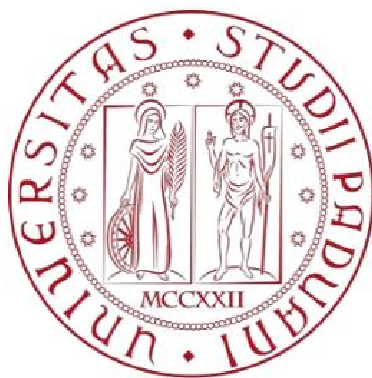

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Second Cycle Degree in

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Evaluation of brain targeted transporter-utilizing prodrugs of neuroprotective agents

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I. Abstract (English)

Neurodegenerative diseases are linked to oxidative stress, making antioxidant (AOX) compounds a viable option for both symptom relief and targeting the disease's root cause. Prodrug, one of the modern drug developments emphasizes enhancing drug delivery. The blood-brain barrier (BBB), as an obstacle for the target organ in neurodegenerative conditions. LAT-1 influx enables the enter of amino acids inside the cells and is expressed in BBB remarkably. LAT1-utilizing prodrugs have shown promise due to LAT1's selective expression in the BBB and its overexpression in some cancers. Designing prodrugs with a phenolic AOX promoiety and LAT1 substrate can enhance targeted delivery. The aim of this study was to investigate the cleavage and release time of antioxidant parent drugs from their LAT1-utilizing prodrugs by bioconversion studies in-vitro. These results showed that most of the studied prodrugs are not able to cleave according to HPLC analysis.

II. Abstract (Italian)

Le malattie neurodegenerative sono collegate allo stress ossidativo, rendendo i composti antiossidanti (AOX) un'opzione praticabile sia per alleviare i sintomi che per colpire la causa principale della malattia. Il profarmaco, uno dei moderni sviluppi farmaceutici, enfatizza il miglioramento della somministrazione del farmaco. La barriera emato-encefalica (BBB), come ostacolo per l'organo bersaglio nelle condizioni neurodegenerative. L'afflusso di LAT-1 consente l'ingresso di aminoacidi all'interno delle cellule ed è espresso in modo notevole in BBB. I profarmaci che utilizzano LAT1 si sono mostrati promettenti grazie all'espressione selettiva di LAT1 nella BBB e alla sua sovraespressione in alcuni tumori. La progettazione di profarmaci con una promoienza fenolica AOX e un substrato LAT1 può migliorare la somministrazione mirata. Lo scopo di questo studio era di indagare la scissione e il tempo di rilascio dei farmaci originari antiossidanti dai loro profarmaci che utilizzano LAT1 mediante studi di bioconversione in vitro. Questi risultati hanno mostrato che la maggior parte dei profarmaci studiati non sono in grado di clivarsi secondo l'analisi HPLC.

III. Introduction

Neurological disorders are the leading cause of physical and cognitive disability worldwide (1). For decades, patients suffering from neurodegenerative disorders have been treated with simple medications that focus on symptom relief rather than targeting the underlying disorder (2). Developing a comprehensive understanding of disease mechanisms is a fundamental task in most clinical laboratories. Modern medicine has primarily developed from our profound understanding of human and animal biology at the molecular and cellular levels (3). This understanding is crucial because identifying the changes that occur in the body is the first step toward curing diseases. Since neurodegenerative diseases are strongly related to oxidative stress, antioxidant (AOX) compounds present a suitable option for addressing both the root cause and relieving the symptoms. Discovering beneficial compounds as AOX activity is the beginning of facing the topic's complexity of drug formulation for gaining high affinity and high bioavailability in the target area in pharmaceutical industry. For this aim, innovative drug development includes various administration routes, nanocarriers, formulation technologies, focused ultrasound-enhanced drug delivery, and prodrugs have

been introduced. Developing prodrugs showed promising aspects of targeting and making it simpler to enhance their imperfection rather than develop entirely new medications (4). Prodrugs represent a significant advancement in pharmaceutical science, designed to optimize the administration and therapeutic effectiveness of drugs. These compounds are administered in an inactive form and become activated only within the target area since they are designed based on the properties of the target area. Neurodegenerative drugs aimed at the brain encounter the challenge of the blood-brain barrier, which tightly regulates the movement of ions and molecules. Given that the blood-brain barrier (BBB) tightly supervises the movement of ions and molecules from the systemic circulation to the brain, drug delivery for neurodegenerative diseases is particularly difficult. For any compounds to exert direct neuroprotective actions they must permeate the blood−brain barrier (BBB). Although there's proof that certain AOX compounds can cross the BBB and have been detected in the brain, increasing the efficacy of AOX drugs remains imperative (5). It has been suggested that transporters are significant factors in determining drug efficacy. If specific transporters are expressed predominantly in one tissue or more abundantly in one tissue compared to others, this characteristic can be exploited for prodrug design. However, Prodrug design indeed faces several challenges, particularly regarding the release of the therapeutic compound from its moiety through enzymatic reactions in the target area. Studies indicate that L-type Amino Acid Transporters (LAT1) are highly expressed in the blood-brain barrier (BBB) and pancreas. L-Type amino acid transporter 1 (LAT1)-utilizing prodrugs have been designed to enhance drug delivery and targeting to the brain or cancer cells. This is because LAT1 is highly and selectively expressed on the blood-brain barrier and is overexpressed in several types of cancer (6). The hypothesis suggests that synthesizing a drug incorporating a phenolic secondary metabolite from plants as an antioxidant (AOX) therapeutic promoiety, which is then attached to a therapeutic moiety, can create a substrate for LAT1. These prodrugs, which

include both the therapeutic promoiety and a LAT1-substrate as the delivery promoiety, can be transported into the desired location via the LAT1 transporter (7).

IV. Literature Review

1. Oxidative Stress

The imbalance between the production of free radicals, which are molecules with unpaired electrons, and their elimination exposes these organs to the risk of oxidative stress. This disproportion may occur in every creature that utilize oxygen molecule as their primary molecule in oxidation reaction. Oxidation reactions release by-products which are called ROS. ROS has a high metabolic activity and participate in reactions if they accumulate in the tissue. This results in the creation of unwanted molecules that often interfere with subsequent reactions. Oxygen-containing reactive species (ROS) is considered the most important free radicals including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) in biology and medicine. This importance has led to the introduction of a new term in modern biology: ROS biology and medicine (3). ROS acts as signals at normal physiological levels, while accumulation of ROS, modifies protein structure, nucleic acids, and lipids then subsequently dysfunction in the latter[1] . Additionally, generating a large array of secondary electrophilic products including α, β-unsaturated aldehydes, ω-6 and ω-3

unsaturated fatty acids, as well as nitro-fatty acids which undergoes covalent reactions. Electrophiles are also observed in the biotransformation of xenobiotics, a process that is highly significant in toxicology (3).

The brain is one of the most vulnerable organs since it has a rich content of lipids that makes it to have weak antioxidant capacity and high oxygen consumption [2]. Detrimental effects of oxidative stress drive the brain to pathologies, such as neurodegenerative diseases including Alzheimer's, Parkinson’s, and Huntington’s disease (4). It has been proposed that ROS and related species mediate inflammatory responses by triggering the release of cytokines. When this response is prolonged, the chronic heterostasis can lay the pathological foundation for numerous illnesses [3]. On the other way, also oxidative stress is the result of inflammatory diseases, atherosclerosis, diabetes, cancers. This is recently established that oxidative stress induces not only neurodegenerative disorders and brain aging but also neuropsychology disorders like anxiety and depression. This is because the cerebal that is part of the central system (CNS) control emotions and utilizing large amount of oxygen and consequently produce a lot of radicals that are ready to react with molecules around then consequently is being affected by oxidative stress [5].

For a healthy body system, free radical production must be depressed which can be accomplished by AOX [8]. Combating ROS has evolved as an evolutionary response to damage in organisms, especially in mammals. This response is facilitated by antioxidants (AOX), which either scavenge ROS or mitigate the damage in tissues (3). Antioxidants protect the body from the harms of free radicals by eliminating or scavenging free radicals when it presents at low concentrations compare to those of an oxidizable substrate. However pathological elements and conditions makes the AOX mechanisms slow or inefficient. There are ways to cope with this issue by synthesizing antioxidants or phytochemicals extracted from plants.

Therefore, discovering new sources of antioxidants (AOX) and introducing them to cells using novel delivery vehicles is an intriguing area of research in the field of pharmacy [8].

In toxicology, antioxidants are understood to follow a hormetic dose-response pattern, where low doses produce beneficial effects, and high doses lead to toxic effects. Unlike most toxins, which have no impact at low doses, hormetic substances can exert positive effects compared to untreated subjects. However, at very low doses, they may cause adverse effects due to deficiency. As the dose increases, beneficial effects emerge, promoting homeostasis. At very high doses, toxicity occurs, as seen with substances like vitamins and micronutrients [9].

2. Blood-brain barrier (BBB)

The blood-brain barrier (BBB) plays a crucial role in maintaining brain homeostasis and protecting neural tissue from pathogens, ensuring the proper function of the central nervous system (CNS). As the CNS is the most vital part of the body, its blood vessels, consisting of endothelial cells (ECs) and mural cells, must differ from peripheral blood vessels to meet its unique protective needs. This protective capability is primarily attributed to the endothelial cells, which possess special physical, transport, and metabolic properties and maintaining by mural cells. ECs are held together by tight junctions (TJs) makes it undergoes intensely low rates of transcytosis as compared with peripheral ECs (10). This restriction is also provided by structures of capillaries. Regards to capillaries structures Continuous fenestrated capillaries are the most restrictive, while discontinuous capillaries are the least restrictive. CNS blood vessels not only possess continuous fenestrated capillaries, but also specific transporters, specific metabolic pathways, and reduced laminin (LAM) expression empowers

endothelial cells (ECs) to regulate homeostasis. Although such restriction protects neural tissue from toxins and inflammation, drug delivery face challenges for therapeutic goals [11].

Two primary types of transporters in ECs of CNS are specifically function in efflux of substrates in up gradient by hydrolysis of ATP and gain nutrients in down gradient concentration. The transporters function selectively to transfer ions and molecules between the blood and the brain. High-resistance paracellular junctions grant these transporters control over permeability. Since efflux transporters require ATP, it is reasonable that there are elevated levels of mitochondria present. That can be also attributed to the existence of some Cytochrome P450 (CYP) enzymes activity that is found in mitochondrial subcellular fraction although they are in microsomes in other organs (10) and low amount of microsome in brain. CYP enzymes expression are affected by some disease due to nuclear receptor on BBB. Some CNS diseases upregulate the receptors and corresponding CYP increase and decreased drug penetration across the BBB. Furthermore, there is believed to be a distinct vascular metabolism in CNS ECs, contributing to barrier formation by modifying the physical properties of molecules, thereby altering their reactivity, solubility, and transport characteristics (11).

3. Prodrugs

The basic aim of prodrug design is to add a moiety or mask undesirable drug properties, such as low solubility in water (as the predominance of water in the human body) or lipid membranes, low target selectivity, chemical in-stability, undesirable taste, irritation or pain after local administration, pre-systemic metabolism and toxicity. In general, the rationale behind the use of prodrugs is to optimize the absorption, distribution, metabolism, excretion,

and unwanted toxicity (ADMET) of the parent drugs (12). The active molecule (the drug) is temporary linked to a carrier also known as a promoiety through a bio-reversible covalent linkage. Once in the body, the carrier-linked prodrug undergoes biotransformation, releasing the parent drug and the carrier. It should be noticed that the carrier should be non-immunogenic and non-toxic (13).

Either moiety or masking approach should make the drug inactive or mostly inactive. Prodrugs are inactive only one or two biotransformation (enzymatic reactions or chemical activation) step away from their active parent drug. Some similar concepts are also recently introduced such as co-drug(prodrug) two pharmacologically active agents coupled together into a single molecule, and act as promoieties of each other, hard drug, soft drug [14].

The major groups of carrier-linked prodrugs are esters and amides. This is because masking amino group probably is the most common target because amino groups 'tendency to ionize under physiological condition makes this group more liable for temporarily masking. Moreover, instability is another issue that make the amino drugs more common target in such a way of development. Primary amines of Amino drugs go under first-pass metabolism due to N-acetylation and oxidation by monoaminoxidase[11]. Other groups of carriers linked prodrugs include phosphates, carbamates, carbonates, oximes, imines and N-Mannich bases. Some prodrugs are transformed metabolically or chemically by hydration (e.g., lactonessuch as some statins), oxidation (e.g., dexpanthenol,nabumetone) or reduction (e.g., sulindac, platinum(IV) complexes) to the active agent. Esters of active agents with carboxyl, hydroxyl or thiol functionalities, and phosphate esters of active agents with hydroxyl or amine functionalities are the most commonly used prodrugs. Approximately half of the prodrugs currently available on the market are activated via enzymatic hydrolysis by ubiquitous esterases (acetylcholinesterase, butyrylcholinesterases, carboxylesterases, arylesterases) which are present throughout the body (12). phosphoric acid ester and amide prodrugs are typically

synthesized from hydroxy or amino groups and are characterized by excellent or adequate chemical stability with rapid biotransformation by phosphatases (13).

4. LAT1

The L-type amino acid transporter 1 (LAT1) is part of the nutrient transporters in central nervous system endothelial cells (CNS ECs). Amino acid transporters are crucial for cell growth and proliferation because they regulate amino acid availability, which in turn affects cellular physiology. As LAT1 is also has a key role in xenobiotics transporters, it is known as drug transporters. Generally, drug transporters play pivotal roles in determining the pharmacokinetics of drugs, including their absorption, distribution, and elimination at the target site (15).

