from opium and poppy straw (Papaver somniferum, opium poppy) (Hubert G et al., 1986). Codeine is a weak analgesic that acts centrally. Its analgesic effect is possible after conversion to morphine, due to its weak affinity for opiate receptors.

Some of the the most common side effects associated with codeine include dizziness (vertigo), headache, nausea, vomiting and constipation. Less common side effects include sleep disorders, rash, urticaria, and rare side effects include thrombocytopenia, euphoria, depression of the respiratory center. Codeine is well absorbed from the digestive system after oral administration. It is usually administered orally, and its bioavailability is similar to that of morphine. Its effect lasts 1.5 – 4.5 hours (Davis et al. 2009). After oral administration, its relative bioavailability is approximately 54% based on a significant effect in the liver. It binds to plasma proteins in 25-30%. Codeine passes into the bloodstream, so possibly into the fetus

3.3.4. Papaverine

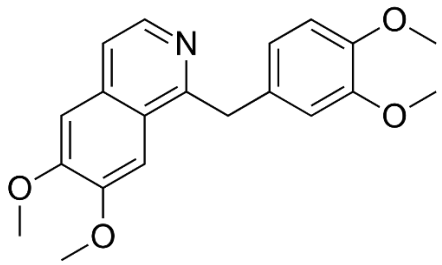


Figure 3 Chemical Structure of Papaverine

The simplest benzylisoquinoline alkaloid which acts as muscle relaxer. After oral administration, it is subject to the first-pass effect, which means that its concentration is greatly reduced before it reaches the systemic circulation. Thebaine mainly mediates stimulatory effects on the central nervous system, such as increased irritability and reflex irritability (excitability) as well as increased motor activity (Eisenreich et al. 2020). When absorbed, it is metabolized in the liver. It is digested in the body through demethylation and then conjugated. Its metabolites are excreted in the urine within 48 hours as 6-hydroxypapaverine and 4- hydroxypapaverine.

It is digested in the body through demethylation and then conjugated. Its metabolites are excreted in the urine within 48 hours as 6-hydroxypapaverine and 4- hydroxypapaverine. A small residue is excluded as pure papaverine. Papaverine has been used for more than 70 years as a vasodilator to treat cerebral and coronary artery vasospasm. It can thus increase the intracellular level of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by inhibiting the corresponding phosphodiesterases in smooth muscle. It can also block calcium ion channels and thus inhibit its release (Guan et al. 2020). Papaverine has been shown to reduce infarct size in ischemic cerebral infarction.

3.3.5. Thebaine

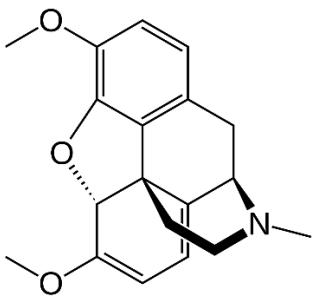


Figure 4 Chemical Structure of Thebaine

Paramorphine or thebaine is pharmacologically active and present in the unripe capsules. It exists at the lowest concentration amongst the five alkaloids of opium poppy. The structural properties of thebaine are like that of morphine and codeine. Biosynthesis of narcotic analgesics are made possible due to thebaine, especially in the production of oxycodone. The alkaloid presents itself with a stimulant effect and is not addictive.

Thebaine mainly mediates stimulatory effects on the central nervous system, such as increased irritability and reflex irritability (excitability) as well as increased motor activity (Eisenreich et al. 2020). The bioavailability of thebaine is reduced by extensive presystolic metabolism. The application of high doses leads to an increased incidence of foetal malformations. The results of studies indicate that its administration can induce teratogenic effects in vivo (Eisenreich et al. 2020).

3.3.6. Noscapine

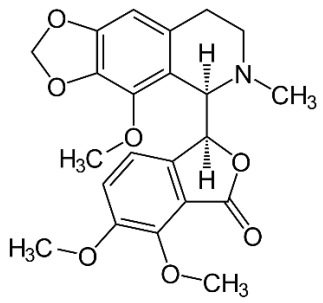


Figure 5 Chemical Structure of Noscapine

Noscapine is the second most abundant alkaloid found in the opium. Classified as phthalideisoquinoline due to its intact tetracyclic structure. Noscapine is responsible for the soothing of gastrointestinal issues, with its quick absorption upon oral administration. It can be detected by urine but leaves the bloodstream quickly.

Noscapine is the second most abundant opium alkaloid and has the lowest toxicity. Unlike morphine, noscapine is not addictive or narcotic. In the 1950s, they discovered its anti-cough properties and since then it has been used as a low-toxic antitussive (Nemati et al. 2020). Noscapine interferes with tubulin polymerization, and this affects microtubule dynamics. Drugs targeting microtubules can inhibit cell proliferation, thereby arresting the cell cycle at the mitosis stage. A study by Nemati et al. (2020) revealed that triazole-based noscapine derivatives may represent valuable cytotoxic agents and are of interest in the field of discovering new anticancer compounds (Nemati et al. 2020).

4. Methodology

The methodology incorporates sample preparation for analysis and the utilization of the HPLC-MS method to develop and test the efficacy of the analytical method to determine the content of morphinans in poppy seeds.

The LC is performed with the HPLC chromatographic system – Ultimate 3000 providing an efficient system for analysis and method development. The MS was performed using the 3200 Q-Trap MS system known for the patented hybrid triple quadrupole/linear ion trap technology. The MS system ensures reliable detection and quantification.

The techniques of HPLC (also known as High Pressure Liquid Chromatography) and MS have separate interfaces as the HPLC operates under high pressure utilizing a pump while the MS functions under high vacuum and low pressure. Therefore, the two techniques are interconnected by an interface that enables the transfer of components from the HPLC column into the ion source of the MS. Combining the two methods ensures that experimental errors are narrowed down and accuracy is improved.

4.1. Materials

4.1.1. Chemicals

The following chemicals are used to analyse the content of alkaloids present in poppy seeds using the HPLC-MS method:

Methanol p.a.	CH_3OH
Sulfuric acid p.a. 3%	H_2SO_4
Diethyl ether p.a.	$C_2H_5\text{-}O\text{-}C_2H_5$
Chloroform p.a.	$CHCl_3$
Ammonium Hydroxide	NH_4OH
Magnesium Sulphate anhydrous pure	$MgSO_4$

4.1.2. Apparatus

- Vacuum evaporator
- Ultrasonic bath
- Apparatus for Separation: Funnel, Stand, Beaker
- Poppy seed Grinder
- Glass Pipette tips
- Analytical Balance

- Apparatus for Filtration: Filter, Vacuum Filter flask, Water suction, Rubber stopper, Separating funnel

4.1.3. Samples

The samples are obtained from various producers from different countries. Out of the 13 producers, 8 originate in the Czech Republic and the rest of the samples are obtained from producers originating in Spain, Austria, USA (2 samples) and Australia. Table 1 below, highlights the sample names assigned to each producer during the experiment.

