Assessment of Stress Biomarkers in the Saliva of Smokers and Nonsmokers via UV Photospectrometry and POMS

By

Maria Fahim

Reg No. 317645

Session 2019

Supervised by

Dr. Asim Waris

A Thesis Submitted to the School of Mechanical and Manufacturing Engineering in partial fulfillment of the

requirements for the degree of

MASTERS of SCIENCE in

Biomedical Sciences

School of Mechanical and Manufacturing Engineering (SMME)
National University of Science and Technology (NUST)
H-12, Islamabad 44000, Pakistan
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Dedication

I dedicate this thesis to my parents, teachers, friends and fellow members without whom it was almost impossible for me to complete my research work

Acknowledgement

All praises be to Allah the Almighty, the Most Gracious, and the Most Merciful for His blessing given to me during my study and in completing this thesis. Peace and salutations are uttered to the beloved Prophet Muhammad SAW, his family and his companions.

I would like to acknowledge my supervisor who made this work possible. I would like to acknowledge and thank my department for allowing me to conduct my research and providing any assistance requested. I would also like to thank the beginning teachers, mentor-teachers and administrators in our university that assisted me with this project.

Abstract

Smoking is one of the major health catastrophes. Smoking is believed to be the major cause of chronic diseases like Cardiovascular complications, stroke, pregnancy issues, respiratory failure, etc. There are three important transdiagnostic emotional factors that make the population vulnerable to initiation of smoking *i.e.* anhedonia anxiety sensitivity, distress tolerance. Research studies for the past five decades have proven the adverse effect of stress on brain physiology and functioning. The human body responds to trauma (physical or non-physical stress) in a definite manner. This response of the body can be qualitatively and quantitatively monitored through several chemicals in the bloodstream, saliva, or urine; responding to the stress, called stress biomarkers *i.e*. Brain-derived neurotrophic factor, cortisol, cytokines *etc*. Saliva delivers an efficient specimen for various diagnostic procedures due to the presence of different biological products and secondary metabolites of xenobiotics and helps in determining the disease progressions as well as therapy outcomes depending on the variations in the markers/triggers. The nature of mindset and mood states are evaluated by a scales designed to rate the behavior of an individual towards the environmental stimuli that may be physical or psychological in nature. This psychological rating scale is known as the profile of mood state (POMS). This scale was initially originated by McNair, Droppleman, and Lorr in 1971. This scale is presented in the form a questionnaire including different questions regarding the mode and feelings of a subject.This research work aims to further elucidate the utilization of UV Photospectrometry for quantitatively assessing POMS and its relation to the stress biomarker.

The samples were collected form the vicinity of the university campus H-12 Islamabad. The samples were processed and stored at the biomedical laboratory of School of Mechanical and Manufacturing Engineering (SMME), NUST. A total of twenty-four (24) male subjects were analyzed. A total of two groups were considered. Group 1 included the non-smoking participants, while group 2 included smoking participants. Simple spitting technique was used for the collection of unstimulated saliva. About 4 ml unstimulated saliva was collected in the sterile falcon tube. Saliva was temporarily stored in cool boxes at 4°C and immediately

shifted to the facility. Centrifugation of the salivary sample was done at 4°C for 5 minutes and 10,000 rpm. Saliva sample was frozen at -80°C until sample collection span was completed.

The mood state of the participants was also evaluated using the profile of mood state technique used initially by McNair, Lorr, and Droppleman in 1971. The total mood disturbance (TMD) score was calculated that ranges from -32 to 200. The questionnaire was accessed from "Mackenzie, B. (2001) Profile of Mood States (POMS) [WWW] Available from: https://www.brianmac.co.uk/poms.htm [Accessed 26/6/2022]". Simulated neural networking (SNN) was applied to the collected data from smokers and non-smokers for accuracy scoring. The required statistical analysis was performed and the data was statistically analyzed through a software "GraphPad Prism 8.0" and the respective graphs were plotted.

UV spectrophotometry studies provided peak plasma concentration peaks at the lower UV range of 190 to 210 nm, but with no significant difference, representing the presence of biological stress markers. The profile of mood state evaluation studies concluded that the smoking participants were presented with a significantly higher level of individual mood profile scores i.e. anger (****, $P<0.0001$), confusion (**, $P<0.0014$), fatigue (*, $P<0.0354$), tension (*, $P<0.0422$) and stress as compared to nonsmoking participants. The vigorous score was significantly high in the nonsmoking individuals (****, $P<0.0001$). Similarly, total mood disturbance score was also significantly high in the smoking participants. The application of artificial neural networking through artificial machine learning scored the accuracy of the results 84% which is a reliable outcome.

The current research work concludes that different stress stimuli including physiological stress and psychological stress tends to initiate/increase the smoking behavior among the community. Likewise, it is also concluded that smoking initiation may not be always triggered in response to stress. Numerous factors i.e. lack of education, negative inspiration, or behavior to impress are also involved. Furthermore, the adaptation of smoking behavior as a result of stressful stimuli is not a valid approach to reduce the noxious/stressful stimuli. The stress may further be exaggerated by smoking.

Keywords: Stress smoking, salivary stress biomarkers, label free detection, UV spectrophotometry, POMS.

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List of Abbreviations

(8-OHdG	8-hydroxy-2′-deoxyguanosine
3HC	trans 3′-hydroxycotinine
CC	Combustible cigarette
COT	Cotinine
CVS	Cardiovascular system
DNA	Deoxyribonucleic acid
ETS	Environmental tobacco smoke
HELIX	Human early-life exposome
HPA	Hypothalamic–pituitary–adrenal axis
HPHC	Harmful and Potentially Harmful Constituent
ISO	International Organization for Standardization
NRC	National research council
NRT	Nicotine replacement therapy
POMS	Profile of Mood State
sAA	Salivary alpha-amylase
sC	Salivary cortisol
sCgA	Salivary chromogranin A
SHSE	Secondhand smoke exposure
TMD	Total mood disturbance
UV	Ultraviolet
WHO	World Health Organization

Chapter 1 Introduction

1.1. Tobacco Smoking

1.1.1. Smoking Impact

Smoking is one of the major health catastrophes. Smoking is believed to be the major cause of chronic diseases like Cardiovascular complications, stroke, pregnancy issues, respiratory failure, etc. Tobacco (*Nicotiana tabacum*, dried leaves through curing) is a harmful substance that is accountable for some oral infections and negative oral ailments. Tobacco is used in two forms, non-smoking and smoking. Tobacco utilization in any form is answerable for illnesses like oral malignant growth, adult periodontitis, and defects of birth such as cleft palate and lip in pediatric population whose mothers consumed tobacco during pregnancy [1]. Smoking is considered a medical issue at present everywhere. It is estimated that smoking will be the reason for 1 death in each of 3 deaths by 2020 [2].

1.1.2. Smoking Tendencies

Different factors are involved in the initiation of smoking that depends on the emotional factors, age groups, gender, *etc*. However there the emotional parameters aid more in smoking tendencies. There are three important transdiagnostic emotional factors that make the population vulnerable to initiation of smoking [3].

a. Anhedonia
b. Anxiety sensitivity
c. Distress tolerance

1.1.2.1. Anhedonia and Smoking

Anhedonia is a psychiatric condition that represents diminished hunger and demonstrates lacking pleasure and enjoyment. Anhedonia also results in decreased pleasure from and interest in the commonly rewarding stimuli. Anhedonia is also manifested by major depressive disorder (MDD). Anhedonia symptom is accompanying other depressive symptoms and recurrently takes place outside depression among psychiatric patients. The effect of reward stimuli is variable in the subject depending on the intensity of anhedonia. The personnel possessing a low level of anhedonia practice elevated amount of enjoyment and the effect of the reward is strong, however, subjects at the top end of the anhedonic spectrum show noticeable scarcities in the level of appetite [4].

Therefore, individuals with anhedonia may be more disposed to seek out drugs and other highly addicting booster drugs or weeds in order to practice a pleasurable experience. Anhedonic individuals are more expected to headway from irregular smoking which is known as *experimentation* to steady smoking patterns due to potential psychopharmacological relation between nicotine and anhedonia [5].

1.1.2.2. Anxiety Sensitivity and Smoking

The term anxiety may be demonstrated as the degree to which individuals consider nervousness and nervousness-related sensations. This has harmful consequences on personal health and psyche. Anxiety is a trigger to panic psychopathology. In addition to this, research also documents anxiety stress's (AS) role in the etiology of major depressive disorder (MDD), Post Traumatic Stress Disorder (PTSD), obsessive-compulsive disorder (OCD), social anxiety disorder (SAD), hypochondrias, chronic pain, and other clinical conditions that may affect the lifestyle of individuals facing emotional consequences. Anxiety-related stress (AS) is a major cause of smoking, hence, there exists a lot of research work that links Anxiety-related stress and smoking. Smoking serves as critically important and provides an instantaneous acute effect on the areas of the bran that have regulatory effect on mood, that may dominate fears about the consequences of smoking affecting the long-term health. The administration of nicotine and tobacco for the time being and quickly diminishes anxiety symptoms and tobacco temperance which may suggest a pharmacological dependence *i.e.* the smokers continue smoking as medication to get a remedy for anxiety and stress [3].

However, even though smoking may temporarily relieve stress, the over longer use of tobacco smoking by its own results in augmented anxiety-related sensations through several mechanisms including perceived and objective, health impairment, nicotine-based withdrawal symptoms, and physical illness.

Sometimes during the maintenance phase of the smoking cycle, once the regular smoking frequency is achieved, high-anxiety stress smokers may be troubled to perform a cease attempt, because these individuals are principally frightened and emotionally sensitive to the nicotine withdrawal-related distressing sensations (e.g., reflex anxiety, concentration difficulty, slowing heart rate; bradycardia) that occur during smoking cessation/moderation [3].

1.1.2.3. Distress Tolerance and Smoking

The ability of a person to manage actual or perceived emotional distress is termed Distress tolerance (DT). Persons with lower Distress tolerance are prone to poor adaptivity response to distress. This poor adaptivity is commonly manifested by avoidance of stressful stimuli and escape from distress-provoking contexts. Contrary to this, individuals with high tolerance to distress may be abler to respond to distress eliciting stimuli and distress adaptively.

Distress tolerance may affect or get affected by several factors involved in self-regulation given as under.

a- Cognitive appraisals of distressing emotional state

b- Cognitive appraisals of distressing physical state

c- Emotional response to distress

d- Interactive responses to distress (Behavior)

e- Attention

People with a qualitatively unique history or more extensive history of passionate experiences may be able to possess a greater ability to cope with the situation and develop a qualitatively discrete kind of perceived or behavioral as well as a strong and deep-rooted response to distress. Distress is coped with by individuals differently depending on their ability to withstand distress. Subjects with more distress tolerance (DT) are less prone to smoking. In contrast to this individuals with low-Distress Tolerance are believed to be more prone to smoking because throughout their lives, they have adopted to employ "low-effort coping skills" for distress [3]. The emotions and smoking are represented by the flow chart given in **Figure 1-1**.

1.1.3. Cigarette smoking stages

Cigarette smokers usually possess a generally specified pattern that includes several stages *i.e.* initiation of smoking, progression of smoking, maintenance, smoking cessation, and relapse [6].

1.1.3.1. Initiation

The first stage of smoking reflects the initial cigarette smoked and later-on supplementary use with irregular frequencies and intensity. This is termed as experimentation (irregular smoking).

1.1.3.2. Progression of Smoking

A substantial number of initiators continue their smoking behavior and intensify their smoking patterns. This escalation eventually progresses to regular smoking, with a frequency ranging from a lower frequency (weekly) to more frequent/daily use.

1.1.3.3. Maintenance

The period of smoking is characterized by a rhythmic consumption of tobacco and smokers adopt a regular and systemized smoking patterns. This is known as the maintenance phase. Smokers at this phase are more prone to addiction/habitualization. The maintenance stage is the critical stage of the smoking pattern which may either further intensify smoking or cause an end to smoking depending on individual variations.

1.1.3.4. Smoking Cessation

There exist individual differences among smokers regarding the frequency and intensity of smoking. Temporary emotional stability may, sometimes, in some users; result in cessation of smoking.

1.1.3.5. Relapse

Addiction is a central behavior of centrally depending on drugs or tobacco that may cause an elevation in the dopamine in the brain.

An individual's chronic exposure to nicotine produces adaptations (neuroadaptations) in the CNS (Central nervous system) *i.e*. mesolimbic dopamine system. Therefore, the level of nicotine must be maintained in the mesolimbic system in order to reserve a steady state mesolimbic (and hedonic) tone. In individuals, different mechanisms of maintaining smoking may variate from subject to subject and promote adaptable smoking patterns with respect to intensity, consistency, and severity. This is an indication of tobacco dependence.

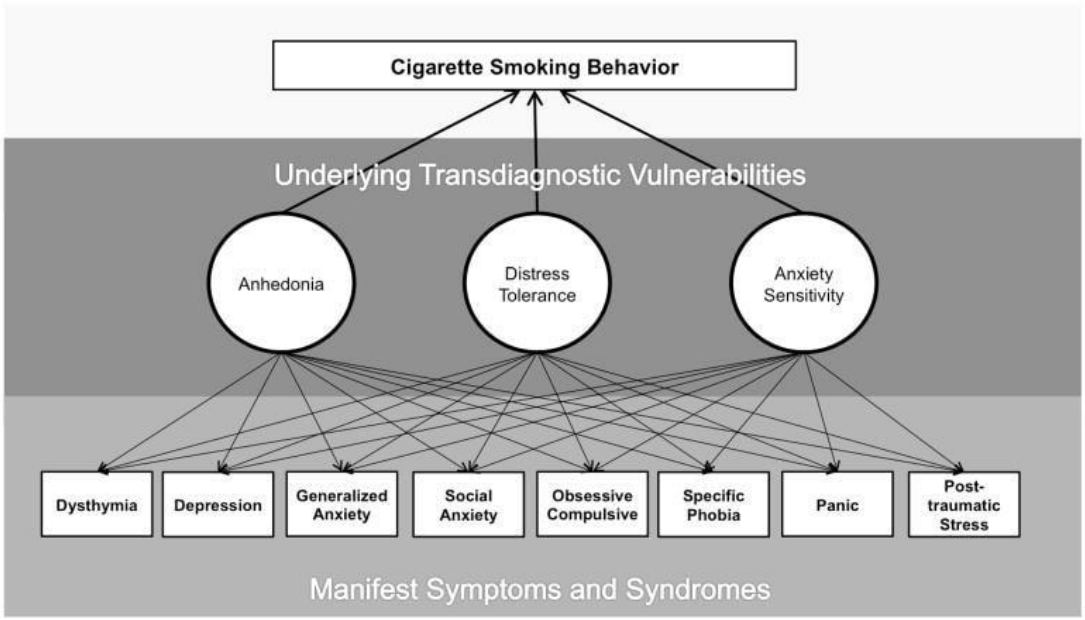


Figure 1-1: Emotional Smoking Comorbidity Explained by Overarching Transdiagnostic Framework [3]

1.1.4. Health Hazards and Consequences of Smoking

The environmental tobacco smoke is mainly categorized into two groups.

A- Sidestream smoke: The burning tip of the cigarette producing the smoke

B- Mainstream smoke: The smoke that is further treated by the individual *i*.e. inhalation, filtration and exhalation.

The vast majority of environmental tobacco smoke is comprised of sidestream smoke. The side-stream smoke is ifferent from mainstream smoke w.r.t quality and chemical nature. In general, the exposure of a non-smoker to tobacco smoke is less compared to a smoker (Active smoker). However, the sidestream smoke is more harmful than mainstream smoke w.r.t the nature of chemicals/combustion products. This due to the fact that the sidestream is produced at a higher temperature of combustion and because sidestream smoke remains unfiltered [7, 8].

The death percentage that can be avoided at non-smoking death rates is provided in **Table 1-1**, which reflects the percentage of high relative risks of smoking for these circumstances.

Table 1-1: The proportion of deaths avoidable for each major disease caused due to smoking [8]

	Males				**Females**			
Age groups (Years)	35–54	55–64	65–74	≥75	35–54	55–64	65–74	≥75
	Percent Avoidance (%)							
Lung carcinoma	93	95	96	96	92	95	96	96
Other carcinomas	43	46	57	54	22	52	51	48
Cerebrovascular disease			54	32			56	41
Coronary heart problem (CHD)	74	67	64	49	80	69	70	56
Atherosclerosis Aneurysm			86	80			85	83
All vascular at ages 35–64	58	60			59	49		
Diabetes mellitus			33	9			35	9
Lung disorders *i*.e. TB and Pneumonia			61	38			43	51
All respiratory at ages 35–64	78	93			84	89		
Pulmonary Obstruction (COPD)			97	96			97	95
All causes	61	66	67	58	44	62	65	60

1.1.4.1. Acute effects

The serious effects of contact to tobacco smoke on healthy nonsmoking adults are primarily associated with physical effects like smell, respiratory mucosa irritation, and psychological impact like annoyance. The nonsmoker (passive smokers) report irritation of eyes as the symptom most commonly, followed by

irritation of the nose, pharynx, and respiratory tract complaints. The intensity of complaints is proportional to the smoke exposure quantitatively. The extent of mucosal irritation is directly proportional to the level and duration of exposure to smoke.

There are different allergens in tobacco smoke. People exposed to tobacco smoke may also face allergic reaction problems[8].

1.1.4.2. Chronic Effects

Prolonged exposure to tobacco smoke is responsible for the chronic effects among the smoking community [8].

