

Univerzita Karlova

1. lékařská fakulta

Study program: Biochemistry and pathobiochemistry



FIRST FACULTY OF MEDICINE Charles University

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A comprehensive metabolomic analysis of natural vanilloids from *Capsicum spp.* and their activation of TRPV1

Komplexní metabolomická analýza přírodních vanilloidů z *Capsicum spp.* a jejich aktivace receptoru TRPV1

Doctoral dissertation

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Praha, 2025

Acknowledgements

A thesis with two supervisors and an indirect third makes for an interesting PhD. While one would hope that every project aligns perfectly, it does not. Despite the divergence from my original project, I cannot thank each of these people enough. Firstly, I want to thank Jan Konvalinka and Pavel Šácha for their patience and continued support throughout the PhD, even though my project veered from the original scope. I want to further thank Jan Konvalinka for the guidance and time despite having an exceptional amount of work as the director of IOCB; I have found inspiration in his drive to push science in a positive and progressive manner. Lastly, I would like to thank everyone from the Konvalinka group that helped me in the beginning of my PhD with cell culture and related work.

Next, and importantly, I would like to thank Tomáš Pluskal. Tomáš was invaluable in shaping me into the scientist I am today. He always pushed me to think about the biological question and how to answer it with good scientific practices. When Tomáš started his lab, I was one of the first two PhD students, and in total there were about 5 of us. It gives me a warm feeling looking back on the beginning and seeing what the lab has become now. Tomáš created an atmosphere that was welcoming and allowed it to shape on its own with healthy guidance. Overall, I aspire to be able to lead like Tomáš with calmness, knowledge, and integrity. While Tomáš greatly supported me, I cannot emphasize enough how much the lab members helped as well.

I want to first thank Lana Mutabdzija for all the support and talks throughout my PhD. I will always appreciate the shared commiseration as a lost first year PhD student in a new lab. I also want to thank the computer scientists that have been in our lab, but especially Roman Bushuiev and Raman Samusevich. I have always admired both Roman's and Raman's intelligence, work ethic, and utmost kindness. Next, I would like to thank Alžběta Kadlecová for being the best and kindest lab manager, you have always gone out of your way for everyone. I would also like to thank Tomas Illiaš, who was always willing to give an extra hand despite how busy he was. Next, I must thank Téo Hebra. I will miss our morning coffees where I was provided with some of the best scientific feedback and inspiration. I also must thank him for all the continued positive, genuine, support. I must also thank Tito Damiani, thank you for everything you have taught me on mass spectrometry and for being "mostly" patient with all the annoying questions when I was first learning. Lastly, everyone in the Pluskal group, even if you are not named, I will never forget all the great times we have had together and the support, especially all my students who helped me complete my projects.

My PhD would not have been possible if it were not for the amazing scientists throughout IOCB. I must mention the chemists, especially Pavel Majer and those in his group. I want to thank two colleagues I am happy to call friends, Martin Hadzima and Róbert Reiberger, for teaching me things that cannot be learned from biologists. This also extends to my other good friend and chemist David Kodr and Tomáš Zimmerman. I must also acknowledge Gabril Birkus who first hired me as a lab technician; if it were not for him, I may have

never had this opportunity. During my time as a lab technician, I was fortunate enough to meet and become friends with Florian Chevrier and Tomáš Lášek, two other chemists who have provided support during my time at IOCB. Lastly, I would like to thank the scientists in Pavlina Rezacková's group. I appreciate all the talks and events you allowed me to take part in despite not being in your group. I would especially like to thank Michal Svoboda for the support during the PhD. Lastly, I would like to thank Katerina Radilová, another friend that I am fortunate to have made during my PhD. Lastly, I would like to thank Vendula Stillerová for all the amazing collaborative help.

Finally, and most importantly, I would like to thank all my supportive friends and family. Firstly, I want to thank my amazing and supportive partner Agata Hrůšová, she was there by my side from the start. Next, I must thank all my friends outside of work, nothing helped like "jedeme na jednou" to take my mind off work. In particular: Ray, Ervins, Martin, Tibor, Ivan, Alba, Yonca, Vivita, Tatiana. Finally, I must thank all my extremely supportive family, who have always put great emphasis on education. Poppy and Grandma Susan for always being available to call, listen and provide support anytime of the day. My granny, for teaching me patience and influencing me to become a scientist. My mother, for always being there for me no matter what and teaching me what it means to be a hard worker, I can never thank you enough for everything you have given me. Last but far from least, my Uncle Jay, I miss you greatly and know that you have always been someone I've looked up to! I love you all and many thanks!

Prohlášení:

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V Praze, 17.04.2025

Joshua Smith

Podpis

Abstract

The transient receptor potential channel vanilloid 1 (TRPV1), also known as the pain and heat receptor, is an important drug target due to its role as a sensory receptor that responds to different stimuli: chemical pain causing agonists, heat and acidity (protons). TRPV1 is activated by several natural pain-causing agonists, such as resiniferatoxin, capsaicin, and double knot toxin. Of these stimuli, capsaicin, a small vanilloid found in *Capsicum spp.* (chilis), is one of the most investigated agonists because of its ability to activate the receptor, but not completely destroy the cell post activation. Because of this, capsaicin has been utilized as an analgesic over-the-counter medicine.

Capsaicin is not the only molecule to agonize TRPV1, but all related molecules found in chilis called capsaicinoids contain the required vanilloid head to agonize TRPV1. Furthermore, capsaicin is often found as a mixture of capsaicinoids, even when sold as an analgesic patch or cream. We hypothesized that capsaicinoids together add to the agonism of TRPV1 and not solely capsaicin. In addition, chilis have been selectively bred to become dangerously spicy, thus it was also hypothesized that this may have resulted in a stronger agonist than capsaicin. Ultimately, to answer these questions, we completed an untargeted metabolomics screening of 40 different chili varieties to identify novel capsaicinoids and then test them on a rat (rTRPV1) activation assay.

Our untargeted metabolomics study resulted in a directed isolation of 18 capsaicinoids, with five novel capsaicinoid structures, eight capsaicinoids reported for the first time from *Capsicum spp.*, and five previously reported capsaicinoids. We tested those 18 compounds, along with five other capsaicinoids and three other related compounds on the rTRPV1 activation assay. It was found that even small deviations from the capsaicin structure result in decreased activity, exemplified by a single hydroxylation on the capsaicin tail reducing TRPV1 potency by 100-fold. Lastly, we tested capsaicin in combination with a non-activating analog and weakly activating capsaicinoids and found that these compounds tend to act as positive modulators. While a more potent molecule than capsaicin was not found, our results from the capsaicin combination assay indicate that TRPV1 activation by capsaicinoids should be considered as a mixture and not just capsaicin.

KEY WORDS

Untargeted metabolomics, TRPV1, Capsaicinoids, Vanilloids, Natural Products, Computational metabolomics

Abstrakt

Přechodný receptorový potenciálový kationtový kanál podrodiny V člen 1 (TRPV1), také známý jako receptor bolesti a horka, je důležitým terapeutickým cílem díky své funkci senzorického receptoru, reagujícího na různé podněty: chemickou bolest způsobenou agonisty, horko a kyselinu (protony). TRPV1 je aktivován několika přirozenými agonisty způsobujícími bolest, jako je resiniferatoxin, kapsaicin a toxin s dvojitým uzlem. Z těchto stimulátorů je kapsaicin, malý vanilloid nacházející se v chilli papričkách (*Capsicum* spp.), jedním z nejvíce prozkoumaných agonistů, protože dokáže aktivovat receptor aniž by zničil samotnou buňku. Z tohoto důvodu se kapsaicin používá jako volně prodejné analgetikum..

Kapsaicin není jedinou molekulou, která agonizuje TRPV1, ale řada příbuzných molekul - kapsaicinoidů - nacházejících se v chilli papričkách, obsahuje vanilloidovou hlavu nutnou pro aktivaci TRPV1. Kapsaicin se často vyskytuje jako směs kapsaicinoidů, i když se prodává jako analgetická náplast nebo krém. Na základě toho jsme vyslovili hypotézu, že všechny kapsaicinoidy společně přispívají k agonismu TRPV1, a nikoli pouze samotný kapsaicin. Navíc chilli papričky prošly procesem selektivního šlechtění za účelem vyšší pikantnosti. Další hypotézou tedy bylo, že šlechtění mohlo vést k produkci ještě silnějšího agonisty než je kapsaicin. Tyto hypotézy jsme testovali pomocí necíleného metabolického screeningu 40 různých odrůd chili, abychom identifikovali nové kapsaicinoidy a a poté provedli testy aktivace s potkaním TRPV1 receptorem (rTRPV1).

Naše necílená metabolická studie vedla k izolaci 18 kapsaicinoidů zahrnujících: 5 úplně nových kapsaicinoidových struktur, 8 kapsaicinoidů poprvé objevených v rostlinách *Capsicum* spp. a 5 již dříve objevených kapsaicinoidů. Těchto 18 látek jsme testovali spolu s pěti dalšími kapsaicinoidy a třemi příbuznými látkami v testu aktivace rTRPV1. Zjistili jsme, že i malé odchylky od struktury kapsaicinu mají za následek sníženou aktivitu, jako například hydroxylace na konci molekuly, která 100krát snižuje účinnost. Na závěr jsme testovali efekt kapsaicinu v kombinaci s neaktivujícími analogy a slabě aktivujícími kapsaicinoidy a zjistili jsme, že tyto sloučeniny mají tendenci působit jako pozitivní modulátory. I když nebyla nalezena silnější molekula než kapsaicin samotný, naše výsledky získané pomocí této kombinace látek naznačují, že aktivace TRPV1 kapsaicinoidy by měla být zkoumána v kontextu směsi látek a nikoli pomocí samotného kapsaicinu.

KLÍČOVÁ SLOVA

Necílená metabolika, TRPV1, kapsaicinoidy, vaniloidy, přírodní látky, výpočetní metabolika

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Abbreviations

TRP — transient receptor potential

TRPC — transient receptor potential canonical

TRPV — transient receptor potential vanilloid

TRPM — transient receptor potential melastatin

TRPA — transient receptor potential ankyrin

TRPP — transient receptor potential polycystin

TRPML — transient receptor potential mucolipin

TRPN — transient receptor potential *Drosophila* NOMPC

TRPY — transient receptor potential yeast

TRPV1 — transient receptor potential cation channel subfamily V member 1

K_v — voltage gated potassium channels

ARD — ankyrin repeat domain

EC₅₀ - median effective concentration

SNPs — nonsynonymous single-nucleotide polymorphisms

PAL — phenylalanine ammonia-lyaseHR-MS

pAMT — putative aminotransferase

VAMT — vanillin aminotransferase

CAD — cinnamyl alcohol dehydrogenase

Pun1 — capsaicin synthase

SHU — Scoville unit or Scoville heat unit

MS — mass spectrometry

HR-MS/MS — high-resolution tandem mass spectrometry

UPLC-MS/MS — ultra high-performance liquid chromatography coupled to high-resolution tandem mass spectrometer

GC — gas chromatography

FBMN — feature-based molecular networking

GNPS — Global Natural Products Social Molecular Networking

EtOAc — ethyl acetate

ED₅₀ — median effective dose

1. Introduction

1.1 TRP proteins and TRPV

Physical stimuli and other chemical signals are often detected in different organisms by transient receptor potential (TRP) channels (Chen et al., 2020). The first TRP channel was discovered in 1969 from a *Drosophila* mutant defective to light sensing (Cosens and Manning, 1969). Since this discovery, over 100 other TRP proteins have been identified (Li, 2017). Mammals only express 28 of these proteins, 27 in humans (Wu et al., 2010). Using homology, the TRP family can be divided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin), and TRPN (*Drosophila* NOMPC) (Li, 2017), **Figure 1**. Recently, a discovery of a new TRP yeast channel named TRPY has established an 8th TRP category (Li, 2017). Since these channel proteins are highly multifunctional, typically acting upon several metabolic pathways upon activation or inhibition, they have been often sought after as promising drug targets (Koivisto et al., 2021). Among these families, one of the most investigated is the TRPV family.

The TRPV name comes from the discovery of the first TRPV channel, transient receptor potential cation channel subfamily V member 1 (TRPV1), which binds to the vanilloid capsaicin (compound **1**, **Figure 2**), but is also sensitive to heat and protons (Caterina et al., 1997). TRPV proteins are all homotetramers with six membrane helices (S1-S6) that form a voltage gated protein-like orientation, with resemblance to voltage-gated potassium channels (K_v); however, they are functionally different from K_v (Seeböhm and Schreiber, 2021; Yu and Catterall, 2004). The TRPV family also shares a similar large cytosolic N-terminus and a shorter C-terminus (Gaudet, 2008). In general, TRPV proteins can be divided into two groups: those that are thermosensitive (TRPV1-4) and those that are Ca^{2+} sensitive (TRPV5 and TRPV6). Regardless, preference for divalent cations, particularly Ca^{2+} , extends to all TRPV channels (Seeböhm and Schreiber, 2021). While the stimuli, distribution and role of TRPV receptors vary greatly from type to type, they all share a common function of the movement of divalent ions, and in the cases of TRPV1-4 also monovalent ions, across the plasma membrane (Seeböhm and Schreiber, 2021; Zhang et al., 2023).

As briefly mentioned above, TRPV channels, and in general TRP channels, have distinctive expression patterns and many different variants which result in channel expression distributed throughout most tissues. This provides a double-edged solution and problem, as TRP channels make great drug targets; however, their wide distribution and post-activation physiological effects result in off-tissue targeting and other downstream negative side effects (Koivisto et al., 2021). TRPV1, the first discovered TRPV channel, is one of the most investigated targets due to its unique role in pain sensation and its established role as a viable drug target.

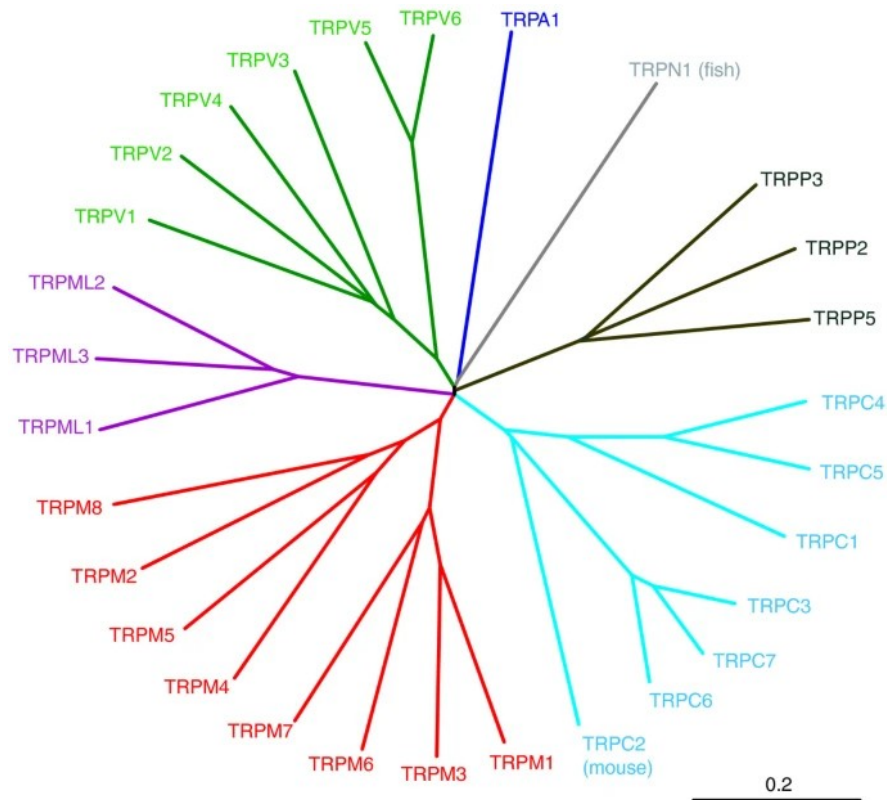


Figure 1 Homology grouping of the different TRP channels. There are many different subtypes of each of these families, which are distributed throughout different types of tissue and with a different variety of functions. The creation of this diagram did not include TRPY. While it is important to be aware of the different types of TRP proteins, due to the scope of this thesis, further details on all the subtypes will not be described here. (Figure was reused from Nilius and Owsianik, 2011).

1.1.1 Discovery of TRPV1

The TRPV1 discovery was largely driven by the effects of capsaicin, a small pain causing vanilloid (compound **1**, **Figure 2**). Before the discovery, it was already known that the receptor was likely located on a nociceptor-type neuron, as these neurons are associated with the signaling of pain (Fields, 1987). Furthermore, it was known that capsaicin was able to both excite and then subsequently inhibit different subtypes of nociceptors, which was expanded to its role as an analgesic (Bevan, 1999). Capsaicin (compound **1**, **Figure 2**) was first thought to act through membrane perturbation resulting in the indirect activation of other receptors (Feigin et al., 1995); however, this indirect action was disputed as there were several studies showing that capsaicin acted in a dose-dependent manner (Szolcsányi and Jancsó-Gábor, 1976, 1975). Further suggestion that capsaicin targets a specific receptor was that nociceptive-like neurons produced a similar response to the vanilloid resiniferatoxin (RTX), (compound **2**, **Figure 2**) (Szallasi and Blumberg, 1989) and that responses to both of these vanilloids were inhibited by capsazepine (compound **3**, **Figure 2**) (Bevan et al., 1992). Capsaicin's target receptor was eventually identified through its use as a molecular probe by agonizing increasingly smaller pools of cDNA from rat dorsal root ganglion until a single cDNA transcript

remained, TRPV1 (Caterina et al., 1997). It was found that this receptor responds to agonists that cause pain (i.e., capsaicin and RTX), heat and protons, thus it was coined as the pain and heat receptor (Caterina et al., 1997). The discovery of TRPV1 by David Julius was one of the key discoveries that led him to receive a Nobel Prize in Physiology or Medicine in 2021 (Andolfo et al., 2022). It took 15 years of building on this discovery to obtain the structure of TRPV1 (Liao et al., 2013), with many studies still being conducted on this complex receptor.

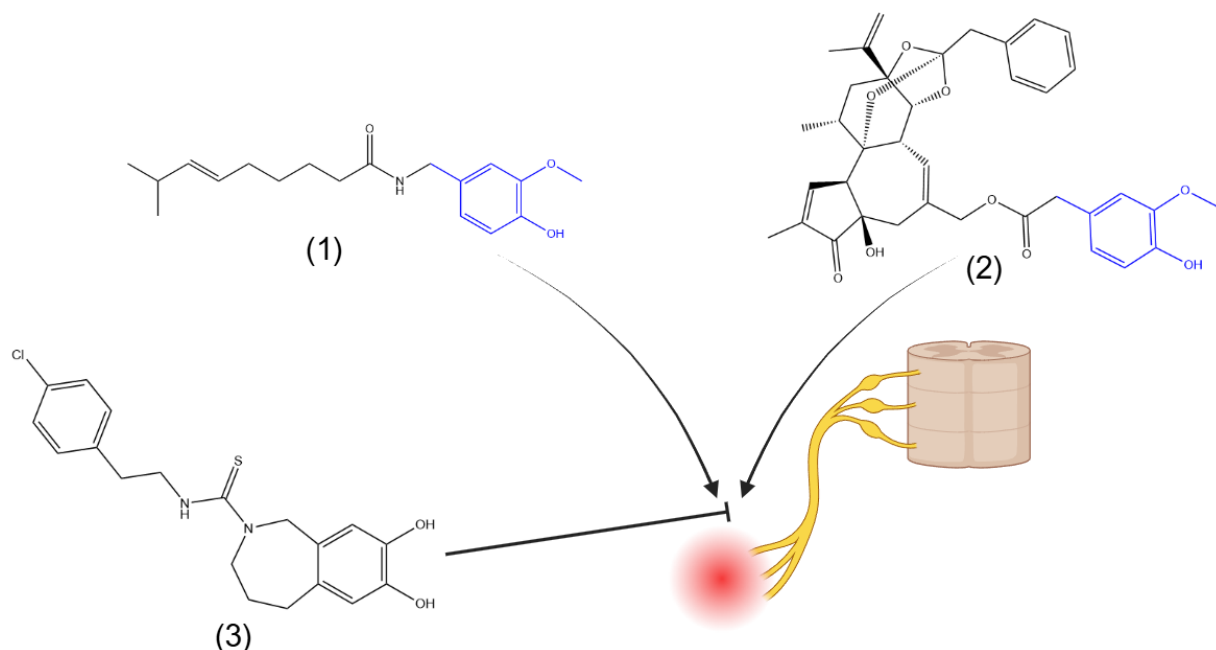


Figure 2 Three important compounds in the discovery of TRPV1. Compound 1, capsaicin, and compound 2, RTX, were two important vanilloids that both agonize TRPV1, which is found on nociceptive receptors and the dorsal root ganglion. The first reported median effective concentration (EC_{50}) values were 711.9 nM for capsaicin and 39.1 nM for RTX (Caterina et al., 1997). Compound 3, capsazepine, was found to be the competitive antagonist of the TRPV1 receptor (Bevan et al., 1992). The highlighted blue regions of capsaicin and RTX are known as the vanilloid head and are important for binding. Created with BioRender.com

1.1.2 Structure and capsaicin binding to TRPV1

Before the complete structure of TRPV1 was determined, only the structures of the N-terminus and C-terminus domains were solved (Lau et al., 2012; Lishko et al., 2007). Both regions reside in the cytosol and have important regulatory functions. The N-terminus contains an ankyrin repeat domain (ARD) and contains several binding sites for both ATP (triphosphates) and calmodulin (**Figure 3**). ATP was found to reduce tachyphylaxis (i.e., diminished activation from repeated exposure) and that without ATP, TRPV1 showed almost no activation after repeated exposure (Lishko et al., 2007). Overall, ATP is considered more of a sensitizing molecule. Conversely, calmodulin, a well-known ubiquitous calcium regulator known to interact with the N-terminal region of TRPV1 (Rosenbaum et al., 2004) was found to be a needed partner to promote tachyphylaxis (Lishko et al., 2007). Calmodulin also interacts with the C-terminal region (**Figure 3**) (Numazaki et al., 2003). Calmodulin bound to Ca^{2+} has a higher affinity for the C-terminus than the N-terminus and

mutations to the C-terminus binding site have led to slower desensitization whereas mutations in the N-terminus have led to abolished desensitization (Lau et al., 2012). Ultimately, the two binding sites of calmodulin indicate different regulatory pathways for TRPV1, which still require further biochemical studies for complete understanding (Lau et al., 2012).

Despite no crystal structure of TRPV1, Julius and his team, upon discovery of TRPV1, leveraged homology search to propose a structure of a 6-transmembrane helix with a cytosolic N- and C- terminus (Caterina et al., 1997; Hardie and Minke, 1993; Montell and Rubin, 1989). Upon cryo-EM analysis of TRPV1, it was undoubtedly confirmed that monomeric TRPV1 contains a six transmembrane repeat with cytosolic N- and C-terminal regions (**Figure 3**) which come together to form a homotetramer (Liao et al., 2013). TRP proteins are known to share at least 16 allosteric binding sites (Yelshanskaya and Sobolevsky, 2022) and TRPV1 is activated by heat, acid, and several different natural agonists, such as capsaicin, RTX, and snake double-knot toxin (Bae et al., 2015), and centipede toxin RhTx (S. Yang et al., 2015). The many different binding sites and variety of agonists make TRPV1 an extremely complex protein, with an incomplete understanding of all the modes of action upon ligand interaction. Of the molecules and activators of TRPV1, capsaicin, and in general, vanilloids, have been thoroughly investigated.

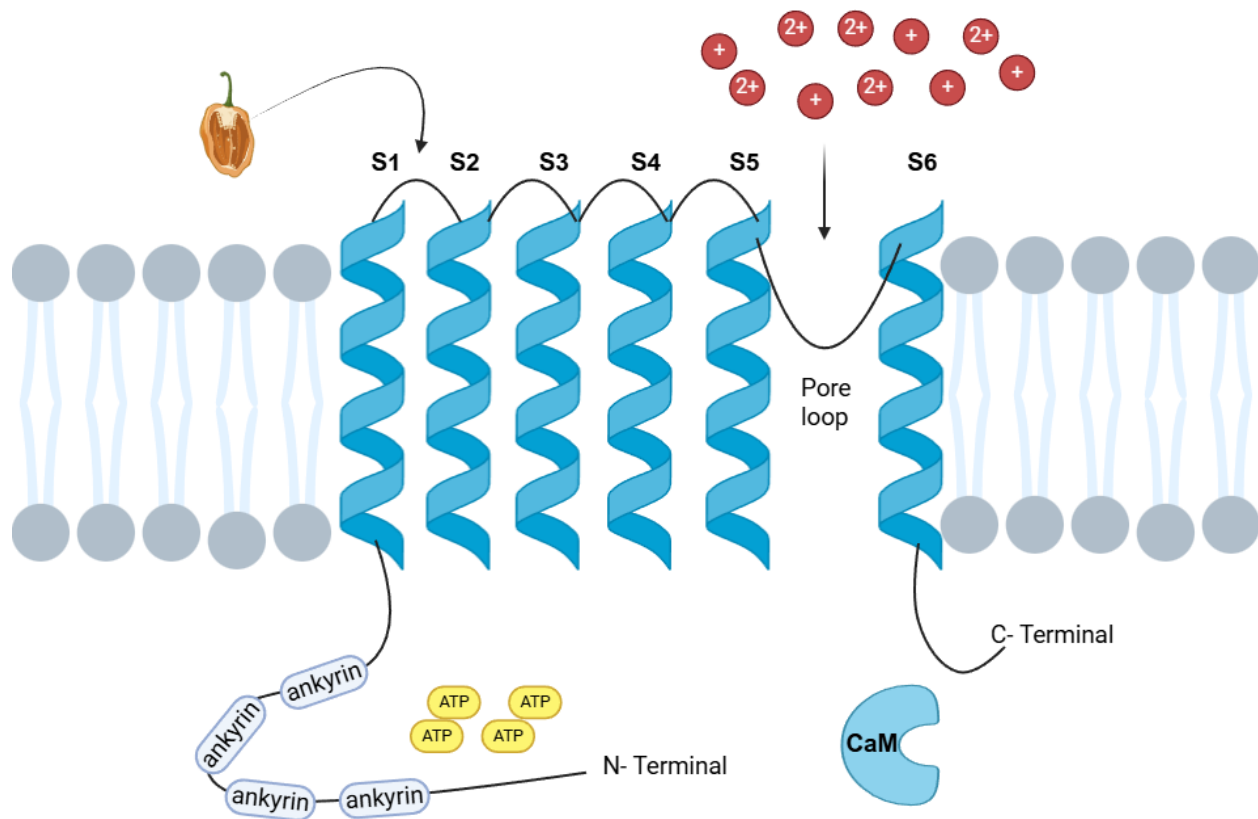


Figure 3 One monomer of TRPV1 shown in a lipid bilayer. TRPV1 is known to be activated by many different natural agonists, however, this study focused on the activation of TRPV1 by chili containing compounds caused by capsaicinoids. TRPV1 activation results in the influx of cations with preference to divalent ions (Caterina et al., 1997). TRPV1 N-terminal

and C-terminal regions also contain important regulatory domains that can bind to ATP or calmodulin. Created with BioRender.com

Capsaicin has been found to bind deep into the transmembrane pocket in a “tails-up, heads-down” configuration, **Figure 4** (Yang and Zheng, 2017). The exact mechanism of how capsaicin enters the channel is not fully understood, but there are assumptions that due to capsaicin’s lipophilic nature, capsaicin is able to first pass through the plasma membrane and enter the protein from the cytosolic side (Domene et al., 2022). The binding pocket of capsaicin is made up of the S3, S4 and the S4-S5 linker (**Figure 4**), where the capsaicin vanilloid head (**Figure 1**), interacts with the S4-S5 which initiates an outward movement and subsequent opening of the S6 activation gate (Yang and Zheng, 2017), **Figure 4**. Initial studies around the capsaicin tail were problematic due to its flexible nature, making it hard to find the exact conformation it took when inside the binding pocket (F. Yang et al., 2015). However, with a series of experiments using synthetic capsaicin analogs with varying alkyl tail lengths, it was found that the tail is important in stabilizing the opening of the channel likely through van der Waals forces (F. Yang et al., 2015). Despite capsaicin binding to TRPV1 causing an activation of the receptor, this state is only partially activated when compared to stronger agonists like RTX or double-knot toxin (Cao et al., 2013).

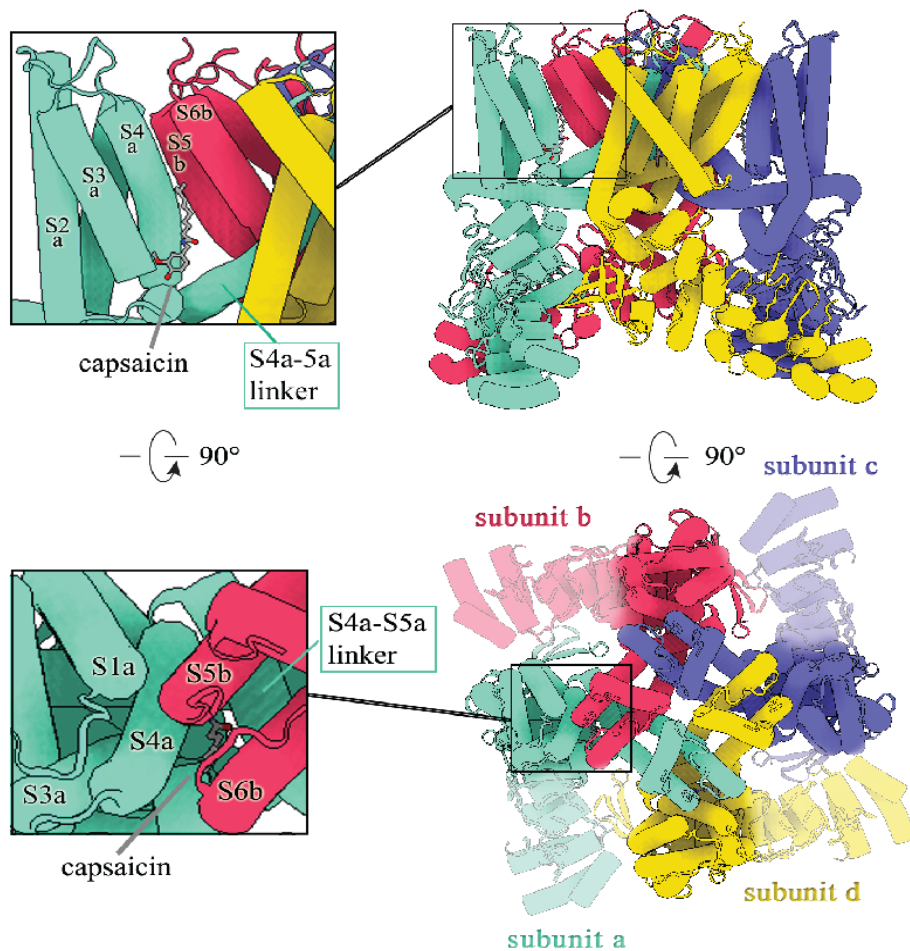


Figure 4 *Capsaicin in the TRPV1 binding pocket*. This structure was created using Protein Imager(Tomasello et al., 2020) from a Cryo-EM structure of TRPV1 in complex with capsaicin (PDB: 7LPB) (Kwon et al., 2021). (Figure was repurposed from Smith et al., 2024))

1.1.2.1 Localization of TRPV1 in the body and its function as a pain receptor

TRPV1 is distributed throughout different tissue types found in the body, but it is predominantly found in primary afferent fibers (i.e., peptidergic sensory neurons) (Alawi and Keeble, 2010), in the peripheral nervous system (e.g., dorsal root ganglion, other ganglion types or small nerves), and in the central nervous system (e.g., thalamus, striatum and amygdala) (Bollimuntha et al., 2011). However, TRPV1 is also found in different organ tissues, such as the pancreas, liver and heart (Bollimuntha et al., 2011). TRPV1 is also found in the gastrointestinal tract (Alawi and Keeble, 2010), and different tissue types related to the cardiovascular system and respiratory system (Seebohm and Schreiber, 2021). Due to this almost cosmopolitan distribution of TRPV1, studying the effects of this receptor is extremely tedious and difficult. For the context of this thesis, focus will be on TRPV1 as a pain receptor.

As previously mentioned, TRPV1 responds to different chemical and noxious stimuli, such as capsaicin, resiniferatoxin, heat, acid (Caterina et al., 1997), double-knot toxin (Bae et al., 2015) and centipede toxin RhTx (S. Yang et al., 2015). The activation of TRPV1 found on the nociceptor fibers (Ochoa and Torebjörk, 1989) results in the influx of cations that initiate the sensation of pain (Willis, 2009). While the pain signal originates in primary afferent neurons, it is transduced to synaptic terminals found in the spinal cord by releasing signaling (e.g., glutamate, substance P and neurokinin A) into the spinal cord dorsal horn (i.e., second order neurons), **Figure 5**. Second order and higher order nociceptive neurons may be activated by these signaling molecules, thus sending a signal of pain to the brain (Willis, 2009), **Figure 5**.

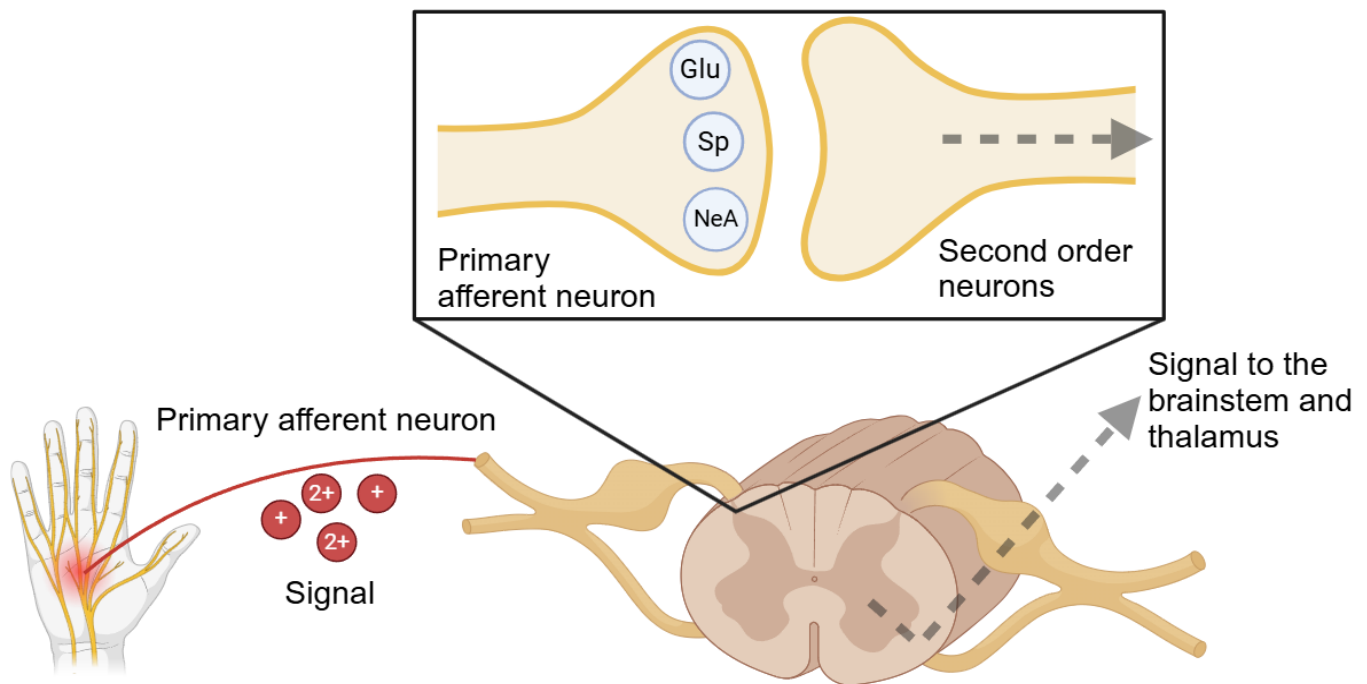


Figure 5 *Simplified diagram of TRPV1 pain signaling.* TRPV1 signaling originates at the site of noxious stimuli, where a signal is transmitted down a primary afferent neuron. Upon reaching the terminal synaptic cleft, the neuron can release further signaling molecules, such as glutamate (Glu), substance p (Sp) and neurokinin A (NeA). These molecules stimulate the second order neurons, which then send a signal to the brain indicating the presence of pain (Price, 2007; Willis, 2009). Created with BioRender.com

1.1.2.2 TRPV1 and taste

One interesting tidbit to mention is the relationship between pain and taste. As capsaicin is found in chilis, a widely used ingredient in many culinary recipes, its accompanying pain is often associated with taste. Despite extensive studies on this relation, there is little evidence that supports the idea that TRPV1 exists in taste cells (Roper, 2014). Two supporting cases for this is the lack of TRPV1 in taste cells by antibody staining (Ishida et al., 2002; Moon et al., 2010) and that mice with a TRPV1 knockout behaved similarly to wild-type mice in salt sensing (Lyall et al., 2004). However, TRPV1 may indirectly affect taste. In another TRPV1 knockout study in mice, it was found that capsaicin can reduce preference for sucrose and inhibit sodium channels in taste cells (Costa et al., 2005). Furthermore, the release of transmitters (**Figure 5**) as a response to TRPV1 activation may result in an increase in the tongue's temperature or by directly interacting with nearby taste cells (Emir, 2017). While TRPV1's complete influence on taste is still being explored, there is no doubt that TRPV1 is present in somatosensory tissue within the oral cavity (Emir, 2017). Conversely to human TRPV1, chickens contain a TRPV1 mutation, which renders them insensitive to capsaicin, therefore allowing the eating of chili seeds without the accompanying pain (Chu et al., 2020).

1.1.2.3 TRPV1 SNPs and capsaicin

Another consideration for TRPV1 induced pain, particularly by capsaicin, is based on the TRPV1 allele among different populations. Nonsynonymous single-nucleotide polymorphisms (SNPs) are often investigated on a population scale to track observable differences in the genotype and the phenotype. One important study to highlight is from Xu and colleagues (Xu et al., 2007), in which they screened six nonsynonymous single-nucleotide polymorphisms of TRPV1 with a capsaicin based activity screening (Xu et al., 2007). It was found that variants TRPV1^{T469I} (rs224534), TRPV1^{I585V} (rs8065080), and TRPV1^{T469I/I585V} had the same activity compared to the wild-type TRPV1, but TRPV1^{P91S} (rs222749) and TRPV1^{I315M} (rs222747) had a greater expression level than other variants (Xu et al., 2007). SNP TRPV1^{P91S} is located near the N-terminal of TRPV1, while TRPV1^{I315M} is found in the ankyrin repeat region of the N-terminal (Xu et al., 2007). Thus, populations with greater expression of these two SNPs may experience greater sensitivity to spicier foods. These findings, in particular, that TRPV1^{I585V} has a similar EC₅₀ to the TRPV1^{wt}, are complicated by the research from Okamoto et al. in which they studied Japanese adults' sensitivity to capsaicin and heat while comparing their TRPV1 SNPs (Okamoto et al., 2018). Okamoto et al. found that there was no relation between SNPs and heat induced pain, but there was an increased sensitivity to capsaicin pain for those with TRPV1^{I585V}. This SNP is found on the 5th alpha-helix of TRPV1. Further complicating the situation, when capsaicin was tested with HEK293 cell lines with recombinant protein expression of TRPV1^{I585V}, it was found to be less sensitive to capsaicin (Wang et al., 2016). Okamoto et al. attributed these differences to their assay testing capsaicin at low-activating thresholds and also pointed out that there can be different expression levels of the SNPs between different tissues compared to testing using recombinant protein screening (Okamoto et al., 2018). Furthermore, the study by Okamoto et al. used a relatively small sample set of only 26 people and pain reporting studies are known to contain self-reporting biases (Boring et al., 2022; Karos et al., 2018).

Capsaicin is often used to induce cough; therefore, several studies have also investigated TRPV1 SNPs' involvement in this matter. One study comparing 470 healthy participants to 301 with asthma found that TRPV1^{I585V} reduced symptoms of wheezing. Their functional studies also showed reduced sensitivity to both capsaicin and heat (Cantero-Recasens et al., 2010). This finding agrees with the previously mentioned study by Wang et al. (2016), where they also found this SNP to reduce sensitivity to capsaicin. Another study found that TRPV1^{I585V} in combination with TRPV1^{P91S} produces an effect on producing two coughs; however, these effects are poorly described along with using a small sample set (Liviero et al., 2020). Further expanding on TRPV1^{I585V} studies: one report of TRPV1^{I585V} found that participants had a faster onset of pain when capsaicin was applied topically to the skin, but were overall less sensitive to the capsaicin (Forstenpointner et al., 2017). The TRPV1^{I585V} variant has also been linked to other phenotypes without consideration of capsaicin. For example, those with painful knee osteoarthritis who have the TRPV1^{I585V} variant were found to be less sensitive to peripheral pain (Valdes et al., 2011). Another comparative study found those with the TRPV1^{I585V}

variant were less prone to experience chronic migraines (Yakubova et al., 2021). These SNPs highlight the diverse range of phenotypes that TRPV1 can produce, thus further highlighting it as a potential drug target.

1.1.3 TRPV1 as a drug target for pain

One of the major attractions of TRPV1 as a drug target, in particular antagonists, is that it provides a novel route of pain relief. That is, many pain relieving drugs target the inflammatory response and signals involving the transmission of pain, while TRPV1 targeted provides pain relief by blocking the initial sensor of the pain (Szallasi et al., 2007). Interestingly, different immunosensing players are known to interact with TRPV1 by either activating or sensitizing TRPV1 (Szallasi et al., 2007). Furthermore, it has been found that the μ -opioid receptor is coexpressed with TRPV1 and that the agonist morphine influences the phosphorylation interaction of TRPV1 (Vetter et al., 2006). It is also known that phosphorylation promotes sensitization (Bhave et al., 2002; Premkumar and Ahern, 2000) and that dephosphorylation favors desensitization (Jung et al., 2004; Mohapatra and Nau, 2005). Ultimately, TRPV1 and modulation of its phosphorylation provide a viable alternative to narcotic and non-narcotic pain relief.

Despite many synthetic antagonistic drugs targeting TRPV1, there have been many drawbacks to their application. One major drawback is that these antagonistic drugs have been found to cause hyperthermia (Szallasi et al., 2007). While minor fevers (i.e., hyperthermia) can be treated with antipyretic drugs (Gavva et al., 2007), more devastating side effects were due to burn injuries which is highlighted by Janssens Mavatrip (JNJ-39439335, compound **4**, **Figure 6**) (Manitpisitkul et al., 2015; Moran and Szallasi, 2018). Merck phase I clinical trial drug MK-2295 (compound **5**, **Figure 6**) also greatly reduced the response to noxious heat but was withdrawn due to fears of burn side effects (Eid, 2011). A phase II drug, AZD1386 (compound **6**, **Figure 6**), was withdrawn due to no significant analgesic effect (Miller et al., 2014). Another promising drug that did not make it past phase II/III was SB-705498 (compound **7**, **Figure 6**) (Asiri and Zaheen Hassan, 2023; Holland et al., 2014) also due to possible hyperthermia (Garami et al., 2020). At the time of writing this thesis, and to the best of our knowledge, there have not been any successful antagonists of TRPV1 to make it to market. Conversely, naturally occurring agonists, in particular capsaicin, have found commercial success in agonistic pain treatment.

