Studies on the Skin Safety of Parabens

By

MD. Golam Muktadir

MPhil Research

Faculty of Health Sciences and Wellbeing

University of Sunderland

Supervised by

Dr. Kalliopi Dodou

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Abstract

Parabens are the most common preservatives in skin care products. Alone or in combination with other compounds they are used as a preservative to protect microbial growth in skin care products. Parabens have been successfully used by the cosmetics and pharmaceutical industries for more than 60 years. Parabens are alkyl esters of p-hydroxybenzoic acid. There are seven types of parabens available in the market. However, mostly four types of parabens are commonly used in cosmetics products; these are methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), and butyl paraben (BP). Those are esterified with an alkyl chain which is structurally reliable for anti-microbial activities. Paraben safety studies proved that parabens are safe for the human body. Therefore, The United States Food and Drug Administration approved parabens as preservatives in skin care products and they set a concentration of 0.4% w/w in a single paraben and 0.8% w/w in a combination of parabens. However, recent studies on the effects of parabens reported that they exhibit estrogenic activity on human skin. Thus, The Danish government banned all paraben use in cosmetics. Although, European Union (EU) government regulatory boards claim that current concentrations of parabens in skin products are safe for cosmetic products. Consequently, there is conflicting evidence over the safety of parabens used as preservatives in skin care products.

This research is presented in three chapters. The **first chapter** explains the feasibility of addressing the safety of parabens through cytotoxicity assay and establishes a ranking order of paraben safety according to their toxicity. This study was conducted with an SRB assay. In th**e second chapter**, the % metabolism of parabens through pig skin has been studied. Pig skin was chosen because it has similar histological and physiological properties to human skin. The permeation of parabens through Franz diffusion cells was determined over 24 hours using HPLC analysis for the quantification of parabens and their metabolite. This permeability study described a comparative analysis of parabens metabolized through pig skin and established a ranking order of paraben permeation according to their rate of metabolism. In **the third chapter**, we investigated the metabolism of parabens in human keratinocyte cells. The recommended concentration of each paraben was incubated with human keratinocyte cells at three different times of incubation. After incubation, parabens and their metabolite were extracted by lysis buffer and quantified using HPLC analysis. During this study, the % metabolism ranking order of these parabens in human keratinocytes was also determined.

Abbreviation

ACC	ASEAN *Cosmetic Committee.*
ACN	Acetonitrile.
ASEAN	The Association of Southeast Asian Nations.
BP	Butyl Paraben.
BZP	Benzyl paraben.
CES1	Carboxylesterase 1.
CE	Capillary Electrophoresis.
CIR	The *Cosmetic Ingredient Review*.
CYP	Cytochromes *P450.*
DMEM	Dulbecco's Modified Eagle Medium.
DNA	Deoxyribonucleic Acid.
EDTA	Ethylenediaminetetraacetic acid.
EP	Ethyl paraben.
EtOH	Ethanol.
EU	European Union.
FDA	U.S. Food and Drug Administration.
GC	Gas Chromatography.
GRAS	Generally Recognized as Safe.
HEPES	Hydroxyethyl Piperazineethanesulfonic acid.
HPLC	High Performance Liquid Chromatography.
hCE1	*Human liver Carboxylesterases 1.*
hiCE	Human intestinal Carboxylesterase.
H_2SO_4	Sulfuric acid.
KERTr	Keratinocyte.
MeCN	Acetonitrile.
MeOH	Methanol.
MW	Molecular weight

MP	Methyl paraben.
NaCl	Sodium chloride.
NaOH	Sodium hydroxide.
OH	Hydroxyl group.
PBS	*Phosphate-buffered saline.*
PP	Propyl paraben.
RNA	Ribonucleic acid.
SRB	Sulforhodamine B
SULT	Sulfotransferases estergen
TCA	*Trichloroacetic acid.*
TFA	*Trifluoroacetic acid.*
UHPLC	Ultrahigh Performance Liquid Chromatography.
UVB	Ultraviolet Radiation.
V/V	Volume / Volume.
WHO	The *World Health Organization.*
W/V	Weight / Volume.

1. Introduction.

1.1 Literature review.

Parabens are a type of preservative that is most used in pharmaceutical, food, and cosmetic/personal care products (Steinberg, 2006). Parabens have a broad spectrum of activity against yeasts, moulds, and bacteria (Mallika *et al.,* 2013). Therefore, parabens are widely used as a preservative in cosmetics and pharmaceuticals for their long self-life (UENO, 2013). Parabens are used alone or in combination to protect against microbial growth (Elder *et al.*, 1984). Combinations of parabens are more useful as preservatives than individual parabens (Steinberg, 2006). There are five types of parabens widely available in the pharmaceutical market as preservatives (Cashman and Warshaw, 2005). It has been allowed to use these on a very small scale as a preservative in skincare and food products (UENO, 2013). In skin care products, the concentration is 0.01% to 0.4% w/w, and in food products, the concentration is 0.1% w/w (FDA, 2014). Parabens are also called para-hydroxybenzoic acids (Cashman and Warshaw 2005). Naturally, those are obtained mainly from fruits and vegetables, for example, cucumbers, cherries, carrots, blueberries, onions, etc (Seeham and Rawaa, 2020). "It is also formed in the human body by the breakdown of some amino acids" (Yang, Zhang, and Wu, 2018). In cosmetics, parabens used, which are found in nature as well as quickly change into natural para-hydroxybenzoic acid in the human body (chemicalSafetyFacts, 2022).

Preservatives can be exposed to the human body through ingestion, inhalation, and dermal absorption. While dermal absorption may be the most important route of paraben exposure due to their widespread use in personal care products, ingestion may also be potent (Dodge *et al* 2015). In pharmaceuticals, parabens act as inactive excipients which may be used as antimicrobial preservatives in oral solid, parenteral, and topical dosage forms. Usually, drug products with active excipients are approved by the FDA (Dodge *et al* 2015). However, “Parabens are considered within a class of compounds referred to as ‘generally recognized as safe’ (GRAS), which are exempt from the usual tolerance requirements in the Federal Food, Drug, and Cosmetic Act” (Cosmetic Ingredient Review, 2008). FDA maintains a database of inactive ingredients that have been used in approved pharmaceutical drug products (Dodge *et al* 2015). The maximum level of paraben potency in pharmaceuticals is Methyl paraben 1.8 mg in a tablet and 0.15% w/v in an oral solution, however, Butyl paraben is 0.04 mg in a tablet and 0.016% w/v in an oral solution (Dodge *et al* 2015). Due to the absorption route of paraben into

the human body, paraben's maximum level acceptance has been different in skin and oral preparations (Tade et al 2018). This research is focussed only on the dermal absorption of parabens via cosmetic skin care products.

Parabens are metabolized into the skin through the subcutaneous layer where four carboxyl esterases are present. These hydrolyze the parabens to para-hydroxybenzoic acid and their relevant side chains (Lobemeier *et al*,1996). In subcutaneous tissue, the shorter chains of carboxyl esterases are more active against parabens. However, in keratinocytes, the longer chains of carboxyl esters are more active against parabens (Lobemeier *et al*, 1996). Parabens absorbed into the body through the epidermis layer of skin are fully metabolized by esterases in the liver and kidney and excreted through the urine and do not accumulate in the body (Cashman and Warshaw, 2005). Parabens can be absorbed and retained into human tissue without hydrolysis of tissue esterases (Oishi, 2004). Human bodies can exposure to a maximum of 76 mg/day for an average person's body weight (Cashman and Warshaw, 2005). The maximum concentration of parabens is exposed per individual through cosmetic and personal care products (Torfs and Brackman, 2021). The human body can expose to paraben through cosmetics and personal products are about approximately 50mg/day, drugs at 25mg/day, and food products at almost 1mg/day (Soni, *et al,* 2001). The European Scientific Committee recommended a safety assessment of parabens used in cosmetic and personal care products. According to Europa. EU (2015) “The maximum total concentration allowed in such consumer products is 8 g of parabens per kg of cosmetic product, with no single paraben having a higher concentration than 4 g/kg”.

1.2 Chemistry of paraben.

1.2.1 Structure of parabens.

Parabens are aromatic compounds that contain an alkyl ester chain of a benzene ring with a hydroxyl group that is situated on the para position of the benzene ring (Ferdinando, *et al* 2000). Therefore, chemically it’s called para-hydroxybenzoic acid or 4-hydroxybenzoic acid, as shown in Figure 1. General formula of paraben is $C_6H_4(OH)COOR$.

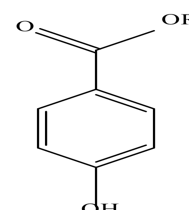


Figure1:
4 Hydroxybenzoic acid
(Chem Draw)

Parabens are an alkyl ester group of chemicals that differ at the para position of the benzene ring by various chemical substitutions (Cashman and Warshaw, 2005). All parabens are

structurally different from each other on the alkyl ester chain. Depending on various chemical substitutions of the alkyl ester group, there are seven types of parabens available in the present market (Darbre and Harvey, 2008). Those are Methyl paraben, Ethyl paraben, Propyl paraben, Butyl paraben, Isopropyl paraben, Isobutyl paraben, and Benzyl paraben. Those structures are shown below.

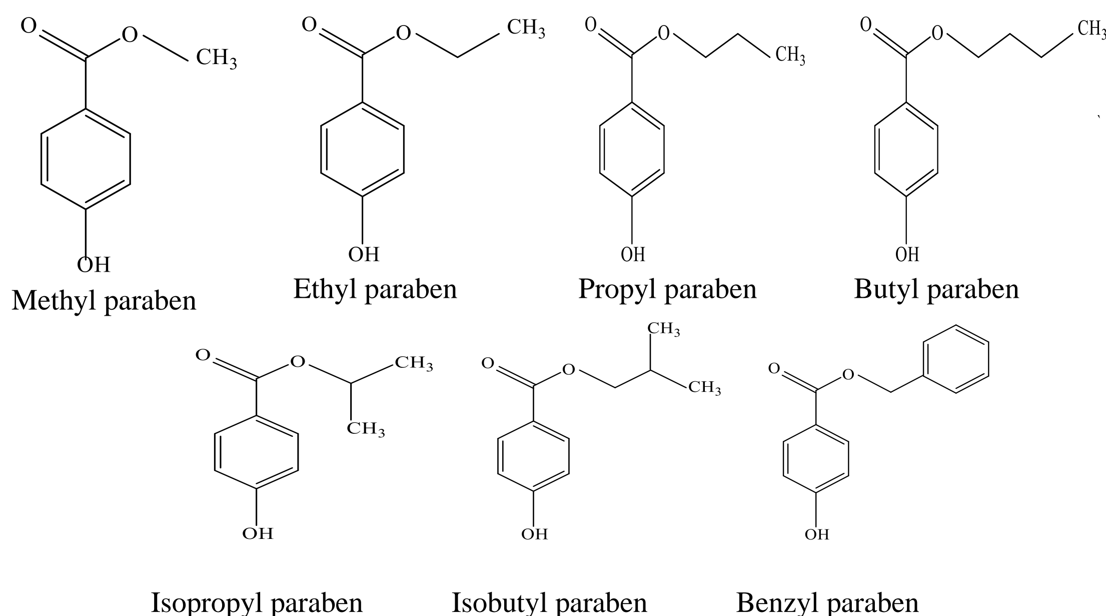


Figure 2: Structure of all parabens (drawn using Chem Draw)

1.2.2 Structural Relationship Activity of Parabens.

Parabens are structurally active against all microorganisms. Para-hydroxybenzoic acids are esterified with an alkyl chain at the C-4 position, which is structurally responsible for the anti-microbial activity of parabens (Uramaru *et al*, 2007). Generally, parabens are more active against most fungi, molds, and gram-positive bacteria but less active against certain bacteria, especially *Pseudomonas* (Elder et al, 1984). Their antimicrobial activity comes from Paraben's alkyl ester chain with key metabolic pathways in the target organisms. The maximum effectiveness of parabens depends on their longer alkyl chains (Cashman and Warshaw, 2005). So that the longer alkyl chains of parabens are more active against shorter alkyl chains. "Enhancement of antimicrobial activity of parabens is achieved by combining through C-4 position at esters with other biocides such as formaldehyde releasers, isothiazolinones, or phenoxyethanol" (Rietschel and Fowler, 2008). In general, microbes or other environmental reactions on pH separate the molecules (Davidson, 2005). So, the anti-microbial properties of

each paraben also depend on pH. The esterified carboxyl group of benzoic acid allows the molecules to remain undissociated up to pH 8.5 compared to the normal dissociation of benzoic acid at pH 5.0 (Davidson, 2005). Benzoic acids can keep the molecules undissociated at pH 2.5 to 4.0 (Davidson, 2005). According to Davidson (2005), parabens are effective at pH levels ranging from 3.0 to 8.0.

In *in vivo* and *in vitro* studies, parabens are fully absorbed through the skin and gastrointestinal tract; they are fully metabolized and rapidly excreted through the urine (Gil *et al*, 2012). Parabens in contact with the skin penetrate the stratum corneum in inverse relation to the ester chain length (Elder *et al*, 1984). The lipophilicity of alkyl parabens and carboxylesterases hydrolyzed into the skin is characterized by the alkyl chain of the ester group (Cashman and Warshaw, 2005). Various in vitro and cell-based studies report that parabens have an estrogenic effect on human skin. Estrogenicity depends on the alkyl chain length of paraben esters. The estrogenicity of parabens increases as the alkyl chain length of the paraben ester increases (Prusakiewicz et al, 2007). Epidermal tissue does contain lower esterase activity than the liver when parabens are absorbed into the body (Prusakiewicz et al, 2007). Epidermal extracts can hydrolyze parabens when applies to the skin (Lobemeier et al, 1996). Therefore, the stability of paraben is higher on the skin. Histamine release from most cells is also activated by parabens. Histamine release activity of parabens is higher with a shorter alkyl ester chain than with a longer ester chain (Uramaru *et al*, 2007).

1.2.3 Physiochemical properties of parabens.

Parabens are simple aromatic carboxylic acids that contain a carboxyl group that is strongly attached to the benzene ring. Therefore, it makes them more stable in the air. According to Davidson (2005), parabens are stable in the air and resistant to cold and heat, including steam sterilization. It has no oxidation reaction at room temperature. It could be melted at 122°C and boiled at 249°C (Davidson 2005). All parabens are solid crystalline white powders that have no odour and no taste, except methyl paraben. "Methyl paraben has a faint characteristic odour (Rossmoore, 1995).

1.2.4 Solubility of parabens.

The chemical substitutions of paraben provide different solubilities and a spectrum of antimicrobial activity against different microbes (Cashman and Warshaw, 2005). Solubility

depends on the length of the alkyl ester chain. The longer chain of alkyl esters has increased oil solubility and decreased water solubility and the greater the oil solubility, the greater the penetration through the epidermis layer of skin (Twist and Zatz,1986). As a result, longer alkyl chains have better penetration through the skin. All parabens have poor aqueous solubility at room temperature but are completely dissolved in solution at temperatures above 60 °C (Giordano et al. 1999). The solubility of Parabens in the aqueous phase is relatively high in shorter alkyl esters chains (Cashman and Warshaw, 2005). When shorter chain parabens are required to dissolve in water that recommends pre-heating the water, if heating is not suitable then using sodium salts increases the solubility (Giordano *et al* 1999).

1.2.5 Bioavailability of parabens.

The bioavailability of parabens is higher in oil-in-water formulations than in water-in-oil formulations (Sasseville, 2004). The bioavailability of all parabens depends on the relative distribution of the oil or water phases in formulations and their various surfactants. According to Cashman and Warshaw (2005), the potential percutaneous absorption of parabens is influenced by the relative distribution of the oil or water phases in formulations. Furthermore, it will be changed by the addition of various surfactants (Esposito *et al,* 2003). Those with longer chains are more soluble in oil, and those with shorter chains are more soluble in water (Cashman and Warshaw, 2005). As a result, shorter-chain parabens require higher concentration to maintain their effectiveness.

1.2.6 Mechanism of action of parabens.

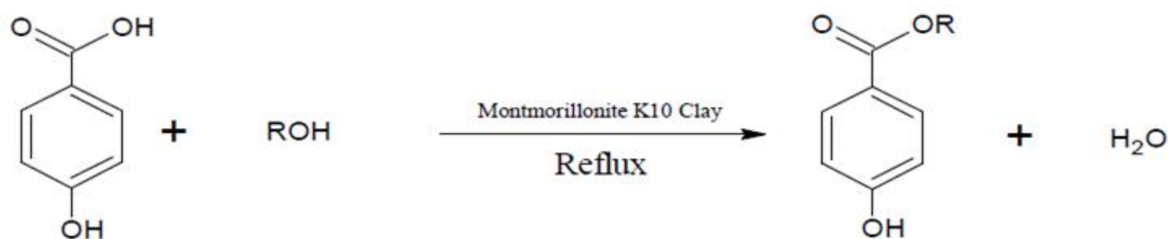
Parabens have excellent antimicrobial properties that have been used in most skin care products and food additives to maintain their effectiveness. They are stable, effective over a wide pH range, and active against a broad spectrum of microbes (Valkova et al, 2001). However, their mechanism of action has not been established. "Various studies have shown that parabens are active at the cytoplasmic membrane" (Davidson, 2005). The cytoplasmic membrane could be disrupted due to the leakage of intracellular compounds. "Leakage" of intracellular RNA (ribonucleic acid) by *Serratia marcescens* in the presence of parabens" (Furr and Russell, 1972). The amount of leakage from the intracellular membrane increases with the increase in the alkyl chain length of the parabens. Nes and Eklund (1983) found that paraben can inhibit DNA and RNA synthesis in *Escherichia coli* and *Bacillus subtilis. Studies on* alanine, serine, phenylalanine, and glucose by whole cells established that paraben reduces amino acids but

not glucose. Parabens reduce the effect of chemical and electrical forces that establish a normal membrane gradient (Nes and Eklund, 1983). Moreover, parabens are also able to inhibit both membrane transport and the electron transport system (Davidson, 2005).

The antimicrobial activity of parabens is also related to the alkyl chain length of the ester group. The antimicrobial activity of parabens is proportional to the alkyl chain length. "The hydrophobicity and octanol-water partition coefficient of parabens increase with the ester chain length" (Jungman, Laugel, and Baillet-Guffroy, 2013). The antimicrobial properties are inversely proportional to water solubility. Parabens with longer alkyl ester chains show better antimicrobial activity (Valkova et al, 2001). Propyl paraben and Butyl paraben are more effective than methylparaben. They are mainly active against fungi but also exhibit high activity against gram-positive bacteria (Cashman and Warshaw, 2005). The amount of paraben dissolved in the water phase determines the preservative's activity (Jungman, Laugel, and Baillet-Guffroy, 2013). Generally, microbial replication occurs in a water phase preparation, and parabens can solubilize in the water phase preparation to kill microorganisms (Steinberg, 2006). Therefore, parabens are mostly used in water-containing preparations such as lotions, creams, shampoo conditioners, and liquid soaps.

1.3 Synthesis of parabens

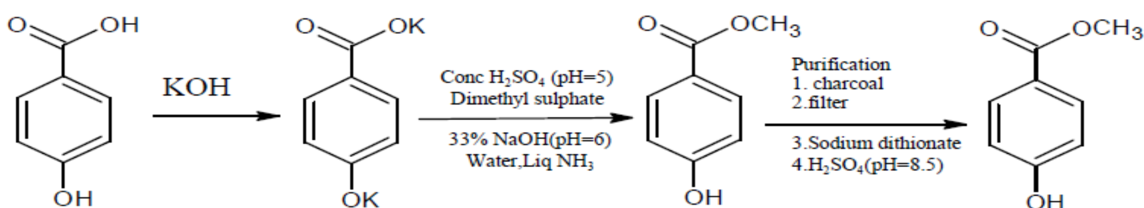
All commercially used parabens are synthetically produced, although some are identical to those found in nature (Hazarika, 2007). According to Valkova et al (2001) Methyl paraben and Propyl paraben are found in nature. Parabens and their derivatives are commonly found in various vegetable foods, such as barley, strawberries, black currants, peaches, carrots, onions, cocoa beans, and vanilla (Anthony, 2015). However, those used in the cosmetics industry as preservatives are always synthesized. Commercial parabens require quite a simple process to synthesize. Usually, they are synthesized by an esterification reaction (Hazarika, 2007). The two reactants involved are 4-hydroxybenzoic acid along with an alcohol such as methanol, ethanol, propanol, or butanol. This synthesis has been done using montmorillonite K10 clay as a heterogeneous catalyst. The alcohols are easily obtainable, but the 4-Hydroxybenzoic acid often needs to be synthesized before being reacted with an alcohol. Furthermore, 4-Hydroxybenzoic acid can also be easily synthesized through a carboxylation reaction between phenol and carbon dioxide (Hazarika,2007).



(Hazarika,2007).

Figure 3: Synthesis of paraben

Shodhganga (2016) reported, "an improved method for the synthesis of 4-hydroxy methyl benzoate using p-hydroxybenzoic acid through dipotasium salt of p-hydroxybenzoic acid and esterification was carried out by dimethyl sulphate and adjusting the pH of the reaction mass by sulphuric acid, NaOH, and ammonia". Thereafter, the crude product was purified by charcoal and sodium dithionate to give p-hydroxy methyl benzoates.

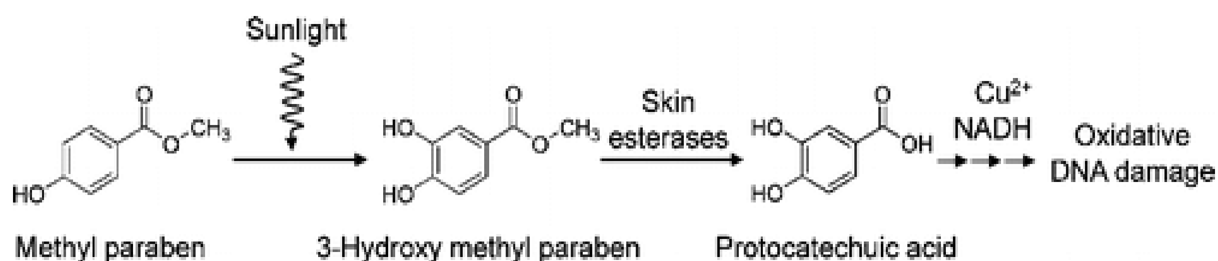


(Shodhganga, 2016)

1.4 Use of parabens in formulations.

In formulation preparation, preservatives are required to avoid microbial contamination and assure a long shelf life. In commercial preparations, preservatives have to be widely used, mostly in those that are low-cost and easy to handle. One of them is paraben, which has a great extent of antimicrobial effect as well as low cost and safety of handling. Parabens are widely used as preservatives in various commercial preparations, such as skin care products and food additives (Jungman, Laugel, and Baillet-Guffroy, 2013). However, recent studies on the effects of parabens on human skin have reported that they exhibit estrogenic activity on human skin and have been found in human breast tissues (Darbre, 2004). Paraben safety studies proved that parabens are safe for the human body. "Toxicology studies in rodents and humans indicate that parabens are practically non-toxic, non-irritating and non-sensitizing" (Elder *et al*, 1984). Therefore, the United States Food and Drug Administration approved parabens as preservatives in topical products (FDA, 2014). The application of parabens to skin that has been exposed to

sunlight causes changes in epidermis cells that could lead to skin cancer (Boberg, 2010). It reacts with the UVB rays from the sun, resulting in increased aging of the skin and DNA damage.



(Boberg, 2010).

Once applied and absorbed into the skin, parabens can bypass the body's metabolic processes and enter the organs and bloodstream (Seko et al 1999). Parabens can cause endocrine disruption by interfering with hormone function and mimicking estrogen in the body by binding to cellular estrogen receptors. Estrogen mimickers can increase gene expression and they cause breast tumour cells to appear (Boberg, 2010). Recently, worldwide regulatory acceptance has tried to establish an alternative as preservatives in topical cosmetics and pharmaceuticals due to their estrogenic effect on human skin (Prusakiewicz et al, 2007).

Different metabolic routes lead to metabolized paraben within the body. Parabens are metabolized systemically and excreted in the urine (Jewell *et al* 2007). Mainly the biotransformation of parabens includes hydrolysis of the ester bond and glucuronidation reactions (Abbas et al 2010). Glucuronidation is the main mechanism in which xenobiotics (a substance that is foreign to the organism and not usually naturally produced) are transformed into water-soluble substrates, that leads to excreted through urine or bile (Sanchez and Kauffman, 2010). In a study, parabens were investigated in liver microsomes and plasma. The results showed that the shorter chained parabens (methyl and ethyl) were stable in plasma and that 95% of the concentration that was added remained after twenty-four hours, while the longer alkyl chained parabens (propyl and butyl) showed the initial concentration to decrease up to 50% during the same time (Abbas et al 2010). The study involving the liver microsomes showed rapid hydrolysis, with the alkyl chain length.

- Studies on the skin safety of parabens

As shown in Figure 4; the metabolic pathway is hydrolysis to 4-hydroxybenzoic acid which is subsequently metabolized to 4-hydroxyhippuric acid, 4-hydroxybenzoyl glucuronide, and 4-hydroxybenzoyl sulphate (Jewell et *al* 2007). Parabens are hydrolyzed by Carboxylesterases; those are present in both microsomal and cytosol fractions of tissues. Human carboxylesterase 1 (hCE1, CES1) and carboxylesterase 2 (hCE2, CES2, hiCE) are serine hydrolases that metabolize ester drugs and xenobiotics. Although both isoforms are present in many tissues, hCE1 and to a lesser extent hCE2 are present in liver microsomes, whereas hCE2 is the esterase predominantly expressed in small intestine microsomes (Imai *et al*., 2006). hCE1 has been shown to prefer substrates with a small alcohol group and a large acyl group, whereas hCE2 hydrolyses lipophilic substrates with a large alcohol group and a small acyl group (Jewell et *al* 2007), The physiochemical properties of the series of parabens with their structure, solubility, and lipophilicity, make these compounds particularly interesting for studies of hydrolysis during absorption (Jewell et *al* 2007). These characteristics were utilized to demonstrate the dominance of hCE1 and hCE2 in the human liver and small intestine (Imai *et al*, 2006), however, the role of carboxylesterase isoforms for paraben hydrolysis has not been evaluated in human skin.

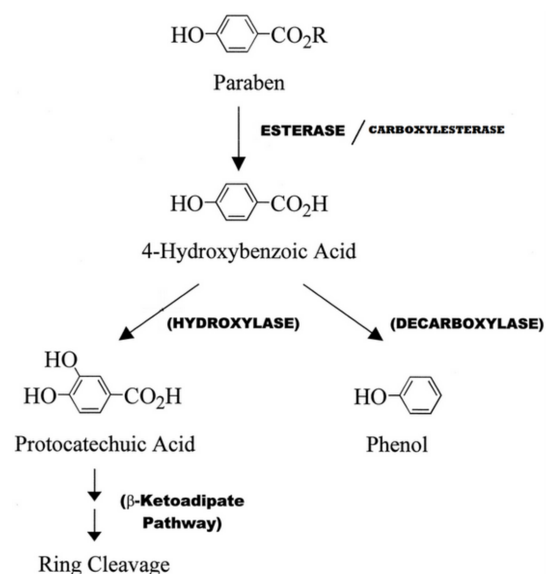


Figure 4: The metabolic pathway of hydrolysis of paraben

(Valkova *et al*,2001)

Skin is the largest organ of the body. Its primary function is to act as a permeability barrier to the surrounding environment. The outer layer of the skin is the stratum corneum, which has an essential role as a barrier to the transport of water and xenobiotics (Imai *et al*, 2013). "The stratum corneum consists of 15–25 layers of corneocytes that are surrounded by intercellular lipids" (Wilkinson, 2008). According to Wilkinson (2008), "each corneocyte is approximately 40 µm in diameter and 0.5µm thick and they represent the end stage of keratinocyte differentiation". Those corneocytes are filled with tight bundles of intracellular keratin that are surrounded by layers of highly cross-linked proteins, which are called cornified cells (Eckhart *et al*, 2013). The skin is no longer fully regarded as an inert barrier capable of retaining internal

moisture while allowing topical chemicals to enter (Wilkinson, 2008). "Numerous enzyme activities have now been identified in several cutaneous tissues (including whole skin, isolated keratinocytes, appendages, and cell lines) which are capable of a considerable variety of chemical transformations of both endo and xenobiotic compounds" (WHO, 2014). According to Imai *et al*, (2013), any xenobiotics which penetrate the skin are biotransformed into harmless or less harmful compounds by various enzymatic activities in the epidermis and dermis. The skin contains a variety of enzymes that are capable of xenobiotic metabolism, including a range of Phase I and Phase II systems. This wide range of Phase I and Phase II metabolic biotransformations can be carried out in the skin, and most dermal metabolism occurs in basal keratinocytes in the epidermis (Imai *et al*, 2013). Constitutive expression of xenobiotic-metabolizing enzymes has been detected in normal human keratinocytes (Imai *et al*, 2013).

