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# Phytochemical Characterization and Sensory Evaluation of *Macropiper excelsum*

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# 1 INTRODUCTION AND OBJECTIVES

Both the gustatory and olfactory systems are necessary for humans to perceive their environment by sensory means. Besides the senses of taste and smell, the trigeminal somatosensory system is also a part of chemosensation (Viana, 2011). Via the trigeminal nerve, perception of touch, temperature, and pain can be initiated by physical stimuli, like heat, cold, and mechanical forces. Also chemical agents, such as piperine, capsaicin, or menthol, are detected via the trigeminal nerve. They provoke sensations described as hot, pungent, tingling, and cooling. Although some of these compounds were designed to act as repellents or irritants by nature (Fitzgerald et al., 1995), humans developed a liking for hot and spicy dishes to a certain extent.

A large number of trigeminal-active compounds can be found in nature, e.g. piperine in pepper (*Piper sp.*), capsaicin in chili (*Capsicum sp.*), or menthol in mint (*Mentha sp.*). In addition to their sensory impressions, some of them play a role in plant defense against environmental stress and herbivores (Crombie, 1955; Scott et al., 2003). Many plants containing in particular trigeminal-active molecules with hot, pungent, cooling, tingling, and/or mouth-watering effects are known for their medical purpose. Extracts of *Spilanthes acmella* were described to have antioxidant, antimicrobial, and local anesthetic activity (Chakraborty et al., 2010; Prachayasittikul et al., 2009). *Heliopsis longipes* (Deciga-Campos et al., 2010) and *Anacyclus pyrethrum* (Patel et al., 1992) are known for their antinociceptive and numbing effects, as well as their usage against toothache. A focused search for plants with such properties resulted in the finding of *Macropiper excelsum*.

The leaves of *M. excelsum*, a shrub growing in New Zealand, are known for their use against toothache (Crowe, 2007). When chewing the leaves, a slightly numbing sensation can be detected. Since the phytochemical profile of this indigenous plant is hardly investigated, the compounds responsible for this effect are unknown. Therefore, one objective of this study was the investigation of the phytochemical and sensory profile of *M. excelsum*. After isolation, identification and structure elucidation of the molecules, they were evaluated sensorially by a trained panel. Selected compounds were additionally tested for their possible masking effects on catechin astringency.

Green tea, brewed from the leaves of *Camellia sinensis*, is one of the most popular beverages worldwide and contains high amounts of catechins, especially

epicatechin, epicatchin-3-gallate, epigallocatechin and epigallocatechin-3-gallate (EGCG) (Cabrera et al., 2003; Chen et al., 2001; Wang et al., 2000). The consumption of these catechins seems to be correlated with a number of health benefits, such as anti-inflammatory (Dona et al., 2003), anti-oxidant (Erba et al., 2005), and cardio-protective effects (Imai and Nakachi, 1995). Besides these beneficial properties, catechins impart the typical astringency and concomitant bitter sensation to green tea. Astringency of EGCG is often described as mouth-drying, puckering, or rough (Narukawa et al., 2010; Scharbert et al., 2004). For few exceptions, such as green tea or red wine, astringency is well accepted or even considered as a positive quality parameter (McDowell et al., 1995). In many cases, however, astringency is perceived as aversive, especially at high levels.

Several solutions to avoid or to reduce off-notes have been developed during the last few years as summarized by Ley (Ley, 2008): removal, scavenging, encapsulation, and masking are only a few examples. However, reduction or removal of these compounds in certain food and beverage applications is not always an option due to their potential health benefits. Sometimes, these compounds are even enriched to obtain so-called “functional foods”. The use of such potential functional ingredients is getting more and more important (Salah et al., 1995; Siro et al., 2008; Vinson and Hontz, 1995).

In order to evaluate complex flavor problems and to identify masking solutions, development of a sensory screening method was required. Pre-tests showed that simple duo tests, as for example used to identify sweetness enhancers or bitter maskers (Ley et al., 2005; Ley et al., 2008a), are not sufficient. They give only a snap shot at a defined time point. Therefore, intensity variation descriptive methodology (IVDM), a time-intensity approach, was used (Lee and Lawless, 1991). The method was established for the evaluation of different descriptors in parallel, which saves time, panel work, and the amount of test substances.

Using IVDM, another objective of this work was the identification and evaluation of compounds that are able to reduce or to mask the astringency of substances like EGCG. In earlier studies, it was found that certain trigeminal-active compounds can induce salivation (Ley and Simchen, 2007). The hypothesis is that weak saliva induction may be correlated with a reduction of astringent sensation due to a clearance of neurons from the astringent molecules. Therfore, the potential of certain trigeminal compounds, especially those which were described to have a mouth-

watering effect, was tested. Furthermore, additional trigeminal-active molecules without an obvious salivating effect were evaluated to validate the existence of specific antagonistic activity.

# 2 BACKGROUND

## 2.1 MECHANISM OF SENSORY PERCEPTION

Odor and taste are among the most crucial factors influencing the selection of food. Additionally, tactile perception and visual appearance are important for the overall impression (Drewnowski, 1997; Köster, 2003). Generally, complex mixtures of volatile and non-volatile compounds determine the flavor of foods. Volatile compounds can be detected orthonasally, which means directly via the nose, or retronasally. This is the case when food is chewed and volatile compounds that are released from the matrix enter the nasal cavity via the nasopharynx. In any case, they will reach the olfactory epithelium and get in contact with the olfactory receptors located there (Burdach and Doty, 1987). Approximately 400 functional odor receptors genes are expressed on the olfactory sensory neurons (OSN), each OSN containing one odorant receptor. The olfactory nerve comprises the axons of the OSNs, which innervate the olfactory bulb, especially discrete structures, called glomeruli (DeMaria and Ngai, 2010).

Non-volatile compounds are detected mainly in the oral cavity. Five basic taste qualities can be discriminated: sour, bitter, salty, sweet, and umami. Gustatory stimuli trigger the behavioral reactions of human and apes. Infants have an innate competency to rate taste qualities hedonically by showing a characteristic facial response. Bitter tasting compounds are disliked instinctively, whereas sweetness is considered as a positive hedonic signal (Steiner and Glaser, 1995).

Sour and bitter taste detection are indispensable to avoid the consumption of unripe fruits and spoiled or potentially toxic food. Salt taste is responsible for the ion and water homeostasis. Sweet and umami taste indicate energy-rich food with high content in carbohydrates and amino acids, primarily monosodium glutamate (MSG). The term umami is derived from the Japanese word “umai”, which means delicious, meaty, or savory (Glendinning, 1994; Ikeda, 2002; Lindemann, 1996).

Apart from the mentioned basic taste qualities, further tastes are under discussion. Calcium taste, for example, is supposed to be mediated by the calcium-sensing receptor (CaSR), which is expressed in taste cells amongst others (Gabriel et al., 2009; Tordoff et al., 2008). Another discussed taste perception is fat taste. Different candidates for the detection of long-chain fatty acids are described in the literature: fatty acid transporter CD36 receptor (Laugerette et al., 2005), G-protein coupled receptor GPR120 (Cartoni et al., 2010; Galindo et al., 2012), and transient receptor

potential channel type M5 (TRPM5) (Liu et al., 2011). Furthermore, the term “kokumi” appears as a description of flavorings that are tasteless themselves but are said to enhance the mouthfeel and thickness of savory applications. γ-Glutamyl peptides and certain sulfur-containing compounds have been described as having a kokumi taste effect (Dunkel et al., 2007; Toelstede et al., 2009; Ueda et al., 1990).

### 2.1.1 Signal Transduction in Taste Buds and Taste Cells

The five basic taste qualities are perceived via three types of taste papillae (fungiform, foliate and circumvallate) on the tongue and palate epithelium. Each of these papillae contains taste buds with 50 to 150 taste receptor cells (TRCs) (Figure 1). In the taste pores, the apical surface of the taste receptor cells gets into contact with the tastants (Chandrashekar et al., 2006).

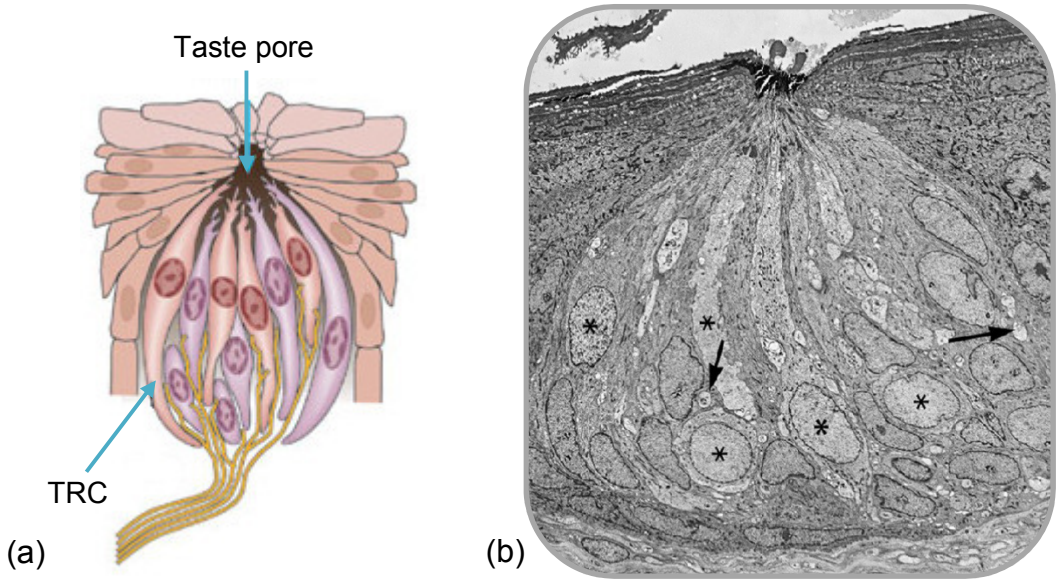



Figure 1: (a) Taste bud containing taste receptor cells (TRC) that get into contact with the tastants via the taste pore (Chandrashekar et al., 2006); (b) Electron micrograph of a rabbit taste bud (longitudinal section) showing taste cells labeled with asterisks and nerve fibers indicated by arrows (Royer and Kinnamon, 1991).

In taste buds, four kinds of taste cells are found: type I cells with supportive functions, type II cells, also referred to as sensory receptor cells, presynaptic (type III) cells, which are suggested as chemoreceptor cells. The fourth type of cells that resemble undifferentiated taste cells are called basal cells (Chaudhari and Roper, 2010; Lindemann, 1996; Roper, 2007).

There are two different ways how taste is mediated. G-protein-coupled receptors (GPCRs) transduce bitter, sweet and umami impressions, while sour and salt taste are supposed to be detected via ion channels (Lindemann, 1996).
Receptor cells express G-protein coupled taste receptors. Any type II cell provides GPCRs for only one taste quality and is therefore narrowly tuned. In contrast, type III cells do not have GPCRs for tastants but apparently respond to sour and salty taste. Even without the ability to receive input directly via GPCRs, presynaptic cells seem to be involved in the sweet, bitter, and sour signal transduction. Additionally, they can express synapses-related proteins and consequently form synapses with afferent nerve fibers (Roper, 2006; Tomchik et al., 2007) (Figure 2).

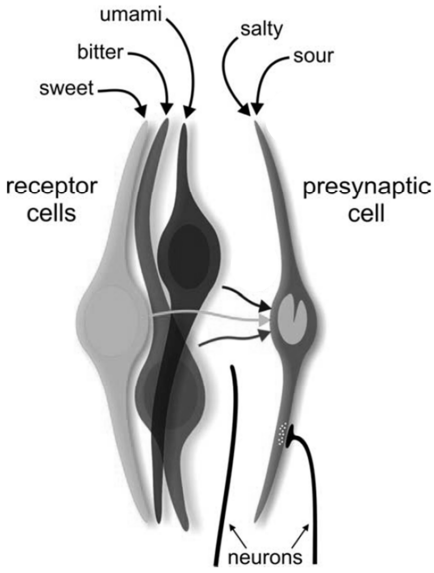



Figure 2: Receptor (type II) cells and presynaptic (type III) cells; two of the four taste cells in taste buds responsible for signal transduction of the five basic taste qualities (Tomchik et al., 2007).

Experimental data provide a strong indication for cell-cell communication within a taste bud (Tomchik et al., 2007). Adenosine triphosphate (ATP) is suggested as a neurotransmitter between the taste cells (Huang et al., 2007). A gustatory stimulus causes the secretion of ATP in type II cells, which then stimulates the presynaptic cells. They transmit the signal to afferent nerve fibers and respond by the release of serotonin (5-HT) (Huang et al., 2008; Tomchik et al., 2007). This last step takes place also, when presynaptic cells are directly interacting with salts and acids, respectively. However, whether salty taste is transduced via type II cells or by type I cells remains uncertain (Chaudhari and Roper, 2010; Vandenbeuch et al., 2008).

The released neurotransmitters interact with receptors on the postsynaptic membrane of the cranial nerves VII, IX, and X. The different nerves innervate various areas of the tongue. The cranial nerve VII, also called chorda tympani nerve, innervates the anterior part of the tongue and the palate. The cranial nerve IX (glossopharyngeal nerve) innervates the posterior third of the tongue. Taste buds in the pharynx and larynx are innervated by cranial nerve X, also named *nervus vagus* (Smith and St John, 1999). The information is communicated to the nucleus tractus solitaries of the medulla, located in the brain stem. Afterwards, signals are conveyed to the primary gustatory cortex (Ogawa et al., 1985) and from there to the central nucleus of the amygdala. At this point, the data reach the lateral hypothalamus and the dopaminergic areas of the midbrain (Simon et al., 2006).

In taste cells, the downstream mechanism for GPCRs is complex (Figure 3). Sweet, bitter, or umami ligands interact with the suitable receptor binding site and activate the heterotrimeric G-protein Gαβγ (McLaughlin and McKinnon, 1992). The Gβγ subunit is separated from the Gα subunit, mainly α-gustducin, and migrates to phospholipase Cβ2 (PLCβ2), which is then activated (Rossler et al., 1998; Zhang et al., 2003). Phospholipids from the cell membrane, e.g. phosphatidyl-inositol-4,5-bisphosphate (PIP2), are cleaved by the activated PLCβ2, resulting in the production of two second messengers, inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ leaves the cell membrane and binds to the $IP_3R3$ receptor on intracellular stores (Asano-Miyoshi et al., 2001). $Ca^{2+}$ is released and results in an increase of cytoplasmic $Ca^{2+}$ levels. This leads to the opening of TRPM5, a transient receptor potential (TRP) channel, followed by a $Na^+$ influx (Perez et al., 2002; Zhang et al., 2003). The intracellular $Ca^{2+}$ and $Na^+$ rise causes a depolarization of the membrane. The combination of depolarization and elevated $Ca^{2+}$ levels opens pores in the cell membrane like the pannexin 1 (Panx1) channel, which than leads to e secretion of ATP.

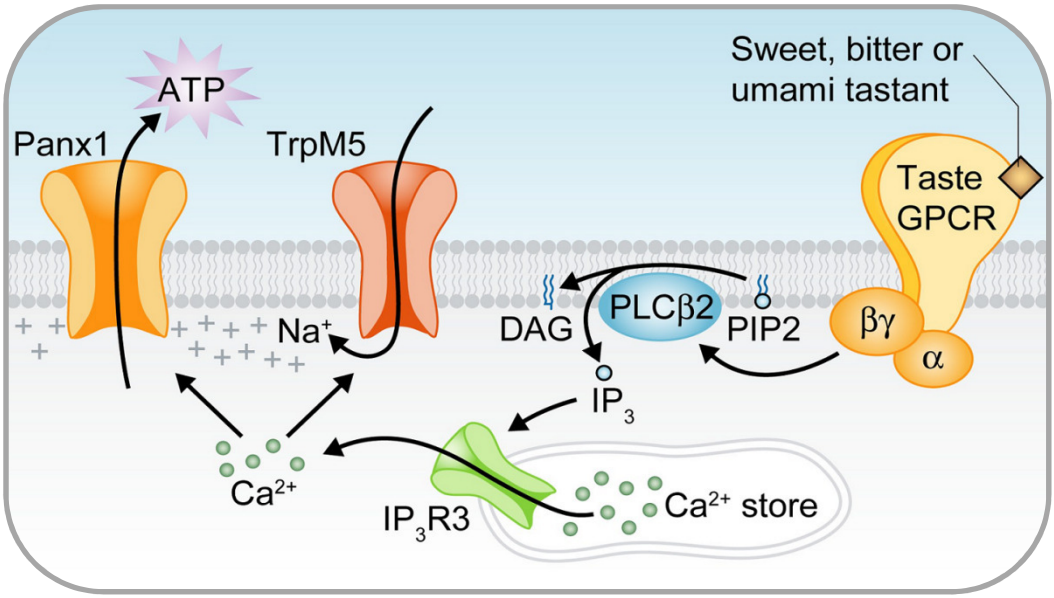



Figure 3: Overview on the molecular mechanisms for detection of the sweet, bitter, and umami taste quality via the GPCR-signaling pathway (Chaudhari and Roper, 2010).

Besides the above-described pathway, a second intracellular path is mentioned in the literature. In sweet taste perception, α-gustducin is linked to an adenylyl cyclase and leads to cyclic adenosine monophosphate (cAMP) production, which inhibits potassium channels due to protein-kinase activation, resulting in cell depolarization. With bitter compounds, however, activated α-gustucin stimulates a phosphodiesterase (PDE), which results in cAMP decrease. This might elevate the intracellular $Ca^{2+}$-concentration via a disinhibition of cyclic nucleotide-inhibited channels (Lindemann, 2001; Margolskee, 2002; Roper, 2007). A cAMP decrease was also shown for umami-taste stimuli (Trubey et al., 2006).

### 2.1.2 Structure and Function of Taste Receptors

GPCRs in general consist of seven transmembrane domains. Taste receptors for sweet and umami are heterodimeric complexes. The presence of a long N-terminal extracellular part is characteristic for the sweet and umami receptor subunits. This extracellular part consist of two domains, the Venus flytrap module (VFTM), which is involved in ligand binding and a cysteine-rich region, the link between the VFTM and the transmembrane domain. For bitter taste, around 25 receptor monomers are mentioned, lacking the long extracellular domain (Pin et al., 2003; Xu et al., 2004; Zhang et al., 2008).

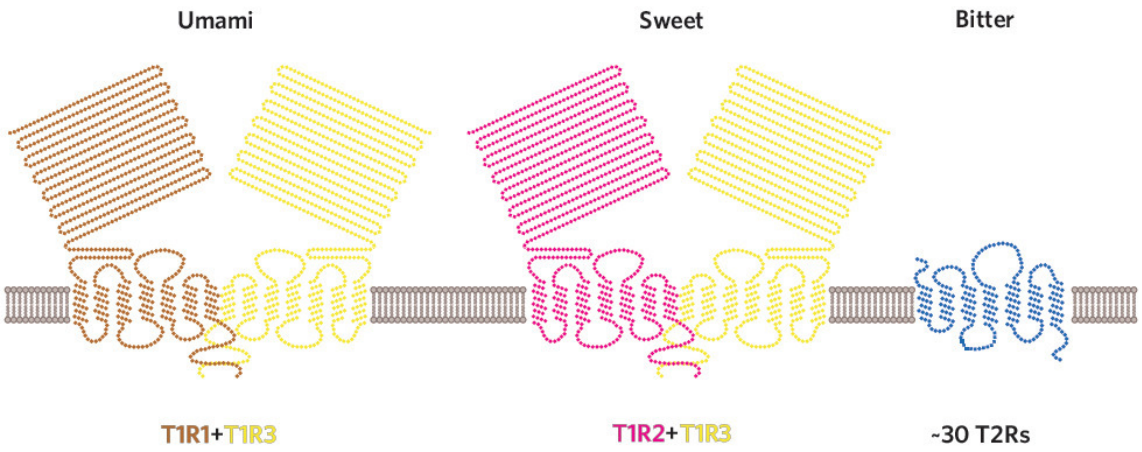



Figure 4: Schematic illustration of the GPCRs for the taste qualities umami (T1R1/T1R3), sweet (T1R2/T1R3), and bitter (25-30 different receptors), modified after Chandrashekar et al., 2006.

*Perception of Bitter Taste*

Bitter sensitivity in humans is very high to prevent the ingestion of potentially harmful or toxic compounds which show a wide structurally variety, such as strychnine or quinine. This high sensitivity is achieved by a family of approximately 25 highly divergent GPCRs, named TAS2Rs. Each of these monomeric receptors reacts explicitly to certain types of compounds (Behrens et al., 2004; Chandrashekar et al., 2006).

(−)-Epicatechin, for example, interacts with the bitter taste receptors TAS2R5, TAS2R4, and TAS2R39 (Soares et al., 2013), and (−)-epigallocatechin gallate with TAS2R14 and TAS2R39 (Yamazaki et al., 2013). Saccharin, denatonium benzoate, nitrosaccharin, acesulfam K, and quinine activate the human bitter receptor hTAS2R44, while at the same time denatonium benzoate interacts with hTAS2R4, 10, 46 and 47. Further examples have been summarized by Ley (2008) and Meyerhof et al. (2010).

In addition to the different bitter taste receptors, a number of receptor variants, caused by so called single nucleotide polymorphisms (SNPs) could be detected (Ueda et al., 2001). This results in inter-individual differences concerning the bitter taste sensitivity in humans. Known examples for this phenomenon are variants in the receptor hTAS2R38 that are supposed to account for differences in sensitivity to phenylthiocarbamide (PTC) and 6-*n*-propylthiouracil (PROP) (Bufe et al., 2005; Kim et al., 2003). People were differentiated in terms of their sensitivity to PROP or PTC. “Non-tasters” do not perceive the bitterness of PROP at all or only very weakly, while

“tasters” describe these compounds as bitter. A minor group among the tasters is even more sensitive and is called “supertasters” (Bartoshuk, 1993; Bartoshuk et al., 1992). Eating habits seem to be also related to the PROP status. It has been reported that PROP-sensitive people reject certain foods, such as the cruciferous vegetables spinach, cabbage, and broccoli because of the contained PTC-like glucosinolates (Sandell and Breslin, 2006).

*Sweetness Perception*

In contrast to bitter taste, the sensitivity for the perception of sweet carbohydrates is lower, because high concentrations of sweet tastants in general indicate a high nutritional value. A heterodimeric receptor complex of TAS1R2 and TAS1R3 forms the human sweet taste receptor (Figure 4) (Li et al., 2002; Nelson et al., 2001). This receptor, showing different binding pockets, responds to a broad variety of structurally diverse sweet tasting molecules (Morini et al., 2005). Compounds that provoke sweet taste include carbohydrates like sucrose, glucose, and fructose, proteins, e.g. thaumatin, brazzein, and monellin (Ming and Hellekant, 1994; Temussi, 2002; van der Wel and Loeve, 1972), steviol glycosides, such as rebaudioside A and rubusoside (Kinghorn and Compadre, 2001), and certain amino acids like glycine and alanine (Solms et al., 1965). Also artificial high intensity sweeteners (HIS) like saccharin, aspartame, and sucralose activate the sweet taste receptor (Roper, 2007; Wiet and Beyts, 1992).

In addition to the above-described sweet taste receptor, a homodimer of TAS1R3 may also act as a low affinity receptor for different natural sugars (Zhao et al., 2003). Research on additional sweet taste receptors is still ongoing.

*Perception of Umami Taste*

Like bitter and sweet, umami taste is also mediated by GPCRs (Lindemann, 1996, 2001). MSG and L-amino acids bind to a heterodimer of TAS1R1 and TAS1R3 (Nelson et al., 2002; Zhao et al., 2003). The involvement of these subunits of the TAS1R receptor family was proven by knock-out experiments (Damak et al., 2003; Zhao et al., 2003). Glutamic acid is present in many foods, e.g. fish and soy sauce, parmesan cheese, fermented beans, and tomatoes (Yamaguchi and Ninomiya, 2000). Combinations of monosodium glutamate and inositol monophosphate (IMP) or guanosin monophosphate (GMP) are known to enhance the umami taste perception

of MSG (Rifkin and Bartoshuk, 1980; Yamaguchi, 1967). The N-terminal extracellular domains of the receptor unit TAS1R1 are crucial for this effect of the 5'-ribonucleotides IMP and GMP (Zhang et al., 2008). Additional receptor candidates for the detection of umami compounds, such as the metabotropic glutamate receptor taste-mGluR4, are under discussion (Bessis et al., 2002; Chaudhari et al., 1996; San Gabriel et al., 2005).

*Salt Taste Perception*

In contrast to sweet, bitter, and umami, the taste qualities salty and sour are assumed to be mediated by ion channels. Sodium is the most relevant stimulus for salt taste, although other ions (potassium, ammonium, lithium) can stimulate the receptor. As soon as such a molecule binds and activates the receptor, cations enter the taste receptor cell and depolarize the membrane (Lindemann, 2001; Roper, 2007). This leads to a release of neurotransmitters, coupled with neuron activation and signal transduction to the brain (Lindemann, 1996). One candidate is the amiloride-sensitive epithelial Na channel, ENaC, which at least partly mediates salty taste (Heck et al., 1984; Lindemann, 2001). In humans, this amiloride-sensitive pathway seems to explain only a minor part of sodium detection (Chandrashekar et al., 2010; Feldman et al., 2003). Other channels, insensitive to amiloride, like the transient receptor potential vanilloid receptor 1 variant, TRPV1t, are mentioned (Lyall et al., 2004; Ruiz et al., 2006). Recent findings show that high-salt taste might share sour- and bitter-sensing pathways, possibly through T2Rs (Oka et al., 2013).

*Perception of Sour Taste*

Protonated organic acids are responsible for the sour taste by permeating the plasma membrane of the taste cell, where they dissociate and acidify the cytosol. At high extracellular proton concentrations, protons cross the cell membrane directly through ion exchangers and channels. It is believed that intracellular protons inhibit proton-sensitive channels and therefore, depolarize the membrane. As a result, voltage-gated $Ca^{2+}$ ion channels open, $Ca^{2+}$ levels in the cell increase and neurotransmitters are released (Chaudhari and Roper, 2010; Richter et al., 2003; Roper, 2007). Several potential receptors for the sensation of sour have been described, for example hyperpolarization-activated cyclic nucleotide-gated channels HCN1 and HCN4 (Stevens et al., 2001) and acid-sensing ion channels (ASIC), e.g. mammalian

degenerin-1 MDEG1 (Ugawa et al., 1998). The transient receptor potential channels, PKD2L1 and PKD1L3 (polycystic kidney disease–like) are most promising among the described candidates (Huang et al., 2006; Kataoka et al., 2008; LopezJimenez et al., 2006; Simon et al., 2006).

### 2.1.3 Trigeminally Active Compounds

Chemosensation, the perception of chemical substances by sensory means, comprises the gustatory and olfactory systems as described before. The trigeminal somatosensory system is also a part of chemosensation and important in food perception (Viana, 2011). Chemical agents as well as physical stimuli (heat, cold, mechanical forces) can evoke sensations of temperatures (thermal), touch (tactile), and pain (nociceptive) via the trigeminal nerve. The trigeminal nerve (cranial nerve V) is divided into three major branches, the ophthalmic (V1), maxillary (V2), and mandibular (V3) nerve (Figure 5). Branches of the ophthalmic nerve innervate the forehead, eye, and tip of the nose, while the maxillary nerve carries information from the cheek, nose, upper lip, and roof of the mouth. The mandibular nerve provides sensations from the lower part of the mouth and the jaw. The three nerves converge on the trigeminal ganglion, also called gasserian or semilunar ganglion, which contains the cell bodies of the sensory nerve fibers. From the trigeminal ganglion, a trigeminal nerve root enters the brainstem with the trigeminal nucleus to the thalamus. Information from the thalamic nuclei is sent to specific areas of the cerebral cortex (Anton and Peppel, 1991; Lundström et al., 2011; Schuenke et al., 2010; Viana, 2011). Pain, for example, is mediated by the lateral pain system including primary and secondary somatosensory cortices (determination of localization, duration and quality) and medial pain system including the anterior cingulate cortex (emotional evaluation) (de Leeuw et al., 2005; Treede et al., 1999).

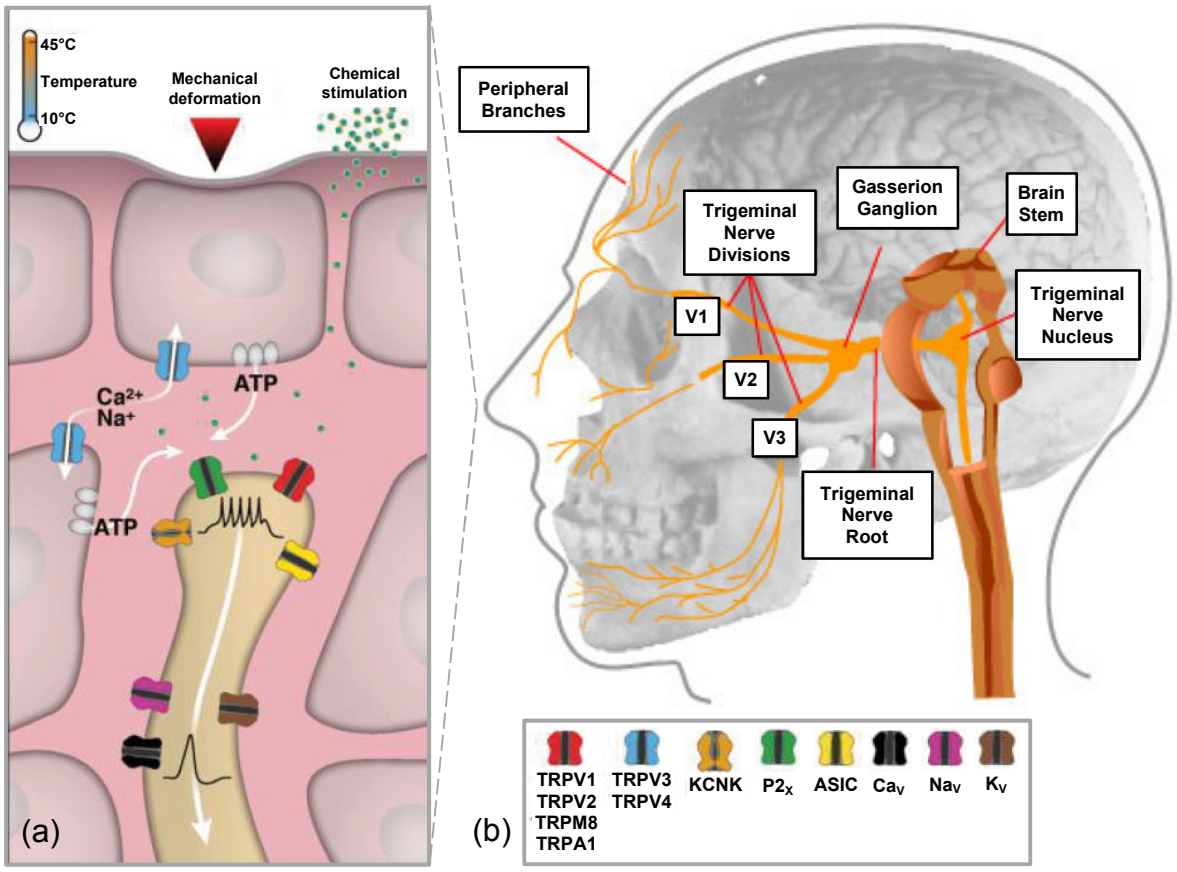



Figure 5: Free nerve endings of the trigeminal nerve that innervate the facial area. The expressed chemosensitive channels are located in sensory nerve endings, skin keratinocytes, and mucosal epithelial cells ((a) picture modified after Viana, 2011; (b) AnaesthesiaUK, 2013).

Branches V1, V2, and V3 of the cranial nerve V are divided into even more branches with many free nerve endings. All these trigeminal cutaneous endings are involved in natural defense and warning mechanism. The epidermis contains nociceptive nerve endings, specialized in noxious irritant detection (Dux et al., 1999; Simone et al., 1998). Chemical agents can cause lachrymation, salivation, sneezing, and coughing (Dourson et al., 2010). Nevertheless, humans developed a liking for hot and spicy dishes. Spices, like black pepper, Szechuan pepper, chili, ginger, or peppermint provoke sensations described as hot, burning, pungent, warm, tingling, fresh, and cooling. Certain compounds in the described foods and spices interact with chemosensitive channels on the trigeminal nerve, after entering the epithelial barrier or tight junctions. Cationic channels that open, or potassium-selective channels that close, lead to a transduction current, depolarization and action potential firing. Voltage gated channels, like potassium ($K_V$), calcium ($Ca_V$) and sodium ($Na_V$) channel are involved in the generation of action potentials (Viana, 2011).

Chemical agonists can activate various receptors (Belmonte and Viana, 2008; Gerhold and Bautista, 2009; Viana, 2011). Therefore, the sensations of a single compound can be complex. The mammalian transient receptor potential (TRP) superfamily includes 28 TRP cation channels in total, divided into six subfamilies. These are TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPC (canonical), TRPP (polycystin), and TRPML (mucolipin) (Clapham, 2003; Nilius and Vennekens, 2010). Each channel contains six transmembrane domains and a cytoplasmic carboxyl and amino terminus (Figure 6).

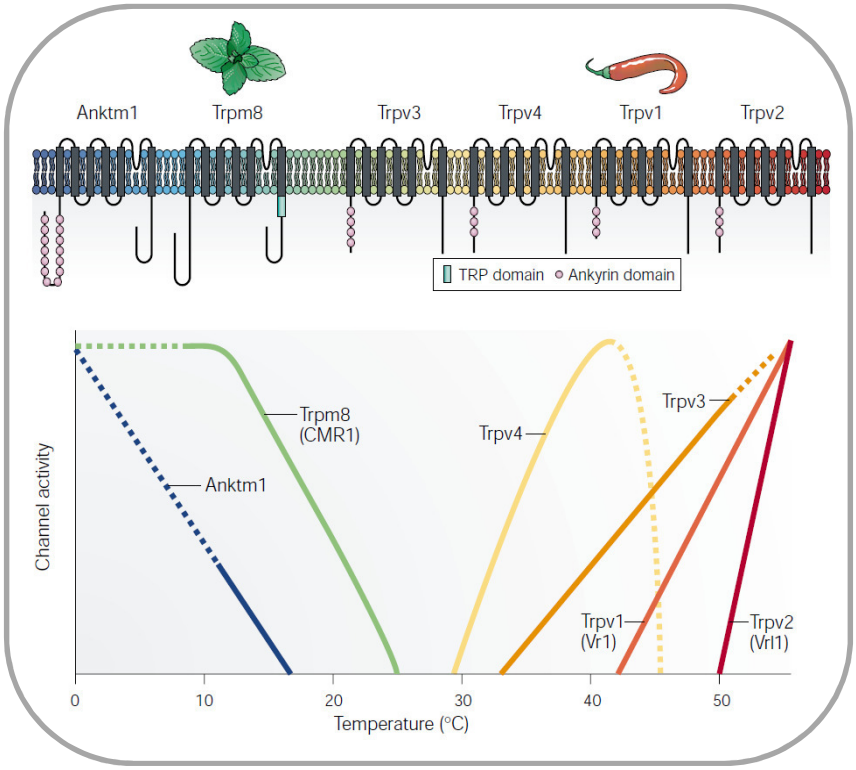



Figure 6: Schematic illustration of thermal TRP channel activation from noxious cold to noxious heat, as well as activation of selected TRP channels by chemical compounds (Patapoutian et al., 2003).

TRPV1, the vanilloid-type $Ca^{2+}$-permeable and nonselective channel, former called VR1 for vanilloid receptor subtype 1, can be activated by heat (>43°C), protons (pH <6.0), and compounds like capsaicin as the probably most known agonist (Caterina et al., 1997; Huang, 2004). Further molecules that can bind to TRPV1 are piperine from *Piper nigrum* (McNamara et al., 2005; Okumura et al., 2010; Ursu et al., 2010), gingerols from *Zingiber officinale* (Dedov et al., 2002), and the potent capsaicin analogue resiniferatoxin. The latter is naturally occurring in the latex of certain

*Cactaceae*, such as *Euphorbia resinifera* and is even more potent than capsaicin (Szallasi and Blumberg, 1989).

Lipophilicity of TRPV1 agonists plays a major role in the determination of pungency. Piperine and capsaicin are described to be medium lipophilic and thus show pungency because of their fast kinetics between the lipid and aqueous phase of the cell membrane (Ursu et al., 2010). One important characteristic of TRPV1 is the fact that the ionic pore properties change under prolonged exposure of agonists (Chung et al., 2008). The permeability of $Ca^{2+}$ increases, which results in a $Ca^{2+}$ overload and at the end in lingering desensitization of nociceptive sensory nerve endings. This is the basis in therapeutic treatment in neuropathic pain (Bley, 2004; Kennedy et al., 2010).

The TRPA1 (ANKTM1) channel can be stimulated by noxious cold (<17°C), as well as by pungent compounds. Chemical activators are isothiocyanates from mustard oil and wasabi, methyl salicylate from winter green, cinnamaldehyde from cinnamon (Bandell et al., 2004; Jordt et al., 2004), as well as allicin and diallyl disulfide from garlic (Baraldi et al., 2010; Bautista et al., 2005; Macpherson et al., 2005). In addition to TRPA1, TRPM8 was identified as a sensor for cold. TRPM8 is activated at temperatures <22-26°C (McKemy et al., 2002; Peier et al., 2002) and by a number of natural and artificial substances, such as L-menthol from peppermint, eucalyptol from eucalyptus oil, Frescolat® ML, WS3, and icilin, evoking a cool sensation (Behrendt et al., 2004; McKemy et al., 2002; Peier et al., 2002; Schäfer et al., 1986). TRPM8-activation can cause also an analgesic effect, which might be another way against chronically sensitized pain (Dhaka et al., 2007; Galeotti et al., 2002; Proudfoot et al., 2006).

Another impression among the trigeminals is the tingling effect. It is also described to be comparable to the sensation of a weakly carbonated solution or mild electric shocks as can be caused by a low voltage battery (Bryant and Mezine, 1999). Tingling is mediated by a number of alkylamides, e.g. hydroxyl-α-sanshool from Szechuan pepper (*Zanthoxylum piperitum*) (Bryant and Mezine, 1999). Further tingling compounds are spilanthol from Jambu (*Acmella oleracea*), and alkamides related to spilanthol, like homospilanthol, achilleamide, and *trans*-pellitorine (Ley et al., 2006). Besides tingling, spilanthol and *trans*-pellitorine are also described to have a mouth-watering effect. This was determined with saliva-inducing tests, where the saliva flow was measured (Ley and Simchen, 2007).

The physiological mechanism of tingling perception has not been fully investigated. According to the literature, the activation of TRPV1 and TRPA1 (Koo et al., 2007; Sugai et al., 2005), and the inhibition of potassium channels, like KCNK3, KCNK9 and KCNK18 (Albin and Simons, 2010; Bautista et al., 2008) are involved in the perception of tingling.

### 2.1.4 Astringency Perception

Astringency is often described as a dry, rough, puckering sensation (Bate-Smith, 1954; Lee and Lawless, 1991) that is elicited by the consumption of various food products. In general, astringency is perceived as aversive and leads to avoidance of ingestion, especially at high levels. There are, however, some exceptions such as green and black tea, red wine and dark chocolate, where astringency is accepted or even considered as a positive quality parameter (McDowell et al., 1995).

So far, the molecular mechanisms of astringency are still unclear and different hypotheses are controversially discussed. According to one theory, astringency is a taste perception because of the activation of the chorda tympani taste nerves (Schiffman et al., 1992). Some of the compounds tested in previous studies, however, are also known for other, non-astringent taste qualities like bitter and sour (Hufnagel and Hofmann, 2008). Therefore, the results may be confounded. Moreover, astringency was perceived on non-gustatory surfaces in the oral cavity and even more clearly by movements of the lips over the gum. This indicates that tactile sensation is also involved in the perception of astringency (Breslin et al., 1993).

The most accepted theory focuses on the fact that numerous astringent molecules are able to complex and/or to precipitate certain proteins in saliva, especially proline-rich basic proteins (Figure 7) (Jobstl et al., 2004). This was, for example, shown for (+)-catechin, (–)-epicatechin, (–)-epicatechin *O*-gallate, epigallocatechin gallate, the procyanidin dimers B1-B8, and trimer C1 (Canon et al., 2013; de Freitas and Mateus, 2001; Sarni-Manchado et al., 1999). Due to the precipitation, the viscosity and the lubricant properties of the saliva are drastically changed, which is probably detected by tactile neurons. This is comprehensible, considering the fact that moving the tongue enhances the sensation (Breslin et al., 1993).

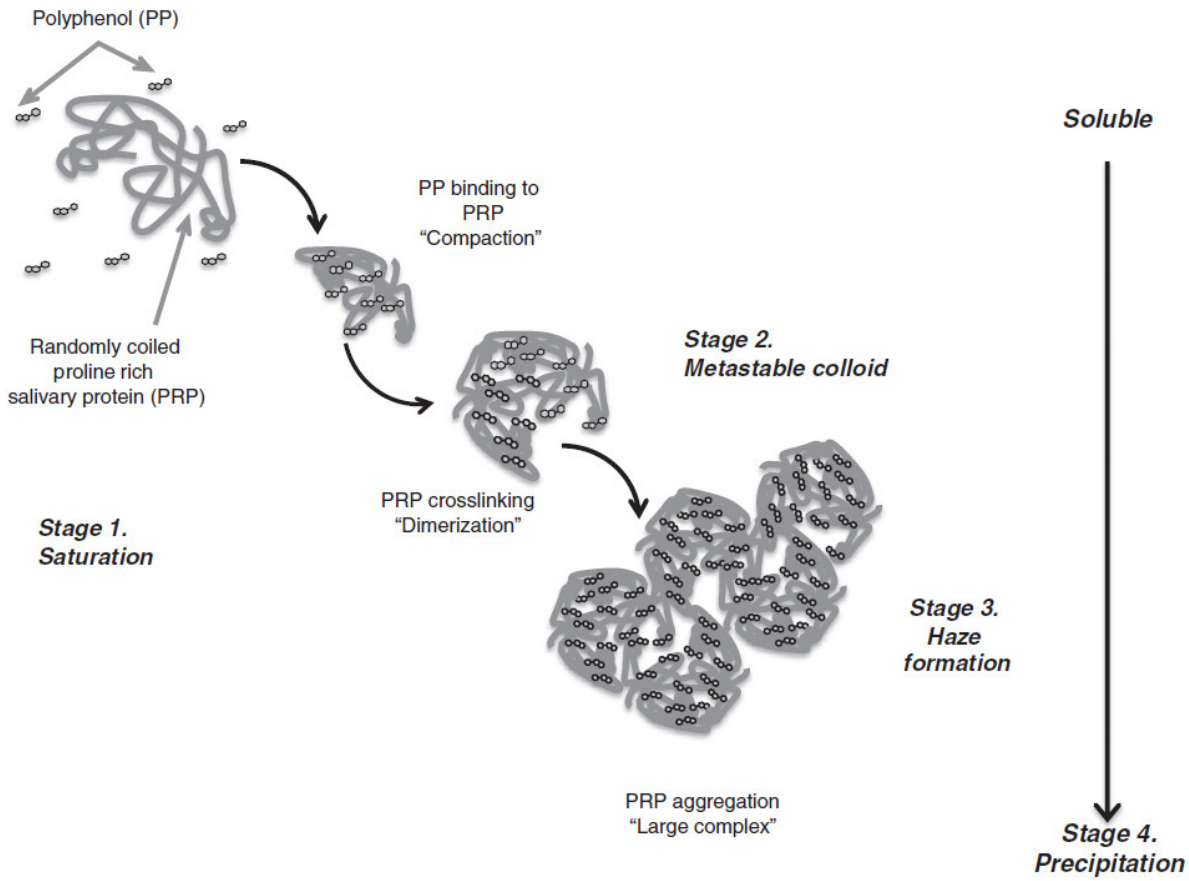


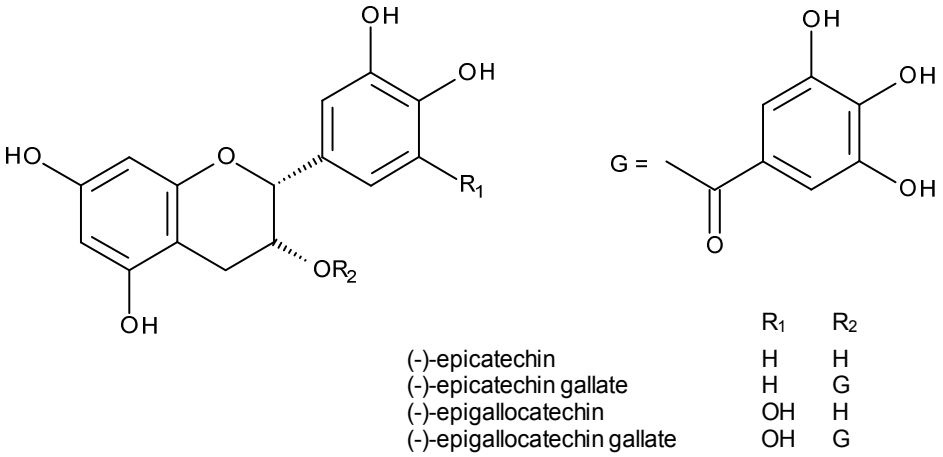

Figure 7: Interaction of polyphenols from food sources and salivary proteins (especially proline-rich proteins) in the mouth, followed by protein precipitation and a reduction of oral lubrication (Ferruzzi et al., 2012).

Recent studies, however, showed that at least some of the astringency elicitors are sensorially active while having no or only low protein binding activity (Rossetti et al., 2009; Schwarz and Hofmann, 2008). Additionally, in time-intensity studies, protein binding and the occurrence of astringency were not correlated (Kallithraka et al., 2001). This indicates that the reduction of salivary lubricity might not be the only mechanism contributing to astringency perception (Lee and Vickers, 2012). There are also some hints that catechins can interact with TRPA1 channels involved in the EGCG-induced $Ca^{2+}$ response in intestinal endocrine cells in mice (Kurogi et al., 2012). Furthermore, one study pointed out that trigeminal rather than gustatory innervation is necessary for astringency perception (Schöbel, 2010; Schöbel et al., 2013). Patients with unilateral lesions of the *Chorda tympani* nerve were not able to perceive the taste qualities sweet, salty, sour, and bitter on the lesioned anterior tongue. Astringency perception of green tea infusion and chest nut powder, however, was equal on both sides of the tongue. More detailed analysis revealed that red wine and green tea astringents (tannins and catechin, respectively) activated isolated

trigeminal ganglion neurons of mice. A G-protein-coupled pathway with different downstream signaling is mentioned in the study; however, further investigations are necessary (Schöbel, 2010; Schöbel et al., 2013). In addition, certain compounds, such as gingerol and sanshool, change the composition of saliva in terms of protein composition (Lorenz et al., 2011). This, of course, could also influence the sensation of astringents in the oral cavity.

A number of astringent compounds have been described in foods. In roasted cocoa nibs, *Theobroma cacao*, different molecules contribute to the perception of astringency. *N*-Phenylpropenoyl amino acids, like (+)-*N*-[3´,4´-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid and (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid are described as purely astringent (Stark and Hofmann, 2005). Flavan-3-ols, including e.g. catechins, such as (+)-catechin and (-)-epicatechin show a bitter and puckering astringent sensation in cocoa, whereas polyphenol glycosides, e.g. quercetin-3-*O*-α-L-arabinopyranoside and naringenin-7-*O*-β-D-glucopyranoside induce a velvety astringency (Stark et al., 2005, 2006). In red wine, flavan-3-ols also contribute to the astringent perception, as well as compounds like hydroxybenzoic acids (e.g. vanillic acid, gallic acid) and procyanidins (e.g. procyanidin B1, B2, B3) (Hufnagel and Hofmann, 2008).

Besides benzotropolones, such as theaflavin, theaflavin acid, and theaflavin-3-gallate, flavon-3-ol glycosides are also important for the astringent sensation in black tea. Among them, rutin (quercetin-3-*O*-rutinoside) was described to possess the lowest astringency threshold among the tested compounds, and has therefore a major influence on the taste of black tea (Scharbert et al., 2004). Flavan-3-ols occur in lower concentrations in black than in green tea (*Camellia sinensis*), because of the fermentation step involved in the production of black tea (Chen et al., 2001). In green tea, flavan-3-ols are the most important compounds for the astringency and bitterness (Narukawa et al., 2010); the major catechins are (-)-epigallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epicatechin (Figure 8) (Chen et al., 2001; Perva-Uzunalic et al., 2006).




| | $R_1$ | $R_2$ |
|---|---|---|
| (-)-epicatechin | H | H |
| (-)-epicatechin gallate | H | G |
| (-)-epigallocatechin | OH | H |
| (-)-epigallocatechin gallate | OH | G |

Figure 8: The major green tea flavanols (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate.

Apart from catechins, certain other polyphenols are described as elicitors for astringency, e.g. tannic acid (Robichaud and Noble, 1990), flavanol glycosides (Schwarz and Hofmann, 2007), tannins (Hofmann et al., 2006), as well as some metal salts such as aluminum sulfate (Peleg et al., 1998) or acids, like tartaric acid (Sowalsky and Noble, 1998).

Intake of polyphenols, especially of flavonoids, as contained e.g. in tea or grape seeds, seems to be correlated with positive health benefits, such as anti-oxidant (Erba et al., 2005; Salah et al., 1995; Vinson and Hontz, 1995), anti-inflammatory (Dona et al., 2003), and cardio-protective (Imai and Nakachi, 1995) properties. In addition, preventive effects against cancer (Fujiki, 1999; Leone et al., 2003; Yang et al., 2002a) are mentioned in the literature. Due to their potential health benefits, these compounds are sometimes even enriched to prepare functional foods (Hasler, 2002; Katan and Roos, 2004; Wu and Wei, 2002). But especially higher concentrations of such compounds cause astringency and bitterness that is perceived as aversive by many people. Therefore, masking solutions are necessary to increase the palatability and the compliance of consumers. Due to the variety of discussed mechanisms and the variety of different astringent compounds, the task to reduce astringency in food is demanding.

### 2.1.5 Reduction of Unpleasant Taste and Flavor

Bitter and astringent sensations are generally perceived as unpleasant and have to be avoided. Many functional ingredients, like polyphenols and catechins that are added to health-promoting foods, show such problems (Barnekow et al., 2007;

Cowart, 1981; Eckert and Riker, 2007). Furthermore, certain pharmaceutically active compounds, e.g. morphine, quinine, and penicillins are known to have an unpleasant bitter taste (Ayenew et al., 2009; Meyerhof, 2005). Improved compliance is expected when reducing this bitterness (Ayenew et al., 2009).

Several strategies have been investigated during the last years to avoid/reduce unpleasant taste or flavor (Ley, 2008). One approach for flavor optimization is the reduction or removal of such compounds. In citrus industry, cleavage of compounds like naringin and therefore debittering of juices is common to enhance the palatability of orange or grapefruit juice (Lee and Kim, 2003; Stinco et al., 2013). This however, is not always an option because of positive health properties of certain bitter substances, especially if they were enriched to obtain a so-called functional food.