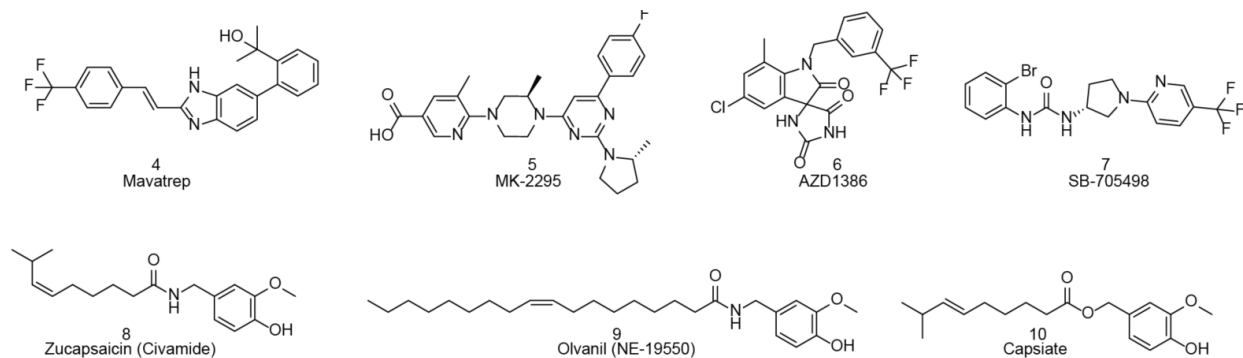


Figure 6 Example antagonistic and agonistic drugs targeting TRPV1. Compounds **4** - **7** are antagonists that did not make it through FDA approval, while compounds **8** and **9** were unsuccessful agonists. However, zucapsaicin achieved some success in Canadian markets. Lastly, compound **10**, capsiate, was explored more as a dietary molecule and struggled to find success as a commercially approved FDA drug.

1.1.3.1 Natural product agonists of TRPV1: vanilloids

Capsaicin can act as a pain reliever due to its ability to internalize TRPV1 at therapeutic concentrations (**Figure 7**). However, since capsaicin is still an agonist used in therapeutic pain-relieving applications, ironically, it can cause burning and stinging at application sites (Altman et al., 1994). Since these side effects do exist, alternative agonists that can produce a similar phenotype, but with reduced TRPV1 activity have been explored. Particularly, vanilloids (e.g., capsaicin and RTX, **Figure 2**), which contain the vanilloid head that initiates the TRPV1 channel opening have been explored. A cis configuration of capsaicin, Civamide, also known as zucapsaicin (compound **8**, **Figure 6**), made it to phase III clinical trials for the treatment of knee pain, but was declined FDA approval (Mobasheri et al., 2024), despite better tolerance than capsaicin (Safat et al., 2014). Civamide did make it through to Canadian markets (Mobasheri et al., 2024) known as Zuacta; however, the drug has since been discontinued due to undisclosed business reasons (“Discontinuation report ZUACTA,” 2020).

Other capsaicin-like molecules have been explored, in particular, olvanil (NE-19550) and capsiate, compounds **9** and **10**, respectively (**Figure 6**). Olvanil, NE-19550, was first synthesized almost 40 years ago (Brand et al., 1987), but was later found to be a naturally occurring substance in *Capsicum oleoresin* (Kobata et al., 2010). Olvanil, unlike capsaicin, can produce similar desensitization effects but results in no hyperalgesia (i.e., increased pain sensation) (Alsalem et al., 2016). Furthermore, olvanil was able to produce anti-inflammatory effects without typical NSAID suppression of arachidonic acid metabolism (Brand et al., 1990). Despite reported potential, NE-19550, nor any other olvanil analog, has made it to any pharmaceutical market. One other capsaicin-like molecule, which technically does not belong to capsaicinoids, is the natural TRPV1 agonist capsiate, compound **10**, **Figure 6**. Capsiate was found in nonpungent cultivars of sweet pepper and differs from capsaicin in the connectivity of the vanilloid head — capsaicin is connected by an amide bond while capsiate is connected by an ester bond (Kobata et al., 1998b). Capsiate was found to activate TRPV1 with a similar potency as capsaicin on direct TRPV1 activation and nociceptive activation when injected but did not produce similar effects when applied externally to the skin surface, eye or mouth (Iida et al., 2003). Most uses for capsiate have not been explored as an analgesic but rather as a metabolism-boosting supplement (Ludy et al., 2012). Ultimately, capsiate has seen market success as unregulated dietary supplements but not as an officially regulated drug. One of the major problems with capsiate, compared to capsaicin, is that capsiate is unstable in polar, aqueous solvents (Iida et al., 2003; Sutoh et al., 2001).

The last natural vanilloid, RTX (compound **2**, **Figure 2**), is found in the spurge *Euphorbia resinifera*, which produces a white latex that causes pain and irritation when touched (Ernesto Fattorusso, Virginia Lanzotti, Orazio Tagliabate-Scafati, Gian Cesare Tron, Giovanni Appendino, 2001). This pain is caused by RTX, a super potent activator, more than capsaicin, of TRPV1 (Caterina et al., 1997). RTX shares the same binding pocket as capsaicin but forms a tighter binding to TRPV1 than capsaicin (Elokely et al., 2016). Unlike capsaicin, which produces a shorter response, RTX results in a slower, prolonged response and therefore continuous influx of calcium ions (Brown, 2016; Kárai et al., 2004). This excess amount of cation influx can result in cytotoxicity and the death of the cell, as depicted in **Figure 7** (Koivisto et al., 2021). This cell toxicity, in combination with RTX's high affinity and selectivity to TRPV1, has made it a unique tool as a “molecular scalpel” (Brown, 2016). This unique way of inducing cell death can be used to ablate neurons, and in turn, has been explored as an intractable pain treatment. At the time of writing, there are two ongoing clinical trials that explore RTX as a “molecular scalpel”: NCT00804154 and NCT0252261. Furthermore, RTX has a larger therapeutic window than capsaicin (Szallasi, 2023); however, RTX contains a complex structure making it harder to produce at a larger scale. Current synthesis takes 15 steps and has less than a 5% yield (Vasilev et al., 2022). Ultimately, despite all the research, only capsaicin has made it through this vigorous process of going from small molecule to medicine.

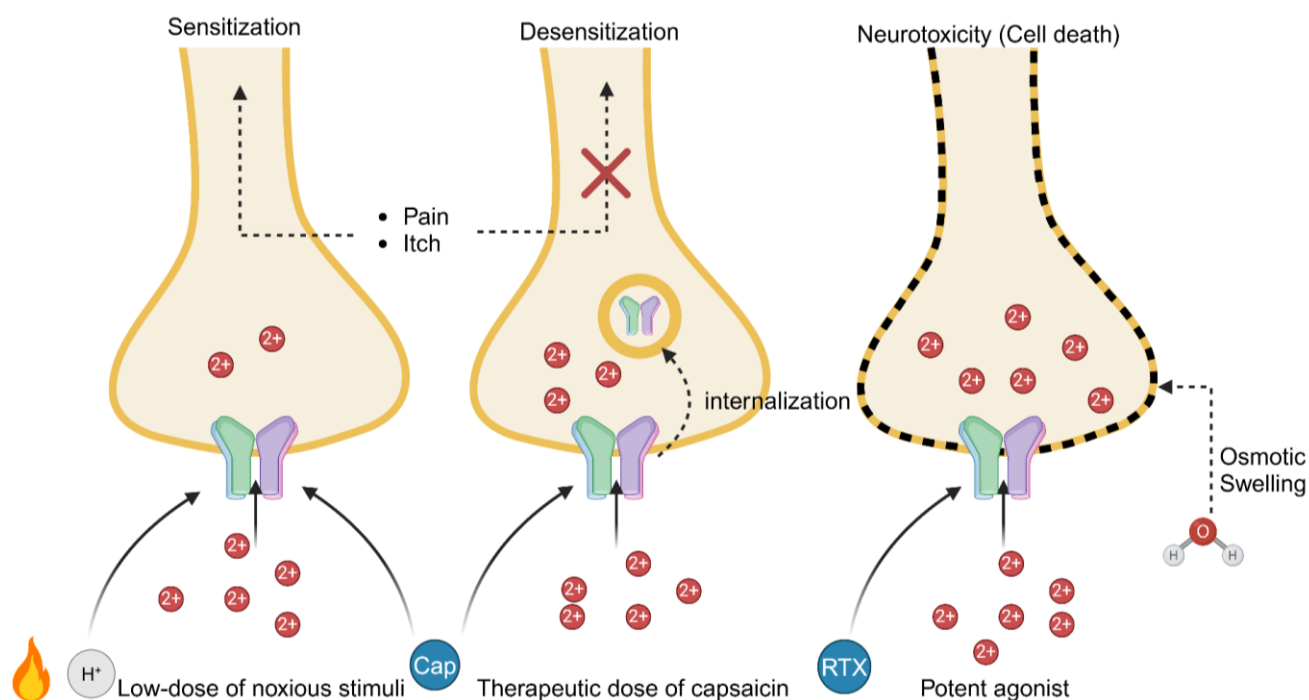


Figure 7 *TRPV1 activation by agonists can be generalized to three dosage levels.* The mildest, sensitization, occurs when a noxious stimulus like capsaicin, heat or acid results in neuronal depolarization, thus signaling pain to the brain. A second dosage is represented by a therapeutic amount of capsaicin, which results in the internalization of TRPV1 (Sanz-Salvador et al., 2012) and therefore desensitization to noxious stimuli. This still requires the activation of the receptor; therefore, a side effect of therapeutic capsaicin can be initial burning and pain. Lastly, molecules like RTX or even supratherapeutic doses of capsaicin can result in a toxic level of calcium influx, which leads to cell death through osmotic swelling. The full mechanism of cell death from TRPV1 influx toxicity is incompletely described (Koivisto et al., 2021).

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1.2 Chilis

Chilis are classified botanically as the fruit of different species of *Capsicum* (*Solanaceae*). There are five domesticated species: *C. annuum* var. *annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* var. *pendulum*, and *C. pubescens*; but there are 26 known species in general (Liu et al., 2023). Not all species produce the pain causing compound capsaicin, such as the sweet bell pepper (*C. annuum*). For those that do produce capsaicin, the compound has been found to be produced in the placenta of the chili. Since the placenta is connected to the seeds and the rest of the reproductive body, capsaicin can spread throughout the entire fruit (i.e., the pericarp), **Figure 8**. The highest concentration of capsaicin is found in oil-like structures on the placenta called blisters (Aza-González et al., 2011; Suzuki et al., 1980). These blisters contain compounds other than capsaicin called capsaicinoids. Capsaicinoids share a similar structure to capsaicin, that is, the vanilloid head, amide neck and acyl tail, **Figure 9**. Since they share a similar structure as capsaicin, they are also able to activate TRPV1 at varying potencies. Ultimately, capsaicin and capsaicinoids have made chilis a highly versatile fruit that has been utilized for many applications other than culinary and pain relieving purposes as previously mentioned, but also in cosmetic applications, repellents, and dietary supplements (Aza-González et al., 2011)

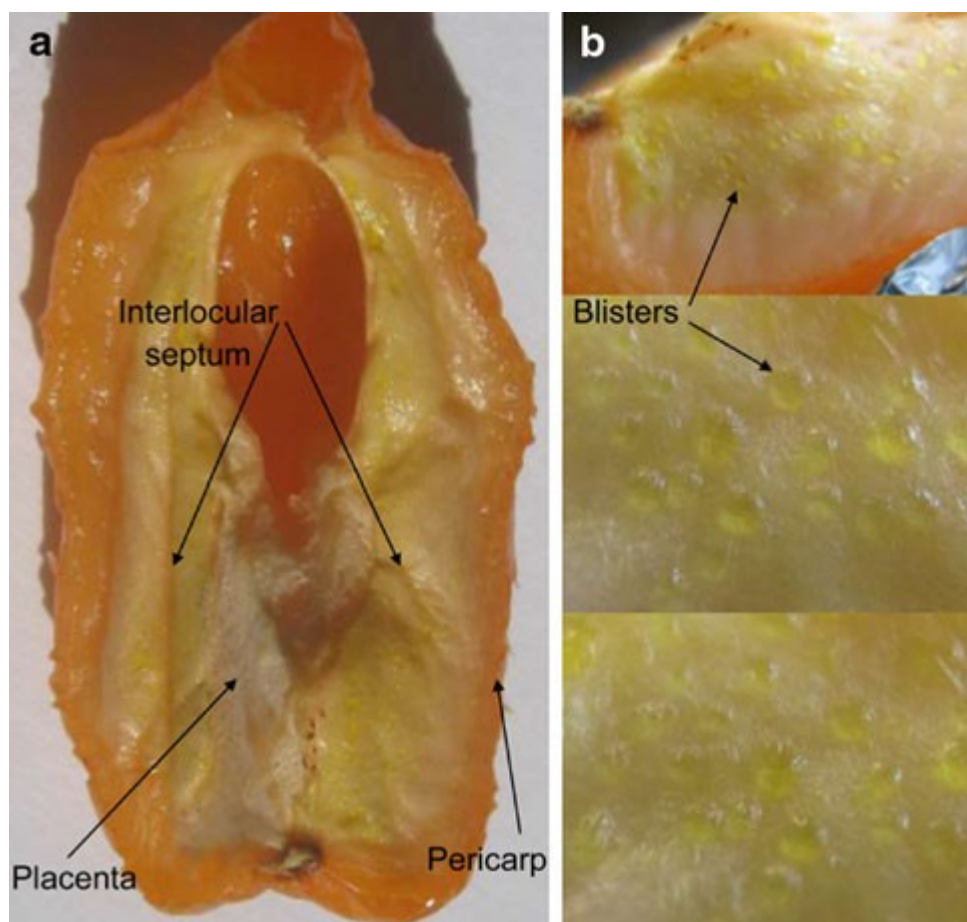


Figure 8 Photo of *Habanero*. This photo from the work of Aza-González et al. (2011) highlights the blisters that form on the placenta of the chili (Aza-González et al., 2011). A locule is a region inside of the ovary with a well-defined enclosed region (Simpson, 2020).

1.2.1 Capsaicin and capsaicinoids — general overview

There are about 25 different capsaicinoids that have been described throughout the literature (**Figure 9** and **Chapter 8, Table 1**) (Attuquayefio and Buckle, 1987; Constant et al., 1996; Higashiguchi et al., 2006; Ishimov et al., 2011; Jurenitsch et al., 1979a; Kobata et al., 2010, 1998b; Ochi et al., 2003; Wang et al., 2020a). Two other capsaicinoids, linvanil and myrvanil, have been putatively identified in *Capsicum oleoresin*, but were not absolutely confirmed (Kobata et al., 2010) and therefore are not included in the list of 25. While Kobata et al. acknowledge that linvanil and myrvanil were only putative candidates based on MS analysis (Kobata et al., 2010), some studies have claimed a structure without confirmation by NMR or reference standard (Ishimov et al., 2011) or were published before the depositing of NMR data to a repository was a standard practice (Jurenitsch et al., 1979b). Ultimately, many capsaicinoid structures published lack reliable reference MS or NMR spectral data. Contemporary practices follow the principle FAIR, “FAIR – Findable, Accessible, Interoperable, and Reusable” standards (Wilkinson et al., 2016), which allows for easier access to reference data. Regardless, while many capsaicinoids have been described, their biosynthetic expression is still not fully understood.

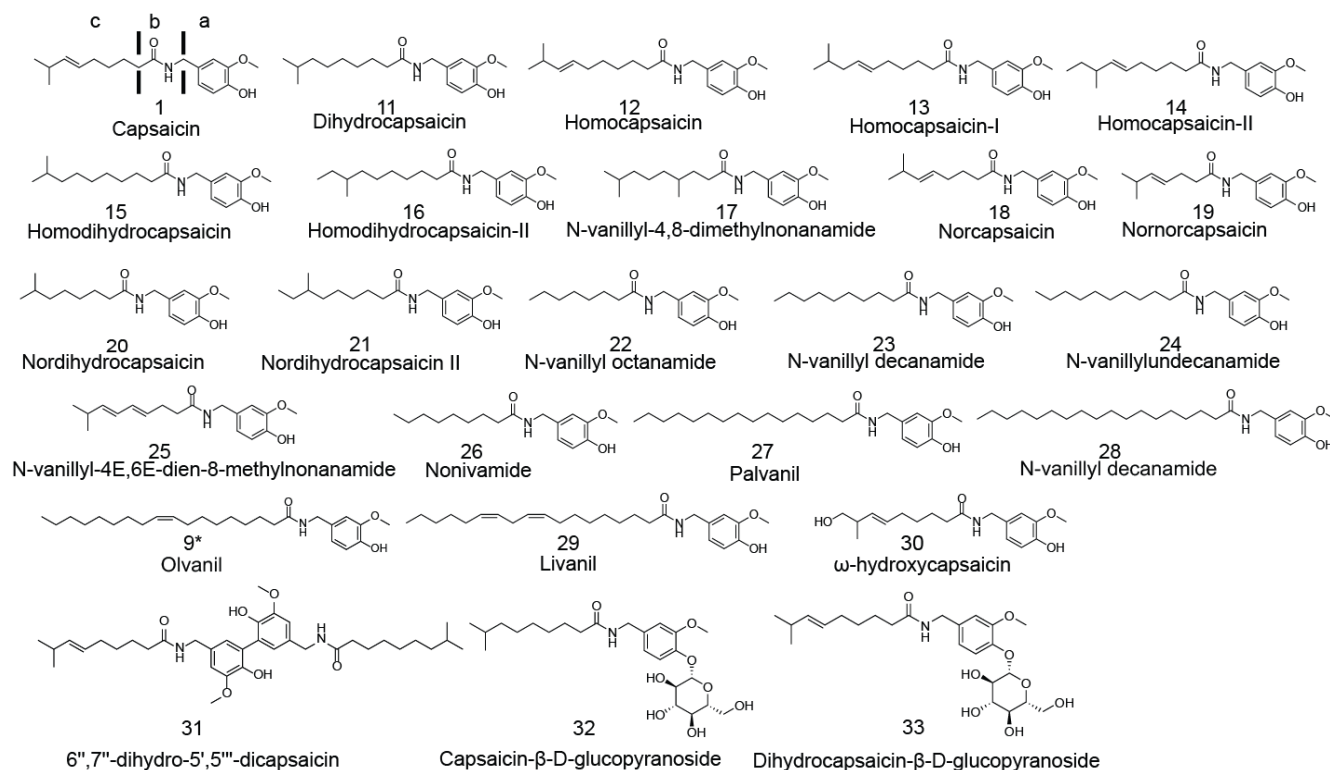


Figure 9 *Previously characterized capsaicinoids from literature.* To date, there are 25 different capsaicinoids described throughout literature. The capsaicinoid structure can be broken into three parts: a — the vanilloid head, b — the amide neck, and c — the acyl tail. Most capsaicinoid structures vary based on their acyl tail by varying degrees of length, oxidation and saturation. Olvanil, structure **9**, was previously mentioned in **Figure 6** and is also known as NE-191550.

1.2.2 Capsaicin and capsaicinoids — biosynthesis

While this thesis is not centered around the biosynthesis of capsaicinoids, an understanding of a few key steps is important for later discussion. Capsaicinoid biosynthesis can be divided into three major steps: formation of the vanilloid head, formation of the acyl tail and then lastly the combination of those two units (**Figure 10**). Synthesis of the vanilloid head begins with compound **34** (**Figure 10**), phenylalanine, which enters the phenylpropanoid pathway and is eventually converted into vanillin, compound **35** (**Figure 10**) (Fujiwake et al., 1982a, 1982b; Sukrasno and Yeoman, 1993). The first enzyme in this pathway to the formation of vanillin, phenylalanine ammonia-lyase (PAL) has also been linked to capsaicin biosynthesis (Phimchan et al., 2014). The more important step to highlight is the conversion of vanillin to vanillylamine, compound **36** (**Figure 10**), by a putative aminotransferase (pAMT) (Curry et al., 1999; Gururaj et al., 2012). The pAMT has been recently further confirmed and suggested to be renamed as vanillin aminotransferase (VAMT) (Nakaniwa et al., 2024). This conversion from vanillin to vanillylamine by pAMT is a key step in the regulation of capsaicinoid biosynthesis. Firstly, based on a metagenomic analysis of pAMT homologs, it was found that evolutionary changes in pAMT may have been needed for vanillylamine formation and the establishment of pungency in chilis (Kusaka et al., 2024). Furthermore, mutations in pAMT have been linked to loss-of-function alleles that have been found in lower pungent chilis (i.e., lower levels of capsaicinoids) (Sano et al., 2023; Tanaka et al.,

2015) or even to the formation of vanillyl alcohol, compound **37** (**Figure 10**) and the subsequent formation of capsiate (Lang et al., 2009). However, it has also been speculated that capsiate, and in general capsinoid biosynthesis, is not solely related to the mutated pAMT, but also requires a cinnamyl alcohol dehydrogenase (CAD) for vanillyl alcohol formation (Sano et al., 2022).

The other important step in capsaicin biosynthesis is the formation of the acyl tail. This begins with valine, compound **38** (**Figure 10**), which is converted into isobutyryl-CoA (compound **39**, **Figure 10**), which enters the fatty acid synthesis pathway where the amino acid is extended and eventually leaves the pathway and becomes compound **40** (**Figure 10**) (Aluru et al., 2003; Thiele et al., 2008). The subsequent acyl tail can then be combined with either the vanillylamine or vanillyl alcohol by capsaicin synthase (Pun1) (Han et al., 2013; Stewart et al., 2005). This gene has also been speculated to be involved in the regulation of capsaicinoid biosynthesis, especially since the expression of this gene can be induced by both wounding and heating of the fruit (Kirke et al., 2018). Despite decades of work on this biosynthetic pathway, the complete understanding of the regulation of capsaicinoid biosynthesis is still not fully understood. Regardless of full understanding, chilis have been able to breed in a selective manner for the creation of “dangerously” spicy varieties.

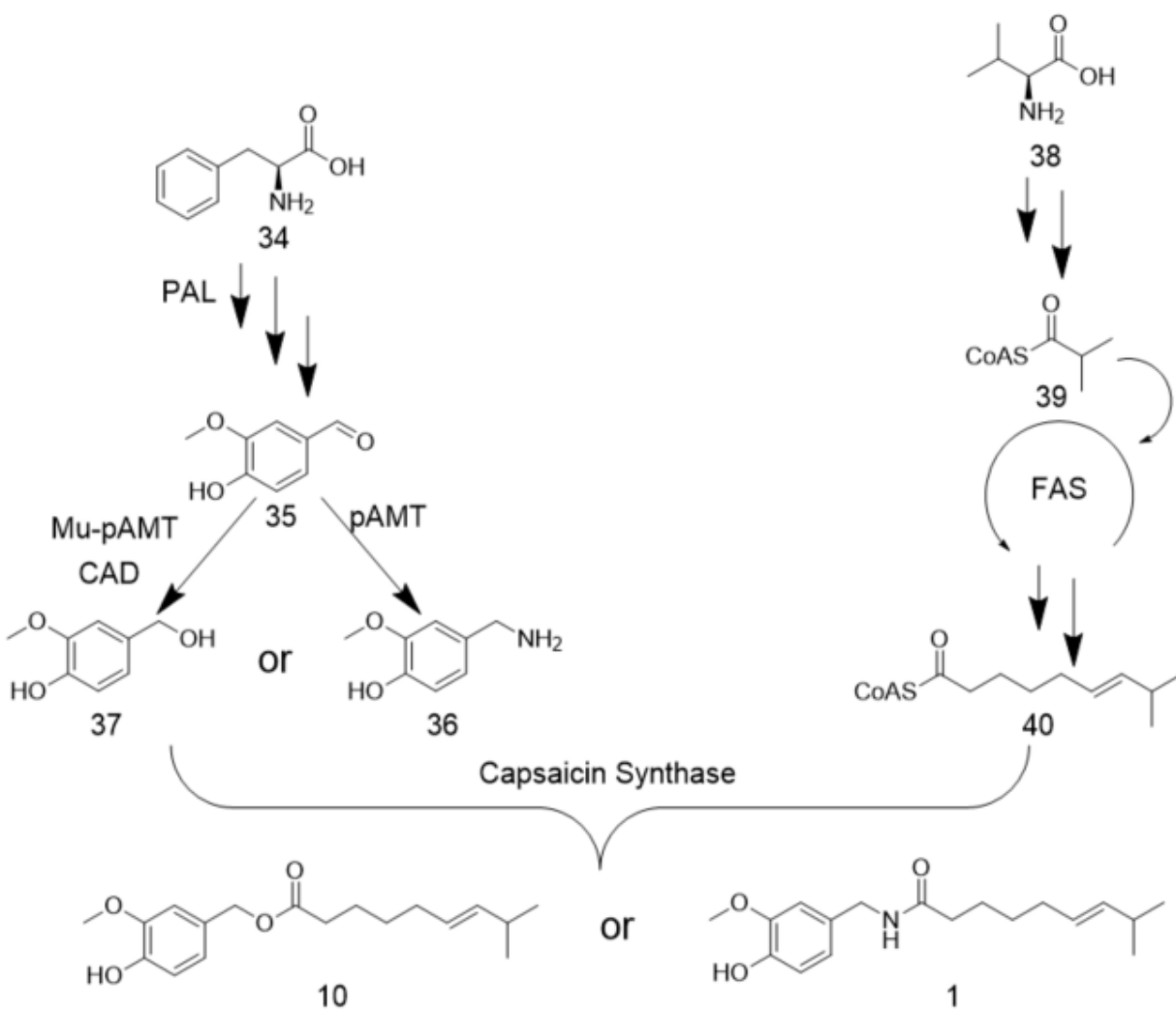


Figure 10 *A brief overview of the biosynthesis of capsaicinoids and capsinoids.* Compounds: **34** — Phenylalanine, **35** — Vanillin, **36** — Vanillylamine, **37** — Vanillyl alcohol, **38** — Valine, **39** — Isobutyryl-CoA, and **40** — 8-methyl-6-nonenoyl-CoA. Other abbreviations: PAL — Phenylalanine ammonia-lyase pMAT — putative aminotransferase, Mu — mutated putative aminotransferase, CAD — cinnamyl alcohol dehydrogenase and FAS — fatty acid synthesis (Aza-González et al., 2011).

1.2.3 Capsaicin and capsaicinoids — the want for more pain

There is an inherent irony in the consumption of spicy foods. Capsaicin, both a molecule that causes pain which should act as an evolutionary deterrent, also acts as an analgesic and, for some, a molecule that brings pleasure (Fu et al., 2018). Directed evolution has been used to selectively breed almost inhumane levels of chilis (Atwal, 2023; Naves et al., 2022). These chilies have been used to make extremely spicy sauces, seasonings and other food products. While incidents are isolated, there has been at least one reported death from consuming excessively spicy foods; one individual experienced cardiopulmonary arrest when participating in the spicy tortilla chip challenge (The Associated Press, 2024). While the individual had a preexisting heart condition (The Associated Press, 2024), this does not reduce the severity of the increasingly dangerous level of spice. Why people like spicy foods and the extent of why different genders or cultures prefer spicy foods are relatively understudied and are harder to make absolute conclusions. Based on a meta-analysis of 8 different studies on gender and spicy food preference, it was found that in 7 out of the 8 studies, males prefer spicy food over females (Siebert et al., 2022). Expanding from genders to cultures, the same meta-analysis suggested that spicy food consumption tends to be higher in some Asian countries, the US and Mexico, but ultimately it depends on person-to-person (Siebert et al., 2022). Of the studies, one by Rozin and Schiller highlights the complexity of chili consumption. They found that Mexicans reported eating several chilis a day, compared to Americans who limited their chili consumption to one or twice a week, and had only a slightly higher — non-significant — threshold for the spice (Rozin and Schiller, 1980). This threshold was based on eating increasingly spicy snacks (Rozin and Schiller, 1980). The similar thresholds, despite the two nationalities consuming a different amount of chilis, suggest that this preference for spicy food may be an acquired taste and that there is an enjoyment of the pain (Rozin and Schiller, 1980).

Why people eat spicy food and whether there is an underlying genetic difference between the preference for spicy and non-spicy food is still not fully understood. In the few studies investigating spice and taste preference, methods utilizing 6-n-propylthiouracil have not provided any conclusive information (Siebert et al., 2022). To the best of our knowledge, there has been no thorough investigation of TRPV1 SNPs and preferences for spicy or non-spicy foods. Most current research has found that chili and spicy food preference is psychological and that the liking may be acquired over time (Rozin and Schiller, 1980). Furthermore, this pain can be related to a psychological enjoyment of the body's defense mechanism (Rozin and Schiller, 1980); this pleasure has been considered a type of “benign masochism” (Fu et al., 2018). While less explored, chili consumption may not be a form of pleasure but rather a result of peer pressure (Gardner and Steinberg, 2005),

which is exemplified by the death of the “one-chip challenge” (The Associated Press, 2024). While the *why question* may be understudied, the method to measure chili’s heat is not.

1.2.3 The Scoville unit and the underestimation from targeted analysis

The first way to determine the heat (i.e. spiciness) of a chili was first developed over 100 years ago. It was based on an ethanolic extract of a chili mixed with glucose and serially diluted to a non-activating amount of extract. The dilutions were then sampled by taste testers and the dilution where pain was no longer experienced was called the Scoville unit or Scoville heat unit (SHU) (Scoville, 1912), **Figure 11**. Thus, a chili with a 250,000 SHU requires a 250,000x dilution to remove the effects of the extract. Since the development of this method, more modern analytical approaches have been developed using gas chromatography (Thomas et al., 1998; Todd et al., 1977) or high-performance liquid chromatography (Collins et al., 1995; Othman et al., 2011). These methods are based on determining the quantity of capsaicin or other capsaicinoids and calculating the SHU based on an intrinsic SHU value for the given analyte and multiplying it by its concentration. The problem with this methodology is that it only takes into consideration a few capsaicinoids, despite most of the known capsaicinoids containing a structure that would allow for TRPV1 binding. Furthermore, it is known that capsaicinoid profiles are inconsistent both inter- and intra-species (Zewdie and Bosland, 2001). Resulting targeted analysis may be limiting in the underestimation of SHUs and the discovery of novel capsaicinoids, especially those that may have been a result of selective breeding. Untargeted metabolomics, the study of the entire metabolome, may offer an advantageous solution to the matter.

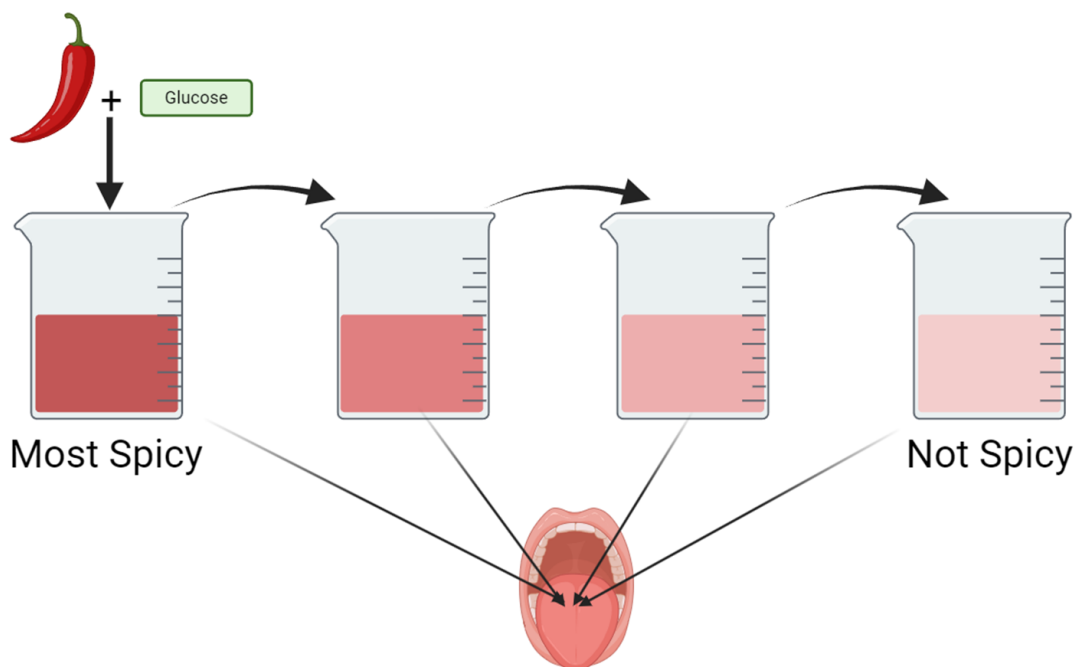


Figure 11 Example of a traditional Scoville measurement. The measurement was based on a series of chili extract dilutions and taste testing (Scoville, 1912). Created with BioRender.com

1.3 Computational metabolomics

Natural products (i.e., secondary metabolites) have long been exploited through both traditional and modern medicines (Atanasov et al., 2021). The identification of pharmacologically relevant secondary metabolites is often an arduous shot-in-the dark process: a) obtain crude extracts from secondary metabolite producing organisms (i.e., plants, yeast, bacteria, and so forth), b) test extracts for bioactivity, c) fractionate biologically active extract(s), d) test fractions for bioactivity, e) repeat the process until a single bioactive compound is identified (Muchiri and van Breemen, 2021). Modern mass spectrometry (MS), in particular high-resolution mass spectrometry (HR-MS) and tandem mass spectrometry (MS/MS), in combination with computational tools, has provided a new approach to this once tedious method of bioactive small molecule discovery (Damiani et al., 2025; Reinhardt et al., 2025; Smith et al., 2024).

1.3.1 Mass spectrometry

Mass spectrometry (MS) is one the essential techniques in a scientist's analytical toolkits. In MS, measurements of target molecules are completed by first ionizing the sample matrix. Ionized molecules are then sent to a detector and measured. The reported units are often presented as a ratio of the mass to the charge of the ion, known as the mass-to-charge ratio (m/z) (Garg and Zubair., n.d.). MS analysis is often used for small molecules, but can be extended to many other applications, such as the analysis of polymers (Hanton, 2001) and proteins (Zhang et al., 2010). In recent years, metabolomic studies (i.e., total metabolome analysis) have seen increased use and success with MS-based applications (Dettmer et al., 2007). One reason for this success is the development of high-resolution mass analyzers with greater resolving power, which allows for accurate mass measurements and chemical formula prediction (Dettmer et al., 2007; Xian et al., 2012). The second major driving force for success has been the development of hybrid MS instruments that allow the combination of different mass analyzers with the capability of tandem mass spectrometry (MS/MS) (Glish and Burinsky, 2008; Heiles, 2021). MS/MS is when a small molecule is selected and fragmented into smaller ions; fragmentation ions can provide structural insights into the parent ion.

The success of high-resolution tandem mass spectrometer-based applications for metabolomic studies is not only due to the mass spectrometer, but also its combination with an ultra-high performance chromatography system (UPLC-MS/MS). UHPLC systems are sometimes substituted with gas chromatography (GC) depending on the analyte. Instruments that couple chromatography provide separation of small molecules before reaching the mass detector giving metabolites additional metadata, such as retention times (i.e., time from injection to detector). By having both an m/z and a retention time, compounds in metabolic studies can be annotated with greater confidence, which is standard practice in targeted metabolomic studies (Reisdorph et al., 2019; Xiao et al., 2012).

1.3.1.1 Targeted and untargeted metabolomics

Targeted and untargeted metabolomics studies are used to investigate the metabolites (i.e. small molecules) in complex biological samples. One of the greatest differences between the two is that untargeted metabolomics investigates all molecules in the samples, even those that are unknown, while targeted metabolomics involves a set of predefined targeted compounds by using a library of standards (Roberts et al., 2012). Because of this, targeted metabolomic studies are often considered hypothesis driven while untargeted metabolomics is hypothesis generating. Furthermore, unlike targeted metabolomics, which looks for specific metabolites and can be completed with a predefined list of molecules, untargeted metabolomic data processing often requires full-fledged software that requires a greater level of experience for successful implementation (**Figure 12**). Therefore, due to the increased complexity of data processing in untargeted metabolomic studies and from the work with unknown metabolites, there have been a plethora of computational tools developed for scientists to help alleviate this complexity (Schrimpe-Rutledge et al., 2016).

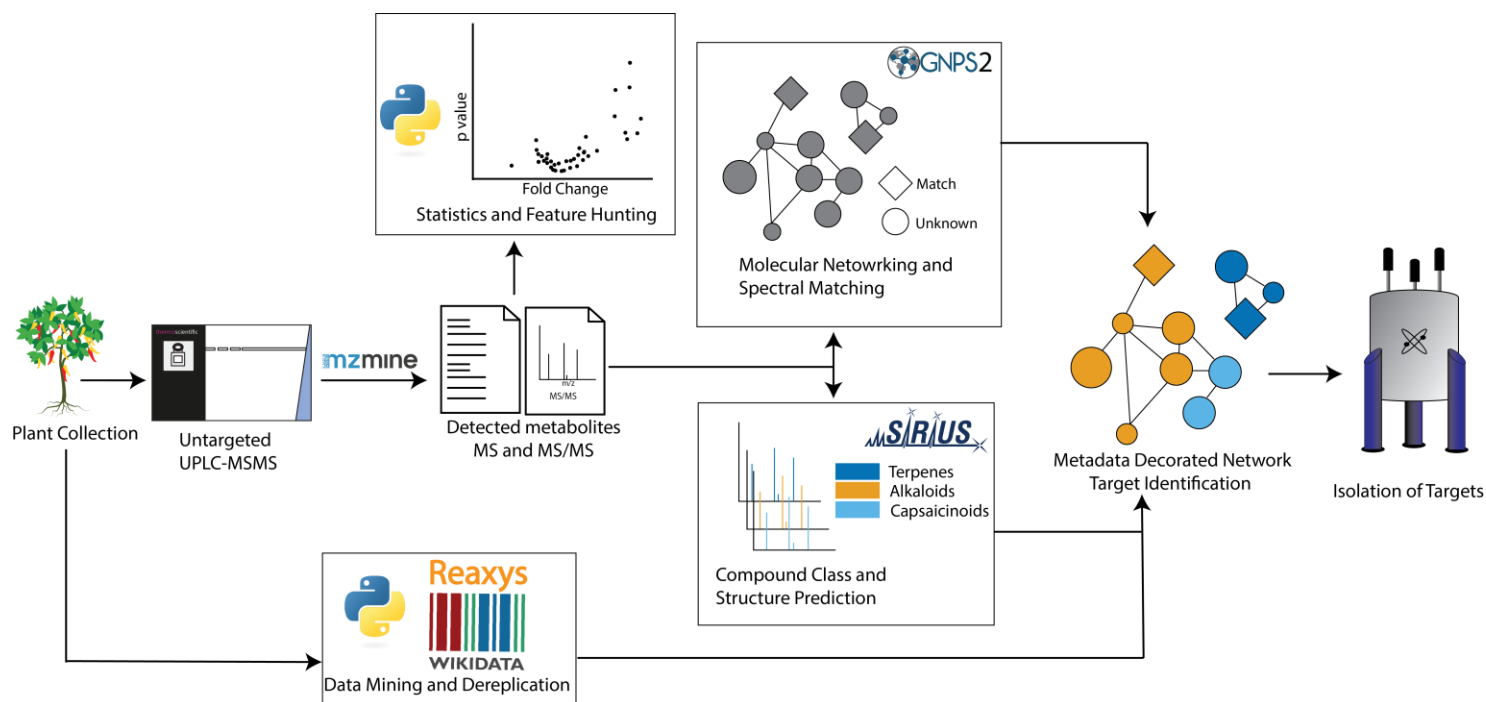


Figure 12 *Untargeted metabolomic workflow that was used in this thesis.* This is just one of many ways that an untargeted metabolomic workflow can be structured. Experimentation begins with sample collection, processing and analysis on the UPLC-MS/MS. Mzmine is used for feature detection (i.e. chromatographic peaks that represent a metabolite) which can be used for downstream untargeted metabolomics software, such as GNPS or SIRIUS. Concurrently, tools like Reaxys and Wikidata can be used to mine information about metabolites that have already been identified. Lastly, Python can be leveraged for this data mining or for other statistical analysis with an mzmine output.

1.3.2 The tools of untargeted metabolomics

As mentioned, untargeted studies often require dedicated software that creates a list of metabolites found in each analyzed sample. Some examples of software are MS-DIAL (Tsugawa et al., 2015), XCMS (Tautenhahn et al., 2012), and mzmine (Schmid et al., 2023). For the rest of this thesis, mzmine will be the only software referenced. One of the major advantages of mzmine is the strong collaborative community that has built other post-data processing software that is compatible with mzmine's output of a feature table. A feature is a meaningful MS signal (i.e, a chromatographic LC-MS peak) that is representative of a metabolite found in the sample, thus a feature table is a list of all meaningful MS signals from all samples in a single table (Heuckeroth et al., 2024). The feature table can be exported for statistical analysis with applications like Metaboanalyst (Pang et al., 2024) or Python. The feature table is also accompanied by a file that contains all the MS/MS information, which is in a format easily adapted to tools for molecular networking, such as feature-based molecular networking (FBMN) on the Global Natural Products Social Molecular Networking (GNPS) platform (Nothias et al., 2020) or MetGem (Olivon et al., 2018) or in-silico compound and molecular class prediction with SIRIUS (Dührkop et al., 2019) and CANOPUS (Dührkop et al., 2020), respectively (**Figure 12**). These tools, when combined, can greatly reduce the complexity of an untargeted metabolomic study, especially one focused on natural product discovery.

1.3.2.1 Feature-based molecular networking

One of the early limitations of MS/MS analysis was the inability to meaningfully interpret fragmentation spectra. MS molecular networking provided an alternative visualization by representing MS/MS features as nodes and clustering based on similar fragmentation patterns (Watrous et al., 2012). The core mathematics behind molecular networking leverages an already established cosine similarity matching that has been used in MS/MS fragmentation matching to MS/MS library and reference standards (Stein and Scott, 1994). However, classical molecular networking had its limitations, namely the inability to utilize MS information, such as retention time or the isotope pattern of the associated MS/MS precursor (Watrous et al., 2012). To alleviate these problems, Dorrestein and colleagues improved upon their previous tools of classical molecular networking by incorporating this missing data. This new method, FBMN, was able to resolve isomers due to the incorporation of the retention time. Furthermore, it was able to utilize the peak area of a feature and not only the individual spectral counts (Nothias et al., 2020). While FBMN greatly improved the state of the art, FBMN does not fully solve the problem of deciphering unannotated features. Therefore, other tools must be used in combination with FBMN to optimize feature annotation (**Figure 12**).