1.1.4.2.1. Cardiovascular Effects

The possible chronic effects of tobacco smoke on cardiovascular health have been analyzed by various researchers in both the smoking and nonsmoking community. The effects of blood pressure, heart rate, and oxygen consumption have been reported. The conditions associated with CVS include coronary artery disease (CHD), aneurysm of aorta, stroke, and peripheral artery disease. These conditions are responsible for a large number of deaths [8].

1.1.4.2.2. Respiratory Effects

Tobacco smoke is in direct contact with respiratory epithelial membranes. Smoke may physically damage the epithelial linings directly as well as the noxious chemical constituents of the ETS can also cause chronic life-threatening conditions. Smoking effects the inner linings of the respiratory mucosa and causes lung disease by damaging the basic functional unit of the lungs; alveoli (the small air sacs) found in the lungs. Chronic smoke contact through the life span can cause some severe lung diseases that include Chronic obstructive pulmonary disease (COPD), which includes chronic bronchitis and emphysema. Cigarette smoke is also a cause of most cases of lung cancer [8].

1.1.4.2.3. Cancer

Plenty of research work have been attended on the potential of tobacco smoke to induce cancer in the subjects due to the presence of potentially harmful carcinogens. The United States Department of Health and Human Services (USSGR) in the year 2014 and the reports issued by the International Agency for Research on Cancer (IARC; humans risk evaluation group for exposure to carcinogens) in the year 2014 have concluded that there exist sufficiently strong evidences to demonstrate that the use of tobacco is a cause of several cancers including lung

carcinoma, lip and tongue cancer, oropharynx and larynx neoplasia, bladder and kidney cancers, esophagus, pancreas, stomach as well as cancer of the cervix and liver.

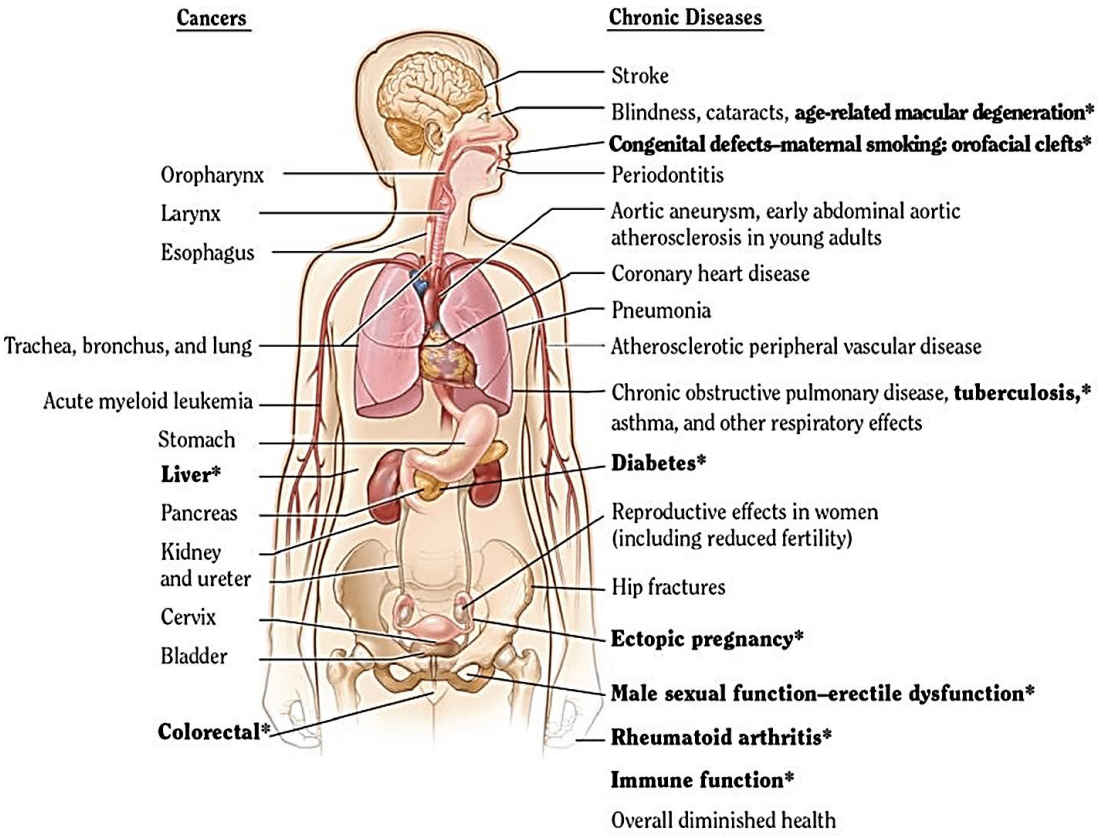


Figure 1-2: Estimation of the anatomic sites and cancers linked to smoking as presented by the U.S. Department of Health and Human Services [8]

1.1.5. Addiction Treatment

The exists a number of evidence that supports simultaneous management for tobacco addiction and other abused substances. The technique for treatments combination is the most effective way to face the problem of concurrent addictions. The percentage of smoking cessation is proportional to the duration of treatment frequency of the follow-up. Smoking cessation has a success rate of 4.6% at a follow-up duration of 6 months. Similarly, the success rate for weekly follow-up is 23.4%.

Smoking cessation has a positive effect that is measurable almost immediately after stopping the cigarette. The time series of smoking cessation in relation to the effects produced are summarized in the phases below.

1. **Phase-1 (prompt effects)**

After 20 minutes of the last cigarette taken, the peripheral vasoconstriction is reduced and blood pressure also decreases. This results in the normalization of the body temperature (especially hands and feet) back to normal.

2. Phase-2

Smoking cigarette causes an elevation in the carbon monoxide (CO) levels in the blood. The CO levels, after 8 hours levels normalizes.

3. Phase-3

After 24 hours of cessation, the chance of a cardiac anomalies is decreased compared to the smoking phase.

4. Phase-4 (Respiratory recovery)

The improvement/recovery to the respiratory system is comparatively late. Approximately 1 to 9 months after exposure, the ciliary function of lungs (responsible for the clearance of mucus from the lungs) returns to normal. The functional ciliary response allows for the proper elimination of particulate matter and mucus, hence reducing the prevalence of microbial infestation. The problems of coughing, sinusitis and nasal congestion, fatigue, and shortness of breath (SOB) are also decreased.

5. Phase-5 (Cardiovascular recovery)

The risk of coronary heart disease will decrease to 50 percent of a chain smoker after 1 year.

6. Phase-6 (Final recoveries)

After 5 to 15 years, the smoker's body is cleared of most of the noxious stimuli that may trigger any cardiovascular defects. The hazard of cardiac stroke is diminished to the level of a nonsmoker.

7. Phase-7 (Full Recovery)

After 15 years, the smoker is physiologically similar to a non-smoker [9].

1.2. Tobacco Constituents

1.2.1. Phytochemcial Properties

A large number of pyridine alkaloids are found in tobacco leaves. Among these alkaloids, the most important one is nicotine. Nicotine is a liquid naturally occurring alkaloid. The nature and percentage of the chemical constituents are variable in different parts of the plant. Based on the location, the distribution of the phytochemicals is given below [10].

1. Leaves

Several other alkaloids include nicotimine, nicoteine, anatalline, anabaine, and nornicotine. A high percentage of organic acids is presented in the leaves. The leaves are also rich in tahacinin, glucosides, iso-quercitrin, chlorogenic, oxalic acids, and caffeic. Carcinogenic substances and terpenic substances are also present. [10]

2. Roots

(+)Nornicotine and anatabine have been isolated from roots.

3. Flowers

Flowers also contain important organic constituents. Quercetin-3-Methyl ether and Quercetin-3,3′-dimethyl ether have been isolated from the flowers.

4. Shoot Apics and Flower Buds

New Gibberellins; the plant regulating growth hormones have been identified in the shoots apices and flower buds. The three novel gibberllins are:

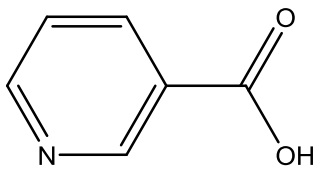
i- Nicotiana (alpha, beta and gamma)

ii- Gibberllin A

iii- Gibberllin A3

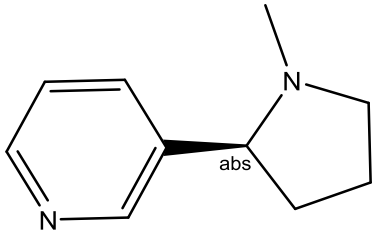
1. Seeds

Tobacco seeds are rich in different types of lipids. The seed oil is reported to contain; cycloartenol 24-daturadiol, cycloartanol, solavetivone, Cholesterol, 24-methylenecholesterol, cholest-7-enol, campesterol, 28-isofucosterol, stigmasterol, sitosterol, lanost-8-enol, lanosterol, 31-norlanosterol, cycloeucalenol, obtusifoliol, 31-norcycloartenol, citrostadienol, granisterol, β-amyrin, 24-methylenecycloartanol, lupeol, and cycloartanol.



NICOTINIC ACID

Figure 1-3: Structural representation of nicotinic acid (parent compound of nicotine)



NICOTINE

Figure 1-4: Structural representation of nicotine; a major constituent of tobacco

The phytochemicals of the tobacco leaf have been screened by researchers through several identification tests listed in **table 2** below.

Table 1-2: identification tests for the phytochemical analysis of tobacco leaf (*Nicotiana tabacum*) [11].

S.No.	Type of Phytochemical	Identification Test	Results
1	Alkaloids	Dragendroff Mayer Wagner	+ + + + + +
2	Tannins	$FeCl_3$	+
3	Phenolic compounds	$FeCl_3$	+
4	Flavonoids	Ammonium	+
5	Terpenoids	Salkowski	+
6	Steroids	Burchard	+
7	Essential oils	NaOH, HCl	+
8	Resins	Turbidity	+
9	Saponins	Froth	+
10	Polypeptides	Biuret	-
11	Quinones	Sulfuric Acid	+

1.2.2. Environmental Tobacco Smoke from Combustible Cigarettes

Environmental tobacco smoke (ETS) is the incineration products of tobacco that are inhaled by smokers. The term was initially used by the National Research Council (NRC). The chemical and physical characteristics of the environmental tobacco smoke were reviewed by the National Research Council. Approximately

3800 chemical constituents have been recognized in tobacco smoke. Most of these compounds are known to cause carcinogenesis. The sidestream smoke is comparatively more hazardous and contains quantitatively more concentrations of ammonia, nicotine, benzene, carbon monoxide, and many other cancer causing agents [7]. Some researchers suggest that the smoke resulted from the burning of tobacco is a chemically complex and dynamic mixture of ingredients containing more than 8000 identified chemical compounds. The smoke of a combustible cigarette is in the form of aerosol that is composed of the following three major components [12].

1- Liquid droplets

2- Carrier gas for the suspension of the liquid droplet

3- Gas-vapor phase surrounding the carrier gas

Cigarette smoke is produced by a combustible cigarette at a combustion temperature of approximately 900°C.

Tobacco smoke has been analyzed by different researchers and organizations to various extents and enlisted the constituents of the smoke. A number of chemicals are enlisted below [12].

1.2.2.1. Criterion 1 Constituents

International Organization for Standardization (ISO) devised methods for the determination of the smoke constituents. The following compounds are included in this list.

Table 1-3: Criterion-1 constituents of tobacco smoke

S.No.	Chemical constituent
1	Total particulate matter
2	Water in the total particulate matter
3	Dry particulate matter free of nicotine
4	Nicotine
5	Benzo[a]pyrene
6	Carbon monoxide

1.2.2.2. Criterion 2 Constituents

The health monitoring authorities and regulators *i.e*. WHO, and Health Canada enlisted the toxic constituents in the Environmental tobacco smoke (ETS). In

addition to the compounds listed in Criteria-1, the constituents (Harmful and Potentially Harmful Constituents; HPHCs) listed in **table-**3 are also included.

Table 1-4: Criterion 2 constituents of tobacco smoke

S. No.	Class	Chemical Constituents
1	Aromatic Amines	1-aminonaphthalene, 3-aminobiphenyl, 2-aminonaphthalene, , 4-aminobiphenyl
2	Aldehydes and ketones	acetaldehyde, acrolein, acetone, butyraldehyde, formaldehyde, crotonaldehyde, propionaldehyde, methyl ethyl ketone
3	Benzene Derivatives	benzene, pyridine, isoprenequinoline, toluene, styrene, catechol, hydroquinone, cresols, resorcinol, phenol
4	Nitrogen containing non-aromatic compounds	nitrogen oxides (NOx), ammonia (NH_3), hydrogen cyanide (HCN), nitric oxide (NO),
5	Nitriles	Acrylonitrile
6	Heavy metals	Selenium (Se), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), and arsenic (As)

1.2.2.3. Criterion 3 Constituents

Criterion 3 list contains toxicants with a recognized biomarker of exposure. These biomarkers are either the metabolic products of the parent compounds inhaled or the parent compounds themselves. These biological markers of exposure to environmental tobacco smoke (ETS) can be utilized clinically to diagnose and study exposure to tobacco smoke.

1.2.2.4. Criterion 4 Constituents

Criterion 4 enlists several chemicals that are produced as a product of combustion under 400°C. These toxicants are not a part of the criterion 2 list.

Table 1-5: Criterion 4 constituents of tobacco smoke

S. No.	Chemical Constituents	Description
1	Acetamide	Formed at around 250 °C

2	Acrylamide	Formed between 120 and 200 °C
3	Propylene oxide	Formed below 400 °C
4	Nitrobenzene, vinyl chloride, and ethylene oxide	Formed below 400 °C

1.2.2.4.1. Acetamide

Acetamide is the pyrolysis product of Amadori compounds. Amodori compounds are formed form the reaction of suars and amino acids. The decomposition of ammonium acetate also genereates aceteamide at a combustion temperature of 250°C.

1.2.2.4.2. Acrylamide

Maillard reaction occurs at a temperature range of 120 and 200 °C during cigarette burning/combustion. This reacton produces acrylamide.

1.2.2.4.3. Propylene Oxide

Dehydration of propylene glycol produces propylene oxide. It used as a humectant in combustible cigarette. It fuctions asaflovouring agent in cigarette.

1.2.2.4.4. Nitrobenzene, vinyl chloride, and ethylene oxide

The source of generation of these three compounds is not clear. The International Agency for Research on Cancer (IARC) has classified ethylene oxide and vinyl chloride as Group-1 carcinogen (recognized carcinogen to humans). Nitrobenzene is classified as Group-2b carcinogen (possibly carcinogenic to humans).

1.2.2.5. Criterion 5 Constituents

Criterion 5 includes those chemicals that are produced as a product of combustion over 400°C. These toxicants are also not incorporated in criterion 1 and criterion 2 lists. These include:

1- benz[a]anthracene

2- dibenz[a,h]anthracene

1.3. Stress, Stress Physiology, and Stress-biomarkers

1.3.1. Stress

The undesirable, difficult, and challenging circumstances or stressors generate a usual psychophysiological response. This response is termed stress. Research studies for the past five decades have proven the adverse effect of stress on brain physiology and functioning. Chronic stress harms the brain's anatomy by

causing atrophy. This leads to a negative impact on cognition and memory and differential response. The intensity of effects depends on the duration of stress, the longer the duration of the stress, the greater the negative impact on the brain leading to psychological alterations

Stress either be acute or chronic. Acute stress means short-term stress with an adaptive state. Chronic stress is high-intensity stress with a longer duration and maladaptive response. This implies harmful effects on bodily mechanisms [13].

1.3.2. Stress Anatomy

Various parts of the body are involved in the induction, maintenance, and cessation of stress. The body organs may either be involved primarily or they may respond secondary to stress and further aggregate stress. The central nervous system including the brain and neurons is the most important locus of stress. However, the following three main body systems may regulate the response to stressful stimuli [14].

A- Central Nervous System (CNS) and Stress

The brain is the primary organ of the CNS that controls the overall coordination and functioning of the body. The hypothalamus is located near the pituitary gland, at the ventral side of the brain. It is a minor region of the brain. The hypothalamic–pituitary–adrenal axis (HPA) has a significant role in response to stress. HPA releases cortisol in reaction to stress. HPA also shows a significant role in depressive illness. HPA produces cortisol; an important stress biomarker. HPA coordinates with the brain, autonomic nervous system (ANS), and immune system through cortisol [15].

B- Autonomic Nervous System (ANS) and Stress

The autonomic nervous system also contributes to the generation and regulation of acute and chronic stress. The autonomic reflexes regulate bodily functions in response to stressful stimuli. The stressful stimuli may be internal or external. The internally instigated factors include conservation of body homeostasis including serum sugar, temperature, removal of excess water from the body, obesity, *etc*. The external stimuli to which ANS responds include environmental, smell, vision, touch, *etc*. The ANS is further categorized into the Sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS) [15]. The autonomic stress biomarkers include:

- Norepinephrine (Noradrenaline)

- Epinephrine (Adrenaline)
- Acetylcholine (ACh)

Epinephrine and norepinephrine are called catecholamines. During stress conditions, the level of acetylcholine decreases while the level of catecholamines increases.