Complexation and encapsulation of relevant molecules is also possible. In these cases the exposure of the taste receptors to bitter compounds is reduced. The principle could be demonstrated for the reduction of (+)-catechin bitterness by using β-cyclodextrin (Gaudette and Pickering, 2012a; Tamamoto et al., 2010) or astringency reduction of polyphenolic extracts by using carboxymethylcellulose (CMC) (Troszynska et al., 2010). However, the bioavailability of such complexes can be reduced, and the approach is not suitable for each application.

Another strategy is the use of congruent flavors e.g., grapefruit flavor, chocolate flavor, or the addition of other flavorings or strong tastants as for example sweeteners like sugar and rebaudioside A (Ares et al., 2009; Gaudette and Pickering, 2012b).

Flavor modifiers, which act on molecular level, are a further possibility to reduce unpleasant sensations. Flavor modifiers are compounds that enhance or decrease the taste or flavor of other compounds and foods (Codex Alimentarius, 2008; Ley et al., 2012). A number of examples are described in the literature. Designated bitter masking compounds are homoeriodictyol (Ley et al., 2005), phloretin (Ley et al., 2008b), zinc sulphate (Keast, 2003), and [2]-gingerdione (Ley et al., 2008a), whereas their bitter reducing efficiency is depending on the bitter compound. Homoeriodictyol acts against the bitterness of quinine, caffeine, and salicin, for example, while [2]-gingerdione affects quinine and caffeine (Ley et al., 2005; Ley et al., 2008a). Matairesinol, a plant lignin, is described to be able to reduce the bitterness of EGCG (Backes et al., 2012). Flavor modifiers can also increase taste sensations as described for the sweet enhancers hesperetin (Ley et al., 2007), phloretin (Krammer et al., 2007), and chlorogenic acid (Bartoshuk et al., 1972). But also sweet inhibition

by using lactisole or gymnemic acid is possible (Jiang et al., 2005; Kurihara, 1969; Schiffman et al., 1999).

The discovery of these substances is challenging because they have to be tested in combination with the flavor or tastant, which has to be modified. Only under this condition agonistic or antagonistic effects can be determined. One sensory method for the discovery of modulating taste effects is a simple duo difference test (Ley et al., 2005; Ley et al., 2008a). By rating the intensity of a solution with and without the test compound on a hedonic scale, the panelists can make a statement about the difference of the two samples.

Depending on the concentration of the compound that has to be tested, the number of test solutions, and the number of panelists, a relatively large amount of substance is required. For this reason, more sophisticated techniques, like the half-site tongue test (Scharbert et al., 2004; Shikata et al., 2000) and comparative taste dilution analysis (cTDA) (Ottinger et al., 2003) can be used. Alternatively to sensory-based methods, biological test systems using cell-based assays, expressing taste receptors, are options for a first selection (Behrens et al., 2004; Brockhoff et al., 2007; Servant et al., 2010).

## 2.2 PRINCIPLES OF SENSORY ANALYSIS

Sensory evaluation is indispensable in the work with flavor and fragrance materials. Human subjects are used as “measuring instruments”. Their sensory performance strongly depends on a number of different factors, e.g. gender, age, health status, drinking and eating habits (Baker et al., 1983; Derntl et al., 2013; Larsson et al., 2000). Elderly people, for example, show a diminished sensitivity in taste and smell (Schiffman, 1977; Schiffman et al., 1979). Furthermore, mood of the testers and stress are factors influencing the subjective assessment of a sample (Chen and Dalton, 2005; Dess and Edelheit, 1998; Ileri-Gurel et al., 2013). Therefore, the panel performance can vary between different individuals and different testing sessions (Meilgaard et al., 2007c). At the same time, there is no instrument available that can provide results comparable to those obtained from humans. A number of analytical methods are known to get detailed data on the chemical composition of a sample, such as GC or HPLC-based methods. Furthermore, different techniques are reported in the literature to mimic human sense of taste and smell. Electronic noses and tongues are devices that are meant to simulate the olfactory and taste system of

human beings. Both apparatus consist of electrochemical non-specific sensors, signal collecting elements, and a software for pattern recognition (Chen et al., 2012; Gardner and Bartlett, 1994; Legin et al., 2002; Persaud and Dodd, 1982; Winquist et al., 1997). Certain compounds in food or beverage applications can be analyzed, which is practical for continuous measurements with high precision and without fatigue of human assessors, e.g. for quality control. This of course saves time, panel work, and money. Nevertheless, the differentiation between relevant and not relevant compounds is not possible and human support is needed. The described systems are based on physical measurements. The high number of different human receptor cells and even more important their selectivity and unrivaled sensitivity cannot be fully replaced by instrumental analysis (Rock et al., 2008). In addition, it requires human assessors to gain information on preference, liking or disliking. Sometimes these human testers are even able to detect compounds that are not detectable via analytical instruments (Acree and Barnard, 1994; Kiefl et al., 2013). Many odorants have a low threshold and therefore, are very active at low concentrations (Blank, 1997). For example, 1-*p*-menthene-8-thiol, a key flavor compound in grapefruit, has a very low odor threshold of 0.1 x $10^{-9}$ g/L in water. With a flavor dilution (FD) factor of 200 in grapefruit juice, it has a significant contribution to the aroma (Buettner and Schieberle, 1999; Demole et al., 1982). 2-Methoxy-3,5-dimethylpyrazine was described as a potent odorant of raw coffee with an odor threshold of 0.4 x $10^{-9}$ g/L water (Czerny and Grosch, 2000).

### 2.2.1 Time-Intensity Measurements

Many taste and flavor compounds such as catechins or high intensity sweeteners show complex sensory profiles. For an adequate description of various taste-actives and their aftertaste properties, a time-intensity profile is fundamental. This is especially important for sweeteners with their sweet lingering after-taste (Obst et al., 2010; Ott et al., 1991; Swartz, 1980), catechins (Narukawa et al., 2010), polyphenol glycosides (Scharbert and Hofmann, 2005), or tannins (Hofmann et al., 2006), showing lingering bitter and astringency sensations, respectively. Therefore, the use of time-intensity (TI) measurements dealing with more than one descriptor is necessary. In early TI methods the panelists were asked to trace the intensity with a pencil on a sheet of paper over the time (Holway and Hurvich, 1937). Guinard et al. developed a computerized procedure for time-intensity measurements (Guinard et

al., 1985). In each of these methods, the intensity of each descriptor is rated and presented as time-intensity curves. Different approaches, such as dual-attribute time-intensity (DATI) (Duizer et al., 1997) and time-intensity profiling (TIP) (Guinard et al., 2002) were described in the following years. DATI allows parallel time-intensity measurements of two descriptors on a two-dimensional plane. The panelists record the changes by moving the mouse on the plane in two directions at the same time, while the data are collected every 3 seconds. TIP enables the evaluation of even more than two attributes, but only in a consecutive way, which is more time consuming. Usually, only one attribute is described per session and the different descriptors of one sample or similar attributes of various samples are compared afterwards.

The intensity variation descriptive methodology (IVDM) is another variation of time-intensity scaling allowing the evaluation of multiple attributes in parallel over a certain period of time (Gordin, 1987). It was originally developed as a modification of the quantitative descriptive analysis procedure (QDA®) (Stone, 1974) that is commonly used in the evaluation of food products. With QDA®, trained panelists measure the intensities of key product attributes, and the results are presented in a spider web as a multidimensional model. The combination of multiple attribute ratings at discrete time points is focused in IVDM. The intensities were rated on line scales, one for each descriptor that occurred at defined time points (Gordin, 1987; Lee and Lawless, 1991). The method was used for documentation of the time-course, e.g. of astringency, puckering, drying, roughing, bitter, and sour sensations of tannic acid, alum, and tartaric acid (Lee and Lawless, 1991), and of the sweet and bitter taste of high intensity sweeteners, like saccharin, aspartame, and acesulfame K (Ayya and Lawless, 1992). Depending on the problem, the number of descriptors and measuring points, the time between the measuring points, and the duration of the test can be varied. With this method, possible relationships between the different descriptors can be observed. By rating them in parallel, there is a closer correlation between the descriptors compared to rating each separately. Especially for complex food samples this trait can be very important.

## 2.3 MACROPIPER EXCELSUM

*M. excelsum*, also called *Piper excelsum*, kawakawa, or New Zealand pepper tree, belongs to the *Piperaceae* family and is a shrub. It can grow up to six meters and is

widespread in New Zealand. Occurrence and distribution of *M. excelsum* is predominant on the North Island of New Zealand and reaches as far as to the northern part of the South Island (Dawson and Lucas, 2009) (Figure 9).

(a)  (b) 

Figure 9: (a) Living specimen of *M. excelsum*; (b) distribution of *M. excelsum* in New Zealand, marked in green (Dawson and Lucas, 2009).

The aromatic leaves are heart shaped and 6 to 12 cm long (Dawson and Lucas, 2009). The orange fruits look like a miniature of sweet corn cobs; their pulp is eaten by the Maori (Colenso, 1880). Besides the fruits, the leaves are traditionally used as tea, as a spice for many dishes, or to prepare popular “Titoki” liqueur (Crowe, 2009). Also drinks are made from the aromatic kawakawa in combination with edible seaweeds as a thickener (Cranwell, 1935).

Kawakawa is also used as a traditional medicinal plant against bladder problems, gonorrhoea, cuts and wounds, abdominal pains or boils (Crowe, 2007; Goldie, 1905). Infusions of the leaves and roots were prepared by the indigenous people against toothache (Armstrong, 1869). Maori and early settlers in New Zealand regarded *M. excelsum* to possess aphrodisiac properties and stimulating effects on the bowls, kidneys, and salivary glands. The leaves were generally considered as less powerful than the seeds (Baber, 1886).

In the essential oil obtained via steam-distillation of the leaves and terminal branches, myristicin was identified as the major component (Briggs, 1941; Briggs et al., 1975). In addition, α-pinene, aromadendrene, γ-cadinene, (-)-cadinene dihydrochloride, palmitic acid, and elemicin were found (Briggs et al., 1975). Camphene, β-

phellandrene, and *n*-hexyl acetate were described tentatively (Briggs et al., 1975). Among the non-volatile compounds, a number of lignans were found. (+)-Excelsin, (+)-epiexcelsin, (+)-demethoxyexcelsin, and sesangolin were isolated from the leaves (Russell and Fenemore, 1973) as well as (+)-diayangambin, also called lirioresinol-C dimethyl ether (Briggs et al., 1968; Russell and Fenemore, 1973). Another lignan, lirioresinol-B dimethyl ether was identified in the wood (Briggs et al., 1968). The structures of the lignans are depicted in Figure 10.

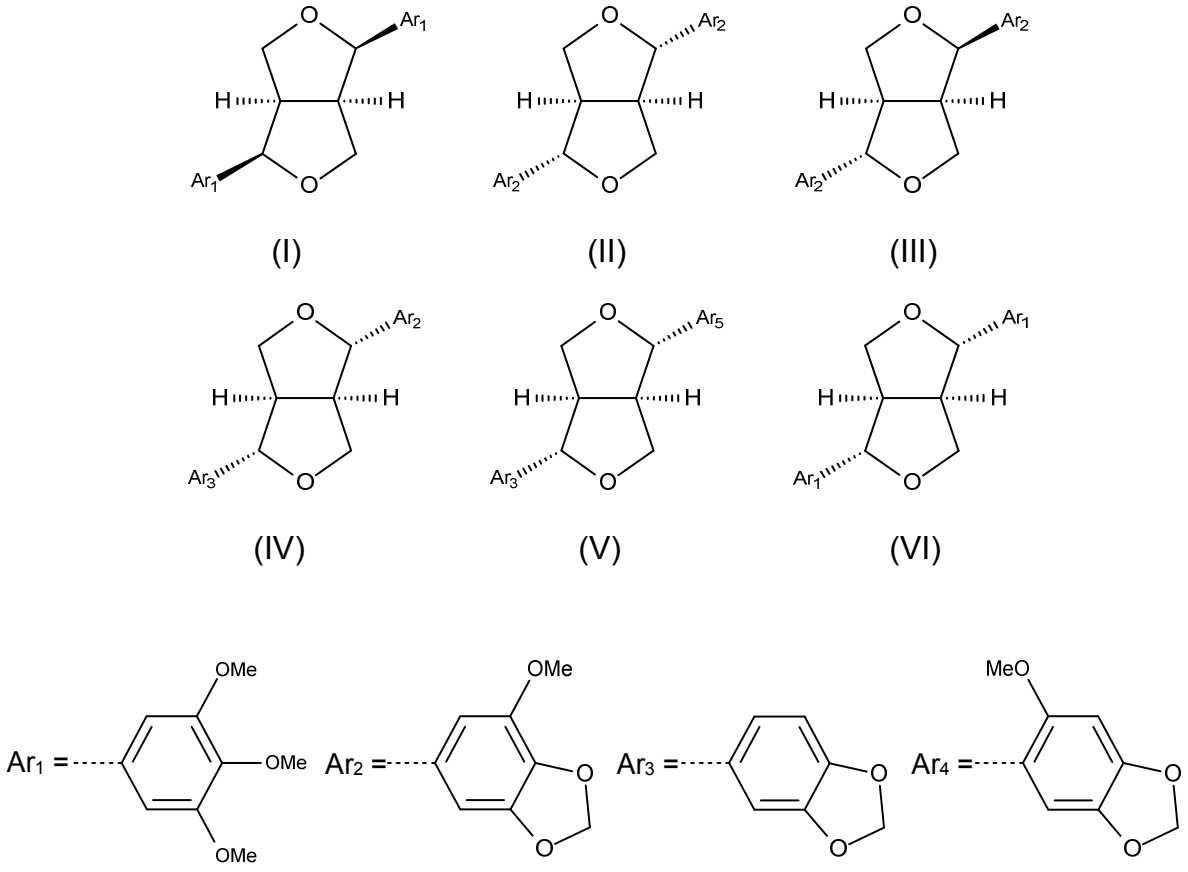



Figure 10: Structures of the lignans (+)-diayangambin (I), (+)-excelsin (II), (+)-epiexcelsin (III), (+)-demethoxyexcelsin (IV), sesangolin (V), and lirioresinol-B (VI) found in *M. excelsum* (Briggs et al., 1968; Russell and Fenemore, 1973).

It is known, that both kawakawa extracts and certain single compounds, which occur in the plant, have antifeedant activity. An extract of the leaves showed activity against ants, as well as greenheaded and brownheaded leafrollers (Russell and Lane, 1993). Furthermore, juvadecene (1-(3,4-methylenedioxyphenyl)-*trans*-3-decene), a juvenile hormone mimic, which was discovered in the roots, has an insecticidal effect (Nishida et al., 1983). Myristicin as the main bioactive ingredient (Russell and Lane, 1993) is also described to have insecticidal properties (Lichtenstein and Casida, 1963;

Srivastava et al., 2001) and exhibited antifeedant and toxic effects against the leaf beetle *Brontispa longissima* (Qin et al., 2010).

Despite these anti-insect properties, a highly specialised native caterpillar, *Cleora scriptaria,* is feeding on the leaves. This causes the characteristic riddled look of *M. excelsum* as seen in Figure 11 (Dawson and Lucas, 2009).

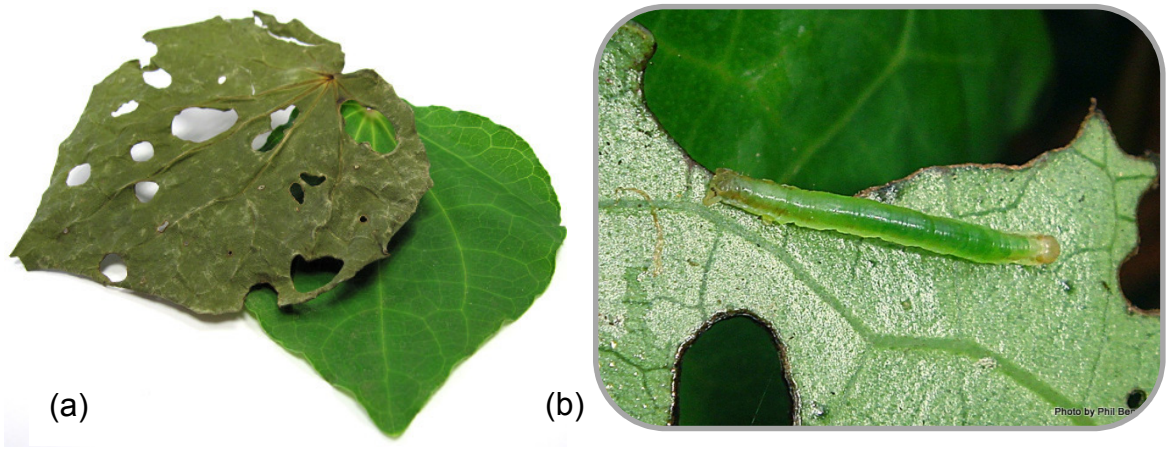



Figure 11: (a) A fresh and undamaged leaf of *M. excelsum* from a potted plant from Germany and a dried leaf originating from New Zealand with holes caused by *Cleora scriptaria* (b) (Bendle, 2013).

The damage however, is apparently not resulting in induction of the production of specific defense compounds repelling *C. scriptaria* (Hodge et al., 1998). Different tests were carried out to determine the feeding preference of the larvae for damaged and undamaged leaves, respectively. None of them showed significant preferences (Hodge et al., 2000a). Even leaf shedding is not affected in response to damage (Hodge et al., 2000b). It can be speculated that some tolerance against the defense mechanism of the host has been developed over time (Hodge et al., 2000a; Levin, 1976).

The Maori name kawakawa can be misleading because of the phonetical similarity to kava-kava (*Piper methysticum*). *P. methysticum* also belongs to the family *Piperaceae.* The two plants seem closely related considering their phylogenetic properties (Figure 12), but have only a distant relationship regarding their composition (Jaramillo and Manos, 2001).

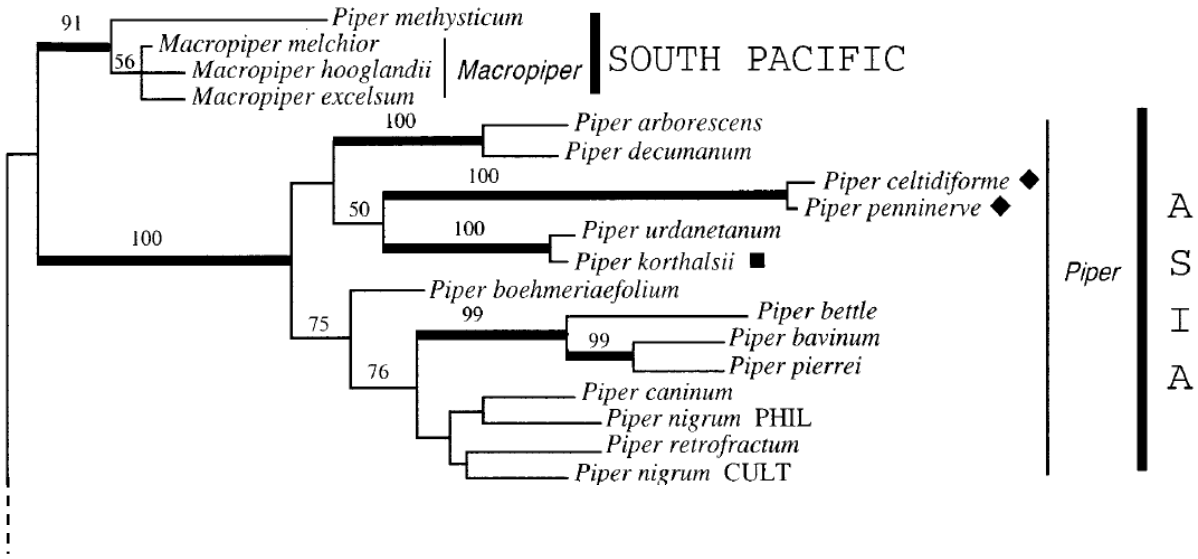



Figure 12: Phylogenetic relationship within the *Piper* species based on the internal transcribed spacers (ITS) sequence data of nuclear ribosomal DNA (Jaramillo and Manos, 2001).

*Piper methysticum* is distributed on the South Pacific Islands, especially on Tonga, Samoa, and Fiji. Preparations made from rhizomes, such as a traditional beverage, are called kava and are consumed in social life and ceremonial occasions (Norton and Ruze, 1994; Singh, 1992). Furthermore, kava is used in folk medicine in the treatment of insomnia, anxiety, and restlessness. It is attributed a relaxation effect without loss of mental vigilance (Foo and Lemon, 1997; Titcomb, 1948). Kavalactones, the active compounds in the piper are responsible for this anxiolytic effect. There are six major kavalactones (Gautz et al., 2006; Simeoni and Lebot, 2002; Smith, 1983), kavain (Borsche and Peitzsch, 1929, 1930), dihydrokavain (Borsche and Peitzsch, 1930), methysticin (Cuzent, 1861; Gobley, 1860), dihydromethysticin (Winzheimer, 1908), yangonin (Borsche and Gerhardt, 1914; Nölting and Kopp, 1874), and desmethoxyyangonin (Klohs et al., 1959).

Several cases with suspected hepatotoxicity in combination with kava-containing products have been reported (Escher et al., 2001; Gow et al., 2003). Kava was therefore banned in various European countries because of its alleged hepatotoxicity (BfArM, 2002, 2007; DAC/NRF, 2011; Richardson and Henderson, 2007). For the traditional consumption of kava, no major adverse effects on human health have been described. The reported hepatotoxic effect when using a standardized extract might be related to production methods (Denham et al., 2002; Escher et al., 2001). Depending on extraction conditions used for industrial purposes, e.g. ethanol-water

or acetone-water mixtures, concentrations and ratios of the lactones differ from traditional preparations, using only water or coconut milk (Denham et al., 2002; Norton and Ruze, 1994). To avoid confusion and possible health-related problems when using kawakawa, the differentiation between the two *Piperaceae* is important and has to be verified analytically.

## 2.4 EXTRACTION, PURIFICATION AND ISOLATION TECHNIQUES

### 2.4.1 Extraction Techniques - Pressurized Solvent Extraction

Traditional extraction techniques, like maceration, percolation, and Soxhlet extraction are widely known. During maceration, the material and a suitable solvent are stored together in a container for several hours up to weeks. The extraction time can be decreased by stirring the mixture, which is called accelerated maceration. Increase of temperature also results in higher extraction efficiency. The extraction process lasts until an equilibrium between the concentration of the compounds in the plant material and in the extract is reached. Filtration of the extract and the use of fresh solvent permit exhaustive extraction. In percolation, the plant material is placed together with the solvent in a vessel containing a perforated chamber. More solvent is filled on top of the material, which flows through the material and leaves the percolator drop-wise at the outlet. Soluble compounds can be extracted exhaustively by refilling the percolator. Filtration is not required because of a filter in front of the outlet. Again, temperature and duration influence the extraction yield (Seidel, 2006).

Soxhlet extraction is a continuous method, where the plant material is filled in a cellulose thimble. The solvent is heated under reflux, condensed and drops in the extraction chamber holding the thimble. When reaching a certain level the condensed solvent is siphoned into a flask beneath together with the solubilized compounds (Soxhlet, 1879).

These classical techniques are usually very time-consuming and require high amounts of solvents (Camel, 2001; Mustafa and Turner, 2011). Various efforts have been made in the past years to improve extraction processes, for example supercritical fluid extraction (Riekkola and Manninen, 1993; Smith, 1999), microwave dissolution (Ganzler et al., 1986; Jocelyn Pare et al., 1994), and pressurized or accelerated solvent extraction (Ramos et al., 2002; Richter et al., 1996). With each of the named techniques, preparation time and the volume of solvents can be reduced.

Pressurized solvent extraction involves higher pressure and temperature and thus enhances the extraction performance compared to ambient conditions. The solid is filled in an extraction cell, mostly stainless-steel, which is placed into an oven (Ramos et al., 2002). If necessary, dispersion agents, such as glass beads and quartz sand, can be added as filling materials to avoid caking and to reduce the dead volume and therefore, the amount of solvents. For the extraction process, solvent is pumped into the cell, which can be heated up to 200°C and pressurized between 35 and 200 bars. After a defined period of time, the extract is discharged by flushing with nitrogen gas and collected in a flask. Further extraction steps with the same solvent or with different solvents with increasing polarity, for example, can be carried out. Using fresh solvents in further extraction cycles, the yield can be increased to a certain level (Mustafa and Turner, 2011; Richter et al., 1996).

One system on the market is the ASE® (accelerated solvent extraction) system from Dionex (Sunnyvale, CA, United States) where the extraction chambers are positioned in a carrousel. Each sample is processed automatically without human assistance. Different types are available, varying in the amount and size of the extraction cells, amount and volume of the collecting vials, and the applied pressure. Another system on the market is the SpeedExtractor from BÜCHI (Flawil, Switzerland). In comparison to Dionex ASE®, fewer cells are available for extraction experiments but at the same time a parallel extraction is possible. By using a number of cells, depending on the apparatus, extractions can be carried out continuously or in parallel with less labor. It is applicable for rapid and reproducible extraction trials.

The above described increase in temperature results in a better solubility and mass transfer of the compounds, while at the same time the surface tension and the viscosity of the solvent decrease. Consequently, the analyte-solvent contact is improved and the extraction can be carried out more rapidly and solvent-saving. The pressure is responsible to keep the solvent liquid even at elevated temperature and to force it into the pores of the extraction material. However, temperature stability of analytes and possible degradation processes under the described conditions have to be kept in mind (Ramos et al., 2002).

### 2.4.2 Sensory-Guided Analysis

#### 2.4.2.1 Aroma Analysis using Gas Chromatography-Olfactometry (GC-O)

For the identification and quantification of aroma substances, gas chromatography coupled with mass spectrometry (GC-MS) is a common technique. With GC-MS, however, it is not possible to detect whether a compound is odor-active or not (Curioni and Bosset, 2002; Delahunty et al., 2006). Many instrumental detectors are less sensitive than the human nose (Acree and Barnard, 1994; Kiefl et al., 2013). A lot of odorants have low thresholds and, therefore, are active at very low concentrations. Thus, the chromatogram does not necessarily reflect the aroma profile of food or extracts (Blank, 1997). A valuable method that combines the separation of volatiles via gas chromatography and the *in-vivo* detection of odor actives by one or more testers was developed in the 1960‘s (Fuller et al., 1964), so-called gas chromatography-olfactometry (GC-O). By splitting the eluent, both the identification via a flame ionization detector (FID) or MS and the odor-activity via olfactometry can be carried out (Figure 13). This allows the correlation of analytical and sensory data (Delahunty et al., 2006). In the following years, improvements have been made regarding certain inconveniences, like a more comfortable position of the evaluator (Fuller et al., 1964) and the humidification of the GC eluent to reduce nose irritations (Dravnieks and O'Donnell, 1971; Fuller et al., 1964).

Figure 13: Gas chromatography-olfactometry (GC-O) with a sniffing port, where the eluted compounds can be directly smelled and evaluated by the assessor, in parallel with a flame ionization detector (FID).

Several GC-O techniques have been developed to evaluate the frequency, quantity, and intensity of an odor (Curioni and Bosset, 2002; Delahunty et al., 2006; van Ruth,

2001). In detection frequency method, a group of panelists evaluate the same sample via GC-O. The number of panelists, who detect the smell of a compound at a certain retention time, is counted. The higher the frequency of detection, the greater is the relative importance, which is probably due to higher intensity. Furthermore, the duration of every odor that occurs can be measured and together with the frequency the peak area can be calculated (Linssen et al., 1993; Pollien et al., 1997).

In aroma extraction dilution analysis (AEDA), a sample is diluted, commonly in a 1:1 or 1:2 series, and evaluated via GC-O until no odor is detected. The last dilution is called the flavor dilution (FD) factor and demonstrates the potency of an odorant (Grosch, 1994). In CharmAnalysis™, another method based on the threshold principle, the time between starting and end of odor detection is taken into account. Times of each dilution are summarized and presented as a chromatogram. The peak areas, also called Charm values, are calculated as $c=d^{n-1}$, where $d$ is the dilution factor and $n$ the number of coincident answers. This dilution factor is similar to the FD factor of AEDA. The Charm value is proportional to the amount of the compound in the sample and thus, demonstrates the quantity of potency (Acree and Barnard, 1994; Acree et al., 1984). The determined odor thresholds can also be used for the calculation of the odor activity value (OAV) (Rothe and Thomas, 1963; Schieberle, 1995). This value represents the importance of a substance in respect to the whole sample. The value is calculated as the quotient between the concentration of a selected compound in the sample and its odor threshold. A value of 10, for example, means that the concentration of a compound in the sample is 10 times higher than its threshold. According to the literature, an OAV >1 suggests that the compound is contributing to the aroma of a sample (Buettner and Schieberle, 2001; Frauendorfer and Schieberle, 2006; Rothe and Thomas, 1963; Tokitomo et al., 2005). One drawback of these dilution methods is the time factor. Depending on the dilutions, assessors, and replications, the analysis can take up days or weeks (Delahunty et al., 2006; van Ruth, 2001).

A further GC-O technique for collecting and processing the data is the intensity technique, where the odor intensity is recorded on a scale. Once a compound is eluted from the column, the maximum odor intensity is determined using the posterior intensity method. In comparison, the dynamic time-intensity method measures the duration of the odor active compound on a 16-point category scale in addition to the maximum intensity. The method is called Osme and the corresponding

chromatogram of replicated ratings Osmegram (Miranda-Lopez et al., 2006). The results are comparable to an instrumental chromatogram (Delahunty et al., 2006; Guichard et al., 1995; van Ruth, 2001). One disadvantage of intensity trials is the required elaborate training of the assessors (Delahunty et al., 2006).
Because of variations between panelists, it is important to use a group of assessors and to carry out replications (Marin et al., 1988).

#### 2.4.2.2 Taste Analysis

Comparable to the AEDA method described-above, taste dilution analysis (TDA) can be considered as its counterpart for non-volatile molecules. Using this HPLC-assisted bioassay, taste-active compounds can be identified. An extract or mixture is separated into a certain number of fractions. The solvent is removed by evaporation or freeze-drying, before each fraction is diluted stepwise and presented to panelists. They determine the threshold by evaluating the samples at increasing concentrations. The dilution, at which the difference between the sample and two blanks is just detectable, is called the taste dilution (TD) factor. A TD factor $\geq$ 1 indicates a taste-active contribution to the flavor (Frank et al., 2000). This screening method was used for the identification of many taste-active compounds in the past, such as falcarindiol as a bitter molecule in carrot products (Czepa and Hofmann, 2003), 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC) and 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC) as cooling agents (Ottinger et al., 2001), and flavon-3-ol glycosides as the main contributors to astringency at black tea consumption (Scharbert et al., 2004).

*The Use of High-Temperature Liquid Chromatography (HTLC)*

Reversed-phase liquid chromatography (RPLC) is a very common technique for separation and analysis of compounds. The need of organic solvents for good separation results is necessary but also brings some disadvantages. They are expensive, both in purchasing and in waste disposal, and are potentially harmful for humans as well as for environment. Thus, the avoidance of harmful solvents in the field of separation is desirable (Kondo and Yang, 2003; Yang, 2007; Yang et al., 2002b). Water would avoid these drawbacks but it is too polar to act as an eluent for RPLC separation at ambient temperature. However, at high temperatures the physicochemical properties of water are changed, making it more non-polar.

Therefore, polar, medium-polar, and even non-polar compounds can be separated with water (Khuwijitjaru et al., 2002). This method is more environmentally friendly and instead of a solvent gradient, a temperature gradient can be used to elute compounds with different polarities (Tran et al., 2001).

A moderate backpressure keeps the solvent liquid, although the boiling point is passed (subcritical water). The use of solvents at temperatures higher than 60°C is named “high-temperature liquid chromatography” (HTLC) (Teutenberg, 2010). Under these conditions the dielectric constant of water is lowered and similar to water/acetonitrile and water/methanol mixtures at ambient temperature (Ramos et al., 2002; Yang et al., 1998). Heated water therefore, behaves like common HPLC solvents regarding their polarities (Yang et al., 1998).

With increasing temperature, the surface tension and the viscosity decrease and, thus, the back pressure can be minimized. This, of course, enables a higher velocity and results in shorter run times and higher sample throughput (Kondo and Yang, 2003; Thompson and Carr, 2002). Additionally, the diffusion and the mass transfer between the analytes and the stationary phase are increased. Consequently, the retention time and peak width are reduced and finally higher efficiency of the chromatography occur (Kondo and Yang, 2003; Thompson and Carr, 2002).

HTLC has many advantages but the main challenge is the column stability at temperatures up to 200°C. Column bleeding, caused by degradation of the stationary phase, is not uncommon with certain materials. Silica-gel based columns that are widely used in HPLC, show problems at higher than 80°C, extreme pH values and at a high content of water. To overcome these limitations, metal-oxide based materials were developed. Zirconium, titanium, and aluminum oxides have a very stable surface (Nawrocki et al., 2004; Teutenberg et al., 2007). Polybutadiene (PBD)-coated zirconia RP-material, for example, is completely stable at temperature up to 200°C for minimum 1300 column volumes (Li et al., 1997) and at a high pH of 12 (Dunlap et al., 2001). However, zirconia is not suitable for every type of separation, as e.g. proteins are irreversibly adsorped on the surface (Dunlap et al., 2001). Another group of column materials, reported to be the most stable, are polymer-based materials. PRP-1 and PLRP-S, both polystyrene divinylbenzene type materials, are the most common polymer columns in subcritical water chromatography. Even though the packing is stable at 200°C, the efficiency is poorer compared to metal-oxide based phases (He and Yang, 2003; Yang, 2007).

Besides the column stability, other factors have to be kept in mind. The strength of the supporting material, such as tubings and seals, is crucial as well. In addition, preheating solvents before entering the column to avoid peak broadening, as well as cooling systems between the column and the detector have to be considered (Coym and Dorsey, 2004).
Due the reduced run times, analytes retain only a short time on the column. Therefore, degradation of analytes is not considered as a major problem as reported in the literature (Yang, 2007).

*Combination of HTLC and Sensory Analysis*

Using a combination of HTLC and sensory analysis, taste-active compounds from foods or botanical extracts, for example, can be discovered. A peak or time-wise fractionation via HTLC with a following tasting of each fraction results in a correlation of analytical and sensory data (Reichelt et al., 2010a; Roloff et al., 2006; Roloff et al., 2009).
For the separation, only food grade solvents, in this study water and ethanol and combinations of them, are used. Elevated temperatures permit a comparable chromatography compared to the use of organic solvents. Polymer-based RP column material with high stability at temperatures up to 200°C under aqueous conditions was chosen. Although lower temperatures are used, column stability is very important to avoid column bleeding and any potential health hazards in the course of the following sensory evaluation. Depending on the ethanol content in the fractions, samples can be tasted directly after separation. By using food grade solvents, evaporation of the solvents to dryness and possible problems with dissolution is no longer required.
In Figure 14, an application example of the investigation of *Eriodictyon angustifolium* is depicted. The upper part (a) shows the HTLC chromatogram of a methanolic extract with the different fractions 1-24. TDA was carried out to estimate the contribution of each fraction to the overall flavor or taste of the extract. A step-wise 1:1 dilution with the HTLC separation and sensory analysis identified fractions 15 and 23 to be very taste active (Reichelt et al., 2010a).

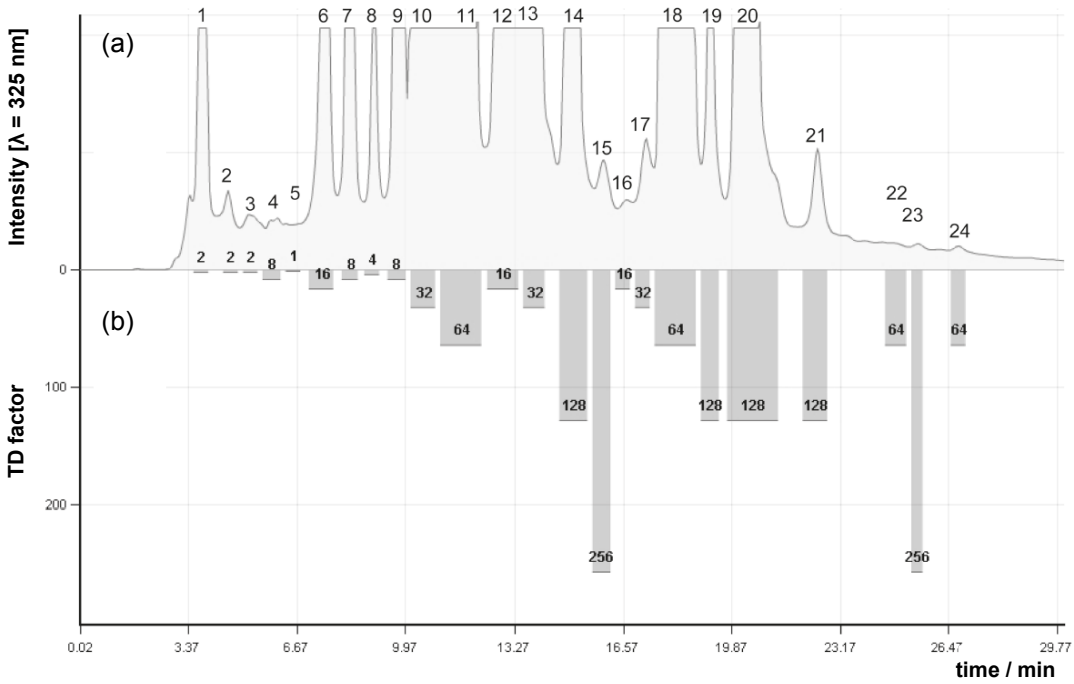



Figure 14: (a) HTLC chromatogram of an methanol extract of *Eriodictyon angustifolium*; (b) TDA of the fractions with the determined taste dilution (TD) factor (Reichelt et al., 2010a).

Identification of taste modulation ability of single fractions is also possible by using HTLC-coupled sensory evaluation. Therefore, the fractions have to be blended with a corresponding test solution, such as sucrose or caffeine solution. In a blind paired comparison test the blended sample and a blank sample, consisting of the corresponding solution only, are tested and compared. The panelists are asked to mark the sample that is sweeter or bitterer, respectively, depending which test solution is used (Reichelt, 2009).

### 2.4.3 Fast Centrifugal Partition Chromatography (FCPC)

#### 2.4.3.1 Principle of Countercurrent Chromatography

In contrast to most classical chromatographic techniques, countercurrent chromatography (CCC) is based on liquid-liquid partition. This means that both phases, the mobile as well as the stationary phase are liquid, non-miscible, and without solid support. Centrifugal force is needed to keep the stationary phase steady. CCC, first described by Ito et al. (1966), provides the retention of the stationary phase by rotation, while the mobile phase is moving through the stationary phase in consequence of the centrifugal field. Therefore, repeated mixing and separations steps occur, which result in an efficient partition process between the phases and a separation of the solutes (Berthod, 2007; Pauli et al., 2008).

An advantage of CCC, compared to conventional liquid chromatography methods, is the fact that there is no loss of sample due to irreversible interactions with a stationary solid phase. Additionally, either phase can be used as the mobile or stationary phase. In case compounds are retained, the phases can be switched during the run (ascending and descending mode). This dual-mode method saves solvent and time, and reduces the amount of sample needed. Another advantage of a liquid stationary and mobile phase is the possibility to mix a variety of solvents and to get a wide polarity range and unique selectivity. Furthermore, the loadability is higher as the compounds interact with the whole liquid column and not only with the surface of the stationary phase. Moreover, no irreversible bindings between analytes and column material can occur. Complex samples and extracts can be separated without previous purifications steps (Berthod, 2007; Berthod et al., 2005; Pauli et al., 2008; Pinel et al., 2007).

Two classes of systems are commercially available for CCC, hydrodynamic and hydrostatic devices (Berthod, 2007; Ito, 1986). The hydrodynamic system contains coiled Teflon® tubes moving in planetary motion around two axes (Figure 15). As a result of the rotation, a centrifugal field with varying intensity occurs. A high field causes a separation of the liquid phases, while in the zones with a low centrifugal field a mixing process follows. Compounds to be separated undergo an efficient partition process between the two phases and throughout the whole length of the coil (Ito, 1981; Ito et al., 1982). High-speed countercurrent chromatography (HSCCC) belongs to the group of hydrodynamic systems and is an improved method regarding separation time, resolution, and loading capacity because of the usage of multi-layer coiled column (Ito et al., 1982).

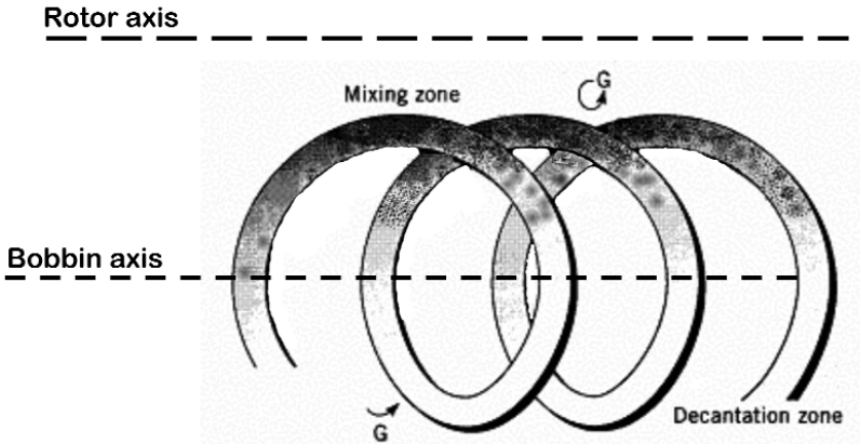



Figure 15: Schematic view of hydrodynamic columns and their liquid motion of the stationary (white) and mobile (black) phase. The bobbin axis with planetary rotation and the central rotor axis are responsible for the cyclic and variable centrifugal field (G) (Berthod et al., 2009).

Droplet countercurrent chromatography (DCCC) was the first hydrostatic system and used only the force of gravity, which results in very long elution times, up to days (Hostettmann et al., 1984; Tanimura et al., 1970). Advanced hydrostatic CCCs are called centrifugal partition chromatography (CPC), using a one-axis centrifugal apparatus with constant rotation and gravity field (Murayama et al., 1982). Rotor discs that are used instead of tubes contain engraved cells and connecting ducts. Depending on the amount of discs, the volume of the solvent systems and the amount of the sample used per run differ. In Figure 16, a fast centrifugal partition chromatography (FCPC) instrument with a rotor and a detailed view of a disc is depicted as example. FCPC, as one of the CPC instruments, can speed up to 1200 to 2000 rpm and uses flow rates of 5 to 20 mL $min^{-1}$. Because of the high rotational speed, the retention of the stationary phase is high, which allows a high flow rate and short run time (Marchal et al., 2003; Pauli et al., 2008).

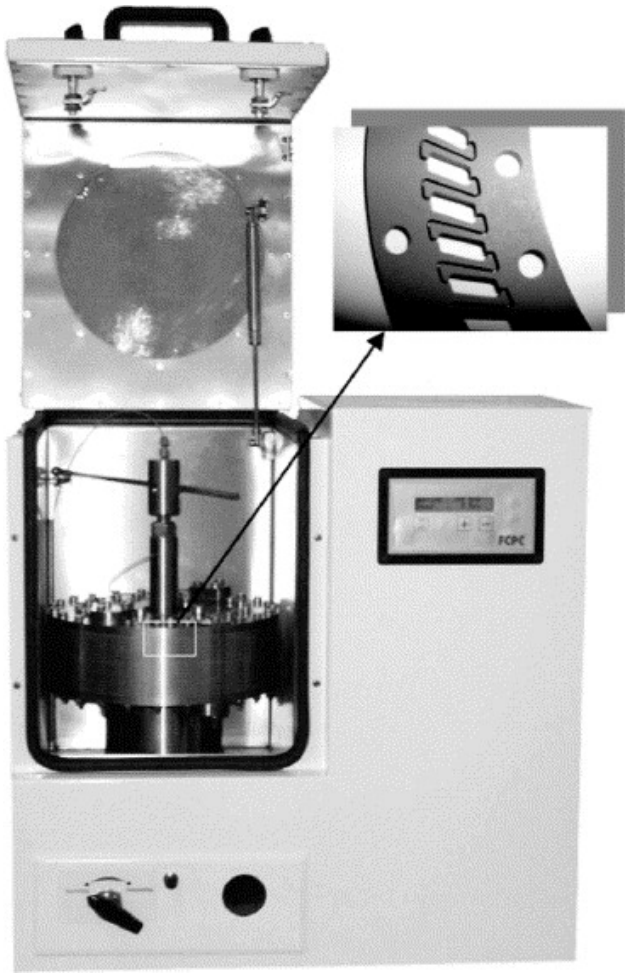



Figure 16: FCPC rotor and separation discs (total volume of 200 mL). Every disc contains a certain amount of cells, which are connected in the bottom and top via ducts. Between two discs, Teflon® gaskets are located (Delannay et al., 2006).

In CPC, the mobile phase percolates through the stationary phase, while the stationary phase remains in the cells due centrifugal forces. When the lighter phase is used as the mobile phase and is pushed through the heavier phase from the bottom to the top against the gravitational field, it is called ascending mode (Figure 17). In the reversed, descending mode, the denser phase acts as the mobile phase, enters the channels on the top and moves in the direction of the gravitational field through the lighter stationary phase (Berthod, 2007; Marchal et al., 2003).

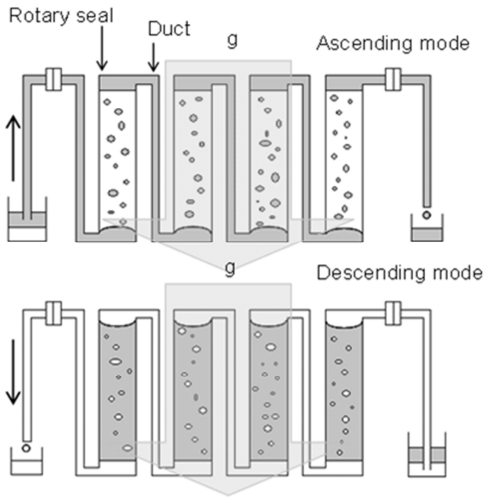



Figure 17: Cells and ducts, engraved in a FCPC disc in the ascending mode with the denser stationary phase in white and the descending mode, whereas the stationary phase is the lighter one in grey (Marchal et al., 2002).

One advantage of hydrostatic compared to hydrodynamic systems is the wider range of solvent systems that can be used for the liquid-liquid partitioning. Additionally, they are more resilient against stationary phase bleeding caused by disruption of the equilibrium. This can happen because of complex sample matrix, e.g. in case the sample contained surface-active compounds, and high sample load, respectively. In hydrodynamic systems, the continuous flow makes cleaning between the runs unneeded. Another advantage of hydrodynamic systems is the availability of analytical systems using volumes below 50 mL (Pauli et al., 2008).

#### 2.4.3.2 Selection of Solvent Systems for FCPC

For FCPC, biphasic solvent systems are used. The choice of the system is fundamental for the whole separation. Before the FCPC run can start, pre-tests have to be carried out to find the adequate system for the respective sample. Usually, three or four solvents are combined to form two immiscible phases. In separation of natural products via CPC, water, ethyl acetate (EtOAc), methanol (MeOH) and *n*-hexane are the most commonly used solvents (Pauli et al., 2008). The HEMWat (*n*-hexane-EtOAc-MeOH-water) system and the so called "*ARIZONA*" approach are examples of such multi-solvent systems (Foucault and Chevolot, 1998; Pauli et al., 2008; Renault et al., 2002). In case of *ARIZONA*, *n*-hexane is substituted by *n*-heptane. Combinations of the two binary systems define 23 solvent systems, named by letters of the alphabet, from A (water/EtOAc, 1:1, v/v) to Z (MeOH/*n*-

heptane, 1:1, v/v), covering a wide polarity range (Table 1). Based on the abbreviation AZ, the method is named after the US state of *ARIZONA*.

Table 1: The *ARIZONA* system for development of the solvent system of the liquid-liquid chromatography.

| System | A | B | C | D | F | G | H | J | K | L | M | N | P | Q | R | S | T | U | V | W | X | Y | Z |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Water** | 1 | 19 | 9 | 6 | 5 | 4 | 3 | 5 | 2 | 3 | 6 | 1 | 5 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| **MeOH** | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 5 | 1 | 6 | 3 | 2 | 5 | 3 | 4 | 5 | 6 | 9 | 19 | 1 |
| **EtOAc** | 1 | 19 | 9 | 6 | 5 | 4 | 3 | 5 | 2 | 3 | 6 | 1 | 5 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| **Heptane** | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 5 | 1 | 6 | 3 | 2 | 5 | 3 | 4 | 5 | 6 | 9 | 19 | 1 |

Once the system is selected, pre-tests can be carried out to identify the optimal solvent ratios. A small amount of the sample is dissolved in the two phases and shaking-flask partition studies have to be carried out. Solvent systems A and Z can be used as anchor points. If the sample is mostly dissolved in the upper phase of A and the lower phase of Z, a ratio in between these two will make an equilibrated partition possible. After shaking the flasks, separation and equilibration of the phases with fast and clear phase separation should occur. This is necessary for a stable system during separation to avoid bleeding. The partition of the analytes can be observed by thin layer chromatography or for more detailed analyses via HPLC. With the concentration of the interesting compounds in the two phases, the partition coefficient (*K*) can be calculated (Ito, 1996; Pauli et al., 2008; Schaufelberger, 1996). *K* represents the concentration in the upper phase divided by lower phase concentration of the compound (Equation 1).

$$K = C_U / C_L$$

$C_U$: concentration ($c$) of the analyte in the upper phase
$C_L$: concentration ($c$) of the analyte in the lower phase

Equation 1: Equation for the calculation of the partition coefficient (*K*).

Solvent systems with a *K*-factor around 1 result in the optimal separation result; values higher than 1 lead to an increase of the run time and peak broadening. With $K < 1$, decreased peak resolution occurs (Ito, 1996; Schaufelberger, 1996).