Sample Name	**Producer**
091121_1T	Czech Republic
091121_2T	Czech Republic
091121_4T-10	Czech Republic
091121_20-ON-F-10	Czech Republic
091121_20-ON-KL	Czech Republic
091121_20-T-F	Czech Republic
091121_20-T-KL	Czech Republic
091121_Benesov-20	Czech Republic
091121_Amatola	Spain
091121_Rako21	Austria
091121_US-0	USA
091121_US-M	USA
091121_21-AUS	Australia

Table 1 Sample Names and Country of Origin of the Poppy Seeds Analysed

4.2. Sample Preparation

Two methods of preparation are utilized – whole poppy seeds and ground poppy seeds. **Whole Poppy Seeds:** 1 g of the sample poppy seeds are collected and placed into test tubes with a cap. **Ground Poppy Seeds:** The sample seeds are ground using a grinder and 1 g of the homogenised ground seeds are weighed into a test tube with a cap.It is to be noted that, only the initial step of obtaining the two samples differs and the preparation of the sample is similar for both types of samples.

25 ml of methanol is added to each of the test tubes containing the samples. The test tubes are capped and placed in an ultrasonic bath for 20 minutes. The methanol is then filtered

using a vacuum filter flask. The filtered portion is collected, and 25 ml of methanol is added to the sample again, following which it is placed in the ultrasonic bath for 20 mins. The procedure of filtration is repeated. This process is repeated for 5 cycles to obtain, 5 fractions of filtered extract. The extracts are then evaporated in a rotary evaporator (vacuum evaporator).

Upon evaporation, each of the distillation residues is shaken with 10ml of 3% sulfuric acid. This step reintroduces the alkaloid salts into an acid phase. The acidic solution is poured into a separating funnel, followed by 15 ml of diethyl ether. The solution is shaken until it is separated. The aqueous layer is reintroduced into the separating funnel and 15 ml of dimethyl ether is added again. The solution is shaken again, and the aqueous phase is separated. This step ensures the removal of fats from the sample.

Ammonium hydroxide is introduced into the aqueous phase and the pH is checked. The pH paper must turn green indicating the desired pH of 8. The alkalization of the aqueous phase converts the alkaloid salts into a free base that is soluble in the chlorinated organic solvent. 15 ml of chloroform is introduced into the extract and shaken in a separating funnel to remove the water-soluble impurities. This step is repeated twice, and the separated chloroform fractions are combined.

Magnesium sulphate is added as a drying agent into the chloroform extract. The extract is filtered and placed in a vacuum evaporator. The residue obtained is dissolved with 1 ml of methanol.

The procedure above is carried out for all 13 samples of poppy seeds. The Spanish variety (091121_Amatola) further undergoes extensive sampling and extractions due to the presence of a high concentration of morphinans.

Ten samples of the Spanish variety of poppy seeds are prepared for analysis. Five consecutive extractions are also performed on the sample of the Spanish variety of poppy seeds. Eighteen different acidic extracting agents are used to test the strength of the extraction agents. The extracts obtained undergo HPLC-MS method analysis to see the efficiency of the extraction agents. Furthermore, samples of the Spanish variety of poppy seeds are also subjected to different temperatures, UV-irradiations and exposed to H_2O_2 and qualitatively and quantitively analysed by the HPLC-MS to understand the influence of the parameters on the level of contamination of alkaloids on poppy seeds.

4.3. Determination of Alkaloids by HPLC-MS Method

The combination of HPLC and sensitive detection MS method aids the qualitative and quantitative determination of opium alkaloids resulting in a highly accurate result.

Analytical conditions of the HPLC-MS system

Instrument:	Mass spectrometer - 3200 Q-Trap (Applied Bio-systems, Canada) HPLC chromatographic system – Ultimate 3000 (Dionex, USA) Analytical HPLC column - Synergi Fusion-RP 10, 100 x 2.0 mm, 2.5 μm (Phenomenex, USA)
Ionization:	ESI + - positive ionization mode
Scan mode:	Full scan – 100-2000 Da
Flow through the column:	200μl/min
Direct infusion:	10μl/min of a sample solution with a concentration of 100 μg/ml (Optimization of MRM transitions)
Column temperature:	40°C
Injection volume:	5μl
Analysis time:	25 min
Ion Spray Voltage:	IS = 5500V
Curtain gas:	CUR = 25 psi
Collision gas:	CAD = medium
Temperature:	TEM = 600°C
Ion source gas 1:	GS1 = 50 psi
Ion source gas 2:	GS2 = 50 psi

Table 2 Analytical conditions for the HPLC-MS method

Parameters of MRM transitions

MORPHINE

Transition 1 – quantitation	**286,1 → 152,2**
Transition 2 – confirmation	**286,1 → 165,2**
"Declustering potential" DP (V)	**51V**
"Entrance Potential" EP (V)	**10V**
Cell Entrance Potential (CEP)	**18V**
Collision Energy (CE); transition 1	**70V**
Collision Energy (CE); transition 2	**45V**
Cell Exit Potential (CXP)	**10V**

CODEINE

Transition 1 – quantitation	**300,1 → 152,1**
Transition 2 – confirmation	**300,1 → 215,1**
"Declustering potential" DP (V)	**40V**
"Entrance Potential" EP (V)	**12V**
Cell Entrance Potential (CEP)	**20V**
Collision Energy (CE); transition 1	**75V**
Collision Energy (CE); transition 2	**35V**
Cell Exit Potential (CXP)	**10V**

NOSCAPINE

Transition 1 – quantitation	**414,1 → 220,1**
Transition 2 – confirmation	**414,1 → 253,1**
"Declustering potential" DP (V)	**40V**
"Entrance Potential" EP (V)	**10V**
Cell Entrance Potential (CEP)	**25V**
Collision Energy (CE); transition 1	**23V**

Collision Energy (CE); transition 2	**30V**
Cell Exit Potential (CEP)	**12V**

PAPAVERINE

Transition 1 – quantitation	**340,1 → 202,1**
Transition 2 – confirmation	**340,2 → 308,3**
“Declustering potential” DP (V)	**55V**
“Entrance Potential” EP (V)	**10V**
Cell Entrance Potential (CEP)	**35V**
Collision Energy (CE); transition 1	**40V**
Collision Energy (CE); transition 2	**50V**
Cell Exit Potential (CEP)	**10V**

THEBAINE

Transition 1 – quantitation	**312,2 → 152,2**
Transition 2 – confirmation	**312,2 → 251,2**
“Declustering potential” DP (V)	**90V**
“Entrance Potential” EP (V)	**12V**
Cell Entrance Potential (CEP)	**35V**
Collision Energy (CE); transition 1	**50V**
Collision Energy (CE); transition 2	**30V**
Cell Exit Potential (CXP)	**8V**

Typical HPLC-MS chromatogram

Chromatographic column

Figure 6 shows a chromatogram obtained by optimizing the analytical method to ensure that the individual components that were monitored in the poppy seeds: morphine, codeine, papaverine, thebaine and noscapine were separated.