1.3.2.2 SIRIUS, CANOPUS and the future

Artificial intelligence and machine learning based solutions have become increasingly prevalent for the annotation of unknown MS features in natural product drug discovery (Gangwal and Lavecchia, 2025;

Mullowney et al., 2023). One of the most relevant is SIRIUS, a support vector machine trained on known molecules' MS/MS fragmentation, which is used to create molecular fingerprints of unknown MS/MS spectra. These fingerprints are then matched to known molecules in databases and act as putative candidates for an unknown feature (Dührkop et al., 2019). Another tool developed by the Böcker group is CANOPUS, a deep neural network that is able to predict molecular classes from the MS/MS fragmentation of unknown metabolites (Dührkop et al., 2020). These tools provide exports that can act as metadata and be overlaid with nodes and networks created by FBMN (**Figure 12**). When combined, untargeted metabolomics studies, especially those investigating natural product discovery, become less convoluted. This thesis highlights the combination of these tools and how they can be applied directly to pharmacologically relevant molecules (**Figure 12**).

2. Hypotheses and objectives of the work

Chilis are a unique food that contains the pain-causing molecule capsaicin. The pain associated with capsaicin is due to the agonism of the transient receptor potential cation channel subfamily V member 1 (TRPV1). Despite this pain when willingly ingested, it has not been a deterrent for consumption amongst the human population. Contrary to expectations, farmers have bred varieties of chili to extreme levels of spice, with consumers regularly participating in contests to consume these potentially dangerous chilis. The pain level of a given chili has been defined by the Scoville value, a measurement of how many dilutions are needed to neutralize the taste of the capsaicin. This measurement has been modernized through the targeted HPLC analysis of capsaicin and its analog dihydrocapsaicin, which is then converted to a Scoville unit. However, this analysis does not consider any other capsaicinoids, molecules related to capsaicin, found in chilies. We hypothesized that directional breeding of spicier varieties may have resulted in stronger agonists to TRPV1 than capsaicin.

Objectives:

1. Complete an untargeted metabolomic study of different spicy chili varieties and identify potential novel capsaicinoids.
2. Isolate target compounds and test them in a TRPV1 activation assay.
3. Create the most comprehensive library of capsaicinoids and their TRPV1 activation.

3. Material and methodology

3.1 Plant collection and sample preparation

To obtain chilis, we began a collaboration with the Botanical Garden of the Faculty of Tropical AgriSciences of the Czech University of Life Sciences in Prague. The garden grows the chilis annually with seed sowing beginning in January or February and the growing of plants continuing into summer and sometimes early fall. Chilis are treated with acetamiprid only in cases of pest infestation. Varieties were confirmed by the botanical garden or verified dealers using morphological traits. Ripe chilis, based on fruit coloration (Po et al., 2018), were harvested on several occasions between the months of August and October. The fruits of 40 different varieties were collected covering a wide range of heat levels (**Chapter 8, Table 3**) and four of the five domesticated chili species. Chilis were grown in an ordinary horticultural substrate fertilized with cow manure. Chilis and sometimes stems were collected at ripeness – based on size, shape and color – and immediately placed on dry ice. Samples were stored at -80°C until further processing, in which they were crushed into a powder with a pestle and mortar under liquid nitrogen, lyophilized and again stored at -80°C until extraction and analysis. It should be noted that several chilis were collected for each variety and pooled together but were not kept as separate biological replicates.

3.2 Chili extraction and capsaicinoid isolation

25 ml of ethyl acetate (EtOAc) was added to 1 g of chili powder and then agitated by an IKA dispenser for 1 minute. This process was completed twice for each variety, with individual extracts pooled, evaporated and then subjected to acetonitrile:hexane partitioning (1:1). The acetonitrile portion was dissolved to 1 mg/ml in acetonitrile (ACN). A portion of the sample was diluted to 0.2 mg/ml and analyzed as a single technical replicate on UPLC-HRMS. Individual capsaicinoids were isolated in two batches. Both consisted of maceration in EtOAc, fractionation on normal-phase flash chromatography and final isolation of the compound with HPLC.

3.2.1 Isolation of individual capsaicinoids

First large-scale extraction. Pooled varieties (see **Chapter 8, Table 3**) of lyophilized chilis were mixed together, cca. 50 g. The mixture was submerged in excess EtOAc and stirred; this was completed 3 times for 2 hours, 4 hours and 16 hours. Each portion of EtOAc was filtered and evaporated under rotary evaporation to leave a crude extract. The evaporated solvent was reused for extraction. Ultimately, all extracts were pooled together, filtered and then fractionated on normal-phase flash chromatography on a CombiFlash Rf 200 system (Teledyne, Thousand Oaks, CA, USA). Flow rate: 30 ml/min. Mobile phase A: Cyclohexane. Mobile phase B: EtOAc. 0 → 45 minutes of isocratic 45% B; 45 → 75 minutes gradient from 45% B to 100% B; 75 → 85

minutes 100% B. Individual fractions were pooled into 7 bigger fractions based on UPLC-MS/MS analysis. Individual capsaicinoids were isolated from the pooled fractions using a 1260 Infinity II Preparative LC system (Agilent), a 19 mm × 250 mm Waters XBridge C18 column and a Milli-Q water:ACN (Fisher) gradient. Gradients were 55 minutes and started at either 25% or 30% ACN and went to 100% ACN.

Second large-scale extraction. The second extraction was like the first but with optimized procedures. Differences include: variety of chilis used (see **Chapter 8, Table 3**), the number of extractions went to 6 times at 1 hr and 30 minutes each but the last extraction was overnight, and 89 g of chilis were used. Other changes were in optimized chromatography as follows: normal-phase flash chromatography. Mobile phase A: cyclohexane. Mobile phase B: EtOAc. 0 → 8.4 minutes isocratic 10% B, 8.4 → 16.8 minutes 10% → 20% B, 16.8 → 34.9 minutes isocratic 20%B, 34.9 → 36.9 minutes 20% → 40%B, 36.9 → 56.9 minutes isocratic 40%B, 56.9 → 70 minutes 40% → 100%B, 70 → 78 minutes 100% B. Fractions were again pooled based on different capsaicinoid distribution. Pooled fractions were again separated into pure compounds on the Agilent preparative HPLC, but with a 10 mm × 250 mm Waters XBridge C18 column. All HPLC methods were fit optimized with XBridge C18 3.5 µm column (Waters) and transferred to the preparative HPLC. Capsaicinoids were isolated using two isocratic hold steps with ACN; depending on the target, the % of ACN differed.

3.3 Dereplication of capsaicinoids

To reduce the possibility of isolating known compounds, Reaxys and Wikidata were queried to obtain all known capsaicinoids. Our pipeline identified 25 previously reported capsaicinoids from *Capsicum* (**Figure 9, Chapter 8, Table 1**). Chemical formulas of known capsaicinoids were leveraged as a matching tool to the predicted SIRIUS chemical formulas and further confirmed annotation by manually investigating MS/MS. Resulting matching metabolites were taken only as putative results, especially due to the alkyl tail isomers that are hard to characterize with MS/MS alone. Two other putative capsaicinoids were excluded, linvanil and myrvanil, which have been suggested to be found in *Capsicum oleoresin* (Kobata et al., 2010).

3.4 UPLC–HRMS analysis

Analysis was completed on an Orbitrap ID-X mass spectrometer (Thermo Fisher) coupled to a Vanquish Duo UHPLC system (Thermo Fisher). The column compartment was set to 40 °C. A Waters Acquity BEH C18 150 × 2.1 mm, 1.7 µm, 130 Å column (Waters Corporation) was used. Injections were completed with 1 µl. For instrument QC, a Waters Vion mix (Waters Corp.) was used. Mobile phase A: LC–MS-grade water + 0.1% MS-grade formic acid. Mobile phase B: LC–MS-grade acetonitrile + 0.1% MS-grade formic acid (Thermo Fisher). The flow rate was set to 0.350 ml/min. 0 minutes 5%B → 0.5 minutes 5%B → 15.5 minutes 100%B → 17.3 minutes 100%B → 17.3 minutes 5%B → 19.3 minutes end of run. The ion source was set to 3000 V, the gas mode was set to static, 50 sheath gas (arb), 10 aux gas (arb), 1 sweep gas (arb). The ion transfer tube's

temperature was set at 325 °C and the vaporizer temperature was 350 °C. The following Orbitrap parameters: resolution — 60,000, scan range of 100–1000 m/z, rf lens — 45%, maximum injection time — 118 seconds, MS/MS — 0.6-second cycles, with 1.0×10^5 signal intensity triggering the MS/MS. An exclusion list was built with fragmented ions in a 5 ppm tolerance for 2 seconds after initial triggering. Isotopes were also excluded. Apex detection was used at a FWHM value of 4 and a 50% window. The MS/MS isolation window was 0.8 m/z, with HCD fragmentation with a fixed energy mode at 35%. The Orbitrap was used for MS/MS detection with a resolution of 15,000 and 80 ms maximum injection time. Capsaicinoid standards (MedChemExpress) were diluted to 10 μ M, capsiate (MedChemExpress) and vanillin (Fluka) to 100 μ M, and 4-hydroxy-3-methoxybenzyl alcohol (Alfa Aesar) to 1 mM, all with LC-MS grade ACN. All raw data have been deposited; see **Chapter 10** for more info.

3.5 Untargeted metabolomics data processing

UPLC–HRMS Thermo Fisher .raw files were converted to .mzML files using MSconvert. Recommended values for MSconvert were used from mzmine documentation (li-chin, 2023). The converted files were processed using mzmine version 4.0.8 (Heuckeroth et al., 2024; Schmid et al., 2023). Processed mzmine data (i.e., the feature table) was exported to .mgf files using both the SIRIUS and GNPS export. Exported files were processed on GNPS's feature-based molecular networking platform and SIRIUS version 4.13.3. All exports from SIRIUS, GNPS and mzmine were post-processed using Python scripts for database dereplication and statistics. All data processing outputs and python scripts are made available for download, see **Chapter 10** for links.

3.5.1 mzmine processing parameters

Mass detection: MS — Centroid at “Noise Levels” set to 1.0×10^4 and MS/MS at 1.0×10^3 .

Chromatogram building: ADAP Chromatogram Builder Module — minimum of 6 consecutive scans, a minimum group intensity of 5.0×10^4 , a minimum absolute height of 1.0×10^5 and m/z tolerance for scan-to-scan matching of 0.001 m/z or 3.5 ppm.

Smoothing: Savitzky–Golay filter with retention time smoothing and 5 points.

Feature deconvolution: Local minimum resolver — chromatographic threshold of 0.95%, a minimum search range of 0.05 (retention time or mobility), 5.0% minimum relative height, a minimum peak height of 1.0×10^5 , a minimum ratio of peak top/edge of 3.00, a peak duration of 0–2 and a minimum of 6 scans. “MS/MS scan pairing” checked — MS1 to MS2 precursor tolerance of 0.0020 or 5 ppm, feature edges as a retention time filter, 25.0 as a minimum relative feature height, 1 minimum required signal and 1% minimum signal intensity.

Isotope grouping: ^{13}C isotope filter — 0.001 m/z tolerance or 2 ppm and a retention time tolerance of 0.03 minutes, monotonic shape checked, keep the most intense isotope signal. Isotope peakfinder — 0.0010 m/z or 2 ppm tolerance, maximum charge of isotope m/z to 1 and search for the single most intense scans

Feature Grouping: Join aligner — m/z tolerance of 0.010 or 3.50 ppm and a retention time tolerance of 0.07 min; m/z and RT weights were set to 1.

Gap Filling: Feature finder — intensity tolerance 15%, 0.0010 m/z tolerance, 3.5 ppm, 0.07 RT tolerance, 6 minimum scans.

Duplicate removal: Duplicate peak filter — new average mode, 0.0010 m/z and 3.0 ppm tolerance and a 0.01 retention time tolerance.

Background subtraction: Feature list blank subtraction — 1 detection in blank, based on peak area with maximum ratio type and features only above a 300 %-fold change were kept.

Adduct identification: Correlation grouping — 0.01 RT tolerance, 0 and 0 were used for minimum feature height and intensity. A minimum of 1 sample was required with 60% overlap and all gap-filled features were excluded. Six points, with 3 points on the edge, were required for feature shape correlation and required 85% Pearson. Ion identity networking — 0.0010 m/z or 3 ppm mass tolerance and a 0 height. Smaller networks without major ion, delete smaller networks: link threshold (set to 4) and delete networks without monomer were all enabled. The ions used were: $[\text{M}]^+$, $[\text{M}+\text{H}]^+$, $[\text{M}+\text{NH}_4]^+$, $[\text{M}+\text{Na}]^+$, $[\text{M}+\text{K}]^+$, $[\text{M}+2\text{H}]^{2+}$ and $[\text{M}-\text{H}_2\text{O}]^+$. An additional ion was manually added to the mzmine list, $[\text{M}+\text{ethylamine}]^+$ (46.0651).

List filtering: Feature list rows filter — 1 isotope per feature. MS/MS scan and feature IDs were reset.

Spectral matching: Spectral library search — using created capsaicinoid library, merged MS/MS spectra, precursor tolerance of 0.0010 or 5 ppm, spectral tolerance of 0.0015 m/z or 10 ppm, removed precursor, minimum of 5 matched signals, weighted cosine similarity based on MassBank and a minimum score of 0.8; all unmatched signals were kept and matched to zero

3.5.2 GNPS and SIRIUS parameters

.mgf files exported from mzmine and uploaded to GNPS and SIRIUS. For GNPS and FBMN processing, the precursor and fragment mass tolerance were set to 0.0075 Da. Minimum cosine pairing was set to 0.65, with 5 minimum matched fragments and 500 Da maximum shift in precursor. Network TopK was set to 15 and the component size was 150; all other parameters were kept default. SIRIUS processing was completed with version 4.13.3. “Orbitrap” was used for the mass analyzer and “bio databases” for compound prediction. m/z values greater than 800 Da were excluded, while all other parameters were kept default.

3.6 Structural characterization of isolated compounds: NMR, UV, ECD and QC

To determine the exact structure of the isolated capsaicinoids, isolated compounds were dissolved in a minimal amount of CD₃CN or DMSO-*d*₆. Capsaicinoids were then analyzed on a 500 or a 600 MHz Bruker Avance III HD spectrometer (Billerica, MA, USA) (¹H at 500.0 or 600.1 MHz and ¹³C at 125.7 or 150.9 MHz). Solvent peaks were used in spectral referencing: CD₃CN δ = 1.94 for ¹H and 1.32 ppm for ¹³C and DMSO-*d*₆: δ = 2.50 for ¹H and 39.52 ppm for ¹³C. To obtain the complete structure of the capsaicinoids, ¹H and ¹³C were completed for 1D experiments and H,H-COSY, H,C-HSQC and H,C-HMBC were completed for 2D correlation experiments. Due to the limited quantity of some samples, not all carbon signals were visible through 1D ¹³C analysis. All structures were obtained using the 2D experiments. All raw data were deposited on the NP-MRD and Zenodo (**Chapter 10**) and all hydrogen and carbon annotations can be found in **Chapter 7**.

In addition to NMR, UV and circular dichroism experiments were completed on all capsaicinoids. For UV determination, a Thermo Fisher Nanodrop One^c spectrophotometer was used with DMSO as a blank. A 1 μ l portion of each sample was analyzed to obtain the UV value. UV values can be found in **Chapter 8, Table 2**. Electronic circular dichroism (ECD) was also completed with the use of a Jasco 1500 spectrometer (Jasco, Tokyo, Japan). Both ECD and absorption spectra were measured from 180 nm to 350 nm in 10 nm/min intervals, with an 8-second response time and 3 accumulations. Samples were dissolved in a small portion of CD₃CN to reach a concentration of about 1.5 mM and then transferred to a cylindrical quartz cell (0.02 cm path). All spectra were corrected for baseline signal.

Lastly, the purity of all isolated compounds and purchased standards was determined. The quality control (QC) was performed using a short 1.5-minute method with a 10%-95% ACN gradient on an ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1x50mm, Waters) and with an ACQUITY UPLC I-Class (Waters) system for analysis. To identify the molecule, the mass spectra exact mass was used and then the area under the curve for the corresponding UV peak (diode array detector) was used to determine the purity of the compound.

3.7 TRPV1 activity assay

A Hek293 cell line stability expressing rTRPV1 was gifted to us from the Whitehead Institute at MIT. This cell line is based on a Tet-on system to induce expression. We called this cell line irTRPV1-FlpIn293 as it was created using the FlpIn system (Thermo Fisher). Cells were grown in Advanced Dulbecco's Modified Eagle Medium (Adv-DMEM/F12, #12634) supplemented with 10% FBS, 1% Pen Strep, and 1% Glutamax (all Gibco, Thermo Fisher Scientific). To induce rTRPV1 expression, doxycycline (Sigma-Aldrich, St. Louis, Mi., USA) was added to the cells at a working concentration of 1 μ g/ml. To help keep the cells in a viable state,

ruthenium red (Merck KGaA, Darmstadt, Germany) was added at a working concentration of 10 mM. After induction, cells were grown for 24 hours and then transferred to a 384-well plate (3674, Corning, Corning, NY, USA). Cells were first washed and detached using 0.5% trypsin–EDTA in PBS (Gibco), resuspended in Adv-DMEM/F12 + 10% FBS + 10 μ M RR, and then counted so that cells could be seeded at 3×10^4 per well. On the next day, the calcium dye, Fluo-4 direct solution (Invitrogen, Thermo Fisher Scientific), was added to each well. The dye was prepared in HHBS buffer at a pH $\sim$ 7.4. Cells with the dye were incubated for 30 minutes at 37 °C and then for an additional 40 minutes at room temperature. Cells incubated with dye were washed using the BlueWasher (BlueCatbio NH Inc., Lebanon, NH, USA). Media was replaced with HHBS + 10 μ M sulfinpyrazone (Merck KGaA) by a Certus (Fritz Gyger AG, Gwatt, Switzerland) dispenser. The well plate with cells was then transferred to FDSS μ Cell (Hamamatsu, Hamamatsu City, Japan) and incubated for 10 more minutes at $\sim$ 30°C before analysis. All compounds, both purchased and isolated, were prepared on a separate plate via an Echo dispenser 650 (Beckman Coulter Inc., Brea, CA, USA). All standards and samples were prepared in DMSO as 10 mM stocks and diluted to a working concentration using HHBS + 10 μ M sulfinpyrazone via Certus. The purchased compounds: capsiate (both MedChemExpress), vanillin (Honeywell Fluka) and 4-hydroxy-3-methoxybenzyl alcohol (Alfa Aesar) and AMG-517 (TargetMol, Boston, MA, USA), all other standards were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Before the addition of compounds, baseline measurement was completed for 40 seconds with data collection every 0.339 seconds. While still recording, cells were then contemporaneously titrated with compounds by the FDSS machine and analyzed for 5 minutes. To obtain max signal, which was used for normalization, 1% Triton X-100 was added (Merck KGaA). Data was normalized from ScreenX 2.0 from the Institute of Molecular Genetics CAS and then analyzed on GraphPad Prism version 10.2.2. All dose/concentration-response curves were plotted using the four parametric logistic function made using least-square fit and separately handled replicates and are represented with the following equation:

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{[(\log EC_{50} \text{ or } \log ED_{50}) - X] * HillSlope}}$$

All other relevant data regarding replicates and statistics can be found in **Chapter 8, Tables 4-6**.

4. Results

4.1 Plant collection and untargeted metabolomics

Experimentation began with the collection of 40 different varieties of chili from the Botanical Garden of the Faculty of Tropical AgriSciences of the Czech University of Life Sciences in Prague (**Figure 13** and **Chapter 8, Table 3**). The collected varieties cover four out of the five domesticated chili species and a wide range of SHU values. All varieties were first extracted on a small scale using EtOAc and analyzed on our UPLC-MS/MS method (**Figure 13**). Raw files were processed with mzmine (Schmid et al., 2023), which resulted in the output of a feature table (i.e., a list of all metabolites). The resulting features were first explored utilizing SIRIUS (Dührkop et al., 2019), an in-silico tool for compound structure prediction based on MS/MS spectra. It also contains a module, CANOPUS (Dührkop et al., 2020), which predicts the compound classes (e.g., alkaloid, terpene, capsaicinoid, etc.) for all features.

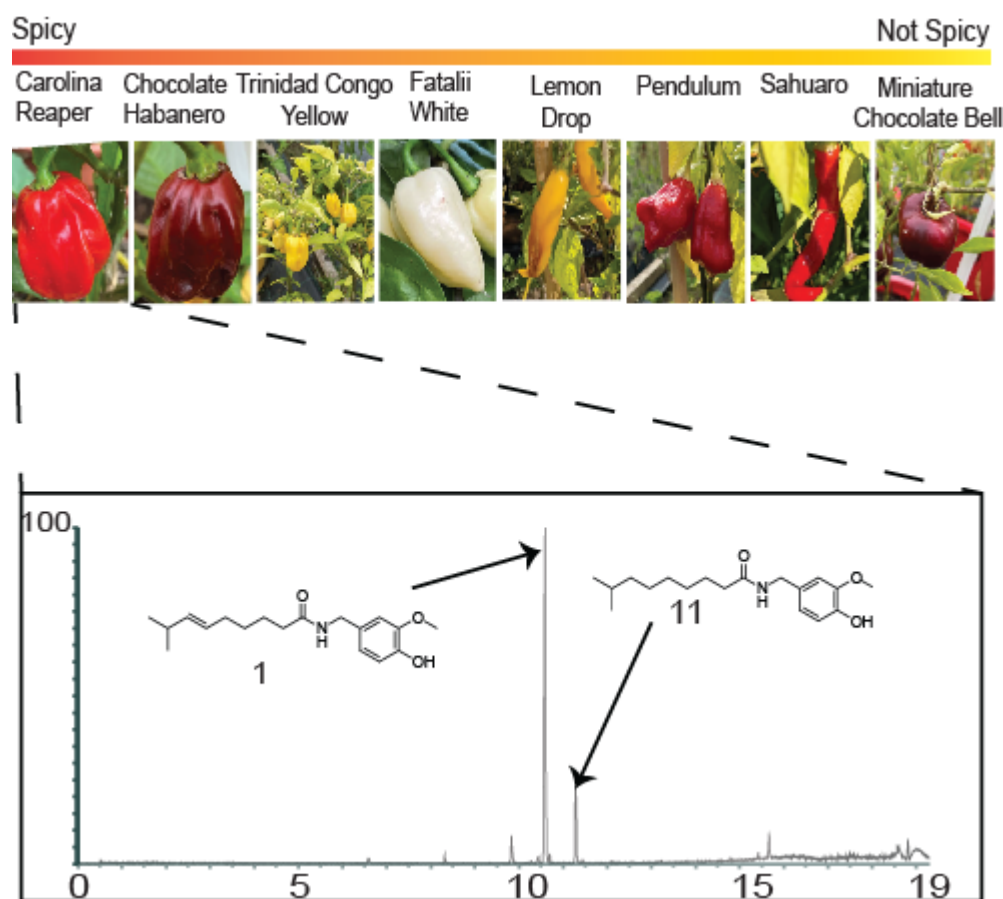


Figure 13 Example of collected chilis and chromatogram. A wide range of varieties with different SHU were collected. The complete list can be found in **Chapter 8, Table 3**. The spiciest chili used was the Carolina Reaper, which at the time of testing had the highest rating of SHU in the world. The Miniature Chocolate Bell variety was one of the few non-pungent varieties. Example UPLC-MS/MS chromatogram of Carolina Reaper extract.

CANOPUS identified the largest molecular class as “N-acyl amines”, precursors corresponding to the capsaicinoid tail, with 12.3% of features identified belonging to that group (**Figure 14 A**). Precursors corresponding to the vanilloid head, phenylethylamines, simple phenolic acids and derivatives of cinnamic acid, counted for about 1-2% of all features (**Figure 14 A**). Capsaicinoids were also one of the largest predicted groups present with 6.85% of feature coverage (**Figure 14 A**). Utilizing features predicted to be capsaicinoids, varieties were clustered together based on feature abundance and visualized with a heat map (**Figure 14 B**). While there was some intra-species clustering, we did not observe any absolute grouping based on a species’ capsaicinoid profile. Most capsaicinoid profiles were unique to the variety; however, capsaicin and then dihydrocapsaicin were typically the most abundant. The variety Lemon Drop was an exception, as it contained more dihydrocapsaicin than capsaicin.

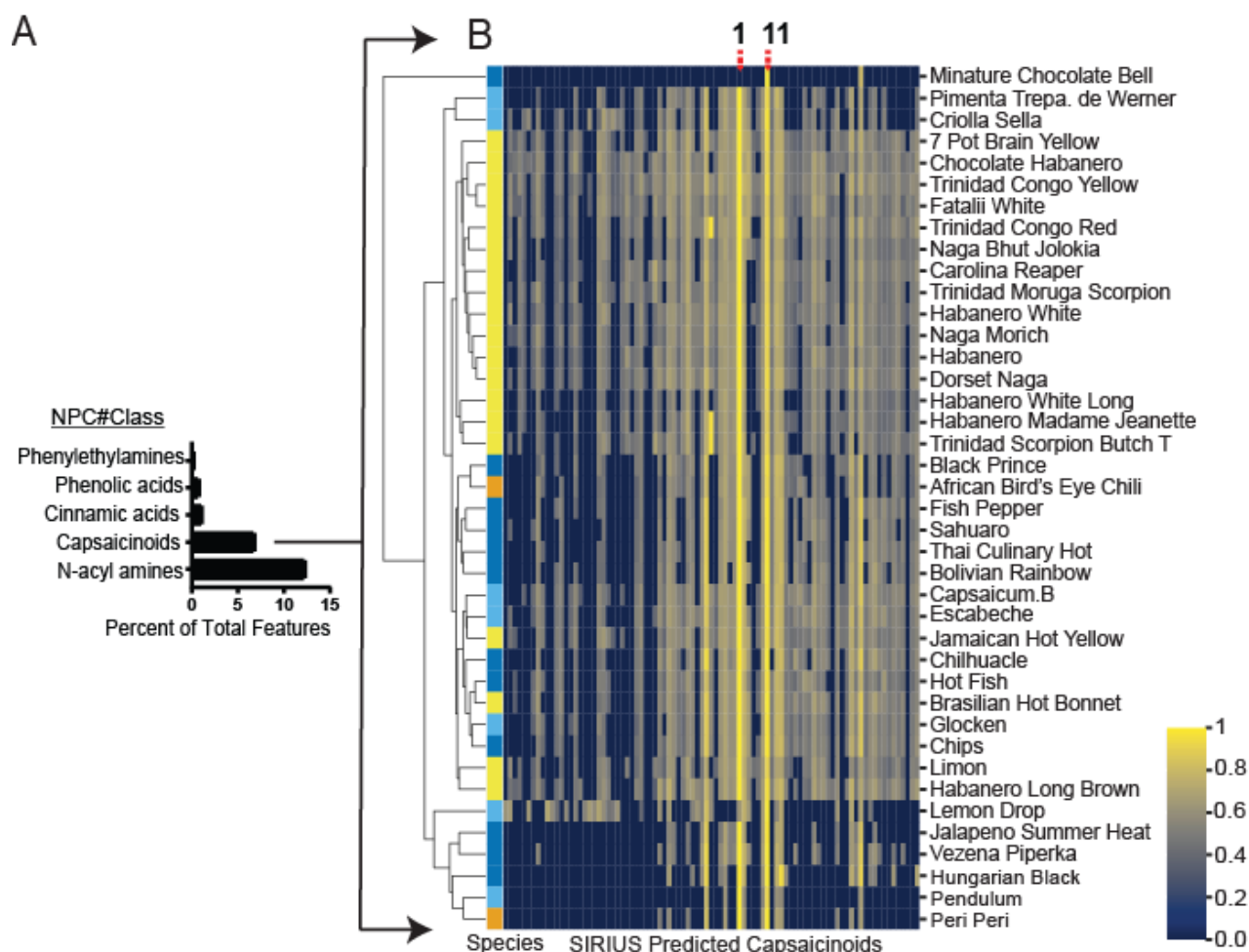


Figure 14 Exploration of untargeted data with SIRIUS and python (A) Five important classes from the CANOPUS predictions as a percentage of all features identified. (B) Clustering and heatmap of all metabolites predicted to be capsaicinoids by CANOPUS. Compound 1 — capsaicin and compound 11 — dihydrocapsaicin are highlighted. Rows were normalized by taking the $\log_{10}$ of each peak area and then dividing it by the most abundant feature per row (i.e. in a

variety). The resulting normalized scale is from 0 to 1. Species are color-coded as follows: yellow (#F0E442) – *C. chinense*, blue (#0072B2) – *C. annuum*, sky blue (#56B4E9) – *C. baccatum*, orange (#E69F00) – *C. frutescens*.

Lastly, a feature-based molecular network was created through the GNPS platform (Nothias et al., 2020), which was used to identify novel capsaicinoids (**Figure 15**). We focused on the network that contained features predicted to be capsaicinoids by CANOPUS. Nodes were further confirmed by checking for the vanilloid head MS/MS fragment of $m/z = 137$ (Wu et al., 2019). This network was leveraged to select targets for isolation based on MS/MS fragmentation indicative of hydroxylation, tail length, or dimerization. These compounds were targeted based on the assumption that they could produce a wide range of TRPV1 activations.

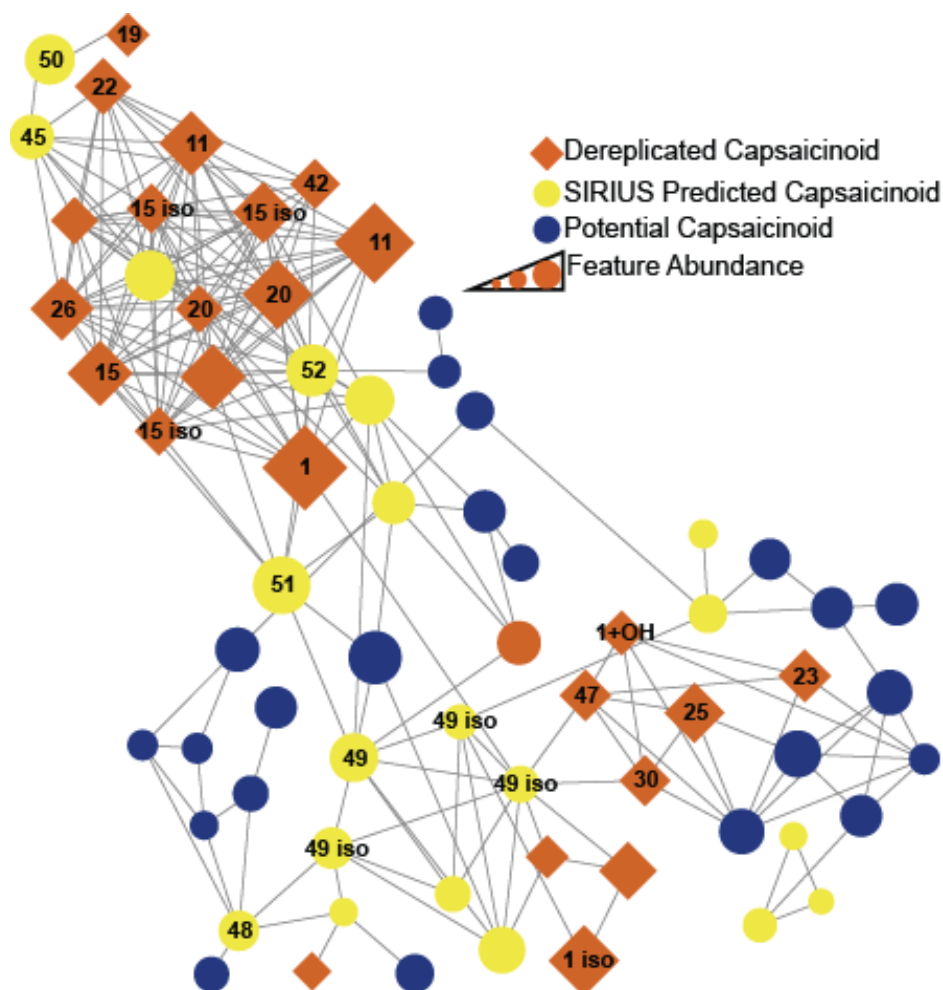


Figure 15 Capsaicinoid network from crude variety screening. Nodes that contained a predicted chemical formula from SIRSUS that matched a chemical formula of a known capsaicinoid were labeled as a dereplicated capsaicinoid. Compounds labeled **iso** had the same mass of a known capsaicinoid but a different retention time. Nodes labeled with **+OH** showed in-source fragmentation indicative of hydroxylation. Potential capsaicinoids were nodes that may have had a fragment ion indicative of a vanilloid head, but also other fragments that possibly suggest a different molecular class.

4.2 Isolation of capsaicinoids

To isolate target capsaicinoids, extraction was scaled up using pooled varieties of chilis that contained the target compounds. Chilis were macerated in EtOAc, followed by normal-phase chromatography and then reverse-phase chromatography to isolate individual compounds. Structures were confirmed with NMR analysis (**Figure 16 A & B**). In total, 18 capsaicinoids were isolated, five with a novel structure (**Figure 16 C** and **Chapter 8, Table 2**). Two of the 18 structures were determined using a mixture of NMR (**Chapter 7**) and MS/MS fragmentation (**Chapter 9**) compared to the confirmed molecules. Ultimately, 26 out of 50 nodes (52%) were annotated with a high degree of confidence (**Figure 16 D**).

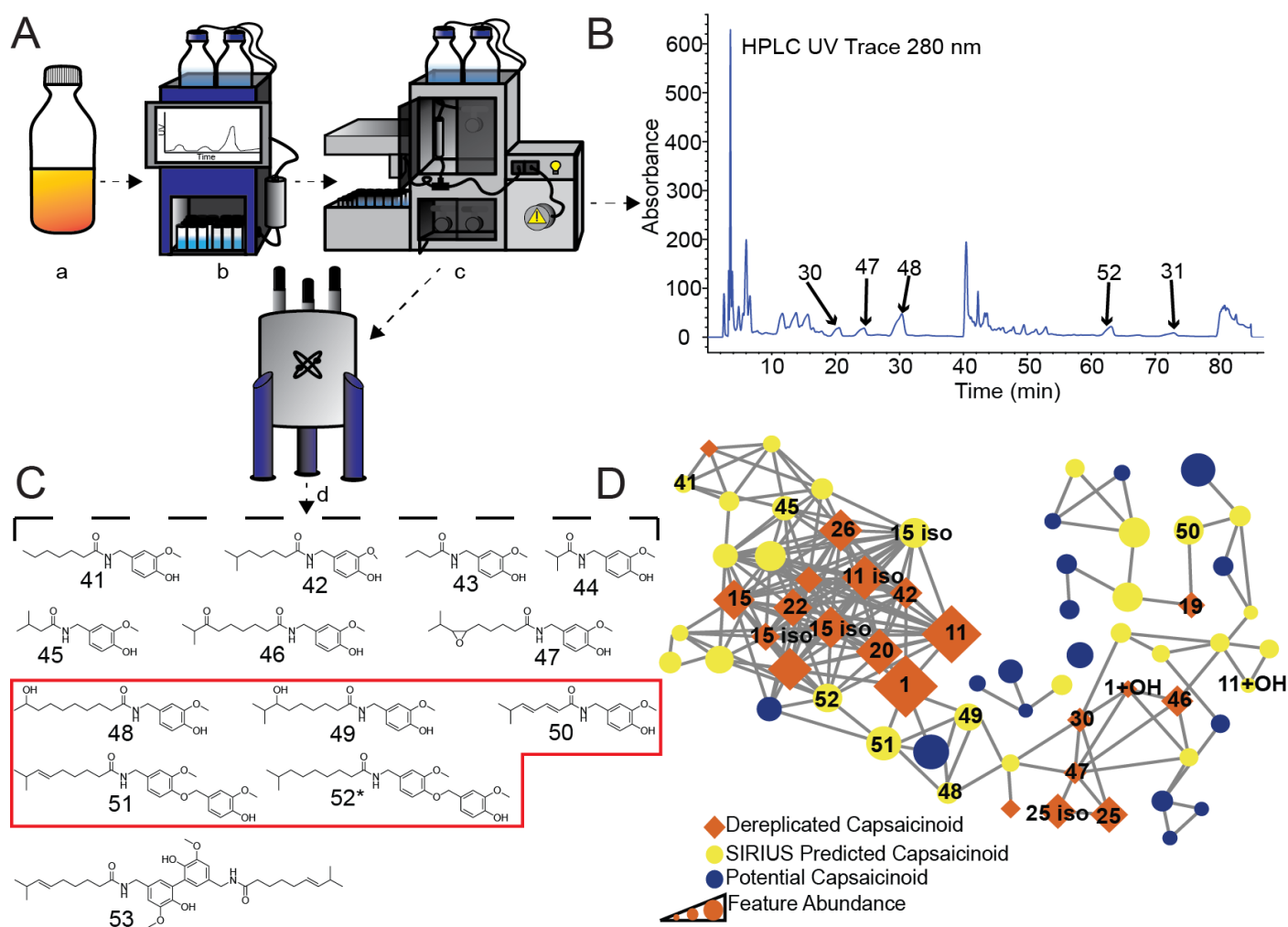


Figure 16 *Isolation of capsaicinoids* (A) Workflow of capsaicinoid isolation: A liquid extraction of chilis (a) was first fractionated by normal-phase flash chromatography (b), then target capsaicinoids were isolated on HPLC (c) and finally the structure was confirmed with NMR (d). (B) HPLC chromatogram from one of the pooled flash fractions highlighting the isolation of individual compounds. (C) Structures of the capsaicinoids isolated in this thesis, the red box highlights

structures that are novel. (D) The molecular network of the pooled chili extraction. Some capsaicinoids were not in this network but are found in smaller networks (not shown).

4.3 rTRPV1 activation assays

A series of rTRPV1 activation assays were performed with a cell line gifted to us from the Whitehead Institute for Biomedical Research (Boston, MA, USA) (Smith et al., 2024). The cell line is based on a Flp-In T-REx-293 system that contains the rTRPV1 gene, which is under a tetracycline repressor and upon the addition of a small molecule (i.e., doxycycline) it induces the expression of rTRPV1. This cell line was coined irTRPV1-FlpIn293. The rTRPV1 response was measured using fluo-4 dye, which binds to intracellular calcium upon rTRPV1 activation. The functionality of the cell line was evaluated using both immunochemical staining with imaging from confocal microscopy and a functional response assay with capsaicin (**Figure 17**).

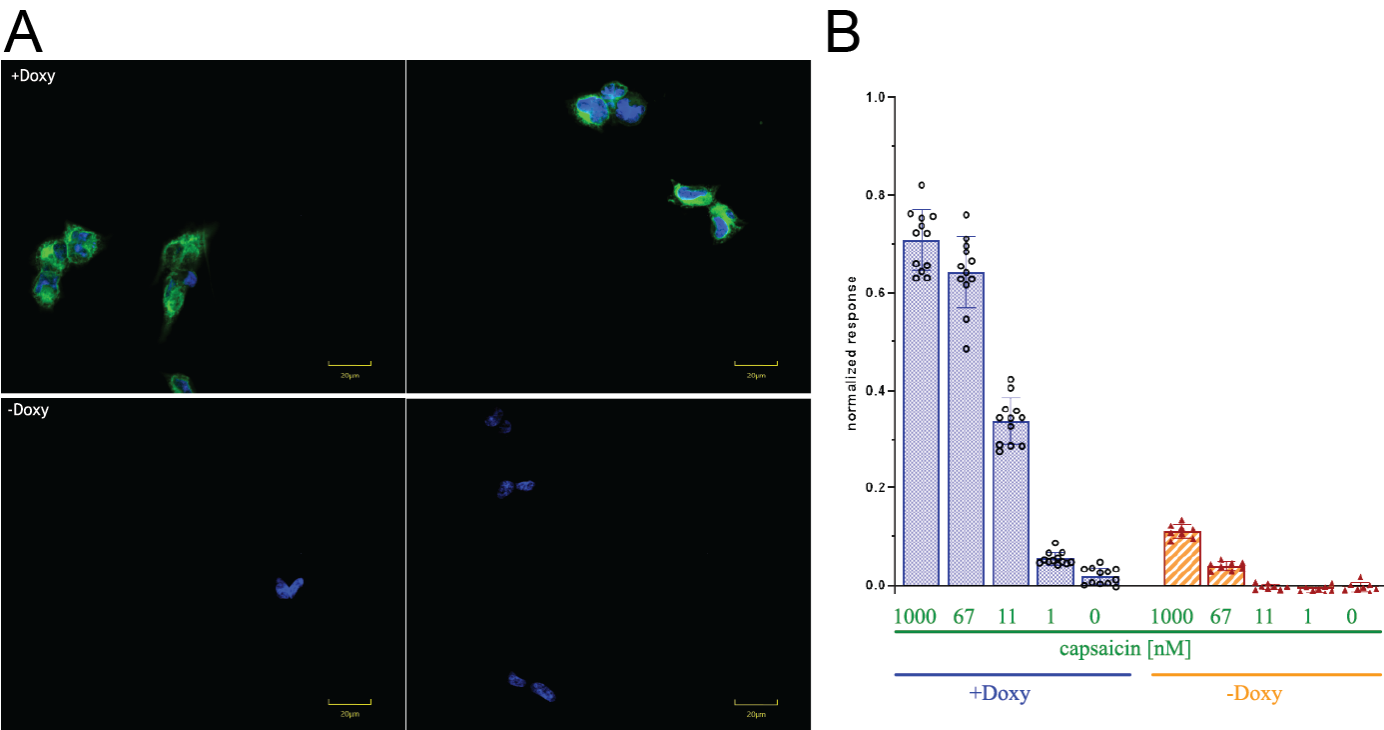


Figure 17 Different experiments testing the functionality of the rTRPV1 receptor. (A) rTRPV1 antibody staining of the irTRPV1-FlpIn293. The top row of cell images shows irTRPV1-FlpIn293+Doxy (induced expression), while the bottom row shows -Doxy. Blue staining is of the nucleus with DAPI, and the green signal is rTRPV1 (Santa Cruz sc-398417 (1:200, 1 hour, RT) + Abcam ab150115 (1:800, 1 hour, RT)) (B) Calcium influx response with different concentrations of capsaicin: the left side in blue is rTRPV1 expressing, while the right side in red is without induced rTRPV1.

4.3.1 Crude extracts

Most experiments completed were using the top of the response curve (**Figure 18 A**) to build either dose- or concentration-response curves. We were curious to determine if any correlation existed between capsaicinoid amounts (i.e., sum of all MS peak areas in each variety) and the median effective dose (ED_{50}) values. We speculated that if capsaicinoids are additive, there should be a positive trend between these two values. When correlating all CANOPUS predicted capsaicinoid features of a variety to its ED_{50} , there was a strong Pearson correlation of 0.86, thus suggesting a possible additive effect of capsaicinoids (**Figure 18 B**). All data for sum intensity and ED_{50} values can be found in **Chapter 8, Tables 3 & 4**.