Transporters are integral membrane proteins that play a crucial role in the uptake and efflux of a variety of substances across cellular membranes. These substances include vital polar endogenous nutrients and a range of xenobiotics, such as drugs. The interaction between drugs and these transporters can significantly affect drug absorption, distribution, metabolism, and excretion (ADME). Transporters belong to the two major superfamilies, solute carrier (SLC) transporters and ATP-binding cassette (ABC) transporters (16).

Extracellular stimuli, such as nutritional compounds or pathogens, either enter cells through receptors or modify the receptors by binding to the domain exposed to the external medium. This binding induces conformational changes in the receptor, thereby activating intracellular proteins [17]. The biomass synthesis rate of cell depends on the transporter activities in gaining exogenous essential amino acids for building the blocks of DNA, nucleic acids, Proteins, lipids

and generate Adenosine Triphosphate (ATP). Among the known transporters responsible for the traffic and balance of amino acids within and between human cells and tissues, system L operates independently of sodium and transports large neutral amino acids [18]. LAT1 belongs to heteromeric amino acid transporters (HATs) which refers to their structure of two subunits that is heavy or ancillary subunit and light or transporter subunit. A functional LAT1 is composed of two encoded proteins by two distinct genes i.e. SLC3A2 gene that encodes protein 4F2hc/CD98 and SLC7A5 gene that encodes light protein CD98. LAT1 preferentially transports branched chains and aromatic amino acids. LAT is highly expressed in brain capillaries (which form the BBB) relative to other tissues[18] . Besides SLC7A5 (also called LAT1) other members of LAT are SLC7A8 (also called LAT2), SLC43A1 (also called LAT3), and SLC43A2 (also called LAT4). LAT1 and LAT2 serve as mandatory amino acid exchangers and LAT3 and LAT4 mediate facilitated amino acid diffusion. LAT1 and LAT2 transport leucine, isoleucine, valine, phenylalanine, methionine, tyrosine, histidine, and tryptophan into cells. Leucine, isoleucine, valine, phenylalanine, and methionine can also be transported into cells by LAT3 and LAT4 [19] . Compounds utilizing LAT1 may mimic natural substrates of LAT1, such as small-size compounds like gabapentin and levodopa, or they may form part of a larger molecule containing structural features recognized by LAT1[18]. LATs operate as asymmetric amino acid exchangers, with the cytoplasmic side demonstrating low apparent affinity. This side regulates the exchange of substrates with high apparent affinity on the extracellular side [16].

LAT1 is important for polar and charged drugs, as they have negligible passive diffusion across biological membranes. Prodrugs that are able to take advantage of such transporters are potential targets in drug design for example levodopa, a prodrug with many challenges till success, which delivers dopamine to the brain for the treatment of Parkinsonism. The

hydrophilic dopamine cannot cross the blood–brain barrier, but levodopa is carried into the brain by LAT1 (21).

LAT1 has been extensively researched for decades, revealing that brain cells and cancer cells express it significantly more than other cells. Consequently, these overexpressed transporters can be exploited to transport therapeutic antioxidants compounds into cells. Therefore, drugs designed to target LAT1 should be utilized for delivering drugs to the central nervous system (CNS) by crossing the blood-brain barrier. Furthermore, it can serve as a marker for detecting cancer cells and inhibiting its function, thereby inducing starvation in cancer cells and suppressing their growth. [17].

One study of Huttunen et al shows that it is important to evaluate secondary transport mechanisms carefully, since they may have a role in pharmacokinetics of LAT1-utilizing prodrugs if LAT1 becomes saturated or un-functional (22).

5. Phenols

Phenolic acids, aromatic secondary metabolites abundant in plant-based foods, are believed to harbour numerous physiological and pharmacological benefits. This diverse group comprises hydroxybenzoic and hydroxycinnamic acids, widely distributed in nature. Hydroxycinnamic acids commonly form simple esters with glucose or hydroxycarboxylic acids. Through both in vitro and in vivo experiments, phenolic acids have demonstrated potent biological effects, including scavenging free radicals, and enhancing antioxidant capabilities. Seven phenolic compounds are taken as AOX agent (neuroprotective agents), Eugenol, *p*-coumaric acid, *trans*-Cinnamic acid, Capsaicin, DOPAC, Caffeic acid, Vanillic acid (23).

5.1 Eugenol

Eugenol (EUG) is present in various medicinally significant plant species. Alternatively, EUG can be synthesized either through the allylation of guaiacol with allyl chloride or by using compounds produced through biotechnological methods involving microorganisms (24). This therapeutic phenolic compound, a volatile oil derived from the lilac flower buds of the Myrtle family, has proven useful in various industrial applications. Recognized as Generally Regarded as Safe (GRAS), it is widely used in food flavouring due to its phenolic hydroxyl group, which imparts a strong pungent aroma, and in medicines. Research indicates that EUG exhibits significant anti-inflammatory, antipyretic, antioxidant, analgesic, antibacterial, antispasmodic, and apoptosis-inducing properties, making it a promising candidate for future pharmaceutical developments (25).

EUG, a lipophilic compound, serves as an antioxidant (AOX) that can effectively scavenge free radicals in lipid-rich organs and subcellular organelles like mitochondria. The brain, which is abundant in mitochondria, is particularly vulnerable to lipid peroxidation and can benefit from lipophilic AOXs such as EUG. In vivo studies exposing animals to Carbon Tetrachloride (CCl4), a common model for induced oxidative stress, showed that EUG did not interfere with the generation and reactions of primary radicals (•CCl3/CClOO•). Instead, it caused hepatotoxicity by oxidizing the lipids of the endoplasmic reticulum. Eugenol's protective effect against CCl4-induced hepatotoxicity is attributed to its interception of secondary radicals derived from oxidized lipids of the endoplasmic reticulum, rather than interfering with the generation and reactions of primary radicals (•CCl3/CClOO•) [26]

Arachidonic acid (AA) is a key constituent of cell membrane lipids as a react with three enzymes, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450). The

primary metabolites of these reactions serve as significant mediators of the inflammatory response by activating NF-κB p56 and MAPK p38. Aspirin, an approved drug for its therapeutic effects on inflammation, and EUG, with its antioxidant (AOX) activity, can mitigate each other's adverse effects if administered as prodrug. The prodrug aspirin eugenol ester (AEE) released in the body as salicylic acid and EUG. The results showed that AEE, overcomes the instability and irritation of each drug by modifying the carboxyl and phenolic hydroxyl groups through an esterification reaction (27).

According to in silico studies on the pharmacokinetic properties of eugenol, it is almost completely absorbed in the human intestine and has low BBB permeability (0.342 logBB). EUG does not inhibit cytochromes CYP2C19, CYP2C9, CYP2D6, or CYP3A4, with the exception of CYP1A2.[28] . Like all antioxidants (AOXs), EUG acts as an antioxidant at low concentrations, reducing ROS-mediated oxidative stress. However, at high concentrations, it functions as a prooxidant, increasing ROS production (29).

EUG inhibits iron and hydroxyl (OH) radicals, demonstrating a half-maximal inhibitory concentration (IC50) (25). Additionally, EUG's antitoxic properties can aid in the detoxification of the kidneys and liver. This molecule has been used for formulation of thousands of drugs [30].

5.2 *p*-coumaric acid

p-Coumaric acid (*p*CA)serves as the precursor for the biosynthesis of downstream compounds such as flavonoids, lignans, stilbenes, condensed tannins, and curcuminoids. This compound is present in both free and conjugated forms in apples, pears, grapes, oranges, tomatoes, berries, and some vegetables and cereals (mostly in conjugated form). Mushrooms, on the other hand, have a high content of free p-coumaric acid. Conjugated forms are

considered more beneficial in terms of bioactivity, including antioxidant, anti-inflammatory, anti-mutagenic, anti-ulcer, and anticarcinogenic properties through regulates some genes and expression of some proteins (31).

P-coumaric acid is regarded as a relatively strong antioxidant and an effective scavenger of reactive oxygen species (ROS) and free radicals. Its antioxidant effects have been demonstrated in cultured endothelial cells exposed to high glucose and free fatty acids, in keratinocytes exposed to UV radiation, and in lens epithelial cells exposed to hydrogen peroxide. Additionally, it has antimicrobial activity, disrupting bacterial cell membranes and intercalating into the grooves of bacterial genomic DNA. Polymeric preparations containing p-coumaric acid have shown both antioxidant and antimicrobial properties, aiding in the regeneration of wounded skin (31).

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) play crucial roles in oxidative stress management. MDA is particularly indicative of oxidative stress in lipid tissues. p-Coumaric acid (PCA) has shown improvements in antioxidant enzyme status, evidenced by enhanced levels of SOD and increased activity of GPx and CAT, while reducing oxidative products like MDA following renal ischemia-reperfusion (I/R) injury. SOD, CAT, and GPx combat oxygen free radicals, suggesting that PCA may lower ROS levels during oxidative stress imbalances. PCA may activate nuclear factor erythroid 2-related factor 2 (Nrf2), which enhances antioxidant capacity in rats. Nrf2 is a transcription factor that controls the Antioxidant Response Element (ARE), leading to the expression of downstream genes like glutathione peroxidases, functionates as reduction hydrogen peroxide to water (32).

p-CA was found to significantly inhibit the formation of the lipid peroxidation by-product, 8-EPI, which subsequently led to a decrease in LDL cholesterol levels in the circulating blood of animals administered CA (32).

A study on the immortalized cell line PC12 suggests that p-coumaric acid (p-CA) inhibited peptidase β-secretase (BACE1), a key enzyme involved in the development of Alzheimer's disease through the formation of β-Amyloid (Aβ) peptide in senile plaques, thereby suppressing Aβ production. Additionally, p-CA enhanced its anti-inflammatory effects by inactivating both the NF-κB and MAPK signalling pathways [32].

5.3 *trans*-Cinnamic acid

Cinnamic acid is a naturally occurring organic compound in plants, characterized by its low toxicity and diverse biological activities. As researchers explore new pharmacologically active substances, cinnamic acid derivatives emerge as significant and promising candidates for drug development due to antioxidant properties of phenolic hydroxyl group [33]. The most frequently found isomer of cinnamic acid is the trans isoform. While cis isomers also exist, they are less common. However, cinnamic acid can be converted to the cis form in certain conditions, such as in the plant Arabidopsis under Ultraviolet light (UV). Cinnamic acid is a naturally occurring aromatic fatty acid found in several plant-based products. It is synthesized in plants through the enzymatic deamination of phenylalanine, a process that is part of the phenylpropanoid pathway (34).

A study by Chandra et al. (2019) delineated that cinnamic acid acts as a ligand of peroxisome proliferator-activated receptor α (PPARα) and induces lysosomal biogenesis by activating PPARα to transcriptionally upregulate TFEB expression. Additionally, the administration of cinnamic acid in mouse models of familial Alzheimer's Disease (AD) significantly reduced

cerebral Aβ plaque accumulation and improved memory features. Therefore, the stimulation of lysosomal biogenesis by cinnamic acid may present therapeutic implications for treating various neurodegenerative disorders arising from the accumulation of toxic protein aggregates. Therefore, positive lysosomal modulations, such as the overexpression of TFEB in astrocytes, can lead to the induction of lysosomal biogenesis. TFEB enhances astroglial uptake of extracellular tau species and reduces tau spreading (35) (36).

Other studies on peroxisome proliferator-activated receptor α (PPARα) have demonstrated that cinnamic acid acts as a potent ligand for PPARα, leading to the upregulation of the master regulator TFEB. Treatment with cinnamic acid has been shown to reduce amyloid plaque pathology and enhance memory in mice. These findings convey that cinnamic acid could have therapeutic potential for mitigating the pathogenesis of Alzheimer's Disease and other conditions caused by abnormal lysosomal storage [37].

Previous studies showed patients with major depressive disorder exerts increased pro-inflammatory cytokines levels, abnormal changes in oxidative stress markers such as malondialdehyde (MDA), superoxide dismutase (SOD), and GST. Therefore, Cinnamic acid with AOX activities can ameliorate depression by decreasing the oxidative markers. Cinnamic acid has been shown to upregulate the suppressor of cytokine signalling 3 in both microglia and astroglia. In the mouse model of Parkinson's Disease (PD), CA was found to activate PPARα, protecting the nigrostriatal axis and improving motor behaviours (38).

The limited intestinal absorption and low bioavailability of cinnamic acid and its derivatives have been reported in several studies. Consequently, new formulations are currently being investigated to improve the entrapment and delivery of these compounds (39).

5.4 Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) exists primarily in the trans isomer form, though it can also be found as a cis isomer. This compound is notable for being found in the genus Capsicum. Pure capsaicin is hydrophobic, colourless, highly pungent, and can range in texture from a crystalline to a waxy solid (40).

This homovanillic acid derivative accounts for the majority of capsaicinoids (70%), while other analogues such as dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, and norcapsaicin are present in lesser amounts. Dihydrocapsaicin is the second most abundant at 22%, followed by nordihydrocapsaicin at 7%, with the other analogues occurring in lower quantities (41).

The impact of capsaicin on thermoregulation was first observed when applying hot pepper extract to dogs' stomachs caused a drop in their rectal temperature through studies conducted by Högyes et al. In terms of pain and inflammation, Nicholas (Miklós) Jancsó perceptively noted that capsaicin induced significant and lasting "desensitization" following exposure to the rat cornea, skin, and airways (42).

