DOCTORAL THESIS

Flavor Potential and Limitations in Novel Protein Sources: An Integrated Sensory and Chemical Analysis Focused on Off-Flavor Identification

Lachinkhanim Huseynli

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Lachinkhanim Huseynli

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Uudsete valguallikate maitseomadused ja piirangud: integreeritud sensoorne ja keemiline analüüs kõrvalmaitsete tuvastamiseks

LACHINKHANIM HUSEYNLI

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List of Publications

The list of the author's publications, based on which the thesis has been prepared:

I **Huseynli, L**., Parviainen, T., Kyllönen, T., Aisala, H. & Vene, K. Exploring the protein content and odor-active compounds of black soldier fly larvae for future food applications. *Future Foods* **7**, 100224 (2023).

II Vene, K., Lumi, E., Alas, M. & **Huseynli, L**. Integrated Sensory, Nutritional, and Consumer Analysis of Sunflower Seed Butter: A Comparative Study of Commercial and Prototype Samples. *Foods* **14**, 1815 (2025).

III **Huseynli, L**., Walser, C., Blumenthaler, L., Vene, K. & Dawid, C. Toward a Comprehensive Understanding of Flavor of Sunflower Products: A Review of Confirmed and Prospective Aroma- and Taste-Active Compounds. *Foods* **14**, 1940 (2025).

IV **Huseynli, L**., Gigl, M., Müller, J., Walser, C., Frank, O., Vene, K., & Dawid, C. Characterization of bitter off-taste stimuli in sunflower press cake using the sensomics approach. (under review)

Author's Contribution to the Publications

Contribution to the papers in this thesis are:

I The author was involved in the study design, sample preparation, data analysis, interpretation of results, and writing of the manuscript.

II The author reviewed and contextualized the findings within the manuscript and was actively involved in drafting and refining the final version.

III The author was responsible for designing the review structure, collecting and analysing the literature, interpreting the findings, and writing the manuscript.

IV The author was involved in the study design, sample preparation, data analysis, interpretation of results, and writing of the manuscript.

Preface

The accelerating global demand for protein, driven by population growth and environmental constraints, has underscored the urgency of developing sustainable protein sources. Numerous alternatives have emerged, including plant-based, single-cell, and insect proteins, offering promising nutritional and ecological advantages. Nevertheless, many of these protein sources remain limited by high production costs, technological constraints, or formulation challenges. Regardless of their origin, a common and critical limitation across most alternatives is their unfavorable sensory properties, particularly off-flavors such as bitterness or astringency, which compromise consumer acceptance and restrict broader market integration.

In the early stages of this research, various protein matrices were evaluated for their sensory potential, including insect-based ingredients known for their distinct volatile profiles. However, due to greater consumer familiarity and application potential, the primary focus of this work was directed toward plant-based protein alternatives.

Although plant-based protein sources such as soy, pea, and oat have been extensively characterized in terms of their functional and sensory attributes, many alternative matrices remain insufficiently explored in terms of flavor chemistry. Among these underutilized sources, sunflower (*Helianthus annuus* L.) press cake stands out as a protein-rich by-product of oil extraction with emerging relevance in food upcycling applications. Despite its potential, no systematic study has been undertaken to characterize its flavor profile or to elucidate the molecular basis of its sensory limitations.

To address this gap, a comprehensive review of potential flavor compounds in sunflower-based products was conducted to provide a broader context and support the interpretation of experimental findings. Building on this foundation, a sensomics approach was applied to investigate the off-flavor of sunflower press cake by identifying key bitter taste-active compounds and evaluating their sensory relevance. Alongside this analytical work, sunflower seeds were also evaluated in a spread formulation to assess their sensory properties and explore real-world application potential.

These findings provide a molecular foundation for mitigation strategies, supporting the development of processing interventions, ingredient optimization or raw material selection criteria that enable the food industry to create more palatable and consumer-acceptable formulations using sunflower-derived proteins.

1 Introduction

1.1 Flavor as a multisensory construct

Few experiences are as universally enjoyed yet deeply complex as the act of tasting food and beverages. But what exactly constitutes flavor, and how do we experience it? These questions bridge the realms of philosophy and science, inviting reflection on both the nature of perception and the mechanisms behind it.

Philosophically, the epistemology of tasting explores what the experience of flavor enables us to know. Is flavor an objective property of food, a real feature independent of perception or is it purely a subjective sensation arising within us? Objectivists argue that flavor resides in the food, a tangible property revealed through tasting, while subjectivists view it as an internal experience shaped entirely by the mind. A more nuanced perspective reconciles these views, suggesting that flavor is neither purely in the food nor solely in the mind but a multisensory construct that emerges from the interaction of sapid, odorous, and tactile properties with the perceiver [1–3].

Scientifically, flavor is often defined as a multisensory phenomenon involving taste (gustatory), smell (olfactory), and trigeminal sensations. Unlike taste and touch, which are localized to the mouth, the receptors responsible for detecting smells are located deep within the nasal cavity. Despite this anatomical separation, smell frequently becomes the defining element of the flavors we perceive during eating. This is because volatile compounds released from foods during chewing and swallowing travel retronasally to the nasal cavity, where they bind to olfactory receptors. These signals are perceived as originating in the mouth, creating a unified perception of flavor [4].

Lim and Johnson's research demonstrated that the perception of odors originating from the mouth relies on the presence of taste rather than tactile stimulation. Their experiments revealed that participants were significantly more likely to experience odors as mouth-based when a congruent taste stimulus was present. This phenomenon highlights a hierarchical relationship: touch captures taste, and taste ultimately captures smell, bringing these sensations into a shared spatial and temporal framework [4,5].

Researchers diverge on whether factors such as visual appearance, sound, and texture should be considered intrinsic components of flavor or merely modulatory influences [6,7]. Growing evidence demonstrates that such sensory cues can profoundly shape how flavor is perceived, often in ways that challenge traditional definitions [8–12]. One perspective argues that flavor encompasses all sensory inputs influencing food perception, making it highly context dependent. Another approach focuses on core intrinsic elements, such as taste and smell, while treating external factors as influences rather than constituents of flavor itself [6,7].

This complexity underscores the idea that "the unification of sensory impressions enabled by the act of eating does not occur at the level of sensation, but rather at the level of perception" [13]. In this view, flavor is a mental construct shaped by expectation, memory, genetics, and environment, transcending its physical attributes. Prior experiences, cultural context, and even the setting in which food is consumed can significantly alter its perception [14].

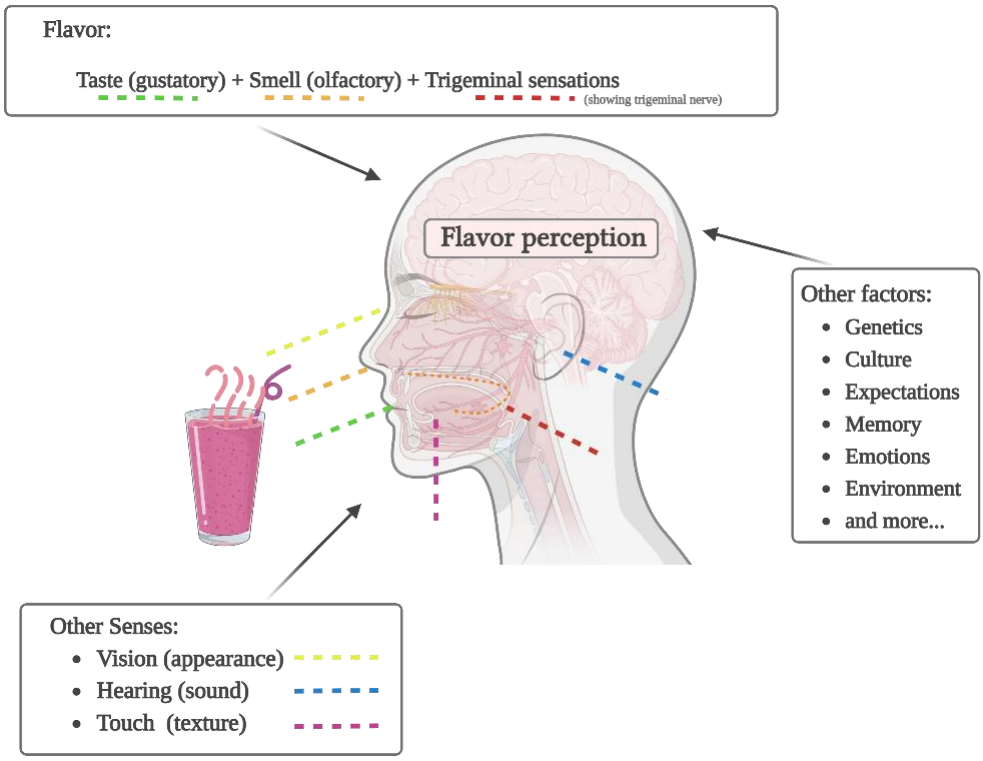

Figure 1. Integrated sensory and modulatory inputs underlying flavor perception. *Flavor perception arises from the multisensory integration of taste (gustatory), smell (olfactory), and trigeminal sensations. This core is further modulated by other sensory inputs such as vision, audition, and somatosensory cues, as well as by internal and external factors including genetics, culture, expectations, memory, emotional state, etc. Image created based on information from Spence et al. (2015) [6], Prescott et al. (2015) [7], Spence et al. (2020) [14], Shepherd et al. (2012) and Castillo et al. (2014) [15,16]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/duh6mxu*

While researchers agree that flavor perception is a multisensory experience, the degree to which various sensory modalities contribute remains contested. Olfaction is widely acknowledged as the dominant factor, with studies suggesting that up to 80–90% of what we perceive as "taste" is actually smell [17]. However, some argue that these figures may hold true only if we consider flavor as merely a combination of taste and smell. When additional senses, such as touch, hearing, and sight, are factored into the equation, the situation becomes far more intricate and interesting [17,18]. Martin Yeomans emphasizes that any attempt to generalize the relative contribution of a single sensory modality to food flavor is inherently flawed, as each food engages distinct and unique interactions across the key sensory systems [19].

The challenge of disentangling the relative contributions of sensory modalities is further complicated by the widespread confusion between the terms "taste" and "flavor." This ambiguity underscores the need for more precise and consistent definitions in both scientific discourse and everyday language [20]. One explanation for this ambiguity is that, in many languages, the same word is used for both taste and flavor, such as in Spanish, German, and Czech [17]. These linguistic overlaps may reflect deep-seated associations formed through learned flavor-taste experiences with specific foods [20].

In conclusion, flavor is both a sensory and philosophical phenomenon, arising from the interplay of objective properties, subjective experiences, and contextual influences (Figure 1). Its perception emerges at the intersection of the physical and the mental, making it one of the most complex and fascinating aspects of human experience. Building on this complexity, Castillo, drawing from Gordon Shepherd's work, illustrates flavor as a multifaceted construct that can be represented by this equation: (smell + taste + mouth sense + sight + sound) × (emotion + memory + decisions + plasticity + language + consciousness) = flavor [15,16].

In the following discussion, we will delve into the components of flavor in detail, focusing primarily on smell and taste, to uncover the intricate mechanisms that shape the core of flavor perception. Understanding these components is crucial for addressing the flavor challenges.

1.2 The role of smell (olfaction) in flavor perception

Research suggests that taste may play a primary role in signaling meal termination, whereas smell appears to be important in guiding food choices, enhancing anticipation, and initiating eating behaviors. This highlights the unique importance of smell in shaping dietary decisions and the eating experience [21].

The sense of smell is uniquely specialized in detecting small airborne molecules known as volatiles. These largely hydrophobic compounds can be perceived at incredibly low concentrations, some detectable at levels of parts per billion (ppb) and others as low as parts per trillion (ppt) [22–24]. This sensitivity is enabled by the human genome, which encodes over 1,000 olfactory receptor genes, representing the largest gene family identified [25]. However, approximately two-thirds of these genes are nonfunctional “pseudogenes.” Despite this, humans can distinguish an astonishing range of odors. Early estimates suggested the ability to differentiate around 10,000 distinct smells, but recent studies have revised this figure significantly, indicating humans can discern at least one trillion distinct olfactory stimuli [26]. This extraordinary capacity highlights the complexity of the olfactory system and its associated receptors.

Smell functions through two distinct pathways: orthonasal and retronasal olfaction (Figure 2). Orthonasal olfaction occurs when odorants enter through the nostrils, enabling the identification of external aromas, shaping anticipation, and influencing food choices based on environmental cues. In contrast, retronasal olfaction takes place during eating. Volatile compounds released from food in the mouth travel through the nasopharynx to the olfactory epithelium [15,23,25,27].

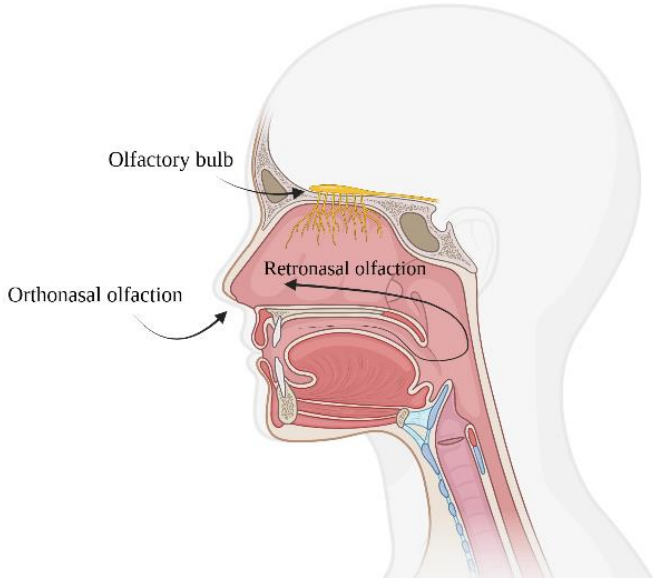

Figure 2. Orthonasal and retronasal pathways of olfactory perception. *Odor perception occurs through two main routes: orthonasal olfaction, which detects odors entering through the nose during sniffing, and retronasal olfaction, which detects volatiles released from food in the mouth and transported to the nasal cavity during eating. Both pathways converge at the olfactory bulb. Image created based on information from Shepherd et al. (2012) [15], Hummel et al. (2015) [23], Shepherd et al. (2006) [25], and Lawless et al. (1991) [27]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/ivyrsqg*

Olfactory perception operates through specialized proteins that recognize and bind odor compounds. Among these are odorant-binding proteins, which transport odorants through nasal mucus, and odorant receptors, which are G-protein-coupled receptors located in the olfactory epithelium. Each olfactory neuron typically expresses a single receptor subtype. These receptors exhibit both selectivity and flexibility, allowing multiple receptors to respond to a single odorant and vice versa. This broad molecular receptive range shapes the sensory response spectrum of neurons. Once odor signals are detected, they are transmitted to the olfactory bulb and then to the primary olfactory cortex, including the piriform cortex. From there, signals are relayed to higher cortical areas such as the orbitofrontal cortex, facilitating the conscious perception of odors and their integration with the limbic system. This integration explains why smells often evoke powerful memories and emotions, given the limbic system's role in generating emotional and motivational responses. Our understanding of these mechanisms extends to how the brain reconstructs these stimuli into a smell map [23,24,28,29].

Understanding the olfactory system's sensitivity and complexity is crucial when addressing flavor challenges. Tackling these issues requires a sophisticated understanding of the interplay between odorants and taste-active compounds. To support this, advanced analytical methods and instruments are essential for identifying key flavor contributors, ultimately paving the way for targeted strategies to improve the sensory profiles of sustainable food products [30].

1.3 The role of taste (gustation) in flavor perception

Taste plays a crucial role in regulating appetite, shaping food choices, and guiding nutrient intake by triggering cephalic phase responses, which are bodily reactions that begin before food enters the stomach. These responses, including salivary, gastric, pancreatic, and intestinal secretions, are driven by sensory cues. As food enters the mouth, it mixes with saliva, which dissolves tastant molecules so they can bind to receptors on taste buds. This interaction sends signals to the brain [31–33].

Taste buds are located within specialized structures called papillae, which are distributed across the tongue, soft palate, pharynx, and epiglottis. These include fungiform papillae on the tongue's surface, foliate papillae along its sides, and circumvallate papillae near the back in a V-shaped formation. The human tongue typically contains approximately 2,000 to 5,000 taste buds. Each taste bud comprises 50 to 100 taste receptor cells (TRCs), which project microvilli into taste pores to interact with dissolved tastants. These cells undergo renewal every 10 to 14 days, allowing for the preservation of taste function despite continual environmental exposure [33–35].

Taste receptor cells are categorized into three primary types (Figure 3). Type I cells serve a glial-like function, maintaining the ionic composition within the taste bud. Type II cells are responsible for detecting sweet, bitter and umami stimuli via G protein-coupled receptors (GPCRs). Type III cells respond to sour stimuli and form synaptic connections with afferent nerve fibers. This structural organization enables the simultaneous detection of multiple taste modalities within individual taste buds, enhancing their function as complex sensory units [33–35].

The primary taste modalities (sweet, salty, sour, bitter, and umami) are mediated by distinct receptor mechanisms, each involving specialized signaling pathways [32,36,37].

Sweet Taste. Sweetness is detected by the heterodimeric GPCR complex T1R2-T1R3. These receptors are located on Type II taste receptor cells and are specialized for detecting sugars and artificial sweeteners. Upon binding to sweet molecules, the T1R2-T1R3 receptor activates a signaling cascade involving G protein subunits, phospholipase Cβ2 (PLCβ2), and inositol triphosphate (IP3). This cascade releases calcium ions from intracellular stores, activating the TRPM5 ion channel, which depolarizes the cell and releases ATP as a neurotransmitter to nearby sensory nerves [32,33,38].

Salty Taste. Saltiness is primarily mediated by epithelial sodium channels (ENaCs) located on taste receptor cells. Sodium ions from salt diffuse through these channels, directly depolarizing the taste cells. This simple ion flux mechanism enables the detection of sodium levels, which are crucial for maintaining electrolyte balance and hydration. Research suggests that other ion channels may also contribute to the perception of high salt concentrations, but ENaCs remain the primary pathway [32,33,39].

Sour Taste Sourness is sensed by Type III taste receptor cells, which detect protons (H^+) from acidic compounds. Proton-sensitive ion channels, such as PKD2L1, enable protons to enter the cell, leading to depolarization and neurotransmitter release. This mechanism is crucial for detecting spoiled or unripe foods, as well as regulating the body's acid-base balance [32,33,40].

Umami Taste. Umami, the savory taste associated with amino acids like glutamate, is detected by the T1R1-T1R3 heterodimeric receptor. This GPCR complex binds to glutamate and other umami molecules, triggering a similar intracellular signaling cascade as sweet and bitter tastes. The detection of umami highlights protein-rich foods, making it an essential component of dietary preference and nutrition [32,33,41].