# 3 MATERIAL AND METHODS

## 3.1 MATERIAL

### 3.1.1 Chemicals

The following solvents were purchased:

- ethanol (absolute puriss. p.a. min. 99.8 %), methanol (ACS spectrophometric grade, ≥99.9 %), water (LC-MS Chromasolv®), acetonitrile (LC-MS Chromasolv®), diethyl ether (puriss. p.a. ≥99.8 %), formic acid (puriss. p.a. ~98 %), chloroform (puriss. p.a. 99.0-99.4 %) (Sigma-Aldrich, Steinheim, Germany),
- dimethylsulfoxide-d6 (99.9 %), methanol-d4 ($CD_3OD$, 99.8 %), chloroform-d1 ($CDCl_3$, 99.8 %) (Deutero GmbH, Kastellaun, Germany),
- *n*-pentane (puriss. p.a. ≥99 %; Riedel-de Haen, Seelze, Germany)

The following materials were obtained commercially:

- kavain, dihydrokavain, yangonin, desmethoxyyangonin, methysticin, dihydromethysticin (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany),
- 3-methylthio propyl isothiocyanate (R C Treatt Ltd, Suffolk, UK),
- myristicin (**1**; ICN Biomedicals Inc., USA),
- elemicin (**2**; Chemos GmbH, Regenstauf, Germany),
- piperine (**7**; Plant Lipids (P) Ltd., Kolenchery, India),
- nonivamide (**31;** Fontarôme Chemical Inc., St. Francis, USA),
- WS3 ((1R,2S,5R)-N-ethyl-2-isopropyl-5-methyl-cyclohexanecarboxamide, **35**; Renessenz LLC, Jacksonville, USA),
- vanillyl butyl ether (>96 %), 6-gingerol, phenylethyl isothiocyanate, 2-nonanol, vitexin, isovitexin, tetramethylsilane (ACS reagent, analytical standard for NMR-spectroscopy, ≥95.5 %), lithium chloride (anhydrous, beads, -10 mesh, ≥99.9 % trace metals basis), hydrocortisone (≥98 %), sand (50-70 mesh particle size) (Sigma-Aldrich, Steinheim, Germany)

The following compounds were provided by Symrise AG (Holzminden, Germany):

- citrus flavor (PN636534),
- 6-paradol (**32**),
- Frescolat® SC1 ((1R,2S,5R)-2-isopropyl-N-(4-methoxyphenyl)-5-methyl-cyclohexanecarboxamide, **33**),

- Frescolat® ML ([(1R,2S,5R)-2-isopropyl-5-methyl-cyclohexyl] 2-hydroxypropanoate, **34**),
- acetoxy-chavicol acetate,
- allyl isothiocyanate

The following compounds were synthesized by Symrise AG (Holzminden, Germany): *trans*-Pellitorine ((2*E*,4*E*)-decadienoic acid *N*-isobutyl amide, **3**) and *cis*-pellitorine ((2*E*,4*Z*)-decadienoic acid *N*-isobutyl amide, **28**) were synthesized according to Ley et al. (2004).

Achilleamid ((2*E*,4*E*)-decadienoic acid *N*-piperidyl amide, **4**), (2*E*)-decenoic acid *N*-isobutyl amide (**26**), decanoic acid *N*-isobutyl amide (**29**), and (3*E*)-nonenoic acid *N*-isobutyl amide (**30**) were prepared as previously described (Ley et al., 2006).

Spilanthol ((2*E*,6*Z*,8*E*)-decatrienoic acid *N*-isobutyl amide, **25**) was isolated from *Heliopsis longipes* extract (Phytos, Inc., San Anselmo, USA) by preparative HPLC.

The amides kalecide ((2*E*,4*E*)-dodecadienoic acid *N*-isobutyl amide, **8**) and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) were synthesized via NaOH-supported amidation of the appropriate acid chlorides with isobutyl amine. The parent acids were prepared by a Wittig-Horner reaction of (2*E*)-decenal or (2*E*)-dodecenal, respectively, with triethylphosphonoacetate and subsequent saponification in KOH and activation by reaction with thionylchloride. Spectral and physical data of the amides (**8**, **9**) correspond to literature data (Abarbri et al., 1998).

The syntheses of methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**), chingchengenamide A ((2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)-*N*-isobutyl-hepta-2,4-dienamide, **11**), piperdardine ((2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)-1-(1-piperidyl)hepta-2,4-dien-1-one, **12**), and dihydropiperlonguminine ((*E*)-5-(1,3-benzodioxol-5-yl)-*N*-isobutyl-pent-2-enamide, **16**) were comparable to the approach used for the compounds **8** and **9**. The corresponding aldehyde was generated starting from piperonal via the Doebner modification of the Knoevenagel condensation. The crucial syntheses of the *E*-configured double bond were achieved by employing a Wittig reaction for compound **16** and a double Wittig reaction for **10**, **11**, and **12** as the key steps.

(2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) was synthesized by the reaction of (2*E*,4*E*)-deca-2,4-dienoyl chloride and the L-alanine methyl ester

hydrochloride and subsequent hydrolysis of the methyl ester to the corresponding free acid (Obst et al., 2013). All compounds had a purity of > 95 % (GC).

The following materials were used as bases for sensory evaluations:

- Vittel® water (Nestlé Waters Deutschland GmbH, Mainz, Germany) was used for preparation of tasting samples,
- vegetable stock was used in a concentration of 12 g per liter boiling water with the following ingredients: fat powder (De Kievit B.V., Meppel, Netherlands) 12.5 %, monosodium glutamate (Helm AG, Hamburg, Germany) 12.5 %, yeast extract, Type Gistex XII MS (Georg Fles GmbH, Hamburg, Germany) 17.5 %, maltodextrin 18-20 (Roquette GmbH, Frankfurt/Main, Germany) 53.12 %, and vegetable stock extract mixture natural (Symrise, Holzminden, Germany) 4.38 %,
- green tea extract (21 % total catechins, 7 % EGCG; Martin Bauer GmbH & Co. KG, Vestenbergsgreuth, Germany),
- grape seed extract 848F exGrape®, OPC40 (>95 % polyphenols, >70 % proanthocyanidins, >40 % oligomeric proanthocyanidins (OPCs); BREKO GmbH, Bremen, Germany),
- epigallocatechin gallate (EGCG, 90 %; Changsha Sunfull Bio-tech Co., Ltd., Changsha, China),
- tannin (75-98 % tannic acid; C.E. Roeper GmbH, Hamburg, Germany),
- ascorbic acid (Nutrilo GmbH, Cuxhaven, Germany),
- sucrose (Nordzucker, Braunschweig, Germany),
- sodium chloride (Merck, Darmstadt, Germany),
- caffeine (Sigma-Aldrich, Steinheim, Germany)

### 3.1.2 Plant Material

For extraction experiments dried leaves, dried fruits, dried seeds, and fresh plant material of *Macropiper excelsum* (M.e.) were used. The dried leaves (M.e. L) were obtained from D.M. Dunningham Ltd., Auckland, New Zealand, dried fruits (M.e. F) from Little Karoo Ltd., Albany, New Zealand, and the seeds (M.e. S) from B&T World Seeds, Aigues-Vives, France. The living plant (M.e. P) was purchased from Rühlemann's Kräuter & Duftpflanzen, Horstedt, Germany. The abbreviations for the different plant material used in this study are summarized in Table 2.

Table 2: Descriptions, abbreviations, and sources of plant material used for the experiments in this study.

| plant material | abbreviation | source |
|---|---|---|
| dried leaves | M.e. L | D.M. Dunningham Ltd., Auckland, New Zealand |
| dried fruits | M.e. F | Little Karoo Ltd., Albany, New Zealand |
| dried seeds | M.e. S | B&T World Seeds, Aigues-Vives, France |
| living plant | M.e. P | Rühlemann's Kräuter & Duftpflanzen, Horstedt, Germany |

## 3.2 TECHNICAL EQUIPMENT

**Mill**

Instrument: Knife mill Grindomix GM 200 (Retsch, Haan, Germany)

**Dry-Mass Scale**

Instrument: Sartorius MA 100 (Sartorius AG, Göttingen, Germany)

Heating program: 15 minutes at 115°C

**Solvent Evaporation**

Rotary evaporator: Laborota 4000 efficiant (Heidolph, Schwabach, Germany)

Vacuum pump: Vacuum pumping unit PC520NT (Vacuubrand, Wertheim, Germany)

Vacuum controller: CVC 3000 (Vacuubrand, Wertheim, Germany)

Water bath: HB digital (Heidolph, Schwabach, Germany)

Temperature: 35-40°C

Parallel evaporator: Syncore®

Vacuum pump: Vacuum pump type V-700

Vacuum controller: Vaccum controller type V-855 (all BÜCHI, Flawil, Switzerland)

Rack size R-12

Temperature: 35-40°C

**High-Resolution Gas Chromatography – Flame Ionization Detector (HRGC-FID)**

Instrument: Carlo Erba Mega II 8575 (C.E. Instruments, Wigan, Great Britain)

Detectors: FID (235°C), FPD (150°C)

**High-Resolution Gas Chromatography – Mass Spectrometry (HRGC-MS)**

| | |
|---|---|
| Instrument: | GC 8000$^{Top}$-Voyager (Thermo Finnigan, Egelsbach, Germany) |
| Detector: | MS (Source: 200 °C, Interface: 240 °C) |

**SpeedExtractor**

| | |
|---|---|
| Instrument: | SpeedExtractor E-916 |
| Cells: | 4 Extraction cells à 120 mL (both BÜCHI, Flawil, Switzerland) |

**High Temperature Liquid-Chromatography (HTLC)**

| | |
|---|---|
| Pump: | Smartline Pump 1050 (Knauer, Berlin, Germany) |
| Injector: | 1000 µL loop; Injection & Switching Valves (Knauer, Berlin, Germany) |
| HPLC oven: | Polartherm Series 9020 semi prep (Selerity Technologies Inc., Salt Lake City, USA) |
| Detector: | Smartline UV Detector (Knauer, Berlin, Germany) |
| Column: | Hamilton PRP-1 reversed phase PS-DVB; 10 µm x 250 mm x 21.5 mm (Hamilton, Bonaduz, Switzerland) |
| Flow rate: | 10 mL min $^{-1}$ |
| Fraction collector: | Foxy® R1 (Teledyne ISCO, Lincoln, USA) |
| Software: | ChromGate® EZChrom Elite 3.3.1 (Knauer, Berlin, Germany) |

**Fast Centrifugal Partition Chromatography (FCPC)**

| | |
|---|---|
| FCPC apparatus: | Bench scale FCPC (Kromaton Technologies, Angers, France) |
| Rotor: | 1000 mL (preparative scale) |
| Thermostat: | Ecoline RE220 (LAUDA, Lauda-Königshofen, Germany) |
| Injector: | Kronlab High Speed Valve (Kronlab Chromatography Technology, Dinslaken, Germany) |
| Injection loop: | 20 mL |
| Pumps: | Smartline pumps 1000 (Knauer, Berlin, Germany) |
| Pulsation damper: | Typ 55073 (BESTA-Technik, Wilhelmsfeld, Germany) |
| Detector: | ELSD SEDEX 75, Evaporative Light Scattering Detector (SEDERE, Alfortville, Cedex, France) |
| Fraction Collector: | Labocol Vario-2000 (Labomatic, Weil am Rhein, Germany) |
| Software: | PrepCon 5 (SCPA GmbH, Weye-Leeste, Germany) |

**Preparative High-Performance Liquid Chromatography (pHPLC)**

| | |
|---|---|
| HPLC apparatus: | Dionex UltiMate 3000 |
| Autosampler: | Dionex UltiMate 3000 autosampler |
| Pumps: | Dionex UltiMate 3000 pumps |
| Software: | Chromeleon |
| Detectors: | Dionex UltiMate 3000 Diode Array Detector (all Dionex, Idstein, Germany) |

**Liquid Chromatography – Mass Spectrometry (LC-MS)**

| | |
|---|---|
| Instrument: | Acquity UPLC: Binary Solvent Manager, Sample Manager, Column Manager (Waters, Eschborn, Germany) |
| Mass spectrometer: | micrOTOF-Q II (Bruker, Bremen, Germany) |
| Detector: | Photo Diode Array Detector (PDA), range: 200-800 nm (Waters, Eschborn, Germany) |
| Software: | Data Analysis (Bruker, Bremen, Germany) |

**Nuclear Magnetic Resonance (NMR)**

| | |
|---|---|
| NMR apparatus: | Varian Unity Inova; 400 MHz<br>Varian Mercury plus; 400 MHz (both Varian, Darmstadt, Germany) |

**Gas Chromatography – Mass Spectrometry Direct Inlet**

| | |
|---|---|
| Instrument: | Shimadzu GC 2010/QP2010 (Shimadzu Corporation, Kyoto, Japan) |
| Detector: | Quadrupol |

**Polarimeter**

| | |
|---|---|
| Instrument: | UniPol L1000 (SCHMIDT+HAENTSCH GmbH & Co., Berlin, Germany) |
| Cuvette: | Micro cuvette, 0.7 mL, 100 mm |

## 3.3 METHODS

### 3.3.1 Extraction of Plant Material

#### 3.3.1.1 Preparation of a Pentane/Diethyl Ether Extract of *M. excelsum*

2 g of dried, cut leaves (M.e. L) were extracted with 30 ml *n*-pentane/diethyl ether (1:1, v/v) and 250 µl internal standard solution (1.050 mg 2-decanol, dissolved in 1 ml *n*-pentane/diethyl ether, 1:1, v/v), for 24 hours under continuous stirring. The plant material was filtered off and rinsed with 10 ml *n*-pentane/diethyl ether (1:1, v/v). The filtrate was concentrated to a final volume of 250 µl using a Vigreux column followed by nitrogen flow.

#### 3.3.1.2 Preparation of an Ethanol/Water Extract of *M. excelsum*

300 g of dried, cut leaves of *M. excelsum* (M.e. L) were extracted three times with 300 mL ethanol/water (4:1, v/v), for one hour each at ambient temperature (20-23°C) under continuous stirring. The plant material was filtered off after every extraction step. The filtrates were combined and the solvent was evaporated to dryness *in vacuo* at 40°C.

#### 3.3.1.3 Preparation of Extracts using the SpeedExtractor

A living specimen of *M. excelsum* (M.e. P) was separated into roots, stems and leaves, before the different parts were cut and milled after freezing with liquid nitrogen to improve homogenization. Additionally, dried leaves (M.e. L), dried seeds (M.e. S), and fruits (M.e. F) of *M. excelsum* from New Zealand were pestled. Each sample was mixed with sand to avoid caking of the plant material and filled in cells of the SpeedExtractor. The extraction was run in 3 cycles at 40°C and 100 bar pressure with ethanol/water (4:1, v/v). Each cycle consisted of a heat-up phase of 1 minute, a constant temperature (hold) period of 13 minutes for the first and 10 minutes each for the following two cycles. The discharge time of the extract, enhanced with a nitrogen flow at five bar, was 10 minutes for the first and 8 minutes, respectively, for the second and third cycle. The extracts of each plant part were combined and dried *in vacuo* at 40°C using the Syncore®.

### 3.3.2 Methods for Volatile Analysis of the Pentane-Diethyl Ether Extract

**High-Resolution Gas Chromatography – Flame Ionization Detector (HRGC-FID)**

| | |
|---|---|
| Column : | DB-WAX 0.25 µm, 60 m x 0.32 mm (Agilent Technologies, Santa Clara, USA) |
| GC-Oven program: | 40°C/5 min//4°C/min//230°C/25 min |
| Carrier gas: | Helium (110 kPa) |
| Flow rate: | 30 mL $min^{-1}$ |
| Injection volume: | 1 µL |
| Injection: | Split 1:10, 215°C |

**High-Resolution Gas Chromatography – Mass Spectrometry (HRGC-MS)**

| | |
|---|---|
| Column: | DB-WAX-ETR 0.5 µm, 30 m x 0.25 mm (Agilent Technologies, Santa Clara, USA) |
| GC-Oven program: | 40°C/5 min//4°C/min//240°C/25 min |
| Carrier gas: | Helium (75 kPa) |
| Flow rate: | 30 mL $min^{-1}$ |
| Injection volume: | 1 µL |
| Injection: | Split 1:10, 200°C |
| Ionization energy: | 70 eV |

For determination of the Kovats retention index, the retention time of each compound was normalized by the retention time of *n*-alkanes, adjacent to the compound (Kovats, 1958; van Den Dool and Kratz, 1963). The Kovats retention index (KI) was calculated as follows (Equation 2):

$$KI = 100 \times \left[ C + \frac{RT - RT_n}{RT_{n+1} - RT_n} \right]$$

KI = Kovats retention index
RT = retention time of the unknown compound
$RT_n$ = retention time of the n-alkane
$RT_{n+1}$ = retention time of the n+1-alkane
C = number of carbon atoms of the n-alkane

Equation 2: Equation for the calculation of the Kovats retention index.

### 3.3.3 High Temperature Liquid-Chromatographic Separation of the Ethanol/Water Extract

| | | | |
|---|---|---|---|
| Stock solution: | 200 mg $mL^{-1}$ in water/ethanol (1:1 v/v) | | |
| Injection volume: | 1000 µL | | |
| Temperature: | 80°C isotherm | | |
| Eluent: | A: water | B: ethanol | |
| Gradient: | 0 min: | 100 % A | 0 % B |
| | 25 min: | 75 % A | 25 % B |
| | 40 min: | 0 % A | 100 % B |
| | 55 min: | 0 % A | 100 % B |
| Flow rate: | 10 mL $min^{-1}$ | | |
| Detection: | UV 210 nm | | |
| Fractionation: | 1.0 min – 49.0 min (à 4 min each fraction) | | |

### 3.3.4 Fast Centrifugal Partition Chromatography

#### 3.3.4.1 Selection of a Solvent System

Several pre-tests, based on the *ARIZONA* approach (Table 1), using distilled water, methanol, ethyl acetate and *n*-heptane, were carried out for the development of a solvent system for FCPC separation. Selected systems, 7 ml each, were mixed in small screw capped glass bottles. The bottles were closed and left overnight for saturation and separation of the two phases. 5 mg of the test extract was mixed with every solvent system until it dissolved. The selection of the most convenient system was based on the following criteria: The extract had to be soluble without any residues; the separation time between the two phases should not take longer than 20 seconds because this enabled a good and clear separation in later FCPC trials. To estimate the partition of the molecules of interest between both phases, the respective partition coefficients *K* were calculated.

#### 3.3.4.2 FCPC – Fractionation of the Ethanol/Water Extract of *M. excelsum*

The complex aqueous-ethanolic extract was first partitioned in a separation funnel into a polar and non-polar phase by using *ARIZONA* system J (water/methanol/ethyl acetate/*n*-heptane 5:2:5:2 v/v/v/v) (Table 1). Both phases were separated and the solvents were removed using a rotary evaporator. The polar phase was directly

separated via preparative HPLC. With the non-polar fraction, method development for FCPC was carried out. Among the tested systems, *ARIZONA* P showed the best results. The required amount of the selected solvent system was prepared and left in separation funnels for 24 hours.

| | |
|---|---|
| Solvent system: | ARIZONA system P |
| | Upper phase: ethyl acetate/*n*-heptane (5:6 v/v) |
| | Lower phase: water/methanol (5:6 v/v) |
| Ascending mode: | Water/methanol as stationary phase |
| Stock solution: | 0.5 g 25 $mL^{-1}$ upper phase (5:6 v/v) |
| Flow rate: | 20 mL $min^{-1}$ |
| Fractionation: | 22 fractions à 25 mL |

### 3.3.5 Preparative HPLC

*Isolation of Compounds **8** and **10** from FCPC Fraction 6*

| | |
|---|---|
| Stock solution: | 80 mg $mL^{-1}$ methanol |
| Injection volume: | 70 µL |
| Column: | Luna C-18 5 µm, 250 mm x 10 mm (Phenomenex, Torrance, USA) |
| Eluent: | Water/methanol (15:85, v/v), isocratic |
| Flow rate: | 3 mL $min^{-1}$ |
| Temperature: | 40°C |
| Pressure: | 110 bar |
| Number of cycles: | 25 |
| Detection: | UV detection at 250 nm |

*Isolation of Compounds **11** and **12** from FCPC Fractions 11-14*

| | |
|---|---|
| Stock solution: | 41.9 mg $mL^{-1}$ methanol |
| Injection volume: | 30 µL |
| Column: | Luna C-18 5 µm, 250 mm x 10 mm (Phenomenex, Torrance, USA) |
| Eluent: | Water/methanol (32:68, v/v), isocratic |
| Flow rate: | 3 mL $min^{-1}$ |
| Temperature: | 40°C |

Pressure: 125 bar
Number of cycles: 45
Detection: UV detection at 250 nm

*Isolation of Compounds **13**-**21** from FCPC Fractions 17-22*

Stock solution: 130 mg $mL^{-1}$ methanol
Injection volume: 20 µL
Column: Luna C-18 5 µm, 250 mm x 10 mm (Phenomenex, Torrance, USA)
Eluent: Water/methanol (48:52, v/v), isocratic
Flow rate: 3 mL $min^{-1}$
Temperature: 40°C
Pressure: 160 bar
Number of cycles: 80
Detection: UV detection at 250 nm

*Isolation of Compound **22** from the Rest Coil after FCPC Fractionation*

Stock solution: 70 mg $mL^{-1}$ methanol
Injection volume: 20 µL
Column: Luna C-18 5 µm, 250 mm x 10 mm (Phenomenex, Torrance, USA)
Eluent: Water/methanol (34:66, v/v), isocratic
Flow rate: 3 mL $min^{-1}$
Temperature: 40°C
Pressure: 130 bar
Number of cycles: 150
Detection: UV detection at 210 nm

*Isolation of Compounds **23** and **24** from the Polar Fraction of the M. excelsum Aqueous-Ethanolic Extract*

Stock solution: 200 mg $mL^{-1}$ methanol
Injection volume: 80 µL
Column: Luna C-18 5 µm, 250 mm x 10 mm (Phenomenex, Torrance, USA)
Eluent: A: water B: methanol

| | | | |
|---|---|---|---|
| Gradient: | 0 min: | 85 % A | 15 % B |
| | 15 min: | 50 % A | 50 % B |
| | 19 min: | 30 % A | 70 % B |
| | 19.1 min: | 0 % A | 100 % B |
| | 26 min: | 0 % A | 100 % B |
| Flow rate: | 3 mL $min^{-1}$ | | |
| Temperature: | 40°C | | |
| Pressure: | 120 bar | | |
| Number of cycles: | 100 | | |
| Detection: | UV detection at 250 nm | | |

### 3.3.6 Liquid Chromatography – Mass Spectrometry

| | | | |
|---|---|---|---|
| Stock solution: | 1 mg $mL^{-1}$ | | |
| Injection volume: | 2 µl | | |
| Column: | Kinetex RP-C18 1.7 µm, 100 mm x 2.1 mm (Phenomenex, Torrance, USA) | | |
| Eluent: | A: acetonitrile + 0.09 % formic acid | | |
| | B: water + 0.1 % formic acid | | |
| Gradient: | 0 min: | 0 % A | 100 % B |
| | 22 min: | 95 % A | 5 % B |
| | 27 min: | 95 % A | 5 % B |
| | 30 min: | 100 % A | 0 % B |
| Temperature: | 50°C | | |
| Flow rate: | 0.55 mL $min^{-1}$ | | |
| Ionization mode: | ESI pos./neg. | | |
| Detection: | UV 200-500 nm (PDA) | | |

### 3.3.7 Gas Chromatography - Mass Spectrometry Direct Inlet

| | |
|---|---|
| Ionization Mode: | Electron Impact (EI) |
| Inlet: | Direct Inlet |
| Temp. program: | 20°C//40°C/min//100°C//80°C/min//350°C/5 min |
| Scan Range: | m/z 25-800 |
| Ionization Energy: | 70 eV |

Software: GCMS Solution, Shimadzu (Shimadzu Corporation, Kyoto, Japan)

### 3.3.8 Sensory Analysis

#### 3.3.8.1 General Conditions

Sensory tests were carried out with healthy and trained panelists without known tasting disorders. Panelists were fully informed about procedure and intention of the project and have given written consent. They were advised not to swallow the samples; samples were tested using the sip and spit method. All test sessions were run in the morning hours with a minimum of 1-2 hours after breakfast. During this time, the panelists were asked not to drink bitter-tasting beverages, such as coffee or black tea. The samples were tested in sensory panel rooms under standardized conditions and were given in randomized order, blinded, and coded with a 3-digit number. For the preparation of the test solutions, Vittel® water was used. The test solutions containing pure EGCG, grape seed extract (GSE), or green tea extract (GTE) were stabilized with 125 mg $kg^{-1}$ ascorbic acid to avoid degradation of catechins. Between two samples the panelists neutralized with Vittel® water, white bread, and white chocolate. The fat contained in the white chocolate was found to help to compensate the mouth drying and puckering perception of astringent compounds.

#### 3.3.8.2 Sensory Analysis of High-Temperature Liquid-Chromatography Fractions

Participants (seven women and three men, ages 22-50 years) were trained on the five basic tastes and with SymScript™ (Symrise AG, Holzminden, Germany), a selection of 100 standard flavor compounds and simple mixtures from the volatile and non-volatile area. Following the training, they evaluated the samples of the high-temperature liquid-chromatography.

After the separation was completed, each fraction was dried to a volume of 1 mL to remove the ethanol. This 1 mL was then diluted in a sucrose solution to yield a volume of 20 mL with a concentration of 3 % sucrose. Each fraction was described by the panel in consensus.

### 3.3.8.3 Flavor Language

For the evaluation and description of samples, it is absolutely necessary for the panelists to use the same terminology. This means they have to be trained on reference compounds with defined descriptions. For setting up a flavor language concerning trigeminally active and astringent compounds, a number of substances were evaluated regarding their sensory properties at different concentrations. Therefore, a panel of six trained and experienced testers (five women and one men, ages 24-45 years) including one flavorist was used. The tested descriptors were cooling, tingling, nasal pungency, hot, burning, pungent, warming, and astringent (Table 3).

Table 3: Tested compounds and their concentrations for the development of a flavor language.

| compound | dosage on water (mg kg$^{-1}$) | descriptor |
|---|---|---|
| WS3 (**35**) | 4, 7, 10 | cooling |
| Frescolat® SC1 (**33**) | 3 | |
| *trans*-pellitorine (**3**) | 10 | tingling |
| allyl isothiocyanate | 2, 4, 10 | nasal pungency |
| phenylethyl isothiocyanate | 0.5, 2 | |
| 3-methylthio propyl isothiocyanate | 0.5, 2 | |
| acetoxy-chavicol acetate | 1, 5, 10 | hot, burning, pungent, warming |
| *cis*-pellitorine (**28**) | 10, 20 | |
| vanillyl butyl ether | 1, 10 | |
| 6-gingerol | 10, 20, 40 | |
| 6-paradol (**32**) | 1, 2, 20 | |
| nonivamide (**31**) | 0.1, 0.2, 0.5 | |
| piperine (**7**) | 15, 25 | |
| tannine | 500 | astringent |
| grape seed extract | 500, 750 | |
| EGCG | 750 | |

### 3.3.8.4 Pre-Tests for Masking Trials

The same panel that had compiled the trigeminal flavor language was trained on bitterness and astringency and performed the pre-testing of potential astringency masking properties of several compounds. The trials were carried out using 750 mg kg$^{-1}$ EGCG with and without the test compound. In consensus, they evaluated a number of mouth-watering compounds, like *trans*-pellitorine ((2*E*,4*E*)-decadienoic acid *N*-isobutyl amide, **3**), achilleamid ((2*E*,4*E*)-decadienoic acid *N*-piperidyl amide, **4**), spilanthol ((2*E*,6*Z*,8*E*)-decatrienoic acid *N*-isobutyl amide, **25**),

(2*E*)-decenoic acid *N*-isobutyl amide (**26**), and (2S)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) in different concentrations on 750 mg $kg^{-1}$ EGCG. Furthermore, *trans*-pellitorine homologues, such as kalecide ((2*E*,4*E*)-*N*-isobutyldodeca-2,4-dienamide, **8**), (2*E*,4*E*)-*N*-isobutyltetradeca-2,4-dienamide (**9**), c*is*-pellitorine ((2*E*,4*Z*)-decadienoic acid *N*-isobutyl amide, **28**), decanoic acid *N*-isobutyl amide (**29**), and (3*E*)-nonenoic acid *N*-isobutyl amide (**30**) were tested. Additional trigeminal compounds, e.g. piperine (**7**), nonivamide (**31**), 6-paradol (**32**), Frescolat® SC1 ((1R,2S,5R)-2-isopropyl-N-(4-methoxyphenyl)-5-methyl-cyclohexanecarboxamide, **33**), Frescolat® ML ([(1R,2S,5R)-2-isopropyl-5-methyl-cyclohexyl] 2-hydroxypropanoate, **34**), and WS3 ((1R,2S,5R)-*N*-ethyl-2-isopropyl-5-methyl-cyclohexanecarboxamide, **35**) were rated as well. Those with a masking effect, even if it was only minor, were chose for further evaluation in the IVDM panel (see chapter 3.3.9.5).

#### 3.3.8.5 Intensity Variation Descriptive Methodology (IVDM)

*Panel Training*

Panelists for the IVDM, 22 in total (14 women and eight men, ages 19-54 years) were trained to evaluate the bitterness and astringency, respectively, of different samples and to differentiate between both descriptors. Caffeine was used as a reference for bitterness only. Grape seed extract (GSE) was perceived as astringent with only a slight bitter taste, while EGCG was described to be astringent as well as bitter. These references were offered to the panel in different concentrations (300, 500, 750, 1000 mg $kg^{-1}$). A number of sessions were carried out to become familiar with both the references and the testing method. In addition, training sessions were carried out using Compusense FCM® Feedback Calibration Method (Findlay et al., 2007). After rating intensities of a sample, panelists got immediate feedback on their performance by comparing their ratings with the previous results of 90 % of the participants.

*Procedure of IVDM*

The panelists took two sips of a sample, 5 ml each. After spitting out, the measurement was started by the panelist and intensities were rated repeatedly after defined time intervals: right at the start (t=0 seconds), after 10 seconds and afterwards repeatedly every 20 seconds for three times. Multiple attributes were rated

at the same time by using an unstructured horizontal line scale (12 cm) with anchors at the endpoints for each descriptor. The left end of the scale was marked with the terms “not bitter/astringent” (0 %), the right end with “extremely bitter/astringent” (100 %). Line scale ratings were reset after each measuring point. A maximum of two samples per session and per day were offered to the panelists. Data were collected and analyzed using Compusense® five. The arithmetic average of all panelists and replications was represented graphically in a time-intensity curve.

For method validation, bitterness and astringency were measured for EGCG concentrations ranging from 45 to 3750 mg $kg^{-1}$. Furthermore, a green tea extract (GTE) at 3600 mg $kg^{-1}$, equaling around 750 mg $kg^{-1}$ catechins, flavored with 0.1 % of a citrus flavor and sweetened with 3 % sucrose, was tested using the following attributes: bitter, astringent, sweet, and citrus.

As the aim was the evaluation and identification of astringency maskers, different concentrations of EGCG (300, 500, 750, 1000 mg $kg^{-1}$) and GSE (500, 750 mg $kg^{-1}$), were tested in combination with potential masking compounds. Therefore, the results of the pre-tests, described above, were consulted. In addition, selected compounds newly found in *M. excelsum*, were also tested for their astringency masking effect. Statistical analysis was carried out as described in the following paragraph.

#### 3.3.8.6 Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey-Kramer HSD test were used to analyze the dose-response curves of EGCG. Data were analyzed using JMP® 10 (SAS, UK). Statistical analyses of the masking tests were carried out via Student’s *t*-test (one-tailed, heteroscedastic). Error bars indicate the standard deviation of all panelists and all replications were calculated in Microsoft® Excel.

# 4 RESULTS AND DISCUSSION

## 4.1 MACROPIPER EXCELSUM

### 4.1.1 Volatile Compounds in *M. excelsum*

The phytochemical profile of *M. excelsum* has hardly been investigated. Only some volatile compounds, like myristicin, elemicin, α-pinene, aromadendrene, γ-cadinene, (-)-cadinene dihydrochloride, camphene, β-phellandrene, *n*-hexyl acetate, and palmitic acid (Briggs, 1941; Briggs et al., 1975) have been described. In order to get more information on the volatile composition, dried leaves of *M. excelsum* were extracted using *n*-pentane/diethyl ether (1:1, v/v) as solvent. The analysis of the extract was carried out via gas chromatography coupled with FID and MS. Identifications were based on the comparison of retention times and mass spectra to those of authentic reference substances. If no reference was available, literature data were used for tentative identifications. A semi-quantification was performed using 2-decanol as internal standard and taking the respective GC-FID response factors into consideration. The HRGC-MS chromatogram of the *n*-pentane/diethyl ether extract with assignments of the main compounds is shown in Figure 18. The identified volatile compounds were grouped according to their chemical classes; they are listed in Tables 4-8 according to decreasing concentrations.

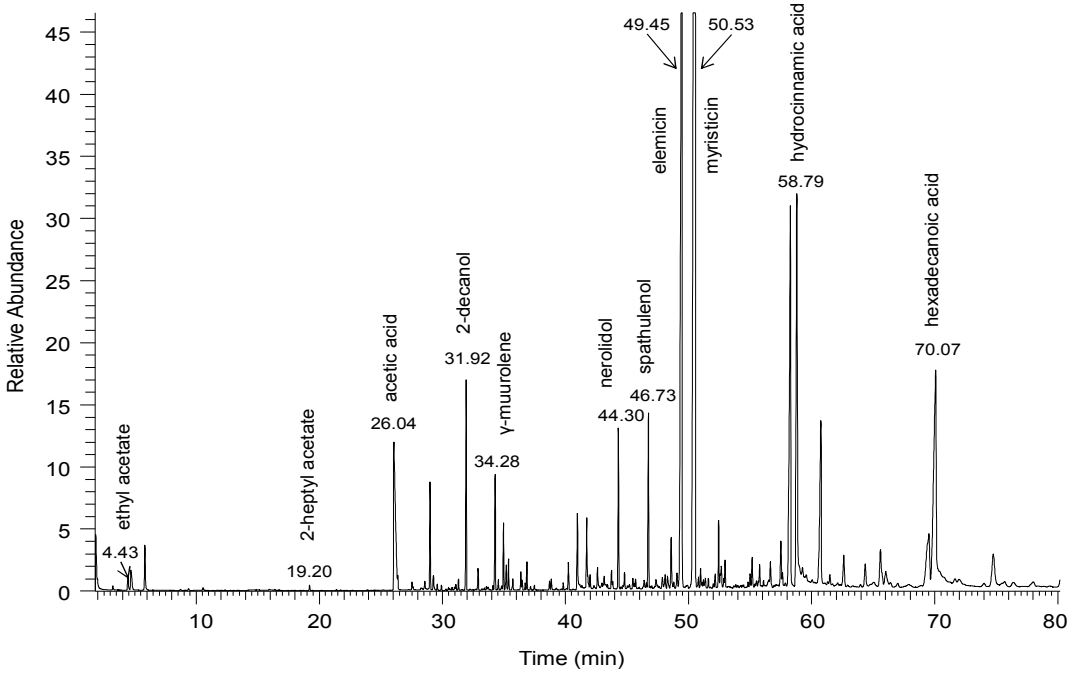



Figure 18: HRGC-MS chromatogram of the *n*-pentane/diethyl ether extract obtained from dried leaves of *M. excelsum*; for conditions see chapter 3.3.2.

Table 4: Terpene hydrocarbons identified in dried leaves of *M. excelsum*.

| terpene hydrocarbons | concentration (mg kg$^{-1}$) | KI | terpene hydrocarbons | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| γ-muurolene[b] | 114 | 1685 | α-caryophyllene[b] | 5 | 1662 |
| germacrene D[a] | 104 | 1707 | β-ylangene[b] | 4 | 1570 |
| α-curcumene[b] | 25 | 1769 | α-muurolene[b] | 3 | 1722 |
| zingiberene[b] | 24 | 1718 | camphene[a] | 3 | 1063 |
| β-bisabolene[b] | 20 | 1724 | aromadendrene[b] | 3 | 1605 |
| δ-cadinene[b] | 18 | 1752 | α-copaene[b] | 3 | 1490 |
| β-cubebene[b] | 18 | 1536 | α-pinene[a] | 1 | 1020 |
| alloaromadendrene[a] | 16 | 1643 | limonene[a] | 0.8 | 1197 |
| α-cubebene[b] | 12 | 1456 | α-ionene[b] | 0.7 | 1690 |
| caryophyllene[a] | 10 | 1594 | calamene[b] | 0.6 | 1830 |
| cadina-4,9-diene[b] | 10 | 1717 | α-ylangene[b] | 0.5 | 1481 |
| elixene[b] | 10 | 1725 | camphor[a] | 0.5 | 1513 |
| γ-cadinene[b] | 10 | 1757 | β-pinene[a] | 0.4 | 1106 |
| β-bourbonene[b] | 9 | 1516 | myrcene[a] | 0.3 | 1157 |
| β-sesquiphellandrene[b] | 8 | 1767 | β-farnesene[a] | 0.3 | 1630 |
| α-bourbonene[b] | 5 | 1495 | sabinene[a] | 0.1 | 1117 |
| β-copaene[b] | 5 | 1589 | p-cymene[a] | 0.02 | 1264 |

[a] identified by comparison of retention time and mass spectrum with those of a reference substance
[b] tentatively identified by comparison of retention time and mass spectrum to literature data

Table 5: Oxygenated terpenes identified in dried leaves of *M. excelsum*.

| oxygenated terpenes | concentration (mg kg$^{-1}$) | KI | oxygenated terpenes | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| spathulenol[b] | 157 | 2129 | linalool[a] | 5 | 1546 |
| nerolidol[a] | 118 | 2039 | elemol[b] | 4 | 2082 |
| germacrene D-4-ol[b] | 80 | 1882 | humulene epoxide II[b] | 3 | 2048 |
| δ-cadinol[b] | 68 | 2194 | iso-borneol[a] | 3 | 1671 |
| caryophyllene oxide[a] | 21 | 1986 | α-bisabolol[a] | 3 | 2217 |
| T-cadinol[b] | 11 | 2173 | cubenol[b] | 2 | 2061 |
| β-bisabolol[b] | 8 | 2146 | terpinen-4-ol[a] | 2 | 1600 |
| viridiflorol[b] | 7 | 2079 | eucalyptol[a] | 0.6 | 1209 |
| T-muurolol[b] | 7 | 2181 | nerol[a] | 0.4 | 1801 |

[a] identified by comparison of retention time and mass spectrum with those of a reference substance
[b] tentatively identified by comparison of retention time and mass spectrum to literature data

Table 6: Norisoprenoids and phenyl propanoids identified in dried leaves of *M. excelsum*.

| norisoprenoids | concentration (mg kg$^{-1}$) | KI | phenyl propanoids | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| dihydroactinidiolide[a] | 30 | 2355 | myristicin[a] | 3513 | 2256 |
| α-ionone[a] | 13 | 1850 | elemicin[a] | 1394 | 2222 |
| hexahydro farnesyl acetone[b] | 8 | 2108 | hydrocinnamic acid[a] | 1209 | 2680 |
| β-ionone[a] | 2 | 1939 | 6-methoxy eugenol[a] | 19 | 2547 |
| dihydro-α-ionone[b] | 0.3 | 1742 | methylhydrocinnamate[b] | 7 | 1823 |

| norisoprenoids | concentration (mg kg$^{-1}$) | KI | phenyl propanoids | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| | | | methyl eugenol[a] | 6 | 2011 |
| | | | cinnamyl alcohol[a] | 4 | 2286 |

[a] identified by comparison of retention time and mass spectrum with those of a reference substance
[b] tentatively identified by comparison of retention time and mass spectrum to literature data

Table 7: Acids and esters identified in dried leaves of *M. excelsum*.

| acids | concentration (mg kg$^{-1}$) | KI | esters | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| hexadecanoic acid[a] | 966 | 2980 | ethyl acetate[a] | 34 | 881 |
| acetic acid[a] | 872 | 1475 | terpinyl acetate[a] | 9 | 1693 |
| *trans*-3-hexenoic acid[a] | 20 | 1980 | 2-heptyl acetate[a] | 4 | 1266 |
| 3-methylbutanoic acid[a] | 4 | 1680 | linalyl acetate[a] | 4 | 1552 |
| heptanoic acid[a] | 4 | 2020 | bornyl acetate[a] | 2 | 1578 |
| nonanoic acid[a] | 4 | 2260 | isobornyl acetate[a] | 2 | 1583 |
| *trans*-2-hexenoic acid[a] | 2 | 2055 | ethyl hexanoate[a] | 1 | 1229 |
| propanoic acid[a] | 2 | 1535 | 2-phenylethyl acetate[a] | 0.9 | 1813 |
| 2-methylpropanoic acid[a] | 1 | 1568 | | | |
| hexanoic acid[a] | 1 | 1921 | | | |
| decanoic acid[a] | 1 | 2400 | | | |
| butyric acid[a] | 0.9 | 1650 | | | |

[a] identified by comparison of retention time and mass spectrum with those of a reference substance

Table 8: Alcohols, aldehydes, and ketones identified in the dried leaves of *M. excelsum*.

| alcohols | concentration (mg kg$^{-1}$) | KI | aldehydes | concentration (mg kg$^{-1}$) | KI |
|---|---|---|---|---|---|
| 2-butanol[a] | 3 | 1019 | nonanal[a] | 0.6 | 1389 |
| 2-heptanol[a] | 0.8 | 1319 | hexanal[a] | 0.5 | 1077 |
| benzyl alcohol[a] | 0.5 | 1874 | *trans*-2-hexenal[a] | 0.1 | 1214 |
| *cis*-3-hexenol[a] | 0.4 | 1381 | **ketones** | **concentration (mg kg$^{-1}$)** | **KI** |
| butanol[a] | 0.3 | 1138 | 6-methyl-5-hepten-2-one[a] | 0.1 | 1333 |
| nonanol[a] | 0.3 | 1661 | | | |

[a] identified by comparison of retention time and mass spectrum with those of a reference substance

In total, 9870 mg kg$^{-1}$ volatile compounds were contained in the dried *M. excelsum* leaves. The mass loss upon drying was determined to be 88%. Taking this into account, the total volatile compounds in the fresh leaves would amount to 1184 mg kg$^{-1}$. This is in the same order of magnitude as the amount of 950 mg kg$^{-1}$ in oil reported to be obtained via steam-distillation from fresh leaves (Briggs et al., 1975).

Around 100 volatile substances were identified in the *n*-pentane/diethyl ether extract of *M. excelsum*. Among them were 34 terpene hydrocarbons, 19 oxygenated terpenes, 12 acids, eight esters, seven phenyl propanoids, six alcohols, five norisoprenoids, three aldehydes, and one ketone. Myristicin (35.6 %), elemicin (14.1 %), hydrocinnamic acid (12.3 %), hexadecanoic acid (9.8 %), and acetic acid (8.8 %) were the five main compounds in the extract and accounted for 80.6 % of the total volatiles. The *n*-pentane/diethyl ether extract was evaluated on a smelling strip and described by five trained testers as follows: herbal, tea, hay, woody, peppery, incense, and carrot.

Except for the previously reported α-pinene, myristicin, aromadendrene, γ-cadinene, camphene, elemicin, β-phellandrene, and hexadecanoic acid (Briggs, 1941; Briggs et al., 1975), all other compounds were found for the first time in *M. excelsum*. They are known from other *Piper* species, e.g. *P. amalago*, *P. hispidum, P. nigrum*, and *P. cubeba* (Kapoor et al., 2009; Parmar et al., 1997; Simeone et al., 2011).

### 4.1.2 Non-Volatile Compounds in *M. excelsum*

In order to investigate the non-volatile compounds in *M. excelsum*, 300 g of the dried leaves were extracted using ethanol/water (4:1, v/v) as solvent. The resulting extract (50 g) was analyzed via LC-MS. The respective chromatogram with assignments of the detected molecular masses is shown in Figure 19.

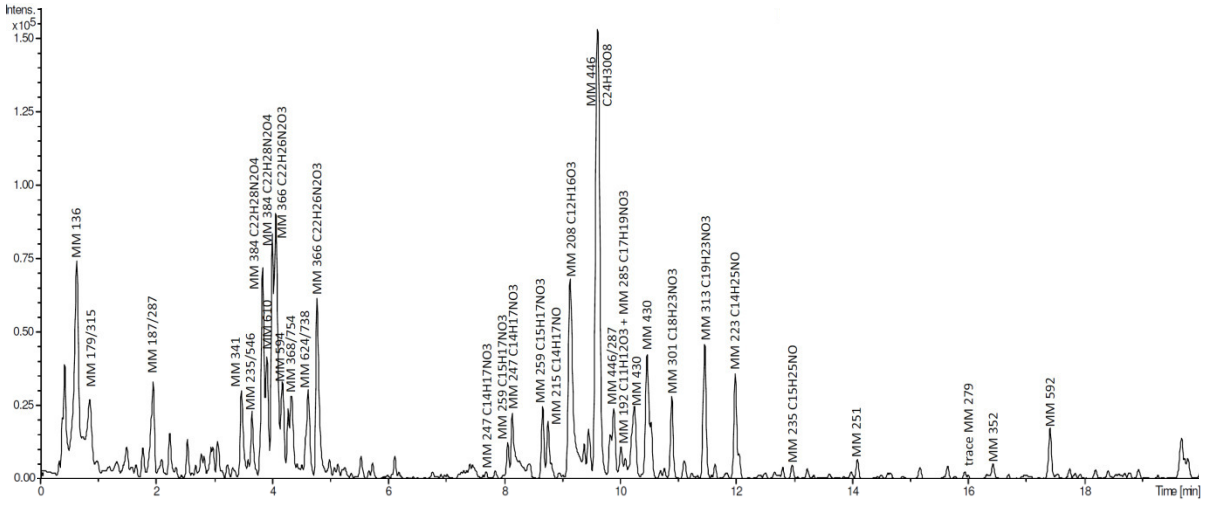
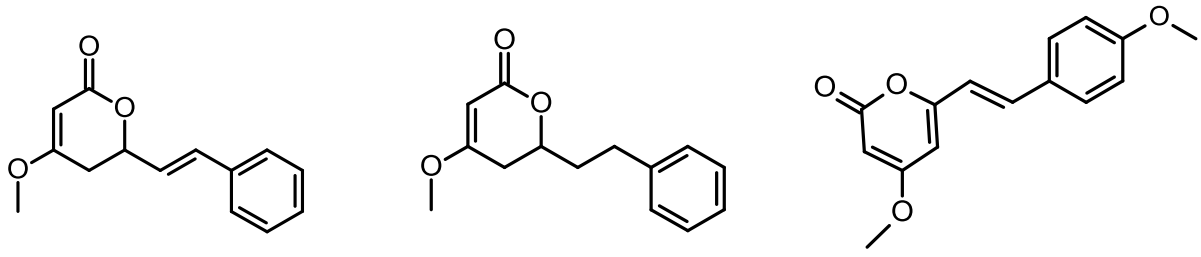


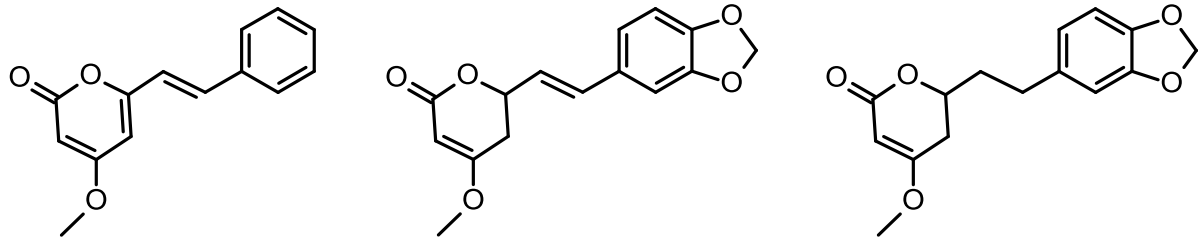

Figure 19: LC-MS chromatogram of the aqueous-ethanolic extract of *M. excelsum*; for conditions see 3.3.6.

#### 4.1.2.1 Authentication and Comparison with *Piper methysticum*

*P. methysticum*, also known as “kava-kava”, belongs to the family *Piperaceae* and contains a number of so-called kavalactones that poss anxiolytic and hepatotoxic effects (Escher et al., 2001; Gow et al., 2003). The name “kawakawa” used by the Maori for *M. excelsum* is very similar to the name “kava-kava” used for *P. methysticum* and may therefore cause confusion. In order to ensure the authenticity of the analyzed plant material prior to any sensory evaluation, the chromatographic and the mass spectrometric data of the six major kavalactones (Figure 20) were compared to those of the substances present in the prepared aqueous-ethanolic extract.



**kavain** **dihydrokavain** **yangonin**



**desmethoxyyangonin** **methysticin** **dihydromethysticin**

Figure 20: Structures of the six major kavalactones kavain, dihydrokavain, yangonin, desmethoxyyangonin, methysticin, and dihydromethysticin, found in *Piper methysticum* (Gautz et al., 2006; Simeoni and Lebot, 2002; Smith, 1983).

The comparison of the retention times, the molecular masses, and the MS-MS fragments of the lactones to those of the compounds in the extract revealed that none of the tested kavalatones were present in the investigated *M. excelsum* extract. As examples, chromatograms and mass spectral data for the three lactones methysticin, dihydrocavain, and desmethoxyyangonin are shown in Figure 21.

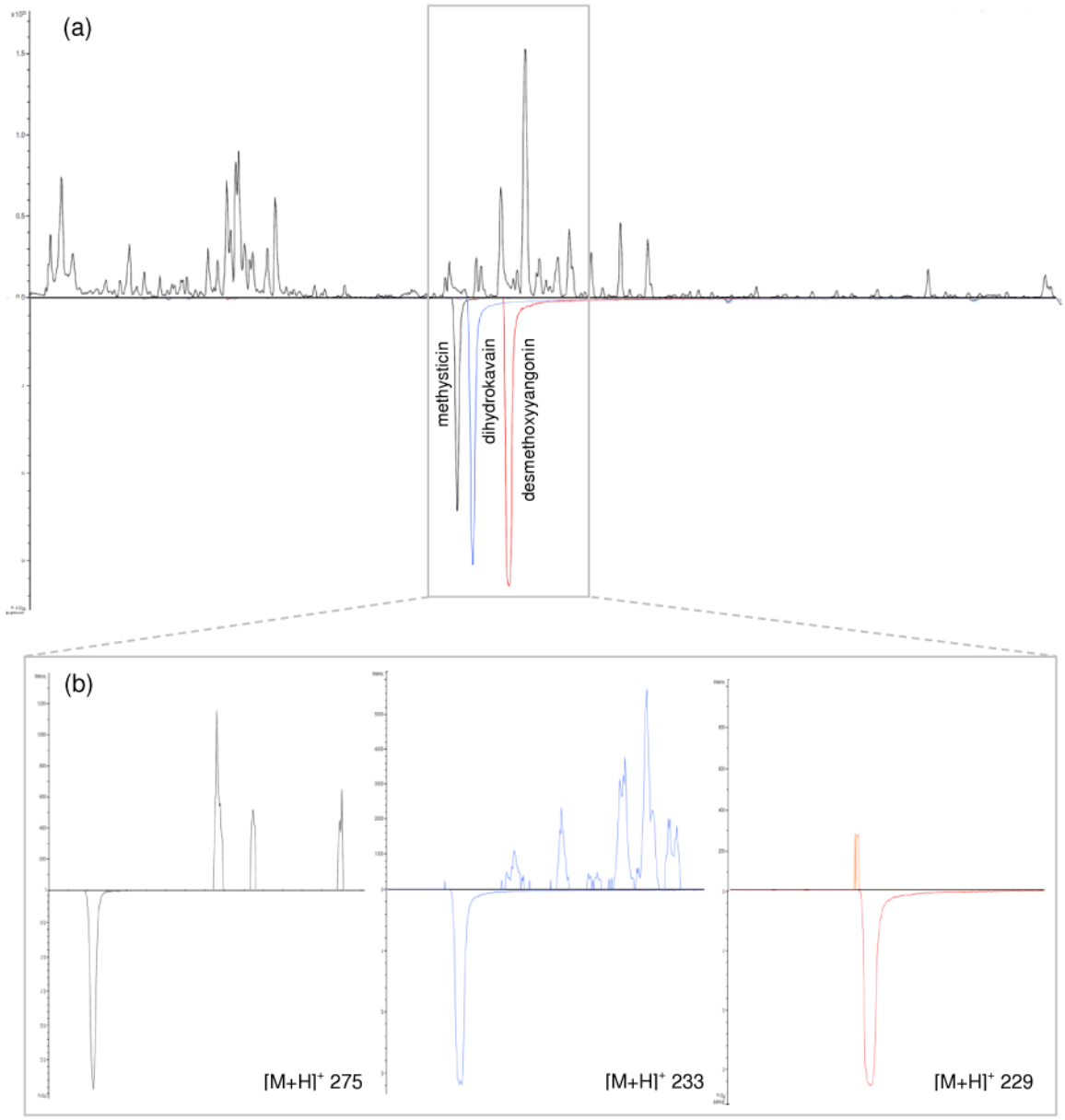



Figure 21: (a) Comparison of LC-MS chromatograms (positive mode) of the aqueous-ethanolic extract of *M. excelsum* and mirrored combined plots of three kavalactones. (b) The extracted ion chromatograms of the molecular mass of methysticin ([M+H]+ 275), dihydrocavain ([M+H]+ 233), and desmethoxyyangonin ([M+H]+ 229) in the extract and the reference compound were mirrored in a time slot between 8 and 11 minutes; for conditions see 3.3.6.