The primary target of capsaicin is the TRPV1 channel. Although this channel can be non-selectively activated by numerous endogenous and exogenous agonists, capsaicin is highly selective and potent in its activation of the TRPV1 channel (43). Capsaicin has been used as anaesthesia in various studies, showing positive outcomes. It has also been utilized in dermal patches to help alleviate peripheral neuropathic pain. These patches have been successful in providing long-lasting effects due to capsaicin's stability in the TRPV1 channel. While capsaicin exhibits adequate absorption through the skin, research suggests that absorption time and levels may vary depending on the specific type of neuropathic disease (44).

Capsaicin is quickly metabolized by hepatic enzymes, primarily through the major action of the microsomal enzyme Cytochrome CYP2C19. It is mainly excreted by the kidneys, with a small portion of the untransformed compound being eliminated in the faeces and urine (45).

Capsaicin exerts its anti-inflammatory effects by inhibiting the production of IL-1β, IL-6, and TNF-α induced by lipopolysaccharide (LPS). This inhibition occurs through the increased expression of Liver X receptor α (LXRα) via the peroxisome proliferator-activated receptor-gamma (PPARγ) pathway (41)

5.5 DOPAC

DOPAC named 3,4-dihydroxyphenylacetic acid is one of the Hydroxybenzoic derivatives hydroxytyrosol, the most effective antioxidant in olive oil. DOPAC is a commercial version of Hydroxytyrosol as it has a structure analogous to DOPAC (46).

Dopamine (DA) is a crucial neurotransmitter and an intermediate product in noradrenaline synthesis. It is involved in motor, sympathetic and parasympathetic, and cognitive functions. Deficits in dopamine within the brain can lead to Parkinson's disease in humans. Its derivative, DOPAC, also plays a key role in Parkinson's disease due to its antioxidant activity. The precursor to DA, NA, and adrenaline is 3,4-dihydroxyphenylalanine (DOPA), the immediate product of the rate-limiting step in catecholamine biosynthesis. Dopamine is deaminated and dehydrogenated by monoamine oxidase (MAO) and aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid (DOPAC) (46).

A study conducted under conditions very similar to in vivo demonstrated that DOPAC has antioxidant activity, although other phenolic acids have greater antioxidant capacities. DOPAC,

at micromolar concentrations, was found to inhibit lipid peroxidation, most likely within biological membranes (47).

DOPAC whose structure is analogous to that of Coumaric acid but lacks a double bond in the aliphatic chain, also exerts antioxidant activity in lipids and sympathetic nervous activity. DOPAC is a phenolic acid catabolite of quercetin, a plant-derived polyphenol containing a catechol moiety. It demonstrates both DPPH radical scavenging and superoxide dismutase-like activities, along with a significant inhibitory effect on superoxide-dependent NBT reduction. DOPAC can absorb in the body more efficiently than quercetin although the former has the lower superoxide-scavenging activity than the latter. Some evidence suggests that DOPAC is a predominant antioxidative catabolite of Q4′G formed by the colonic microbiota in the large intestine (48).

DOPAC like other phenolic compounds showed the ROS generating ability, but incubation of The mouse hepatoma cell line Hepa1c1c7, showed indirect antioxidant of DOPAC by activating some gene expression of several drug metabolizing enzymes including catalytic subunit GCLC, NAD(P) H: quinone oxidoreductase 1 (NQO1) and , hemeoxygenase 1 (HO-1) and some increase in mRNA of cytochrome P450 1A1 (CYP1A1) phase1. This dose dependently result suggests there might be different pathways for inducing different drug metabolizing enzymes. DOPAC also can induce the expression of phase II drug-metabolizing enzymes such as glutathione S-transferase (49).

5.6 Caffeic acid

Caffeic acid (CAF), 3,4-dihydroxycinnamic acid, plants-derived molecule, classified as a hydroxycinnamate and phenylpropanoid. CAF has been found in blueberries, apples, cider, coffee and propolis. It is a carcinogenic inhibitor and has a high antioxidant effect and some

antimicrobial activity. Besides, other positive effect on heart diseases and atherosclerosis has been seen. Toxicity of Caffeic acid and its derivatives are low (50).

According to a study by Spagnol et al., it can be concluded that CAF exhibited significant antioxidant activity. The results demonstrated the relative efficiency of CAF in scavenging reactive species, particularly $O2 •−$ and $HOCl/OCl−$, which are overproduced during infectious processes. Thus, in both the peroxidative and chlorinating pathways of myeloperoxidase, there is evidence that CAF may act as a substrate for myeloperoxidase and subsequently interact with $HOCl/OCl−$ (51). Among Caffeic acid derivatives, caffeic acid phenyl ester shows important biological activities, including neuroprotective activity by modulating the Nrf2 and NF-κB pathways, promoting antioxidant enzyme expression and inhibition of proinflammatory cytokine expression (52).

According to studies has been proved by A. khan et al, that caffeic acid has strong antioxidative properties and prevents the mice brain from Aβ-induced oxidative stress (53).

Caffeic acid markedly upregulated the expression of neuronal growth factors, markers in the brains of Aβ-injected mice such as brain-derived neurotrophic factor (BDNF) has shown promising rescuing effects against neurodegenerative diseases (54).

5.7 Vanillic acid

Vanillic acid (4-hydroxy-3-methoxy benzoic acid) is an aromatic phenolic acid with a molecular weight of 168.14 g/mol and a slightly yellow appearance. Known for its pleasant creamy odour, Vanillic acid is the oxidized form of vanillin. It is naturally present in high concentrations in plants such as sweet basil, vanilla beans, dried fruits, and Angelica sinensis,

and can also be biosynthesized by a strain of Ochrobactrum anthropi. Vanillic acid role extends far beyond flavouring, as its nutraceutical potential is increasingly being recognized and explored (55).

Studies have shown that lower concentrations of vanillic acid exhibit significantly higher antioxidant activity, whereas higher concentrations demonstrate reduced antioxidant activity. At a high dose of 2 mg/ml, Vanillic acid has been found to have genotoxic effects on DNA. However, at reasonable low concentrations, Vanillic acid can protect DNA and chromosomes from oxidative damage. greatly recovered enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase), non-enzymatic antioxidants (vitamin C, vitamin E), and lipid peroxidation products (thiobarbituric acid reactive compounds, lipid hydroperoxides, conjugated dienes) (56).

Ebinger, in 1976, identified Vanillic acid as one of the endogenous biochemicals derived from catecholamines. It was also identified in various parts of the human brain (cortex, striatum, thalamus, cerebellum, brain stem, and hypothalamus) and cerebrospinal fluid (CSF) The cannabinoid type 1 (CB1) receptor binding affinity of vanillic acid is very high. Vanillic acid has the highest estimation score for blood–brain barrier penetration. inhibition of signalling pathways of the nitric oxide (NO)–protein kinase G (PKG) pathway by Vanillic acid from ginseng root has protect cardiovascular disease (56).

Pharmacokinetic properties in go.drug bank indicate that Vanillic acid acts as a substrate for CYP450 1A2 and neither as a substrate nor an inhibitor for CYP450 2C9, CYP450 2D6, and CYP450 2C19. Vanillic acid is excreted in the urine after consuming coffee, chocolate, or green tea, originating from caffeic acid (57) .

A new formulation combining Vanillic acid and Apocynin has been developed as a promising method to enhance the bioavailability and aqueous solubility of both compounds. This is achieved by encapsulating them with Hydroxypropyl betacyclodextrin (HPBCD). The HPBCD inclusion complex with AP-VA has been synthesized and characterized for its physicochemical properties, which could be advantageous for pharmacological applications (58).

studies on an in vivo AD mouse model have shown that VA treatment reduces Aβ1-42-induced neuronal apoptosis and neuroinflammation, as well as improves synaptic and cognitive deficits. Additionally, research on the HT22 cell line demonstrated that vanillic acid mitigates the harmful effects of oxidative stress by reducing Aβ accumulation and BACE-1 (β-site APP-cleaving enzyme-1) expression, with no recorded toxicity on the cell line (59)

V. Experimental Part

1. Aim of studies

The aim of the present study was to explore whether the antioxidant parent drugs would cleave from their LAT1-utilizing prodrugs and how long it would take to be released. This biotransformation studies would give more information about the proper drug delivery into the brain of the used LAT1-prodrug approach. If the delivery is more efficient and targeted, the dose can be adjusted so that we have improved efficacy with fewer side-effects.

2. Material and methods

2.1 Reagents

All reagents and solvents used in the analytical studies were commercial, high purity of analytical grade or ultra-gradient high performance liquid chromatography (HPLC)-grade. Acetonitrile for HPLC LC-MC grade manufactured by VWR international S.A.S made in COREE DU SUD, Formic acid 99-100% manufactured by VWR international S.A.S made in COREE DU SUD. Phosphoric acid 85% TAMBRO medlab OY, Rajatorpantie 41B, 01640 Vantaa, Dimethyl Sulfoxide HPLC grade.

The studied prodrugs were synthesized in the Transporter-mediated Targeted Drug Delivery - Reserch Group, School of Pharmacy, University of Eastern Finland. Deionized water used in all the experiments was passed through a Milli-Q water purification system (17-18 MΩ/cm).

2.2 Instrumentation

Instrumentation and Chromatographic Conditions. Analytical Column ZORBAX SB-C18 Agilent 4.6*150mm 5micron diameter, SupelcSIL column 15 cm*4.6mm.5µm, High-performance-Liquid-Chromatography Agilent 1100-series**.** Developing an assay method for release studies of each prodrug has been performed with 1100-series Agilent HPLC consisting of a degasser (G1379A), a binary pump (G1312A), an autosampler (G1313A), a column oven (G1316A), and a diode array detector (G1315A).

Chromatographic analysis was conducted using isocratic separation mode for 1116,1134, 1097, 1095, 1098,1125,and gradient system for *p*-coumaric acid and 1118, Vanillic acid and 1129. An injection volume of 10 μL was used for all standards and samples.

You need to explain how the standards were prepared. Sample prepared for blank samples were diluted (5:1000) with water-ACN mixture (1:1).

2.3 Biological materials

For the bioconversion study, the immortalized mouse microglia cell line (BV2) was cultured and the cells were collected as pellets from confluent dishes. The cells were homogenized in 50 mM Tris-buffer saline (TBS) (pH 7.4) by sonication. After homogenizing the cells, the S9 subcellular fractions were collected by centrifuging the homogenates at 10 000 x g for 20 minutes at 4 °C. Mouse immortalized microglia cell line (BV2) was a generous gift from Prof. Tarja Malm (University of Eastern Finland).

Protein concentrations were determined from the lysed cell samples with Bio-Rad Protein Assay that is based on the Bradford protein assay method. The absorbance was measured at 595 nm using Bovine serum albumin (BSA, Sigma-aldrich) as a standard protein by a multiplate reader (EnVision 2104, Perkin Elmer, Inc., Waltham, MA, USA). 10 µl of cell lysate was pipetted as a triplicate in a 96-well plate with 200 µl Bio-Rad reagent dilution (1:10 in water)

2.4 Prodrugs

The prodrugs and their parent drugs with their molecular weights are presented in table 1. The prodrug structure consisted of the antioxidant parent drug bound via either an ester bond or amide bond to amino acid promoiety that targeted LAT1. Thus, according to the hypothesis, the prodrugs should be able to bind to the LAT1 transporter and be transported into cell and release the antioxidant inside the cells via enzymatic reactions by either esterase or amidase depending on the prodrug bond. The structures of prodrugs are not published yet therefore they are not presented in this thesis

Table 1. Properties of the studied prodrugs

Antioxidant (with the CAS number) and its molecular weight	Prodrug code, used prodrug bind and molecular weight
Eugenol CAS. 97-53-0 MW=164.20 g/mol	UEF-PD-1095 Ester bond MW=355.39 g/mol
Capsaicin CAS.404-86-4 MW=305.42 g/mol	UEF-PD-1098 Amid bond MW=4966.60 g/mol
Vanillic acid CAS. 121-34-6 MW=168.15 g/mol	UEF-PD-1129 Amid bond MW=330.34 g/mol
trans- Cinnamic acid CAS.140-10-3 MW=148.16 g/mol	UEF-PD-1134 Amid bond MW=310.35
trans- Cinnamic acid CAS.140-10-3 MW=148.16 g/mol	UEF-PD-1097 Ester bond Mw=311.34 g/mol
DOPAC CAS. 102-32-9	UEF-PD-1120 Amid bond

MW=168.15 g/mol	MW=330.34 g/mol
Caffeic acid CAS. 331-39-5 MW=180.16	UEF-PD-1125 Amid bond MW=342.35 g/mol
p-Coumaric acid CAS. 501-98-4 MW=164.16	UEF-PD-1118 Amid bond MW=326.35 g/mol
p-Coumaric acid CAS. 501-98-4 MW=164.16	UEF-PD-1116 Ester bond MW=327.34 g/mol

*CAS (Chemical Abstracts Service) Registry Number

2.5 HPLC assay method development

Selectivity was checked by examining chromatogram of two blank sample of prodrugs and parent drugs (therapeutic moiety) by running separately to check for no simultaneous peak at a specific Retention Time (RT). The prodrug chromatogram could have one or two peaks. One of the peaks should overlap with the peak of parent drug chromatogram. Parent drugs had only one peak at the specific RT. For all methods the BV2 fresh sample taken from freezer ran with the method and got the RT earlier than 1 minute. Also, in the case of closeness of RT of parent drugs and prodrugs, the blank mixture of both parent and prodrugs ran to check any overlap. The peak of BV2 was also checked. Details of the developed HPLC methods can be found in table 2.

