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Detection of stress- and taste-related compounds in human saliva

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Abstract

Saliva is made up by 99% water and only 1% other components including plasma-derived ions, proteins, hormones and so on. Hence, even though they are included in only 1% composition of saliva, those components have a vital role as biomarkers for such a huge range of molecules.

The hypothesis for this thesis is that it is possible to detect cortisol-like compound (CLC) and taste-related compound (TRC) in saliva using NMR approach. With CLCs, cortisol, cortisone, aldosterone, and corticosterone are performed with several experiments. With TRCs, adenosine-related compounds and other taste compounds are analysed.

Through a series of experiments, 5.8 ppm region in 1D spectra is suggested as the most promising region to detect CLC in human and animal saliva. NMR approaching can also distinguish sample's genders, as well as detect if a person uses corticosteroid.

Salmon extraction using saliva together with TCA are used in an attempt to extract flavouring compounds. On one hand, TCA extraction result shows greater stability than extraction result performing with saliva. On the other hand, saliva quality and condition have such an influence in the extraction result. In short, the possibility to track the concentration of many TRCs in human saliva over time is confirmed.

Abbreviations

Acronym	Description
µL	Microlitre
ACTH	Adrenocorticotropic Hormone
ADP	Adenosine 5’-iphosphate
AMP	Adenosine 5’-monophosphate
ANS	Autonomic Nervous System
ATP	Adenosine 5’-triphosphate
BMRB	Biological Magnetic Resonance Data Bank
CLC	Cortisol-like compound
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CRF	Corticotropin-Releasing Factor
D_2O	Deuterated water
DEPT	Distortionless Enhancement by Polarization Transfer
Epp. tube	Eppendorf tube
g	Gram
G	G-force or Relative Centrifugal Force (RCF)
GAS	General Adaptation Syndrome
GLC	Gas-Liquid Chromatography

HMBC	Heteronuclear Multiple Bond Correlation
HMDB	The Human Metabolome Database
HPA	Hypothalamic–Pituitary–Adrenal axis
hrs	Hours
HSQC	Heteronuclear Single Quantum Correlation
Hx	Hypoxanthine
IMP	Inosine monophosphate
Ino	Inosine
mM	Millimolar or millimole per litre
mmol	Millimole
NMR	Nuclear Magnetic Resonance
NTNU	Norwegian University of Technology and Science
PaNS	Parasympathetic Nervous System
PCA	Principal Component Analysis
PNS	Peripheral Nervous System
ppm	Part Per Million
Sal	Saliva
SalmA	Salmon brand that was used for this project
SNS	Somatic Nervous System
SRF	Somatotropin-Releasing Factor

SyNS	Sympathetic Nervous System
TCA	Trichloroacetic acid
TOCSY	TOtal Correlation SpectroscopY
TSP	3-(Trimethylsilyl)propanoic acid
Xan	Xanthine

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1. Introduction

Stress has become one of the most common terms that has been mentioning in everyday life. It is a serious problem, especially in developed countries where numerous stress-causing factors can be found in almost every aspect of such a busy life. When someone says "I am stressed", it simply exposes how she or he feels. More than just an emotional expression, stress is a disorder indicated by the significant change of some metabolite molecules in our body in order to response to the change of various factors. The result of stress response in human and animal is releasing of what is called "stress hormones" in which cortisol is the most well-known (Ranabir and Reetu, 2011).

Cortisol can be detected in mostly everywhere in our body such as tissue, blood, hair scalp, urine, etc. Cortisol in blood is usually used to demonstrate level of human stress (Lightman and Terry, 2014). However, blood sampling is invasive. It could trigger an increase in secretory of stress hormones as well. Overcoming the problem, saliva has its measurement non-invasive and simply preparative, which is potential to replace the blood measuring (Granger et al., 2007, Groschl and Rauh, 2006).

Saliva plays such an important role in our body not only because of its heterogeneous composition but also the interaction between the components to maintain oral healthiness. Those saliva components contribute to many functions that can be divided into 11 groups; for example: taste, speech, digestion, or buffer capacity (Milanowski et al., 2017, Almeida et al., 2008).

During initial stage of food ingestion, saliva plays an important role to activate the sense of taste that allows identification of nutrients or toxic compounds (Pedersen et al., 2002). On the other hand, Norway is

famous for Atlantic salmon. With locally good source of salmon, salmon is set as suitable study subject for this thesis. Thus, salmon extracted in saliva is used as a model to studied taste-related compounds.

There are several methods used to conduct the study of biofluids in general, and saliva in specific including HPLC, GLC, or column chromatography. They are all powerful tools for chemical analysis, but they also have their own light and dark sides. Prevail over limitation of those mentioned methods, NMR spectroscopy therefore turns out to be a great technique that can be an alternative for researches (Silwood et al., 2002).

1.1 Stress

In Japan, for instance, overwork-related disorders usually come together with two terms "karoshi" (過労死), meaning "death from overwork"; and "karojisatsu" (過労自殺), meaning "suicide due to overwork (Bannai and Tamakoshi, 2014). Both are consequences of overwork, which is one the major causes of stress at work. Still, what stress really is?

1.1.1 History and definition

The most important term about every single event in all living creatures is "homeostasis", meaning "keep the balance" inside the body. The importance of maintaining the balance of internal milieu inside the body in order to adapt to the change of environment is the key to keep life moving on. The term was firstly used by Canon in 1929 (Landis, 1930, Everly and Lating, 2013). As McEwen and Wingfield (2003) described, stress is often known as a threat to this homeostasis of the body. Simpler way to explain, stress is emotional and physical tension responding to a threatened event to the body. That event is called "stressor", the factor that causes stress. And reaction of the body to the

stressor is “stress response”. Sometimes stress is exercised as both stressor and stress response but in this work, stress is referred to response (McEwen and Wingfield, 2003, Lupien et al., 2015, Lupien et al., 2007, Selye, 1975a, Selye, 1975b, Hans Selye, 1998).

Two types of stressor are classified based on impact density of a threat: real (absolute) stressor and implied (relative) stressor. Real stressors such as tsunami or earthquake raise such a significant stress response in all or a great number of individuals facing the threat resulting in absolute stress response. Whereas relative stressor, which is milder and not necessary lead to physiological response, triggers stress response in only a part of community of which very relied on each individual. As a common term, stress is usually known as distress, meaning bad stress. There is also eustress (good stress). Alike relative stress response, it is difficult to consider which stress is good and which one is bad because they are based on various cognitive interpretation of each individual to a given situation. Depending on biological response to stressor, stress development exhibits as acute and chronic stress that will be explained in the next part. (McEwen and Wingfield, 2003, Lupien et al., 2015, Lupien et al., 2007, Selye, 1975a, Selye, 1975b, Hans Selye, 1998, Schneiderman et al., 2005)

Modern term “stress” was first physiologically described as “general adaptation syndrome” by Hans Selye, a Hungarian scientist, in 1936. As he described, stress had non-specific responding property and was results of many different physical stimuli such as pain, cold, heat, etc. (Jackson, 2014, Selye, 1936). However, in Selye’s experiments, he had focused only on physiological aspect of stress. Obviously, stress is much more than that. According to the book “Controlling stress and tension”, stressors are divided into two categories: psychosocial stressors and biogenic stressors (Girdano et al., 2012). Psychosocial stressors are those do not directly give rise to stress response but rather through a

progression. Biogenic stressors, such as caffeine, nicotine, physical factors, etc., are instantly the root for elicitation of stress response. Certainly, biogenic stressors are not the majority. Plus, there are many psychological studies of stress pointed out that psychological stressors have such a momentous effect on both physiological and psychological stress responses. As this logical aspect, there are two types of stress responses that are physiological stress responses and psychosocial stress responses. Physiological stress (also called physical stress responses) includes pain, stomach-ache, headache, etc. Some common psychological stress responses are cognitive problem, anxiety, and emotional disorder. Because of that, managing stress responses have become the main factor to maintain both physical and mental health. (Suzuki and Ito, 2013, Mason, 1968c, Lazarus and Folkman, 1984, Everly and Lating, 2013)

1.1.2 Biology of stress

Stress, as mentioned earlier, is understood as stress response that is neither result nor cause of progression but the progression itself. To understand clearly how progression of stress happens in the body, the basic knowledge of anatomical nervous system, which involves in stress response, is necessary.

Central nervous system (CNS) and peripheral nervous system (PNS) are two basic nervous systems in human body (figure 1). CNS includes spinal cord and brain. Brain functions are further classified into three levels:

- Highest level - neocortex with the most sophisticated component which is responsible for imagination, logic or decision.

- Limbic system, second level, with numerous neural structures such as hypothalamus, pituitary gland, or hippocampus, has function as control centre for emotion.
- Lowest level – reticular formation and brain stem maintain vegetative functions like heartbeat, respiration.

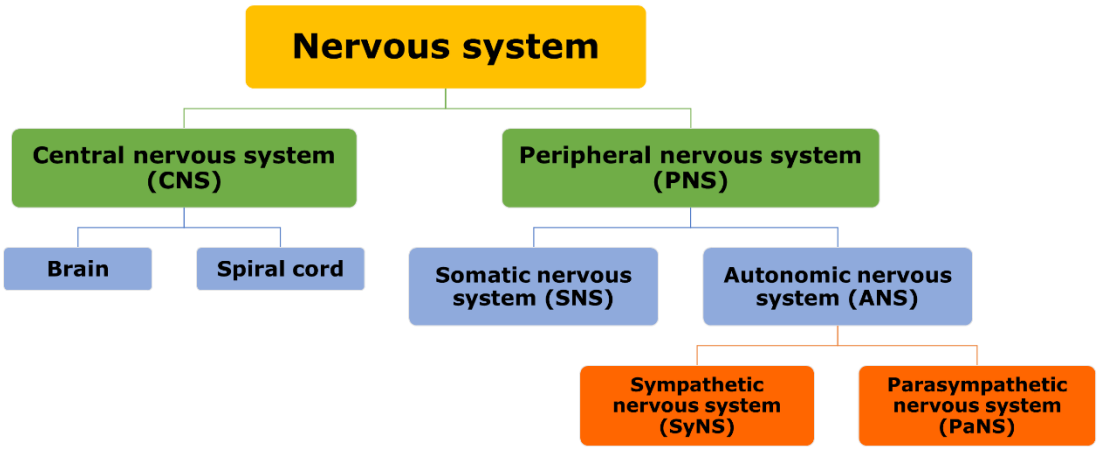

Figure 1: Basic nervous system of human body

All neurons that do not belong to CNS are classified as PNS and are sub-grouped into somatic nervous system (SNS) and autonomic nervous system (ANS), shown in figure 1. SNS is carrier of signals from and to CNS while ANS carries impulses related to homeostasis maintenance and regulation of environment inside the body. There are two sub-categorises of ANS that are sympathetic (SyNS), functioning as preparation for movement of the body; and parasympathetic (PaNS), functioning as relaxation and restoration of the body. They seem to function oppositely but sometimes they work as complementary to each other (PUDMED HEALTH (NCBI) 2018, Everly and Lating, 2013).

When psychosocial stimuli stress the body, there are three physiological responses that actually occur consisting of (1) neural axes, (2) neuroendocrine axis, and (3) endocrine axes. The neural axes are the first and the quickest responses of the body to stressors regarding the

fact that this pathway is based completely on neural system, mostly on ANS (Widmaier et al., 2004). The effects of neural axes in reply to stressors are immediate, however not chronic. In responding to chronic and prolonged stress, the body uses neuroendocrine and endocrine axes in which the body releases huge levels of hormones signals (LeBlanc, 1976).

In neuroendocrine stress response axis, adrenal medulla plays the most important role in characteristics of both ANS and endocrine. The term "fight-or-flight" is another well-known which its meaning is refer to neuroendocrine process (Cannon, 1914, Landis, 1930). As that, first nervous impulses are stimulated at dorsomedial amygdala complex passing to lateral and posterior hypothalamic regions in limbic system of the brain. From that, it continues passing down through spiral cord and ends up at adrenal medulla, in adrenal gland located at the superior poles of kidneys, where it initiates the hormonal release of catecholamines. There are two types of catecholamines secreted by neuroendocrine axis: norepinephrine (noradrenaline) and epinephrine (adrenaline) (Roldán et al., 1974, Everly and Lating, 2013). Catecholamines are not only released by adrenal medulla but also by SyNS and catecholaminergic neurons in the brain. Effects of catecholamines to the body are increase in cardio muscular, rise in blood pressure, greater level of glucose, etc. leading to the maximising possibility of mobilisation that helps setting the body ready for "fight-or-flight" situation (Sabban, 2009).

Hypothalamic–pituitary–adrenal (HPA) axis is the term of stress response pathway which involves hypothalamic and pituitary regions in limbic nervous system of the brain and hormonal releasing organ adrenal glands of the kidney. Therefore, it includes both neuroendocrine and endocrine axes. If the neural axes are the quickest stress response, endocrine axes would be the most chronic and prolonged response

(MASON, 1968b). The most well-established endocrine axis in stress response is the adrenal cortical axis. Three other axes that also participate in stress response to be mentioned are somatotropic axis, thyroid axis, and posterior pituitary axis (Mason, 1968a, McCabe and Schneiderman, 1985).

The initial start of cortical axis is septal-hippocampal complex in limbic region. From the origination, neural impulses pass down to median eminence of the hypothalamus triggering the release of corticotropin-releasing factor (CRF). CRFs descend to anterior pituitary by hypothalamic-hypophyseal portal system. The result of sensitiveness of anterior pituitary with CRFs is the stimulation of adrenocorticotropic hormone (ACTH) in systemic circulation. These all happen in the brain of CNS. ACTH travels in that part of cardiovascular system until it reaches the target adrenal cortex of the kidney. Here it excites zona's cells of the cortex to synthesise and release glucocorticoids and mineralocorticoids. Actually, cortisol and corticosterone are glucocorticoids whilst aldosterone and deoxycorticosterone are mineralocorticoids to be mentioned. Those corticosteroids are released into systemic circulation resulting in various effects of the body. Glucocorticoids lead to increases of gluconeogenesis, urea production, releasing of free fatty acids, association with stress-related feelings, etc. Mineralocorticoids have effects on higher absorption of sodium and chloride in renal tubules as well as lower level of their excretion in some glands and gastrointestinal tract. Beside affect the production of glucocorticoids, ACTH also stimulates the production of some other hormones such as testosterone and catecholamines (Henry, 1992, Henry and Stephens, 2011, Aguilera et al., 2001, Rochefort et al., 1959, Blank and Spiess, 2009, Everly and Lating, 2013, McCabe and Schneiderman, 1985).

Corticosteroids and catecholamines are the basic and also the most well-researched in stress study. In addition to them, some other hormones released during stress response are:

1. Growth hormones. Growth hormones aka somatotropic hormones are released by anterior pituitary in responded to somatotropin-releasing factor (SRF) passed down by systemic circulation with similar pathway to descend CRF (Everly and Lating, 2013, Ranabir and Reetu, 2011).
2. Vasopressin. Like CRF, vasopressin is another hormone released from hypothalamus in respond to acute stress. ACTH can be stimulated by vasopressin as well (Aguilera et al., 2008, Ranabir and Reetu, 2011).
3. Thyroid hormones. Thyroid hormones are synthesised by thyroid axis. It starts with releasing thyrotropin-releasing factor at hypothalamus. The signal passes to anterior pituitary resulting in releasing thyroid-stimulating hormones into systemic circulation. These factors trigger the synthesis of thyroid in thyroid gland (Everly and Lating, 2013).

These mentioned hormones and even other stress hormones work together in respond to acute and chronic stress events. In general, acute stress is usually good for the body. When the body gets threatened, acute stress occurs leading to systematic changes in the body including, for instance, nervous, endocrine, or immune systems (Selye, 1978). First, the SNS and HPS axis produce stress hormones aka cortisol and catecholamines promoting ready energy for immediate use. Then energy spreads out through to organs by higher blood pressure. This is corelated to “flight-or fight” situation which CNS tend to produce isolated response leading to integrated coping responses to stressors. This has big meaning for adaptive ability and ability to escape dangerous situation of

animal. This acute stress, in fact, does not last long (Schneiderman et al., 2005).

In Selye's report, he also first brought out three stages of "general adaptation syndrome" (GAS) development, or three stages of stress in short. The initial stage is known as alarm reaction followed by resistance stage and ended with exhaustion stage (Selye, 1936, Selye, 1975b). The first two stages can be repeated many times in life of someone. But when stressor is too powerful, or the adaptive ability is depleted; then comes the third stage. The body then enters chronic stress. Chronic stress is usually a distress that associated with slower recovery of the body to wound or surgery, and vulnerability of immune system as poorer antibody to vaccine or more infection from virus and microorganism. Hence, chronic stress is a big issue to elder people with weaker immune system (Schneiderman et al., 2005, Everly and Lating, 2013).

1.1.3 Cortisol and cortisol-like compounds (CLC)

Cortisol and cortisol-like compounds (CLCs) are responsible for various functions in our body. Above all of that, these compounds are most well-known with responsory to stress. In this thesis, there are cortisol and other three CLCs to be used: cortisone, aldosterone, corticosterone. All CLCs belongs to corticosteroids group.

Cortisol and corticosterone are glucocorticoids whereas aldosterone is a mineralocorticoid; all are secreted when the body is stress. Cortisone is an inactive form that is transferred to active hydrocortisone (NCBI, 22786). Hydrocortisone is medical name of cortisol and this name will be used from this moment in this thesis. Hydrocortisone is the major stress hormone to be find in human and some animals whereas corticosterone, also a glucocorticoid, is major stress hormone found in rodent and other animals.

The monoisotopic mass of four CLCs are shown in the table 1 that will be used for lateral calculation (observed from PubChem-NCBI).

Table 1: Monoisotopic mass of CLCs

CLC	**Monoisotopic mass (Da)**
Hydrocortisone/ Cortisol	362.209320
Cortisone	360.193665
Aldosterone	360.193674006
Corticosterone	346.214409448

In general, methods that are used for cortisol detection can be divided into 4 groups: chromatographic techniques; immunoassays for cortisol detection; electrochemical immunosensing; and point-of-care (POC). Cortisol secretion of individuals depends on day–night cycle and field environment hence its detection at POC is deemed essential to provide personalized healthcare. Chromatographic techniques have been traditionally used to detect cortisol. The issues relating to assay formation, system complexity, and multistep extraction/purification limits its application in the field. In order to overcome these issues and to make portable and effective miniaturized platform, various immunoassays sensing strategies are being explored. However, electrochemical immunosensing of cortisol is considered as a recent advancement towards POC application. Highly sensitive, label-free and selective cortisol immunosensor based on microelectrodes are being integrated with the microfluidic system for automated diurnal cortisol monitoring useful for personalized healthcare. Although the reported sensing devices for cortisol detection may have a great scope to improve portability, electronic designing, performance of the integrated sensor, data safety and lifetime for point-of-care applications (Kaushik et al.,

2014, McVie et al., 1979, Vieira et al., 2014, Few et al., 1986, Perogamvros et al., 2009). Some of CLCs concentration that has been detected by other methodologies rather than NMR are presented in appendix 1 as an extra information.

1.2 Biofluid metabolomic

Human biofluids which are common used for research purposes are blood, urine, plasma, and saliva which include different components with different amount of each such as water, macronutrients, volatile compounds, amino acids, etc. (Dame et al., 2015)

Urine has been used widely for metabolomics researches. It is the easiest biofluid that can be obtained its volume in hundred-millilitres. However, as liquid-form-waste of human body, urine contains many downward products from all metabolism progresses. Many of those downward products may be harmful and have not been well characterised. Urea presents with high concentration in urine together with other compounds (Bouatra et al., 2013) On the other hand, blood is one of the most important biofluid in the body as it plays vital role in transport metabolites in the body. It is well-researched subject especially in medical field. However, blood sampling is invasive as well as possibility to have negative impact on both sample and the body.

Therefore, saliva turns out to be a potential candidate to alternate other biofluids. Saliva is a complex fluid, which influences oral health through specific and nonspecific physical and chemical properties. Saliva is produced and secreted from salivary glands. Components of saliva are most predominant (99%) by water. A variety of plasma-derived inorganic anions and cations can be found in oral fluid, such as sodium, potassium, magnesium, calcium, chloride, bicarbonate, phosphate and ammonia. Many proteins/peptides manifest antimicrobial activities, such

as lactoperoxydase, lactoferrin, immunoglobulins, lysozyme, agglutinins, chitinases and mucins. Other proteins like enzymes (e.g., α-amylase, carbonic anhydrase), hormones (e.g., growth factors, cortisol, catecholamine, progesterone, testosterone), free carbohydrates (glucose, mannose, galactose), lipids (e.g., cholesterol), amino and fatty acids are also present in saliva. The interactions between salivary components are crucial to fulfil the functions of oral healthiness's maintenance. They can be classified into eleven groups involving digestion, taste, speech, lubrication, lavage/cleansing, buffering action, promotion of re-mineralization, antibacterial activity, antioxidant capacity, tissue repair and maintenance of oral mucosa (Tiwari, 2011, Pedersen et al., 2002, Milanowski et al., 2017, Schenkels et al., 1995).

1.3 Taste

Taste is subjective, what taste great for one person might not taste great for another person. There is no such a way to determine "great taste". However, when it comes to a scientific point of view, there are certain measurement methods. As scientific point of view, taste is made of various of compounds with different structures and sizes. Those compounds can be grouped as five basic tastes which are sweetness, bitterness, sourness, saltiness, and savoury taste.

There are taste buds in the tongue which are responsible for tasting function with the help of saliva. And compound that can be tasted needs a typical structure that fix and bind to receptor protein in taste bud.

Sweetness comes from compounds that have AH-B structure. The AH-B concept says that a compound with a correct angel formed by two AH and B electronegative groups creates sweet taste. The two electronegative groups must have 0.3 nm between them. The other groups in compound corelate to the intensity of sweetness. Some

common types of sugar having this structure are glucose, fructose, xylitol, etc. Bitterness is from α-acids such as hops, from phenolic compounds, or from salt with ionic diameter bigger than 0.658 nm (ionic diameter of potassium bromide). Whereas the salt with smaller ionic diameter than 0.658 nm gives salty taste. Sourness, in fact, is related to the high amount of hydrogen ion, meaning high acidity. Therefore, most of organic acids are the ones to claim for sourness in food and beverage. Some representatives of sour compound are citric acid (from citrus) , acetic acid (vinegar), or tartaric acid (grape) (Coultate, 2016).

Another name that people use to call savoury taste is umami, meaning "delicious" in Japanese. Umami taste is classified as one in five basic taste recently. It was known as small group of specific taste before the confirmation study rather than one basic taste. Commonly, glutamate aka glutamic acid is recalled as the most popular umami-related compound (Chen et al., 2009, Vandenbeuch and Kinnamon, 2016).