"In Phase, I metabolism, xenobiotics interact via oxidation, reduction, or hydrolysis and in Phase II metabolism, these functionalized compounds are conjugated to compounds such as glucuronic acid, sulphate, glycine, and glutathione or further metabolized by epoxyhydrases and other oxidoreductases to increase their molecular weight and water solubility" (Wilkinson, 2008). During Phase I metabolism, further metabolism can result in increased toxicity from chemicals (Wilkinson, 2008). The Phase I enzymes are a number of cytochromes (CYP) P450 isoenzymes, esterases, dehydrogenases, reductases, and Phase II enzymes are glucuronide, sulphate, acetate, and glutathione transferases that are present in dermal tissue; they contribute to the skin's tolerance of oxidative stress caused by topical exposure to chemicals and solar radiation (Wilkinson 2008).

Paraben esters are used in over 22,000 cosmetics as preservatives at concentrations up to 0.8% w/w (mixtures of parabens) or up to 0.4% w/w (single paraben) (Campbell, Yoon, and Clewell, 2015). They include methyl paraben, ethyl paraben, propyl paraben, isopropyl paraben, butyl paraben, isobutyl paraben, and benzyl paraben. Some articles report the daily use of cosmetic products containing parabens at 17.76g for adults and 378mg for infants (Ozaki *et al* 2013). The Cosmetic Ingredient Review (CIR) Expert Panel (2008) reported a low grade of paraben toxicity at concentrations that are used in cosmetics, according to acute, sub chronic, and chronic toxicity tests using a range of exposure routes. The use of parabens has been extensively studied to evaluate male reproductive toxicity. CIR (2008) demonstrated in an *in vitro* study that sperm was not viable at concentrations as low as 6mg/ml of Methylparaben,

❖ Studies on the skin safety of parabens

8mg/ml of Ethylparaben, 3 mg/ml of Propyl paraben, and 1 mg/ml Butylparaben, but an *in vivo* study of 0.1% or 1.0% Methylparaben or Ethylparaben in the diet of mice reported no spermatotoxic effects. The present review discusses the evidence that parabens may not be as safe as initially thought and suggests that the interaction between parabens and mitochondrial function in the testis may be key in explaining the contribution of parabens to a decrease in reproductive capacity (Renata *et al* 2009). The CIR (2008) considers that these concentrations may have a possible adverse effect on the safety of cosmetic products in which parabens are useful as preservatives. However, there is no range of paraben concentration yet to be disclosed that is considered safe for cosmetic products.

In recent studies, parabens may cause adverse side effects. One of the most significant is that they have estrogenic activity in human skin. This has been linked to an increased risk of developing breast cancer (Darbre and Harvey, 2008). Additional adverse effects are prolonged exposure can cause the possibility of the growth of malignant melanoma (skin cancer) and infertility (Boberg, 2010). “Estrogen is a major etiological factor in the growth and development of the majority of human breast cancers” (Oishi, 2004). Brunei University's Department of Biology and Biotechnology discovered that the most serious concern about parabens is their ability to mimic estrogen in laboratory animals (Boberg, 2010). These risks are further aggravated by the ability of parabens can be absorbed by human skin without being degraded by esterase enzymes (Imai *et al*, 2013). This research also exposes that any parabens at a low concentration, with long-term exposure to the body, can also be absorbed and retained in tissues without metabolism by esterases. In the structures, all paraben contains a benzene ring. This ring allows parabens to be detectable by UV at very low concentrations (as they have a good chromophore). While they have no ionic functional groups, they are lipophilic. “Some accumulation in fatty tissues of the body would be expected due to the lipophilic nature of the structures” (Lobemeier *et al*, 1996).

The extensive application of parabens in commercial products increases the probability of exposure to the human body through dermal contact, ingestion, or inhalation. Various in vitro and cell-based assays have shown parabens can compete with estrogens for binding to the estrogen receptors. Other assays have shown parabens to be estrogenic, albeit much less potent than estradiol. Each study reported an increase in estrogenicity with an increasing chain length of paraben ester. Therefore, in 2011, the Danish government banned the use of parabens in

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personal care products based on the possibility of high systemic absorption from an immature metabolism and skin barrier dysfunction however in 2006 the European Union (EU) government regulatory boards examined parabens and most have agreed that current concentrations of parabens in skin product are safe for consumer use as well as set up limits in cosmetic products at concentrations of 0.4% w/w for any individual paraben and 0.8% w/w for total paraben concentrations not only that United States, FDA and the Cosmetic Ingredient Review (CIR) also agreed with EU (Mark, *et al* 2013). However, the Association of Southeast Asian Nations (ASEAN) Cosmetics Committee (ACC) decided at its recent meeting to ban the use of parabens as preservatives in cosmetics due to their adverse effect on the human body (Chamicalwatch, 2015). Therefore, there is conflicting evidence on the safety of the use of parabens as preservatives (with respect to potential tumorigenic effects). As a result, there is a need for further investigations.

1.5 Analytical methods for paraben detection and quantification.

Many analytical techniques can be used for the analysis of parabens. However, the most used technique is high-performance liquid chromatography (HPLC). whereas ultrahigh performance liquid chromatography (uHPLC) is used in fewer sources. A reversed-phase system with gradient elution with a mixture of water and organic solvents is frequently used. "Gas Chromatography (GC) and capillary electrophoresis (CE) has been less reported techniques for the analysis of paraben, and single determinations have been made using spectrophotometric or voltametric methods" (Irena *et al* 2014). An HPLC method can be used for the identification and quantitative determination of the parabens within a range of pharmaceutical formulations. "It is assumed that the content of the formulations would be determined using one chromatographic system" (Zabrzewska, Chyła, and Bogdan, 2014)**.** Most parabens are highly soluble in many organic solvents such as alcohol, acetone, ether, etc. This solubility of paraben makes reversed phase HPLC an ideal analytical technique for the separation/identification of paraben (Coral *et al* 2005).

Before the HPLC analysis, a suitable extraction method is required to determine to extract parabens from the water-based formulations. (Fenhua, Dandan, and Zhimin 2016). Several extractions and separation techniques have been developed which include shaking and sonication extraction, solid-phase extraction, and solid-phase microextraction. Solid phase extraction is an extensively used sample preparation technique for the separation or extraction

of selected analytes in a variety of matrices (Rebbeck, et al 2006). “This technique has benefits such as preconcentration of trace analytes from larger volumes of samples and online coupling with various detectors” (Hashemi, Shamsipur, and Fattahi, 2015). Therefore, during this research, HPLC chromatographic analysis was used to determine paraben and their metabolites from the extraction and diffusion study.

- Studies on the skin safety of parabens

2. Aims of the research

People use cosmetics daily to protect their skin from the sun and keep skin fresh and healthy. Nowadays, cosmetic users are becoming more conscious of the safety of cosmetics. Commercially used skin care products must contain safe chemicals without harmful impact on human skin. The expectation of long shelf-life and microorganism-free products require the use of preservatives, and parabens are one of them. Due to reported adverse reactions, parabens have been banned from some countries. However, FDA and CIR stated certain concentrations of paraben are safe for human skin. Despite the controversy surrounding parabens, they are still used as preservatives in most skin care formulations due to their efficient antimicrobial properties. In this research, our major goal was the safety evaluation of parabens in human skin as well as to challenge those "safe" concentrations of parabens approved by the FDA and CIR.

The overall **aim** of this work has been investigated via the following objectives:

- ❖ To investigate the feasibility of addressing the safety of parabens used in cosmetics through cytotoxicity assay in human keratinocyte cells. During this study, this toxicity will be analyzed with an SRB study. **(Chapter 1)**
- ❖ To investigate the solubility of parabens in a culture medium to assess appropriate toxicity in human skin. **(Chapter 1)**
- ❖ To investigate the percentage (%) metabolism of paraben via pig skin. As pig skin has similar histological and physiological properties to human skin. This permeation of parabens will be evaluated through the Franz diffusion study. **(Chapter 2)**
- ❖ To investigate paraben metabolism in human keratinocytes through cell extracts at three different times of incubation. After incubation, parabens and their metabolite will be extracted by lysis buffer. **(Chapter 3)**
- ❖ To establish the ranking order of parabens according to their toxicity and metabolism in human keratinocyte cells. **(Chapters 1 & 3)**

In this research, a cytotoxicity assay will be conducted with methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), and benzyl paraben (BzP). However, the permeation and extraction studies will be performed with the most used parabens which are methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), and butyl paraben (BP).

3. Chapter 1: Determination of paraben toxicity in human keratinocytes cell line using a cytotoxicity assay.

Abstract

Parabens are a group of alkyl esters of 4-hydroxybenzoic acids. It has been successfully used as a preservative in skin care products since the 1920s. They are esterified with an alkyl chain which is structurally responsible for anti-microbial activity. Paraben safety studies proved that parabens are safe for the human body. Therefore, The United States Food and Drug Administration approved parabens as preservatives in skin care products and they set a concentration of 0.4% w/w in a single paraben and 0.8% w/w in a combination of parabens. However, recent studies on the effects of parabens reported that they exhibit estrogenic activity on human skin. Therefore, the Danish government banned all paraben use in cosmetics, but European Union (EU) government regulatory boards claim that current concentrations of parabens in skin products are safe for cosmetic products. Therefore, paraben's use in skin care products is controversial.

This study demonstrated the feasibility of addressing the safety of parabens in skin care products such as methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), and benzyl paraben (BzP) was investigated by evaluating the recommended concentration that is claimed to be safe for consumer products. From this study, we established a ranking order of paraben toxicity. The toxicity of parabens was analyzed in human keratinocytes through a cytotoxicity assay. The study was conducted with an SRB assay. During this experiment, we also investigated the solubility of parabens in an aqueous solution at different temperatures. This study showed parabens at low concentrations were less toxic; increasing the concentration of parabens increased toxicity, and toxicity was also increased with longer alkyl ester chains. This toxicity analysis through SRB assay was a novel finding of our research.

3.1 Aim & objectives.

Parabens are the alkyl ester of 4-hydroxybenzoic acid/p-hydroxybenzoic acid and the most common and effective preservatives in cosmetics industries (Ferdinando, *et al* 2000). Their alkyl ester chains are attached to a benzene ring that; is structurally responsible for antimicrobial activity. Longer alkyl ester chains are more active than shorter alkyl ester chains paraben (Valkova et al, 2001). In this research, we used five common parabens such as methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), and benzyl paraben (BzP) which are commercially used in cosmetics /skin care products. All parabens are structurally different from each other on the alkyl ester chain as shown in Figure 2. Paraben safety studies on humans and animal show; they are safe and effective for skin care products. FDA and CIR both agree and recommend a set of concentrations for paraben use in cosmetics. England and European Union are following FDA and CIR guidelines while using a paraben in skin care products. FDA continues collecting safety data sheets from different research on parabens.

Recent studies of parabens have shown estrogenic activity on human skin (Darbre, 2004). However, some other studies reported paraben is safe (Elder *et al* 1984). Considering toxicity, paraben safety studies so far are inconclusive. Once parabens enter the human body, immediately absorbed, and metabolized, then excreted from the body (Cashman and Warshaw, 2005). "Numerous acute toxicity studies, as well as sub-chronic and chronic oral studies, confirm their low toxicity, non-sensitivity, and non-irritability" (Elder *et al* 1984). However, *in vitro,* and *in vivo* studies have shown estrogen agonist properties of parabens in the epidermis layer of human skin (Prusakiewicz et al, 2007). Paraben exhibits its estrogenic effects by binding with estrogen receptors to inhibit estrogen sulfotransferases (SULTs) enzyme in the epidermis layer of the skin as shown in figure 5 (Prusakiewicz et al, 2007). Topical application of paraben can also lead to skin cancer. Parabens exposed to sunlight cause changes in epidermis cells that could lead to skin cancer (Boberg, 2010). On the other hand, estrogenicity in early pregnancy studies indicated that paraben may not be as potent as previously reported (Shaw and deCatanzaro, 2009). Therefore, the cosmetics regulatory body approved paraben; and set a specific concentration for paraben used in skin care products.

In this study, a range of paraben concentrations was evaluated for toxicity levels in skin care products. Those concentrations were analyzed in a human keratinocyte cell line. Human

Keratinocytes are the most common type of skin cell, as they make up the structural component of the epidermis (ATCC 2021). Keratinocytes can be extracted from different parts of the body. It consists of the epidermis (the outermost layer of the skin) layer of human skin which constitutes 90% of epidermal skin cells (ThermoFisher SCIENTIFIC, 2020). The basic role of those keratinocytes is essential for skin repair (Piipponen, Li, and Landén 2020). Keratinocyte cells are cultured to grow enough for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging, drug testing). In the culture process, cells are grown under controlled conditions, generally outside their natural environment (Segeritz and Vallier 2017). Cell confluency has to be 70-80% around the flask or container for any drug treatment or subculture (LONZA, 2021).

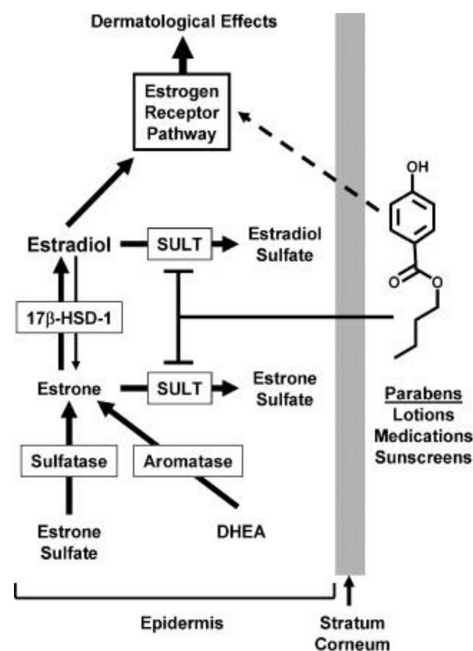


Figure 5: Estrogenic activity in skin

(Prusakiewicz et al, 2007)

In this study, paraben toxicity was performed in Sulforhodamine B (SRB) cell cytotoxicity assay. This is one of the most widely used methods to detect cell viability or drug cytotoxicity. Since 1990 this method has been developed and used for cytotoxicity assay (Skehan *et al.*, 1990). SRB can bind with cellular protein and measure the total biomass in drug components (Orellana and Kasinski, 2016). “SRB is a bright pink aminoxanthene dye that can form an electrostatic complex with basic amino acid residues of proteins in slightly acidic conditions, but it can dissociate under basic conditions” (Biotrend, 2020). Different types of cells including cancerous and non-cancerous cell lines have been scanned for Drug toxicity through this method. In addition, this assay has less interference in testing compounds due to independent cell metabolic activity (Biotrend, 2020). SRB binds with the cell line and releases the incorporated dye after washing from stained cells which are directly proportional to the cell biomass (Biotrend, 2020). It can be measured at 565 nm (Orellana and Kasinski, 2016). SRB cell cytotoxicity assay is simple, accurate, reproducible, and sensitive. “This is an excellent and efficient method for *in vitro* cytotoxicity studies as well as high-throughput drug screening that can detect between 5,000-50,000 cells per well” (Biocompare, 2022).

- Studies on the skin safety of parabens

3.2 Method and Materials

3.2.1 Determination of the saturation solubility of parabens in Ethanol

3.2.1.1 Instruments

Evolution™ 300 UV-Vis Spectrophotometer (Thermo Fisher Scientific. Hempstead, UK), M501 – Single Beam Scanning UV/Visible Spectrophotometer (Camspec Analytical instruments Ltd, Leeds, UK), cuvette, Electronic weight balance (Ohaus), electronic weight balance (GRAM, UK), Electronic weight balance (Ohaus Pioneer, Thetford, UK,) Thermometer (Thermo Fisher Scientific), Volumetric flask, Glass pipette, Filter paper, Syringe filter, supernatant sample vial, Baker and Pyrex glass tube.

3.2.1.2 Chemicals

Methyl paraben (Alfa Aesar, Heysham UK), Ethyl paraben (Alfa Aesar, Heysham UK), Propyl paraben (Alfa Aesar, Heysham UK), Butyl paraben (Alfa Aesar, Heysham UK), Benzyl paraben (Alfa Aesar, Heysham UK), ≥99% Ethanol (sigma Aldrich, UK).

3.2.1.3 Stock solution preparation (ethanol)

100 mg of paraben was dissolved in 100 ml ethanol to prepare a stock solution at 1 mg/ml concentration.

3.2.1.4 Preparation of calibration curve for parabens in ethanol

Stock solutions were diluted in 20 ml volumetric flasks to the following concentrations: 0.05 mg/ml, 0.01 mg/ml, 0.005 mg/ml, 0.001 mg/ml, and 0.0005 mg/ml. The absorbance of the standard solutions was measured using an M501 - Single Beam Scanning UV/Visible Spectrophotometer at λ max 229 nm for all parabens.

3.2.1.5 Saturation solubility of parabens in ethanol

Excess amounts of parabens were dissolved in stock solution (ethanol) to measure the saturation solubility via the shake flask method. 10 ml of paraben stock solution was transferred into pyrex glass flasks. Then, an excess amount of paraben (two or more full spatulas) was added to the same pyrex glass flasks until precipitation was formed, then gently shaken. After that, the flasks were placed in a 37°C water bath shaker for 24 hours. Then, 5 ml of sample was withdrawn from each flask and passed through a 0.2 µm filter to collect the supernatant. The

collected supernatant was diluted 1 in 100 and its absorbance was measured using an M501 - Single Beam Scanning UV/Visible Spectrophotometer.

3.2.2 Determination of saturation solubility of parabens in Epilife Medium with 5% (v/v) ethanol.

3.2.2.1 Instruments

Agilent Technologies 1290 infinity LC system (University of Sunderland, UK), Agilent infinity DAD detector, Agilent Zabox C18 UHPLC column, Sanyo CO2 Incubator (IR sensor), freezer (Liebherr, UK), Biological safety Laminar flow hood (Thermo Scientific, UK electronic weight balance (GRAM, UK), Ultrasonic heated water bath (Thermo Fisher Scientific, UK), Thermometer (Thermo Fisher Scientific, UK), Volumetric flask, Syringe filter, supernatant sample vial, pipette, Baker, and Falcon tube.

3.2.2.2 Chemicals

Methyl paraben (Alfa Aesar, Heysham UK), Ethyl paraben (Alfa Aesar, Heysham UK), Propyl paraben (Alfa Aesar, Heysham UK), Butyl paraben (Alfa Aesar, Heysham UK), Benzyl paraben (Alfa Aesar, Heysham UK), Acetonitrile (MeCN) (sigma-Aldrich, UK) HPLC grade, Trifluoroacetic acid (TFA) (sigma-Aldrich, UK), Epilife® Medium (sigma-Aldrich, UK), ≥99% Ethanol (sigma-Aldrich, UK) and Distilled water.

3.2.2.3 Sample Preparation in Epilife Medium with 5% (v/v) ethanol.

The sample was prepared in a 10ml volumetric flask. 10 mg of parabens were dissolved in 10 ml Epilife medium with 5% (v/v) ethanol to a target concentration of 1 mg/ml.

3.2.2.4 Preparation of calibration standards by HPLC.

Samples were diluted in a 5 ml volumetric flask to the following concentrations 0.2 mg/ml, 0.4 mg/ml, 0.5mg/ml, 0.6 mg/ml, 0.8mg/ml, and 1 mg/ml. After that, those were filtrated and diluted with 30% Acetonitrile MeCN in distilled water to be analyzed through the HPLC system at 210 nm of UV wavelength according to the Chromatographic parameter.

3.2.2.5 HPLC Analytical Assay

Throughout the whole project, an Agilent Technology 1290 infinity LC system was used to obtain the data. The system was fitted with an Agilent Infinity DAD detector running to detect a UV wavelength of λ 210nm. The system was fitted with an Agilent Zabox C18 UHPLC

column length of 50mm×2.1mm×1.7µm and the temperature was maintained at 40°C. The mobile phase A consisted of 0.1% TFA with a solution of Acetonitrile MeCN and distilled water at 90:10 as well as the mobile phase B consisted of 0.1% (v/v) TFA with a solution of Acetonitrile MeCN and distilled water in 10:90 using a gradient elution Table 1 with a flow rate of 0.3 ml/min. Throughout all experiments, the injection volume was set to 1.0 µl and the overall run time was 13 minutes. The post-run time was set to 1 minute.

Table 1: Gradient profile for HPLC Method.

Time (Minutes)	Mobile phase B: 0.1% TFA in MeCN: Water (10:90)	Mobile phase A: 0.1% TFA in MeCN: Water (90:10)
0	0	100
2	0	100
11	40	60
12	40	60
13	0	100

3.2.2.6 Saturation solubility of paraben in Epilife Medium with 5% (v/v) ethanol.

An excess amount of paraben was dissolved in Epilife medium with 5% (v/v) ethanol to measure the saturation solubility by the HPLC system. An excess amount of paraben was added to the 1 mg/ml of sample in a 5 ml volumetric flask. Then, placed into a heated water bath at different temperatures for 24 hours. After that supernatant sample was taken with filtration at a 0.2 µm filter and measured saturation concentration through HPLC Chromatographic parameter.

3.2.3 Method for cytotoxicity assay

3.2.3.1 Instruments

xMark™ Microplate Absorbance Spectrophotometer (BIO-RAD, UK), Sanyo CO2 Incubator (IR sensor), freezer (Liebherr, UK), Biological safety Laminar flow hood (Thermo Scientific, UK), Harrier 15/80 Centrifuge (MSE), microscope (Olympus, UK), Hemocytometer (Marienfeld, UK), 75-cm^2 culture flasks (Thermo Fisher Scientific, UK), 96-Well Cell Culture Plate (Thermo Fisher Scientific, UK) Electronic weight balance (GRAM, UK), Heated water bath (Thermo Fisher Scientific, UK), Thermometer (Thermo Fisher Scientific, UK), Volumetric flask, Syringe filter, supernatant sample vial, pipette, Baker and Falcon tube.

3.2.3.2 Chemicals

Methyl paraben (Alfa Aesar, Heysham UK), Ethyl paraben (Alfa Aesar, Heysham UK), Propyl paraben (Alfa Aesar, Heysham UK), Butyl paraben (Alfa Aesar, Heysham UK),), Benzyl paraben (Alfa Aesar, Heysham UK), Acetonitrile (MeCN) (sigma-Aldrich, UK) HPLC grade, Trifluoroacetic acid (TFA) (sigma-Aldrich, UK), Epilife® Medium (sigma-Aldrich, UK), Dulbecco′s Modified Eagle′s Medium (DMEM) medium (sigma-Aldrich, UK), Trypsin, Phosphate buffer Solution (PBS) (sigma-Aldrich, UK), Ethanol (sigma-Aldrich, UK), disinfectant liquid (sigma-Aldrich, UK)and Distilled water.

3.2.3.3 Cell line (Human Keratinocyte)

CCD-1106 KERTr (KERTr) cell lines obtained via the ATCC Cell were cultured in Epilife supplemented with human keratinocyte growth supplement (Life Technologies) and primocin (100 µg/ml). CCD-1106 cells were routinely cultured in 75cm^2 culture flasks. the cells were maintained in a humidified incubator at 37°C and 5% CO_2. All culture media (Epilife, DMEM, trypsin, and PBS) and CCD 1106 KERTr cells were warmed at 37°C temperature for 30 minutes in a heated water bath. Cells were allowed to reach 80% confluency before routine sub-culture, according to the following procedure: the cells were washed in prewarmed PBS then Trypsin was added to the culture flask and placed in the incubator at 37°C for 5 minutes to remove adherent cells. After the cells were detached from the flask, a prewarmed DMEM medium was added to the flask to transfer cells to a falcon tube. Cells were centrifuged at 1000 rpm and plated in a new flask with Epilife medium to incubate overnight.

3.2.3.4 Sample preparation and treatment

CCD-1106 cells were cultured in a 96-well plate where cells were counted using a hemocytometer and diluted with the required amount of epilife medium with to prepared 20 ml solution, each well contain 100 ul culture medium and incubate overnight to grow cells in a 96-cell plate to treating with parabens. For treatment with cell line, parabens were dissolved in epilife medium with 5% (v/v) ethanol to prepare paraben solution. Cells were incubated for 24 hours in a humidified incubator at 37°C and 5% CO_2 with 0 to 0.5% (v/v) concentrations of each paraben by added of 100ul paraben solution to create a final volume of 200ul /well in 96-well cell culture plate. Ethanol alone at a concentration of 5% (v/v) was used as vehicle control.

Each sample was prepared with 5% (v/v) ethanol in Epilife Media. The solubility of paraben in Epilife medium with 5% (v/v) ethanol at 40°C was used in this method. Parabens were treated in a cell line with their saturation concentrations at 40°C and their effect on keratinocyte stability was monitored. Saturated concentrations mg/ml were converted into percentages shown in table 2.

Table 2: Saturated concentration of each paraben in 5% (v/v) ethanol, at 40 ℃

Name	Saturation Concentration (mg/ml)	Saturation Concentration (g/ml)	Saturation Concentration % (w/v)
Methyl paraben	9.763	0.00976	0.97
Ethyl paraben	7.884	0.00788	0.78
Propyl paraben	6.211	0.00621	0.62
Butyl paraben	5.486	0.00548	0.54
Benzyl paraben	5.102	0.00510	0.51

3.2.3.5 SRB assay

TCA-fixed cells were stained for 30 minutes with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed, and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. The residual wash solution was removed by sharply flicking plates over a sink, which ensured the complete removal of the rinsing solution. After being rinsed, the plate was allowed to dry at room temperature overnight (or 1-2 hours at 60°C) until no standing moisture was visible. Bound dye was solubilized with 10 *mM* unbuffered Tris base (pH 10.5) for 20 minutes on a gyratory shaker and Absorbance was measured at 570 nm by using the xMark™ Microplate Absorbance Spectrophotometer.

3.3 Result and Discussion

3.3.1 Analysis of Saturation solubility of parabens in ethanol.

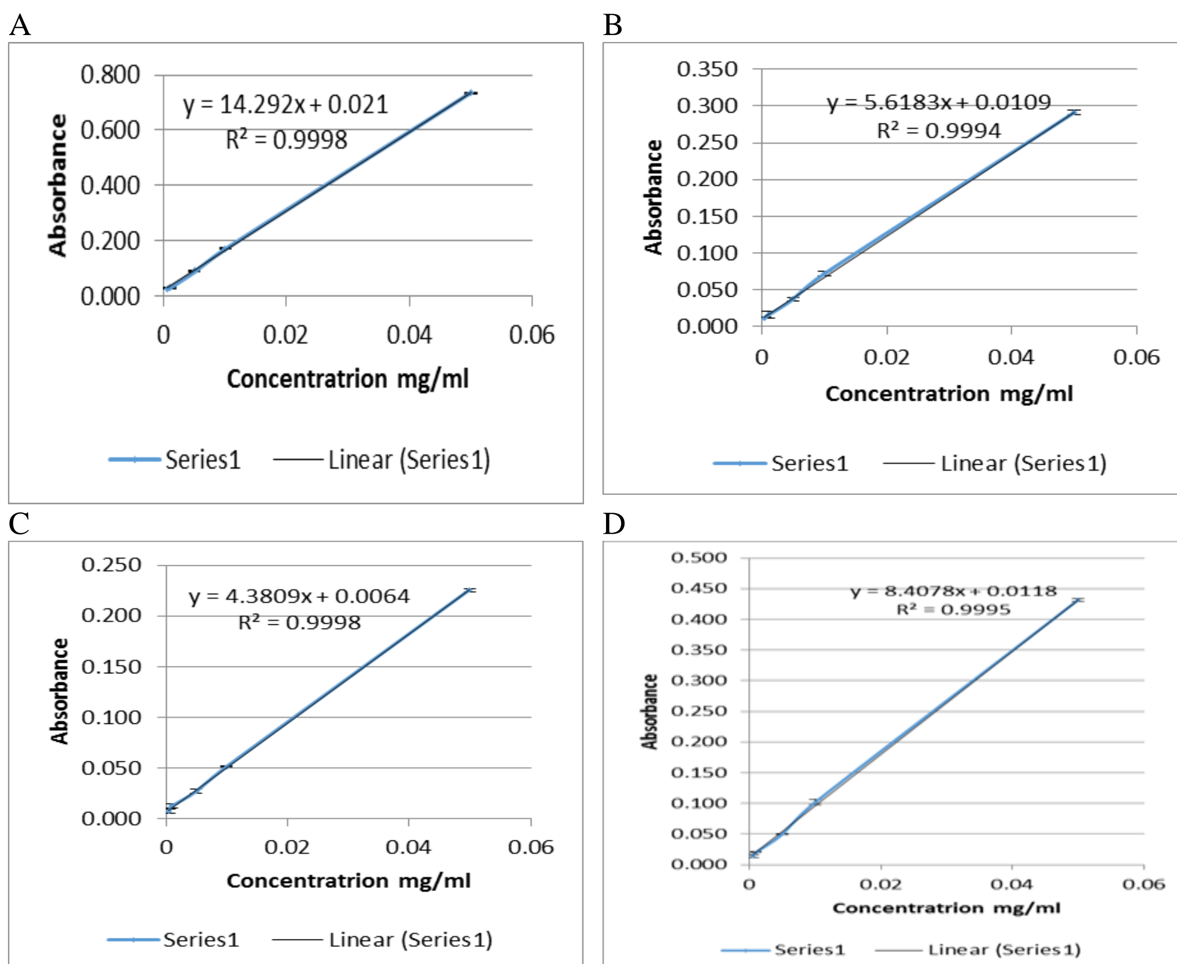
The chemical substitutions of parabens provide different solubility and spectrum of antimicrobial activity against different microbes (Cashman and Warshaw, 2005). Solubility depends on the length of the alkyl esters chain which means a longer chain of alkyl esters has increased oil solubility and decreased water solubility and the greater the oil solubility, the greater the penetration through the epidermis layer of the skin (Twist and Zatz 1986). Parabens are highly soluble in alcohol, acetone, and a number of other organic solvents (Cashman and Warshaw, 2005). In this study, the saturation solubility of parabens was determined in ethanol.