#### 4.1.2.2 Sensory-Guided Analysis of the Non-Volatile Compounds via HTLC

After authentication of the plant material, the aqueous-ethanolic extract obtained from the dried leaves of *M. excelsum* was evaluated at a concentration of 500 mg $kg^{-1}$ on water, sucrose solution (5 %), sodium chloride solution (0.5 %), and a vegetable stock. The panelists described the sample as slightly bitter, slightly sweet, and

herbal, and perceived trigeminal effects with slightly tingling, cooling, as well as hot sensations.

For the identification of the trigeminal-active constitutents, the extract was fractionated via high-temperature liquid chromatography (HTLC) (Reichelt et al., 2010a; Roloff et al., 2006; Roloff et al., 2009). Each fraction was sensorially evaluated on a 3 % sucrose solution. The HTLC chromatogram and the descriptions of the panelists are depicted in Figure 22.

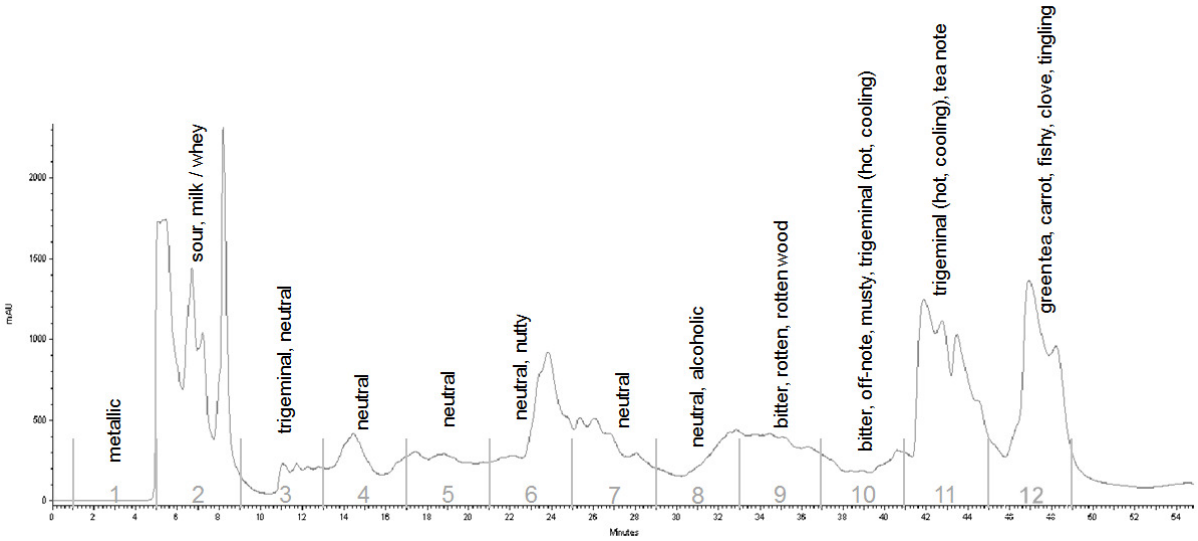



Figure 22: HTLC chromatogram of the aqueous-ethanolic extract of *M. excelsum* and sensory description of each fraction (n=10), for conditions see 3.3.3.

While the first fractions were described as more or less neutral with some metallic, sour, and milky notes, fractions 10, 11, and 12 turned out as the most interesting, since they showed trigeminal effects. This was the first time that trigeminal sensations, like tingling, hot, and cooling were detected via this approach based on HTLC-fractionation. Until now, the use of this method has only been described for the detection of sweet and bitter taste, as well as sweet enhancing and bitter masking properties (Reichelt et al., 2010a; Reichelt et al., 2010b).

For a detailed elucidation of the compounds, LC-MS analysis of each of the fractions 10, 11 and 12, showing trigeminal effects, was carried out (Figure 23).

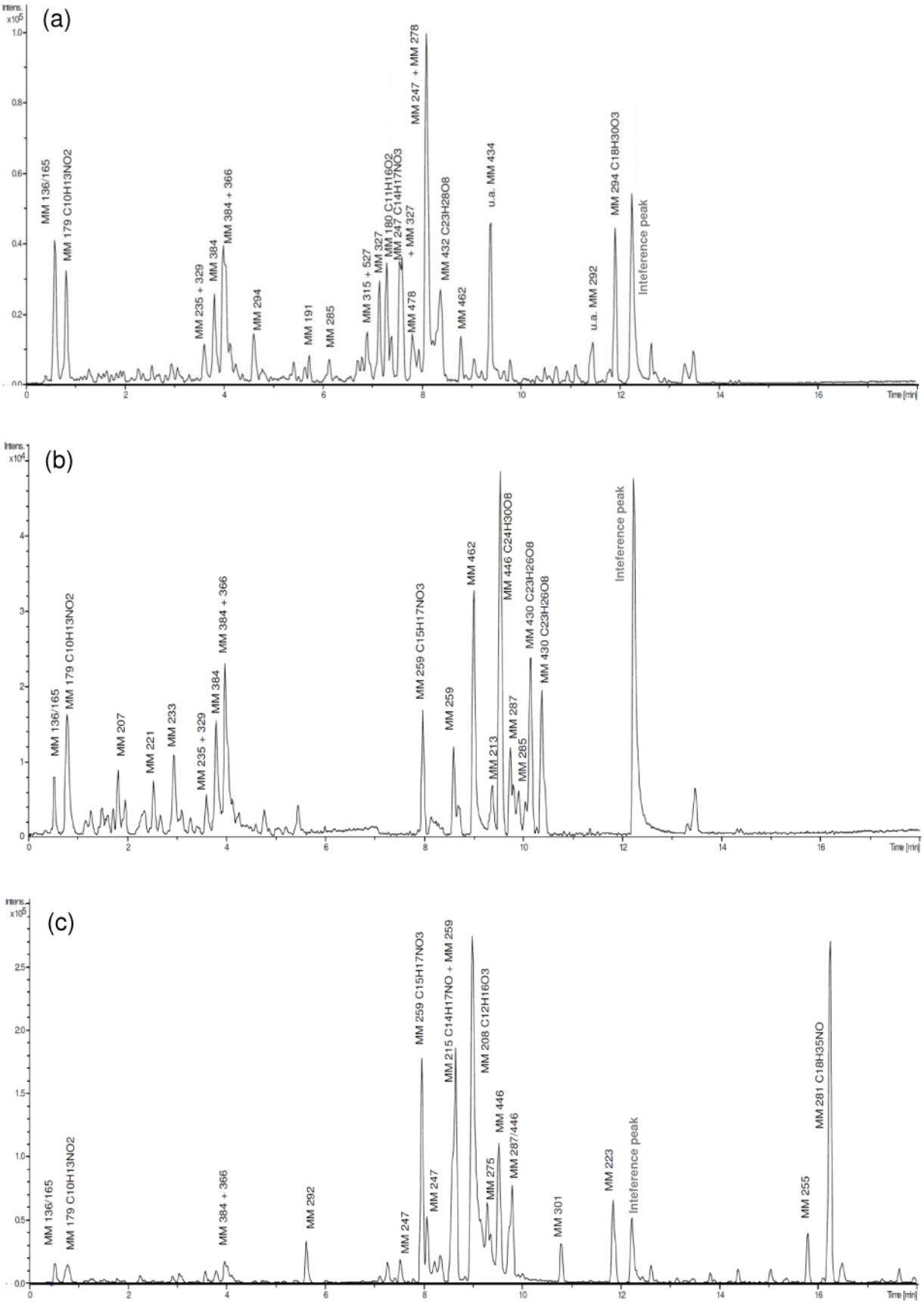



Figure 23: LC-MS chromatograms (positive mode) of fractions 10, 11, and 12 (a-c) of the HTLC. The peak at 12.3 minutes is the known MS contaminant tetrabutylammonium (Weber et al., 2012); for conditions see 3.3.6.

Fraction 10 contained molecules with molecular masses of 247 (**5**, **6**), 278, 294, 366, 384, and 434 g $mol^{-1}$, fraction 11 with 259 (**14**, **15**), 285 (**7**), 430 (**18**-**21**), 446 (**22**), and 462 g $mol^{-1}$, and fraction 12 with 208 (**2**), 215 (**13**), 223 (**3**), 259 (**14**, **15**), 284, and 446 (**22**) g $mol^{-1}$. As the fractions were still very complex, no clear relationship between individual compounds and the described trigeminal sensations could be made. Therefore, further trials had to be carried out for improved purification and finally isolation of the compounds of interest.

#### 4.1.2.3 Identification of Compounds in *M. excelsum* Extract by Comparison with Reference Molecules

In the crude extract (Figure 19), myristicin (**1**) and elemicin (**2**), which were already described in *M. excelsum* (Briggs, 1941; Briggs et al., 1975), were identified based on mass spectra. For the unambiguous identification of the compounds, comparison of elution times, as well as MS-MS fragments with reference substances were carried out.

In addition to these two compounds, a number of molecules with *N*-containing structures were detected. Taking into account literature data on related *Piper* species, it was suggested that these compounds belong to the structural class of amides. Based on this assumption, a selection of commercially available amides, namely *trans*-pellitorine (**3**), achilleamid (**4**), *trans*-fagaramid (**5**), *cis*-fagaramid (**6**), and piperine (**7**) were analyzed via LC-MS. The resulting data were compared to the elution times and MS-MS spectra of the corresponding candidates in the extract. For all five compounds, which were so far not described in *M. excelsum*, the data were in accordance with the tested references (Figure 24). The structures, the measured and calculated molecular masses, as well as the mass fragments, analyzed via direct inlet by GC-MS, are shown in Table 9.

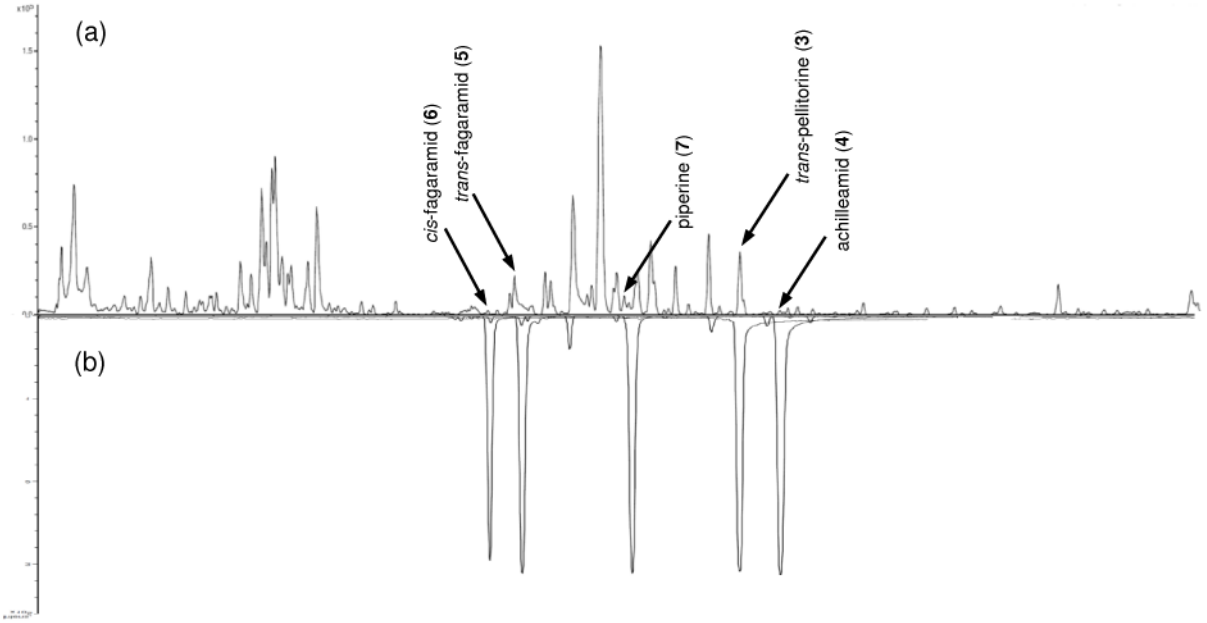
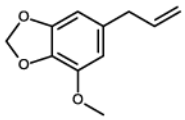
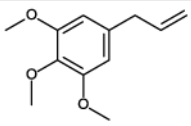
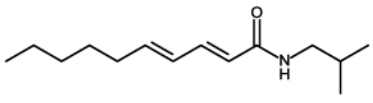
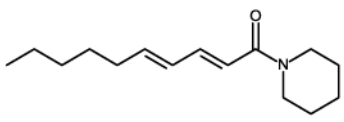
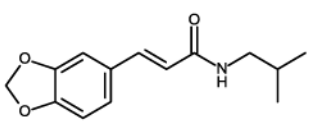
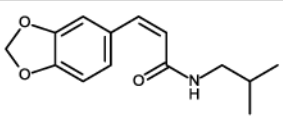
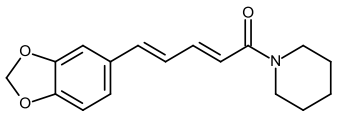



Figure 24: Comparison of the LC-MS chromatograms (positive mode) of the aqueous-ethanolic extract of *M. excelsum* (a) and combined plots of reference compounds *cis*-fagaramid (**6**), *trans*-fagaramid (**5**), piperine (**7**), *trans*-pellitorine (**3**), and achilleamid (**4**) (b); for conditions see 3.3.6.

Table 9: Structures, calculated and measured mass, and mass fragments (GC-MS, direct inlet) of reference compounds identified in *M. excelsum.*

| compound | structure | HR-MS $[M + H]^+$ (calculated) | HR-MS $[M + H]^+$ | m/z mass ion (percentages) |
|---|---|---|---|---|
| myristicin (**1**) |  | 193.0859 | 193.0879 | 192(100), 165(26), 161(28), 133(24), 131(28), 119(35), 91(48), 77(22), 65(33), 39(24) |
| elemicin (**2**) |  | 209.1172 | 209.1176 | 208(100), 193(77), 133(62), 118(44), 105(57), 103(29), 91(47), 79(55), 77(57), 39(31) |
| *trans*-pellitorine (**3**) |  | 224.2009 | 224.2017 | 223(37), 152(38), 151(100), 96(69), 95(27), 81(65), 57(29), 55(27), 41(42), 29(28) |
| achilleamid (**4**) |  | 236.2009 | 236.2037 | 235(46), 192(80), 178(31), 164(60), 138(41), 84(100), 81(67), 69(33), 67(23), 41(28) |
| *trans*-fagaramid (**5**) |  | 248.1281 | 248.1295 | 247(64), 191(30), 190(82), 176(20), 175(100), 145(60), 135(11), 117(28), 89(44), 63(14) |
| *cis*-fagaramid (**6**) |  | 248.1281 | 248.1314 | 247(47), 191(28), 190(77), 176(18), 175(100), 145(59), 135(11), 117(28), 89(44), 63(14) |

| compound | structure | HR-MS $[M + H]^+$ (calculated) | HR-MS $[M + H]^+$ | m/z mass ion (percentages) |
|---|---|---|---|---|
| piperine (**7**) |  | 286.1438 | 286.1456 | 286(34), 285(100), 202(28), 201(97), 174(25), 173(43), 172(24), 143(31), 115(76), 84(30) |

*trans*-Pellitorine (**3**) has been found in many *Piper* species, for example in *P. chaba* (Matsuda et al., 2009), *P. nigrum* (Kapoor et al., 2009; Subehan et al., 2006), and *P. sarmentosum* (Likhitwitayawuid et al., 1987), but also in other plants, like *Anacyclus pyrethrum* (Gulland and Hopton, 1930) and *Asarum heterotropoides* (Perumalsamy et al., 2010).

*trans*-Fagaramid (**5**) and *cis*-fagaramid (**6**) were identified in *P. chaba* (Matsuda et al., 2009; Okumura et al., 2010), but also in *P. tuberculatum* (Vasques da Silva et al., 2002) and *Fagara macrophylla* (Kubo et al., 1984).

Piperine (**7**) occurs in *P. nigrum* (Subehan et al., 2006), *P. guineense* (Adesina et al., 2003), and *P. longum* (Iwashita et al., 2007), achilleamid in *P. nigrum* (Kapoor et al., 2009) as well as *Artemisia dracunculus* (Saadali et al., 2001).

#### 4.1.2.4 Fast Centrifugal Partition Chromatography (FCPC) of *M. excelsum* Extract

For the isolation and identification of the unknown compounds, fast centrifugal partition chromatography (FCPC) was carried out. To improve the separation, the aqueous-ethanolic extract obtained from *M. excelsum* was first divided into a polar and a non-polar fraction by liquid-liquid extraction. For separation, solvent system *ARIZONA* J was used with water, methanol, ethyl acetate, and *n*-heptane at a ratio of 5:2:5:2 (v/v/v/v). Further FCPC method development was carried out using the non-polar, dried fraction. Based on the *ARIZONA* approach, solvent systems A, D, J, N, S, W, and Z were tested at the beginning to limit the range of the different solvent combinations. Among them, systems N and S worked best and showed fast and clear separation, as well as equilibration of the phases. For optimization, additional systems in between the two (O, P, Q, R) were tested and the coefficient factors were calculated. Based on these results, “P” was chosen as the best solvent system for the separation of the unknown compounds in *M. excelsum*. A scheme of the applied process is demonstrated in Figure 25.

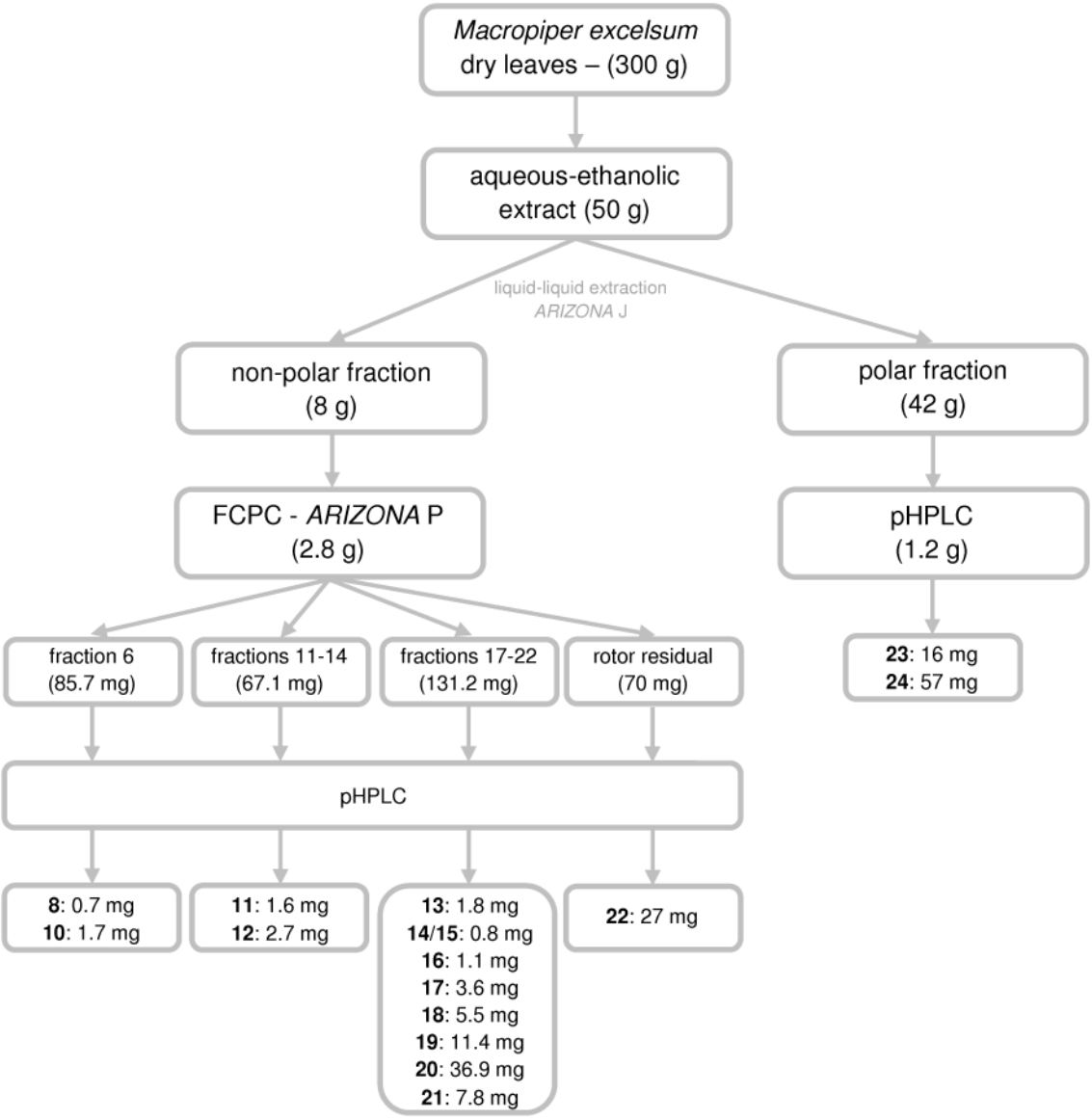



Figure 25: Overview of the employed procedure involving extraction of *M. excelsum* leaves, FCPC separation (for conditions see 3.3.4.2), and isolation via pHPLC (for conditions see 3.3.5).

For each FCPC run, 0.4 g of the non-polar fraction of the extract was dissolved in 20 mL ethyl acetate/*n*-heptane (5:6 v/v) and fractionated in ascending mode (Figure 26). In total, seven runs were completed and thus 2.8 g of the non-polar part of the extract was used.

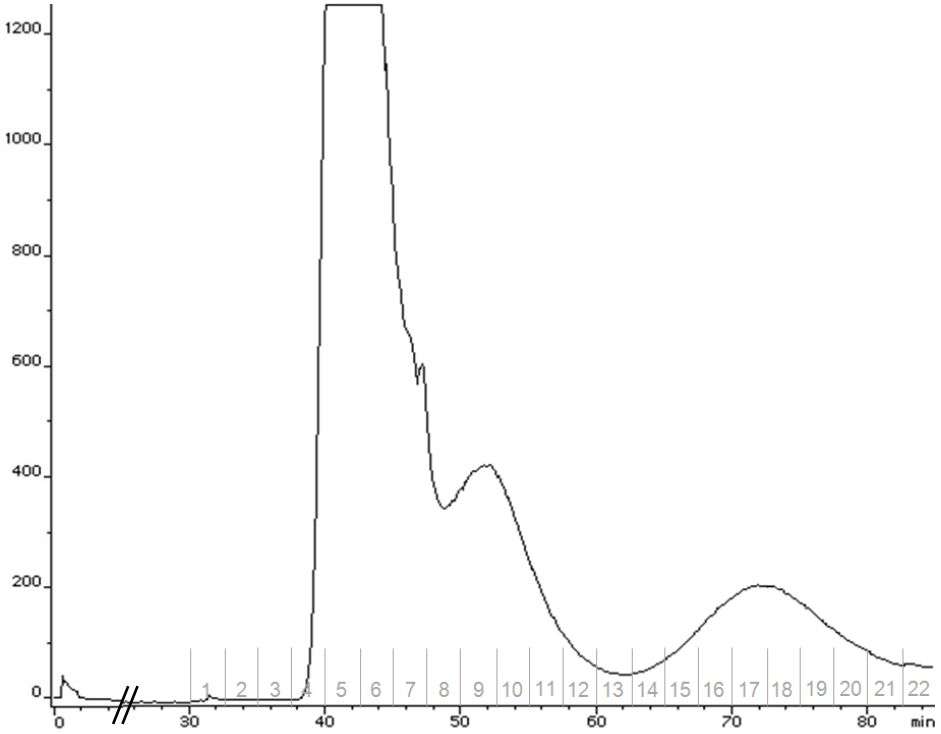



Figure 26: FCPC chromatogram of the non-polar phase of the aqueous-ethanolic extract of *M. excelsum* by using the solvent system *ARIZONA* P; 22 fractions à 2.5 minutes; ELSD, for conditions see 3.3.4.2.

At the end of the run, the solvent of the stationary phase left in the rotor was collected in a vessel. The solvent of each fraction and of the residue in the rotor was evaporated *in vacuo* and subjected to LC-MS analysis. The molecular masses of the compounds identified in the fractions and the residue in the rotor are listed in Table 10.

Table 10: Overview of the compounds detected in the FCPC fractions and the residue in the rotor.

| FCPC fraction | molecular weights of the main compounds (g mol$^{-1}$) |
|---|---|
| 4 | 192 (myristicin, **1**) |
| 5 | 192 (myristicin, **1**) |
| 6 | 251 (**8**), 260 (**10**), 279 (**9**) |
| 7 | 208 (elemicin, **2**), 223 (*trans*-pellitorine, **3**) |
| 8 | 208 (elemicin, **2**), 223 (*trans*-pellitorine, **3**) |
| 9 | 208 (elemicin, **2**), 223 (*trans*-pellitorine, **3**) |
| 10 | 208 (elemicin, **2**), 313 (**12**) |
| 11 | 313 (**12**), 276 |
| 12 | 301 (**11**), 313 (**12**) |
| 13 | 301 (**11**), 313 (**12**) |
| 14 | 301 (**11**), 313 (**12**) |
| 15 | 285 (piperine, **7**), 301 (**11**), 430 (**18-21**) |
| 16 | 285 (piperine, **7**), 301 (**11**), 430 (**18-21**) |
| 17 | 287, 301 (**11**), 430 (**18-21**) |
| 18 | 215 (**13**), 287, 430 (**18-21**) |
| 19 | 215 (**13**), 287, 430 (**18-21**) |
| 20 | 259 (**14, 15**), 215 (**13**), 430 (**18-21**) |
| 21 | 259 (**14, 15**), 215 (**13**), 430 (**18-21**) |
| 22 | 259 (**14, 15**), 215 (**13**), 430 (**18-21**) |
| residue in rotor | 446 (**22**) |

#### 4.1.2.5 Isolation of Compounds from FCPC Fractions via Preparative HPLC

Since FCPC did not yield pure compounds, selected FCPC fractions (6, 11-14, 17-22) were used for further purification via preparative HPLC (pHPLC). Each pHPLC fraction was analyzed via LC-MS. On the basis of these results, the fractions, marked in green (Figures 28, 30, 32, 34, and 36), were collected to obtain higher amounts for NMR analysis and sensory evaluation.

Investigation of FCPC-fraction 6:

The LC-MS chromatogram of FCPC-fraction 6 with the molecular masses of the isolated compounds and the corresponding pHPLC chromatogram are shown in Figure 27 and Figure 28.

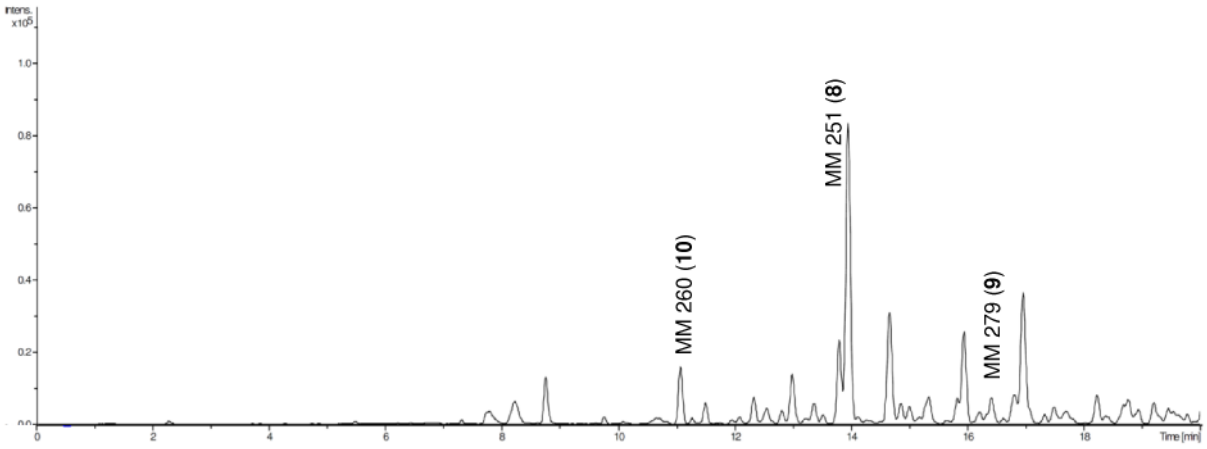



Figure 27: LC-MS chromatogram of FCPC fraction 6; for conditions see 3.3.6.

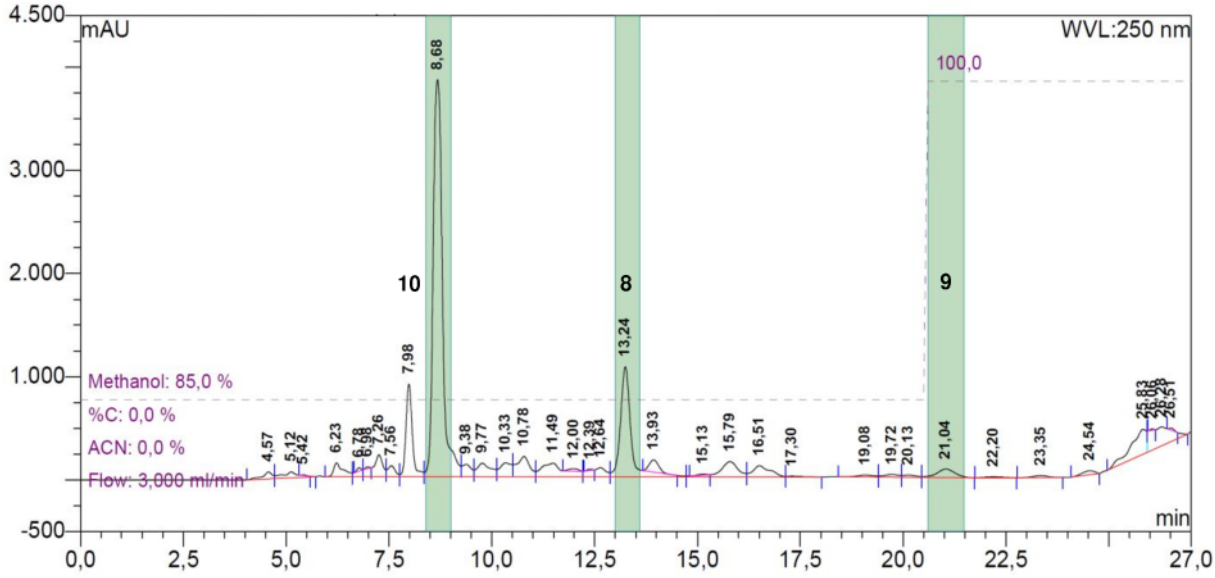



Figure 28: pHPLC chromatogram of FCPC fraction 6 with the collected fractions highlighted in green; UV detection at 250 nm; for conditions see 3.3.5.

In FCPC fraction 6, two molecules (**10**, **8**) could be isolated via pHPLC in sufficient amounts. Compound **9** was present in the FCPC fraction only in traces; the respective pHPLC fraction contained just 0.4 mg of **9** together with unknown impurities.

Investigation of FCPC-fractions 11-14:

Because of similar compositions, FCPC fractions 11-14 were combined (Figure 29) and the isolation of compounds **11** and **12** was carried out under the same conditions (Figure 30).

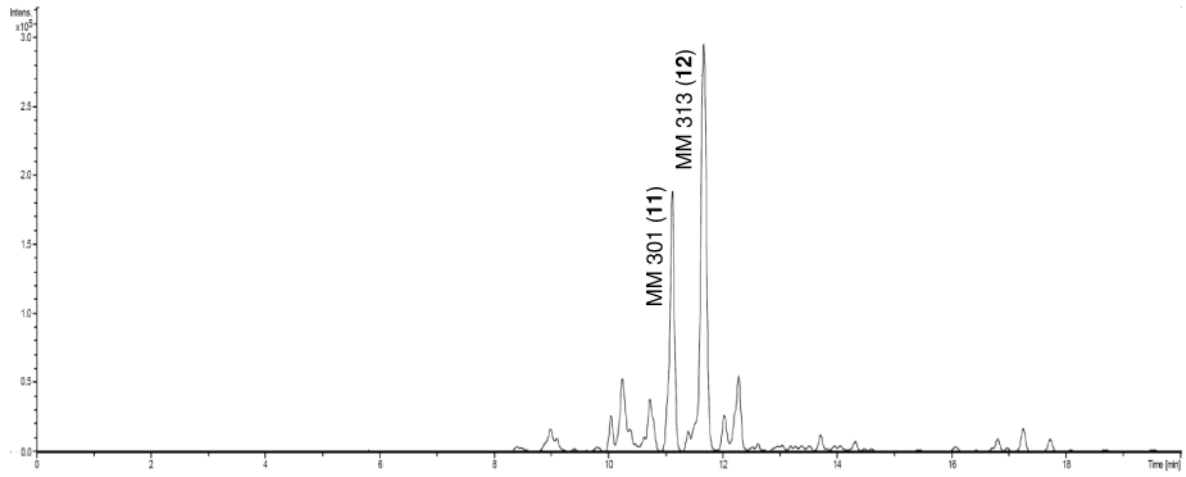



Figure 29: LC-MS chromatogram of the combined FCPC fractions 11-14; for conditions see 3.3.6.

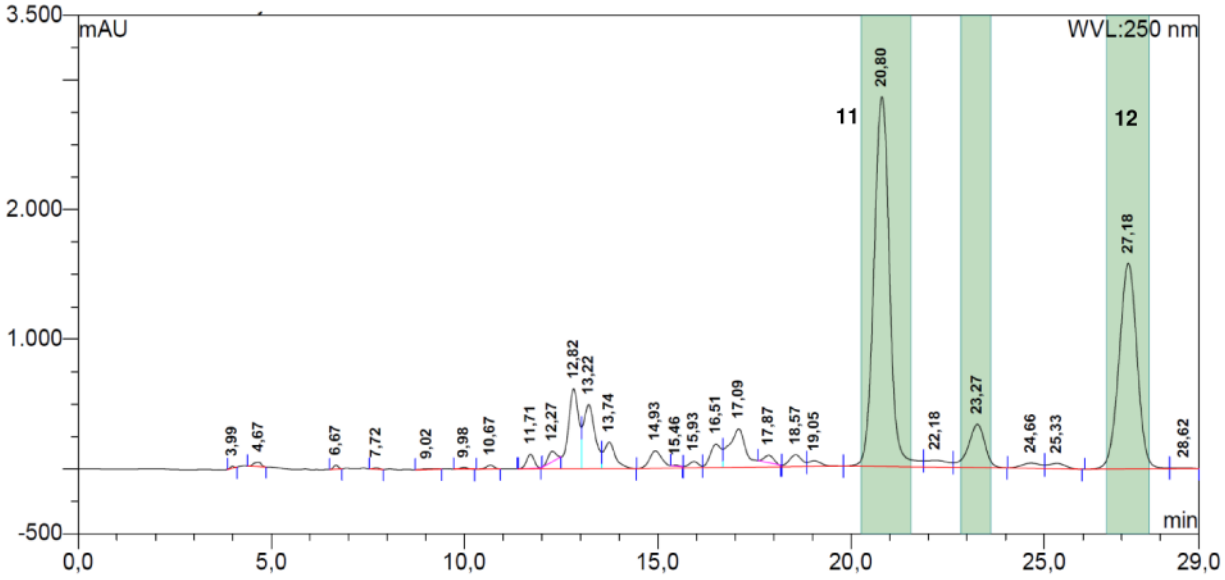



Figure 30: pHPLC chromatogram of FCPC fractions 11-14 with the collected fractions highlighted in green; UV detection at 250 nm; for conditions see 3.3.5.

Three fractions of the pHPLC run were collected; fraction 1 contained compound **11** and fraction 3 compound **12**. pHPLC fraction 2 contained more than one unknown substance and was not further cleaned up or investigated.

Investigation of FCPC-fractions 17-22:

FCPC fractions 17-22 were more complex but showed comparable compositions (Figure 31). Therefore, they were combined followed by pHPLC. During isolation, nine molecules were obtained (**13**-**21**).

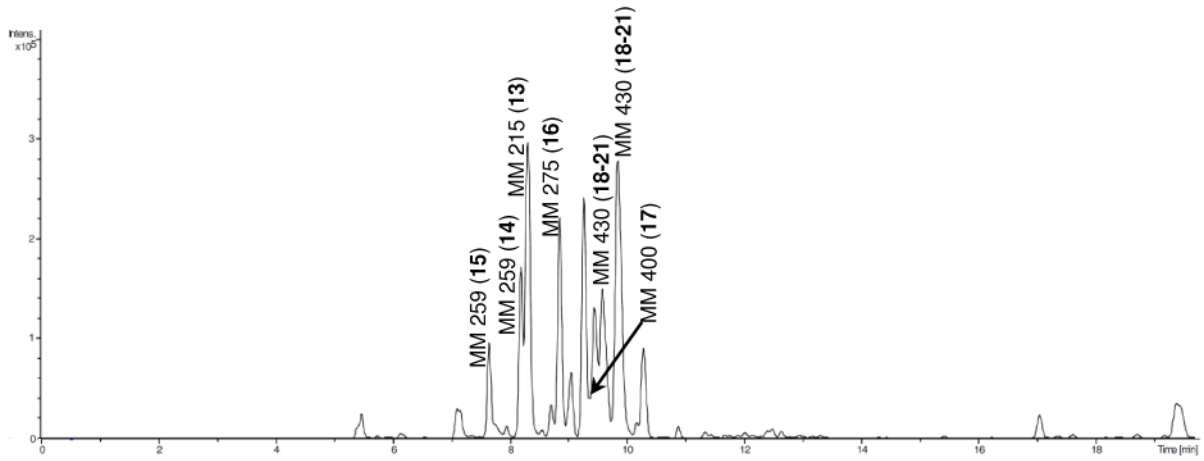



Figure 31: LC-MS chromatogram of the combined FCPC fractions 17-22; for conditions see 3.3.6.

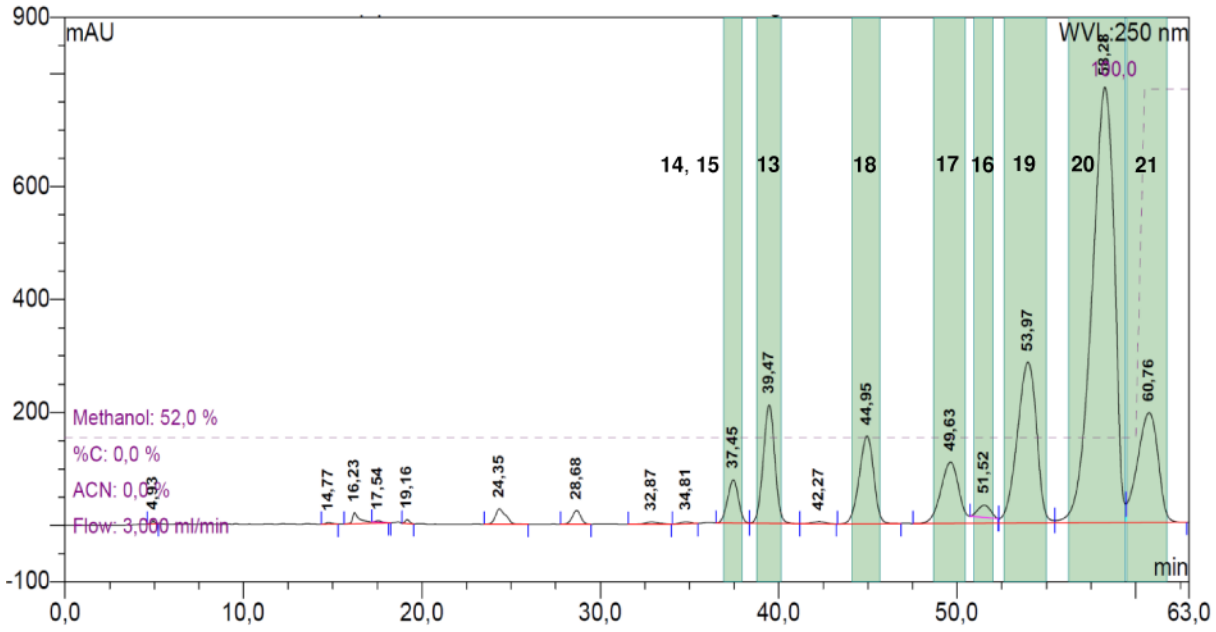



Figure 32: pHPLC chromatogram of FCPC fractions 17-22 with the collected fractions highlighted in green; UV detection at 250 nm; for conditions see 3.3.5.

Under the conditions described in chapter 3.3.5, separation of nearly all of the target molecules in fractions 17-22 was possible (Figure 32). Only pHPLC fraction 1 contained two compounds (**14** and **15**) with the molecular masses of 259 g mol$^{-1}$. Fraction 2 contained compound **13**, fraction 3 **18**, fraction 4 **17**, fraction 5 **16**, fraction 6 **19**, faction 7 **20**, and fraction 8 **21**.

Investigation of the residue in the rotor:

The residue, left in the rotor after the FCPC run was finished, mainly contained compound number **22** with a molecular mass of 446 g mol$^{-1}$ (Figure 33). The molecule was also purified via pHPLC (Figure 34).

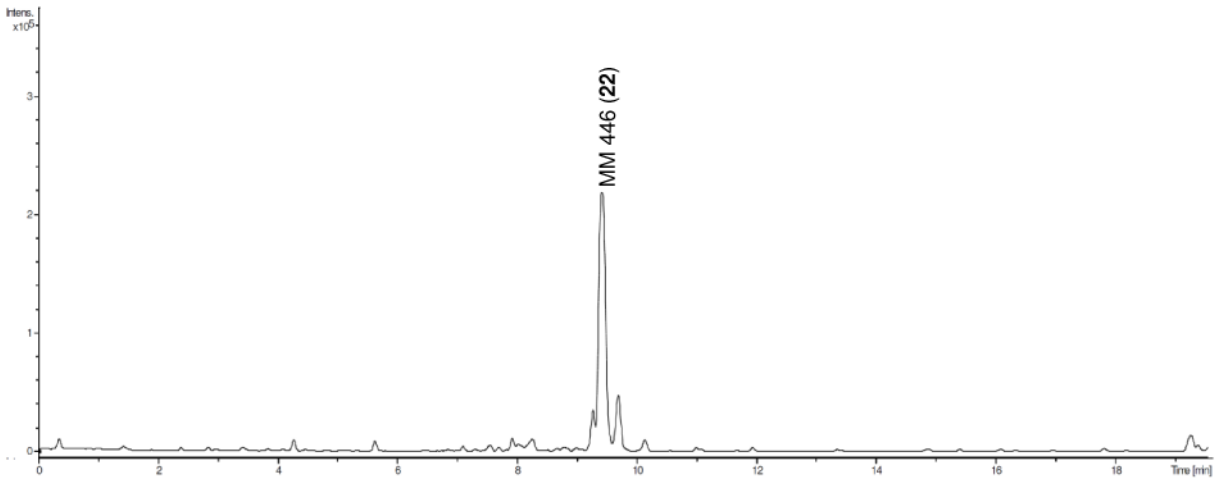



Figure 33: LC-MS chromatogram of the residue, left in the FCPC rotor; for conditions see 3.3.6.

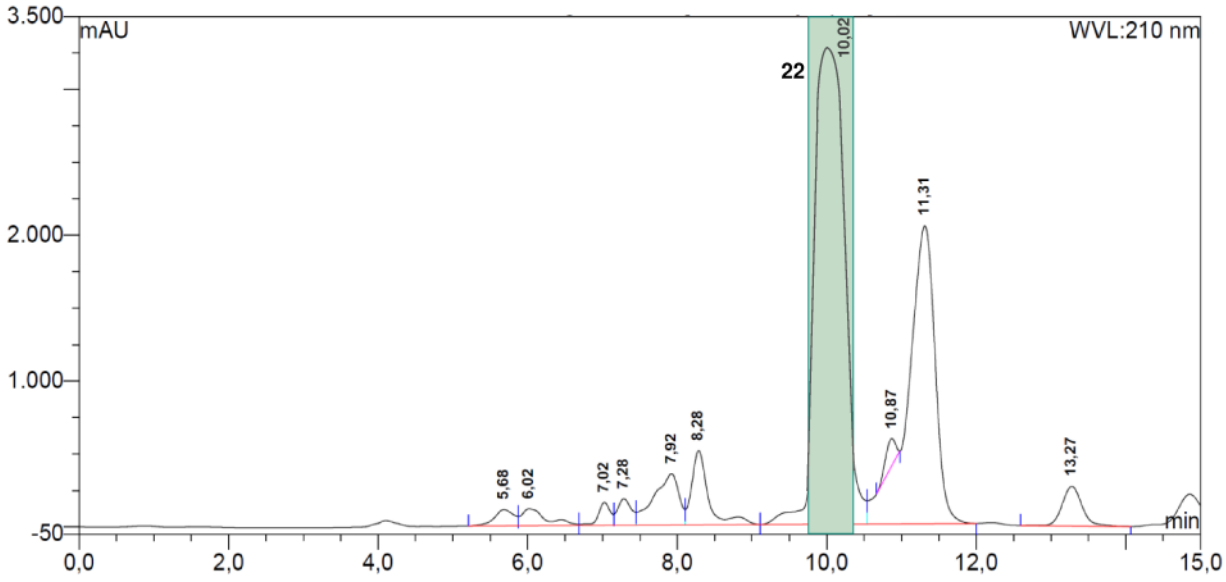



Figure 34: pHPLC chromatogram of the residue in the rotor after the FCPC run with the collected fraction highlighted in green; UV detection at 210 nm; for conditions see 3.3.5.

Investigation of the polar fraction:

The polar fraction of the aqueous-ethanolic extract of *M. excelsum* (Figure 25) was directly used for isolation via pHPLC. The LC-MS chromatogram with the molecular

masses of the isolated compounds and the corresponding pHPLC chromatogram are shown in Figure 35 and Figure 36.

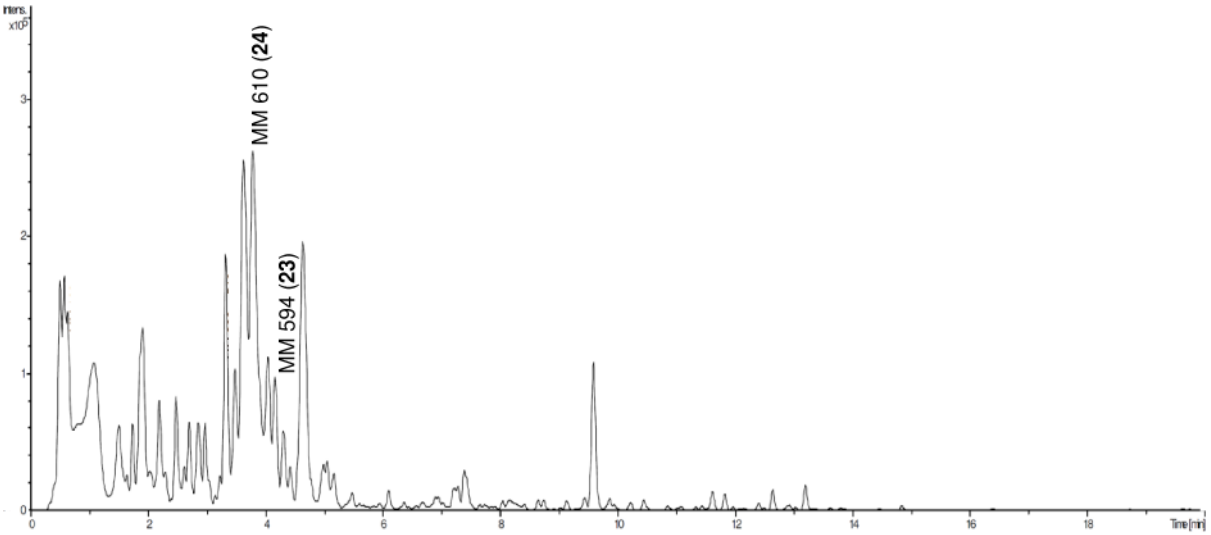



Figure 35: LC-MS chromatogram of the polar fraction of the aqueous-ethanolic extract of *M. excelsum*; for conditions see 3.3.6.

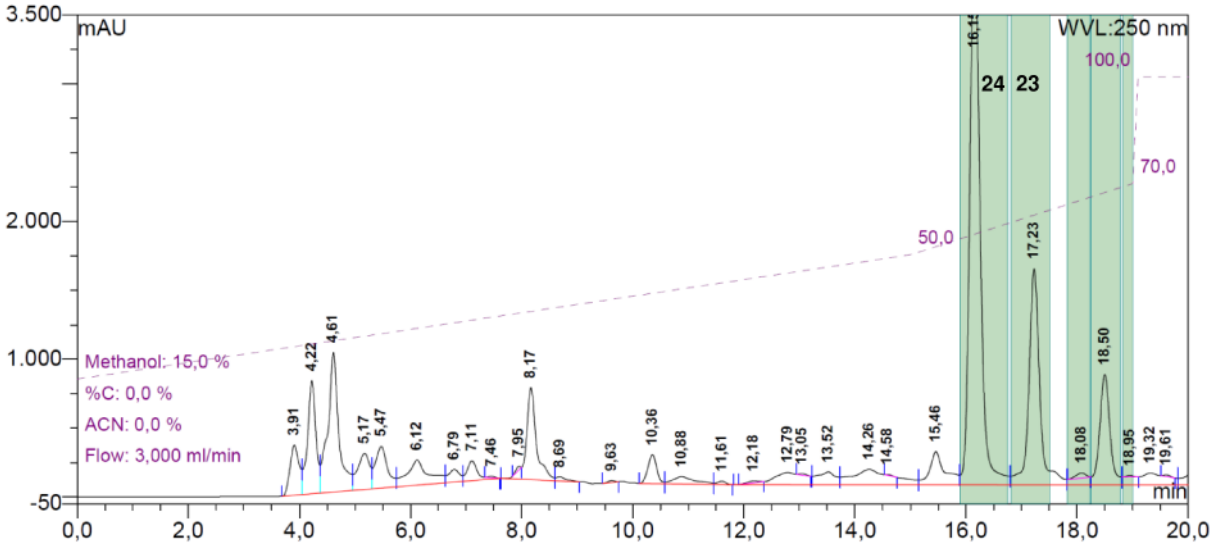



Figure 36: pHPLC chromatogram of the polar fraction of the aqueous-ethanolic extract of *M. excelsum* with the collected fractions highlighted in green; UV detection at 250 nm; for conditions see 3.3.5.