C- Immune System and Stress

The immunological biomarkers that are regulated by CNS and ANS are also responsible for the immune system's response to stress. The human body's immune system fights to negate stress. The stress that the immune system fights may be physical or psychological. The injury or infection to the body is stressful stimuli and these produce fight and flight responses. The immune system dysregulation may increase the inflammatory responses. The nature of responses and intensity of the response is variable from individual to individual [16]. The immunological stress biomarkers include:

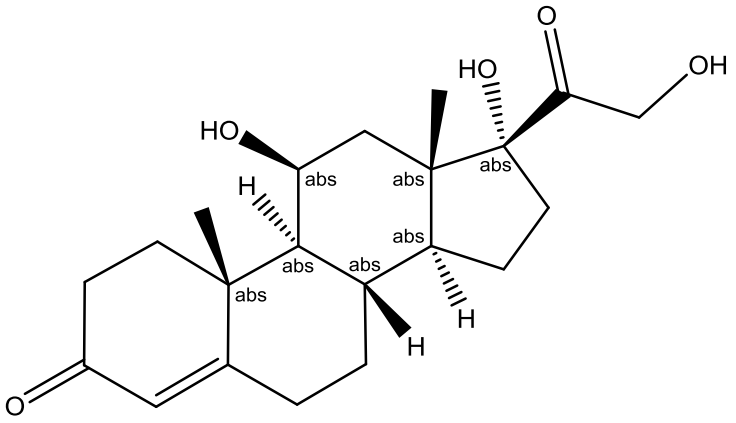
- Cytokines: they include interleukin 1 (IL-1) and interleukin 6 (IL-6) and control immune cells differentiation.
- Tumor necrosis factor-alpha (TNF-alpha): it is also called pro-inflammatory cytokines
- C-Reactive Protein (CRP): CRP is injected to the blood stream by the liver in response to stress.
- Natural killer cells (NK)

1.3.3. Stress-Biomarkers

The human body responds to trauma (physical or non-physical stress) in a definite manner. This response of the body can be qualitatively and quantitatively monitored through several chemicals in the bloodstream, saliva, or urine; responding to the stress, called stress biomarkers.

1.3.3.1. Cortisol

The corticotropin-releasing hormone (CRH) is secreted by the hypothalamic-pituitary-adrenal axis (HPA-axis). The anterior pituitary is further stimulated by CRH and persuades the release of a tropic polypeptide adrenocorticotropic (ACTH) hormone. ACTH prompts the secretion of adrenal steroid known as cortisol [17].



CORTISOL

Figure 1-5: Structural representation of cortisol (HPA stress biomarker)

1.3.3.1.1. Synthesis

The synthesis of cortisol that occurs in zona fasciculata of the adrenal cortex of the kidney, is carried out form cholesterol precursor and acts as the primary glucocorticoid. Cortisol is then secreted by the influence of biochemical stress.

1.3.3.1.2. Significance

The time to measure the blood cortisol has significant importance. This is due to the fact that it is decreased in the evening time and increases in the early morning. Similarly, the blood cortisol level initial phase of sleep is also decreased.

1.3.3.1.3. Properties

Cortisol occurs as both complexed and free forms. The unbound/free cortisol is lipophilic and possess a low molecular weight. The entrance of cortisol to the cell takes place through passive transport, therefore, easily examined in the body fluids. The quantification of cortisol can be done by obtaining samples from hair samples, saliva, and urine [17].

1.3.3.1.4. Salivary Cortisol

The estimation of cortisol through saliva samples increases and provides useful results using salivary cortisol as a stress biomarker. Comparing the basal physiological values in healthy subjects and the physiological stress response in stress (burnout) subjects, the results show that the level of cortisol and heart rate of the stressful subjects are comparatively higher during the first hour after wakening from sleep. Therefore, after awakening from sleep, the measurement of free cortisol should be deliberated a possible biomarker for assessing stress intensity [18].

1.3.3.1.5. Hair Cortisol

Hair cortisol levels measurement is a probable neonatal chronic stress clinical biomarker for infants, the hair cortisol levels also provide an excellent dimension for monitoring chronic stress during the first year of childhood. The scalp hair cortisol quantification is a biomarker for long-term cortisol measurement in children. Similarly, the hair cortisol is also a suitable biomarker for chronic stress determination in pregnant women [19].

1.3.3.1.6. Urinary Cortisol

Urinary cortisol is also an indicator of chronic stress. The first-day urine of the burnout subject after sleep contains free cortisol that can be utilized as a chronic stress biomarker. However, reduced free urinary cortisol is seen in stressed patients as compared to the healthy subjects. Hypocortisolism is observed in the stressed patient.

1.3.3.2. Adrenocorticotropic Hormone (ACTH)

Corticotrophin-releasing hormone (CRH) plays a very significant role in the autonomic, endocrine, and immune systems. CHR stimulates the release of ACTH. Adrenocorticotropic hormone can also be used as a ration to estimate chronic stress. ACTH is released by the pituitary gland in stressful conditions [20].

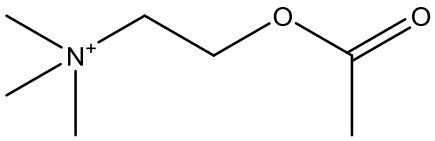
1.3.3.3. Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor (BDNF) plays a very vital part in the maintenance of the physiological activity of the nervous system and the development, regulation, and survival of neurons. Research studies suggest that a significantly lowered level of BDNF is observed in the stress (burnout) subjects. When compared to the healthy subjects, the cognition factor is also decreased [21].

1.3.3.4. Catecholamines

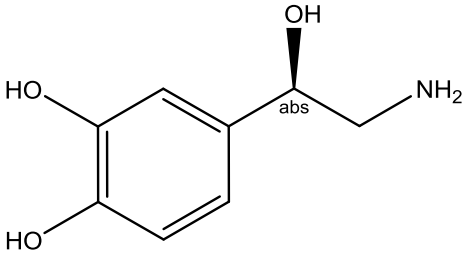
Catecholamines are major autonomic biomarkers. They control the sympathetic nervous system. They are also considered the major biomarkers for autonomic nervous system activity. The synthesis of catecholamines (adrenaline and noradrenaline) occurs in the centrally allocated noradrenergic neurons and is released into the bloodstream principally by the adrenal medulla. The exposure of individuals to stressful conditions results in the elevation of adrenaline and noradrenaline levels that further induces physiological alterations in the normal physiology *i.e.* cardiovascular system (CVS), gastrointestinal system (GIT), respiratory system (RS), nervous system, *etc*.

Acetylcholine is another autonomic stress biomarker. It is a neurotransmitter of primary importance in the parasympathetic nervous system. It has an opposite action to the catecholamines, hence the level of acetylcholine is decreased in stressful situations [17].



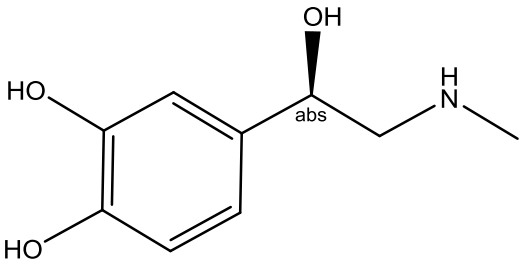
ACETYLCHOLINE

Figure 1-6: Structural representation of acetylcholine (autonomic stress biomarker)



NOR EPINEPHRINE

Figure 1-7: Structural representation of norepinephrine (autonomic stress biomarker)



EPINEPHRINE

Figure 1-8: Structural representation of epinephrine (autonomic stress biomarker)

1.3.3.5. Cytokines

Cytokines play a significant role in immune responses by promoting the growth of cell involved in immunity and cell to cell communication in the immune system. This group of biomarkers includes growth factors, interferon, and interleukins. Immune biomarkers including the cytokines involved in inflammation (IL-6, IL-1b), tumor necrosis factor alpha (TNF-α), cytokines involved in inflammatory (IL-10), and natural killer cells (NK cells) also act as stress indicators.

1.3.3.6. Fibrinogen and C-Reactive Protein

The is a significant relationship between daily stressors, inflammatory markers, and chronic stress. The levels of c-reactive protein are intensified in severe stress levels and infections of various origins [22].

1.3.3.7. Other Biomarkers

1.3.3.7.1. Metabolic Stress Biomarkers

The metabolic biomarkers are also utilized for examining the relationship of chronic stress as a result of long term diseases. These biomarkers include fasting glucose level, glycosylated hemoglobin (HbA1c), glucose tolerance test, triglycerides, and cholesterol levels [22].

1.3.3.7.2. Endocrine Stress Biomarkers

The intensity of chronic stress is also evaluated by screening blood samples for considering the endocrine stress hormones. The hormones include prolactin, oxytocin, Growth Factor (GF), estradiol, and Dehydroepiandrosterone Sulfate (DHEA-S) [23].

1.3.3.7.3. Antioxidant Stress Biomarkers

A complex array of non-enzymatic and enzymatic antioxidants is also present in the human body. They are involved in the antioxidant defense system. They act as antidotes for free radicals and scavenge reactive oxygen species (ROS). These antioxidants also provide a quantitative approximation of the level of stressful stimuli. These biomarkers include; glutathione peroxidase (GTPx), superoxide dismutase (SOD), Ascorbic Acid, and malondialdehyde (MDA) [24].

1.4. Saliva and its Role in Biological Measures

Saliva delivers an efficient specimen for various diagnostic procedures due to the presence of different biological products and secondary metabolites of xenobiotics and helps in determining the disease progressions as well as therapy outcomes depending on the variations in the markers/triggers [25].

1.4.1. Factors Affecting Saliva Composition and Quantity

The volume of saliva produced is dependent and different internal and external factors. Internal factors include the normal physiology of the oral cavity that is based on the genetic expression of the salivary glands, diseased conditions, and other syndromes that lead to low production of saliva. The external factors include physical stimuli *i.e*. drugs (pharmacologically used or abused) and psychological aspects *i.e*. environmental stress.

In the same manner, the composition of saliva is also different among the individuals depending on different factors *i.e* gender, age, and diet. Each of these factors greatly affects the screening process. For example, the quantity of saliva produced and the rate of flow are dependent on the age of the patients. Saliva quantity and flow are decreased in old age. The position of the mouth while collecting saliva has a significant role. There are different salivary glands *i.e*.

a- Sublingual glands b- Submandibular glands

c- Parotid Glands d- Other minor glands

Parotid glands are believed to produce a larger amount of proline-rich proteins and alpha-amylase. Similarly, a small quantity of alpha-amylase is produced by submandibular glands. A large number of glycoproteins and mucous cells are produced by sublingual glands.

1.4.2. Saliva Sampling

Obtaining saliva is an easy and painless process providing a preferred sampling technique. However, minor errors in saliva collection may lead to altered results. Therefore, in order to reduce the chance of error, the process of saliva collection must be optimized. These factors may include the quantity of saliva collected, time of saliva collection, normal or stimulated collection, heat and light exposure, storage temperature, the time elapsed from collection to processing, and several other factors aforementioned. The correct selection of the subjects is of primary importance. A saliva sample should not be stored for more than 6 hours in a refrigerator, otherwise, the integrity and composition are slowly degraded. Therefore it is suggested that specimens should be frozen and stored at -80 ºC. Specimen stored at this temperature can be safely used for up to 2 years [26].

In the same way, the processing methods for preparing samples also affect the salivary products by altering the pH and osmolarity as well as physically and chemically interacting with the added ingredients. The protocols and standards used to analyze the samples also modify results.

1.4.3. Saliva and Biomedical Technology

Saliva is a source of more than 3000 peptides and proteins that can be utilized as biomarkers for a number of circumstances. Salivary proteome has been used to diagnose and predict disease progressions of serious ailments *i.e*. oral squamous cell carcinoma, oral inflammatory diseases, periodontal diseases, cystic fibrosis, hepatitis, diabetes mellitus, *etc*.

Stress progression and intensity can also be assessed with the biomarkers delivered by salivary secretions. Cortisol is the often reported biomarker of stress that can be analyzed from saliva. Several kinds of literature are available that support the fact that salivary cortisols are elevated greatly depending on the intensity and duration of physical and psychological stress. 3-methoxy-4-hydroxyphenylglycol is a metabolite of adrenaline. It is also an important stress biomarker. Certain other markers include alpha-amylase, chromogranin-A, testosterone, *etc*.

1.5. Biomarkers of Smoking Exposure and Assays

1.5.1. Biomarkers of Exposure to Cigarette Smoke

The determination of tobacco smoke biomarkers is an important aspect of measuring the negative effects of cigarette smoke exposure. Several products of tobacco combustion can be detected in the human body secretions or bloodstream that can quantify tobacco use. List of smoke biomarkers is given below.

Table 1-6: list of combustion product of tobacco and their respective biomarkers in the body

S.No.	Tobacco combustion product	Biomarker	Sample
1	Nicotine	Cotinine	Serum
2	Carbon monoxide (CO)	Carboxyhemoglobin (COHb)	Serum
3	Nicotine	Total Nicotine	Urine
4	2-aminonaphthalene, o-toluidine, and 4-aminobiphenyl	Parent amines	Urine
5	Crotonaldehyde	HMPMA; 3-hydroxy-2-methylpropyl mercapturic acid	Urine
6	Acrolein	3-hydroxypropylmercapturic acid	Urine
7	Butadiene (1,3)	MHBMA; 1-hydroxy-2-(N-acetyl-cysteineyl)-3-butene	Urine
8	Acrylamide	AAMA; acrylamide mercapturic acid	Urine
9	Acrylamide	CEMA; 2-	Urine

		cyanoethylmercapturic acid	
10	Benzene	S-PMA; S-phenylmercapturic acid	Urine
11	NNN	Total NNN	Urine
	Benzo[a]pyrene and pyrene	1-OHP; Total 1-hydroxypyrene	Urine
	NNK	Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)	Urine

1.5.2. Commonly Used Assays for Biomarkers Detection

The biomarkers can be qualitative and quantitatively investigated through various analytical techniques. The type of technique used depends upon the nature of the sample and the type of analyte. Some of the most commonly used techniques include:

- Enzyme-linked immunosorbent assay (ELISA
- Time-resolved immune fluorometric assay
- Radioimmunoassay
- Other enzyme assays
- NicAlertR analysis
- LC-MS-MS; Liquid Chromatography with tandem mass spectrometry
- HPLC; High-performance liquid chromatography electrochemical findings
- Spectrophotometric techniques

1.5.2.1. UV Spectrophotometric Assay

UV spectroscopy of stress biomarkers is carried out in the spectroscopic range of 190-400 nm. At near UV wavelengths, different stress biomarkers show primary and secondary absorption peaks depending on their molecular structure.

Cortisol provides a maximum absorption peak at λmax: 247 nm. Serotonin has a complex UV spectrum and gives out four maximum absorption peaks at λmax 201, 224, 278, and 298 nm. The catechol biomarkers possess mutual structural similarities and contain amine groups *i.e*. Dopamine, Norepinephrine, and Epinephrine. They reveal unique absorption peaks at λmax 200, 219, and 273 nm. Neuropeptide Y creates characteristic absorption peaks at λmax 229 and 276nm.

Brain-derived neurotrophic factor generates a distinctive absorption peak at λmax: 190nm. Serotonin provides a particular absorption peak at λmax: 203 nm [27, 28].

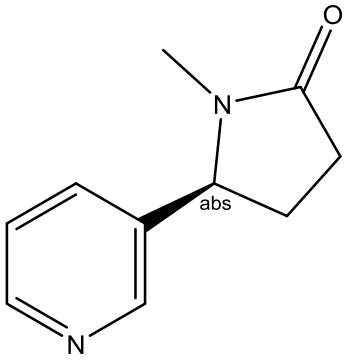
Nicotine provides a characteristic absorption peak at λmax: 260 nm (220-280 nm range) [29]. The maximum absorbance wavelengths are summarized in **table 7.**

Table 1-7: The maximum absorbance wavelengths of different stress biomarkers.

S. No.	**Biomarker**	**Max Absorption Wavelength (λmax)**
1	Cortisol	**247**
2	Epinephrine	**204**, 217, 278
3	Norepinephrine	**204**, 217, 278
4	Dopamine	**204**, 217, 278
5	Serotonin	**220**
6	Neuropeptide Y	**190**, 227, 276
7	Brain-derived neurotrophic factor	**190**
8	Alpha-amylase	**282**

1.6. Cotinine

Cotinine (COT) is the chief metabolite of Nicotine which is the product of oxidation carried out in the liver by the Cytochrome P450 Enzyme system. The CYP2A6 member of subfamily A is specifically involved in nicotine metabolism and results in the production of cotinine [1].



COTININE

Figure 1-9: Chemical structure of cotinine; the metabolic product of nicotine and a smoking biomarker

Cotinine is utilized as a biological marker for contact with tobacco smoke. Among the nicotine metabolites, it has comparatively a longer half life *i.e.* 12-15

hours. This makes it a suitable biomarker of smoking. Cotinine can be detected in the saliva, blood, or urine by various assays. Urine is a more sensitive matrix to detect cotinine because urine contains four to six times higher concentrations of cotinine as compared to blood and saliva. [30]

1.6.1. Cotinine Levels

The level of cotinine in different samples obtained from smokers is listed below in **table 7**.

Table 1-8: Concentration of cotinine (ng/mL) in samples obtained from various routes [31]

S. No.	Sample	Cotinine level (ng/mL)	Smoking severity
1	Blood	<10 ng/Ml	No active smoking
		10 ng/mL to 100 ng/mL	Reduced smoking or modest passive exposure
		≥300 ng/mL	Heavy smoking
2	Urine	-	-
		11 ng/mL to 30 ng/mL	Reduced smoking or modest passive exposure
		500 ng/mL or more	Active smokers
3	Saliva	-	-
		1 ng/mL and 30 ng/mL	Reduced smoking or modest passive exposure
		100 ng/mL or more	Active smokers

1.6.2. Cotinine in Various Samples

The concentration of cotinine is increased in saliva by 15% to 40% because of minimal protein binding, the small molecular size, and high water solubility. Therefore, the assessment of cotinine in the saliva is more convenient regarding sample collection, non-invasive technique, and well-tolerated procedure. In blood, cotinine has a longer half-life. It does not require hydration adjustment among smokers [32].