Optimized analytical conditions

An HPLC-MS method was developed that met the stated requirement for ideal separation, however, the analysis time was around 25 minutes. The total analysis time can be reduced by adjusting the conditions, while maintaining the complete separation of all the components. The analysis conditions were as follows and the separation is captured in the chromatogram (Figure 6).

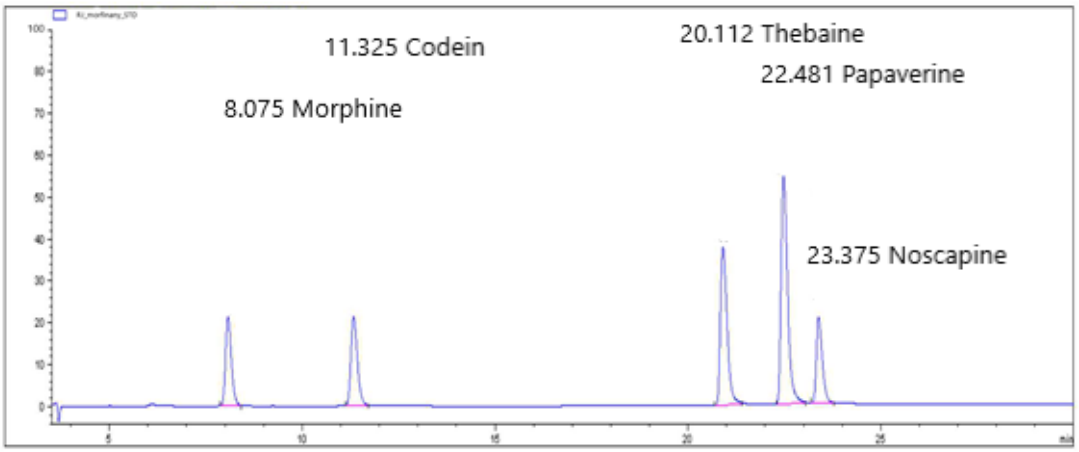


Figure 6 Separation of Chromatogram with optimized analytical conditions

Analyte Retention time

- Morphine 8.08 min.
- Codeine 11.33 min
- Thebaine 20.11 min
- Papaverine 22.48 min
- Noscapine 23.38 min

Optimized HPLC-MS analytical conditions:

Chromatographic system: HPLC Ultimate 300 RS, Dionex, USA

Mass spectrometer: 3200 QTRAP, AB-Sciex, Canada

Chromatographic column and conditions:

Column:	ACQUITY UPLC BEH C18 (50 mm x 2.1 mm x 1.7 µm)
Pre-column:	ACQUITY UPLC BEH C18 (5 mm x 2.1 mm x 1.7 µm)
Injection volume:	2 µl
Weak wash solution:	Deionized water/methanol (90/10)
Strong washing solution:	Methanol
Mobile phase A:	0.1% acetic acid in deionized water
Mobile phase B:	0.1% acetic acid in methanol
Mobile phase flow rate:	0.6 ml/min
Mobile phase gradient:	0 min (10% B) – 4 min (100% B) – 7 min (100% B) – 7.1 min (10% B) – 10 min (10% B).

4.4. Validation of HPLC-MS Method

The developer HPLC/MS method has been validated and important validation parameters are represented in Table 3

	LOD (ng/ml)	LOQ (ng/ml)	Linearity (ng/ml)	Precision %	Accuracy %
Morphine	1.2	4	1 - 5000	0.85	1.65
Codeine	0.1	0.3	0.1 - 5000	1.25	1.75
Papaverine	0.03	0.1	0.1 - 5000	0.70	0.95
Thebaine	2.5	8.3	3 - 5000	0.55	0.90
Noscapine	0.03	0.01	0.1 - 5000	0.65	1.25

Table 3 HPLC-MS Validation parameters

Interference:

By choosing a selective MRM transition and testing them with spiked real samples and spiked "blanks", it was verified that there was no interference.

Intra-day and inter-day precision:

The range of intra-day and inter-day precision was 0.20–1.25%. Precision was determined by the least-squares method, and the relative standard deviation was determined to be 98.25–99.20% and 0.40–1.65%.

Samples Protection and Storage:

Samples were stored at -20°C in a freezer protected from light. The LOD and LOQ were 2.5 and 8.3 ng/mL, respectively.

5. Results

The samples prepared following the methodology mentioned in Chapter 4 are analysed with the HPLC-MS method. The contents of the opium alkaloids morphine, codeine, noscapine, thebaine and papaverine are determined quantitatively.

The results of the content of morphinans of the 13 poppy seed varieties are shown in Table 4.

Origin	Sample Name	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
CZ	091121_1T	0,483	0,012	0,198	0,050	0,466
CZ	091121_2T	0,761	0,026	0,580	0,021	2,180
CZ	091121_4T-10	5,605	0,081	0,140	0,029	108,800
CZ	091121_20-ON-F-10	1,111	0,230	0,030	0,013	27,620
CZ	091121_20-ON-KL	0,047	< LOD	0,002	< LOD	0,072
CZ	091121_20-T-F	0,242	0,018	0,022	0,003	21,010
CZ	091121_20-T-KL	0,055	< LOD	0,010	0,001	18,320
CZ	091121_Benesov-20	0,068	0,017	0,113	0,008	0,667
ES	091121_Amatola	41,970	0,201	0,254	0,048	46,370
AT	091121_Rako21	0,257	0,162	0,063	0,008	0,230
US	091121_US-0	0,168	0,034	0,529	0,117	1,614
US	091121_US-M	0,656	1,786	0,001	0,018	10,790
AU	091121_21-AUS	0,073	0,002	9,095	0,002	0,542

Table 4 Experimental values obtained of the Content of Alkaloids in Whole Poppy seeds analysed by HPLC-MS

Table 5 displays the results of the Spanish variety (091121_Amatola) of poppy seeds samples that underwent 10 analyses, prepared by grinding the seeds carried out at a controlled temperature of 20°C.