However, in this thesis we will not focus mainly on those basic tastes. Instead, the freshness is more attractive to discover. Freshness is not really a taste but in food ingestion, it is obvious that the tongue can taste freshness. Freshness in indicated by K-index, which has been named as one of the best indicator for measuring freshness (Tejada, 2009). K-index is calculated with the formula:

$$\boldsymbol{k-index}\ (\%) = \frac{\boldsymbol{Hx + Ino}}{\boldsymbol{ATP + ADP + IMP + Hx + Ino}} \times \mathbf{100}$$

ATP and its degraded products after the fish dies are used for calculation because ATP is the energy molecule of animal (including human and fish). ADP pluses one phosphate group and energy to synthesize one ATP and vice versa. As the animal dies, there is no supply of energy require to create more ATP and to operate the muscle. Therefore, ATP is degraded

backward to ADP and so on. All reactions are catalysed by endogenous enzymes (Kitaoka, 2014, Hong et al., 2017). The brief process follows:

$$ATP \rightarrow ADP \rightarrow IMP \rightarrow Ino \rightarrow Hx$$

Based on the process, Hx and Ino is the two major endings of ATP degradation in fish, thus, appear as devisors in the calculating formula. It means that there is inverse proportion between Hx plus Ino and all ATP degradation products. Before ADP is conversed to IMP, there is another reaction where ADP becomes AMP then AMP becomes IMP. However, AMP does not have significant meaning in calculation K-index of fish as ATP is immediately degraded to IMP in high speed. Values of K-index has been mentioned to change among different fish species (Tejada, 2009, Tejada et al., 2007). K-index does not only mean of freshness measuring but also function as indicator for fish consumption. According to a Japanese study, fish with K-index lower than 20 % can be used for raw consumption aka sushi (Hamada-Sato et al., 2005). K-value around 40-50 % is considered as premium quality for cooking whereas K-value around 70–80 % is set as limitation for salmon consumption (Erikson et al., 1997).

1.4 Salmon source

Three fillets of premium skinless and boneless loins of Atlantics salmon were purchased 3 days after being slaughtered at local stores. The salmon were from brand SALMA®, belonging to Bremnes Seashore AS company – a Norwegian supplier.

All fillets were brought to laboratory and were stored at 4 ^{0}C during experimental days.

1.5 Nuclear Magnetic Resonance (NMR)

NMR spectroscopy is a powerful analytical tool for structural as well as quantitative study of chemical and biochemical compounds. It has been intensively used for studying biofluids (Lindon et al., 1999). While methods like gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), or column chromatography (CC) have some disadvantages for human saliva researches, NMR spectroscopy appears as a potential alternative that is briefly summarised in table 2 (Silwood et al., 2002, Emwas et al., 2015, Gebregiworgis and Powers, 2012, Dona et al., 2016).

Table 2: Strength and limitation of NMR compared to other methods in human saliva study

	NMR	**Other methods (GLC, HPCL, CC)**
Advantage	• Non-invasive • Un-targeted detection • Minimal sample preparation • Simultaneous information • Non-destructive sample	Higher sensitivity
Disadvantage	Lower sensitivity	• Time consuming • Intensive labour work • Partial characterization • Sample-destructive

In NMR sample preparation for biofluids, it is needed sometimes to remove proteins by precipitation before NMR run as proteins are not stable and easy to degrade (Gebregiworgis and Powers, 2012). For the purpose of quantifying components of the NMR sample, a reference with known concentration is added into the sample, a role of external standard. The most common external standard compound used in biofluid research is 3-(trimethylsilyl) propanoic acid (TSP) located at 0 ppm in spectra. Another well-used standard is deuterated 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (Dona et al., 2016). TSP is supplemented as external standard for all experiments in this thesis.

Data from NMR spectroscopy studies are globally shared on Human Metabolome Database (HMDB), BioMagResBank (BMRB), The Madison Metabolomics Consortium Database, and Birmingham Metabolite Library (Dona et al., 2016, Gebregiworgis and Powers, 2012). The data that has been used for this thesis is obtained from HMDB. Unit used for chemical shifts in NMR spectra is parts per million (ppm).

1.5.1 One-Dimensional (1D) NMR spectroscopy

One of the most useful NMR techniques is 1D-^{1}H NMR. It provides such a huge information of signals. It is fast and easy to acquire but it has narrow range of chemical shifts, spectral width around 20 ppm. This such a small range of spectrum leads to highly overlapping of peaks and intensive signals. 1D-^{1}H NMR spectrum improves its sensitivity and resolution corresponding with magnetic field strength (Gebregiworgis and Powers, 2012, Emwas et al., 2015).

1.5.2 Two-Dimensional (2D) NMR spectroscopy

Overcoming the weakness of 1D-^{1}H NMR spectra, 2D NMR spectra are introduced as powerful methods to conduct researches. Among all 2D NMR methods, HSQC, HMBC, TOCSY are the most common used in

metabolomic studies. 2D ^{1}H-^{13}C HSQC, standing for Heteronuclear Single-Quantum Correlation Spectroscopy, correlates hydrogen with carbon atoms over one bond. Whereas correlations of hydrogen and carbon between two to three bonds are indicated by ^{1}H-^{13}C HMBC - Heteronuclear Multiple Bond Correlation. Total Correlation Spectroscopy (TOSCY) is useful to provide structural information of indirect correlated atoms. These threes work accompanied to each other for all aspects of the studied substances (Gebregiworgis and Powers, 2012, Dona et al., 2016).

1.6 Objectives of the project

The aim of this project is to develop method of stress- and taste-related molecules detection in human saliva using NMR spectroscopy.

The main task of the project will be (1) to detect in human saliva cortisol-like compounds (CLCs) and to explore the possibility of interaction between CLC and other molecules present in saliva; and (2) to isolate taste-related compounds (TRCs) via simulated version of food ingestion in human saliva and food particles (salmon).

The result of the research may help find an efficient way to detect stress in both humans and animals. It also helps with better understood of how TRCs are affected by different quality of food.

The outline of this thesis project is spread out through 4 main parts:

1. To have better understanding of CLC, CLC's structures are assigned via theirs 1D and 2D NMR spectra.
2. From the understanding, it is expected to see through titration experiments a better view of remarkable signals of CLC in human saliva.
3. A group of student's saliva are collected at different times; before and after lunch to detect if CLC could be found in their saliva. It is interested to see if there is any difference in their saliva by collecting conditions.
4. Taste-related molecules are studied by comparison salmon extract in TCA versus salmon extract in saliva. The result of this may help determine variation in food tasting at different level of freshness.

2. Methodology

2.1 Experimental design

To fulfil thesis main requirements, experiments were designed as followed:

1. To have better understanding about spectrum signals of CLC and how they were showed in NMR spectra, CLC stocks were assigned with NMR solvents which were D_2O and deuterated acetone.
2. Different concentrations of CLC stock were admixed with saliva as another NMR assignment. The spectra obtained were used to study CLC-saliva interaction.
3. Saliva from 2 groups of students were collected: saliva of the first group was collected in the morning before having any meal while the other was collected in the afternoon after lunch. The expectation was to confirm the CLC-saliva interaction and to know if diverse conditions of saliva would have any effect on CLC signals.
4. To test how different time of storage fish changed its freshness or K-index, we conducted salmon extraction in saliva. The experiment was done with a series of sampling the same salmon at different post-mortem days. The mentioned salmon was also extracted using TCA as a reference data for comparison. Salmon samples were stored from 3^{rd} day, when the fish was on market and being purchased, to 18^{th} day. Days were used for experiment were day 6 (T6), day 7 (T7), day 8 (T8), day 11 (T11), day 14 (T14), and day 18 (T18).

2.2 Chemicals and Equipment

Chemicals and equipment that were used in this project were shown in table 3 and 4 correspondingly.

Table 3: Chemicals used in the project and their suppliers

Chemical	**Supplier**
3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D)	Armar Chemic
Acetone 0.03% TMS	ACROS organics
Aldosterone	Sigma-Aldrich
ATP	MP Biomedicals
Corticosterone	Sigma-Aldrich
Cortisone	Sigma-Aldrich
Deuterated water (D_2O, 99.9%)	Cambridge Isotope Laboratories Inc.
Hydrocortisone	Sigma-Aldrich
IMP	MP Biomedicals
Inosine	ACROS organics
Sodium Hydroxide (NaOH)	Sigma-Aldrich
Trichloroacetic acid (TCA)	Merck Millipore
Xanthine	ACROS organics

Besides those chemicals and equipment mentioned in table 3 and 4, several software was necessary for result analysing: TopSpin 3.5pl7 (Bruker, Germany), ChemBioDraw Ultra 14.0 (Perkin Elmer), AMIX (Bruker), and The Unscrambler 10.4 (CAMO, Norway). TopSpin and AMIX licenses were provided by Bruker the company. ChemBioDraw Ultra 14.0 was licensed by NTNU. And the Unscrambler was powered by help of Professor Turid Rustad (NTNU).

Table 4: Equipment used and its supplier

Equipment	Supplier
Homogeniser	RETSCH MM400
Incubator	INFORS HT Multitron
Centrifuge	KUBOTA 3500
NMR spectrometer	Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe
NMR sample tube	5 mm Wilmad - LabGlass

2.3 CLC stock solution

Two standard stock solutions of aldosterone were prepared using deuterated acetone and deuterated water. Hydrocortisone, cortisone and corticosterone stock solutions were prepared using deuterated water. The final concentrations of all stock solutions together with compounds solubility are shown in the table 5.

Preparation of standard solutions were carried out like follows:

Aldosterone in water: 540 µL stock + 60 µL TSP

Aldosterone in acetone: 150 µL stock + 450 µL acetone

Hydrocortisone: 200 µL stock + 340 µL D_2O +60 µL TSP

Cortisone: 540 µL stock + 60 µL TSP

Corticosterone: 540 µL stock + 60 µL TSP

Table 5: CLC stock solutions and their solubility.

	Solvent	Concentration used [mg/mL]	Solubility [mg/mL]
Aldosterone	Water	0.02	0.051[a] (HMDB0000037, HMDB)
	Acetone	4	
Hydrocortisone (Cortisol)	Water	0.2	0.32 (HMDB0000063, HMDB)
Cortisone	Water	0.2	0.28 [b] (HMDB0002802, HMDB)
Corticosterone	Water	0.2	0.2 (HMDB0001547, HMDB)

[a] at 37^0C

[b] at 25^0C

2.4 CLC titration preparation

The titration of saliva with aldosterone, hydrocortisone, cortisone and corticosterone were performed as showed in table 6.

Sample preparation started with saliva collection, 1 mL saliva was required for each sample, followed by centrifugation at 20000 x G for 10 min at 8 ^{0}C. 500 µL of the clear saliva was transferred into new Epp. tube before certain amount of D_2O and stock solution were added. The mixture was centrifuged again for 10 min to remove all precipitant. 495 µL of the clear mixture was mixed with 55 µL TSP buffer to form final NMR sample. Temperature at -20 ^{0}C was used for sample storage.

Table 6: Volumes of saliva, D_2O, stock for the CLC titration

Saliva [µL]	D_2O [µL]	CLC stock [µL]	Mixture volume [µL]
500	480	20	1000
500	460	40	1000

2.5 Standard saliva sampling

Saliva was collected right before experiment for its freshness and its quality for better isolation. 1 mL of saliva was needed for each sample. This sampling also played a role of standard control for all other experiments related to saliva.

Saliva was centrifuged at 20000 x G for 10 min at 8 ^{0}C. Precipitated part was removed from sample. 500 µL saliva then was diluted with ratio 1:1 with 500 µL D_2O followed by centrifugation for 10 min to remove the remaining pellet. 600 µL sample was transferred to Epp. tube and had extra centrifugation if saliva was still not necessarily clear. Storage of sample was at -20 ^{0}C.

2.6 Salmon extraction in saliva (SalmA-Sal)

0.5 g salmon was weighted and minced. It was put into 2 mL Epp. tube with 4 mini glass balls adding 1 mL saliva. It was horizontally homogenised followed by incubating at 37 ^{0}C for 10 min. The sample was centrifuged at 20000 x G for 10 min. 500 µL clear supernatant was transferred into a new 1.5 mL Epp. tube together with 500 µL D_2O followed by another 10 min centrifugation. 600 µL mixture was transferred into another Epp. tube with optionally 5 min extra

centrifugation. Sample was stored at -20 ^{0}C until NMR would be conducted.

2.7 Salmon extraction in TCA (SalmA-TCA)

5 g of salmon was mixed and homogenizes with 30 mL extraction medium (7.5 % TCA). The mixture was filtered using Whatman filter paper after being centrifuged for 10 min at 8 ^{0}C at 6500 x G. The solution was then adjusted to pH 7.0 using 9M NaOH. Sample was stored at -20 ^{0}C before further process. This procedure was modified from the protocol from another Atlantic salmon study via NMR approach (Shumilina et al., 2015).

2.8 Other experiments

2.8.1 Salmon-TCA-saliva (SalmA-TCA-Sal) interaction

Extraction of salmon in TCA (SalmA-TCA) was secondary extracted via saliva, called SalmA-TCA-Sal. Ratio 1:1 was used for mixing SalmA-TCA and saliva or D_2O. As limited extracted amount of SalmA-TCA, 500 µL saliva and 300 µL D_2O were used. The mixture was centrifuged with speed of 20000 x G for 10 min at 8 ^{0}C before its clear solution being transferred into new Epp. tube for storage. The SalmA-TCA-Sal sample was stored in -20 ^{0}C freezer till needed.

2.8.2 Titration of metabolite

Titration samples were first followed procedure of salmon extraction in saliva. 1D ^{1}H spectrum of all the samples were acquired as reference. Then different amounts of stock solution were added into samples before they were acquired once more time.

2.8.3 Student saliva

One group of students, age in range 22 - 28 years old, was collected saliva since 2016. The saliva was collected in the morning before breakfast and was stored at -80 ^{0}C before conducting experiment.

The other group of saliva was collected in 2018 from students with age in range 22 -28 years old. The collection of saliva was performed after lunch and its storage was at -20 ^{0}C.

All saliva samples were prepared using similar protocol as standard saliva sampling and stored at -20 ^{0}C before NMR experiment.

2.9 Sample preparation for NMR analysis

For SalmA-TCA experiments, 10 mM TSP in 20mM sodium phosphate NMR buffer in deuterated water was used instead of 1mM TSP buffer. The ration for this would follow 60 µL TSP: 540 µL SalmA-TCA. The final concentration of TSP in NMR sample was 1mM.

For all other experiments, samples were each mixed with 1 mM TSP in 20 mM phosphate buffer in D2O with volume ratio 55 µL TSP: 495 µL sample, filling up 550 µL final NMR mixture. pH was equal to 7.0 and the sample had 0.1 mM final concentration of TSP in NMR tube.

Final sample was placed in 5mm NMR tube and was stored at -20^{0}C.

2.10 NMR experiments

Experiments were acquired at 300K using Bruker Avance 600-MHz spectrometer to acquire 1D ^{1}H, 2D ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and DEPT NMR spectra. All the mentioned above NMR experiments were acquired with the standard Bruker pulse sequences noesygppr1d;

mlevgpphprzf; hsqcetgpprsisp2.2; hmbcgplpndprqf and deptsp135 respectively.

Number of scans were diverse from 48 from 256, receiver gains (RG) were from 32 to 144 (shown in appendix 2)

2.11 NMR processing

Software TopSpin 3.7pl7 (Bruker, Germany) was exercised to process all spectra. Both registered experiments and available NMR databases (BMRB, HMDB) were used for performance of NMR assignments.

The spectra were calibrated against TSP signal at 0 ppm in both ^{1}H and ^{13}C dimensions. Other signals of chemical shifts were integrated proportional to this TSP signal.

2.12 Analysis

2.12.1 CLC assignment

For CLC structure and chemical shifts assignment, structure of CLCs with theoretical proton and carbon chemical shifts were predicted via BioChem Draw Ultra 14.0 (accessed through NTNU). Then, all carbon atoms in the molecule were numbered. In this thesis, order of carbons did not follow common numbering approaches, numbering was isolated as following these steps:

1. Number all methyl [$-CH_3$] groups
2. Number carbons surrounding CH_3 groups that were one and two bonds from carbon of CH_3 groups
3. Number the rest from one side to the other side of the compound (from left to right)

After numbering carbon, the next step was checking baseline of the spectra and calibration in 1D ^{1}H spectra. High field region of CH_3 groups were needed to be found. CH_3 groups signal were integrated and calibrated as equal to 3.

Next, 2D ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC were used to assign cross peaks corresponding to the CH_3 groups. Before that, baseline of these spectrum needed to be checked and calibration was necessary. DEPT spectrum was used to assign CH_3, CH_2, CH and C groups whereas 2D ^{1}H-^{1}H TOCSY was used to clarify hydrogen in some complicated regions. CH_3, CH and C groups gave possitive signals in DEPT spectrum whistl CH_2 group was negative. All information was manipulated to build some fragments around CH_3 groups. Those fragments finally were put together via ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HMBC.

2.12.2 Statistical analysis

TopSpin was used to process all spectra followed by data analysis via AMIX 3.9.15 (Bruker) software. The outcome from AMIX, an integral table, was then processed with The Unscrambler software resulted in performance of PCA.

3. Result

3.1 Error calculating

3.1.1 Pipetting error

Five samples of SalmA-Sal experiment were used to calculated error of NMR sample preparation. When 495 µL of sample were mixed with 55 µL of 1 mM TSP stock solution in a 1.5 mL Epp. tube. The tube was weighed, and the standard deviation was calculated via Microsoft Excel (Microsoft Office 2016). The result was error during NMR sample preparation shown in the table 7.

Table 7: Weight and standard deviation of samples

Sample no.	**Weight [g]**
SalmA-Sal T7	0.566
SalmA-Sal T8	0.569
SalmA-Sal T11	0.566
SalmA-Sal T14	0.567
SalmA-Sal T18	0.566
Mean	**0.5668**
Standard Deviation	**0.0013**
Standard Deviation [%]	**0.23**

3.1.2 TSP concentration uncertainty

The concentration of 0.1 mM and 1 mM TSP was used for calculating metabolite concentration as an external standard. The concentration error of TSP detected came from the error of NMR samples preparation plus the possible fluctuation of shim during acquiring NMR spectrum. Calculating standard deviation of TSP signal integral gave estimation of united uncertainty of other metabolites. Therefore, standard region of ^{1}H NMR spectrum (0.01/-0.01) was integrated for samples with shared parameters of NS, RG and TSP concentration. As calculated, estimated error of 1 mM TSP samples was 4.486 % and that of 0.1 mM TSP sample was 2.762 % (appendix 3).

3.1.3 NMR – S/N measurement

One of essential ratio for accurate integrations is the signal-to-noise ratio (S/N). It was calculated and performed by TopSpin. According to Maniara, the detection of metabolites is possible only if the S/N ration is higher than 3. And if S/N ration is larger than 10, it is able to quantify these metabolites (Maniara et al., 1998).

3.1.4 Integration uncertainty

For estimation of integration uncertainty, each NMR signals was integrated three times and the standard deviation was calculated for the concentration of each metabolite. This could be found in appendix 4.

3.1.5 Metabolite quantification

Metabolite concentration in saliva was calculated using the formula:

$$C^{Met} = k \times \left(\frac{\eta^{TSP}}{\eta^{Met}}\right) \times \left(\frac{I^{Met}}{I^{TSP}}\right) \times C^{TSP}$$

Where k – dilution of sample in NMR tube

η – number of hydrogen atoms in TSP and metabolite

I – integral values of TSP or metabolite (since the signals for each metabolite was integrated three times, the I is the average value of three measurements

C^{TSP} - molar concentration of TSP in NMR tube.

3.2 Saliva composition

Saliva played role of reference for all experiments. Concentration of saliva's components were important that helped understand results of target samples. Some key components of saliva were first determined using 1D-^{1}H NMR spectrum. Those signals were labelled and shown in figure 2.

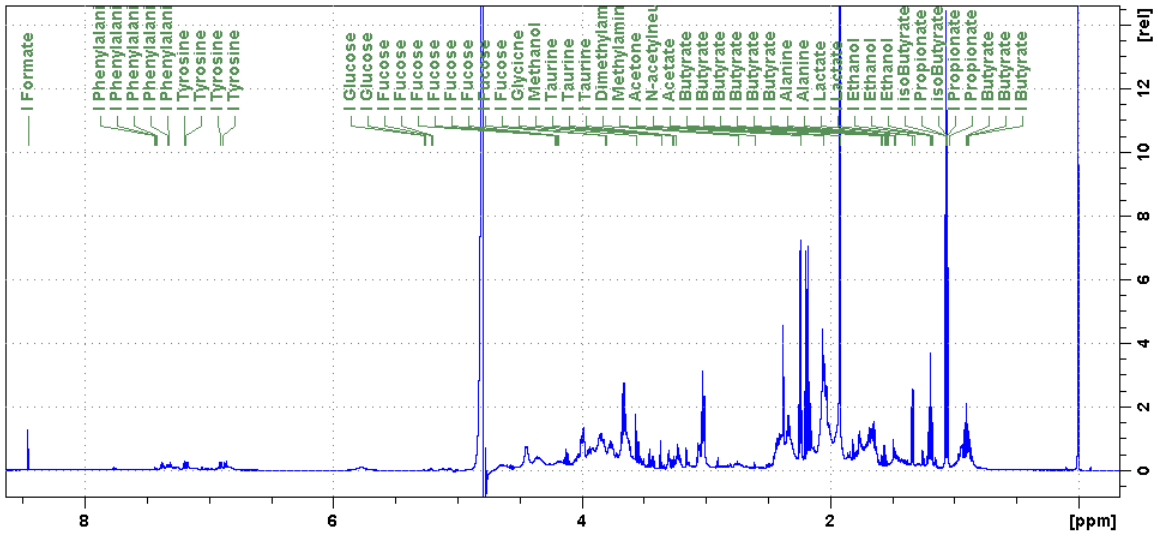

Figure 2: Saliva composition shown by 1D-^{1}H NMR spectrum

There was the highest singlet peak of acetate at 1.92 ppm. Formate was distinguished by a singlet at 8.458. Many signals were too complicated to isolate; therefore, the focus was on some key signals such as formate, lactate, acetate, and alanine. Concentration of all saliva components were fluctuated and relied on various conditions of the body. The concentration of those compounds changed during the day (see section 1.2). This could be taken into account for further discussion of results.

3.3 CLC assignment with stock solutions

3.3.1 Hydrocortisone

Chemical fomula of hydrocortisone is $C_{21}H_{30}O_5$. It contains four rings including three 6-C rings fused with one 5-C ring and one tail. It has three hydroxyl [-OH] groups attached directly to two neighbour rings (C17 and C10) next to the tail with containing the other one at C21 at the end of the tail. There are two ketone [=O] groups which one attaches directly to the left ring at C12 and the other near the end of the compound at C20. Structure of hydrocortisone and its carbon numbered were showed in figure 3.