Expanding on the previous comparison, other groupings of features were compared to the ED_{50} values. Keeping only capsaicin and dihydrocapsaicin, a similar correlation was obtained, Pearson = 0.85 (**Figure 18 C**). To determine how much capsaicin and dihydrocapsaicin may have been influencing the original correlation of all capsaicinoids (**Figure 18 B**), we kept all capsaicinoids but removed all feature areas corresponding to capsaicin and dihydrocapsaicin (**Figure 18 D**). To determine this, even without those two compounds, a strong relation between capsaicinoid content and ED_{50} values remained, Pearson = 0.77. Lastly, all feature areas were grouped together (**Figure 18 E**) with or without capsaicinoids (**Figure 18 F**). Both situations produced strong correlations: all features — Pearson = 0.78 and all features without capsaicinoids — Pearson = 0.71.

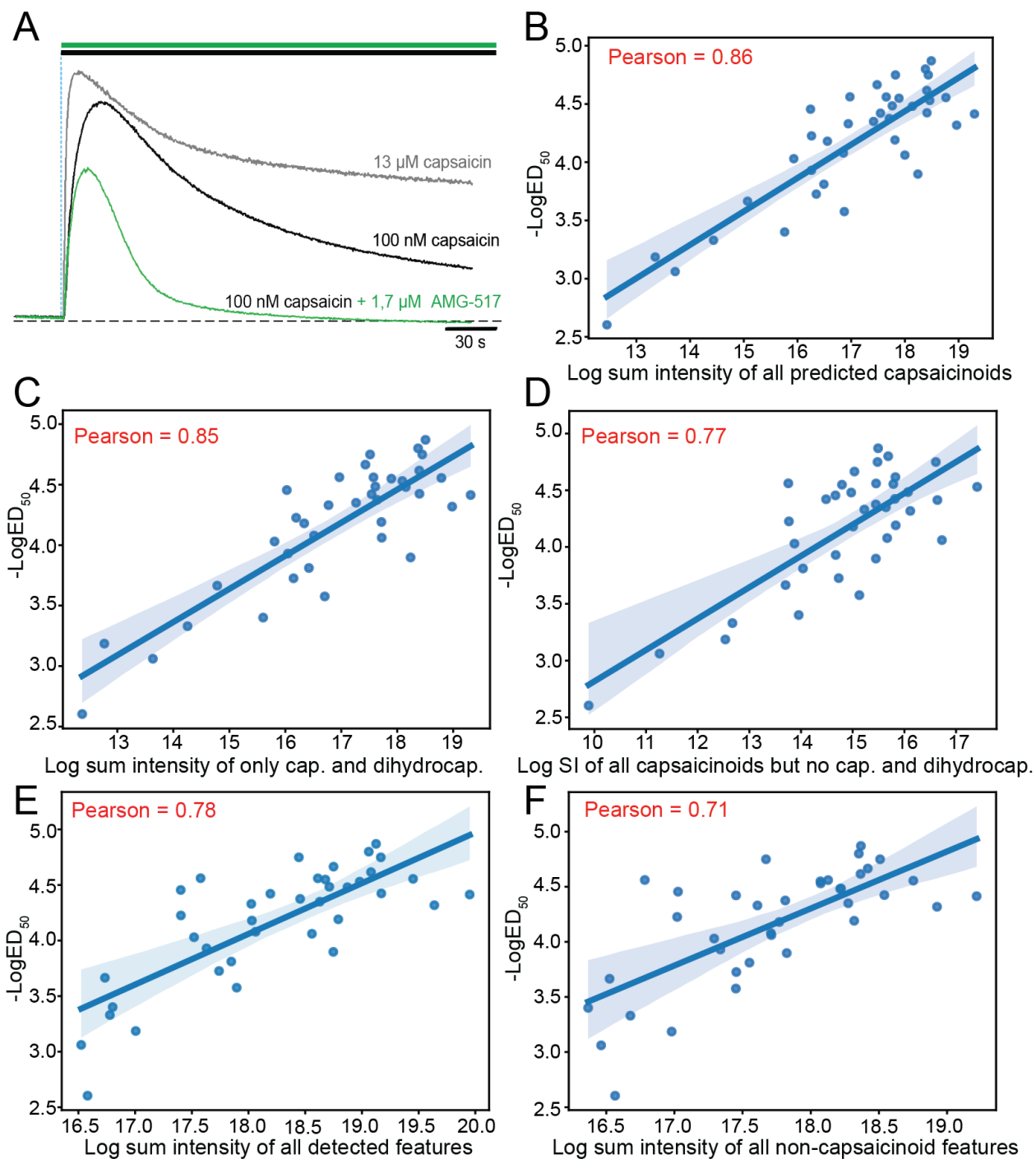


Figure 18 *Traces and dose curve experiments* (A) Example traces that were used for all subsequent experiments, tops of responses were used to build dose-response curves. All compounds or mixtures were applied contemporaneously. (B) Pearson correlation of crude extract capsaicinoid content compared to their corresponding $-\text{LogED}_{50}$ values. Each point on the curve is one chili variety, the x-axis is the sum intensity of the MS peak area for all predicted capsaicinoids, and the y-axis is $-\text{LogED}_{50}$. Varieties S7 and S25 were removed from data analysis; this removal did not change the overall positive slope of the line. All sum intensity data can be found in **Chapter 8, Table 3** and all crude extract $-\text{LogED}_{50}$ values can be found in **Chapter 8, Table 4**. Other graphs use different combinations of the Log sum intensity of different feature groupings within a given variety compared to its corresponding $-\text{LogED}_{50}$. (C) Comparison of MS sum feature area for only capsaicin and dihydrocapsaicin, Pearson = 0.85. The sum area of these two features consists of $[\text{M}+\text{H}]^+$, $[\text{M}+\text{Na}]$, $[\text{M}+\text{ethylamine}]^+$ and $[2\text{M}+\text{H}]^+$ adducts. (D) This plot is similar to panel B, but all peak areas corresponding to capsaicin and dihydrocapsaicin were removed, leaving all other capsaicinoids, Pearson = 0.77. (E) This curve uses all features found in each variety, Pearson = 0.78. (F) This is a similar correlation to panel E, but all capsaicinoid feature areas were removed, Pearson = 0.71.

4.3.2 Individual compounds

After testing crude extracts, individual capsaicinoids, both purchased and isolated, were tested on rTRPV1 activation. All EC_{50} values obtained from the experiments can be found in **Chapter 8, Table 2** and more detailed data on the curves can be found in **Chapter 8, Table 5**. Capsaicinoids tested had a wide range of rTRPV1 activation; several were selected and highlighted in **Figure 19 A**. Compounds that contain similar structures (i.e., similar number of carbons and saturation), such as nordihydrocapsaicin (**20**), had similar EC_{50} values to capsaicin (**Figure 19 A & B**). Compounds that have a modification to the vanilloid head (**53**) or have a shorter acyl tail (**43**) had reduced potency to rTRPV1 when compared to rTRPV1 (**Figure 19 A & B**). Lastly, compounds with a tail longer than capsaicin, capsaicinoid **9**, had similar potency to capsaicin but reduced efficacy (**Figure 19 A & B**). All compounds were tested in combination with AMG-517, a known antagonist of rTRPV1. Data indicated that the antagonist was successfully blocking the capsaicinoid from activating the receptor (**Figure 19 C**).

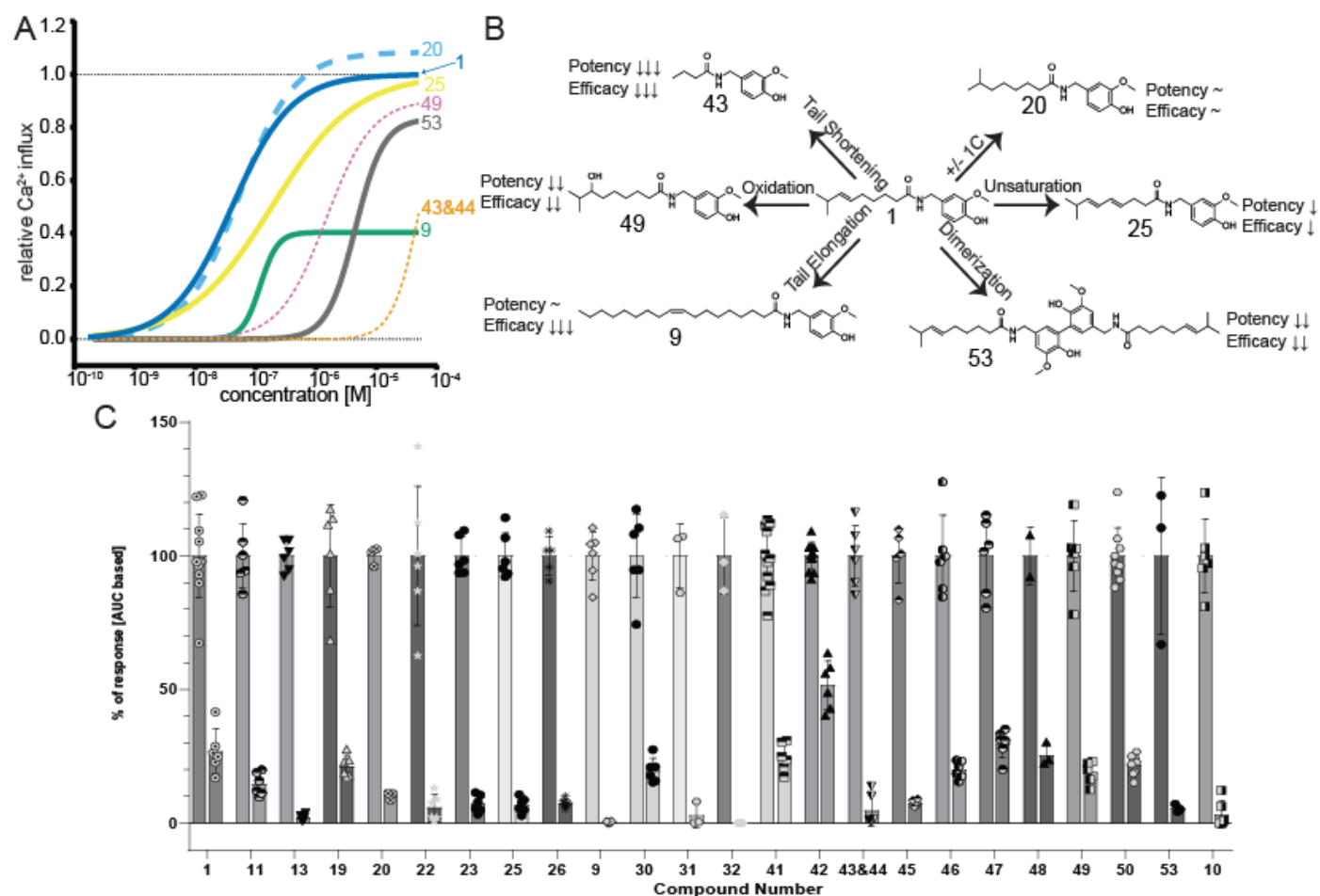


Figure 19 Individual capsaicinoids *rTRPV1* activation and inhibition. (A) Select curves that highlight the wide range of *rTRPV1* activation due to differences in capsaicinoid structure. All EC_{50} values can be found in **Chapter 8, Table 2** and all other information about the curves can be found in **Chapter 8, Table 5**. (B) Summary of how changes in capsaicin's structure affect *rTRPV1* activation. (C) All compounds were mixed at a concentration close to their EC_{50} value with 1.7 μM AMG-517. Cells were also treated without AMG-517 for comparison. Unlike other experiments, the area under the response curve was used for inhibition evaluation. As both the capsaicinoid and inhibitor were added contemporaneously, the capsaicinoid may have outcompeted the inhibitor in certain cases.

4.3.3 Modulation assays

Lastly, to determine if capsaicinoids were interacting with each other, non-potent to non-activating analogs and related capsaicinoids were tested in combination with capsaicin. To determine the working concentration, capsaicin at its EC_{10} , EC_{50} and EC_{90} was tested in combination with a full-dose response curve of a related capsaicinoid (Data not shown). Curves of capsaicinoids with capsaicin added to them were compared to curves without to determine the modulation amount. Following this, a full capsaicin dose-response curve was then tested with determined modulating amounts of analog. Most non-active compounds resulted in positive modulation of capsaicin rTRPV1 activation (**Figure 20**). Vanillin (compound **35**), a small precursor to capsaicin biosynthesis, produced the largest modulation with capsaicin being 10x more potent when added (**Figure 20**). All information on modulation assays can be found in **Chapter 8, Table 6**.

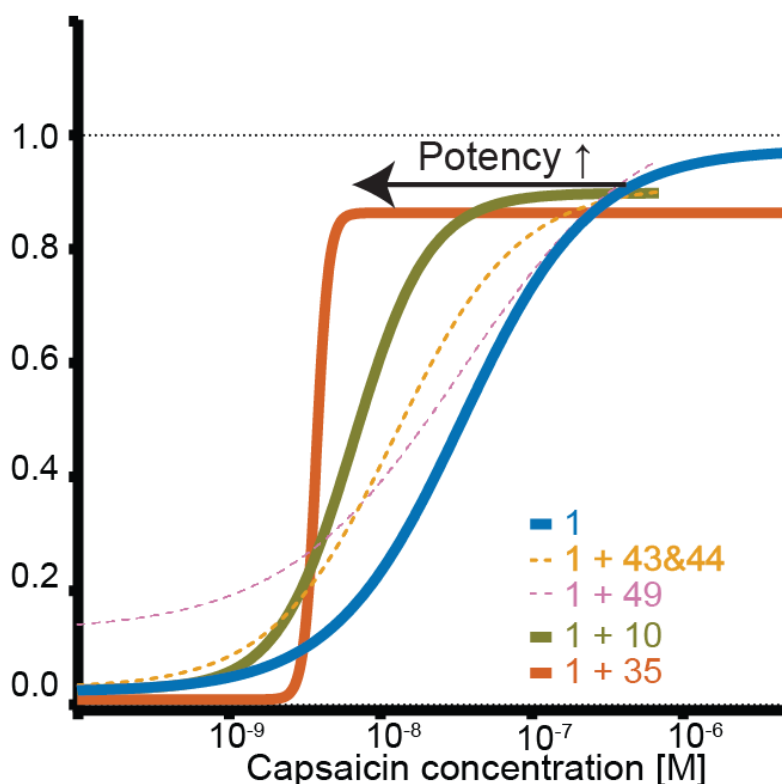


Figure 20 Dose-response curves of capsaicin in combination assay. Most non-active to low-potency analogs or related capsaicinoids acted as positive modulators of capsaicin. Concentrations of capsaicinoids that were mixed with capsaicin: **43** and **44** – 1.7 μ M, **49** – 0.2 μ M, **10** – 1.7 μ M, **35**– 1 μ M. Detailed information on curves can be found in **Chapter 8, Table 6**.

5. Discussion

5.1 Untargeted metabolomic exploration of novel vanilloids in chilis

It was not surprising that the N-acyl amines are some of the most abundant compounds in the chili extracts as these molecules are precursors to the capsaicinoid tail (**Figure 9 & 10**). Conversely, there were only a few compounds that were predicted to be phenylethylamines, simple phenolic acids and derivatives of cinnamic acid; molecules that correspond to capsaicinoid head. We speculate that vanilloid head-like molecules may be the limiting factor in the biosynthesis and diversification of capsaicinoids. This idea is supported by a previous study which found that phenylethylamines are quickly converted into capsaicinoids (Ogawa et al., 2015). Surprisingly, there were no molecules predicted to be capsinoids.

Capsinoids are structurally related compounds to capsaicinoids and are also found in *Capsicum*. The first discovered capsinoid was capsiate (compound **10**) from a non-pungent variety of *C. annuum* (Kobata et al., 1998b). The difference between capsaicinoids and capsinoids is based on the connection to the vanilloid head: capsaicinoids are connected from their acyl tail to the vanilloid head by an amide bond while capsinoids are condensed together to form an ester bond. Because of the ester bond, capsinoids are more susceptible to degradation in polar solvents but should be stable in non-polar solvents like our extraction solvent ethyl acetate (Sutoh et al., 2001). Thus, the lack of capsinoids in our sample should not be caused by the extraction solvent. As mentioned, the pAMT to produce vanillyl alcohol (i.e., capsinoid precursor) may require a point mutation (Lang et al., 2009); however, trace amounts of vanillyl alcohol were found in some varieties (1000-fold lower signal intensity than a 1 mM standard). This would support the hypothesis that a CAD is needed for vanillyl alcohol formation as it was found in the same sample along with capsaicinoids. One last consideration is that capsinoids easily fragment in-source under our ionization methods. Capsiate's base peak signal is based on a vanilloid head fragment and contains only a small $[M+H]^+$ peak (**Chapter 8, Table 2**). Since capsinoids easily fragment in-source, it may result in too low intensity or a nonexistent $[M+H]^+$ peak, which would result in no MS/MS detection and no subsequent CANOPUS prediction nor molecular networking. Tuning a particular ionization method for capsinoids should be considered for future studies.

When exploring the molecular network in **Figure 15**, several nodes were found that indicated most alterations of the capsaicinoid structure occurred on the tail, which is expected based on the diversity of N-acyl amines and what has been published in previous studies. Upon completion of this study, 28 out of the 70 nodes (40%) were annotated. Other unannotated capsaicinoids likely reflect further variations of the molecules identified in this thesis.

5.2 Isolated capsaicinoids

As previously stated, 18 capsaicinoids were isolated, five with a structure reported for the first time, and several other structures that were only obtained through synthesis (see **Figure 16**). As expected, these compounds varied with different degrees of tail length, hydroxylation, and —surprisingly— some with a modified vanilloid head. In total, 26 out of 50 nodes (52%) were annotated. As with the previous network, the remaining unannotated nodes are likely capsaicinoids with varying tail length, saturation, or hydroxylation.

Capsaicinoids **19**, **22** and **41–45** were expected to have different tail lengths based on SIRIUS formula prediction and manual MS/MS spectral investigations, which were confirmed with the isolation of targets and NMR analysis. Both capsaicinoids **19** and **22** have been previously identified (Gannett et al., 1988; Wang et al., 2020b), and capsaicinoids **41**, **44** and **45** have only been chemically synthesized and have never been reported from a chili species (Ježo, 1975; Nelson, 1919; F. Yang et al., 2015). Capsaicinoid **43** was previously identified through enzymatic synthesis (Kobata et al., 1998a). Only capsaicinoid **42**, which was synthesized, had tentative identification in *C. chinense* and *C. annuum* without any NMR or reference standard confirmation (Buitimea-Cantúa et al., 2020; Stipcovich et al., 2018; Sweat et al., 2016; Takahashi et al., 1976).

Several capsaicinoids had an oxidized tail: **30**, **46**, **47**, **48**, and **49**. The first identified hydroxylated capsaicinoid, ω -hydroxycapsaicin **30**, was first reported as a product of capsaicin P450 oxidation (Reilly et al., 2003) but was later isolated from *C. annuum* (Ochi et al., 2003). More recently, ω -hydroxycapsaicin was found to be a product of microbial transformation of capsaicin (Deng et al., 2022). Similar to ω -hydroxycapsaicin, capsaicinoid **46** was linked to human cell line oxidation, while capsaicinoid **47** has only been known to be a product of microbial P450 processing or other epiphytic modulation (Ma et al., 2021; Migglautsch et al., 2018; Xin et al., 2017). To the best of our knowledge, capsaicinoids **48** and **49** are structures first reported in this project.

Two capsaicinoids contained an additional unsaturation, capsaicinoid **25** and **50**. Capsaicinoid **50** is firstly reported in our work, while capsaicinoid **25** was previously found in *C. annuum* (Wang et al., 2020b).

Several nodes identified contained possible modifications to the vanilloid head, but most of those compounds were low abundant and contained MS/MS spectra with hard-to-generate structures. Conversely, capsaicinoids **51** and **52** were isolated for the first time and contained a unique double vanilloid head structure. We postulate that these capsaicinoids are likely enzymatic products as photocatalyzed products would resemble insertion-like capsaicinoids as in **31**. Capsaicinoid **51** was confirmed with NMR, while capsaicinoid **52** was confirmed with MS/MS annotation based on capsaicinoid **51**. Ultimately, while the compounds were identified in multiple harvests, the structure is inherently unstable based on NMR and MS measurements.

Lastly, two dimers, capsaicinoids **31** and **53**, were isolated. Capsaicinoid **53**, 5,5'-dicapsaicin, structure was determined through NMR, while capsaicinoid **31** was determined based on MS/MS differences. Capsaicinoid **53** has never been reported from direct *Capsicum* isolation, however, it has been found as a product of different reactions (Tateba and Mihara, 1991)(Bernal and Barceló, 1996)(Henderson et al., 1999)(Reilly et al., 2013; Tian et al., 2019)(Martínez-Juárez et al., 2004).

We tried to determine the absolute configuration of chiral capsaicinoids **30**, **48** and **49** (Data not shown) using standard circular dichroism experiments. No absolute structures were confirmed from the experiments, which was likely caused by the flexibility between the chromophore and the chiral center itself(Saito and Schreiner, 2020).

Finally, all capsaicinoids and standards were tested for purity, which can be found in **Chapter 8, Table 2**. Most of the compounds were found to have a purity of 90% or greater. Impurities consisted of unknown unrelated compounds, isomers of the target compound or capsaicinoids (about 100-1000 times less intense than the target compound, assuming they have similar ionization efficiency). Of concern, the dimers did indicate degradation into monomers, which may influence subsequent EC₅₀ values. The most concerning was capsiate, which was analyzed to be 50% purity, while the manufacturer reported > 94% (Alomone Labs). QC testing occurred several months after initial rTRPV1 assaying and the capsiate is known to be unstable in DMSO (Sutoh et al., 2001), the testing and storage solution. We suggest in future studies to use an alternative solvent or to prepare capsinoid samples at the time of testing.

5.3 TRPV1 activation assays

We tested all crude extracts, isolated compounds and several purchased standards on the rTRPV1 assay. rTRPV1 shares high homology with human TRPV1 (hTRPV1), with 83% sequence similarity (Neuberger et al., 2023). Furthermore, rTRPV1 and hTRPV1 respond similarly to capsaicin activation. Noticeable differences between rTRPV1 and hTRPV1 are that rTRPV1 is less sensitive to the antagonists capsazepine and ruthenium red (McIntyre et al., 2001). While all results discussed below should be repeated on the hTRPV1 receptor to confirm activity, our results should be comparable to hTRPV1 activity based on the aforementioned.

5.3.1 TRPV1 activation by crude extracts

Most of the crude extracts resulted in rTRPV1 activity with a response of 60% or greater; however Miniature Chocolate Bell (S25) elicited only partial activation at highest concentration tested. This slight

activation can be due to a lower capsaicinoid concentration; however, the inclusion of an inhibitor or negative modulator in the mixture cannot be ruled out.

The positive correlation comparing crude extract activity to capsaicinoid concentration, **Figure 18 B**, indicated that there may be some additive effects from all capsaicinoids present. A positive correlation was found even when different MS feature groupings were compared, indicating that there may be non-vanilloids influencing rTRPV1 activation or that, in general, spicier varieties are more metabolically active than non-pungent varieties. Subsequent studies should consider increasing both the number of varieties and replicates to obtain a more jointly normally distributed representation (Schober et al., 2018). This correlation was taken only as a suggestion that there may be an additive effect; thus, capsaicinoids were tested in combination to confirm this speculation.

5.3.2 TRPV1 activation by individual compounds

Capsaicinoids' least susceptible region of change is the acyl tail moiety; however, it is known that the shortening of the tail does reduce potency (Yin et al., 2019). Following the tail, the amide neck can tolerate changes, but alterations can affect binding and potency (Vu et al., 2020; Yin et al., 2019), which is exemplified by molecules like piperine (Dong et al., 2019; McNamara et al., 2005) or gingerols (Dedov et al., 2002; Yin et al., 2019). The most sensitive region to change is the vanilloid head (Walpole et al., 1996). Despite the extensive structural-activity relationship of capsaicinoids and TRPV1 and the many studies utilizing synthetic analogs (Lee et al., 2004, 2003; Park et al., 2004; Ryu et al., 2008), naturally occurring capsaicinoids have remained underexplored. This thesis has helped fill in those gaps in natural capsaicinoid rTRPV1 activation (**Chapter 8, Table 2**).

Of the 24 compounds tested, excluding capsaicin, only six of them have been previously screened for TRPV1 activity. Our literature studies indicated that capsaicinoids **22**, **41** and **43** have been previously tested for TRPV1 activation (Yin et al., 2019). Capsaicinoids **11**, **26** and **9** were purchased standards that have been previously tested, but on dorsal root ganglia, which express TRPV1. The dorsal root ganglia testing produced slightly higher median effective concentration (EC_{50}) values of 0.19 μ M, 0.55 μ M and 0.17 μ M (Walpole et al., 1993). Differences in our results from their experiments are expected since testing occurred on a different system. Lastly, capsaicinoids **23** and **26** have already been tested in a $^{45}\text{Ca}^{2+}$ uptake assay which used rTRPV1-expressing Chinese hamster ovary cells and resulted in EC_{50} values of 0.061 μ M and 0.072 μ M, respectively (Cho et al., 2012). Differences in EC_{50} values between our results and the previous study differed almost threefold in magnitude, but still within an acceptable range considering differences in the system and experimentation. To the best of our knowledge, there have been no other tests of capsaicinoids reported herein.

5.3.2.1 Modification of the tail

It is well established that the vanilloid head is key for later stages in TRPV1 gating (Chu et al., 2020; Dong et al., 2019; Fan Yang et al., 2015), but the tail is needed to both initiate the binding of capsaicin and stabilize this open state (Fan Yang et al., 2015). Capsaicin's tail was found to first enter towards the S3 and S4 segments of the binding site (Fan Yang et al., 2015; Yang and Zheng, 2017), followed by the binding of the neck which allows the vanilloid head to interact with the S4-S5 linker (Vu et al., 2020; Yang and Zheng, 2017; Yin et al., 2019). The binding to the vanilloid head initiates a movement of the S4-S5 linker which is coupled to the movement of the S6 helix, with which the capsaicin tail interacts and thus stabilizes the opening (Fan Yang et al., 2015; Yang and Zheng, 2017).

Several capsaicinoids (**11**, **25** and **26**) shared the same number of carbons as capsaicin but contained changes in connectivity or saturation, which resulted in a small loss of TRPV1 activity (**Figure 19 A&B**). Since these changes do not greatly affect the overall tail polarity, van der Waals interactions needed for channel stabilization remain relatively unperturbed. Conversely, capsaicinoids (**30** and **46-49**) that contained the same number of carbons but were hydroxylated had EC₅₀ values reduced by 10- to 150- fold depending on the location of the hydroxylation. Since the TRPV1 channel opening is stabilized by the hydrophobic tail and requires S6 helix movement for activation, it is likely that the polar hydroxyl group reduces the van der Waals interactions and limits the movement of the S6 helix. Capsaicinoids (**13**, **20** and **26**) which differed by a single carbon atom had comparable EC₅₀ values to capsaicin, 1-6 times less potent. Of the compounds, nordihydrocapsaicin had the most similar EC₅₀ value (0.05 μ M) to capsaicin and dihydrocapsaicin (**Figure 19 A&B**).

Other tail differences were marked by capsaicinoids with 2-5 fewer carbons and changes in connectivity, which resulted in 15-fold lower activity to almost non-active molecules. N-vanillyl octanamide (**22**) was the only exception with three to five times less potency than capsaicin. Interestingly, capsaicinoids **9** and **43/44**, both differed by six carbons when compared to the capsaicin structure and shared partial antagonism to TRPV1; however, capsaicinoid **9** had similar potency to capsaicin. It is known that short-tailed capsaicinoids, like **43** and **44**, are unable to achieve the same opening and stabilization as capsaicin (F. Yang et al., 2015). Capsaicinoid **9**'s (olvanil) bulky tail may result in an unfavorable conformation inside the binding pocket which reduces the interaction and stabilization with the S6 helix (Vu et al., 2020). Ultimately, EC₅₀ values obtained for individual capsaicinoids were appropriate based on previous literature findings.

5.3.3 TRPV1 modulation assays

Often, capsaicin-containing products are sold as mixtures of capsaicinoids and not solely capsaicin. The capsaicinoid mixture will vary greatly in both amount and composition depending on the variety and the chosen extraction method. Since a trend between capsaicinoid content and ED_{50} values was observed, a series of experiments were conducted to determine if capsaicinoids act in a synergistic manner. Modulation experiments consisted of adding a non-activating or low-potency capsaicinoid or analog to a capsaicin with a full dose-response curve (**Chapter 8, Tables 3 & 4**). Capsaicin curves with potential modulators were compared to the capsaicin curve without. Depending on the left shift of the curve with the modulator, we considered the effects to be either additive or synergistic (**Chapter 8, Table 6**) (Tallarida, 2011; Zhao et al., 2010). Our results suggest that, in general, less potent analogs tend to act as positive modulators and increase the overall potency of capsaicin. In particular, compounds **43**, **44**, **10**, and **35** resulted in the greatest modulation of capsaicin rTRPV1 activation (**Figure 20** and **Chapter 8, Table 6**).

It was previously shown that a similar capsaicin modulation from ATP was linked to G-protein-coupled purinergic receptors (Tominaga et al., 2001). However, it is unlikely that our modulation is a result of non-TRPV1 interactions. Since our irTRPV1–FlpIn293 overexpressed rTRPV1, it was expected that the amount of rTRPV1 would outweigh any endogenous expression of other TRPV1-modulating proteins. Furthermore, only the tops of traces were considered (**Figure 18 A**); therefore, we were investigating immediate responses from capsaicinoid activation. While we do not attribute TRPV1 modulation to other receptors, further studies are needed to confirm this.

TRPV1 channel opening begins with the binding of the vanilloid head of capsaicin ((Jara-Oseguera et al., 2019; Yang et al., 2018); however, a single molecule cannot fully activate the channel on its own (Liu et al., 2019). Furthermore, it has been found that the channel gating is key on the cytosolic side for full opening and ion permeability (Jara-Oseguera et al., 2019; Yang et al., 2018). Because of the importance of opening at the receptor's gate, it may be possible for a single molecule of capsaicin to evoke a full channel response if there is the presence of another vanilloid that can influence the initial gating. TRPV1 is also an allosteric protein, and in general, the family of TRPV proteins is known to share 16 different ligand-binding sites (Yelshanskaya and Sobolevsky, 2022). While it is likely that these small molecules are interacting in the known vanilloid pocket, it cannot be completely ruled out that these small molecules are interacting with a known or unknown allosteric site. Ultimately, it appears that these small non-pungent vanilloids, compounds **43**, **44**, **10**, and **35**, either collectively aid the facilitation of the channel opening or increase its stability in the open state through the vanilloid head. In future studies, the exact mechanism of this positive modulation needs to be further investigated.

6. Conclusions

Untargeted metabolomic studies have been previously combined with biological readouts, but the combination of untargeted metabolomics to identify novel ligands with a specific biological target remains a relatively underexplored area. A novel pipeline was applied for the investigation of TRPV1 activating capsaicinoids and resulted in the creation of the single largest library of naturally occurring vanilloids from *Capsicum sp.* Most capsaicinoids contain an unmodified vanilloid head with variations mostly occurring on the tail either by varying length or with the presence or absence of oxidation or double bonds. We did identify two capsaicinoids with a modified head, but these molecules were unstable based on repeated analysis and were not further investigated.

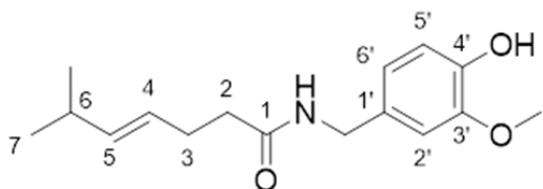
While TRPV1 agonist discovery was the main goal of this thesis, our untargeted metabolomics study expanded further than this original scope. We found that the capsaicinoid fingerprint between varieties was inconsistent, thus making them unreliable for identification. This finding agrees with the previous targeted studies claiming that capsaicinoids are not a viable option for chemotaxonomic investigation (Zewdie and Bosland, 2001). Furthermore, this inconsistency should be considered when capsaicinoids are implemented in medicinal products. The untargeted screening identified very few molecules that were predicted by CANOPUS to be vanilloid-like precursors. This lack of analogs may be a limiting factor in both capsaicinoid biosynthesis and diversity.

The combination of crude extract ED_{50} values combined with untargeted metabolomics, to the best of our knowledge, has not been explored before. This combination suggests that capsaicinoids are potentially acting in either an additive or synergistic manner. When testing individual capsaicinoids, we found that the slightest deviation from the capsaicin structure reduced the activity of a given capsaicinoid. In particular, the shortening of the tail resulted in an inactive analog, which agrees with previous studies on capsaicinoids. Long-tail capsaicinoids, while able to activate TRPV1, unlike short-tailed analogs, are less efficient at channel opening than capsaicin. Of the findings, the reduction of TRPV1 activity by a single hydroxylation raises poignant considerations when storing capsaicinoids (i.e., oxidation should be avoided to maintain potency). Despite the reduced potency, we found that these molecules can modulate capsaicin potency.

Our initial hypothesis that capsaicinoids may interact with each other in an additive manner was supported when testing non-activating to weakly-activating capsaicinoids in combination with capsaicin. Non-activating ligands can act like antagonists, which was not the case with capsaicinoids. In fact, non-activating capsaicinoids acted as positive modulators to TRPV1. Of the non-activating, vanillin, which is found in chilis, may add to the overall potency of a given variety. While chilis contain mostly capsaicin and dihydrocapsaicin,

all capsaicinoids should be considered when determining the SHU. Further studies are needed to understand the full mechanism behind this modulation.

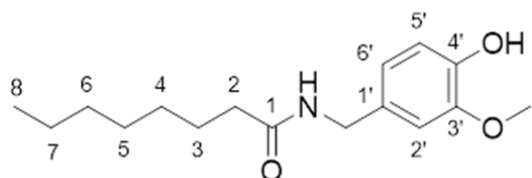
7. NMR



Nornorcapsaicin **19**, Capsicum8-P4,P5

^1H NMR (500.0 MHz, CD_3CN) δ = 6.86 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.74 (d, 1H, $J_{5',6'} = 8.0$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.65 (bs, 1H, NH), 6.42 (bs, 1H, OH), 5.34–5.47 (m, 2H, H4 and H5), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'–CH₂), 3.82 (s, 3H, OCH₃), 2.16–2.26 (m, 5H, H2, H3 and H6).

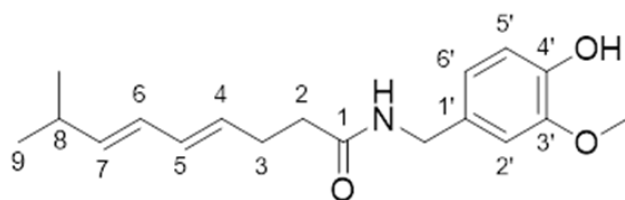
^{13}C NMR (125.7 MHz, CD_3CN) δ = 172.8 (C1), 148.1 (C3'), 146.1 (C4'), 139.2 (C5), 132.3 (C1'), 126.7 (C4), 121.0 (C6'), 115.4 (C5'), 112.1 (C2'), 56.6 (OCH₃), 43.3 (1'–CH₂), 37.0 (C2), 31.7 (C6), 29.4 (C3), 22.8 (C7).



22, Capsicum8-P10

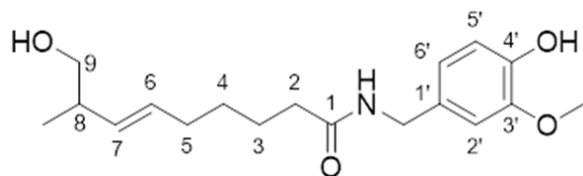
^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 7.9$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.65 (bs, 1H, NH), 6.42 (bs, 1H, OH), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'–CH₂), 3.82 (s, 3H, OCH₃), 2.13 (m, 2H, H2), 1.57 (m, 2H, H3), 1.21–1.33 (m, 8H, H4, H5, H6 and H7), 0.88 (m, 3H, H8).

^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.6 (C1), 148.1 (C3'), 146.1 (C4'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 56.6 (OCH₃), 43.2 (1'–CH₂), 36.9 (C2), 32.5 (C6), 29.9 (C4 or C5), 29.8 (C4 or C5), 26.6 (C3), 23.3 (C7), 14.4 (C8).

**25, Capsicum8-P14**

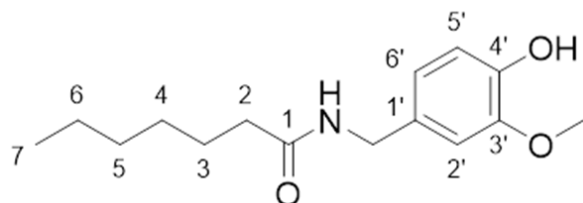
^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.74 (d, 1H, $J_{5',6'} = 8.0$, H5'), 6.70 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.67 (bs, 1H, NH), 6.44 (bs, 1H, OH), 5.93–6.05 (m, 2H, H5 and H6), 5.53–5.60 (m, 2H, H4 and H7), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 2.20–2.35 (m, 5H, H2, H3 and H8), 0.98 (d, 6H, $J_{9,8} = 6.7$, H9).

^{13}C NMR (125.7 MHz, CD_3CN) δ = 172.8 (C1), 148.1 (C3'), 146.1 (C4'), 140.8 (C7), 132.3 (C1'), 132.1 (C5), 131.7 (C4), 128.3 (C6), 121.0 (C6'), 115.4 (C5'), 112.1 (C2'), 56.6 (OCH₃), 43.3 (1'-CH₂), 36.5 (C2), 31.8 (C8), 29.3 (C3), 22.6 (C9).

**ω-hydroxycapsaicin 30, Capsicum11-P8**

^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.70 (ddt, 1H, $J_{6',5'} = 8.1$, $J_{6',2'} = 2.0$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.69 (bs, 1H, NH), 6.44 (bs, 1H, 4'-OH), 5.44 (dtd, 1H, $J_{6,7} = 15.4$, $J_{6,5} = 6.7$, $J_{6,8} = 1.0$, H6), 5.32 (ddt, 1H, $J_{7,6} = 15.4$, $J_{7,8} = 7.4$, $J_{6,5} = 1.3$, H7), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 3.26–3.34 (m, 2H, H9), 2.56 (t, 1H, $J_{\text{OH},9} = 5.9$, 9-OH), 2.11–2.22 (m, 3H, H2 and H8), 2.00 (m, 2H, H5), 1.57 (m, 2H, H3), 1.35 (m, 2H, H4), 0.93 (d, 3H, $J_{\text{CH}_3,8} = 6.8$, 8-CH₃).

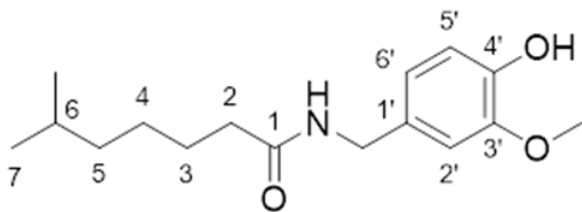
^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.6 (C1), 148.2 (C3'), 146.0 (C4'), 134.4 (C7), 132.4 (C1'), 130.9 (C6), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 67.82 (C9), 56.6 (OCH₃), 43.2 (1'-CH₂), 40.4 (C8), 36.6 (C2), 33.0 (C5), 29.7 (C4), 26.0 (C3), 17.1 (8-CH₃).



41, Capsicum10-P3

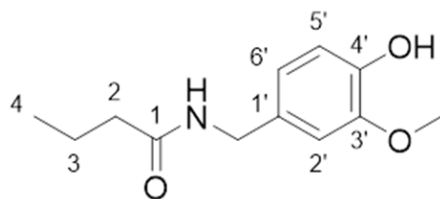
^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.0$, H5'), 6.70 (ddt, 1H, $J_{6',5'} = 7.9$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.65 (bs, 1H, NH), 6.41 (bs, 1H, OH), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 2.13 (m, 2H, H2), 1.56 (m, 2H, H3), 1.26–1.33 (m, 6H, H4, H5 and H6), 0.88 (m, 3H, H7).

A small amount of the sample did not allow for ^{13}C NMR measurement.

**42, Capsicum8-P9**

^1H NMR (500.0 MHz, CD_3CN) δ = 6.86 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.74 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.6$, H6'), 6.65 (bs, 1H, NH), 6.41 (bs, 1H, OH), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 2.13 (m, 2H, H2), 1.47–1.60 (m, 3H, H3 and H6), 1.29 (m, 2H, H4), 1.18 (m, 2H, H5), 0.86 (d, 6H, $J_{7,6} = 6.7$, H7).

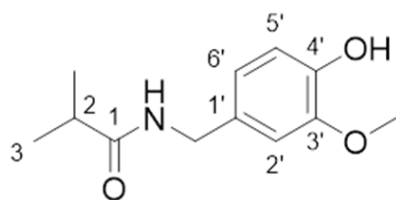
^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.7 (C1), 148.2 (C3'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 56.6 (OCH₃), 43.2 (1'-CH₂), 39.4 (C5), 36.9 (C2), 28.6 (C6), 27.7 (C4), 26.8 (C3), 22.9 (C7).

**43, Capsicum10-P3**

Structural analysis was performed for a mixture of compounds **43** and **44**.

^1H NMR (500.0 MHz, CD_3CN) δ = 6.86 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.69–6.72 (m, 1H, H6'), 6.64 (bs, 1H, NH), 6.41 (bs, 1H, OH), 4.23 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 2.13 (m, 2H, H2), 1.60 (sextet, 2H, $J_{3,2} = 7.4$, H3), 0.91 (t, 3H, $J_{4,3} = 7.4$, H4).

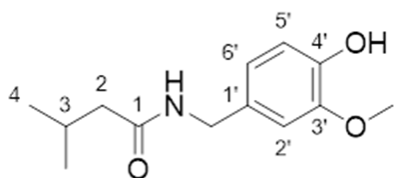
^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.5 (C1), 148.3 (C3'), 146.1 (C4'), 132.5 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 56.6 (OCH₃), 43.2 (1'-CH₂), 38.8 (C2), 19.9 (C3), 14.0 (C4).

**44, Capsicum10-P3**

Structural analysis was performed for a mixture of compounds **43** and **44**.

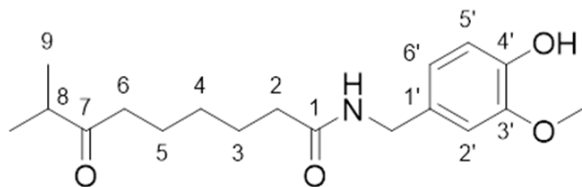
^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.69–6.72 (m, 1H, H6'), 6.64 (bs, 1H, NH), 6.41 (bs, 1H, OH), 4.23 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'–CH₂), 3.82 (s, 3H, OCH₃), 2.37 (septet, 1H, $J_{2,3} = 6.8$, H2), 1.08 (d, 6H, $J_{3,2} = 6.9$, H3).

^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.5 (C1), 148.3 (C3'), 146.1 (C4'), 132.5 (C1'), 120.8 (C6'), 115.4 (C5'), 111.9 (C2'), 56.6 (OCH₃), 43.1 (1'–CH₂), 35.9 (C2), 19.9 (C3).

**45, Capsicum10-P6**

^1H NMR (500.0 MHz, CD_3CN) δ = 6.86 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.1$, $J_{6',2'} = 2.0$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.66 (bs, 1H, NH), 6.41 (bs, 1H, OH), 4.23 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 5.9$, 1'–CH₂), 3.82 (s, 3H, OCH₃), 2.00–2.04 (m, 3H, H2 and H3), 0.91 (m, 6H, H4).