Bitter Taste. Bitter taste plays a critical role in survival by detecting potentially harmful substances and triggering aversive responses that influence dietary behavior [42–44]. Nevertheless, the association between bitterness and toxicity is not absolute, as some bitter compounds are non-toxic, while certain toxic substances may lack bitterness altogether [45]. The detection of bitterness is mediated by a family of approximately 25 GPCRs, which are expressed on Type II taste receptor cells [43,46]. These receptors are highly sensitive to a broad range of chemically diverse compounds, including plant alkaloids, flavonoids, and synthetic chemicals.

Upon binding to a bitter compound, T2Rs initiate a signaling cascade involving the G protein gustducin, which activates phospholipase Cβ2 (PLCβ2). This activation leads to the generation of inositol triphosphate (IP3), triggering the release of calcium ions from intracellular stores. The resulting calcium influx activates TRPM5 channels, causing cell depolarization and the release of ATP as a neurotransmitter [32,33].

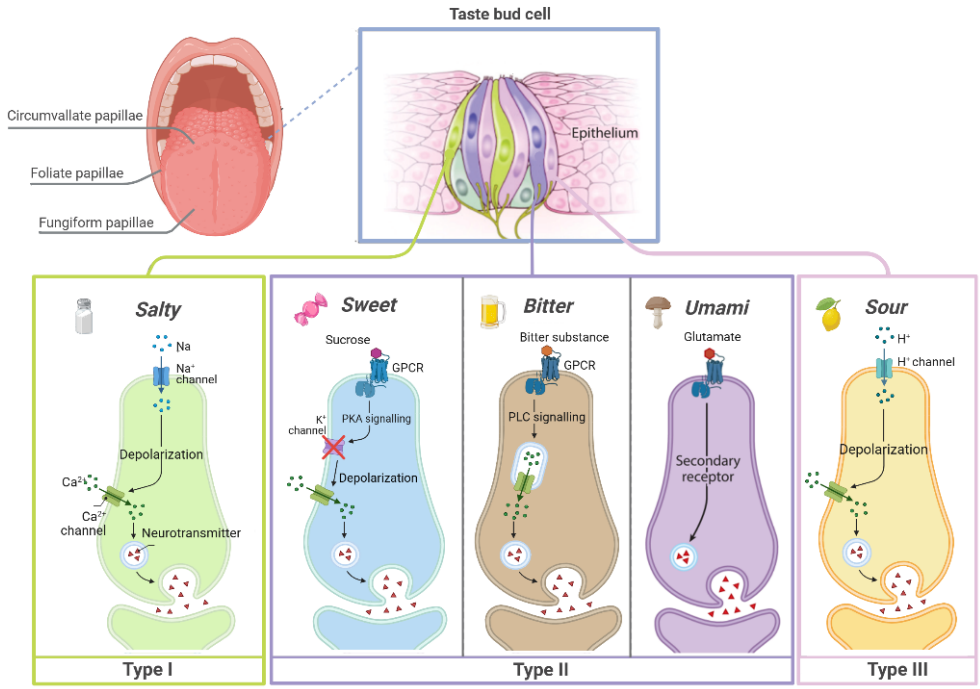

Figure 3. Taste transduction pathways for basic taste modalities. *Different taste qualities are detected by specialized taste cells within taste buds. Type I cells primarily mediate salty taste, Type II cells detect sweet, bitter, and umami via G protein-coupled receptors (GPCRs), and Type III cells respond to sour stimuli through proton channels. Signal transduction involves depolarization, neurotransmitter release, and activation of downstream pathways. Adapted with minor modifications from Forestell et al. (2024) [37] and Gravina et al. (2013) [32], including the addition of tongue taste bud cell localization and color-coded differentiation of taste modalities. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/w2gqscn.*

Importantly, the perception of bitterness varies among individuals. Genetic polymorphisms in T2R genes contribute to variations in sensitivity and response to bitter compounds. For example, differences in the TAS2R38 gene influence the ability to detect compounds such as phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), which can, in turn, shape dietary

preferences and nutritional behavior [43,47]. Based on these genetic differences, individuals are often categorized as supertasters, medium tasters, or non-tasters, with supertasters exhibiting greater sensitivity to bitterness due to both specific gene variants and a higher density of fungiform papillae on the tongue.

Further diversity in bitter perception arises from the functional specialization of T2R receptors. Some, like TAS2R3, TAS2R5, TAS2R8, TAS2R13, TAS2R41, TAS2R49, and TAS2R50, respond selectively to individual compounds and are classified as specialists. Others, including TAS2R10, TAS2R14, and TAS2R46, interact with numerous ligands and are considered generalists. To date, TAS2R42, TAS2R45, TAS2R48, and TAS2R60 are considered orphan receptors since no known agonist has been reported for them so far [43,48,49].

Taste receptors are also now known to be expressed in various non-gustatory organs, where they act as molecular sensors rather than mediators of conscious taste. The main receptor families TAS1Rs (sweet and umami) and TAS2Rs (bitter) have been identified in the gut, lungs, pancreas, cardiovascular system, immune cells, liver, brain, and other tissues. In these locations, they regulate diverse processes such as hormone secretion, immune responses, metabolic activity, and cell signaling. In contrast, salt and sour receptors show minimal expression outside traditional taste pathways. Collectively, the widespread distribution of taste receptors challenges the narrow view of them as gustatory tools, revealing their broader significance in maintaining homeostasis [34,50].

1.3.1 Emerging taste modalities

In addition to the established primary tastes, ongoing research has identified several potential "emerging tastes" such as fat, kokumi, and metallic taste, etc. Although not universally accepted as basic tastes, these modalities are studied for their distinct physiological and perceptual roles in gustation. To qualify as a primary taste, a sensation must meet specific criteria: ecological relevance, activation by a specific class of chemicals, detection via specialized receptors, transmission through gustatory nerves, perception as a distinct sensory quality, and the ability to elicit a physiological or behavioral response. Emerging tastes often fulfil some but not all of these requirements, fueling continued debate about their classification [51–53].

Fat, or oleogustus, is proposed as a sixth basic taste due to its detection of nonesterified fatty acids (NEFAs) via CD36 and GPR120 receptors on taste cells. This sensory input may allow the body to monitor fat intake, supporting metabolic regulation [53]. Similarly, kokumi compounds such as γ-glutamyl peptides do not produce a taste on their own but enhance other taste qualities like umami, salty, and sweet. These effects are mediated through calcium-sensing receptors (CaSR) on taste cells, contributing to overall flavor complexity [52,54].

1.3.2 Taste evolution over the human lifespan: biological and environmental drivers

Taste preferences and sensitivities evolve across the lifespan, beginning in utero and continuing into old age. Prenatal exposure to dietary flavors via the amniotic fluid, shaped by the maternal diet, can influence postnatal taste preferences, particularly for sweet and umami tastes, which are associated with energy-dense and protein-rich foods. Postnatal exposure through breast milk further reinforces these early preferences, while innate aversions to bitter compounds, linked to evolutionary toxin avoidance, may diminish over time with repeated exposure [55,56].

Pregnancy introduces another critical period of altered taste perception. Hormonal fluctuations, especially during the first trimester, increase sensitivity to bitterness, potentially serving as a protective mechanism. As pregnancy progresses, there is typically an increased

preference for sweet and salty tastes, likely reflecting elevated metabolic and electrolyte demands [37,56].

With aging, taste sensitivity tends to decline, particularly for sweet, salty, and umami tastes, which can diminish food enjoyment and contribute to nutritional deficiencies. This decline is often compounded by medication use and health conditions. Therefore, enhancing food flavor is recommended to support adequate nutrition in older adults [37,55,56].

1.4 Trigeminal sensations

While taste and smell are well-established as primary contributors to flavor, the trigeminal system, often referred to as chemesthesis, plays an equally significant, though less recognized, role. Trigeminal nerve endings are distributed throughout the oral cavity, nasal passages, cornea, and respiratory tract, where they detect chemical and mechanical irritants. This system responds to compounds such as capsaicin (found in chili peppers) and menthol (found in peppermint), producing sensations like burning, cooling, tingling, or stinging. Another key trigeminal sensation is astringency, a dry, puckering feeling commonly elicited by polyphenol-rich foods such as red wine or unripe fruit. Astringency arises from the interaction of tannins with salivary proteins, leading to decreased lubrication and an increased tactile friction effect, which is perceived through mechanoreceptors and trigeminal fibers rather than taste buds [32,33,37,57,58]. Astringency can be softened with polysaccharides, compounds that form complexes with polyphenols, reducing their interaction with salivary proteins [59].

Trigeminal activation not only adds to oral sensation but also modulates gustatory processing. For instance, capsaicin has been shown to enhance sensitivity to sweet and salty stimuli while potentially suppressing bitter perception. These effects are mediated through shared neural pathways and receptor interactions, including transient receptor potential (TRP) channels, which are involved in both taste and chemesthetic signaling. Depending on concentration, chemesthetic stimuli can either amplify or dampen taste perception, contributing to the dynamic interplay between taste and somatosensation [57,58].

Beyond the direct sensory inputs of taste, smell, and chemesthetic responses, flavor perception is further modulated by complex interactions between aroma and taste compounds and the surrounding food matrix. These interactions influence the release, availability, and accessibility of these compounds, adding a physicochemical dimension to the overall flavor experience.

1.5 Flavor-matrix interactions and sensory perception dynamics

Besides the well-known synergistic, masking, or suppressing effects that flavor compounds can have on each other, flavor-matrix interactions introduce an entirely different layer of complexity. The internal chemistry between flavor active compounds shapes perception at a molecular level; the interaction between these compounds and the surrounding food matrix determines how, when, and where those perceptions arise [60].

These interactions occur through both chemical and physical mechanisms, broadly classified as non-covalent binding, covalent reactions, and phase partitioning [61,62]. Together, these mechanisms influence how readily flavor molecules reach our olfactory and gustatory receptors. Many aroma compounds are hydrophobic, causing them to associate strongly with non-polar matrix regions. Lipid-rich phases are well known for dissolving hydrophobic aroma molecules, reducing their vapor-phase release. Likewise, hydrophobic sites on proteins can bind volatile compounds. As a result, non-polar aromas can become embedded in protein interiors

or adsorbed to protein surfaces via hydrophobic interactions. These interactions are usually reversible (non-covalent) and lead to flavor retention. Flavor is held in the matrix, thereby lowering its gas-phase concentration and reducing its immediate sensory impact [60,62–64].

Certain flavor compounds can form covalent bonds with matrix components, leading to irreversible binding [62]. A classic example of this is carbonyl amine reactions, where aldehydes can react with the amino groups of proteins. This permanently ties up the aroma as a new conjugate, effectively removing it from the flavor pool. Such irreversible binding can diminish desirable aromas, especially during processing or storage, but can also be leveraged to capture and eliminate off-flavors [61–63].

The propensity of a flavor molecule to leave the matrix and enter the vapor phase depends on its volatility and how it partitions between phases [65]. Flavors are distributed among water, lipid, air, and solid phases according to thermodynamic equilibria. In this context, a key parameter is the partition coefficient between the aqueous and other matrices [63–65].

1.6 Protein alternatives in sustainable and innovative food systems

The challenge of sustainably feeding a global population projected to reach 9.8 billion by 2050 has placed increasing pressure on current food systems, particularly those dependent on animal agriculture [66,67]. Although traditional livestock production has long played a central role in human nutrition, it has become a focal point of concern due to its extensive environmental, ethical, and health implications. Confronting these issues while still meeting future nutritional demand, without overexploiting natural resources or causing irreversible ecological damage has become a central challenge [68–71].

Environmentally, animal agriculture is a major contributor to climate change, accounting for approximately 14.5% of global greenhouse gas emissions through the release of carbon dioxide, methane, and nitrous oxide [70]. It is also associated with deforestation, water scarcity, biodiversity loss, and inefficient land use. Notably, over half of the world's cultivable land is used for food production, and a substantial portion of that supports feed crops for animals rather than direct human consumption [72].

Beyond ecological strain, ethical concerns surrounding animal welfare have become increasingly prominent as awareness of industrial farming practices has spread. At the same time, consumer interest in health has intensified scrutiny of meat-heavy diets, which have been linked to chronic diseases such as heart conditions, obesity, and hypertension [68,70–73]. These combined challenges have contributed to a growing consumer movement to reduce animal-based foods in their diets and increased interest in alternative protein sources.

The term "alternative protein" is sometimes narrowly interpreted as plant-based or non-animal sources, but its usage is broader and more nuanced across various contexts. As defined by Grossmann and Weiss (2021) [66], alternative proteins are those derived from sources that have a low environmental impact and are intended to replace established protein sources.

According to Kumar et al. (2023) [69], four primary types of alternative proteins have been widely recognized for their sustainability, nutritional efficiency, and scalability: in vitro meat, edible insects, single-cell proteins, and plant-based proteins (Figure 4).

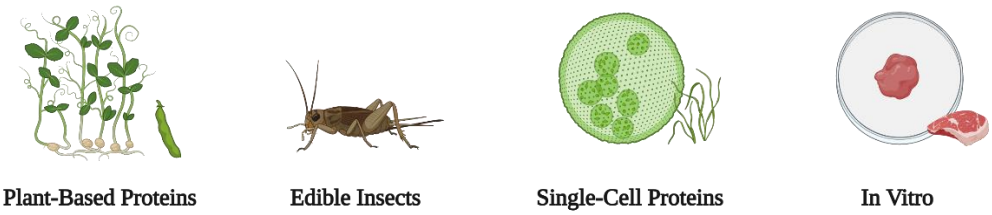

Figure 4. Emerging protein sources for sustainable food production. *Alternative proteins include plant-based proteins, edible insects, single-cell proteins (such as algae, fungi, bacteria), and cultured meat produced in vitro. Image created based on concepts from Kumar et al. (2023) [69]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/okx3no6*

1.6.1 In vitro meat

Also referred to as cultured meat, lab-grown meat, or clean meat, is produced through tissue engineering technologies by culturing animal muscle cells outside the animal body. The process involves collecting stem cells through biopsy or from slaughtered animals and growing them in bioreactors under controlled conditions. This approach offers the potential to produce real meat without slaughter and with a significantly reduced environmental footprint. However, in vitro meat is still in the early stages of commercial development, facing high production costs, scalability issues, and uncertainty regarding long-term consumer acceptance [66,69,73,74].

1.6.2 Edible insects

Insects, including species such as crickets, locusts, and mealworms, have been consumed by humans for millennia and are part of traditional diets in many cultures. They offer high protein content, favorable amino acid profiles, and efficient feed conversion ratios, requiring far less water, land, and energy compared to conventional livestock. Despite these benefits, edible insects face significant cultural and psychological barriers in many Western countries, including food neophobia and consumer disgust. Their future success relies on normalization, processing into familiar formats, and addressing regulatory concerns [69,74–76].

1.6.3 Single-Cell Proteins (SCPs)

Single-cell proteins are derived from microbial sources, including microalgae, fungi, and bacteria. These microorganisms can be cultivated in controlled environments and are often processed into protein-rich biomass suitable for human consumption. SCPs have historically been used in food and feed applications to enhance nutritional content, stability, and shelf life. Notable examples include mycoprotein, spirulina, and other microbial biomass products. Their advantages include high growth rates, minimal land use, and adaptability to various production systems. However, regulatory frameworks and consumer familiarity are still evolving [69,74,75,77,78].

1.6.4 Plant-based proteins

These proteins originate from a wide range of plant sources, including legumes (e.g., lentils, peas, chickpeas), oilseeds (e.g., flaxseed, canola, hempseed, sunflower), cereals (e.g., oats, wheat, rice), and pseudo-cereals (e.g., chia, amaranth) [79]. Plant-based proteins are increasingly favored due to their low environmental impact, broad availability and favorable nutritional

profile. Compared to animal-based proteins, they require less water and land, generate fewer greenhouse gas emissions, and raise fewer ethical concerns related to animal welfare [80]. Additionally, many plant-derived ingredients are rich in fiber, vitamins, antioxidants, and bioactive compounds that contribute to improved health outcomes, including cholesterol reduction, cardiovascular benefits, and blood pressure regulation [73,81,82]. They can be consumed in their natural form or processed into meat analogs that mimic the flavor, texture, and appearance of animal-based products. Consumer acceptance is generally higher compared to insects or SCPs [66–68,72,75].

The development of meat analogs has significantly broadened the appeal of plant-based proteins. Through technologies such as extrusion, protein texturization, and advanced ingredient formulation, manufacturers are now able to replicate the fibrous texture and visual characteristics typically associated with animal-derived meat products [75,76,81,82].

Despite these advances, plant-based proteins face significant challenges that limit their widespread application. Among these challenges, flavor consistently emerges as the most critical factor influencing consumer acceptance. For flexitarian and omnivore consumers, the flavor often outweighs concerns related to texture or appearance, as these products are directly compared to traditional meat [72,73,83].

Characteristic off-odors and undesirable tastes, such as beany, grassy, bitter, and astringent notes, originate from both the intrinsic composition of plant materials and the processing methods applied [67,68,82,83]. These off-notes are attributed to various classes of compounds, more frequently to aldehydes, alcohols, ketones, and sulfur-containing compounds, as well as to saponins, phenolics, peptides, and lipid oxidation byproducts [73]. These compounds often bind tightly to the protein matrix, reducing their release and ultimately diminishing sensory perception. Even efforts to improve solubility and texture by applying enzymatic hydrolysis can unintentionally intensify flavor challenges by generating bitter peptides [72,73].

Several strategies have been proposed to reduce off-flavors in plant proteins. These include the use of highly purified protein isolates, breeding of low off-taste genotypes, and the application of enzymatic inhibitors. Techniques such as enzymatic hydrolysis, germination, fermentation, and chemical or physical modification can alter the surface properties of proteins to reduce flavor binding and improve sensory quality [67,72,75,81,82]. Innovations like electromagnetic and high-pressure processing are also gaining attention for their potential to enhance sensory profiles while maintaining protein functionality. Despite this progress, flavor improvement still lags behind developments in texture and mouthfeel of protein alternatives. Many mitigation strategies remain highly specific to the protein source, and no universal approach has yet been established [72,73].

Research on plant-based proteins has primarily focused on market-dominant sources, such as soy and pea proteins; however, novel proteins, including sunflower, are beginning to attract interest due to their promising functional and nutritional properties [84–86]. This growing interest sets the stage for the following chapter, which will delve into the potential of sunflower as a protein source for sustainable food product development and the specific flavor-related challenges involved.

1.7 Overview of sunflower and its byproducts as a protein alternative

The sunflower (*Helianthus annuus* L.) belongs to the family Asteraceae and has its origins in North America [87]. Sunflowers are cultivated for their fruits, providing essential nutrients for human consumption and serving as a resource for livestock feed [88]. The term “seed” refers to the whole fruit, while the “kernel” describes the dehulled sunflower seed [89] (Figure 5).

Sunflower seeds are rich in tocopherols and polyphenols, which contribute to their notable antioxidant potential, surpassing that of many other commonly consumed seeds, such as flax, chia, and sesame [90,91]. The sunflower seeds also contain health-promoting unsaturated fatty acids, proteins, fiber, vitamins, and minerals, which are beneficial to nerves, muscles, bones, and blood in humans. In traditional medicine, sunflower seeds are used to treat cancer due to their antioxidant properties and selenium content, which may help induce apoptosis in cancer cells [92,93].