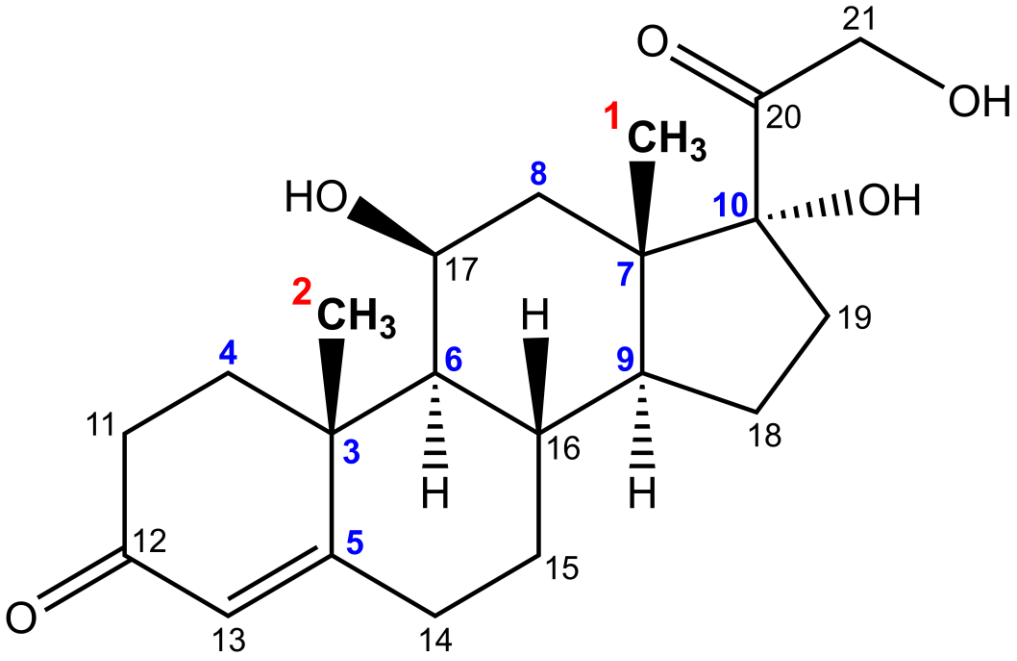

Figure 3: Hydrocortisone structure and its carbon order

A prediction of hydrocortisone obtained was used as tentative reference for study of NMR spectrum of hydrocortisone. The fully tentative reference of chemical shifts was showed in figure 4.

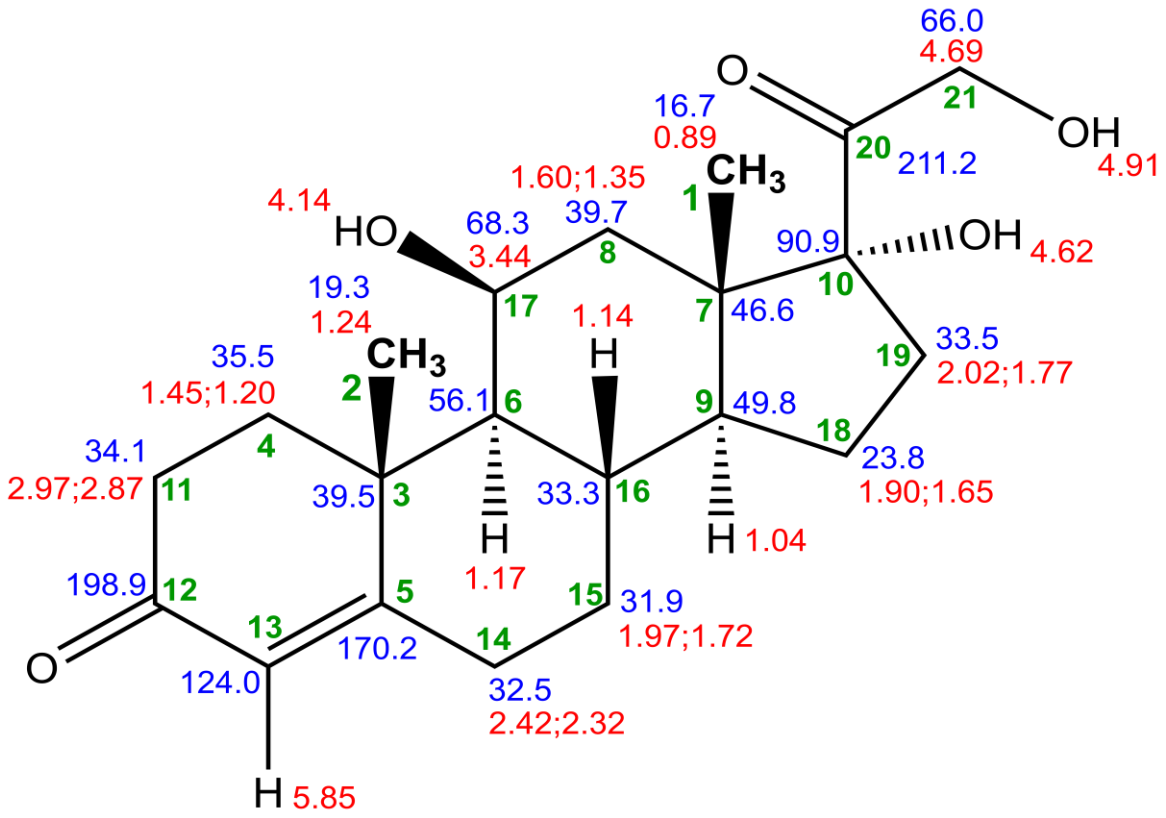

Figure 4: Prediction of chemical shifts for Hydrocortisone. Red presents proton shift and blue presents carbon shift.

Hydrocortisone contained two methyl-CH_3 groups in which gave highest peaks and clearest signals in NMR spectrum. This made CH_3 group the most important and most calibrated signal for analysing other signals of other atoms in the molecule showed in the same spectrum (figure 5).

There were some distinguished regions in the spectrum. The most condensed region was from 0.84 ppm to 2.70 ppm in 1D ^1H dimention and 18-74 ppm for Carbon. This differentiation was caused by the functional groups resulting one region of $CH/CH_2/CH_3/$, one region of OH.

As shown in figure 5, there were two large signals at 0.85 ppm and 1.41 ppm. Compared to prediction of 0.89 ppm for C1 and 1.24 ppm for C2, it was concluded that the signal at 0.85 ppm belonged to C1 and that at

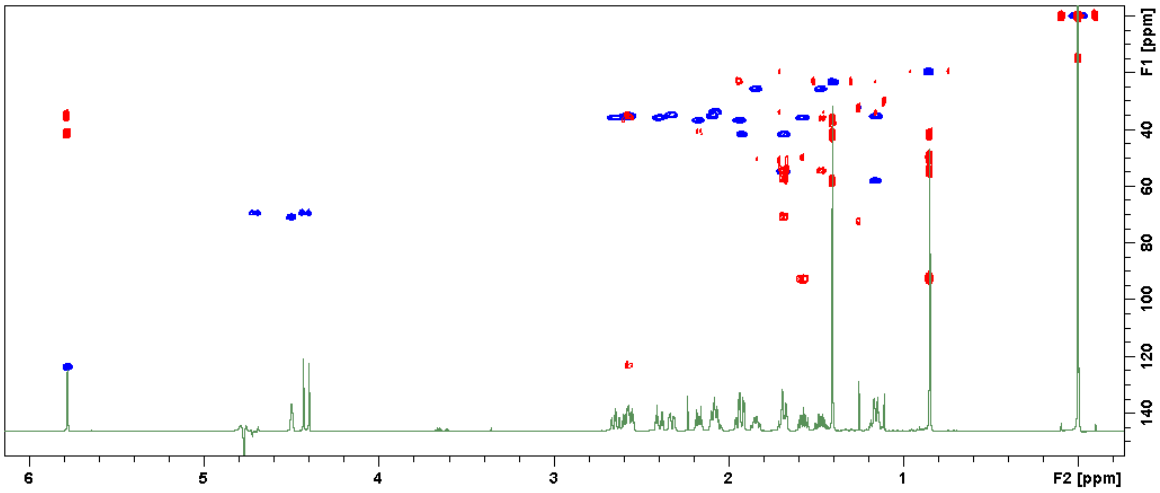

Figure 5: 1D-^{1}H and 2D spectra of Hydrocortisone. Green shows proton shifts in 1D spectrum. Blue shows 2D-HSQC and red shows 2D-HMBC spectra.

1.41 ppm belonged to C2. Based on found C1 and C2, eight carbons were assigned consisting of C3, C4, C5, C6 related to C1 signal; and C7, C8, C9, C10 relavent to C2 signal. There was also a notable signal at 5.78 ppm in 1D spectrum. There were some signals farther around 180 – 220 ppm. Based on confirmation of ten carbon signals that had been confirmed and theoritical chemical shifts, the 2D and 1D NMR spectra (figure 5) of this compound were acquired and the assignment was fully reported in the appendix 5.

3.3.2 Cortisone

Chemical formula of cortisone is $C_{21}H_{28}O_5$ with two hydrogen atoms less than hydrocortisone's formula. Cortisone has very similar structure compared to hydrocortisone with no difference in carbon backbone. Therefore, its carbon number was exact to that of hydrocortisone.

It has four rings structure fused with each other including three 6-C rings, one 5-C ring and one tail. It has two hydroxyl [-OH] groups attached to C10 and C21 at the tail of the compound. There are three ketone [=O]

groups which link to C12, C17 and C20. Its formula differentiates itself with hydrocortisone formula at C17 in which C17 in cortisone has a ketone [=O] group instead of hydroxyl [-OH] group in hydrocortisone. Cortisone structure and its carbon numbers were shown in figure 6.

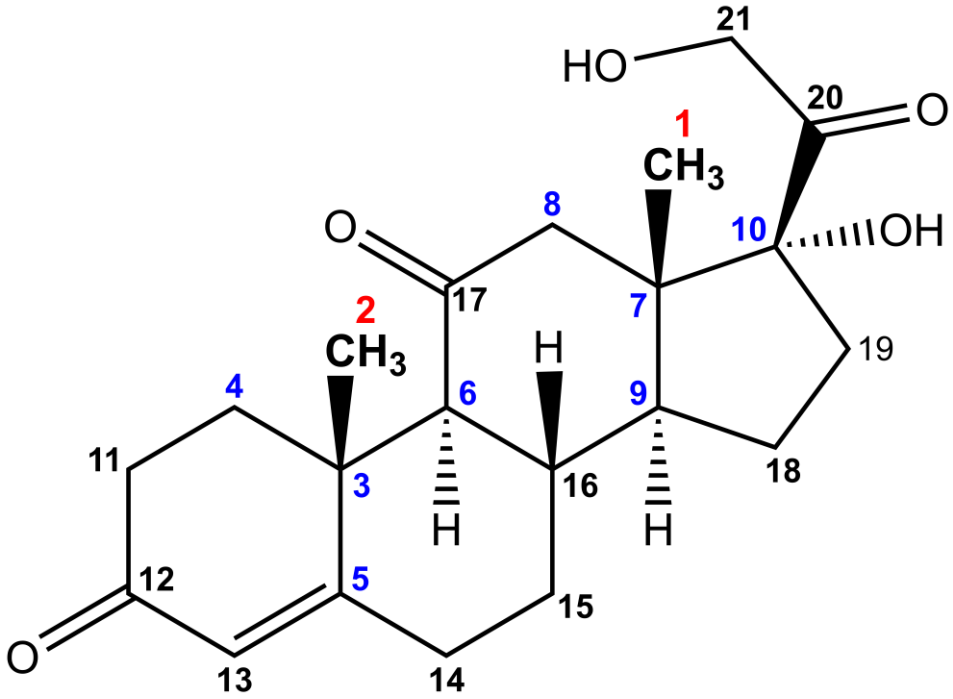

Figure 6: Cortisone struture and its carbon order

Because of the ketone group at C17 that had been mentioned above, the chemical shifts of atoms in the centre of the compound were expected to be shifted while atoms located far to the edge of the compound remained unchanged in prediction of chemical shifts of cortisone compared to that of hydrocortisone. Cortisone with this prediction of chemical shifts was shown in figure 7.

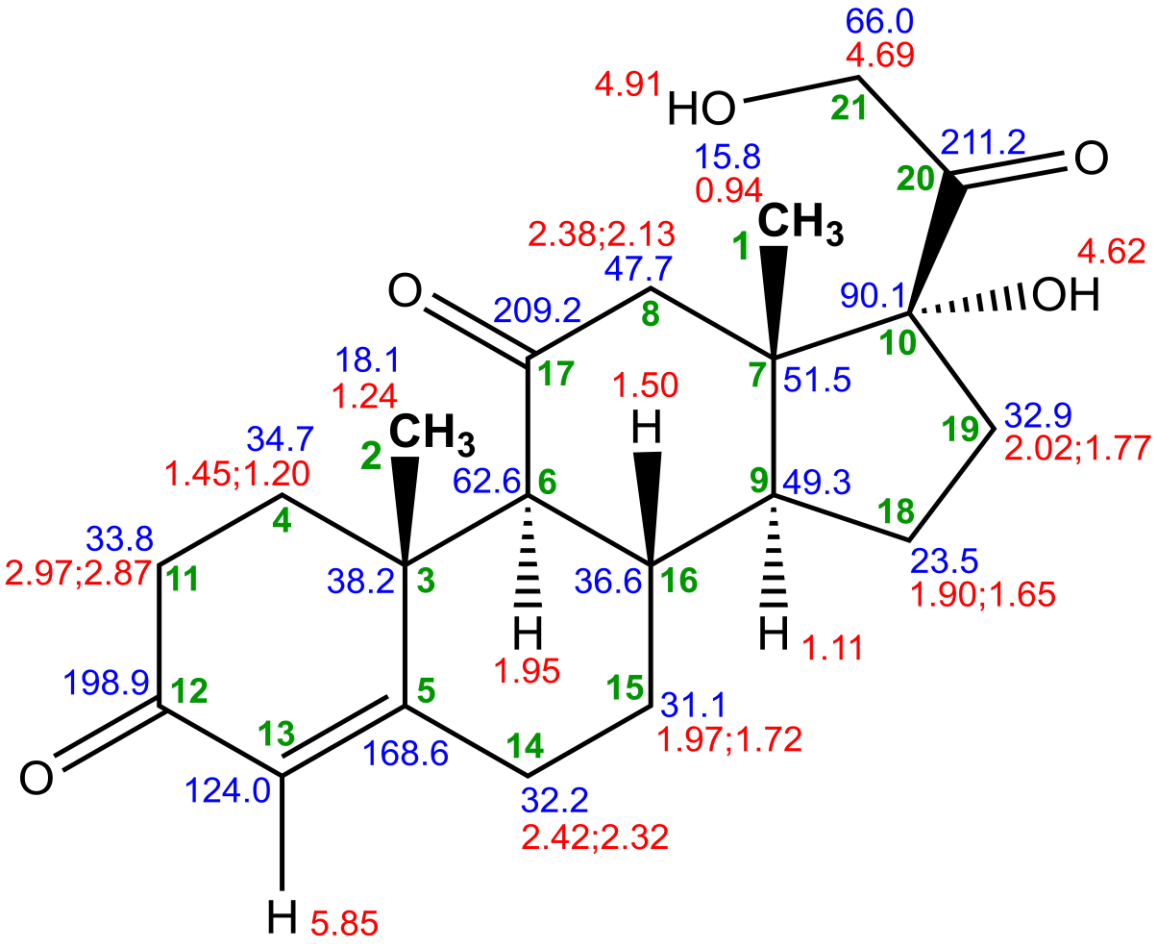

Figure 7: Tentative carbon (blue) and proton (red) shifts of Cortisone

Signals of cortisone were most condensed in the region 1.20–2.95 ppm and 16.2-94 ppm in 1D-^{1}H NMR spectra and 2D HSQC, HMBC respectively. These were mostly signals from carbon bonding with hydrogen in the centre of compound. There were also some signals near 200 ppm in 2D HMBC spectra and signals far to 4.35 ppm and 5.83 ppm in 1D ^{1}H spectra caused by carbons that was far from the centre of the compound or carbons that had double bonds. 1D ^{1}H NMR spectra; 2D TOCSY, HSQC and HMBC spectra of cortisone was shown in figure 8.

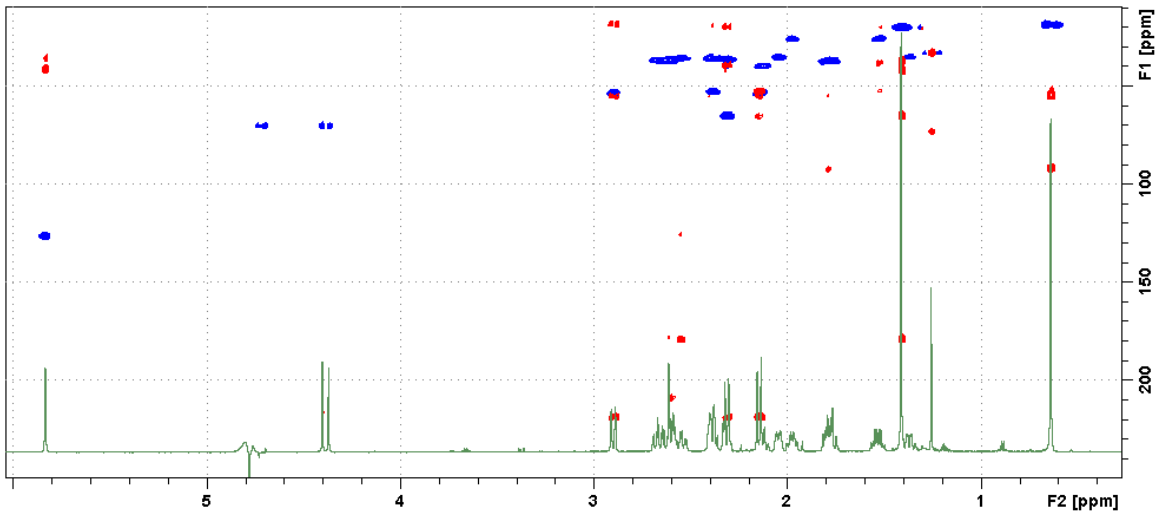

Figure 8: 2D HSQC (blue) and HMBC (red) and 1D (green) NMR spectra of Cortisone

The spectra were assigned, and the recorded positions of chemical shifts were presented in appendix 6.

3.3.3 Aldosterone

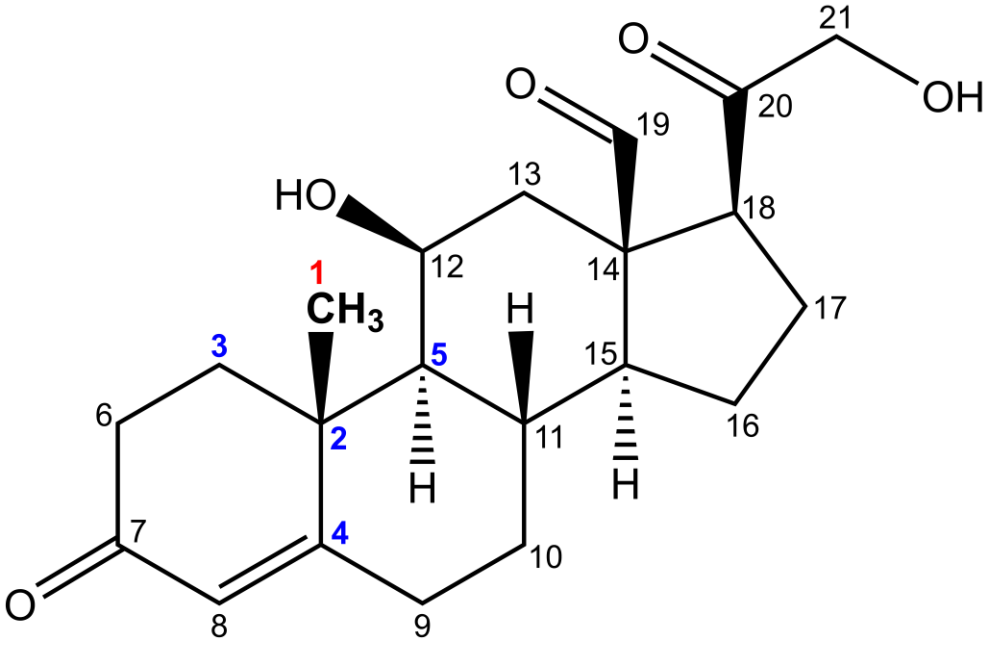

Figure 9: Structure of Aldosterone with carbon number

Aldosterone has formula $C_{21}H_{28}O_5$ which alike formula of cortisone. But there is only one methyl [-CH_3] in chemical structure of aldosterone leading to re-arrangement in carbon order compared to hydrocortisone and cortisone. Structure and carbon numbers were shown in figure 9.

Besides, water solubility of aldosterone is very low, at 0.0512 mg/mL according to Drugbank resource. The spectra of aldosterone had proofed that it was insoluble in water but well soluble in acetone as there was not many signals detected in spectra of aldosterone in D_2O. This assignment was conducted with aldosterone in deuterated acetone.

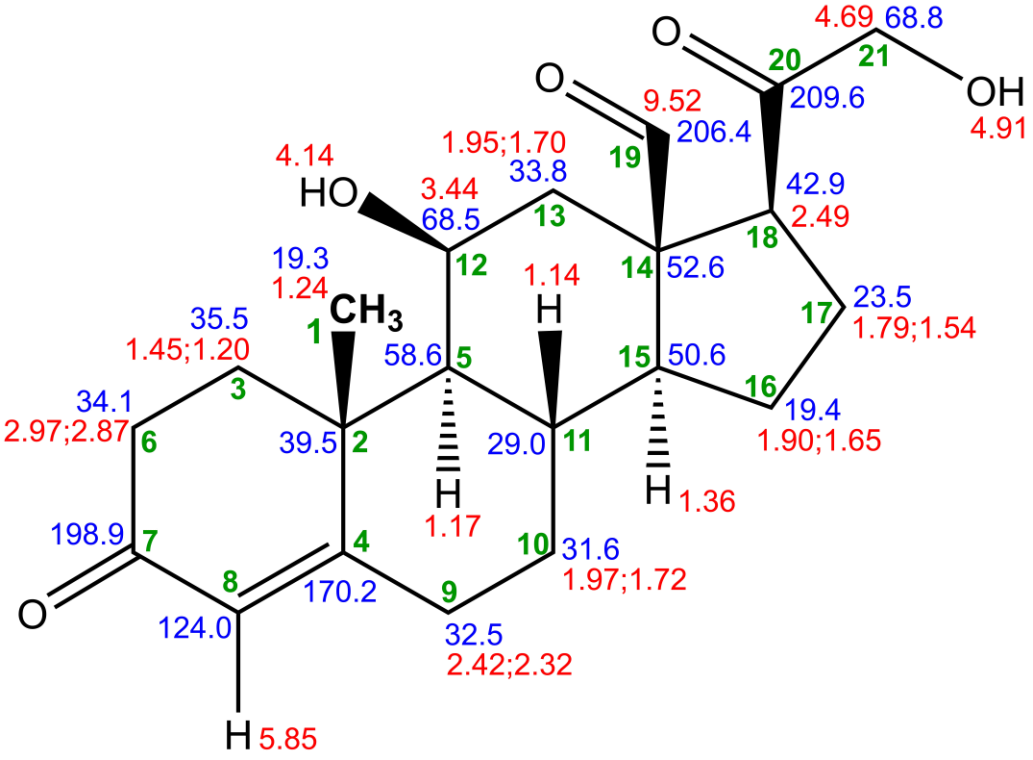

Figure 10: Structure of Aldosterone with theoretical chemical shifts of proton (red) and carbon (blue)

C1 and C19 in aldosterone was at position of C2 and C1 in other CLCs used in this project. C19 had double bonds to an oxygen forming a ketone [=O] group, which was one of the three ketone groups in aldosterone together with C7 and C20. Two hydroxyl [-OH] groups were attached to the compound via C12 and C21. These led to the divergence

in predicted chemical shifts of atoms near C19 in aldosterone compared to those in hydrocortisone. The other atoms that were far from C19 remained unchanged in tentative chemical shifts. The tentative chemical shifts were shown in figure 10.