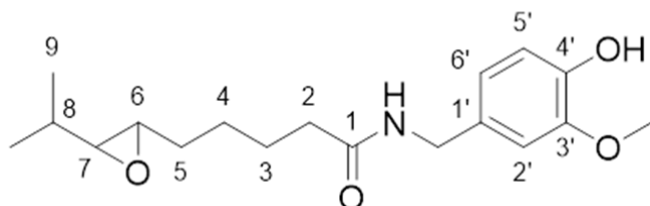
^{13}C NMR (125.7 MHz, CD_3CN) δ = 172.9 (C1), 148.1 (C3'), 146.0 (C4'), 132.5 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 56.6 (OCH₃), 46.2 (C2), 43.2 (1'–CH₂), 27.0 (C3), 22.7 (C4).

**46, Capsicum-P10-16**

^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.70 (ddt, 1H, $J_{6',5'} = 8.1$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.7$, H6'), 6.65 (bs, 1H, NH), 6.42 (bs, 1H, OH), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'–CH₂), 3.82

(s, 3H, OCH₃), 2.59 (septet, 1H, $J_{8,9} = 6.9$, H8), 2.45 (t, 2H, $J_{6,5} = 7.3$, H6), 2.13 (m, 2H, H2), 1.57 (m, 2H, H3), 1.50 (m, 2H, H5), 1.26 (m, 2H, H4), 1.02 (d, 6H, $J_{9,8} = 6.9$, H9).

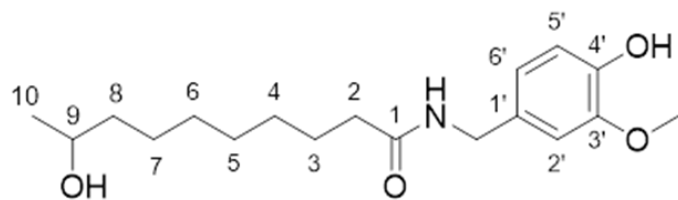
¹³C NMR (125.7 MHz, CD₃CN) δ = 215.4 (C7), 173.5 (C1), 148.1 (C3'), 146.1 (C4'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 56.6 (OCH₃), 43.2 (1'-CH₂), 41.3 (C8), 40.7 (C6), 36.7 (C2), 29.5 (C4), 26.4 (C3), 24.2 (C5), 18.5 (C9).



47, Capsicum-P10-17

¹H NMR (500.0 MHz, CD₃CN) δ = 6.86 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 2.0$, $J_{6',CH_2} = 0.7$, H6'), 6.66 (bs, 1H, NH), 6.42 (bs, 1H, OH), 4.23 (d, 2H, $J_{CH_2,NH} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 2.66 (m, 1H, H6), 2.39 (m, 1H, H7), 2.15 (m, 2H, H2), 1.37–1.65 (m, 7H, H3, H4, H5 and H8), 0.95 (d, 3H, $J_{9,8} = 6.7$, H9), 0.91 (d, 3H, $J_{9,8} = 6.9$, H9).

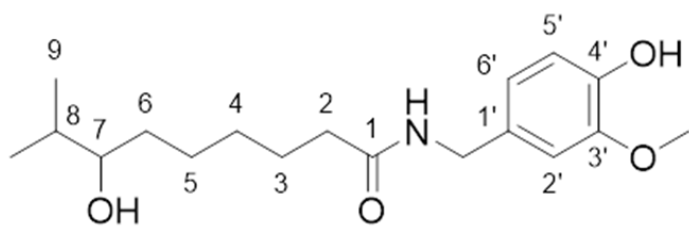
¹³C NMR (125.7 MHz, CD₃CN) δ = 173.4 (C1), 148.1 (C3'), 146.0 (C4'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.1 (C2'), 64.6 (C7), 58.1 (C6), 56.6 (OCH₃), 43.3 (1'-CH₂), 36.7 (C2), 32.6 (C5), 31.4 (C8), 26.4 (C3 or C4), 26.3 (C3 or C4), 19.3 and 18.6 (C9).



48, Capsicum11-P9

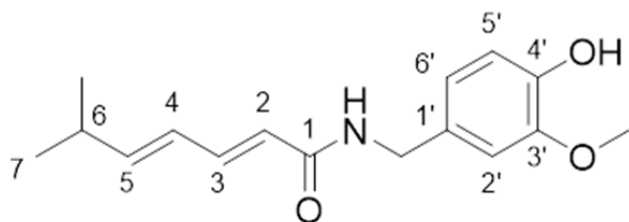
¹H NMR (500.0 MHz, CD₃CN) δ = 6.85 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.0$, H5'), 6.70 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 2.0$, H6'), 6.67 (bs, 1H, NH), 6.45 (bs, 1H, 4'-OH), 4.22 (d, 2H, $J_{CH_2,NH} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 3.64 (m, 1H, H9), 2.48 (m, 1H, 9-OH), 2.13 (m, 2H, H2), 1.56 (m, 2H, H3), 1.35 (m, 2H, H8), 1.24–1.31 (m, 8H, H4, H5, H6 and H7), 1.07 (d, 3H, $J_{10,9} = 6.1$, H10).

¹³C NMR (125.7 MHz, CD₃CN) δ = 173.6 (C1), 148.1 (C3'), 146.0 (C4'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 67.8 (C9), 56.6 (OCH₃), 43.2 (1'-CH₂), 40.1 (C8), 36.9 (C2), 30.3, 30.1 and 29.9 (C4, C5 and C6), 26.6 (C3 or C7), 26.5 (C3 or C7), 23.9 (C10).

**49, Capsicum11-P11**

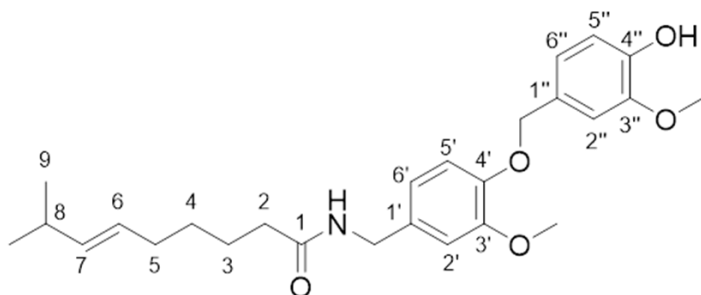
^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 1H, $J_{2',6'} = 1.9$, H2'), 6.75 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.71 (ddt, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 1.9$, $J_{6',\text{CH}_2} = 0.6$, H6'), 6.67 (bs, 1H, NH), 6.44 (bs, 1H, 4'-OH), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'-CH₂), 3.82 (s, 3H, OCH₃), 3.22 (m, 1H, H7), 2.37 (bd, 1H, $J_{\text{OH},7} = 5.5$, 7-OH), 2.14 (m, 2H, H2), 1.52–1.61 (m, 3H, H3 and H8), 1.24–1.46 (m, 6H, H4, H5 and H6), 0.86 and 0.85 (2 × d, 2 × 3H, $J_{9,8} = 6.6$, H9).

^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.6 (C1), 148.1 (C3'), 146.0 (C4'), 132.4 (C1'), 121.0 (C6'), 115.4 (C5'), 112.0 (C2'), 76.5 (C7), 56.6 (OCH₃), 43.2 (1'-CH₂), 36.9 (C2), 34.8 (C6), 34.4 (C8), 30.0 (C4), 26.6 (C3 or C5), 26.6 (C3 or C5), 19.3 and 17.6 (C9).

**50, ISO-276**

^1H NMR (500.0 MHz, $\text{DMSO}-d_6$) δ = 8.33 (t, 1H, $J_{\text{NH},\text{CH}_2} = 5.9$, NH), 7.01 (dd, 1H, $J_{3,2} = 15.1$, $J_{3,4} = 10.6$, H3), 6.82 (d, 1H, $J_{2',6'} = 2.0$, H2'), 6.70 (d, 1H, $J_{5',6'} = 8.0$, H5'), 6.64 (dd, 1H, $J_{6',5'} = 8.0$, $J_{6',2'} = 2.0$, H6'), 6.14 (dd, 1H, $J_{4,5} = 15.4$, $J_{4,3} = 10.5$, H4), 6.07 (dd, 1H, $J_{5,4} = 15.3$, $J_{5,6} = 6.4$, H5), 5.98 (d, 1H, $J_{2,3} = 15.1$, H2), 4.22 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 5.9$, 1'-CH₂), 3.73 (s, 3H, OCH₃), 2.40 (m, 1H, H6), 1.00 (d, 6H, $J_{7,6} = 6.8$, H7).

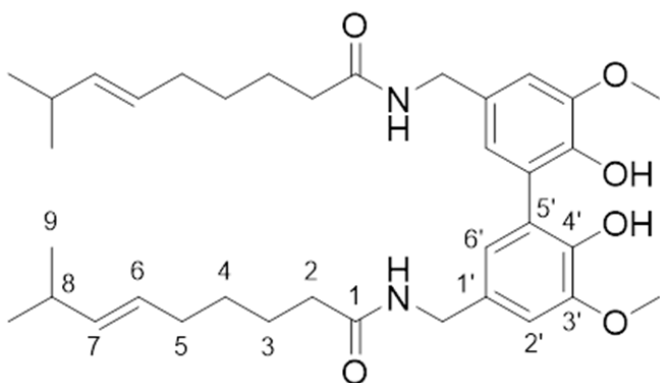
Small amount of the sample did not allow for ^{13}C NMR measurement.



51, JS-ISO-21-20-442

^1H NMR (600.1 MHz, CD_3CN) δ = 7.03 (d, 1H, $J_{2'',6''} = 1.9$, H2''), 6.92 (d, 1H, $J_{5',6'} = 8.1$, H5'), 6.86–6.88 (m, 2H, H2' and H6''), 6.80 (d, 1H, $J_{5'',6''} = 8.1$, H5''), 6.76 (dd, 1H, $J_{6',5'} = 8.1$, $J_{6',2'} = 2.0$, H6'), 6.69 (bs, 1H, NH), 5.33–5.42 (m, 2H, H6 and H7), 4.94 (s, 2H, 1''– CH_2), 4.24 (d, 2H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'– CH_2), 3.85 (s, 3H, 3''– OCH_3), 3.78 (s, 3H, 3'– OCH_3), 2.22 (m, 1H, H8), 2.15 (m, 2H, H2), 1.97 (m, 2H, H5), 1.57 (m, 2H, H3), 1.34 (m, 2H, H4), 0.95 (d, 6H, $J_{9,8} = 6.8$, H9).

^{13}C NMR (150.9 MHz, CD_3CN) δ = 173.6 (C1), 150.7 (C3'), 148.2 (C3''), 148.2 (C4'), 147.0 (C4''), 138.7 (C7), 133.8 (C1'), 130.0 (C1''), 127.8 (C6), 122.3 (C6''), 120.4 (C6'), 115.5 (C5''), 114.9 (C5'), 112.9 (C2''), 112.5 (C2'), 71.8 (1''– CH_2), 56.7 (3''– OCH_3), 56.3 (3'– OCH_3), 43.1 (1'– CH_2), 36.7 (C2), 32.9 (C5), 31.8 (C8), 30.0 (C4), 26.1 (C3), 23.0 (C9).

**53**, Capsicum11-24

^1H NMR (500.0 MHz, CD_3CN) δ = 6.85 (d, 2H, $J_{2',6'} = 2.0$, H2'), 6.69 (d, 2H, $J_{6',2'} = 2.1$, H6'), 6.68 (bs, 2H, NH), 5.31–5.41 (m, 4H, H6 and H7), 4.26 (d, 4H, $J_{\text{CH}_2,\text{NH}} = 6.0$, 1'– CH_2), 3.85 (s, 6H, OCH_3), 2.21 (m, 2H, H8), 2.13 (m, 4H, H2), 1.95 (m, 4H, H5), 1.57 (m, 4H, H3), 1.32 (m, 4H, H4), 0.93 (d, 12H, $J_{9,8} = 6.8$, H9).

^{13}C NMR (125.7 MHz, CD_3CN) δ = 173.3 (C1), 148.6 (C3'), 144.2 (C4'), 138.5 (C7), 131.0 (C1'), 127.6 (C6), 126.0 (C5'), 122.7 (C6'), 110.8 (C2'), 56.7 (OCH_3), 43.5 (1'– CH_2), 36.7 (C2), 32.8 (C5), 31.8 (C8), 29.9 (C4), 26.0 (C3), 23.1 (C9).

8. Tables

Table 1

Metadata for all capsaicinoids previously isolated from *Capsicum* or used in this study.
Struc. is short for structure and ref. is short for reference.

Chemical Name	Struc. No.	SMILES	Molecular Formula	Isolation Source	NMR Data Ref.	Ref.
Capsaicin	1	<chem>COC1=CC(CNC(=O)CCCC\C=C\C(C)C)=CC=C1O</chem>	C18H27NO3	--	NP0047867	--
Dihydrocapsaicin	11	<chem>COC1=C(O)C=CC(CNC(=O)CCCCCCC(C)C)=C1</chem>	C18H29NO3	--	--	--
Homocapsaicin	12	<chem>COC1=C(O)C=CC(CNC(=O)CCCCC\C=C\C(C)C)=C1</chem>	C19H29NO3	C. annuum L. var. annuum C. annuum L.; C.	--	(Jurenitsch et al., 1979)
Homocapsaicin-I	13	<chem>COC1=CC(CNC(=O)CCCC\C=C\CC(C)C)=CC=C1O</chem>	C19H29NO3	annuum L. var. annuum C. annuum	--	(Jurenitsch et al., 1979; Wang et al., 2020)
Homocapsaicin-II	14	<chem>CCC(C)\C=C\CCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C19H29NO3	L. var. annuum	--	(Jurenitsch et al., 1979)
Homodihydrocapsaicin; homodihydrocapsaicin I	15	<chem>COC1=C(O)C=CC(CNC(=O)CCCCCCCC(C)C)=C1</chem>	C19H31NO3	C. annuum L. var. annuum	--	(Jurenitsch et al., 1979)
Homodihydrocapsaicin-II	16	<chem>CCC(C)CCCCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C19H31NO3	C. annuum L.; C. annuum L. var. annuum C. annuum L.; C.	--	(Jurenitsch et al., 1979; Wang et al., 2020)
N-vanillyl-4,8-dimethylnonanamide	17	<chem>COC1=CC(CNC(=O)CCC(C)CCCC(C)C)=CC=C1O</chem>	C19H31NO3	annuum L. var. annuum	--	(Jurenitsch et al., 1979; Wang et al., 2020)

Norcapsaicin	18	<chem>COC1=C(O)C=CC(CNC(=O)CCC\C=C\C(C)C)=C1</chem>	C17H25NO3	<i>C. annuum</i> L.; <i>C. annuum</i> L. var. <i>annuum</i>	--	(Jurenitsch et al., 1979; Wang et al., 2020)
Nornorcapsaicin	19	<chem>COC1=C(O)C=CC(CNC(=O)CC\C=C\C(C)C)=C1</chem>	C16H23NO3	<i>C. annuum</i> L. (from Uzbekistan)	NP03419 21	(Ishimov et al., 2011)
Nordihydrocapsaicin; 7-methyl-N-vanillyloctamide; Norhydrocapsaicin	20	<chem>COC1=C(O)C=CC(CNC(=O)CCCCC(C)C)=C1</chem>	C17H27NO3	<i>C. annuum</i> var. Yatsubusa; <i>C. annuum</i> L. (from Uzbekistan)	--	(Attuquayefio and Buckle, 1987; Jurenitsch et al., 1979)
Nordihydrocapsaicin II	21	<chem>CCC(C)CCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C18H29NO3	<i>C. annuum</i> L. (from Uzbekistan)	--	(Ishimov et al., 2011)
N-vanillyl octanamide; Caprylsaeure-vanillylamid; Caprylic acid vanillylamide	22	<chem>CCCCCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C16H25NO3	<i>C. annuum</i> L. (from Uzbekistan); <i>C. annuum</i> L. var. <i>annuum</i>	NP03419 22	(Ishimov et al., 2011; Jurenitsch et al., 1979)
N-vanillyl decanamide; Vanillyl N-decoylamide	23	<chem>CCCCCCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C18H29NO3	<i>C. annuum</i> L.; <i>C. annuum</i> L. var. <i>annuum</i>	NP03419 23	(Jurenitsch et al., 1979; Wang et al., 2020)
N-Vanillylundecanamide; N-Vanillyl-undecanamid; N-(4-Oxy-3-methoxybenzyl)-undecylsaeureamid; N-(4-hydroxy-3-methoxybenzyl)-undecamide	24	<chem>CCCCCCCCCCC(=O)NCC1=CC=C(O)C(OC)=C1</chem>	C19H31NO3	<i>C. annuum</i> L. (from Uzbekistan)	--	(Ishimov et al., 2011)

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N-vanillyl-4E,6E-dien-8-methylnonanamide	25	<chem>COC1=CC(CNC(=O)CC\C=C\C=C\C(C)C)=CC=C1O</chem>	C18H25NO3	<i>C. annuum</i> L.	--	(Wang et al., 2020)
Nonivamide; N-(4-hydroxy-3-methoxybenzyl)nonanamide	26	<chem>CCCCCCCCC(=O)NCC1=CC(OC)=C(O)C=C1</chem>	C17H27NO3	<i>Capsicum</i> .	--	(Constant et al., 1996)
Palvanil	27	<chem>CCCCCCCCCCCCCCCC(=O)NCC1=CC(OC)=C(O)C=C1</chem>	C24H41NO3	(oleoresin) <i>Capsicum</i> (oleoresin)	--	
Stevanil; N-Vanillyloctadecanamide; N-(4-hydroxy-3-methoxybenzyl)octadecanamide; N-vanillyl-stearamide; N-Vanillyl-stearamid; N-Vanillyl-stearinamid; Stearinsaeure-vanillylamid	28	<chem>CCCCCCCCCCCCCCCCC(=O)NCC1=CC(OC)=C(O)C=C1</chem>	C26H45NO3	<i>Capsicum</i> (oleoresin)	NP0341924	(Kobata et al., 2010)
Olvanil; N-(3-methoxy-4-hydroxybenzyl)oleamide; N-vanillyloleamide	9	<chem>CCCCCCCC\C=C/CCCCCCCC(=O)NCC1=CC(OC)=C(O)C=C1</chem>	C26H43NO3	<i>Capsicum</i> (oleoresin)	--	(Kobata et al., 2010)
Livanil; (Z,Z)-N-[(4-hydroxy-3-methoxyphenyl)methyl]-octadeca-9,12-dienamide; N-vanillyl-9Z,12Z-octadecadienamide; N-vanillyllinoleamide	29	<chem>CCCCC\C=C/C\C=C/CCCCCCCC(=O)NCC1=CC(OC)=C(O)C=C1</chem>	C26H41NO3	<i>Capsicum</i> (oleoresin)	--	(Kobata et al., 2010)
ω -Hydroxycapsaicin	30	<chem>COC1=C(O)C=CC(CNC(=O)CCCC\C=C\C(C)CO)=C1</chem>	C18H27NO4	<i>C. annuum</i>	--	(Ochi et al., 2003)
6",7"-dihydro-5',5'''-dicapsaicin	31	<chem>CC(C)CCCCCCC(=O)NCC1=CC(=C(C(=C1)C2=CC(=CC(=C2O)OC)CNC(=O)CCCC=CC(C)C)O)OC</chem>	C36H54N2O6	<i>C. annuum</i>	NP0341925	(Ochi et al., 2003)

Capsaicin-β-D-glucopyranoside	32	<chem>COC1=CC(CNC(=O)CCCC\C=C\C(C)C)=CC=C1O[C@@H]1O[C@H](CO)[C@@H](O)[C@H](O)[C@H]1O</chem>	C24H37NO8	<i>C. annuum</i> var. 'High Heat'	NP0341926	(Higashiguchi et al., 2006)
Dihydrocapsaicin-β-D-glucopyranoside	33	<chem>COC1=CC(CNC(=O)CCCCCCC(C)C)=CC=C1O[C@@H]1O[C@H](CO)[C@@H](O)[C@H](O)[C@H]1O</chem>	C24H39NO8	<i>C. annuum</i> var. 'High Heat'	NP0341934	(Higashiguchi et al., 2006)
N-heptanoate vanillylamide	41	<chem>COC(C=C(CNC(CCCCC)=O)C=C1)=C1O</chem>	C15H23NO3	Newly isolated	NP0341935	--
Nornordihydrocapsaicin	42	<chem>COC(C=C(CNC(CCCCC(C)C)=O)C=C1)=C1O</chem>	C16H25NO3	Newly isolated	NP0341927	--
N-vanillylbutyramide	43	<chem>COC(C=C(CNC(CCC)=O)C=C1)=C1O</chem>	C12H17NO3	Newly isolated	NP0341928	--
N-vanillyl-isobutyramide	44	<chem>COC(C=C(CNC(C(C)C)=O)C=C1)=C1O</chem>	C12H17NO3	Newly isolated	NP0341929	--
N-Isovalerylvanillylamin	45	<chem>COC(C=C(CNC(CC(C)C)=O)C=C1)=C1O</chem>	C13H19NO3	Newly isolated	NP0341930	--
N-(4-hydroxy-3-methoxybenzyl)-8-methyl-7-oxononanamide	46	<chem>COC(C=C(CNC(CCCCC(C(C)C)=O)=O)C=C1)=C1O</chem>	C18H27NO4	Newly isolated	NP0341931	--
Epicapsaicin	47	<chem>COC(C=C(CNC(CCCCC1C(O1)C(C)C)=O)C=C2)=C2O</chem>	C18H27NO4	Newly isolated	NP0341932	--
N-vanillyl-9-hydroxydecanamide	48	<chem>COC(C=C(CNC(CCCCCCCC(O)C)=O)C=C1)=C1O</chem>	C18H29NO4	Newly isolated	NP0341936	--
N-vanillyl-7-hydroxy-8-methylnonamide	49	<chem>COC(C=C(CNC(CCCCC(O)C(C)C)=O)C=C1)=C1O</chem>	C18H29NO4	Newly isolated	--	--
N-vanillyl 6-Methyl-2,4-heptadienamide	50	<chem>COC(C=C(CNC(/C=C/C=C/C(C)C)=O)C=C1)=C1O</chem>	C16H21NO3	Newly isolated	NP0341933	--
N-vanillyl-capsaicin ether	51	<chem>COC(C=C(CNC(CCCC/C=C/C(C)C)=O)C=C1)=C1OCC2=CC(OC)=C(C=C2)O</chem>	C26H35NO5	Newly isolated	--	--
N-vanillyl-dihydrocapsaicin ether	52	<chem>COC(C=C(CNC(CCCCCC(C)C)=O)C=C1)=C1OCC2=CC(OC)=C(C=C2)O</chem>	C26H37NO5	Newly isolated	--	--
5,5'-dicapsaicin	53	<chem>O=C(NCC1=CC(OC)=C(C(C2=CC(CNC(CCCC/C=C/C(C)C)=O)=CC(OC)=C2O)=C1)O)CCCC/C=C/C(C)C</chem>	C36H52N2O6	Newly isolated	--	--
Capsiate	10	<chem>CC(C)C=CCCCC(=O)OCC1=CC(=C(C=C1)O)OC</chem>	C18H26O4	<i>C. annuum</i> L.	--	(Kobata et al., 1998)

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Vanillin	35	<chem>COC1=C(C=CC(=C1)C=O)O</chem>	C8H8O3	--	--	--
4-Hydroxy-3-methoxybenzyl alcohol	37	<chem>COC1=C(C=CC(=C1)CO)O</chem>	C8H10O3	--	--	--

Table 2

Summary of properties of used compounds; RT – UPLC-HRMS retention time, m/z [M+H]⁺ – base peak mass², UVmax – maximum UV absorbance, EC50 - median effective concentration on TRPV1.

¹ Novel capsaicinoids

² Base peak represents the m/z value at the apex of the [M+H]⁺ peak. For compounds 24, 39 and 41, the neutral losses are the most abundant base peak ion. Neutral losses result in capsaicin for capsaicinoid 24 or the vanilloid head for compounds 39 and 41.

³ Compounds were isolated in a mixture.

⁴ n.d. - values were not able to be determined.

⁵ Compounds were not tested due to instability.

Compound number	Name	Purchased or isolated	Amount [mg]	Description	RT (min)	m/z [M+H] ⁺	UVmax	Purity	EC50 [μM]
1	Capsaicin	Purchased	--	White powder	10.54	306.2064	--		0.04
11	Dihydrocapsaicin	Purchased	--	White powder	11.25	308.2219	--	100%	0.05
13	Homodihydrocapsaicin I	Purchased	--	Yellow powder	12.31	322.2382	--	100%	0.32
19	Nornorcapsaicin	Isolated	0.24	Yellow-orange residue	8.93	278.1749	283.5	93.95%	0.81
20	Nordihydrocapsaicin	Purchased	--	Off-white powder	10.61	294.2066	--	100%	0.05
22	N-vanillyl octanamide	Isolated	1.13	Yellow oily residue	9.67	280.1906	284	100%	0.15
23	N-Vanillyldecanamide	Purchased	--	White powder	11.65	308.224	--	100%	0.2
25	N-vanillyl-4E,6E-dien-8-methylnonanamide	Isolated	1.29	Yellow oily residue	10.09	304.1908	247	94.59%	0.14
26	Nonivamide	Purchased	--	White powder	10.74	294.2067	--	100%	0.18

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9	Olvanil	Purchased	--	White powder	16.23	418.3317	--	98%	0.12
30	ω -Hydroxycapsaicin	Isolated	0.43	Yellow residue	7.18	322.2016	284	94.60%	5.56
31	6",7"-Dihydro-5',5"-dicapsaicin	Isolated	0.62	Yellow residue	13.88	611.4063	247	79.94%	10.27
32	Capsaicin β -D-glucopyranoside	Purchased	--	White powder	8.81	306.2069 ²	--	100%	4.69
41	N-Heptanoate vanillylamide	Isolated	0.22	White residue	8.72	266.1748	243.5	76.68%	1.81
42	Nornordihydrocapsaicin	Isolated	0.28	White residue	9.5	280.1906	284	86.73%	1.04
45	N-isovalerylvanillylamin	Isolated	0.51	Yellow residue	6.4	238.1436	284	93.80%	2.84
46	N-(4-Hydroxy-3-methoxybenzyl)-8-methyl-7-oxononanamide	Isolated	0.55	Yellow residue	7.94	322.2014	283.5	92.77%	0.56
47	Epicapsaicin	Isolated	0.26	Yellow residue	8.08	322.2017	284.5	85.40%	0.6
48 ¹	N-vanillyl-9-hydroxydecanamide ¹	Isolated	0.41	Yellow residue	7.35	324.2169	284	100%	6.33
49 ¹	N-vanillyl-7-hydroxy-8-methylnonamide ¹	Isolated	0.87	Yellow residue	7.66	324.217	284	100%	1.46
50 ¹	N-vanillyl 6-Methyl-2,4-heptadienamide ¹	Isolated	0.31	Yellow residue	9.03	276.1595	258.5	93.41%	1.2
51 ¹	N-vanillyl-capsaicin ether ¹	Isolated	< 1.0	Yellow residue	11.58	442.2591	--	— ⁵	— ⁵
52 ¹	N-vanillyl-dihydrocapsaicin ether ¹	Isolated	< 1.0	Yellow residue	12.19	444.2745	--	— ⁵	— ⁵

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53	5,5'-Dicapsaicin	Isolated	0.21	Yellow residue	13.37	609.3901	243.5	79.71%	4.46
10	Capsiate	Purchased	--	White powder	13.51	137.0596 ²	--	50.20%	490.9
35	Vanillin	Purchased	--	White powder	5.62	153.0545	--	100%	n.d. ⁴
37	4-Hydroxy-3-methoxybenzyl alcohol	Purchased	--	Off-white powder	3.87	137.0596 ²	--	93.19%	n.d. ⁴
43, 44 ³	N-Vanillylbutyramide / N-vanillyl-isobutyramide	Isolated	0.4	White residue	5.43/5.48	224.128	244.5	89.77%	n.d. ⁴

Table 3

Summary of chili varieties analyzed in the present study.

¹Samples used in the first large-scale extraction.

²Samples used in the second large-scale extraction .

³The values of Scoville Units presented in this table were obtained from either the Botanical Garden of the Faculty of Tropical AgriSciences of the Czech University of Life Sciences in Prague or an established chili dealer. Capsaicinoid profiles and how they are measured when determining the Scoville Units (27) are susceptible to many factors of variance. Ultimately, Scoville Units are never absolute and are only a way to gauge a variety's heat level.

⁴Capsaicinoid Intensity is the log of the sum precursor intensity of all SIRIUS predicted capsaicinoid features.

⁵The log sum intensity for features corresponding only to capsaicin and dihydrocapsaicin.

⁶Capsaicinoid Intensity Reduced is the log of the sum precursor intensity of all features predicted to be capsaicinoids by SIRIUS but the peak area corresponding to capsaicin and dihydrocapsaicin were removed.

⁷The log sum intensity of all features.

⁸The log sum intensity for all features that were not predicted to be capsaicinoids by SIRIUS.

	Common Name	Species	Scoville Units (SHU) ³	Capsaicinoid Intensity ⁴	Only capsaicin and dihydrocapsaicin ⁵	Capsaicinoid Intensity Reduced ⁶	All detected features ⁷	All non-capsicinoid features ⁸
						13.96		
S1	Lemon Drop	Capsicum baccatum	15,000–30,000	15.76	15.60		16.80	16.36
S2	Escabeche	C. baccatum	12,000–17,000	17.82	17.72	15.83	18.79	18.29
S3	Capsaicum.B	C. baccatum	≤ 200,000	17.76	17.61	16.07	18.71	18.20

S4	Pimenta Trepaderra de Werner	C. baccatum	70,000–100,000	16.26	16.19	13.77	17.40	17.01
S5	Glocken	C. baccatum	5,000–30,000	16.87	16.70	15.13	17.90	17.44
S6	Criolla Sella	C. baccatum	25,000–30,000	16.49	16.42	14.04	17.85	17.54
S7	Black Prince	C. annuum	10,000–30,000	16.43	16.29	14.55	17.41	16.93
S8	Thai Culinary Hot ²	C. annuum	25,000–40,000	16.35	16.15	14.73	17.74	17.45
S9	Fish Pepper	C. annuum	5,000–30,000	16.56	16.34	15.01	18.03	17.76
S10	Chips	C. annuum	N/A	16.95	16.77	15.23	18.02	17.60
S11	Hot Fish	C. annuum	10,000–20,000	17.48	17.43	15.03	18.75	18.40
S12	Sahuaro	C. annuum	500	16.26	16.05	14.67	17.63	17.33
S13	Trinidad Moruga Scorpion ¹	C. chinense	800,000–1,000,000	18.49	18.50	15.49	19.12	18.29
S14	Brasilian Hot Bonnet	C. chinense	100,000–350,000	17.42	17.26	15.64	18.63	18.26
S15	Habanero	C. chinense	100,000–350,000	18.14	18.15	14.97	18.87	18.16
S16	Naga Morich ¹	C. chinense	≤ 1,040,000	18.44	18.45	15.48	19.17	18.45
S17	Dorset Naga ²	C. chinense	1,000,000– 1,500,000	18.41	18.40	15.82	19.17	18.48
S18	Chilhuacle	C. annuum	5,000–15,000	16.85	16.51	15.67	18.06	17.70
S19	Pendulum	C. baccatum	5,000–15,000	12.45	12.37	9.89	16.58	16.56
S20	Peri Peri	C. frutescens	30,000–100,000	13.72	13.63	11.26	16.52	16.46
S21	Hungarian Black African Bird's Eye	C. annuum	500–2,500	13.35	12.77	12.54	17.01	16.98
S22	Chili	C. frutescens	80,000–150,000	15.93	15.81	13.87	17.52	17.29
S23	Jalapeno Summer Heat ²	C. annuum	10,000	14.44	14.25	12.67	16.78	16.68
S24	Bolivian Rainbow Miniature Chocolate	C. annuum	10,000–30,000	16.24	16.03	14.67	17.40	17.02
S25	Bell	C. annuum	0	8.11	7.96	6.19	16.99	16.99
S26	Vezena Piperka	C. annuum	≤ 10,000	15.07	14.78	13.70	16.73	16.52
S27	Carolina Reaper ^{1,2}	C. chinense	1,150,000– 2,200,000	18.38	18.37	15.69	19.06	18.29
S28	Habanero Madame Jeanette	C. chinense	100,000–350,000	18.00	17.72	16.73	18.56	17.66
S29	Habanero White Long	C. chinense	≤ 300,000	16.98	16.97	13.75	17.58	16.75
S30	7 Pot Brain Yellow Trinidad Congo	C. chinense	≤ 1,400,000	18.41	18.39	15.83	19.08	18.30
S31	Red ^{1,2}	C. chinense	250,000–350,000	18.46	18.09	17.41	18.98	18.02
S32	Limon	C. chinense	15 000–30 000	17.55	17.54	14.48	18.19	17.40

S33	Trinidad Congo Yellow ¹	C. chinense	100,000–350,000	19.30	19.31	16.64	19.95	19.12
S34	Trinidad Scorpion Butch T ^{1,2}	C. chinense	1,000,000–1,500,000	17.82	17.51	16.61	18.44	17.63
S35	Naga Bhut Jolokia	C. chinense	≤ 1,000,000	17.89	17.90	14.79	18.68	18.03
S36	Jamaican Hot Yellow ²	C. chinense	≤ 100,000	17.65	17.58	15.45	18.61	18.10
S37	Chocolate Habanero	C. chinense	≤ 500,000	18.96	18.98	16.12	19.64	18.84
S38	Habanero White ²	C. chinense	300,000–450,000	18.24	18.24	15.45	18.75	17.73
S39	Fatalii White ²	C. chinense	125,000–325,000	18.76	18.79	15.79	19.45	18.67
S40	Habanero Large Brown ¹	C. chinense	100,000–350,000	17.71	17.64	15.45	18.46	17.78

Table 4

Detailed overview of properties of dose-response curves of extracts (S1-S40).

Each curve consists of at least 7 concentration points in triplicates. Least square non-linear fit with separately handled, each replication was used to determine the curves. Two curves (S7 and S25) were manually removed from the correlation because of low potency (S25) or poor fit (S7). ED50 = median effective dose

extract no.	Variety name	Top	ED50	logED50	HillSlope	R squared
S1	Lemon Drop	0.842	3.98E-04	-3.401	0.942	0.967
S2	Escabeche	0.827	6.44E-05	-4.191	0.962	0.985
S3	Capsaicum.B	0.743	3.28E-05	-4.484	1.243	0.876
S4	Pimenta Trepaderra de Werner	0.664	5.94E-05	-4.226	1.522	0.979
S5	Glocken	0.866	2.66E-04	-3.576	0.873	0.942
S6	Criolla Sella	0.689	1.54E-04	-3.811	1.015	0.942
S7	Black Prince	0.496	1.78E-06	-5.749	0.597	0.751
S8	Thai Culinary Hot	0.820	1.88E-04	-3.726	0.830	0.947
S9	Fish Pepper	0.735	6.60E-05	-4.180	1.127	0.983
S10	Chips	0.745	4.68E-05	-4.330	1.505	0.974
S11	Hot Fish	0.762	2.16E-05	-4.665	1.291	0.944
S12	Sahuaro	0.690	1.18E-04	-3.930	0.999	0.969
S13	Trinidad Moruga Scorpion	0.752	1.35E-05	-4.870	0.903	0.968
S14	Brasilian Hot Bonnet	0.730	4.46E-05	-4.350	1.328	0.989
S15	Habanero	0.824	3.31E-05	-4.480	0.969	0.985
S16	Naga Morich	0.673	1.78E-05	-4.749	1.234	0.947

S17	Dorset Naga	0.770	3.77E-05	-4.424	0.956	0.898
S18	Chilhuacle	0.733	8.34E-05	-4.079	10.860	0.969
S19	Pendulum	0.928	2.49E-03	-2.604	1.185	0.984
S20	Peri Peri	0.888	8.69E-04	-3.061	0.596	0.953
S21	Hungarian Black	0.703	6.51E-04	-3.186	1.088	0.946
S22	African Bird's Eye Chili	0.650	9.34E-05	-4.030	1.054	0.917
S23	Jalapeno Summer Heat	0.792	4.68E-04	-3.330	0.983	0.955
S24	Bolivian Rainbow	0.602	3.51E-05	-4.455	2.038	0.959
S25	Miniature Chocolate Bell	0.137	2.50E-03	-2.603	73.280	0.885
S26	Vezena Piperka	0.722	2.16E-04	-3.665	1.241	0.897
S27	Carolina Reaper	0.760	1.59E-05	-4.800	0.998	0.964
S28	Habanero Madame Jeanette	0.840	8.70E-05	-4.061	0.474	0.918
S29	Habanero White Long	0.741	2.75E-05	-4.561	0.598	0.901
S30	7 Pot Brain Yellow	0.811	2.42E-05	-4.616	0.793	0.922
S31	Trinidad Congo Red	0.697	2.95E-05	-4.530	1.490	0.857
S32	Limon	0.705	3.79E-05	-4.421	1.312	0.847
S33	Trinidad Congo Yellow	0.901	3.86E-05	-4.414	0.702	0.942
S34	Trinidad Scorpion Butch T	0.666	1.78E-05	-4.749	0.704	0.921
S35	Naga Bhut Jolokia	0.640	2.83E-05	-4.549	1.831	0.972
S36	Jamaican Hot Yellow	0.653	2.75E-05	-4.560	0.926	0.945
S37	Chocolate Habanero	0.851	4.80E-05	-4.318	0.707	0.948
S38	Habanero White	0.978	1.27E-04	-3.898	0.513	0.918
S39	Fatalii White	0.705	2.79E-05	-4.555	1.255	0.942
S40	Habanero Large Brown	0.730	4.22E-05	-4.375	1.795	0.950

Table 5

Detailed overview of the activity of isolated compounds and standards on rTRPV1 receptor measured by calcium influx assay.

Values are normalized to capsaicin. The curves were fitted using at least 10 concentration points measured as triplicates in two or more experiments. The least square non-linear fit was used to calculate the confidential intervals and other values. The comparison of models by using extra sum-of-squares F tests was used to determine statistical difference to capsaicin curve (EC50 = median effective concentration, CI = confidence interval, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

No.	Name	Top	Top 95%CI	EC50	EC50 95%CI	Hill Slope	Hill Slope 95%CI	Log EC50	R squared
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1	Capsaicin	0.9951		0.9273 to 1.072	3.98E-08		2.600e-008 to 6.381e-008	0.892	0.6583 to 1.317	-7.4	0.9572
11	Dihydrocapsaicin	1.0330	-	0.9840 to 1.089	4.87E-08	-	3.687e-008 to 6.657e-008	0.925	0.7166 to 1.231	-	0.9782
13	Homodihydrocapsaicin I	1.1220	*	1.007 to 1.295	3.17E-07	****	1.632e-007 to 7.651e-007	0.543	0.4268 to 0.6875	-	0.9653
19	nornorcapsaicin	0.7781	****	0.7197 to 0.8556	8.07E-07	****	6.179e-007 to 1.181e-006	1.514	1.016 to 3.059	-	0.9369
20	Nordihydrocapsaicin	1.0840	**	1.026 to 1.149	5.34E-08	-	3.890e-008 to 7.646e-008	0.952	0.7276 to 1.324	-	0.9770
22	N-vanillyl octanamide	0.9366	**	0.8960 to 0.9805	1.47E-07	****	1.203e-007 to 1.790e-007	1.685	1.228 to 2.581	-	0.9820
23	N-Vanillyldecanamide	1.1430	**	1.033 to 1.301	1.98E-07	****	1.047e-007 to 4.524e-007	0.561	0.4391 to 0.7213	-	0.9659
25	N-vanillyl-4E,6E-dien-8-methylnonanamide	0.9428	-	0.8844 to 1.014	1.41E-07	****	9.707e-008 to 2.155e-007	0.710	0.5719 to 0.8901	-	0.9769
26	Nonivamide	1.0460	-	0.9771 to 1.132	1.82E-07	****	1.259e-007 to 2.810e-007	0.849	0.6575 to 1.124	-6.74	0.9764
9	Olvanil	0.4035	****	0.3589 to 0.4483	1.19E-07	****	7.418e-008 to ???	2.961	1.143 to ???	-	0.7535
30	ω -hydroxycapsaicin	0.9003	**	0.8468 to 0.9628	5.56E-06	****	4.703e-006 to 6.694e-006	1.433	1.237 to 1.672	-	0.9853
31	6",7"-dihydro-5',5'''-dicapsaicin	0.9067	-	0.8032 to 1.098	1.03E-05	****	7.474e-006 to 1.742e-005	1.051	0.8352 to 1.300	-	0.9847
32	Capsaicin β -D-glucopyranoside	1.0160	-	0.9522 to 1.094	4.69E-06	****	3.913e-006 to 5.761e-006	1.396	1.161 to 1.706	-	0.9892
41	N-heptanoate vanillylamide	1.1360	****	1.100 to 1.176	1.81E-06	****	1.633e-006 to 2.018e-006	1.651	1.430 to 1.904	-	0.9945
42	nornordihydrocapsaicin	0.9481	-	0.9000 to 1.004	1.04E-06	****	8.582e-007 to 1.295e-006	1.199	0.9836 to 1.489	-	0.9883
43&44	N-vanillylbutyramide / N-vanillyl-isobutyramide	n.d.									
45	N-Isovalerylvanillylamin	0.7995	****	0.7679 to 0.8342	2.84E-06	****	2.517e-006 to 3.204e-006	1.528	1.309 to 1.805	-	0.9922
46	N-(4-hydroxy-3-methoxybenzyl)-8-methyl-7-oxononanamide	0.8420	***	0.7905 to 0.9023	6.02E-07	****	4.807e-007 to 7.655e-007	1.757	1.154 to 2.959	-	0.9712
47	epicapsaicin	1.1010	**	1.038 to 1.178	1.11E-06	****	8.768e-007 to 1.456e-006	0.983	0.8160 to 1.196	-	0.9883
48	N-vanillyl-9-hydroxydecanamide	0.9653	-	0.8031 to 1.390	6.33E-06	****	3.645e-006 to 2.064e-005	0.972	0.6429 to 1.443	-	0.9503

49	N-vanillyl-7-hydroxy-8-methylnonamide	0.9056	**	0.8537 to 0.9702	1.46E-06	****	1.186e-006 to 1.877e-006	1.133	0.9167 to 1.415	-	0.9882
50	N-vanillyl 6-Methyl-2,4-heptadienamide	0.7776	****	0.7308 to 0.8358	1.20E-06	****	9.415e-007 to 1.596e-006	0.996	0.8176 to 1.226	-	0.9881
53	5,5'-dicapsaicin	0.8330	****	0.7836 to 0.8890	4.46E-06	****	3.848e-006 to 5.265e-006	1.850	1.462 to 2.524	-	0.9861
10	capsiate	n.d.			4.91E-04	****	???	1.808	1.362 to ???	-	0.9666
35	vanillin	n.d.									
37	4-Hydroxy-3-methoxybenzyl alcohol	n.d.									
8	Zucapsaicin	1.0230	-	0.9636 to 1.093	6.19E-08	-	4.321e-008 to 9.366e-008	0.782	0.6174 to 1.009	-	0.9752

Table 6

Detailed overview of the activity of compounds in combination with capsaicin on rTRPV1 receptor measured by calcium influx assay.