Sunflowers are broadly categorized into two types based on seed composition. Oilseed sunflower seeds contain a minimum of 40% lipids and are processed into oils such as mid-oleic, high-oleic, high-stearin, or high-palmitic sunflower oils, each with specific applications [94–98].

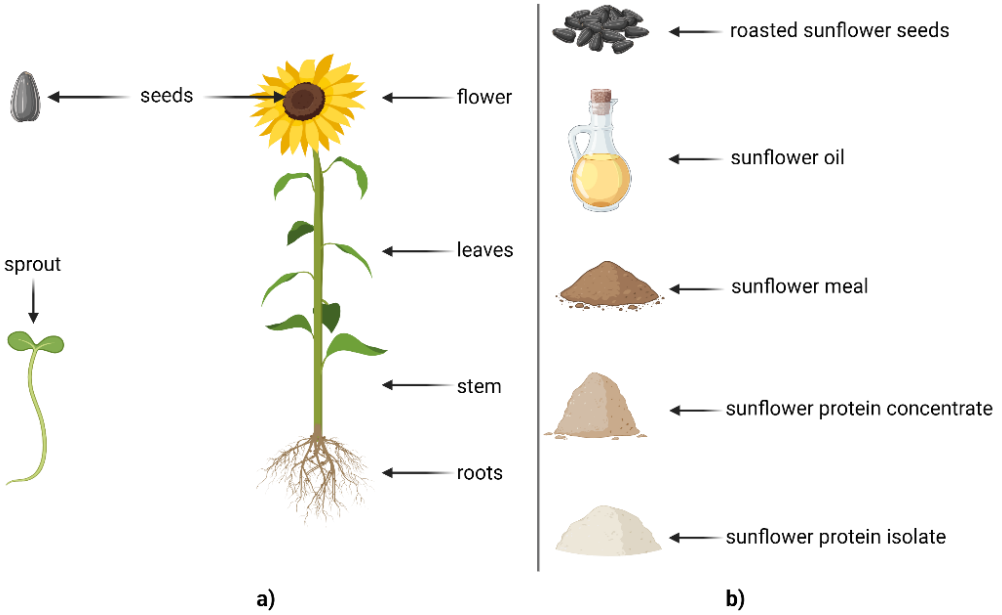

Figure 5. Sunflower plant anatomy and seed-derived products*. a) Parts of a sunflower plant and b) products derived from sunflower seeds: whole seeds, oil, meal, protein concentrate, and protein isolate based on Puttha et al. [86]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/f45e251*

Non-oilseed sunflowers are used for human intake (in confectionery) or as feed [88]. Variations in the chemical composition of the seeds may arise from growth conditions, biotic/abiotic stress, or storage practices, even within the same genotype [99]. The climate and seasonal conditions can also lead to variations in fatty acid content, such as the total lipid content and the concentration of oleic acid, which are higher under warmer temperature conditions. Additionally, processing conditions such as high temperatures and high pressures significantly impact the nutritive value of sunflower seeds [93].

Sunflowers are mainly cultivated for oil production, ranking as the third most cultivated oilseed crop in the world, after soybeans (364 million tons) and rapeseed (71 million tons), with a production volume of 57 million tons per year [100]. The oil extraction process yields a substantial amount of sunflower meal, which is composed of proteins, fibers, lignins, polyphenols, and minerals [101]. In 2019, global production of sunflower meal reached approximately 21.85 million tons [102]. With a protein content ranging from 19.9 to 44.9%

[103], sunflower meal holds promise as a protein source to help meet global nutritional needs [104]. However, it is still primarily utilized in animal feed and fertilizers.

In recent years, there has been an increasing interest in the valorization of sunflower meal or press cake as a sustainable source of plant protein for food applications [105–107]. A few studies suggest that sunflower proteins require fewer odor-masking agents than pea proteins, making them a more favorable choice in product formulations [108]. Additionally, sunflower flour is also considered blander than soybean flour, further enhancing its appeal [109]. However, a bitter taste has been reported in some food applications using sunflower-based proteins, which may negatively affect consumer acceptance [110,111]. To support the broader use of sunflower proteins, it is crucial to identify and mitigate these off-flavor compounds to improve sensory acceptability.

To address these sensory challenges more effectively at a molecular level, the sensomics approach can be used successfully to identify key off-flavor compounds [112].

1.8 Sensomics approach for the identification of flavor-active compounds

The sensomics approach is a sensory-driven analytical strategy designed to decode the key molecular determinants of flavor in food by identifying both key aroma and taste-active compounds [30]. Unlike conventional profiling methods that simply catalog detected molecules, sensomics approach integrates sensory-guided fractionation with advanced analytical chemistry to identify those compounds that significantly contribute to human perception. Through the combination of instrumental analysis and sensory validation, the approach directly links chemical composition to sensory impact via reconstitution and omission experiments [30,113,114].

Historically, aroma research dates back to the 19th century, with early work focusing on single impact compounds such as vanillin and benzaldehyde. However, the introduction of gas chromatography in the 1960s enabled the identification of thousands of volatiles, revealing that food aroma is a result of complex mixtures rather than single molecules. Today, more than 12,000 volatile compounds have been identified in foods, but only a small fraction actively contribute to aroma perception [30].

The sensomics approach emerged in the early 2000s as a response to the need for a more precise, perception-driven flavor analysis, building on the limitations of broader techniques like volatilomics [30,113,114]. The methodology begins with the extraction of flavor-active substances using appropriate solvents to isolate both volatile and non-volatile compounds. For aroma analysis, odor-active compounds are often separated using solvent-assisted flavor evaporation (SAFE), steam distillation, or headspace techniques [115,116]. Taste-active compounds, on the other hand, are subjected to fractionation using reversed-phase and hydrophilic interaction liquid chromatography, liquid-liquid extraction, ultrafiltration, and gel permeation chromatography. These processes generate fractions enriched in specific classes of compounds [116–118].

To identify sensory-relevant fractions, different screening techniques are used. For volatiles, gas chromatography-olfactometry (GC-O) combined with aroma extract dilution analysis (AEDA) is applied to assign flavor dilution (FD) factors [119]. For non-volatiles, taste dilution analysis (TDA) is conducted. In TDA, fractions are serially diluted and tasted in triangle or duo tests against blanks, and the taste dilution (TD) factor is calculated based on the concentration at which the taste becomes perceptible [120]. Fractions with the highest FD or TD factors are prioritized for further isolation.

Structural elucidation of active compounds is performed using techniques such as gas chromatography–mass spectrometry (GC-MS), liquid chromatography–tandem mass spectrometry (LC-MS/MS), and one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy [30].

To assess the relevance of identified compounds, activity values are calculated: odor activity values (OAVs) for aroma compounds and dose-over-threshold (DoT) factors for taste compounds. These values represent the ratio of a compound's concentration in the food to its sensory threshold. Compounds with OAVs or DoT values of 1 or greater are considered key contributors to the overall flavor. In some cases, even compounds with lower values may contribute through synergistic or additive interactions [116,118,121].

Quantitative analysis of aroma-active volatiles is typically conducted using stable isotope dilution analysis (SIDA) with isotope-labeled internal standards, which provide high precision by correcting for matrix effects and processing losses [30]. For taste-active compounds, targeted quantification is typically performed using LC-MS/MS methods with external calibration or internal standards [113,114,122].

The final and critical step in the sensomics workflow is the validation of identified compounds through recombination and omission experiments. In recombination studies, all key compounds are mixed in their natural concentrations and compared with the original food to assess sensory similarity. Omission experiments, in which individual compounds or compound classes are excluded, reveal the unique contribution of each to the overall profile [30,113,123]. This experimental design confirms the relationship between the presence of the compound and flavor perception.

The methodology also forms the basis for emerging fields, such as sensoproteomics, which combines sensory analysis with proteomic techniques to understand taste-active peptides [124,125].

As part of this work, the sensomics approach provides the instrumental foundation for elucidating the molecular contributors to off-taste in protein-rich matrices. It has been successfully employed to characterize taste-active components in linseed oil [126], hazelnuts [127], poppy seeds [128], asparagus [129], rapeseed protein isolates [130,131], and pea protein isolates [118]. In most of these studies, the focus was placed on mapping taste-active compounds associated with bitterness. Bitter compounds pose a particular challenge due to their structural diversity and broad range of sensory properties.

1.9 Bitter compounds in food and their structural and sensory characteristics

To date, over 1000 bitter compounds have been cataloged [132]. These appear naturally in plants, animals, and fungi or are formed during storage and processing through reactions such as hydrolysis, oxidation, and fermentation. They span multiple chemical classes (Figure 6), including alkaloids, phenolics, terpenoids, amino acids, peptides, glucosinolates, fatty acid derivatives, and others [44,48].

Despite this diversity, bitter compounds often share specific physicochemical traits. They tend to be low in molecular weight, hydrophobic, and structurally rigid, often featuring bulky side chains such as aromatic rings or branched aliphatic groups. These characteristics enhance their ability to interact with the hydrophobic binding pockets of TAS2R receptors. Interestingly, their structural versatility also allows them to function variably, acting as agonists for some TAS2Rs and antagonists for others, depending on the context [48,49,133].

Amino acids and peptides represent some of the most structurally diverse and broadly distributed bitter compounds. Several L-amino acids, particularly those with sulfur-containing

or non-polar side chains, exhibit inherent bitterness. Examples include histidine, leucine, isoleucine, valine, phenylalanine, methionine, tyrosine, cystine, and tryptophan. Although common in foods, their impact on perceived bitterness is minimal due to high recognition thresholds. Intact proteins are typically not bitter; however, enzymatic hydrolysis exposes hidden residues, intensifying bitterness as more hydrophobic segments are released [44,48].

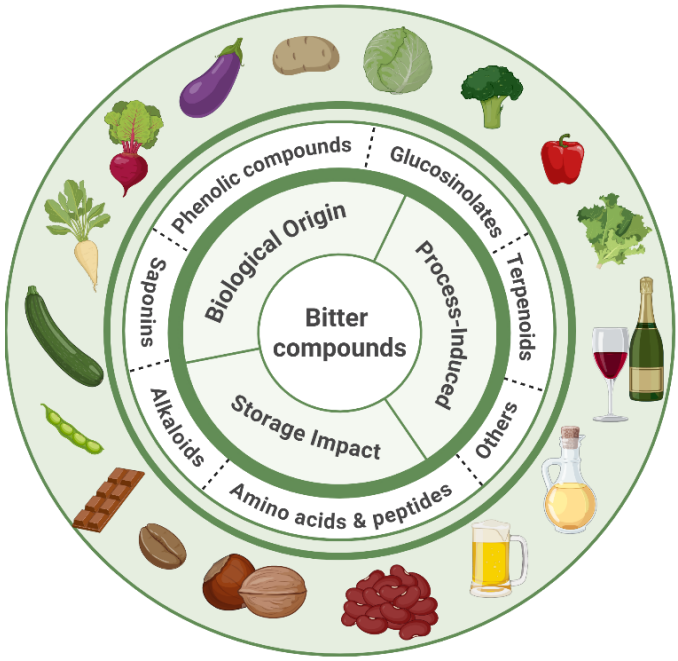

Figure 6. Schematic classification of bitter compounds based on origin and chemical structure*. Bitter compounds are classified based on their origin, biological formation, process-induced generation, or storage-related changes, and grouped into key chemical classes commonly found in food systems. Image created based on Yan et al. (2023), Chu et al. (2024), and Li et al. (2023) [48,134,135]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/cab87lo*

Alkaloids are nitrogen-containing compounds known for their strong bitterness and wide distribution in foods such as coffee, tea, chocolate, and some seeds and vegetables. Key alkaloids include caffeine, theobromine, morphine, berberine, and hypoxanthine. Caffeine and theobromine contribute to the stimulating and bitter qualities of coffee and cocoa [44]. While moderate intake of dietary alkaloids is typically safe and sometimes beneficial, excessive or uncontrolled consumption can pose risks, especially in sensitive individuals or under certain health conditions [48,136].

Phenolic compounds are a chemically diverse class characterized by the phenol group, consisting of one or more hydroxyl groups attached to an aromatic ring. These play a major role in the bitterness and astringency of many plant-based foods [137]. Phenolics include simple molecules, as well as complex flavonoids and tannins, whose bitterness is influenced by molecular weight and structure [48,137]. Their low bitterness thresholds make them powerful flavor contributors, even in small amounts.

Structurally, terpenoids are derived from repeating isoprene (C_5) units, forming linear or cyclic frameworks that may include oxygen-containing functional groups. Terpenoids, responsible for both aroma and bitterness, are particularly prominent in citrus fruits and herbs. Naringin, found in grapefruit, is especially bitter and detectable at low concentrations. Other notable terpenoids include limonin and artemisinin [44,48].

Certain free fatty acids, especially long-chain unsaturated ones such as oleic acid and linoleic acid, can elicit a bitter taste. This is attributed to their ability to interact with bitter taste receptors when released during lipid hydrolysis or oxidation [48,72,138,139].

2 The Aims of this Dissertation

The overall objective of this dissertation is to advance the understanding of off-flavor in protein alternatives, with a specific focus on sunflower-based proteins, building on prior work exploring off-flavor formation in alternative protein sources (including insect-based). This objective is addressed through studies that focus on the flavor challenges of sunflower as a protein source:

1. A critical review of existing literature on volatile and non-volatile compounds in sunflower products, with a focus on their potential flavor relevance and identification of current knowledge gaps.
2. Application of a sensomics approach to identify key taste-active compounds responsible for undesirable taste impressions, particularly bitterness, in sunflower press cake.
3. Quantification of compounds to assess their sensory relevance and contribution to off-taste perception in sunflower press cake.
4. Exploration of a novel food application by developing a sunflower-based butter formulation, evaluating its sensory characteristics, and assessing its potential as a plant-based spread.

3 Materials and Methods

The work presented here builds on broader research focused on protein alternatives. One line of investigation explored insect-based protein alternatives, with particular attention to off-flavors (**Publication 1**). In parallel, sunflowers were investigated as a promising plant-based protein alternative due to their nutritional potential and consumer acceptability, eventually becoming the central focus of this dissertation. This line of research included collaborative work on the development of sunflower-based food products, such as spread formulations (**Publication 2**), as well as a comprehensive review of volatile and non-volatile compounds with potential sensory relevance in sunflower-derived matrices (**Publication 3).** These prior efforts laid the foundation for the experimental study discussed here (**Publication 4**), which aimed to identify and characterize the key contributors to undesirable flavor, particularly bitterness, in sunflower press cake. All materials and methods described below are derived from **Publication 4** and are included here to ensure clarity and ease of reference for the reader. The methods from **Publications 1–3** are not included in this section but can be found in the original articles, which are presented in the appendix of this dissertation.

3.1 Chemicals

All solvents and reagents used in this study were sourced from commercial suppliers. Acetonitrile (ACN) and methanol (MeOH) were obtained from J.T. Baker (Deventer, The Netherlands), while acetone, ethyl acetate, and *n*-pentane were purchased from BDH Prolabo (Briare, France), and formic acid from Merck (Darmstadt, Germany). Additional chemicals, including dimethyl sulfoxide-d_6 (DMSO), deuterium oxide (D_2O), oleic acid, linoleic acid, palmitic acid, stearic acid, α-linolenic acid, [$^{13}C_{18}$]-linoleic acid, hydrochloric acid, anhydrous pyridine, *L*-cysteine methyl ester hydrochloride, phenylethyl isothiocyanate, and various sugars (*D*-glucose, *D*-galactose, *D*-mannose, *D*-xylose, *D*-ribose, *D*-apiose), were purchased from Sigma-Aldrich (Steinheim, Germany). Isopropyl alcohol was sourced from Honeywell (Seelze, Germany). Lipid derivatives including (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid, 2-hydroxyoleic acid, and 18-hydroxyoleic acid were obtained from Larodan AB (Solna, Sweden). The acetonitrile used for high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis was LC-MS grade (Honeywell, Seelze, Germany). Prior to use, acetone, ethyl acetate, and *n*-pentane were distilled. All other solvents were of HPLC-grade. The water for chromatographic separation was purified by using an Advantage A10 water System (Millipore, Molsheim, France). For sensory experiments, bottled water (Evian) was adjusted to pH 5.9 with formic acid. Commercial sunflower protein powders were purchased from Amazon (Germany). The sunflower press cake used in this study was obtained from SUNFLY OU (Estonia).

3.2 Sequential solvent extraction

A total of 300 g of sunflower press cake was extracted with methanol/water (70:30, v/v, 1500 mL) three times by stirring for 30 min at room temperature, followed by centrifugation (4 min, 5000 rpm) and filtration. The filtrates were collected and combined, separated from the solvent by vacuum evaporation at 40 °C, and lyophilized to obtain the MeOH/H2O extractable (fraction F1). The residue was further extracted with MeOH (1500 mL, fraction F2), followed by ethyl acetate (1500 mL, fraction F3) and n-pentane (1500 mL, fraction F4). The extracted solvent

fractions (F1−F4) were freeze-dried twice to remove trace amounts of solvents and stored at –20 °C until they were used for a comparative taste profile analysis. Method developed based on the literature [118].

3.3 Fractionation of F1 fraction by solid-phase extraction

For solid-phase extraction (SPE) fractionation, an aliquot (1 g) of fraction F1 was dissolved in 40 mL of water and sonicated at room temperature for 10 min. The resulting solution was separated on a Chromabond C18 ec cartridge (45 µm, 70 mL/10 g, Macherey-Nagel, Düren, Germany). The cartridge was preconditioned with methanol (2 × 70 mL), followed by water (2 × 70 mL) prior to sample application. Elution was performed with water (2 × 70 mL) to obtain fraction F1-1, methanol/water (30:70, v/v, 2 × 70 mL) to obtain fraction F1-2, methanol/water (50:50, v/v, 2 × 70 mL) to obtain fraction F1-3, methanol/water (70:30, v/v, 2 × 70 mL) to obtain fraction F1-4, and methanol (2 × 70 mL) to obtain fraction F1-5. The collected fractions were separated from the solvent by vacuum evaporation at 40 °C, lyophilized twice, and stored at –20 °C until used for subsequent chemical and sensory analyses.

3.4 Separation of fraction F1-4 by preparative high-performance liquid chromatography

The fraction F1-4 was dissolved in a mixture of H_2O/ACN (80:20, v/v; 320 mg in 6 mL) by ultrasonication at room temperature (10 min). The sample was membrane filtered and injected (300 µL) into a Nucleodur C18 Pyramid column (250 × 21 mm, 5 µm, 110Å, Macherey-Nagel, Düren, Germany) equipped with a guard column of the same type. The separation was carried out at a 20 mL/min flow rate using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) as the mobile phases. The effluent was monitored using a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) operating at Gain 12. The gradient flow was as follows: 0 min, 15% B; 3 min, 15% B; 28 min, 60% B; 30 min, 100% B; 32 min, 100% B; 35 min, 15% B; 40 min, 15% B. In total, 17 fractions were collected (F1-4-1 to F1-4-17), freed from solvent under vacuum at 40 °C, lyophilized twice, and then kept at –20 °C until further analysis.