Figure 11 below would show 1D ^{1}H, 2D HSQC, HMBC, TOSCY and DEPT spectra of aldosterone. Signals separated themselves into two parts in the spectra with lots of complicated signals to compare with other CLCs spectra in this thesis. The presence of acetone was expected to be reason of this increase in number of signals. There were two high signals at 1.29 ppm that belonged to CH_3 group of C1. The greatest signal at 2.053 ppm was one of acetone signals. Beside the condensed region, other signals were less difficult to isolate. A signal at 5.80 ppm was notable as signal from proton of C8.

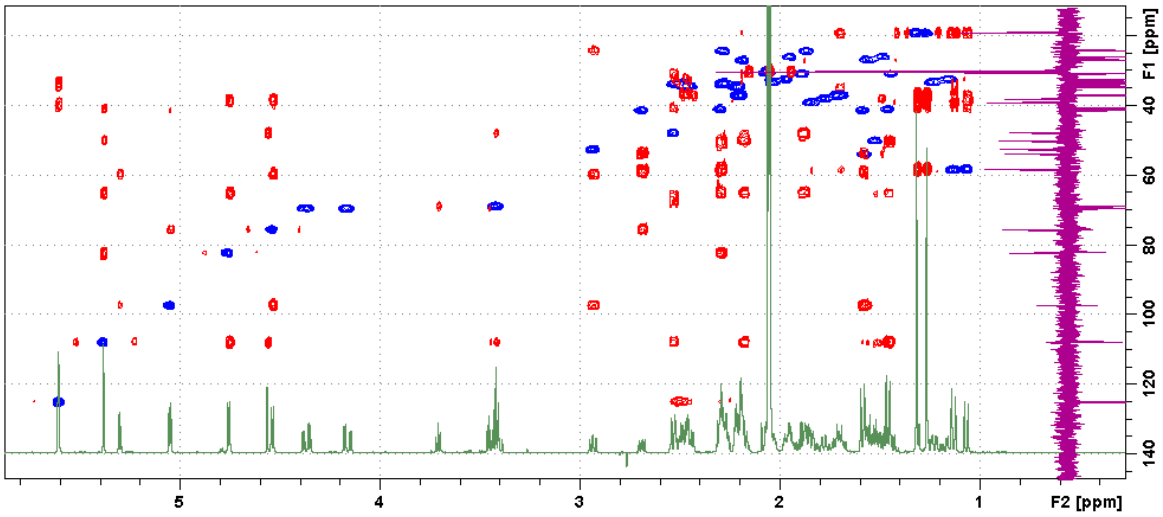

Figure 11: 2D HSQC (blue), HMBC (red), DEPT (violet) and 1D (green) NMR spectra of Aldosterone in deuterated acetone

There were some difficulties to isolate and to assign all chemical shift signals of aldosterone due to the complicated and interfered signals between acetone and aldosterone in the same sample mixture. All suggestive detected signals were shown in appendix 7.

3.3.4 Corticosterone

Corticosterone has the formula $C_{21}H_{30}O_4$. It had similar carbon order as hydrocortisone and cortisone do due to two methyl [-CH_3] groups and similar carbon backbone. What makes corticosterone as variance of the two CLCs is that its C10 do not link to hydroxyl [-OH] group but a direct bond to hydrogen instead. This explains why corticosterone has one oxygen less than other CLCs formulas. This difference also leads to the variation in its chemical shifts compared to other CLCs. Figure 12 below showed the information of structure, carbon numbering, and theoretical proton and carbon chemical shifts.

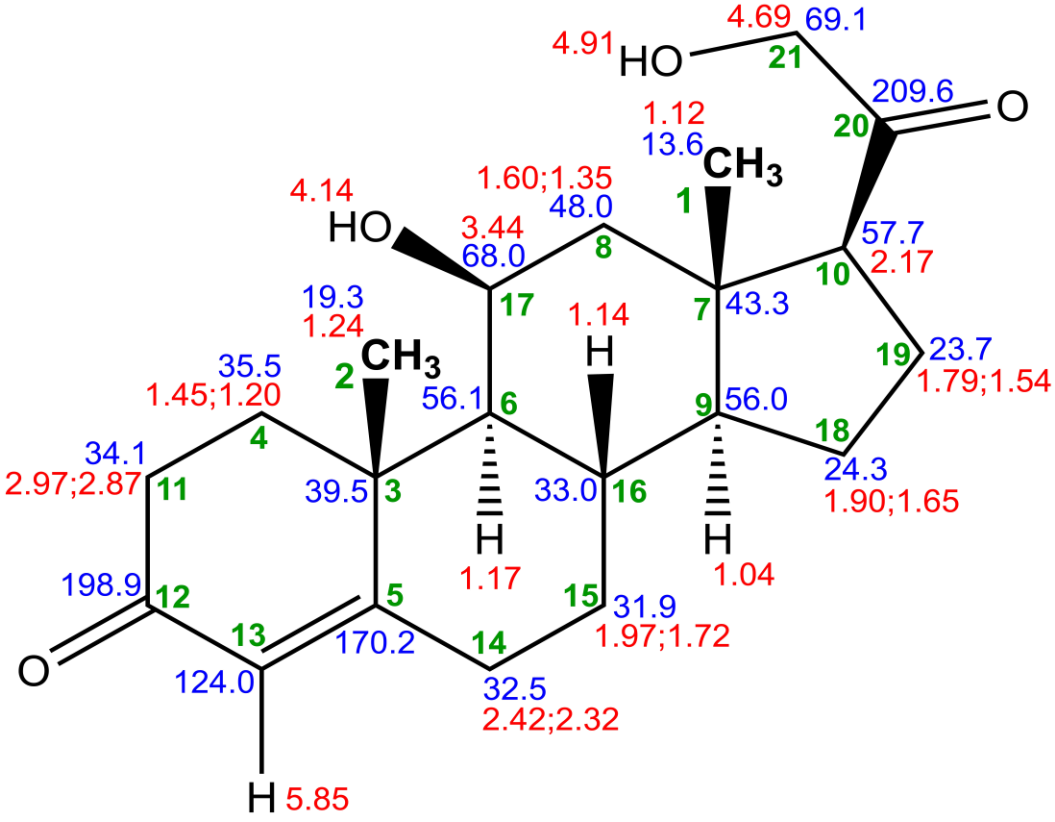

Figure 12: Structure of Corticosterone with carbon number (green), predicted chemical shifts of proton (red) and carbon (blue)

Unlike aldosterone spectra with numerous signals, corticosterone spectra had so few signals that resulted in lack of information to assign which signal belonging to which carbon. However, some remarkable

signals could be isolated. Three of those signals were: C1 - 0.83;18.16 ppm, C2 - 1.40;23.28 ppm, and C13 - 5.78 ppm. The report of all assigned chemical shifts were shown in appendix 8.

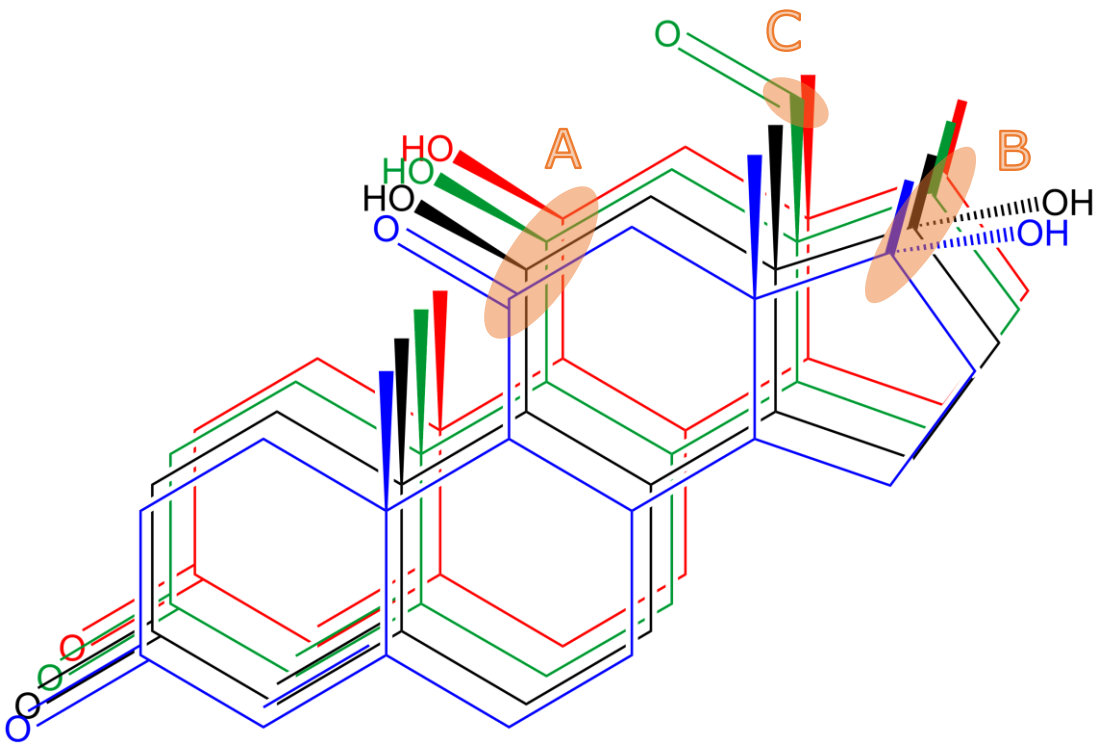

Figure 13: Regions which make CLCs diversed from each other by linking to different functional groups
The tails of CLCs are not shown in the figure. CLCs are presented in order Cortisone (blue), Hydrocortisone (black), Aldosterone (green), and Corticosterone (red).

A short notice for this 3.3 section, the similarity in CLCs structure is that they all have four rings structure (three 6-carbons rings and one 5-carbons ring) and one tail attached to 5-carbons ring. Shown in figure 13, it could be seen that four CLCs are differentiated from each other by chemical groups linked to two carbons in region A and region B, which belong to the two rings structure in the centre and near the tail of each molecule. In case of aldosterone, there is third divergence with having

only one methyl group as the other methyl group in other three CLCs is a ketone group in aldosterone making the variance in carbon order (region C).

Through this CLCs assignment, it is obvious that the signal around 5.8 ppm plays important role in assigning CLCs in saliva. As shown in all the structure figures of CLCs and the figure 13 above, they are slightly diverse in carbon-linked groups in some the middle positions of the compound, whether in the centre or at the carbon where it connects to the tail end of the compound. Many carbons near those positions have their one or two protons. These protons create different angles to each other. As the bonds in carbon change, the angles between their protons change. It leads to the signal shifting of these protons in NMR spectra. Some carbons containing no proton, but it directly links to methyl groups or links to carbons whose have theirs change in proton signals, are also impacted resulted by theirs shifts of signal. The proton signal at 5.8 ppm somehow stands out from that. It has a bond to a carbon whose it is not significantly affected by the derivative variances. There are two explanations for this:

- The carbon has double bonds to the neighbour carbon.
- The carbon has the other neighbour carbon having a ketone group.

The structure of this is shown in figure 14. As seen, both carbon's neighbours have their own double bonds and have no proton linked to them. Therefore, no proton angle that could make change in signal are formed in that area. This structure makes the signal of proton at 5.8 ppm significantly stable.

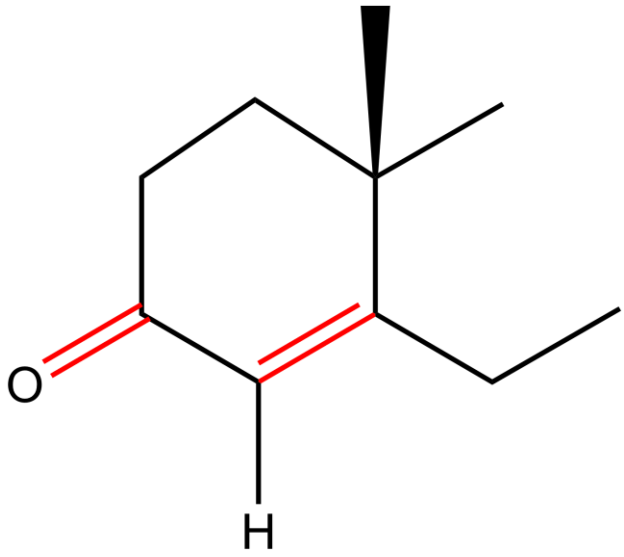

Figure 14: Highlighted structure that causes the stable signal at 5.8 ppm

3.4 Human saliva titration with CLC standard

3.4.1 Hydrocortisone titration

There were final concentrations of 0.00994 mM in 20 µL hydrocortisone NMR tube and that of 0.01988 mM in 40 µL ones. The 1D-^{1}H NMR spectra of hydrocortisone titration was shown in the figure 15.

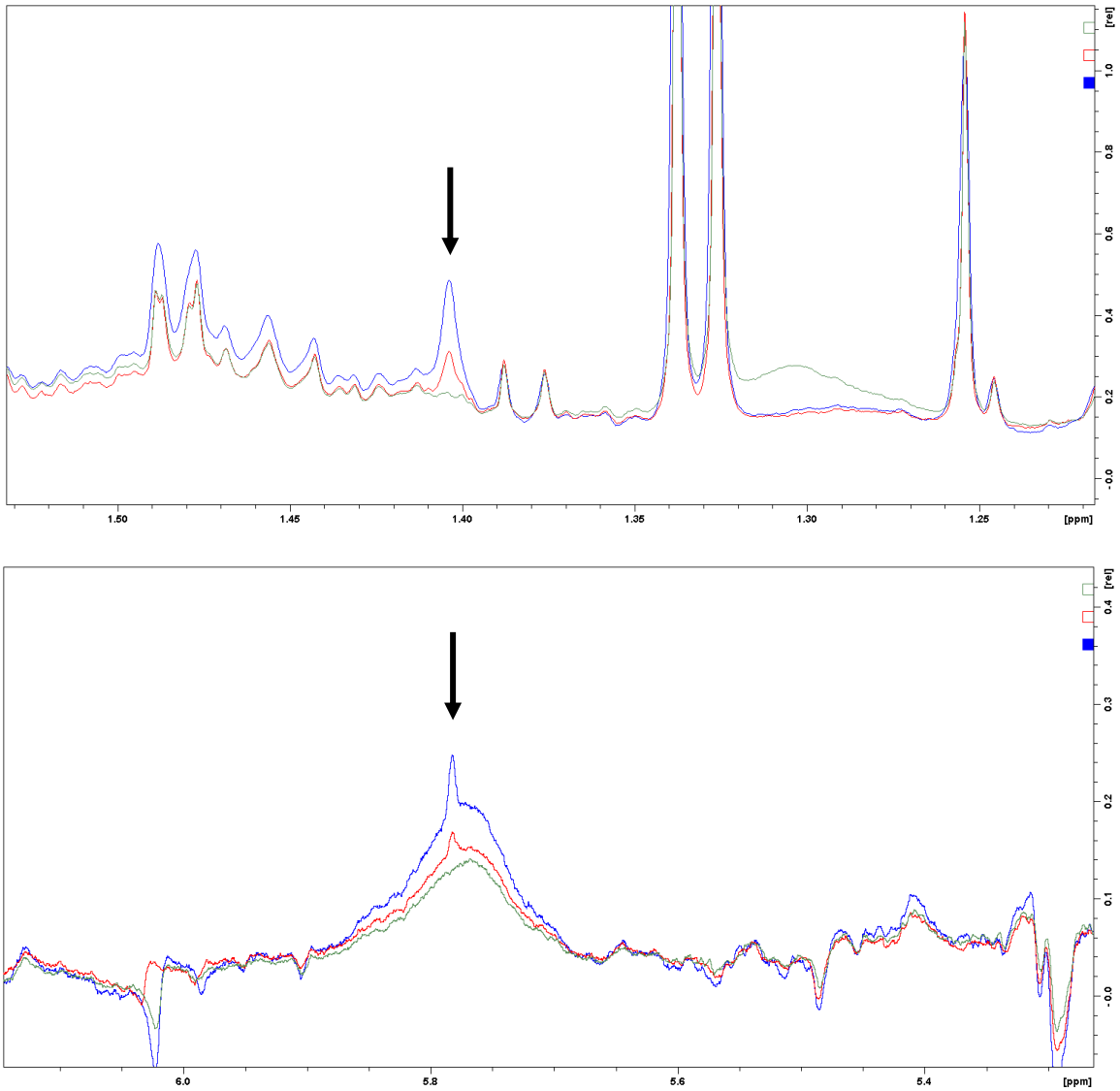

Figure 15: Two titration regions found in Hydrocortisone.
Green is reference saliva without added stock, red and blue are 20 µL and 40 µL stock added samples.

Two titration areas had been indicated from the spectrum: 1.405 ppm and 5.782 ppm. In the previous assignment, one of the methyl groups of hydrocortisone (C2) had been assigned at 1.41 ppm while 5.78 ppm has been similar to position of hydrogen at C13. The 1.41 ppm and 5.78 ppm regions were integrated, and results were shown in figure 16. The values of integral were shown in appendix 9.

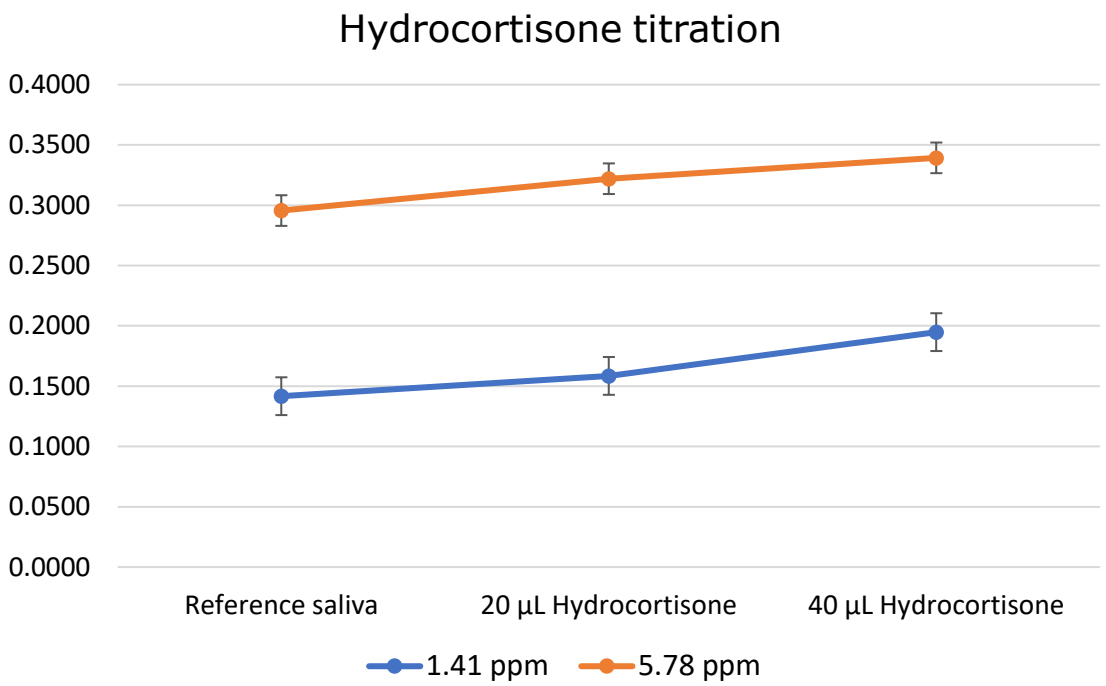

Figure 16: Results of two titration regions found in Hydrocortisone

From the results, there was not well related between reference, 20 µL hydrocortisone and 40 µL hydrocortisone added samples as the increasing lines were different. Region of 1.41 ppm had lower increase from standard to 20 µL hydrocortisone added sample and higher rise from 20 µL hydrocortisone added sample to 40 µL hydrocortisone added one. Whereas, the trendline of 5.78 ppm was steadily increased. The integral region at 5.78 ppm had around 0.15 more value than the integral at 1.41 ppm. The chart showed that the lines of both titration regions were mostly parallel.

3.4.2 Cortisone titration

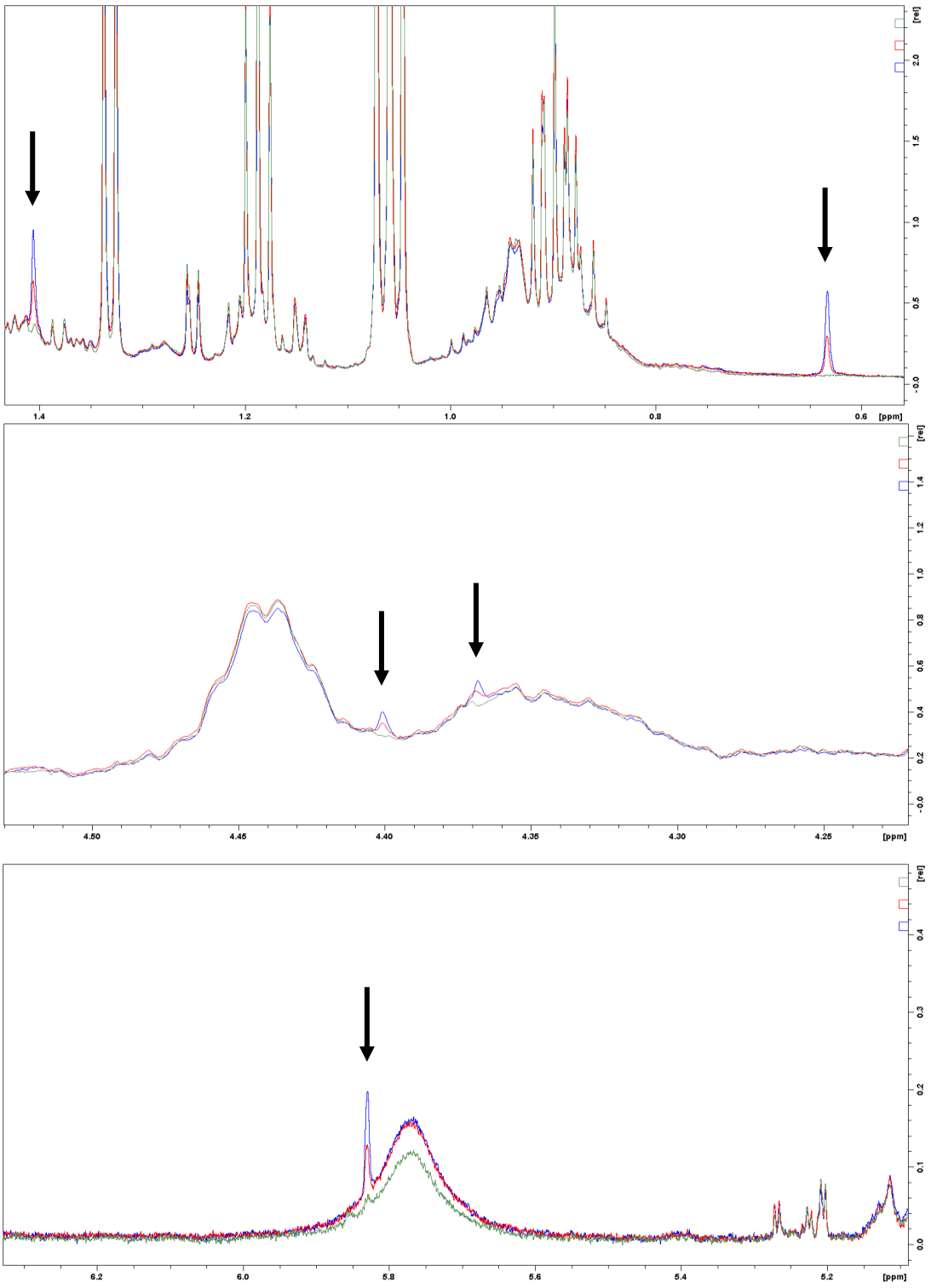

Figure 17: Titration regions found in Corticosone.
Green is reference saliva without added stock, red and blue are 20 µL and 40 µL stock added samples.