Analysis No	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
1	40.371	0.172	0.147	0.029	40.178
2	46,659	0,913	0,226	0,041	46,370
3	42.904	0,133	0,319	0,163	41,783
4	42,847	0.561	0,528	0,097	41,489
5	41,961	0,231	0,281	0,044	39,399
6	37,473	0,223	0.372	0,054	41,492
7	44,878	0,228	0,315	0,067	44,369
8	43,605	0,319	0,278	0.053	40,339
9	36,382	0,406	0,623	0,012	38,112
10	42.183	0,168	0,234	0,038	42,221

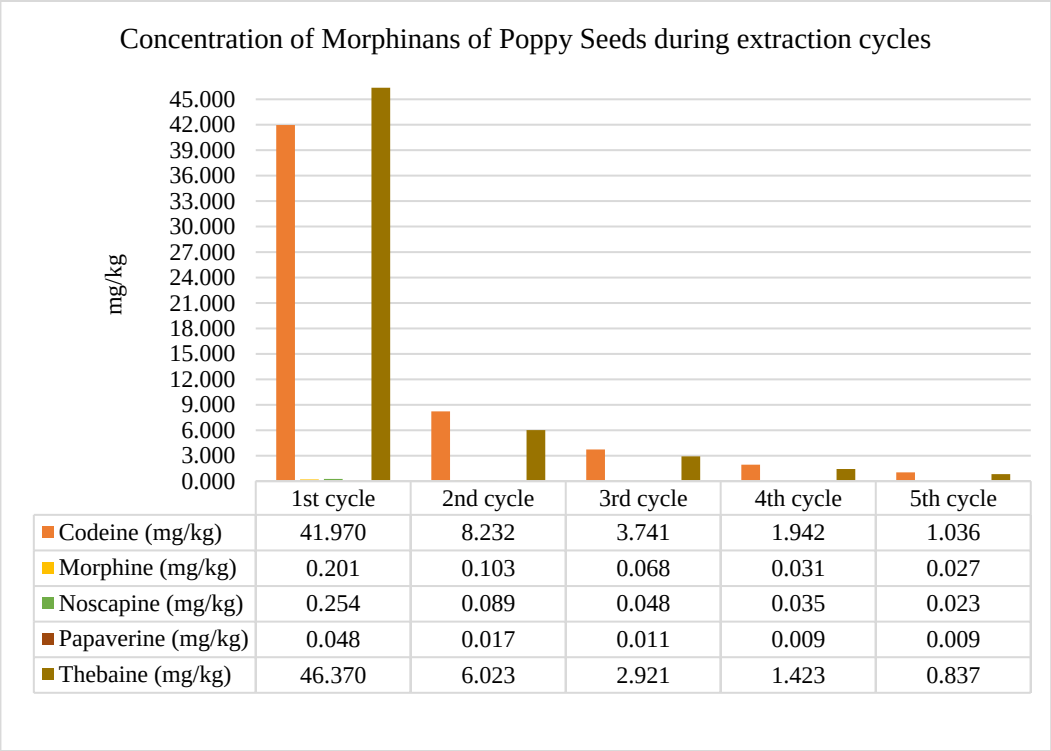
Table 5 Experimental values obtained of the content of Alkaloids present in the Spanish Variety (091121_Amatola) for 10 samples/analysis) conducted by the HPLC-MS method

From Table 4, it is observed that the quantity of alkaloids is distributed unevenly among the varieties of poppy seeds. Experimental values shown in Table 5 indicate the variation of concentration of opium alkaloid contamination in the samples collected from the primary source of the Spanish variety of poppy seeds.

Higher content of codeine and thebaine in contrast with the significantly lower values of morphine, noscapine and papaverine are observed across all the samples analysed. Moreover, there is no statistically significant difference between the methods of using whole seeds and ground seeds.

5.1. Alkaloid extraction cycle

Figure 7 indicates the results of 5 extractions conducted with Sulfuric acid performed on the Spanish variety of poppy seeds at the constant temperature of 20°C.



	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle
Codeine (mg/kg)	41.970	8.232	3.741	1.942	1.036
Morphine (mg/kg)	0.201	0.103	0.068	0.031	0.027
Noscapine (mg/kg)	0.254	0.089	0.048	0.035	0.023
Papaverine (mg/kg)	0.048	0.017	0.011	0.009	0.009
Thebaine (mg/kg)	46.370	6.023	2.921	1.423	0.837

Figure 7 Graphical representation of the Experimental values of Morphinan content of Poppy Seed of the Spanish variety during 5 consecutive extractions of a single sample with Sulfuric acid