The single concentrations (column concentration) were added to a dilution series representing an expanded dose–response curve of capsaicin.

Values are normalized to capsaicin. The curves were fitted using at least 10 concentration points measured as triplicates. The least square non-linear fit with detection and elimination of outliers was used to calculate the confidential intervals and other values. The comparison of models by extra sum-of-squares F tests was used to determine statistical difference between capsaicin curve and combinations' curves (EC50 = median effective concentration, CI = confidence interval, * p<0.05, ** p< 0.01, *** p < 0.001, **** p < 0.0001)

Comp. is compound, Sig. Dec. is significant decrease, inter. is interaction.

Comp. No.	EC ₅₀ [μM]	concentration in combo [μM]	Bottom	Top	Top 95% CI	EC ₅₀ [μM]	EC ₅₀ 95% CI	Hill Slope	Hill Slope 95% CI	logEC50	R ²	EC ₅₀ Fold Change	sig. dec. of EC ₅₀	elevated bottom	Type of inter.
1	0.04		0.018	0.964	0.9265 to 1.004	0.0347	2.784e-008 to 4.292e-008	1.055	0.9042 to 1.238	-7.460	0.9377				

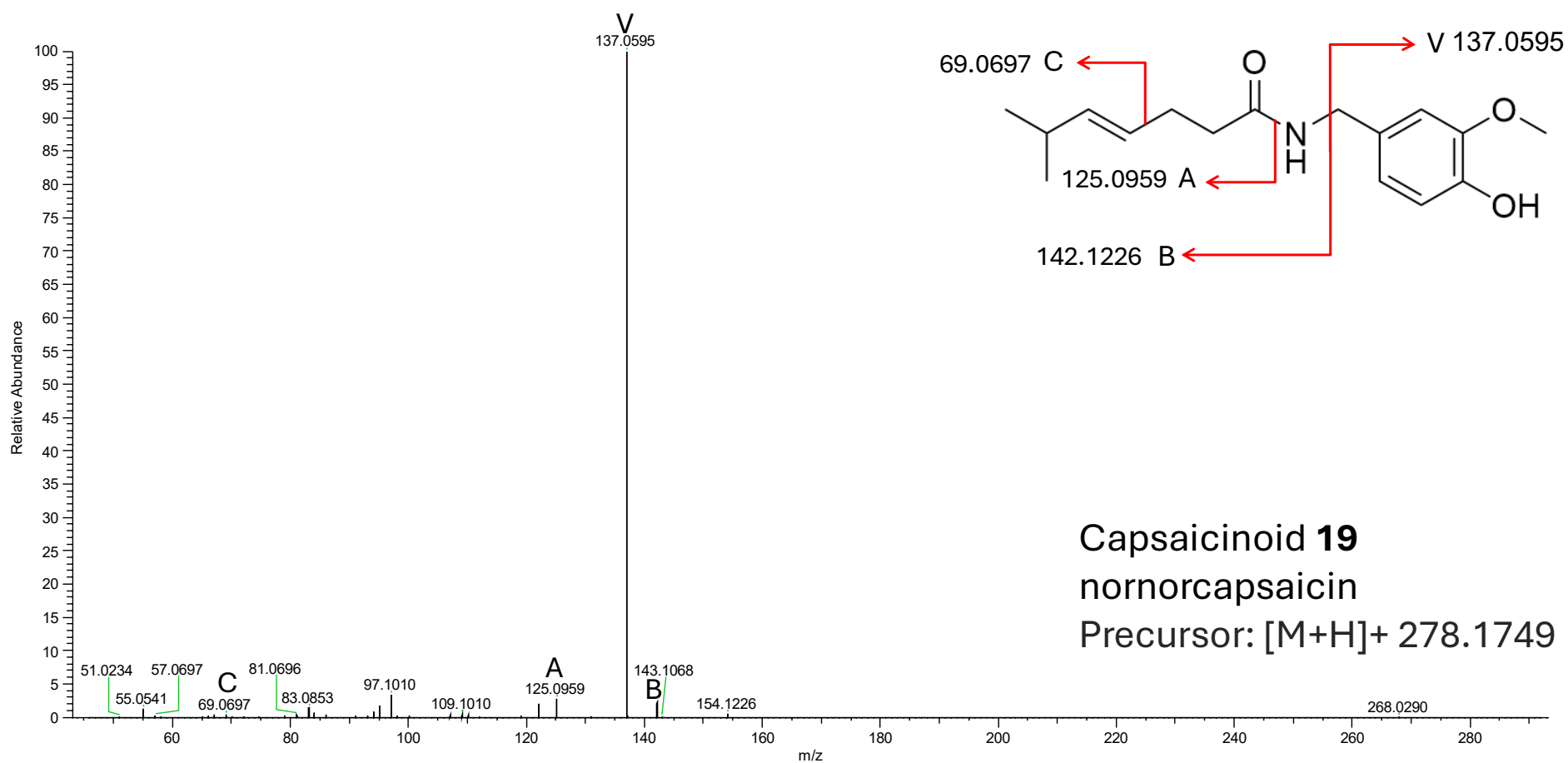
Smith 74

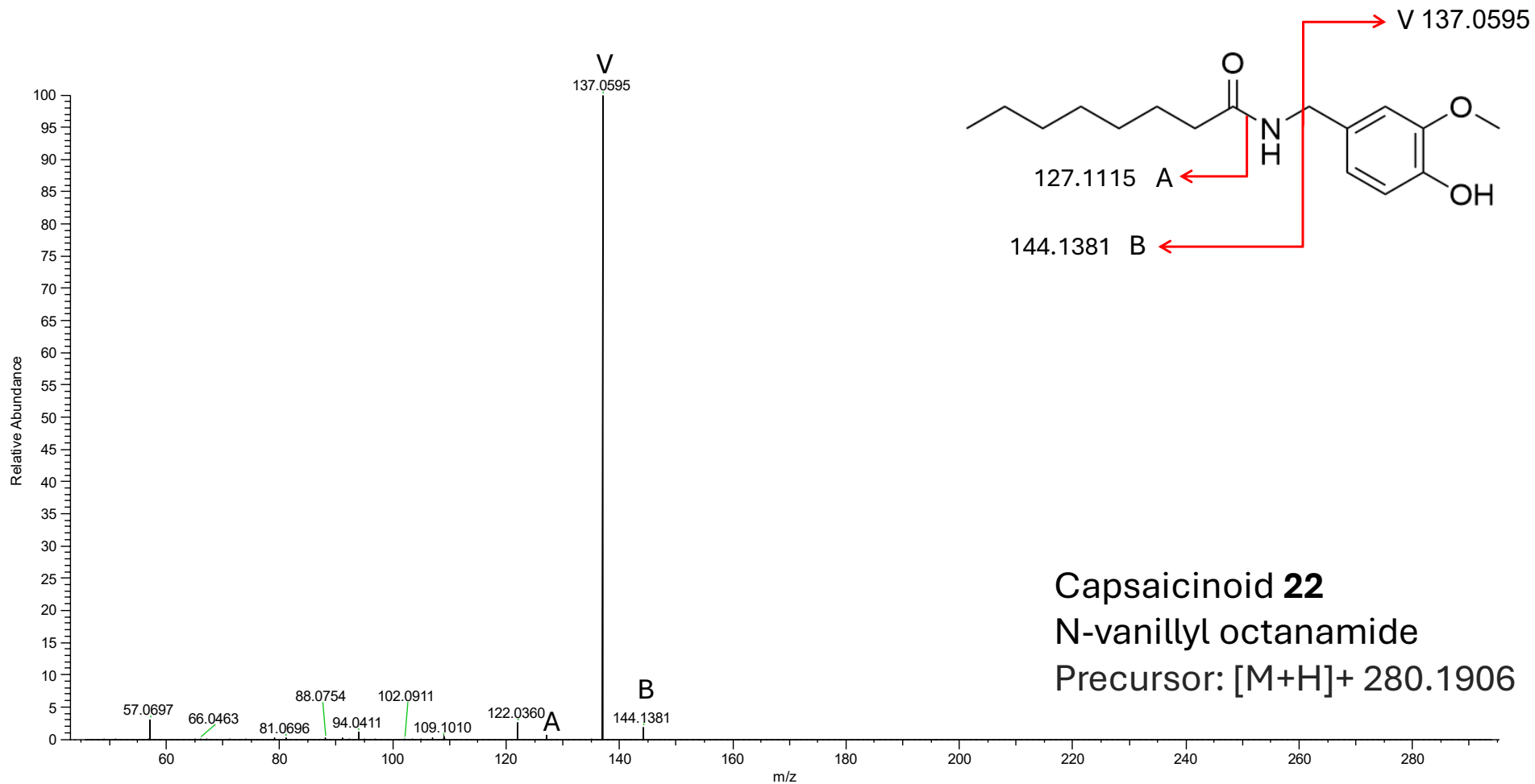
30	5.56	1.67	0.475	0.877	0.7972 to 0.9958 0.8066 to	0.0041	*	1.628e- 009 to 1.665e- 008 ??? to 3.358e- 008 2.490e- 008 to 5.907e- 008 3.747e- 008 to 5.868e- 008 1.347e- 008 to 2.346e- 008 1.346e- 008 to 2.365e- 008 1.001e- 008 to 5.448e- 008 3.199e- 008 to 5.555e- 008 7.188e- 009 to 1.164e- 008 1.966e- 009 to 3.550e- 009 1.182e- 008 to 2.050e- 008	1.163	0.4922 to ???	-8.391	0.6623	8.5293	yes	yes	additive /synergy
30	5.56	0.50	0.009	0.847	0.9203	0.0188	*	0.9051 to	2.931	1.547 to ???	-7.593	0.9916	1.8427	yes	no	synergy
31	10.27	1.67	0.042	0.955	0.8693 to 1.047	0.0393	-	0.9051 to 1.903	1.257	0.9051 to 1.903	-7.406	0.9762	0.8831	no	no	-
31	10.27	0.50	0.000	0.968	0.9222 to 1.013	0.0461	-	0.9527 to 1.302	1.081	0.9527 to 1.302	-7.337	0.9948	0.7528	no	no	-
45	2.84	0.21	0.038	0.967	0.9211 to 1.026	0.0175	****	0.9682 to 1.604	1.216	0.9682 to 1.604	-7.758	0.9840	1.9874	yes	no	synergy
48	6.33	0.21	0.036	0.872	0.8318 to 0.9278	0.0172	***	1.077 to 2.009	1.412	1.077 to 2.009	-7.763	0.9815	2.0116	yes	no	synergy
49	1.46	0.21	0.138	1.063	0.9451 to 1.265	0.0205	-	0.4290 to 0.8761	0.608	0.4290 to 0.8761	-7.688	0.8964	1.6909	no	yes	additive
49	1.46	0.08	0.054	0.995	0.9263 to 1.083	0.0419	-	0.9641 to 1.503	1.200	0.9641 to 1.503	-7.378	0.9757	0.8285	no	no	-
19	#####	1.67	0.012	0.961	0.9257 to 1.004	0.0090	****	0.9495 to 1.523	1.197	0.9495 to 1.523	-8.047	0.9824	3.8636	yes	no	synergy
35	n.d.	1.00	0.022	0.837	0.7944 to 0.8797	0.0027	****	1.173 to 4.616	1.666	1.173 to 4.616	-8.571	0.9270	12.9066	yes	no	synergy
35	n.d.	0.10	0.000	0.863	0.8236 to 0.9117	0.0151	****	1.084 to 1.997	1.389	1.084 to 1.997	-7.820	0.9943	2.2906	yes	no	synergy

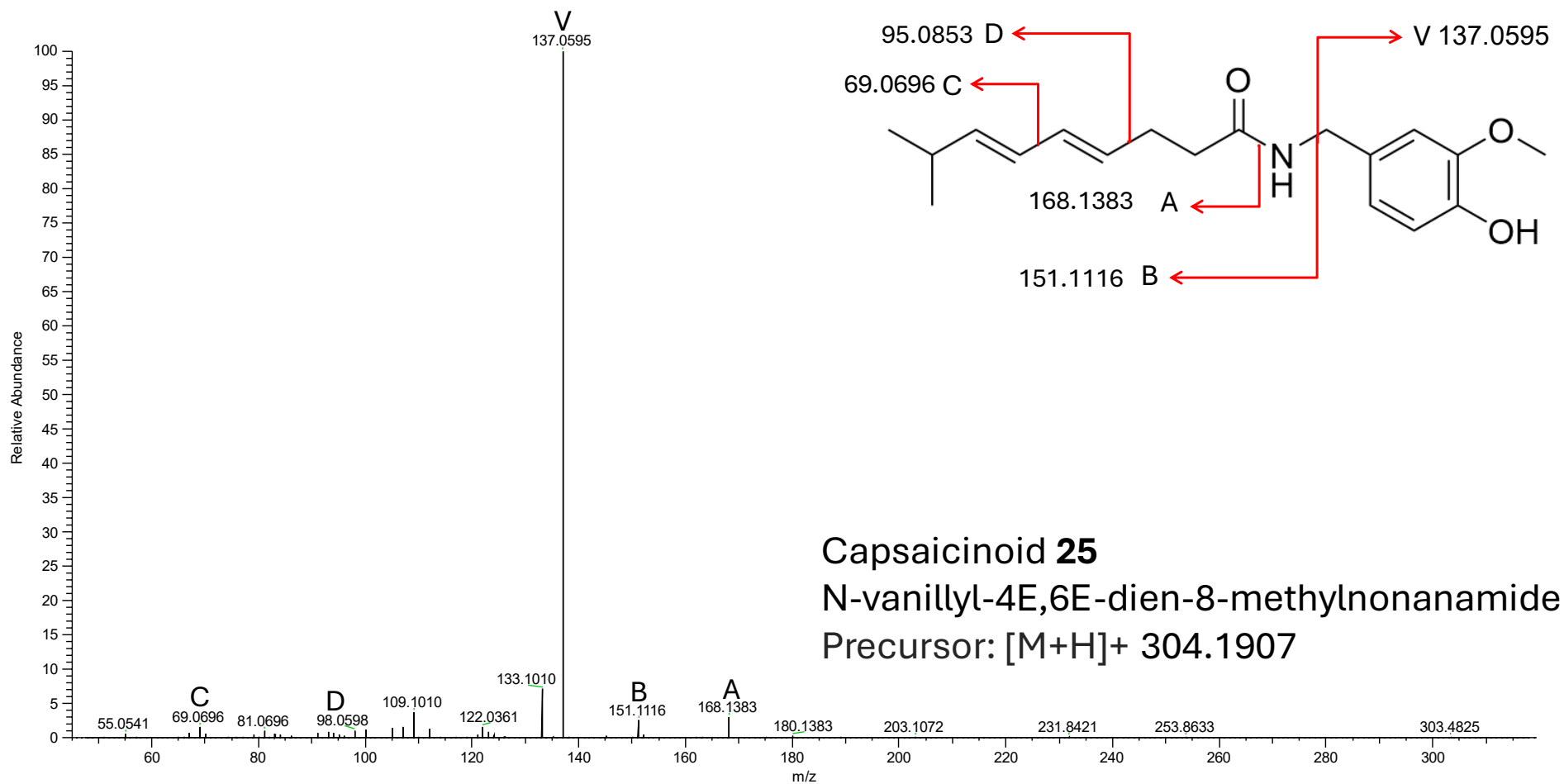
Smith 75

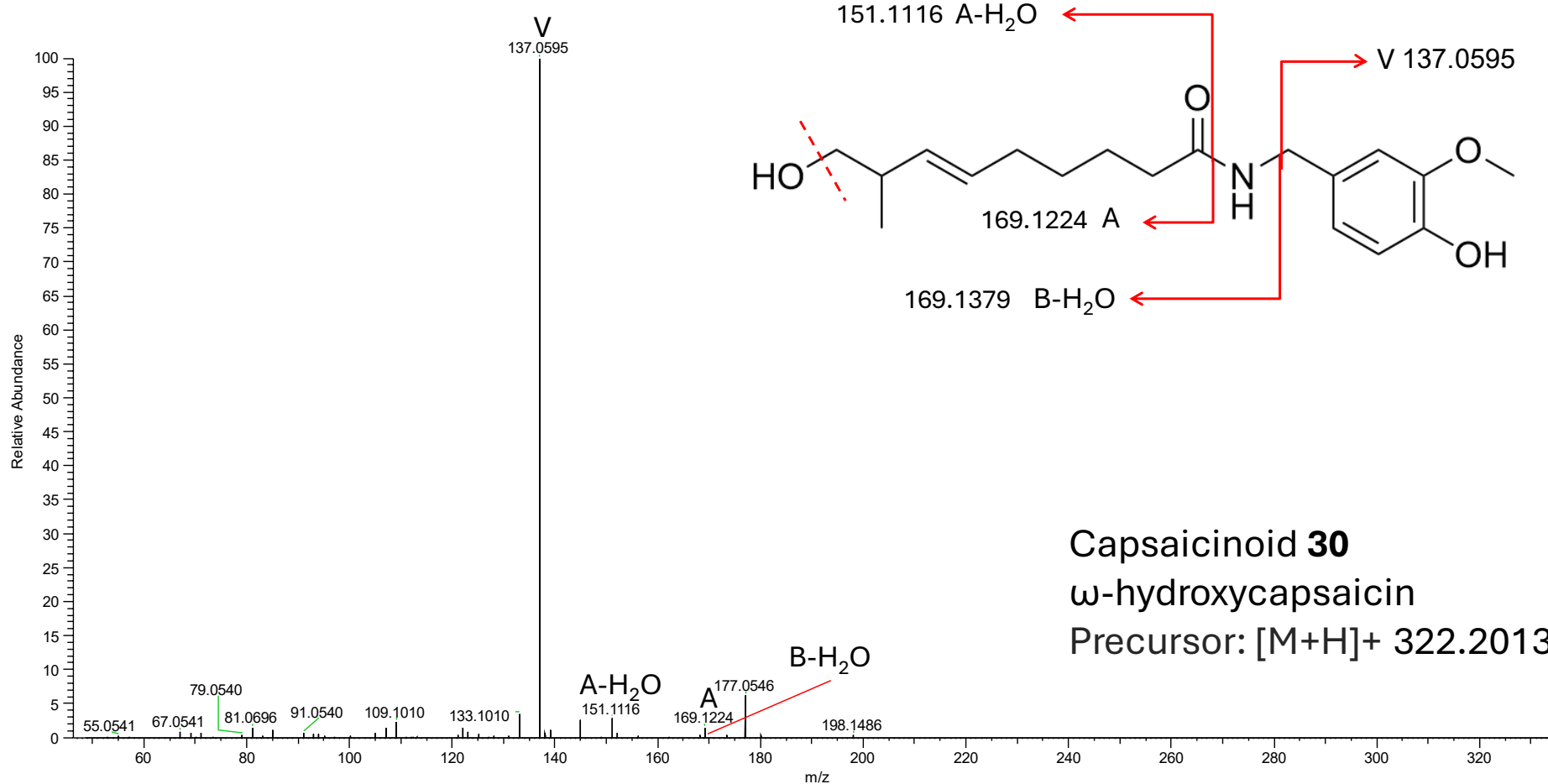
35	n.d.	0.05	0.000	0.990	0.9161 to 1.060	0.0432	*	3.291e- 008 to 6.175e- 008	0.923	0.8064 to 1.166	-7.365	0.9885	0.8033	no	no	-
37	n.d.	1.04	0.021	0.874	0.8427 to 0.9093	0.0114	****	9.538e- 009 to 1.399e- 008	1.566	1.288 to 1.903	-7.942	0.9876	3.0368	yes	no	synergy
43&44	n.d.	20.00	0.026	1.010	0.9478 to 1.096	0.0210	*	1.540e- 008 to 3.072e- 008	0.935	0.7501 to 1.186	-7.678	0.9782	1.6514	yes	no	synergy
43&44	n.d.	1.67	0.065	0.959	0.8691 to 1.089	0.0162	*	9.431e- 009 to 2.978e- 008	0.673	0.4567 to 1.019	-7.792	0.9760	2.1474	yes	no	synergy