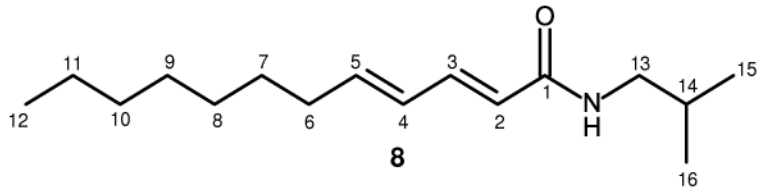
Two compounds, **24** in pHPLC fraction 1 and **23** in fraction 2, were isolated from the polar fraction. The other collected fractions contained more than one unknown compound and were not further cleaned up or investigated.

All isolated compounds described above were analyzed by NMR for complete structure elucidation.

### 4.1.2.6 Structure Elucidation of Isolated Compounds via NMR Spectroscopy

The structures of the isolated molecules were elucidated by 1D and 2D NMR experiments ($^{1}$H spectra, $^{13}$C spectra, $^{1}$H,$^{13}$C gHSQC, $^{1}$H,$^{13}$C gHMBC and $^{1}$H,$^{1}$H gCOSY spectra).

Taking into account the molecular masses and the already identified *trans*-pellitorine in FCPC fraction 6, compounds **8** and **9** seemed to be homologues with chain elongations by two and four methylene groups, respectively. Compound **8** with a molecular mass of 251 g mol$^{-1}$ and a molecular formula of $C_{16}H_{29}NO$ was isolated from *M. excelsum*. The structure was elucidated by NMR spectroscopy (Tables 11, 12), and verified by synthesis. Comparison of $^{1}$H and $^{13}$C NMR data of the isolated compound **8** and the data of the synthesized sample, as well as literature data (Tables 11, 12) confirmed the structure to be kalecide (Figure 37). Kalecide has been found in *P. chaba* (Matsuda et al., 2009), *P. guineense* (Gbewonyo and Candy, 1992), *Zanthoxylum gillettii* (Adesina and Reisch, 1988), and *Echinacea angustifolia* (Lopes-Lutz et al., 2011).




**8**

Figure 37: Kalecide ((2*E*,4*E*)-dodecadienoic acid *N*-isobutyl amide, **8**) from FCPC fraction 6 of *M. excelsum*.

Table 11: $^{1}$H NMR data of kalecide (**8**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Abarbri et al., 1998).

| | **isolated 400 MHz, $CD_3OD$** | **synthesized 400 MHz, $CD_3OD$** | **reported 200 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 5.92, d (15.1) | 5.92, dt (0.6, 15.2) | 5.73, d (15) |
| 3 | 7.11, dd (10.5, 15.1) | 7.11, dd (10.2, 15.1) | 7.16, dd (10, 15) |
| 4 | 6.19, dd (10.2, 15.5) | 6.19, ddq (0.5, 10.4, 15.0) | 6.03-6.1, m (7, 10, 15) |
| 5 | 6.09, dt (6.7, 15.2) | 6.09, dt (6.7, 15.2) | 6.03-6.1, m (7, 10, 15) |
| 6 | 2.17, q (7.1) | 2.17, q (7.6) | 2.1, dt (6.0, 7.0) |
| 7 | 1.43, m | 1.44, q (6.9, 7.5) | 1.22-1.41, m |
| 8/9 | 1.31, m | 1.31, m | 1.22-1.41, m |
| 10 | 1.31, m | 1.31, m | 1.22-1.41, m |
| 11 | 1.31, m | 1.31, m | 1.22-1.41, m |
| 12 | 0.90, t (7.1) | 0.90, t (7.1) | 0.84, t (7) |

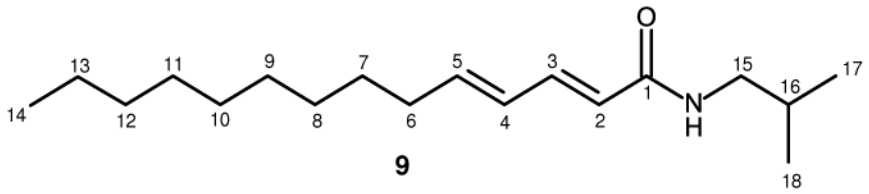
| | isolated 400 MHz, $CD_3OD$ | synthesized 400 MHz, $CD_3OD$ | reported 200 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 13 | 3.06, d (6.9) | 3.06, d (6.9) | 3.13 t, (6.5) |
| 14 | 1.79, thept (6.8) | 1.79, m (6.9) | 1.7-1.87, m |
| 15/16 | 0.92, d (6.7) | 0.92, d (6.7) | 0.87, d (6.6) |

Table 12: $^{13}C$ NMR data of kalecide (**8**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Abarbri et al., 1998).

| | isolated 100 MHz, $CD_3OD$ | synthesized 100 MHz, $CD_3OD$ | reported 50 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** |
| 1 | 169.0, C | 169.2, C | 166.6 |
| 2 | 122.8, CH | 123.0, CH | 122.0 |
| 3 | 142.0, CH | 142.2, CH | 141.1 |
| 4 | 129.6, CH | 129.8, CH | 128.3 |
| 5 | 143.8, CH | 144.0, CH | 143.0 |
| 6 | 33.7, CH2 | 33.9, CH2 | 33.0 |
| 7 | 29.7, CH2 | 30.1, CH2 | 28.8 |
| 8/9 | 30.3, CH2 | 30.3, CH2 | 29.1, 29.3 |
| 10 | 32.6, CH2 | 33.0, CH2 | 31.8 |
| 11 | 23.3, CH2 | 23.7, CH2 | 22.6 |
| 12 | 14.1, CH3 | 14.5, CH3 | 14.0 |
| 13 | 47.8, CH2 | 48.1, CH2 | * |
| 14 | 29.5, CH | 29.8, CH | 28.6 |
| 15/16 | 20.2, CH3 | 20.5, CH3 | 20.2 |

* The $^{13}C$ NMR data in position 13 is missing in the cited literature.

Because of the small amount of **9** in the fraction, the assumed molecule, (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide, was synthesized. For the identification of **9** in the extract, elution time and MS-MS fragments were compared to the trace compound. The data were congruent and therefore one homologue with the molecular weight of 279 g $mol^{-1}$ and the molecular formula of $C_{18}H_{33}NO$ was identified (Figure 38). Reported sources of **9** are e.g. *P. chaba* (Matsuda et al., 2009; Okumura et al., 2010), *P. guineense* (Adesina et al., 2003), and *P. nigrum* (Ee et al., 2009).




**9**

Figure 38: (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) from FCPC fraction 6 of *M. excelsum*.

In addition, NMR data of the synthetic molecule **9** and data from literature were compared. As shown in Table 13, 1D and 2D spectroscopic data were in accordance as well.

Table 13: $^{13}C$ and $^{1}H$ NMR data of synthesized (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) compared to literature data (Abarbri et al., 1998).

| | **synthesized 100 MHz, $CDCl_3$** | **reported 200 MHz, $CDCl_3$** | **synthesized 100 MHz, $CDCl_3$** | **reported 200 MHz, $CDCl_3$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 166.4 C | 166.0 | | |
| 2 | 121.7 CH | 121.7 | 5.80, d (15.1) | 5.78, d (15) |
| 3 | 141.3 CH | 141.2 | 7.19, dd (9.8, 15.0) | 7.22, dd (10.0, 15) |
| 4 | 128.2 CH | 128.0 | 6.13, dd (9.8, 15.2) | 6.01-6.23, m |
| 5 | 143.3 CH | 143.0 | 6.06, dt (6.3, 15.1) | 6.01-6.23, m |
| 6 | 33.0 CH2 | 32.8 | 2.14, q (6.7) | 2.17, dt (6.6, 7) |
| 7 | 28.8 CH2 | 28.7 | 1.41, m (7.3) | 1.26-1.4, m |
| 8/9/10/11 | 29.2-29.5 CH2 | 29.0-29.4 | 1.27, m | 1.26-1.4, m |
| 12 | 31.9 CH2 | 31.8 | 1.27, m | 1.26-1.4, m |
| 13 | 22.7 CH2 | 22.4 | 1.27, m | 1.26-1.4, m |
| 14 | 14.1 CH3 | 14.0 | 0.88, t (6.9) | 0.91, t (6.7) |
| 15 | 46.9 CH2 | 46.8 | 3.17, dd (6.1, 6.8) | 3.2, dd (6.4, 6.6) |
| 16 | 28.6 CH | 28.5 | 1.80, m (6.7) | 1.76-1.86, m |
| 17/18 | 20.1 CH3 | 20.0 | 0.93, d (6.7) | 0.95, d (6.6) |

Another compound isolated from fraction 6 was **10** with a molecular weight of 260 g $mol^{-1}$ and a molecular formula of $C_{15}H_{16}O_4$. The structure was elucidated by 1D and 2D NMR information.

The carbonyl ether C-1 with a characteristic $^{13}C$ shift of $\delta_C$ = 169.4 ppm showed $^{1}H,^{13}C$ long range correlation with $H_3$-15 of the methoxy group ($\delta_{C/H}$ = 52.0/3.71 ppm). Further, C-1 showed gHMBC cross correlations with H-2 and H-3 from COSY sequence A. The two double bonds in COSY sequence A are *trans*-configured, shown by the vicinal coupling constants of $^{3}J_{2,3}$ = 15.4 Hz, $^{3}J_{4,5}$ = 15.0 Hz and $^{3}J_{3,4}$ =

9.9 Hz. The six-membered aromatic ring system (C-8 to C-14) showed the characteristic chemical shifts and $^{1}H,^{13}C$ long range correlations, as well as COSY correlations between H-13 and H-14 presented in Figure 39 and Table 14. Further, the quaternary carbon C-8 showed long range correlation with H-6 and H-7, as well as C-9 and C-14 with H-7 connecting the aromatic ring system over C-8 with COSY sequence A. The quaternary aromatic carbons C-10 and C-12 showed characteristic $^{13}C$ chemical shifts of $\delta_c$ = 149.1 and 147.2 ppm to support the acetal at this position. In addition, C-10 and C-12 showed gHMBC cross correlation with the methylene group $H_2$-11in the acetal to give the structure of **10** as presented in Figure 39.

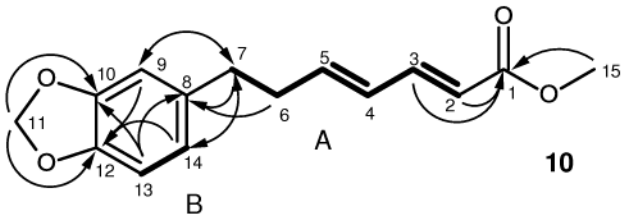



Figure 39: Methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**) from FCPC fraction 6 of *M. excelsum*, presented with the NMR COSY sequences in bold and selected gHMBC correlations (arrows).

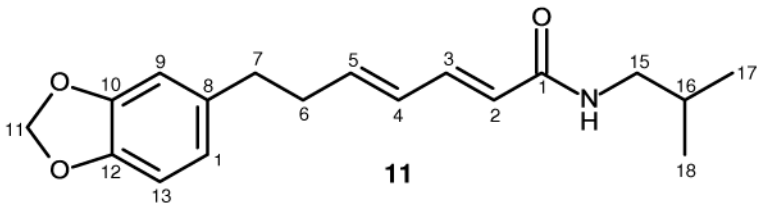
As **10** has not been previously described in the literature, the proposed compound was synthesized for confirmation, and the NMR data were compared with the isolated compound (Table 14).

Table 14: $^{13}C$ and $^{1}H$ NMR data of methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**) isolated from *M. excelsum* compared to the synthetic sample.

| | **isolated 100 MHz, $CD_3OD$** | **synthesized 100 MHz, $CD_3OD$** | **isolated 400 MHz, $CD_3OD$** | **synthesized 400 MHz, $CD_3OD$** |
|---|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 169.4, C | 169.3, C | | |
| 2 | 119.9, CH | 119.9, CH | 5.81, d (15.4) | 5.80, d (15.4) |
| 3 | 146.7, CH | 146.7, CH | 7.23, dd (9.9, 15.4) | 7.23, dd (9.8, 15.4) |
| 4 | 130.1, CH | 130.1, CH | 6.24, dd (9.9, 15.0) | 6.22, dd (9.8, 15.2) |
| 5 | 145.1, CH | 145.1, CH | 6.18, dt (6.4, 15.0) | 6.16, dt (6.4, 15.1) |
| 6 | 35.8, CH2 | 35.8, CH2 | 2.45, m | 2.43, m |
| 7 | 36.2, CH2 | 36.2, CH2 | 2.67, t (7.6) | 2.65, m |
| 8 | 136.4, C | 136.4, C | | |
| 9 | 109.8, CH | 109.8, CH | 6.69, d (1.7) | 6.68, dd (0.5, 1.8) |
| 10 | 149.1, C | 149.1, C | | |
| 11 | 102.0, CH2 | 102.1, CH2 | 5.88, s | 5.87, s |
| 12 | 147.2, C | 147.2, C | | |

| | isolated 100 MHz, $CD_3OD$ | synthesized 100 MHz, $CD_3OD$ | isolated 400 MHz, $CD_3OD$ | synthesized 400 MHz, $CD_3OD$ |
|---|---|---|---|---|
| position | $\delta_c$ (ppm) | $\delta_c$ (ppm) | $\delta_H$ (ppm), mult (*J* in Hz) | $\delta_H$ (ppm), mult (*J* in Hz) |
| 13 | 109.0, CH | 109.0, CH | 6.70, d (8.0) | 6.70, dd (0.4, 8.0) |
| 14 | 122.3, CH | 122.3, CH | 6.63, dd (1.7, 7.8) | 6.62, ddt (0.6, 1.8, 7.9) |
| 15 | 52.0, CH3 | 52.0, CH3 | 3.71, s | 3.70, s |

In FCPC fractions 11-14, two molecules were isolated: compound **11** (MW 301 g $mol^{-1}$) with the molecular formula of $C_{18}H_{23}NO_3$ and compound **12** (MW 313 g $mol^{-1}$) with the molecular formula of $C_{19}H_{23}NO_3$. After 1D and 2D NMR analysis and comparison of the isolated compounds with data from literature (Tables 15-18), chingchengenamide A (**11**, Figure 40) and piperdardine (**12**, Figure 41) were identified. Based on the identified structures, synthetic samples were prepared for unambiguous identification. Both molecules can be found in different *Piper* species: **11** was described in *P. falconeri* (Parmar et al., 1993) and *P. boehmeriaefolium* (Tang et al., 2011), and **12** in *P. tuberculatum* (De Araujo-Junior et al., 1997), *P. nigrum* (Friedman et al., 2008), and *P. sintenensis* (Chen et al., 2002), respectively.




**11**

Figure 40: Chingchengenamide A ((2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)-*N*-isobutyl-hepta-2,4-dienamide, **11**) from FCPC fractions 11-14 of *M. excelsum*.

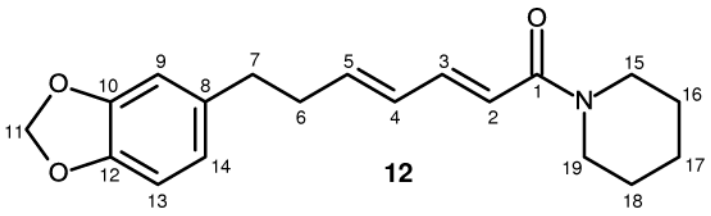
Table 15: $^1$H NMR data of chingchengenamide A (**11**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Facundo et al., 2004).

| | isolated 400 MHz, $CD_3OD$ | synthesized 400 MHz, $CD_3OD$ | reported 400 MHz, $CDCl_3$ |
|---|---|---|---|
| position | $\delta_H$ (ppm), mult (*J* in Hz) | $\delta_H$ (ppm), mult (*J* in Hz) | $\delta_H$ (ppm), mult (*J* in Hz) |
| 1 | | | |
| 2 | 5.91, dt (0.5, 15.2) | 5.91, dt (0.6, 15.2) | 5.77, d (15.0) |
| 3 | 7.08, dd (10.5, 15.2) | 7.08, dd (10.4, 15.2) | 7.17, dd (10.5, 15.0) |
| 4 | 6.18, ddq (0.7, 1.1, 10.5, 15.2) | 6.18, ddq (0.7, 1.2, 10.4, 15.2) | 6.14 |
| 5 | 6.08, dt (6.7, 15.2) | 6.09, dd (6.7, 15.2) | 6.06, td (7.4, 15.0) |
| 6 | 2.43, q (6.8) | 2.43, q | 2.42, q (7.4) |
| 7 | 2.66, t (7.6) | 2.66, t | 2.66, t (7.4) |

| | isolated 400 MHz, $CD_3OD$ | synthesized 400 MHz, $CD_3OD$ | reported 400 MHz, $CDCl_3$ |
|---|---|---|---|
| position | $\delta_H$ (ppm), mult (*J* in Hz) | $\delta_H$ (ppm), mult (*J* in Hz) | $\delta_H$ (ppm), mult (*J* in Hz) |
| 8 | | | |
| 9 | 6.69, p (0.5, 1.8) | 6.69, dd (0.4, 1.7) | 6.62, sl |
| 10 | | | |
| 11 | 5.88, s | 5.88, s | 5.92, s |
| 12 | | | |
| 13 | 6.70, d (7.9) | 6.70, dd (0.5, 7.7) | 6.84, d (7.8) |
| 14 | 6.63, ddt (0.5, 1.8, 7.9) | 6.63, ddt (0.6, 1.8, 7.9) | 6.60, dl (7.8) |
| 15 | 3.06, d (6.9) | 3.06, d (6.9) | 3.15, t (6.7) |
| 16 | 1.79, thept (6.9) | 1.79, m (6.9) | 1.79, m |
| 17/18 | 0.91, d (6.7) | 0.91, d (6.7) | 0.92, d (6.7) |

Table 16: $^{13}C$ NMR data of chingchengenamide A (**11**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Facundo et al., 2004).

| | isolated 100 MHz, $CD_3OD$ | synthesized 100 MHz, $CD_3OD$ | reported 100 MHz, $CDCl_3$ |
|---|---|---|---|
| position | $\delta_c$ (ppm) | $\delta_c$ (ppm) | $\delta_c$ (ppm) |
| 1 | 169.1, C | 169.2, C | 166.61, C |
| 2 | 123.4, CH | 123.4, CH | 122.51, CH |
| 3 | 142.0, CH | 142.0, CH | 141.35, CH |
| 4 | 130.3, CH | 130.4, CH | 129.10, CH |
| 5 | 142.8, CH | 142.8, CH | 141.70, CH |
| 6 | 36.1, CH2 | 36.2, CH2 | 35.16, CH2 |
| 7 | 36.0, CH2 | 36.0, CH2 | 35.16, CH2 |
| 8 | 136.6, C | 136.6, C | 135.29, C |
| 9 | 109.8, CH | 109.8, CH | 109.02, CH |
| 10 | 149.0, C | 149.1, C | 147.79, C |
| 11 | 102.0, CH2 | 102.1, CH2 | 100.99, CH2 |
| 12 | 147.2, C | 147.2, C | 145.94, C |
| 13 | 109.0, CH | 109.0, CH | 108.96, CH |
| 14 | 122.3, CH | 122.4, CH | 121.38, CH |
| 15 | 48.0, CH2 | 48.1, CH2 | 47.18, CH2 |
| 16 | 29.8, CH | 29.8, CH | 28.82, CH |
| 17/18 | 20.5, CH3 | 20.5, CH3 | 20.33, CH3 |




**12**

Figure 41: Piperdardine ((2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)-1-(1-piperidyl)hepta-2,4-dien-1-one, **12**) from FCPC fractions 11-14 of *M. excelsum*.

Table 17: $^{1}$H NMR data of piperdardine (**12**) isolated from *M. excelsum* compared to the synthetic sample and literature data (De Araujo-Junior et al., 1997).

| | **Isolated 400 MHz, $CD_3OD$** | **synthesized 400 MHz, $CD_3OD$** | **reported 400 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 6.43, d (14.8) | 6.43, d (14.8) | 6.26, d (14.8) |
| 3 | 7.12, dd (10.8, 14.8) | 7.12, ddd (0.7, 10.8, 14.8) | 7.21, dd (10.8, 14.8) |
| 4 | 6.26, dd (10.8, 15.2) | 6.27, ddtd (0.6, 1.3, 10.8, 15.2) | 6.18, dd (10.8, 15.1) |
| 5 | 6.09, dt (6.9, 15.2) | 6.09, dt (6.9, 15.1) | 6.06, dt (7.0, 15.1) |
| 6 | 2.43, q (7.3) | 2.44, q (7.3) | 2.42, dt (7.0, 7.3) |
| 7 | 2.66, t (7.5) | 2.66, t (7.5) | 2.66, t (7.3) |
| 8 | | | |
| 9 | 6.69, d (1.7) | 6.69, dd (0.4, 1.7) | 6.66, d (1.5) |
| 10 | | | |
| 11 | 5.88, s | 5.88, s | 5.92, s |
| 12 | | | |
| 13 | 6.70, d (7.9) | 6.70, dd (0.5, 7.8) | 6.70, d (7.9) |
| 14 | 6.63, dd (1.7, 7.8) | 6.63, ddt (0.6, 1.8, 7.8) | 6.61, dd (1.5, 7.9) |
| 15 | 3.58, m | 3.58, m | 3.48, br s |
| 16 | 1.57, m | 1.57, s | 1.45-1.60, m |
| 17 | 1.69, p (5.6) | 1.68, m | 1.61-1.70, m |
| 18 | 1.57, m | 1.57, s | 1.45-1.60, m |
| 19 | 3.58, m | 3.58, m | 3.61, br s |

Table 18: $^{13}$C NMR data of piperdardine (**12**) isolated from *M. excelsum* compared to the synthetic sample and literature data (De Araujo-Junior et al., 1997).

| | **isolated 100 MHz, $CD_3OD$** | **synthesized 100 MHz, $CD_3OD$** | **reported 100 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** |
| 1 | 167.8, C | 167.9, C | 165.8, C |
| 2 | 119.9, CH | 119.9, CH | 119.3, CH |
| 3 | 144.4, CH | 144.4, CH | 142.7, CH |
| 4 | 130.8, CH | 130.8, CH | 129.7, CH |
| 5 | 142.9, CH | 142.9, CH | 141.1, CH |
| 6 | 36.1, CH2 | 36.2, CH2 | 35.2, CH2 |
| 7 | 36.0, CH2 | 36.0, CH2 | 35.2, CH2 |
| 8 | 136.5, C | 136.6, C | 135.4, C |
| 9 | 109.8, CH | 109.8, CH | 109.0, CH |
| 10 | 149.0, C | 149.1, C | 147.8, C |
| 11 | 102.0, CH2 | 102.1, CH2 | 101.0, CH2 |
| 12 | 147.2, C | 147.2, C | 145.9, C |
| 13 | 109.0, CH | 109.0, CH | 108.4, CH |
| 14 | 122.3, CH | 122.3, CH | 121.4, CH |
| 15 | 44.5 or 48.1 CH2 | 44.5, CH2 | 43.4, CH2 |
| 16 | 26.9 or 27.8, CH2 | 26.9 or 27.9, CH2 | 25.7, CH2 |
| 17 | 25.5, CH2 | 25.6, CH2 | 24.9, CH2 |
| 18 | 26.8 or 27.8, CH2 | 26.9 or 27.9, CH2 | 26.9, CH2 |
| 19 | 44.5 or 48.1 CH2 | 44.5, CH2 | 47.1, CH2 |

Further molecules were isolated from FCPC fractions 17-22 of the *M. excelsum* extract. Compound **13** with a molecular mass of 215 g $mol^{-1}$ and a molecular formula of $C_{14}H_{17}NO$ was identified as *N*-(*trans*-cinnamoyl)piperidine (Figure 42). NMR data of the isolated compound were compared with a synthesized sample and literature data (Tables 19, 20); both, the $^{1}H$ and $^{13}C$ data were in accordance. The compound occurs in *P. chaba* (Matsuda et al., 2009) and *P. nigrum* (Kapoor et al., 2009; Subehan et al., 2006), for example.

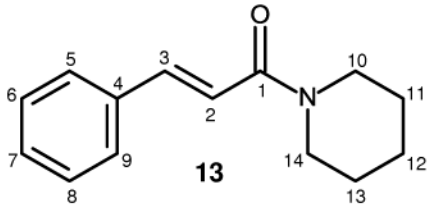



Figure 42: *N*-(*trans*-cinnamoyl)piperidine ((*E*)-3-phenyl-1-(1-piperidyl)prop-2-en-1-one, **13**) from FCPC fractions 17-22 of *M. excelsum*.

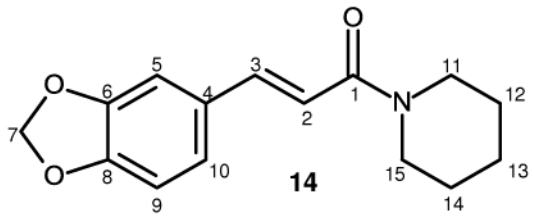
Table 19: $^{1}H$ NMR data of *N*-(*trans*-cinnamoyl)piperidine (**13**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Leung et al., 2010).

| | **isolated 400 MHz, $CD_3OD$** | **synthesized 400 MHz, $CD_3OD$** | **reported 400 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 6.90, d (15.5) | 6.90, d (15.5) | 6.91, d (15.5) |
| 3 | 7.64, d (15.5) | 7.64, d (15.5) | 7.64, d (15.5) |
| 4 | | | |
| 5/9 | 7.52, m | 7.52, m | 7.51-7.53, m |
| 6/8 | 7.36, m | 7.35, m | 7.32-7.38, m |
| 7 | 7.36, m | 7.35, m | 7.32-7.38, m |
| 10/14 | 3.63, d (30.6) | 3.63, d | 3.57-3.66, m |
| 11/13 | 1.65, m | 1.62, 1.69, m | 1.59-1.67, m |
| 12 | 1.65, m | 1.62, 1.69, m | 1.59-1.67, m |

Table 20: $^{13}C$ NMR data of *N*-(*trans*-cinnamoyl)piperidine (**13**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Leung et al., 2010).

| | **isolated 100 MHz, $CD_3OD$** | **synthesized 100 MHz, $CD_3OD$** | **reported 100 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** |
| 1 | 165.3, C | 165.4, C | 165.3 |
| 2 | 117.7, CH | 117.8, CH | 117.7 |
| 3 | 142.1, CH | 142.1, CH | 142.1 |
| 4 | 135.5, C | 135.5, C | 135.4 |
| 5/9 | 127.6, CH | 127.7, CH | 127.6 |
| 6/8 | 128.7, CH | 128.8, CH | 128.7 |
| 7 | 129.4, CH | 129.4, CH | 129.4 |
| 10/14 | 43.4, 47.0, CH2 | 43.4, 47.0, CH2 | 43.3, 47.0 |
| 11/13 | 25.6, 26.8, CH2 | 25.6, 26.8, CH2 | 25.6, 26.7 |
| 12 | 24.6, CH2 | 24.7, CH2 | 24.6 |

The structure of another amide, **14**, was elucidated by NMR spectroscopy and comparison with data of the synthetic molecule and those found in literature (Tables 21, 22). The identified compound, ilepcimide (Figure 43), has a molecular weight of 259 g $mol^{-1}$ and a molecular formula of $C_{15}H_{17}NO_3$. The compared shifts are mostly congruent, only small differences in the $^{13}C$ shifts in positions 12-14 can be observed. This is due to the fact, that the isolated sample was a mixture of compound **14** as the minor compound and **15**. Compound **15** was assumed to be an isomer of **14**. Thus, the chemical shifts were assigned from the 2D NMR data and not all multipletts were unambiguously assigned. In case of the synthesized sample, direct $^{13}C$ shifts were used. Ilepcimide (**14**) is described in *Piper* species, such as *P. chaba* (Matsuda et al., 2009) and *P. nigrum* (Ee et al., 2009).




**14**

Figure 43: Ilepcimide ((*E*)-3-(1,3-benzodioxol-5-yl)-1-(1-piperidyl)prop-2-en-1-one, **14**) from FCPC fractions 17-22 of *M. excelsum*.

Table 21: $^{1}$H NMR data of ilepcimide (**14**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Correa et al., 2010).

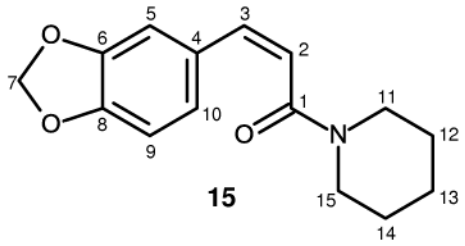
| | **isolated 400 MHz, $CDCl_3$** | **synthesized 400 MHz, $CDCl_3$** | **reported 400 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 6.73, d (15.3) | 6.74, d (15.3) | 6.73, d (15.4) |
| 3 | 7.56, d (15.4) | 7.56, d (15.4) | 7.56, d (15.5) |
| 4 | | | |
| 5 | 7.03, td (0.4, 1.7) | 7.03, dt (0.5, 1.8) | 7.03, d (1.7) |
| 6 | | | |
| 7 | 5.99, s | 5.95, s | 5.97, s |
| 8 | | | |
| 9 | 6.80, d (8.0) | 6.79, d (8.1) | 6.79, d (8.0) |
| 10 | 7.00, ddd (0.5, 1.7, 8.0) | 6.99, ddd (0.6, 1.7, 8.0) | 6.99, dd (1.7, 8.0) |
| 11 | 3.62, m | 3.62, m | 3.60, br s |
| 12 | 1.60, m | 1.64, m | 1.59, m |
| 13 | 1.60, m | 1.64, m | 1.65, m |
| 14 | 1.60, m | 1.64, m | 1.59, m |
| 15 | 3.62, m | 3.62, m | 3.60, br s |

Table 22: $^{13}$C NMR data of ilepcimide (**14**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Correa et al., 2010).

| | **isolated 100 MHz, $CDCl_3$** | **synthesized 100 MHz, $CDCl_3$** | **reported 100 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** |
| 1 | 165.3, C | 165.4, C | 165.2 |
| 2 | 115.6, CH | 115.6, CH | 115.5 |
| 3 | 142.0, CH | 141.9, CH | 141.6 |
| 4 | 130.3, C | 129.9, C | 129.6 |
| 5 | 106.3, CH | 106.3, CH | 106.1 |
| 6 | 148.1, C | 148.2, C | 147.9 |
| 7 | 101.2, CH2 | 101.4, CH2 | 101.3 |
| 8 | 148.6, C | 148.8, C | 148.5 |
| 9 | 108.6, CH | 108.5, CH | 108.2 |
| 10 | 123.5, CH | 123.6, CH | 123.3 |
| 11 | 43.2 or 46.9, CH2 | 43.3 or 47.0, CH2 | 43.1 |
| 12 | 24.5 or 26.0, CH2 | 24.7 or 25.6 or 26.8, CH2 | 25.4 |
| 13 | 24.5 or 26.0, CH2 | 24.7 or 25.6 or 26.8, CH2 | 24.5 |
| 14 | 24.5 or 26.0, CH2 | 24.7 or 25.6 or 26.8, CH2 | 26.5 |
| 15 | 43.2 or 46.9, CH2 | 43.3 or 47.0, CH2 | 46.6 |

The *trans*-isomer of **14**, compound **15**, was also found in *M. excelsum*. Based on the NMR data, the assumed molecule was synthesized for comparison. Additionally, literature data were used. Based on the comparison of the $^{1}$H and $^{13}$C spectra

(Tables 23, 24), **15** was identified as (*Z*)-antiepilepsirine (Figure 44). *P. capense* has been described as a further plant source for this compound (Kaou et al., 2010).




**15**

Figure 44: *Z*-Antiepilepsirine ((*Z*)-3-(1,3-benzodioxol-5-yl)-1-(1-piperidyl)prop-2-en-1-one, **15**) from FCPC fractions 17-22 of *M. excelsum*.

Table 23: $^{1}$H NMR data of (*Z*)-antiepilepsirine (**15**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Kaou et al., 2010).

| | **isolated 400 MHz, $CDCl_3$** | **synthesized 400 MHz, $CDCl_3$** | **reported 600 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 5.92, d (12.5) | 5.92, d (12.5) | 5.92, d (12.4) |
| 3 | 6.50, d (12.5) | 6.51, dt (0.5, 12.6) | 6.52, d (12.4) |
| 4 | | | |
| 5 | 6.92, d (1.7) | 6.92, dt (0.4, 1.8) | 6.92, d (1.5) |
| 6 | | | |
| 7 | 5.95, s | 5.95, s | 5.95, s |
| 8 | | | |
| 9 | 6.75, d (8.1) | 6.75, d (8.1) | 6.75, d (8.2) |
| 10 | 6.85, ddd (0.6, 1.7, 8.0) | 6.85, ddd (0.6, 1.7, 8.0) | 6.84, dd (1.5, 8.2) |
| 11 | 3.35, 3.62 m,t | 3.35, 3.61, m | 3.35, m |
| 12 | 1.58, m | 1.56, m | 1.54, m |
| 13 | 1.29, m | 1.28, m | 1.27, m |
| 14 | 1.58, m | 1.56, m | 1.54, m |
| 15 | 3.35, 3.62 m t | 3.35, 3.61, m | 3.60, m |

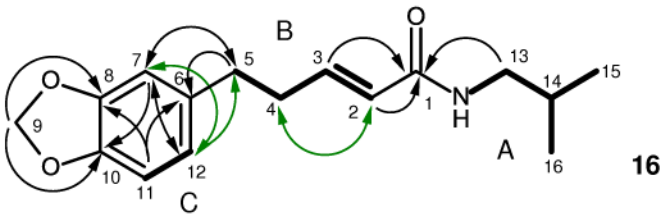
Table 24: $^{13}$C NMR data of (*Z*)-antiepilepsirine (**15**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Kaou et al., 2010).

| | **isolated 100 MHz, $CDCl_3$** | **synthesized 100 MHz, $CDCl_3$** | **reported 150 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** |
| 1 | 167.3, C | 167.3, C | 167.3, C |
| 2 | 121.9, CH | 121.9, CH | 121.9, CH |
| 3 | 132.4, CH | 132.4, CH | 132.3, CH |
| 4 | 129.8, C | 129.8, C | 129.8, C |
| 5 | 108.2, 108.2, CH | 108.2, 108.2, CH | 108.2, CH |
| 6 | 147.8, C | 147.8, C | 147.8, C |
| 7 | 101.2, CH2 | 101.2, CH2 | 101.1, CH2 |

| | isolated 100 MHz, $CDCl_3$ | synthesized 100 MHz, $CDCl_3$ | reported 150 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** |
| 8 | 147.6, C | 147.6, C | 147.6, C |
| 9 | 108.2, CH | 108.2, CH | 108.2, CH |
| 10 | 123.1, CH | 123.1, CH | 123.1, CH |
| 11 | 42.0 or 47.3, CH2 | 42.0 or 47.3, CH2 | 47.3, CH2 |
| 12 | 24.5 or 25.2, CH2 | 24.5 or 25.2, CH2 | 26.1, CH2 |
| 13 | 26.1, CH2 | 26.1, CH2 | 24.4, CH2 |
| 14 | 24.5 or 25.2, CH2 | 24.5 or 25.2, CH2 | 25.2, CH2 |
| 15 | 42.0 or 47.3, CH2 | 42.0 or 47.3, CH2 | 42.0, CH2 |

Compound **16** with a molecular mass of 275 g $mol^{-1}$ and a molecular formula of $C_{16}H_{21}NO_3$ was also isolated from *M. excelsum*. The structure was elucidated by 1D and 2D NMR data and verified by synthesis to be dihydropiperlonguminine (**16**, Figure 45).

The amide carbonyl C-1 ($\delta_C$ = 165.8 ppm) showed gHMBC correlations with H-13 from the iso-butanyl side chain of the amide in COSY sequence A, as well as with H-2 and H-3 of the *trans*-double bond ($^3J_{2,3}$ = 15.2 Hz) in COSY sequence B presented in Figure 45. Furthermore, the six-membered aromatic ring (C-6 to C-12) showed the expected chemical shifts (Tables 25, 26) as well as gHMBC correlations and the COSY correlation between C-11 and C-12. The connectivity of the aromatic system with COSY sequence B was established by the long range correlations of H-4 and H-5 with C-6, as well as mutual long range correlations of C-7 and C-12 with H-5. The methyl acetal $CH_2$-9 ($\delta_{C/H}$ = 100.8/5.92 ppm) showed the expected correlations with C-8 ($\delta_C$ = 147.6 ppm) and C-10 ($\delta_C$ = 145.8 ppm).




**16**

Figure 45: Dihydropiperlonguminine (**16**) presented with the NMR COSY sequences in bold and gHMBC correlations with the key correlations in green arrows.

The NMR data of the isolated and synthesized dihydropiperlonguminine (**16**) are in good accordance with the data described in literature, except for the assignment of the quaternary carbons C-2 and C-12. The selected assignment was, however,

strongly supported by the unambiguously identified mutual $^{1}H,^{13}C$ gHMBC correlations of CH-2 and CH-4 and on the other side of CH-12 with CH-7 and CH-5 presented in Figure 45 in green arrows.

The compound has been identified, for example, in *P. chaba* (Matsuda et al., 2009), *P. guineense* (Adesina et al., 2003), *P. longum* (Tabuneng et al., 1983), and *Fagara macrophylla* (Kubo et al., 1984).

Table 25: $^{1}H$ NMR data of dihydropiperlonguminine (**16**) isolated from *M. excelsum* compared to those of the synthetic sample and to literature data (Bernard et al., 1995).

| | **isolated 400 MHz, $CDCl_3$** | **synthesized 400 MHz, $CDCl_3$** | **reported 500 MHz, acetone-*d6*** |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | | | |
| 2 | 5.77, dt (1.5, 15.2) | 5.77, dt (1.5, 15.2) | 5.96, dt (1.5, 15,2) |
| 3 | 6.84, dt (6.9, 15.2) | 6.84, dt (6.9, 15.3) | 6.75, dt (7.5, 15.2) |
| 4 | 2.45, m | 2.45, dtd (1.5, 7.0, 8.8) | 2.44, dq (1.5, 7.5) |
| 5 | 2.69, m | 2.68, dd (6.8, 8.7) | 2.69, t (7.5) |
| 6 | | | |
| 7 | 6.67, dd (0.4, 1.7) | 6.67, dd (0.5, 1.7) | 6.76, d (1.7) |
| 8 | | | |
| 9 | 5.92, s | 5.92, s | 5.95, s |
| 10 | | | |
| 11 | 6.73, dd (0.4, 7.8) | 6.72, d (7.9) | 6.75, d (7.9) |
| 12 | 6.62, ddt (0.6, 1.7, 7.9) | 6.62, ddt (0.6, 1.7, 7.8) | 6.69, dd (1.7, 7.9) |
| 13 | 3.15, dd (6.1, 6.9) | 3.14, dd (6.1, 6.9) | 3.16, t (6.9) |
| 14 | 1.79, thept (6.7) | 1.79, dp (6.7, 13.4) | 1.77, non (6.9) |
| 15/16 | 0.92, d (6.7) | 0.92, d (6.7) | 0.89, d (6.9) |

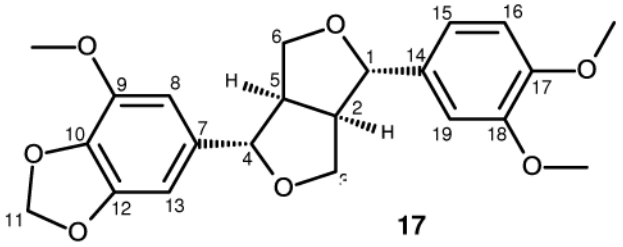
Table 26: $^{13}C$ NMR data of dihydropiperlonguminine (**16**) isolated from *M. excelsum* compared to the synthetic sample and literature data (Navickiene et al., 2000).

| | **isolated 100 MHz, $CDCl_3$** | **synthesized 100 MHz, $CDCl_3$** | **reported 50 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** |
| 1 | 165.8, C | 165.8, C | 166.0, C |
| 2 | 124.2, CH | 124.3, CH | 120.8, CH* |
| 3 | 143.3, CH | 143.3, CH | 142.6, CH |
| 4 | 34.1, CH2 | 34.1, CH2 | 33.8, CH2 |
| 5 | 34.4, CH2 | 34.5, CH2 | 34.1, CH2 |
| 6 | 134.9, C | 134.9 C | 134.6, C |
| 7 | 108.8, CH | 108.7 CH | 107.9, CH |
| 8 | 147.6, C | 147.6 C | 145.5, C |
| 9 | 100.8, CH2 | 100.8 CH2 | 100.5, CH2 |
| 10 | 145.8, C | 145.7 C | 147.5, C |
| 11 | 108.2, CH | 108.3 CH | 108.4, CH |
| 12 | 121.1, CH | 121.2 CH | 124.2, CH* |

| | isolated 100 MHz, $CDCl_3$ | synthesized 100 MHz, $CDCl_3$ | reported 50 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** |
| 13 | 46.8, CH2 | 46.9 CH2 | 46.7, CH2 |
| 14 | 28.6, CH | 28.6 CH | 28.3, CH |
| 15/16 | 20.1, CH3 | 20.1, CH3 | 19.9, CH3 |

* Diverse assignment of the quaternary carbons C-2 and C-12 in comparison with the literature.

Additionally, five lignans were isolated and identified in the FCPC fractions 17-22 of *M. excelsum*. For compound **17**, the determined molecular weight was 400 g $mol^{-1}$ with a molecular formula of $C_{22}H_{24}O_7$. In accordance with $^1H$ and $^{13}C$ NMR spectra (Tables 27, 28) and 2D data, the structure was assigned as 6-[(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-4-methoxy-1,3-benzodioxole (Figure 46). Two similar compounds were found in literature: one with similar stereochemistry but different substituent at position C-17, (+)-methoxypiperitol (De Carvalho et al., 1987) and a second with a different stereochemistry, 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan (Solis et al., 2005). Therefore, both literature references were compared and supported the assignment of **17**.




**17**

Figure 46: 6-[(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-4-methoxy-1,3-benzodioxole (**17**) from FCPC fractions 17-22 of *M. excelsum*.

Table 27: $^1H$ NMR data of 6-[(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-4-methoxy-1,3-benzodioxole (**17**) isolated from *M. excelsum* compared to literature data (De Carvalho et al., 1987; Solis et al., 2005).

| | isolated 400 MHz, $CDCl_3$ | (+)-methoxypiperitol 60 MHz, $CDCl_3$ | 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan 500 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 4.74, d (4.8) | 4.70, d (5.0) | 4.42, d (7.3) |
| 2 | 3.07, m | 3.05, m | 2.91, m |
| 3 | 3.89, m, 4.26, dd (6.6, 9.2) | 3.73-3.85, m, 4.07-4.40, m | 4.12, d (9.6), 3.83, d (9.6) |

| | isolated 400 MHz, $CDCl_3$ | (+)-methoxypiperitol 60 MHz, $CDCl_3$ | 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan 500 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 4 | 4.72, d (4,7) | 4.70, d (5.0) | 4.83, d (5.3) |
| 5 | 3.07, m | 3.05, m | 3.32, m |
| 6 | 3.89, m, 4.25, dd (6.6, 9.2) | 3.73-3.85, m, 4.07-4.40, m | 3.34, m, 3.84, d (9.4) |
| 7 | | | |
| 8 | 6.55, dd (0.6, 1.5) | 6.55, s | 6.6, s |
| 9 | | | |
| 10 | | | |
| 11 | 5.96, s | 5.95, s | 5.98, s |
| 12 | | | |
| 13 | 6.53, dd (0.5, 1.5) | 6.55, s | 6.52, s |
| 14 | | | |
| 15 | 6.87, ddd (0.6, 1.9, 8.2) | 6.8, 6.85, m | 6.89, dd (1.9, 8.1) |
| 16 | 6.84, d (8.2) | 6.8, 6.85, m | 6.84, d (8.1) |
| 17 | | | |
| 18 | | | |
| 19 | 6.90, d (1.9) | 6.8, 6.85, m | 6.93, d (1.9) |
| 9 OCH3 | 3.91, s | 3.9, s | 3.93, s |
| 17 OCH3 | 3.88, s | 5.8* | 3.88, s |
| 18 OCH3 | 3.90, s | 3.87, s | 3.9, s |

* In the cited literature, position $OCH_3$-17 has a hydroxyl group instead of the methoxy group

Table 28: $^{13}C$ NMR data of 6-[(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-4-methoxy-1,3-benzodioxole (**17**) isolated from *M. excelsum* compared to literature data (De Carvalho et al., 1987; Solis et al., 2005).

| | isolated 100 MHz, $CDCl_3$ | (+)-methoxypiperitol 60 MHz, $CDCl_3$ | 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan 125 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** |
| 1 | 85.7, CH | 85.4 | 87.6, CH |
| 2 | 54.1, 54.4, CH | 53.6 | 54.4, CH |
| 3 | 71.7, CH2 | 71.3 | 70.9, CH2 |
| 4 | 85.9, CH | 85.4 | 82.0, CH |
| 5 | 54.1, 54.4. CH | 54.0 | 50.1, CH |
| 6 | 71.7, CH2 | 71.3 | 69.6, CH2 |
| 7 | 135.8, C | 135.6 | 132.9, C |
| 8 | 105.5, CH | 105.4 | 104.8, CH |
| 9 | 143.6, C | 143.2 | 143.5, C |
| 10 | 134.6, C | 134.2 | 134.1, C |
| 11 | 101.5, CH2 | 101.0 | 101.4, CH2 |
| 12 | 149.1, C | 148.7 | 148.8, C |
| 13 | 100.0, CH | 99.7 | 99.9, CH |
| 14 | 133.4, C | 132.4 | 133.5, C |
| 15 | 118.2, CH | 118.5 | 118.5, CH |

| | isolated 100 MHz, $CDCl_3$ | (+)-methoxypiperitol 60 MHz, $CDCl_3$ | 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan 125 MHz, $CDCl_3$ |
|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** |
| 16 | 111.0, CH | 114.1 | 110.9, CH |
| 17 | 148.6, C | 145.0 | 149.2, C |
| 18 | 149.1, C | 146.5 | 148.7, C |
| 19 | 109.1, CH | 108.5 | 109.0, CH |
| 9 OCH3 | 56.7 | 56.3 | 56.6 |
| 17 OCH3 | 55.9 | * | 55.9 |
| 18 OCH3 | 55.9 | 55.5 | 55.9 |

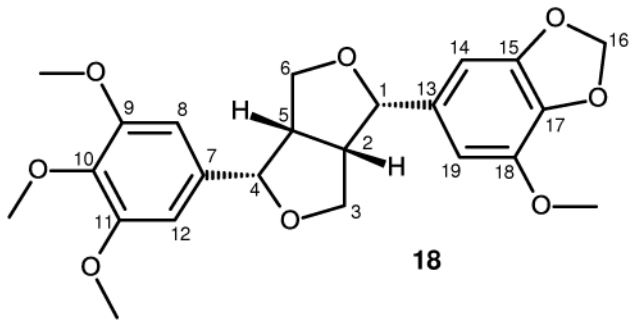
* There is no $^{13}C$ signal, because of a hydroxyl instead of a methoxy group in position $OCH_3$-17

The $^1H$ shifts of the isolated compound in comparison to the reported data of (+)-methoxypiperitol (De Carvalho et al., 1987) show a difference at position $OCH_3$-17, due to the hydroxyl group instead of the methoxy group. In the $^{13}C$ spectra, this modification was shown in positions C-16 to C-18 (114.1 ppm, 145.0 ppm, 146.5 ppm).

By comparing the data to the 3,4,5´-trimethoxy-3´,4´-methylenedioxy-7,9´:7´,9 diepoxylignan, having a different stereochemistry, the NMR data differ from position C-1 to C-7. The molecule described by Solis et al. was found in *P. fimbriulatum* (Solis et al., 2005).

On the basis of the parts which were comparable to the isolated compound, the molecular structure of **17** was in accordance and therefore led to the identification of 6-[(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-4-methoxy-1,3-benzodioxole.

The other four isolated lignans from the FCPC fractions 17-22 had a molecular weight of 430 g $mol^{-1}$ and a molecular formula of $C_{23}H_{26}O_8$. The structures of the compounds were confirmed by comparison of the $^1H$ NMR data with the data of a publication describing the four isomers in *Artemisia absinthium* (Greger and Hofer, 1980). As there are no $^{13}C$ data presented in this publication, further literature was used (Ahmed et al., 2002; Solis et al., 2005). For molecule **18**, (+)-diasesartemin (Figure 47), no $^{13}C$ data were so far published and only $^1H$ NMR shifts were available for comparison (Table 29).




**18**

Figure 47: (+)-Diasesartemin (4-methoxy-6-[(1*R*,3a*R*,4S,6a*R*)-tetrahydro-4-(3,4,5-trimethoxyphenyl)-1H,3H-furo[3,4-c]furan-1-yl]-1,3-benzodioxole, **18**) from FCPC fractions 17-22 of *M. excelsum*.

Table 29: $^{13}C$ and $^{1}H$ NMR data of (+)-diasesartemin (**18**) isolated from *M. excelsum* compared to literature data (Greger and Hofer, 1980).

| | **isolated 100 MHz, $CDCl_3$** | **isolated 400 MHz, $CDCl_3$** | **reported 100 MHz, $CDCl_3$** |
|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 84.1, CH | 4.88, d (5.5) | 4.89, d (4.5) |
| 2/5 | 49.5, 49.5, CH | 3.17, m | 3.18, m |
| 3/6 | 68.8, 68.9, CH2 | 3.56, 3.71, dd (2.4, 6.9, 9.6, 9.8), 3.57, 3.74, dd (2.1, 6.8, 9.7, 9.8) | 3.68, dd (9.5), 3.59, dd (9.5) |
| 4 | 84.0, CH | 4.90, d (5.5) | 4.89, d (4.5) |
| 7 | 134.3, C | | |
| 8/12 | 103.2, CH | 6.59, d (0.7) | 6.57, s |
| 9/11 | 153.2, C | | |
| 10 | 137.1, C | | |
| 13 | 133.5, C | | |
| 14 | 100.6, CH | 6.56, dd (0.7, 1.4) | 6.55, s |
| 15 | 148.9, C | | |
| 16 | 101.4, CH2 | 5.98, s | 5.96, s |
| 17 | 134.6, C | | |
| 18 | 143.6, C | | |
| 19 | 105.7, CH | 6.58, dd (0.8, 1.4) | 6.57, s |
| 9/11 OCH3 | 56.2, CH3 | 3.89, s | 3.89 |
| 10 OCH3 | 60.9, CH3 | 3.85, s | 3.86 |
| 18 OCH3 | 56.6, CH3 | 3.92, s | 3.93 |

For each of the other three isolated isomers, $^{1}H$ and $^{13}C$ data were in good agreement with the compared literature data. Compound **19** was identified as (+)-sesartemin (Figure 48), compound **20** as (+)-episesartemin B (Figure 49), and compound **21** as (+)-episesartemin A (Figure 50). All NMR data are presented in Tables 30-32.