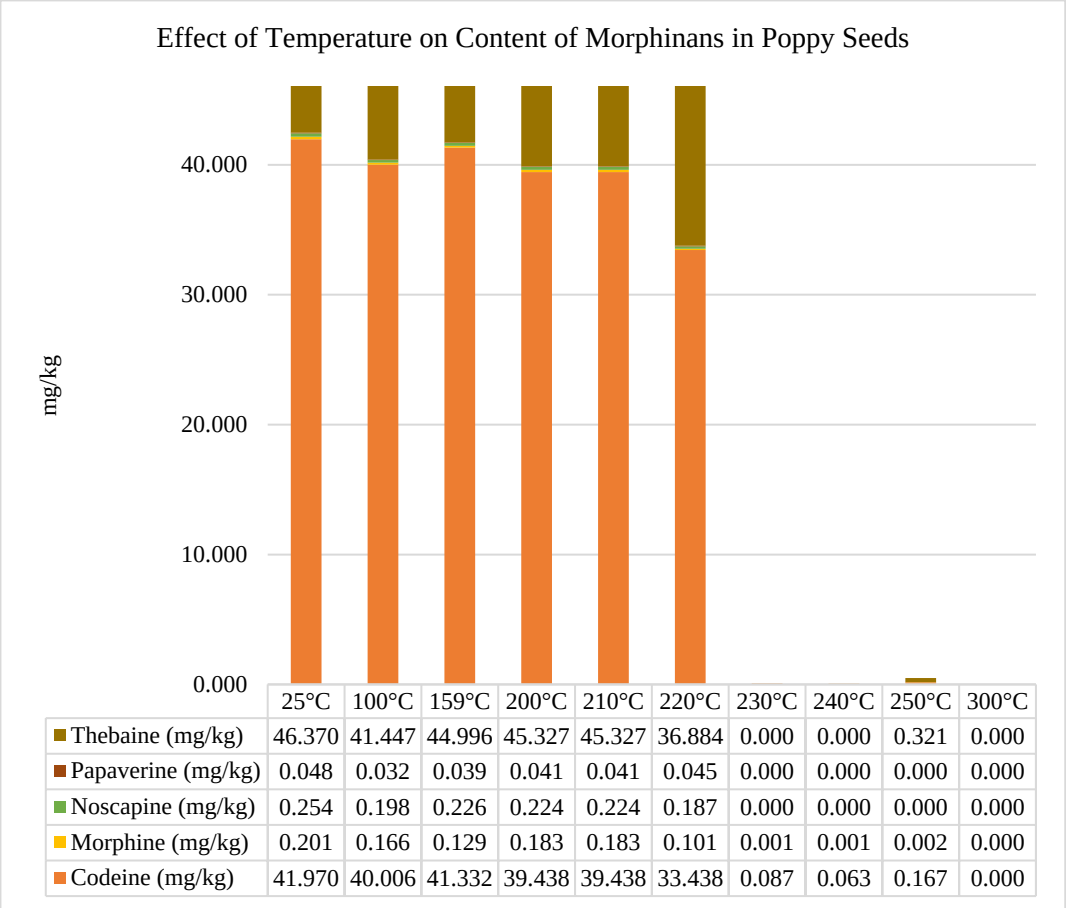
Opium Alkaloids	**Percentage decrease from Cycle 1 to Cycle 5 of Extraction (%)**
Codeine	97.53
Morphine	86.57
Noscapine	90.95
Papaverine	96.46
Thebaine	98.20

Table 6 Percentage decrease from extraction cycle 1 to cycle 5 of the opium alkaloids

Figure 7 helps demonstrate the significant reduction of opium alkaloid levels with the increment of extractions conducted with H_2SO_4 (Sulfuric acid) solution on the same sample. A substantial decline of 80.37% of codeine and 87.01% of thebaine is observed from extraction cycle 1 to extraction cycle 2.

5.2. Effect of Temperature on Morphinans concentration of Poppy

Figure 8 shows the effects of temperature on the concentration of the opium alkaloids present on the surface of the Spanish variety of poppy seeds.

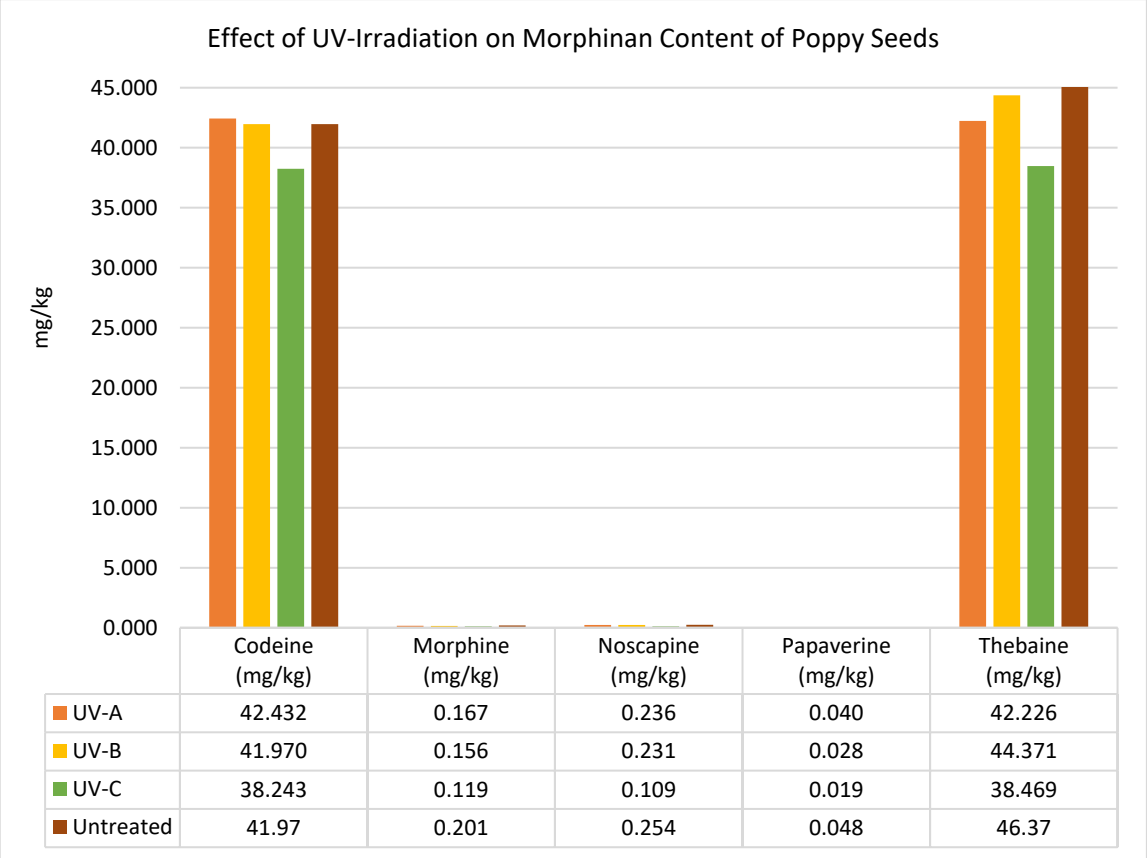


	25°C	100°C	159°C	200°C	210°C	220°C	230°C	240°C	250°C	300°C
Thebaine (mg/kg)	46.370	41.447	44.996	45.327	45.327	36.884	0.000	0.000	0.321	0.000
Papaverine (mg/kg)	0.048	0.032	0.039	0.041	0.041	0.045	0.000	0.000	0.000	0.000
Noscapine (mg/kg)	0.254	0.198	0.226	0.224	0.224	0.187	0.000	0.000	0.000	0.000
Morphine (mg/kg)	0.201	0.166	0.129	0.183	0.183	0.101	0.001	0.001	0.002	0.000
Codeine (mg/kg)	41.970	40.006	41.332	39.438	39.438	33.438	0.087	0.063	0.167	0.000

Figure 8 Graphical representation of the Experimental values of Morphinans found in Spanish Variety of Poppy Seeds when exposed to varying Temperatures from 25°C to 300°C

From Figure 8 it can be concluded that there is only trace level of reduction in the levels of alkaloids at temperatures between 25°C - 200°C. Furthermore, beyond 200°C we can see a steep drop in the levels of opium alkaloids. Finally, at 300°C the levels of all alkaloids are below LOD indicated by 0 value on the data table.