Sample of 20 µL cortisone added and 40 µL cortisone added had the final concentration in the tube 0.009995 mM and 0.01999 mM correspondingly. The 1D-^{1}H NMR spectra of cortisone titration was shown in the figure 17.

Four titration regions were found in cortisone samples and their integrals and results were shown by diagram in figure 18. More detailed values of integral were shown in appendix 9.

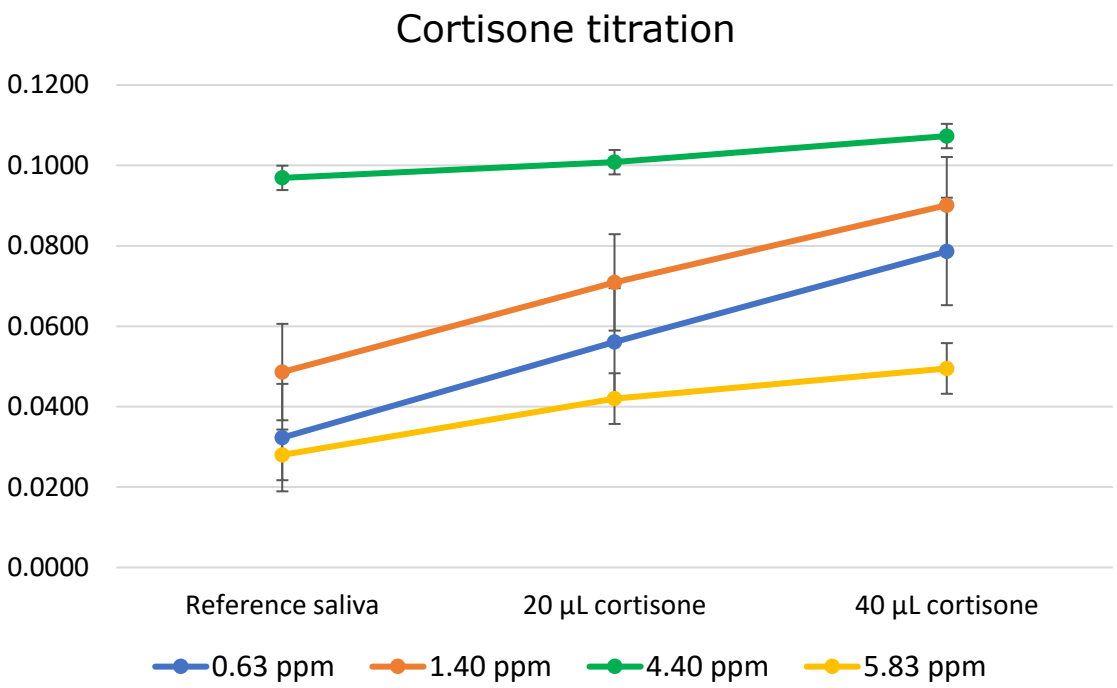

Figure 18: Cortisone titration result

Among four CLCs, cortisone was the one having most titration regions that were easy to detect. Four titration regions were 4.40 ppm, 1.40 ppm, 0.63 ppm, and 5.83 ppm sorted by the descending of their values of integrals. Trendlines of 1.40 ppm and 0.63 ppm were mostly parallel with more increased in values. The other two lines were less likely, but still, parallel with small changes in values.

It was believed that the titrations at 0.63 ppm and 1.40 ppm corresponding to two methyl groups of cortisone. Hence, it was explainable that theirs values trend was parallel. Titration at 5.83 ppm was supposed to be C13, the special region that had been discussed in 3.2 section. In the three-dimensional structure of cortisone, the tail was nearer to the methyl group in which only C21 had protons located in the tail. This was reason for titration region at 4.40 ppm that belonged to C21 signals.

3.4.3 Aldosterone titration

The final concentrations of aldosterone in NMR tube were 0.1998 mM for 20 µL stock added and 0.3996 mM for 40 µL stock added. The calculation was based on 4 mg of aldosterone diluted in 1 mL of deuterated acetone. Due to the difficulty of dissolving aldosterone in D_2O, the spectra of aldosterone in D_2O was not used for studying its structure and chemical shifts but it was used for this titration assignment. The 1D-^{1}H NMR spectra of aldosterone titration was shown in the figure 19.

There are three regions in the aldosterone spectra where the titration was clear detected: 1.19 ppm, 1.25 ppm, and 5.80 ppm (figure 19). Even though the signal of aldosterone in water was not good, but it also could tell the mostly identical signals with titration ones. Signal integrals at 1.254 ppm were signal of methyl group of C1 in aldosterone structure while 5.803 ppm titration area related to hydrogen of C8 which was obviously distinguishing itself to the other regions.

The integrals of reference saliva, aldosterone 20 µL and 40 µL samples at all titrated region were show in figure 20 and appendix 9.

Titration region at 5.80 ppm rose gradually from blank sample to 20 µL aldosterone added sample followed by small increase for 40 µL aldosterone added sample (see figure 20). Titration at 1.25 ppm and

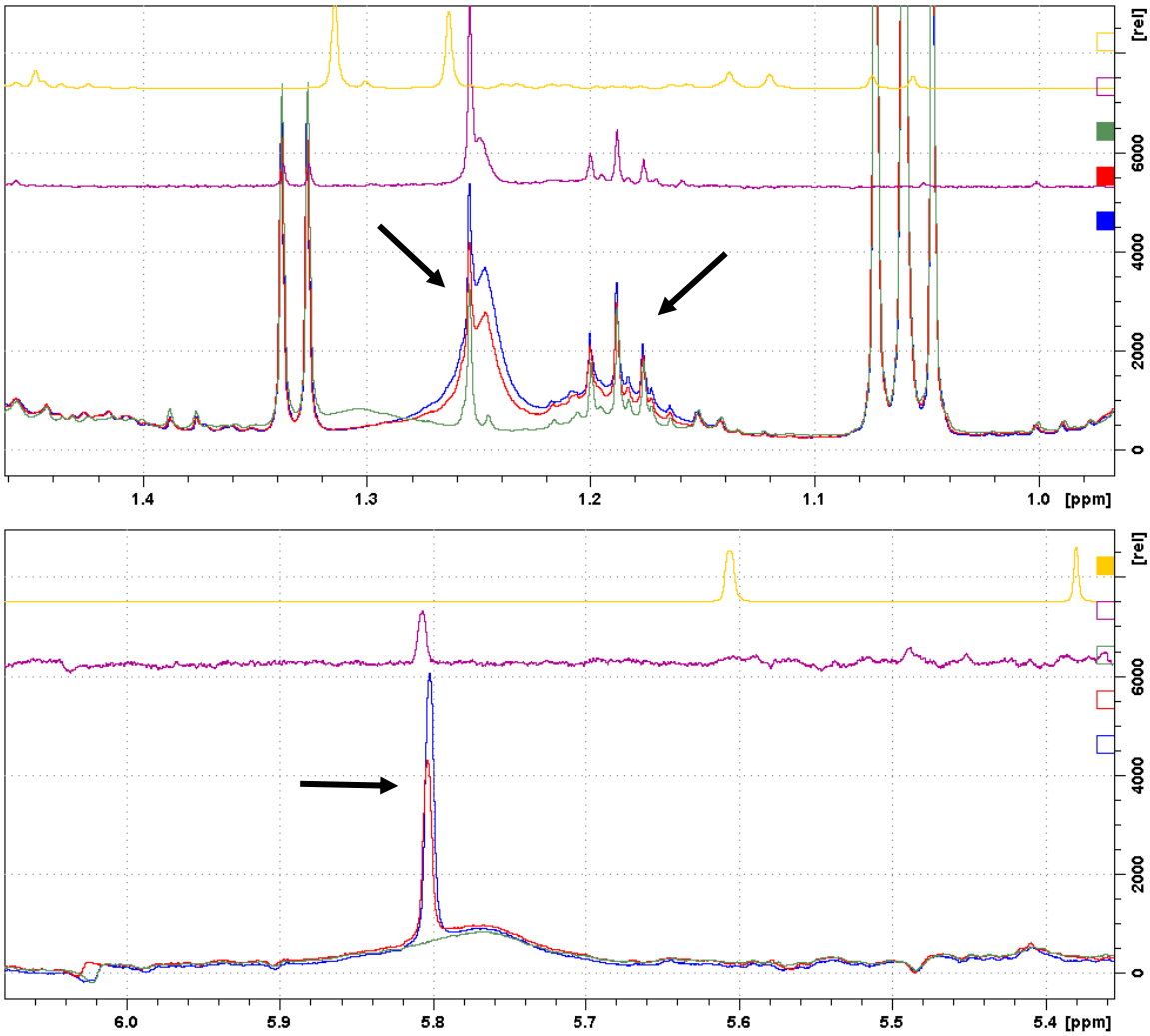

Figure 19: Titration regions of Aldosterone.
On the top, yellow indicates Aldosterone in deuterated acetone, violet is Aldosterone diluted in water. Green is reference saliva, red and blue are 20 µL and 40 µL stock added samples respectively.

1.19 ppm had very similar values with their integrals for added 20 µL and 40 µL aldosterone samples. However, there was a big difference in integrals of both 1.19 ppm and 1.25 ppm in blank sample. With error calculated by Excel (Microsoft office, 2016), error bars of both titration regions were overlapped which could be the reason for this difference in their integrals. It was suggested that titration at 1.25 ppm was due to methyl group of C1 and titration at 5.80 ppm was from C8.

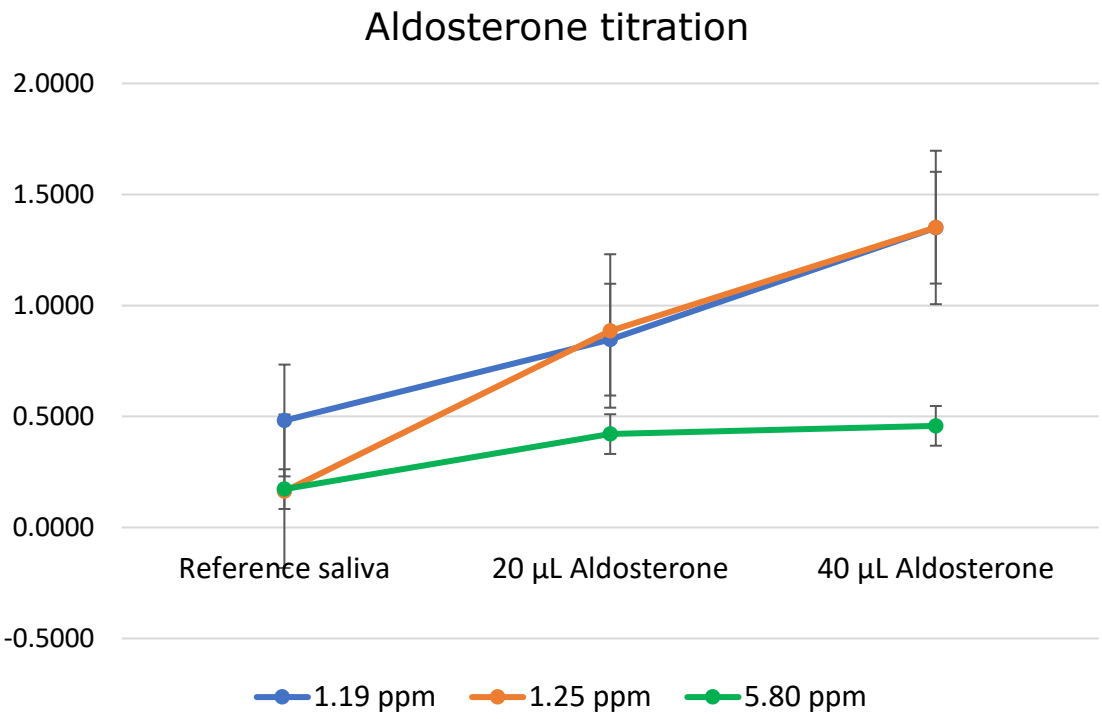

Figure 20: Aldosterone titration result

3.4.4 Corticosterone titration

Similarly, concentrations of corticosterone were calculated. Sample with 20 µL stock added had concentration of 0.010398 mM and concentration of sample with 40 µL stock added was 0.020796 mM.

Three titration regions had been isolated, shown in figure 21. Titration regions of corticosterone were detected and integrated. Results were shown in figure 22 below and appendix 9.

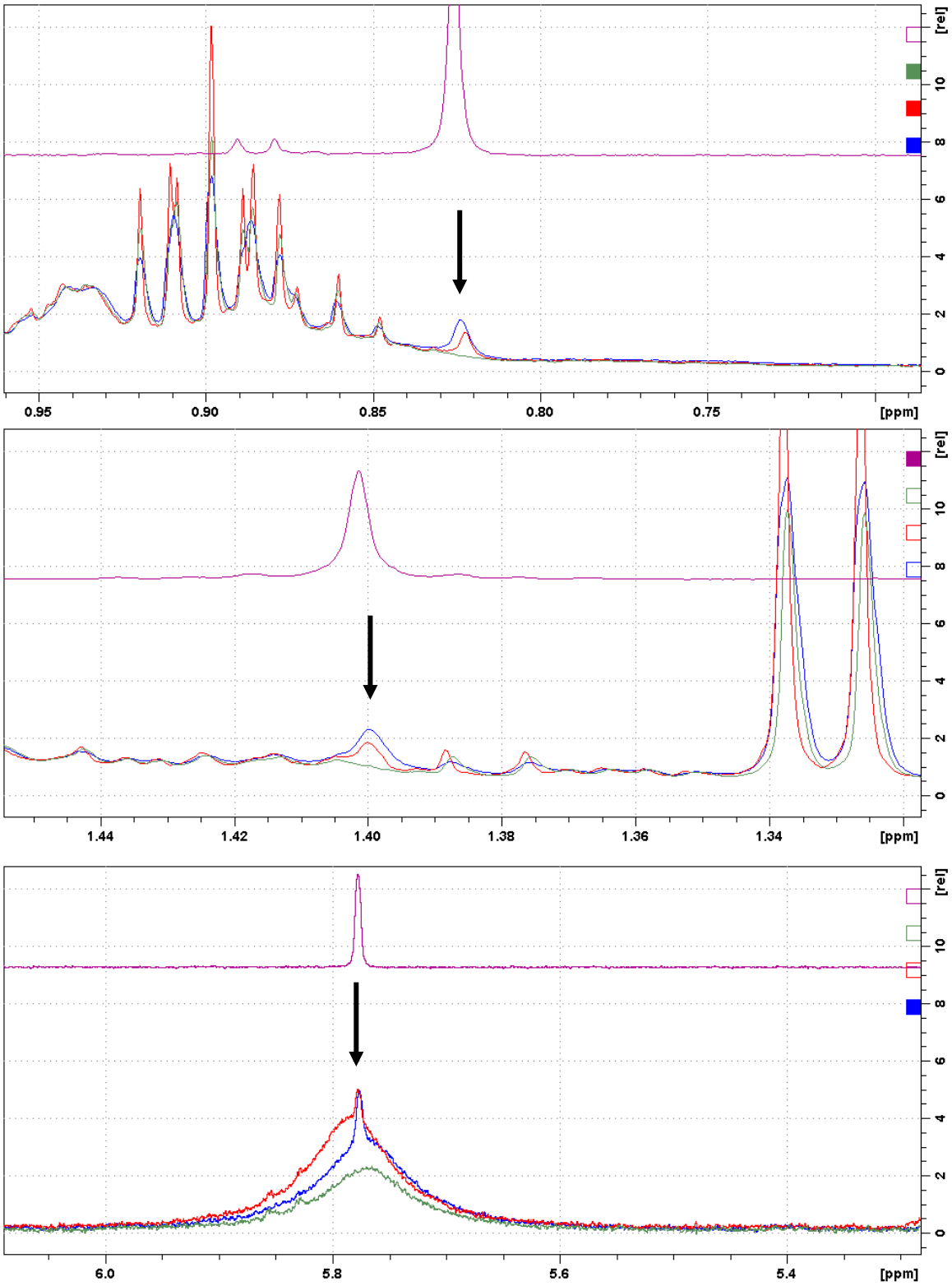

Figure 21: Corticosterone titration regions.
Reference saliva is shown in green, red and blue are 20 µL and 40 µL stock added samples. Corticosterone in water is shown in violet.

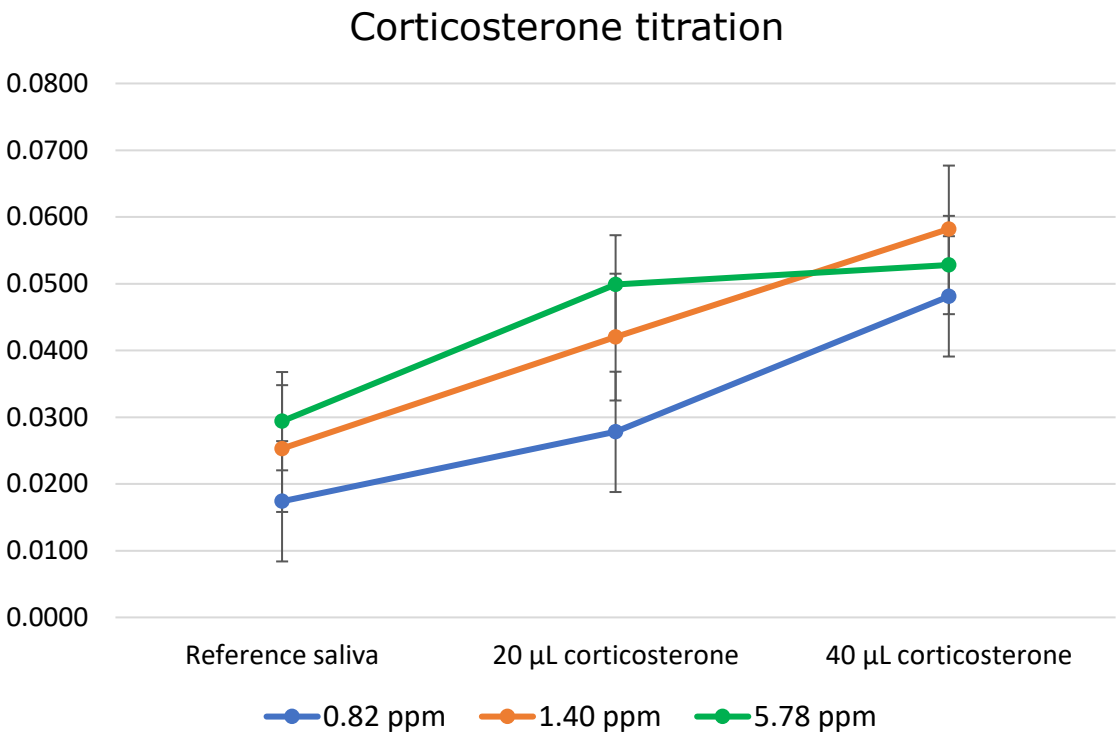

Figure 22: Results of Corticosterone titration

Three titrated regions were 0.82 ppm, 1.40 ppm and 5.78 ppm. In general, integral of 0.82 ppm region had smallest value while the highest value belonged to 5.78 ppm integral. The trendlines of 0.82 ppm and 1.40 ppm were nearly similar in shape with steadily increased. Also, integrals at 1.40 ppm and 0.82 ppm regions had their values expanded twice compared between reference and 40 µL added samples. The titration at 5.78 ppm increased greater between blank sample and sample with 20 µL stock and increased smaller between 20 µL with 40 µL stock containing samples. It was notable that 0.82 ppm and 1.40 ppm titration points came from two methyl groups of the compound whereas the other one at 5.78 ppm was from C13.

A brief summary for this section, CLCs titration assignment has indicated that titration peaks are influenced by methyl groups and the remarkable

region (see 3.2 section). Methyl groups of all CLCs are titration detected, except one methyl group of hydrocortisone (C1 at 0.85 ppm). For CLCs with two methyl groups, the integrals of C2 (around 1.4 ppm) have greater values than the other. The methyl group leading to titration in hydrocortisone is also its C2 (at 1.41 ppm) while the only methyl group of aldosterone also leads to a titration point there (at 1.25 ppm). Hence, it is believed that signal of methyl protons from carbon at that position, centre of the compounds, is more powerful than those from the other methyl carbon.

The clearest observation is the titration at around 5.8 ppm resulted from the special region that has been described earlier. In general, the titration integrals at 5.8 ppm tend to show more variance between 20 µL stock added samples and reference sample than that between 20 µL and 40 µL stock added samples. Whereas, values of methyl groups tend to grow gradually. In addition, the signal at 5.8 ppm region seems to interact with a big molecule leading to a broad signal at the base. Thus, to have integration of this region, the whole board complex is integrated then it is splitted to smaller integrals.

3.5 Study of student saliva

Among all signals in 1D spectra of CLCs, those at titration regions were hypothesised to be locatable regions for CLCs in saliva. To test the hypothesis, saliva from two groups of student were analysed. All saliva spectra were processed as described in session 2.12.2 using titration regions obtained from session 3.4 including 1.19 ppm, 1.25 ppm, 1.41 ppm and 5.78 ppm.

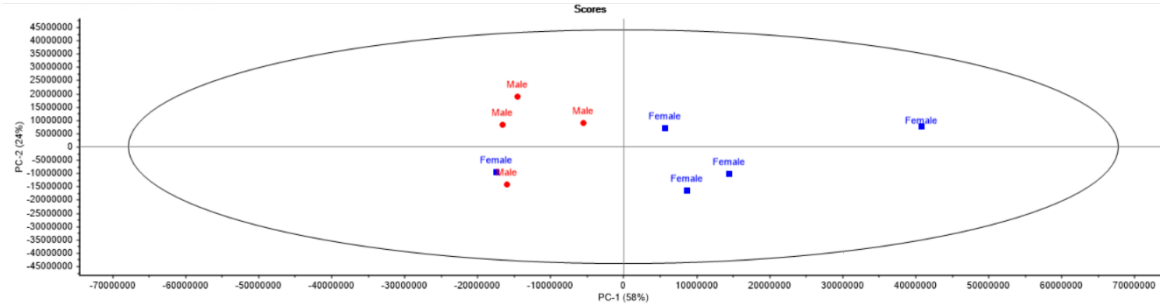

Figure 23: PCA scores of 2016 group of student with gender female (blue) and male (red)

The group of saliva collected in 2016 was analysed and its PCA plot was shown in figure 23. Saliva was sub-grouped into "Female", shown in blue and "Male", shown in red. It could be seen the scores gathered into two crowds respective to gender. Female group was completely on the right while male group was on the left.