An analytical essay for the saturation solubility was carried out using a UV/Visible Spectrophotometer. 1mg/ml of each paraben was prepared as stock solution. The λ max of each stock solution was measured using an Evolution™ 300 UV-Vis Spectrophotometer. Those are shown in table 3.

Table 3: λ max for all parabens.

Paraben	Concentration mg/ml	λ max (nm)
Methyl paraben in Ethanol	1 mg/ml	229
Ethyl paraben in Ethanol	1 mg/ml	229
Propyl paraben in Ethanol	1 mg/ml	229
Butyl Paraben in Ethanol	1 mg/ml	229
Benzyl Paraben in Ethanol	1 mg/ml	229

An absorbance *vs* Concentration beer-lambert graph was plotted, and the linear regression equation was determined for all paraben in figure 6.



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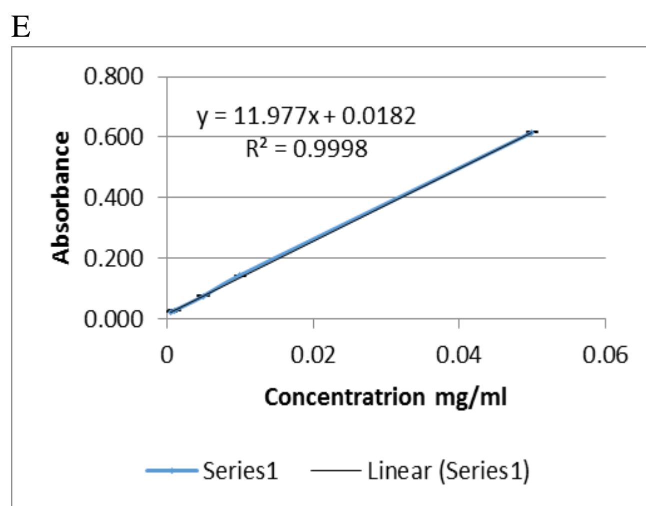


Figure 6: Beer-Lambert calibration plots for the standard solutions: A) Methyl Paraben, B) Ethyl Paraben, C) Propyl paraben, D) Butyl paraben, E) Benzyl paraben was made at a concentration range from 0.0005 mg/ml to 0.05 mg/ml in UV/Visible Spectrophotometer and plotted absorbance (n=3) *vs* Concentration.

Through this method saturation solubility was determined by using an excess amount of paraben dissolved in ethanol through an absorbance *vs* Concentration beer-lambert graph. The mean solubility, Standard deviation, and % relative Standard deviation for all parabens is shown in table 4. This study demonstrated that parabens were soluble in ethanol and ethanol was an excellent solvent and the solubility of parabens was very high.

Table 4: Saturation solubility for parabens in ethanol.

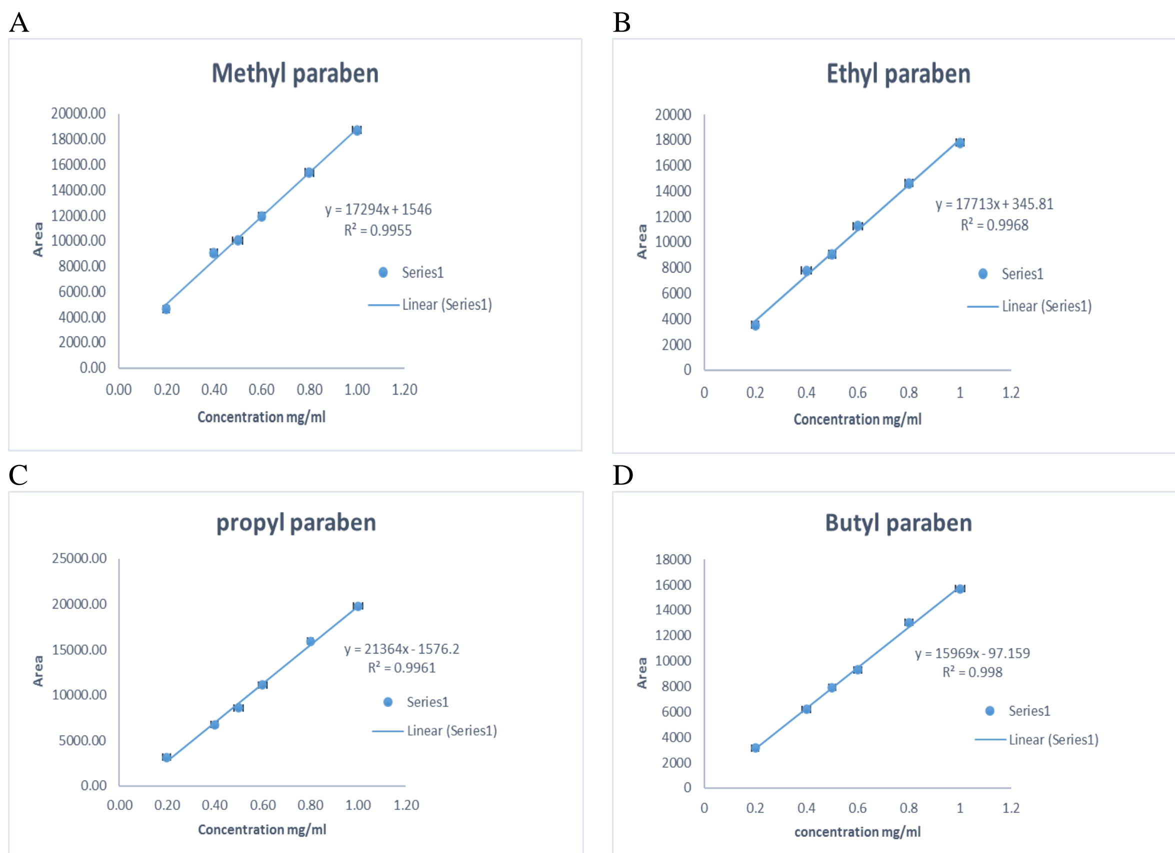
Paraben	Concentration g/ml			Mean g/ml	Standard deviation g/ml	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	0.153	0.421	0.310	0.295	0.1347	45.70%
Ethyl paraben	0.677	0.679	0.705	0.687	0.0156	2.27%
Propyl paraben	0.446	0.759	1.033	0.746	0.2937	39.36%
Butyl paraben	0.168	0.218	0.226	0.204	0.0314	15.39%
Benzyl paraben	0.217	0.284	0.311	0.271	0.0484	17.86%

3.3.2 Analysis of Saturation solubility of parabens in Epilife medium with 5% (v/v) ethanol.

Solubility plays an important role in permeation study; better solubility can have better absorption (Segeritz and Vallier 2017). Parabens are poorly soluble in an aqueous solution due

to lipophilicity (Twist and Zatz 1986). Epilife medium is an aqueous solution (Thermo Fisher Scientific, 2021). The solubility of paraben increases with a shorter ester chain in an aqueous solution (Cashman and Warshaw, 2005). Parabens are inadequately dissolved in an aqueous solution at room temperature (Giordano, *et al,* 1999). Pre heat is required to enhance the solubility of paraben in an aqueous solution (Ferdinando, *et al,* 2000). In this chapter, we examined parabens solubility in epilife medium which acts as an aqueous solution. To enhance solubility, ethanol was used in epilife medium, and the heat was applied for 24 hours. Parabens were highly soluble in ethanol. During this study, we investigated paraben solubility in epilife medium with 5% (v/v) ethanol at three different temperatures which were 15°C (room temperature), 40°C, and 60°C.

In this study, parabens were dissolved in epilife medium with 5% (v/v) ethanol and placed into a heated water bath at 15^0C (room temperature), 40°C, and 60^0C temperatures for 24 hours. The concentration of each paraben was determined through Area *vs* Concentration equation where the mean value (n=3) was considered shown in figure 7. Samples were prepared with a concentration range from 0.2 mg/ml to 1mg/ml diluted with 30:70 Acetonitrile MeCN and distilled water solution.



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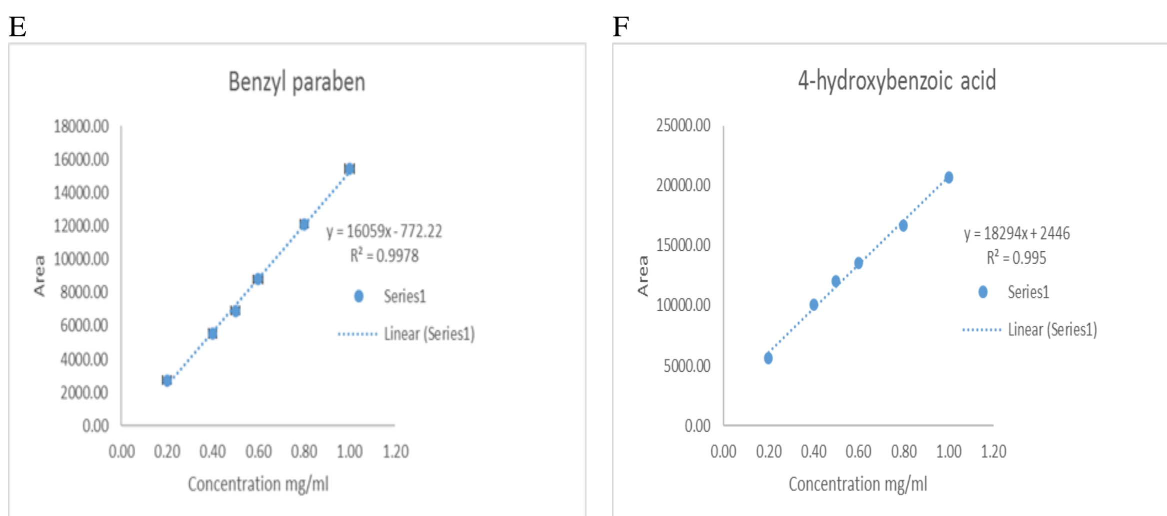

Figure 7: Calibration standard A) Methyl Paraben, B) Ethyl Paraben, C) Propyl paraben, D) Butyl paraben, E) Benzyl paraben and F) 4-Hydroxybenzoic acid was made at a concentration range from 0.2 mg/ml to 1mg/ml in HPLC parameter. Standards were made to volume in Epilife medium with 5% (v/v) ethanol. Mean (n=3) area against paraben concentration.

Saturation solubility of paraben in epilife medium with 5% (v/v) ethanol at three different temperatures at 15^{0}C (room temperature), 40^{0}C, and 60^{0}C was measured during this study through Area *vs* Concentration linear regression equation. Figure 7 demonstrated the linearity and equations of each paraben. Excess amount of paraben dissolved in epilife medium with 5% (v/v) ethanol at room temperature 15^{0}C for 24 hours to a measured saturated concentration of methyl paraben was 6.94 mg/ml, ethyl paraben was 5.05 mg/ml, propyl paraben was 3.89 mg/ml, butyl paraben was 2.77 mg/ml and benzyl paraben was 1.91 mg/ml shown in table 5. We also investigated 40^{0}C solubility of paraben in epilife medium with 5% (v/v) ethanol. Increasing the temperature resulted in increased solubility of parabens in an aqueous solution, this observation is in congruence with other studies (Ferdinando, *et al*, 2000). In this study, saturated solubility of parabens was measured at 400C which was 9.76 mg/ml of Methyl paraben, 7.88 mg/ml of Ethyl paraben, 6.21 mg/ml of propyl paraben, 5.48 mg/ml of Butyl paraben and 5.10 mg/ml of Benzyl paraben shown in table 6. At 60^{0}C temperature, the solubility of parabens has been increased. However, we also measured the solubility of paraben at above 600C which was 11.01 mg/ml of Methyl paraben, 9.12 mg/ml of Ethyl paraben, 7.09 mg/ml of Propyl paraben, 6.21 mg/ml of Butyl paraben and 5.90 mg/ml of Benzyl paraben shown in table 7.

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Table 5: Saturation Concentration of paraben at room temperature 15^0C

Paraben	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	7.125	6.989	6.698	6.94	0.22	3.17%
Ethyl paraben	5.032	5.233	4.891	5.05	0.17	3.36%
Propyl paraben	3.994	3.648	4.033	3.89	0.21	5.39%
Butyl paraben	2.725	2.559	3.011	2.77	0.23	8.32%
Benzyl paraben	1.885	1.714	2.121	1.91	0.20	10.49%

Table 6: Saturation Concentration of paraben at room temperature 40^0C

Paraben	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	9.701	10.031	9.556	9.76	0.24	2.45
Ethyl paraben	8.163	7.646	7.839	7.88	0.26	3.29
Propyl paraben	6.407	6.299	5.933	6.21	0.25	4.02
Butyl paraben	5.731	5.388	5.314	5.48	0.22	4.01
Benzyl Paraben	5.244	4.839	5.215	5.10	0.23	4.51

Table 7: Saturation Concentration of paraben at room temperature 60^0C.

Paraben	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	11.254	10.904	10.864	11.01	0.21	1.91
Ethyl paraben	9.355	9.111	8.899	9.12	0.23	2.52
Propyl paraben	7.345	7.031	6.879	7.09	0.24	3.38
Butyl paraben	6.449	6.201	5.986	6.21	0.23	3.71
Benzyl paraben	6.118	5.955	5.641	5.90	0.24	4.07

The size of the alkyl ester chain was inversely proportional to the solubility of parabens in all studied solvents. The lipophilicity of paraben increases with increased alkyl ester (Cashman and Warshaw, 2005). The saturation concentration of each paraben was used for treatment with the cell line, where epilife medium was used to culture the cell line. "EpiLife® greatly extends the in vitro lifespan of human epidermal keratinocytes compared to other serum-free media

systems" (ThermoFisher Scientific, 2016). Ethanol was an excellent solvent to improve solubility in epilife medium. This study demonstrated solubility decrease with an increased alkyl ester of paraben in epilife medium with 5% (v/v) ethanol. The mean (n=3) solubilities, standard deviations, and % relative standard deviations of parabens are shown in Tables 5, 6, and 7.

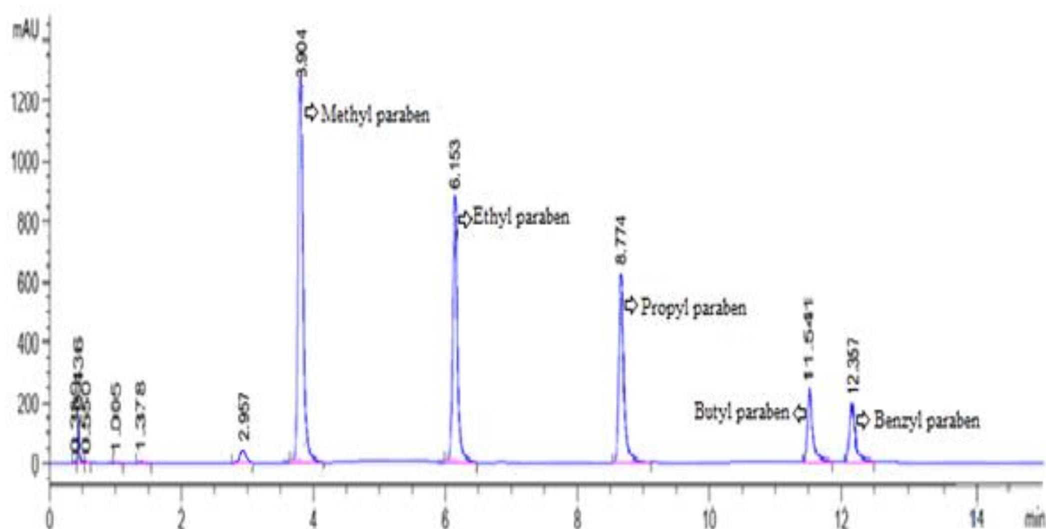

Figure 8: Test mix of parabens Including methyl paraben, ethyl paraben, propyl paraben, butyl paraben, and benzyl paraben at saturated concentration diluted with 30% Acetonitrile MeCN in distilled water run through HPLC. The chromatographic condition was performed as the above method.

During this experiment, we also prepared a combination of all parabens including methyl paraben 9.76 mg/ml, ethyl paraben 7.88 mg/ml, propyl paraben 6.21 mg/ml, butyl 5.48 mg/ml, and benzyl paraben 5.10 mg/ml in Epilife medium with 5% (v/v) ethanol as test mix to analyze with HPLC. Clear differentiation in peak elution was observed (figure 8). The chromatographic elution showed a retention time of methyl, ethyl, propyl, butyl, and benzyl paraben at approximately 3.90 min, 6.15 min, 8.77 min,11.54 min, and 12.35 min respectively at wavelength 210 nm. Due to the shorter ester chain, methyl paraben elutes earlier (Imamovic *et al* 2012). In parabens, the alkyl ester chain increased with increased retention time in HPLC analysis. In this study, each paraben was diluted with 30% Acetonitrile MeCN in distilled water to prepare the test mix and run through HPLC chromatographic condition shown in figure 8.

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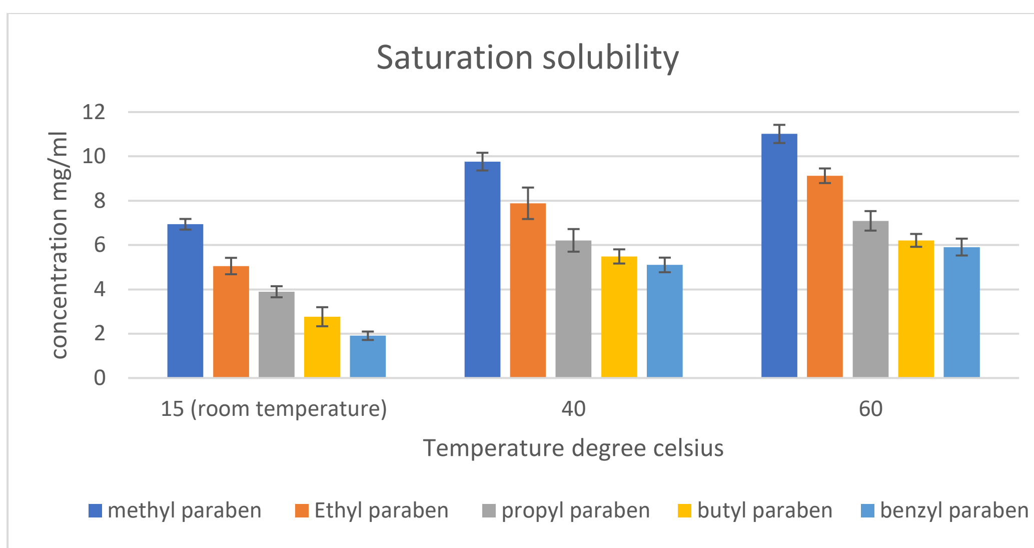


Figure 9: Saturation Solubility of paraben at three different temperatures at 15^{0}C (room temperature), 40°C, and 60^{0}C through HPLC analysis. Data are mean values +/- SD and 3 replicates respectively methyl, ethyl, propyl, butyl, and benzyl paraben from this experiment.

During this study, saturation concentration was involved in cytotoxicity assay preparation. Therefore, we used the solubility of paraben at 40^{0}C. The temperature of human skin is 35-40^{0}C (Lee *et al* 2019). For Compatible with human skin, we selected solubility of paraben in epilife medium with 5% (v/v) ethanol at 40^{0}C. Comparative analysis of saturation solubility of paraben in epilife medium with 5% (v/v) ethanol at 15^{0}C (room temperature), 40^{0}C, and 60^{0}C is shown in figure 9. This experiment proved that solubility increased with decreased alkyl ester chain paraben as well as the solubility improved at high temperatures in an aqueous solution.

3.3.3 Studies on cytotoxicity by exposure to parabens

Parabens have a great extent of antimicrobial effect as well as due to the low cost and safety of handling parabens are widely used as a preservative in various commercial preparation such as skin care products and food additives (Cashman and Warshaw 2005). However, recent studies on the effects of parabens on human skin reported that they exhibit estrogenic activity on human skin and have been found in human breast tissues (Darbre, 2004). Paraben safety studies proved that parabens are safe for the human body. "Toxicology studies in rodents and humans indicate that parabens are practically non-toxic, non-irritating and non-sensitizing” (Elder *et al* 1984). Therefore, the United States Food and Drug Administration approved parabens as preservatives in topical products (FDA, 2014). But recently, paraben potency, stability, and

worldwide regulatory acceptance made them an attractive choice as preservatives in topical cosmetics and pharmaceuticals due to the estrogenic effect on human skin (Prusakiewicz et al, 2007).

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (Thermo Fisher Scientific, 2021). “The advantage of using cell lines in scientific research is their homogeneity and associated reproducibility of results that can be obtained from using a batch of clonal cells” (Segeritz and Vallier 2017). Cells require to mature enough to treat with any substance for biological activity (Briske-Anderson, Finley, and Newman 1997). Treatment has proceeded when cell confluency is more than 80-90% in the culture flask (LONZA, 2021). Usually, a cell has matured within Passage 5-15 (number of the subculture) (Briske-Anderson, Finley, and Newman 1997). In this study, CCD-1106 KERTr (KERTr) cell line was prepared at passage (number of the subculture) 8-11. Cell density was also measured through a Hemocytometer of each flask to prepare the cell for this treatment shown in Table 4. Measurement of Cell density is important to maintain the cell quantity of each flask (Segeritz and Vallier 2017). Cells were cultured at 75cm^2 culture flasks and treated in 96 well plates.

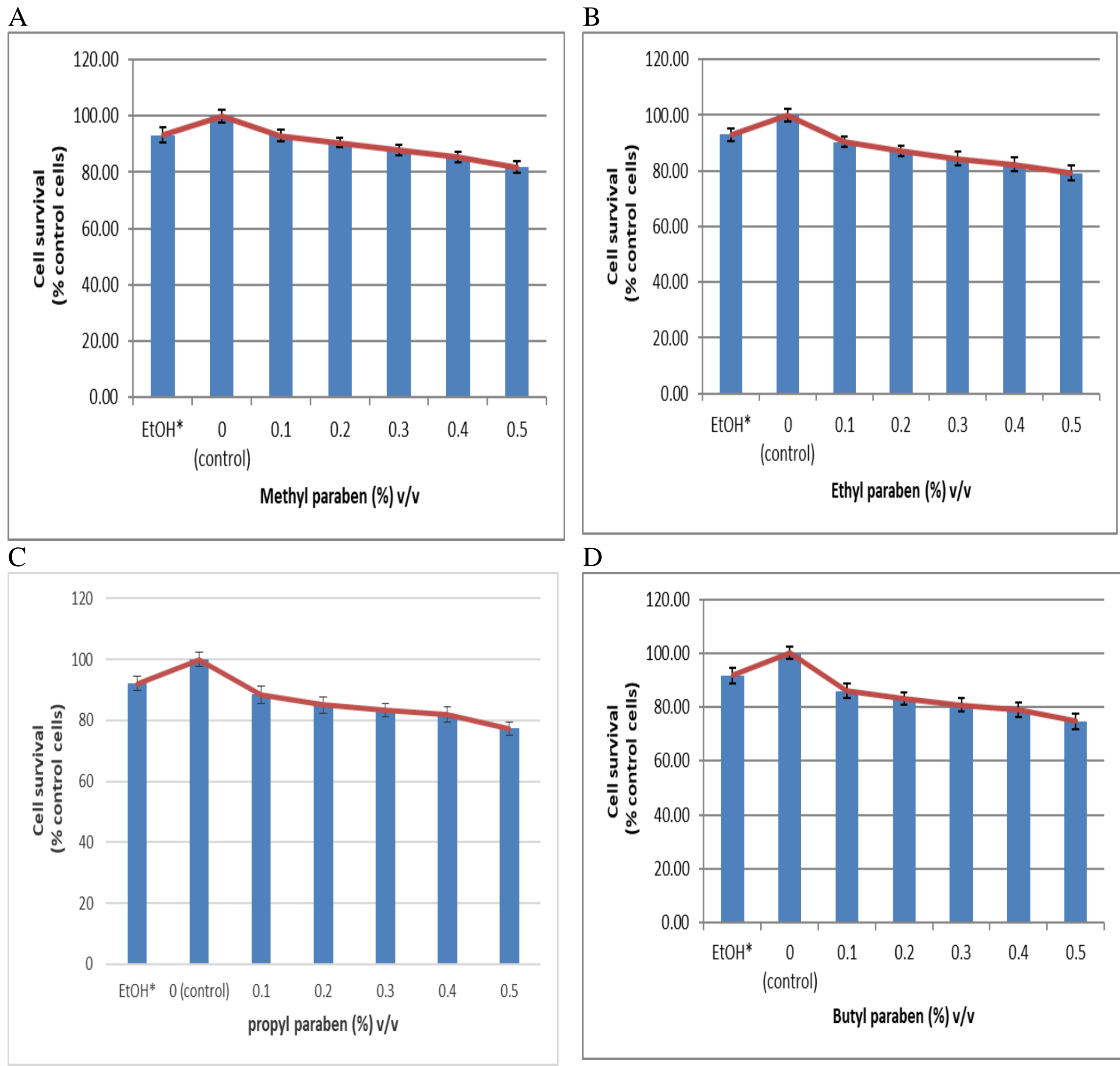
Table 4: Cell density of each paraben in 75-cm^2 culture flasks.

Name	The average number of cells	Target cell density	Total Volume	Dilution	Passage
Methyl paraben	2.1×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	3.2	8
Ethyl paraben	2.3×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	2.9	8
Propyl paraben	1.9×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	3.5	9
Butyl paraben	2.2×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	3.1	9
Benzyl paraben	2.1×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	3.2	10
4-hydroxybenzoic acid	1.9×10^5 cell/ml	3.4×10^4 cell/ml	20 ml	3.5	11

Throughout this method toxicity level of parabens including methyl paraben, ethyl paraben, propyl paraben, butyl paraben, and benzyl paraben was determined by a cytotoxicity assay. In this experiment CCD 1106 human cell was used to culture and treat with diluted concentrations 0% v/v, 0.1 % v/v, 0.2 % v/v, 0.3 % v/v, 0.4 % v/v and 0.5 % v/v of each paraben and prepared for cellular cytotoxicity studies. Parabens were dissolved in culture media (epilife medium) with 5% v/v ethanol to prepare those concentrations. In this study, SRB is a bright pink aminoxanthene dye that binds to cell protein. Therefore, cells were grown in 96-well plates and treated with paraben for 24 hours at a range of concentrations. Then, the cellular protein was

fixed to the well, stained with dye, and finally, the dye was solubilized in Tris buffer, and absorbance was measured by using the xMark™ Microplate Absorbance Spectrophotometer.