5.3. Effects of UV-irradiation on Morphinans content in Poppy

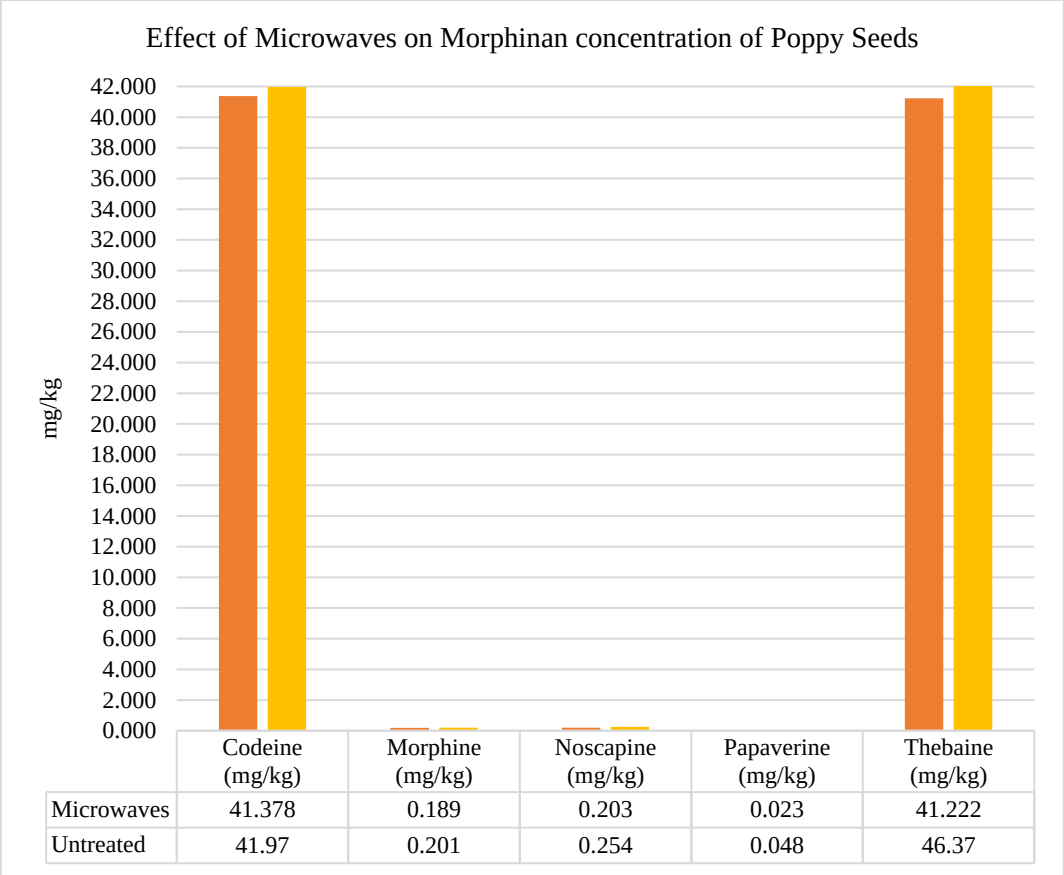


	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
UV-A	42.432	0.167	0.236	0.040	42.226
UV-B	41.970	0.156	0.231	0.028	44.371
UV-C	38.243	0.119	0.109	0.019	38.469
Untreated	41.97	0.201	0.254	0.048	46.37

Figure 9 Graphical Representation of the Experimental Values of Morphinans of Poppy Seed of the Spanish Variety when exposed to UV-Radiations type UV-A, UV-B and UV-C compared to untreated poppy seeds

Figure 9 suggests that there is negligible effect on morphinan concentrations when the samples are exposed to UV-A and UV-B irradiations, but UV-C irradiation is the most effective in reducing the concentration of morphinans compared to UV-A and UV-B radiation waves.

5.4. Effect of Microwaves on Morphinans concentration of Poppy



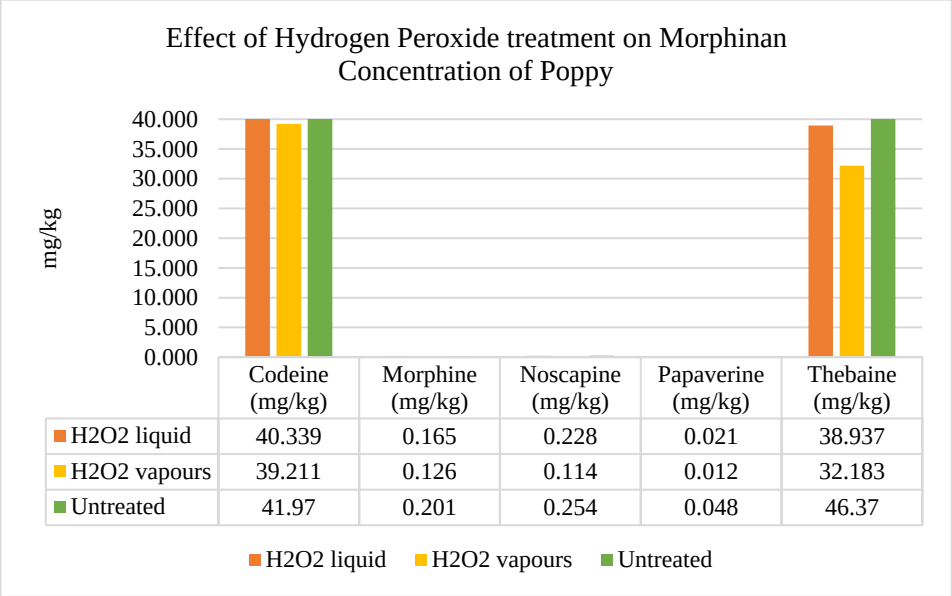
	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
Microwaves	41.378	0.189	0.203	0.023	41.222
Untreated	41.97	0.201	0.254	0.048	46.37

Figure 10 Graphical representation of the Experimental values of Morphinans on Poppy Seeds of the Spanish Variety when exposed to Microwaves (treated in a Microwave Oven) compared to untreated poppy seeds

The results from Figure 10 indicate only a marginal decrease in opium alkaloids when treated by microwaves in the microwave oven. Despite the decrease in concentrations of morphine, codeine, noscapine, papaverine and thebaine in the sample of poppy seeds treated with microwaves, the percentage of decrease is negligible, averaging at 1.075% decrease in the alkaloid content.