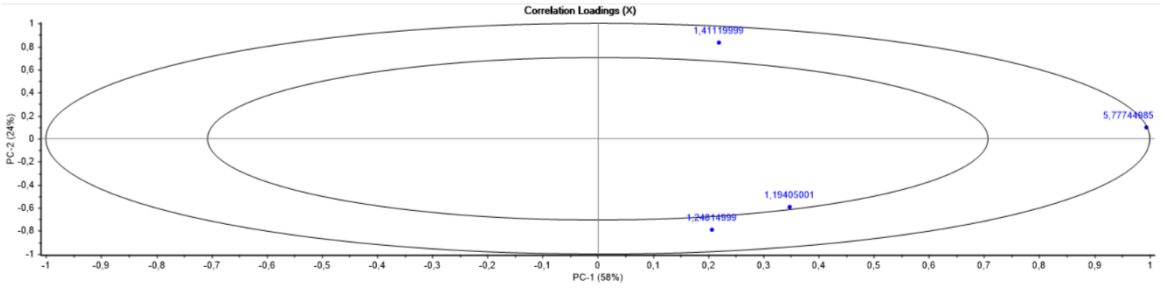

Figure 24: Correlation loadings of 2016 group of student

The figure 24 shown Correlation Loadings plot of 2016 student. The variables outer of the smaller ring were important for specify which

corresponding regions could be recognisable region for CLCs. Here, the signals at all the four regions were marked as significant signals to detect CLCs in saliva. All signals appeared on the right of the correlation loadings that meant female group had more of those signals than male group. 1.19 ppm was slight inside the smallest circle which meant it was not significant. In opposite, 5.78 ppm was highly significant indicator.

However, there was a female point laid together with male ones. which was collected from the only vegetarian in the student group. She also took antihistamine and Symbicort Turbuhaler. Symbicort Turbuhaler is a medicine used for asthma and chronic obstructive pulmonary disease (COPD). Two medicinal ingredients of this are budesonide and formoterol fumarate dihydrate. Formoterol is to bronchodilator while budesonide belongs to glucocorticoid (glucocorticosteroid), one of the two main sub-classes of corticosteroid (AstraZeneca, 2016, Kuna and Kuprys, 2002). This was supposed to be reason of the distinctive point between the girl and the rest of female group.

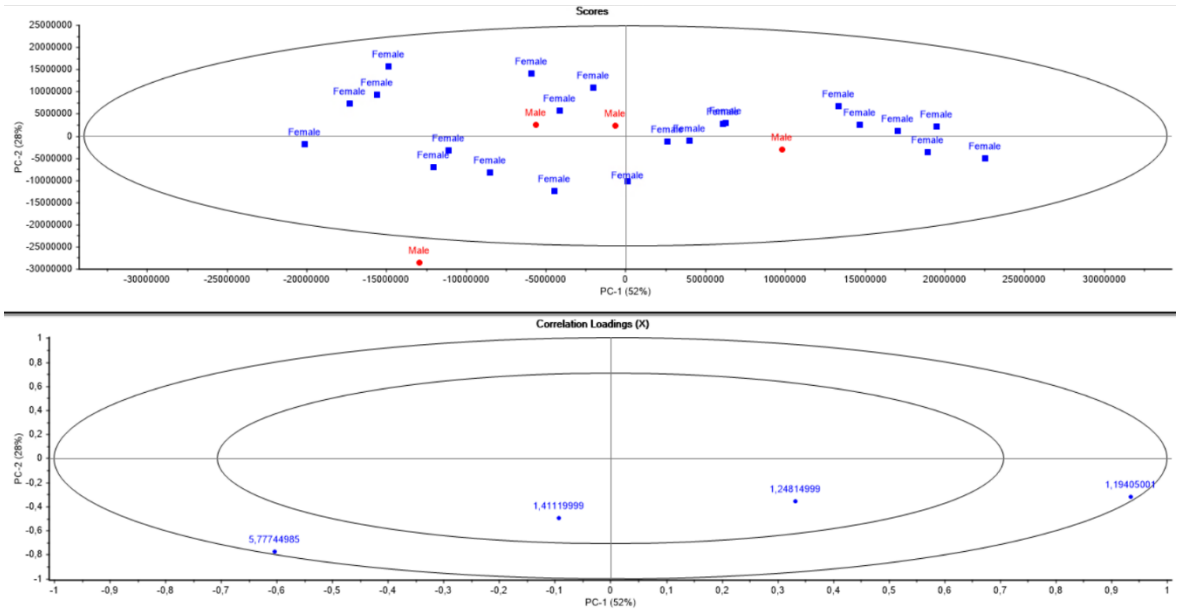

Figure 25: PCA plot of 2018 group of student

Figure 25 indicated PCA plot of student's saliva collected in 2018. As shown, female group (blue points) was spreaded out the cloud which

were mixed with red points of male group. Here, it was not possible to group the sample according to their genders which was unlike the result from 2016 group. The correlation loadings were separated in to two part, two values on the left and two on the right which meant insufficient to have any conclusion.

The student saliva of 2018 group was collected after lunch while that of 2016 group was collected in the morning before eating. Hence, the food that has been eaten by the student group in 2018 is believed to cause this difference in grouping the points correlated to student's gender.

Because none of titration areas from session 3.4 could be detected in saliva of both student groups, the hypothesis in the beginning of this session is failed. However, reflecting back into session 3.4, almost titration regions of reference samples do not have any signal as well. The titration signals are seen in stock added samples rather than the non-added samples. One special thing is the broad signal at 5.8 ppm. This broad signal might come from overlapping or covering signal of

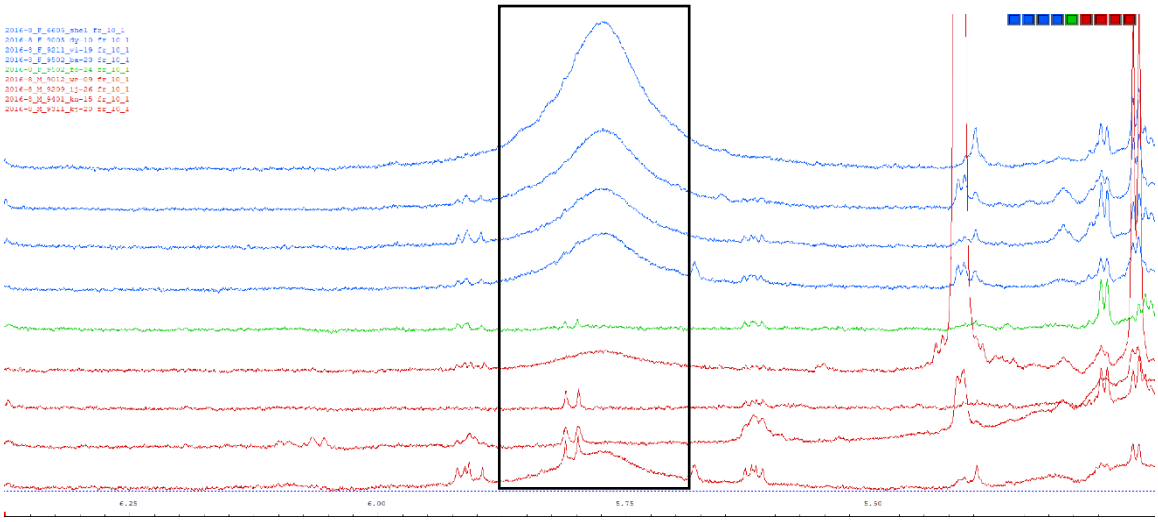

Figure 26: Signal at 5.8 ppm of female (blue), male (red) and corticosteroid user (green)

CLCs at the region which seems to appear slightly more in female than in male, shown in figure 26. In 2016 group, female tends to have higher and greater board signal at 5.8 ppm region. Whereas male tends to have much lower or no broad signals with interference of a double peaks. Here, the girl who took medicine containing corticosteroid also has no broad signal with a doublet signal, like male samples. From this situation, two things that could be observed here:

1. Women are more sensitive with specific regions in CLCs than men.
2. It is possible to identify person who use corticosteroids.

Since signal of CLCs at 5.8 ppm is a singlet, that doublet in this assignment is not the titration signal of CLCs but it could be related to CLCs signal.

Plus, in a general 1D-^{1}H spectrum, signals are usually overlapped causing the difficulty in detect a specific one, especially the region from 0.8 ppm to water signal at 4.8 ppm. All titration regions of CLCs obtained from the previous session are in the condensed region of signals, the only exception is region at 5.8 ppm. All of these figure out the vital role of this region as a key region for studying of salivary CLCs.

Finally, as ingestion of the food would affect the properties of saliva in term of composition and its quantification, shown by result of the student group in 2018, sampling of saliva is suggested to be in the morning and before any meal.

3.6 Taste-related compounds in SalmA extract

3.6.1 Adenosine-related compounds

3.6.1.1 Day 6 to day 18

As explained earlier in this thesis, K-index is a standard guideline for isolating freshness of the fish (see section 1.3). Therefore, K-values of both SalmA-TCA and SalmA-Sal were calculated and exhibited in figure 27.

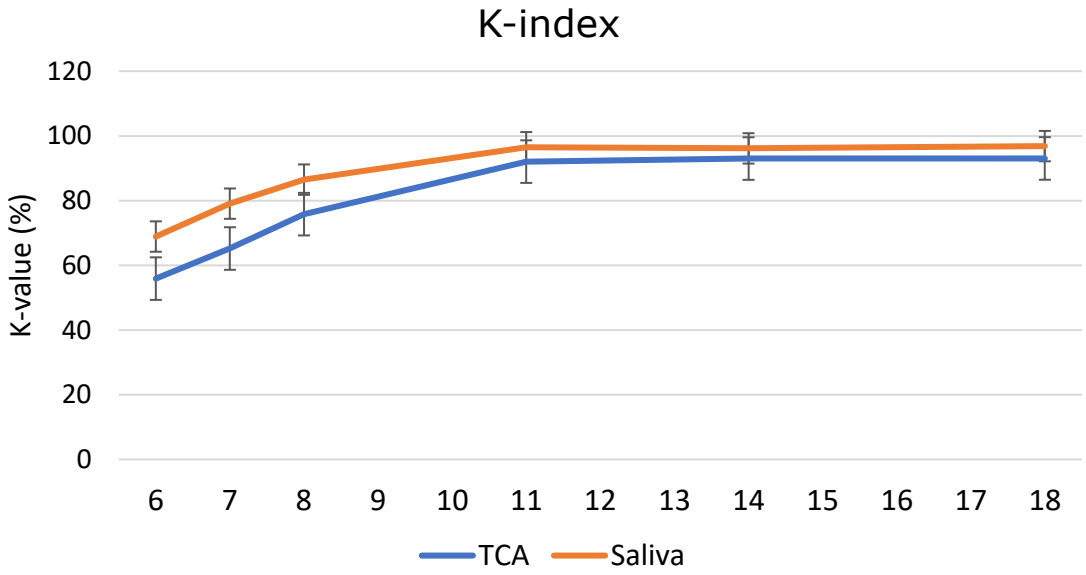

Figure 27: K-index calculated from day 6 to day 18

As seen above, K-index of SalmA-TCA had generally lower value compared to that in SalmA-Sal extract. All K-index was above limitation for raw consumption (lower than 20 %) as the lowest K-value was 56 % and 69 % for SalmA-TCA and SalmA-Sal correspondingly. Since day 7 and 8, both K-values of two samples were above threshold limit for edibility (above 70-80 %). From day 11, both SalmA-TCA and SalmA-Sal were mostly similar in K-values. At day 18, SalmA-TCA had K-value at 93 % and SalmA-Sal had that at 97%.

Followed K-index, adenosine-related compounds that were components of K-index's calculated formula. The data were all presented as amount of metabolite detected in 100 g of SalmA. The unit to go with all values in these data was mmol.

In NMR spectra, Hx signal presented at 8.193 ppm with a singlet peak. Amount of Hx has steadily rise in SalmA-Sal. It increased steadily as well in SalmA-TCA from day 6 to day 14 before speeding up to day 18. At day 18, SalmA-TCA had much higher amount of Hx than SalmA-Sal did. The result of Hx was presented by figure 28.

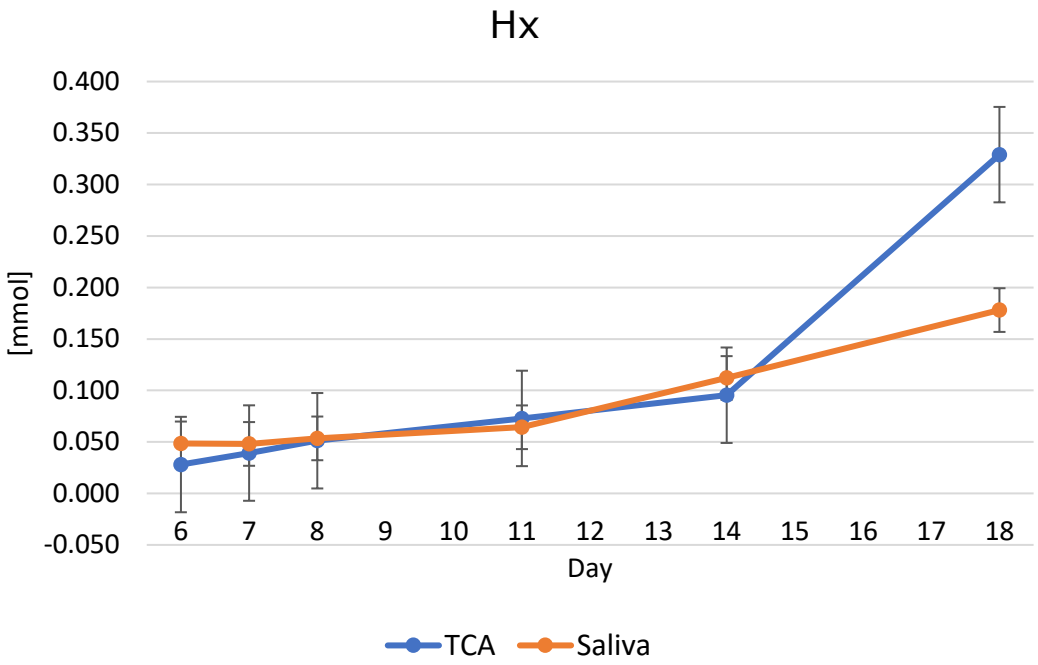

Figure 28: Hypoxanthine in SalmA-Sal and SalmA-TCA based on data from day 6 to day 18

Different from amount of Hx, amount of Ino from day 6 to day 11 had its amount increased more than 150 % in SalmA-TCA and more than 190 % in SalmA-Sal. After that, amount of Ino in both decreased. Amount of Ino in SalmA-TCA at day 18 was same as it at day 6 whereas amount if Ino in SalmA-Sal at day 18 was slightly higher than it at day 6. The amount of Ino in SalmA-Sal fluctuated from day 6 to day 8 as it increased to day 7 and decreased to day 8. In general, the amount of

Ino in SalmA-TCA was all higher than that in SalmA-Sal as well as they had same trendline of increasing followed by decreasing (figure 29). Signal of Ino was presented as singlet at 8.352 ppm.

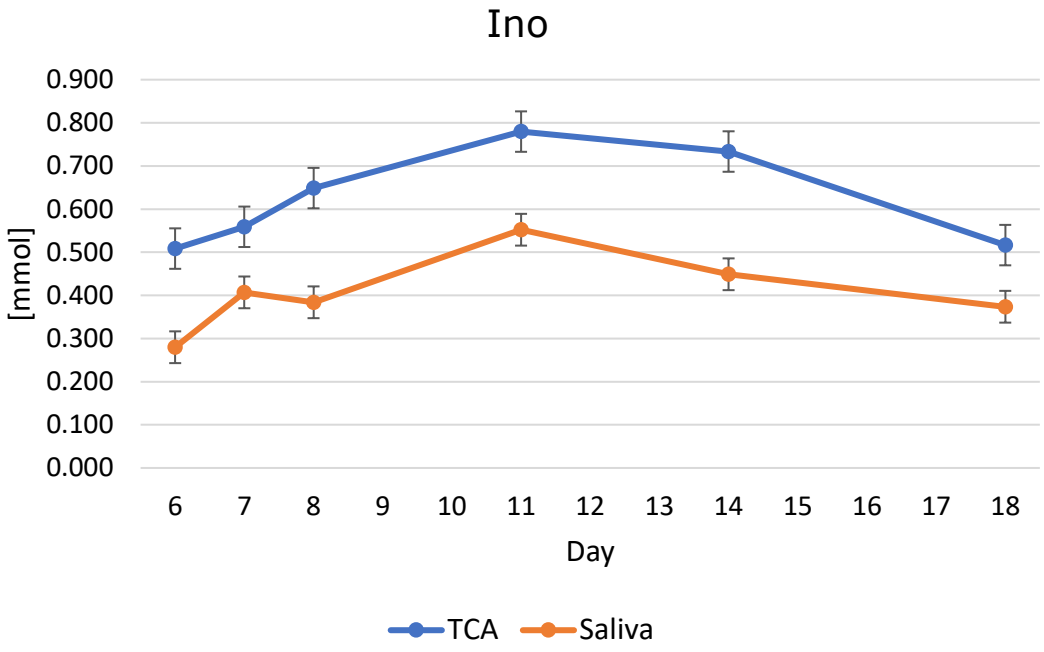

Figure 29: Result of Ino isolated from day 6 to day 18

Contrasting from both of Hx and Ino, amount of IMP in SalmA-TCA tended to fall whilst IMP in SalmA-Sal tended to rise, shown in figure 30. From day 6 to day 11, amount of IMP in SalmA-TCA gradually fell more than 9 times followed by slowly drop to day 18. IMP in SalmA-Sal rose slightly from day 6 to day 18. At day 6, amount of IMP in SalmA-TCA was 96 times higher than that in SalmA-Sal but at day 18, both were mostly the same.

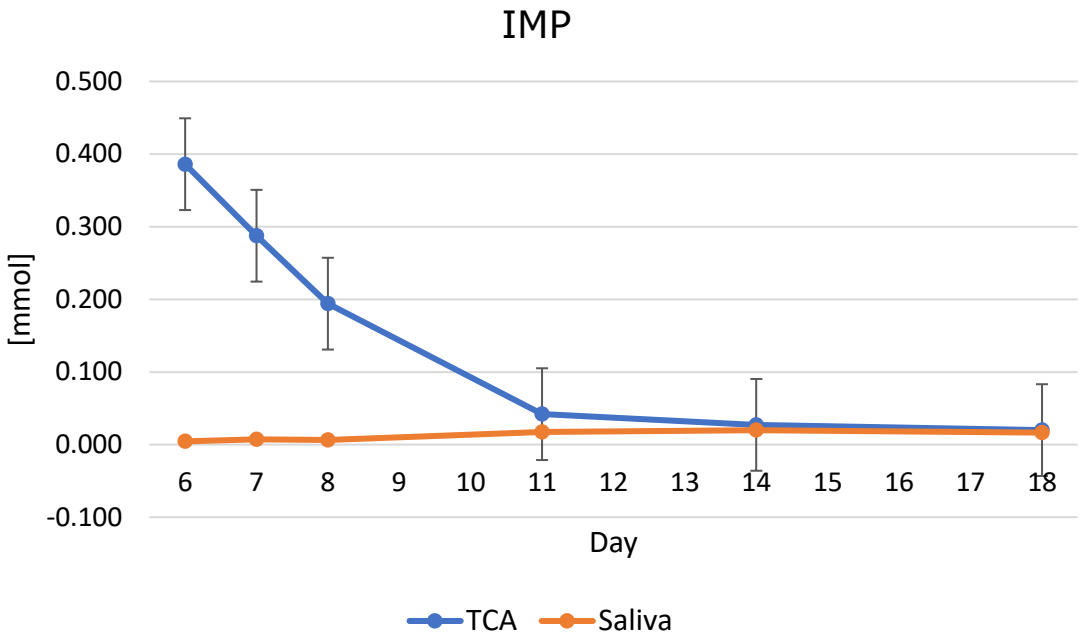

Figure 30: IMP detected in SalmA extracts

ATP and ADP were not included in this calculating as signals of both were sometimes separated themselves in the spectra, but sometimes they presented themselves as shared signal with one region only (Sitter et al., 1999). In K-value calculation, there was not important whether ATP and ADP present as one factor or two factors because they all resulted in the unchanged values of K-index. However, it could not be presented as individual indicator.

As seen in data charts of Hx and Ino above, there were different in the shape of their trendlines while the isolated amounts of SalmA in both TCA and saliva extracts had similar trends. Differently, the trendline of SalmA-TCA was contrary to SalmA-Sal's trendline in which one rose where the other fell. Additionally, there was a such a huge gap in amount of IMP in both kind of extracts at earlier days.

3.6.1.2 SalmA-TCA-Saliva

At beginning, SalmA was extracted independently in TCA and saliva resulted in interesting distinction of amount IMP isolated. Therefore, it was hypothesis that putting SalmA-TCA extract to saliva to see if there was any change could be marked. Four replicates of SalmA-TCA were chosen for mixing with each of saliva or D_2O as reference. One of these four dupes was shown in figure 31.

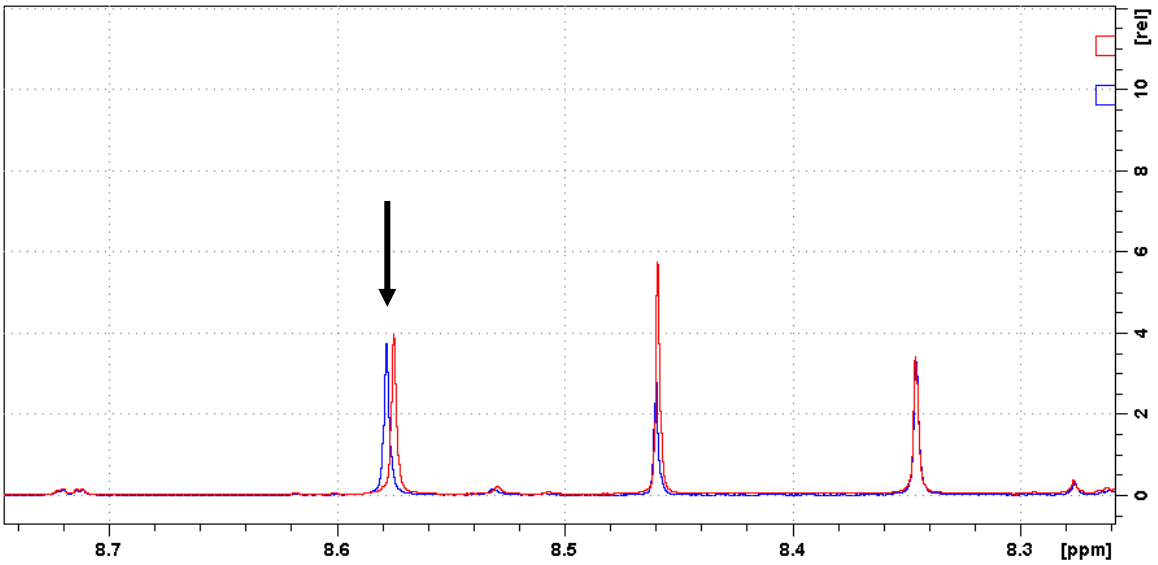

Figure 31: SalmA-TCA extracted with D_2O (blue) and saliva (red)

As seen, there was no change detected. Hence, it could be concluded that IMP was not affected by adding SalmA-TCA extract to saliva. This might be the consequence of TCA inhibiting saliva activity as saliva did not influence amount of IMP when TCA presented in the mixture. Moreover, IMP difference might be caused by interaction of some compounds in salmon tissue with saliva because the significant low amount of IMP happened in presence of salmon tissue but not salmon extract.