During this experiment, the greater the absorbance, the greater amount of protein was present and a higher % of cells survived during the 24 hours treatment period. Control cells (untreated) represent 100% cell survival during this time frame. This study demonstrated; that CCD 1106 human cells were treated with paraben to take an absorbance through xMark™ Microplate Absorbance Spectrophotometer at 570 nm. Samples were measured at each concentration from 96 cell plates in six replicates as well as the number of 96 plates was 3. Data were mean values (n=3), then made an average to calculate the % control cell survival of each replicate shown in figure 10. Finally mean of % control cell survival and standard deviation were calculated to plot on the chart. During this Study, Ethanol control was also determined.



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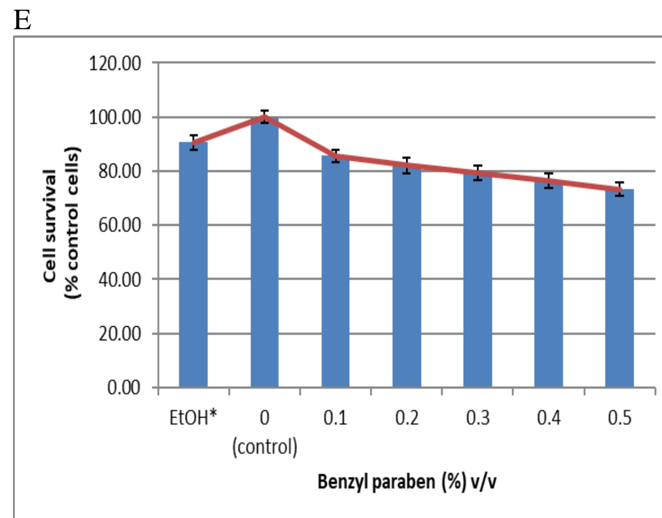

Figure 10: Cytotoxicity assay for all parabens. CCD-1106 cells were treated with appropriate paraben at a concentration of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 % (v/v) for 24 hr, and the anti-proliferative effect determined using the SRB assay. Values were expressed as a percentage of the control cell growth determined in the cell culture medium in the presence of a vehicle (5% ethanol). Data were mean values (n=3), +/- SD, and 6 replicates from one experiment.

In this cytotoxicity study, concentration was prepared in an epilife medium with 5% v/v ethanol. Paraben was relatively non-toxic at low concentrations (although ethanol alone was slightly toxic at the concentration on cell survival). Ethanol has enhanced solubility in epilife medium during the sample preparation. The toxicity level of all parabens is shown in figure 10. This study has shown the toxicity of butyl paraben was slightly higher and methyl paraben was relatively less toxic. However, % of cell survival was acceptable during this concentration. Almost 80% of the cell survived at a given concentration in all parabens. The concentration range was very close to each other. In this study, our aim was safety assessment of FDI and CIR recommended concentration in a human keratinocyte cell line. In single paraben the recommended centration of 0.4% (v/v) was safe. In methyl paraben nearly 86 % of cells survived at 0.4% concentration. Although 83%, 82%, 79%, and 77% of cells survived in ethyl paraben, propyl paraben, butyl paraben, and benzyl paraben respectively at a recommended concentration where 90-93% of cells survived at only ethanol EtOH control (without paraben in epilife medium with 5% v/v ethanol) as shown in figure 10. Throughout this study 0 (Control) was the absolute epilife medium.

During this cytotoxicity study, we also evaluated the toxicity of 4-hydroxybenzoic acid in a human keratinocyte cell line. Cells were prepared in 96 cell plates at passage 11. Then, sample

was treated with these cells at concentration respectively 0% v/v, 0.2% v/v, 0.4% v/v, 0.6% v/v, 0.8% v/v and, 1% v/v to incubated for same condition as other parabens. Before the sample preparation, the solubility of 4-hydroxybenzoic acid was determined in an epilife medium with 5% v/v ethanol at 40^0C as shown in table 5. Saturation solubility was recorded at 10.432 mg/ml from Area *vs* Concentration equation where data were mean value (n=3) shown in figure 7 and converted to percentage to treat with CCD1106 human keratinocyte cells as shown in table 6. After that, an SRB assay was applied to determine the toxicity of 4-hydroxybenzoic acid as shown in figure 11. 4-hydroxybenzoic acid was relatively non-toxic. During this study, approximately 80% of the cell survived at maximum concentration.

Table 5: Saturation concentration of 4-hydroxybenzoic acid at 40^0C

Metabolite	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
4-hydroxy benzoic acid	10.682	10.231	10.379	10.431	0.23	2.21

Table 6: Saturation concentration of 4-hydroxybenzoic acid conversion to percentage.

Name	(In 5% ethanol) Concentration (mg/ml)	(In 5% ethanol) Concentration (g/ml)	(In 5% ethanol) Concentration % (w/v)
4 hydroxybenzoic acid	10.432 mg/ml	0.0104 g/ml	1.04 %

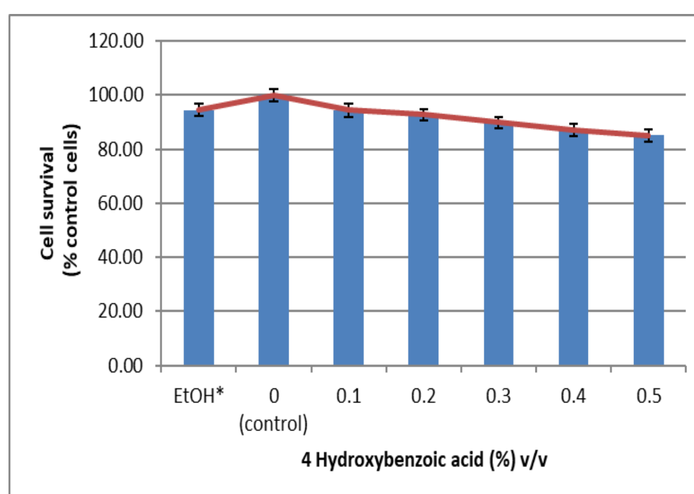

Figure 11: Cytotoxicity assay for 4-hydroxybenzoic acid (metabolite). CCD-1106 cells were treated with 4-hydroxybenzoic acid at a concentration of 0, 0.2, 0.4, 0.6, 0.8, and 1 % (v/v) for 24 hr, and the anti-proliferative

effect was determined using the SRB assay. Values were expressed as a percentage of the control cell growth determined in the cell culture medium in the presence of a vehicle (5% ethanol). Data were mean values (n=3), +/- SD, and 6 replicates from one experiment.

This experiment showed that alkyl chain length increases the toxicity of parabens. This cytotoxicity assay observation in human keratinocytes was a novel finding in our research. Paraben safety assessment in personal care products has already proven that parabens are safe at low concentrations (Elder, *et al* 1984). However, the controversy of paraben in cosmetics is still on. During this study, it was proved again FDI, and CIR recommended concentration is safe for human skin and the toxicity level of all parabens was acceptable. Paraben effectiveness depends on the alkyl ester chain, longer ester chain parabens are more active than shorter chain parabens (Uramaru *et al* 2007). In reverse, longer chain parabens were more toxic (Cashman and Warshaw 2005). Therefore, a combination of parabens is used to maintain a balance between toxicity and effectiveness in cosmetic products (Steinberg, 2006). Some reports have shown a combination of parabens is most useful in skin care products (Lee et al 2018).

3.4 Conclusion

Nowadays, parabens are the most controversial preservative in skin care products. Several studies have proven that parabens are safe however, in some cases, they have hazard for human health. Parabens are most popular due to their excellent antimicrobial activity, low cost, and available in the market. It is important to ensure that topical antimicrobials and preservatives guarantee both product and consumer safety. Parabens in contact with the human cell characterized the lipophilicity of alkyl ester. During this study, paraben toxicity was investigated in the human keratinocyte cell line through a cytotoxicity assay. This study shows parabens at low concentrations are safe and increasing the concentration of paraben would increase cytotoxicity. Shorter ester chain parabens are less toxic, but their antimicrobial effectiveness is also lower. Whereas longer ester chain parabens are more toxic and more effective. However, 4- hydroxybenzoic acid (metabolite of paraben) is relatively non-toxic. Combinations of parabens would be useful to maintain low toxicity alongside the required antimicrobial effectiveness in skin care products. This study showed that FDI and CIR recommended concentrations in skin care products were relatively safe due to their low toxicity. The cytotoxicity ranking order we observed was methyl paraben < ethyl paraben < propyl paraben < butyl paraben.

4. Chapter 2: Determination of % metabolism of parabens via pig skin using Diffusion cell study.

Abstract

Parabens are widely used preservatives in cosmetic, food, and pharmaceutical products. They are stable, effective over a wide pH range, and active against a broad spectrum of microbes. The most used parabens in the cosmetic industry are methyl paraben, ethyl paraben, propyl paraben, and butyl paraben as well as their combinations. However, at present paraben's use in cosmetic products is questionable due to their alleged adverse effect on human skin. Our previous study (Chapter 1) showed the safety evaluation of all parabens via cytotoxicity assays in human keratinocytes. The shorter alkyl chain parabens such as methyl paraben were safer than other parabens. The toxicity of paraben was increased with an increased alkyl ester chain. Whereas the longer alkyl chain parabens are more effective preservatives in cosmetics. Determination of paraben toxicity in human keratinocytes via cytotoxicity assay was a novel finding of our research.

In this study, we investigated the % metabolism of parabens through pig skin. Pig skin has similar histological and physiological properties to human skin. This study demonstrated the permeation of parabens through Franz diffusion cells over 24 hours using HPLC analysis for the quantification of parabens and their metabolite. The more soluble parabens showed higher permeation through the skin. Solubility depends on the alkyl ester chain; shorter chain parabens are more soluble in an aqueous solution. As a result, the permeation of methyl paraben was higher than others. The rank of % permeation of parabens through the skin was Methyl paraben> Ethyl paraben> Propyl paraben> Butyl paraben. Through this experiment we also established the rank of % metabolism of parabens which was Methyl paraben> Ethyl paraben> Propyl paraben> Butyl paraben.

4.1 Aim & Objectives.

Parabens are antimicrobial preservatives widely used in pharmaceutical, cosmetic and food industries. The alkyl chain connected to the ester group defines some important physicochemical characteristics of these compounds (Darbre and Harvey, 2008). Methyl paraben (MP), Ethyl paraben (EP), Propyl paraben (PP), and Butyl Paraben (BP) are mostly used to protect bacterial growth in skin care products (Esposito *et al* 2003). Those structures are shown in Figure 2. Parabens are also able to inhibit both membrane transport and the electron transport system (Davidson, 2005). The amount of paraben dissolved in the water phase determines the preservative's activity (Jungman, *et al,* 2013). Generally, microbial replication occurs in a water phase preparation, and parabens can be soluble in the water phase to kill microorganisms (Steinberg, 2006). Therefore, parabens alone or in combination are mostly used in water phase preparations such as lotions, creams, shampoo, conditioners, liquid soaps, and various cosmetic products which have high water concentrations.

Nowadays the most common technique for measuring *in vitro* dermal absorption of a test substance in a formulation is the Franz diffusion cell system. As shown in figure 12, in the Franz cell diffusion system, the skin membrane is mounted in between the donor compartment and the receptor compartment as a barrier (Bartosova and Bajgar, 2012). "Diffusion cells may be Static or flow-through" (Bartosova and Bajgar, 2012). The Static diffusion cell samples in the receptor chamber are replaced with new perfusate at each time point (Franz, 1975). On the other hand, "Flow-through cells can pass through the receptor chamber with a pump to collect flux by repetitively collecting perfusate" (Bronaugh and Stewart, 1985). Static diffusion cells can adjust based on the skin surface according to the mount of the skin membrane which can be horizontally or vertically. "The

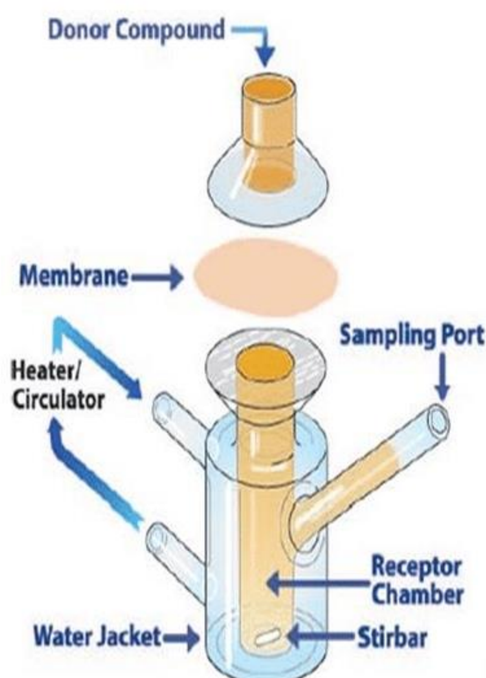

Figure 12: Franz diffusion cell system.

(Particle Sciences, 2018)

majority of skin absorption studies are conducted using horizontal cells, where the skin surface is exposed to the air" (Bartosova and Bajgar, 2012). However, in drug delivery systems, vertical cells are more common which requires immersion of both surfaces of the skin preparation (Bartosova and Bajgar, 2012). Brain *et al (*1998) In the Franz diffusion system, receptor chambers are made with inert non-adsorbing material that consists of approximately 0.5 – 10 ml of volume, and surface areas of skin membrane thickness are approximately 0.2 – 2 cm^2. Multiple samples *(i.e.,* minimum of six) are able to carry out in one experiment through the multi-station Franz diffusion system.

According to Particle Sciences, (2018) synthetic membranes such as a tissue construct, or biological sample (*i.e.,* cadaver skin) can be used in the Franz diffusion cell system. Diffusion of drug samples across the membrane to sequentially collect from a receptor compartment and analyzed through usually Mass spectroscopy or HPLC.

4.2 Method and materials

4.2.1 Method for saturation solubility in PBS.

4.2.1.1 Instruments.

Evolution™ 300 UV-Vis Spectrophotometer (Thermo Fisher Scientific. Hempstead, UK), M501 - Single Beam Scanning UV/Visible Spectrophotometer (Camspec Analytical instruments Ltd, Leeds, UK), Agilent Technologies 1290 infinity LC system, Vertical Franz Diffusion Cell (BRS Plc., co., Durham, UK.). Agilent infinity DAD detector, Eclipse XDB-C18 rapid resolution column, weight balance, and Glass apparatus. Electronic weight balance (Ohaus Pioneer, Thetford, UK), heated water bath, Thermometer, Volumetric flask, Glass pipette, Filter paper, Syringe filter, supernatant sample vial, Beaker and Pyrex glass tube.

4.2.1.2 Chemicals.

Methyl paraben (Alfa Aesar, Heysham UK), Ethyl paraben (Alfa Aesar, Heysham UK), Propyl paraben (Alfa Aesar, Heysham UK), Butyl paraben (Alfa Aesar, Heysham UK), Phosphate buffer solution (PBS) pH 7.4 (Sigma-Aldrich. UK), Methanol (HPLC grade) (Sigma-Aldrich. UK), Phosphoric acid (Loughborough, UK) and distilled water.

4.2.1.3 Sample preparation.

5 mg of each paraben was weighed on an analytical balance and dissolved in 50 ml of PBS (pH 7.4) to a concentration of 0.1 mg/ml.

4.2.1.4 Preparation of calibration curve for parabens.

Stock solutions were diluted in 10 ml volumetric flasks to the following concentrations: 0.05 mg/ml, 0.01 mg/ml, 0.005 mg/ml, 0.001 mg/ml and 0.0005 mg/ml. The absorbance of the standard solutions was measured using an M501 - Single Beam Scanning UV/Visible Spectrophotometer at their respective λ max as shown in Table 1.

4.2.1.5 Saturation solubility of parabens.

Excess amounts of parabens were dissolved in PBS to measure the saturation solubility via the shake flask method. 10 ml of paraben stock solution was transferred into Pyrex glass flasks. Then, an excess amount of paraben (two or more full spatulas) was added to the same Pyrex glass flasks and gently shaken. The flasks were placed in a 37°C water bath shaker for 24 hours. After that, 5 ml of sample was withdrawn from each flask and passed through a 0.2 µm filter to collect the supernatant. The collected supernatant was diluted 1 in 10 before measuring its UV absorbance using an M501 - Single Beam Scanning UV/Visible Spectrophotometer.

4.2.2 Method for In-vitro skin permeation (Franz diffusion Cell) studies.

4.2.2.1 Integrity check.

The integrity of the skin membrane was checked through a visual inspection that conformed to *in vitro* permeation via Franz diffusion cell studies. In addition, integrity was checked through a flux of tritiated water (100µl/cm^3), and the cut-off for acceptability was 6×10^{-3} cm/h.

4.2.2.2 Skin preparation.

Domestic Yorkshire Landrus breed pigs (Barr Farm) were used. Excised skin from the shoulder of the animal was wrapped in an aluminum foil, placed in a polyethylene bag then stored at -20°C in a freezer. On the day of the experiment, it was removed from the freezer and allowed to defrost at room temperature. Then the skin was placed on a multi-station Franz diffusion system with the dermal side down. Each dermatome was 1000 µm in thickness without cutting the skin hair follicles.

4.2.2.3 Donor solution preparation.

30 mg of parabens were weighed and dissolved in 24 ml of PBS to prepare the donor solution. These were diluted with PBS to give approximately 50% saturated concentration for each paraben as shown in table 3.

4.2.2.4 Parabens permeation by Franz cell studies.

In a multi-station Franz diffusion system (BRS Plc., co., Durham, UK.), one donor was attached to seven ground glass Franz diffusion cells with an absorption surface area of 0.79 cm^2. The skin sample was mounted in between the donor and receptor chambers of the cell with the dermis in contact with the receptor medium. The 1.5 ml volume of the receptor chamber was filled up with PBS that was continuously stirred with a magnetic stirrer in an incubator at 37°C for 1h. 50% w/v saturated donor solution was applied, and each cell contained 300 µl donor solution. After that, the adequate amount (100 µl for MP and 150 µl for EP, PP, and BP receptively) of receptor fluid dose was withdrawn from the receptor chamber for each time point (t) and replaced with fresh preheated PBS at 37°C. The Franz cell was placed back into the incubator with continued stirring and samples were collected from the receptor fluid at different time intervals (t= 0, 1, 2, 3, 4, 8, 20, and 24 hours). Those samples were stored in a glass vial. At end of the study, the remaining solution was also collected in a sample vial for further analysis, and the receptor chamber was also rinsed. All the samples were stored in a freezer at 5°C as described in a previous study.

4.2.2.5 Determination of paraben in the skin and skin surface.

At the end of the permeation study, the paraben trace remaining on the skin surface of each donor chamber was washed with PBS. Those samples were also stored in plastic vials at 5°C, before HPLC analysis.

The skin was removed from the Franz cell and placed in a plastic vial with 1 molar KOH (2mL). The skin was dissolved thorough stirring and sonication (Hilsonic Ltd ultrasonic bath, Wirral, UK), and any remaining skin particulate was removed via filtration (syringe and syringe filter, glass microfiber filters, Whatman). The resulting solutions were neutralized (pH 7.4) using glacial acetic acid and stored in plastic vials at 5°C before their paraben levels were measured by HPLC.

4.2.2.6 HPLC Analytical Assay.

HPLC analysis was performed using a (4.6 mm × 150 mm × 5µm) Zorbax Eclipse XDB-C18 rapid resolution column (Agilent Technologies, Santa Clara CA) and the autosampler temperature was maintained at 25°C throughout. HPLC parameters were: Solvent A was 0.1% (v/v) phosphoric acid in distilled water and solvent B was 0.1% (v/v) phosphoric acid in

methanol. The flow rate was 1.0 ml/min. The solvent gradient program was 25% solvent B at time 0.0 minute, 25% solvent B at time 1.0 minute, 70% solvent B at time 5.0 minute, 70% solvent B at time 10.0 minute, 25% solvent B at time 10.1 and 25% solvent B at time 15.0 minute. During the sample analysis, the injection volume was 5 µl and the overall sample run time was 15 minutes. This HPLC system flitted through an Agilent infinity DAD detector which was performed to detect 254 nm of UV wavelength.

4.2.2.7 Preparation of a calibration standard by HPLC.

0.1mg/ml of paraben was prepared in methanol (HPLC grade). Then diluted to the following concentration (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) in 10 ml volumetric flask and analyzed through HPLC system at 254 nm of UV wavelength.

4.3 Results and Discussion

4.3.1 Analysis of the Saturation solubility of parabens in Phosphate buffer solution (PBS).

"Solubility of paraben depends on their alkyl ester of a benzene ring" (Cashman and Warshaw, 2005). Solubility of Parabens in the aqueous phase is much better in shorter alkyl esters chain. Phosphate buffer solution acts as an aqueous phase (Twist and Zatz, 1986). This study has determined the saturation solubility of paraben in phosphate buffer solution (PBS) at pH 7.4. We used a phosphate buffer tablet for preparing the PBS solution. 0.1mg/ml of each paraben was prepared as stock solution. The λ max of each solution was measured using an Evolution™ 300 UV-Vis Spectrophotometer. Those are shown in table 1.

Table 1: λ max for parabens in PBS.

Paraben	Concentration mg/ml	λ max (nm)
Methyl paraben with PBS	0.1 mg/ml	255
Ethyl paraben with PBS	0.1 mg/ml	255
Propyl paraben with PBS	0.1 mg/ml	255
Butyl Paraben with PBS	0.1 mg/ml	255

Saturation solubility of paraben was measured through absorbance *vs* Concentration beer-lambert equation where the mean value (n=3) was considered. The absorbance *vs* Concentration beer-lambert equations are shown in figure 13.

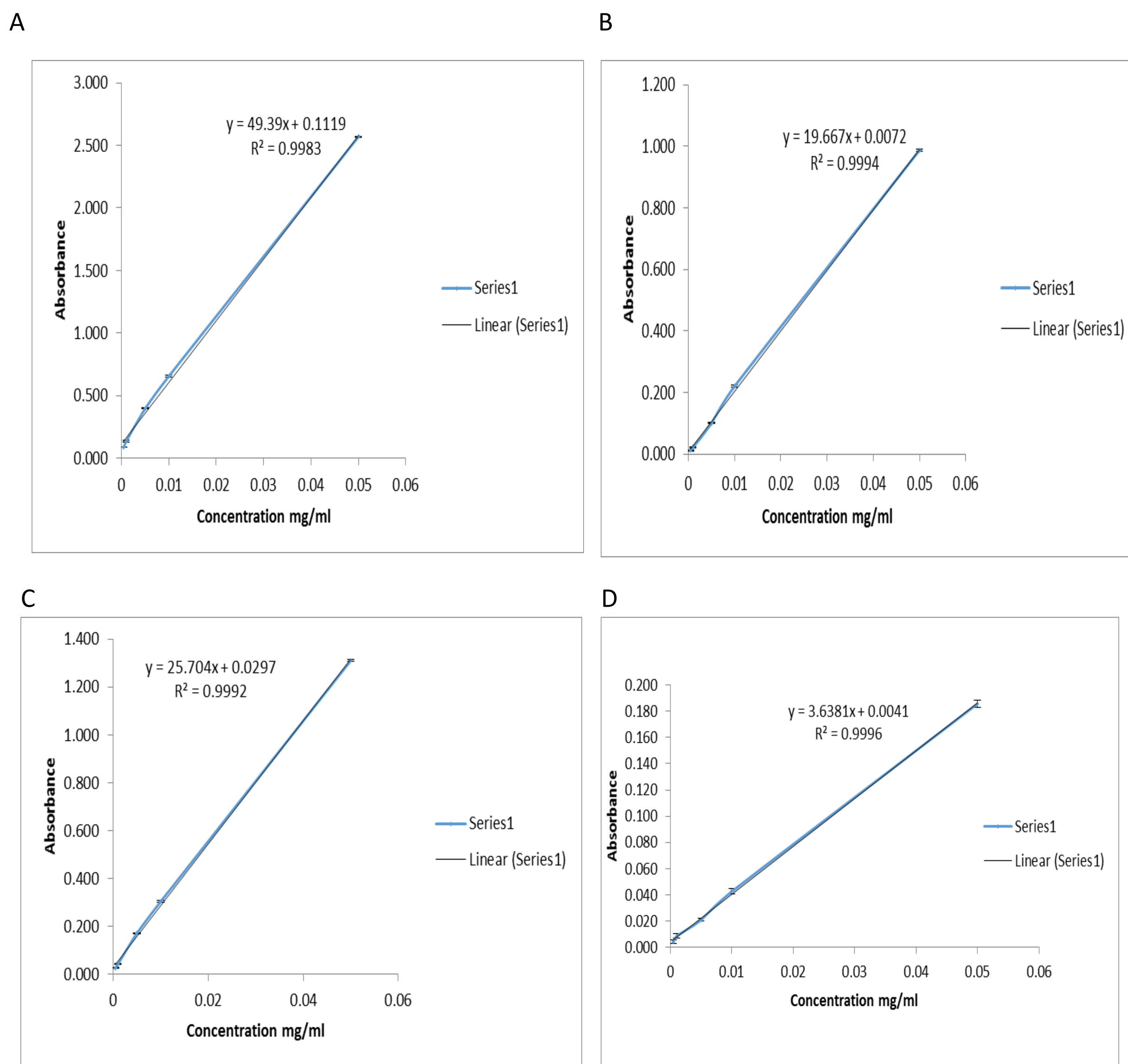

Figure 13: Calibration standard of A) Methyl paraben, B) Ethyl paraben, C) Propyl paraben and D) Butyl paraben was made at a concentration range from 0.0005 mg/ml to 0.05 mg/ml in HPLC system. Standards were made to volume with PBS at pH 7.4. Mean (n=3) Absorbance against paraben concentration.

Tajarobi, et al (2011) determined the solubility of parabens in phosphate buffer as 2.7, 1.6, 0.56, and 0.16 mg/mL for Methyl paraben (MP), Ethyl Paraben (EP), Propyl paraben (PP) and Butyl paraben (BP), respectively. This study shows that saturation solubility was determined

by using an excess amount of paraben dissolved in PBS through an Absorbance *vs* Concentration linear regression equation. The solubility of paraben in our study was nearly similar to that of Tajarobi, et al (2011). The mean (n=3) solubility, standard deviation, and % relative standard deviations of all parabens are shown in table 2. This study demonstrated that parabens were soluble in PBS and the solubility of methyl paraben was higher than butyl paraben.

Table 2: Saturation solubility for parabens in PBS

Paraben	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	2.01	2.77	3.01	2.59	0.52	20.1%
Ethyl paraben	1.57	1.05	1.31	1.31	0.26	19.8%
Propyl paraben	1.01	0.74	10.91	0.88	0.14	15.9%
Butyl paraben	0.56	0.43	0.61	0.53	0.09	16.9%

We also analyzed the donor solution of each paraben through HPLC to measure % the solubility of a paraben. For this experiment, we used the calibration standard of each paraben by HPLC which is shown in figure 15. A calibration standard was used to quantify the unknown concentration of the donor solution. The measured % saturated solubility of paraben in PBS was 49.61%, 53.51%, 48.52%, and 53.77% for Methyl paraben (MP), Ethyl Paraben (EP), Propyl paraben (PP), and Butyl paraben (BP), respectively as shown in table 3. So, this study showed approximately 50% of paraben was saturated during the diffusion study.

Table 3: % saturation of paraben in donor solutions.

Paraben	Saturation solubility	Concentration in Donor solution (HPLC)	% saturation
Methyl paraben	2.56 mg/ml	1.27 mg/ml	49.61 %
Ethyl paraben	1.31 mg/ml	0.701 mg/ml	53.51 %
Propyl paraben	0.88 mg/ml	0.427 mg/ml	48.52 %
Butyl paraben	0.53 mg/ml	0.285 mg/ml	53.77 %

4.3.2 Analysis of In vitro skin permeation (Franz cell) study with parabens.

Permeation studies of paraben have been done through pig ear skin previously (Caon *et al* 2010). Pig skin and human skin exhibits similar properties (Dodou *et al* 2014). In our study, Methyl paraben (MP), Ethyl Paraben (EP), Propyl paraben (PP), and Butyl paraben (BP) were used to evaluate permeation through pig skin. To confirm the elution of each paraben a test mix of parabens was analyzed to ensure clear differentiation in peak elution shown in Figure 14.