3.4.1 Identification of fatty acids and fatty acid oxidation products in fraction F1-4-15 and F1-4-16

Fractions were subjected to untargeted screening using ultrahigh performance liquid chromatography–time-of-flight–mass spectrometry (UPLC-TOF-MS) to enable the identification of compounds within the fractions. To confirm these preliminary identifications, analyses were carried out in parallel with reference standards, applying an established method described in earlier studies [118,128]. The retention times and mass spectral data obtained from the present analysis were consistent with those of the reference compounds reported in previous research [118,128].

3.4.2 Isolation and identification of the bitter compound in fraction F1-4-12

Fraction F1-4-12 was dissolved in H_2O/ACN mixture (80:20, v/v; 10 mg/mL) and, after membrane filtration, fractionated by HPLC using a semipreparative Luna Phenyl-Hexyl column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), equipped with a guard column of the same type. Chromatographic separation was achieved using a binary gradient of 0.1% formic acid in H_2O as solvent A and 0.1% formic acid in ACN as solvent B with a flow rate of 4.7 mL/min.

Details of the solvent gradient are provided in Table 1. For detection, DAD detector model MD-2010 Plus (Jasco, Groß-Umstadt, Germany) was used.

The subfraction that contained the bitter target compounds (F1-4-12-5) was collected in multiple HPLC runs, combined, separated from the solvent under vacuum at 40 °C, and then lyophilized twice. For further purification this subfraction was dissolved in ACN/H_2O (30:70, v/v; 1mg/mL) and fractionation was performed with an analytical Luna Phenyl-Hexyl column (250 × 4.60 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) with a flow rate of 1 mL/min and using 0.1% formic acid in H_2O as solvent A and 0.1% formic acid in MeOH as solvent B. DAD detector model MD-2010 Plus (Jasco, Groß-Umstadt, Germany) was used for detection. Separation was performed by using the solvent gradient method mentioned in Table 1.

Table 1. Semi-preparative and analytical HPLC gradient used for fraction F1-4-12.

semi-preparative gradient		**analytical gradient**	
Time (min)	**%B / %A**	**Time (min)**	**%B / %A**
0	30 / 70	0	50 / 50
3	30 / 70	2	50 / 50
25	45 / 55	29	62 / 38
28	100 / 0	32	100 / 0
30	100 / 0	34	100 / 0
32	30 / 70	36	50 / 50
35	30 / 70	40	50 / 50

The fraction containing the bitter compound (referred to as compound **15**) was accumulated from several HPLC runs, evaporated, and lyophilized before conducting structure elucidation via MS/MS following hydrolysis, TOF-MS, and NMR analyses, as well as sensory threshold analysis.

3.4.3 Determination of monosaccharide constituents via ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) following acidic hydrolysis

The monosaccharide constituents of **15** were determined after acidic hydrolysis based on the following protocol reported earlier [140]. Therefore, the isolated compound **15** was dissolved in a mixture of ACN/H_2O (3:7, v/v, 100 µL) and then treated with hydrochloric acid (6 mol/L, 1 mL), followed by heating at 100 °C for 60 minutes. After heating, the mixture was dried under a stream of nitrogen.

The resulting residue was suspended in 2 mL of H_2O and extracted three times with 2 mL of ethyl acetate. The aqueous layer, containing the monosaccharides, was again evaporated to dryness. This residue was dissolved in anhydrous pyridine (100 µL), and a solution of L-cysteine methyl ester hydrochloride (500 µL, 2 mg/mL) was added. The mixture was placed in a thermo shaker (PHMT-PSC24N, Grant Bio, Cambridge, UK) at 60 °C with a speed of 1400 rpm for 60 min. Subsequently, phenylethyl isothiocyanate (50 µL) was added to the solution, and the resulting mixture was shaken again at 60 °C for 60 min. The mixture was dried under a stream of nitrogen, reconstituted in a mixture of ACN/H_2O (1:1, v/v, 500 µL), transferred to an autosampler vial, and an aliquot (1 µL) was subjected to UHPLC–MS/MS analysis.

Mass spectrometry detection was conducted using a QTRAP 6500 mass spectrometer (AB Sciex, Darmstadt, Germany) operated in positive electrospray ionization (ESI+) mode with the following settings: ion spray voltage at 5500 V (ESI^+), curtain gas at 35 psi, nebulizer gas at 55 psi, heater gas at 65 psi, collision-activated dissociation high, and source temperature at 500 °C. The MS system was coupled to a Shimadzu Nexera X2 UHPLC (Shimadzu, Duisburg, Germany). The system consisted of two pumps (LC-30AD), a degasser (DGU-20A5R), an autosampler (SIL-30AC), a column oven (CTO-30A), and a controller (CBM-20A). Data were acquired using Analyst 1.6.3 (AB Sciex, Darmstadt, Germany).

For all reference compounds, individual MS/MS parameters were first tuned and optimized on the UHPLC–MS/MS system after derivatization. After optimizing instrument settings with reference compounds, the derivatized monosaccharides were analyzed using the mass transitions mentioned in Table 2.

Table 2. Optimized UHPLC–MS/MS parameters for selected derivatized monosaccharides, including mass transitions and fragmentation settings.

sugar	Q1/Q3 (m/z)	DP (V)	CE (V)	CXP (V)
D-Glucose	461.0 / 298.1	86	17	6
D-Galactose	461.1 / 298.2	71	17	6
D-Mannose	461.0 / 298.1	71	17	6
D-Xylose	430.9 / 268.0	76	17	12
D-Ribose	430.9 / 268.0	71	29	11
D-Apiose	430.9 / 268.0	76	17	9

Chromatography was carried out utilizing a Phenomenex Kinetex F5 column (100 × 2.1 mm i.d., 100 Å, 1.7 µm, Phenomenex, Aschaffenburg, Germany) kept at 40 °C. Compound elution was performed with a flow rate of 0.4 mL/min. The mobile phase consisted of (A) 1% aqueous formic acid and (B) ACN (1% formic acid) with the following gradient: 0 min, 5% B; at 3 min, 5% B; at 5 min, 20% B; at 25 min, 25% B; at 27 min, 100% B; at 30 min, 100% B; at 31 min, 5% B; at 35 min, 5% B. Identification of the monosaccharide constituents D-glucose and D-apiose, present in the isolated bitter compound **15** from fraction F1-4-12-5 achieved by comparing the observed retention times and mass transitions with those of reference compounds.

3.5 Sensory analysis

3.5.1 Sample preparation and sensory panel training

The 12 panelists (6 females and 6 males, aged 22–30) voluntarily participated in the sensory evaluation and provided informed consent to take part in the present research. None of the panelists reported any history of taste disorders. They were familiar with the sensory analysis methodologies used and were able to evaluate various chemosensory attributes. Sensory training utilized aqueous reference solutions (2.0 mL, pH 5.9), including sucrose (50 mmol/L) for sweet, L-lactic acid (20 mmol/L) for sour, NaCl (20 mmol/L) for salty, caffeine (1 mmol/L) for bitter, and monosodium L-glutamate (3 mmol/L) for umami taste perception [128]. The sensory evaluations were performed at 22−25 °C in a sensory panel room. Nose clips were used during all sensory analyses to avoid cross-model interactions with odor-active compounds.

3.5.2 Taste profile analysis

An aliquot (6g) of the sunflower press cake was suspended in water (100 mL, pH 5.9) and presented to the trained panel [118]. To prevent sedimentation, the suspension was stirred during the sensory test. The trained panelists were asked to evaluate the taste attributes sweet, bitter, umami, salty, astringent, and sour on a scale from 0 (not detectable) to 5 (strongly detectable). The same preparation and evaluation procedure was applied to the reference sample and the commercially bought sunflower proteins. Additionally, an aliquot of fraction F1 and its subfractions were taken up in bottled water (25 mL, pH 5.9) at natural concentrations and evaluated by the trained sensory panelists regarding bitterness, sweetness, sourness, saltiness, umami, and astringency.

3.5.3 Taste dilution analysis

Subfractions F1-4-1 to F1-4-17, isolated from an aliquot (320 mg) of fraction F1-4, were individually dissolved in 20 mL of bottled water (pH 5.9) and sequentially diluted 1:1 (v/v) with bottled water. The dilution series was presented to the panel in ascending concentrations, and the taste dilution (TD) factor for bitterness was determined by asking the sensory panel to indicate the point at which the first detectable difference between the sample and the control (bottled water, pH 5.9).

3.5.4 Human taste recognition thresholds

The two-alternative forced choice (2-AFC) test was used to determine the threshold concentration at which the bitter taste quality of the compound was just detectable [118]. For this purpose, the purified substances were dissolved in bottled water at increasing concentrations. The individual recognition thresholds were determined by calculating the geometric mean of the first falsely and the last correctly identified concentrations. The taste threshold for the sensory panel was estimated by averaging the threshold values obtained from each panelist.

3.6 High-performance liquid chromatography

The HPLC analyses were conducted using an instrument setup (Jasco, Groß-Umstadt, Germany) that consisted of a binary pump system PU-2087 Plus, a DG-4400 degasser, and a Rheodyne injection valve (model Rh 2807*i* type Rheodyne, Bensheim, Germany). The effluent was monitored using an MD-2010 Plus diode array detector (Jasco, Groß-Umstadt, Germany) operating within a wavelength range of 200−500 nm, along with a Sedex LT-ELSD detector Model 80 (Sedere, Alfortville, France). Chromatographic separations were achieved using a preparative Nucleodur C18 Pyramid column (250 × 21 mm, 5 µm, 80 Å, Macherey-Nagel, Düren, Germany), a semipreparative Luna Phenyl-Hexyl column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), and a Luna Phenyl-Hexyl column (250 × 4.60 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), all equipped with a guard column of the same type. Data was recorded and managed using Galaxie Chromatography Software, version 1.10.0.5590.

3.6.1 UPLC-TOF-MS system and parameters

High-resolution mass spectra were obtained by injecting 2 µL aliquots of all analytes in ACN/H_2O (80:20, v/v) into an Acquity UPLC core system (Waters, Manchester, UK). This system included a binary solvent manager, a sample manager, and a column oven. Chromatography separations

were conducted on a BEH C18 column (150 × 2,1 mm, 1.7 µm, 130 Å, Waters, Manchester, UK) at a flow rate of 0.4 mL/min and a column temperature of 40 °C with 0.1% formic acid in H_2O (v/v) as solvent A and 0.1% formic acid in ACN (v/v) as solvent B. For the initial screening of fractions, the gradient started at 5% B and increased to 100% B within eight minutes and remained isocratic for five minutes. The methods used were previously reported in the literature [118,128].

High-resolution mass spectra were acquired on a Synapt G2-S HDMS (Waters, Manchester, UK) in positive and negative ESI resolution modes using a capillary voltage of 2.5 kV and –1.7 kV, respectively; 50 V sampling cone; 4.0 kV extraction cone; 150 °C source temperature; 450 °C desolvation temperature, 2 and 30 L/h cone gas, and 800 L/h desolvation gas.

The mass spectrometer was calibrated across a range of m/z 50−1200 using a sodium formate solution (0.5 mmol/L) in isopropanol/H_2O (90:10, v/v). The mass data were lock mass-corrected by infusing a solution of leucine enkephalin (1 ng/µL, m/z 556.2771, $[M+H]^+$ and m/z 554.2615, $[M-H]^-$) at 10 µL/min. The data processing was performed using MassLynx 4.2 (Waters, Manchester, UK).

3.7 Quantification of fatty acids and fatty acid oxidation products using UHPLC–DMS–MS/MS

The reference compounds were obtained commercially, and to ensure accurate quantification, two different commercially available internal standards structurally similar to the analytes were selected: [$^{13}C_{18}$]-linoleic acid (IS1) was used as the internal standard for fatty acids, while 18-hydroxyoleic acid (IS2) served as an internal standard for the oxylipins. MS/MS parameters for each analyte and internal standard were optimized individually in ESI negative ionization mode to monitor the fragmentation of pseudomolecular ions [141].

3.7.1 Solvent extraction for quantification

To perform the quantification in triplicate, 3 × 500 mg of sunflower press cake, along with a mixture of MeOH/H_2O (50:50, v/v, 5 mL), and the following internal standard solutions were added to a cryogenic tube (10 mL, VWR Chemicals, Fontenay-sous-Bois, France): 25 µL of [$^{13}C_{18}$]-linoleic acid (IS1, 0.5 mM in MeOH), 25 µL of 18-hydroxyoleic acid (IS2, 0.5 mM in MeOH). The extraction was carried out using an Analogue Orbital Shaker 3005 (GFL, Burgwedel, Germany) for 1 h at 300 U/min. The extracts were membrane-filtered (Minisart RC 15, 0.45 µm, Sartorius AG, Göttingen, Germany) and subsequently injected into the LC−DMS−MS/MS system [141].

3.7.2 Calibration curve

The exact concentration of the analytes was verified by quantitative NMR (qNMR), and a stock solution (0.2 mM) was prepared in MeOH. This stock solution was diluted to 0.1, 0.05, 0.025, 0.0125, 0.0063, 0.0031, 0.0016, 0.0008, and 0.0004 mM. Next, 1 mL of each dilution was mixed with 10.1 µL of an internal standard solution mixture. Each prepared sample was analyzed in triplicate using UHPLC–DMS–MS/MS. Then, calibration curves were prepared by plotting the peak area ratio of the analyte to the internal standard versus the concentration ratio of each analyte to the internal standard. A linear regression was performed for quantitation, using MultiQuant version 3.03 (Sciex, Darmstadt, Germany) [118].

3.7.3 UHPLC–DMS–MS/MS system and parameters

The MS/MS analysis was performed using a QTrap 6500+ mass spectrometer equipped with a SelexION + DMS cell (Sciex, Darmstadt, Germany) in the negative ionization mode, as reported in the literature [118,141]. The ion mobility parameters included isopropanol as the chemical modifier at the flow rate of 363.6 µL/min (low), an SV of 3500 V, a DMS temperature of 225 °C (medium), and a DMS offset of 3 V. The declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were optimized using commercial references of methanolic solutions of the analytes and internal standards [141].

The mass spectrometer was operated in MRM full scan mode (ion-spray voltage: –4500 V for ESI negative ionization) with the following parameters: temperature, 450 °C; gas 1, 55 psi; gas 2, 65 psi. The MS/MS system was coupled to a Shimadzu LC system Nexera X3 (Shimadzu, Duisburg, Germany) consisting of a Shimadzu LC-40D pump, a Shimadzu DGU-405 degasser, a Shimadzu SIL-40C autosampler, a Shimadzu CTO-40C column oven AC, and a Shimadzu SCL-40 control unit.

Sample injections (1 µL) were followed by chromatography on a Kinetex C18, (150 × 10 mm, 1.7 µm; Phenomenex, Aschaffenburg, Germany) with a binary gradient using 5 mM NH_4Ac in H_2O (pH 5) as solvent A and 5 mM NH_4Ac in H_2O (pH 5)/ACN/isopropanol (5:55:40, v/v/v) as solvent B (flow rate of 0.35 mL/min): 0 min, 15% B; 0.5 min, 15% B; 2 min, 30% B; 6 min, 50% B; 17 min, 71% B; 19 min, 100% B; 21 min, 100% B; 22 min, 15% B; 24 min, 15% B. The instrument was controlled using the Analyst 1.6.3 software (Sciex, Darmstadt, Germany). Data analysis was performed using Microsoft Excel (Microsoft Office, 2016) and Multiquant (version 3.0.3, Sciex, Darmstadt, Germany).

3.8 Quantification of bitter compound 15 using UHPLC-MS/MS

3.8.1 Sample preparation for quantification

The sunflower press cake (1 g, n = 3) was weighed into bead beater tubes (5 mL, CKMix, Bertin Technologies, Montigny-le-Bretonneux, France), and a mixture of methanol and water (50:50, v/v, 5 mL) was added. Extractive grinding was performed at 6000 rpm for 3 × 30 s with 30 s breaks in between using a bead beater (Precellys Homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France). The samples were centrifuged (10 min, 6000 rpm), and the clear supernatant was separated. The residue was extracted using the same protocol a total of five times. The combined supernatants were evaporated to dryness under nitrogen and reconstituted in a mixture of methanol and water (50:50, v/v, 400 µL), then transferred to autosampler vials, and stored at –20 °C until further analysis.

3.8.2 UHPLC-MS/MS system and parameters

Mass spectrometry was conducted using the QTRAP 6500 system described earlier, operated in ESI negative mode with the following ion source parameters: ion spray voltage at –5500 V (ESI-), curtain gas at 35 psi, nebulizer gas at 55 psi, heater gas at 65 psi, collision-activated dissociation medium, and source temperature at 500 °C. The MS system was coupled to a Shimadzu Nexera X2 UHPLC (Shimadzu, Duisburg, Germany) as mentioned earlier. The MS/MS parameters of compound 15 were tuned and optimized, resulting in the characteristic Q1/Q3 transitions of m/z 607.2/161.0 (DP = –140 V, CE = –46 V, CXP = –17 V) as the quantifier, and m/z 607.2/178.9 (DP = –140 V, CE = –44 V, CXP = –21 V) as the qualifier. Chromatography was performed using a Kinetex Biphenyl column (100 × 2.1 mm, 1.7 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) maintained at 40 °C. Aliquots (1 µL) were injected into the system at a flow rate of 0.4 mL/min

and using 0.1% formic acid in water and 0.1% formic acid in methanol as solvents A and B, respectively, with the following gradient: 5% B held for 1 min, increased in 2.5 min to 60 % B, held at 60% B for 2 min, increased in 1 min to 100% B and held at 100% for 1 min, then decreased in 1.5 min to 5% B, and re-equilibrated for 1.5 min at 5% B.

3.8.3 Standard calibration curve

For quantification, a stock solution of bitter tastant 15 was prepared in a mixture of acetonitrile and water (50:50, v/v). The concentration was determined using quantitative ^{1}H-NMR spectroscopy. The stock solution (10.96 mmol/L) was then diluted successively (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256) using the same solvent mixture. All dilutions were analyzed by means of UHPLC-MS/MS using a scheduled (20 s window) multiple reaction monitoring (MRM) method. Then, the peak area was plotted against the concentrations, and an external calibration curve was established with linear regression ($y = 2667.8x + 386458$, $R^2 = 0.9992$), which was used for quantification of **15** in the sunflower press cake.

3.9 Nuclear magnetic resonance spectroscopy

NMR spectra were recorded using a Bruker Avance Neo 600 MHz system (Bruker, Rheinstetten, Germany) equipped with a cryo-TCI probe at 300 K. The samples were prepared in 100 x 3 mm NMR tubes (Hilgenberg, Münnerstadt, Germany). Data acquisition and processing were carried out using TopSpin 4.1.1 (Bruker, Rheinstetten, Germany) and MestReNova 11.0.4 (Mestrelab Research, La Coruña, Spain). Chemical shifts were referenced to the residual solvent signals of DMSO-d_6 or D_2O.