3.6.1.3 Confirmation experiment

To test for reproduction of the experiment as well as to test if the observation from IMP result was accurate, another salmon tissue was extracted with TCA and saliva. The spectra of adenosine-related compound regions were zoom in, shown in figure 32.

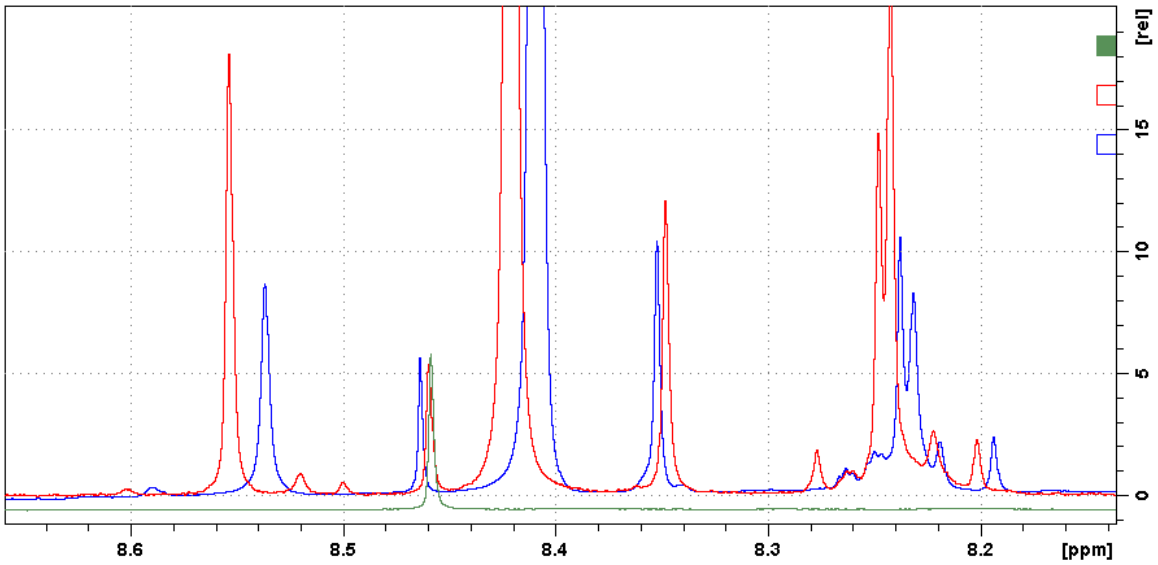

Figure 32: Adenosine-related compounds in 1D spectrum. Reference saliva presents as green, SalmA-TCA is shown in red and SalmA-Sal is shown in blue.

As described in section 3.5.1.1, in early days after being slaughtered, the amount of Hx in both SalmA-TCA and SalmA-Sal was nearly similar while the amount of Ino in SalmA-TCA was slightly higher than that in SalmA-Sal. Plus, IMP had its amount in SalmA-TCA significantly higher than it in SalmA-Sal. It was shown clearly in figure 32 above that all Hx, Ino and IMP signals were just as precise as described. Through this, the difference of IMP amount in both TCA and saliva extracts were confirmed.

3.6.1.4 Titration

Study of adenosine-related compounds could not be complete without titration assignments performed with ATP, IMP, Ino, and Xan. First to be

mentioned was ATP titration shown in figure 33. The region at 8.53 ppm contained the overlapped signals of ATP and ADP (Sitter et al., 1999). Therefore, ATP and ADP in reference SalmA-Sal sample and 20 µL ATP added ones had two peaks that were partially overlapped. But in 30 µL ATP added sample, only one big signal was detected. The explanation for this was that ATP signal was large enough to fully overlapped ADP signal resulted in combined signals of both. The concentration of ATP stock was prepared as 11.45 mg/mL.

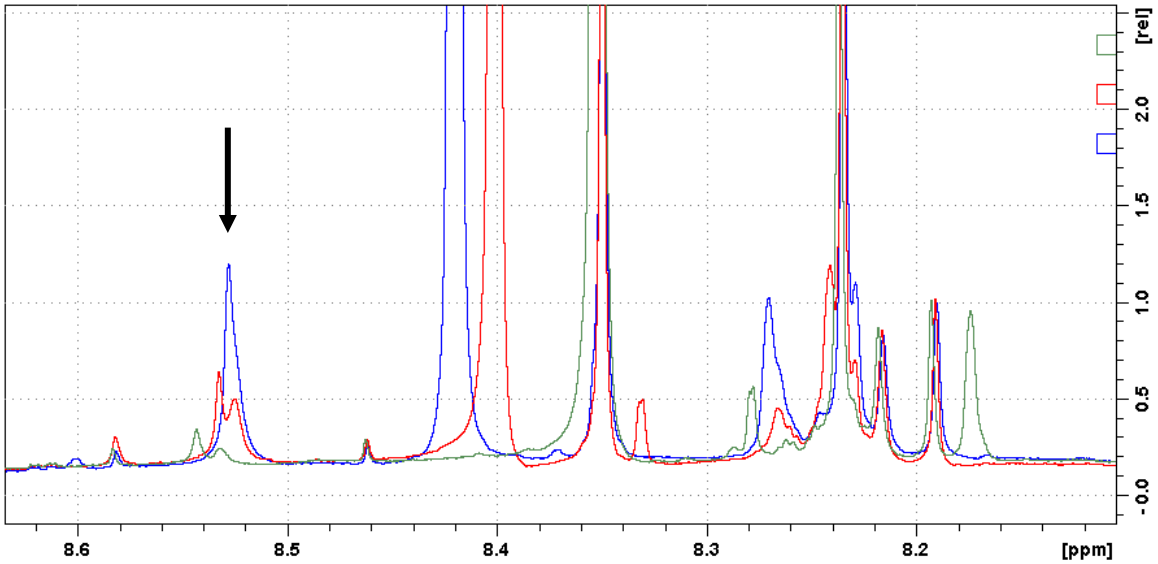

Figure 33: ATP titration result view from 1D spectra. Reference saliva is shown in green, 20 µL and 30 µL added ATP samples are shown in red and blue.

Integral of ATP was measured for the whole region in which might include ADP as well. Integrals of ATP had value of 0.1076 for reference sample, 0.3521 for 20 µL stock added sample, and 0.5998 for 30 µL stock added sample.

It required more effort to detect IMP titration as the amount of IMP extract in SalmA-Sal was very low (see previous sections). Titration spectra of IMP was shown in figure 34. In the figure, there was an extra spectrum of solid IMP stock added to SalmA-Sal extract. This was because titration signals of IMP were not so significant that needed an

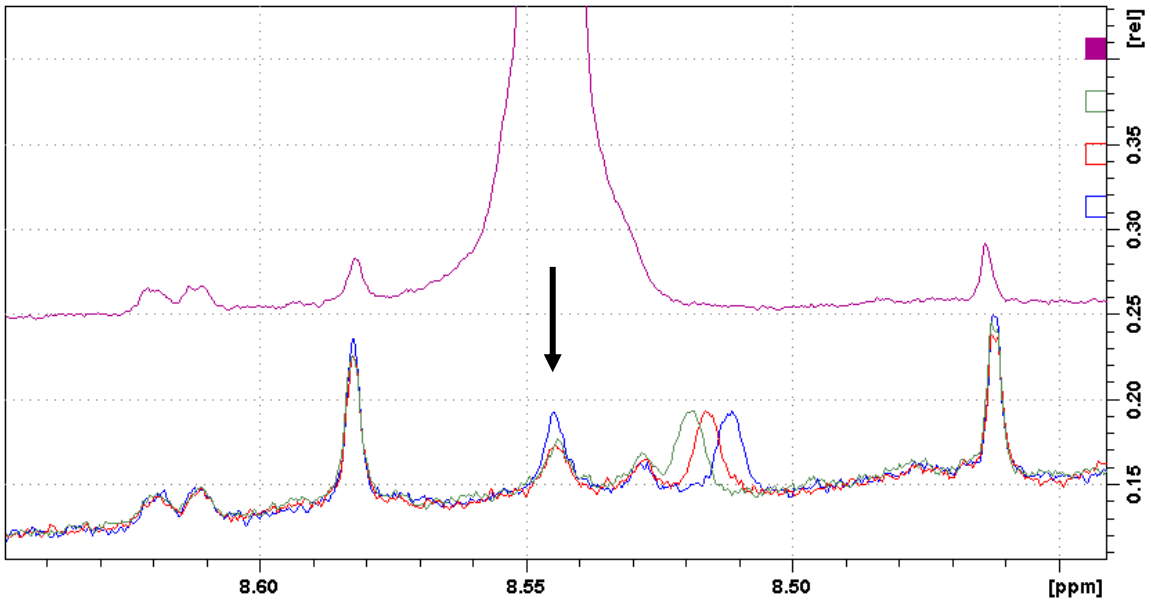

Figure 34: Result of IMP titration

Green shows reference saliva. Red and blue show 10 µL and 40 µL added IMP samples. Violet shows IMP added to sample as solid form.

external information. It could be seen in the figure that the difference between sample with and without 10 µL IMP stock added was not detectable. With 40 µL IMP stock added, it was titration region to be isolated. Initial IMP found in reference sample was 0.0103 whilst IMP found in 10 µL added sample had integral of 0.0119 and that in 40 µL added sample was 0.0178. It could be seen in the figure that there was no noticeable change between reference IMP and 10 µL added IMP. The possible explanation for this could due to the very low amount of IMP leading to the difficulty plus big error in preparation stock solution; as the stock of IMP was prepared with diluting of 0.44 mg IMP stock powder into 1 mL D_2O.

Ino titration was assigned due to the high amount present in the extract. The result was shown in figure 35. Ino stock was prepared by diluting 12 mg IMP purified powder in 1 mL D_2O. The integrals of Ino in reference extract, 10 µL added and 20 µL stock added were 0.6994, 2.2148 and 3.7640 respectively. With adding 10 µL Ino stock, the integral of Ino increased approximately 3 folds. With adding 20 µL stock, it increased

5.4 folds. If the initial Ino was removed from the calculating, then the added amount of Ino in 20 µL was double that in 10 µL.

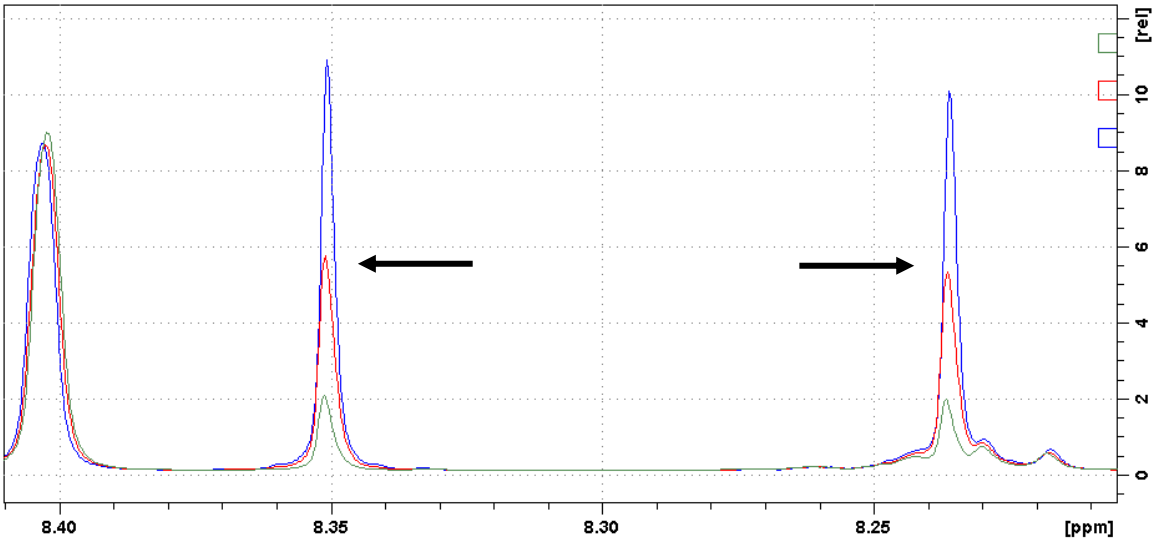

Figure 35: Signal peaks of Inosine resulting in titration assignment Reference saliva is in green. 10 µL added Ino is red and 20 µL added Ino sample is in blue.

Similar to IMP, amount of Hx in SalmA-Sal extract was low in general. This also led to the same big error and difficulty in Hx stock solution preparation. 0.504 mg purified Hx was weighted and diluted in 1 mL D_2O.

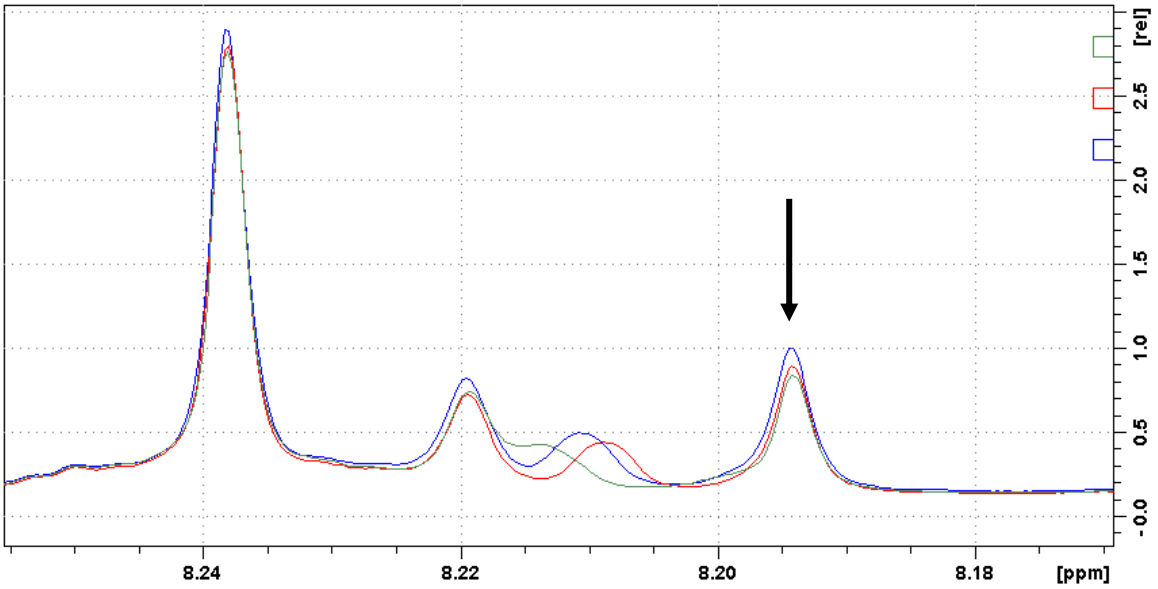

Figure 36: Hx titration shown in 1D spectra Reference is shown in green. 10 µL added Hx is in red and 20 µL added Hx sample is in blue.

20 µL and 40 µL Hx stock solution were added into reference sample and result was shown in figure 36. As seen, there was not significant increase in Hx signal. However, it was still noticeably increased signal. Integrals of Hx were measured and results were 0.2594, 0.2673, and 0.2951 correlated with reference, 20 µL, and 40 µL stock added samples.

Hx and IMP have so small amount that they become hard to detect and isolate. ATP signal is commonly overlapped with ADP signal. Whereas, IMP has the greatest signal among all the adenosine-related molecules. Based on the results obtained from section 3.5.1.1, it is suggested that performance of titration should be done for sample with two weeks post mortem when the amount of Hx and IMP rise up and the amount of Ino is still high. Plus, it is recommended to prepare higher concentration of Hx and IMP to have better titration view. This is because the initial hypothesis that if small amounts of stock (10 µL or 20 µL) are added into the reference sample, the concentration of metabolite would increase double. As that purpose, Ino titration shows its suitability with high concentration and nice result.

3.6.2 Other taste-related compounds

In this section, results of some other taste-related compounds comparison between SalmA-TCA and SalmA-Sal would be discussed. All data were presented with mmol unit courted based on the amount found in 100g fish.

3.6.2.1 Glutamate

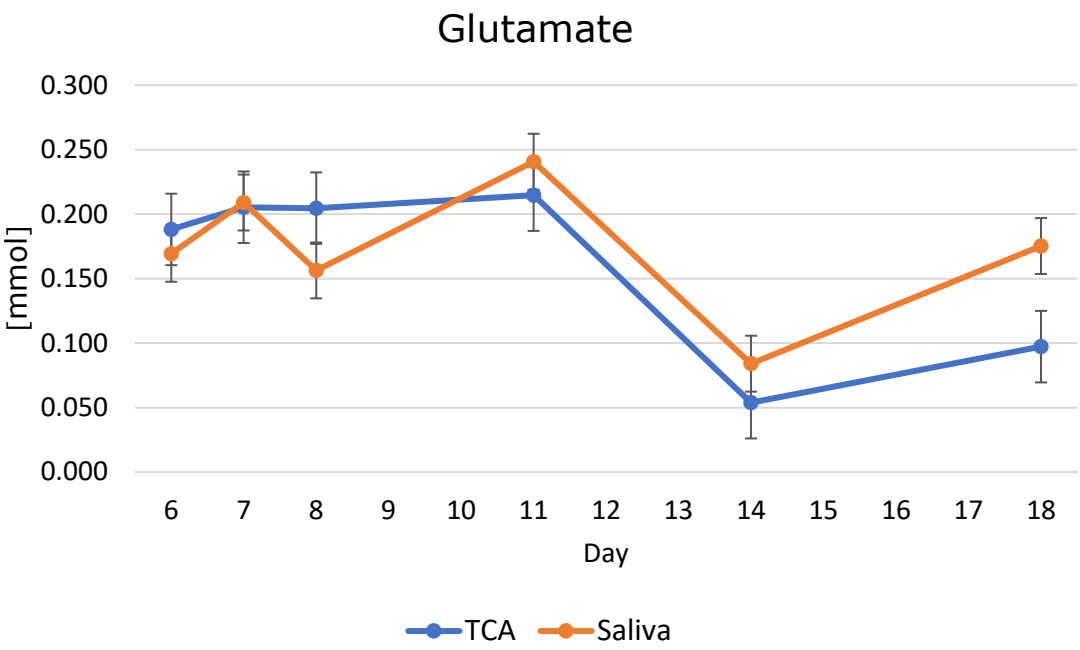

Figure 35: Glutamate detected in SalmA-Sal and SalmA-TCA from day 6 to day 18

Representative for umami taste, amount of glutamate was calculated. Signal of glutamate was presented as multiplet at 2.36 ppm and result of glutamate calculating was shown in figure x. Glutamate found in SalmA-TCA extract tended to increase slowly from day 6 to day 11 before dropping to day 14 followed by increasing to day 18. On the other hand, glutamate found in SalmA-Sal extract fluctuated from day 6 to day 8 then it rose to day 11. After day 11, glutamate in SalmA-Sal had the same trend with SalmA-TCA. From day 5 to day 11, SalmA-TCA inclined to have higher concentration than SalmA-Sal did but after that, SalmA-Sal had higher concentration of glutamate. This meant that the umami taste that could be tasted was clearest at day 11 and lowest at day 14.

3.6.2.2 TMA

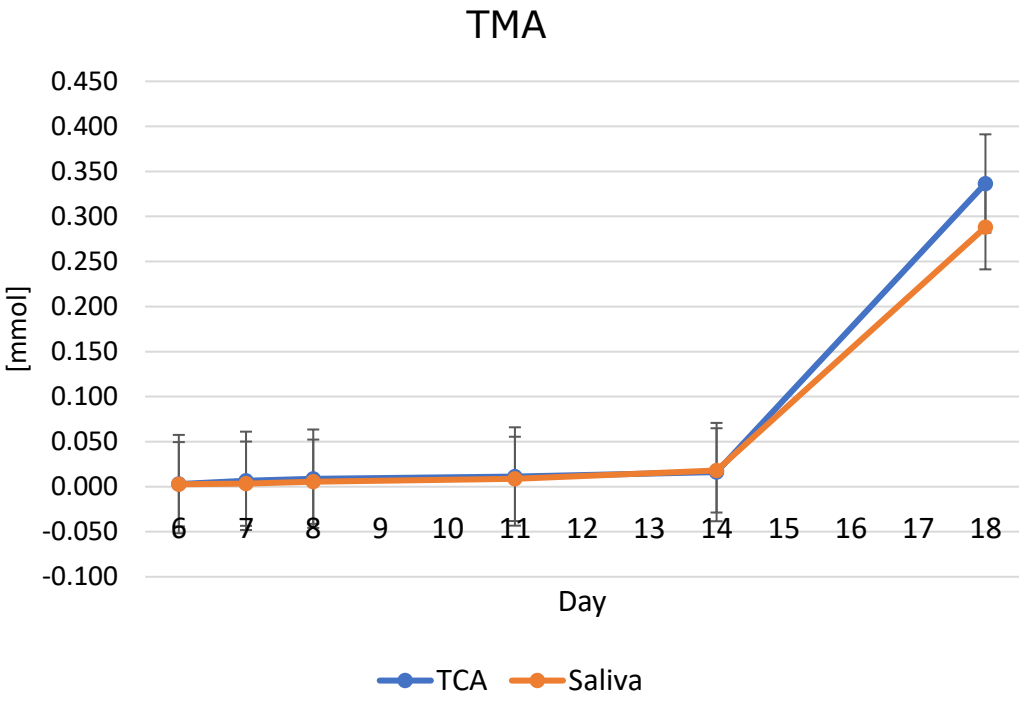

Figure 36: TMA result measured from day 6 to day 18

TMA responsible for "fishy odour" was integrated at 2.9 ppm presented by a single peak. Result after calculating was exhibited in figure 38. From the chart, TMAs extracted in SalmA-TCA and SalmA-Sal had mostly identical values from day 6 to day 14. This period, TMA's concentration rose slowly followed by a rapid lift to day 18 where SalmA-TCA had differently higher value than SalmA-Sal did. Therefore, it was concluded that the "fish smell" was not strong within two weeks after being slaughtered, after two weeks the fish would increase its smell significantly and it could be so strong that it could not be consumed.