1.7. Mood Profiling and Artificial Intelligence

1.7.1. Profile of Mood State

The nature of mindset and mood states are evaluated by a scales designed to rate the behavior of an individual towards the environmental stimuli that may be physical or psychological in nature. This psychological rating scale is known as the profile of mood state (POMS). This scale was initially originated by McNair, Droppleman, and Lorr in 1971. This scale is presented in the form a questionnaire including different questions regarding the mode and feelings of a subject.

The POMS is composed of different mood profiles including Confusion, Anger, Depression, Tension, Fatigue, and Vigour. The first 5 mood states are considered negative mood profiles while the last mood state (vigour) is considered a positive mood profile. Each mood profile is evaluated by a number of questions. Each question is answered in different levels of intensities. Based on these intensities, each question is scored and calculated to measure the different mood profiles of a person [33].

1.7.2. Artificial Intelligence

1.7.2.1. Machine Learning

Artificial intelligence (AI) is applied to interpret the outcomes of a dataset by various software in a more sophisticated manner employing programs, computer languages and machine (computers, processors, and databases). This type of artificial intelligence is known as machine learning (ML). there are four basic categories of machine algorithm *i.e*. reinforced machine learning algorithm, unsupervised machine learning algorithm, semi-supervised machine learning algorithm, and supervised machine learning algorithm. The supervised ML will be used in this research work. The algorithms are supplied with training data being labelled. The variables that are required to be assessed are defined. Specification of input and output algorithm is performed. Machine learning works on the basis of computer programming languages. There are five major types of programming languages *i.e*. functional programming language, procedural programming language, logic programming language, scripting programming language, and object-oriented programming language. Logic programming language will be utilized in this research work [34].

Machine learning provides an excellent prediction in the trends and ups and downs of operational patterns. They are of great significance in the field of business, marketing, and research.

1.7.2.2. Neural Networking

Neural networking is a sort of artificial intelligence/machine learning. The behavior and response of the human brain is simulated by computer programs known as neural networks (NN) in order to identify different patterns and solve various common problems. It is also termed as artificial neural networking (ANN) or simulated neural networking (SNN).

There are three main types of neural networks *i.e*. artificial neural network, recurrent neural networks, and convolution neural networks. Neural networks are utilized to perform cognitive functions inspired by the neuronal networking in human brain [35].

1.7.2.3. Confusion Matrix

Confusion matrices (CF) are utilized in computer programming languages to assess the performance, precision, and accuracy of classification algorithms. The performance of a classification algorithm is summarized and visualized in a tabular form. CF may either be used for binary classification including two sets of data and multiclass classification issues. CF table contains actual values and predicted values [36]. The general representation of the confusion matrix is provided in **Figure 1-6**.

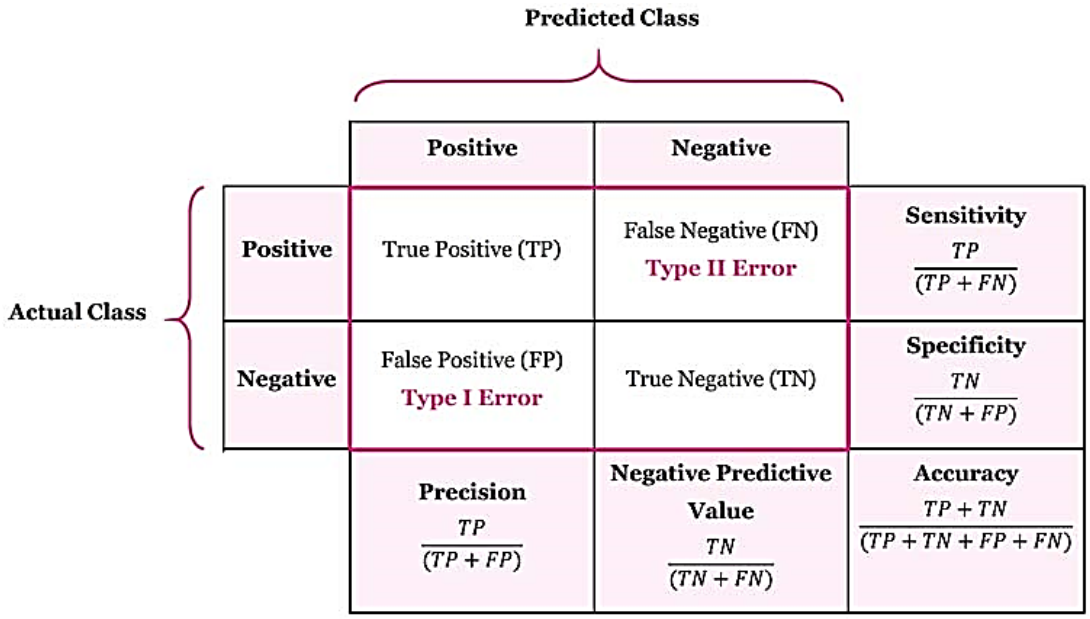


Figure 1-10: Confusion matrix tabular form representing actual and predicted values and accuracy calculation [36]

Chapter 2 Literature Review

Cotinine has been suggested as one of the most important and stable metabolites of nicotine by various researchers to be assessed for evaluating the intensity of smoking or exposure to environmental tobacco smoke. To date, various studies have been conducted to elucidate different techniques and assays for finding the cotinine concentration in samples obtained from various body secretions. Likewise, several research works have also been conducted to study the relationship between cotinine levels and stress/anxiety biomarkers to direct their mutual relationship. The negative effects of smoking can also be monitored through cotinine concentrations and respective biomarkers of tissue damage.

Studies on the stability of cotinine and its relative concentration in different samples have also been conducted. Avila-Tang et. al. [30] also suggest cotinine to be a stable biomarker of smoking/secondhand smoke exposure (SHSE). Besides saliva, blood, and urine; hair and toenails can also be used to investigate cotinine levels. As compared to carbon monoxide as a biomarker, cotinine has relatively high specificity and sensitivity. Therefore, it provides a reliable indication of recent nicotine intake. However, nicotine replacement therapy (NRT) through nicotine gum and patches of nicotine can affect cotinine significance. During NRT, cotinine cannot be utilized as a smoking cessation indicator because cotinine and nicotine are present at the same levels as in smokers. In this case, the urinary NNAL; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol has been reported not to be affected by NRT and a reliable smoking exposure biomarker [37]. The cotinine level determination provides consistent information about the exposure of individuals to cigarette smoke. However, plasma cotinine levels can highly be affected by various factors including the sum of cigarettes per day, the intensity of addiction (Fagerstrom test), purity of tobacco, *etc*. According to a research the cotinine concentration in smokers reached from 0 to 838 ng/ml. The cotinine concentration in smokers who consumed a total of 20 cigarettes per day raged from 55 to 684 ng/ml. the cotinine concentration in nonsmokers ranged from 0 to 46 ng/ml. The median saliva cotinine concentration has no variations between gender and was similar in females (2.4 ng/ml) and in males (3.0 ng/ml). Similarly, age variations were also not effective in changing the cotinine concentrations [38].

Cotinine cut-off values (concentrations) in different body secretions have serious importance in differentiating non-smokers, passive as well as active smokers. One study suggests that the urinary concentration of cotinine, in passive smokers, is lower than 50 µg/L of urine. While the concentration of cotinine is generally more than 100 µg/L in smokers [39]. Salivary cotinine cut-off values have also been set. Polanska et. al. suggests that 10 ng/mL of saliva is a suitable cut-off value for active smoking (specificity 95%, sensitivity 96%). While 1.5 ng/mL of saliva is the cut-off value for passive smoking (specificity 71%, sensitivity 63%) [40]. Cotinine level monitoring in subjects who have stopped smoking is critical for setting cut-off values for smoking exposure. The cotinine level is in the range of low ng/mL in the blood of nonsmokers. The mean serum concentration of cotinine of nonsmokers exposed passively turns out to be 1.1 ng/mL. Similarly, the serum cotinine level of former smokers with passive exposure turns out to be 1.7 ng/mL [41].

Cotinine is the primary metabolite of nicotine. Another cotinine's structural analog; trans 3′-hydroxycotinine (3HC), can also be utilized for estimating nicotine intake. The ratio of trans 3′-hydroxycotinine to cotinine is used for calculating the rate of metabolism of nicotine. It is called the nicotine metabolite ratio [42]. Studies suggest that the urinary level of cotinine should be corrected for creatinine clearance as the kidney function can profoundly alter the results [43].

Various animal (mice) models have also been used to learn smoking patterns, addiction, and plasma concentrations of smoking and stress biomarkers. Certain additives in cigarettes can also alter the concentration of cotinine in body fluids. A C57Bl/6J mouse model was utilized to study the effect of menthol on smoking addiction and subsequent cotinine levels. Menthol proves to be a promotor of smoking and causes a roughly 1.5-fold increase in cotinine levels in plasma [44].

Reactive oxygen species has tendency of causing mutilation to DNA through oxidation and result in the production of 8-OHdG; 8-hydroxy-2′-deoxyguanosine which is used as a biological marker of oxidative stress. The relationship between cotinine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) has also been studied [45]. Secondhand smoke (SHS) is also associated with adversarial effects on the respiratory system. Various researchers evaluated the negative impact of smoking on the lung's physiology. Tobacco smoke is considered to be a major cause of emphysema (chronic obstructive pulmonary disease) inducement [41]. The effects of

various pollutants including environmental tobacco smoke (ETS) have been studied in the Human early-life exposome (HELIX) project [46].

The salivary biomarkers for assessing the intensity of exposure to stress and their relation to smoking tendencies have also been studied. Three important stress biomarkers of saliva *i.e.* salivary alpha-amylase (sAA), salivary cortisol (sC), and salivary chromogranin A (sCgA) have been analyzed regarding stress-dependent alterations [47]. Salivary investigation of stress biomarkers is preferred because of standardized sample collection, non-invasive techniques, and easy handling of the samples. The standardized collection of saliva is ensured by different collection devices [48]. Suzuki et. al. [49] concluded that regardless of the mood state, the salivary interleukin (IL-1β) levels were considerably higher in smokers than non-smokers. There was a positive correlation between fatigue score and secretory immunoglobulin A (SIgA). The correlation between tumor necrosis factor (TNF-α) level and the the Brinkman index (BI *i.e.* The number of smoked cigarettes per day multiplied by the number of smoking years) also stands positive.

Ultraviolet spectrometry has also been suggested to be a convenient, quick, simple, and reliable method of analyzing cotinine through different studies. Ray et al [28] detected the multiple biomarkers (Dopamine, Serotonin, Cortisol, and Neuropeptide) in different bodily fluids i.e., Sweat, Saliva, Urine, and Blood. The authors used Ultra Violet (UV) Spectrometry for the detection of biomarkers in the range of 190-400 nm and their wavelengths depending upon the molecular structure. They performed UV spectrometry on the target sample. The absorption peaks whose wavelength helps to identify the biomarker presence and their peak amplitude showed their concentration. Cortisol levels showed only single-peak around 247 nm, which is close to the physiological range and the α-amylase absorption peak is 247 nm.

Biomarkers of stress are expressed in various body secretions at different levels of concentration. Different sampling routes provide several merits and demerits regarding the level of chemical moiety detection, stability, sampling technique, sample storage and processing, *etc*. Human saliva provides an excellent medium for biomarker detection due to repeated sampling, ease of collection, noninvasive collection, easy processing, and detection of a single chemical moiety and metabolites [29]. The mutual relation of nicotine dependence and cortisol responses has also been studied. One study reports that the severity of nicotine

dependence is inversely related to cortisol levels. Changes in the level of cortisol in response to the stressor were not observed in nicotine dependence of a higher level. This concluded that the different nicotine dependence levels/severity may be explained by different mechanisms. The stress response system is highly triggered in subjects with lower nicotine dependence level and vice versa [50]. Salivary determination of Cotinine has been used as a novel technique due to its non-invasive and easy nature. Cotinine has relatively a longer half-life (cotinine; approx. 20 hours, nicotine; approx. 2 hours) and acts as a short-term biomarker of nicotine following tobacco smoke exposure. The fluctuations in the concentration of cotinine in body fluids are negligible during the active period of smoking. This delivers a steady determination of serum, saliva, and urine levels. The measurement of cotinine provides a reliable investigation of tobacco smoke exposure but the duration of smoke exposure and the intake of other tobacco smoke combustion products cannot be determined through cotinine assessment [51]. The method of sample collection and type of specimen may affect the detection of cotinine levels. The level of cotinine in unstimulated saliva is greater as compared to the saliva obtain through the stimulation technique. This is because of alteration in the pH due to altered flow rate. Cotinine is transferred from blood plasma to saliva through passive diffusion. The pH of the medium greatly affects the level of salivary cotinine [52]. The salivary cotinine levels play a significant role in determining the recent exposure to tobacco smoke (*i.e.* 3to 4 days). Moreover, it can also determine the smoking status of the individual *i.e.* passive smoker, occasional smoker, active smoker, or non-smoker. Similarly, salivary cotinine can also help to predict plasma and urinary cotinine levels indirectly [53-55].

Similarly, Fatemeh et al [56] and Satvinder et al [57] also detected the stress biomarkers i.e., α-amylase, Salivary Catalase, and Cotinine levels in smokers and nonsmokers by retrospective cohort study and comparative study respectively. Fatemeh et al [56] detected the catalase, α-amylase, and Vitamin C by the spectrometer and got the results that stress biomarkers such as salivary α-amylase levels are high while catalase levels are lower but statistically insignificant. While Satvinder et al [57] also evaluates the effect of cigarette smoking on levels of antioxidant and also the cotinine levels in smokers and nonsmokers. They also got insignificant results among the groups by evaluating the salivary catalase and salivary α-amylase. While their results were significant when compared with

cotinine levels. Sharma et al [58] assessed the levels of cotinine in smokers, passive smokers, and nonsmokers' saliva and urine by liquid chromatographic technique and mass spectrometry. Their results showed that cotinine levels are considerably higher in smokers in comparison to nonsmokers and passive smokers.

The lifestyle of individuals suffering from chronic stress is also effected. They adopt obesity triggering lifestyles *i.e*. staying passive, eating high calorie foods. In this way, the risk of diseases related with high obesity is also increased [59]. Several research studies have concluded that chronic stress is produces hypercortisolism. Hypercortisolism subsequently causes obesity related problems such as diabetes mellitus, cardiovascular risks, and psychological effects (anhedonia) [60]. Increased thickness of carotid intima media is also linked with stress and obesity. This is a clinical marker for early cardio vascular disease. Any defect in the hypothalamic–pituitary–adrenal (HPA) leads to the induction obesity and obesity liked problems in normal and non-obese individuals. These studies suggest that stress is correlated with both mental and physical abnormalities that lead to negative quality of life. These alterations are also observed in minority communities facing esteem discriminations. Cortisol awakening response is also observed in African Americans due to this effect. Therefore, obesity and obesity related disease prevention protocols also involve manipulation of stress related factors [61, 62].

The response to acute stress and chronic stress is also evaluated. During the exposure to acute and chronic stress different physiological responses of the body are activated to counter face stimuli. Most of these responses are instigated by the the hypothalamic-pituitary-adrenal axis at the base of the brain. Apart from cortisol, salivary alpha amylase is also considered as a valid and reliable biological marker of stress exposure. In order to cope with the environmental stress, human body is designed to adopt certain changes to protect itself from the negative impact of these noxious stimuli. The autonomic nervous system (ANS) plays a significant role. The efferent sympathetic-adrenal-medullary (SAM) system plays primary role in this regard. The easy measurement of cortisol and salivary amylase from saliva makes it a suitable marker of stress [63]. Sympathetic and parasympathetic system has got specific effects on the cardiovascular system. These effects can be utilized to study the relationship of stress and its impact on the cardiovascular system. Heart rate variation is an efficient parameter in this regard. Apart from the heart rate monitoring, other autonomic nervous system parameters may also be used to assess

this relationship. *i.e*. the concentration of catecholamines like adrenaline and noradrenaline in the central nervous system. The utilization of these markers from the central nervous system require invasive techniques that utilize syringe to draw the spinal fluids from the spinal cord [63].

The level of cortisol and salivary amylase is also effected on the time to measure their concentrations. Different research articles have concluded that the concentration of these biological markers is prominently high in morning time when the subjects are facing some king of stress either in the form of physical stimuli or psychological factors. These two stress biomarkers have a distinctive diurnal profile. The salivary cortisol demonstrates peak plasma concentrations after 30 minutes of awakening from the sleep. However, in divergence to cortisol, the plasma concentration of salivary alpha amylase abruptly drops within the first 30 minutes of awakening from sleep. The sAA values slowly rise up during the course of the day depending upon different environmental factors. Therefore, it is important to keep in mind the exact time to monitors these values [64]. This should also be taken into account that several samples of saliva should be collected at different times of the day or on different days in order to get a fully understanding of the serum biochemical changes in response to stress.

There is also clear documentation of the effect of gender on the salivary stress biomarkers. Studies have been conducted on the nursing subjects working at hospitals. They are exposed to high level of burnout stress. It was concluded that over the period of day, male nursing population show less pronounced elevation the salivary stress biomarkers (*i.e*. salivary cortisol and salivary alpha amylase). While female nursing population showed a more noticeable response [65]. Variations may be there among the concentrations of different biomarkers with respect to the nature of stress. For example, the diurnal patterns of salivary alpha amylase are observed to present in patients suffering from asthma, while the cortisol levels were not affected. Similarly, the trajectories of salivary alpha amylase were flatter in those pregnant women who had a history of previous miscarriage as compared to those who did not face the problem of miscarriage [66].