Quantitative NMR spectroscopy (qHNMR). For quantification, data were recorded on a Bruker AV III 400 MHz system, which was equipped with a Broadband Observe BBFOplus probe. The concentration of the target compounds was determined with the external reference L-tyrosine (5.21 mmol/L) via the ERETIC II.

4 Results and Discussion

The research presented in this thesis was initiated with the aim of identifying and characterizing off-flavor contributors in protein alternatives. This growing category includes both insect and plant-derived sources. While insect-based proteins were examined in an earlier study focusing on off-flavor formation **(Publication 1**), the primary focus of this dissertation centers on plant-derived sources due to their broader consumer acceptance. Within this category, a range of candidates, including legumes, cereals, and oilseeds, were extensively explored, from which sunflower (*Helianthus annuus* L.) protein sources (press cake, protein isolates, defatted meal) emerged as particularly relevant yet insufficiently studied. Previous work on sunflower-based food products, such as spread formulations **(Publication 2**), has demonstrated both the matrix's complexity and its potential for broader food applications, further supporting the relevance of deeper investigation into its use as a protein source and its associated sensory characteristics.

Although sunflower use in protein-rich applications has been increasing, limited data exist regarding the taste-active constituents and overall flavor profile of these ingredients. To bridge this gap, an in-depth literature review **(Publication 3**) was conducted to characterize the non-volatile and volatile compounds that may be relevant or potentially contribute to the flavor of sunflower-derived protein sources.

Furthermore, the review highlights how factors such as genotype, growth conditions, and environmental stress may influence the biochemical composition of sunflower matrices and, consequently, their flavor potential. To facilitate clearer analysis, the study grouped the identified compounds into major classes, including lipids, amino acids, sugars, minerals, vitamins, phenols, and flavonoids. It further examined the reported concentrations, taste thresholds, and dose-over-threshold values, where available, for all listed compounds to support their potential relevance. Beyond these classifications, the review aimed to frame the current state of knowledge for future exploration by identifying not only what is known but also what remains uncertain.

Additionally, the review also critically discusses the methodological approaches applied for the identification of volatiles and highlights key limitations, particularly the absence of sensory validation steps, which restricts the ability to confirm their actual contribution to perceived flavor. Importantly, as explicitly stated in the review, the presence of compounds in the dataset does not in itself indicate sensory activity. Instead, the review offers a comprehensive chemical overview of sunflower-based matrices that may contain flavor-active constituents.

The review also notes that while some studies briefly mention the presence of off-flavors in sunflower protein alternatives, these claims are often made without accompanying sensory or analytical evidence. For this reason, the review also emphasizes that sensomics approach and food matrix interactions are essential to confirm the actual contribution of individual compounds to perceived flavor.

A review of the potential taste-active compounds in sunflower products served as a foundation for the experimental investigations presented in **Publication 4**. In this study, key bitter compounds in sunflower press cake were identified and characterized using a sensomics approach. These findings may guide future product reformulation strategies aimed at enhancing the palatability of sunflower protein-based products.

4.1 Identification of bitter off-taste compounds in sunflower press cake using the sensomics approach (Publication 4)

As an initial step, a comprehensive taste profile analysis was conducted on a reference sample selected from commercially available sunflower protein sources, which served as a baseline for evaluating sensory attributes. On a scale from 0 (not detectable) to 5 (strongly detectable), the panelists were asked to rate the intensity of bitterness, sweetness, sourness, umami, saltiness, and astringency. Based on the established reference point, the trained sensory panel evaluated the sunflower press cake using the same taste profile analysis, comparing the sample directly with the reference (Figure 7). Sensory analysis of the sunflower press cake showed that bitterness and astringency exhibited the highest intensity ratings, with scores of 2.5 and 2.4, respectively, followed by sourness with a score of 0.8. In comparison, sweetness and saltiness were perceived with lower intensity, each scoring 0.4, whereas umami was perceived with the lowest intensity score of 0.3.

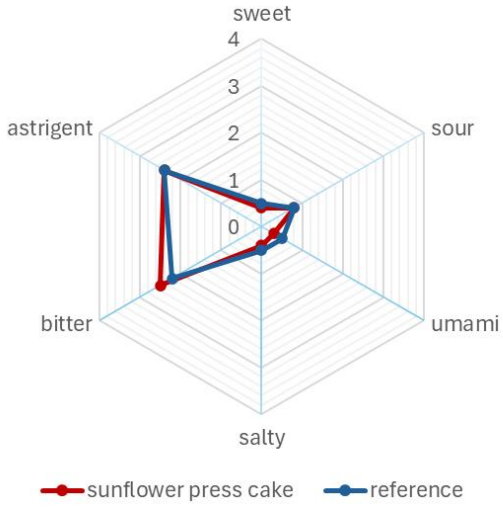

Figure 7. Taste profile analysis of sunflower press cake compared to the reference. *Radar plot comparing the sensory taste profiles of sunflower press cake (red) and a reference sample (blue). Taste attributes (sweet, sour, umami, salty, bitter, and astringent) were evaluated on a 5-point scale (0 = not perceived, 5 = strongly perceived) by a trained panel.*

To assess how sunflower press cake compares to other sunflower-derived proteins in terms of metabolite composition, two commercially available sunflower protein isolates were included in the analysis. All three samples were analyzed through untargeted screening with UPLC-TOF-MS. The resulting data revealed a high degree of overlap, indicating compositional similarities among the products (see Appendix 1, Figure S1).

To complement the chemical data, a sensory profile analysis was performed to evaluate and compare the taste characteristics of the samples. The sensory evaluation showed that overall taste profiles were broadly similar across all samples. Bitterness and astringency were the most prominent attributes, with press cake sample showing a bitterness rating of 2.5. This finding is consistent with previous reports describing bitterness as a common sensory characteristic of sunflower protein matrices [110,111]. The sensory data are presented in Figure 8.

It is important to acknowledge that the composition and sensory characteristics of sunflower protein sources can vary depending on their botanical origin and processing conditions [142,143]. Although the samples analyzed in this study exhibited high compositional similarity, such findings should be interpreted with caution, as variability among protein sources remains a critical consideration in food formulation and sensory research.

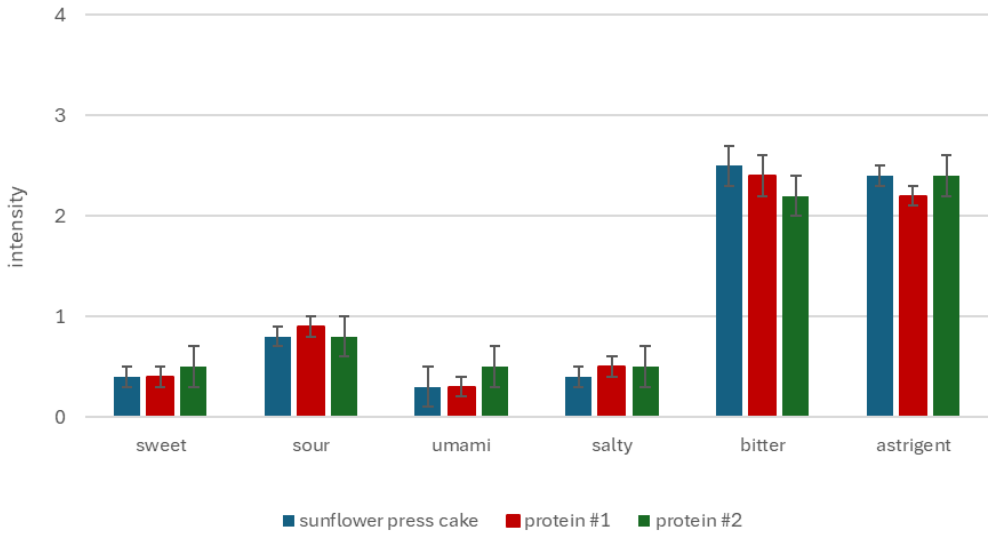

Figure 8. Comparison of the taste profiles of commercially available sunflower proteins with sunflower press cake*. Sensory evaluation was conducted by a trained panel using a five-point intensity scale (0 = not detectable, 5 = strongly detectable). Protein #1 and protein #2 refer to commercially obtained sunflower proteins used in this study.*

Given the prominent bitterness detected during sensory analysis, a targeted analytical strategy was employed to identify the key bitter compounds in sunflower press cake. Sensomics, a widely used approach in characterizing taste-active compounds in various protein-rich matrices [118,131], was subsequently applied to elucidate the molecular drivers of the off-taste in this sample (Figure 9). The identification process began with sequential solvent extraction, followed by solid-phase extraction for further fractionation. These steps enabled the isolation of bitter-active fractions, which were subsequently analyzed using HPLC and further techniques for compound identification and sensory validation.

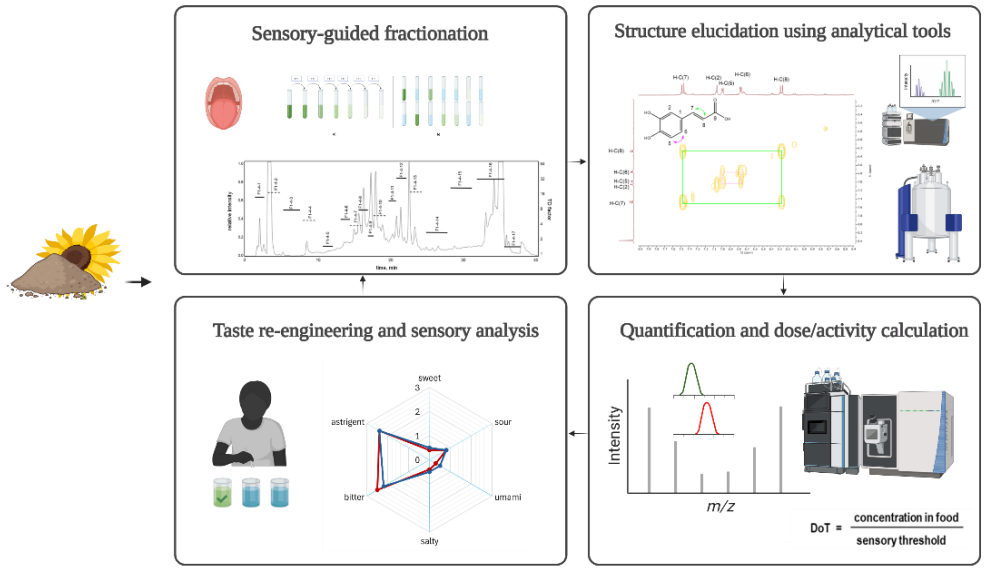

Figure 9. Workflow of the sensomics approach for identifying key taste-active compounds in sunflower press cake. *The workflow begins with sensory-guided fractionation, followed by structure elucidation using advanced analytical tools, and quantification calculations to estimate sensory relevance. In the final step, taste re-engineering and sensory evaluation are used to confirm the impact of identified compounds through recombination and omission testing. Image created based on concepts from Granvogl et al. (2022) [30], Meyer et al. (2016) [113], Hillmann et al. (2012) [114], and Frank et al. (2022) [120]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/a7c6h8o*

4.1.1 Sequential solvent extraction of sunflower press cake followed by sensory analysis

The sunflower press cake was extracted sequentially with a series of solvents, beginning with MeOH/H2O (F1), followed by MeOH (F2), ethyl acetate (F3), and n-pentane (F4). Each extraction step was performed three times, and after each round, the solvent was filtered to separate the liquid extract from the solid residue. From the collected fractions, the solvent was removed using a rotary evaporator and a freeze-dryer to ensure the complete elimination of residual solvents. The resulting dried samples were then subjected to sensory analysis.

Fractions were dissolved in their natural concentrations in water and analyzed using comparative taste profile analysis (Table 3). Among the four fractions, fraction F1, with an intensity score of 2.5, showed the highest bitterness compared with fractions F2–F4. Due to its pronounced bitterness, fraction F1 was further fractionated to identify the key bitter molecules.

To evaluate whether residual bitter compounds remained in the matrix after extraction, the insoluble residue was also tasted. In a consensus panel, six sensory-trained panelists were asked to compare the bitterness of the untreated sunflower press cake with that of the insoluble residue. All panelists rated the residue (0.4) as less bitter than the original product. According to these results, it could be assumed that key bitter-tasting compounds had been successfully extracted from the matrix through sequential solvent extraction.

Table 3. Sensory evaluation of sunflower press cake fractions isolated by sequential solvent extraction. *The panelists were asked to rate aqueous solutions of the natural concentrations of the fractions F1–F4. The intensity of the individual taste descriptors was rated by a trained panel on a scale from 0 (not detectable) to 5 (strongly detectable).*

taste intensities for individual fractions					
taste attributes	**press cake**	**F1**	**F2**	**F3**	**F4**
sweet	0.4	0.3	0.1	0.2	0.2
sour	0.8	0.6	0.2	0.5	0.3
umami	0.3	0.2	0.1	0.2	0.1
salty	0.4	0.2	0.1	0.2	0.3
bitter	2.5	2.5	1.0	1.1	0.9
astringent	2.4	1.8	1.0	1.1	1.1

4.1.2 Solid-phase extraction of fraction F1 followed by sensory evaluation

To identify the fraction with key bitter compounds, F1 was separated further with RP-18 solid-phase extraction into five subfractions: F1-1 (H_2O, 100%), F1-2 (MeOH/H_2O, 30:70), F1-3 (MeOH/H_2O, 50:50), F1-4 (MeOH/H_2O, 70:30), and F1-5 (100% MeOH). Solvent was removed from all subfractions using a rotary evaporator, followed by two rounds of freeze-drying.

To evaluate the sensory contribution of each subfraction, a comparative taste profile analysis was conducted using a trained panel. Bitterness intensity rated for F1-2 and F1-3 were 2.1 and 2.4, respectively, while F1-4 exhibited the highest bitterness with a score of 3.0 (Figure 10). Due to this elevated intensity, F1-4 was selected for further compound identification.

The subfraction F1-4 was obtained using MeOH/H_2O (70:30), which coincides with the polarity employed in the initial extraction step. This fraction rated more bitter than the original sunflower press cake, likely due to the absence of matrix effects that may mask or bind bitter compounds in the intact matrix [144].

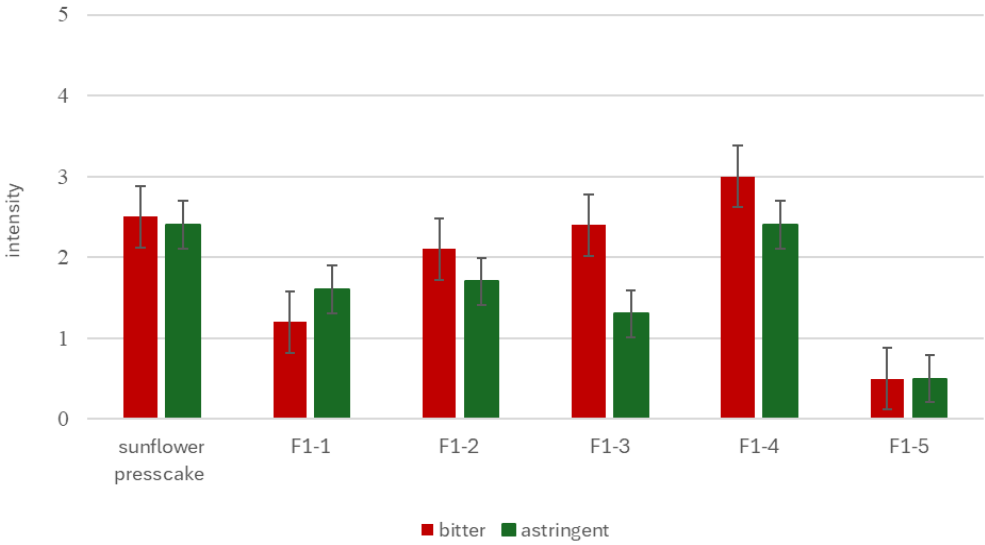

Figure 10. Sensory analysis of solid-phase extraction fractions F1-1 to F1-5 isolated from sunflower press cake. *The panelists were asked to rate the sensory attributes on a scale from 0 (not detectable) to 5 (strongly detectable).*

4.1.3 Isolation and purification of taste-active compounds from fraction F1-4 via preparative HPLC

To isolate the bitter compounds from fraction F1-4 in a more purified form, a preparative reversed-phase HPLC method was developed, coupled with evaporative light scattering detection (ELSD). Multiple chromatographic runs were carried out to collect sufficient material for sensory testing, resulting in 17 subfractions (F1-4-1 to F1-4-17), and each subfraction was freed from solvent.

The sensory activity of each subfraction was evaluated using taste dilution analysis, following the approach originally described by Frank et al. (2001) [120]. This approach is designed to identify the subfractions with the highest sensory activity, thereby narrowing down the focus for compound identification in a targeted, activity-guided manner.

To carry out this analysis, each subfraction was dissolved in Evian® water (pH 5.9) at four times its natural concentration. An aliquot was then serially diluted in a 1:1 ratio to create a dilution series. Panelists evaluated each dilution step in ascending order using a duo test, comparing each sample to Evian® as a blank reference. To avoid visual bias, the solutions were presented in amber glass tubes to mask any color differences.

The dilution level at which no perceptible difference from the blank could be detected is defined as the taste dilution (TD) factor, which reflects the relative sensory potency of each subfraction. The TD factor of each sample was calculated from the arithmetic mean of all perceptible dilution levels across panelists (Figure 11).

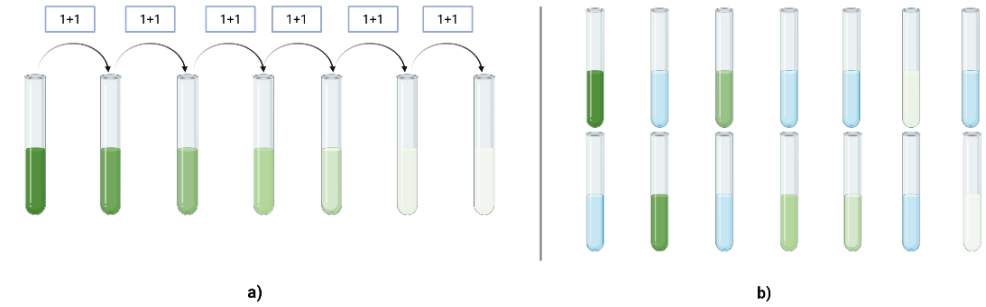

Figure 11. Visualization of the taste dilution analysis*. a) Preparation of serial dilutions: each subfraction was subjected to stepwise dilution (1:1 ratio) using water to generate a defined dilution series. This process was applied to all fractions to ensure consistency across sensory evaluation. b) Sensory evaluation: diluted samples were presented in randomized order to trained panelists, each paired with a blank (Evian® water, pH 5.9) to control for bias. Panelists evaluated samples in ascending concentration. If all test samples were correctly distinguished from the blank, additional dilution levels were introduced to refine the taste dilution factor calculation. Based on Frank et al. (2001) [120]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/vdo424n*

The sensory profiles of the subfractions varied across samples; while some were predominantly astringent, others elicited a bitter taste. This variation may reflect the presence of different taste-active compounds within the F1-4 fraction. The elution of bitter-active subfractions throughout the chromatogram suggests that these compounds span a broad range of polarities, further indicating the chemical diversity underlying the observed sensory effects.