5.5. Effect of H_2O_2 treatment on Morphinans concentration of Poppy

Figure 11 represents the values of concentration of morphinans in the Spanish variety of poppy seeds treated with vapour and liquid hydrogen peroxide.



	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
H2O2 liquid	40.339	0.165	0.228	0.021	38.937
H2O2 vapours	39.211	0.126	0.114	0.012	32.183
Untreated	41.97	0.201	0.254	0.048	46.37

Figure 11 Graphical Representation of the Experimental values Opium Alkaloids of Poppy Seeds of the Spanish Variety when treated with Hydrogen Peroxide in Liquid and Vapour phase compared to Untreated poppy seeds

Figure 11 shows that the vapour treatment of hydrogen peroxide can significantly reduce the alkaloids found on poppy seeds as compared to the liquid treatment of hydrogen peroxide. Significant decline in concentrations of alkaloids in vapour hydrogen peroxide treated poppy seeds with regards to initial concentrations of untreated poppy seeds.

5.6. Concentration of Alkaloids extracted using various extraction agents

Extraction agent	Codeine (mg/kg)	Morphine (mg/kg)	Noscapine (mg/kg)	Papaverine (mg/kg)	Thebaine (mg/kg)
Acetic acid no1	37.642	0.172	0.223	0.041	27.471
Acetic acid no2	39.368	0.159	0.194	0.027	38.578
Apple acid no1	37.658	0.163	0.152	0.022	38.112
Apple acid no2	34.394	0.184	0.163	0.029	37.274
Ascorbic acid no1	18.639	0.070	0.076	0.013	18.472
Ascorbic acid no1	16.117	0.084	0.013	0.023	9.345
Citric acid no1	38.477	0.187	0.192	0.035	41.263

Citric acid no2	40.365	0.168	0.164	0.033	39.154
Formic acid no1	41.351	0.186	0.235	0.052	42.333
Formic acid no2	39.428	0.195	0.178	0.039	38.687
H_2SO_4 no1	41.970	0.201	0.254	0.048	46.370
H_2SO_4 no2	39.492	0.174	0.193	0.059	42.667
H_3PO_4 no 2	38.065	0.173	0.141	0.039	41.109
H_3PO_4 no1	36.238	0.166	0.134	0.034	40.221
Tartaric acid no1	36.221	0.123	0.169	0.051	38.268
Tartaric acid no2	39.461	0.196	0.154	0.026	34.376
Untreated	41.970	0.201	0.254	0.048	46.370
Vinegar no1	35.117	0.154	0.195	0.015	34.274

Table 7 Experimental values of Opium Alkaloids of Poppy Seeds of the Spanish Variety when treated with different Extraction Solvents (acids)

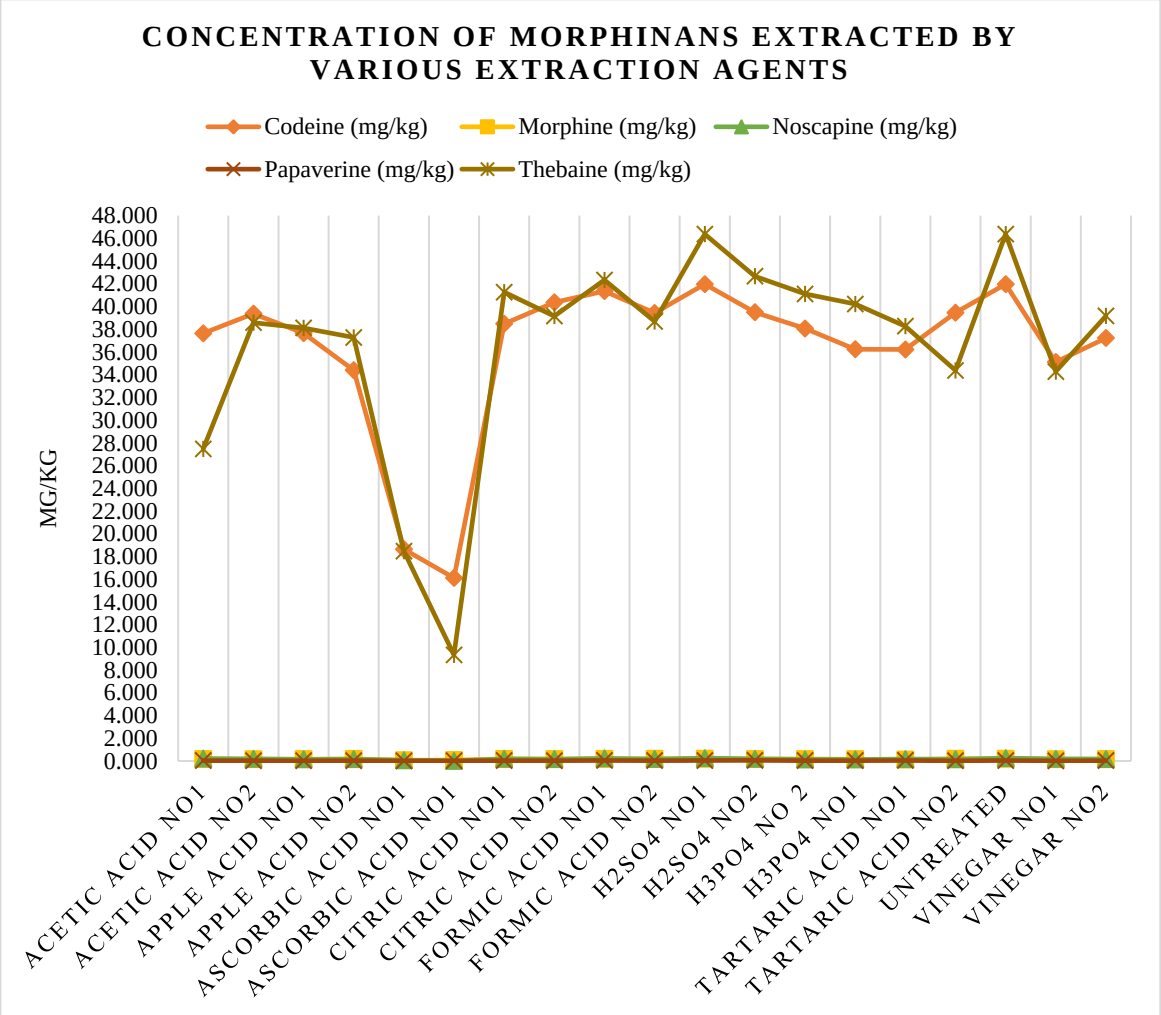


Figure 12 Graphical representation of the distribution of Concentration of Morphinans extracted from Spanish Variety of Poppy seeds by various Extraction Agents

Table 8 represents the extraction agents utilized to extract morphinans and the value of morphinans extracted from the poppy seed samples. The graphical representation in Figure 12 visually represents the strength of the extracting agents.