Response to the stress is a protective feedback of the body. The protective feedback is responsible for the release of cortisol from the zona fasciculata in the adrenal cortex. Cortisol then causes metabolic changes to the body in order to bring back the normal functioning of the body [67]. Continuous exposure of the body to

the stressful stimuli lead to modulation in the physiology of the Hypothalamic-Pituitary-Adrenal axis known as *passivation*. Sometimes, due to prolonged exposure to stress, the HPA may not be restored to normal functioning. Therefore, abnormally high levels of cortisol are observed in the serum and saliva. This situation is mostly observed in patient suffering from post-traumatic-stress-disorder (PTSD) [68].

Salivary cortisol monitoring is preferred by various researchers due several benefits over other techniques. The collection of salivary samples is suggested to be a non-invasive technique. Moreover, this technique is practically more applicable with ease. A large number of samples can be collected in a home based setting easily. Similarly, in comparison to other samples *i.e*. urinary cortisol and blood cortisol, the salivary cortisol is more promptly available [69].

The profile of mood state (POMS) is an important psychological parameter to assess different mood states. The POMS was initially developed by McNair et al. in 1971 [70]. It is a questionnaire comprising of 5-point adjective rating scale and 65 questions/items. This questionnaire has been developed to assess several mental states including depressive-dejection, tension-anxiety, fatigue-inertia, vigor activity, Confusion Bewilderment, and anger-hostility (coded by D, T, F, V, C and A respectively) [71]. The POMS questionnaire required approximately 7 minutes for a normal person to complete. However, a time of 15 to 20 minutes was required by ill person (cancer patient) to fill it. A study was conducted on 83 cancer patients aimed to assess the effect of their current medical condition of their lifestyle and mood sate as well as the efficacy of the treatment conducted [72]. Time required to fill POMS is a crucial factor. It suggested that the time required for assessing a subject can be reduced without losing the effectiveness of the questionnaire or the information contained in it. POMS has been utilized in the clinical practice for about half a century. During the course of time, various changes have been made in the format [73].

There exists several studies to investigate the relationship between the physical state of an individual and his/her mental state [74]. A study suggests that physical exercise can be useful to maintain a healthy mental state and promotes stress-less life [75]. However, the intensity of exercise may also effect the mental effects variably. A physical activity of moderate level can positively affect the mood state of a person. While a physical activity of intense nature can negatively affect the mood state. However, in general moderate exercise can effectively result in the

depressive symptoms in older population [76]. Sports performance also predominantly effect the mood states and ultimately the quality of life. A major development to the sports psychology was made in 1974 by Morgan, and his colleagues. Morgan studied the mood states in the athletes of elite level and suggested that they possessed a high level of mood states based on the POMS screenings [77]. Studies have been conducted to elaborate POMS for sports psychology further. It is suggested that mood and sports can be used to assess different parameters including the use of POMS for differentiating non-athletes from athletes, athletic performance, and effect of mood in determining the athletic performance [78].

POMS has also been utilized to study the mental state of cancer patients and adaptation of cancer patients to this disease. Guadagnoli *et. al*. in 1989 suggested that an efficient psychological monitoring questionnaire can be developed for hospitalized/ill patients. An improved POMS monitoring system can take 4-5minutes without altering the efficacy and integrity of the contents [71]. World health organization (WHO) has suggested that 10% of the world population if living in stress induced depression [79]. Studies have also been conducted in the COVID-19 pademic situation to address the stress impacts on the physical and mental health of individuals quarantined in homes and other facilities. Yasemin Afacan 2021 with the help of POMS suggests that incorporation of biophilic designs to homes *i.e*. addition of greenery, gardens, plant and tress in the architectural designs of homes can significantly improve the mood states [80].

The use of tobacco smoke and relapse behaviors have also been studies with the approach of profile of mood state. A study designed a total of 72 different variables to assess confusion, depression and anxiety with the help variable intensities *i.e*. agree, relax, confusion, and anxious scales. It was concluded that the exposure of the specific minority community (*i.e*. African-american) to socio-cultural stressors can induce smoking behavior as well as a negative impact on the mental state [81]. Treatment efficacies of different drugs like varenicline have also been evaluated among the smoking minorities and linked with the psychological parameters through POMS [82]. A study suggests that smokers subjected to a high level of discrimination intensity are more prone to face higher level of smoking withdrawal symptoms as compared to those faced low discrimination [83]. Smoked cannabis also variably effects the mood state and mental health. Matheson *et. al*.

concluded that the cognitive performance of the individuals is not significantly affected by smoked cannabis. On the other hand, a statistically more significant effect was monitored on the mood state through POMS [84]. Tetrahydrocannabinol (THC) is thought to be involved in these mood alterations. THC is believed to act at specific receptors in the brain known as the cannabinoid type-1 (CB1) receptor. Moreover, the intensity of these effects is also influenced by the intensity of the cannabis smoking *i.e*. the quantity of smoke inhaled, the duration of holding the smoke inside the lungs *etc*. [85].

Sound mental health is a sign of good health. Mental health has been declared as a developmental priority worldwide by WHO [86]. The workers' health is greatly affected by stress and serve as a risk factor for mental health. Females workers working at hospitals at night are presented with high level of salivary cortisol as compared to normal female workers. Similarly, male workers reveal lower cortisol alterations that female workers which represents gender variations [87]. Studies has also been performed on the difference between cortisol responses to physical and psychological stressors. It was concluded that physical and psychological stressors produced invariable results for cortisol levels. The comparative working memory capacity was also not effected by the kind of stressor and the level of cortisol [88].

Aim and Objectives of the Study

This research work aims to further elucidate the utilization of UV Photospectrometry for quantitatively assessing the stress biomarker and its relation to POMS.

The aim and objectives of this research work are:

- To study the relationship between smoking and stress
- To investigate the effect of cigarette smoking on salivary stress biomarkers
- To assess the cotinine level in the smokers and nonsmokers
- To utilize UV photospectrometery technique for analyzing the smoke biomarkers
- To utilize UV photospectrometery technique for analyzing the Stress biomarker *i.e.* cortisol

Chapter 3 Methodology

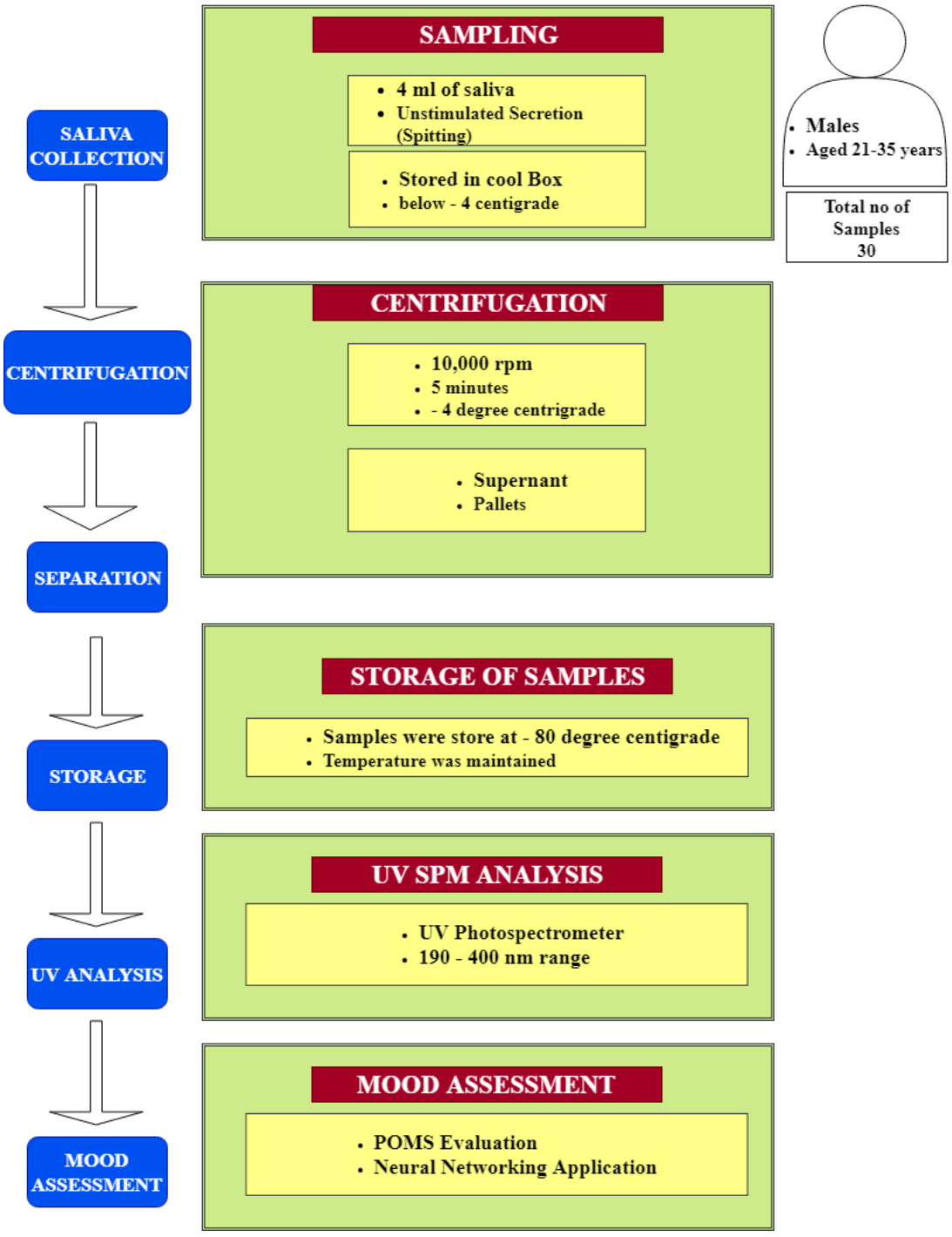


Figure 3-1: Flow chart for Methodology used in this study

3.1. Study Design

3.1.1. Study setting

This research study was conducted at National University of Science and Technology (NUST), Islamabad. The samples were collected form the vicinity of the university campus H-12 Islamabad. The samples were processed and stored at the biomedical laboratory of School of Mechanical and Manufacturing Engineering (SMME), NUST.

3.1.2. Sample Population

Subjects residing in Islamabad were included in the study. The participants were randomly selected based upon the eligibility criteria. All of the subjects were cigarette smokers. All the participants belong to the age bracket of 21-35 with approximately similar mean age. A total of twenty-four (24) male subjects were analyzed. A total of two groups were considered.

i- Group-I

ii- Group-II

Group 1 included the non-smoking participants, while group 2 included smoking participants. Among the total participants, 12 were non-smokers while 12 were smokers. The nonsmokers or unexposed group were selected on the assumption that they have never smoked.

3.1.2.1. Exclusion Criteria

The exclusion criteria for samples selection of this research as mentioned below.

- No one among the participants was having any known systemic illness. All of them were healthy volunteers.
- Participants using ay medications/drugs were also not included in the study
- Participants prone to drug allergies were not included in the study
- Participants addicted to alcohol were not entertained in this study
- New smokers that have started smoking for less than a year were also not included in the study.
- Female subjects were not included in the study to avoid gender differences regarding the mood state profiling

- Subjects who smoked cigarette in other forms i.e. cigar, huqqa were not included in the study
- Participants who has a history psychiatry disease in the family were excluded from the study

3.2. Materials

SAMPLE COLLECTION

Sterile falcon tube, ice box, hand sanitizers

SAMPLE PROCESSING AND STORAGE

Centrifugation machine, Chiller Storage (-80°C)

SAMPLE PREPARATION

Test tubes, test tube racks, pipette, micropipette, flasks, graduated cylinder

ANALYSIS

UV Photospectrometer

MACHINE LEARNING

Python's Seaborn library, Google Colab, Jupyter notebook, ADAM optimizer

3.3. Methodology

3.3.1. Saliva Collection

Group 1 (smokers) were instructed to smoke at least 5 times a day for the past 2 months. Simple spitting technique was used for the unstimulated saliva collection. Before the collection of Saliva, all the subjects were barred from oral stimulation. The participants were allowed to sit in a proper position before collecting the saliva samples. They were instructed not to take any cigarette 1 hour prior to sample collection. Saliva was allowed to pool at the floor of oral cavity. A total of 4 ml saliva (unstimulated) was collected in the sterilized falcon tube. The collection of saliva was made after almost two hours of fasting to diminish food effects on the collected samples. Saliva was temporarily stored in cool boxes at 4°C and immediately shifted to the facility [57].

3.3.2. Saliva Processing and Storage

The salivary sample were centrifuged for 5 minutes with a temperature maintained at 4°C for 5 minutes and 10,000 rpm for the removal of cellular and extracellular fragments. After centrifugation, the supernatant solution was separated. Saliva sample was stored at -80°C until further analysis [57].

3.3.3. UV Photospectrometry Analysis

The UV is tuned in the range of 190 nm to 400 nm. The cuvettes for sample analysis were thoroughly washed with double distilled water (ddH_2O). DDH_2O is taken as a reference standard. Saliva sample with an amount of 1ml was taken in another cuvette. Initially DDH_2O is run through UV analysis to get the blank medium readings. In the second step, salivary stress biomarkers were screened in the same nanometer range and the saliva sample (smokers and non-smokers) was run through the same way. Same procedure was followed for the smoker saliva and nonsmoker saliva samples. The required parameters to be analyzed were recorded and compiled [28].

3.3.4. Profile of Mood State (POMS)

The mood state of the participants was evaluated using the profile of mood state technique used initially by McNair, Lorr, and Droppleman in 1971. The positive and negative levels of them was calculated [70, 89]. The questionnaire was comprised of approximately 65 questions about the mental state. The participants were asked about the feelings of their last week experience. Each question/statement/word asked was replied in five (5) levels of quantitative/qualitative extremities given in **Table 3-1**. The total mood disturbance (TMD) score was calculated that ranges from -32 to 200. The scoring detail for each set of feeling (mood profile) is given in **Table 3-2**.

No time limit was set for answering the questions. The participants replied the preset questions in a friendly and calm environment. They were provided information about the importance of this research work and there was no discrimination/interference of their personal life was involved.

The POMS questionnaire was obtained online from the following linked website.

POMS Questionnaire: https://www.brianmac.co.uk/poms.htm

Mackenzie, B. (2001) Profile of Mood States (POMS) [WWW] Available from: https://www.brianmac.co.uk/poms.htm [Accessed 26/6/2022]

Table 3-1: Different sets of mood profiles in POMS questionnaire for TMD scoring

S. NO.	Mood Profile	Score range
1.	Confusion	0 to 28
2.	Anger	0 to 48
3.	Depression	0 to 60
4.	Tension	0 to 36
5.	Fatigue	0 to 28
6.	Vigour	0 to 32
Total score (TMD)		-32 to 200

Table 3-2: Different levels of extremes for answering the POMS questionnaire

S. NO.	Grade/level	Extremity	Score
1	First	Not at all	0
2	Second	A little	1
3	Third	Moderately	2
4	Forth	Quite a bit	3
5	Fifth	Extremely	4

Table 3-3: Questionnaire statements of the profile of mood state

S. NO.	FEELINGS	REPLY
1.	Tense	Replied in the Five levels of extremeties *i.e.* 1. Not at all 2. A little 3. Moderately 4. Quite a bit 5. Extremely
2.	Friendly	
3.	Angry	
4.	Unhappy	
5.	Worn out	
6.	Clear headed	
7.	Confused	
8.	Lively	
9.	Sorry for things done	
10.	Listless	
11.	Shaky	
12.	Peeved	

13.	Sad	
14.	Considerate	
15.	Active	
16.	Grouchy	
17.	On edge	
18.	Blue	
19.	Panicky	
20.	Energetic	
21.	Hopeless	
22.	Unworthy	
23.	Relaxed	
24.	Spiteful	
25.	Uneasy	
26.	Sympathetic	
27.	Restless	
28.	Fatigued	
29.	Unable to concentrate	
30.	Helpful	
31.	Discouraged	Replied in the Five levels of extremeties *i.e.* 1. Not at all 2. A little 3. Moderately 4. Quite a bit 5. Extremely
32.	Annoyed	
33.	Resentful	
34.	Lonely	
35.	Nervous	
36.	Miserable	
37.	Cheerful	
38.	Muddled	
39.	Bitter	
40.	Anxious	
41.	Exhausted	
42.	Ready to fight	
43.	Gloomy	
44.	Good natured	

45.	Desperate	
46.	Rebellious	
47.	Sluggish	
48.	Helpless	
49.	Bewildered	
50.	Weary	
51.	Alert	
52.	Furious	
53.	Deceived	
54.	Efficient	
55.	Full of pep	
56.	Trusting	
57.	Bad tempered	
58.	Forgetful	
59.	Worthless	
60.	Carefree	
61.	Guilty	
62.	Terrified	
63.	Vigorous	
64.	Bushed	
65.	Uncertain about things	

3.3.4.1. Scoring Steps

The total mood disturbance (TMD) score was calculated automatically just by clicking the "Analyze" button on the website mentioned above. However, the following steps were involved in calculating the POMS scores.

STEP-1:

The data was analyzed for scoring the depression, tension, anger, confusion, fatigue, and vigour.

STEP-1:

The scores for depression, tension, anger, confusion, and fatigue was added and finally vigour score was subtracted to get the final TMD core.

3.3.5. Machine Learning Algorithm and Neural Networking

3.3.5.1. Machine Learning Algorithms

The data was arranged in MS Excel into the CSV file format. The prediction of the results was made initially by machine learning algorithms. The data was arranged according to classes and each class was labeled through codes in the CSV file. The arrangement of the data and data codes are provided in **Table 4-1**.