Among the 17 subfractions of F1-4 analyzed for their taste dilution (TD) factors (Figure 12), subfractions F1-4-12, F1-4-15, and F1-4-16 exhibited the most intense bitterness, with TD factors of 32, 28, and 32, respectively. These subfractions were therefore selected for further analysis, as their sensory properties indicated the presence of potent bitter compounds and underscored the need for detailed chemical characterization.

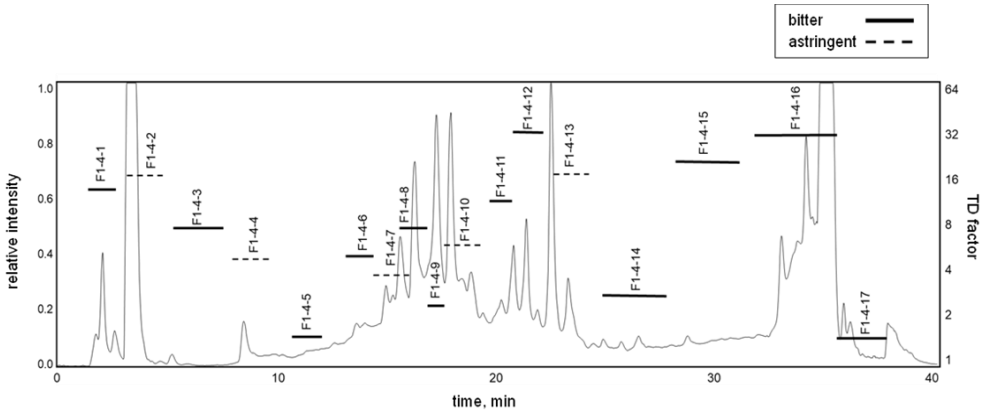

Figure 12. RP-HPLC-ELSD chromatogram of sample F1-4 with corresponding taste dilution factors for subfractions F1-4-1 to F1-4-17*. Subfractions exhibiting bitterness are marked with solid lines, while those associated with astringency are indicated by dashed lines.*

Following this analysis, the subfractions were screened using UPLC-TOF-MS to determine their compound complexity and to assess whether further subfractionation was necessary. Screening results suggested that the bitter fractions F1-4-15 and F1-4-16 may contain fatty acids and their oxidation products. Therefore, these compounds were further characterized using LC-TOF-MS (ESI-) analysis, which revealed pseudomolecular ions ([M-H]-) with m/z values of 329.2329, 329.2330, 329.2332, 295.2277, 295.2274, and 293.2113. Based on their elution times and fragmentation patterns, the compounds correspond to oxidation products previously identified in matrices such as pea protein [118] and poppy seeds [128], where they have been associated with bitterness. Furthermore, known taste-active compounds and free fatty acids were screened and analyzed against reference standards using LC-MS/MS and UPLC-TOF-MS. The results indicated that trihydroxyoctadecenoic acids and hydroxyoctadecenoic acids, found in various plant-based products, were also present in sunflower press cake [118,128,141]. The identified compounds are listed in Table 4.

Table 4. Identified free fatty acids and their oxidation products in sunflower press cake.

	compound name	molecular formula
Trihydroxyoctadecenoic acid	**1.** 9,12,13-trihydroxyoctadec-10-enoic acid	$C_{18}H_{34}O_5$
	2. 9,10,11-trihydroxyoctadec-12-enoic acid	$C_{18}H_{34}O_5$
	3. 11,12,13-trihydroxyoctadec-9-enoic acid	$C_{18}H_{34}O_5$
Hydroxy derivative	**4.** (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid	$C_{18}H_{32}O_3$
	5. (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid	$C_{18}H_{32}O_3$
	6. (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	$C_{18}H_{32}O_3$
	7. (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	$C_{18}H_{32}O_3$
Oxo derivative	**8.** (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid	$C_{18}H_{30}O_3$
Free fatty acid	**9.** α-linolenic acid	$C_{18}H_{30}O_2$
	10. Linoleic acid	$C_{18}H_{32}O_2$
	11. Oleic acid	$C_{18}H_{34}O_2$
	13. Palmitic acid	$C_{16}H_{32}O_2$
	14. Stearic acid	$C_{18}H_{36}O_2$
Monohydroxy fatty acid	**12.** 2-hydroxyoleic acid	$C_{18}H_{34}O_3$

Among plant-based materials, sunflower-derived products are especially prone to oxidation due to their high concentrations of polyunsaturated fatty acids (PUFAs) [94–98]. This biochemical vulnerability makes the formation of oxidation products not only likely but also expected during processing and storage.

The identification of fatty acid oxidation derivatives in sunflower press cake as contributors to bitter off-taste may originate from various oxidative pathways (Figure 13). Understanding the mechanisms behind these transformations is essential for controlling and improving the sensory qualities of sunflower-based food ingredients. The oxidation can proceed through three primary pathways: autoxidation, enzymatic oxidation, and photo-oxidation, each contributing uniquely to the formation of primary and secondary oxidation products [145–147].

Autoxidation represents a non-enzymatic, radical-mediated pathway initiated spontaneously when PUFAs interact with molecular oxygen. This process is particularly active in sunflower systems due to their high linoleic acid content, which contains bis-allylic methylene groups that are highly reactive toward hydrogen abstraction. Exposure to oxygen, metal ions (e.g., Fe^{2+} from plant tissues), and elevated temperatures can significantly accelerate autoxidation [146,148,149].

The process is typically divided into three distinct phases: initiation, propagation, and termination. During initiation, hydrogen atoms are abstracted from bis-allylic positions of unsaturated fatty acids by reactive species such as hydroxyl radicals, hydroperoxyl radicals (HOO•), or transition metal ions [145,149]. These initiators can originate from thermolysis, enzymatic activity, light exposure, or naturally occurring oxidative stress, all of which contribute to the formation of lipid radicals (L•). These lipid radicals rapidly react with molecular oxygen to form peroxyl radicals (LOO•), marking the beginning of the propagation phase. In this phase, peroxyl radicals continue the chain reaction by abstracting hydrogen atoms from neighboring lipids, generating new lipid radicals and lipid hydroperoxides (LOOH) [145,146,148]. These hydroperoxides serve as unstable intermediates that can break down into aldehydes, ketones, hydroxylated fatty acids, and volatile compounds, many of which contribute to sensory deterioration and nutritional loss. The termination phase occurs when two radicals, either L•, LOO• or their combinations, interact to form non-radical products, effectively halting the chain reaction. However, termination is often outpaced by propagation, especially under oxidative stress, leading to a significant accumulation of degradation products [145,146,150]. Despite being a non-enzymatic process, autoxidation can yield a wide variety of compounds, many of which overlap with those produced via enzymatic routes.

Simultaneously, ***enzymatic oxidation*** may occur, particularly in matrices that have not undergone extensive heat treatment, such as mechanically pressed or mildly processed plant materials. In such conditions, enzymes such as lipoxygenase (LOX) remain functionally active. LOX enzymes catalyze the regio- and stereoselective insertion of molecular oxygen into polyunsaturated fatty acids, particularly linoleic acid (18:2) and α-linolenic acid (18:3), both of which contain a (*Z,Z*)-1,4-pentadiene system essential for LOX recognition [147]. This enzymatic oxidation yields 9- and 13-hydroperoxyoctadecadienoic acids from linoleic acid as primary products [146].

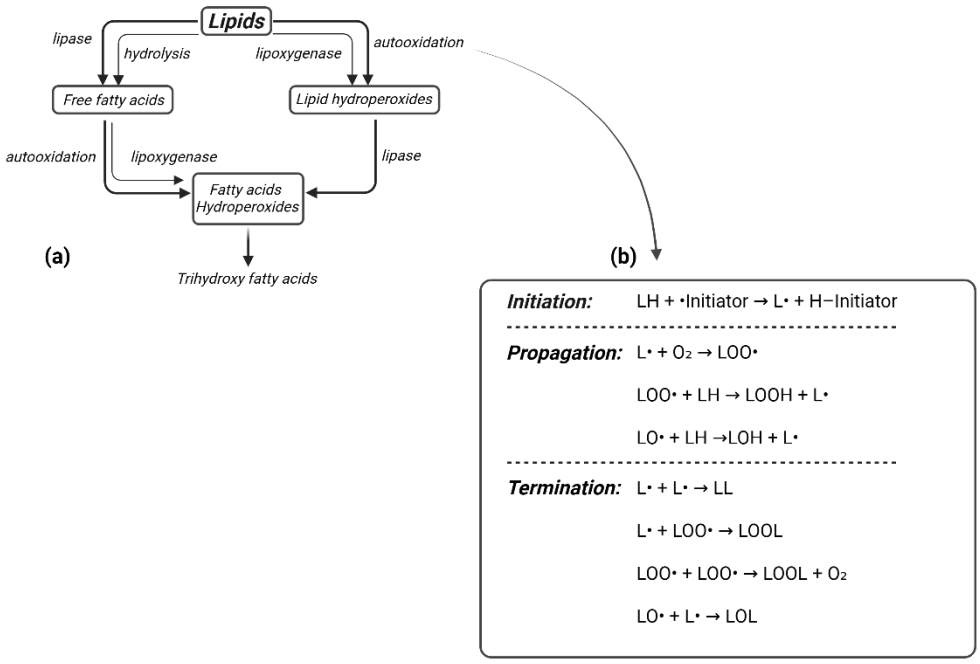

Figure 13. Schematic representation of fatty acid hydroperoxide formation via enzymatic and auto-oxidation pathways. *a) Diagram illustrates the general mechanisms of lipid oxidation, including enzymatic oxidation, primarily initiated by lipase and lipoxygenase activity, and non-enzymatic (auto-oxidation) processes. b) This panel details the classical auto-oxidation mechanism, involving initiation, propagation, and termination steps that collectively lead to the formation of fatty acid hydroperoxides. Based and adapted with permission from Baert et al. (2012), Pajunen et al. (2008), Geng et al. (2023), Shurson et al. (2015), and Saldana et al. (2013) [145–149]. Created in BioRender. Huseynli, L. (2025) https://BioRender.com/pjm9lpj*

These hydroperoxides can be further metabolized through enzymatic pathways involving peroxygenase, hydroperoxide lyase, and epoxide hydrolase, leading to the production of hydroxy fatty acids, epoxides, aldehydes, and trihydroxy derivatives. These transformations are typically stereoselective, providing molecular signatures that distinguish them from autoxidation products. Additional oxidative enzymes involved in plant lipid metabolism include α-dioxygenases, which contribute to the formation of unique hydroxy and oxo fatty acid species [151]. The activity of these enzymes is closely tied to plant defense and stress signaling mechanisms, often becoming upregulated in response to tissue damage, pathogen attack, or abiotic stress [147]. Among common fatty acid substrates, linoleic acid is significantly more susceptible to enzymatic oxidation than oleic acid (18:1), as the latter lacks bis-allylic hydrogens required for efficient LOX activity. Linoleic acid has been shown to oxidize up to 30 times faster than oleic acid under comparable conditions. α-Linolenic acid (18:3) is also highly prone to oxidation, 3–4 times more reactive than linoleic acid [147]. Enzymatic oxidation is especially prominent when raw materials are fresh or have been stored under mild conditions, allowing the endogenous enzymes to remain intact and functionally active.

Though less emphasized in many food systems, ***photooxidation*** may occur if the material is exposed to light in the presence of natural photosensitizers, such as chlorophyll or riboflavin. This process involves the generation of singlet oxygen ($^1O_2$), which reacts with unsaturated fatty acids through a non-radical, direct oxygenation mechanism, leading to

hydroperoxides that form at positions not typically targeted by LOX or autoxidation. In practical settings, this pathway usually has a minor impact compared to the other two, unless the material is stored in transparent or semi-translucent containers [147,152].

Due to its biologically rich and minimally processed nature, sunflower press cake serves as a complex system where multiple oxidative pathways likely co-exist. The oxidation products identified in sunflower press cake, including various hydroxy-, oxo-, and trihydroxy-octadecadienoic acids, can be traced back to specific oxidative pathways based on their molecular structures and stereochemical characteristics. Trihydroxy derivatives such as 9,10,11-trihydroxyoctadec-12-enoic acid, and 11,12,13-trihydroxyoctadec-9-enoic acid are consistent with products formed through lipoxygenase (LOX)-initiated peroxidation, enzymatic epoxidation and hydrolysis by epoxide hydrolases. The formation of 9,12,13-trihydroxyoctadec-10-enoic acid also fits within this enzymatic cascade, arising from regioisomeric hydroperoxides derived from 13-hydroperoxyoctadecadienoic acid. The diversity of these trihydroxy isomers suggests activity from both 9- and 13-LOX pathways. In contrast, hydroxylated fatty acids such as (10*E,*12*E*)-9-hydroxyoctadeca-10,12-dienoic acid and (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid may result from either enzymatic reduction of 9-hydroperoxyoctadecadienoic acid or from autoxidative cleavage processes, with the presence of *Z/E* isomers suggesting at least partial non-enzymatic contributions. The isomeric forms of 13-hydroxyoctadecadienoic acid, including (9*E*,11*E*)- and (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, are classically associated with LOX activity, yet the isomeric diversity observed here may indicate the influence of uncontrolled oxidation. Furthermore, the detection of (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid suggests enzymatic oxidation beyond hydroxide intermediates, most likely involving alcohol dehydrogenase acting on 13-HODE precursors. Collectively, these findings may suggest that both enzymatic and autoxidative processes were concurrently active during oxidation in sunflower press cake, resulting in a structurally diverse set of lipid-derived compounds reflective of overlapping biochemical mechanisms [145–147,150].

As discussed above, hydroxyoctadecadienoic acids (**4**–**7**, Figure 14) are formed via 9-/13-LOX activity and subsequent peroxygenase-mediated reduction [151,153,154]. The bitterness threshold of these compounds [128], along with that of related 2-hydroxy derivatives formed via the α-oxidation pathway in other plants [151,155,156], is already established in the literature [118].

To consolidate these findings, *E/Z* isomer configurations were confirmed by comparing analyte retention times with commercial reference standards, focusing on structural elucidation (Figure 14).

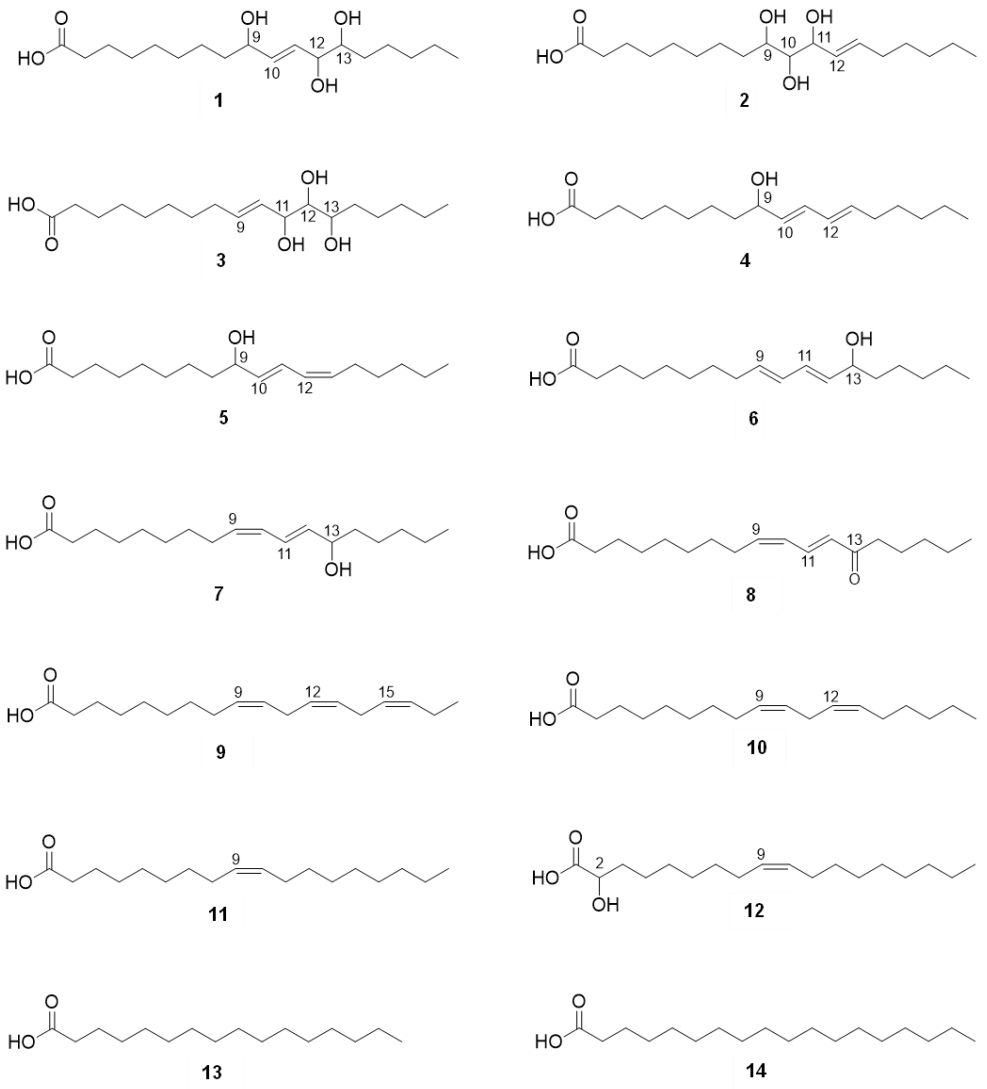

Figure 14. Chemical structures of identified compounds from sunflower press cake. *9,12,13-trihydroxyoctadec-10-enoic acid (**1**), 9,10,11-trihydroxyoctadec-12-enoic (**2**), 11,12,13-trihydroxyoctadec-9-enoic acid (**3**), (10E,12E)-9-hydroxyoctadeca-10,12-dienoic acid (**4**), (10E,12Z)-9-hydroxyoctadeca-10,12-dienoic acid (**5**), (9E,11E)-13-hydroxyoctadeca-9,11-dienoic acid (**6**), (9Z,11E)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), (9Z,11E)-13-oxooctadeca-9,11-dienoic acid (**8**), α-linolenic acid (**9**), linoleic acid (**10**), oleic acid (**11**), 2-hydroxyoleic acid (**12**), palmitic acid (**13**), stearic acid (**14**).*

4.1.4 Structural elucidation of the targeted compound in fraction F1-4-12

Among the isolated fractions, the other highest TDA factor was observed in fraction F1-4-12. This fraction displayed a significantly more complex composition, requiring further fractionation. Therefore, fraction F1-4-12 was further fractionated by semi-preparative HPLC, and eight fractions were collected. The potential target compound was contained in fraction F1-4-12-5, which was further purified with analytical HPLC for final structure elucidation (Figure 15).