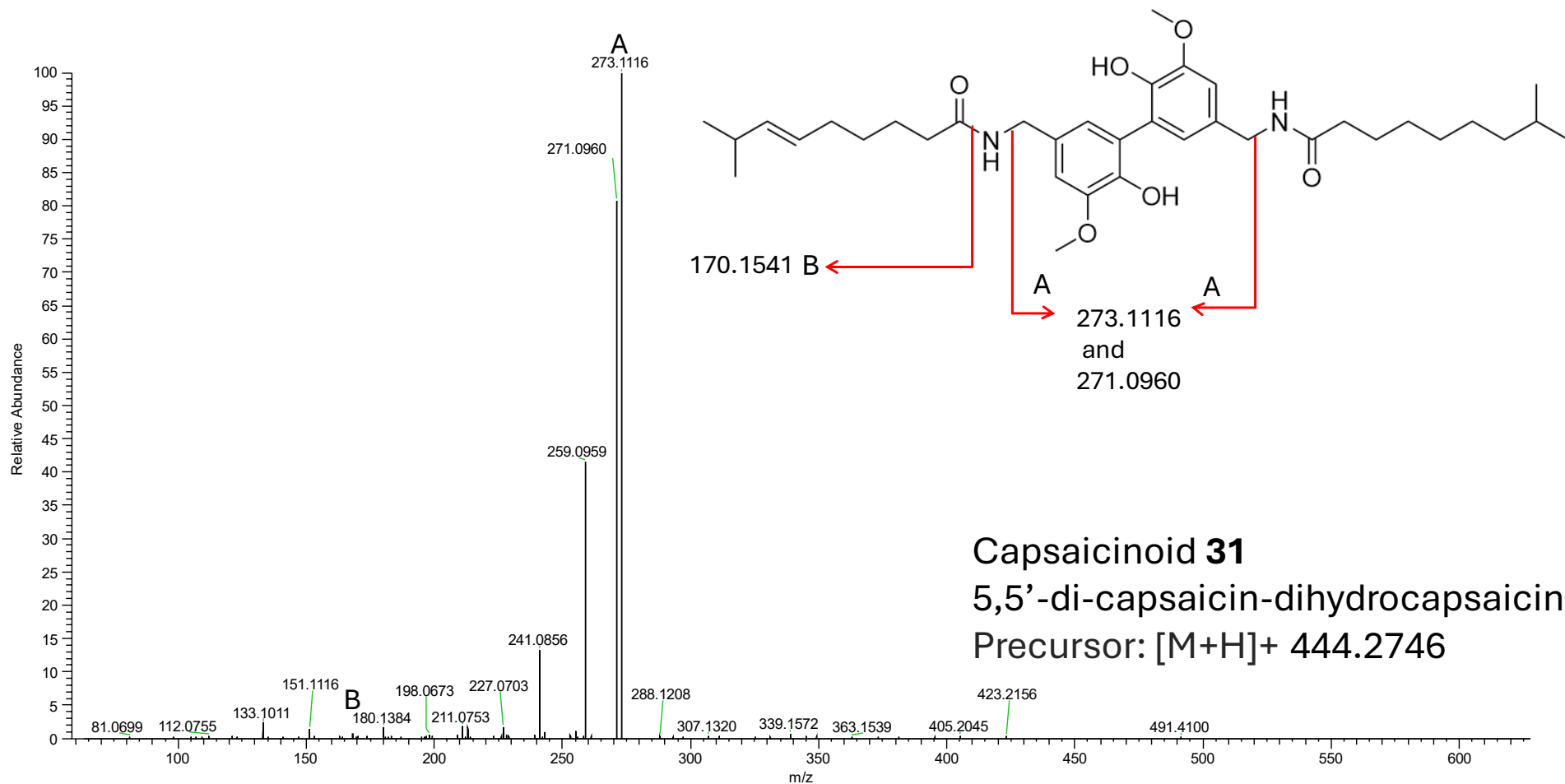
9. MS/MS annotation

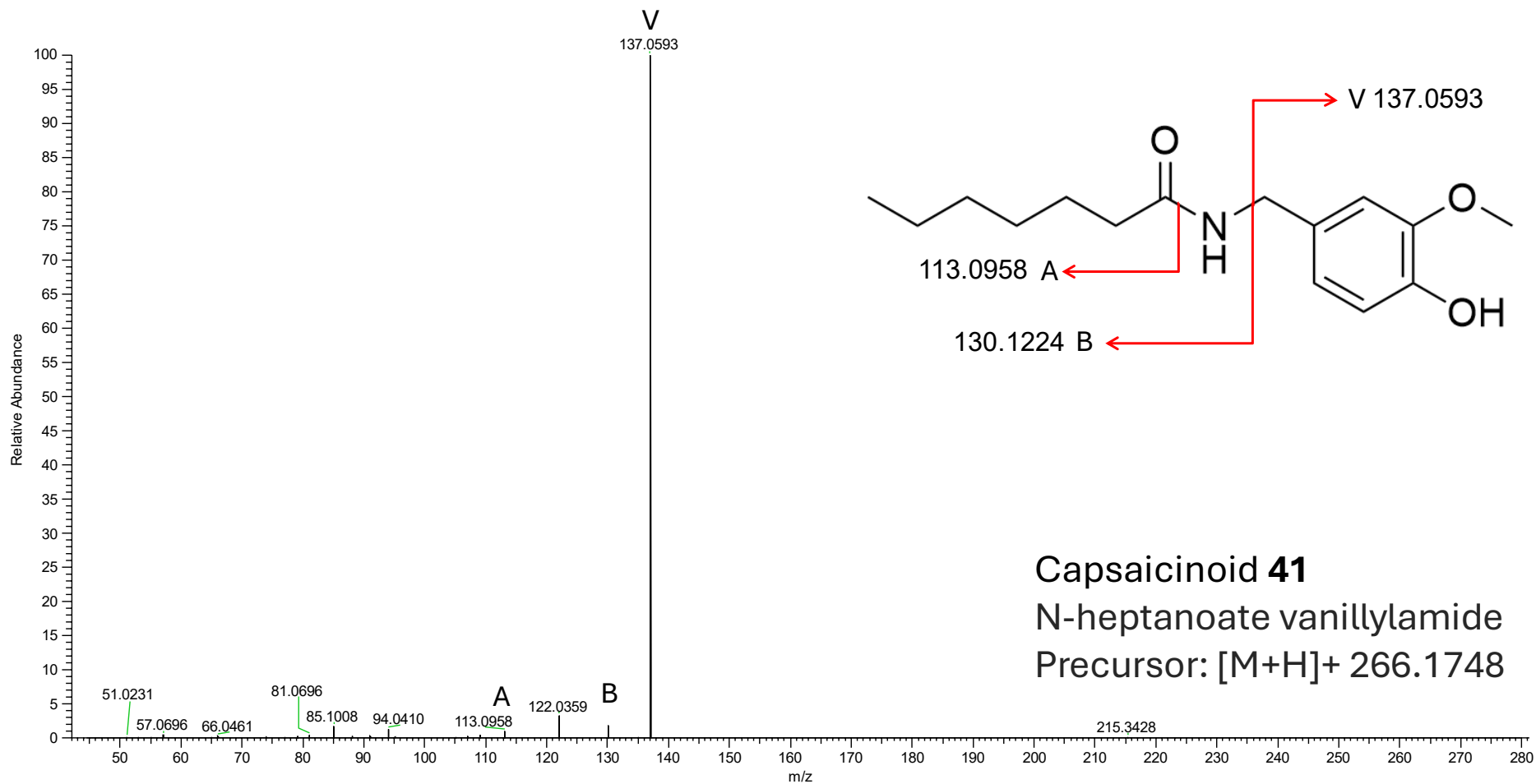


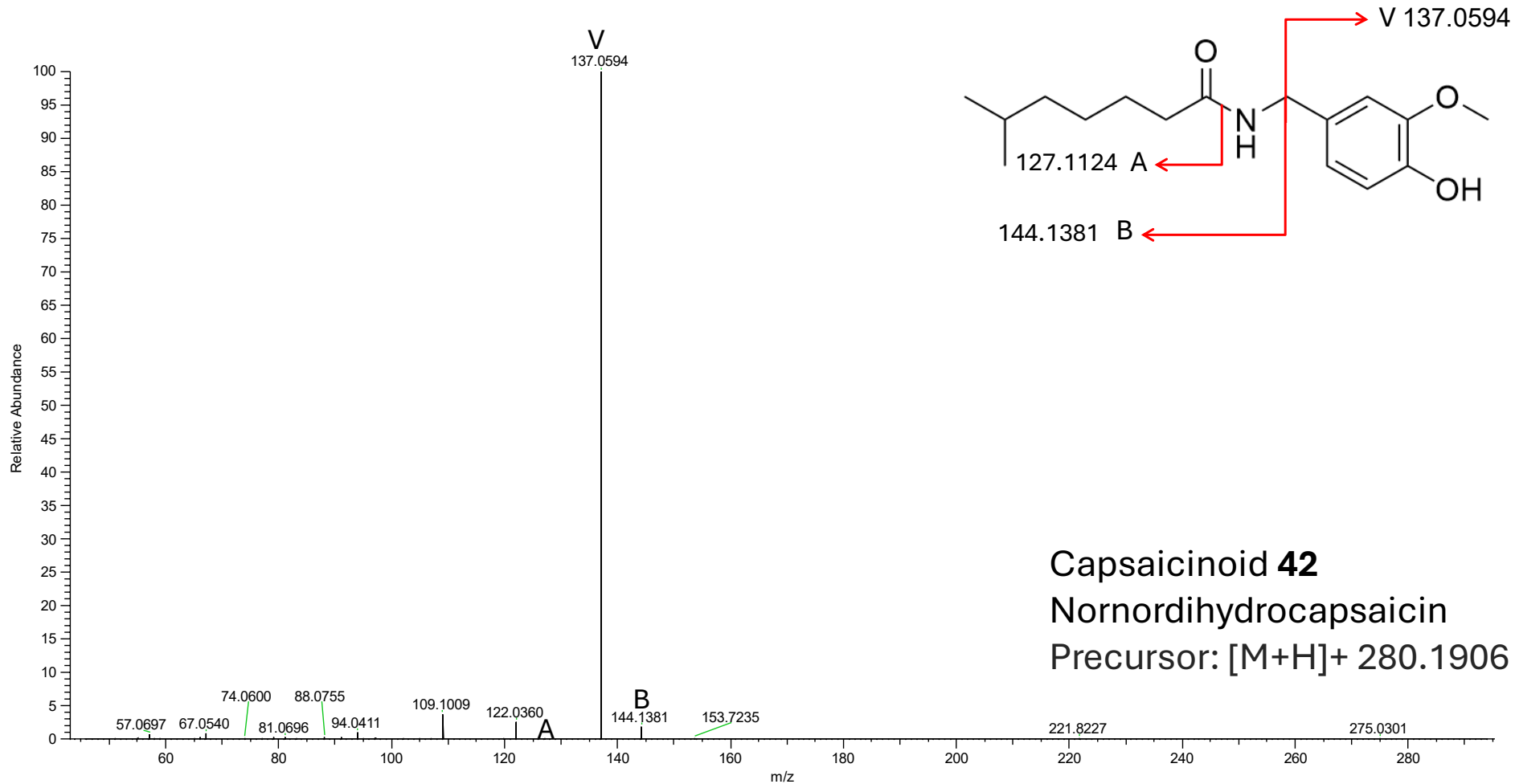


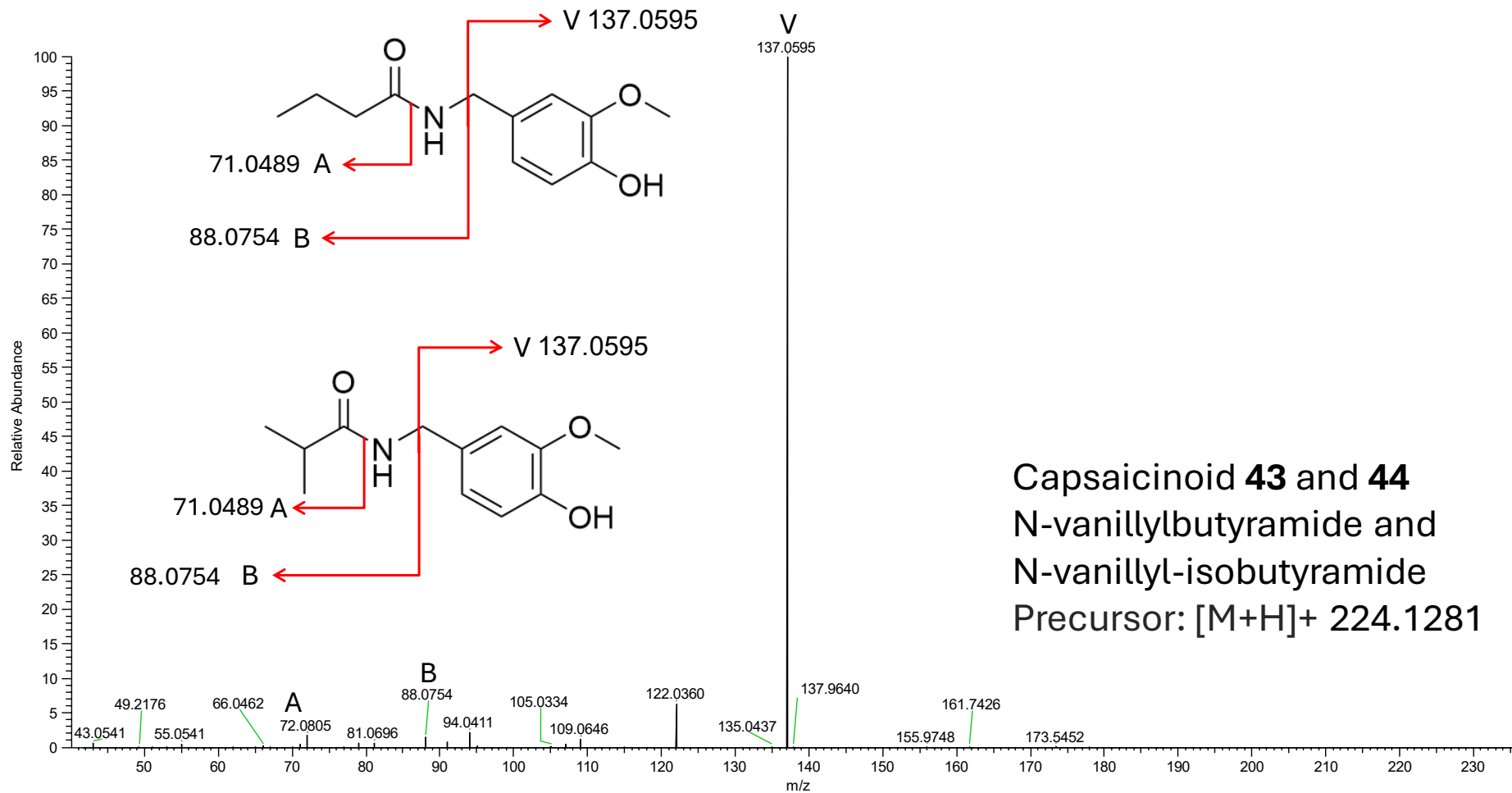




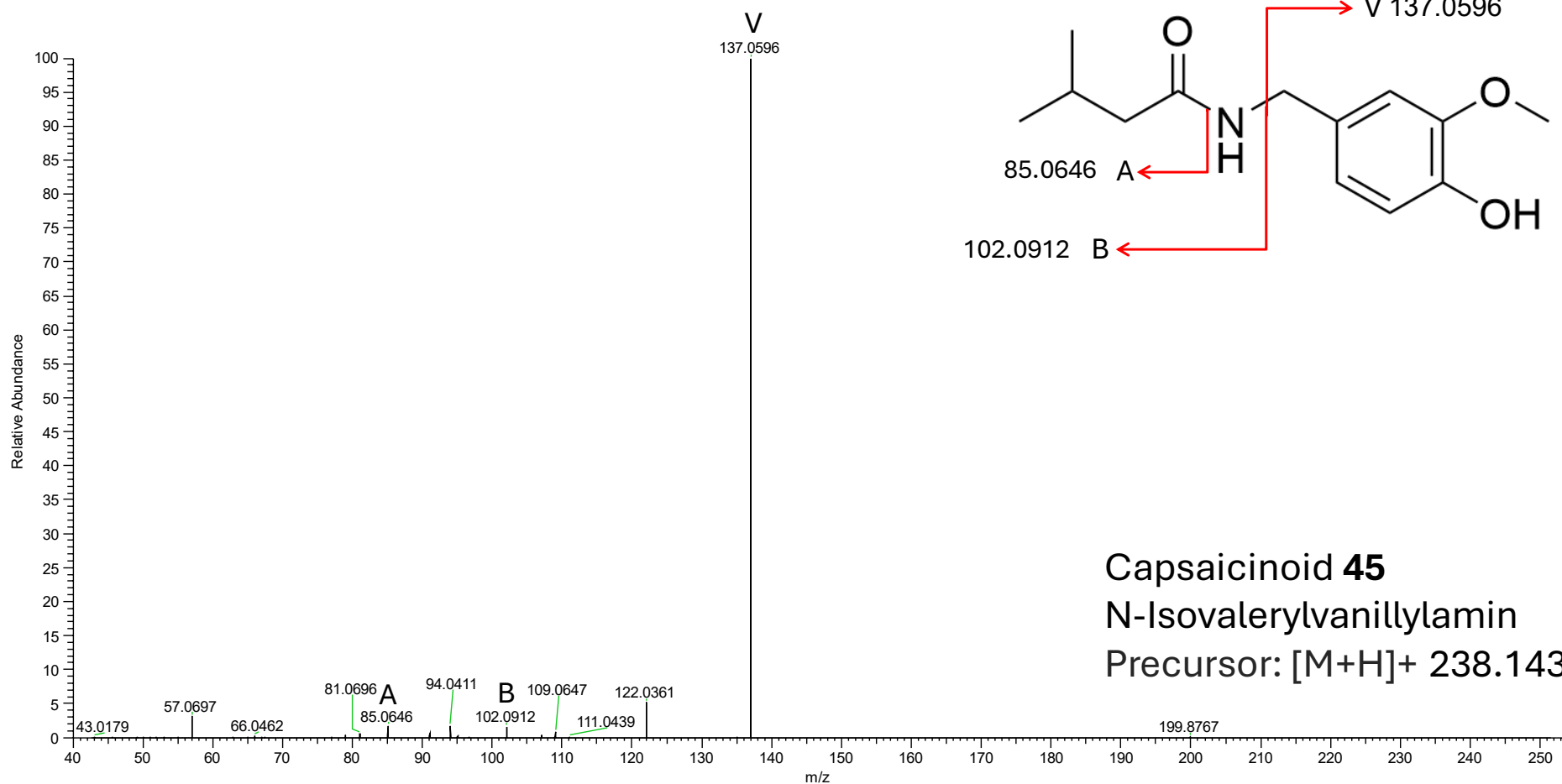




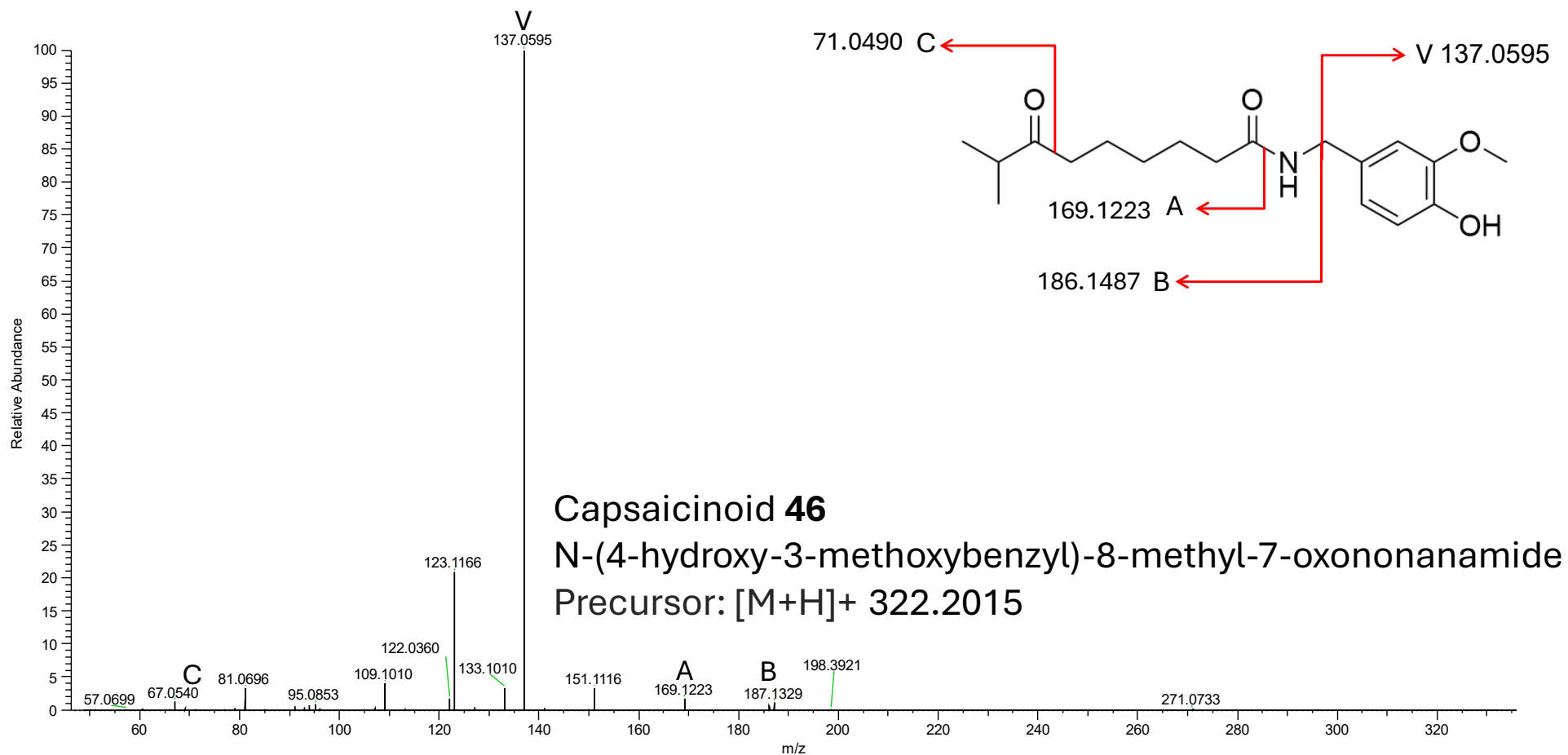


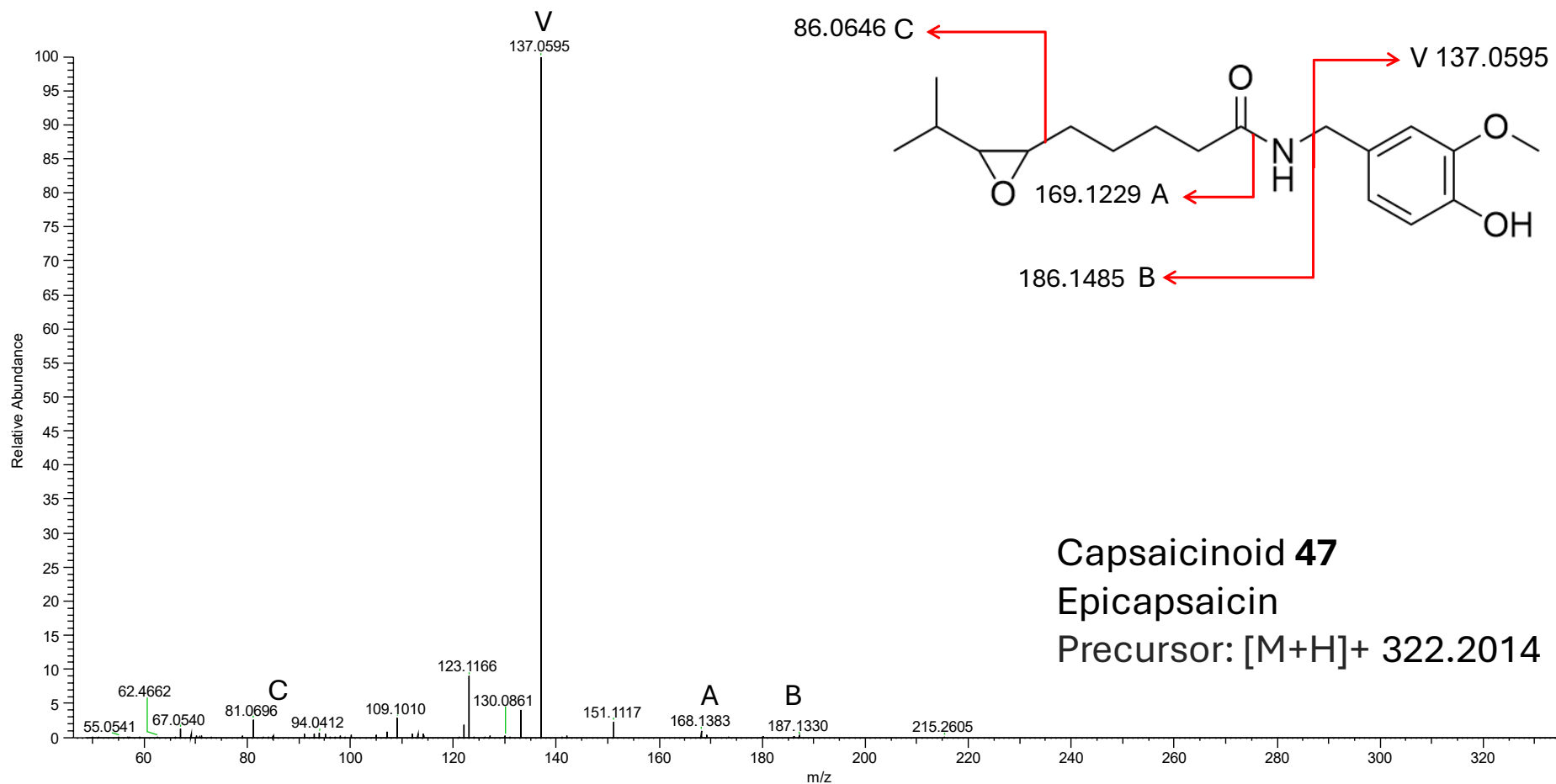


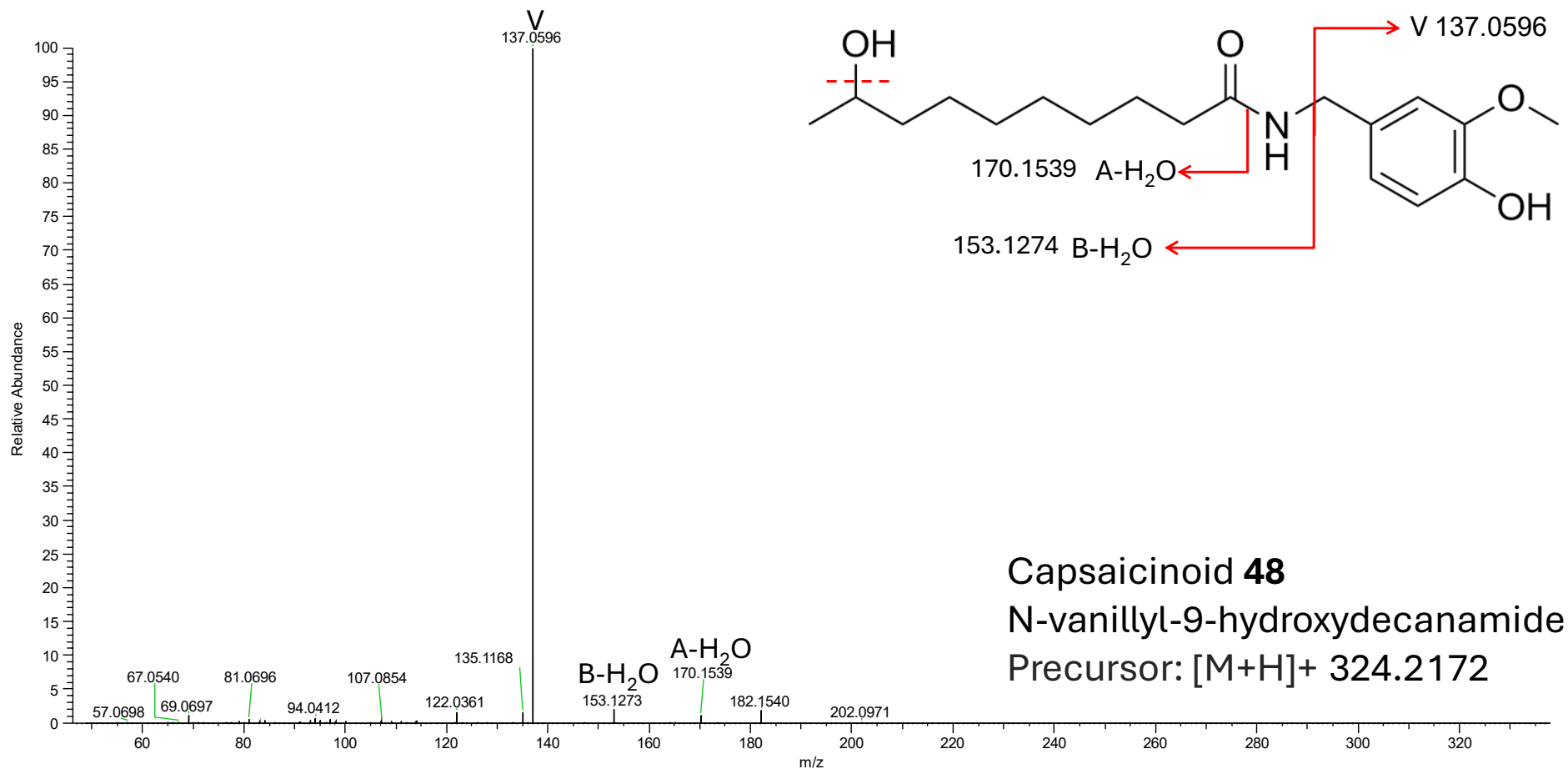
Capsaicinoid **43** and **44**
N-vanillylbutyramide and
N-vanillyl-isobutyramide
Precursor: [M+H]⁺ 224.1281

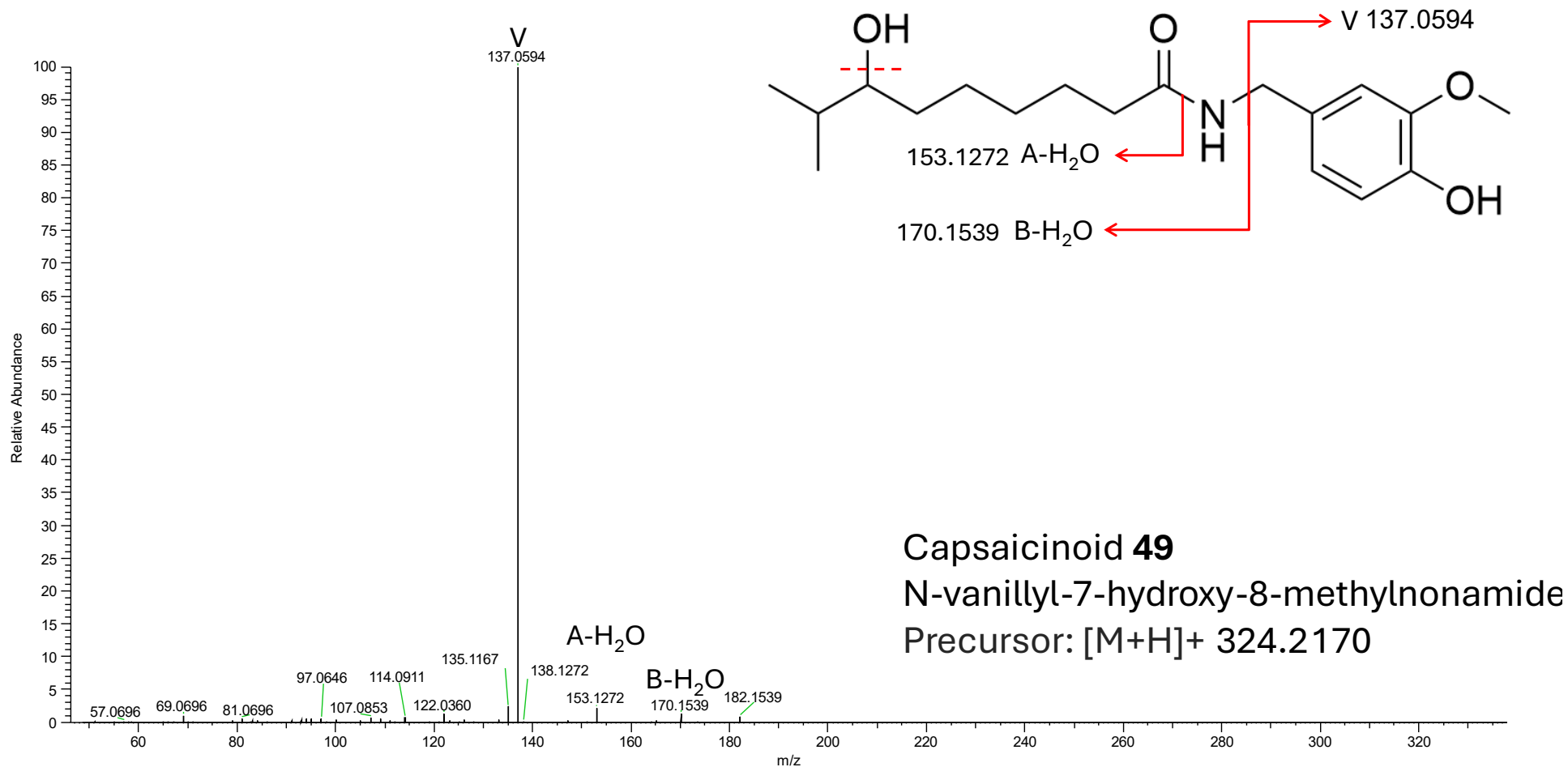


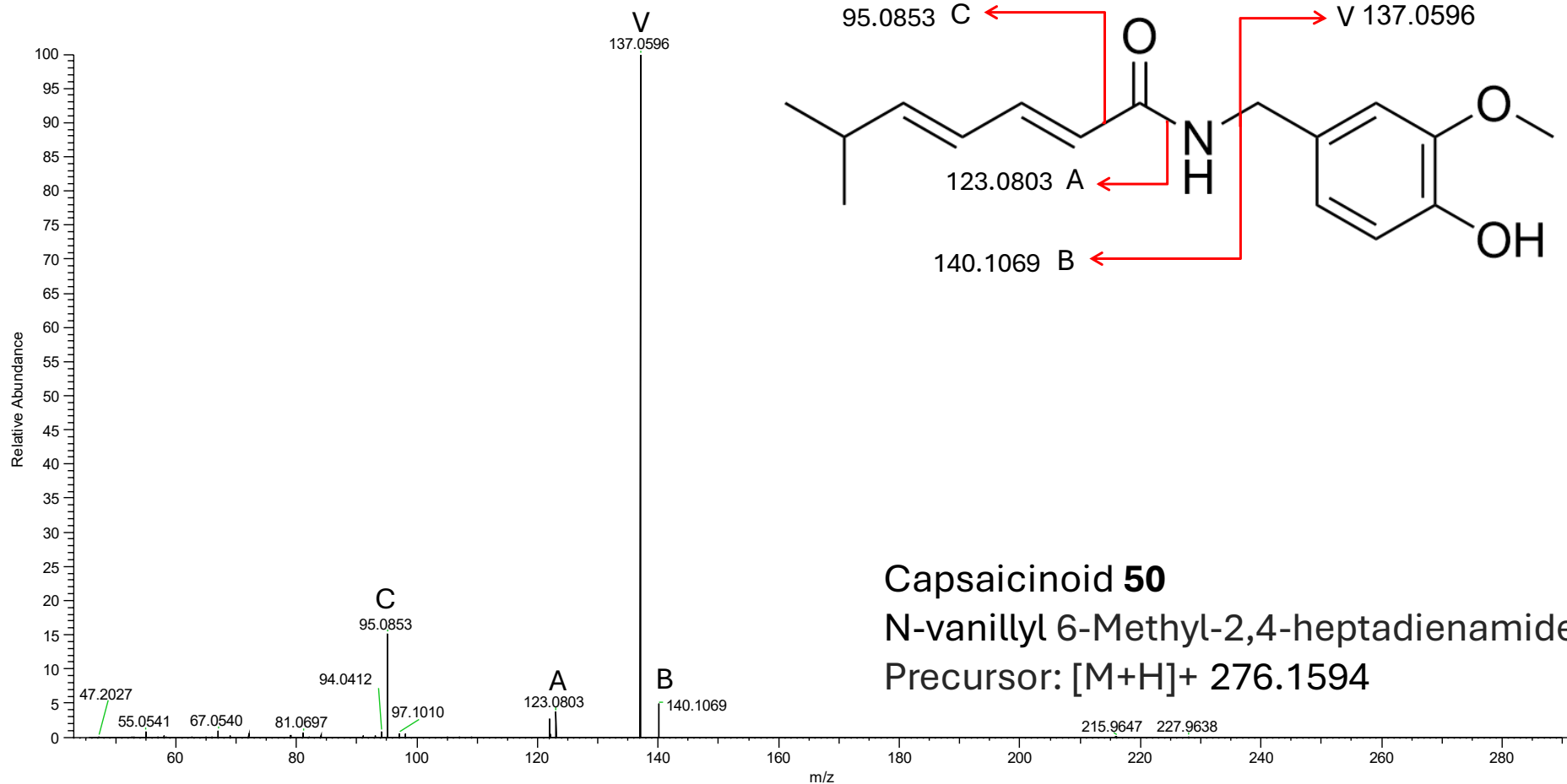
Capsaicinoid **45**
N-Isovalerylvanillylamin
Precursor: $[M+H]^+$ 238.1437

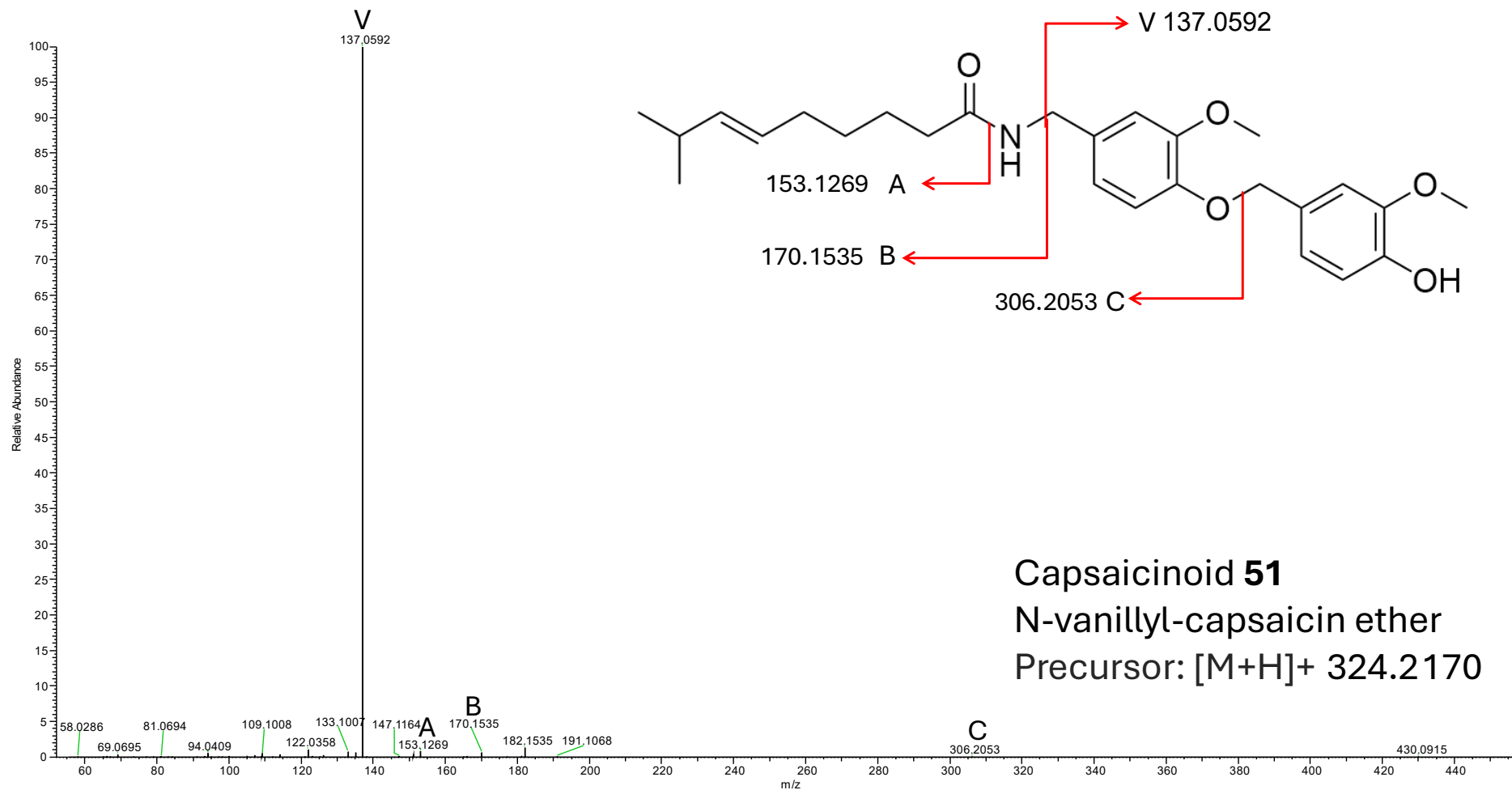


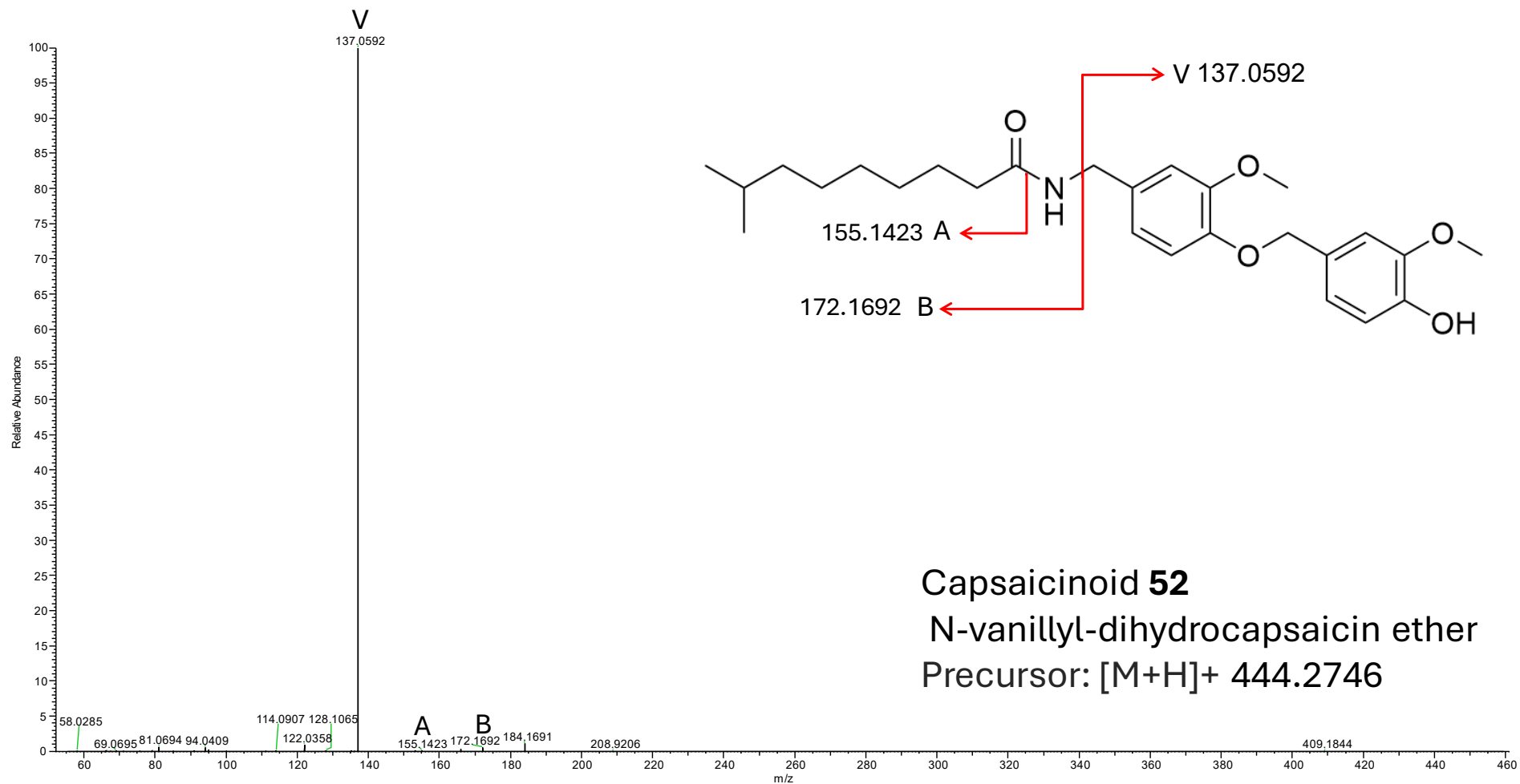


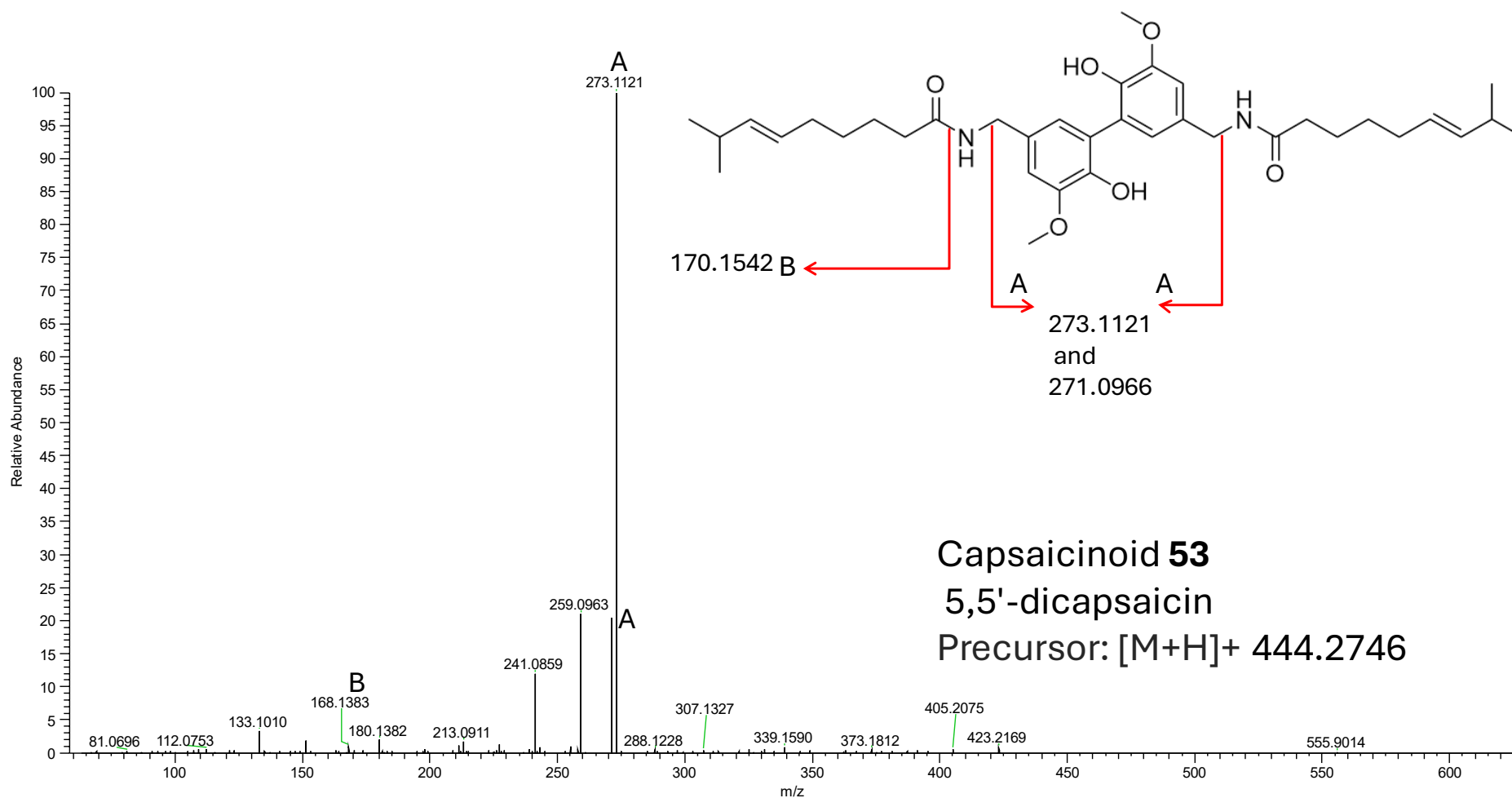












10. Data Availability

All scripts for post-processing mzmine data can be found at

<https://github.com/jdsmith145/Capsaicinoids>. Raw data from the metabolomics study, including all batch files and processed data files can be found in the MassIVE repository under reference number MSV000096223. All NMR spectra were reported to the NP-MRD (np-mrd.org); individual identifiers can be found in **Chapter 8, Table 1**. All MS data, along with all mzmine exports, molecular network analysis and NMR data were also deposited at Zenodo doi: 10.5281/zenodo.15088148. Lastly, all data related to the biological assays can also be found at Zenodo doi: 10.5281/zenodo.14204350.

11. References

1. Alawi, K., Keeble, J., 2010. The paradoxical role of the transient receptor potential vanilloid 1 receptor in inflammation. *Pharmacol. Ther.* 125, 181–195.
2. Alsalem, M., Millns, P., Altarifi, A., El-Salem, K., Chapman, V., Kendall, D.A., 2016. Antinociceptive and desensitizing effects of olvanil on capsaicin-induced thermal hyperalgesia in the rat. *BMC Pharmacol. Toxicol.* 17, 31.
3. Altman, R.D., Aven, A., Holmburg, C.E., Pfeifer, L.M., Sack, M., Young, G.T., 1994. Capsaicin cream 0.025% as Monotherapy for Osteoarthritis: A double-blind study. *Semin. Arthritis Rheum.* 23, 25–33.
4. Aluru, M.R., Mazourek, M., Landry, L.G., Curry, J., Jahn, M., O'Connell, M.A., 2003. Differential expression of fatty acid synthase genes, Acl, Fat and Kas, in Capsicum fruit. *J. Exp. Bot.* 54, 1655–1664.
5. Andolfo, I., Alper, S.L., Iolascon, A., 2022. Nobel prize in physiology or medicine 2021, receptors for temperature and touch: Implications for hematology. *Am. J. Hematol.* 97, 168–170.
6. Asiri, Y.I., Zaheen Hassan, M., 2023. An overview of ion channels therapeutics in the treatment of pain. *Arab. J. Chem.* 16, 105180.
7. Atanasov, A.G., Zotchev, S.B., Dirsch, V.M., Supuran, C.T., 2021. Natural products in drug discovery: advances and opportunities. *Nat. Rev. Drug Discov.* 20, 200–216.
8. Attuquayefio, V.K., Buckle, K.A., 1987. Rapid sample preparation method for HPLC analysis of capsaicinoids in capsicum fruits and oleoresins. *J. Agric. Food Chem.* 35, 777–779.
9. Atwal, S., 2023. Pepper X dethrones Carolina Reaper as world's hottest chilli pepper [WWW Document]. Guinness World Records. URL <https://www.guinnessworldrecords.com/news/2023/10/pepper-x-dethrones-carolina-reaper-as-worlds-hottest-chilli-pepper-759706> (accessed 3.30.25).
10. Aza-González, C., Núñez-Palenius, H.G., Ochoa-Alejo, N., 2011. Molecular biology of capsaicinoid biosynthesis in chili pepper (*Capsicum* spp.). *Plant Cell Rep.* 30, 695–706.
11. Bae, C., Jara-Oseguera, A., Krepiy, D.V., Ryu, J., Kim, J.I., Swartz, K.J., 2015. Surface

- characterization and membrane interaction of double-knot toxin, an activator of TRPV1 channels. *Biophys. J.* 108, 36a.
12. Bernal, M.A., Barceló, A.R., 1996. 5,5'-Dicapsaicin, 4'-O-5-Dicapsaicin Ether, and Dehydrogenation Polymers with High Molecular Weights Are the Main Products of the Oxidation of Capsaicin by Peroxidase from Hot Pepper. *J. Agric. Food Chem.* 44, 3085–3089.
 13. Bevan, S., 1999. Capsaicin and pain mechanisms, in: *Pain and Neurogenic Inflammation*. Birkhäuser Basel, Basel, pp. 61–80.
 14. Bevan, S., Hothi, S., Hughes, G., James, I.F., Rang, H.P., Shah, K., Walpole, C.S., Yeats, J.C., 1992. Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.* 107, 544–552.
 15. Bhawe, G., Zhu, W., Wang, H., Brasier, D.J., Oxford, G.S., Gereau, R.W., 4th, 2002. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* 35, 721–731.
 16. Bollimuntha, S., Selvaraj, S., Singh, B.B., 2011. Emerging roles of canonical TRP channels in neuronal function. *Adv. Exp. Med. Biol.* 704, 573–593.
 17. Boring, B.L., Ng, B.W., Nanavaty, N., Mathur, V.A., 2022. Over-rating pain is overrated: A fundamental self-other bias in pain reporting behavior. *J. Pain* 23, 1779–1789.
 18. Brand, L., Berman, E., Schwen, R., Loomans, M., Janusz, J., Bohne, R., Maddin, C., Gardner, J., Lahann, T., Farmer, R., 1987. NE-19550: a novel, orally active anti-inflammatory analgesic. *Drugs Exp. Clin. Res.* 13, 259–265.
 19. Brand, L.M., Skare, K.L., Loomans, M.E., Reller, H.H., Schwen, R.J., Lade, D.A., Bohne, R.L., Maddin, C.S., Moorehead, D.P., Fanelli, R., 1990. Anti-inflammatory pharmacology and mechanism of the orally active capsaicin analogs, NE-19550 and NE-28345. *Agents Actions* 31, 329–340.
 20. Brown, D.C., 2016. Resiniferatoxin: The evolution of the “molecular scalpel” for chronic pain relief. *Pharmaceuticals (Basel)* 9, 47.
 21. Buitimea-Cantúa, G.V., Velez-Haro, J.M., Buitimea-Cantúa, N.E., Molina-Torres, J., Rosas-Burgos, E.C., 2020. GC-EIMS analysis, antifungal and anti-aflatoxigenic activity of *Capsicum chinense* and *Piper nigrum* fruits and their bioactive compounds capsaicin and piperine upon *Aspergillus parasiticus*. *Nat. Prod. Res.* 34, 1452–1455.
 22. Cantero-Recasens, G., Gonzalez, J.R., Fandos, C., Duran-Tauleria, E., Smit, L.A.M., Kauffmann, F., Antó, J.M., Valverde, M.A., 2010. Loss of function of transient receptor potential vanilloid 1 (TRPV1) genetic variant is associated with lower risk of active childhood asthma. *J. Biol. Chem.* 285, 27532–27535.
 23. Cao, E., Liao, M., Cheng, Y., Julius, D., 2013. TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* 504, 113–118.
 24. Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816–824.
 25. Chen, Y., Mu, J., Zhu, M., Mukherjee, A., Zhang, H., 2020. Transient receptor potential channels and inflammatory bowel disease. *Front. Immunol.* 11, 180.
 26. Cho, Y., Kim, M.S., Kim, H.S., Ann, J., Lee, J., Pearce, L.V., Pavlyukovets, V.A., Morgan, M.A., Blumberg, P.M., Lee, J., 2012. The SAR analysis of TRPV1 agonists with the α -methylated B-region. *Bioorg. Med. Chem. Lett.* 22, 5227–5231.
 27. Chu, Y., Cohen, B.E., Chuang, H.-H., 2020. A single TRPV1 amino acid controls species sensitivity to capsaicin. *Sci. Rep.* 10, 8038.
 28. Collins, M.D., Wasmund, L.M., Bosland, P.W., 1995. Improved method for quantifying capsaicinoids in *Capsicum* using high-performance liquid chromatography. *HortScience* 30, 137–139.
 29. Constant, H.L., Cordell, G.A., West, D.P., 1996. Nonivamide, a Constituent of *Capsicum*

- oleoresin. *J. Nat. Prod.* 59, 425–426.
30. Cosens, D.J., Manning, A., 1969. Abnormal electroretinogram from a *Drosophila* mutant. *Nature* 224, 285–287.
 31. Costa, R.M., Liu, L., Nicoletis, M.A.L., Simon, S.A., 2005. Gustatory effects of capsaicin that are independent of TRPV1 receptors. *Chem. Senses* 30 Suppl 1, i198–200.
 32. Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez, M., O'Connell, M.A., 1999. Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Sci.* 148, 47–57.
 33. Damiani, T., Smith, J., Hebra, T., Perković, M., Čičak, M., Kadlecová, A., Rybka, V., Dračinský, M., Pluskal, T., 2025. Computational metabolomics reveals overlooked chemodiversity of alkaloid scaffolds in *Piper fimbriatum*. *Plant J.* 121, e70086.
 34. Dedov, V.N., Tran, V.H., Duke, C.C., Connor, M., Christie, M.J., Mandadi, S., Roufogalis, B.D., 2002. Gingerols: a novel class of vanilloid receptor (VR1) agonists: Gingerols: a novel class of VR1 agonists. *Br. J. Pharmacol.* 137, 793–798.
 35. Deng, Y., Wang, Y., Huo, X., Deng, S., Jin, L., Zhang, H., Yu, Z., Ning, J., Ma, X., Wang, C., 2022. Microbial transformation of capsaicin by several human intestinal fungi and their inhibitory effects against lysine-specific demethylase 1. *Phytochemistry* 202, 113365.
 36. Dettmer, K., Aronov, P.A., Hammock, B.D., 2007. Mass spectrometry-based metabolomics: MASS SPECTROMETRY-BASED METABOLOMICS. *Mass Spectrom. Rev.* 26, 51–78.
 37. Discontinuation report ZUACTA [WWW Document], 2020. . www.drugshortagescanada.ca. URL (accessed 3.27.25).
 38. Domene, C., Darré, L., Oakes, V., Gonzalez-Resines, S., 2022. A potential route of capsaicin to its binding site in the TRPV1 ion channel. *J. Chem. Inf. Model.* 62, 2481–2489.
 39. Dong, Y., Yin, Y., Vu, S., Yang, F., Yarov-Yarovoy, V., Tian, Y., Zheng, J., 2019. A distinct structural mechanism underlies TRPV1 activation by piperine. *Biochem. Biophys. Res. Commun.* 516, 365–372.
 40. Dührkop, K., Fleischauer, M., Ludwig, M., Aksenov, A.A., Melnik, A.V., Meusel, M., Dorrestein, P.C., Rousu, J., Böcker, S., 2019. SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. *Nat. Methods* 16, 299–302.
 41. Dührkop, K., Nothias, L.-F., Fleischauer, M., Reher, R., Ludwig, M., Hoffmann, M.A., Petras, D., Gerwick, W.H., Rousu, J., Dorrestein, P.C., Böcker, S., 2020. Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. *Nat. Biotechnol.* 39, 462–471.
 42. Eid, S.R., 2011. Therapeutic targeting of TRP channels--the TR(i)P to pain relief. *Curr. Top. Med. Chem.* 11, 2118–2130.
 43. Elokely, K., Velisetty, P., Delemotte, L., Palovcak, E., Klein, M.L., Rohacs, T., Carnevale, V., 2016. Understanding TRPV1 activation by ligands: Insights from the binding modes of capsaicin and resiniferatoxin. *Proc. Natl. Acad. Sci. U. S. A.* 113, E137–45.
 44. Emir, T.L.R. (Ed.), 2017. *Neurobiology of TRP Channels*. CRC Press, Boca Raton : CRC Press, 2017.
 45. Ernesto Fattorusso, Virginia Lanzotti, Orazio Tagliatela-Scafati, Gian Cesare Tron, Giovanni Appendino, 2001. Bisnorsesquiterpenoids from *Euphorbia resinifera* Berg. and an Expedient Procedure to Obtain Resiniferatoxin from Its Fresh Latex. *Eur. J. Org. Chem.* [https://doi.org/10.1002/1099-0690\(20021\)2002:1<71::AID-EJOC71>3.0.CO;2-C](https://doi.org/10.1002/1099-0690(20021)2002:1<71::AID-EJOC71>3.0.CO;2-C)
 46. Feigin, A., Aronov, E., Bryant, B., Teeter, J., Brand, J., 1995. Capsaicin and its analogs induce ion channels in planar lipid bilayers. *Neuroreport* 6, 2134–2136.
 47. Fields, H.L., 1987. *Pain* McGraw-Hill. New York.
 48. Forstenpointner, J., Förster, M., May, D., Hofschulte, F., Cascorbi, I., Wasner, G.,

- Gierthmühlen, J., Baron, R., 2017. Short Report: TRPV1-polymorphism 1911 A>G alters capsaicin-induced sensory changes in healthy subjects. *PLoS One* 12, e0183322.
49. Fujiwake, H., Suzuki, T., Iwai, K., 1982a. Capsaicinoid formation in the protoplast from the placenta of *Capsicum* fruits. *Agricultural and biological chemistry* 46, 2591–2592.
 50. Fujiwake, H., Suzuki, T., Iwai, K., 1982b. Intracellular distributions of enzymes and intermediates involved in biosynthesis of capsaicin and its analogues in *Capsicum* fruits. *Agricultural and biological chemistry* 46, 2685–2689.
 51. Fu, Y., Deng, F., Yang, S., Xu, J., 2018. A bite of “masochism”: The psychological issues of eating spicy food. *Xin Li Ke Xue Jin Zhan* 26, 1651–1660.
 52. Gangwal, A., Lavecchia, A., 2025. Artificial intelligence in natural product drug discovery: Current applications and future perspectives. *J. Med. Chem.* 68, 3948–3969.
 53. Gannett, P.M., Nagel, D.L., Reilly, P.J., Lawson, T., Sharpe, J., Toth, B., 1988. Capsaicinoids: their separation, synthesis, and mutagenicity. *J. Org. Chem.* 53, 1064–1071.
 54. Garami, A., Shimansky, Y.P., Rumbus, Z., Vizin, R.C.L., Farkas, N., Hegyi, J., Szakacs, Z., Solymar, M., Csenkey, A., Chiche, D.A., Kapil, R., Kyle, D.J., Van Horn, W.D., Hegyi, P., Romanovsky, A.A., 2020. Hyperthermia induced by transient receptor potential vanilloid-1 (TRPV1) antagonists in human clinical trials: Insights from mathematical modeling and meta-analysis. *Pharmacol. Ther.* 208, 107474.
 55. Gardner, M., Steinberg, L., 2005. Peer influence on risk taking, risk preference, and risky decision making in adolescence and adulthood: an experimental study. *Dev. Psychol.* 41, 625–635.
 56. Garg, E., Zubair., M., n.d. Mass Spectrometer.
 57. Gaudet, R., 2008. TRP channels entering the structural era: TRP channels entering the structural era. *J. Physiol.* 586, 3565–3575.
 58. Gavva, N.R., Bannon, A.W., Hovland, D.N., Jr, Lehto, S.G., Klionsky, L., Surapaneni, S., Immke, D.C., Henley, C., Arik, L., Bak, A., Davis, J., Ernst, N., Hever, G., Kuang, R., Shi, L., Tamir, R., Wang, J., Wang, W., Zajic, G., Zhu, D., Norman, M.H., Louis, J.-C., Magal, E., Treanor, J.J.S., 2007. Repeated administration of vanilloid receptor TRPV1 antagonists attenuates hyperthermia elicited by TRPV1 blockade. *J. Pharmacol. Exp. Ther.* 323, 128–137.
 59. Glish, G.L., Burinsky, D.J., 2008. Hybrid mass spectrometers for tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* 19, 161–172.
 60. Gururaj, H.B., Padma, M.N., Giridhar, P., Ravishankar, G.A., 2012. Functional validation of *Capsicum frutescens* aminotransferase gene involved in vanillylamine biosynthesis using *Agrobacterium* mediated genetic transformation studies in *Nicotiana tabacum* and *Capsicum frutescens* calli cultures. *Plant Sci.* 195, 96–105.
 61. Han, K., Jeong, H.-J., Sung, J., Keum, Y.S., Cho, M.-C., Kim, J.-H., Kwon, J.-K., Kim, B.-D., Kang, B.-C., 2013. Biosynthesis of capsinoid is controlled by the Pun1 locus in pepper. *Mol. Breed.* 31, 537–548.
 62. Hanton, S.D., 2001. Mass spectrometry of polymers and polymer surfaces. *Chem. Rev.* 101, 527–569.
 63. Hardie, R.C., Minke, B., 1993. Novel Ca²⁺ channels underlying transduction in *Drosophila* photoreceptors: implications for phosphoinositide-mediated Ca²⁺ mobilization. *Trends in neurosciences* 16, 371–376.
 64. Heiles, S., 2021. Advanced tandem mass spectrometry in metabolomics and lipidomics-methods and applications. *Anal. Bioanal. Chem.* 413, 5927–5948.
 65. Henderson, D.E., Slickman, A.M., Henderson, S.K., 1999. Quantitative HPLC Determination of the Antioxidant Activity of Capsaicin on the Formation of Lipid Hydroperoxides of Linoleic Acid: A Comparative Study against BHT and Melatonin. *J. Agric. Food Chem.* 47, 2563–2570.