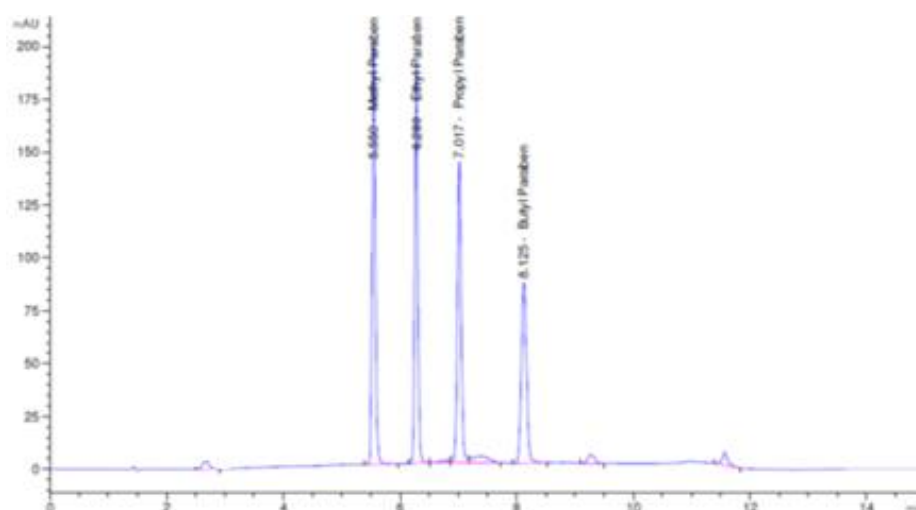

Figure 14: Test mix of four parabens Including methyl paraben, ethyl paraben, propyl paraben, and butyl paraben at a concentration of approximately 0.1mg/ml. The chromatographic condition was performed as the above method.

Imamovic et al (2012) determined the retention time of methyl, ethyl, and propyl paraben at approximately 4.33, 5.49, and 7.68 min respectively at wavelength 254 nm. Due to method differences in HPLC, the retention time was slightly different. In our study, the calibration curve for each paraben exhibited excellent linearity (Figure 5). The calibration standard of each paraben was used for quantification analysis of unknown concentrations collected during the diffusion studies.

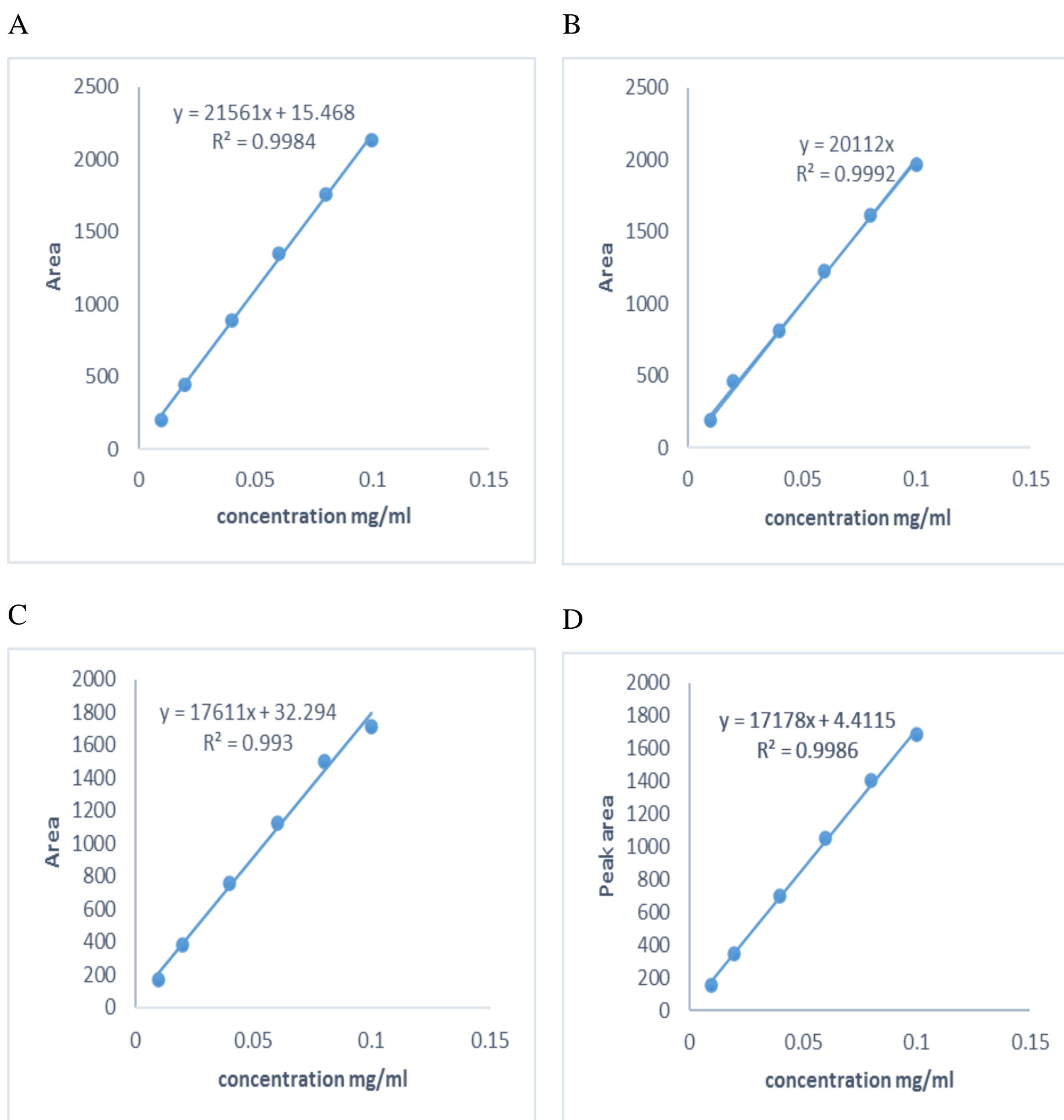

Figure 15: Calibration standard of A) Methyl paraben, B) Ethyl paraben, C) Propyl paraben and D) Butyl paraben was made at a concentration range from 0.01mg/ml to 0.1 mg/ml in HPLC system. Standard was made to volume with HPLC grade Methanol. Mean (n=3) Area against paraben concentration.

In our study, approximately 50% of the saturation solubility of each paraben in PBS was used to maintain sink conditions. Methyl paraben showed higher permeation than the other parabens. A longer chain of alkyl esters has decreased water solubility and lower penetration through the epidermis layer of the skin (Twist and Zatz, 1986). Butyl paraben had lower permeation due to its longer alkyl aster chain. Permeation profile was Methyl paraben > Ethyl paraben > Propyl

paraben > Butyl paraben in Figure 16. The different solubility and lipophilicity of the molecules could impact the permeation profile (Caon *et al*, 2010).

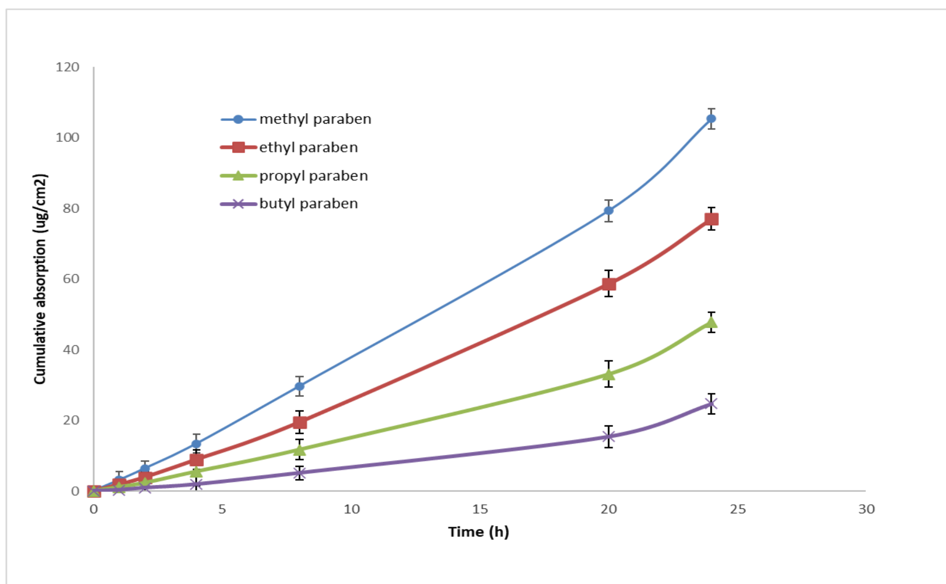

Figure 16: Permeation profile of paraben via pig skin of Methyl paraben, Ethyl paraben, Propyl paraben, and Butyl paraben. Paraben concentration in the receptor solution was measured at each time point (t = 0, 1, 2, 3, 4, 8, 20 and 24h). Values are expressed as Cumulative permeation (µg/cm2) during 24 hours. Data are mean values +/- SD, 7, 7, 4, and 4 replicates respectively methyl, ethyl, propyl, and butyl paraben from the experiment.

Diffusion data were plotted as mean (methyl paraben for 7 replicates, ethyl paraben for 7 replicates, propyl paraben for 4 replicates, and butyl paraben for 4 replicates.) cumulative amount of paraben was collected in the receptor chambers per unit area ($\mu g/cm^2$), as a function of time (h) and surface area of pig skin was 0.79 $cm^{2.}$ Permeation parameters are shown in table 4. The amount of substance permeated per unit area per unit time is called flux. The flux ($\mu g/cm^2/h$) was evaluated from the ratio of the cumulative absorption through a skin surface at each time point (slope of the cumulative absorption vs time plot). The steady flux is expressed as J ($\mu g/cm^2/h$). The lipophilicity of a substance increases with a decrease in the permeation flux (Caon *et al* 2010). A longer alkyl ester chain has higher lipophilicity (Twist and Zatz, 1986). Therefore, methyl paraben presented the highest steady flux (7.1 ± 0.24) followed by ethyl paraben, and propyl paraben, while butyl paraben had the lowest flux (2.21 ± 0.17) due to their lipophilicity as shown in table 4. The log p value represents the lipophilicity of parabens.

The flux values were lower than Caon *et al* (2010) which could be due to different membrane thicknesses or solutions. In Caon *et al* (2010) pig ear skin with a surface area of 1.77 cm^2 was used. The time taken to achieve diffusion during each time interval was referred to as the lag time. Lag time was calculated to form the intercept of the cumulative absorption vs time plot. Lag time is expressed as L_t (hours). In this study, Lag time was recorded as small for methyl paraben 0.98 ± 0.44 hours then ethyl paraben 1.14 ± 0.31 hours, ethyl paraben 1.23 ± 0.37 hours, and butyl paraben 2.06 ± 0.21 hours as shown in table 4. Lag time was reported at 1.2 ± 0.3, 1.3 ± 0.2, 1.4 ± 0.4, and 2.3 ± 0.2 for methyl paraben, ethyl paraben, propyl paraben, and butyl paraben respectively in pig ear skin (Caon *et al* 2010). The lag time differences between studies were due to the thickness of the skin membranes they used. The thinner the membrane, the shorter the lag time (Neri et al 2022). However, Akomeah et al. (2007) evaluated "methyl paraben, ethyl paraben, propyl paraben, and butyl paraben permeation through the human epidermis and the lag time values were 0.288 ± 0.093 and 0.450 ± 0.109 h, respectively". This study was conducted with pig skin with a surface area of 0.79 cm^2 and PBS was used as a solvent, the solution thus could higher lag time compared to human dermis and epidermis.

Table 4: Permeation parameters of parabens in pig skin.

Permeation parameter	Methyl paraben	Ethyl paraben	Propyl paraben	Butyl paraben
	Mean ± SD	Mean ±SD	Mean ±SD	Mean ± SD
Flux (µg/cm²/h)	7.1 ± 0.24	4.85 ± 0.41	3.39 ± 0.31	2.21 ± 0.17
Lag time (h)	0.98 ± 0.44	1.14± 0.31	1.23 ± 0.37	2.06 ± 0.21
Permeation coefficient Kp×10^{-3}	3.3 ± 0.1	2.3 ± 0.2	1.7 ± 0.2	0.8 ± 0.1
Log p	1.93	2.27	2.81	3.53
Molecular weight (g/mol)	152.15	166.17	180.20	194.23
r^2	0.998	0.994	0.981	0.941
Cumulative absorption after 24h (µg/cm2)	105.4 ± 2.9	77.1 ± 3.1	47.8 ± 2.8	24.7 ± 2.9
Percentage permeation after 24h	28.3% ± 0.7	19.7% ± 0.4	11.4 % ± 0.7	6.8% ± 0.2

❖ Studies on the skin safety of parabens

This study also presented a permeation coefficient Kp (cm/h) value which was higher for methyl paraben than ethyl paraben, propyl paraben, and butyl paraben as shown in table 4. Permeation coefficients were obtained to steady flux divided by the initial concentration of each paraben. This study shows that methyl paraben had higher permeation after 24h than other parabens shown in table 4. Approximately 28.3% of methyl paraben (MP), 19.7% of Ethyl paraben (EP), 11.4% of propyl Paraben (PP), and 6.8% of Butyl paraben (BP) were permeated for 24h as shown in table 4. The permeability profile of this study was MP>EP>PP>BP. After 24 h permeation study, skin membrane, donor chamber wash, and receptor chamber wash were collected for measured mass balance, and their % recovery is shown in tables 5, 6, 7, and 8.

In this permeation study, Cumulative absorption (µg/cm2) after 24h methyl paraben was higher than the other three parabens as shown in table 4. The percentage recovery of each paraben was obtained from their total mass in receptor fluid, receptor chamber, donor chamber, and skin membrane to the concentration of applied dose in donor solution. Ethyl paraben had a better recovery than methyl, ethyl, and butyl paraben. So % recovery of methyl paraben was 90.7%, ethyl paraben was 91.5%, propyl paraben was 90.1% and butyl paraben was 85.7% as shown in the table 5,6,7 and 8.

Table 5: Mass balance and total % recovery of methyl paraben

Cell no	Applied dose concentration in donor solution. (µg)	Cumulative paraben in receptor fluid after 24h (µg) (t= 24h)	Mass remains in the donor chamber (µg)	Mass remains in the receptor chamber (µg)	Mass remains on the skin membrane (µg)	Total (µg)	% Recovery
1	381	148.9	95.29	18.55	69.8	332.54	87.1%
2	381	155.6	93.15	25.01	71.31	345.07	90.6%
3	381	144.3	101.1	24.2	91.15	360.75	94.7%
4	381	131.8	112.1	16.16	90.12	350.18	91.9%
5	381	138.4	95.18	19.18	93.65	346.41	90.9%
6	381	137.4	95.18	17.2	95.5	345.28	90.6%
7	381	137.9	105.1	23.15	72.55	338.7	88.9.%
Total Mass Balance Mean						**345.56**	**90.7%**

Table 6: Mass balance and total % recovery of Ethyl paraben

Cell no	Applied dose concentration as donor solution (µg)	Cumulative paraben in receptor fluid after 24h (µg) (t= 24h)	Mass remains in the donor chamber (µg)	Mass remains in the receptor chamber (µg)	Mass remains on the skin membrane (µg)	Total (µg)	% Recovery
1	210	85.05	64.97	11.6	20.6	182.22	86.8%
2	210	83.21	64.82	13.18	21.16	182.37	86.8%
3	210	96.19	79.15	7.2	17.22	199.76	95.1%
4	210	92.74	61.35	11.57	22.13	187.79	89.4%
5	210	85.12	72.85	8.22	25.92	192.11	91.5%
6	210	93.08	85.18	11.37	15.49	205.12	97.7%
7	210	90.69	81.42	10.23	14.4	196.74	93.7%
Total Mass Balance			Mean			**192.3**	**91.5%**

Table 7: Mass balance and total % recovery of Propyl paraben.

Cell no	Applied Dose concentration as donor solution (µg)	Cumulative paraben in receptor fluid after 24h (µg) (t= 24h)	Mass remains in the donor chamber (µg)	Mass remains in the receptor chamber (µg)	Mass remains on the skin membrane (µg)	Total (µg)	% Recovery
1	129	86.32	14.5	2.6	18.34	121.76	94.4%
2	129	81.68	11.1	1.9	16.51	111.19	86.2%
3	129	88.67	14.2	3.5	13.31	119.68	92.8%
4	129	83.17	11.5	2.8	14.76	112.23	87.0%
Total Mass Balance			Mean			**116.21**	**90.1%**

- Studies on the skin safety of parabens

Table 8: Mass balance and total % recovery of Butyl paraben

Cell no	Applied Dose concentration as donor solution (µg)	Cumulative paraben in receptor fluid after 24h (µg) (t= 24h)	Mass remains in the donor chamber (µg)	Mass remains in the receptor chamber (µg)	Mass remains on the skin membrane (µg)	Total (µg)	% Recovery
1	87	41.92	9.6	1.6	19.34	72.46	83.3%
2	87	39.78	9.4	2.7	13.51	65.39	75.2%
3	87	44.67	17.2	5.6	21.31	88.78	102.04%
4	87	37.87	11.5	3.6	18.76	71.73	82.4%
Total Mass Balance Mean						**74.59**	**85.7%**

The in vitro permeation profiles of parabens (including Methyl paraben, ethyl paraben, Propyl paraben, and butyl paraben) across the pig skin confirmed that parabens are permeable through the skin for 24 h. Higher the solubility, the higher the diffusion of paraben (Esposito *et al,* 2003). Methyl paraben permeated higher due to its higher solubility in an aqueous solution. In this study, solubility ranking in Phosphate-buffered saline (PBS) was Methyl paraben> Ethyl paraben> propyl paraben> Butyl paraben. As the following structure is shown in figure 1 methyl paraben has a shorter side chain and butyl paraben was a longer side chain. The results indicate that methyl paraben was highly permeable through the skin. On the other hand, butyl paraben has lower permeability due to its longer alkyl ester chain.

In this study, the plateaued permeation curves indicated that permeated amount remains constant at a particular time and a further dose would require in the donor chamber to increase the permeation amount continuing until the end of the experiment at 24h. The permeation of paraben gradually improved during a time interval of 0 to 24 h. The detection of the dosing compound in the skin membrane and the receptor solution confirmed the metabolism of the compound by the skin enzyme (Dodou *et al* 2014). In this study, we also calculated the % metabolism of each paraben during the 24h of the experiment. The overall % metabolism of Methyl paraben (MP) was 12.6%, Ethyl paraben (EP) was 9.3%, Propyl paraben (PP) was 7.0%

and Butyl paraben (BP) was 4.5% as shown in figure 17. As per the following results, methyl paraben was highly metabolized during 24h and butyl paraben was shown lower. So, the ranking of % metabolism via pig skin was Methyl paraben > Ethyl paraben > Propyl paraben > Butyl paraben. The amount of paraben permeated via pig skin during 24 hours shown in table 9.

Table 9: The amount of paraben permeated via pig skin during 24 hours.

Name	Applied dose	Permeated dose	% metabolism
Methyl paraben	381 µg	48.001 µg	12.6 %
Ethyl paraben	381 µg	35.42 µg	9.3 %
Propyl paraben	381 µg	26.67 µg	7.0 %
Butyl paraben	381 µg	17.14 µg	4.5 %

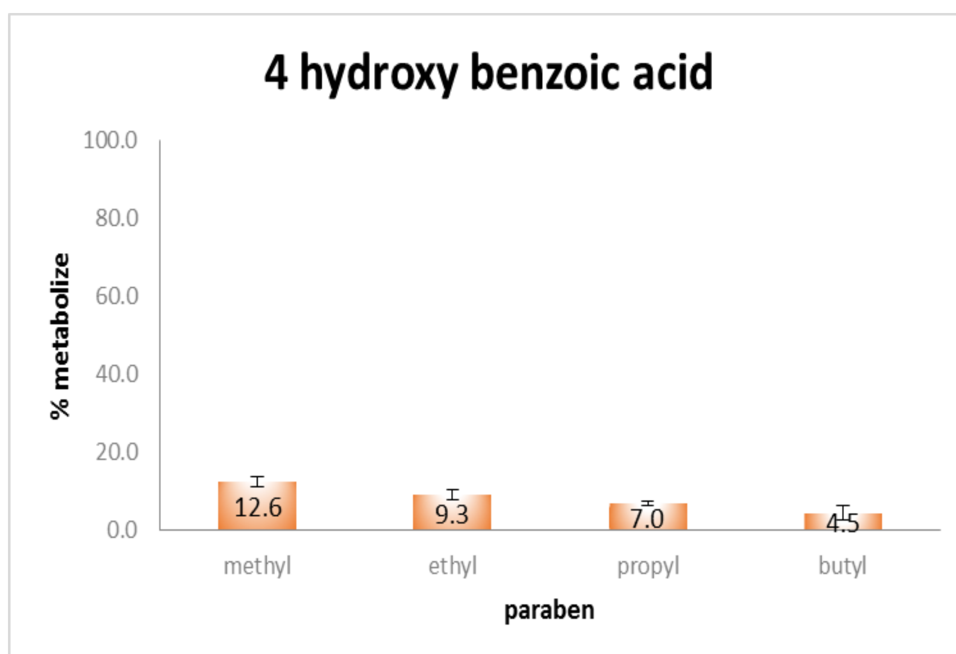

Figure 17: the amount of % metabolism of paraben during 24h via the pig skin membrane. Paraben was absorbed at each time point (t = 0, 1, 2, 3, 4, 8, 20 and 24h). Data are mean values +/- SD, 7, 7, 4, and 4 replicates respectively methyl, ethyl, propyl, and butyl paraben from the experiment.

In our study, we used pig skin as a model membrane. "A review of numerous studies recommended that the permeability of a compound through both human and pig skin showed a correlation coefficient of 0.88 with an intra species standard coefficient of variation in skin permeability of 21% for pig and 35% for human" (Barbero and Frasch, 2009). Pig skin and

human skin exhibits have a smaller difference from a functional point of application (Dodou *et al,* 2014). “Pig skin has less variability than the human skin model as well as both pig and human are good models for skin permeability” (Barbero and Frasch, 2009). Pig skin is a good model for human skin permeability due to its histological and physiological properties are almost similar to human skin (Ian and Robert, 1992). So that in this experiment we used pig skin as a permeation barrier for human skin permeability.

4.4 Conclusion

In cosmetic formulations, preservatives are the most important substances which protect microbial growth as well as provide to long shelf life, and parabens are one of them. A good cosmetic product mandates safe and effective preservatives. However, the safety of paraben is questionable due to its adverse effects on human health according to some studies. This study demonstrated the permeability of paraben via pig skin. Pig skin has similar histological and physiological properties to human skin. Parabens are applied on the skin and determined comparative diffusion between them. Due to its low molecular weight, methyl paraben diffused more than other parabens even though is less lipophilic ie more soluble in an aqueous solution. So, the permeation of methyl paraben was higher than other parabens as well as % metabolism was also higher. During the toxicity study, butyl paraben was relatively more toxic however, its permeation through the skin was less than others because of its higher molecular weight. Our aim has to rank the order of the % metabolism of parabens which was Methyl paraben > Ethyl paraben > Propyl paraben > Butyl paraben.

5. chapter 3: The *in-vitro* investigation of paraben metabolism in human keratinocytes.

Abstract

Parabens are a group of alkyl esters that are widely used as preservatives in pharmaceutical and cosmetic formulations. Parabens have a broad spectrum of activity against yeasts, moldss, and bacteria. They are used alone or in combination with other compounds to protect microbial growth. Longer ester chain parabens are more effective than shorter ester chain parabens. There are four major parabens commercially used in skin care products: Methyl paraben, Ethyl paraben, Propyl Paraben, and Butyl paraben. The 1st and 2nd chapters of this research demonstrated the safety of parabens, using a cytotoxicity assay and the extent of metabolism of each paraben in pig skin, using the Franz diffusion study. The toxicity of paraben increased with increased ester chain. Shorter alkyl chain paraben was safer. Determination of paraben toxicity in human keratinocytes through cytotoxicity assay was a novel finding of our research. % Metabolism of parabens in pig skin showed metabolism of the shorter alkyl ester parabens was higher (methyl paraben) than other parabens.

In this Chapter, we investigated paraben metabolism in human keratinocyte cell extracts at three different times of incubation. CCD1106 human keratinocyte cells were prepared at passage (number of the subculture) 7 (P7). The FDA and CIR recommended concentration of 0.4% w/w of each paraben was applied in the cell line and was allowed to incubate for 24 hours, 48 hours, and 72 hours. After incubation, parabens and their metabolite were extracted by lysis buffer, and the paraben/ metabolite concentrations were determined using HPLC.

This experiment showed that parabens were metabolized in the human skin and the longer the incubation time the higher the % metabolism. This study also demonstrated shorter chain paraben was highly metabolized whereas, certain concentration has a low metabolic effect on human skin. Solubility in aqueous solution increased with decreasing alkyl ester chain paraben alongside an increased metabolic rate. The % metabolism ranking in human cell was Methyl paraben> Ethyl paraben> Propyl paraben> Butyl paraben.

5.1 Aim & Objectives.

Parabens are widely used preservatives in the cosmetic industry due to their excellent antimicrobial activity (Steinberg, 2006). They are structurally active against all kinds of microbes (Mallika *et al*, 2013). There are seven parabens available in the market. Due to their effectiveness, availability, and low cost, mainly four parabens are commonly used in skin care products; Methyl paraben, Ethyl paraben, Propyl paraben, and Butyl paraben shown in figure 2. Those are used singly or in combination with other parabens as a preservative in cosmetics such as makeup, moisturizers, hair care products, and shaving products (Elder *et al*,1984). Their antimicrobial activity comes from the alkyl ester chain with key metabolic pathways in the target organisms (Uramaru *et al* 2007). Longer ester chain parabens are more active than shorter chain parabens (Cashman and Warshaw, 2005). It has been used in a very low range as preservatives in cosmetic and food products. FDA and CIR recommend 0.4% w/w as single and 0.8% w/w as combination paraben used in skin care products. This research was conducted with a safety evaluation of recommended concentration in human skin.

Paraben activities examine through *in vivo* and *In vitro* studies. Parabens are fully absorbed through the skin and gastrointestinal tract; those are fully metabolized and rapidly excreted through the urine (Gil *et al*, 2012). Several toxicity studies of these compounds have been carried out in humans and animals; no marked toxicity was found as they are rapidly absorbed, metabolized, and excreted from the body (Suzanne, *et al* 2010). However, various in vitro and cell-based studies also reported that parabens have an estrogenic effect on human skin. Estrogenicity increases with the increased alkyl chain length of paraben esters (Prusakiewicz et al, 2007). Esterases are active for hydrolysis of parabens to 4- hydroxybenzoic acid (Suzanne, *et al* 2010). 4- hydroxybenzoic acid is the metabolite for all parabens. Lobemeier *et al* (1996) explained that parabens are metabolized in the skin's subcutaneous layer where four carboxyl esterases are present: these hydrolyze paraben to para-hydroxybenzoic acid and their relevant side chains. Parabens can be absorbed and retained into the human tissue without hydrolysis of tissue esterases (Oishi, 2004).

Different metabolic routes could metabolize paraben into the body. Mainly the biotransformation of parabens includes the hydrolysis of the ester bond and glucuronidation reaction which is called xenobiotics (Abbas *et al* 2010). Xenobiotics are biotransformed into harmless or less harmful compounds by various enzymatic activities in the epidermis and

dermis (Imai *et al*, 2013). The skin contains a variety of enzymes that are capable of xenobiotic metabolism, including a range of Phase I and Phase II systems. This wide range of Phase I and Phase II metabolic biotransformations are able to be carried out in the skin, and most dermal metabolism occurs in basal keratinocytes in the epidermis (Wilkinson, 2008). Constitutive expression of xenobiotic-metabolizing enzymes has been detected in normal human keratinocytes (Imai *et al*, 2013).