The results from Figure 12 and Table 8 suggests that Sulfuric acid No 1 and Formic acid No 1 are the strongest extraction agents while Apple Acid No 1 and Ascorbic acid No 1 are the weakest extraction agents. But it should be noted that Apple Acid No 1 is significantly more effective than ascorbic acid in extracting the morphinans from poppy seeds. Moreover, during the experiment, the first extraction with ascorbic acid caused rotting within 24 hours and the second extraction resulted in rotting within 48 hours.

6. Discussion

The thesis aims to develop an analytical method to determine the content of alkaloids present in poppy seeds. The HPLC-MS method was successful in analysing the various samples of poppy and determining the concentration of the opium alkaloids morphine, thebaine, papaverine, noscapine and codeine. The method validation indicates the ideal parameters to receive chromatography without interference and ideal separation. It was also observed that we have to further adjust the conditions to reduce the existing analysis time of 25 minutes while ensuring the separation of compounds is maintained. The HPLC-MS analysis combines two systems, to provide a robust, efficient and effective method to analyse the samples.

From the thirteen samples collected, further parametric studies were conducted on the Spanish variety due to the difference in concentration of the opium alkaloids. The Spanish variety was able to indicate the effects of each parameter on the concentrations of the morphinans. The uneven distribution of the concentration of opium alkaloids can be attributed to the surface contamination of the poppy seeds. During harvest or growth, some of the poppy varieties may have been damaged by pests or diseases or improper harvesting techniques. Some of the samples including the Spanish variety crossed the maximum level of 20 mg/kg of morphinans set by the EC 1881/2006.

EC Regulations play an important role in ensuring that the poppy cultivated for food products has trace amounts of alkaloids, but as we have seen in the previous chapters, due to the lack of stringent laws, consumption of poppy-based food products has given false drug positives. Without the establishment of strict laws, the ban on the opium poppy will continue to be established in many parts of the world, which can negatively affect the countries like the Czech Republic that export most of the poppy cultivated.

By preparing samples through two methods, we were able to observe and conclude that, there are statistical differences between the value of alkaloids extracted from whole seeds and ground seeds. The two methods produce similar results as poppy seeds themselves do not have significant levels of alkaloids in them, rather the pore dust from the capsule or latex released from the mature capsules or straw can contaminate the seeds.

Sulphuric acid was observed to be the most efficient agent to extract the surface contaminants. It is also observed that the most suitable solvent for alkaloids is ether and chloroform due to the high level of solubility of morphine, codeine, thebaine, noscapine and

papaverine. By performing five extraction cycles we can see the strength of the acid in removing the surface contaminants.

Heat treatment of poppy seeds indicates positive results in reducing the content of alkaloids present. This is especially useful in the food industries to remove significant concentrations of poppy seeds while preparing confectionaries and bakery goods. This helps establish a practical method to remove contaminants without having to utilize laboratory chemicals. It is to be noted that the concentration of alkaloids decreases at temperatures above 200°C.

An interesting observation made was the effect of various wavelength irradiation on the samples of poppy seeds. Treating the sample with microwave using a microwave oven had no significant impact on the concentration of alkaloids. This can be attributed to the longer wavelength and lesser frequency of the microwave. Longer wavelengths result in lesser excitement of the photons that are needed to generate energy.

On the other hand, UV-irradiation had a considerable effect compared to the microwave due to its shorter wavelength, higher frequency and therefore larger energy emitted. The order of wavelength in ascending order for UV-rays are as follows: UV-C<UV-B<UV-A. Therefore, the shorter wavelength can generate more energy through photons, thereby making it more efficient in removing the alkaloids from the sample.

Treating the poppy seeds with two phases of hydrogen peroxide, liquid and vapour phase showed a significant difference. Hydrogen peroxide in vapour form is more effective than in the liquid phase.

Ascorbic acid is a mildly acidic solution with a mild reducing capacity. The acid was unable to remove a significant amount of surface-level contaminants. This could be due to the heat sensitivity of the acid, as ascorbic acid is highly light and heat sensitive and degrades upon contact with light and heat. Therefore, the inability of ascorbic acid not only to remove morphinans from the sample but also microbial activity noticed in the samples (rot development after day 1 and day 2) shows that ascorbic acid probably underwent decomposition.

Testing various extracting agents also indicated the effectiveness of vinegar as an extracting agent. Similar to the hypothesized practical application of heat treatment, washing poppy seeds with vinegar before consumption or preparation of food can effectively reduce the content of morphinans on a large scale.

The experiment also shed light on the efficiency of formic acid and sulphuric acid as effective extracting agents.

The origin of the source of poppy seeds plays a huge role in determining the concentration of alkaloids present in the poppy seeds. It is further influenced by the harvesting method, the irrigation patterns and the damages caused by pests and diseases alike.

The HPLC-MS analysis is efficient, effective and accurate with minimal error percentage. Therefore, we can develop a robust analytical method to understand the morphinans present in poppy seeds.

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7. Conclusion

- An efficient, highly analytical, and accurate HPLC-MS method was developed to determine the morphinan content in poppy seeds.
- The hypothesis that poppy seeds are contaminated only on the surface by latex residues containing morphine, codeine, thebaine, noscapine and papaverine is proved true since there was no statistical difference between the two methods (whole and ground poppy seeds) of sample preparation.
- Though the method was developed and validated, it can be concluded that further adjustments to the parameters of the HPLC-MS method are required to reduce the processing time and therefore the efficiency of the analytical method.
- The hypothesis that altering physical-chemical parameters such as temperature, pH, and exposing poppy seeds to microwaves, UV-Radiation and hydrogen peroxide can influence the parametric value of the alkaloid contaminants in the poppy seed is confirmed to be true.
- The physical-chemical parameters can influence the content of residual opium alkaloids present in poppy-based food products.

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9. List of abbreviations and symbols

HPLC-MS	High Performance Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
MRM	Multiple Reaction Monitoring
UV-Irradiation	Ultraviolet Irradiation
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
H_3PO_4	Phosphoric Acid
P.A	Practical Grade

10. List of figures and tables