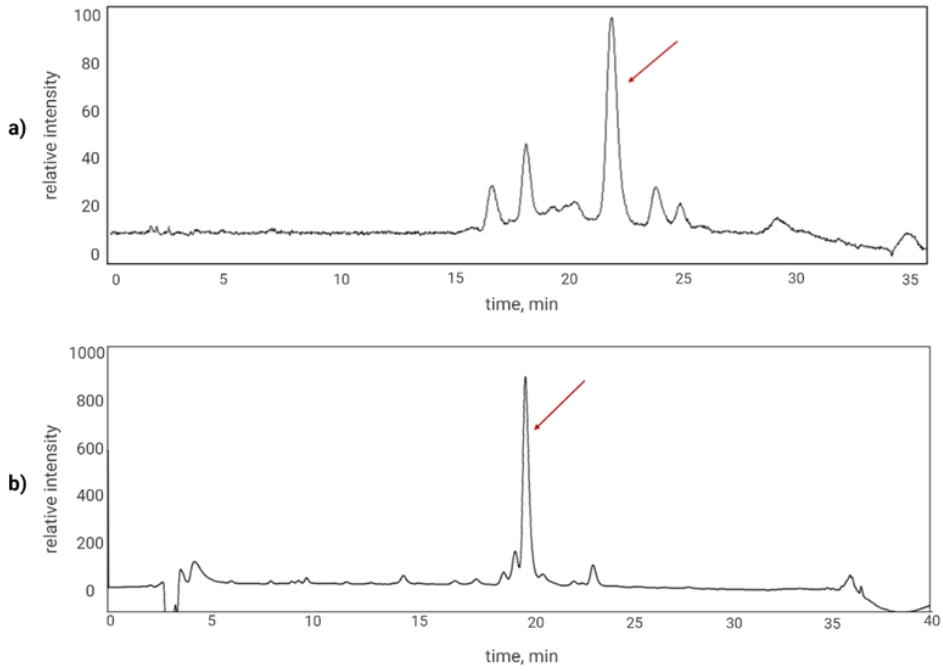

Figure 15. The illustration of the two-step purification approach used for the isolation of the targeted compound. *a) Semi-preparative HPLC chromatogram showing the separation of fraction F1-4-12. The red arrow indicates the peak corresponding to compound F1-4-12-5, which was not entirely pure. b) Analytical HPLC chromatogram with UV detection of fraction F1-4-12-5, showing improved resolution and a dominant peak corresponding to the targeted compound.*

To ensure the isolated compound in sub-fraction F1-4-12-5 was suitable for structural analysis, its purity was assessed using UPLC-TOF-MS. The spectrum (Figure 16) showed a single, well-defined molecular ion peak, with no significant background signals, confirming the absence of detectable co-eluting impurities. This level of purity indicated that the compound was ready for detailed structural characterization.

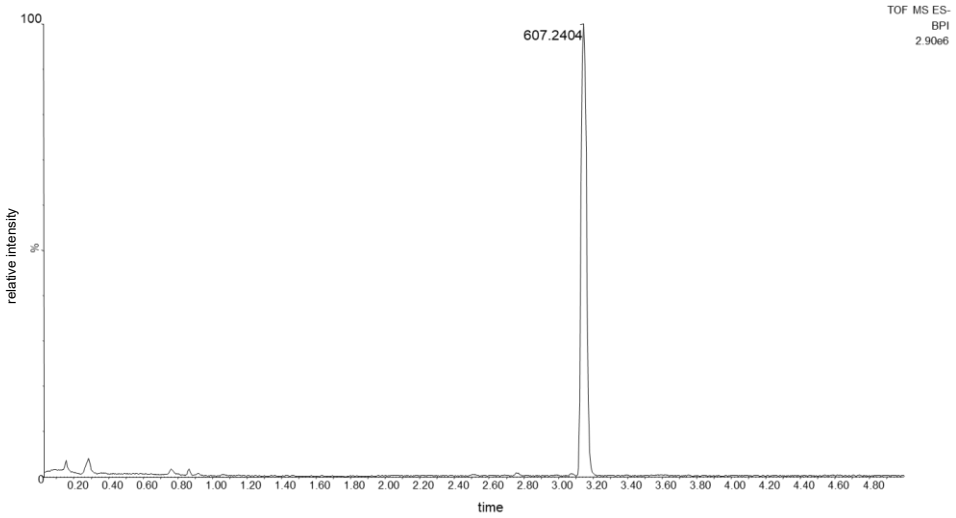

Figure 16. UPLC-TOF-MS spectrum of the targeted compound (15) from fraction F1-4-12-5.

After the isolation of the target compound by iterative HPLC fractionation, the structure of **15** was determined by TOF-MS and 1D-/2D-NMR experiments. First, high-resolution mass spectra were acquired, which showed a mass-to-charge (*m/z*) ratio of 607.2404, resulting in a predicted elemental composition of $C_{30}H_{39}O_{13}$ ($[M-H]^-$) in ESI negative mode. The calculated m/z ratio (607.2396 m/z for $C_{30}H_{39}O_{13}$, $[M-H]^-$) was in good agreement with the measured value indicated by a mass error of only 1.58 ppm. Additionally, MS^e spectra showed characteristic fragment ions, reported earlier [157] with *m/z* ratios of 179.0348, 161.0240, and 135.0444, all of which point to caffeic acid (179.0349 *m/z*, $[M-H]^-$) as a putative constituent of **15** (Figure 17).

To further elucidate the structure of **15**, NMR experiments were carried out. The ^{1}H- and ^{13}C-NMR spectra of compound **15** exhibited signals typical of a trans-caffeoyl moiety, which can be identified via the AMX spin system consisting of the proton resonances H-C(9`), H-C(8`), and H-C(5`) at 6.76 ppm, 6.98 ppm and 7.05 ppm. The characteristic coupling pattern, along with the coupling constants of the aromatic signals 8.2 Hz (doublet), 8.2/1.9 Hz (doublet of doublets), and 1.9 Hz (doublet), were in agreement with the values reported in the literature [158]. The distinctive trans olefinic protons signals resonating at 6.26 ppm and 7.47 ppm, with a coupling constant of 15.8 Hz (doublet), supported by 9 carbon signals C-1`–C-9` (δ 166.1, 114.2, 146.2, 124.7, 114.8, 145.9, 150.8, 122.2, 116.1) in the ^{13}C NMR spectrum, confirmed the presence of caffeic acid as a substructure of **15** (Figure 18, B).

In addition, the ^{1}H-NMR spectrum displayed eight proton signals in the aliphatic region, integrating twelve protons. The signals resonating at 0.59 ppm and 1.21 ppm could be assigned as methyl groups (H-(9), H-C(10)), integrating for 3 protons each. Two diastereotopic methylene groups resonating at 1.92-1.95 ppm/2.05-1.14 ppm (H-C(2)) and 1.58/2.24-2.30 ppm (H-C(8)), as well as three methine protons at 1.88-1.93 ppm (H-C(3)), 2.41 ppm (H-C(5)), and 4.39 ppm (H-C(1)) were assigned using the heteronuclear (C,H) single quantum coherence (HSQC) and homonuclear (H,H) correlated spectroscopy (COSY) experiments.

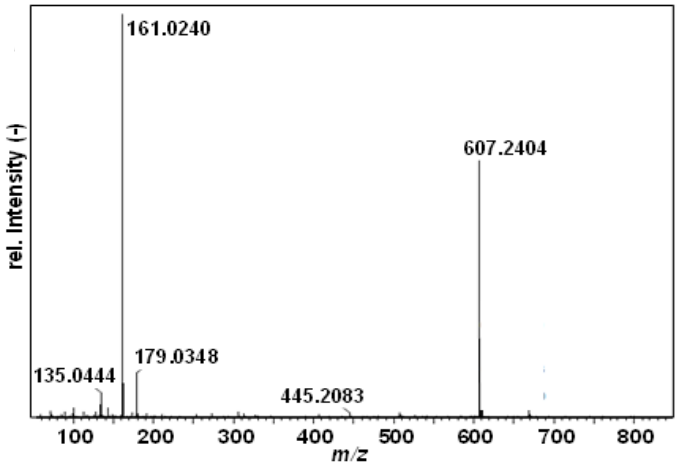

Figure 17. MS^e (20-40 eV, ESI- mode) spectrum of bitter tastant 15 isolated from sunflower press cake.

The presence of an exocyclic double bond, resonating at 4.81/5.01 ppm (H-C(7)), was assigned using heteronuclear multiple bond correlation spectroscopy (HMBC), optimized for $^2J_{C,H}$ and $^3J_{C,H}$ couplings. The correlation of proton signals H-C(1), H-C(3), H-C(5), H-C(7), H-C(8), H-C(9), and H-C(10) with carbon C-5 (50.4 ppm) cumulatively indicated the presence of a pinocarveol moiety. This assignment is supported by the identification of the two quaternary carbon atoms C-4 at 40.1 ppm and C-6 at 151.2 ppm, which were determined through ^{13}C-NMR in combination with the absence of HSQC correlations. The pinocarveol group was further supported by the respective carbon signals C-1–C-7 (δ 72.6, 32.2, 39.6, 40.1, 50.4, 151.2, 114.1, 27.2, 1.2, 0.6), closely resembling reports from the literature [159] (Figure 18, C).

In addition to caffeic acid and pinocarveol, the presence of two carbohydrate moieties in **15** was indicated by the presence of two anomeric proton signals at 4.37 ppm (H-C(1``)) and 4.79 ppm (H-C(1```)). It is presumed that these residues consist of a hexose and a branched-chain pentose, evidenced by the ^{13}C-NMR spectrum, which showed 11 aliphatic carbon signals, including one quaternary carbon (C-3```) at 79.6 ppm and three methylene carbon resonances at 67.4 ppm, 63.6 ppm, and 73.8 ppm (Table 5).

The carbohydrates were ultimately identified as D-glucose and D-apiose after acidic hydrolysis, followed by chemical derivatization with L-cysteine methyl ester and phenylisothiocyanate via LC-MS/MS analysis, as described previously [140]. The β-glycosidic linkage of the glucose was indicated by the coupling constant of 7.9 Hz of H-C(1``), and the anomeric configuration of the apiose moiety was determined to be β based on a comparison of the ^{13}C-NMR data for **15** with those of α- and β-D-apiofuranoside [160] and coupling constants of 2.8 Hz (H-C(1```)) consistent with the reported data for β-D-apiofuranoside (J = 2.6 Hz) [161–163].

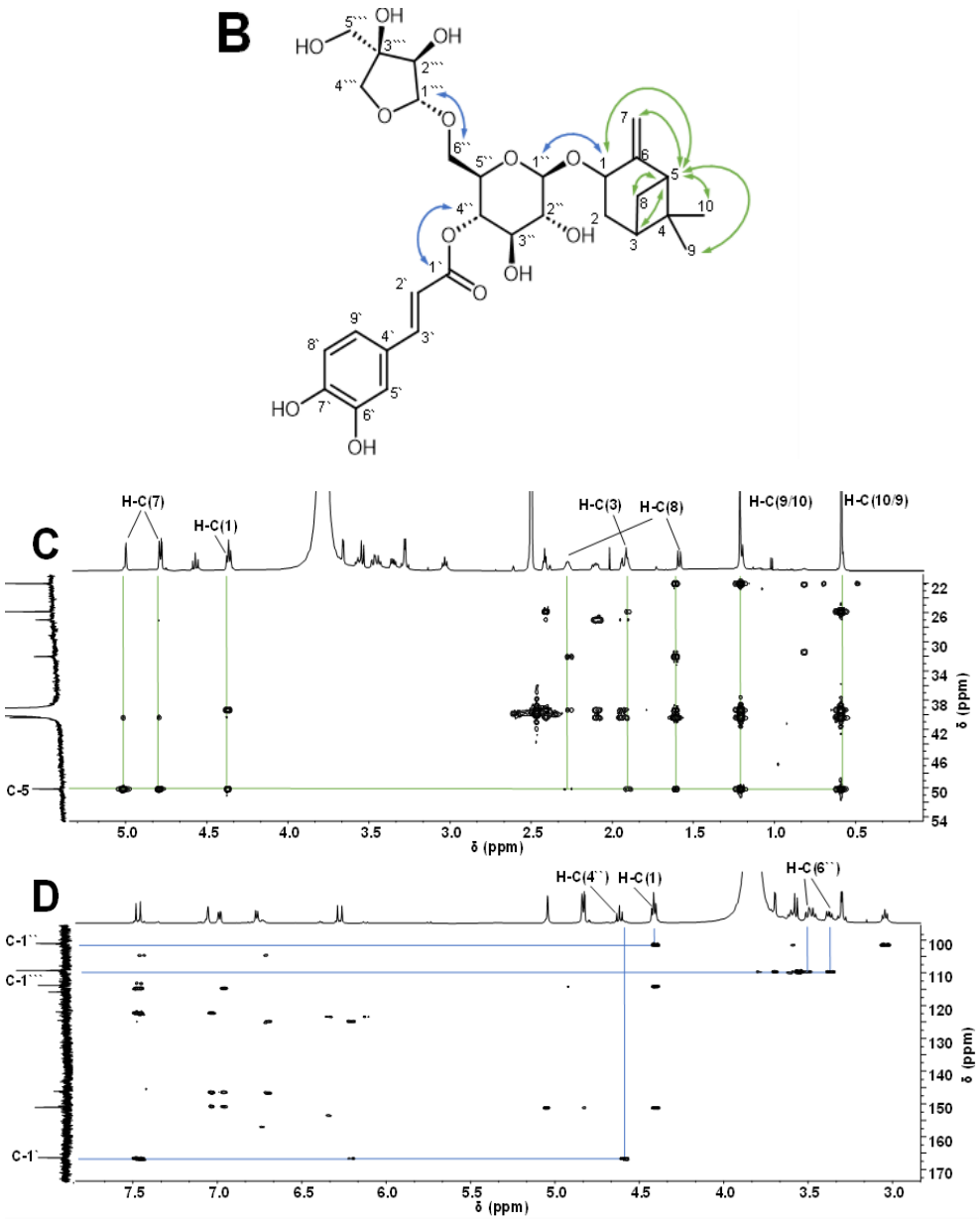

Figure 18. MS^e (20-40 eV, ESI- mode) spectrum of bitter tastant 15 isolated from sunflower press cake*. B) Chemical structure of 15 showing key correlations for structure elucidation via NMR spectroscopy. C) Excerpts of the HMBC spectrum (600/150 MHz, DMSO-d_6, 300 K) of 15 indicate the presence of a pinocarveol moiety and D) the connection of individual substructures.*

Table 5. ^{1}H- and ^{13}C-NMR assignments (600/150 MHz, DMSO-d_6, 300 K) of pinocarveol D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside.

	position	δ_C (ppm)	HSQC	δ_H (ppm)	M (*J*, Hz)
pinocarveol	1	72.6	[CH]	4.39	d (*J* = 7.3 Hz)
	2	32.2	[CH_2]	1.92–1.95 2.05–2.14	m m
	3	39.6	[CH]	1.88–1.93	m
	4	40.1	[C]	-	-
	5	50.4	[CH]	2.41	t (*J* = 5.4 Hz)
	6	151.2	[C]	-	-
	7	114.1	[CH_2]	4.81 5.01	s s
	8	27.2	[CH_2]	1.58 2.24–2.30	d (*J* = 9.5 Hz) m
	9	1.21	[CH_3]	25.9	s
	10	0.59	[CH_3]	22.3	s
caffeic acid	1`	166.1	[C]	-	-
	2`	114.1	[CH]	6.26	d (*J* = 15.8 Hz)
	3`	146.2	[CH]	7.47	d (*J* = 15.8 Hz)
	4`	124.7	[C]	-	-
	5`	114.8	[CH]	7.05	d (*J* = 2.2 Hz)
	6`	145.9	[C]	-	-
	7`	150.8	[C]	-	-
	8`	122.2	[CH]	6.98	dd (*J* = 1.9, 8.3 Hz)
	9`	116.1	[CH]	6.76	d (*J* = 8.2 Hz)
β-D-glucose	1``	101.8	[CH]	4.37	d (*J* = 7.8 Hz)
	2``	73.6	[CH]	3.04	t (*J* = 8.5 Hz)
	3``	74.1	[CH]	3.45	t (*J* = 9.5 Hz)
	4``	72.1	[CH]	4.59	t (*J* = 8.5 Hz)
	5``	73.2	[CH]	3.58–3.62	m
	6``	67.4	[CH_2]	3.36 3.49	dd (*J* = 6.3, 11.6 Hz) dd (*J* = 2.4, 11.6 Hz)

position		δ_C (ppm)	HSQC	δ_H (ppm)	M (*J*, Hz)
β-D-apiose	1```	109.7	[CH]	4.79	d (*J* = 2.8 Hz)
	2```	76.0	[CH]	3.66	d (*J* = 2.5 Hz)
	3```	79.6	[C]	-	-
	4```	63.6	[CH_2]	3.26–3.31	m
	5```	73.8	[CH_2]	3.55 3.76	d (*J* = 9.5 Hz) d (*J* = 9.5 Hz)

Finally, the connections of the individual substructures were determined via the HMBC correlations of H-C(1) of pinocarveol (4.39 ppm) with C-1`` (101.8 ppm) of glucose, the proton at position H-C(4``) of glucose (4.59 ppm) and C-1` of caffeic acid (169.1 ppm), as well as the methylene protons H-C-(6``) of glucose (3.36/3.49 ppm) with C(1```) of apiose (109.7 ppm), as highlighted in Figure 18, (D). Consequently, compound **15** was identified as pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (Figure 19).

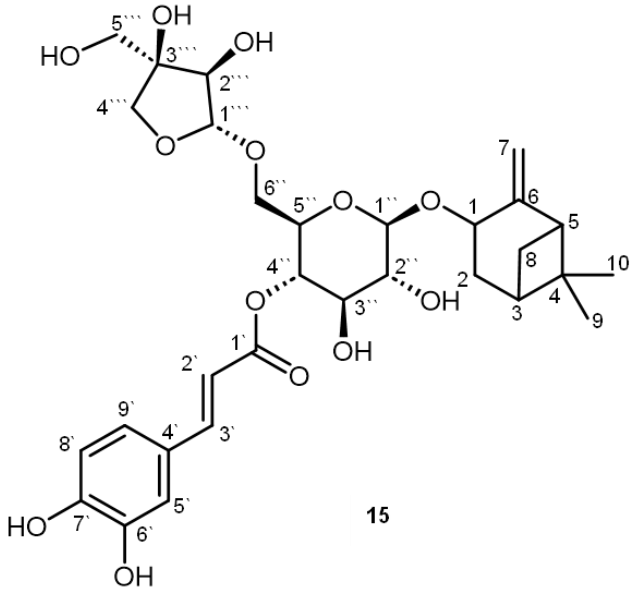

Figure 19. Pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside (15).

4.1.5 Sensory activity of identified bitter compounds

Following the structural elucidation of the compounds present in the bitter subfraction, their sensory relevance was assessed. This evaluation began with a consideration of the bitter taste properties of known compounds in sunflower press cake, using reported taste threshold concentrations from recent literature as a reference [118,128]. These thresholds were determined using a 3% aqueous ethanol solution in a two-alternative forced choice (2-AFC) test to address the solubility challenges of hydrophobic compounds.