2.6 HPLC method validation

HPLC methods were validated to ensure reliable measurements. System suitability, linearity, specificity, accuracy, precision, precision, lower limit of quantification (LLOQ) and limit of detection (LOD) were studied. System suitability was studied with six different injections of 50 µM solutions and evaluated by the relative standard deviation (RSD%) of peak areas and retention times from 6 different injections. Linearity is determined by injecting a series of standards of 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM diluted stock solutions. The linearity graph is plotted using Microsoft Excel (Concentration vs. Peak Area Response) and evaluated based on standard curve correlation coefficient of constructed standard line. Accuracy and precision were studied with three parallel samples of three concentrations: 1, 50 and 100 µM. The validation criteria are according to guidelines on bioanalytical method validation by European medicines. The acceptance criteria have been done in-house and it is based on ICH guidelines. Samples were prepared freshly by dilution of stock solutions in dimethyl sulfoxide(DMSO) (10mM) by dissolving in water : ACN (1:1).

2.7 Bioconversion studies

Bioconversion, or in other words enzymatic stability, was studied in the mouse immortalized microglia (BV2) subcellular fraction (S9). Samples were prepared so that the concentration of the drug in the incubation mixture was 100µM and protein concentration 1000 µg/ml. 10 mM prodrug DMSO solution was added to 50 mM Tris-HCl (pH 7,4) and biomaterial. Mouse brain samples were incubated at 37 °C at time points: 1, 15, 30, 60, 120 180, 240, 360. Samples

were collected in several time points and reaction was stopped with the ice-cold ACN. Samples were centrifuged for 5 minutes at 12 000 rpm (20 °C) and the supernatants were run by HPLC.

2.8 Half life

The time required for a quantity to reduce to half its initial value, using the observed rate constant. The formula relates the observed rate constant to the slope of a linear plot in a logarithmic decay process.

$Kobs$= $k_{observed}$ =(-2.303)*slope

$$\frac{t1}{2} = == LN(2)/kobs$$

Among the studied prodrugs, two prodrugs 1134 and 1098 have shown the remaining of prodrug after bioconversion reached 50% of the starting dose.

VI. Result

1. Method development

Table 2. method development

<table>
<tr><th>COMPOUND NAME</th><th>RETENTION TIME</th><th>COLUMN</th><th>MOBILE PHASE A</th><th>MOBILE PHASE B</th><th>FLOW RATE</th><th>WAVELENGTH</th><th>TEMP</th></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>P-COUMARIC ACID</td><td>3.904</td><td>C18</td><td>80 H2O+0.1%H3PO4
PH=5</td><td>20
ACN</td><td>1
ml/min</td><td>286</td><td>22°C</td></tr>
<tr><td>1116</td><td>3.096</td><td>C18</td><td>80 H2O+0.1%H3PO4
PH=5</td><td>20
ACN</td><td>1
ml/min</td><td>318</td><td>22°C</td></tr>
<tr><td>1118

P-COUMARIC ACID</td><td>6.570

8.782</td><td>Supelcol</td><td>Gradient system
H2O+0.1% NH4CH3CO2
PH=4.7
<table><tr><th>Time</th><th>%B(ACN)</th></tr><tr><td>1</td><td>10</td></tr><tr><td>2</td><td>10</td></tr><tr><td>5</td><td>70</td></tr><tr><td>10</td><td>80</td></tr><tr><td>17</td><td>10</td></tr></table></td><td>Gradient
ACN</td><td>0.5</td><td>318</td><td>22°C</td></tr>
<tr><td>CIN

1134</td><td>2.9

6.4</td><td>C18</td><td>55
H2O+0.1%FA</td><td>45
ACN</td><td>0.6</td><td>255</td><td>22°C</td></tr>
<tr><td>CIN

1097</td><td>5.081

2.709</td><td>C18</td><td>62
H2O+0.1%FA</td><td>38
ACN</td><td>1</td><td>280</td><td></td></tr>
<tr><td>DOPAC

1120</td><td>3.457

6.473</td><td>Supelcol</td><td>45
H2O+0.1%FA

70</td><td>55
CAN

30</td><td>1.00</td><td></td><td>22°C</td></tr>
</table>

<table>
<tr><td></td><td></td><td></td><td>H_2O+0.1%FA</td><td>ACN</td><td>0.6</td><td></td><td>22°C</td></tr>
<tr><td>Vanillic acid

1129</td><td>3.034

6.118</td><td>supelcol</td><td>Gradient system<table>
<tr><td>Time</td><td>%B(ACN)</td></tr>
<tr><td>1</td><td>10</td></tr>
<tr><td>2</td><td>10</td></tr>
<tr><td>5</td><td>10</td></tr>
<tr><td>10</td><td>60</td></tr>
<tr><td>16</td><td>60</td></tr>
<tr><td>18</td><td>60</td></tr>
<tr><td>20</td><td>10</td></tr>
</table></td><td></td><td>0.7</td><td>266nm</td><td>22°C</td></tr>
<tr><td>EUGENOL

1095</td><td>2.501

5.011</td><td>C18</td><td>58
H_2O+0.1%FA</td><td>42
ACN</td><td>1</td><td>280nm</td><td>22°C</td></tr>
<tr><td>CAPSAICIN

1098</td><td>5.915

2.457</td><td>C18</td><td>47 %
FA</td><td>53%
ACN</td><td>1</td><td>280nm</td><td>22°C</td></tr>
<tr><td>CAFFEIC ACID

1125</td><td>3.991

5.167</td><td>C18</td><td>40%
FA</td><td>60%
ACN</td><td>1</td><td>312nm</td><td>22°C</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

2. Method validation

2.1 System suitability

System suitability was studied to verify the suitability of the analytical method for analysis. System suitability was studied by calculating the relative standard deviation (RSD%) of peak areas and retention times from six different injections. Validation for the parent drugs which the peak is before t0 has not performed Dopac, vanillic acid, caffeic acid. Syst1m suitability results are presented in table 3.

Table 3. System suitability validation results

Compound	RSD % of Retention times	RSD % of Peak area
p-coumaric acid	0.451679	0.779864
1116	0.29613	0.463821
p-coumaric acid	0.075789	1.725744
1118	0.039132	0.642614

trans- Cinnamic acid	0.116806	1.231299394
1134	0.057853	1.391041
trans- Cinnamic acid	0.039568	0.671344
1097	0.184827	0.428786
1020	0.340631	1.71746818
1129	0.312569	1.637148
Eugenol	0.088829	0.528933
1095	0.12579	1.65865
Capsaicin	1.42244E-14	1.099957
1098	0	0.663445
1025	0.154473	0.602339
Criteria	≤ 0.5 %	≤ 2 %

2.2 Linearity

The standard curve was calculated from the response of the HPLC peak areas to each concentration. It was visually examined as well as with calculated values. Standard curves of studied sub- 53 stances are presented in figure 9. The R2 -value of linear regression of standard curve was calculated. Correlation coefficient was > 0.95 (table 9) and method was therefore found to be linear with all five compounds.

Table 4. Linearity results. Values outside the criteria are marked with star (*).

Criteria	**$R^2 \geq 0,95$**
p-coumaric acid	0.9978
1116	0.9945
p-coumaric acid	0.9699

1118	0.9883
trans- Cinnamic acid	0.9989
1134	0.9968
trans- Cinnamic acid	0.9994
1097	0.9902
1020	0.9648
1029	0.9867
Eugenol	0.9999
1095	0.9761
Capsaicin	0.9983
1098	0.9992
1025	0.9607

2.3 Accuracy

Accuracy describes how close measured and theoretical values are to each other. Accuracy was calculated using the formula

$$\text{Accuracy \%} = \frac{\text{measured concentration x 100}}{\text{theoretical concentration}}$$

Table 5. Accuracy validation results. Values outside the criteria are marked with star (*).

compounds	**Accuracy %**		
	1 µM	**50 µM**	**100 µM**
p-coumaric acid	102.9524	102.3413	64.53123
1116	100.8075	97.17845	103.7999
p-coumaric acid	351.3755*	120.3195*	103.2244
1118	79.74729*	98.33359	89.54482*
trans- Cinnamic acid	not detected	56.16287*	96.5131

1134	98.45139	98.41591	100.0568
trans- Cinnamic acid	87.05274*	100.445	94.7343*
1097	10.06311*	58.38386*	52.13118*
1020	not detectable	102.9129	92.8553*
1029	167.7038*	106.0947*	101.4608
Eugenol	72.49909*	96.92673	97.24108
1095	14.91311*	113.8804*	81.92968*
Capsaicin	119.8594*	111.8437*	100.9038
1098	89.14387*	105.1136	102.0464
1025	not detectable	98.10686	86.34112*
	Criteria	95-105%	

2.4 precision

Precision describes how close parallel values are to each other. RSD % of three parallel samples was calculated in concentrations

Table 5. precision   stars are not in criteria

compounds	RSD %		
	1 µM	**50 µM**	**100 µM**
P- coumaric acid	7.762071	5.652859	8.91519
1116	3.646423	1.017793	6.699616
p-coumaric acid	2.73178	1.446952	0.190599
1118	5.951397	8.932544	7.632641

trans- Cinnamic acid	not detected	3.617085	0.442264
1134	21.62162*	3.210135	1.83115
trans- Cinnamic acid	6.501754	1.448411	0.222388
1097	14.62833	3.162095	0.446785
1020	not detectable	6.758625	8.897145
1029	16.43421*	1.504421	10.91328
Eugenol	35.06285*	2.384164	1.330495
1095	39.9016*	2.070168	0.891937
Capsaicin	2.063597	3.301132	0.664681
1098	6.735522	0.794734	0.857543
1025	not detectable	9.565388	0.773549
Criteria ≤ 15 %			

3. Bioconversion studies

3.1 Bioconversion studies for prodrug 1116

The chromatogram of bioconversion shows two peaks at 3.1min, identified as a prodrug, and 3.9min, identified as the parent compound. Remaining amount of prodrug and released parent drug has been calculated based on the areas of the first sample triplicates (1 minute after starting the incubation) (table 6). Replicate A shows an overall increase while some fluctuations occurred between 1st to 240 minutes. After 240 minutes, a significant increase in area. Replicate B shows a steady increase from 2 to 2.20 throughout the period. Replicate C shows an overall increase to 2.24 while the growth has fluctuated slightly.

To sum up all replicates in both bioconversion studies showed an overall increase that we can conclude that the release did not happen.

Time points	A	B	C
1	100	100	100
15	87.08543	102.0928	109.0594
30	96.13065	98.81222	104.1546
60	99.34673	116.1199	116.5032
120	94.72362	106.5611	115.4934
180	97.88945	114.819	111.5984
240	102.4623	124.4344	118.7536
360	137.6884	148.0769	175.4183

Table 6. Remaining prodrug 1116 for triplicates A,B,C as percentage (%)