3.6.2.3 Acetate

Acetate single peak at 1.92 ppm was integrated and results were shown in figure 39. Amount of acetate in SalmA-Sal was fluctuated, especially

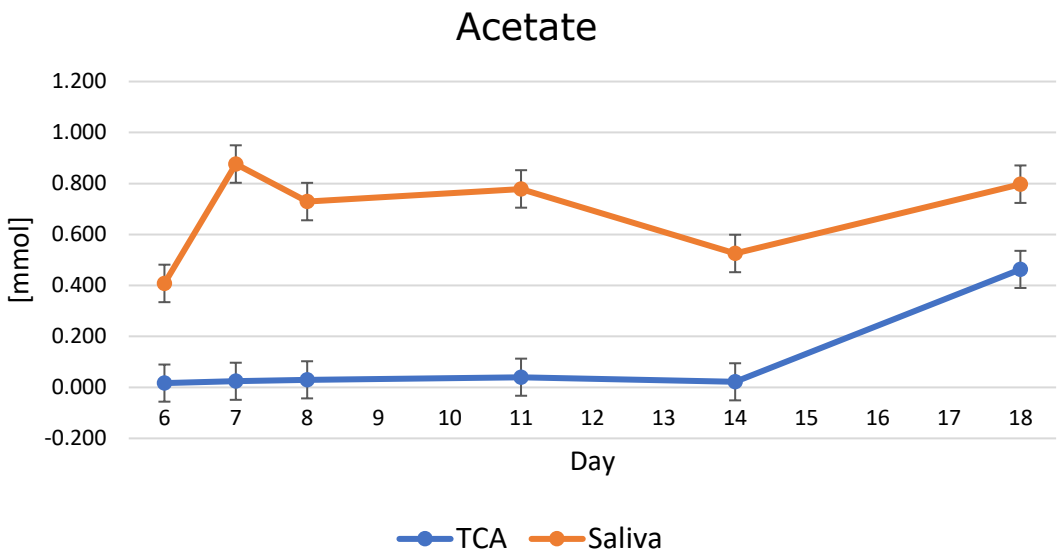

Figure 37: Acetate isolated in SalmA-Sal and SalmA-TCA

a rapid increase at day 7. Amount of acetate in SalmA-TCA was, oppositely, stably increase from day 6 to day 14. After that, it increased significantly to day 18. In general, SalmA-Sal always had higher concentration than SalmA-TCA did.

3.6.2.4 Glucose

Signal of glucose was a doublet at 5.24 ppm. Integrals of all samples were isolated, and results was shown in figure 40. Glucose in SalmA-TCA was slightly unstable from day 6 to day 8; it rose to day 11 then dropped down nearly 10 times to reach lowest value at day 18. Glucose in SalmA-Sal, on the other hand, was fluctuated from day 6 to day 11. After day 11, it fell to its lowest concentration of 0.014 mmol, three times less than glucose in SalmA-TCA at similar day. All detected amounts of glucose in SalmA-TCA were higher than that in SalmA-Sal.

Glucose was one the basic sugar form in which it had sweet taste. From result shown in the figure, it also meant that the sweetness of SalmA was decrease regarding long storage of the fish.

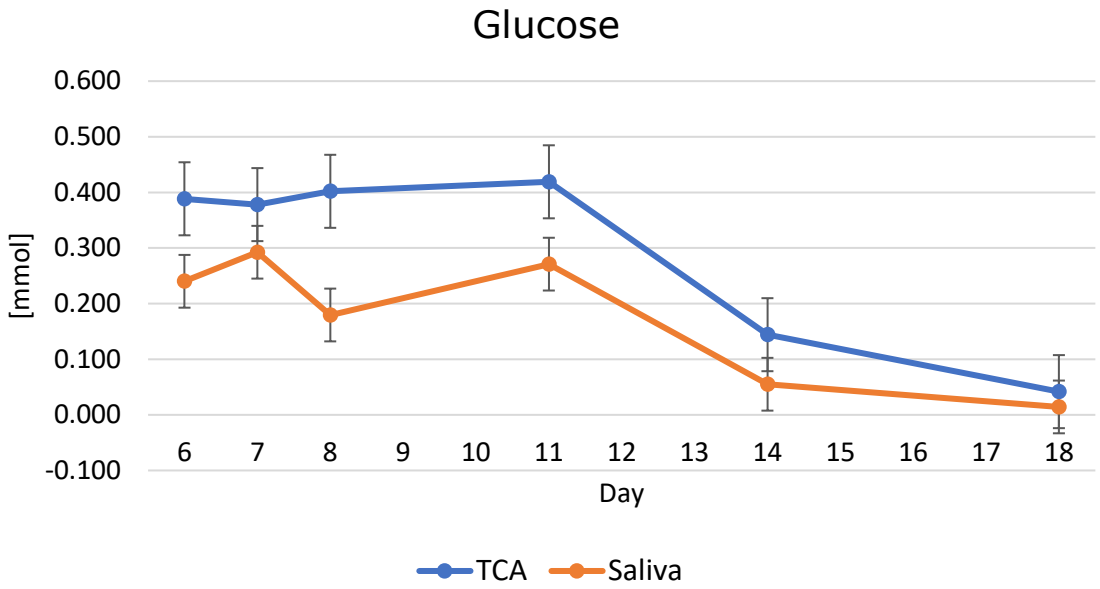

Figure 38: Glucose measured in salmon extracted in saliva and TCA

3.6.2.5 Alanine

Alanine integrals were measured at 1.48 ppm and results after being calculated were shown in figure 41. Alanine in SalmA-TCA tented to increase in general even though it slightly dropped in value from day 11 to day 14. In contrast, Alanine in SalmA-Sal was so fluctuated that it was impossible to figure out its trendline. Alanine increased and decreased alternatively. But when it was to compare beginning day and ending day of experiment, the trendline of alanine in SalmA-Sal was still a rise.

Similar to glucose taste, alanine taste was sweet. But different from glucose as a sugar molecule, alanine was an α-amino acid. As the after fish was dead, fish's protein started to degrade to amino acids (Konikova et al., 1975). This explained the increase in alanine during the experiment.

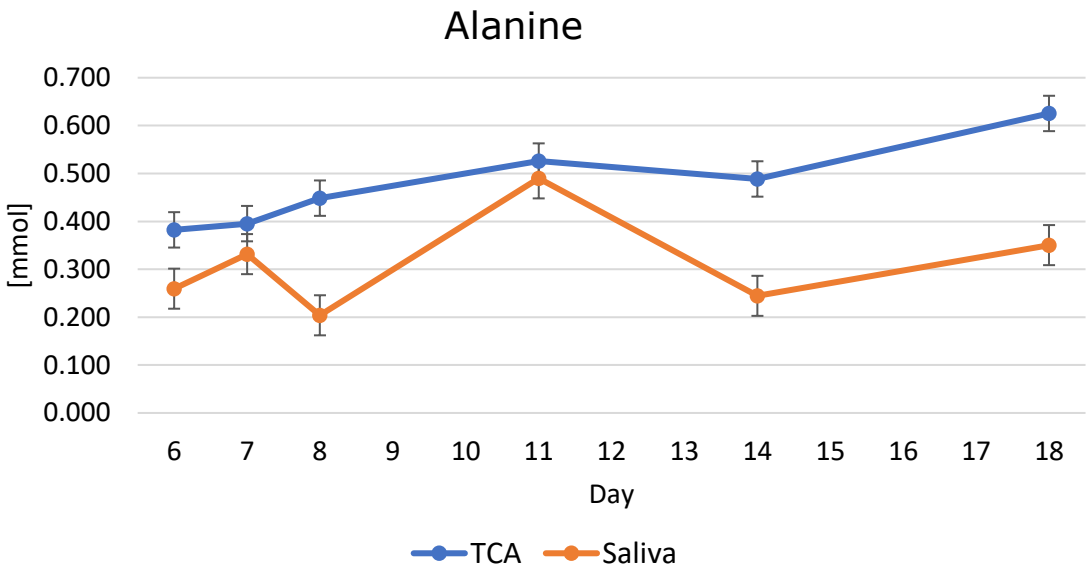

Figure 39: Alanine measured in

In section 3.2, some major components of saliva had been named including acetate, glucose, glutamate, and alanine. Because saliva quality did have huge effect on analysing results, acetate was chosen to test condition of saliva from day 6 to day 11. The result of acetate was indicated in figure 42. Amount of acetate was presented in mmol found in 1 mL saliva. The trendline of saliva's acetate had very similar shape to the trendline of acetate detected in SalmA-Sal from day 6 to day 11. It could, therefore, inferred that the fluctuated trends of some compounds composing in both saliva and salmon were due to saliva condition. It confirmed the great impact of saliva to the result.

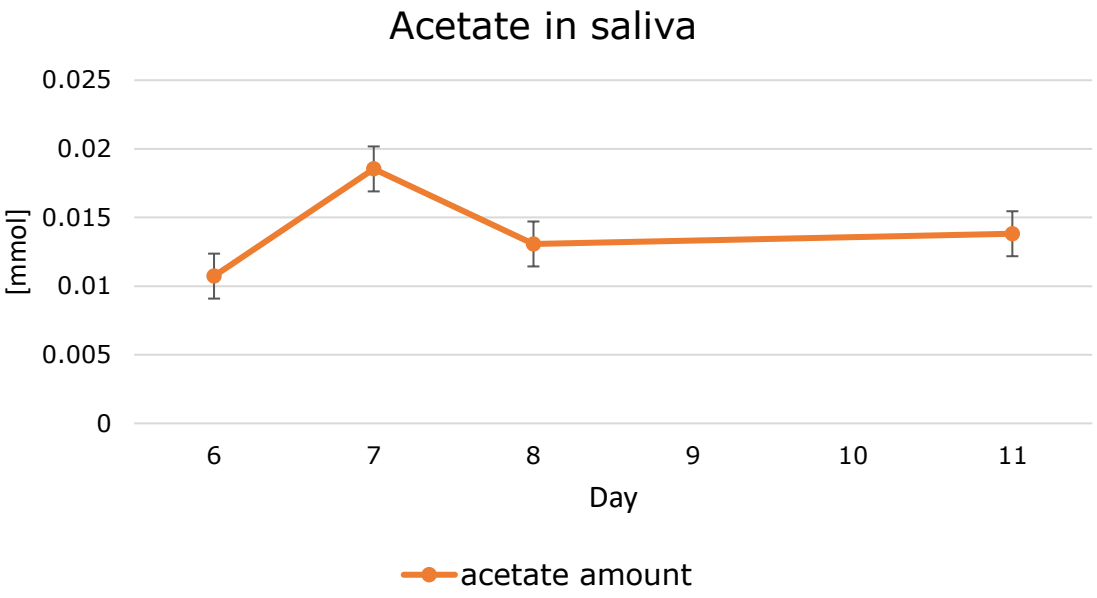

Figure 40: Salivary acetate calculated from day 6 to day 11

4. Conclusion

This thesis aimed to use NMR approach to detect CLCs and TRCs in human saliva. Saliva was analysed and its major components were identified successfully. Signals of crucial position in structure of CLCs, which had impact on chemical properties of CLCs, were determined. There were two kinds of essential signals came from methyl groups and from ketone group on the first ring to the left of the compounds.

These signals shown theirs important in study of CLCs clearly in titration assignment where theirs signal regions corresponded to most titration regions. With mass signals spreading along 0.8 ppm to 4.8 ppm, the noticeable signal at 5.8 ppm turned out to be potential indicator to CLCs. However, this signal was interfered with a broad signal in the same region. The broad signal be inclined to appear more in women than men. It also was found in both saliva reference, CLCs titrated saliva and saliva from public source.

CLCs in public samples presented by student saliva was not detected in this thesis due to insufficiency of data. However, two important results were discovered. First, it was possibility to divide saliva samples into groups based on gender. This result was obtained from saliva of group 2016 student whereas samples from group 2018 student did not show this feature. The explanation for this difference was the conditions of saliva at the time it was collected. Another positive result, via NMR approach, it was the able to distinguish person using corticosteroid.

In this thesis, adenosine-related compounds and other tasting compounds in salmon were successfully detected in saliva. This was an *in vitro* model of actual tasting process of salmon ingestion. Comparison salmon extracted in TCA and saliva indicated the similarity in trendline of adenosine-related compounds such as Hx or Ino. In contrast, IMP

shown its noteworthy distinction as opposite trendline between the two extractions. In general, TCA extract seemed to have higher quantity of salmon-related metabolites than saliva extract had. But acetate and glutamate presented more in SalmA-Sal extract than SalmA-TCA extract. The fluctuation of presenting metabolites were caused by the conditional inconstancy of saliva itself.

In the future, it is necessary to focus on these aspects:

- More research focusing on the highlighted region in CLCs structure and which molecule interacting with it
- Determining a doublet at the region 5.8 ppm
- Using more kinds of fish and food for study of taste
- Using larger size of samples to have more accuracy as it might have some varieties in result
- More research focusing on IMP (from salmon) growing trend in saliva

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Appendix 1: Hydrocortisone-like compounds found in human

CLC	Concentration	Method used	Reference
Hydrocortisone	2.5 - 10 µg/100mL	GC-ECD	(P. A and R, 1975)
	0.39 – 400 ng/L	LC/MS - MS	(Perogamvros et al., 2009)
	52 ng/100mL (median)	HPLC - MS/MS	(Vieira et al., 2014)
	86 ng/100mL (median)	radioimmunoassay	(Vieira et al., 2014)
Cortisone	0.78 – 200 ng/L	LC/MS - MS	(Perogamvros et al., 2009)
	278 ng/100mL (median)	HPLC - MS/MS	(Vieira et al., 2014)
Aldosterone	0-5.4 µg/100mL	direct radioimmunoassay with modified	(Few et al., 1986)
	2.5 ng/100mL	radioimmunoassay	(McVie et al., 1979)
	4.0-18 ng/100mL	GC - ECD	(P. A and R, 1975)
Corticosterone	0.08 - 0.8 µg/100mL	GC - ECD	(P. A and R, 1975)

Appendix 2: Numbers of scan and RGs of all samples

Sample	NS	RG
Aldosterone in acetone	96	144
Aldosterone in water	96	32
Hydrocortisone	128	181
Cortisone	48	28.5
Corticosterone	48	28.5
Aldosterone ref	256	90.5
Aldosterone 20 µL	256	90.5
Aldosterone 40 µL	256	90.5
Hydrocortisone ref	256	90.5
Hydrocortisone 20 µL	256	90.5
Hydrocortisone 40 µL	256	90.5
Cortisone ref	256	90.5
Cortisone 20 µL	256	64
Cortisone 40 µL	256	64
Corticosterone ref	256	64
Corticosterone 20 µL	256	64
Corticosterone 40 µL	256	64
Standard-Sal T6	23	22.6
Standard-Sal T7	176	128
Standard-Sal T8	176	128
Standard-Sal T11	176	128
Standard-Sal T14	176	128

Standard-Sal T18	176	128
SalmA-Sal T6	128	128
SalmA-Sal T7	176	128
SalmA-Sal T8	176	128
SalmA-Sal T11	176	128
SalmA-Sal T14	176	128
SalmA-Sal T18	176	128
SalmA-TCA T6	48	144
SalmA-TCA T7	48	144
SalmA-TCA T8	48	144
SalmA-TCA T11	48	144
SalmA-TCA T14	48	144
SalmA-TCA T18	48	144
Hydrolysis-Sal-ref	256	64
Hydrolysis-TCA-50C	256	64
Hydrolysis-TCA-RT	256	64
Hydrolysis-NaOH-50C	256	64
Hydrolysis-NaOH-RT	256	64
IMP-SalmA-TCA-Sal-1	176	128
IMP-SalmA-TCA-Sal-2	176	128
IMP-SalmA-TCA-Sal-3	176	128
IMP-SalmA-TCA-Sal-4	176	128
IMP-SalmA-TCA- ref-1	176	128
IMP-SalmA-TCA- ref-2	176	128

IMP-SalmA-TCA- ref-3	176	128
IMP-SalmA-TCA- ref-4	176	128
Check-Sal-ref	256	90.5
Check-SalmA-TCA	48	144
Check-SalmA-Sal	256	90.5
SalmA-Sal-ATP-ref	48	90.5
SalmA-Sal-ATP-20 µL	48	90.5
SalmA-Sal-ATP-30 µL	48	90.5
SalmA-Sal-IMP-ref	48	90.5
SalmA-Sal-IMP-10 µL	48	90.5
SalmA-Sal-IMP-40 µL	48	90.5
SalmA-Sal-Xan-ref	48	90.5
SalmA-Sal-Xan-20 µL	48	90.5
SalmA-Sal-Xan-40 µL	48	90.5
SalmA-Sal-Ino-ref	256	64
SalmA-Sal-Ino-10 µL	256	64
SalmA-Sal-Ino-20 µL	256	64

Appendix 3: TSP concentration error

Samples	NS	RG	TSP absolute integral
1 mM TSP in final samples for SalmA-TCA			
SalmA-TCA T6	48	144	211695095.1
SalmA-TCA T7	48	144	213532375
SalmA-TCA T8	48	144	208003753.3
SalmA-TCA T11	48	144	206805713.9
SalmA-TCA T14	48	144	193479841.1
SalmA-TCA T18	48	144	192340267.1
Mean			**15063434.7778**
Standard deviation			**416035.3327**
SD in percentage			**2.761888897**
0.1 mM TSP in final samples for other samples			
SalmA-Sal-ATP-ref	48	90.5	15783065
SalmA-Sal-ATP-20 µL	48	90.5	15049935
SalmA-Sal-ATP-30 µL	48	90.5	15702764
SalmA-Sal-IMP-ref	48	90.5	14718912
SalmA-Sal-IMP-10 µL	48	90.5	14607468
SalmA-Sal-IMP-40 µL	48	90.5	14823782
SalmA-Sal-Xan-ref	48	90.5	15004528
SalmA-Sal-Xan-20 µL	48	90.5	15081699
SalmA-Sal-Xan-40 µL	48	90.5	14798760
Mean			**204309507.6**
Standard deviation			**9165490.457**
SD in percentage			**4.486081223**

Appendix 4: Standard deviation in percentage of metabolite quantification detected by ^{1}H NMR.

Sample	Standard deviation (%)			
	IMP	Ino	Hx	ATP + ADP
SalmA-Sal T6	1.4	0.1	3.0	1.6
SalmA-Sal T7	2.3	0.2	1.2	1.0
SalmA-Sal T8	3.4	4.8	2.6	2.0
SalmA-Sal T11	1.1	1.9	1.5	1.6
SalmA-Sal T14	0.4	0.6	0.7	2.6
SalmA-Sal T18	0.2	1.5	0.9	9.4
SalmA-TCA T6	2.3	0.8	4.5	4.3
SalmA-TCA T7	3.2	0.6	3.7	11.4
SalmA-TCA T8	4.8	0.2	4.7	2.4
SalmA-TCA T11	2.9	0.3	2.0	2.1
SalmA-TCA T14	1.3	0.4	1.3	7.1
SalmA-TCA T18	4.7	0.6	4.9	5.1
Check-SalmA-TCA	0.6	0.2	1.1	0.1
Check-SalmA-Sal	0.2	1.3	3.2	7.8

Appendix 5: Chemical shifts of Hydrocortisone assignment

Carbon number	Group	ppm	ppm
1	CH_3	19.69	0.85 (s;3) C8 - 41.73 C7 - 49.97 C9 - 54.75 C10 - 92.53
2	CH_3	23.34	1.41 (s;3) C4 - 36.9 C3 - 41.8 C6 - 58.2 C5 - 181.8
3	C	41.8	[a]
4	CH_2	36.9	1.94 (m; 2) 2.17 (m; 1)
5	C	181.8	[a]
6	CH	58.2	1.16 (m; 2.5)
7	C	49.97	[a]
8	CH_2	41.73	1.68 (m; 2) 1.93 (m; 2)
9	CH	54.75	1.68 (m; 2)
10	C	92.53	[a]
11	CH_2	35.9	2.4 2.64 (m; 3)
12	C	207.8	[a]
13	CH	123.57	5.78

14	CH_2	35.36	2.56 (m;3) 2.08 (m; 2)
15	CH_2	34.9	2.32 1.16 (m; 2.5)
16	CH	33.9	2.08 (m; 2)
17	CH	70.84	4.5
18	CH_2	25.89	1.47 1.84
19	CH_2	35.97	2.56 (m;3) 1.57
20	C	216.3	[a]
21	CH_2	69.4	4.42

[a] – ppm not applicable as there is no H

Appendix 6: Chemical shifts of each carbon number of cortisone

Carbon	Group	ppm	ppm
1	CH_3	18.16	0.64 C7 - 53.96 C8 - 52.92 C9 - 52.2 C10 - 91.43
2	CH_3	19.59	1.41 C3 - 41.04 C4 - 36.67 C5 - 178.3 C6 - 64.8
3	C	41.04	[a]
4	CH_2	36.67	2.6 (m;4) 1.78 (m;2)
5	C	178.3	[a]
6	CH	64.8	2.31 (m;2)
7	C	53.96	[a]
8	CH_2	52.92	2.13 (m;2) 2.89
9	CH	52.2	2.4 (m;2)
10	C	91.43	[a]
11	CH_2	35.7	2.31 (m;2) 2.6 (m;4)
12	C	208.26	[a]
13	CH	125.86	5.83
14	CH_2	35.2	2.4 (m;2)

			2.6 (m;4)
15	CH_2	25.4	1.98 1.53
16	CH	39.24	2.13 (m;2)
17	C	217.8	[a]
18	CH_2	34.9	1.34 2.05
19	CH_2	36.62	2.6 (m;4) 1.78 (m;2)
20	C		[a]
21	CH_2	69.7	4.38

[a] – ppm not applicable as there is no H

Appendix 7: Chemical shifts of aldosterone in deuterated acetone

Carbon	Group	ppm	ppm
1	CH_3	18.9	1.26 C2 – 38.8 C3 – 36.7 C4 – 170.3 C5 – 58.0
2	C	39.8	[a]
3	CH_2	36.7	1.70 2.21
4	C	170.3	[a]
5	CH	58.0	1.13
8	CH	124.6	5.61

[a] – ppm not applicable as there is no H

Appendix 8: Chemical shifts of corticosterone

Carbon	Group	ppm	ppm
1	CH_3	18.16	0.83 C7 – 46.6 C8 – 49.24 C9 – 59.76 C10 – 61.91
2	CH_3	23.28	1.40 C3 – 41.87 C4 – 36.9 C5 – 182.15 C6 – 58.55
3	C	41.87	[a]
4	CH_2	36.9	1.93 2.18
5	C	182.15	[a]
6	CH	58.55	1.19
7	C	46.6	[a]
8	CH_2	49.24	1.67 2.07 (m;3)
9	CH	59.76	1.26
10	CH	61.91	2.6 (m;3)
12	C	182.1	[a]
13	CH	123.49	5.78

[a] – ppm not applicable as there is no H

Appendix 9: Integrals of CLCs titration

Hydrocortisone	1.41 ppm	5.78 ppm
Reference saliva	0.1417	0.2956
20 µL Hydrocortisone	0.1585	0.3220
40 µL Hydrocortisone	0.1948	0.3393

Cortisone	0.63 ppm	1.40 ppm	4.40 ppm	5.83 ppm
Reference saliva	0.0323	0.0486	0.0969	0.0280
20 µL cortisone	0.0561	0.0709	0.1008	0.0420
40 µL cortisone	0.0786	0.0901	0.1073	0.0495

Aldosterone	1.19 ppm	1.25 ppm	5.80 ppm
Reference saliva	0.4819	0.1632	0.1728
20 µL Aldosterone	0.8461	0.8851	0.4206
40 µL Aldosterone	1.3503	1.3513	0.4577

Corticosterone	0.82 ppm	1.40 ppm	5.78 ppm
Reference saliva	0.0174	0.0253	0.0294
20 µL corticosterone	0.0278	0.042	0.0499
40 µL corticosterone	0.0481	0.0582	0.0528