Python's Seaborn library (programming language; type: logical) was used for the data randomization. The complete data was distributed into testing and training sets (75% training sets and 25% testing sets). Randomization script was used in python in order to randomize the data. Programming was done through Jupyter notebook. The results were trained and predicted initially through Support Vector Machine (SVM). The tuning of hyperparameter and other supervised leaning machine algorithms was performed to increase the accuracy of results.

3.3.5.2. Simulated Neural Networking and Model Development

Simulated neural networking (SNN) was applied to the collected data from smokers and non-smokers. These artificial mimicking of the human brain and behavior was used in order to full understanding of the TMD scoring and its relationship with the smoking and non-smoking population.

The training and prediction of the results was initially performed through machine learning algorithms. Neural networks were subsequently applied to make the results more precise. A 3-layer Artificial/Simulated Neural Network was trained and possible results were obtained through ADAM optimizer. Programming was done through Google Colab. ESP32 Micro-controller was used for model deployment. The board can utilize micropython script but with a slow rate. Therefore, code was converted to C++ code and uploaded to ESP32 in order to boost the speed.

3.4. Statistical Analysis

This research work included 24 male subjects to be analyzed, in which 12 were smokers and 12 were nonsmokers. The required statistical analysis was performed and the data was statistically analyzed through a software "GraphPad Prism 8.0" and the respective graphs were plotted. The data was compared through unpaired t-test and the required post-hoc analysis (Sidak's multiple comparisons test)

was performed. Data with probability values of $p \leq 0.05$ were considered statistically significant.

Chapter 4 Results

4.1. Results

4.1.1. Raw Data

Table 4-1: Raw Data Collected from the Participants (Nonsmokers are Represented by Class Code 0 and Smoker by 1)

Class	Anger	Confusion	Depression	Fatigue	Tension	Vigorous	TMD Score	Wavelength	Abs
0	9	4	11	10	7	30	11	190	7.472
0	10	11	22	11	7	27	34	197	9.224
0	12	9	21	9	6	27	30	196	8.796
0	16	8	24	9	9	29	37	200	9.279
0	13	5	24	7	9	30	28	192	7.356
0	14	7	23	7	9	28	32	193	8.619
0	13	8	24	6	9	30	30	194	9.206
0	11	8	19	5	13	29	27	196	9.354
0	14	8	19	5	11	30	27	194	7.708
0	11	5	17	4	11	25	23	200	8.809
0	11	6	15	4	12	28	20	200	9.065
0	11	10	22	5	12	29	31	192	9.671
1	17	13	29	10	15	24	60	194	9.147
1	20	13	26	13	13	11	74	195	9.759
1	15	10	17	5	12	23	36	198	9.549
1	12	8	20	6	13	17	42	193	9.643
1	18	14	29	11	16	18	70	196	7.641
1	13	10	16	12	13	15	49	190	5.181
1	16	13	19	17	14	17	62	196	6.268
1	24	19	26	14	13	22	74	196	6.268
1	22	10	20	11	15	19	59	192	9.353
1	30	14	31	11	10	24	72	195	9.441
1	19	15	23	9	14	17	63	190	9.489
1	14	11	18	8	11	18	44	191	9.205

A total of 24 saliva samples were collected from different participants who complied with the inclusion criteria of the study protocol. Among these 24 samples, 12 were smokers and 12 were nonsmokers. Nonsmokers were denoted by code 0 and smokers were represented by code 1. Each one of the 24 participants was individually interviewed for assessing their mood profile. Similarly, the saliva samples collected from them was analyzed through UV Photospectrometry and readings were recorded as presented in **Table 4-1.**

4.1.2. Profile of Mood State (POMS)

Data regarding the mood profile evaluation was collected by asking a total of 65 questions, contributing to develop the individual mood profile *i.e.* anger, confusion, depression, fatigue, tension and vigorous. Anger was noted to be statistically extremely significant in nonsmokers as compared to smokers ($P \leq 0.0001$, ****). A very significant difference was observed for confusion among smoking group ($P \leq 0.0014$, **). Depression among the nonsmokers and smokers showed no significant difference. Similarly, fatigue and tension was also significantly high in smokers as compared to nonsmokers ($P \leq 0.05$, *). The nonsmoking population exhibited an extremely significant vigorous nature compared to smokers ($P \leq 0.0001$, ****).

The comparative results for general statistics of nonsmokers and smokers is represented in the **Table 4-2**. ANOVA results are represented in the **Table 4-3**. The results of statistical analysis using post-hoc multiple comparison test (Sidak's multiple comparisons test) are presented in **Table 4-4**. The results are graphically represented in **Figure 4-1**.

Table 4-2: Profile of Mood State (POMS) General Statistics

Mood Profile	Non Smokers			Smokers		
	Mean	**SEM**	**n**	**Mean**	**SEM**	**N**
Anger	12.08	0.57	12	18.33	1.48	12
Confusion	7.41	0.61	12	12.5	0.85	12
Depression	20.08	1.18	12	22.83	1.50	12
Fatigue	6.83	0.69	12	10.58	0.96	12
Tension	9.58	0.64	12	13.25	0.49	12
Vigorous	28.50	0.45	12	18.75	1.13	12

Table 4-3: Profile of Mood State Statistical Analysis Using ANOVA

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	1032	5	206.4	F (5, 132) = 19.09	P<0.0001
Row Factor	4655	5	931.0	F (5, 132) = 86.12	P<0.0001
Column Factor	138.1	1	138.1	F (1, 132) = 12.77	P=0.0005
Residual	1427	132	10.81		

Table 4-4: Profile of Mood State Statistical Analysis Using Post-hoc Multiple Comparison Test (Sidak's multiple comparisons test)

Mood State	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
ANGER	-6.250	-9.835 to -2.665	Yes	****	<0.0001
CONFUSION	-5.083	-8.669 to -1.498	Yes	**	0.0014
DEPRESSION	-2.750	-6.335 to 0.8355	No	ns	0.2293
FATIGUE	-3.750	-7.335 to -0.1645	Yes	*	0.0354
TENSION	-3.667	-7.252 to -0.08118	Yes	*	0.0422
VIGOROUS	9.750	6.165 to 13.34	Yes	****	<0.0001

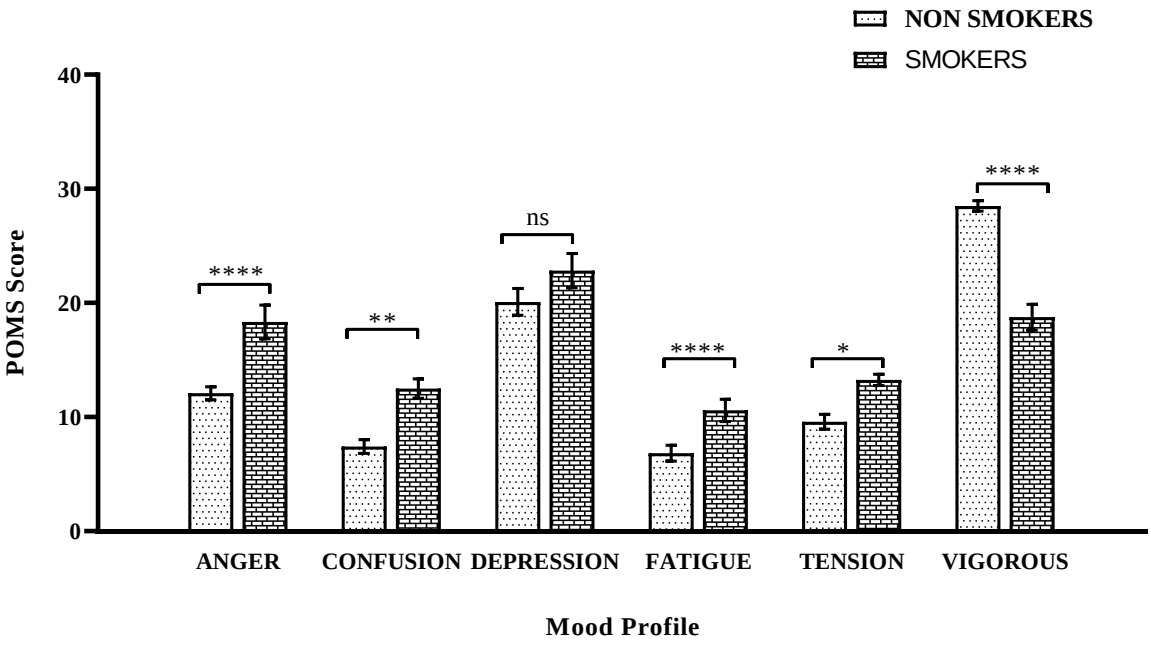


Figure 4-1: Profile of Mood State Comparison for Nonsmokers and smoker and their respective statistical difference in the mood profile

4.1.3. Total Mood Disturbance (TMD) Score

Total mood disturbance score was calculated by adding all the negative mood profiles (anger, confusion, depression, fatigue, tension) and finally subtracting the vigorous score. The smokers showed a significantly high total mood disturbance score of 58.75 ± 3.80 as compared to nonsmoker group which showed a mean value of 27.50 ± 2.00. the unpaired t-test signified these results with *P* value of ≤ 0.05. The general statistics for TMD score is presented in **Table 4-5.** Results of the t-test are presented in **Table 4-6**. The results are graphically represented in the **Figure 4-2**.

Table 4-5 Total Mood Disturbance General Statistics

Mood Profile	**Non Smokers**			**Smokers**		
	Mean	**SEM**	**n**	**Mean**	**SEM**	**n**
Total Mood Disturbance (TMD Score)	27.50	2.00	12	58.75	3.80	12

Table 4-6 Total Mood Disturbance Statistical Analysis Using Unpaired t-test

General Statistics	
Mean of column B	58.75
Difference between means (B - A) ± SEM	31.25 ± 4.295
95% confidence interval	22.34 to 40.16
R squared (eta squared)	0.7064
Mean of column B	58.75
F test to compare variances	
F, DFn, Dfd	3.621, 11, 11
P value	0.0432
P value summary	*
Significantly different (P < 0.05)?	Yes

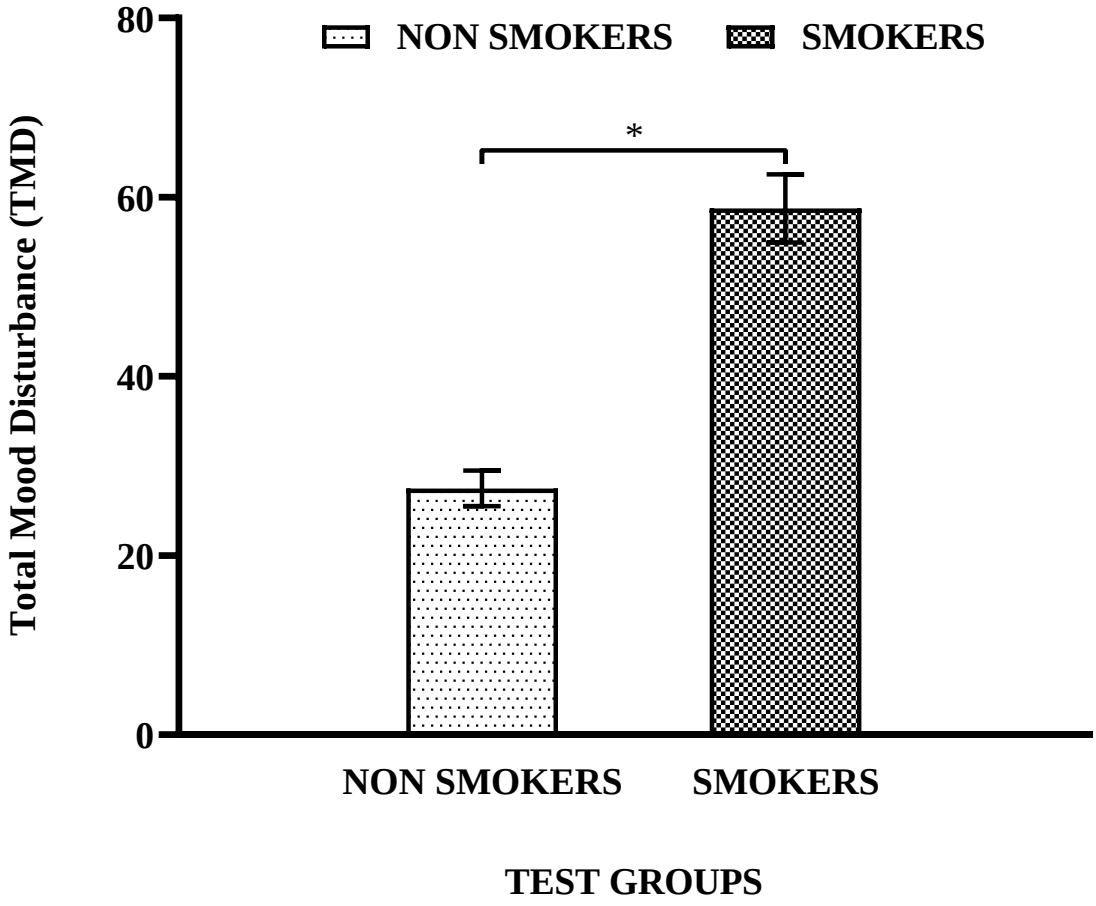


Figure 4-2: Total Mood Disturbance comparison for nonsmokers and smokers and their respective statistical difference in TMD Score

4.1.4. UV Photospectrometric Analysis

The saliva samples were analyzed through UV spectrophotometry. The maximum absorption was obtained in the lower nanometer range of ultra-violet spectrum *i.e*. 190-200 nm. The salivary stress biomarkers also give the peak UV absorption values in the early UV range. However, salivary stress biomarkers were not significantly different between the nonsmokers and smokers. The UV absorbtion readings for nonsmoking groups are provided in the **Table 4-7**. The UV absorbtion readings for smokers are provided in the **Table 4-8**. The results are graphically represented in the **Figure 4-3**. UV absorption peaks given by individual saliva samples obtained from smokers and nonsmokers combined and the absorbance difference are provided in **Figure 4-4**, **Figure 4-5**, **Figure 4-6**, **Figure 4-7**, and **Figure 4-8**.

Table 4-7: UV Photospectrometry analysis of Saliva Samples obtained from nonsmoker participants and their maximum absorbance against wavelength

NON-SMOKERS ANALYSIS		
S. No.	**Wavelength (nm)**	**Maximum Absorbance**
1.	190	7.472
2.	192	7.356
3.	192	9.671
4.	193	8.619
5.	194	9.206
6.	194	7.708
7.	196	8.796
8.	196	9.354
9.	197	9.224
10.	200	9.279
11.	200	8.809
12.	200	9.065

Table 4-8: UV Photospectrometry analysis of saliva samples obtained from smoker participants and their maximum absorbance against wavelength

SMOKERS ANALYSIS		
S. No.	**Wavelength (nm)**	**Maximum Absorbance**
1.	190	5.181
2.	190	9.489
3.	191	9.205
4.	192	9.353
5.	193	9.643
6.	194	9.147
7.	195	9.759
8.	195	9.441
9.	196	7.641
10.	196	6.268
11.	196	6.268
12.	198	9.549

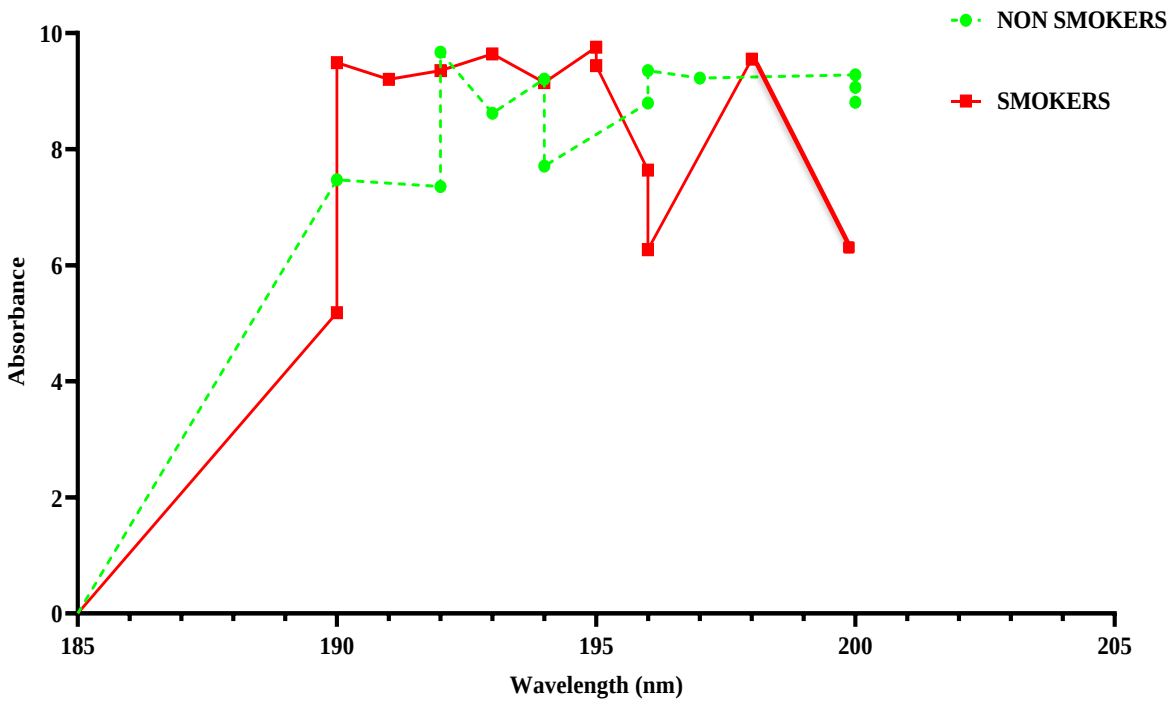


Figure 4-3: Maximum Absorbance of UV light by the individual saliva samples of nonsmokers and smokers against their respective wavelength (nm). The nonsmokers are represented by green dashed line while smokers are represented by red solid line.