66. Heuckeroth, S., Damiani, T., Smirnov, A., Mokshyna, O., Brungs, C., Korf, A., Smith, J.D., Stincone, P., Dreolin, N., Nothias, L.-F., Hyötyläinen, T., Orešič, M., Karst, U., Dorrestein, P.C., Petras, D., Du, X., van der Hooft, J.J.J., Schmid, R., Pluskal, T., 2024. Reproducible mass spectrometry data processing and compound annotation in MZmine 3. *Nat. Protoc.* 1–45.
67. Higashiguchi, F., Nakamura, H., Hayashi, H., Kometani, T., 2006. Purification and structure determination of glucosides of capsaicin and dihydrocapsaicin from various *Capsicum* fruits. *J. Agric. Food Chem.* 54, 5948–5953.
68. Holland, C., van Drunen, C., Denyer, J., Smart, K., Segboer, C., Terreehorst, I., Newlands, A., Beerahee, M., Fokkens, W., Tsitoura, D.C., 2014. Inhibition of capsaicin-driven nasal hyper-reactivity by SB-705498, a TRPV1 antagonist: Intranasal SB-705498 in humans. *Br. J. Clin. Pharmacol.* 77, 777–788.
69. Iida, T., Moriyama, T., Kobata, K., Morita, A., Murayama, N., Hashizume, S., Fushiki, T., Yazawa, S., Watanabe, T., Tominaga, M., 2003. TRPV1 activation and induction of nociceptive response by a non-pungent capsaicin-like compound, capsiate. *Neuropharmacology* 44, 958–967.
70. Ishida, Y., Ugawa, S., Ueda, T., Murakami, S., Shimada, S., 2002. Vanilloid receptor subtype-1 (VR1) is specifically localized to taste papillae. *Brain Res. Mol. Brain Res.* 107, 17–22.
71. Ishimov, U.Z., Ziyavitdinov, Z.F., Sagdiev, N.Z., 2011. Minor constituents of total capsaicinoids from *Capsicum annuum*. *Chem. Nat. Compo.* 46, 1006–1007.
72. Jara-Oseguera, A., Huffer, K.E., Swartz, K.J., 2019. The ion selectivity filter is not an activation gate in TRPV1-3 channels. *Elife* 8. <https://doi.org/10.7554/eLife.51212>
73. Ježo, I., 1975. Synthese des Capsaicins und einiger seiner Modellderivate. *Chem. zvesti* 29, 714–718.
74. Jung, J., Shin, J.S., Lee, S.-Y., Hwang, S.W., Koo, J., Cho, H., Oh, U., 2004. Phosphorylation of vanilloid receptor 1 by Ca²⁺/calmodulin-dependent kinase II regulates its vanilloid binding. *J. Biol. Chem.* 279, 7048–7054.
75. Jurenitsch, J., David, M., Heresch, F., Kubelka, W., 1979a. Nachweis und Identifizierung neuer Scharfstoffe in *Capsicum*-Früchten. *Planta Med.* 36, 61–67.
76. Jurenitsch, J., David, M., Heresch, F., Kubelka, W., 1979b. Nachweis und Identifizierung neuer Scharfstoffe in *Capsicum*-Früchten. *Planta Med.* 36, 61–67.
77. Kárai, L.J., Russell, J.T., Iadarola, M.J., Oláh, Z., 2004. Vanilloid receptor 1 regulates multiple calcium compartments and contributes to Ca²⁺-induced Ca²⁺ release in sensory neurons. *J. Biol. Chem.* 279, 16377–16387.
78. Karos, K., Alleva, J.M., Peters, M.L., 2018. Pain, please: An investigation of sampling bias in pain research. *J. Pain* 19, 787–796.
79. Kirke, J., Kaplan, N., Velez, S., Jin, X.-L., Vichyavichien, P., Zhang, X.-H., 2018. Tissue-preferential activity and induction of the pepper capsaicin synthase PUN1 promoter by wounding, heat and metabolic pathway precursor in tobacco and tomato plants. *Mol. Biotechnol.* 60, 194–202.
80. Kobata, K., Kawamura, M., Toyoshima, M., Tamura, Y., Ogawa, S., Watanabe, T., 1998a. Lipase-Catalyzed Synthesis of Capsaicin Analogs by Amidation of Vanillylamine with Fatty Acid Derivatives. *Biotechnol. Lett.* 20, 451–454.
81. Kobata, K., Saito, K., Tate, H., Nashimoto, A., Okuda, H., Takemura, I., Miyakawa, K., Takahashi, M., Iwai, K., Watanabe, T., 2010. Long-chain N-vanillyl-acylamides from *Capsicum oleoresin*. *J. Agric. Food Chem.* 58, 3627–3631.
82. Kobata, K., Todo, T., Yazawa, S., Iwai, K., Watanabe, T., 1998b. Novel Capsaicinoid-like Substances, Capsiate and Dihydrocapsiate, from the Fruits of a Nonpungent Cultivar, CH-19 Sweet, of Pepper (*Capsicum annuum* L.). *J. Agric. Food Chem.* 46, 1695–1697.
83. Koivisto, A.-P., Belvisi, M.G., Gaudet, R., Szallasi, A., 2021. Advances in TRP channel

- drug discovery: from target validation to clinical studies. *Nat. Rev. Drug Discov.* 21, 41–59.
84. Kusaka, H., Nakasato, S., Sano, K., Kobata, K., Ohno, S., Doi, M., Tanaka, Y., 2024. An evolutionary view of vanillylamine synthase pAMT, a key enzyme of capsaicinoid biosynthesis pathway in chili pepper. *Plant J.* 117, 1453–1465.
 85. Kwon, D.H., Zhang, F., Suo, Y., Bouvette, J., Borgnia, M.J., Lee, S.-Y., 2021. Heat-dependent opening of TRPV1 in the presence of capsaicin. *Nat. Struct. Mol. Biol.* 28, 554–563.
 86. Lang, Y., Kisaka, H., Sugiyama, R., Nomura, K., Morita, A., Watanabe, T., Tanaka, Y., Yazawa, S., Miwa, T., 2009. Functional loss of pAMT results in biosynthesis of capsinoids, capsaicinoid analogs, in *Capsicum annuum* cv. CH-19 Sweet. *Plant J.* 59, 953–961.
 87. Lau, S.-Y., Procko, E., Gaudet, R., 2012. Distinct properties of Ca²⁺-calmodulin binding to N- and C-terminal regulatory regions of the TRPV1 channel. *J. Gen. Physiol.* 140, 541–555.
 88. Lee, J., Kang, S.-U., Choi, H.-K., Lee, J., Lim, J.-O., Kil, M.-J., Jin, M.-K., Kim, K.-P., Sung, J.-H., Chung, S.-J., Ha, H.-J., Kim, Y.-H., Pearce, L.V., Tran, R., Lundberg, D.J., Wang, Y., Toth, A., Blumberg, P.M., 2004. Analysis of structure-activity relationships for the “B-region” of N-(3-acyloxy-2-benzylpropyl)-N’-[4-(methylsulfonylamino)benzyl]thiourea analogues as vanilloid receptor antagonists: discovery of an N-hydroxythiourea analogue with potent analgesic activity. *Bioorg. Med. Chem. Lett.* 14, 2291–2297.
 89. Lee, J., Lee, J., Kang, M., Shin, M., Kim, J.-M., Kang, S.-U., Lim, J.-O., Choi, H.-K., Suh, Y.-G., Park, H.-G., Oh, U., Kim, H.-D., Park, Y.-H., Ha, H.-J., Kim, Y.-H., Toth, A., Wang, Y., Tran, R., Pearce, L.V., Lundberg, D.J., Blumberg, P.M., 2003. N-(3-Acyloxy-2-benzylpropyl)-N’-[4-(methylsulfonylamino)benzyl]thiourea Analogues: Novel Potent and High Affinity Antagonists and Partial Antagonists of the Vanilloid Receptor. *J. Med. Chem.* 46, 3116–3126.
 90. Liao, M., Cao, E., Julius, D., Cheng, Y., 2013. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504, 107–112.
 91. li-chin, R.S.S.H., 2023. https://mzmine.github.io/mzmine_documentation/index.html [WWW Document]. Data handling (supported formats). URL https://mzmine.github.io/mzmine_documentation/data_conversion.html
 92. Li, H., 2017. TRP channel classification. *Adv. Exp. Med. Biol.* 976, 1–8.
 93. Lishko, P.V., Procko, E., Jin, X., Phelps, C.B., Gaudet, R., 2007. The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* 54, 905–918.
 94. Liu, F., Zhao, J., Sun, H., Xiong, C., Sun, X., Wang, X., Wang, Z., Jarret, R., Wang, J., Tang, B., Xu, H., Hu, B., Suo, H., Yang, B., Ou, L., Li, X., Zhou, S., Yang, S., Liu, Z., Yuan, F., Pei, Z., Ma, Y., Dai, X., Wu, S., Fei, Z., Zou, X., 2023. Genomes of cultivated and wild *Capsicum* species provide insights into pepper domestication and population differentiation. *Nat. Commun.* 14, 5487.
 95. Liu, T.-Y., Chu, Y., Mei, H.-R., Chang, D., Chuang, H.-H., 2019. Two vanilloid ligand bindings per channel are required to transduce capsaicin-activating stimuli. *Front. Mol. Neurosci.* 12, 302.
 96. Liviero, F., Campisi, M., Scarpa, M.C., Mason, P., Guarnieri, G., Maestrelli, P., Pavanello, S., 2020. Multiple single nucleotide polymorphisms of the transient receptor potential vanilloid 1 (TRPV1) genes associate with cough sensitivity to capsaicin in healthy subjects. *Pulm. Pharmacol. Ther.* 61, 101889.
 97. Ludy, M.-J., Moore, G.E., Mattes, R.D., 2012. The effects of capsaicin and capsiate on energy balance: critical review and meta-analyses of studies in humans. *Chem. Senses* 37, 103–121.

98. Lyall, V., Heck, G.L., Vinnikova, A.K., Ghosh, S., Phan, T.-H.T., Alam, R.I., Russell, O.F., Malik, S.A., Bigbee, J.W., DeSimone, J.A., 2004. The mammalian amiloride-insensitive non-specific salt taste receptor is a vanilloid receptor-1 variant: A VR-1 variant mammalian non-specific salt taste receptor. *J. Physiol.* 558, 147–159.
99. Ma, B., Wang, Q., Han, B.-N., Ikeda, H., Zhang, C., Xu, L.-H., 2021. Hydroxylation, Epoxidation, and Dehydrogenation of Capsaicin by a Microbial Promiscuous Cytochrome P450 105D7. *Chem. Biodivers.* 18, e2000910.
100. Manitpisitkul, P., Mayorga, A., Shalayda, K., De Meulder, M., Romano, G., Jun, C., Moyer, J.A., 2015. Safety, tolerability and pharmacokinetic and pharmacodynamic learnings from a double-blind, randomized, placebo-controlled, sequential group first-in-human study of the TRPV1 antagonist, JNJ-38893777, in healthy men. *Clin. Drug Investig.* 35, 353–363.
101. Martínez-Juárez, V.M., Ochoa-Alejo, N., Lozoya-Gloria, E., Villarreal-Ortega, M.L., Ariza-Castolo, A., Esparza-García, F.J., Calva-Calva, G., 2004. Specific synthesis of 5,5'-dicapsaicin by cell suspension cultures of capsicum annum var. annum (chili Jalapeño chigol) and their soluble and NaCl-extracted cell wall protein fractions. *J. Agric. Food Chem.* 52, 972–979.
102. McIntyre, P., McLatchie, L.M., Chambers, A., Phillips, E., Clarke, M., Savidge, J., Toms, C., Peacock, M., Shah, K., Winter, J., Weerasakera, N., Webb, M., Rang, H.P., Bevan, S., James, I.F., 2001. Pharmacological differences between the human and rat vanilloid receptor 1 (VR1). *Br. J. Pharmacol.* 132, 1084–1094.
103. McNamara, F.N., Randall, A., Gunthorpe, M.J., 2005. Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *Br. J. Pharmacol.* 144, 781–790.
104. Migglautsch, A.K., Willim, M., Schweda, B., Glieder, A., Breinbauer, R., Winkler, M., 2018. Aliphatic hydroxylation and epoxidation of capsaicin by cytochrome P450 CYP505X. *Tetrahedron* 74, 6199–6204.
105. Miller, F., Björnsson, M., Svensson, O., Karlsten, R., 2014. Experiences with an adaptive design for a dose-finding study in patients with osteoarthritis. *Contemp. Clin. Trials* 37, 189–199.
106. Mobasher, A., Rannou, F., Ivanavicius, S., Conaghan, P.G., 2024. Targeting the TRPV1 pain pathway in osteoarthritis of the knee. *Expert Opin. Ther. Targets* 28, 843–856.
107. Mohapatra, D.P., Nau, C., 2005. Regulation of Ca²⁺-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J. Biol. Chem.* 280, 13424–13432.
108. Montell, C., Rubin, G.M., 1989. Molecular characterization of the *Drosophila* trp locus: a putative integral membrane protein required for phototransduction. *Neuron* 2, 1313–1323.
109. Moon, Y.W., Lee, J.-H., Yoo, S.B., Jahng, J.W., 2010. Capsaicin receptors are colocalized with sweet/bitter receptors in the taste sensing cells of circumvallate papillae. *Genes Nutr.* 5, 251–255.
110. Moran, M.M., Szallasi, A., 2018. Targeting nociceptive transient receptor potential channels to treat chronic pain: current state of the field: Nociceptive TRP channels. *Br. J. Pharmacol.* 175, 2185–2203.
111. Muchiri, R.N., van Breemen, R.B., 2021. Affinity selection-mass spectrometry for the discovery of pharmacologically active compounds from combinatorial libraries and natural products. *J. Mass Spectrom.* 56, e4647.
112. Mullowney, M.W., Duncan, K.R., Elsayed, S.S., Garg, N., van der Hooft, J.J.J., Martin, N.I., Meijer, D., Terlouw, B.R., Biermann, F., Blin, K., Durairaj, J., Gorostiola González, M., Helfrich, E.J.N., Huber, F., Leopold-Messer, S., Rajan, K., de Rond, T.,

- van Santen, J.A., Sorokina, M., Balunas, M.J., Beniddir, M.A., van Bergeijk, D.A., Carroll, L.M., Clark, C.M., Clevert, D.-A., Dejong, C.A., Du, C., Ferrinho, S., Grisoni, F., Hofstetter, A., Jespers, W., Kalinina, O.V., Kautsar, S.A., Kim, H., Leao, T.F., Masschelein, J., Rees, E.R., Reher, R., Reker, D., Schwaller, P., Segler, M., Skinnider, M.A., Walker, A.S., Willighagen, E.L., Zdrazil, B., Ziemert, N., Goss, R.J.M., Guyomard, P., Volkamer, A., Gerwick, W.H., Kim, H.U., Müller, R., van Wezel, G.P., van Westen, G.J.P., Hirsch, A.K.H., Linington, R.G., Robinson, S.L., Medema, M.H., 2023. Artificial intelligence for natural product drug discovery. *Nat. Rev. Drug Discov.* 22, 895–916.
113. Nakaniwa, R., Misawa, Y., Nakasato, S., Sano, K., Tanaka, Y., Nakatani, S., Kobata, K., 2024. Biochemical aspects of putative aminotransferase responsible for converting vanillin to vanillylamine in the capsaicinoid biosynthesis pathway in *Capsicum* plants. *J. Agric. Food Chem.* 72, 559–565.
 114. Naves, E.R., Scossa, F., Araújo, W.L., Nunes-Nesi, A., Fernie, A.R., Zsögön, A., 2022. Heterosis for capsaicinoids accumulation in chili pepper hybrids is dependent on parent-of-origin effect. *Sci. Rep.* 12, 14450.
 115. Nelson, E.K., 1919. VANILLYL-ACYL AMIDES. *J. Am. Chem. Soc.* 41, 2121–2130.
 116. Neuberger, A., Oda, M., Nikolaev, Y.A., Nadezhdin, K.D., Gracheva, E.O., Bagriantsev, S.N., Sobolevsky, A.I., 2023. Human TRPV1 structure and inhibition by the analgesic SB-366791. *Nat. Commun.* 14, 1–10.
 117. Nilius, B., Owsianik, G., 2011. The transient receptor potential family of ion channels. *Genome Biol.* 12, 218.
 118. Nothias, L.-F., Petras, D., Schmid, R., Dührkop, K., Rainer, J., Sarvepalli, A., Protsyuk, I., Ernst, M., Tsugawa, H., Fleischauer, M., Aicheler, F., Aksenov, A.A., Alka, O., Allard, P.-M., Barsch, A., Cachet, X., Caraballo-Rodriguez, A.M., Da Silva, R.R., Dang, T., Garg, N., Gauglitz, J.M., Gurevich, A., Isaac, G., Jarmusch, A.K., Kameník, Z., Kang, K.B., Kessler, N., Koester, I., Korf, A., Le Gouellec, A., Ludwig, M., Martin, H. C., McCall, L.-I., McSayles, J., Meyer, S.W., Mohimani, H., Morsy, M., Moyne, O., Neumann, S., Neuweber, H., Nguyen, N.H., Nothias-Esposito, M., Paolini, J., Phelan, V.V., Pluskal, T., Quinn, R.A., Rogers, S., Shrestha, B., Tripathi, A., van der Hooft, J.J.J., Vargas, F., Weldon, K.C., Witting, M., Yang, H., Zhang, Z., Zubeil, F., Kohlbacher, O., Böcker, S., Alexandrov, T., Bandeira, N., Wang, M., Dorrestein, P.C., 2020. Feature-based molecular networking in the GNPS analysis environment. *Nat. Methods* 17, 905–908.
 119. Numazaki, M., Tominaga, T., Takeuchi, K., Murayama, N., Toyooka, H., Tominaga, M., 2003. Structural determinant of TRPV1 desensitization interacts with calmodulin. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8002–8006.
 120. Ochi, T., Takaishi, Y., Kogure, K., Yamauti, I., 2003. Antioxidant activity of a new capsaicin derivative from *Capsicum annuum*. *J. Nat. Prod.* 66, 1094–1096.
 121. Ochoa, J., Torebjörk, E., 1989. Sensations evoked by intraneural microstimulation of C nociceptor fibres in human skin nerves. *J. Physiol.* 415, 583–599.
 122. Ogawa, K., Murota, K., Shimura, H., Furuya, M., Togawa, Y., Matsumura, T., Masuta, C., 2015. Evidence of capsaicin synthase activity of the Pun1-encoded protein and its role as a determinant of capsaicinoid accumulation in pepper. *BMC Plant Biol.* 15, 93.
 123. Okamoto, N., Okumura, M., Tadokoro, O., Sogawa, N., Tomida, M., Kondo, E., 2018. Effect of single-nucleotide polymorphisms in TRPV1 on burning pain and capsaicin sensitivity in Japanese adults. *Mol. Pain* 14, 1744806918804439.
 124. Olivon, F., Elie, N., Grelier, G., Roussi, F., Litaudon, M., Touboul, D., 2018. MetGem software for the generation of molecular networks based on the t-SNE algorithm. *Anal. Chem.* 90, 13900–13908.

125. Othman, Z.A., Ahmed, Y.B.H., Habila, M., Ghafar, A.A., 2011. Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography. *Molecules* 16, 8919–8929.
126. Pang, Z., Lu, Y., Zhou, G., Hui, F., Xu, L., Viau, C., Spigelman, A.F., MacDonald, P.E., Wishart, D.S., Li, S., Xia, J., 2024. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res.* 52, W398–W406.
127. Park, H.-G., Choi, J.-Y., Choi, S.-H., Park, M.-K., Lee, J., Suh, Y.-G., Cho, H., Oh, U., Kim, H.-D., Joo, Y.H., Kim, S.-Y., Park, Y.-H., Jeong, Y.S., Choi, J.K., Kim, J.K., Jew, S.-S., 2004. N-4-methansulfonamidobenzyl-N'-2-substituted-4-tert-butyl-benzyl thioureas as potent vanilloid receptor antagonistic ligands. *Bioorg. Med. Chem. Lett.* 14, 1693–1696.
128. Phimchan, P., Chanthai, S., Bosland, P.W., Techawongstien, S., 2014. Enzymatic changes in phenylalanine ammonia-lyase, cinnamic-4-hydroxylase, capsaicin synthase, and peroxidase activities in capsicum under drought stress. *J. Agric. Food Chem.* 62, 7057–7062.
129. Po, L.G., Siddiq, M., Shahzad, T., 2018. Chili, Peppers, and Paprika, in: *Handbook of Vegetables and Vegetable Processing*. John Wiley & Sons, Ltd, Chichester, UK, pp. 633–660.
130. Premkumar, L.S., Ahern, G.P., 2000. Induction of vanilloid receptor channel activity by protein kinase C. *Nature* 408, 985–990.
131. Price, D.D., 2007. Spinothalamic Tract Neurons, in Deep Dorsal Horn, in: Schmidt, R.F., Willis, W.D. (Eds.), *Encyclopedia of Pain*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 2278–2282.
132. Reilly, C.A., Ehlhardt, W.J., Jackson, D.A., Kulanthaivel, P., Mutlib, A.E., Espina, R.J., Moody, D.E., Crouch, D.J., Yost, G.S., 2003. Metabolism of capsaicin by cytochrome P450 produces novel dehydrogenated metabolites and decreases cytotoxicity to lung and liver cells. *Chem. Res. Toxicol.* 16, 336–349.
133. Reilly, C.A., Henion, F., Bugni, T.S., Ethirajan, M., Stockmann, C., Pramanik, K.C., Srivastava, S.K., Yost, G.S., 2013. Reactive intermediates produced from the metabolism of the vanilloid ring of capsaicinoids by p450 enzymes. *Chem. Res. Toxicol.* 26, 55–66.
134. Reinhardt, J.K., Craft, D., Weng, J.-K., 2025. Toward an integrated omics approach for plant biosynthetic pathway discovery in the age of AI. *Trends Biochem. Sci.* <https://doi.org/10.1016/j.tibs.2025.01.010>
135. Reisdorph, N.A., Walmsley, S., Reisdorph, R., 2019. A perspective and framework for developing sample type specific databases for LC/MS-based clinical metabolomics. *Metabolites* 10, 8.
136. Roberts, L.D., Souza, A.L., Gerszten, R.E., Clish, C.B., 2012. Targeted metabolomics. *Curr. Protoc. Mol. Biol.* Chapter 30, Unit 30.2.1–24.
137. Roper, S.D., 2014. TRPs in taste and chemesthesis. *Handb. Exp. Pharmacol.* 223, 827–871.
138. Rosenbaum, T., Gordon-Shaag, A., Munari, M., Gordon, S.E., 2004. Ca²⁺/calmodulin modulates TRPV1 activation by capsaicin. *J. Gen. Physiol.* 123, 53–62.
139. Rozin, P., Schiller, D., 1980. The nature and acquisition of a preference for chili pepper by humans. *Motiv. Emot.* 4, 77–101.
140. Ryu, H., Jin, M.-K., Kim, S.Y., Choi, H.-K., Kang, S.-U., Kang, D.W., Lee, J., Pearce, L.V., Pavlyukovets, V.A., Morgan, M.A., Tran, R., Toth, A., Lundberg, D.J., Blumberg, P.M., 2008. Stereospecific high-affinity TRPV1 antagonists: chiral N-(2-benzyl-3-pivaloyloxypropyl) 2-[4-(methylsulfonylamino)phenyl]propionamide analogues. *J. Med. Chem.* 51, 57–67.

141. Saito, F., Schreiner, P.R., 2020. Determination of the absolute configurations of chiral alkanes – an analysis of the available tools. *European J. Org. Chem.* 2020, 6328–6339.
142. Sałat, K., Jakubowska, A., Kulig, K., 2014. Zucapsaicin for the treatment of neuropathic pain. *Expert Opin. Investig. Drugs* 23, 1433–1440.
143. Sano, K., Nakasato, S., Nagata, K., Kobata, K., 2023. A single amino acid substitution alters the vanillylamine synthesis activity of *Capsicum pAMT*. *Biochem. Biophys. Res. Commun.* 680, 86–92.
144. Sano, K., Uzawa, Y., Kaneshima, I., Nakasato, S., Hashimoto, M., Tanaka, Y., Nakatani, S., Kobata, K., 2022. Vanillin reduction in the biosynthetic pathway of capsiate, a non-pungent component of *Capsicum* fruits, is catalyzed by cinnamyl alcohol dehydrogenase. *Sci. Rep.* 12, 12384.
145. Sanz-Salvador, L., Andrés-Borderia, A., Ferrer-Montiel, A., Planells-Cases, R., 2012. Agonist- and Ca²⁺-dependent desensitization of TRPV1 channel targets the receptor to lysosomes for degradation. *J. Biol. Chem.* 287, 19462–19471.
146. Schmid, R., Heuckeroth, S., Korf, A., Smirnov, A., Myers, O., Dyrland, T.S., Bushuiev, R., Murray, K.J., Hoffmann, N., Lu, M., Sarvepalli, A., Zhang, Z., Fleischauer, M., Dührkop, K., Wesner, M., Hoogstra, S.J., Rudt, E., Mokshyna, O., Brungs, C., Ponomarov, K., Mutabdzija, L., Damiani, T., Pudney, C.J., Earll, M., Helmer, P.O., Fallon, T.R., Schulze, T., Rivas-Ubach, A., Bilbao, A., Richter, H., Nothias, L.-F., Wang, M., Orešič, M., Weng, J.-K., Böcker, S., Jeibmann, A., Hayen, H., Karst, U., Dorrestein, P.C., Petras, D., Du, X., Pluskal, T., 2023. Integrative analysis of multimodal mass spectrometry data in MZmine 3. *Nat. Biotechnol.* 41, 447–449.
147. Schober, P., Boer, C., Schwarte, L.A., 2018. Correlation coefficients: Appropriate use and interpretation. *Anesth. Analg.* 126, 1763–1768.
148. Schrimpe-Rutledge, A.C., Codreanu, S.G., Sherrod, S.D., McLean, J.A., 2016. Untargeted metabolomics strategies-challenges and emerging directions. *J. Am. Soc. Mass Spectrom.* 27, 1897–1905.
149. Scoville, W.L., 1912. Note on capsicums. *J. Am. Pharm. Assoc.* (1912) 1, 453–454.
150. Seeböhm, G., Schreiber, J.A., 2021. Beyond hot and spicy: TRPV channels and their pharmacological modulation. *Cell. Physiol. Biochem.* 55, 108–130.
151. Siebert, E., Lee, S.-Y., Prescott, M.P., 2022. Chili pepper preference development and its impact on dietary intake: A narrative review. *Front. Nutr.* 9, 1039207.
152. Simpson, M.G., 2020. *Plant systematics*, 3rd ed. Academic Press, San Diego, CA.
153. Smith, J.D., Stillerová, V.T., Dračinský, M., Gaustad, H.L.A., Lorenzi, Q., Smrčková, H., Reinhardt, J.K., Liénard, M.A., Bednářová, L., Šácha, P., Pluskal, T., 2024. Discovery and isolation of novel capsaicinoids and their TRPV1-related activity. *bioRxiv*. <https://doi.org/10.1101/2024.10.29.620944>
154. Stein, S.E., Scott, D.R., 1994. Optimization and testing of mass spectral library search algorithms for compound identification. *J. Am. Soc. Mass Spectrom.* 5, 859–866.
155. Stewart, C., Jr, Kang, B.-C., Liu, K., Mazourek, M., Moore, S.L., Yoo, E.Y., Kim, B.-D., Paran, I., Jahn, M.M., 2005. The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. *Plant J.* 42, 675–688.
156. Stipcovich, T., Barbero, G.F., Ferreiro-González, M., Palma, M., Barroso, C.G., 2018. Fast analysis of capsaicinoids in Naga Jolokia extracts (*Capsicum chinense*) by high-performance liquid chromatography using fused core columns. *Food Chem.* 239, 217–224.
157. Sukrasno, N., Yeoman, M.M., 1993. Phenylpropanoid metabolism during growth

- and development of *Capsicum frutescens* fruits. *Phytochemistry* 32, 839–844.
158. Sutoh, K., Kobata, K., Watanabe, T., 2001. Stability of capsinoid in various solvents. *J. Agric. Food Chem.* 49, 4026–4030.
 159. Suzuki, T., Fujiwake, H., Iwai, K., 1980. Intracellular localization of capsaicin and its analogues, capsaicinoid, in *Capsicum* fruit 1. Microscopic investigation of the structure of the placenta of *Capsicum annuum* var. *annuum* cv. *Karayatsubusa*. *Plant and Cell Physiology* 21, 839–853.
 160. Sweat, K.G., Broatch, J., Borrer, C., Hagan, K., Cahill, T.M., 2016. Variability in capsaicinoid content and Scoville heat ratings of commercially grown Jalapeño, Habanero and Bhut Jolokia peppers. *Food Chem.* 210, 606–612.
 161. Szallasi, A., 2023. Resiniferatoxin: Nature's precision medicine to silence TRPV1-positive afferents. *Int. J. Mol. Sci.* 24, 15042.
 162. Szallasi, A., Blumberg, P.M., 1989. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* 30, 515–520.
 163. Szallasi, A., Cortright, D.N., Blum, C.A., Eid, S.R., 2007. The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept. *Nat. Rev. Drug Discov.* 6, 357–372.
 164. Szolcsányi, J., Jancsó-Gábor, A., 1976. Sensory effects of capsaicin congeners. Part II: Importance of chemical structure and pungency in desensitizing activity of capsaicin-type compounds. *Arzneimittelforschung* 26, 33–37.
 165. Szolcsányi, J., Jancsó-Gábor, A., 1975. Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. *Arzneimittelforschung* 25, 1877–1881.
 166. Takahashi, M., Osawa, K., Ueda, J., Okada, K., 1976. Anodic Synthesis of Acid Parts in Capsaicinoids and Spectroscopic Analysis. II. PHARMACEUTICAL SOCIETY OF JAPAN 96, 1000–1004.
 167. Tallarida, R.J., 2011. Quantitative methods for assessing drug synergism. *Genes Cancer* 2, 1003–1008.
 168. Tanaka, Y., Sonoyama, T., Muraga, Y., Koeda, S., Goto, T., Yoshida, Y., Yasuba, K., 2015. Multiple loss-of-function putative aminotransferase alleles contribute to low pungency and capsinoid biosynthesis in *Capsicum chinense*. *Mol. Breed.* 35. <https://doi.org/10.1007/s11032-015-0339-9>
 169. Tateba, H., Mihara, S., 1991. Photochemical Oxidative Dimerization of Capsaicin in an Aqueous Solution. *Agric. Biol. Chem.* 55, 873–874.
 170. Tautenhahn, R., Patti, G.J., Rinehart, D., Siuzdak, G., 2012. XCMS Online: a web-based platform to process untargeted metabolomic data. *Anal. Chem.* 84, 5035–5039.
 171. The Associated Press, 2024. Family files lawsuit in case of teen who died after eating spicy chip [WWW Document]. [npr.org](https://www.npr.org).
 172. Thiele, R., Mueller-Seitz, E., Petz, M., 2008. Chili pepper fruits: presumed precursors of fatty acids characteristic for capsaicinoids. *J. Agric. Food Chem.* 56, 4219–4224.
 173. Thomas, B.V., Schreiber, A.A., Weisskopf, C.P., 1998. Simple method for quantitation of capsaicinoids in peppers using capillary gas chromatography. *J. Agric. Food Chem.* 46, 2655–2663.
 174. Tian, K., Zhu, J., Li, M., Qiu, X., 2019. Capsaicin is efficiently transformed by multiple cytochrome P450s from *Capsicum* fruit-feeding *Helicoverpa armigera*. *Pestic. Biochem. Physiol.* 156, 145–151.
 175. Todd, P.H., Jr, Bensinger, M.G., Biftu, T., 1977. Determination of pungency due to capsaicin by gas-liquid chromatography. *J. Food Sci.* 42, 660–665.

176. Tomasello, G., Armenia, I., Molla, G., 2020. The Protein Imager: a full-featured online molecular viewer interface with server-side HQ-rendering capabilities. *Bioinformatics* 36, 2909–2911.
177. Tominaga, M., Wada, M., Masu, M., 2001. Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. *Proc. Natl. Acad. Sci. U. S. A.* 98, 6951–6956.
178. Tsugawa, H., Cajka, T., Kind, T., Ma, Y., Higgins, B., Ikeda, K., Kanazawa, M., VanderGheynst, J., Fiehn, O., Arita, M., 2015. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nat. Methods* 12, 523–526.
179. Valdes, A.M., De Wilde, G., Doherty, S.A., Lories, R.J., Vaughn, F.L., Laslett, L.L., Maciewicz, R.A., Soni, A., Hart, D.J., Zhang, W., Muir, K.R., Dennison, E.M., Wheeler, M., Leaverton, P., Cooper, C., Spector, T.D., Cicuttini, F.M., Chapman, V., Jones, G., Arden, N.K., Doherty, M., 2011. The Ile585Val TRPV1 variant is involved in risk of painful knee osteoarthritis. *Ann. Rheum. Dis.* 70, 1556–1561.
180. Vasilev, V.H., Spessert, L., Yu, K., Maimone, T.J., 2022. Total Synthesis of Resiniferatoxin. *J. Am. Chem. Soc.* 144, 16332–16337.
181. Vetter, I., Wyse, B.D., Monteith, G.R., Roberts-Thomson, S.J., Cabot, P.J., 2006. The mu opioid agonist morphine modulates potentiation of capsaicin-evoked TRPV1 responses through a cyclic AMP-dependent protein kinase A pathway. *Mol. Pain* 2, 22.
182. Vu, S., Singh, V., Wulff, H., Yarov-Yarovoy, V., Zheng, J., 2020. New capsaicin analogs as molecular rulers to define the permissive conformation of the mouse TRPV1 ligand-binding pocket. *Elife* 9, e62039.
183. Walpole, C.S., Bevan, S., Bloomfield, G., Breckenridge, R., James, I.F., Ritchie, T., Szallasi, A., Winter, J., Wrigglesworth, R., 1996. Similarities and differences in the structure-activity relationships of capsaicin and resiniferatoxin analogues. *J. Med. Chem.* 39, 2939–2952.
184. Walpole, C.S.J., Wrigglesworth, R., Bevan, S., Campbell, E.A., Dray, A., James, I.F., Masdin, K.J., Perkins, M.N., Winter, J., 1993. Analogs of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 3. The hydrophobic side-chain “C-region.” *J. Med. Chem.* 36, 2381–2389.
185. Wang, R., Liu, Y., Sun, S., Si, Y., Liu, X., Liu, X., Zhang, S., Wang, W., 2020a. Capsaicinoids from hot pepper (*Capsicum annuum* L.) and their phytotoxic effect on seedling growth of lettuce (*Lactuca sativa* L.). *Nat. Prod. Res.* 34, 1597–1601.
186. Wang, R., Liu, Y., Sun, S., Si, Y., Liu, X., Liu, X., Zhang, S., Wang, W., 2020b. Capsaicinoids from hot pepper (*Capsicum annuum* L.) and their phytotoxic effect on seedling growth of lettuce (*Lactuca sativa* L.). *Nat. Prod. Res.* 34, 1597–1601.
187. Wang, S., Joseph, J., Diatchenko, L., Ro, J.Y., Chung, M.-K., 2016. Agonist-dependence of functional properties for common nonsynonymous variants of human transient receptor potential vanilloid 1. *Pain* 157, 1515–1524.
188. Watrous, J., Roach, P., Alexandrov, T., Heath, B.S., Yang, J.Y., Kersten, R.D., van der Voort, M., Pogliano, K., Gross, H., Raaijmakers, J.M., Moore, B.S., Laskin, J., Bandeira, N., Dorrestein, P.C., 2012. Mass spectral molecular networking of living microbial colonies. *Proc. Natl. Acad. Sci. U. S. A.* 109, E1743–52.
189. Wilkinson, M.D., Dumontier, M., Aalbersberg, I.J.J., Appleton, G., Axton, M., Baak, A., Blomberg, N., Boiten, J.-W., da Silva Santos, L.B., Bourne, P.E., Bouwman, J., Brookes, A.J., Clark, T., Crosas, M., Dillo, I., Dumon, O., Edmunds, S., Evelo, C.T., Finkers, R., Gonzalez-Beltran, A., Gray, A.J.G., Groth, P., Goble, C., Grethe, J.S., Heringa, J., 't Hoen, P.A.C., Hooft, R., Kuhn, T., Kok, R., Kok, J., Lusher, S.J., Martone, M.E., Mons, A., Packer, A.L., Persson, B., Rocca-Serra, P., Roos, M., van Schaik, R., Sansone, S.-A., Schultes, E., Sengstag, T., Slater, T., Strawn, G., Swertz, M.A., Thompson, M., van der Lei, J., van Mulligen, E., Velterop, J., Waagmeester, A.,

- Wittenburg, P., Wolstencroft, K., Zhao, J., Mons, B., 2016. The FAIR Guiding Principles for scientific data management and stewardship. *Sci. Data* 3, 160018.
190. Willis, W.D., Jr, 2009. The role of TRPV1 receptors in pain evoked by noxious thermal and chemical stimuli. *Exp. Brain Res.* 196, 5–11.
 191. Wu, L.-J., Sweet, T.-B., Clapham, D.E., 2010. International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol. Rev.* 62, 381–404.
 192. Wu, T., Yuan, X., Wu, X., Tang, Y., Lin, H., Che, Z., Zhou, W., Li, W., 2019. Rapid Determination of Capsaicin and Dihydrocapsaicin in Fermented Pepper Paste by Direct Analysis in Real Time Mass Spectrometry. *Food Anal. Methods* 12, 32–40.
 193. Xian, F., Hendrickson, C.L., Marshall, A.G., 2012. High resolution mass spectrometry. *Anal. Chem.* 84, 708–719.
 194. Xiao, J.F., Zhou, B., Ransom, H.W., 2012. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *Trends Analyt. Chem.* 32, 1–14.
 195. Xin, X.-L., Wang, Y., Fan, G.-J., Chen, L., Sun, C.-P., 2017. Biotransformation of capsaicin by *Penicillium janthinellum* AS 3.510. *Phytochem. Lett.* 19, 210–214.
 196. Xu, H., Tian, W., Fu, Y., Oyama, T.T., Anderson, S., Cohen, D.M., 2007. Functional effects of nonsynonymous polymorphisms in the human TRPV1 gene. *Am. J. Physiol. Renal Physiol.* 293, F1865–76.
 197. Yakubova, A., Davidyuk, Y., Tohka, J., Khayrutdinova, O., Kudryavtsev, I., Nurkhametova, D., Kamshilin, A., Giniatullin, R., Rizvanov, A., 2021. Searching for predictors of migraine chronification: A pilot study of 1911A>G polymorphism of TRPV1 gene in episodic versus chronic migraine. *J. Mol. Neurosci.* 71, 618–624.
 198. Yang, F., Xiao, X., Cheng, W., Yang, W., Yu, P., Song, Z., Yarov-Yarovoy, V., Zheng, J., 2015. Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nat. Chem. Biol.* 11, 518–524.
 199. Yang, F., Xiao, X., Cheng, W., Yang, W., Yu, P., Song, Z., Zheng, 2015. Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nature chemical biology* 11, 518–524.
 200. Yang, F., Xiao, X., Lee, B.H., Vu, S., Yang, W., Yarov-Yarovoy, V., Zheng, J., 2018. The conformational wave in capsaicin activation of transient receptor potential vanilloid 1 ion channel. *Nat. Commun.* 9, 2879.
 201. Yang, F., Zheng, J., 2017. Understand spiciness: mechanism of TRPV1 channel activation by capsaicin. *Protein Cell* 8, 169–177.
 202. Yang, S., Yang, F., Wei, N., Hong, J., Li, B., Luo, L., Rong, M., Yarov-Yarovoy, V., Zheng, J., Wang, K., Lai, R., 2015. A pain-inducing centipede toxin targets the heat activation machinery of nociceptor TRPV1. *Nat. Commun.* 6, 8297.
 203. Yelshanskaya, M.V., Sobolevsky, A.I., 2022. Ligand-binding sites in vanilloid-subtype TRP channels. *Front. Pharmacol.* 13, 900623.
 204. Yin, Y., Dong, Y., Vu, S., Yang, F., Yarov-Yarovoy, V., Tian, Y., Zheng, J., 2019. Structural mechanisms underlying activation of TRPV1 channels by pungent compounds in gingers. *Br. J. Pharmacol.* 176, 3364–3377.
 205. Yu, F.H., Catterall, W.A., 2004. The VGL-kanome: a protein superfamily specialized for electrical signaling and ionic homeostasis. *Sci. STKE* 2004, re15.
 206. Zewdie, Y., Bosland, P.W., 2001. Capsaicinoid profiles are not good chemotaxonomic indicators for *Capsicum* species. *Biochem. Syst. Ecol.* 29, 161–169.
 207. Zhang, G., Annan, R.S., Carr, S.A., Neubert, T.A., 2010. Overview of peptide and protein analysis by mass spectrometry. *Curr. Protoc. Protein Sci.* Chapter 16, Unit16.1.
 208. Zhang, M., Ma, Y., Ye, X., Zhang, N., Pan, L., Wang, B., 2023. TRP (transient receptor potential) ion channel family: structures, biological functions and therapeutic interventions for diseases. *Signal Transduct. Target. Ther.* 8, 261.

209. Zhao, L., Au, J.L.-S., Wientjes, M.G., 2010. Comparison of methods for evaluating drug-drug interaction. *Front. Biosci. (Elite Ed.)* 2, 241–249.

List of publications of the doctoral student in this order:

1. Publications in extension, which are the basis of the dissertation

With impact factor:

*Co-first authorship

1. Damiani, Tito, **Smith, Joshua*** et al. "Computational metabolomics reveals overlooked chemodiversity of alkaloid scaffolds in *Piper fimbriulatum*." *The Plant Journal* (2025): e70086. **IF = 7.1**
2. Heuckeroth, Steffen; 16 others, **Smith, Joshua**; Pluskal, Tomáš. "Reproducible mass spectrometry data processing and compound annotation in MZmine 3." *Nature Protocols* (2024): 1-45 **IF = 14.8**

Without impact factor:

1. **Smith, Joshua***; 10 others. "Discovery and isolation of novel capsaicinoids and their TRPV1-related activity." *bioRxiv* (2024): 2024-10. (under review, *European Journal of Pharmacology*)

2. Publications in extension not related to the topic of the dissertation

With impact factor:

1. Pimková Polidarová, Markéta; 15 others; **Smith, Joshua**; Birkus, Gabriel. "Synthesis and Biological Evaluation of Phosphoester and Phosphorothioate Prodrugs of STING Agonist 3', 3'-c-Di (2' F, 2' dAMP)." *Journal of Medicinal Chemistry* 64.11 (2021): 7596-7616. **IF = 7.3**
2. Vařena, Zdeněk; 13 others; **Smith, Joshua**; Birkus, Gabriel. "Design, Synthesis, and Biochemical and Biological Evaluation of Novel 7-Deazapurine Cyclic Dinucleotide Analogues as STING Receptor Agonists." *Journal of Medicinal Chemistry* 65.20 (2022): 14082-14103 **IF = 7.3**