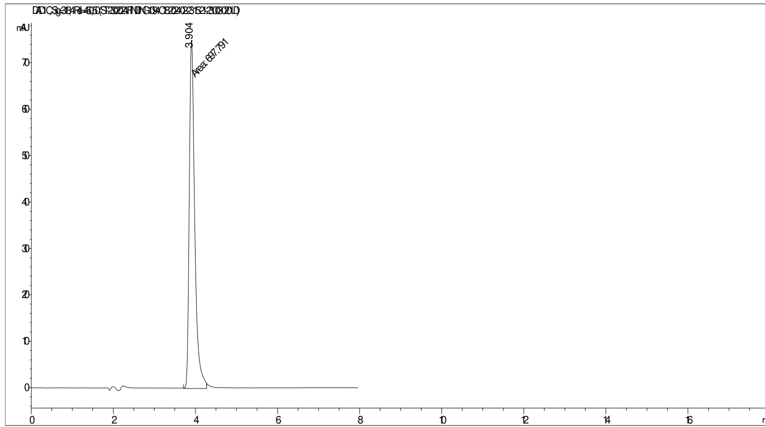


Figure 1 p-cumaric acid parent drug of 1116 method:101223 1118 sara 8min

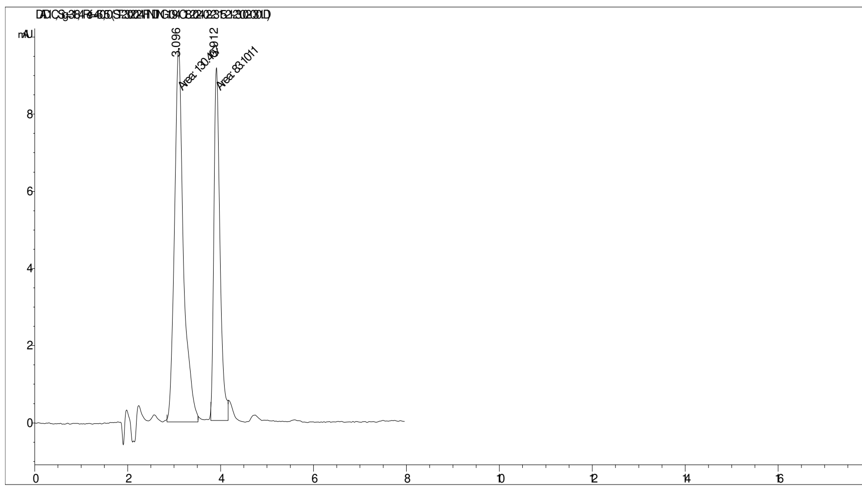


Figure 2 prodrug 1116 sample method 101223sara8min

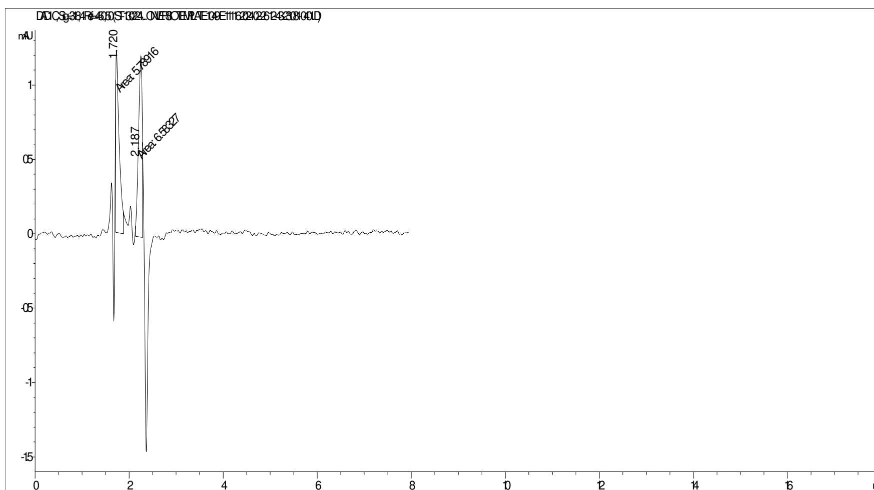


Figure 3 BV2 tris hcl 101223sara1118 8min

3.2 Bioconversion studies for prodrug 1118

The chromatogram of bioconversion illustrates two peaks, at 4.5 minutes (overlapping with t_0), and at 8.7 minutes for the prodrug. The peak at 4.5 minutes overlaps with the acetonitrile peak(t_0) with the injection signal (t_0) with a varying area between 75-80 milli absorbance unit (mAU). Integration for the peak at 8.8 minutes for three replicates indicates an ascending trend line for the remaining prodrug compared to the first time point sample.

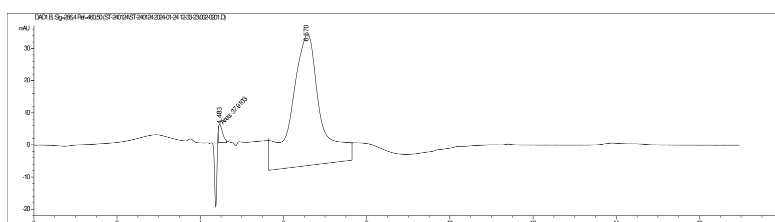


Figure 4 p-coumaric acid

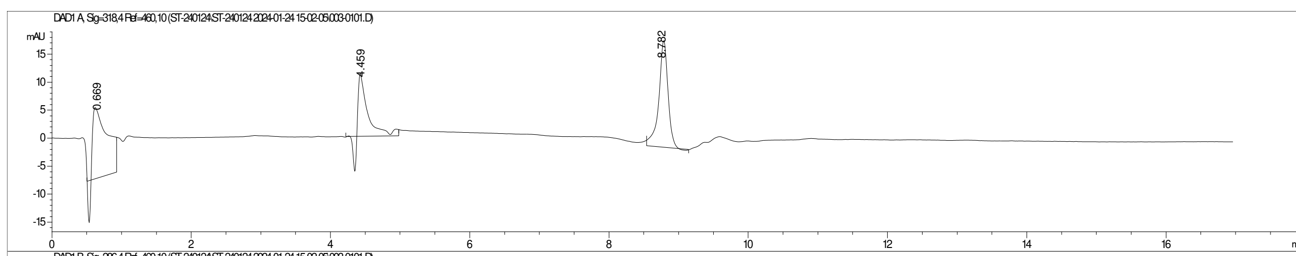


Figure 5 1118

	A	B	C
1	100	100	100
15	122.6545	98.35729	116.4414
30	122.1968	104.1068	114.8649
60	133.1808	124.846	128.6036
120	132.9519	129.5688	134.009
180	157.4371	136.5503	136.9369
240	149.8856	140.8624	150.4505
360	283.524	146.6119	165.0901

Table 7. Remaining amount of prodrugs 1118 for triplicates A,B,C as percentage (%).

3.3. Bioconversion studies for prodrug 1134

The bioconversion studies for prodrug 1134 showed two peaks at 6.0, identified as the parent compound, and 2.9 minutes, identified as the prodrug. Integration of signal area and calculation of remaining based on the concentration of first time point has been done for all the peaks. The area of the parent drug is increasing steadily, and simultaneously the prodrug signal is decreasing. The remaining prodrug at 60 minutes was less than 50%. The half-life for prodrug

is 1.53494 minutes. The remaining of prodrug after 6 hours of injection is 4.1% with Standard deviation of 1.9% .

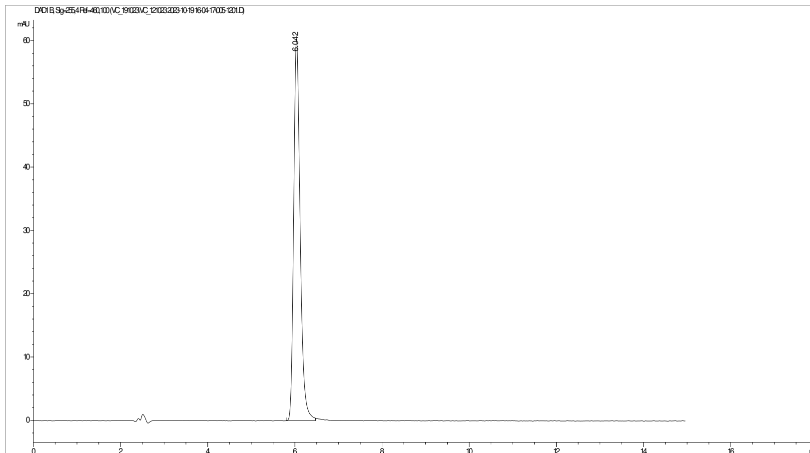


Figure 6 cinnamic acid

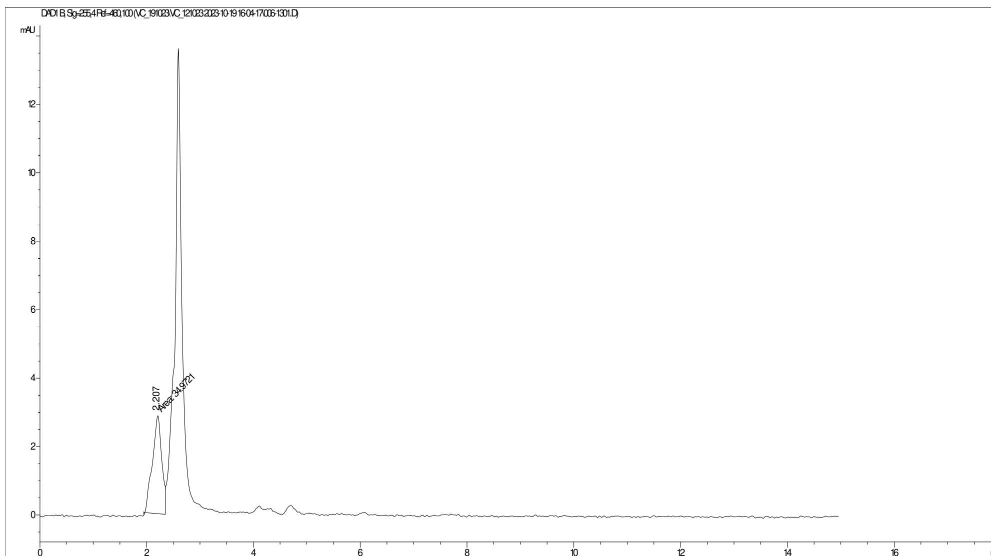


Figure 7. Prodrug 1134

min	A	B	C
1	100	100	100
15	60.71429	75.35885	53.28597
30	50.66964	50.47847	43.69449
60	20.75893	32.77512	25.0444
120	3.571429	26.07656	13.85435
180	7.366071	16.02871	11.19005
240	4.241071	17.46411	8.170515
360	5.133929	5.263158	1.953819

Table 8. remaining of prodrugs 1134 for triplicates A,B,C

3.4 Bioconversion studies for prodrug 1097

Two peaks at 1.3 and 2.5 min were observed in the chromatography of bioconversion for prodrug 1097 with its suitable method. With all three replicates, the prodrug signal is growing, and no released parent drug was detected. Therefore, the release from prodrug did not occur.

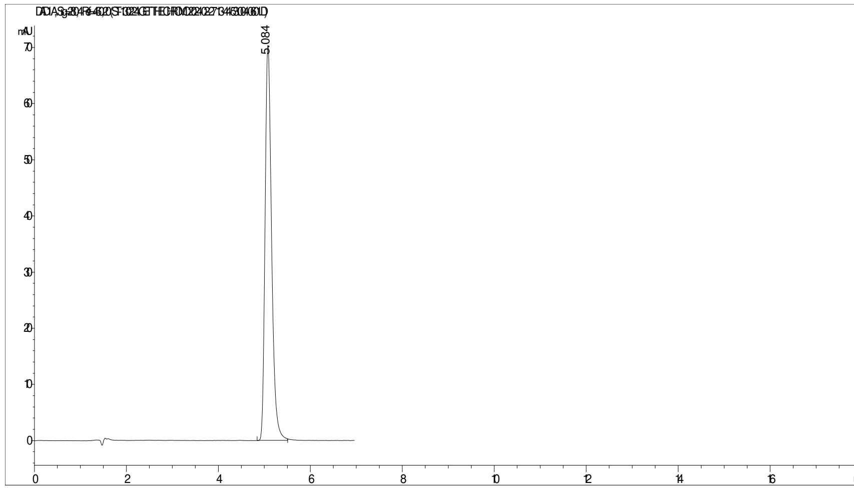


Figure 8. Cinnamic acid

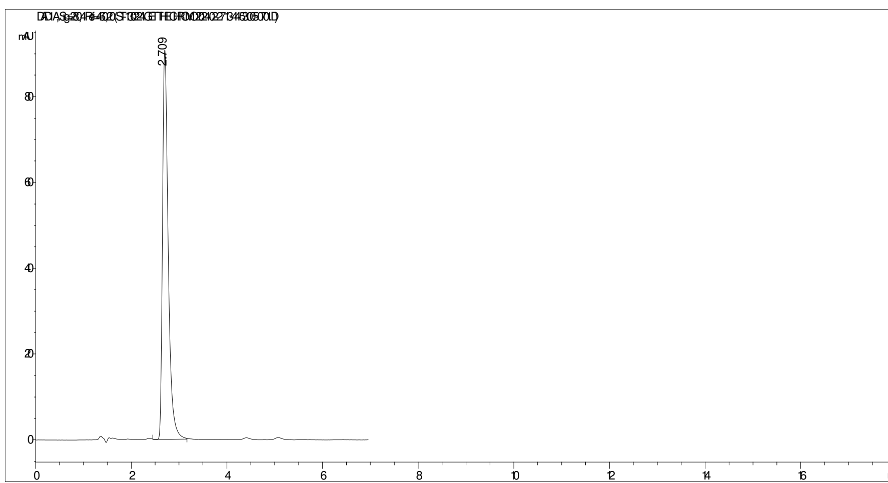


Figure 9. Prodrug 1097

time	A	B	C
1	100	100	100
15	94.72661	93.21983	109.7494
30	91.76185	91.87575	105.9094
60	99.22236	85.63321	109.2665
120	96.59781	91.78614	103.1961
180	97.83718	97.04301	112.0487
240	97.37546	99.49223	119.1998
360	101.0693	101.9713	127.0407

Table 10. Remaining amounts of prodrug 1097 for triplicates A,B,C as percentage (%).

3.5 Bioconversion studies for prodrug 1020

Chromatogram of bioconversion with prodrug 1120 showed two peaks . Integration of signal area and calculation of remaining based on the concentration of first time point has been done for all the peaks. Peak at 6.4 was assumed to be prodrug but the area was so slowly increasing from 7 mAU to 10 mAU during the 6 hour-incubation. Peak at parent drug retention time (3.5 minutes) signal is increasing as well. Replicates showed similar patterns in fluctuation too.