In in-vitro studies of paraben extraction in a cell, an extract is used to detect paraben in human skin. There is a different type of extraction procedure are available to extract from cells such as Lysis buffer, 80% methanol (MeOH) (-80^{0}c), acetonitrile (ACN) and phenol-*chloroform* isoamyl alcohol, etc (Dietmair S *et al* 2010) (Ser *et al* 2015). The most commonly used procedure is 80% methanol (MeOH) (-80^{0}c) for western blot and metabolite extraction (Ser *et al* 2015). However, lysis buffer is also effective for the extraction of target molecules. Methanol (MeOH) is toxic to human skin, lysis buffer is a detergent that disrupts to break open the cells (Shehadul, Aditya, and Selvaganapathy, 2017). There are not many studies on the extraction of paraben in cell extract through lysis buffer. Cell lysis reacts with the outer boundary or cell membrane broken down or destroyed to release inter-cellular materials from the cell (Shehadul, Aditya, and Selvaganapathy, 2017). The lysis method is used for the ease of purification steps, the target molecules for analysis, and quality of final products" (Harrison, 1991). The successful extraction of metabolites is a critical step in metabolite profiling. The most important step in metabolite extraction is the quenching of metabolism (Sapcariu *et al* 2014). Once the metabolic processes have been quenched, the next step is to lyse the cells (Shehadul, Aditya, and Selvaganapathy, 2017). A schematic representation of paraben and their

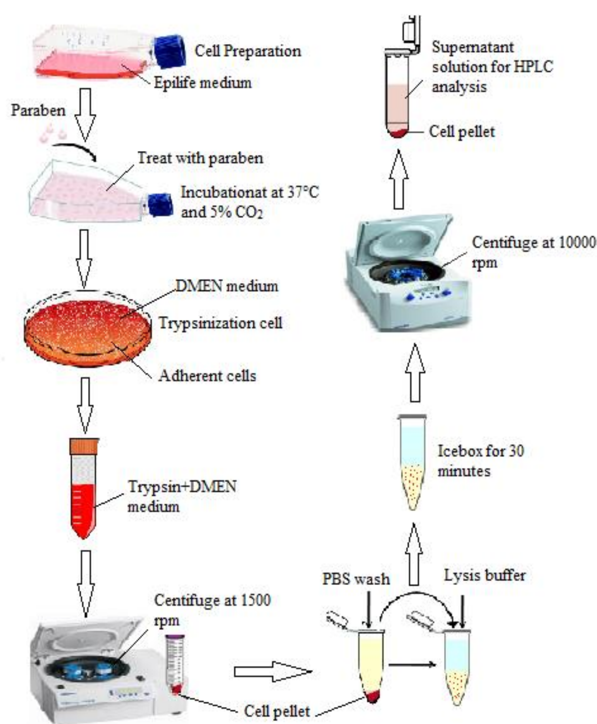

Figure 18: Parabens and metabolite extraction by lysis buffer

metabolite extraction procedure is shown in figure 18. A better understanding of extraction parameters will allow optimization of metabolite extraction protocols.

In this study, paraben and their metabolite are extracted from human keratinocyte cells by lysis buffer. Normally, paraben concentration is applied to the cell and incubated at three different times to quenching of metabolism. FDA and CIR recommended 0.4% of single paraben and 0.8% of a combination of paraben on human skin (Jerry, Miyoung, and Harvey 2015). The research will conduct with FDA and CIR-approved concentrations in single paraben for safety evaluation in cosmetic products. Cell preparation is also important for extracting the compound. Healthy cells can make a difference in the extraction process. A strong and healthy cell can absorb a molecule quickly (Ser *et al* 2015). In the extraction procedure, samples are centrifuged properly to collect the maximum number of cells. In this study, a sample was prepared by filtration with a 0.2µm filter and analyzed through HPLC.

Many analytical techniques can be used for the analysis of paraben and its metabolite. However, the most frequently used technique is HPLC. Whereas fewer sources report the use of the uHPLC technique. A reversed-phase system with gradient elution by a mixture of water and organic solvents is often used (Irena *et al* 2014). Parabens are highly soluble in an organic solvent such as Alcohol, Acetone, Acetonitrile, etc (Giordano *et al* 1999). Solubility property makes reversed-phase HPLC an ideal separation/identification technique for analysis of paraben (Coral *et al* 2005). Before the HPLC analysis, a suitable extraction method must be determined to remove paraben from water-based formulation (Fenhua, Dandan, and Zhimin 2016). This study will demonstrate four major parabens Methyl, Ethyl, Propyl, and Butyl are determined from cell extract by HPLC analytical technique.

5.2 Materials and Methods

5.2.1 Instruments

Agilent Technologies 1290 infinity LC system (University of Sunderland, UK), Agilent infinity DAD detector, Agilent Zabox C18 UHPLC column, Sanyo CO2 Incubator (IR sensor), freezer (Liebherr, UK), Biological safety Laminar flow hood (Thermo Scientific, UK), Harrier 15/80 Centrifuge (MSE), microscope (Olympus, UK), Hemocytometer (Marienfeld, UK), 75-cm^2 culture flasks (Thermo Fisher Scientific, UK), electronic weight balance (GRAM, UK),

Heated water bath (Thermo Fisher Scientific, UK), Thermometer (Thermo Fisher Scientific, UK), Volumetric flask, Syringe filter, supernatant sample vial, pipette, Baker and Falcon tube.

5.2.2 Chemicals

Methyl paraben (Alfa Aesar, Heysham UK), Ethyl paraben (Alfa Aesar, Heysham UK), Propyl paraben (Alfa Aesar, Heysham UK), Butyl paraben (Alfa Aesar, Heysham UK), Acetonitrile (MeCN) (sigma-Aldrich, UK) HPLC grade, Trifluoroacetic acid (TFA) (sigma-Aldrich, UK), Epilife® Medium (sigma-Aldrich, UK), Dulbecco′s Modified Eagle′s Medium (DMEM) medium (sigma-Aldrich, UK), Trypsin, Phosphate buffer Solution (PBS) (sigma-Aldrich, UK), HEPES Buffer (sigma-Aldrich, UK), Triton x-100 surfactant (sigma-Aldrich, UK), EDTA (sigma-Aldrich, UK), Sodium chloride (NaCl) (sigma-Aldrich, UK), ≥99% Ethanol (sigma-Aldrich, UK) and Distilled water.

5.2.3 Cell line (Human Keratinocyte).

CCD-1106 KERTr (KERTr) cell lines obtained via the ATCC Cell were cultured in Epilife supplemented with human keratinocyte growth supplement (Life Technologies) and primocin (100 µg/ml). CCD-1106 cells were routinely cultured in 75cm^2 culture flasks. the cells were maintained in a humidified incubator at 37°C and 5% CO_2. All culture media (Epilife, DMEM, trypsin, and PBS) and CCD 1106 KERTr cells were warmed at 37°C temperature for 30 minutes in a heated water bath. Cells were allowed to reach 80% confluency before routine sub-culture, according to the following procedure: the cells were washed in prewarmed PBS then Trypsin was added to the culture flask and placed in the incubator at 37°C for 5 minutes to remove adherent cells. After the cells were detached from the flask, a prewarmed DMEM medium was added to the flask to transfer cells to a falcon tube. Cells were centrifuged at 1000 rpm and plated in a new flask with Epilife medium to incubate overnight.

5.2.4 Lysis buffer preparation

The purpose of lysis buffer was to disrupt the exterior environment of a cell causing it to break open and release its contents. It was prepared with a chemical composition at pH 7.4 including 1% V/V Triton X-100, 50 mM of HEPES, 150 mM of NaCl, and 1mM EDTA. Triton X-100 was used as a surfactant or detergent that disrupts to break open the cells and NaCl was needed to prevent non-specific interactions within the cells. EDTA was added to inhibit and protect

against oxidative damage. HEPES was used to prevent the denaturation of cell contents. After preparation, it was immediately used and kept in a frozen condition.

5.2.5 Sample preparation and treatment

Each sample was prepared with 5% (v/v) ethanol in Epilife Media. The solubility of paraben in Epilife medium with 5% (v/v) ethanol at 40 ^{0}C was Methyl 9.76 mg/ml, Ethyl 7.88 mg/ml, Propyl 6.21 mg/ml and 5.48 mg/ml (shown in chapter 1). Parabens were weighed in electronic weight balance and dissolved in Epilife media that contained 5% (v/v) Ethanol. 0.4% (v/v) of paraben was applied in the cell line. As FDA and CIR allowed 0.4% of single paraben as a preservative in cosmetic products. We prepared paraben solution with 5% (v/v) ethanol for each paraben to make final concentration 0.4% (v/v) (Table 1). The cell density of each flask was measured through a hemocytometer while cell preparation. After preparation, it was treated and incubated at three different times such as 24, 48, and 72 hours. Then extracted paraben from the cell line and diluted with 30% of acetonitrile in distilled water for HPLC analysis.

Table 1: Treatment of parabens in cell line

Name	Saturation Concentration (in Epilife media with 5% v/v Ethanol) at 40 ^{0}C.	The volume of paraben in a 10ml volumetric flask (5% v/v Ethanol)	Volume media (Solution) 10ml (5% v/v Ethanol)	Final Concentration (%) w/v	The molecular weight of parabens	Final Concentration (mM)
Methyl paraben	9.76 mg/ml	4.1 ml	5.9 ml	0.4%	152.15 g/mol	26.28 mM
Ethyl paraben	7.88 mg/ml	5.1 ml	4.9 ml	0.4%	166.17 g/mol	24.07 mM
Propyl paraben	6.21 mg/ml	6.4 ml	3.6 ml	0.4%	180.2 g/mol	22.19 mM
Butyl paraben	5.48 mg/ml	7.4 ml	2.6 ml	0.4%	194.227 g/mol	20.59 mM

5.2.6 Cell extraction by Lysis buffer

The cell was extracted after incubation by using a lysis buffer. After incubation media was taken out from the flask and then washed with prewarmed PBS then Trypsin was added to the flask and placed in an incubator at 37°C for 5 minutes to remove adherent cells. After the cells

were detached from the flask, prewarmed DMEM medium was added to the flask and transferred in a falcon tube to centrifuge at 1500 rpm. When cells were precipitated, the media was carefully removed and washed with PBS. Then 500 µl Lysis buffer was added and mixed with the cell then kept in Icebox for 30 minutes to break open the cells and solubilize any parabens/metabolites without saponifying them. After that centrifuged at 10000 rpm to remove cell debris and filtrated with a 0.2µm size filter to prepare supernatant for HPLC analysis.

5.2.7 HPLC Analytical Assay

Throughout the whole project, an Agilent Technology 1290 infinity Lc system was used to obtain the data. The system was fitted with an Agilent Infinity Dad detector running to detect a UV wavelength of λ 210nm. The system was fitted with an Agilent Zabox C18 UHPLC column length of 50mm×2.1mm×1.7µm and the temperature was maintained at 40°C. The mobile phase A consisted of 0.1% TFA with a solution of Acetonitrile MeCN and distilled water in 90:10 as well as the mobile phase B consisted of 0.1% TFA with a solution of Acetonitrile MeCN and distilled water in 10:90 using a gradient elution Table 2 with a flow rate of 0.3ml/min. Throughout all experiments, the injection volume was set to 1.0 µl and the overall run time was 13 minutes. The post-run time was set to 1 minute.

Table 2: Gradient profile for HPLC Method.

Time (Minutes)	Mobile phase B: 0.1% TFA in MeCN: Water (10:90)	Mobile phase A: 0.1% TFA in MeCN: Water (90:10)
0	0	100
2	0	100
11	40	60
12	40	60
13	0	100

5.2.8 Preparation of calibration standards by HPLC

1 mg/ml stock solution of each paraben was prepared by dissolving in Epilife medium with 5% (v/v) ethanol. Then prepared the following concentration (0.2mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and1 mg/ml) in 5 ml volumetric flask. After that, those were filtrated and diluted with 30% Acetonitrile MeCN in distilled water to be analyzed through the HPLC system at 210 nm of UV wavelength according to the Chromatographic parameter.

5.3 Results and Discussion

5.3.1 Analysis of the Saturation solubility of parabens in Epilife medium with 5% v/v ethanol at 40°C

Different cell extraction techniques show different recoveries (Ivanisevic, *et al* 2013). “Simple modifications, such as changing the temperature or composition of the extraction solvent have impacts on the extraction process" (Dettmer, *et al* 2011). For parabens and their metabolite extraction, we used lysis buffer as the extraction solvent. How much paraben was metabolized after applying it to human cells (CCD1106) was measured during those extractions. We examined the four most commonly used parabens in skin care products, Methyl paraben, Ethyl paraben, Propyl paraben, and Butyl paraben. The solubility of paraben in an aqueous solution has decreased with a higher ester side chain (Twist and Zatz, 1986). Butyl paraben was less soluble than methyl paraben in the aqueous phase (shown in table 3). Due to Lipophilicity, parabens are poorly soluble in the aqueous phase (Cashman and Warshaw, 2005). Epilife medium (culture media) is an aqueous solution (Thermo Fisher Scientific, 2021). Throughout this study, to improve solubility in an Epilife medium we used ethanol as a co-solvent.

As discussed in first chapter, each paraben was dissolved in an Epilife medium with 5% ethanol at 40°C. The concentration of paraben was determined through Area *vs* Concentration Beer-lambert equation where the mean value (n=3) was considered (Figure 7). Samples were prepared with a concentration range from 0.2 mg/ml to 1mg/ml diluted with 30:70 Acetonitrile MeCN and distilled water solution to measure in HPLC parameter. Saturation solubility of paraben in Epilife medium with 5% (v/v) ethanol was measured during this study through Area *vs* Concentration beer-lambert equation as discussed in chapter 1. Figure 7: demonstrates the linearity of the calibration graphs. During this study, Solubility of paraben in epilife medium at 40⁰C was used because this is close to human skin temperature. The average temperature of skin has between 36⁰C to 39⁰C for adult Male and Female (lee et al 2019). Saturation solubility for parabens in Epilife medium with 5% (v/v) ethanol at 40⁰C shown in table 3.

Table 3: Saturation solubility for parabens in Epilife medium with 5% (v/v) ethanol at 40⁰C.

Paraben	Concentration mg/ml			Mean mg/ml	Standard deviation	% RSD
	Sample 1	Sample 2	Sample 3			
Methyl paraben	9.701	10.031	9.556	9.76	0.24	2.45 %
Ethyl paraben	8.163	7.646	7.839	7.88	0.26	3.29 %

Propyl paraben	6.407	6.299	5.933	6.21	0.25	4.02 %
Butyl paraben	5.731	5.388	5.314	5.48	0.22	4.01 %

5.3.2 Analysis of Paraben and metabolite by cell extract in HPLC.

Many analytical techniques can be used for the analysis of parabens; however, the most frequently used technique is high-Performance liquid chromatography (HPLC). Whereas fewer sources report using ultrahigh Performance liquid chromatography (UHPLC). Gas chromatography (GC) and Capillary electrophoresis (CE) have been reported much less frequently and single determinations have been made by using Spectrophotometric or voltametric methods (Irena *et al* 2014). An HPLC method can be used for the identification and quantitative determination of the parabens in various formulations (Coral *et al* 2005). Parabens are highly soluble in alcohol, acetone, and some other organic solvents (Twist and Zatz, 1986). In this study, we used ethanol to enhance the solubility of paraben in an Epilife medium. Solubility properties make reversed-phase HPLC an ideal separation/identification technique for the analysis of parabens (Coral *et al* 2005).

In this study, we also prepared a combination of four parabens including Methyl paraben 9.76 mg/ml, ethyl paraben 7.88 mg/ml, propyl paraben 6.21 mg/ml, and butyl 5.48 mg/ml in Epilife medium with 5% (v/v) ethanol as test mix to analyzed with HPLC. Clear differentiation in peak elution was observed (Figure 19). The chromatographic elution showed a retention time of methyl, ethyl, propyl, and butyl paraben at approximately 5.3, 7.5, 9.6, and 11.5 minutes respectively at wavelength 210 nm. Due to the shorter ester chain, methyl paraben elutes earlier than the longer ester chain butyl paraben (Imamovic *et al* 2012).

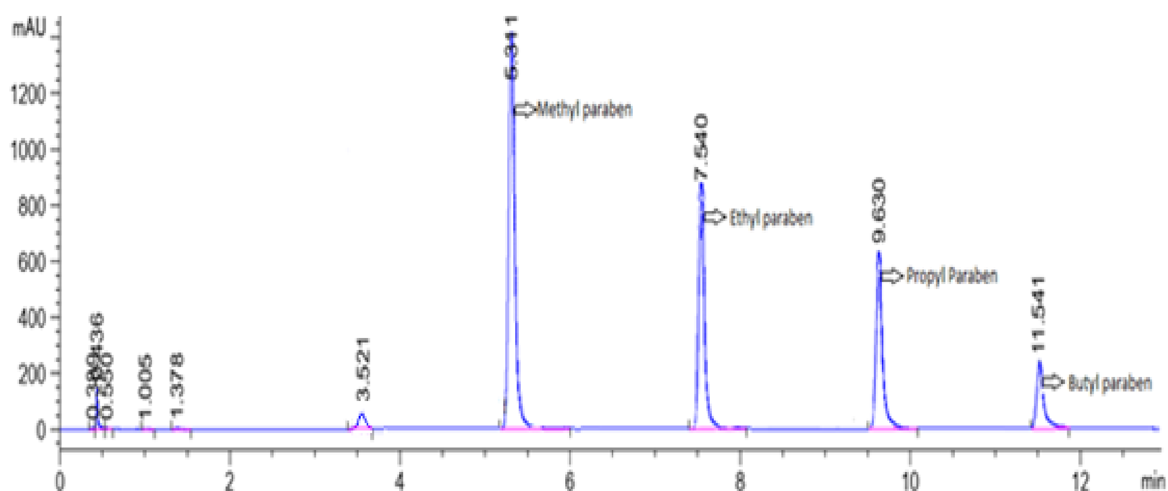

Figure 19: Test mix of four parabens Including methyl paraben, ethyl paraben, propyl paraben, and butyl paraben at saturated concentration diluted with 30% Acetonitrile MeCN in distilled water run through HPLC. The chromatographic condition was performed as the above method.

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (Thermo Fisher Scientific, 2021). "The advantage of using cell lines in scientific research is their homogeneity and associated reproducibility of results that can be obtained from using a batch of clonal cells" (Segeritz and Vallier 2017). Cells require to mature enough to treat with any substance for biological activity (Briske-Anderson, Finley, and Newman 1997). Treatment will proceed with cell confluency of more than 80-90% in the culture flask (LONZA, 2021). Usually, the cell has matured within Passage 5-15 (number of the subculture) (Briske-Anderson, Finley, and Newman 1997). In this study, CCD-1106 KERTr (KERTr) cell line was prepared at passage (number of the subculture) 7 (P7). Cell density in each flask was also measured using a Haemocytometer (Table 4). Measurement of Cell density is important to maintain cell quantity in each flask (Segeritz and Vallier 2017).

Table 4: Cell density of each paraben in 75-cm^2 culture flasks.

Name	The average number of cells	Target cell density	Total Volume	Dilution
Methyl paraben	2.3×10^5 cell/ml	3.4×10^4 cell/ml	30 ml	4.4
Ethyl paraben	2.4×10^5 cell/ml	3.4×10^4 cell/ml	30 ml	4.2
Propyl paraben	2.3×10^5 cell/ml	3.4×10^4 cell/ml	30 ml	4.4
Butyl paraben	2.2×10^5 cell/ml	3.4×10^4 cell/ml	30 ml	4.6

In this study, we used Epilife as culture media which contained a growth supplement and antibiotic/anti-fungal reagent. During the culture, each flask maintained 10ml of culture media. Generally, cells require 4 to 6 weeks to prepare for any experiment (LONZA, 2021). Trypsin and DMEN media are used to remove adherence and proliferation of the cell. DMEN media contains a different combination of L-glutamine and sodium pyruvate (SIGMA-ALDRICH, 2021). FDA and CIR recommend 0.4% for individual paraben in skin care products. So that we used 0.4% of each paraben to treat the CCD-1106 KERTr (KERTr) cell line. Throughout this study, Paraben concentration in cell culture was defined in millimolar, the concentration of paraben including Methyl paraben was 26.28 mM, Ethyl paraben was 24.07mM, Propyl paraben was 22.19 mM and Butyl paraben was 20.59mM applied for this study which was equivalent to 0.4%. After application, those were incubated for 24 hours, 48 hours, and 72 hours to extract paraben and their metabolite by using lysis buffer as shown in figure 20.

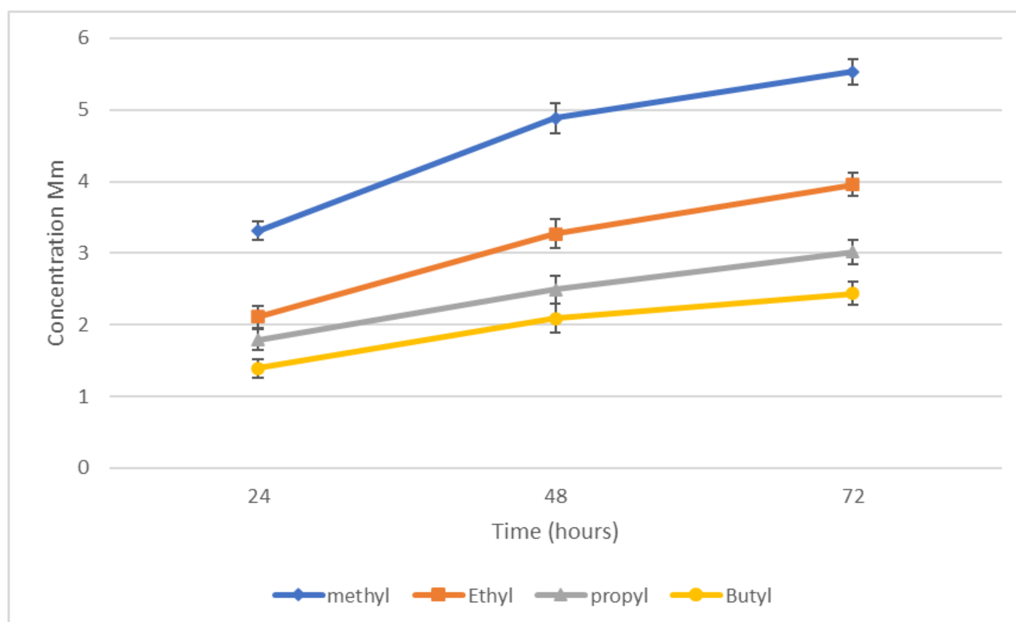

Figure 20: Concentration extract through lysis buffer each paraben includes Methyl paraben, Ethyl paraben, Propyl paraben, and Butyl paraben treated with CCD-1106 KERTr (KERTr) human keratinocyte cell line and incubated at three different times at 24 hours, 48 hours, and 72 hours by HPLC analysis. Concentration as express in millimolar (mM). Data are mean values +/- SD and 3 replicates respectively methyl, ethyl, propyl, and butyl paraben from this experiment.

During this study, parabens were treated and incubated in 75cm^2 culture flasks at 37°C with 5% CO_2. "Human skin contains CO_2 which acts as a pH buffer to allow for gas, nutrient and metabolites fluctuations without causing pH changes" (LONZA, 2021) and a temperature range from 35°C to 40°C (Segeritz and Vallier 2017). So, the ideal condition for the cell maintains in an incubator at 37^{0}c with 5% CO_2 for better effect. (Segeritz and Vallier 2017). Throughout the study, we recorded respectively at 24 hours methyl paraben 3.31 mM, ethyl paraben 2.11 mM, propyl paraben 1.79 mM, and Butyl paraben 1.39 mM, at 48 hours methyl paraben 4.89 mM, ethyl paraben 3.27 mM, propyl paraben 2.49 mM, and Butyl paraben 2.09 mM, and at 72 hours methyl paraben 5.53 mM, ethyl paraben 3.96 mM, propyl paraben 3.02 mM, and Butyl paraben 2.44 mM after incubated and extracted by lysis buffer. Methyl paraben was metabolized slightly higher than other parabens due to solubility in Epilife media as well as more incubation time also enhanced the rate of metabolism of each paraben. This study showed that metabolized paraben after extraction was found Methyl paraben > ethyl paraben > propyl paraben > butyl paraben. During this study, amount of paraben was extract through lysis buffer at 24 hours, 48 hours and 72 hours shown in table 5, 6, and 7.

Table 5: Amount of paraben extracted through lysis buffer at 24 hours.

Name	Applied dose			Total volume	Applied Amount	Extraction dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.504 mg/ml	3.31 mM	5.04mg	12.59 %
Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.351 mg/ml	2.11 mM	3.51 mg	8.77 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.322 mg/ml	1.79 mM	3.22 mg	8.06 %
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.270 mg/ml	1.39 mM	2.70 mg	6.75%

Table 6: Amount of paraben extracted through lysis buffer at 48 hours.

Name	Applied dose			Total volume	Applied Amount	Extracted dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.744 mg/ml	4.89 mM	7.45 mg	18.61 %
Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.543 mg/ml	3.27 mM	5.43 mg	13.58 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.449 mg/ml	2.49 mM	4.49 mg	11.22 %
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.406 mg/ml	2.09 mM	4.06 mg	10.15 %

Table 7: Amount of paraben extracted through lysis buffer at 72 hours.

Name	Applied dose			Total volume	Applied Amount	Extracted dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.744 mg/ml	4.89 mM	7.45 mg	18.61 %
Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.543 mg/ml	3.27 mM	5.43 mg	13.58 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.449 mg/ml	2.49 mM	4.49 mg	11.22 %
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.406 mg/ml	2.09 mM	4.06 mg	10.15 %

In this study, we calculated the % extraction of each paraben through lysis buffer shown in figure 21. In extraction approximately methyl paraben was 12.59 % in 24 hours, 18.61 % in 48 hours, and 21.04 % in 72 hours, ethyl paraben was 8.77 % in 24 hours, 13.58 % in 48 hours, and 16.45 % in 72 hours, propyl paraben was 8.06 % in 24 hours11.22 % in 48 hours, and 13.61 % in 72 hours. Butyl paraben was 6.75 % in 24 hours, 10.15 % in 48 hours, and 11.85 % in 72 hours. Paraben extraction was increased with increased time as well as short ester chain paraben was higher than the longer ester chain. The extraction from the cell line depends on cell density, temperature, cell proliferation, and interaction with the cell line (Sapcariu *et al* 2014). A healthy cell can have better extraction (Ser *et al* 2015). The quantity of cells also has a greater impact

on the extraction process (Ser *et al* 2015). This study demonstrated paraben has a prolonged effect on the human cell. it was incubated with a cell line for a longer time, to be more absorbed and resulting in better extraction. This is our novel founding during this experiment.

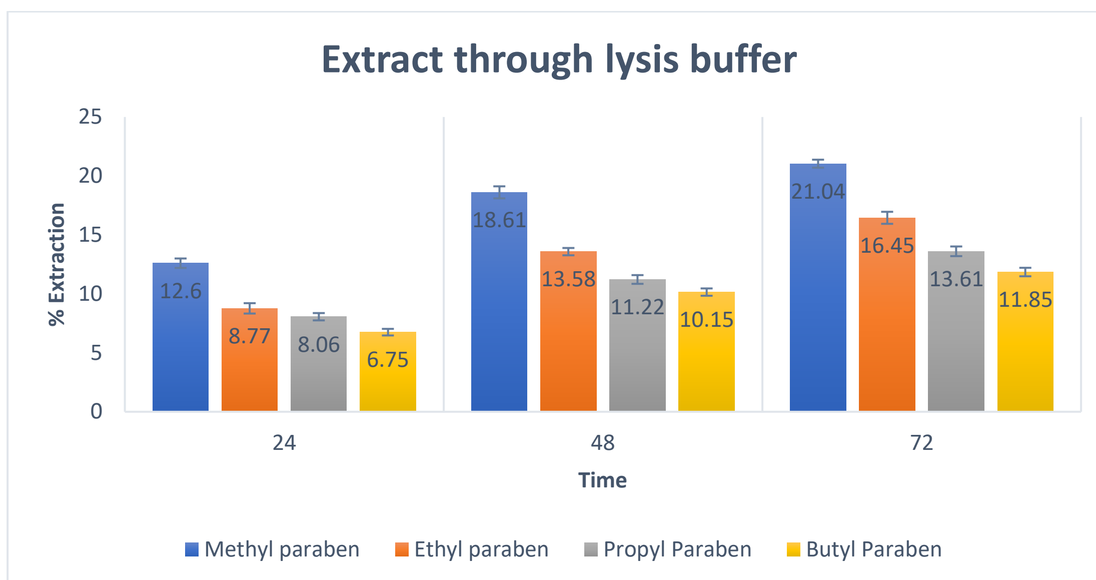

Figure 21: % Concentration of each paraben extract through lysis buffer after incubation at three different times at 24 hours, 48 hours, and 72 hours by HPLC analysis. Data are mean values +/- SD and 3 replicates respectively methyl, ethyl, propyl, and butyl paraben from this experiment.

There are different types of extraction procedures are available to extract from cells such as Lysis buffer, 80% methanol (MeOH), acetonitrile (ACN) and phenol-*chloroform* isoamyl alcohol, etc (Dietmair S *et al* 2010) (Ser *et al* 2015). In this study, we used a lysis buffer to extract paraben and its metabolites from a cell. The material extracted from the cell depends on lysis buffer composition (Shehadul, Aditya, and Selvaganapathy 2017). There are many types of lysis buffer, most are commercially available, however, but some are required to prepare with chemical composition for target molecules extraction (Sepmag 2021). In this experiment, lysis buffer was prepared with a composition of HEPES, Triton X-100, NaCl, and EDTA. Cell lysis is used to disrupt the exterior environment of the cell membrane but preserves target molecules (Sepmag 2021). Which material must extract also depends on which type, or composition of lysis buffer require (Shehadul, Aditya, and Selvaganapathy 2017). Due to lipophilicity, paraben was extracted through this lysis buffer which acts as a surfactant or detergent. "Detergents react with cell membrane forming pores on the surface of membrane resulting in the release of intracellular components" (Shehadul, Aditya, and Selvaganapathy 2017).