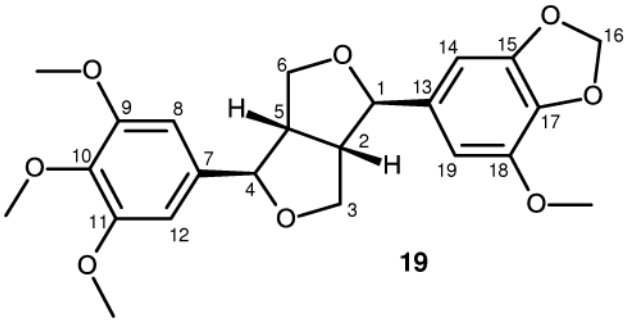



Figure 48: (+)-Sesartemin (6-[(3*R*,3a*S*,6*R*,6a*S*)-6-(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl]-4-methoxy-1,3-benzodioxole, **19**) from FCPC fractions 17-22 of *M. excelsum*.

Table 30: $^{13}C$ and $^{1}H$ NMR data of (+)-sesartemin (**19**) isolated from *M. excelsum* compared to literature data (Ahmed et al., 2002).

| | **isolated 100 MHz, $CDCl_3$** | **reported 500 MHz, $CDCl_3$** | **isolated 400 MHz, $CDCl_3$** | **reported 125 MHz, $CDCl_3$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1/4 | 85.8, 86.0, CH | 85.8, 86.0, CH | 4.72, d (4.3), 4.73, d (4.6) | 4.74, d (4.5) |
| 2/5 | 54.4, CH | 54.3, CH | 3.07, m | 3.08, m |
| 3/6 | 71.8, 72.0, CH2 | 71.8, 72.0, CH2 | 3.90, 3.91, dd (3.7, 3.8, 9.1, 9.2), 4.27, 4.29, dd (6.7, 6.8, 8.8, 8.9) | 3.90, 3.93, dd (3.6, 9.2), 4.27, 4.30, dd (6.7, 9.2) |
| 7 | 136.7, C | 136.7, C | | |
| 8/12 | 102.9, CH | 102.8, CH | 6.57, d (0.5) | 6.57, s |
| 9/11 | 153.4, C | 153.4, C | | |
| 10 | 137.5, C | 137.5, C | | |
| 13 | 135.8, C | 135.8, C | | |
| 14 | 100.1, CH | 100.0, CH | 6.53, dd (0.6, 1.4) | 6.53, d (1.5) |
| 15 | 149.1, C | 149.1, C | | |
| 16 | 101.5, CH2 | 101.5, CH2 | 5.96, d (1.5) | 5.97, s |
| 17 | 134.7, C | 134.6, C | | |
| 18 | 143.7, C | 143.6, C | | |
| 19 | 105.6, CH | 105.6, CH2 | 6.55, dd (0.6, 1.4) | 6.55, d (1.5) |
| 9/11 OCH3 | 56.20, CH3 | 56.2, CH3 | 3.87, s | 3.88, s |
| 10 OCH3 | 60.84, CH3 | 60.8, CH3 | 3.84, s | 3.85, s |
| 18 OCH3 | 56.73, CH3 | 56.7, CH3 | 3.92, s | 3.93, s |

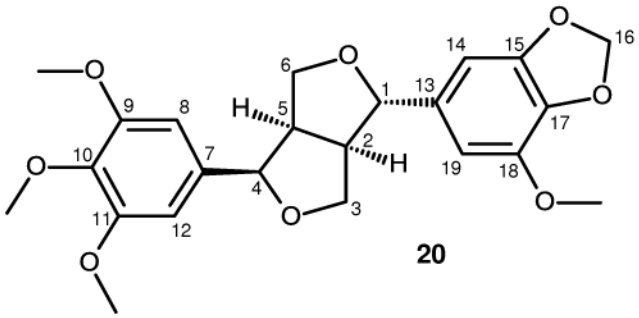
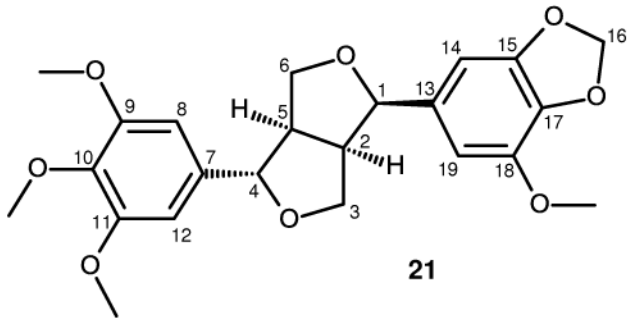



Figure 49: (+)-Episesartemin B (6-[(3*S*,3a*R*,6*R*,6a*R*)-6-(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl]-4-methoxy-1,3-benzodioxole, **20**) from FCPC fractions 17-22 of *M. excelsum*.

Table 31: $^{13}C$ and $^{1}H$ NMR data of (+)-episesartemin B (**20**) isolated from *M. excelsum* compared to literature data (Ahmed et al., 2002).

| | **isolated 100 MHz, $CDCl_3$** | **reported 500 MHz, $CDCl_3$** | **isolated 400 MHz, $CDCl_3$** | **reported 125 MHz, $CDCl_3$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 87.7, CH | 87.9, CH | 4.41, d (7.0) | 4.42, d (7.0) |
| 2 | 54.6, CH | 54.8, CH | 2.88, dddd (1.3, 6.3, 6.8, 9.0) | 2.89, m |
| 3/6 | 69.7, 71.0, CH2 | 70.0, 71.3, CH2 | 3.33, m, 3.87, m, 4.13, dd (1.2, 9.5) | 3.34, m, 3.88, m, 4.14, dd (0.9, 9.4) |
| 4 | 82.1, CH | 82.4, CH | 4.85, d (5.4) | 4.86, d (5.6) |
| 5 | 50.0, CH | 50.2, CH | 3.33, m | 3.36, m |
| 7 | 134.0, C | 134.2, C | | |
| 8/12 | 102.5, CH | 102.8, CH | 6.58, d (0.7) | 6.58, s |
| 9/11 | 153.2, C | 153.4, C | | |
| 10 | 136.8, C | 137.1, C | | |
| 13 | 135.8, C | 136.0, C | | |
| 14 | 100.2, CH | 100.4, CH | 6.55, dd (0.4, 1.4) | 6.56, d (1.5) |
| 15 | 149.0. C | 149.3, C | | |
| 16 | 101.5, CH2 | 101.7, CH2 | 5.96, d (1.5) | 5.97, s |
| 17 | 134.7, C | 135.0, C | | |
| 18 | 143.6, C | 143.9, C | | |
| 19 | 105.5, CH | 105.8, CH | 6.57, dd (0.4, 1.4) | 6.57, d (1.4) |
| 9/11 OCH3 | 56.1, CH3 | 56.4, CH3 | 3.88, s | 3.89, s |
| 10 OCH3 | 60.9, CH3 | 61.1, CH3 | 3.85, s | 3.87, s |
| 18 OCH3 | 56.6, CH3 | 56.9, CH3 | 3.91, s | 3.93, s |




**21**

Figure 50: (+)-Episesartemin A (6-[(3*R*,3a*R*,6*S*,6a*R*)-6-(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl]-4-methoxy-1,3-benzodioxole, **21**) from FCPC fractions 17-22 of *M. excelsum*.

Table 32: $^{13}C$ and $^{1}H$ NMR data of (+)-episesartemin A (**21**) isolated from *M. excelsum* compared to literature data (Solis et al., 2005).

| | **isolated 100 MHz, $CDCl_3$** | **reported 500 MHz, $CDCl_3$** | **isolated 400 MHz, $CDCl_3$** | **reported 500 MHz, $CDCl_3$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 82.1, CH | 81.9, CH | 4.83, d (5.4) | 4.83, d (5.1) |
| 2 | 50.1, CH | 50.0, CH | 3.33, m | * |
| 3/6 | 69.7, 71.0, CH2 | 69.7, 70.9, CH2 | 3.33, m, 3.86, m, 4.14, dd (1.2, 9.5) | 3.85-3.91, m, 4.14, d (9.6) |
| 4 | 87.8, CH | 87.8, CH | 4.42, d (7.1) | 4.41, d (7.3) |
| 5 | 54.6, CH | 54.5, CH | 2.91, dddd (1.4, 6.0, 7.2, 8.9) | * |
| 7 | 136.8, C | 136.7, C | | |
| 8/12 | 103.0, CH | 102.8, CH | 6.59, d (2.5) | 6.51, s |
| 9/11 | 153.4, C | 153.4, C | | |
| 10 | 137.6, C | 137.4, C | | |
| 13 | 132.9, C | 132.9, C | | |
| 14 | 99.9, CH | 99.8, CH | 6.52, dd (0.8, 1.4) | 6.52, d (0.9) |
| 15 | 148.9, C | 148.8, C | | |
| 16 | 101.4, CH2 | 101.4, CH2 | 5.97, m | 5.98, s |
| 17 | 134.2, C | 134.1, C | | |
| 18 | 143.6, C | 143.5, C | | |
| 19 | 105.0, CH | 104.8, CH | 6.59, d (2.5) | 6.59, d |
| 9/11 OCH3 | 56.2, CH3 | 56.1, CH3 | 3.88, s | 3.89, s |
| 10 OCH3 | 60.8, CH3 | 60.8, CH3 | 3.84, s | 3.84, s |
| 18 OCH3 | 56.7, CH3 | 56.6, CH3 | 3.93, s | 3.93, s |

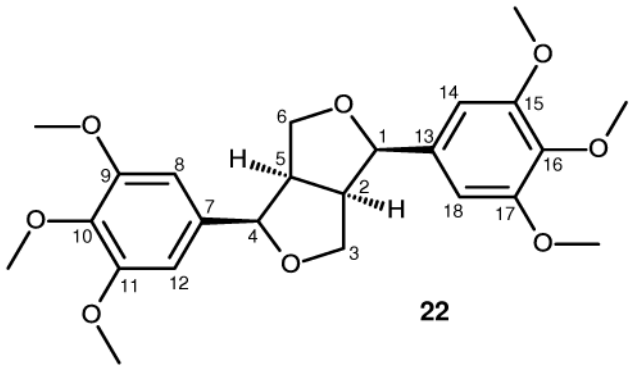
* Data not given in the cited literature

Additionally, the specific rotation was measured for **20** and **21** in chloroform at 23°C. Both compounds showed a positive rotation and the values were comparable to literature data (Greger and Hofer, 1980). For compound **20** the measured rotation was $[\alpha]_D$ + 132° (concentration = 0.22 g/100 mL), reported $[\alpha]_D$ + 127° (chloroform,

20°C, concentration = 0.4-0.5 g/100 mL) and for **21** the measured rotation was $[\alpha]_D$ + 128° (concentration = 0.39 g/100 mL), and reported $[\alpha]_D$ + 115° (chloroform, 20°C, concentration = 0.4-0.5 g/100 mL). The specific rotation was not measured for **18** and **19** due to the low amount available after pHPLC clean-up. **19** and **20** were found in *Achillea holosericea* (Ahmed et al., 2002), besides *Artemisia absinthium*. **21** occurs additionally in *P. fimbriulatum* (Solis et al., 2005).

Another lignan was directly isolated as the main component from the solvent left in the rotor after finishing the FCPC run. The 1D and 2D NMR data resulted in a molecule named (+)-diayangambin (**22**, Figure 51) with a molecular formula of $C_{24}H_{30}O_8$ (MW 446 g $mol^{-1}$). The measurement of the specific rotation and literature comparison (Table 33), confirmed the proposed structure. The specific rotation of $[\alpha]_D$ + 305° (concentration = 0.32 g/100 mL) was measured in chloroform at 23°C and was in good agreement with literature data ($[\alpha]_D$ + 289°, chloroform, 18°C) (Russell and Fenemore, 1973).

It is the only compound isolated in this study that is already described as a constituent of *M. excelsum* (Russell and Fenemore, 1973). Further sources are *P. fimbriulatum* (Solis et al., 2005) and *P. aborescens* (Duh et al., 1990).




**22**

Figure 51: (+)-Diayangambin ((3*R*,3a*R*,6*R*,6a*R*)-3,6-bis(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan, **22**), isolated from *M. excelsum* extract.

Table 33: $^{13}C$ and $^{1}H$ NMR data of (+)-diayangambin (**22**) isolated from *M. excelsum* compared to literature data (Solis et al., 2005).

| | **isolated 100 MHz, DMSO-*d6*** | **reported 500 MHz, $CDCl_3$** | **isolated 400 MHz, DMSO-*d6*** | **reported 500 MHz, $CDCl_3$** |
|---|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1/4 | 83.1, CH | 84.0, CH | 4.86, d (4.9) | 4.92, d (4.9) |
| 2/5 | 48.6, CH | 49.4, CH | 3.30, m | 3.21, m |
| 3/6 | 68.2, CH2 | 68.8, CH2 | 3.44, 3.50, dd (2.0, 7.0, 9.3, 9.4) | 3.59, 3.74, dd (1.3, 9.6) |
| 7/13 | 135.0, C | 134.5, C | | |
| 8/12/14/18 | 103.2, CH | 103.0, CH | 6.64, s | 6.61, s |
| 9/11/15/17 | 152.7, C | 153.2, C | | |
| 10/16 | 136.2, C | 136.9, C | | |
| 9/11/15/17 OCH3 | 55.8, CH3 | 56.0, CH3O | 3.78, s | 3.89, s |
| 10/16 OCH3 | 60.0, CH3 | 60.8, CH3O | 3.67, s | 3.85, s |

Using the polar fraction of the aqueous-ethanolic *M. excelsum* extract (Figure 25), two flavonoid glycosides were isolated (**23** and **24**). In addition to the NMR analysis of **23** and **24**, vitexin and isovitexin were measured in comparison (Figure 52, Table 34) to determine the position of the glucosides of the isolated molecules. Vitexin and isovitexin were chosen because of the presumed similarity to **23** and **24**.

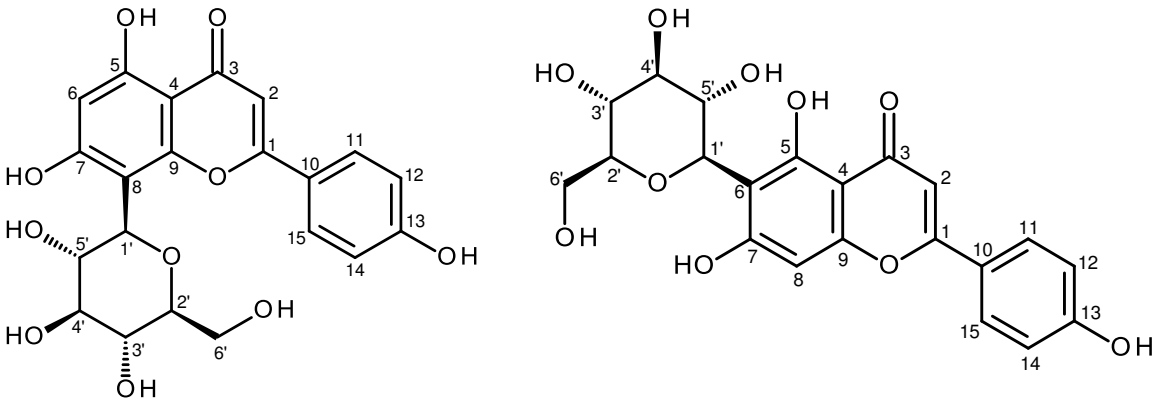


Figure 52: Vitexin and isovitexin, used as reference molecules for the confirmation of the glucosidation pattern of **23** and **24**.

Table 34: $^{13}C$ and $^{1}H$ NMR data of vitexin and isovitexin, measured for the identification of **23** and **24**.

| | **vitexin 100 MHz, $CD_3OD$** | **isovitexin 100 MHz, $CD_3OD$** | **vitexin 400 MHz, $CD_3OD$** | **isovitexin 400 MHz, $CD_3OD$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 166.4, C | 166.2, C | | |
| 2 | 103.6, CH | 103.9, CH | 6.56, s | 6.57, s |
| 3 | 183.9, C | 184.0, C | | |
| 4 | 104.9, C | 105.2, C | | |
| 5 | 162.6, C | 162.0, C | | |
| 6 | 100.5, CH | 109.2, C | 6.22, s | |
| 7 | 167.5, C | 164.9, C | | |
| 8 | 105.5, C | 95.2, CH | | 6.48, s |
| 9 | 158.3, C | 158.7, C | | |
| 10 | 123.6, C | 123.1, C | | |
| 11/15 | 130.2, CH | 129.4, CH | 7.95, m | 7.81, m |
| 12/14 | 117.4, CH | 117.0, CH | 6.93, m | 6.91, m |
| 13 | 163.1, C | 162.8, C | | |
| 1' | 75.6, CH | 75.3, CH | 5.01, d (9.9) | 4.90, d (9.9) |
| 2' | 83.0, CH | 82.6, CH | 3.48, dd (1.7, 3.3) | 3.42, ddd (2.3, 5.3, 9.8) |
| 3' | 72.8, CH | 71.8, CH | 3.61, m | 3.49, m |
| 4' | 80.6, CH | 80.1, CH | 3.52, t (8.9) | 3.49, m |
| 5' | 73.0, CH | 72.6, CH | 4.12, t (8.2) | 4.17, ddd (2.4, 6.9, 9.8) |
| 6' | 63.3, CH2 | 62.9, CH2 | 3.78, dd (5.5, 12.0), 3.93, d (11.9) | 3.75, dd (5.2, 12.1), 3.88, dd (2.2, 12.1) |

As shown for vitexin and isovitexin, the positions C-6 and C-8 in the $^{13}C$-spectra were crucial for the identification of the glucoside binding position in **23** and **24**. With a chemical shift of $\delta_C$ = 100.53 ppm at C-6 and $\delta_C$ = 105.48 ppm at C-8, the data are comparable to the corresponding spectra of **23** (Table 35) with the glucoside located at position C-8. Thus, compound **23** was identified as vitexin-2''-O-glucoside (Figure 53) with a molecular weight of 594 g $mol^{-1}$ and a molecular formula of $C_{27}H_{30}O_{15}$. Comparison with the literature NMR data was congruent. The specific rotation of $[\alpha]_D$ + 8° (concentration = 0.15 g/100 mL) was measured in ethanol at 23°C. However, no specific rotation was found in literature. The compound was described in different plant sources, e.g. in *Adonis aleppica* (Pauli and Junior, 1995), passion flower *Passiflora palmeri* (Ulubelen et al., 1984), *Cryptocoryne usteriana* (Franke et al., 2006), *Podocarpus totara* (Markham et al., 1985), and in beet *Beta vulgaris* (Isayenkova et al., 2006).

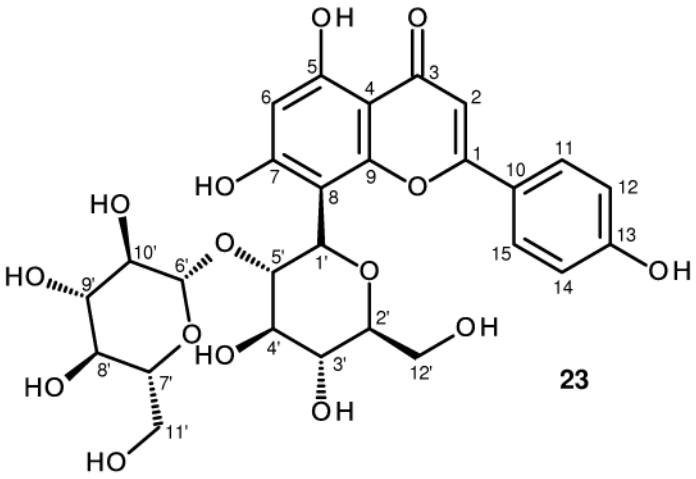
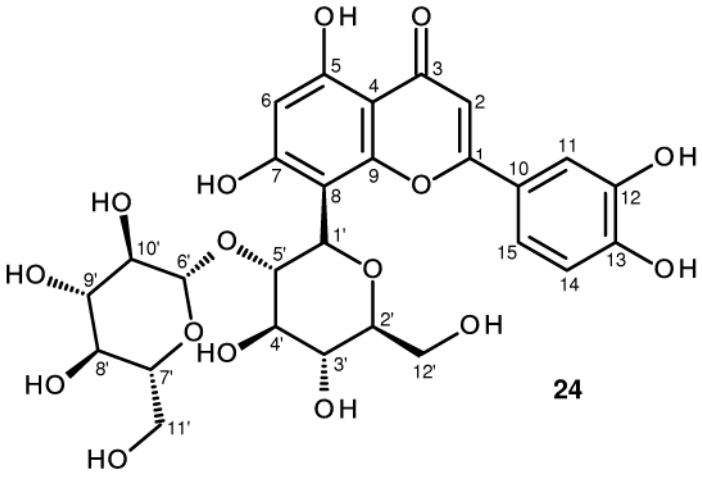



Figure 53: Vitexin-2"-O-glucoside (Flavosativaside, **23**) from the polar fraction of the aqueous-ethanolic *M. excelsum* extract.

Table 35: $^{13}C$ and $^{1}H$ NMR data of vitexin-2"-O-glucoside (**23**) isolated from *M. excelsum* compared to literature data (Isayenkova et al., 2006).

| | **isolated 100 MHz, $CD_3OD$** | **reported 100 MHz, $CD_3OD$** | **isolated 400 MHz, $CD_3OD$** | **reported 400 MHz, $CD_3OD$** |
|---|---|---|---|---|
| **position** | **$\delta_C$ (ppm)** | **$\delta_C$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 166.6, 167.0, C | 166.3, 166.5, C | | |
| 2 | 103.8, 104.0, CH | 103.8, 104.0, CH | 6.61, s | 6.64 |
| 3 | 184.2, 184.3, C | 184.2, 184.3, C | | |
| 4 | 105.5, 105.6, C | 105.5, 105.6, C | | |
| 5 | 162.7-163.0, C | 162.7-163.0, C | | |
| 6 | 99.5, 100.8, CH | 99.5, 100.8, CH | 6.25, 6.26, s | 6.29, 6.3 |
| 7 | 164.6, 164.9, C | 164.6, 164.9, C | | |
| 8 | 104.5, 105.1, C | 104.5, 105.1, C | | |
| 9 | 158.3, 158.5, C | 157.7, 158.3, C | | |
| 10 | 123.6, C | 123.7, C | | |
| 11/15 | 129.7, 130.2, CH | 129.7, 130.1, CH | 7.86, 7.96, m | 7.9, 8.03 |
| 12/14 | 117.1, CH | 117.0, CH | 6.95, m | 6.99 |
| 13 | 162.7-163.0, C | 162.7-163.0, C | | |
| 1' | 73.6, 74.8, CH | 73.6, 74.9, CH | 5.05, d (10.0), 5.14, d (9.9) | 5.1, 5.18 |
| 2' | 82.9, 83.0, CH | 82.9, CH | 3.48, m | 3.5, 3.55 |
| 3' | 71.8, 72.1, CH | 72.1, 72.3, CH | 3.56, t (7.7), 3.74, m | 3.6, 3.72 |
| 4' | 79.8, 80.3, CH | 79.8, 80.2, CH | 3.74, m | 3.77, 3.8 |
| 5' | 81.7, 82.4, CH | 81.7, 82.4, CH | 4.32, m | 4.37 |
| 6' | 105.8, 106.2, CH | 105.8, 106.1, C | 4.2, d (7.7), 4.33, d (8.6) | 4.24, 4.35 |
| 7' | 77.7, 77.9, CH | 77.7, 77.9, CH | 2.71, dt (3.3, 9.0), 2.89, dt (4.4, 9.5), 3.16, m | 2.76, 2.93 |
| 8' | 71.1, 71.3, CH | 71.1, 71.3, CH | 3.07, t (9.3), 3.16, m | 3.11, 3.20 |
| 9' | 77.1, 77.6, CH | 77.1, 77.5, CH | 2.71, dt (3.3, 9.0), 2.89, dt (4.4, 9.5), 3.16, m | 3.17, 3.25 |
| 10' | 75.8, 76.1, CH | 75.8, 76.1, CH | 2.95, m, 3.0, t (8.4) | 2.99, 3.04 |
| 11' | 62.3, 62.6, C | 62.3, 62.5, C | 3.31, m | 3.32-3.43 |
| 12' | 62.6, 62.9, C | 62.6, 62.9, C | 3.74, 3.92, m | 3.81-4.00 |

With regard to the 1D and 2D NMR spectra (Table 36), the other flavonoid glycoside was shown to contain one additional hydroxyl group at the B-ring of the flavonoid compared to **23**. Compound **24** is described therefore as orientin-2''-O-glucoside (Figure 54) with a molecular mass of 610 g $mol^{-1}$ and a molecular formula of $C_{27}H_{30}O_{16}$. The specific rotation of $[\alpha]_D$ + 5° (concentration = 0.51 g/100 mL) was measured in ethanol at 23°C. However, no specific rotation was found in literature. The compound occurs in plants, such as *Setaria italica* (Gluchoff-Fiasson et al., 1989), *Podocarpus totara* (Markham et al., 1985), and *Trollius europaeus* (Maciejewska-Rutkowska et al., 2007). However, no NMR spectra were found in the literature for direct comparison of the spectroscopic data. Therefore, the $^{1}H$- and $^{13}C$-information of the isolated vitexin-2''-O-glucoside (**23**) was used, as already described in Table 35. The compared shifts were in accordance, just the data at the positions C-11 to C-15 varied, which was due to the different chemical environment of the several substitution patterns (Table 36).




**24**

Figure 54: Orientin-2''-O-glucoside (Flavocannabiside, **24**) from the polar fraction of the aqueous-ethanolic *M. excelsum* extract.

Table 36: $^{13}C$ and $^{1}H$ NMR data of orientin-2''-O-glucoside (**24**) compared to vitexin-2''-O-glucoside (**23**), both isolated from *M. excelsum.*

| | **24** 100 MHz, $CD_3OD$ | **23** 100 MHz, $CD_3OD$ | **24** 400 MHz, $CD_3OD$ | **23** 400 MHz, $CD_3OD$ |
|---|---|---|---|---|
| **position** | **$\delta_c$ (ppm)** | **$\delta_c$ (ppm)** | **$\delta_H$ (ppm), mult (*J* in Hz)** | **$\delta_H$ (ppm), mult (*J* in Hz)** |
| 1 | 166.5, 166.6, C | 166.6, 167.0, C | | |
| 2 | 103.8, 104.0, CH | 103.8, 104.0, CH | 6.54, 6.56, s | 6.61, s |
| 3 | 184.2, 184.3, C | 184.2, 184.3, C | | |
| 4 | 105.5, 105.6, C | 105.5, 105.6, C | | |
| 5 | 162.6, 162.9, C | 162.7-163.0, C | | |
| 6 | 99.5, 100.8, CH | 99.5, 100.8, CH | 6.25, 6.26, s | 6.25, 6.26, s |
| 7 | 164.7, 164.9, C | 164.7, 164.9, C | | |
| 8 | 104.6, 105.1, C | 104.5, 105.1, C | | |
| 9 | 157.7, 158.3, C | 158.3, 158.5, C | | |
| 10 | 124.1, 124.1, C | 123.6, C | | |
| 11 | 114.4, 115.1, CH | 129.7, 130.2, CH | 7.39, m, 7.56, d (2.0) | 7.86, 7.96, m |
| 12 | 147.1, 147.2, C | 117.1, CH | | 6.95, m |
| 13 | 151.0, 151.1, C | 162.7-163.0, C | | |
| 14 | 116.8, 116.9, CH | 117.1, CH | 6.92, d (8.6) | 6.95, m |
| 15 | 120.6, 120.9, CH | 129.7, 130.2, CH | 7.38, m, 7.51, dd (2.1, 8.3) | 7.86, 7.96, m |
| 1` | 73.7, 74.8, CH | 73.6, 74.8, CH | 5.05, d (10.0), 5.15, d (9.8) | 5.05, d (10.0), 5.14, d (9.9) |
| 2` | 82.6, 82.9, CH | 82.9, 83.0, CH | 3.51, m | 3.48, m |
| 3` | 71.3, 72.0, CH | 71.8, 72.1, CH | 3.51, 3.81, m | 3.56, t (7.7), 3.74, m |
| 4` | 79.7, 80.3, CH | 79.8, 80.3, CH | 3.81, m | 3.74, m |
| 5` | 81.6, 82.3, CH | 81.7, 82.4, CH | 4.35, m | 4.32, m |
| 6` | 105.8, 106.1, CH | 105.8, 106.2, CH | 4.21, d (7.8), 4.34, d (7.7) | 4.2, d (7.7), 4.33, d (8.6) |
| 7` | 77.7, 77.9, CH | 77.7, 77.9, CH | 3.17, m | 3.16, m |
| 8` | 71.1, 71.3, CH | 71.1, 71.3, CH | 3.08, t (9.3), 3.17, m | 3.07, t (9.3), 3.16, m |
| 9` | 77.1, 77.5, CH | 77.1, 77.6, CH | 2.73, dt (3.1, 9.0), 2.9, dt (3.5, 9.0) | 2.71, dt (3.3, 9.0), 2.89, dt (4.4, 9.5) |
| 10` | 75.8, 76.1, CH | 75.8, 76.1, CH | 2.95, 3.0, m | 2.95, m, 3.0, t (8.4) |
| 11` | 62.3, 62.6, CH2 | 62.3, 62.6, C | 3.32, m | 3.31, m |
| 12` | 62.6, 63.1, CH2 | 62.6, 62.9, C | 3.81, m, 3.98, dd (1.9, 12.1) | 3.74, 3.92, m |

The calculated and measured molecular weights and the UV data of all isolated compounds described above (**8**, **10**-**24**), are summarized in Table 37.

Table 37: Calculated and measured molecular weights of the isolated compounds **8** and **10**-**24** via high resolution mass spectrometry (HR-ESI-MS), mass fragments (m/z), and UV data.

| compound | HR-MS (calc) | HR-MS | molecular formula $[M+H]^+$ | *m/z* | UV $\lambda_{max}$ (nm) |
|---|---|---|---|---|---|
| **8** | 252.2322 | 252.2330 | $C_{16}H_{30}NO$ | 196.1708 | 220, 260 |
| **10** | 261.1121 | 261.1112 | $C_{15}H_{17}O_4$ | 128.0628, 135.0451 | 200, 260 |
| **11** | 302.1751 | 302.1737 | $C_{18}H_{24}NO_3$ | 135.0454 | 200, 220, 260 |
| **12** | 314.1751 | 314.1752 | $C_{19}H_{24}NO_3$ | 135.0451 | 200, 265 |
| **13** | 216.1383 | 216.1395 | $C_{14}H_{18}NO$ | 103.0553, 131.0507 | 210, 220, 230, 280 |
| **14** | 260.1281 | 260.1282 | $C_{15}H_{18}NO_3$ | 89.0382, 125.0299, 175.0408 | 220, 230, 290, 320 |
| **15** | 260.1281 | 260.1290 | $C_{15}H_{18}NO_3$ | 89.0386, 125.0301, 175.0411 | 215, 270, 300 |
| **16** | 276.1594 | 276.1588 | $C_{16}H_{22}NO_3$ | 135.0466 | 200, 215, 285 |
| **17** | 401.1595 | 401.1575 | $C_{22}H_{25}O_7$ | 151.0762, 165.0553 | 220 |
| **18** | 431.1700 | 431.1697 | $C_{23}H_{27}O_8$ | 165.0560 | 220 |
| **19** | 431.1700 | 431.1684 | $C_{23}H_{27}O_8$ | 165.0555 | 220 |
| **20** | 431.1700 | 431.1695 | $C_{23}H_{27}O_8$ | 165.0561, 203.0714 | 220 |
| **21** | 431.1700 | 431.1670 | $C_{23}H_{27}O_8$ | 165.0550, 203.1701 | 225 |
| **22** | 447.2013 | 447.2002 | $C_{24}H_{31}O_8$ | 181.0868, 204.0788, 219.1013 | 210, 270 |
| **23** | 595.1657 | 595.1623 | $C_{27}H_{31}O_{15}$ | 271.0574, 313.0680, 433.1097 | 220, 270, 300, 330 |
| **24** | 611.1607 | 611.1602 | $C_{27}H_{31}O_{16}$ | 287.0549, 329.0653, 449.1080 | 220, 250, 265, 290, 340 |

In summary, a total of 24 molecules were found in *M. excelsum*. Among them, only myristicin (**1**), elemicin (**2**), and (+)-diayangambin (**22**) were already described as constituents of *M. excelsum* (Briggs, 1941; Briggs et al., 1975; Russell and Fenemore, 1973); all others were identified in kawakawa for the first time. Methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**) was so far not described in literature. The remaining molecules are known from other plant sources.

The LC-MS chromatogram in Figure 55 gives an overview of the identified compounds (**1**-**24**) in the aqueous-ethanolic extract of *M. excelsum*.

Nearly all main compounds in the medium-polar section of the chromatogram were unambiguously identified, additionally two flavonoid-glucosides from the polar section.

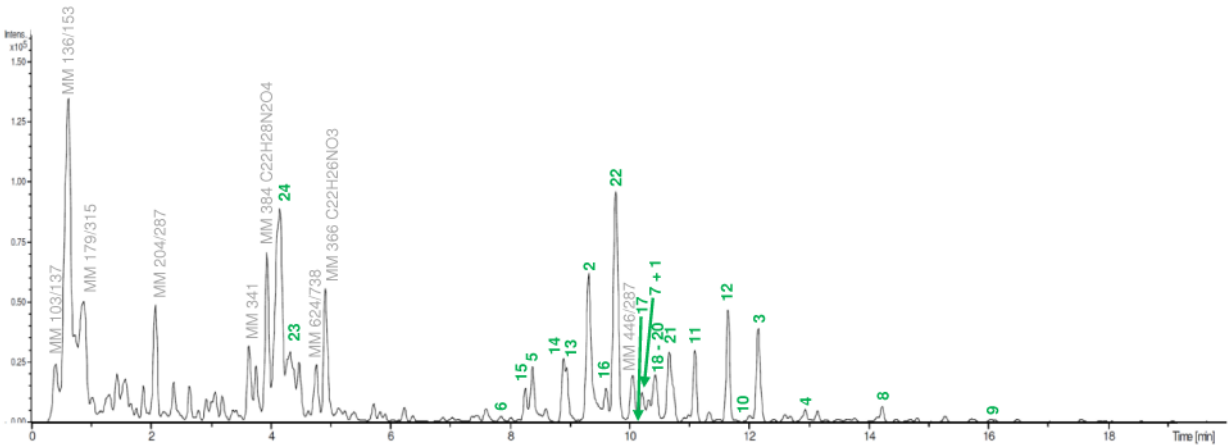



Figure 55: LC-MS chromatogram (positive mode) of the aqueous-ethanolic extract of *M. excelsum* with the isolated and identified compounds; for conditions see 3.3.6.

#### 4.1.2.7 Comparison of the Contents of Non-Volatile Comounds in Different Parts of the *M. excelsum* Plant

The objective of this part of the study was to determine the contents of the non-volatile compounds not only in the dried leaves of *M. excelsum* but also in other parts of the plant. Therefore, a living specimen, grown in Germany, was separated into roots, stems, and leaves (M.e P). Additionally, dried fruits (M.e. F) and seeds (M.e. S) were purchased from New Zealand. The different parts were extracted with ethanol and water (4:1) as described in chapter 3.3.1.3 and analyzed via LC-MS. For direct comparison of fresh and dried leaves, a sample of the dried leaves from New Zealand (M.e. L) was extracted in parallel under the same conditions.

*trans*-Pellitorine (**3**), its two homologues kalecide (**8**) and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**), and another amide, ilepcimide (**14**), as well as two of the lignans, (+)-episesartemin B (**20**) and (+)-diayangambin (**22**) were chosen for the quantification. The quantification was carried out using hydrocortisone as external standard, on the basis of peak areas and taking into account the respective response factors determined for the reference substances.

The samples from New Zealand (M.e. F, M.e. S, and M.e. L) were delivered as dried material and therefore the exact mass loss during drying was not known. To compare the different parts, the concentrations were calculated on the basis of the dried plant material. For the parts of the fresh plant, the mass losses was determined in duplicate and used as basis for the calculations. The average mass losses were 85.7 % (roots), 85.3 % (stems), and 88.1 % (leaves). The results are summarized in Table 38.

Table 38: Concentrations of *trans*-pellitorine (**3**), kalecide (**8**), (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**), ilepcimide (**14**), (+)-episesartemin B (**20**), and (+)-diayangambin (**22**) in different parts (dried material) of the *M. excelsum* plant.

| content (mg kg$^{-1}$) / sample | 3 | 8 | 9 | 14 | 20 | 22 |
|---|---|---|---|---|---|---|
| roots (M.e. P) | 1284 | 110 | 11 | 2505 | 63 | 313 |
| stems (M.e. P) | 76 | 5 | < 1 | 223 | 82 | 390 |
| leaves (M.e. P) | 5 | < 2 | < 2 | 5 | 269 | 1007 |
| dried leaves (M.e. L) | 32 | 5 | < 2 | 36 | 680 | 1814 |
| dried fruits (M.e. F) | 953 | 125 | 15 | 1026 | 147 | 366 |
| dried seeds (M.e. S) | 693 | 108 | 12 | 843 | 331 | 723 |

In the different parts of the plant, the amides (**3**, **8**, **9**, and **14**) were found at the highest concentrations in the dried roots (approximately 1284 mg kg$^{-1}$ of **3** and 2505 mg kg$^{-1}$ of **14**), followed by the dried stems and leaves. For the lignans, the highest concentrations were found in the leaves (269 mg kg$^{-1}$ of **20** and 1007 mg kg$^{-1}$ of **22**).

The measured compounds were present in higher amounts in the leaves from New Zealand (M.e. L) than in the leaves harvested from a plant grown in Germany (M.e. P). This variation may be explained by the fact, that the dried leaves were grown and harvested in their natural habitat in New Zealand. Different climatic conditions, soil, age of the plants, and herbivores may have influenced the composition of the plant ingredients.

The amounts of the amides in the dried fruits and seeds were high and in a similar range as in the roots. The concentrations of the lignans were higher than in the roots and more comparable to the stems and leaves, respectively. As example, a comparison of the LC-MS chromatograms obtained from the dried seeds and the dried leaves from New Zealand is shown in Figure 56.

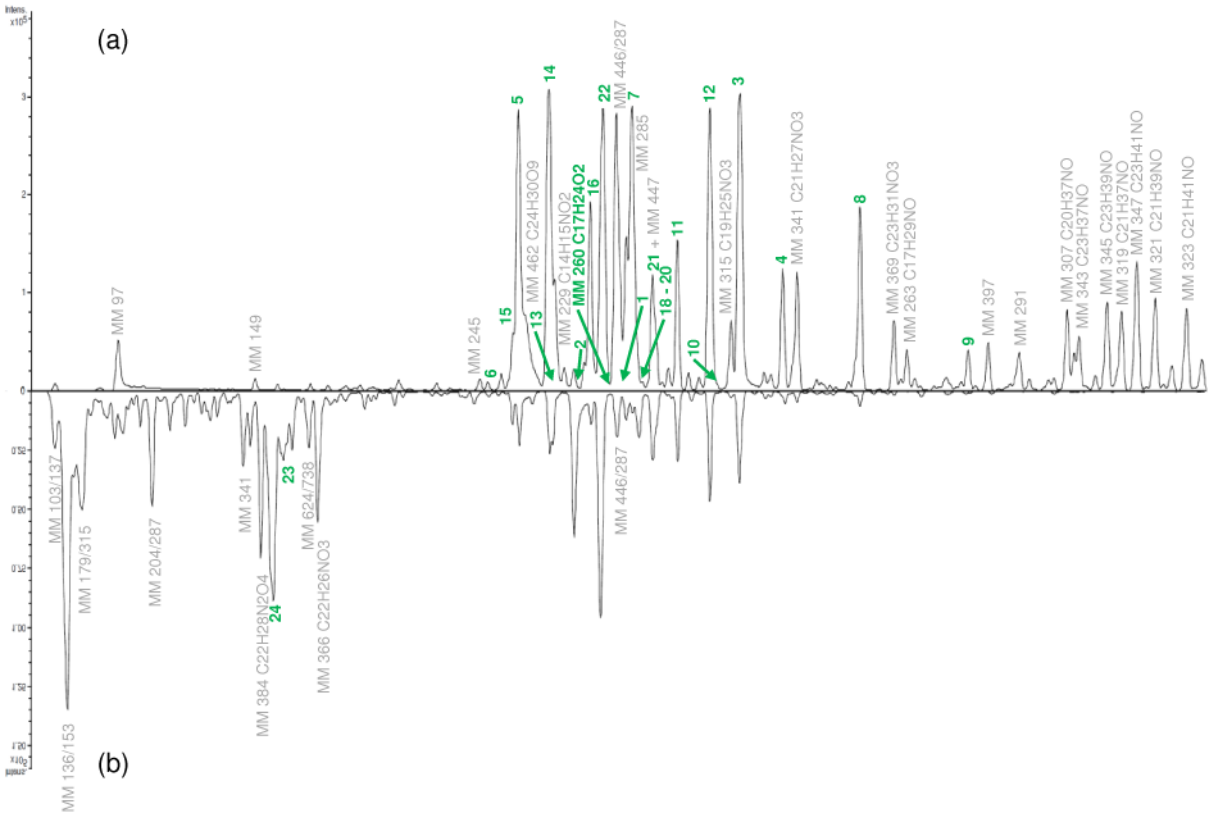



Figure 56: Comparison of the LC-MS chromatograms of the aqueous-ethanolic extracts of *M. excelsum* dried seeds (a) and dried leaves from New Zealand (b) with the isolated and identified compounds; for conditions see 3.3.6.

In the medium-polar part of the chromatogram, every molecule that was identified in the leaves could also be determined in the seeds. Additionally, a further compound was found in the seeds with a molecular weight of 260 g mol$^{-1}$ and a molecular formula of C17H24O2. The experimental data fit well with data described for juvadecene, a juvenile hormone mimic discovered in *M. excelsum* (Nishida et al., 1983). For a final identification, however, more work would be necessary.

The two flavonoid glycosides vitexin-2''-O-glucoside (**23**) and orientin-2''-O-glucoside (**24**), which were isolated from the leaves, were not found in the seeds. Additional unknown non-polar *N*-containing compounds were found in this extract with molecular weights between 307 and 347 g mol$^{-1}$.

The results demonstrated that the tested compounds differ in their concentrations depending on the analyzed part of *M. excelsum*. Yet, only a few molecules were not present in every part of the plant.

### 4.1.3 Sensory Evaluation of the Compounds Identifid in *M. excelsum*

#### 4.1.3.1 Development of a Common Flavor Language

The crude extract as well as the HTLC fractions obtained from *M. excelsum* showed trigeminal effects. Therefore, panelists were trained regarding the identification and evaluation of trigeminal-active compounds, using corresponding reference substances. A number of compounds (Table 3, 3.3.8.3) were tested and classified according to their trigeminal sensation. The molecules, for which a single sensation could be unambiguously described by all panelists, were defined as the respective reference compounds (Table 39).

For cooling, tingling, and nasal pungency the determination was relatively easy. WS3 was described as cooling without any off-notes at a concentration of 10 mg $kg^{-1}$. Menthol, one of the best known cooling compounds, was not used as a reference because of its additional minty aroma. *trans*-Pellitorine showed a tingling effect at 10 mg $kg^{-1}$ and allyl isothiocyanate was described as typically nasal pungent at 7 mg $kg^{-1}$. While reference compounds could be found for the named descriptors, panelists were not able to clearly differentiate between the descriptors hot, burning, pungent, and warming, when testing several other molecules. Depending on the substance and the concentration, the descriptors were used differently and no agreement was possible. Thus, two references, nonivamide (0.5 mg $kg^{-1}$) and piperine (15 mg $kg^{-1}$), were defined as the standards for the four named trigeminal sensations.

As the descriptor astringent is crucial for further tests on astringency masking and the molecular mechanism of action is not fully understood, it is listed in Table 39 together with the trigeminal-active compounds. Both tannin and a commercial grape seed extract showed a clear astringency at the tested concentrations and were therefore chosen as references. EGCG was also evaluated, but the additional bitter taste excluded the compound as a reference to describe clear astringent sensation.

Table 39: Selected compounds and their concentrations for the description of trigeminal and astringency sensations (* no explicit attribution to a single trigeminal description).

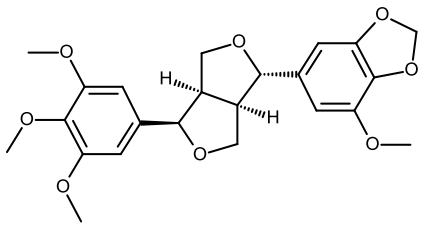
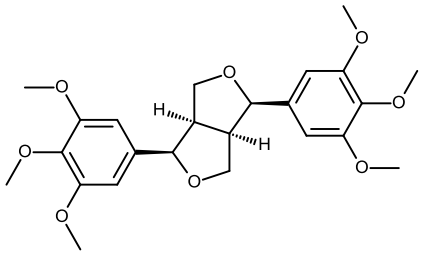
| compound | dosage on water (mg kg$^{-1}$) | descriptor |
|---|---|---|
| WS3 (**35**) | 10 | cooling |
| *trans*-pellitorine (**3**) | 10 | tingling |
| allyl isothiocyanate | 7 | nasal pungency |
| nonivamide* (**31**) | 0.5 | miscellaneous (hot, burning, pungent, warming) |
| piperine* (**7**) | 15 | |
| tannin | 500 | astringent |
| grape seed extract | 750 | |

#### 4.1.3.2 Sensory Description of the Single Compounds

As mentioned in chapter 4.1.2.2, the crude extract of *M. excelsum* was perceived by the panelists as bitter, sweet and herbal, and showed trigeminal effects. Sensory analysis via HTLC revealed fractions 10, 11, and 12 as the most interesting regarding the trigeminal effects. However, each of these fractions was still quite complex and no direct correlation between individual compounds and the sensory description could be made. Hence, the identities of the compounds were elucidated after isolation via FCPC, followed by pHPLC. For some compounds, the amounts isolated were not sufficient for sensory evaluation and therefore only used for structure elucidation. In those cases where synthetic samples were available, these were used to evaluate the sensory properties of the molecules. The data are summarized in Tables 40-44. The molecules were grouped according to their chemical structures in lignans, flavonoid glycosides, piperidides, isobutyl amides, and esters. The intrinsic taste of each compound was evaluated at least at two concentrations, 10 and 50 mg kg$^{-1}$ in water. Only *trans*-pellitorine (**3**) was tested at lower concentration levels as it was already described as strongly tingling at 10 mg kg$^{-1}$. Flavor descriptions of most molecules were not reported in literature so far.

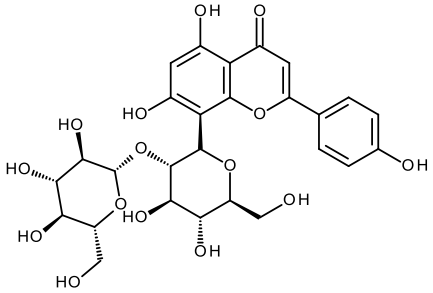
The two tested lignans, (+)-episesartemin B (**20**) and (+)-diayangambin (**22**), showed primarily trigeminal activity. **20** was described as burning, hot, cooling, and numbing, while **22** showed tingling, mouth-watering, and cooling effects (Table 40). For both compounds the effects were not very strong and therefore no further investigations were carried out.

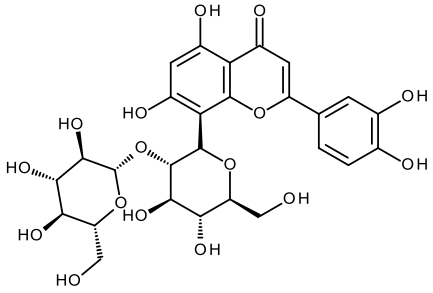
Table 40: Flavor descriptions of selected lignans identified in *M. excelsum*.

| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| (+)-episesartemin B (**20**)  | 10<br>50 | - slightly burning/hot<br>- slightly bitter, slightly burning, numbing, cooling |
| (+)-diayangambin (**22**)  | 10<br>50 | - tingling (delayed), mouth-watering, fresh/ cooling<br>- tingling (delayed), mouth-watering, irritating |

Vitexin- and orientin-2''-*O*-glucoside (**23**, **24**) were slightly woody at 10 mg kg$^{-1}$, and showed fatty, fruity, bitter, and astringent notes at 50 mg kg$^{-1}$ (Table 41). The two flavonoid glycosides differ in one methoxy group at the B-ring. This variation seems to make no difference in sensory perception under the tested conditions.

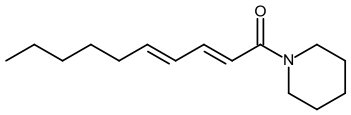
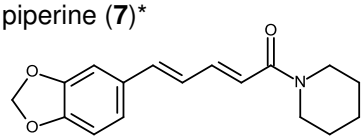
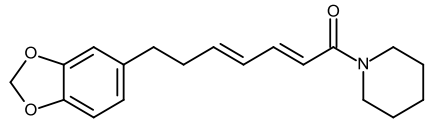
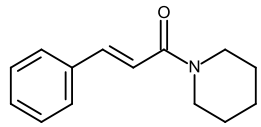
Table 41: Flavor descriptions of the flavonoid glycosides identified in *M. excelsum*.

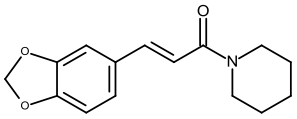
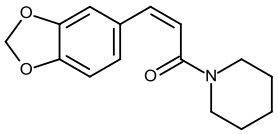
| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| vitexin-2''-*O*-glucoside (**23**)  | 10<br>50 | - slightly woody, bitter<br>- slightly fatty, fruity, slightly mouth-watering, tingling |

| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| orientin-2''-*O*-glucoside (**24**)  | 10<br>50 | - slightly woody<br>- slightly astringent, bitter, mouth-watering |

At 10 mg kg$^{-1}$ most of the tested piperidides (**12**-**15**) were described to be neutral or to have weak flavor impressions (Table 42). Only piperine (**7**) and achilleamid (**4**) showed already pungent, throat irritating, and hot sensations. At 50 mg kg$^{-1}$, some molecules developed trigeminal sensations, beside a few bitter and umami tastes. As the cooling effect of **15** and the umami taste of **13** and **14** were only weak, no further tests were carried out.