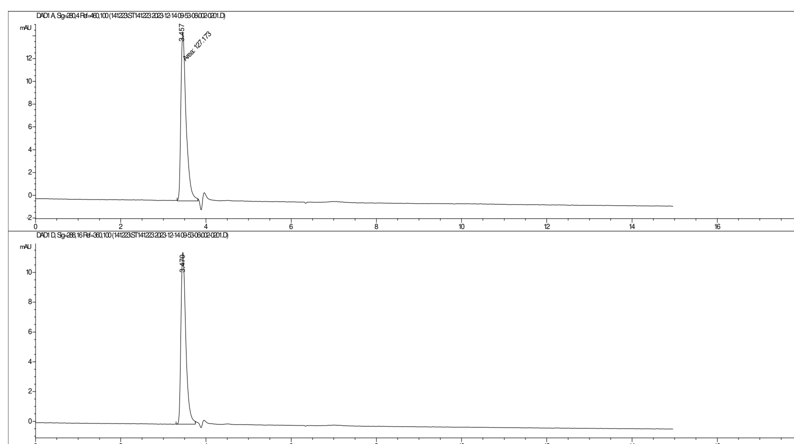


Figure 10. Dopac

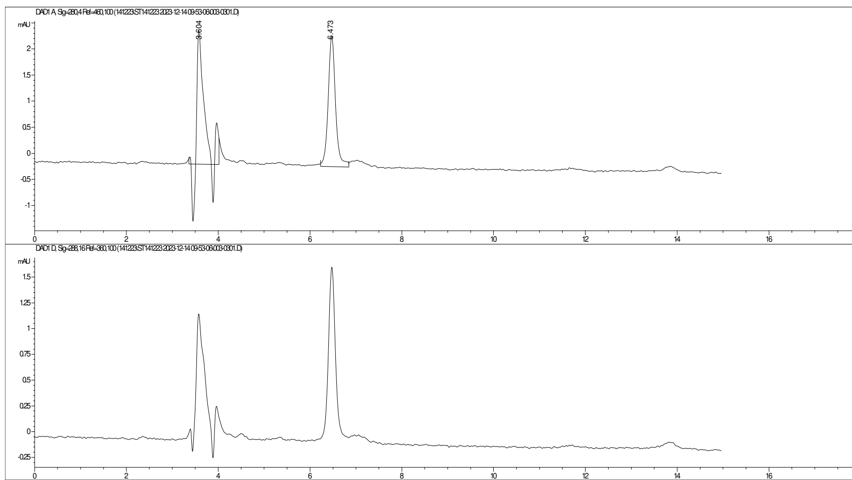


Figure 11. 1120

time	A	B	C
1	100	100	100
15	119.5559	113.5714	113.6659
30	130.0132	125.5102	126.4093
60	144.6256	145.5782	149.9829
120	180.1451	184.3027	206.1155
180	213.7537	235.1701	211.9235
240	262.4243	260.4048	269.9693
360	334.9522	316.9388	307.3796

Table 15. Remaining amounts of prodrug 1120 for triplicates A, B, C as percentage (%).

3.6 Bioconversion studies for prodrug 1129

The bioconversion studies show three peaks at 3.04, and 6.1 minutes. The peak at 6.1 is assumed to be prodrug because it is at the same time as prodrug in the method created for 1129. Remaining of concentration of metabolites and prodrug has been calculated. Growing trend line of remaining prodrug is indicated by figures from three replicates so the promoiety and parent drug remained intact. The forth peak, which might be a metabolite, appeared at 60 minutes, incubation time, at 7.8 minutes, retention time, that increased from 2.1 to 9 mAU.

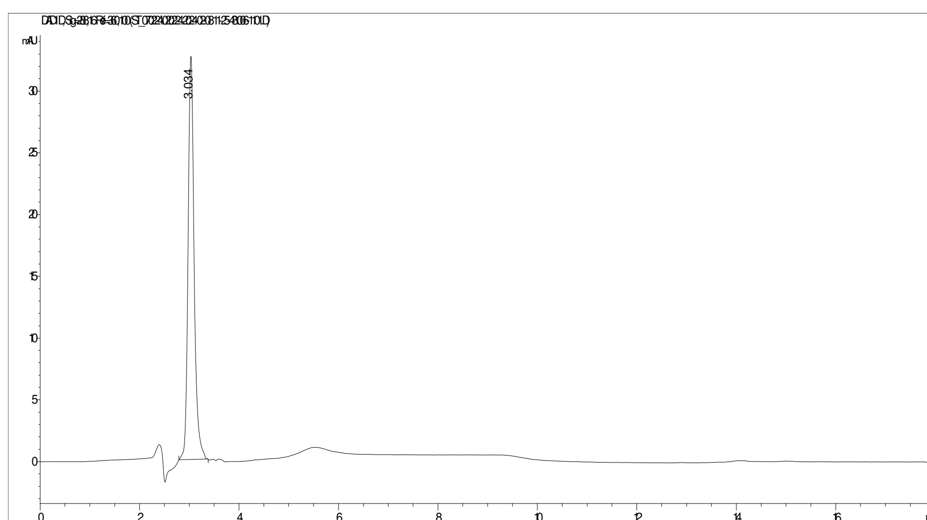


Figure 12. Vanillic acid

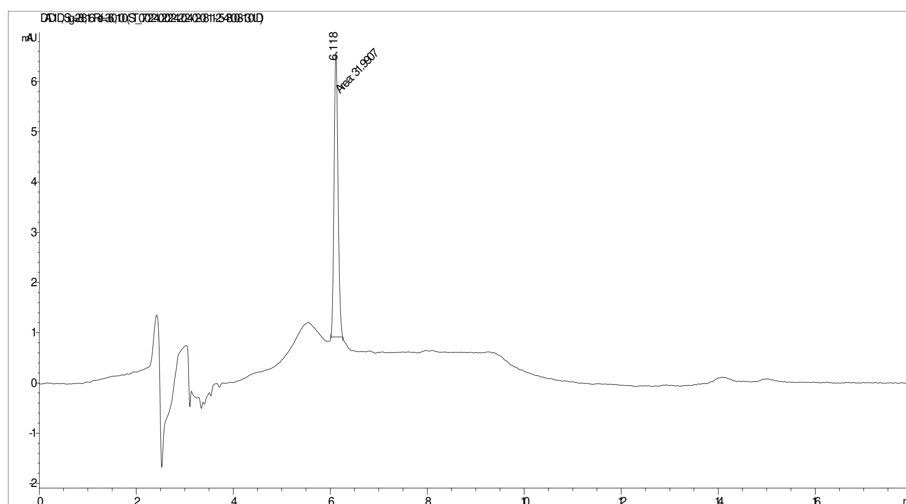


Figure 13. 1129

	A	B	C
1	100	100	100
15	103.5714	121.7391	104.3478
30	110.7143	130.4348	126.087
60	107.1429	126.087	137.3913
120	139.2857	152.1739	153.913
180	153.5714	174.7826	186.9565
240	189.2857	191.3043	191.3043
360	228.5714	252.1739	260.8696

Table 11. Remaining of prodrugs 1129 for triplicates A,B,C as percentage (%).

3.7 Bioconversion studies for prodrug 1095

The bioassay for this prodrug has shown two peaks at 2.5 min and 5 min. Integration of signal area and calculation of remaining based on the concentration of first time point has been done for all the peaks. Peaks at 2.5 min and 5 min increasing unsteady. 1095 retained in the column for 4.7 minutes and integration has been done for this peak which indicates increasing of prodrug in enzymatic reaction by calculation of remaining prodrug.

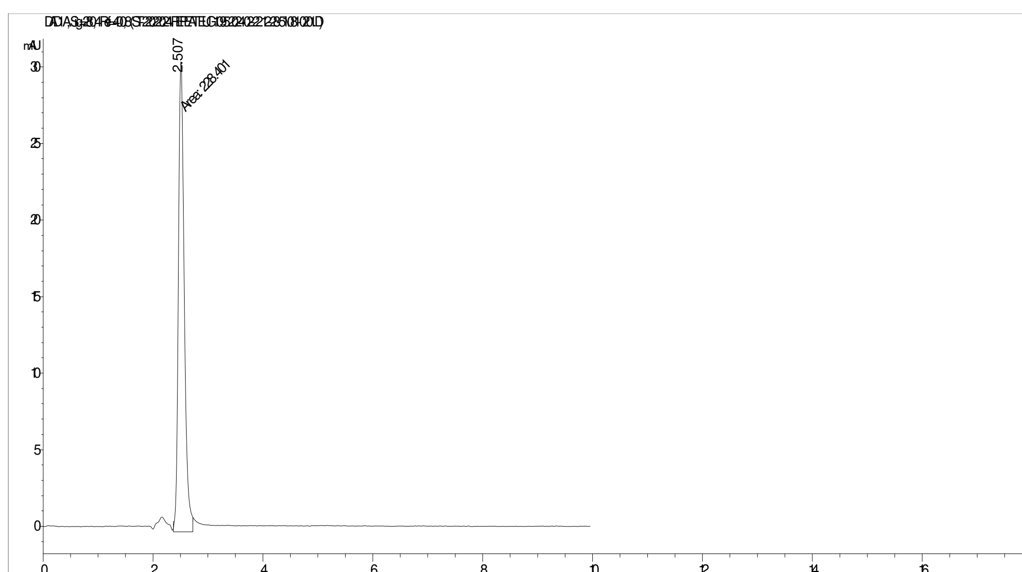


Figure 14. Eugenol

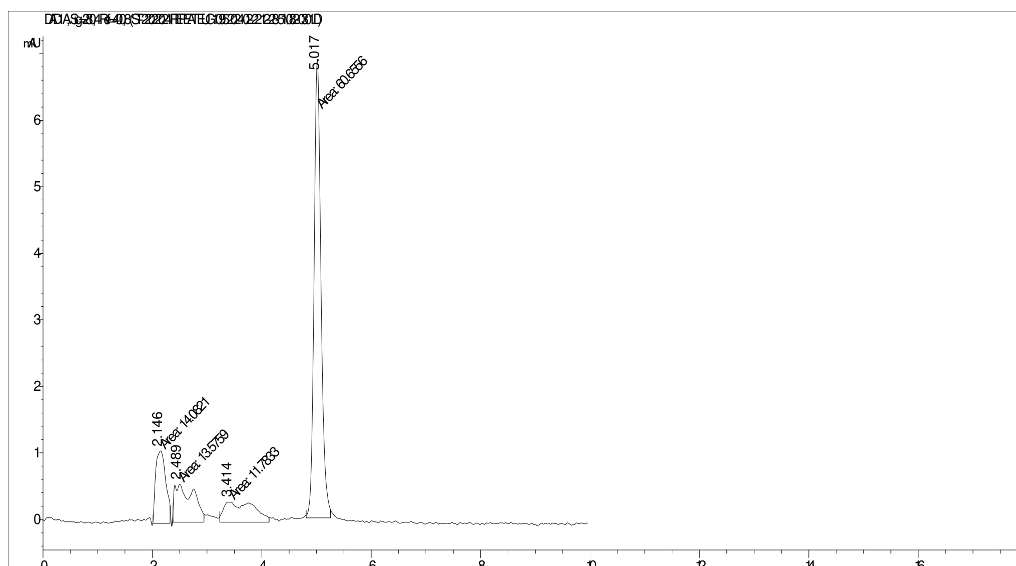


Figure 15. 1095

time points	A	B	C
1	100	100	100
15	104.6314	99.73187	103.4637
30	114.0098	103.6668	97.92388
60	106.766	101.0551	102.6298
120	106.0382	103.4231	100.8304
180	110.9303	104.607	105.6747
240	104.0365	104.4677	96.6436
360	110.4054	107.8803	102.3183

Table 12. Remaining amounts of prodrug 1095 for triplicates A,B,C as percentage (%).

3.8 Bioconversion studies for prodrug 1098

Two chromatographic peaks showing three metabolites of bioconversion eluted at 2.5 min, 3 min and 6 min. Integration of signal area and calculation the remaining has been done for the peaks. The peak at 2.5 min identified as the prodrug. The graphs of this peak for three technical replicates had a descending trend line that implies that the prodrug is releasing parent drug in vitro. The calculated half-life among replicant was different. Only one replicate' remaining amounts of prodrug reached 50%.