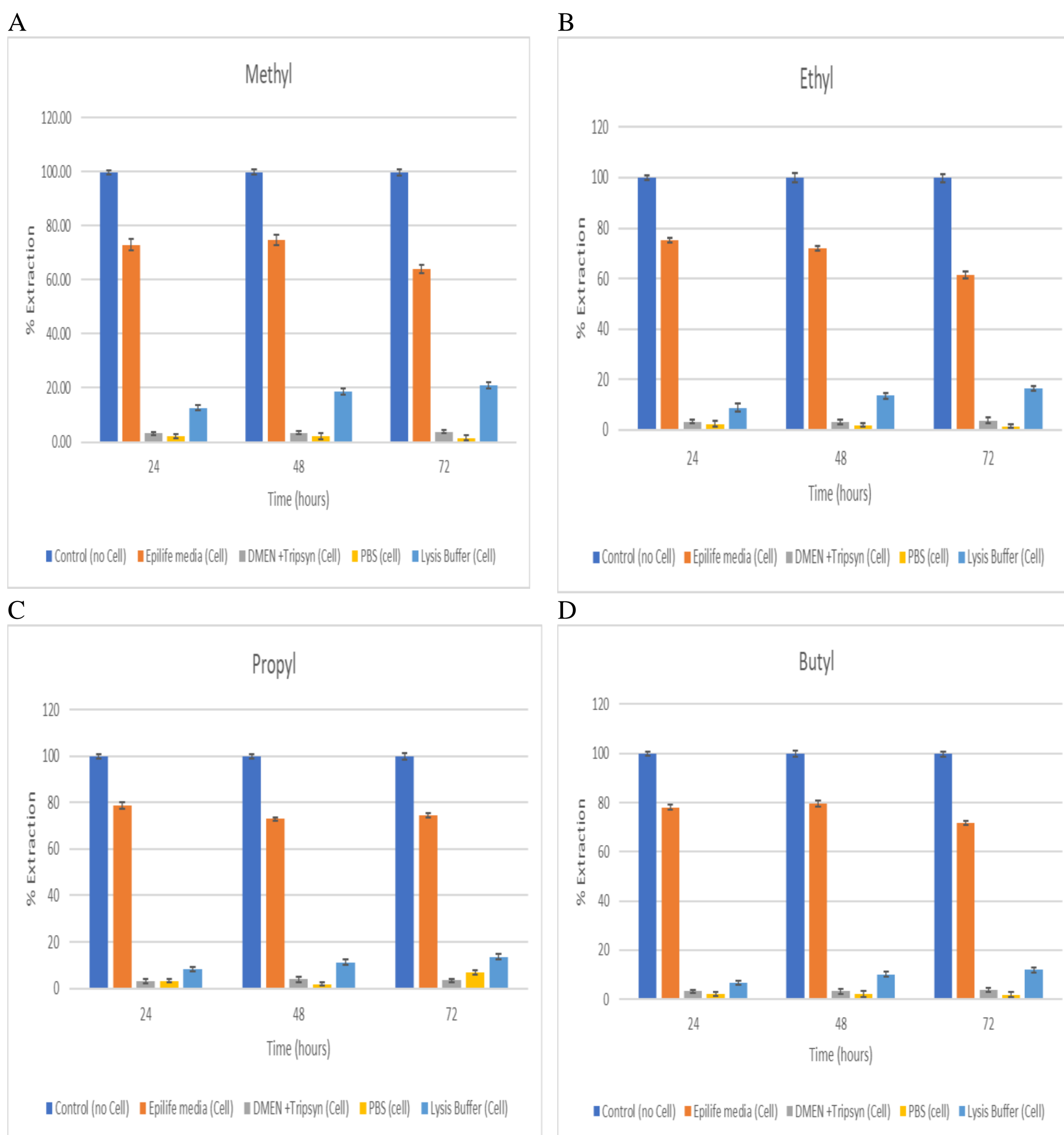

Figure 22: Extraction parameter of each paraben includes A) Methyl paraben, B) Ethyl paraben, C) Propyl paraben, and D) Butyl paraben in different phases such as Control (no cell), Epilife medium, DMEN+Trypsin, PBS, and Lysis buffer at 24 hours, 48 hours, and 72 hours of incubation by HPLC analysis. Concentration expresses as a percentage. Data are mean values +/- SD and 3 replicates in this experiment.

In this study, we analyzed different phases of extraction parameters of each paraben including Methyl paraben, Ethyl paraben, Propyl paraben, and Butyl paraben in different phases such as Control (no cell), Epilife medium, DMEN+Trypsin, PBS, and Lysis buffer at 24 hours, 48 hours, and 72 hours of incubation as shown in figure 22. For methyl paraben 26.28 mM was applied, and after 24 hours of incubation, approximately 73% paraben was recorded in culture

media which was an Epilife medium, as well as 3.16 % and 2.09% paraben was found in DMEN+Trypsin and PBS wash. Similarly, after 48 hours and 72 hours of incubation, a large amount of paraben was found in Epilife medium approximately 75 % and 64 %. Where only 12.59 %, 18.61 %, and 21.04 % of methyl paraben were found respectively at 24, 48, and 72 hours in lysis buffer after extraction. For ethyl paraben 24.07 mM was applied, after 24 hours of incubation, almost 76% paraben was recorded in Epilife media. In DMEN+Trypsin and PBS were found at approximately 4 % and 3%. In 48 and 72 hours of incubation, approximately 72 % and 62 % of paraben were recorded in the Epilife medium. Whereas 8.77 %, 13.58 %, and 16.45 % of paraben were found respectively at 24, 48, and 72 hours in lysis buffer after extraction. For propyl paraben 22.19 mM was applied, after incubation, almost 70-80% paraben was found in the Epilife medium. In DMEN+Trypsin and PBS was found approximately 3-4% during at 24 to 72 hours. However, 8.06 %, 11.22 %, and 13.61 % of paraben were found respectively at 24, 48, and 72 hours in lysis buffer after extraction. And butyl paraben 20.59 mM was applied after incubation nearly80% paraben was found in the Epilife medium. In DMEN+Trypsin and PBS was found 3-4% of paraben in 24 to 72 hours. Although,6.75%, 10.15 %, and 11.85 % of paraben were found respectively at 24, 48, and 72 hours in lysis buffer after extraction. During this experiment, we observed adequate amount of parabens were incorporated into a cell which was extracted through lysis buffer as shown in figure 21 and figure 22. This study also exhibited control which was the same concentration of parabens dissolved in Epilife media without cell line at a similar condition.

Table 8: Total % recovery of parabens after 24 hours of incubation.

Name	Applied dose. (mM)	Paraben in media. (mM)	Paraben in cell-washed media. (mM)		Extracted paraben from the cell. (mM)	Total % Recovery (mM)
		Epilife Media	DMAN media	PBS	Lysis buffer	
Methyl paraben	26.28	19.17	0.83	0.55	3.31	90.79
Ethyl Paraben	24.07	18.11	0.81	0.58	2.11	89.77
Propyl Paraben	22.19	17.46	0.71	0.69	1.79	93.05
Butyl Paraben	20.59	16.09	0.66	0.41	1.39	90.09

Table 9: Total % recovery of parabens after 48 hours of incubation.

Name	Applied dose. (mM)	Paraben in media. (mM)	Paraben in cell-washed media. (mM)		Extracted paraben from the cell. (mM)	Total % Recovery (mM)
		Epilife Media	DMAN media	PBS	Lysis buffer	
Methyl paraben	26.28	19.66	0.88	0.59	4.89	99.01
Ethyl Paraben	24.07	17.36	0.71	0.47	3.27	90.61
Propyl Paraben	22.19	16.18	0.84	0.39	2.49	89.68
Butyl Paraben	20.59	16.37	0.65	0.41	2.09	94.80

Table 10: Total % recovery of parabens after 72hours of incubation.

Name	Applied dose. (mM)	Paraben in media. (mM)	Paraben in cell-washed media. (mM)		Extracted paraben from the cell. (mM)	Total % Recovery (mM)
		Epilife Media	DMAN media	PBS	Lysis buffer	
Methyl paraben	26.28	16.81	0.88	0.37	5.53	89.76
Ethyl Paraben	24.07	16.75	0.91	0.33	3.96	91.19
Propyl Paraben	22.19	16.51	0.75	0.81	3.02	95.04
Butyl Paraben	20.59	14.77	0.79	0.39	2.44	89.31

Different extraction methods result in different recoveries (Ivanisevic *et al* 2013). In this study, we examined the total % recovery of each paraben such as Methyl, Ethyl, Propyl, and Butyl paraben after 24 hours, 48 hours, and 72 hours of incubation at 37°C and 5% CO_2 shown in tables 8, 9 and 10. This study also demonstrated, each phase of extraction. Methyl paraben was found at 90.79% at 24 hours, 99.01% at 48 hours, and 89.76% at 72 hours of incubation. Ethyl paraben was found at 89.77% at 24 hours, 90.61% at 48 hours, and 91.19% at 72 hours of incubation. Then, Propyl paraben was recorded at 93.05% at 24 hours, 89.68% at 48 hours, and 95.04% at 72 hours of incubation. Finally, Butyl paraben was found at 90.09% at 24 hours, 94.80 % at 48 hours, and 89.31% at 72 hours of incubation. The procedure for extraction plays

an extremely important role in the determination of the range of recovery (Ser *et al* 2015). Parabens were prepared with Epilife medium then trypsin was used to remove adherent cells from the surface and add DMEN media to proliferation cell after that it was washed with PBS and finally extracted paraben through lysis buffer. "Better understanding of extraction parameters will allow optimization of extraction protocols" (Dietmair *et al* 2010).

The quantity of extraction also varies on the chemical composition of the extraction solvent (Huichang *et al* 2013). Throughout this study, a large amount of paraben was retained on Epilife media, small trace was recorded from DMEN + Trypsin and PBS. A recent review reported that washing is a common practice to improve analytical assessment such as signal-to-noise ratio however it removes extract compounds outside the cell (Leon, *et al* 2013). In this study, PBS was used as a washing solvent. PBS is non-toxic and prevents cells from rupturing or shrivelling up due to osmosis (Martin, *et al* 2006). Trypsinization is the most popular detachment technique in cell culture, approximately 95% of cells are detached from the culture plate (Yuta *et al* 2019) which has an impact on this recovery. This study also reported solubility of paraben in Epilife medium was extremely poor in longer ester chain paraben however it was holding paraben to incorporate into the cell. In Lysis buffer, a modest amount of paraben was detected during this study. This study also demonstrated the amount of 4 Hydroxybenzoic acid (metabolite) metabolized into the keratinocyte cell extracted from each paraben through lysis buffer at 24 hours,48 hours and 72 hours shown in table 11, 12 and 13.

Table 11: Amount of 4 Hydroxybenzoic acid was metabolized at 24 hours.

Name	Applied dose			Total volume	Applied Amount	Extraction dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.157 mg/ml	1.03 mM	1.57 mg	3.91 %
Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.111 mg/ml	0.67 mM	1.11 mg	2.78 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.083 mg/ml	0.46 mM	0.83 mg	2.09 %
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.062 mg/ml	0.32 mM	0.62 mg	1.55 %

Table 12: Amount of 4 Hydroxybenzoic acid was metabolized at 48 hours.

Name	Applied dose			Total volume	Applied Amount	Extraction dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.181 mg/ml	1.19 mM	1.81 mg	4.51 %

Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.133 mg/ml	0.80 mM	1.33 mg	3.33 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.115 mg/ml	0.64 nM	1.15 mg	2.89%
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.081 mg/ml	0.42 mM	0.81 mg	2.01 %

Table 13: Amount of 4 Hydroxybenzoic acid was metabolized at 72 hours.

Name	Applied dose			Total volume	Applied Amount	Extraction dose		Extraction amount	% extraction
Methyl paraben	0.4% w/v	4mg/ml	26.28 mM	10 ml	40 mg	0.198 mg/ml	1.30 mM	1.98 mg	4.95 %
Ethyl paraben	0.4% w/v	4mg/ml	24.07 mM	10 ml	40 mg	0.148 mg/ml	0.89 mM	1.48 mg	3.71 %
Propyl paraben	0.4% w/v	4mg/ml	22.19 mM	10 ml	40 mg	0.124 mg/ml	0.69 mM	1.24 mg	3.11 %
Butyl paraben	0.4% w/v	4mg/ml	20.59 mM	10 ml	40 mg	0.095 Mg/ml	0.49 mM	0.95 mg	2.37 %

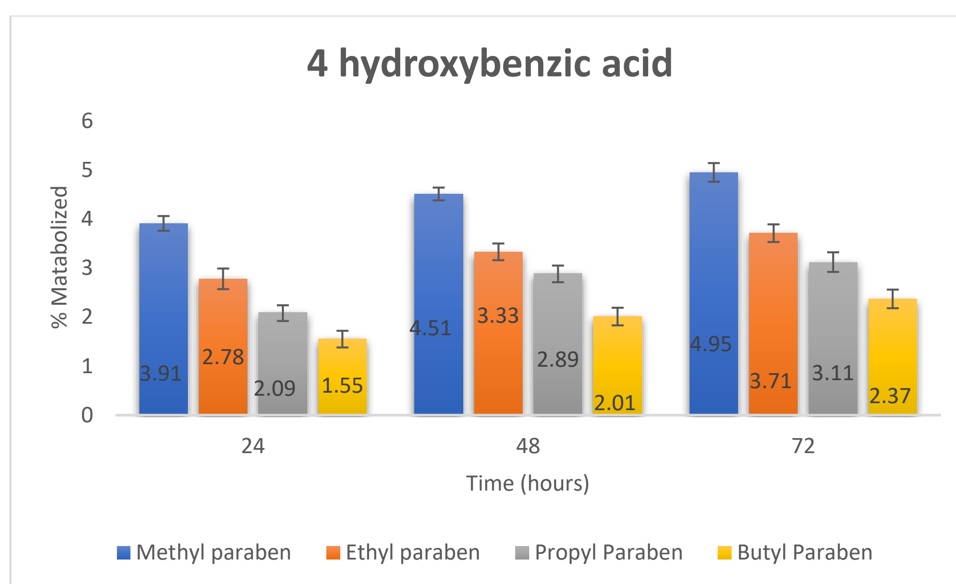

Figure 23: % Concentration of metabolite extract through lysis buffer after incubation at three different times at 24 hours, 48 hours, and 72 hours by HPLC analysis. Data are mean values +/- SD and 3 replicates respectively methyl, ethyl, propyl, and butyl paraben from this experiment.

Throughout this study, we also investigated % the metabolism of paraben to 4 hydroxybenzoic acids. A high amount of metabolite was detected at 72 hours of incubation and methyl paraben was highly metabolized. Parabens can be absorbed and retained into the human tissue without hydrolysis of tissue esterases (Oishi 2004). Solubility of Parabens in the aqueous phase is much better in shorter alkyl esters chains (Cashman and Warshaw 2005). This study demonstrated

highly soluble paraben was more metabolized. Incubation time was a greater impact on metabolite extraction; increased incubation time has increased metabolism. In this study, we applied 0.4% of parabens dissolved in a cell. A small amount of concentration will have low metabolized into the skin (Cashman and Warshaw 2005). Parabens in contact with the skin penetrate the stratum corneum in inverse relation to the ester chain length (Elder *et al* 1984). In our experiment, methyl paraben was more highly metabolized than butyl paraben as well as increased metabolism rate with increased incubation time shown in figure 24. The approved concentration from FDA and CIR was safe for the human body. During this study low amount of paraben was metabolized at a low concentration. The rank of % metabolism in cell extract was Methyl paraben > Ethyl paraben > Propyl paraben > Butyl paraben.

5.4 Conclusion

Parabens have excellent antimicrobial properties that have been used in most skin care products to maintain their effectiveness. They are stable, effective over a wide pH range, and active against all broad spectrums of microbes. There are four most used preservatives Methyl paraben, ethyl paraben, propyl paraben, and butyl paraben in skin care products. Butyl parabens are more active however, it has more toxic than others. Therefore, a combination of parabens has useful in cosmetics products to maintain both microbial activity and toxicity. This study has analyzed FDA and CIR recommended single paraben concentration of 0.4 % w/w in human keratinocyte cells through extraction. Methyl paraben has highly metabolized but lower antibacterial activity, in reverse butyl paraben, has low metabolized but higher antibacterial activity. Metabolism of parabens in the human cell also increases with the increased duration of time on human skin. Parabens are applied to the skin and remain for a longer time than a probability have more metabolize into the skin however, 0.4% of individual parabens have a very low metabolic effect on human skin this is also a novel finding in our research. This study also demonstrated the ranking of metabolism which is Methyl paraben > Ethyl paraben > Propyl paraben > Butyl paraben.

6. Discussion

Overall, the objectives were successfully achieved, with a few novel findings were reported. Paraben safety evaluation in human skin was slightly tricky to carry out as an *In-vitro* analysis. In this research, our main purpose was to review the current concentration of paraben in skin care products which has been approved by the Food and Drug Administration (FDA) and the Cosmetic Ingredient Review (CIR), by reason of some countries and organisation are against this statement. In fact, there is no sufficient evidence has been established yet to clarify the safety or adverse effect of this recommended concentration. Therefore, we set up different types of method to analyzed that concentration. Throughout those method preparation, we considered a few questions regarding current controversy such as; are parabens safe in cosmetic products? In what concentrations are they safe and how does paraben metabolise during permeation studies via human skin? However, due to health and safety regulations as well as lack of availability and high cost, we used pig skin instead of human skin. Pig skin is a good model for human skin permeability due to its histological and physiological properties are almost similar to human skin (Ian and Robert, 1992). As we also found some articles which have used pig skin as an *in-vitro* model for human skin permeability. Paraben safety evaluation was successfully studied through those methods.

In this research, we determined paraben toxicity in human keratinocyte cell line through cytotoxicity assay and determination of % metabolism of parabens via Pig skin trough Franz diffusion cell, as well as *In-vitro* analysis of parabens in recommended concentration in the human keratinocyte cell line. The Food and Drug Administration (FDA) and The Cosmetic Ingredient Review (CIR) propose a concentration of 0.4% w/w for single parabens and 0.8% w/w for a combination of parabens safe in skin care products (Campbell, Yoon. and Clewell, 2015). While there is not enough evidence to support this concentration in the skin. Paraben safety studies have shown that parabens are not completely safe as they have an estrogenic effect on human skin (Darbre, 2004) and in contact with UV can cause skin cancer (Boberg, 2010); however, some articles said this estrogenic effect may not be as potent as previously reported (Shaw and deCatanzaro, 2009). Although they do not have evaluated any particular concentrations on human skin. During this study, we investigated 0.4% v/v of single paraben in a human keratinocyte cell line. This keratinocyte cell was obtained from the epidermis layer of human skin (ATCC, 2022). This cell line was known as the CCD1106 KERTr cell (ATCC,

2022). In Franz diffusion study, Paraben was permeated through Landras breed pig's shoulder skin which was collected from Yorkshire. In the permeation study, a multi-station Franz diffusion system was used to determine % of the metabolism of parabens.

6.1 Saturation solubility of paraben in Epilife medium with 5% v/v ethanol.

For the investigation of paraben concentration and toxicity analysis, solubility was a very important factor. Parabens have different solubility in different solvents (Cashman and Warshaw, 2005). Those are highly soluble in an organic solvent but poorly soluble in an aqueous solution (Giordano *et al* 1999). Solubility depends on the length of the alkyl esters chain which means a longer chain of alkyl esters increases oil solubility and decreases water solubility (Twist and Zatz, 1986). Parabens are lipophilic in nature. Due to lipophilicity, paraben has less soluble in an aqueous solution (Cashman and Warshaw, 2005). This lipophilicity increases with increases in the alkyl ester chain (Cashman and Warshaw, 2005). In this study, it has been proved that solubility of paraben has reduced with longer alkyl ester chain paraben in an aqueous solution as discussed in chapter 1. Methyl paraben (MP) has a short alkyl ester chain and Butyl paraben (BP) has a longer alkyl ester chain paraben. So, we observed solubility of Methyl paraben was slightly higher than Butyl paraben in an aqueous solution. Temperature also plays an important role in the solubility of paraben. Paraben has poorly soluble at room temperature while the solubility of parabens has increased with increased temperature in aqueous solution (Giordano *et al* 1999).

In this study, parabens were dissolved in an epilife medium which was a cell culture medium. Epilife medium act as an aqueous solution (Thermo Fisher Scientific, 2016). It consists of essential and non-essential amino acids, vitamins, other organic compounds, trace minerals, and inorganic salts (Thermo Fisher Scientific, 2016). Human keratinocyte growth supplement (Life Technologies) and primocin (100 µg/ml) were added to prepare this epilife medium for CCD1106 KERTr cell culture. In the 1st and 3rd chapter, the solubility of paraben in the epilife medium is described. During this study, due to lipophilicity, parabens were poorly soluble in epilife medium. To enhance this solubility to prepare a required concentration, we used 5% (v/v) ethanol. Ethanol is an organic solvent and paraben has highly soluble in ethanol (Paruta, 1969). Methyl paraben (MP), Ethyl paraben (EP), Propyl paraben (PP), Butyl paraben (BP), and Benzyl paraben (BzP) were involved in this experiment.

The saturation solubility of parabens in ethanol (EtOH) was also determined during this study to clarify how good ethanol is as a solvent in paraben. This study was performed with excess amounts of parabens dissolved in ethanol and after filtration supernatant was collected to determine by using a UV/Visible Spectrophotometer. This study showed ethanol was an excellent solvent and the solubility of parabens was very high as discussed in 1st chapter. Paraben was highly dissolved in ethanol due to the polarity of ethanol and the hydroxyl group (-OH) has responsibility for this polarity of ethanol (Paruta, 1969). Therefore, ethanol was used to enhance the solubility of parabens in epilife medium. Human keratinocyte cell lines were cultured in an epilife medium, and it was act as an aqueous solution in this experiment. Our aim was parabens were treated with CCD1106 KERTr human keratinocyte cell in presence of epilife medium as solvent.

In this study, the solubility of paraben in epilife medium with 5% (v/v) ethanol was also determined. Due to poor solubility in the epilife medium, we combined 5% (v/v) ethanol to improve this solubility. The alkyl ester chain has responsible to reduce this solubility in the epilife medium due to its lipophilicity. For this study, we required an excellent solvent that is used to improve this solubility to prepare appropriate concentration and we used ethanol. However, ethanol has a toxic effect on CCD1106 KERTr human keratinocyte cells (ATCC, 2022). Nevertheless, the toxicity effect in human keratinocyte cells was slightly less in ethanol than in other organic solvents (Tapani *et al*, 1996). Therefore, only 5% (v/v) ethanol was used in the epilife medium to enhance the solubility of parabens. Solubility can also be enhanced with higher temperature (Giordano *et al* 1999).

In this experiment, we also measured three different temperatures in an epilife medium with 5% (v/v) ethanol for this solubility study since it has poorly soluble in epilife medium at room temperatures. Although, 5% (v/v) ethanol was not enough to prepare the appropriate concentration. Increasing ethanol concentration will increase the toxicity in human cells as they have a toxic effect on human cells. Therefore, the temperature was involved in achieving those concentrations. The aqueous solubility of paraben has very poor at room temperature while those will be completely dissolved in solution at temperatures above 60°C (Giordano *et al* 1999). In this study, three different temperatures including 15℃ (room temperature), 40℃, and 60℃ were involved. Used a standard thermometer to check those temperatures. During this research, the solubility of paraben in epilife medium with 5% (v/v) ethanol at 15℃ (room

temperature), 40°C, and 60°C were also discussed in 1st chapter. Where we found parabens were poorly soluble at room temperature at 15°C. However, this solubility has improved with temperature increases (Giordano *et al* 1999). At 40°C temperatures was ideal to prepare appropriate concentrations in human cells.

6.2 Determination of paraben toxicity using a cytotoxicity assay.

In cytotoxicity assay, we applied saturation solubility of paraben in epilife medium with 5% (v/v) ethanol at 40°C. As the human Skin can contain 35- 40°C. (Lee *et al* 2019). For compatibility with human skin, we selected the solubility of paraben in an epilife medium with 5% (v/v) ethanol at 40°C. Saturation concentrations of methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), and benzyl paraben (BzP) were dissolved in an epilife medium with 5% (v/v) ethanol at 40°C to prepared sample with appropriate concentrations in this experiment. Paraben toxicity was evaluated through this cytotoxicity assay. This is one of the most widely used methods to detect cell viability or drug toxicity (Skehan *et al.*, 1990). This method will evaluate how much parabens are toxic in human cells via cell survival. This study was performed with an SRB assay. SRB can bind with cellular protein and measure the total biomass in drug components (Orellana and Kasinski, 2016). "SRB is a bright pink aminoxanthene dye that can bind with cell lines and release the incorporated dye after washing from stained cells which are directly proportional to the cell biomass" (Biotrend, 2020). In this study, 96-Well Cell Culture Plate was used to carry out this SRB assay. Where we can evaluate different sets of concentrations in one plate in the same environment.

For this study, we bought CCD1106 KERTr human keratinocytes as frozen due to their availability in the market (ATCC, 2022). Then, cells were cultured with epilife medium to grow enough to treat with paraben. CCD-1106 cells were routinely cultured in 75cm^2 culture flasks. The cells were maintained in a humidified incubator at 37°C and 5% CO_2. Usually, cells have matured within Passage 5-15 (number of the subculture) (Briske-Anderson, Finley, and Newman 1997). In this study, cells were treated with paraben concentration within Passage 8-11 and each passage has been taken place when cell confluency was more than 80% in 75cm^2 culture flasks. The cell will be grown healthy and proliferation during the culture when cell confluency will be more than 80-90% within the culture flask (Lonza, 2021). In this study, cell density was also measured to maintain the growth of this cell as described in 1st and 3rd

chapters. Calculating cell density was important, which helped to maintain an equal amount of cells in each plate for all parabens to identify exact toxicity. Hemacytometer was used to measure those cell densities to treat with paraben in a 96-well cell culture plate. After treatment SRB assay was applied to detect paraben toxicity by using the xMark™ Microplate Absorbance Spectrophotometer at 570nm.

During this cytotoxicity assay, the toxicity of paraben in appropriate concentrations has been evaluated by using this SRB assay. Throughout this method toxicity level of parabens including methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), and benzyl paraben (BzP) was determined by this cytotoxicity studies. Our goal was how much parabens are toxic at 0.4% v/v as recommended in cosmetics. Therefore, we set a range of concentration concentrations as 0% (v/v), 0.1 % (v/v), 0.2 % (v/v), 0.3 % (v/v), 0.4 % (v/v) and 0.5 % (v/v) of each paraben in 96-well cell plate where parabens were dissolved in culture media (epilife medium) with 5% v/v ethanol to dilute those concentrations. Toxicity was measured in those concentrations through cell survival after treatment with paraben. During this experiment, the percentage (%) of cell survival was determined in those concentrations as shown in 1st chapter. Higher cell survival implies lower toxicity of parabens.

Parabens were measured at each concentration from 96 cell plates in six replicates, Data were mean values (n=3), then made an average to calculate the % cell survival of each replicate. Finally, the mean of % cell survival and standard deviation were calculated to plot on the chart described in 1st chapter. During this Study, 5% (v/v) ethanol as control was also determined to verify the toxicity of ethanol in human keratinocytes. During this study, the Metabolite of parabens (4-hydroxybenzoic acid) was also analyzed in terms of the toxicity of paraben as discussed in chapter 1. Evaluation of ethanol control, pure epilife medium, and metabolite (4-hydroxy benzoic acid) were assisted to detect accurate toxicity of each paraben. In this study, it has been confirmed methyl paraben (MP) is less toxic than other parabens. However, % of cell survival was acceptable in recommended concentration; and 4-hydroxybenzoic acid (metabolite of paraben) itself was relatively non-toxic. The toxicity of paraben increases with an increasing longer alkyl ester chain paraben (Uramaru *et al* 2007). We found toxicity level of parabens during this experiment was methyl paraben (MP) > ethyl paraben (EP) > propyl paraben (PP) > butyl paraben (BP) > Benzyl paraben (BzP), as well as The Food and Drug Administration (FDA) and The Cosmetic Ingredient Review (CIR), recommended

concentration as single paraben 0.4% (v/v) was completely safe in term of toxicity in human skin was our novel findings in this research.