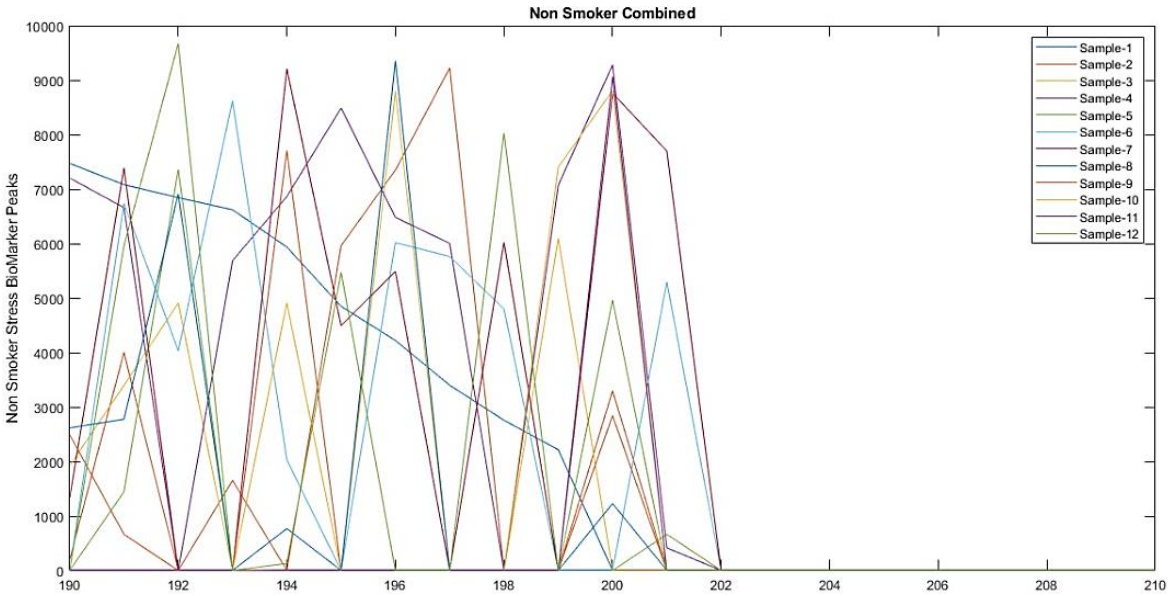


Figure 4-4: Various UV Absorbance peaks given by different stress biomarkers present in the individual saliva samples of non-smokers

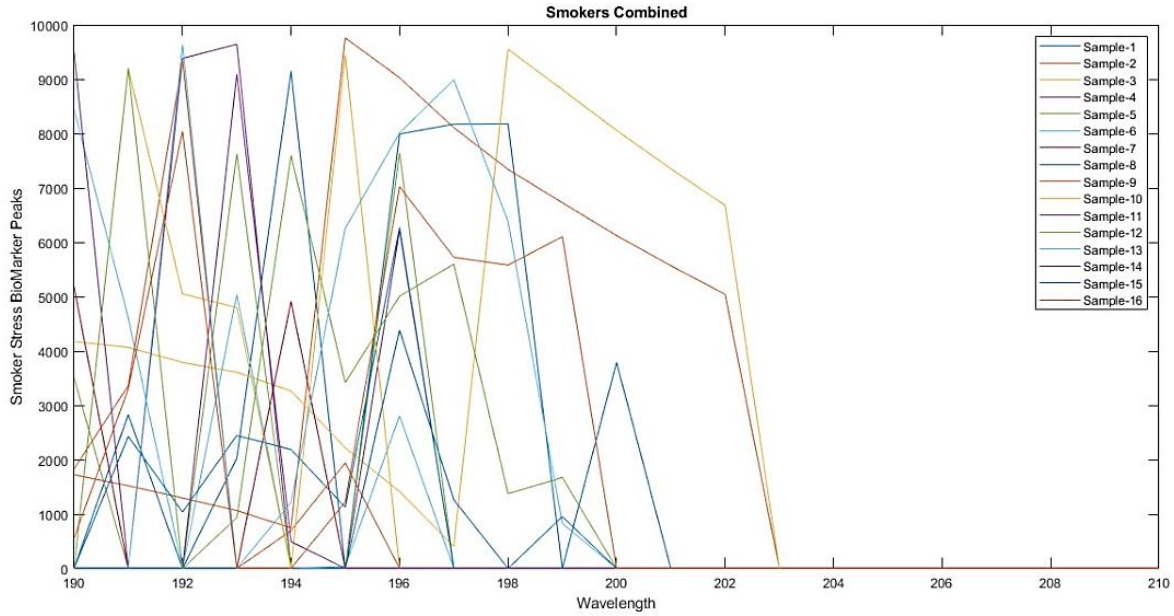


Figure 4-5: Various UV Absorbance peaks given by different stress biomarkers present in the individual saliva samples of smokers

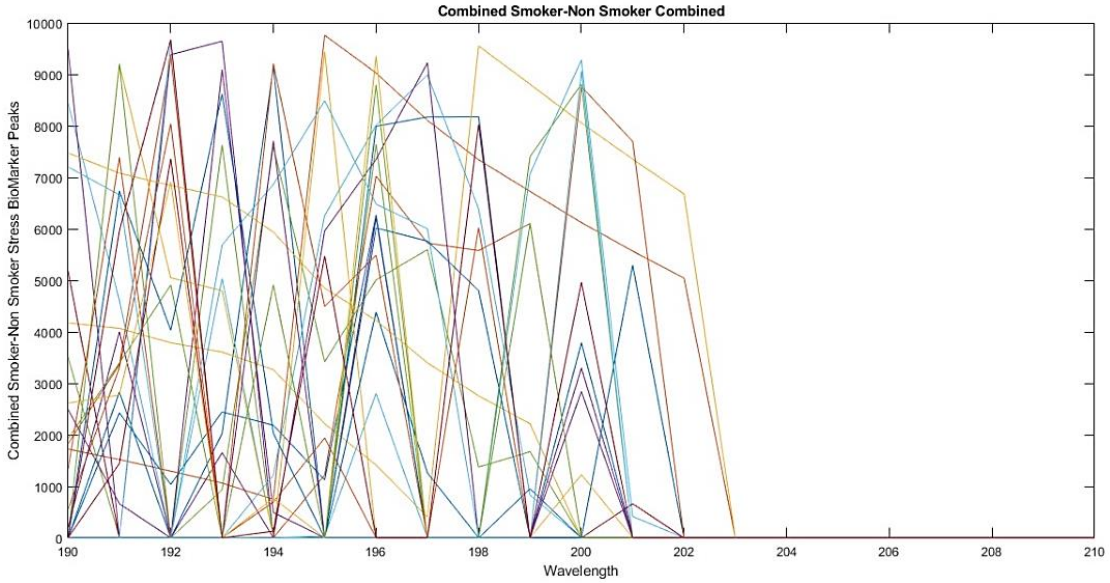


Figure 4-6: Various UV Absorbance peaks given by different stress biomarkers present in the individual saliva samples of non-smokers and smokers

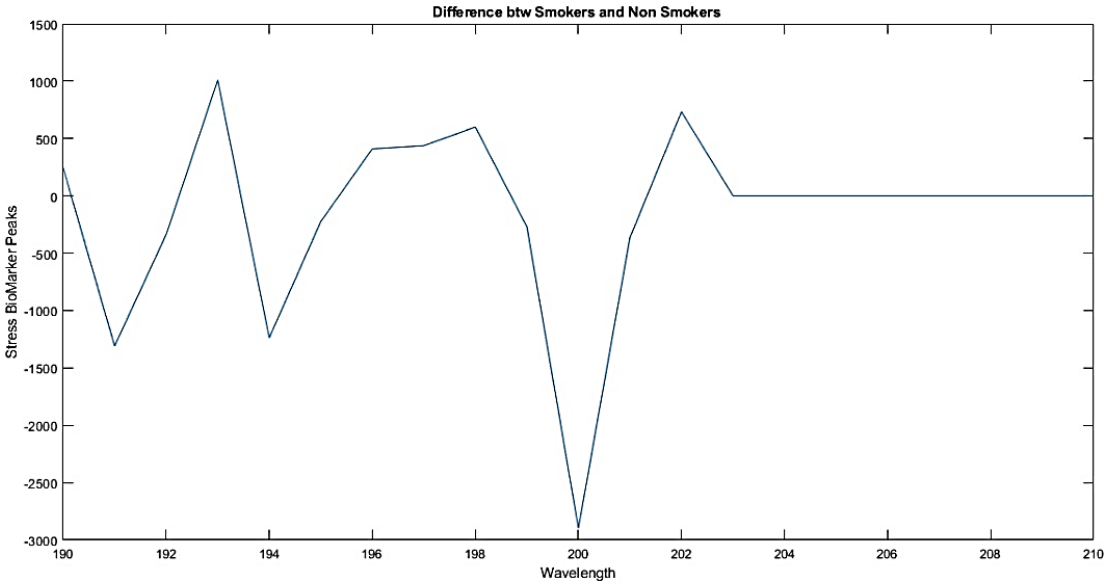


Figure 4-7: Difference between the UV Absorbance peaks of smokers and non-smokers

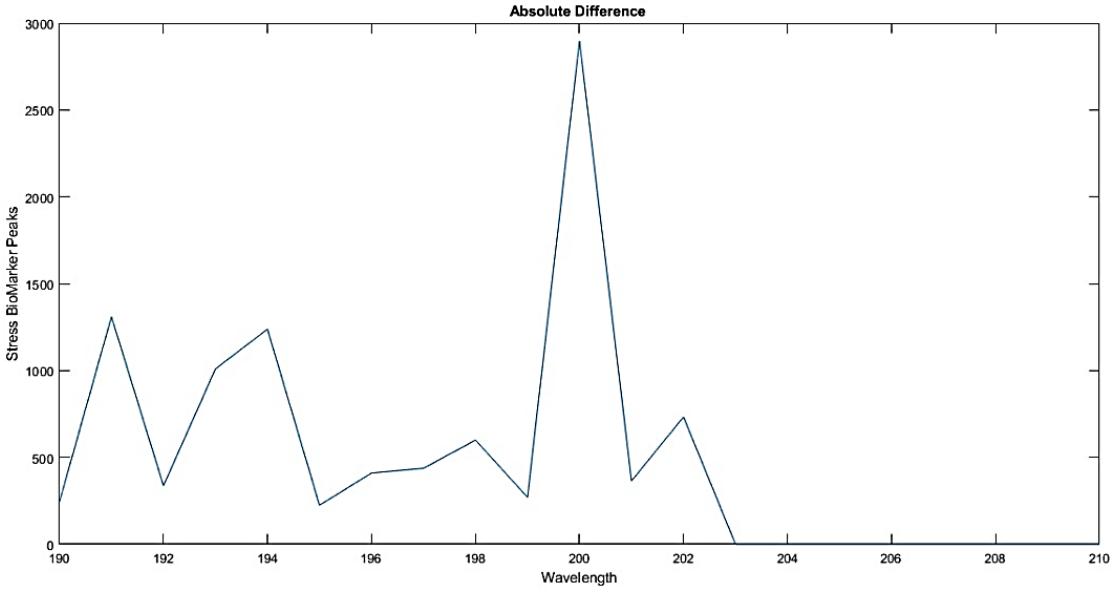


Figure 4-8: Absolute difference between the UV Absorbance peaks of smokers and non-smokers

4.1.5. Neural Networking

The results were trained and predicted initially through Support Vector Machine (SVM). The interpretation of the raw data obtained for mood profiling was performed to validate and enforce the reliability of results. The accuracy obtained through training the algorithm was 84% on the testing data. These were good and accurate results. However, the after performing the hyperparameter tuning, there was no effect observed on the accuracy of the results. Therefore, 84% accuracy was considered final results for neural networking analysis. The results for SVM are presented in the **Figure 4-4 and Figure 4-5**. These findings strongly backing the fact that smoking population are prone to the adverse impact of psychological and physical stress.

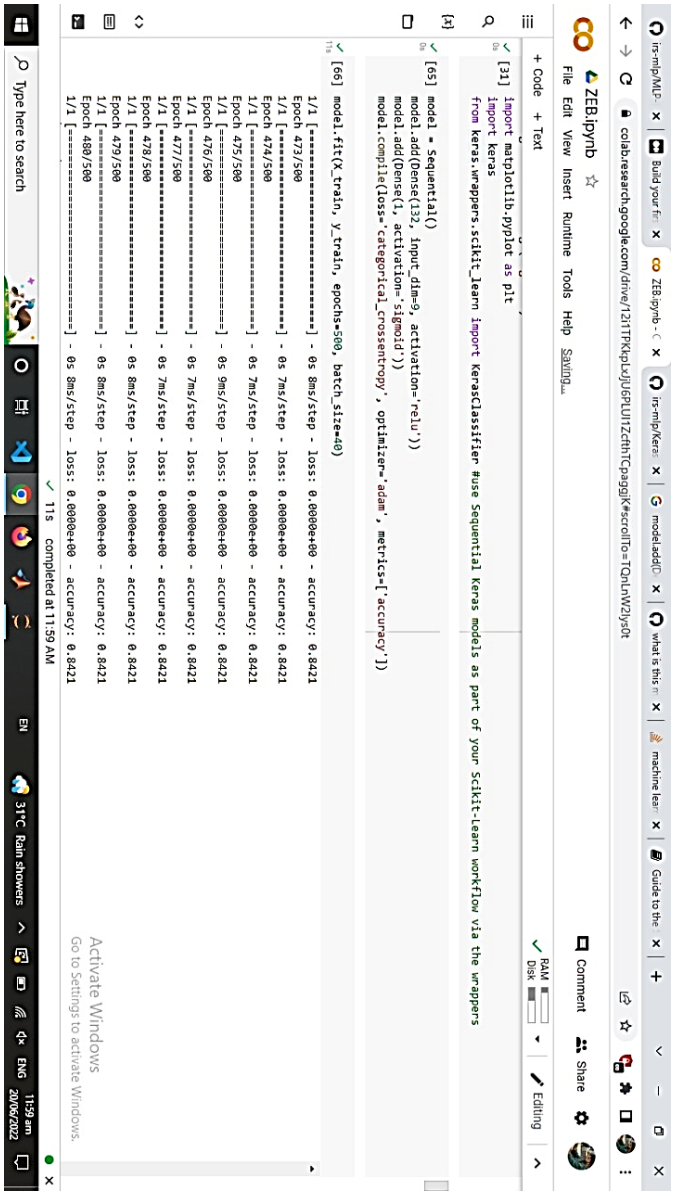


Figure 4-9: Results and accuracy neural networking

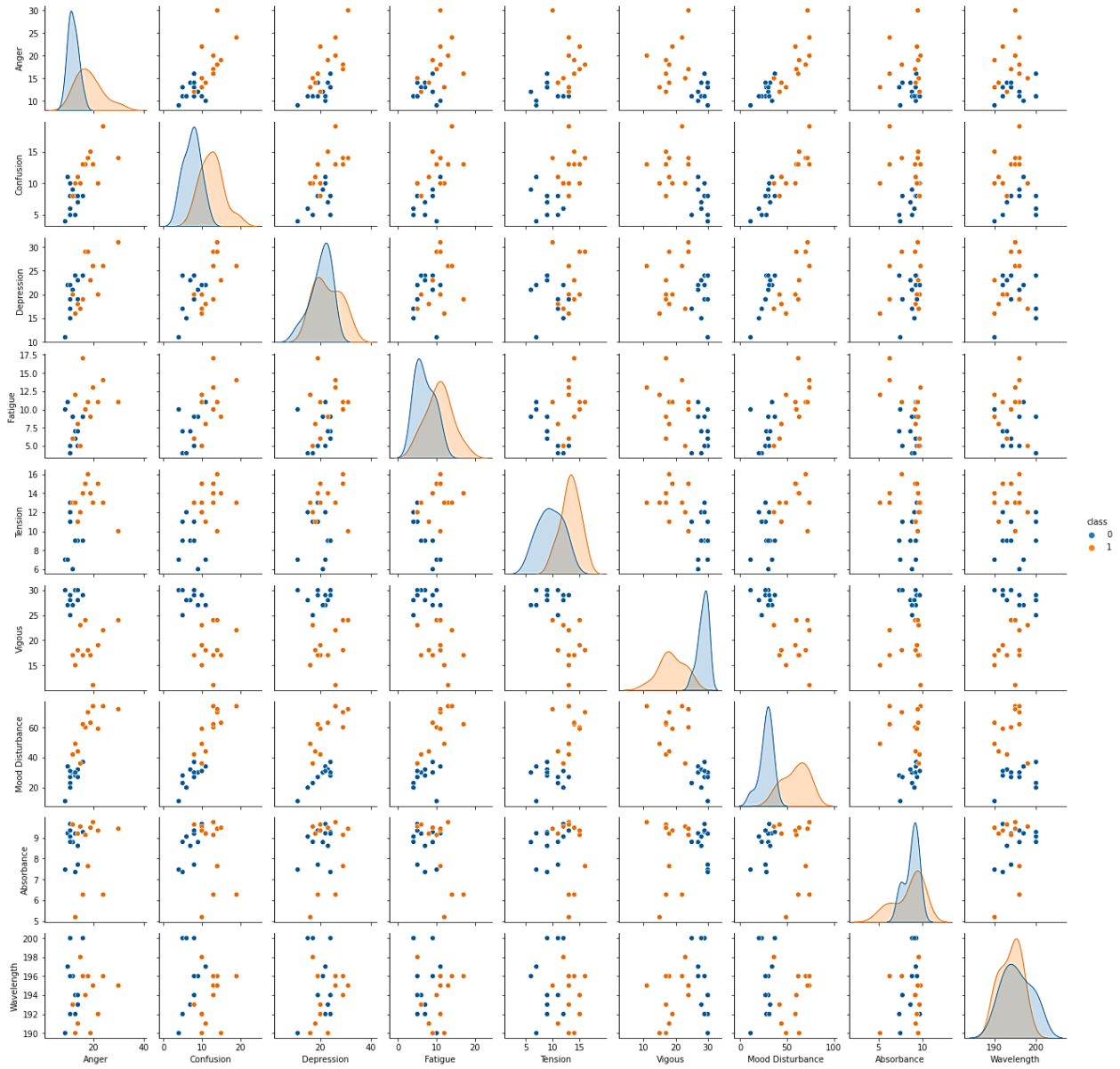


Figure 4-10: Results of the Neural networking (data visualization using Seaborn) on the mood disturbance and stress biomarkers of smokers and nonsmokers. Nonsmokers are encoded by 0 and presented by blue dots while smokers are encoded by 1 and presented by red dots.