Table 42: Flavor descriptions of the piperidides identified in *M. excelsum*.

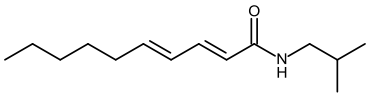
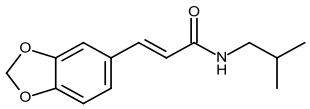
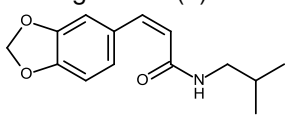
| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| achilleamid (**4**)*  | 10<br>20<br>50 | - throat irritating, hot, cardboard<br>- hot, tingling, mouth-watering<br>- bitter, fatty, nutty, dry, delayed hot |
| piperine (**7**)*  | 10<br>20<br>50 | - pungent<br>- strong pungent, burning<br>- strong pungent, burning, hot |
| piperdardine (**12**)*  | 10<br>50 | - slightly pyrazinic, slightly dry/astringent, slightly tingling<br>- bitter, hot (peppery) |
| *N*-(*trans*-cinnamoyl)piperidine (**13**)*  | 10<br>50 | - neutral, slightly hot (few panelists)<br>- umami, hot (delayed) |

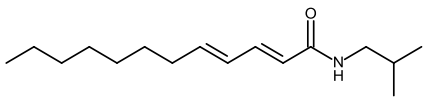
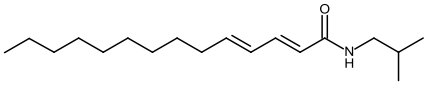
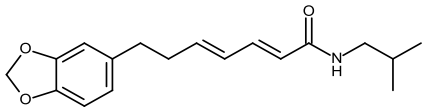
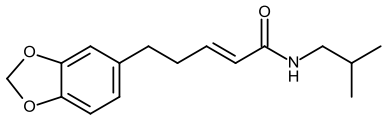
| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| ilepcimide (**14**)*  | 10<br>50 | - neutral, slightly tingling (delayed)<br>- hot (delayed), irritating, slightly umami |
| *Z*-antiepilepsirine (**15**)*  | 10<br>50 | - neutral<br>- bitter, dry, slightly irritating and cooling |

* Synthetic sample

Among the isobutyl amides isolated from *M. excelsum*, a number of molecules had trigeminal effects. *trans*-Fagaramid (**5**) was described as burning, *cis*-fagaramid (**6**) and *trans*-pellitorine (**3**) as tingling, and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) as tingling, as well as hot and cooling. Furthermore, descriptions such as coumarinic (**5**), bitter (**6**, **16**), soy-like (**3**), fatty, waxy, and rancid (**9**, **8**) were used for the evaluation of the isobutyl amides (Table 43). Compounds **5** and **16** showed an umami taste, and **9** cooling activity. But in all cases the intensity was not strong enough compared to known umami and cooling compounds. Therefore, no further tests were done.

Table 43: Flavor descriptions of the isobutyl amides identified in *M. excelsum*.

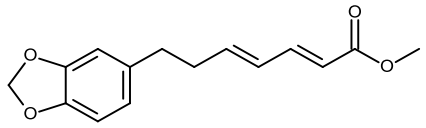
| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| *trans*-pellitorine (**3**)*  | 5<br>10 | - tingling, mouth-watering, soy note<br>- strong tingling, strong mouth-watering, soy note, numbing |
| *trans*-fagaramid (**5**)*  | 10<br>20<br>50 | - slightly coumarinic and tingling<br>- coumarinic, benzaldehyde-like<br>- coumarinic, benzaldehyde-like, slightly burning, slightly umami, mouth-watering |
| *cis*-fagaramid (**6**)*  | 10<br>20<br>50 | - neutral, slightly glue<br>- slightly bitter, slightly dry, slightly trigeminal<br>- bitter, astringent, mouth-watering, tingling |

| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| kalecide (**8**)*<br>  | 10<br>50 | - fatty, rancid, soapy<br>- fatty, oily, rancid, slightly irritating |
| (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**)*<br>  | 10<br>50<br>100 | - neutral, slightly irritating<br>- slightly irritating (punctual), fatty, waxy<br>- slightly tingling, hot, cooling |
| chingchengenamide A (**11**)*<br>  | 10<br>50 | - slightly throat irritation, dry<br>- neutral, slightly sweet |
| dihydropiperlonguminine (**16**)*<br>  | 10<br>50 | - slightly dry, slightly bitter<br>- bitter (long-lasting), umami |

* Synthetic sample

One ester, methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**), was described sensorially for the first time. At a concentration of 10 mg kg$^{-1}$, the sample was described as neutral by the trained panel. At 50 mg kg$^{-1}$, umami, slightly bitter, salty, and burning sensations were perceived. The molecule was also tested on salt and MSG but without a positive taste effect.

Table 44: Flavor description of ester **10** identified in *M. excelsum*.

| compound | concentration (mg kg$^{-1}$) | flavor description |
|---|---|---|
| methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**)*<br>  | 10<br>50 | - neutral<br>- umami, slightly bitter, slightly salty, slightly burning |

The isolated and tested compounds of the aqueous-ethanolic extracts of *M. excelsum* showed various taste impressions. However, nearly all of them had at least slightly trigeminal effects. A combination of the molecules probably caused the taste impressions that were described in the crude extract and the corresponding HTLC fractions (chapter 4.1.2.2).

## 4.2 INTENSITY VARIATION DESCRIPTIVE METHODOLOGY (IVDM)

For further sensory testing, selected molecules were evaluated in terms of their astringency-masking effects. In order to identify and to describe masking effects for complex tastants such as epigallocatechin gallate (EGCG), a screening tool was necessary to evaluate different candidates. The method should save time, panel work and the amount of test substances. Paired comparison, duo-trio, or triangle tests are commonly used for the identification of differences between samples (Meilgaard et al., 2007a, b). For long-lasting effects, these methods are often not sufficient, since samples are compared only at one time point and not over a longer period of time. Another problem is the build-up effect when comparing various astringent samples. Due to these difficulties, methods for sensory evaluation over time, like time-intensity approaches, seem better suitable (Kobue-Lekalake et al., 2012; Lee and Lawless, 1991; Robichaud and Noble, 1990; Valentova et al., 2002). Hence, a time-intensity design, based on intensity variation descriptive methodology (IVDM) according to Lee and Lawless (1991) was used as a screening tool for the identification of flavor-modulating effects.

In the following two figures, schematic diagrams of the test method are depicted. With IVDM, the focus is on consecutively monitoring the intensities of more than one attribute over time. This protocol, in combination with panel training, allows the analysis of several descriptors in parallel.

Figure 57 shows a computer screen of the unstructured line scales, one for each descriptor that has to be evaluated.

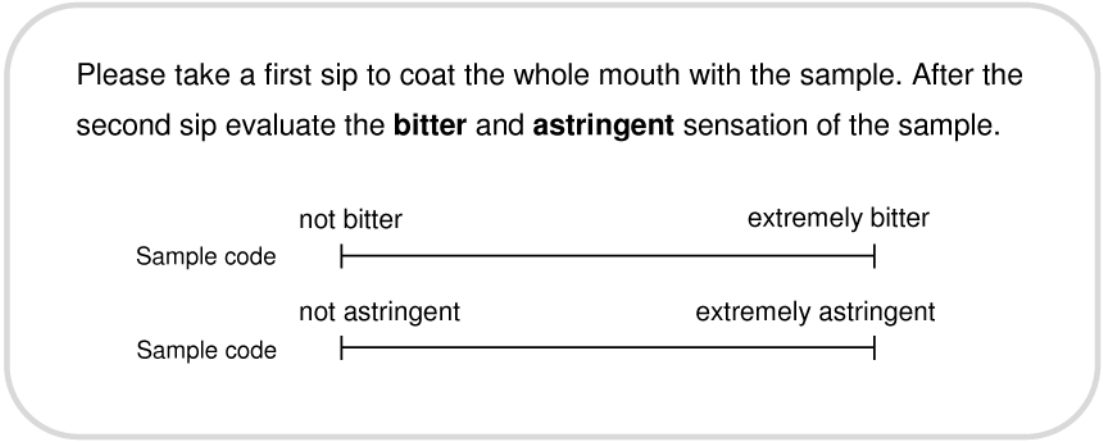



Figure 57: Unstructured line scales for the different descriptors to rate the intensity.

In Figure 58, the resulting time-intensity curves are shown for the bitterness and astringency of 500 mg $kg^{-1}$ EGCG + 500 mg $kg^{-1}$ caffeine. The panelists rated the intensities at defined time points, at 0 seconds, after 10 seconds, and repeatedly after every 20 seconds.

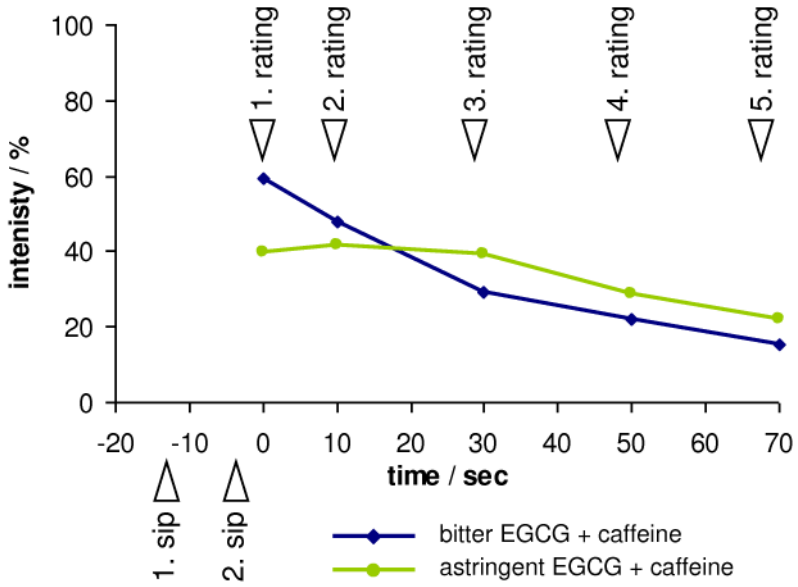



Figure 58: Schematic diagram of the IVDM method: after two sips, the intensities of bitter and astringent for 500 mg $kg^{-1}$ EGCG + 500 mg $kg^{-1}$ caffeine were rated.

Experience showed that the test method is easier to handle and less exhaustive compared to the TI method. Furthermore, the repeated appearance of the line scales makes the panelists aware of the sensations again at every interval.

### 4.2.1 Differentiation between Descriptors

In green tea or beverages prepared with green tea extract (GTE), various catechins, such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC) and (-)-epicatechin gallate (ECG), play important roles and contribute to the complex flavor sensation (Narukawa et al., 2010). EGCG as the most abundant catechin in green tea (Chen et al., 2001; Perva-Uzunalic et al., 2006) was used as the reference compound for masking tests. Besides astringency, it shows a strong bitterness that is also perceived as unpleasant by several people, especially at higher concentrations (Narukawa et al., 2010).

Tasting sessions were carried out, using a combination of typical bitter and astringent compounds to show that the panel was reproducibly able to differentiate between bitterness and astringency. EGCG, grape seed extract (GSE), and caffeine were used in different combinations and concentrations. Each test solution that contained EGCG, GTE, or GSE was stabilized with 125 mg $kg^{-1}$ ascorbic acid. Without stabilization, the catechin-containing test solutions turned orange-brownish after 45 minutes due to oxidation of polyphenols. Ascending concentrations of ascorbic acid were tested to avoid this problem. Concentrations lower than 125 mg $kg^{-1}$ were not sufficient for stabilizing the test solution over the period the panelists came for testing. The employed amount of 125 mg $kg^{-1}$ ascorbic acid had no sensory influence.

The combination of EGCG, which is bitter and astringent, and a purely bitter tasting compound, such as caffeine, was used to demonstrate the panelists´ performance. In one trial, panelists rated the bitterness and astringency of 500 mg $kg^{-1}$ EGCG with and without 500 mg $kg^{-1}$ caffeine, at which astringency did not change between both samples, while the bitter taste increased significantly ($p<0.05$) with added caffeine (Figure 59).

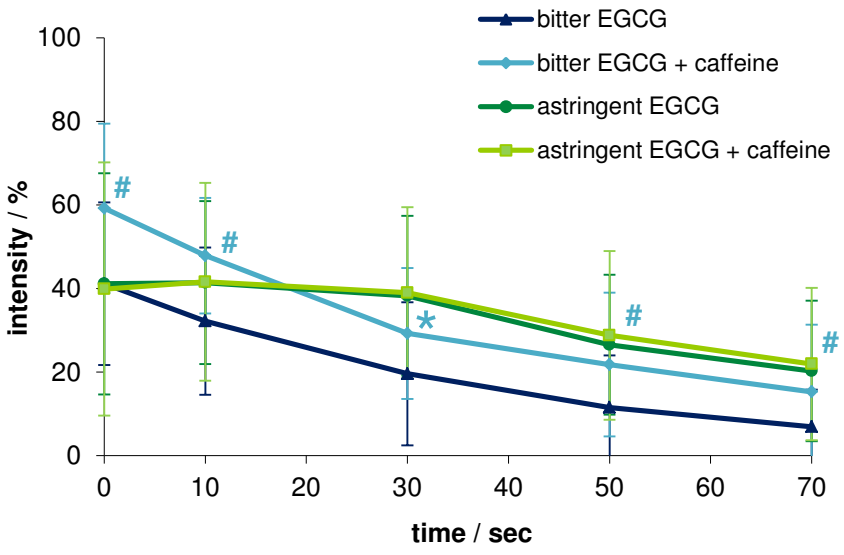



Figure 59: Time-intensity curves of 500 mg $kg^{-1}$ EGCG with and without 500 mg $kg^{-1}$ caffeine for bitter and astringent; n=15, one replication, significant differences *$p<0.1$, #$p<0.05$.

By adding 500 mg kg$^{-1}$ GSE, which is mainly astringent, to the same amount of EGCG, the astringency increased significantly ($p<0.05$), whereas the bitterness was not affected (Figure 60).

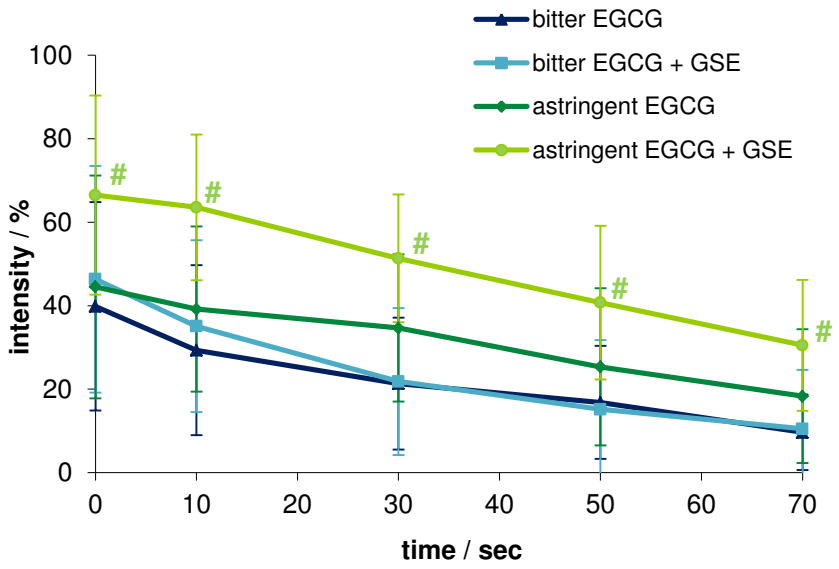



Figure 60: Time-intensity curves of 500 mg kg$^{-1}$ EGCG with and without 500 mg kg$^{-1}$ GSE for bitter and astringent; n=15, one replication, significant differences $^{\#}p\leq0.05$.

These data of the training session showed that the panelists were able to differentiate between the two attributes bitterness and astringency.

### 4.2.2 Dose-Response of Individual Taste Qualities of EGCG

Besides the differentiation of the descriptors, the distinction of different concentrations is also important for the identification of masking effects. To make sure that the panelists will detect potential masking properties of the test compounds, they have to be able to distinguish between different concentrations. Therefore, panel tests included ratings of 12 different concentrations of EGCG, ranging from 45 mg kg$^{-1}$ to 3750 mg kg$^{-1}$. The resulting time-intensity curves for bitterness and astringency are displayed in Figure 61 for every concentration and over the time of 70 seconds.

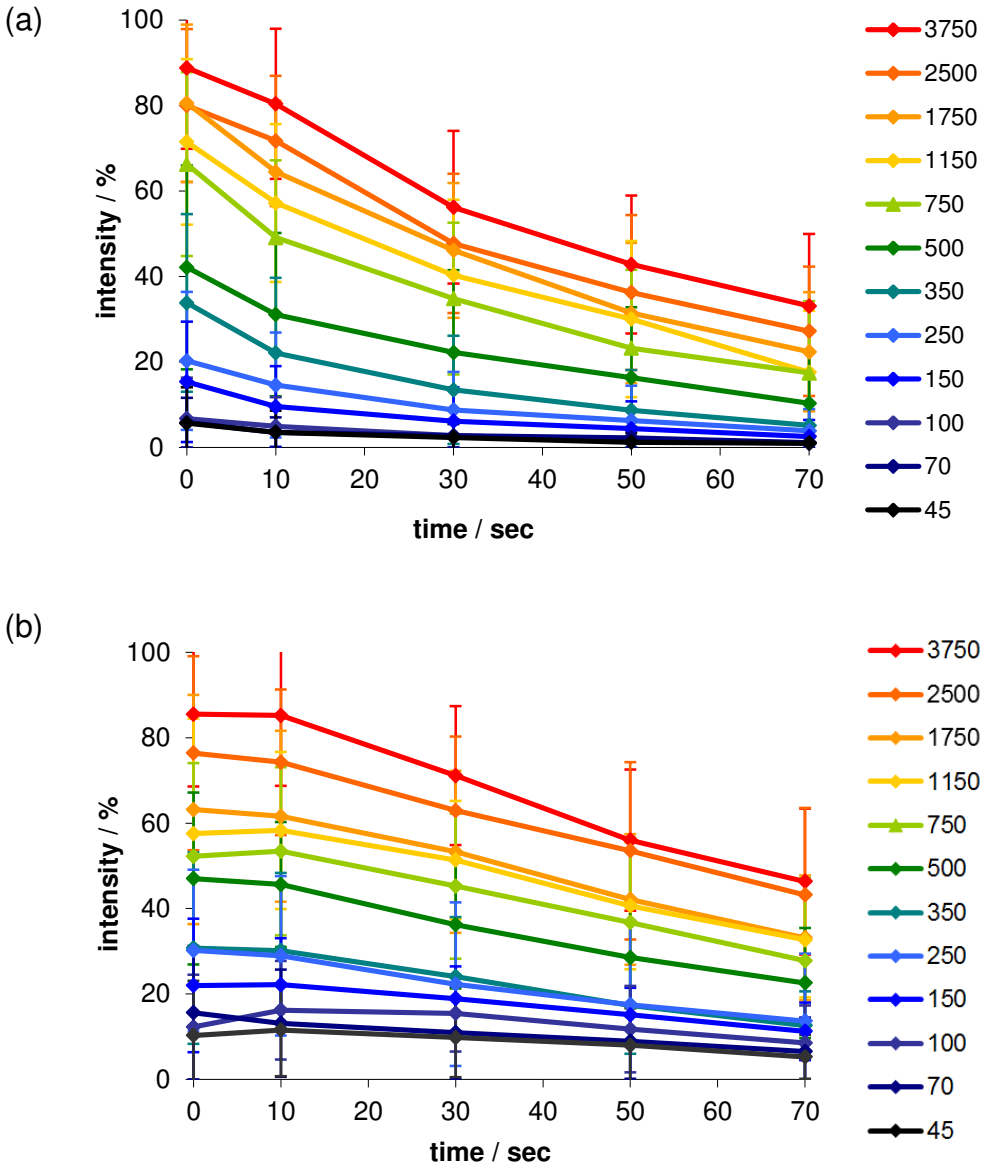



Figure 61: Time-intensity relationship of EGCG for the descriptor bitter (a) and astringent (b) at different concentrations (45 – 3750 mg $kg^{-1}$) from 0 to 70 seconds; n=15.

The time-intensity curves showed a clear dose-response relationship. As expected, 3750 mg $kg^{-1}$ was rated with the highest bitter and astringent intensity, while decreasing concentrations showed a stepwise lower intensity. Differentiability of the tested concentrations is shown in Table 45.

Table 45: Comparison for all pairs of the tested EGCG concentrations by using Tukey-Kramer HSD. Concentrations, which are not marked with the same letter are significantly different ($p<0.1$).

| | bitter | | | | | astringent | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| time/s / c(EGCG) | 0 | 10 | 30 | 50 | 70 | 0 | 10 | 30 | 50 | 70 |
| **3750 mg kg$^{-1}$** | A | A | A | A | A | A | A | A | A | A |
| **2500 mg kg$^{-1}$** | A B | A B | A B | A B | A B | A B | A B | A B | A B | A B |
| **1750 mg kg$^{-1}$** | A B C | B C | A B | A B C | B | B C | B C | B C | A B C | B C |
| **1150 mg kg$^{-1}$** | B C | C D | B C | B C | B C | B C | B C D | B C | B C | C |
| **750 mg kg$^{-1}$** | C | D | C | C D | B C | C | C D | C D | C D | C D |
| **500 mg kg$^{-1}$** | D | E | D | D E | C D | C D | D | D | D E | D E |
| **350 mg kg$^{-1}$** | D | E F | D E | E F | D | D E | E | E | E F | E |
| **250 mg kg$^{-1}$** | E | F G | E | E F | D | D E F | E F | E | F | E |
| **150 mg kg$^{-1}$** | E | G | E | F | D | E F | E F G | E | F | E |
| **100 mg kg$^{-1}$** | E | G | E | F | D | E F | E F G | E | F | E |
| **70 mg kg$^{-1}$** | E | G | E | F | D | E F | F G | E | F | E |
| **45 mg kg$^{-1}$** | E | G | E | F | D | F | G | E | F | E |

The differences between the consecutive concentrations in the higher and lower concentration range, for bitterness i.e. 1750 to 3750 mg kg$^{-1}$ and 45 to 150 mg kg$^{-1}$ at 10 seconds, respectively, are detectable but not significant. The bitterness and astringency were differentiated significantly ($p<0.1$) for medium concentrations rated around 50 % intensity (250-1150 mg kg$^{-1}$). The time-intensity curves for the bitter ratings (Figure 61a) declined more rapidly compared to the time-intensity curves for the descriptor astringent (Figure 61b). Especially for the first two measuring points this effect is distinct. The intensities for astringency in the time slot from 0 to 10 seconds were nearly constant for all tested concentrations, and the decrease, starting after 20 seconds, is not as steep as for the bitter ratings. These data confirm the long-lasting effects of bitterness and astringency, with the latter being more pronounced, requiring longer measuring times.

Based on these results, the optimal dosage of EGCG for masking trials was selected. The optimal dosage should not be too weak for the panelists to detect possible reduction effects, and not too strong, in order not to miss possible enhancing effects. Therefore, the reference concentration for the following tests was selected to be around the 50 % intensity level, in the present case at 750 mg kg$^{-1}$. The test duration was set at 70 seconds to have a screening tool for masking/reduction of long-lasting aftertaste. As soon as a compound showed a masking effect, further tests could be carried out, for example, for a longer period.

### 4.2.3 Inter- and Intra-Individual Differences of Panelists

Sensory responses vary between individuals. They may be impacted, for example, by age, gender, smoking, drinking, or eating habits. Different salivary response after intake of astringent stimuli can affect the acceptance and liking of phenol-rich foods (Dinnella et al., 2011).

The variations of the ratings between different panelists and between different sessions of one panelist were analyzed for the selected reference concentration. In Figure 62a, the time-intensity curves for the bitterness of 750 mg $kg^{-1}$ EGCG of five panelists are given (inter-individual). The intensities differed around 20 and 30 % between the lowest and highest ratings at the selected time points (e.g. 37 and 76 % at 0 seconds, 20 and 42 % at 30 seconds). The arithmetic mean and the standard deviation of the selected panelists were calculated and are shown in Figure 62b. The standard deviation is in the range of 8 to 17 % and is comparable to the standard deviation of the astringency description (Figure 63b). The single time-intensity curves for astringency are shown in Figure 63a. Between the different panelists, the curves differ to a certain extent but the overall shapes are comparable.

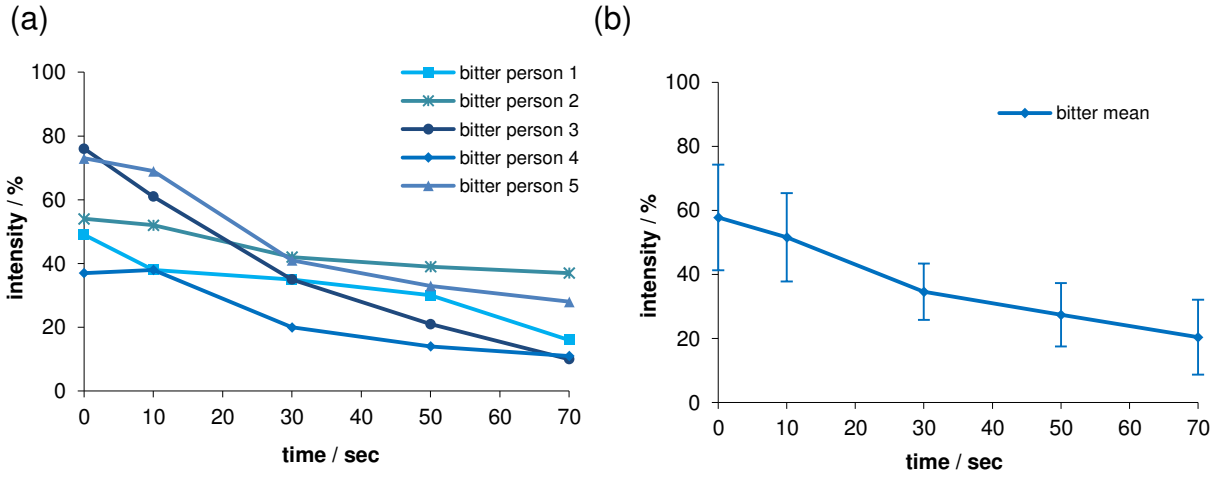



Figure 62: (a) Time-intensity curves of five panelists in one session for the bitterness ratings of 750 mg $kg^{-1}$ EGCG to present the inter-individual differences. (b) Mean and standard deviation of the results of the five panelists.

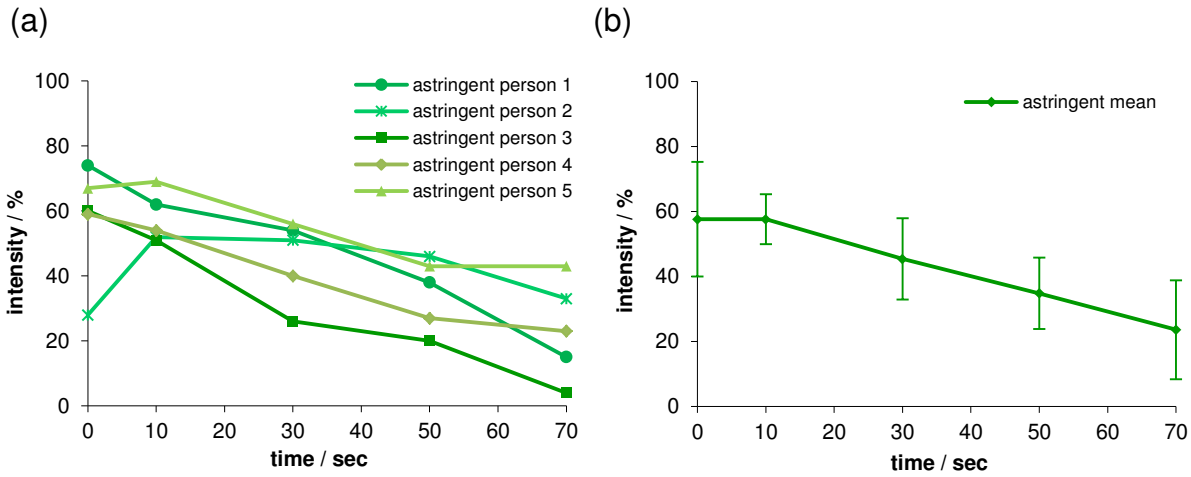



Figure 63: (a) Time-intensity curves of five panelists in one session for the astringency ratings of 750 mg $kg^{-1}$ EGCG to present the inter-individual differences. (b) Mean and standard deviation of the results of the five panelists.

Besides the group performance, the reproducibility of the results of every single panelist is also important. Therefore, the time-intensity curves of a randomly selected panelist are exemplarily shown for three separate sessions. The single curves for the bitter ratings of 750 mg $kg^{-1}$ EGCG are similar and differ only by a maximum of 10 % over the whole period (Figure 64a). The standard deviation of the arithmetic mean is 5 %, which is very low (Figure 64b). Nearly the same statement can be made for the astringency description (Figure 65a, b). Only at the last two measuring points, the distance between the curves and consequently the standard deviation, increased to 10 % compared to 5 % at the previous time points. The intra-individual difference of the selected panelist is low and thus the reproducibility very high. The same results can be seen for the other panelists (data not shown).

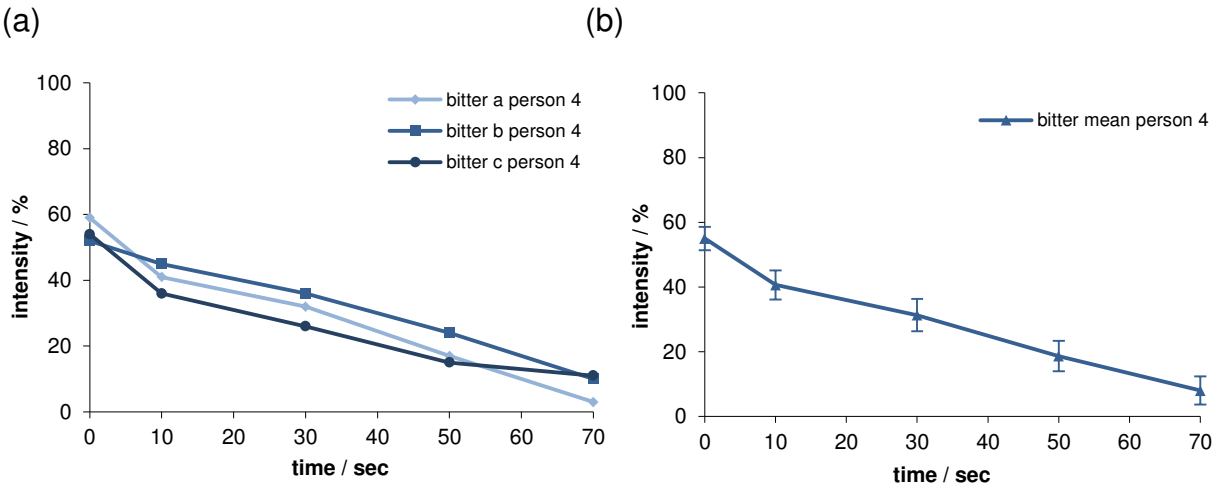



Figure 64: (a) Bitter time-intensity curves of 750 mg $kg^{-1}$ EGCG (three sessions) of a single panelist to present the intra-individual differences. (b) Mean and standard deviation of the results from the three sessions.

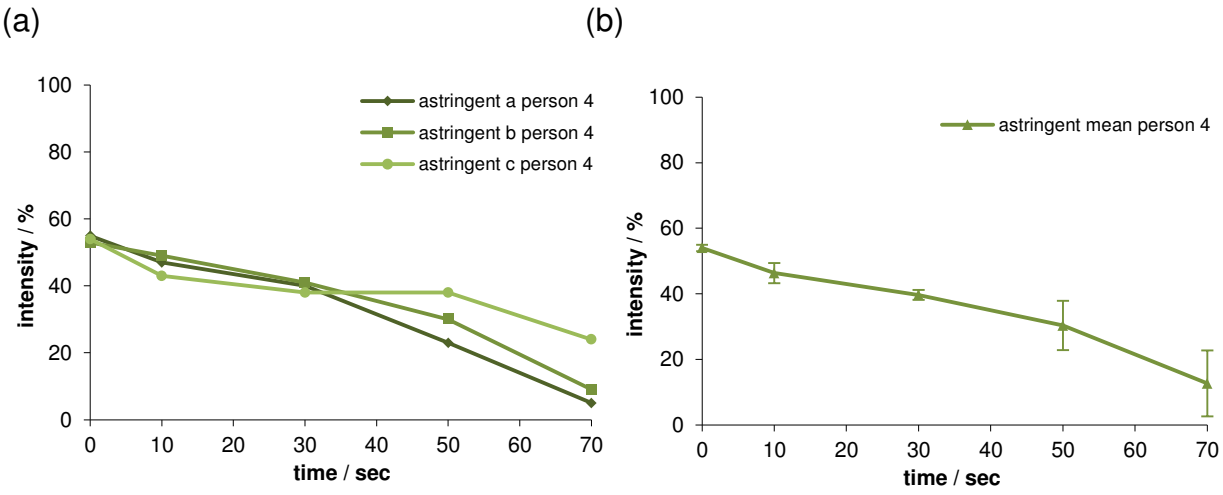



Figure 65: (a) Astringency time-intensity curves of 750 mg $kg^{-1}$ EGCG (three sessions) of a single panelist to present the intra-individual differences. (b) Mean and standard deviation of the results from the three sessions.

### 4.2.4 Complex Flavored Beverage

As an example for a more complex system, a sweetened, citrus-flavored green tea beverage was evaluated via IVDM. Panelists were asked to rate the intensities of bitterness, astringency, sweetness, and citrus flavor in parallel (Figure 66). For the plain green tea beverage, without sucrose and flavor, the attributes sweet and citrus were negligible as expected, while the astringency and bitterness intensities were rated at nearly equal intensities (around 50 %) at the first time point (Figure 66a). From 0 to 30 seconds, the range between the two descriptors increased by up to 17 percentage points (pp) and this remained constant until the end of the

measurement. During the time course of 70 seconds, the bitterness decreased to 12 % intensity, faster than the astringency with 25 % at the last measuring point. With added sucrose and citrus flavor, the sweet and citrus taste increased by maximal 31 pp from 3 % to 34 % and by 35 pp from 2 % to 37 %, for the first measuring point (Figure 66b), compared to the plain sample. In comparison, the bitterness decreased by maximal 18 pp and the astringency by about 7 pp at the same time.

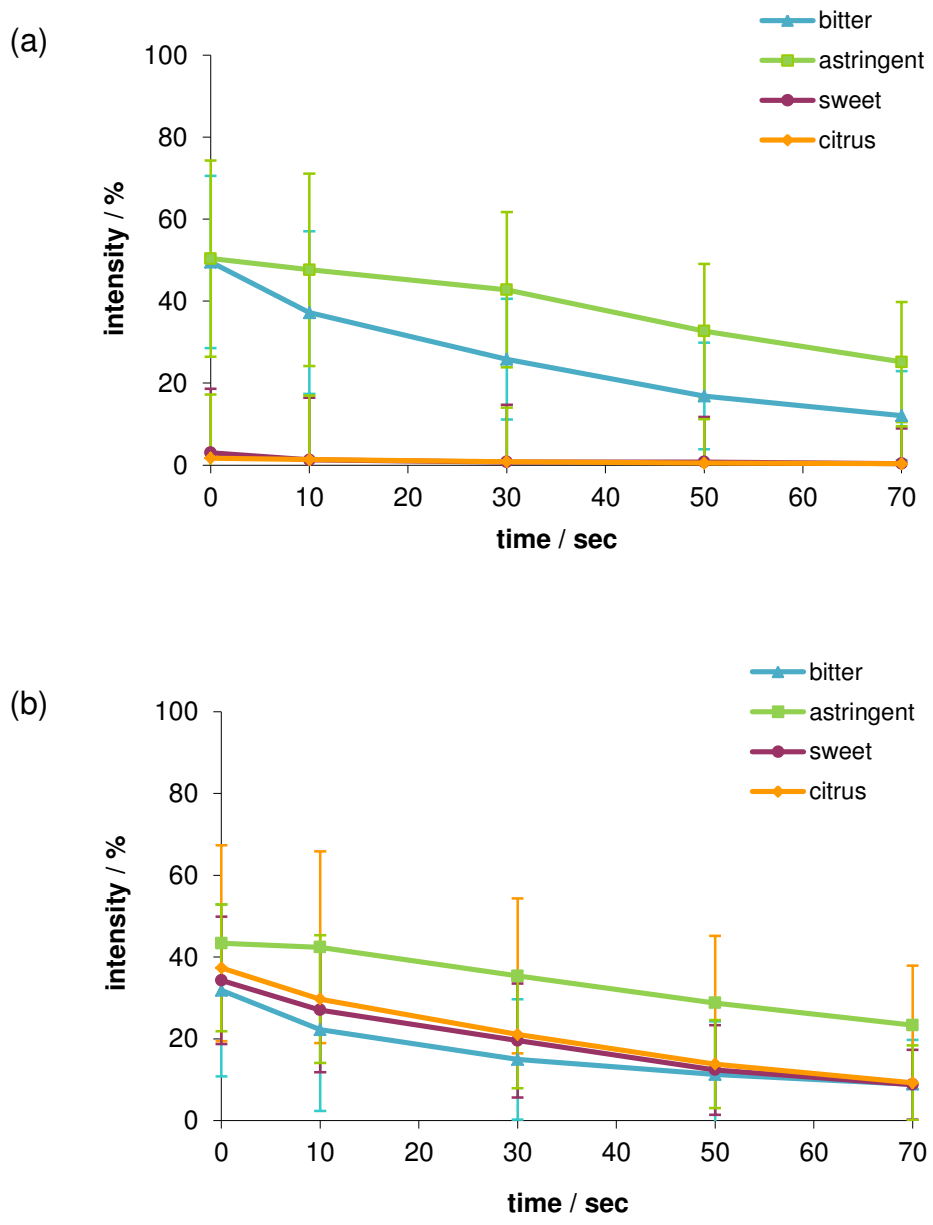



Figure 66: Time-intensity curves of a green tea beverage for the descriptors bitter, astringent, sweet and citrus over 70 seconds; (a) non-flavored and without sucrose; (b) flavored and sweetened; n=15; 3 replications.

When using the IVDM protocol, panelists were capable of differentiating between four descriptors and the corresponding intensities in a green tea beverage (Figure 66).

The addition of sugar and the concomitant increase of sweetness, respectively, led to a significant ($p<0.1$) decrease of bitterness. This phenomenon is known for certain non-caloric and caloric sweeteners, such as sucralose and sucrose (Ares et al., 2009). By testing all attributes at the same time, possible interactions can be more easily determined. The results are comparable to the data and curve shapes created by Lee and Lawless (1991). They evaluated the aftertaste of the astringent compounds tannic acid, alum, and tartaric acid by using six different descriptors at the same time, whereby the long-lasting effect was clearly shown.

The above described sensory tests indicate that the panel was reproducibly able to differentiate between bitterness and astringency of typically bitter and astringent compounds. Furthermore, various EGCG concentrations could be distinguished by the panelists, which is fundamental for the masking trials. 750 mg $kg^{-1}$ EGCG with a described 50 % astringent intensity was set as the reference concentration and 70 seconds as the test duration of the planned IVDM tests.

### 4.2.5 Pre-Tests for Masking Trials

Research suggests that trigeminal chemosensors are involved in astringency detection (Schöbel, 2010; Schöbel et al., 2013). Therefore, it should be possible to find compounds, which can influence these chemosensors and consequently reduce astringency. By using trigeminal-active compounds, this theory might be proven.

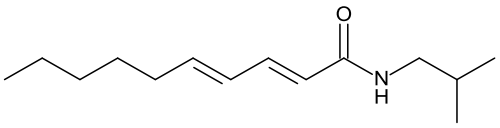
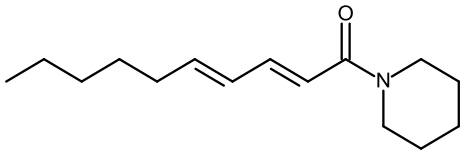
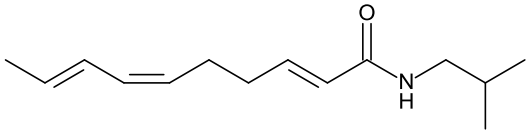
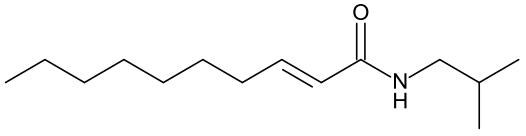
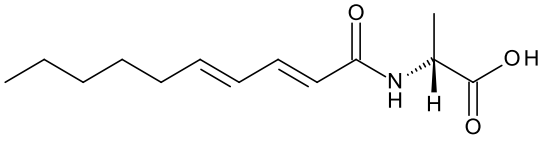
In earlier studies, it was found that certain trigeminal-active compounds can induce salivation (Ley et al., 2006; Ley and Simchen, 2007). In this work, it was hypothesized that weak saliva induction may be correlated with a reduction of astringency sensation. In order to validate this hypothesis, selected compounds of three different groups were tested. The first group contained molecules having a mouth-watering effect, like *trans*-pellitorine (**3**) (Table 46). A second group comprised molecules structurally related to *trans*-pellitorine (**3**) without salivating effects (Table 47). This group was chosen to test whether the potential masking effects are due to these mouth-watering properties or due to so far not known molecular interactions. Additional trigeminal compounds, showing neither mouth-watering effects nor pellitorine-like structures, were tested in a third group (Table 48).

Selected representatives of the newly isolated substances from *M. excelsum* were assigned to the three groups. Among the mouth-watering compounds, *trans*-pellitorine (**3**) and achilleamid (**4**) were in the first group. Further saliva-inducing

compounds not found in *M. excelsum*, such as spilanthol (**25**) and (2*E*)-decenoic acid *N*-isobutyl amide (**26**), were tested as well (Ley et al., 2006). Additionally, the newly synthesized (2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**), described as strong mouth-watering and tingling at 50 mg $kg^{-1}$, was also evaluated in this group (Table 46).

In a number of pre-tests, each compound was tested at least at two concentrations on 750 mg $kg^{-1}$ EGCG against 750 mg $kg^{-1}$ EGCG only by a small expert panel in open discussion. The concentrations were selected in a range around their detection level. At this level, possible masking effects are not disturbed by off-notes caused by intrinsic taste or flavor, which can lead to bias.

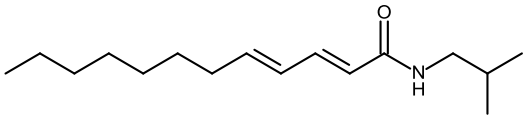
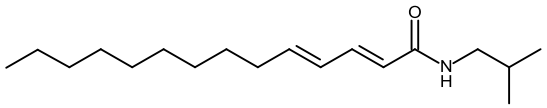
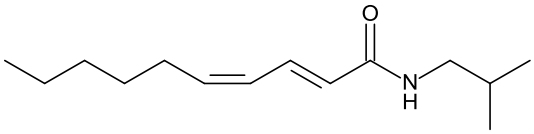
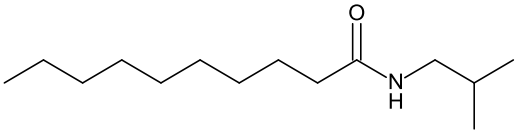
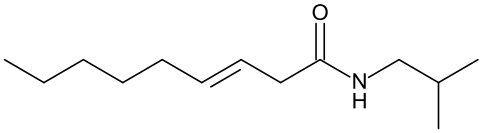
Table 46: Compounds with a saliva-inducing effect (**3**, **4**, **25**-**27**), which were evaluated in pre-tests regarding their masking effect of EGCG astringency (group 1).

| compound | concentration (mg $kg^{-1}$) | results |
|---|---|---|
| *trans*-pellitorine (**3**)  | 1<br>2<br>3<br>5<br>8 | no effect[a]<br>slight masking effect[b]<br>slight masking effect[b]<br>masking effect[c]<br>masking effect[c] |
| achilleamid (**4**)  | 5<br>20 | no effect[a]<br>no effect[a] |
| spilanthol (**25**)  | 1<br>2<br>5 | no effect[a]<br>slight masking effect[b]<br>no effect[a] |
| (2*E*)-decenoic acid *N*-isobutyl amide (**26**)  | 10<br>20 | no effect[a]<br>no effect[a] |
| (2S)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**)  | 50<br>100<br>250 | masking effect[c]<br>masking effect[c]<br>slight masking effect[b] |

[a] < 33 %, [b] 33-50 %, [c] > 50 % of the panelists described reduced astringency compared to reference

In a second step, structurally related compounds of *trans*-pellitorine, like kalecide (**8**) and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**), without known saliva-inducing effects, were tested. Sometimes a small structural variation, for example the geometrical configuration of a compound, can change the trigeminal sensation. As shown for *trans*-pellitorine (**3**), the *cis*-isomer (**28**) lost the tingling sensation and was described as pungent and warming (Ley et al., 2004; Ley et al., 2006). Decanoic acid *N*-isobutyl amide (**29**), described as tingling at 10 mg $kg^{-1}$, also belongs to the group of structurally related compounds, as well as the numbing (*3E*)-nonenoic acid *N*-isobutyl amide (**30**) (Table 47).

Table 47: Structurally-related molecules of *trans*-pellitorine (**8**, **9**, **28**-**30**), which were evaluated in pre-tests regarding their masking effect of EGCG astringency (group 2).

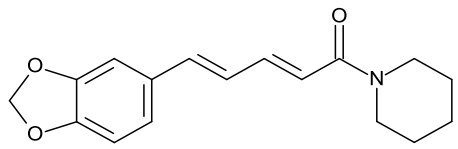
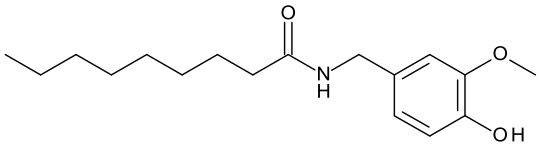
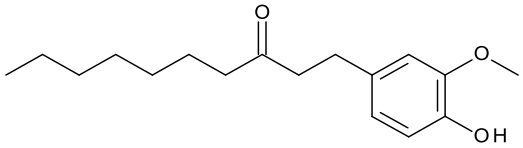
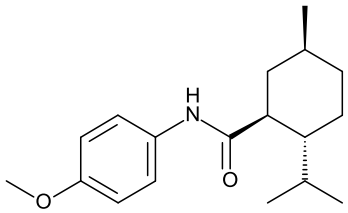
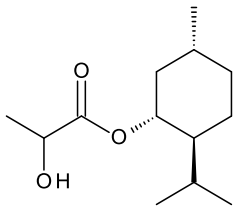
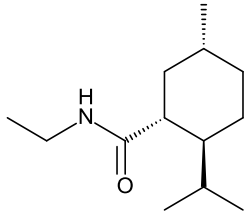
| compound | concentration (mg $kg^{-1}$) | results |
|---|---|---|
| kalecide (**8**)  | 10<br>50 | no effect[a]<br>no effect[a] |
| (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**)  | 10<br>50 | no effect[a]<br>no effect[a] |
| c*is*-pellitorine (**28**)  | 1<br>2<br>5 | slight masking effect[b]<br>masking effect[c]<br>masking effect[c] |
| decanoic acid *N*-isobutyl amide (**29**)  | 10<br>20 | no effect[a]<br>no effect[a] |
| (3*E*)-nonenoic acid *N*-isobutyl amide (**30**)  | 2<br>10<br>20 | no effect[a]<br>no effect[a]<br>no effect[a] |

[a] < 33 %, [b] 33-50 %, [c] > 50 % of the panelists described reduced astringency compared to reference

Further trigeminal-active compounds, like the hot, burning, pungent, and warming compounds piperine (**7**), nonivamide (**31**), and 6-paradol (**32**), and the cooling

compounds Frescolat® SC1 (**33**), Frescolat® ML (**34**), and WS3 (**35**) were evaluated as well (Table 48).

Table 48: Further trigeminal-active compounds (**7**, **31**-**35**), which were evaluated in pre-tests regarding their masking effect of EGCG astringency (group 3).

| compound | concentration (mg kg$^{-1}$) | results |
|---|---|---|
| piperine (**7**)  | 1<br>5 | no effect[a]<br>no effect[a] |
| nonivamide (**31**)  | 0.05<br>0.1<br>0.2 | no effect[a]<br>no effect[a]<br>no effect[a] |
| 6-paradol (**32**)  | 0.2<br>1<br>10 | no effect[a]<br>no effect[a]<br>no effect[a] |
| Frescolat® SC1 (**33**)  | 0.05<br>0.1<br>0.5<br>1 | no effect[a]<br>no effect[a]<br>no effect[a]<br>no effect[a] |
| Frescolat® ML (**34**)  | 0.2<br>0.3<br>0.5<br>1 | slight masking effect[b]<br>slight masking effect[b]<br>no effect[a]<br>no effect[a] |
| WS3 (**35**)  | 0.1<br>0.2<br>1<br>2<br>5 | no effect[a]<br>no effect[a]<br>no effect[a]<br>no effect[a]<br>no effect[a] |

[a] < 33 %, [b] 33-50 %, [c] > 50 % of the panelists described reduced astringency compared to reference

As shown in Table 46, the mouth-watering compounds *trans*-pellitorine (**3**), spilanthol (**25**), and (2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) indicated astringency-masking effects, while achilleamid (**4**) and (2*E*)-decenoic acid *N*-isobutyl amide (**26**) did not. Furthermore, one structurally related molecule, *cis*-pellitorine (**28**), as well as the cooling agent Frescolat® ML (**34**) hinted to reduce the astringency of 750 mg $kg^{-1}$ EGCG (Tables 47, 48). The compounds that showed astringency reduction were chosen for tasting sessions in the IVDM panel.

### 4.2.6 Screening for Masking Effects against Bitterness and Astringency of EGCG in the IVDM Panel

The panel was asked to rate the bitterness and astringency of the EGCG test solution with and without a potential masking compound. The results of both time-intensity curves were compared statistically to determine the reduction of bitterness and astringency after addition of the test compound. The test was repeated and the arithmetic mean was determined and analyzed via Student's *t*-test.

By adding 5 mg $kg^{-1}$ of the mouth-watering compound *trans*-pellitorine (**3**, Table 46) to 750 mg $kg^{-1}$ EGCG, the absolute bitterness ratings were reduced at the first rating by 6 pp and during the further time course only weakly by about 1 to 3 pp (relative decrease by 3-23 %, Figure 67, Table 49). However, the absolute intensity of astringency was lowered significantly by 7.5 to 11.5 pp over the whole measuring time (relative decrease of 18-33 %; Figure 67, Table 49).