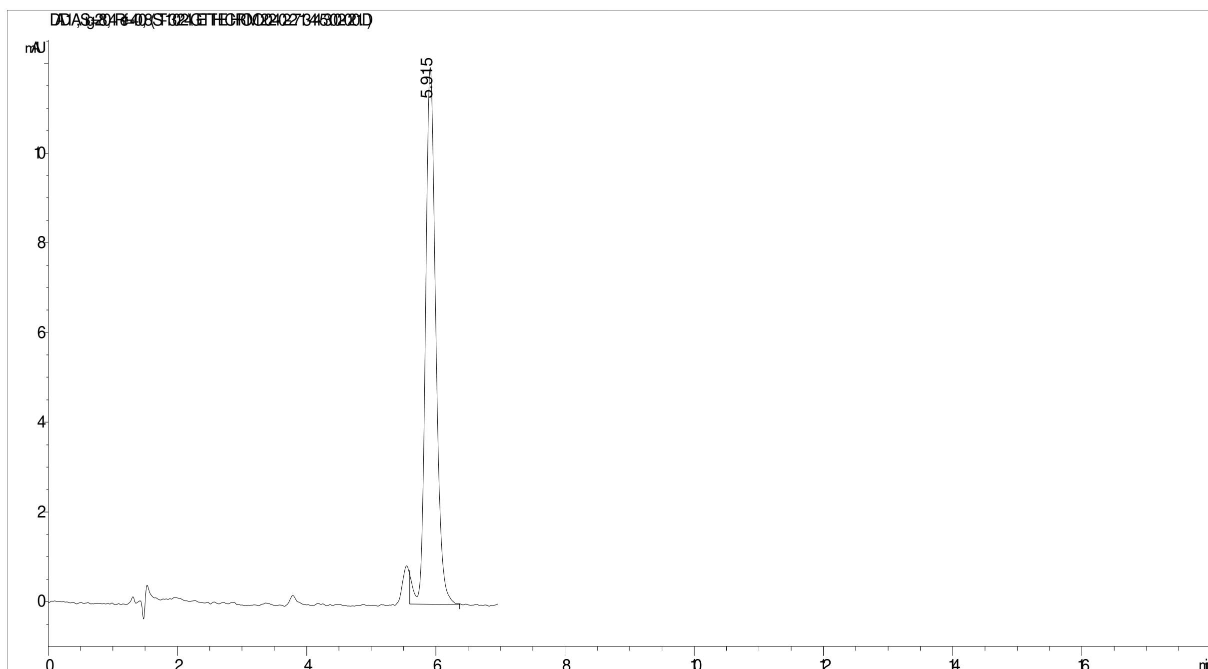


Figure 16. Capsaicin

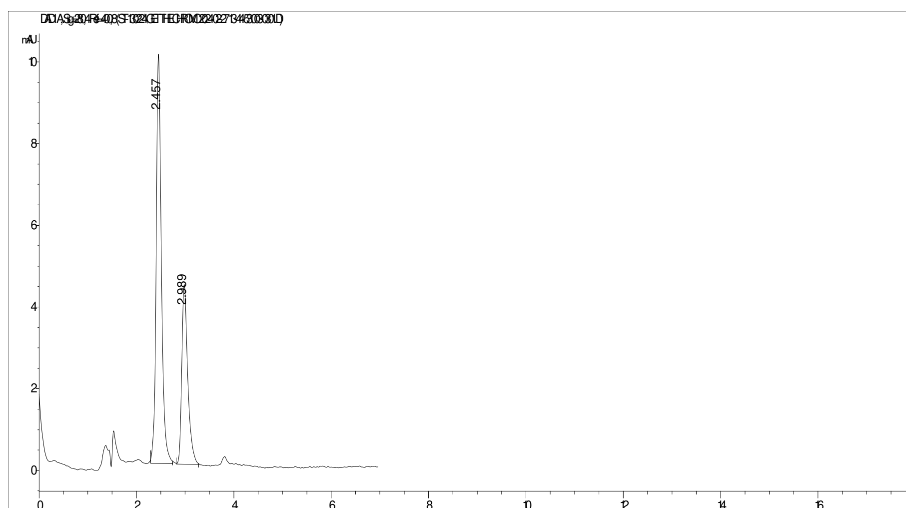


Figure 17. 1098

min	A	B	C
1	100	100	100
15	69.73684	94.444444	82.857143
30	71.05263	96.969697	90
60	75	95.959596	85.714286
120	77.63158	97.979798	105
180	96.05263	71.212121	108.57143
240	82.89474	46.464646	62.857143
360	67.10526	43.939394	73.571429

Table 13. Remaining amounts of prodrug 1098 for triplicates A,B,C as percentage (%).

3.9 Bioconversion studies for prodrug 1025

The bioconversion study of prodrug 1125 showed two signals at 4 min and 5.1 min alike the retention times for the prodrug in the method. However, the peak at 4 min (t_0) is too close to the parent drug peak. Therefore, only the signal at 5.1 minutes has been integrated as intact prodrug. The assay did not show any compound or metabolite at the retention time of parent drug. Integration of signal area and calculation of remaining prodrug based on the concentration of first time point has been done for all the peaks, which is a growing trend for all replicates.

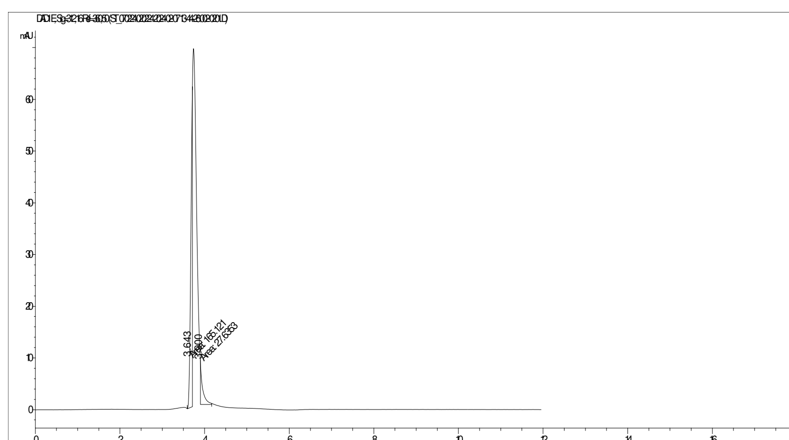


Figure 18. Caffeic acid

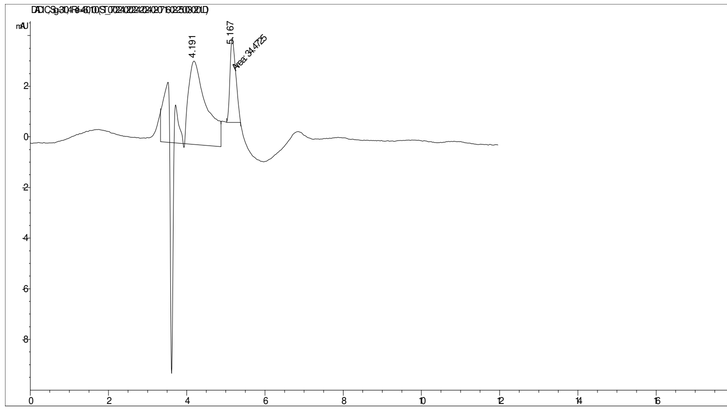


Figure 19. 1125

	A	B	C
1	100	100	100
15	99.2126	110.9091	95.37037
30	102.3622	112.7273	102.7778
60	99.2126	116.3636	82.40741
120	105.5118	117.2727	108.3333
180	108.6614	109.0909	132.4074
240	125.1969	137.2727	89.81481
360	158.2677	150	135.1852

Table 16. Remaining amounts of prodrug 1125 for triplicates A, B,C as percentage (%).

VII. Discussion

This study has focused on bioconversion studies of nine LAT1-utilizing brain-targeted prodrugs, namely 1116, 1118 (derived from *p*-coumaric acid), 1134, 1097 (cinnamic acid), 1020 (DOPAC), 1029 (vanillic acid), 1095 (eugenol), 1098 (capsaicin), 1025(caffeic acid) synthesized at the University of Eastern Finland. To achieve this aim, nine HPLC-method was developed for the detection of the prodrug and potentially the parent drug.

The method development was carried out at different conditions changing parameters, such as flow rate, temperature, various percentages of organic content in the mobile phase and buffer pH. All prodrugs consisted of similar amino acid promoiety, but they had different functional groups and exhibited different pKa values. The method development was carried out with simple mobile phases including either phosphoric acid buffer, acetic acid buffer, or formic acid buffer as mobile phase A and acetonitrile as mobile phase B. Regarding to principle work of chromatography, components in the samples have different attractions or affinity to the stationary and mobile phase due to different level of polarity of the components. Basically, the compounds were conjugated molecules which can be cleaved because of the acidic or basic properties of the mobile phase. Some compounds were too hydrophilic or too hydrophobic and therefore the available columns were not suitable for them because of the non-polarity of the stationary phase of the reverse phase column. The studied prodrugs were mainly hydrophobic, so they elute too early. Other instrumental limitation is that the mobile phase pH below2 or upper 7 may break down the prodrug and get better peaks but the range of suitable buffer pH for the column was 2.6 to 7 otherwise the stationary phase of column will dissolve in extreme pH environments (60) (61).

The successfully developed methods were validated with data complying with ICH guidelines regarding selectivity, suitability, linearity, accuracy and precision. HPLC method for the

bioconversion studies should have selectivity for parent drug, prodrug, and those signals should not interfere with the biological signal of the target tissue. For all methods the BV2 fresh sample taken from freezer ran with the method and got the RT earlier than 1 minute (62).

Some of parent drugs of prodrugs 1120, 1129, 1125 were eluted at the same time as t_0 acetonitrile signal so the validation of the method has not been performed for those caffeic acid, vanillic acid and DOPAC. As mentioned earlier, the prodrugs we studied are quite hydrophilic. Because of their high hydrophilicity, they tend to elute too quickly when using a reverse-phase column.

In vitro bioconversion studies showed that 7 prodrugs, namely 1116, 1118, 1097, 1095, 1129, 1120,1125, did not released their parent drugs in mouse microglia cell homogenate. There were some differences in Retention Time of prodrugs after bioconversion with the method that could be justified because of difference biological background of BV2 and the blank samples in the method development. There is some fluctuation among the triplicates that is due to technical errors or conditions for example acetonitrile evaporation after a while.

On the other hand, bioconversion studies of prodrug 1134 and 1198 showed the release of their parent drugs. The release kinetics of 1134 implied that half of the parent drug can be released within 2 minutes and only 4% of prodrug remained intact after 6 hours. The low standard deviation among the replicates stands for a credit of the release occurrences. The release kinetics of 1098 instead implied a slower bioconversion rate; the prodrug released its parent drugs but intact prodrug remained with variable content (44-74%). Curiously, both of these prodrugs were connected to their parent drugs via amide bonds.

Hydrolases, such as esterases, amidases, and phosphatases, are the most frequently utilized enzymes for the bioactivation of prodrugs, playing a role in activating about half of the prodrugs on the market. Another enzyme group involved in prodrug activation is the cytochrome P450 enzymes (CYPs), though they are used less often compared to hydrolases (63). In addition, several cytochrome P450 (CYP) enzymes responsible for drug metabolism, along with drug transporters, have been discovered to be overexpressed and hyperactive in human epileptic brain regions, especially at the blood-brain barrier (BBB) (64).

Carboxylesterases (CES) are crucial enzymes that facilitate the hydrolysis of esters, amides, carbamates, and thioesters, while also playing a key role in converting prodrugs and soft drugs into their active forms. The brain contains low amounts of metabolic enzymes, such as CYPs and CES, leading to less biotransformation activity in the brain. For example, carboxylesterase 2 (CES2) is a microsomal enzyme primarily expressed in the small intestine and colon, with lower levels observed in the brain (65).

The activation enzyme for the studied prodrugs has not yet been discovered. This is not a rare situation for instance, the activating enzymes for valacyclovir were identified eight years after the drug's approval, while those for dabigatran etexilate were discovered nearly twelve years after its initial development (66). Cutting-edge technologies have been greatly advanced in identifying prodrug-activating enzymes, improving the understanding of enzymes' tissue distribution, individual variability, and genetic polymorphisms, and therefore aiding in optimizing the prodrug design in regards to targeting to desired site and rate of release (4).

However, during the prodrug development several requirements need to be taken into account, including that the release of promioety and therapeutic parent drug should not occur in distribution phase before taken up to the target site. The cleavage of prodrug to the active therapeutic moiety and non-active and non-immunogenic promoiety (13) (67).

Moreover, in vitro testing, which involves studying drugs using isolated cells or tissues in controlled environments, provides valuable initial insights into the drug's mechanisms of action and potential efficacy. However, in vitro studies are limited by their inability to replicate the complex interactions and systemic effects present in a living organism. Furthermore, each enzyme work in a specific condition e.g. the presence of cofactors and specific pH are necessary to take into account to optimize the conditions for enzymatic activity. Nevertheless, only in vivo testing involving studies of drugs within a whole living organism, such as animal models, can validate the in vitro bioconversion results and ensure that the prodrugs are enabled to release their parent drugs in realistic biological context (68) (69).

VIII. Conclusion

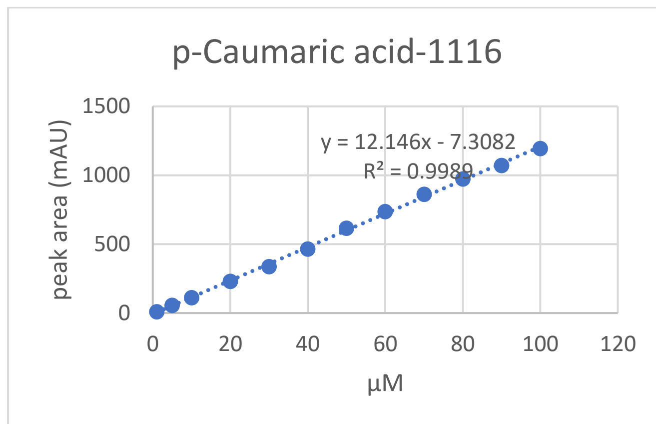
Out of nine prodrugs studied in this research, two, specifically Prodrugs 1134 and 1098, were successfully released from the parent drug. Notably, Prodrug 1098 was released in just five minutes, indicating a relatively short release time. Even more significantly, Prodrug 1134 was released within two minutes, suggesting an exceptionally rapid release. A proper prodrug should release the parent drug neither too soon nor too late. Otherwise, patients will need to administer the medication frequently. The method validations for prodrugs 1129, 1095, and

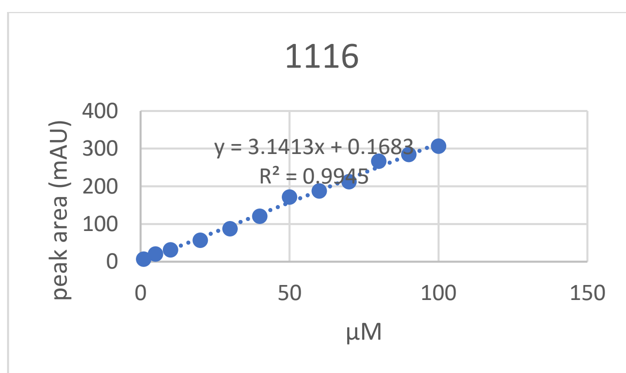
1120 are approaching the criteria limits. It is recommended that future work explores more complex mobile phases to improve the results.