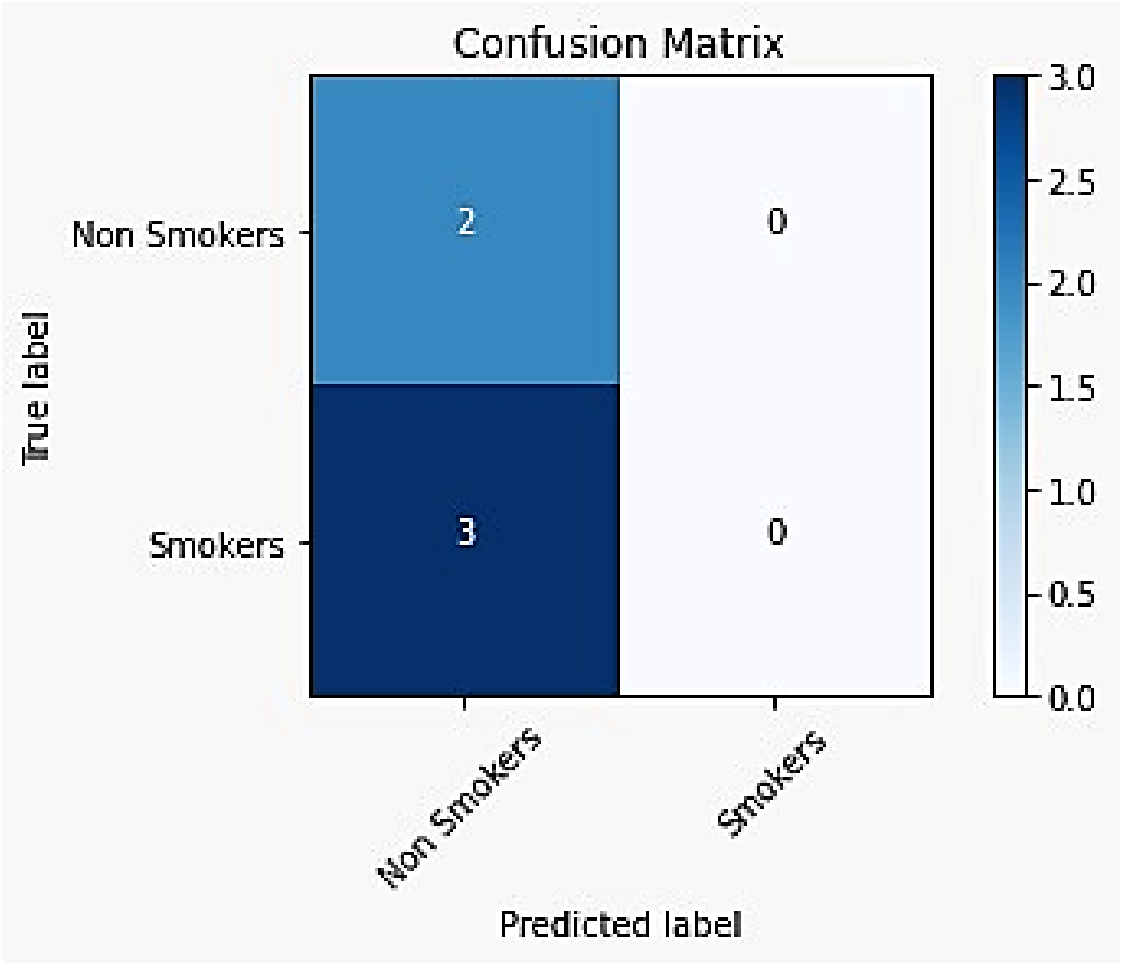


Figure 4-11: Binary classification confusion matrix for calculating the accuracy of neural network algorithm in machine learning method during training and validation

Chapter 5 Discussion

5.1. Discussion

This research work studied the effect of negative or positive environmental factors in the form of physical stress or psychological social complications on the mental alterations among the randomly selected subjects, classified into smokers and nonsmokers based on the inclusion and exclusion criteria and ultimately the inclination towards adopting smoking behavior and tobacco addiction to cope those stimuli. POMS calculated statistically significant confusion, anger, fatigue, and tension among the smoking subjects as compared to the nonsmoking group. However, the subjects indistinctively responded to the queries fabricating depressive mood profile *i.e*. no significant difference was observed between the smokers and nonsmokers in respect of assessing depression. Similarly, nonsmokers were evaluated to be more vigorous and energetic with a pleasant mood personality as compared to smokers. The results for vigorous profiling was also extremely significant.

Profound work has been performed regarding the mood escalations among the population and the initiation of smoking in order to muddle through the negative mood variabilities. Studies suggest that smoking behavior is escalating in nature *i.e*. it start and stops which is further dependent on the mental moral of the subjects and other factors including age, gender, education, social, and environmental effects [90]. It is suggested by certain studies that women are more prone to the relapse after abstinence of smoking behavior that may be explained by the low confrontation strength to the symptoms of nicotine withdrawal [91]. The study was enforced by the Shiffman–Jarvik Withdrawal Scale, Profile of Mood States, and Urge to Smoke Scale [92]. Negative reinforcement approach of mind play key role in the denial of abstinence and relapse initiation *i.e*. the smokers start thinking about the nicotine withdrawal symptoms that make them vulnerable to chain smoking [93]. Several studies propose that individuals that are more prone to negative mood profile *i.e*. confusion, anger, fatigue, cigarette cravings are more likely to adopt smoking behavior [94]. It is suggested that chronic smokers with a low mood profile experience the nicotine withdrawal symptoms within a few hours after even thinking about the nicotine withdrawal symptoms [95]. However certain controversial studies

suggest that the profile of mood state has no significance difference regarding the smoking behavior initiation [92].

The current research work also analyzed the saliva samples obtained from smoking and nonsmoking participants for screening the salivary stress biomarkers using ultraviolet spectrophotometry technique and correlated the smoking patterns with the mood state profiles. All the samples were screened at a specified UV wavelength range (190-400nm). The screening was not performed using a specific maximum absorption wavelength (lambda max; λ_{max}) for individual stress biomarker, however a cumulative absorption was observed. There was no significant difference observed while using UV analytic approach. Although UV analysis provides a more convenient and easy approach but slight undesirable alterations in the predefined protocols may lead to indefinite results because a high sensitivity is needed to study biomarkers in biological fluids. Various traditional methods have been defined for biomarker detections that include mass spectrometry (MS), X-ray diffraction (XRD), liquid or gas chromatography (LC/GC), and nuclear magnetic resonance (NMR) [47]. Both labelled and label free detection have been utilized by various researchers for analytic purposes. Labelled detection provided specificity and precision of results by attaching a recognition label in the form of a heavy metal/nanoparticles to the molecule of interest. However, labeled detection are more time consuming and costly. Label free detection involve optics for analyzing the moieties. These techniques include spectroscopy, florescence, and photonics [96].

Various other factors also affect the analysis outcomes. For instance, the method of saliva collection should also be observed. It is important to use the same method for all the subjects [97]. Saliva collection is also main cause of unsystematic errors that is usually ignored in general. It was observed in a study previously conducted that dehydroepiandrosterone, estradiol, testosterone, and progesterone levels were significantly increased using cotton collection method while salivary immunoglobulin was significantly decreased. However, salivary cortisol and cotinine levels were not affected [98]. The passive drooling method of saliva collection is usually preferred by researchers which can avoid the unsystematic errors. The cotton swab method is not suggested to be used for unstimulated collection because of stimulation of the salivary glands with the physical contact of the cotton itself [99]. The spitting method has also got downsides of its own as compared to the drooling

method. There has been observed a significant amount of bacteria in the saliva obtained through spitting that further tends to alter the results [100]. Storage of saliva and every biological fluid is a key factor in the reliability of results. It is advised that saliva after collection should be immediately store below -20°C. Otherwise heat and other environmental factors will negatively impact the biological products [100]. Additionally, storing the samples at -80°C will keep the integrity of the constituents intact for years without or very little alterations.

Furthermore, the concentrations of certain biological stress markers may be very low in saliva samples that make it more challenging to be screened using UV spectroscopic approach. The salivary concentration of salivary cortisol is found to be 100 times less in saliva as compared to the rest of the body fluids *i.e*. urine, blood, sweat [101]. Serotonin is cannot be detected in the saliva samples with a very low effective concentration (less than 1 pg/mL) while it can be detected in blood, interstitial fluids, and urine [47, 102]. However, salivary alpha amylase (AA) can be a better alternative for detection as a biological stress marker due to its higher concentration comparatively [103]. Neural stimuli to the acinar cells results in the production and release of alpha amylase by the parotid glands in the oral cavity. The salivary concentrations of alpha amylase ranges from 0.6−2.6 mg/mL, which is an enough concentration from optimum detection through various analytic techniques [104]. Psychological stressful stimuli are the major instigation factors for the release of alpha amylase. Chronic stimulation of adrenergic nervous system also prompts its release [105]. Epinephrine is primarily release by the adrenal medulla while nor epinephrine is released by the sympathetic nerve endings. Epinephrine is detectable in the plasma with a concentration of 0−0.15 nM as well as in the urine with a detectable concentration of 0−0.1 μM. Norepinephrine is having a concentration of 0−0.3.5 nM in blood and 0.09−0.5 μM in urine. Their analysis in the rest of the boy fluids is still under review. They are released in response to high degree stress with a fight or flight situation [106, 107]. Certain other stress biomarkers are also present intensively in plasma and urine with considerable concentrations but their undetectable availability in saliva make them a subject of further struggle for researchers. Cardiac troponin T (CCT) is a protein originated in the cardiac muscles in response to cardiac stress/anomalies with concentration of 1−8 pg/mL in the plasma and act as a main biomarker representing troponin [108]. Brain derived

neutropic factor (BDNF) is also a protein derived from sympathetic nerve terminals with a detectable serum concentration of 3.0−43 ng/mL and salivary concentration of 0.067−2.74 ng/mL. BDNF is triggered by various mental conditions like depression, neuropsychiatric disorder, and schizophrenia. This makes it also a good salivary stress biomarker.

Additionally, psychological parameters may be responsible for biased or unreliable results. The subjects participating in the study despite the pre-advised instructions may be exposed to stressful stimuli to a variable extent which may cause issues. Numerous commonly occurring psychological factors may cause variable amount of stress biomarkers production fluctuating the results *i.e*. watching suspense movies, public speaking, dental procedures, sports competition, examinations, doing adventures *etc*. change the salivary alpha-amylase and cortisol levels in healthy young adults [109]. Similarly, cortisol has been evidenced to maintain a steady concentration in response to these inconsistent factors as compared to alpha amylase which act more rapidly [109]. However, exposure to dental treatment stresses subjects to secrete a higher concentration of salivary cortisol and immunoglobulin as compared to alpha amylase [110].

Different profile of mood states (POMS) also result in different concentrations of salivary stress biomarkers including alpha amylase and salivary cortisol in special. Exposure of individuals to acute stress cause the activation of sympathetic-adrenal-medullary (SAM) and hypothalamus-pituitary axis (HPA) activation [111]. Different researchers used Trier Social Stress Test (TSST) in order to induce stress among the participants in a systemized way for studying the nature and extent of stress biomarkers produced during the various stages of the test. It was observed that a higher concentration of cystatin S, glutathione S-transferase, alpha-amylase and light chain immunoglobulin (IgA), and prolactin-inducible protein (PIP) were produced [112]. TSST also results in a 50% elevated levels of interlukin-6 (IL-6). The levels were observed for about 20 minutes after the test [113].

Hence, the variable concentrations in the body fluids, their stability *i.e*. half-life, nature of the stressful stimuli causing their release, storage and the method of detection comprehensively impact the results of the analysis. Likewise, the adaptation of smoking behavior may not be always in response to dealing the

physical or psychological stress. Sometimes, a smoker may initiate smoking because of certain other factors *i.e*. lack of education, negative inspiration from community or social media, or behavior to impress others especially in the young population *etc*. these factors can significantly deviate the expected outcomes of the research work regarding the salivary stress biomarkers analysis.

Chapter 6 Conclusion

The current research work concludes that different stress stimuli including physiological stress *i.e*. work burden, disease conditions, environmental effects and psychological stress *i.e*. work pressure, family issues, financial problems, social problems tends to initiate/increase the smoking behavior among the community. UV spectrophotometry studies provided peak plasma concentration peaks at the lower UV range of 190 to 210 nm, but with no significant difference, representing the presence of biological stress markers. The profile of mood state evaluation studies concluded that the smoking participants were presented with a significantly higher level of individual mood profile scores *i.e.* anger (****, $P<0.0001$), confusion (**, $P<0.0014$), fatigue (*, $P<0.0354$), tension (*, $P<0.0422$) and stress as compared to nonsmoking participants. The vigorous score was significantly high in the nonsmoking individuals (****, $P<0.0001$). Similarly, total mood disturbance score was also significantly high in the smoking participants. The application of artificial neural networking through artificial machine learning scored the accuracy of the results 84% which is a reliable outcome. Likewise, it is also concluded that smoking initiation may not be always triggered in response to stress. Numerous factors *i.e*. lack of education, negative inspiration, or behavior to impress are also involved. Furthermore, the adaptation of smoking behavior as a result of stressful stimuli is not a valid approach to reduce the noxious/stressful stimuli. The stress may further be exaggerated by smoking.

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Appendices

Appendix-1

POMS QUESTIONNAIRE

NAME: _____ AGE: _____

SMOKER/NON-SMOKER: _____ Subject No: _____

S. NO.	FEELINGS	Response				
		Not at all	Minute	Discreetly	A lot	Excessively
1.	Tense	0	1	2	3	4
2.	Friendly	0	1	2	3	4
3.	Angry	0	1	2	3	4
4.	Unhappy	0	1	2	3	4
5.	Worn out	0	1	2	3	4
6.	Clear headed	0	1	2	3	4
7.	Confused	0	1	2	3	4
8.	Lively	0	1	2	3	4
9.	Sorry for things done	0	1	2	3	4
10.	Listless	0	1	2	3	4
11.	Shaky	0	1	2	3	4
12.	Peeved	0	1	2	3	4
13.	Sad	0	1	2	3	4
14.	Considerate	0	1	2	3	4
15.	Active	0	1	2	3	4
16.	Grouchy	0	1	2	3	4
17.	On edge	0	1	2	3	4
18.	Blue	0	1	2	3	4
19.	Panicky	0	1	2	3	4
20.	Energetic	0	1	2	3	4
21.	Hopeless	0	1	2	3	4

22.	Unworthy	0	1	2	3	4
23.	Relaxed	0	1	2	3	4
24.	Spiteful	0	1	2	3	4
25.	Uneasy	0	1	2	3	4
26.	Sympathetic	0	1	2	3	4
27.	Restless	0	1	2	3	4
28.	Fatigued	0	1	2	3	4
29.	Unable to concentrate	0	1	2	3	4
30.	Helpful	0	1	2	3	4
31.	Discouraged	0	1	2	3	4
32.	Annoyed	0	1	2	3	4
33.	Resentful	0	1	2	3	4
34.	Lonely	0	1	2	3	4
35.	Nervous	0	1	2	3	4
36.	Miserable	0	1	2	3	4
37.	Cheerful	0	1	2	3	4
38.	Muddled	0	1	2	3	4
39.	Bitter	0	1	2	3	4
40.	Anxious	0	1	2	3	4
41.	Exhausted	0	1	2	3	4
42.	Ready to fight	0	1	2	3	4
43.	Gloomy	0	1	2	3	4
44.	Good natured	0	1	2	3	4
45.	Desperate	0	1	2	3	4
46.	Rebellious	0	1	2	3	4
47.	Sluggish	0	1	2	3	4
48.	Helpless	0	1	2	3	4
49.	Bewildered	0	1	2	3	4
50.	Weary	0	1	2	3	4
51.	Alert	0	1	2	3	4
52.	Furious	0	1	2	33	4

53.	Deceived	0	1	2	3	4
54.	Efficient	0	1	2	3	4
55.	Full of pep	0	1	2	3	4
56.	Trusting	0	1	2	3	4
57.	Bad tempered	0	1	2	3	4
58.	Forgetful	0	1	2	3	4
59.	Worthless	0	1	2		4
60.	Carefree	0	1	2	33	4
61.	Guilty	0	1	2	3	4
62.	Terrified	0	1	2	3	4
63.	Vigorous	0	1	2	3	4
64.	Bushed	0	1	2	3	4
65.	Uncertain about things	0	1	2	3	4