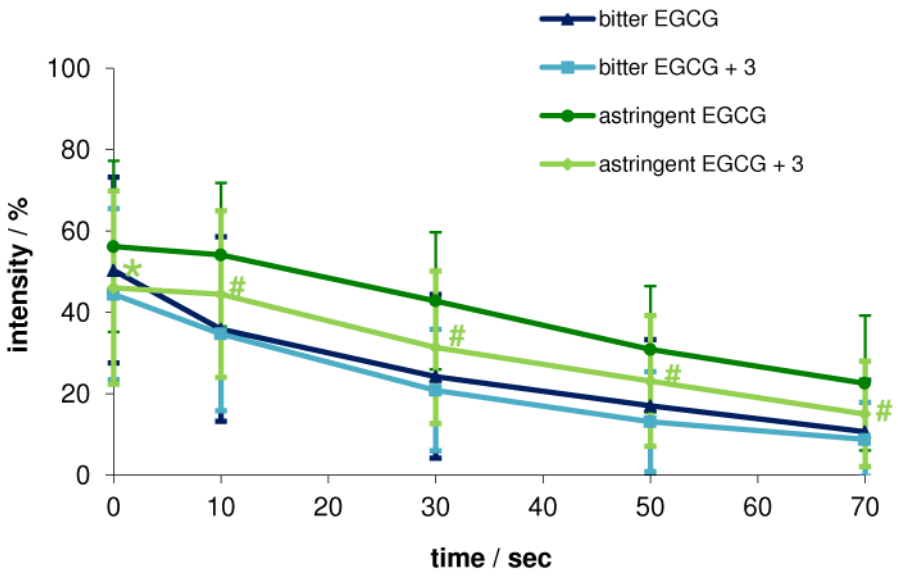



Figure 67: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 5 mg kg$^{-1}$ *trans*-pellitorine (**3**) for bitter and astringent; n=15, two replications, significant differences *p≤0.1, $^{\#}$p≤0.05.

After this distinct astringency-masking effect was detected, the dose-response relationship of the base and test compound was evaluated in more detail. First, the concentration of *trans*-pellitorine (**3**) was varied (1, 3, 5, and 8 mg kg$^{-1}$), while the EGCG was fixed at 750 mg kg$^{-1}$. In a second step, the EGCG concentration was changed in the range of 300 to 1000 mg kg$^{-1}$, while the concentration of **3** was set at 5 mg kg$^{-1}$. The results are presented in Table 49.

Table 49: Dose-response relationship of different *trans*-pellitorine (**3**: 1, 3, 5 mg kg$^{-1}$) and EGCG (300, 500, 750, 1000 mg kg$^{-1}$) concentrations and their masking effect for bitterness and astringency in percentage points (pp); n=15.

| | **bitter masking/pp** | | | | | **astringency masking/pp** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| time/s<br>c(EGCG) | 0 | 10 | 30 | 50 | 70 | 0 | 10 | 30 | 50 | 70 |
| | | | | | **3 (1 mg kg$^{-1}$)** | | | | | |
| **300 mg kg$^{-1}$ [a]** | -4.4 | -3.8 | 0.1 | -1.9 | -2.6 | -3.4 | -2.4 | -1.8 | -3.1 | -2.7 |
| **750 mg kg$^{-1}$ [b]** | -4.0 | -2.3 | -1.0 | -1.0 | -0.8 | -2.0 | -1.5 | 1.6 | -1.1 | -0.2 |
| | | | | | **3 (3 mg kg$^{-1}$)** | | | | | |
| **500 mg kg$^{-1}$ [a]** | -1.5 | 2.3 | 3.6 | 0.0 | 0.8 | 0.6 | -1.4 | -1.1 | 1.0 | -0.3 |
| **750 mg kg$^{-1}$ [b]** | 0.4 | -1.4 | -2.3 | -1.2 | -0.3 | -1.4 | -1.6 | -1.7 | -2.5 | -4.2 |
| | | | | | **3 (5 mg kg$^{-1}$)** | | | | | |
| **300 mg kg$^{-1}$ [a]** | -1.4 | -0.7 | -4.4$^{\#}$ | -3.2$^{\#}$ | -3.5* | 0.3 | -1.3 | -4.3 | -1.0 | -2.8 |
| **500 mg kg$^{-1}$ [a]** | 1.8 | 0.0 | -1.9 | 0.0 | -1.8 | 1.9 | 3.1 | 2.2 | -3.1 | -5.9 |
| **750 mg kg$^{-1}$ [b]** | -5.9 | -1.2 | -3.4 | -3.9 | -1.9 | -10.1* | -9.7$^{\#}$ | -11.5$^{\#}$ | -7.8$^{\#}$ | -7.6$^{\#}$ |
| **1000 mg kg$^{-1}$ [b]** | -0.5 | -4.3 | -6.3* | -5.6 | -4.6 | -2.9 | -5.0 | -8.7$^{\#}$ | -8.3$^{\#}$ | -7.1$^{\#}$ |

[a] One replication. [b] Two replications. Significant differences * p<0.1, $^{\#}$ p<0.05

The results at a concentration of 8 mg $kg^{-1}$ *trans*-pellitorine (**3**) are not shown because of the strong tingling and mouth-watering sensation that occurred noticeable for all panelists. There was an effect, however, this strong sensations might have influenced the blinded test design. For 750 mg $kg^{-1}$ EGCG plus 1, 3, or 5 mg $kg^{-1}$ **3**, a significant masking effect was only determined for the highest concentration with up to 11 pp. Therefore, a *trans*-pellitorine (**3**) concentration of 5 mg $kg^{-1}$ was selected to be tested on 300, 500, and 1000 mg $kg^{-1}$ EGCG as well. An astringency-masking effect was also shown on 1000 mg $kg^{-1}$ EGCG with a reduction of up to 9 pp and was significant ($p<0.05$) at 30, 50, and 70 seconds. Apparently, a specific ratio between EGCG and **3** is necessary to gain masking effects. To verify the hypothesis that the concentration ratios of both compounds have to be adapted, 300 mg $kg^{-1}$ EGCG + 1 mg $kg^{-1}$ **3** and 500 mg $kg^{-1}$ EGCG + 3 mg $kg^{-1}$ **3** were tested (Table 49). But even these adapted combinations did not show a masking effect. Therefore, only specific base-to-compound-ratios seem to fulfill the requirements for astringency-masking effects. This hypothesis, of course, was only tested to a limited extent for the above-described scenario and cannot be generalized for every other compound. Although a number of different concentrations and ratios were tested, more combinations are possible.

Spilanthol (**25**), as a further known salivation-causing alkamide was also tested using the IVDM approach to evaluate its astringency masking potential. The results obtained for **25** are not as obvious as for the previous test compound. After three replications, no significant masking effect occurred, except for one measuring point at 30 seconds (Figure 68). 2 mg $kg^{-1}$ **25** showed only a hint towards bitter and astringency masking. Two more concentrations, 1 and 5 mg $kg^{-1}$, were tested as well but without any effect.

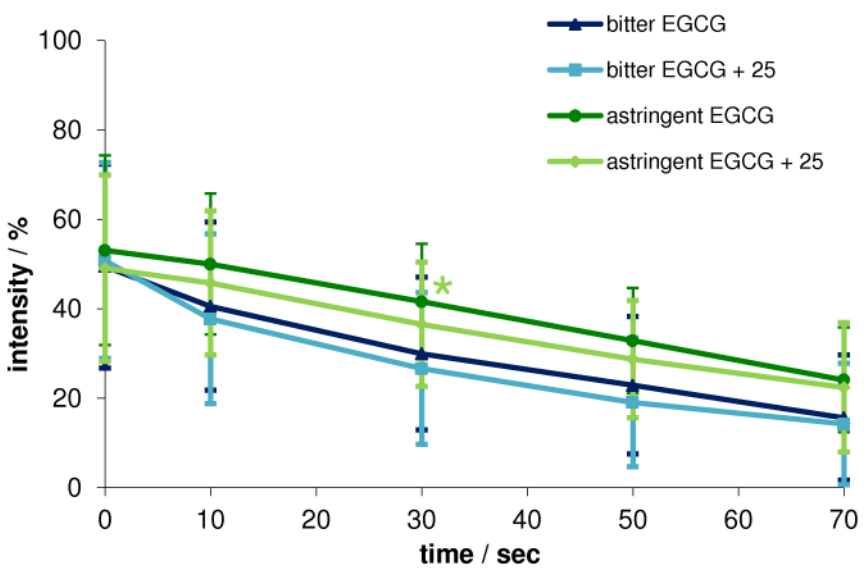



Figure 68: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 2 mg kg$^{-1}$ spilanthol (**25**) for bitter and astringent; n=15, three replications, significant differences * p≤0.1.

Also (2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**, Table 46) showed positive effects in pre-tests and was evaluated using the IVDM method. This new molecule was specifically synthesized for this masking study. The synthesis was based on the one for the *trans*-pellitorine (**3**) molecule; however it is more hydrophilic and better water soluble due to its amino acid moiety (Obst et al., 2013). The concentration of **27** was varied (50, 100, and 250 mg kg$^{-1}$), while the concentration of EGCG was fixed at 750 mg kg$^{-1}$. The results are presented in Table 50 and exemplarily for 50 mg kg$^{-1}$ in Figure 69.

Table 50: Dose-response relationship of different (2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) concentrations (50, 100, 250 mg kg$^{-1}$) on 750 mg kg$^{-1}$ EGCG and their masking effects for bitterness and astringency in percentage points (pp); n=15.

| | | **bitter masking/pp** | | | | | **astringency masking/pp** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| time/s<br>c(EGCG) | | 0 | 10 | 30 | 50 | 70 | 0 | 10 | 30 | 50 | 70 |
| **750 mg kg$^{-1}$** | **27 (50 mg kg$^{-1}$)** | -2,5 | -4,4 | -2,6 | -2,6 | -4,1 | -3,8 | -5,5* | -9,5$^{\#}$ | -6,7$^{\#}$ | -5,8* |
| | **27 (100 mg kg$^{-1}$)** | 3,1 | -0,2 | -3,3 | -2,0 | -3,4 | -6,3 | -7,8* | -8,1* | -5,5 | -3,1 |
| | **27 (250 mg kg$^{-1}$)** | 1,3 | 0,9 | 0,2 | 0,4 | -0,5 | 0,2 | -3,4 | -1,0 | -2,4 | -4,7 |

[a] Two replication. [b] Three replications. Significant differences * p<0.1, $^{\#}$ p<0.05

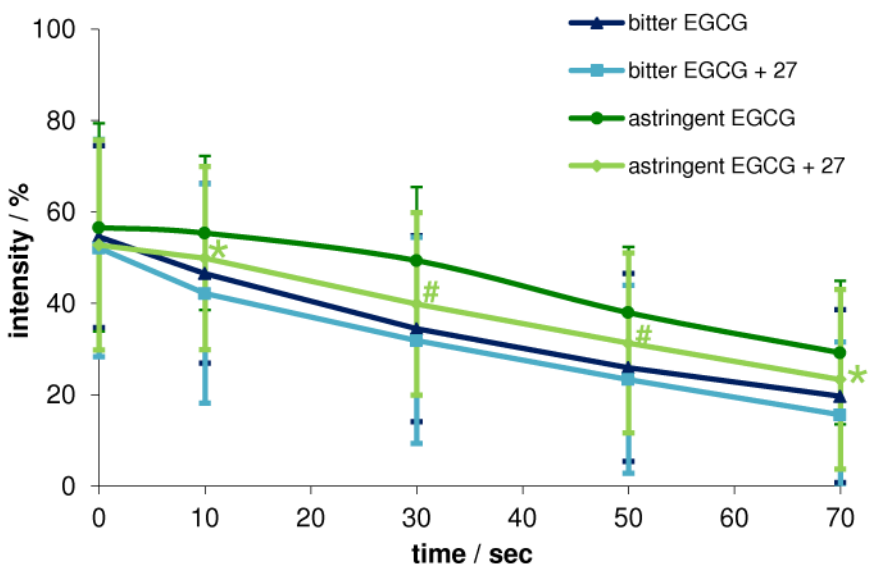



Figure 69: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 50 mg kg$^{-1}$ (2*S*)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) for bitter and astringent; n=15, three replications, significant differences * $p \leq 0.1$, $^{\#}$ $p \leq 0.05$.

A significant masking effect was found for 50 mg kg$^{-1}$ with up to 10 pp. Astringency reduction was also shown on 100 mg kg$^{-1}$ with up to 8 pp and significant ($p<0.1$) at 10 and 30 seconds. This higher concentration that was chosen for the tests is due to the approximately ten-fold lower activity of **27** compared to **3**. In contrast to *trans*-pellitorine (**3**), however, its derivative **27** did not show a trigeminal effect or off-notes at this concentration.

Achilleamid (**4**) was not able to reduce the astringency at the tested concentrations and ratios between the base and the test compound. Neither 5 mg kg$^{-1}$ nor 20 mg kg$^{-1}$ showed any effects when added on 750 mg kg$^{-1}$ EGCG (data not shown). The monosaturated (2*E*)-decenoic acid *N*-isobutyl amide (**26**) was described as pungent, warming, and mouth-watering, the latter one, however, less intense compared to *trans*-pellitorine (**3**) and spilanthol (**25**) (Ley et al., 2006). In pre-tests, no masking effect was perceived by the panelists at the tested concentrations of 10 and 20 mg kg$^{-1}$. Thus, no IVDM trials were carried out.

As a structurally related molecule, the geometrical isomer of *trans*-pellitorine (**3**), *cis*-pellitorine (**28**) was evaluated regarding its activity to mask EGCG off-tastes. While the addition of 1 mg kg$^{-1}$ **28** allowed only a weak reduction of astringency of 4 to 5 pp, 2 mg kg$^{-1}$ **28** resulted in a masking effect of up to 10 pp. At a higher concentration (5 mg kg$^{-1}$), the effect decreased again (Table 51). Similar to **3**, the intrinsic taste

and/or trigeminal effect increased at higher concentrations. **28** exhibited pungency at these concentrations, which may disturb the panelists' ratings for astringency and bitterness. The results of the IVDM tests are exemplarily shown for **28** (2 mg kg$^{-1}$) in Figure 70.

Table 51: Dose-response relationship of different *cis*-pellitorine (**28**) concentrations (1, 2, 5 mg kg$^{-1}$) on 750 mg kg$^{-1}$ EGCG and their masking effect for bitterness and astringency in percentage points (pp); n=15; two replications.

| | | **bitter masking/pp** | | | | | **astringency masking/pp** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| time/s<br>c(EGCG) | 0 | 10 | 30 | 50 | 70 | 0 | 10 | 30 | 50 | 70 |
| **750 mg kg$^{-1}$** | **28 (1 mg kg$^{-1}$)** | | | | | | | | | |
| | -4.7 | -1.8 | 1.5 | 2.0 | -0.7 | -1.0 | -4.9 | -4.9 | -4.2 | -5.3* |
| | **28 (2 mg kg$^{-1}$)** | | | | | | | | | |
| | -1.0 | 1.7 | -4.9 | -5.4 | -4.4 | -9.6 | -10.0* | -9.7 | -8.3* | -5.2 |
| | **28 (5 mg kg$^{-1}$)** | | | | | | | | | |
| | -1.4 | -0.2 | 0.7 | -0.3 | -1.8 | -6.1 | -3.7 | -4.8 | -4.8 | -4.7 |

Significant differences * p<0.1.

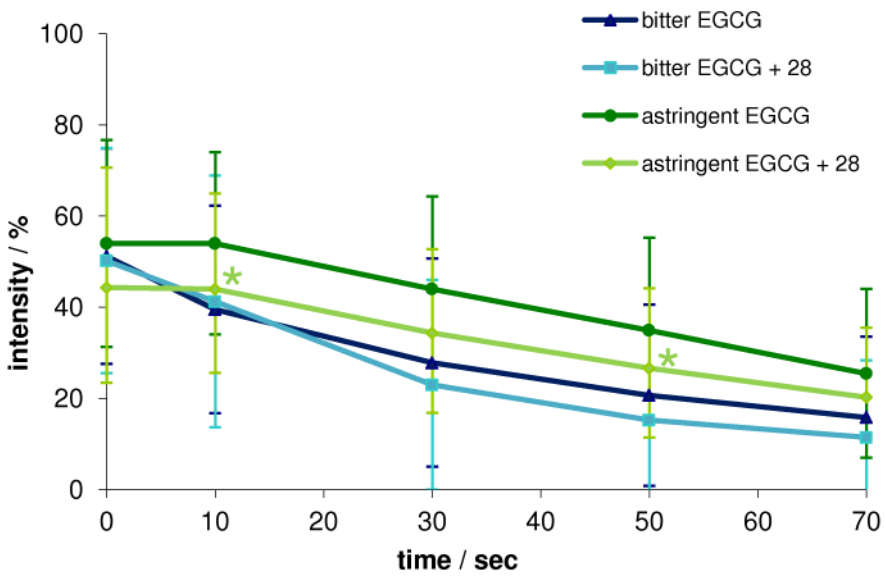



Figure 70: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 2 mg kg$^{-1}$ *cis*-pellitorine (**28**) for bitter and astringent; n=15, two replications, significant differences * p≤0.1.

Since two *trans*-pellitorine homologues, kalecide (**8**) and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**), were among the isolated and identified compounds from *M. excelsum*, the two molecules were tested on EGCG, as well.

At the used concentrations (10, 50, 100 mg kg$^{-1}$), kalecide (**8**) and (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) with chain elongations by two and four carbon atoms, respectively, showed no masking effect on 750 mg kg$^{-1}$ EGCG. As an example, the time-intensity measurements of **9** are presented in Figure 71. The rated astringency is nearly similar with and without **9**, while the bitterness even slightly increased by adding the tested molecule.
Among the additionally evaluated derivatives (**29**, **30**), none of them showed positive effects.

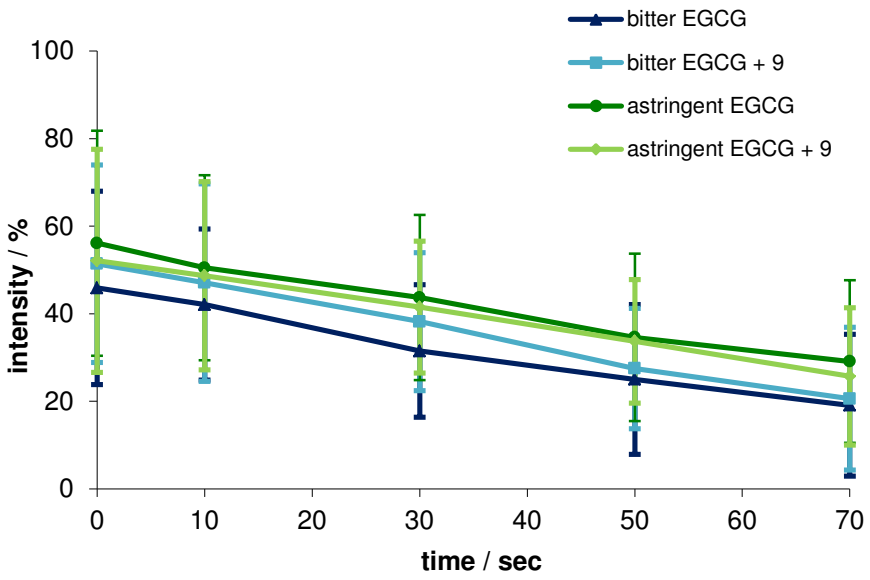



Figure 71: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 10 mg kg$^{-1}$ (2*E*,4*E*)-tetradecadienoic acid *N*-isobutyl amide (**9**) for bitter and astringent; n=15, one replication.

In the group of the trigeminal-active compounds with cooling, pungent, warming, or hot effects (Table 48), only Frescolat® ML (**34**) was able to reduce the astringency of EGCG and was therefore tested using IVDM (Figure 72). Astringency reduction was described over the whole period of time, while significant ($\leq$0.1) at 30 and 50 seconds. The bitterness was not affected at 300 µg kg$^{-1}$ of **34**.

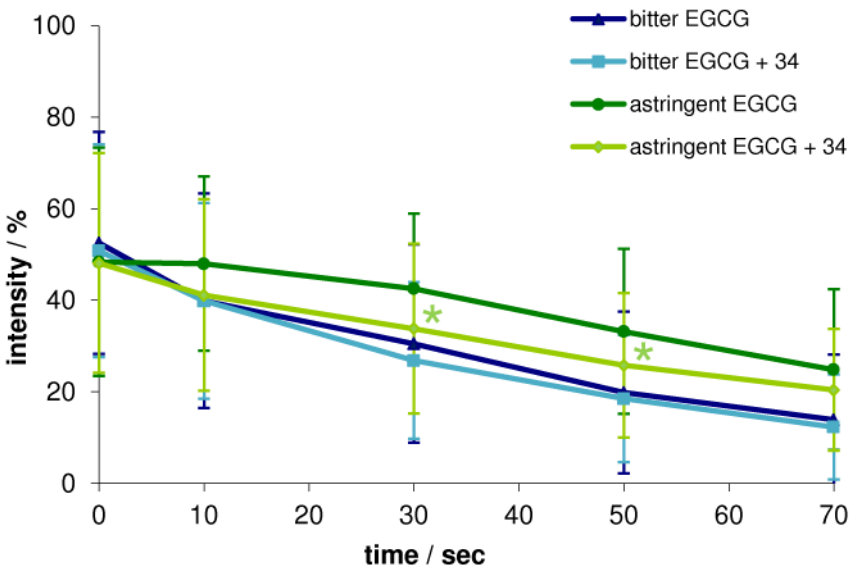



Figure 72: Time-intensity curves of 750 mg kg$^{-1}$ EGCG without and with 300 µg kg$^{-1}$ Frescolat® ML (**34**) for bitter and astringent; n=15; one replication, significant differences * $p \leq 0.1$.

### 4.2.7 Effects of trans-Pellitorine on Other Bases than EGCG

For the exploration of *trans*-pellitorine (**3**) as a general astringency masker, further astringents were evaluated. Therefore, a green tea extract (GTE) and a grape seed extract (GSE) were evaluated.

The astringency and bitterness of EGCG plays an important role in beverages made with GTE. For that reason, the effect of *trans*-pellitorine (**3**) was also tested on a more complex mixture. In GTE other catechins, such as epigallocatechin and epicatechin-3-gallate contribute to the typical flavor sensation in addition to EGCG (Narukawa et al., 2010). **3** (5 mg kg$^{-1}$) was tested on 3600 mg kg$^{-1}$ GTE, equaling about 750 mg kg$^{-1}$ catechins. A clear masking effect, significant for all but one time point, for the bitterness and astringency was found (Figure 73). Additionally, a concentration of 3 mg kg$^{-1}$ *trans*-pellitorine (**3**) on 3600 mg kg$^{-1}$ GTE did not show any effect. In comparison to the tests on pure EGCG, the results are similar. Under the tested concentrations, 5 mg kg$^{-1}$ of **3** seems to be the best masking concentration.

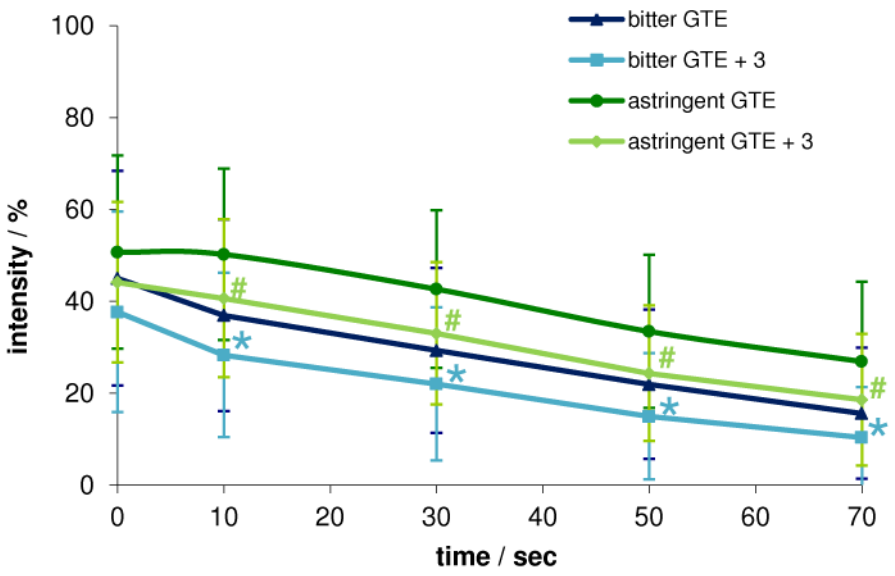



Figure 73: Time-intensity curves of 3600 mg kg$^{-1}$ (GTE) without and with 5 mg kg$^{-1}$ *trans*-pellitorine (**3**) for bitter and astringent; n=15; two replications, significant differences * $p \leq 0.1$, # $p \leq 0.05$.

Besides on EGCG and GTE, **3** was also tested on a GSE to explore the general use of *trans*-pellitorine as a masker for astringent compounds. Phenolic substances in grape seeds comprise, among others, oligomeric proanthocyanidins (OPCs), which are mostly di- or trimers of catechins. These OPCs are known for their antioxidant activity (Jayaprakasha et al., 2001; Meyer et al., 1997), as well as for their astringent sensation (Monteleone et al., 2004; Vidal et al., 2004). The intensity of astringency at 0 seconds was rated at around 50 % and thus in a similar range to the rating of EGCG at the same time point. As shown in Figure 74, the addition of **3** (5 mg kg$^{-1}$) did not result in modulations of the bitterness and astringency ratings.

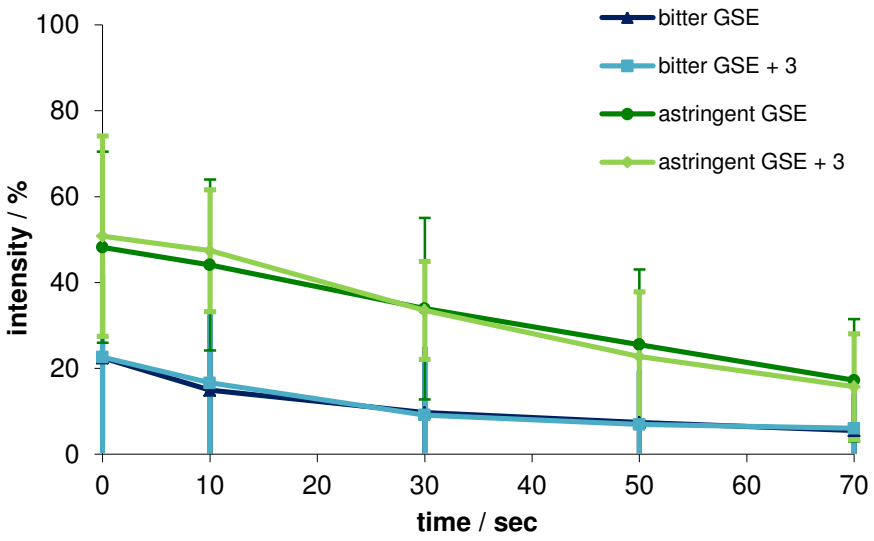



Figure 74: Time-intensity curves of 750 mg $kg^{-1}$ GSE without and with 5 mg $kg^{-1}$ *trans*-pellitorine (**3**) for bitter and astringent; n=15; one replication.

In order to confirm this unexpected result and to investigate the possible role of the relative ratio rather than the absolute concentrations, another test was carried out using 500 mg $kg^{-1}$ GSE and 5 mg $kg^{-1}$ *trans*-pellitorine (**3**). Again, no masking effect was detected (data not shown). The OPCs in GSE might act differently in terms of astringency causation compared to the catechins in GTE, especially EGCG. As the molecular mechanism of astringency is still unclear, it is also possible, that the astringency of GSE develops differently compared to the astringency of catechins.

It can be summarized that IVDM permits the evaluation of the aftertaste sensation of complex taste profiles in a very time-saving manner. Therefore, the workload of the panelists and the risk of adaptation to the test base can be reduced. Using this protocol, panelists were capable to differentiate between up to four descriptors, especially between bitterness and astringency and their intensities in simple EGCG model solutions. In accordance to Lee and Lawless (1991), only few tests are necessary for screening proposes to evaluate the aftertaste of various descriptors over time.

Depending on the problem, the number of descriptors and measuring points, the time between the measuring points, and the duration of the test can be varied. Another advantage is the observation of relationships between different descriptors. By rating them in parallel, there is a closer correlation between the descriptors compared to

rating each separately. Especially for complex food samples, this trait is very important and possible interactions can be more easily determined.

The starting question for the astringency masking study was whether an enhanced salivation effect causes the clearance of neurons from EGCG. Some of the compounds that were described to have a strong mouth-watering effect showed an astringency-masking effect in a defined concentration range, as shown for *trans*-pellitorine (**3**), while others, e.g. (2*E*)-decenoic acid *N*-isobutyl amide (**26**), did not. Therefore, no definite conclusion can be drawn, if a compound has masking activity on astringency based on their salivating effect only (activity-activity relationship). Moreover, one of the tested structurally related molecules but not mouth-watering, showed also a reduction of EGCG astringency. *cis*-Pellitorine (**28**), the geometric isomer of **3**, was able to reduce the ECGC astringency, while (2*E*)-decenoic acid *N*-isobutyl amide (**26**) and decanoic acid *N*-isobutyl amide (**29**), which differ only in the amount of double bonds compared to **3**, showed no reduction. Thus, neither the salivating activity nor the structural relationship alone seems to be responsible for masking effects. Furthermore, additional trigeminal-active compounds were tested, whereby the cooling agent Frescolat® ML (**34**), was able to reduce the astringency of EGCG. This might support another hypothesis, the existence of specific antagonistic activity towards orphan EGCG receptors. Moreover, it was shown that the influence of the type of astringents is crucial as well. A clear masking effect was shown for the astringency of EGCG and the catechin-based green tea extract (GTE) by using *trans*-pellitorine (**3**) as the test compound, while no reduction occurred on grape seed extract (GSE).

Among the identified and tested compounds in *M. excelsum*, only *trans*-pellitorine (**3**) was able to reduce the astringency of EGCG. The two homologues (**8**, **9**), the trigeminal-active piperine (**7**), as well as the saliva-inducing achilleamid (**4**) were not able to reduce the astringency under tested conditions.

It can be summarized that no obvious structure-activity relationship was found with the tested compounds and astringents at the tested concentrations. The results indicate an apparently specific base-to-compound interaction, but the molecular mechanism of these effects remains still unclear. To know the molecular mechanism of astringency would bring forward focused research on astringency masking. A number of papers in the past indicate different approaches, how astringency occurs. Some authors suggest that astringent molecules are able to complex and to

precipitate certain proteins in saliva, which changes the viscosity and lubricity. These changes might be detectable by tactile neurons (Breslin et al., 1993; Jobstl et al., 2004). In contrast, other research groups suggest astringency perception without protein binding (Rossetti et al., 2009; Schwarz and Hofmann, 2008). This refers to an involvement of the trigeminal system in astringency perception. First evidences were presented by Schöbel (2010, 2013), who showed that astringent chemicals, e.g. EGCG, can stimulate isolated trigeminal ganglion neurons. An interaction of catechins and mouse TRPA1 channels is also mentioned in the literature (Kurogi et al., 2012).

With regard to the literature findings and the results of this study, an interaction of astringent molecules with trigeminal neurons and their activation seems to be possible and might be one mechanism of astringency perception.

# 5 SUMMARY

The leaves and fruits of *Macropiper excelsum,* also called kawakawa, are traditionally eaten by the Maori and exhibit a slight numbing and tingling sensation when chewed. *M. excelsum* belongs to the *Piperaceae* family like *Piper longum* and *Piper nigrum,* which are well known for their trigeminal-active compounds. However, not much is known about the constituents of kawakawa. Due to occasional reports in the literature that tingling and numbing compounds, e.g. *trans*-pellitorine, stimulate mild salivation, and therefore may help to counteract astringency of certain phytochemicals such as epigallocatechin gallate (EGCG), *M. excelsum* was phytochemically investigated to identify the compounds which are responsible for its promising sensory properties.

To investigate the volatile part, a pentane/diethyl ether extract of the dried leaves from New Zealand was analyzed. The smell of this extract was described as herbal, tea, hay, woody, peppery, and incense. About 100 substances of different structural classes were found, like terpene hydrocarbons, oxygenated terpenes, norisoprenoids, phenyl propanoids, acids, esters, alcohols, and aldehydes. Only a few volatile compounds (e.g. α-pinene, myristicin, aromadendrene, γ-cadinene, camphene, elemicin, hexadecanoic acid, β-phellandrene) were already described in *M. excelsum*. The other compounds were newly identified in the leaves of kawakawa. Myristicin, elemicin, hydrocinnamic acid, hexadecanoic acid, and acetic acid were the main compounds in the extract and accounted for 80.6 % of the total volatiles.

For the identification of the non-volatile constituents, the dried leaves were extracted with ethanol and water. During sensory evaluation, the crude extract was described as slightly bitter, slightly sweet, and herbal by the panelists and showed the expected trigeminal effects. When doing sensory-guided analysis via HTLC, these trigeminal effects occurred again in some fractions. As these fractions were still quite complex, further purification steps were carried out via FCPC and pHPLC, followed by structure elucidation via NMR.

Besides the already described constituent diayangambin (**22**), five further known lignans, like (+)-diasesartemin (**18**) and (+)-episesartemin A (**21**), and two flavonoid glycosides, vitexin-2''-O-glucoside (**23**) and orientin-2''-O-glucoside (**24**), were identified. Thirteen amides, such as *trans*-pellitorine (**3**), achilleamid (**4**), kalecide (**8**), and chingchengenamide A (**11**), were shown to be present in *M. excelsum* for the first time. Methyl (2*E*,4*E*)-7-(1,3-benzodioxol-5-yl)hepta-2,4-dienoate (**10**) has not been described in the literature before. When comparing the phytochemical

compositions of the different parts of a living specimen of *M. excelsum* grown in Germany, the amides were found at the highest concentrations in the roots, followed by the stems and leaves, while the highest concentrations of the lignans were measured in the leaves.

The intrinsic tastes of the isolated molecules were determined at 10 and 50 mg $kg^{-1}$ on water. Some molecules were described only as bitter, dry, and woody (**6**, **16**, **23**, **24**), but most of the isolated compounds showed some trigeminal effects. *trans*-Pellitorine (**3**), achilleamid (**4**), and (+)-diayangambin (**22**) were described as tingling and mouth-watering, and *(Z)*-antiepilepsirine (**15**) as cooling. *trans*-Fagaramid (**5**), piperine (**7**) and (+)-episesartemin B (**20**) were described as hot and burning. A combination of the trigeminal-active substances is probably responsible for the taste impression of the plant, the crude extract, and the HTLC fractions. As the effect of some of the compounds was relatively weak, only a subset of molecules, such as **3**, **4**, **7**, **8**, and **9**, was evaluated in terms of their astringency-masking effects.

For the evaluation of complex taste impressions such as astringency and the corresponding potential masking compounds, the use of a specific sensory screening tool was necessary. Due to the long-lasting aftertaste of many astringent compounds such as EGCG, a time-intensity approach, based on intensity variation descriptive methodology (IVDM) was established. With IVDM, the intensity of different descriptors can be rated in parallel over a certain period of time. Only a few tests are necessary to evaluate possible masking effects of a compound, which minimizes testing time, panel workload and the amount of substances. Additionally, by rating the descriptors at the same time, the relationship between them is observed.

After evaluation of the *M. excelsum* compounds, *trans*-pellitorine (**3**) remained as the most active astringency masker on EGCG, the most abundant catechin in green tea. Therefore, further mouth-watering compounds as well as structural analogues of pellitorine were investigated. Additionally, trigeminal-active molecules for cooling, heating, and pungent were tested. Besides *trans*-pellitorine (**3**), the hydrophilic (2S)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propanoic acid (**27**) showed also significant astringency-masking effects on EGCG. For compounds having similar mouth-watering activity, e.g. (2*E*)-decenoic acid *N*-isobutyl amide (**26**), no masking effect was found. Thus, no conclusion about a general relationship between a salivating effect and masking possibility of EGCG off-notes can be drawn. Structurally related molecules of **3**, such as *cis*-pellitorine (**28**) and decanoic acid *N*-isobutyl amide (**29**)

were tested; however, they also showed no unambiguous relationship between structures and masking effect. *cis*-Pellitorine (**28**) was able to reduce the ECGC astringency, while **29** showed no reduction. Further trigeminal-active molecules, such as Frescolat® SC1 (**33**), Frescolat® ML (**34**), WS3 (**35**), nonivamide (**31**), piperine (**7**), and 6-paradol (**32**) were tested as well. Among them, the cooling agent Frescolat® ML (**34**) was also able to reduce EGCG astringency. Again, no obvious structure-activity relationship within the tested set of molecules was found. In consideration of the existing literature, these results contribute to the hypothesis that astringent molecules can interact with and activate trigeminal neurons as one possible mechanism of astringency perception.

Besides the masking compound, the type of astringents has to be taken into account as well. *trans*-Pellitorine (**3**) was able to reduce the astringency of EGCG, however, did not work on grape seed extract (GSE). Thus, a specific base-to-compound relationship seems to be important.

As a result of this study, the most important actives causing the chemosensation of *M. excelsum* could be identified. Furthermore, sensory evaluation of trigeminal-active compounds as astringency maskers lead to a proof that *trans*-pellitorine is able to reduce significantly the astringency of EGCG. However, the investigation of structural and sensorial analogues demonstrates the specificity of certain trigeminal-active compounds in their ability to mask unpleasant taste.

# 6 ZUSAMMENFASSUNG

Die Blätter und Früchte des neuseeländischen Pfefferbaums *Macropiper excelsum,* Kawakawa, werden traditionell von den Maori verzehrt und bewirken beim Kauen einen leicht betäubenden und kribbelnden Effekt. *M. excelsum* gehört zur Pflanzenfamilie der *Piperaceae*, wie auch *Piper longum* und *Piper nigrum*, die für ihre trigeminal-wirksamen Inhaltsstoffe bekannt sind. In der Literatur ist jedoch nicht viel über die einzelnen Inhaltsstoffe von Kawakawa zu finden. Vereinzelte Literaturstellen weisen darauf hin, dass kribbelnde und betäubende Substanzen, wie *trans*-Pellitorin, einen leichten Speichelfluss hervorrufen. Dieser Speichelfluss könnte der Adstringenz von Pflanzeninhaltsstoffen, wie Epigallocatechingallat (EGCG), entgegenwirken. Das phytochemische Profil von *M. excelsum* wurde hinsichtlich der sensorischen und maskierenden Eigenschaften der Moleküle untersucht.

Zur Aufklärung der flüchtigen Verbindungen wurde ein Pentan/Diethylether-Extrakt aus den getrockneten Blättern aus Neuseeland hergestellt und analysiert. Der Geruch des Extraktes wurde bei der sensorischen Bewertung mit den Begriffen krautig, Tee, Heu, holzig, pfeffrig und Weihrauch-artig beschrieben. Die Analyse brachte ca. 100 Substanzen verschiedener Stoffklassen, wie beispielsweise Terpenkohlenwasserstoffe, oxygenierte Terpene, Norisoprenoide, Phenylpropanoide, Säuren, Ester, Alkohole und Aldehyde hervor. Nur wenige davon (z.B. α-Pinen, Myristicin, Aromadendren, γ-Cadinen, Camphen, Elemicin, Hexadecansäure, β-Phellandren) sind für *M. excelsum* beschrieben. Alle anderen Verbindungen wurden zum ersten Mal in Kawakawa identifiziert. Myristicin, Elemicin, Hydrozimtsäure, Hexadecansäure und Essigsäure waren mit zusammen 80.6 % der flüchtigen Stoffe die Hauptkomponenten im Extrakt.

Zur Identifizierung der nichtflüchtigen Substanzen wurden die getrockneten Blätter zunächst mit Ethanol/Wasser extrahiert. Der Extrakt wurde als leicht bitter, süß und krautig beschrieben und zeigte zusätzlich trigeminale Effekte. Diese trigeminalen Effekte konnten nach der Auftrennung über HTLC in einigen Fraktionen wiedergefunden werden. Nachdem diese Fraktionen immer noch sehr komplex waren, wurden weitere Aufreinigungsschritte mittels FCPC und pHPLC durchgeführt.

Neben dem schon bekannten Diayangambin wuden fünf weitere Lignane, z.B. (+)-Diasesartemin (**18**) und (+)-Episesartemin A (**21**) und zwei Flavonoidglycoside, Vitexin-2''-O-glucosid (**23**) und Orientin-2''-O-glucosid (**24**), identifizert. 13 Amide, wie *trans*-Pellitorin (**3**), Achilleamid (**4**), Kalecid (**8**) und Chingchengenamid A (**11**)

wurden zum ersten Mal in *M. excelsum* gefunden. (2*E*,4*E*)-7-(1,3-Benzodioxol-5-yl)-2,4-heptadiensäuremethylester (**10**) wurde bislang nicht in der Literatur beschrieben. Beim Vergleich der verschiedenen Pflanzenteile einer Lebendpflanze von *M. excelsum*, die in Deutschland herangezogen wurde, konnten die höchsten Gehalte der Amide in den Wurzeln gefunden werden, gefolgt von den Ästen und Blättern. Die höchsten Konzentrationen der Lignane waren hingegen in den Blättern zu finden.

Die Geschmackseindrücke der isolierten Moleküle wurden bei zwei Konzentrationen, 10 und 50 mg $kg^{-1}$, auf Wasser bestimmt. Einige Stoffe waren bitter, austrocknend und holzig (**6**, **16**, **23**, **24**) und fast alle trigeminal-aktiv. *trans*-Pellitorin (**3**), Achilleamid (**4**) und (+)-Diayangambin (**22**) wurden als kribbelnd und mundwässernd beschrieben, *(Z)*-Antiepilepsirin (**15**) als kühlend, und Piperin (**7**) und (+)-Episesartemin B (**20**) als scharf und brennend. Eine Kombination der trigeminal-wirksamen Verbindungen ist wahrscheinlich für die sensorische Wahrnehmung des Extraktes und der verschiedenen HTLC-Fraktionen verantwortlich. Da der sensorische Effekt der meisten Substanzen relativ schwach war, wurden nur einzelne Moleküle (**3**, **4**, **7**, **8**, **9**) für weitere Tests herangezogen. Diese wurden hinsichtlich ihrer mögliche Wirkung, Adstringenz zu maskieren, getestet.

Um komplexe sensorisch Problemstellungen und mögliche Maskierungslösungen bewerten zu können, ist ein Verfahren zur gleichzeitigen Betrachtung mehrerer sensorischer Parametern nötig. Daher war es nötig, eine geeignete sensorische Methode zu identifizieren und zu etablieren. Aufgrund des langanhaltenden Nachgeschmacks von adstringierenden Substanzen ist die Bewertung der Deskriptoren über die Zeit notwendig. Als Verfahren wurde auf die bestehende Methode „intensity variation descriptive methodology“ (IVDM) zurückgegriffen. Dabei werden die Intensitäten verschiedener Deskriptoren parallel über eine bestimmte Zeitspanne gemessen. Durch das Methodendesign sind nur wenige Tests für die Evaluierung möglicher maskierender Stoffe nötig, was wiederum die Dauer, die Arbeitslast und die Menge an Testsubstanzen reduziert. Zusätzlich wird durch die Abfrage mehrerer Deskriptoren zur gleichen Zeit deren Beziehung zueinander stärker einbezogen.

Von denen in *M. excelsum* identifizierten und getesteten Substanzen, zeigte *trans*-Pellitorin (**3**) die beste Wirksamkeit in Bezug auf die Adstringenzmaskierung von EGCG, dem Hauptcatechin in Grüntee. Aus diesem Grund wurden weitere Substanzen mit mundwässernden Effekten, aber auch dem Pellitorin strukturell

ähnliche Substanzen mittels IVDM evaluiert. Zusätzlich wurden trigeminal-wirksame Moleküle mit kühlenden, scharfen oder brennenden Eigenschaften getestet. Neben *trans*-Pellitorin (**3**) zeigte (2S)-2-[[(2*E*,4*E*)-deca-2,4-dienoyl]amino]propansäure (**27**) signifikant maskierende Effekte auf die Adstringenz von EGCG. Bei Molekülen mit ähnlich starken mundwässernden Eigenschaften, wie beispielsweise *N*-Isobutyl-2*E*-decensäureamid (**26**), konnten allerdings keine Effekte nachgewiesen werden. Daher war es nicht möglich, einen generellen Zusammenhang zwischen den speichelanregenden Eigenschaften eines Stoffes und dessen Fähigkeit die Adstringenz von EGCG zu reduzieren aufzuzeigen.

Strukturell ähnliche Substanzen von *trans*-Pellitorin (**3**), wie *cis*-Pellitorin (**28**) und *N*-Isobutyl-decansäureamid (**29**) wurden auch geprüft, allerdings konnte kein eindeutiger Zusammenhang zwischen Struktureigenschaften und Maskierungseffekten hergestellt werden. Die Adstringenz von EGCG konnte mittels *cis*-Pellitorin (**28**) reduziert werden, wohingegen *N*-Isobutyl-decansäureamid (**29**) keinen positiven Effekt zeigte. Von den weiteren getesteten trigeminal-aktiven Stoffen Nonivamid (**31**), Piperin (**7**), 6-Paradol (**32**), WS3 (**35**), Frescolat® SC1 (**33**) und Frescolat® ML (**34**), konnte nur letzterer die Adstringenz von EGCG senken. Im Allgemeinen lässt sich daher sagen, dass es keine offensichtliche Struktur-Aktivitäts-Beziehung gibt. Die Ergebnisse unterstützen die in der Literatur beschriebene Hypothese, dass bei der Wahrnehmung von Adstringenz trigeminale Neuronen beteiligt sind.

Neben der maskierenden Substanz spielt der adstringierende Stoff eine wichtige Rolle. *trans*-Pellitorin (**3**), welches die Adstringenz von EGCG reduzieren konnte, zeigte keinerlei Maskierungseffekt auf Traubenkernextrakt. Es scheint also auf eine ganz bestimmte Kombination zwischen dem Maskierer und dem zu maskierenden Stoff anzukommen.

Als Ergebnis dieser Arbeit konnten die wichtigsten aromarelevanten Inhaltsstoffe von *M. excelsum* identifiziert werden. Darüber hinaus ergab die Bewertung der trigeminal-wirksamen Stoffe, dass vor allem *trans*-Pellitorin die Adstringenz von EGCG signifikant reduzieren kann. Die Untersuchung von weiteren mundwässernden Substanzen und strukturellen Analogen des Pellitorins zeigte allerdings die Spezifität bestimmter trigeminaler Substanzen bezogen auf ihre maskierenden Eigenschaften von Fehlgeschmäckern.

# 7 LITERATURE

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# 8 ABBREVIATIONS

| | |
|---|---|
| AEDA | aroma extraction dilution analysis |
| ANOVA | analysis of variance |
| ASE | accelerated solvent extraction |
| ASIC | acid-sensing ion channels |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| br s | broad singlet |
| *c* | concentration |
| cAMP | cyclic adenosine monophosphate |
| CaSR | calcium-sensing receptor |
| CCC | countercurrent chromatography |
| $CDCl_3$ | chloroform-d1 |
| $CD_3OD$ | methanol-d4 |
| CMC | carboxymethylcellulose |
| CPC | centrifugal partition chromatography |
| cTDA | comparative taste dilution analysis |
| d | doublet |
| DAG | diacylglycerol |
| DATI | dual-attribute time-intensity |
| DCCC | droplet countercurrent chromatography |
| EC | (-)-epicatechin |
| ECG | (-)-epicatechin gallate |
| EGC | (-)-epigallocatechin |
| EGCG | (-)-epigallocatechin gallate |
| EI | electron impact |
| EtOAc | ethyl acetate |
| FCPC | fast centrifugal partition chromatography |
| FD | flavor dilution |
| FID | flame ionization detector |
| G | centrifugal field |
| GC-MS | gas chromatography – mass spectrometry |
| GC-O | gas chromatography – olfactometry |
| gCOSY | gradient correlation spectroscopy |
| gHMBC | gradient heteronuclear multiple bond coherence |
| gHSQC | gradient heteronuclear single quantum coherence |
| GMP | guanosin monophosphate |
| GPCR | G-protein-coupled receptor |
| GSE | grape seed extract |
| GTE | green tea extract |
| hep | heptet |
| HIS | high intensity sweeteners |
| HRGC-FID | high-resolution gas chromatography – flame ionization detector |
| HSCCC | high-speed countercurrent chromatography |
| HTLC | high-temperature liquid-chromatography |
| Hz | hertz |
| IMP | inositol monophosphate |
| $IP_3$ | inositol-1,4,5-trisphosphate |
| ITS | internal transcribed spacers |
| IVDM | intensity variation descriptive methodology |

| | |
|---|---|
| *J* | coupling constant |
| *K* | partition coefficient |
| KI | Kovats retention indices |
| LC-MS | liquid chromatography – mass spectrometry |
| m | multiplet |
| M.e. | *Macropiper excelsum* |
| M.e. F | *Macropiper excelsum* dried fruits |
| M.e. L | *Macropiper excelsum* dried leaves |
| M.e. P | *Macropiper excelsum* living plant |
| M.e. S | *Macropiper excelsum* dried seeds |
| MeOH | methanol |
| MHz | megahertz |
| MSG | monosodium glutamate |
| MW | molecular weight |
| n.d. | not detected |
| NMR | nuclear magnetic resonance |
| OAV | odor activity value |
| OPC | oligomeric proanthocyanidin |
| OSN | olfactory sensory neurons |
| PBD | polybutadiene |
| PDA | photo diode array |
| PDE | phosphodiesterase |
| pHPLC | preparative high-performance liquid chromatography |
| PIP2 | phosphatidyl-inositol-4,5-bisphosphate |
| PKD1L3 | polycystic kidney disease-like |
| PLCβ2 | phospholipase Cβ2 |
| pp | percentage points |
| ppm | parts per million |
| PROP | 6-*n*-propylthiouracil |
| PTC | phenylthiocarbamide |
| q | quartet |
| QDA® | quantitative descriptive analysis procedure |
| RPLC | reversed-phase liquid chromatography |
| s | singlet |
| SNPs | single nucleotide polymorphisms |
| t | triplet |
| TDA | taste dilution analysis |
| TI | time-intensity |
| TIP | time-intensity profiling |
| TRC | taste receptor cell |
| TRP | transient receptor potential |
| TRPA | ankyrin type |
| TRPC | canonical type |
| TRPM | melastatin type |
| TRPML | mucolipin type |
| TRPP | polycystin type |
| TRPV | vanilloid type |
| VFTM | venus flytrap module |
| $\delta_C$ | carbon chemical shift |
| $\delta_H$ | proton chemical shift |

# 9 FIGURES

# 10 TABLES

# CURRICULUM VITAE

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## BERUFSERFAHRUNG

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|---|---|
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## BILDUNGSWEG

| | |
|---|---|
| 2009 – 2014 | Doktorarbeit an der Technische Universität München, Lehrstuhl für Allgemeine Lebensmitteltechnologie, Prof. Dr. K.-H. Engel, in Zusammenarbeit mit Symrise AG<br>*"Phytochemical Characterization and Sensory Evaluation of Macropiper excelsum"* |
| 2007 – 2009 | MSc Ernährungswissenschaft, Technische Universität München Note: 1,4; Masterarbeit: *"Sensory characterization of Stevia extracts"* (in Zusammenarbeit mit Symrise GmbH & Co. KG) |
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