6.3 Determination of parabens metabolism via pig skin using Diffusion cell study.

In this research, parabens were permeated through pig skin in the Franz diffusion study to determine percentage (%) metabolism. “A review of numerous studies recommended that the permeability of a compound through both human and pig skin showed 0.88 of correlation coefficient with an intra species, the difference of standard coefficient in skin permeability of 21% for pig and 35% for human” (Barbero and Frasch, 2009). Pig skin and human skin exhibits have a smaller difference from a functional point of application (Dodou *et al,* 2014). "Pig skin has less variability than the human skin model as well as both pig and human are good models for skin permeability" (Barbero and Frasch, 2009). So, in this experiment, we used pig skin as a permeation barrier instead of human skin permeation. Parabens have permeated through pig skin as a model membrane in this study.

For this experiment, parabens were dissolved in PBS to prepared samples including methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), and butyl paraben. Saturation solubility of paraben in PBS was also determined during this study as discussed in the 2nd chapter. PBS was used in this experiment; due to its non-toxic properties and often maintains a pH of 7.4 which close to the human skin (Bronaugh, and Stewart, 1985). Approximately 50% of the saturated concentration of each paraben was applied to the prepared donor solution in this Franz diffusion study. In a multi-station Franz diffusion system (BRS Plc., co., Durham, UK.), one donor was attached to seven ground glass Franz diffusion cells with an absorption surface area of 0.79 cm^2. The skin sample was mounted in between the donor and receptor chambers of the cell with the dermis in contact with the receptor medium. The 1.5 ml volume of the receptor chamber was filled up with PBS that was continuously stirred with a magnetic stirrer in an incubator at 37°C, while the donor chamber contains a 300 µl solution. This is because small volumes allow evaporation, thus producing time-dependent alterations in formulation composition that change solubility in the vehicle and poor stirring may also be associated with efficient fluid mixing throughout the bulk of the chamber (Shiow-fern. et al, 2010). After that, an adequate amount of receptor fluid was withdrawn from the receptor chamber at each time point (t) and replaced with fresh preheated PBS. During this study, samples were collected from the receptor fluid at different time intervals (t= 0, 1, 2, 3, 4, 8, 20,

and 24 hours). At end of this study, the remaining solution was also collected in a sample vial and the receptor chamber was also washed with PBS to prepare a sample (Dodou *et al* 2014). All samples were analyzed with HPLC chromatography.

In this permeability study, parabens were permeated through the Pig skin membrane in static Franz diffusion cells for 24 hours and samples were collected at each time point (t = 0, 1, 2, 3, 4, 8, 20, and 24h). The amount of substance permeated per unit area per unit time is called flux (Bartosova and Bajgar, 2012). The flux (µg/cm²/h) was calculated from the ratio of the cumulative absorption through a skin surface at each time point (slope of the cumulative absorption vs time plot) as shown in the 2nd chapter. Data were mean values. The steady flux is expressed as J_s (µg/cm²/h) (Bartosova and Bajgar, 2012). The time taken to achieve diffusion during each time interval was referred to as the lag time (Bartosova and Bajgar, 2012). Lag time was calculated to form the intercept of the cumulative absorption vs time plot. Lag time is expressed as L_t (hours) (Bartosova and Bajgar, 2012). During this study, we also obtained permeation coefficient K_p from the speed of permeation (cm/h).

In this study, Methyl paraben (MP) showed higher permeation flux than other parabens. The permeability of paraben depends on their solubility; the higher solubility of paraben has higher permeation through pig skin. In a literature review, we observed permeation studies of paraben via pig skin have a similar outcome to this research (Caon *et al* 2010). Permeation flux was lower in higher alkyl ester paraben. This is because high lipophilicity and molecular weight (MW) of paraben I.e., longer ester chain parabens have higher MW. Increasing MW would decrease the diffusion coefficient of the molecule (paraben) and therefore its flux via the skin and the lipophilicity of a substance increases to decrease the permeation flux (Caon *et al* 2010). Therefore, butyl paraben (BP) has a lower permeation flux recorded during this study. After the permeation study, skin membrane, donor chamber wash, and receptor chamber wash were also collected for measured mass balance in their % recovery as discussed in the 2nd chapter. The *in vitro* permeability profiles of parabens were methyl paraben (MP) > ethyl paraben (EP) > propyl paraben (PP) > butyl paraben (BP). The steady state of paraben diffusion was conducted under perfect sink conditions.

6.4 Determination of paraben metabolism in human keratinocytes through cell extraction

In this experiment, the *in-vitro* investigation of paraben and their metabolite in human keratinocytes through cell extract was also performed. During this study, paraben concentration was applied to the cell and incubated at three different times to quenching metabolism. This study was conducted with 0.4% (v/v) of single paraben dissolved in epilife medium with 5% (v/v) ethanol at 40^0C. CCD1106 human keratinocytes cell was cultured in 75-cm^2 culture flasks to prepare for this experiment. Cell preparation was also important for extracting a compound from cells. Healthy cells can make a difference in the extraction process. A strong and healthy cell can absorb a molecule quickly (Ser *et al* 2015). In this study, the cells were prepared at passage (number of the subculture) 7 and maintained in a humidified incubator at 37°C and 5% CO_2 during the cell culture for strong and healthy cells. The cell density of each flask was also maintained using a Haemocytometer as described in 3rd chapter. Measurement of Cell density was also important to maintain cell quantity in each flask (Segeritz and Vallier 2017). In the extraction procedure, samples were centrifuged properly to collect the maximum amount of cells.

There is a different type of extraction procedure are available to extract from cells such as Lysis buffer, 80% methanol (MeOH) (-80^0c), acetonitrile (ACN) and phenol-*chloroform* isoamyl alcohol, etc (Dietmair S *et al* 2010) (Ser *et al* 2015). The most commonly used procedure is 80% methanol (MeOH) (-80^0c) for western blot and metabolite extraction (Ser *et al* 2015). However, Methanol (MeOH) is highly toxic to human skin (Paruta, 1969). In this study, paraben and their metabolite were extracted from human keratinocyte cells by lysis buffer. There are not many studies on the extraction of paraben in cell extract through lysis buffer. Cell lysis reacts with the outer boundary or cell membrane broken down or destroyed in order to release inter-cellular materials from the cell (Shehadul, Aditya, and Selvaganapathy, 2017). The lysis method is used for the ease of purification steps, the target molecules for analysis, and quality of final products" (Harrison, 1991).

There are many types of lysis buffer, most are commercially available, however, some are required to prepare with chemical composition for target molecules extraction (Sepmag 2021). In this experiment lysis buffer was prepared with a composition of HEPES, Triton X-100, NaCl, and EDTA. Cell lysis is used to disrupt the exterior environment of the cell membrane

but preserves target molecules (Sepmag 2021). Which material has to extract also depends on which type, or composition of lysis buffer require (Shehadul, Aditya, and Selvaganapathy 2017). This lysis buffer was used because, it can isolate the molecule (paraben) in a stable condition. Lysis buffer acts as a surfactant or detergent. "Detergents react with cell membrane forming pores on the surface of membrane resulting in the release of intracellular components" (Shehadul, Aditya, and Selvaganapathy 2017). During this study, methyl paraben (MP), ethyl paraben (EP), Propyl paraben (PP), and Butyl paraben (BP) were treated and extracted through this lysis buffer.

In the extraction procedure, cells were extracted after incubation at 24 hours, 48 hours, and 72 hours by using a lysis buffer. After incubation medium was taken out from the flask and then washed with prewarmed PBS then Trypsin was added to the flask and placed in an incubator at 37°C for 5 minutes to remove adherent cells. After the cells were detached from the flask, prewarmed DMEM medium was added to the flask and transferred in a falcon tube to centrifuge at 1500 rpm. When cells were precipitated, the media was carefully removed and washed with PBS. Then 500 µl Lysis buffer was added and mixed with the cell then kept in Icebox for 30 minutes to break open the cells and solubilize any parabens/metabolites without saponifying them. After that centrifuged at 10000 rpm to remove cell debris and filtrated with a 0.2µm size filter to prepare supernatant for HPLC analysis.

In this study, paraben metabolism in CCD1106 human keratinocytes cells at recommended concentration of 0.4% v/v (single paraben) was investigated. Therefore, parabens were dissolved in epilife medium with 5% (v/v) ethanol at 40°C diluted to 0.4% (v/v). Cells were prepared and treated with 0.4% (v/v) of paraben at 24 hours, 48 hours, and 72 hours. Parabens can be absorbed and retained into the human tissue without hydrolysis of tissue esterases (Oishi 2004). After this treatment cells were extracted using an extraction procedure with lysis buffer. During this experiment, we observed methyl paraben (MP) was highly metabolized than other parabens and increased incubation time with increased metabolism as described in the 3rd chapter. The procedure for extraction plays an extremely important role in the determination of the range of recovery (Ser *et al* 2015). In this study, we also calculated the total % recovery of parabens through different phases of extraction such as Epilife medium, DMEN+Trypsin, PBS washed, and Lysis buffer at 24 hours, 48 hours, and 72 hours of incubation as shown in

the 3rd chapter. This recovery shows a mass balance in this study. We also examined paraben concentration under the same condition without cells.

The quantity of extraction also varies on the chemical composition of the extraction solvent (Huichang *et al* 2013). In this study, lysis buffer was prepared with a composition of HEPES, Triton X-100, NaCl, and EDTA. Throughout this study, a large amount of paraben was retained on epilife medium, small trace was recorded from DMEN + Trypsin and PBS. A recent review reported that washing is a common practice to improve analytical assessment such as signal-to-noise ratio however it removes extract compounds outside the cell (Leon, *et al* 2013). PBS was used as a washing solvent. “PBS is non-toxic and prevents cells rupturing or shrivelling up due to osmosis” (Martin, *et al* 2006). Trypsinization is the most popular detachment technique in cell culture, approximately 95% of cells are detached from the culture plate (Yuta *et al* 2019). In Lysis buffer, a modest amount of paraben was detected during this study.

In this study, the % metabolite of paraben was also determined. This study demonstrated highly soluble paraben was more metabolized. Incubation time was a greater impact on metabolite extraction; increased incubation time has increased metabolism. This is our novel finding of our research. Solubility of Methyl paraben (MP) was higher in epilife medium than in other parabens during this study. Metabolism level of parabens were methyl paraben (MP) > ethyl paraben (EP) > propyl paraben (PP) > butyl paraben (BP). Overall, 0.4% of paraben has a low metabolic rate in human keratinocyte cells was observed during this extraction study. So, it has been confirmed that the approved concentration from The Food and Drug Administration (FDA) and The Cosmetic Ingredient Review (CIR) in single paraben was relatively safe for human skin.

7. Conclusion and future work

In conclusion, despite the controversy around parabens, they are widely used as preservatives in skin care products due to their effective antimicrobial activity. They are stable, effective over a wide pH range, and active against all broad spectrums of microbes. Their antimicrobial activity depends on their alkyl ester chain length. Longer alkyl ester chains of parabens are more active against shorter alkyl ester chains parabens. However, longer ester chain parabens have a higher estrogenic effect on human skin as well as are toxic. Toxicity increases with increased alkyl ester chain. Therefore, a combination of parabens could be a useful option as

preservatives in cosmetics products to maintain microbial activity and toxicity. The most commonly used parabens in cosmetic products are methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), and butyl paraben (BP). According to FDA and CIR guidelines, 0.4% w/w in a single paraben and 0.8% w/w in combination parabens are allowed in skin care products.

In this research, we found that parabens at low concentrations are safe, and increasing the concentration of parabens, increased their cytotoxicity. This study shows longer ester chain parabens have more toxicity than shorter alkyl chain parabens. Permeability also depends on the solubility of paraben. Higher the solubility, the higher the permeation of paraben through the skin. Permeation of methyl paraben was higher than other parabens as well as % metabolism was also higher in the diffusion study. During this research, parabens had a low metabolic effect in human cells at low concentrations. Metabolism of parabens in the human cell also increased with increased duration of time on human skin observed during this study. The FDI and CIR recommended concentration has relatively safe for human skin and 4-hydroxybenzoic acid (metabolite of paraben) has relatively non-toxic. The toxicity level of paraben has methyl paraben (MP) < ethyl paraben (EP) < propyl paraben (PP) < butyl paraben (BP). However, the metabolism of paraben has reversed in human skin. Metabolism of paraben was methyl paraben (MP) > ethyl paraben (EP) > propyl paraben (PP) > butyl paraben (BP).

This study does not involve a combination of paraben concentration in human skin. In further studies, a combination of paraben concentrations would be investigated. Then, the determination of % metabolism via human skin using diffusion cell studies would also be analyzed in future work. Next, recombinant enzyme stability assays in human skin would be a handy analysis to evaluate the toxicity of paraben in future studies. Finally, concentration-dependent skin toxicity studies of parabens using markers of toxicity would also lead to the future work of this research.

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Appendix I: Cell and Culture medium Description.

CCD 1106 KERTr (Cell)

Cell Description:

Organism: Homo sapiens, Human

Tissue: Skin.

Cell Type: keratinocyte; Human papillomavirus 16 (HPV16) E6/E7 transformed.

Morphology: Epithelial.

Product format: Frozen.

Biosafety Level: 2

Storage Conditions: Liquid nitrogen vapor phase.

Epilife® Medium

"A sterile, liquid medium prepared with 60 µM calcium chloride for the long-term, serum-free culture of human epidermal keratinocytes and human corneal epithelial cells. This basal medium requires the addition of an appropriate growth supplement prior to use. EpiLife® greatly extends the in vitro lifespan of human epidermal keratinocytes compared to other serum-free media systems. This basal media is animal origin free and contains essential and non-essential amino acids, vitamins, other organic compounds, trace minerals, and inorganic salts. It does not contain antibiotics, antimycotics, hormones, growth factors, or proteins. EpiLife® medium is HEPES and bicarbonate buffered and is designed for use in an incubator with an atmosphere of 5% CO_2 and 95% air" (ThermoFisher SCIENTIFIC, 2016). Epilife Medium was storage in a freezer which contains 2°C to 8°C.

Dulbecco′s Modified Eagle′s Medium (DMEM) medium

"Dulbecco′s Modified Eagle′s Medium (DMEM) is a modification of Basal Medium Eagle (BME) that contains four-fold concentrations of the amino acids and vitamins. Each of these media contains a different combination of L-glutamine and sodium pyruvate. This DMEM-Hi glucose medium is a 1x complete medium with sodium pyruvate added. It also differs from the original DMEM-Hi formulation wherein pyridoxine is substituted for pyridoxal. Pyridoxal is an unstable component of media. This medium requires supplementation with L-glutamine or L-alanyl-L-

glutamine" (SIGMA-ALDRICH, 2016). DMEM Medium was storage in a freezer which contains 2ºC to 8ºC.

Trypsin

"Trypsin may be used to remove adherent cells from a culture surface. Cells are most commonly removed from the culture substrate by treatment with trypsin or trypsin/EDTA solutions. Trypsin concentration in 1x working solutions can range from 0.025–0.5%, depending on trypsin activity or potency, incubation times, and cell lines. Incubating cells with too high a trypsin concentration for too long a time period will damage cell membranes and kill the cells" (SIGMA-ALDRICH, 2016). Trypsin was storage in a freezer which contains 2ºC to 8ºC.

Appendix II: Sample preparation for cytotoxicity assay.

Methyl paraben prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) Volume to prepare 5 ml (ml)	(in 5% ethanol) Volume of media (ml)	Epilife media used in cell culture (ml)
0	0	0	0	0
0.1	0.97	0.5	4.4	0.1
0.2	0.97	1	3.9	0.1
0.3	0.97	1.5	3.4	0.1
0.4	0.97	2.1	2.8	0.1
0.5	0.97	2.6	2.5	0.1

Ethyl paraben prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) Volume to prepare 5 ml (ml)	(in 5% ethanol) Volume of media (ml)	Epilife media used in cell culture (ml)
0	0	0	0	0
0.1	0.78	0.6	4.3	0.1
0.2	0.78	1.3	3.6	0.1
0.3	0.78	1.9	3	0.1
0.4	0.78	2.6	2.3	0.1
0.5	0.78	3.2	1.7	0.1

Propyl paraben prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) **Volume to prepare 5 ml (ml)**	(in 5% ethanol) **Volume of media (ml)**	**Epilife media used in cell culture (ml)**
0	0	0	0	0
0.1	0.62	0.8	4.1	0.1
0.2	0.62	1.6	3.3	0.1
0.3	0.62	2.4	2.5	0.1
0.4	0.62	3.2	1.7	0.1
0.5	0.62	4	0.9	0.1

Butyl paraben prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) **Volume to prepare 5 ml (ml)**	(in 5% ethanol) **Volume of media (ml)**	**Epilife media used in cell culture (ml)**
0	0	0	0	0
0.1	0.54	0.9	4	0.1
0.2	0.54	1.8	3.1	0.1
0.3	0.54	2.8	2.1	0.1
0.4	0.54	3.7	1.2	0.1
0.5	0.54	4.6	0.3	0.1

Benzyl paraben prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) **Volume to prepare 5 ml (ml)**	(in 5% ethanol) **Volume of media (ml)**	**Epilife media used in cell culture (ml)**
0	0	0	0	0
0.1	0.51	0.9	4	0.1
0.2	0.51	1.9	3	0.1
0.3	0.51	2.9	2	0.1
0.4	0.51	3.9	1	0.1
0.5	0.51	4.9	0	0.1

4-hydroxy benzoic acid prepared for Cytotoxicity assay.

Final concentration (%)	(in 5% ethanol) Concentration (% w/v)	(in 5% ethanol) Volume to prepare 5 ml (ml)	(in 5% ethanol) Volume of media (ml)	Epilife media used in cell culture (ml)
0	0	0	0	0
0.2	1.04	0.9	4.0	0.1
0.4	1.04	1.9	3.0	0.1
0.6	1.04	2.8	2.1	0.1
0.8	1.04	3.8	1.1	0.1
1	1.04	4.8	0.1	0.1

Appendix III: Calculation for Cytotoxicity assay.

Take an average abs 570 nm (mean value) 0 control.

% control cell survival = each wall (96 cell wall) ÷ Average abs 570 nm of 0 control × 100

		methyl-paraben (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Ab (570 nm) average	2.17	2.19	2.09	2.069	2.015	1.975	1.899
	2.093	2.249	2.049	2.014	1.997	1.893	1.789
	2.077	2.256	2.068	2.023	1.941	1.872	1.819
	2.064	2.297	2.016	1.961	1.939	1.903	1.829
	1.999	2.173	2.14	2.025	1.909	1.875	1.796
mean		2.233					
% control cell survival	97.18	98.07	93.60	92.66	90.24	88.45	85.04
	93.73	100.72	91.76	90.19	89.43	84.77	80.12
	93.01	101.03	92.61	90.60	86.92	83.83	81.46
	92.43	102.87	90.28	87.82	86.83	85.22	81.91
	89.52	97.31	95.84	90.69	85.49	83.97	80.43
mean % control cell survival	93.18	100.00	92.82	90.39	87.74	85.25	81.79
SD	2.75	2.28	2.08	1.73	1.98	1.88	1.96

		ethyl-paraben (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Ab (570 nm)	1.934	1.997	1.768	1.701	1.609	1.599	1.511
	1.901	2.094	1.837	1.784	1.729	1.691	1.608
	1.863	2.01	1.799	1.718	1.694	1.605	1.577
	1.818	1.971	1.818	1.772	1.716	1.688	1.631
	1.836	1.986	1.868	1.789	1.741	1.692	1.641
mean		2.0116					
% control cell survival	96.14	99.27	87.89	84.56	79.99	79.49	75.11
	94.50	104.10	91.32	88.69	85.95	84.06	79.94
	92.61	99.92	89.43	85.40	84.21	79.79	78.40
	90.38	97.98	90.38	88.09	85.31	83.91	81.08
	91.27	98.73	92.86	88.93	86.55	84.11	81.58
mean % control cell survival	92.98	100.00	90.38	87.13	84.40	82.27	79.22
SD	2.35	2.40	1.88	2.01	2.62	2.41	2.60

		propyl-paraben (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Ab (570 nm)	2.145	2.374	2.011	1.927	1.898	1.849	1.769
	2.231	2.475	2.102	2.066	2.024	1.997	1.899
	2.137	2.36	2.102	2.069	1.994	1.985	1.838
	2.262	2.414	2.193	2.019	1.997	1.954	1.843
	2.216	2.319	2.149	2.074	2.023	1.989	1.884
mean		2.3884					
% control cell survival	89.81	99.40	84.20	80.68	79.47	77.42	74.07
	93.41	103.63	88.01	86.50	84.74	83.61	79.51
	89.47	98.81	88.01	86.63	83.49	83.11	76.96
	94.71	101.07	91.82	84.53	83.61	81.81	77.16
	92.78	97.09	89.98	86.84	84.70	83.28	78.88
mean % control cell survival	92.04	100.00	88.40	85.04	83.20	81.85	77.32
SD	2.30	2.48	2.83	2.60	2.17	2.57	2.12

		butyl-paraben (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Ab (570 nm)	1.368	1.505	1.397	1.309	1.277	1.252	1.205
	1.468	1.536	1.329	1.287	1.259	1.238	1.138
	1.416	1.598	1.283	1.232	1.208	1.182	1.128
	1.459	1.541	1.319	1.289	1.217	1.179	1.115
	1.395	1.574	1.336	1.321	1.296	1.269	1.205
mean		1.5508					
% control cell survival	88.21	97.05	90.08	84.41	82.34	80.73	77.70
	94.66	99.05	85.70	82.99	81.18	79.83	73.38
	91.31	103.04	82.73	79.44	77.90	76.22	72.74
	94.08	99.37	85.05	83.12	78.48	76.03	71.90
	89.95	101.50	86.15	85.18	83.57	81.83	77.70
mean % control cell survival	91.64	100.00	85.94	83.03	80.69	78.93	74.68
SD	2.73	2.32	2.66	2.20	2.45	2.66	2.80

		benzyl-paraben (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
Ab (570 nm)	0.429	0.488	0.417	0.409	0.386	0.365	0.342
	0.439	0.499	0.419	0.406	0.396	0.379	0.365
	0.466	0.502	0.439	0.423	0.411	0.395	0.375

	0.445	0.498	0.422	0.394	0.379	0.377	0.363
	0.449	0.473	0.408	0.388	0.379	0.361	0.357
mean		0.492					
% control cell survival	87.20	99.19	84.76	83.13	78.46	74.19	69.51
	89.23	101.42	85.16	82.52	80.49	77.03	74.19
	94.72	102.03	89.23	85.98	83.54	80.28	76.22
	90.45	101.22	85.77	80.08	77.03	76.63	73.78
	91.26	96.14	82.93	78.86	77.03	73.37	72.56
mean % control cell survival	90.57	100.00	85.57	82.11	79.31	76.30	73.25
SD	2.78	2.41	2.30	2.78	2.75	2.72	2.47

		4-hydroxybenzoic acid (%) - 24hr treatment					
	EtOH*	**0 (control)**	**0.2**	**0.4**	**0.6**	**0.8**	**1**
Ab (570 nm)	2.119	2.19	2.119	2.058	1.938	1.885	1.831
	2.113	2.249	2.098	2.001	1.931	1.867	1.812
	2.047	2.256	2.011	1.953	1.941	1.872	1.724
	2.104	2.297	2.016	1.976	1.839	1.802	1.751
	2.005	2.173	2.04	1.925	1.864	1.785	1.749
mean		2.233					
% control cell survival	94.89	98.07	94.89	92.16	86.79	84.42	82.00
	94.63	100.72	93.95	89.61	86.48	83.61	81.15
	91.67	101.03	90.06	87.46	86.92	83.83	77.21
	94.22	102.87	90.28	88.49	82.36	80.70	78.41
	89.79	97.31	91.36	86.21	83.48	79.94	78.33
mean % control cell survival	93.04	100.00	92.11	88.79	85.20	82.50	79.42
SD	2.22	2.28	2.20	2.27	2.13	2.03	2.05

Appendix IV: Sink condition of Franz Diffusion study.

Paraben	Saturation solubility In PBS (mg/ml)	Concentration in donor solution (mg/ml)	Volume of Donor chamber (ml)	Amount applied in donor chamber (mg)	Volume of Receptor chamber (ml)	Theoretically maximum concentration in Receptor chamber (mg/ml)
Methyl	2.56	1.27	0.3	0.381	1.5	0.254
Ethyl	1.31	0.70	0.3	0.210	1.5	0.140
Propyl	0.88	0.427	0.3	0.128	1.5	0.085
Butyl	0.53	0.285	0.3	0.085	1.5	0.057

This study defines that the Sink condition not more the 10% of saturation solubility of paraben in PBS. “A dissolution test is said to be performed under sink conditions if the concentration of the drug in the bulk of the dissolution medium does not exceed 10% of the solubility of the drug” (Aulton and Taylor, 2018).

Appendix V: Conversion of mg/ml to percentage.

	(in 5% ethanol)	(in 5% ethanol)	(in 5% ethanol)
Name	**Concentration (mg/ml)**	**Concentration (g/ml)**	**Concentration % (w/v)**
Methyl paraben	9.763	0.00976	0.97
Ethyl paraben	7.884	0.00788	0.78
propyl paraben	6.211	0.00621	0.62
butyl paraben	5.486	0.00548	0.54

Appendix VI: Conversion of percentage to Millimolar (Mm).

Name	% (v/v) Concentration	Molecular weight	Calculation	Mm Concentration
Methyl paraben	0.4	152.15 g/mol	$\frac{0.4\times1000\times1000}{152.15\times100}$	26.28
Ethyl paraben	0.4	166.17 g/mol	$\frac{0.4\times1000\times1000}{166.17\times100}$	24.07
Propyl Paraben	0.4	180.2 g/mol	$\frac{0.4\times1000\times1000}{180.2\times100}$	22.19
Butyl paraben	0.4	194.227 g/mol	$\frac{0.4\times1000\times1000}{194.227\times100}$	20.59

Appendix VII: Sample preparation for Cell extract.

Name	(In 5% ethanol) **Concentration (% w/v)**	(In 5% ethanol) **prepare concentration (% v/v)**	(In 5% ethanol) **Volume to prepare 10 ml**	(In 5% ethanol) **Volume of media**	Final Concentration **Mm**
Methyl paraben	0.97	0.4	4.1	5.9	26.28
Ethyl paraben	0.78	0.4	5.1	4.9	24.07
Propyl paraben	0.62	0.4	6.4	3.6	22.19
Butyl paraben	0.54	0.4	7.4	2.6	20.59

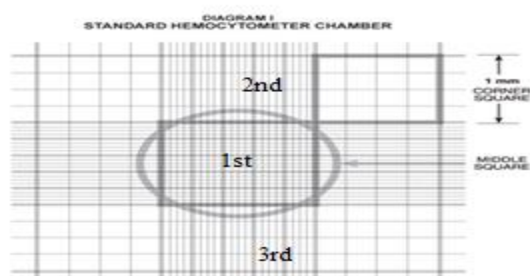
Appendix VIII: Lysis buffer preparation.

Name	Volume to Prepare 10 ml		Final concentration %	Final concentration
	Concentration	volume		
HEPES	2.38 ml	7.62 ml	1.19%	50 mM
Triton X-100	2 ml	8 ml	1%	1%
EDTA	0.06 ml	9.94 ml	0.03%	1 mM
NaCl	8.26 ml	1.74 ml	0.87%	150 mM

For preparation of Lysis buffer all compounds were dissolved in distilled water. After combined all chemical to prepared 10 ml solution with pH 7.4.

Appendix IX: Cell density (cell count).

For cell density we used hemocytometer. 10 µl of cell medium was used on hemocytometer and observed through microscope for cell count. In passage 7 we take an average cell count of each hemocytometer chamber. Example calculation for Methyl paraben.



Calculation:

1st count 22 cells
2nd count 24 cells
3rd count 23 cells
So Average 23 cells

It was 10000 dilution of cell line in 10 ml.
So that 23×10000
So $=2.3\times10^5$ cell / µl
Target cell density 3.4×10^4 cell / µl (Standard cell treatment)
So that 2.3×10^5 cell / µl $\div$ 3.4×10^4 cell / µl
$=6.76$
Volume we prepared = 30 ml
So that $30\div6.76$
= 4.4 ml (dilution).
Similarly, cell density was counted with Ethyl, Propyl, and Butyl paraben.

End