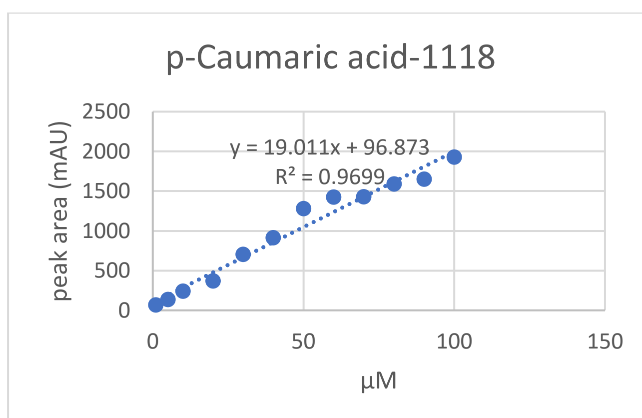
Fortunately, polymeric columns, such as PLRP-S have brought broad range of pH work and offer significant advantages for analyses of difficult samples, such as our case studied prodrugs. For in-vitro bioconversion studies, it is strongly recommended to conduct the biotransformation of prodrugs under more complex conditions that better mimic real physiological environments, as the S9 fraction lacks co-factors and NADPH, which are essential for accurate metabolic simulations.

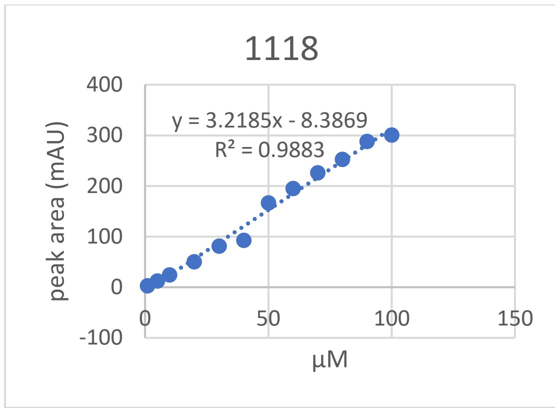
Figures 20-34

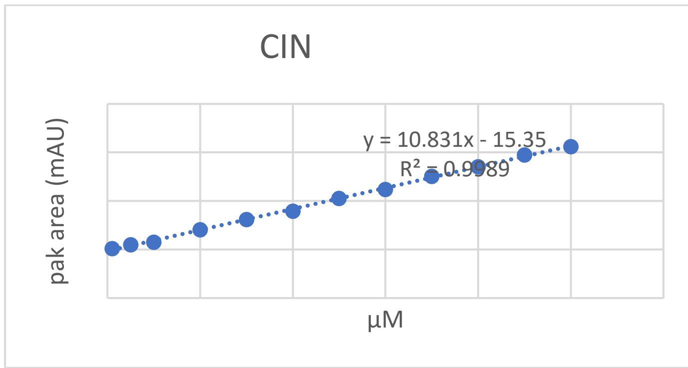
Standard curves of studied substances

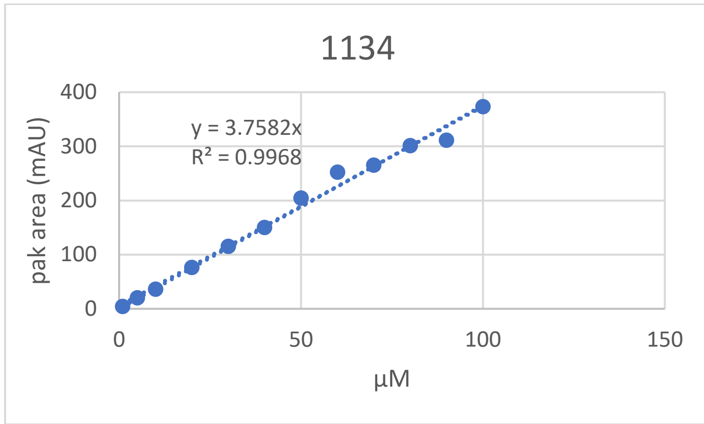


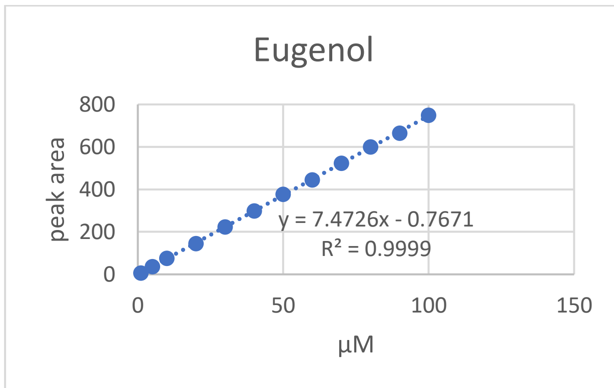


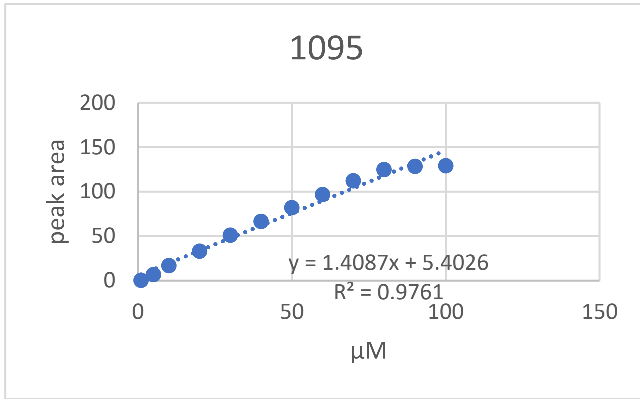


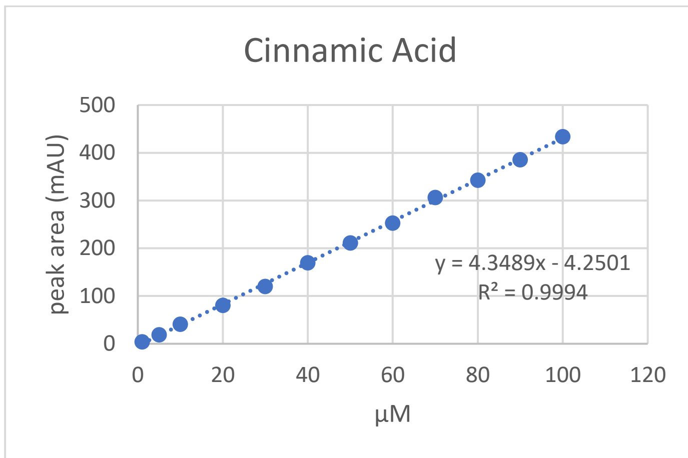

1118
y = 3.2185x - 8.3869
R² = 0.9883
peak area (mAU)
µM
400
300
200
100
0
-100
0
50
100
150

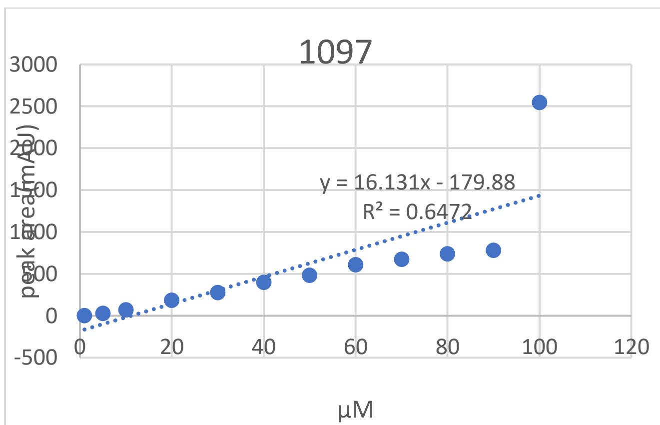

CIN
y = 10.831x - 15.35
R² = 0.9989
pak area (mAU)
µM

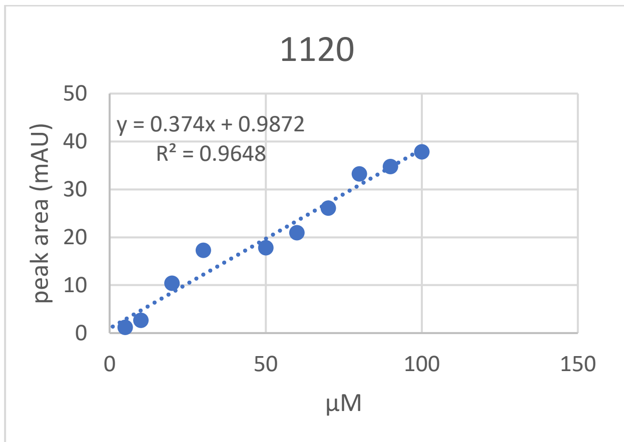

1134
y = 3.7582x
R² = 0.9968
pak area (mAU)
0
100
200
300
400
0
50
100
150
µM

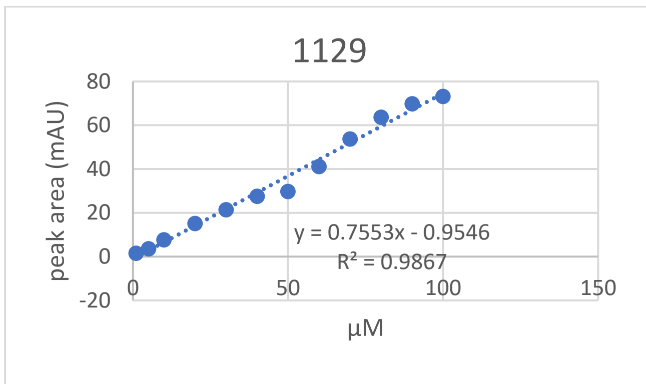

Eugenol
y = 7.4726x - 0.7671
R² = 0.9999
peak area
0
200
400
600
800
0
50
100
150
µM

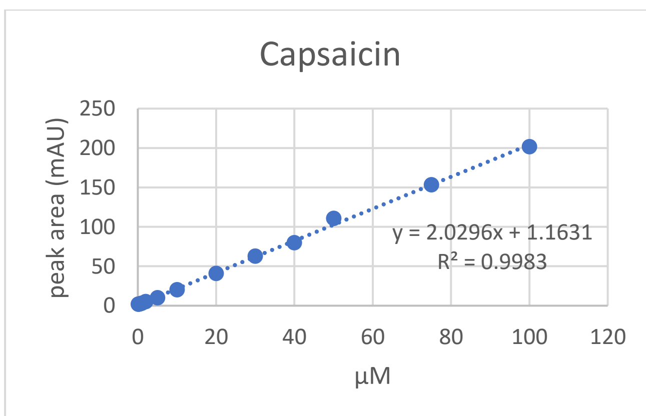

1095
peak area
200
150
100
50
0
y = 1.4087x + 5.4026
R² = 0.9761
0
50
100
150
µM

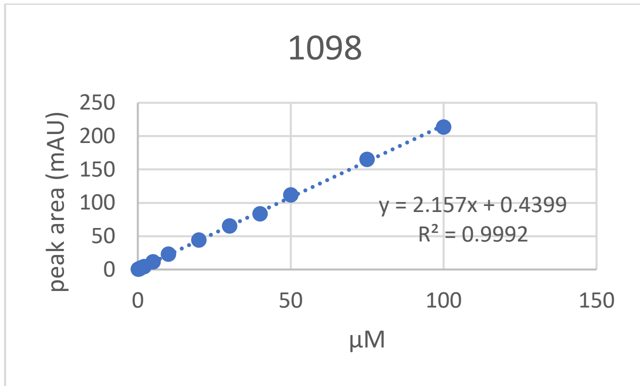

Cinnamic Acid
peak area (mAU)
500
400
300
200
100
0
y = 4.3489x - 4.2501
R² = 0.9994
0
20
40
60
80
100
120
µM

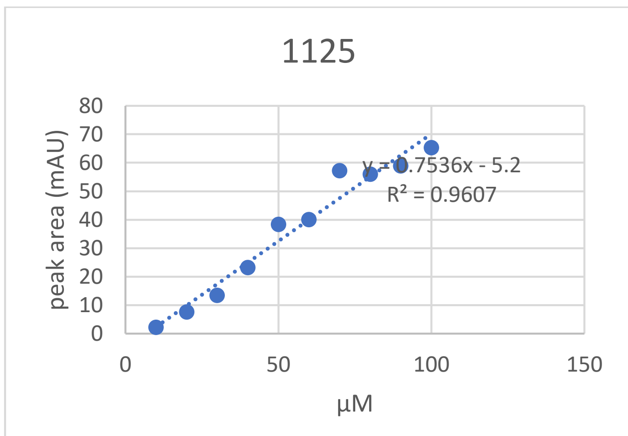

1097
3000
2500
2000
1500
1000
500
0
-500
y = 16.131x - 179.88
R² = 0.6472
0
20
40
60
80
100
120
µM


1120
y = 0.374x + 0.9872
R² = 0.9648
peak area (mAU)
µM
0
10
20
30
40
50
0
50
100
150


1129
y = 0.7553x - 0.9546
R² = 0.9867
peak area (mAU)
µM
-20
0
20
40
60
80
0
50
100
150


Capsaicin
y = 2.0296x + 1.1631
R² = 0.9983
peak area (mAU)
µM
0
50
100
150
200
250
0
20
40
60
80
100
120


1098
peak area (mAU)
250
200
150
100
50
0
0
50
100
150
µM
y = 2.157x + 0.4399
R² = 0.9992


1125
peak area (mAU)
80
70
60
50
40
30
20
10
0
0
50
100
150
µM
y = 0.7536x - 5.2
R² = 0.9607

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