The reported thresholds for the compounds listed in Table 6 include a notably low bitter threshold for 2-hydroxyoleic acid and slightly higher thresholds for 9,12,13-trihydroxyoctadec-10-enoic acid and its isomeric counterparts (9,10,11-trihydroxyoctadec-12-enoic acid and 11,12,13-trihydroxyoctadec-9-enoic acid) each at 0.13 mmol/L. Previously reported threshold values indicate that α-linolenic acid has a relatively lower bitter threshold compared to free fatty acids such as linoleic acid, oleic acid, palmitic acid, and stearic acid, as reported in this study [118,128].

Interestingly, several other studies have reported bitterness-masking effects for similar lipid-derived compounds, particularly at subthreshold concentrations or when interacting with other bitter compounds. Specifically, linoleic acid was shown to increase the bitterness detection threshold of caffeine in humans, indicating a suppressive effect on sensitivity to bitterness [164]. Furthermore, oleic and other long-chain fatty acids were found to mask the bitterness of quinine and similar nitrogenous bitterants by forming insoluble complexes through hydrogen bonding and hydrophobic interactions [165]. This dual behavior suggests that the sensory impact of these compounds can be context-dependent and may vary based on concentration, interaction with other bitterants, or matrix composition. Therefore, both their direct bitterness and their modulatory potential should be considered when evaluating their role in off-flavor development and when designing strategies for bitterness mitigation.

For the newly identified compound **15**, no previously reported threshold value was available in the literature. To determine its sensory potency, a bitter threshold analysis was performed using the same method described above (2-AFC in 3% aqueous ethanol) based on the literature [166]. Each panelist's threshold was calculated as the geometric mean of the last incorrectly identified concentration and the first correctly identified concentration using the equation below:

$$c_p = \sqrt{c_1 \cdot c_0}$$

c_p = threshold concentration of panelist
c_1 = first correctly identified concentration
c_0 = last incorrectly identified concentration

The overall panel threshold was then determined as the geometric mean of all individual values by using the equation below:

$$c_S = \sqrt[n]{\prod_{i=1}^{n} c_{p;i}}$$

c_s= overall threshold concentration of the panel
n= number of panelists

Based on the calculations, the estimated threshold value for pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside was 0.42 mmol/L.

The threshold concentration of a compound provides valuable insight into its sensory impact. A low threshold value may indicate that even a small amount of the compound is sufficient to elicit a bitter perception, suggesting a high sensory potency. In contrast, a high threshold potentially implies that larger concentrations are required before bitterness is

perceived, meaning the compound has lower sensory relevance [48,167,168]. From a formulation perspective, compounds with low bitter thresholds pose a greater challenge, as they may negatively impact taste even at trace levels. In the case of sunflower press cake, the presence of such potent bitter compounds could be a major contributor to the bitterness of the final product. However, it's important to note that threshold values can vary depending on the food matrix and compound interactions. In complex mixtures, bitterness can be either enhanced or suppressed due to synergistic or masking effects. Therefore, interpreting threshold values in isolation may not fully capture the real sensory impact of a product, making it crucial to consider the chemical environment and possible interactions [138,168,169].

4.1.6 Quantification of bitter compounds in sunflower press cake and calculation of DoT factors

The free fatty acids (FFAs) identified as predominant in the analyzed sunflower cake were oleic acid, linoleic acid, palmitic acid, stearic acid, and α-linolenic acid, with respective concentrations of 96.64 mmol/kg, 52.56 mmol/kg, 47.31 mmol/kg, 28.36 mmol/kg, and 2.32 mmol/kg. Although there is a lack of directly comparable quantitative data from other sunflower cake samples, this distribution is consistent with the literature, where oleic and linoleic acids are regularly reported as the most abundant FFAs in various sunflower matrices in various sunflower matrices [94,96–98,170]. Traditional sunflower oil, which has been widely cultivated, contains moderate levels of oleic acid (14–39%) and high levels of linoleic acid, typically over 50%, reaching up to 61% in some cases. However, the fatty acid composition of sunflower oil is highly variable depending on the breeding strategies implemented. For instance, high oleic sunflower cultivars are developed to contain more than 75% oleic acid, whereas high stearic–high oleic variants feature approximately 15–20% stearic acid. Other fatty acids, such as palmitic acid, generally fall within the range of 4.6–7% in different sunflower oil types, while α-linolenic acid remains consistently low, usually below 0.1% in most varieties [94–98,171–177].

The concentrations of trihydroxyoctadecenoic acids (THOAs) and hydroxyoctadecadienoic acids (HODEs) were significantly lower in sunflower press cake, with values ranging between 0.07 to 0.33 mmol/kg and 0.03 to 0.61 mmol/kg, respectively. Ricinoleic acid was also detected and quantified in sunflower press cake at an average concentration of 0.64 mmol/kg. Although it has been described in the literature as a bitter compound [139] and its specific bitter threshold remains unknown. Consequently, it was not included in the assessment of taste-active compounds. Further research is required to clarify its sensory relevance.

In addition to fatty acids and their oxidation products, the second most intense bitter fraction, identified by the TDA, was fraction F1-4-12. This fraction was shown to contain pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (**15**) with a bitter threshold concentration of 0.42 mmol/L. To demonstrate the importance of this compound to the overall bitter off-taste of sunflower press cake, UHPLC-MS/MS quantification was performed. The analysis of sunflower press cake revealed a concentration of 1.41 mmol/kg of compound **15**. Since this compound was isolated and described for the first time, there are no reference concentration ranges available in the literature. However, isolating protein from plant sources often leads to an enrichment of secondary plant metabolites. These secondary metabolites frequently possess a bitter off-taste and are typically present in concentrations similar to that of compound **15** in the final protein isolates, as reported in the literature [118,131,141].

Dose-over-threshold (DoT) factors were calculated by taking the ratio of the concentration of the taste threshold for each specific tastant [125,127,178–180], in order to assess the bitter taste impact of compounds **1**–**15**. The DoT reflects taste relevance, with values above 1 indicating a direct contribution to bitterness. The DoT factor was calculated using the following equation:

$$DoT = \frac{concentration\ in\ food}{sensory\ threshold}$$

The calculation of DoT factors revealed that oleic acid (DoT 98.6) exhibited the highest bitter impact among the tested sunflower meal, followed by linoleic acid (DoT 56.5), palmitic acid (DoT 58.4), stearic acid (DoT 35.0), and α-linolenic acid (DoT 8.3). Among the oxidized fatty acids, THOAs, particularly 9,12,13-trihydroxyoctadec-10-enoic acid (DoT 1.1) and 11,12,13-trihydroxyoctadec-9-enoic acid (DoT 2.5) exhibited the bitter impact.

In complex food systems such as sunflower press cake, compounds, especially those with DoT values below 1, may still participate in cumulative interactions with other bitter molecules [181]. This cumulative effect can shift the sensory profile, even if individual components are each below the detection limit. To definitively establish the sensory relevance of such compounds, recombination and omission tests are essential [30]. These approaches help verify whether the identified compounds are truly responsible for the bitterness by reconstructing the taste profile or by assessing the impact of removing specific compounds. Without such targeted validation, conclusions based solely on threshold and concentration data may remain suggestive but not conclusive.

To date, no detailed quantitative analysis has been conducted on the key bitter compounds present in sunflower press cake. The high DoT-values observed for FFAs such as oleic acid, linoleic acid, palmitic acid, stearic acid, and α-linolenic acid, along with the THOAs 9,12,13-trihydroxyoctadec-10-enoic acid and 11,12,13-trihydroxyoctadec-9-enoic acid, align with the significant amount of residual oil retained in sunflower press cake, ranging from 7% to 16.6%, depending on the extraction process [105,174,182,183]. This residual oil contains free fatty acids and their oxidation products and consequently may contribute to the observed bitterness of sunflower press cake.

Given this lipid-rich environment, the extent of oxidation may be influenced by the presence of naturally occurring antioxidants. Previous studies have reported the presence of tocopherols, including α- and γ-tocopherol, in sunflower oil, which can inhibit free radical propagation and slow down lipid oxidation [184]. These antioxidants are highly effective even at low concentrations. In particular, γ-tocopherol has demonstrated greater oxidative stability across a broader range of conditions [184]. However, the degree to which these tocopherols remain active in sunflower press cake and their potential role in limiting oxidation have not been systematically investigated.

Table 6. Bitter taste threshold concentrations and calculated DoT factors of compounds quantified in sunflower press cake.

compound no.	compound name	bitter threshold concentration [mmol/L]	DoT factor
1	9,12,13-trihydroxyoctadec-10-enoic acid	0.13 [b]	1.1
2	9,10,11-trihydroxyoctadec-12-enoic acid	0.13 [b]	0.5
3	11,12,13-trihydroxyoctadec-9-enoic acid	0.13 [b]	2.5
4	(10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid	0.35 [a, b]	0.08
5	(10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid	0.79 [a, b]	0.1
6	(9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.97 [a]	0.6
7	(9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.79 [a,b]	0.8
8	(9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid	0.79 [a]	0.8
9	α-linolenic acid	0.28 [a]	8.3
10	linoleic acid	0.93 [a]	56.5
11	oleic acid	0.98 [a]	98.6
12	2-hydroxyoleic acid	0.06 [b]	0.2
13	palmitic acid	0.81 [a]	58.4
14	stearic acid	0.81 [a]	35.0
15	pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside	0.42	3.4

[a] Taste threshold taken from Lainer et al. (2020) [b] Taste thresholds are taken from Gläser et al. (2020)

Additionally, this study identified a novel bitter tastant (**15**), representing a previously unrecognized class of bitter compounds. Pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside showed a DoT value of 3.4, indicating a direct contribution to the overall bitterness of sunflower press cake. Therefore, it can be concluded that, in addition to fatty acids and their oxidation products, compound **15** contributes to the bitter off-flavor in sunflower press cake. Detailed sensory reconstitution, as well as omission experiments, will be performed in a future study to elucidate the precise contribution of individual constituents to the overall off-flavor profile of sunflower press cake and investigate how these compounds are generated during the food processing of sunflower seeds.

In summary, the application of the sensomics approach has provided detailed insights into the bitter off-taste profile of sunflower press cake, a promising by-product for sustainable protein sourcing. By identifying key bitter compounds, including free fatty acids and their oxidation products, this study has established the primary contributors to the off-flavor challenges that limit the broader acceptance of sunflower press cake in food applications.

These findings offer a pathway to mitigating bitterness through targeted processing and formulation strategies, paving the way for sunflower press cake to become a more palatable and viable option in addressing the global protein demand. Future research could focus on refining processing techniques to reduce these compounds while preserving nutritional value, further enhancing palatability and consumer acceptance of sunflower press cake.

5 Conclusion

This dissertation contributes to the expanding field of protein alternatives by addressing the growing demand for sustainable nutrition and the sensory barriers that continue to limit consumer acceptance. Among these, bitterness remains one of the most persistent and challenging attributes to overcome.

Focusing on sunflower (*Helianthus annuus* L.) press cake, a protein-rich by-product of oil extraction with promising application potential, this study employed a sensomics approach to investigate its bitter off-flavor. Several compounds previously reported to elicit bitterness in other plant-based protein sources, such as trihydroxyoctadecenoic acids, hydroxyoctadecadienoic acids, and common free fatty acids (oleic acid, linoleic acid, α-linolenic acid, palmitic acid, and stearic acid), were identified as contributors to the bitter taste profile of sunflower press cake.

The study also led to the identification of a previously unreported bitter compound, pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside, representing a novel taste-active compound in plant-based proteins.

The quantitative and dose-over-threshold analysis confirmed that several of the identified compounds occur at concentrations relevant to human sensory perception, reinforcing their direct role in the off-flavor profile.

Ultimately, this work contributes to the ongoing effort to make sustainable protein alternatives more acceptable to consumers by addressing the critical barrier of undesirable taste. Future research should build on these findings by validating the sensory relevance of identified compounds through omission and recombination testing as well as by exploring processing strategies that balance nutritional value with improved flavor.

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To the ones who passed through my life and left their mark, thank you for touching me in ways that shaped who I am. Every one of you, in your own way, helped me arrive at this moment. Now, with a heart full and pride in hand, I'm ready for the next journey.

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Abstract

Flavor Potential and Limitations in Novel Protein Sources: An Integrated Sensory and Chemical Analysis Focused on Off-Flavor Identification

Protein alternatives are gaining momentum as sustainable solutions to meet the increasing global demand for nutrition. These sources include cultured meat, single-cell proteins, edible insects, and plant-based options, each with varying degrees of technological maturity, scalability, and consumer acceptance. Despite their potential, many protein alternatives face major sensory challenges, with off-flavors being a common barrier to broader adoption.

This dissertation addresses these sensory limitations across multiple protein sources, with a primary focus on plant-derived ingredients, particularly sunflower-based matrices, due to their growing relevance and consumer application potential.

The research is structured around four complementary studies. The first study examines off-flavor characteristics in insect-based protein alternatives, identifying key volatile compounds that impact consumer acceptance. The remaining studies focus on plant-based alternatives, with a particular emphasis on sunflower-derived ingredients.

The second study evaluates sunflower's potential in food formulations through the development of a sunflower butter spread, assessing both functional performance and sensory perception in a real-world application.

The third study presents an in-depth review of volatile and non-volatile compounds reported in sunflower-based protein sources aimed at identifying potentially flavor-active constituents. Given the limited sensory research in this area, the review compiles compounds from diverse studies, grouping them into major chemical classes and evaluating their reported concentrations and sensory thresholds where available. Furthermore, the review highlights major gaps in literature, including the absence of sensory validation and inconsistencies in analytical methods, emphasizing that the presence of a compound does not necessarily confirm its sensory activity.

Building on these findings, the final and central study applies a targeted analytical approach to identify the key contributors to the bitterness of sunflower press cake and to assess their sensory relevance through a combination of chemical and sensory analyses. This investigation led to the identification of both previously reported bitter compounds and a novel molecule that had not been described in sunflower-based matrices. This sensomics-based study forms the core of the dissertation and contributes to a deeper understanding of the flavor chemistry of sunflower-derived proteins.

Together, these findings advance our understanding of flavor chemistry and the sensory challenges facing protein alternatives while highlighting the unique limitations of sunflower press cake as a food ingredient. By combining analytical precision with sensory validation, this work lays the foundation for creating more palatable and consumer-friendly protein alternatives.

Lühikokkuvõte

Uudsete valguallikate maitseomadused ja piirangud: integreeritud sensoorne ja keemiline analüüs kõrvalmaitsete tuvastamiseks

Valgu alternatiivid koguvad üha enam populaarsust kui jätkusuutlikud lahendused rahuldamaks kasvavat ülemaailmset toitumisvajadust. Nende allikate hulka kuuluvad kultiveeritud liha, üherakulised valgud, söödavad putukad ja taimsed alternatiivid igaühel neist on erinev tehnoloogiline valmidus, skaleeritavus ja tarbijate vastuvõetavus. Vaatamata nende potentsiaalile seisavad paljud valgu alternatiivid silmitsi oluliste sensoorsete väljakutsetega, kus kõrvalmaitsed on laialdasema kasutuselevõtu peamisteks takistusteks.

Käesolev doktoritöö käsitleb sensoorseid piiranguid mitmesuguste valguallikate puhul, keskendudes eelkõige taimset päritolu koostisosadele, eriti päevalillepõhistele, kuna nende olulisus ja rakendusvõimalused tarbimistoodetes on kasvamas.

Uurimistöö koosneb neljast teineteist täiendavast osauuringust. Esimene uurimus käsitleb putukapõhiste valgu alternatiivide kõrvalmaitse omadusi, eesmärgiga tuvastada peamised lenduvad ühendid, mis mõjutavad tarbijate aktsepteeritavust. Ülejäänud uuringud keskenduvad taimsetele alternatiividele, keskendudes päevalillepõhistele koostisosadele.

Teine uurimus hindab päevalille potentsiaali toiduvalemites päevalillevõi määrde arendamise kaudu, hinnates selle funktsionaalset toimivust ja sensoorset tajumist reaalses rakenduses.

Kolmas uurimus esitab põhjaliku ülevaate lenduvatest ja lendumatutest ühenditest, mida on kirjeldatud päevalillepõhistes valgutoodetes, eesmärgiga tuvastada potentsiaalselt maitseaktiivseid ühendeid. Arvestades sensoorsete uuringute vähesust selles valdkonnas, koondab ülevaade ühendeid erinevatest uuringutest, rühmitades need peamistesse keemilistesse klassidesse ning hinnates nende kontsentratsioone ja sensoorseid lävendeid. Lisaks juhib ülevaade tähelepanu olulisematele puudujääkidele teaduskirjanduses, sealhulgas sensoorsete valideerimismeetodite ja analüüsimeetodite järjekindluse puudumisele, pannes rõhku sellele, et ühendi olemasolu ei kinnita veel selle sensoorset aktiivsust.

Viimase uurimuse eesmärk oli rakendada sihipärast analüütilist lähenemisviisi, et tuvastada päevalillepresskoogi kibeduse peamised põhjustajad ning hinnata nende sensoorset tähtsust, kombineerides keemilist ja sensoorset analüüsi. Uuringu käigus tuvastati nii varem kirjeldatud kibedaid ühendeid kui ka üks uus molekul, mida ei ole seni päevalillepõhistes maatriksites kirjeldatud. Sensoomikale tuginev lähenemine moodustab doktoritöö keskse osa ning annab olulise panuse päevalillevalgul põhineva maitsekeemia paremasse mõistmisse.

Kokkuvõttes aitavad need leiud süvendada meie arusaama maitsekeemiast ja sensoorsetest väljakutsetest, millega seisavad silmitsi valgualternatiivid. Uuring toob esile ka päevalillepresskoogi kui toidu koostisosa spetsiifilised piirangud. Kombineerides analüütilise täpsuse sensoorsete kinnitustega, loob see töö aluse maitsvamate ja tarbijasõbralikumate valgupõhiste toodete arendamiseks.

Appendix 1

Supplementary materials

Supplementary Tables

Supplementary table to Publication 4

Table S1: Quantification of Fatty Acids and Fatty Acid Oxidation Products. *Tuning parameters and CoV values for fatty acids and oxylipins to be quantified for the LC-DMS-MS/MS analysis. DP [V] is the Declustering Potential, EP [V] the Entrance Potential, CE [V] the Collision Energy, CXP [V] the Collision Exit Potential, and CoV (V) the Compensation Voltage. Tuning parameters were established by colleague Alexandra Knobloch; her valuable contribution is gratefully acknowledged.*

Analyte	Q1 [Da]	Q3 [Da]	DP [V]	EP [V]	CE [V]	CXP [V]	CoV (V)
oleic acid	281,066	281	-80	-10	-16	-29	-17
	281,066	263,1	-80	-10	-28	-21	-17
α-linolenic acid	276,993	276,9	-110	-10	-10	-21	-20
	276,993	259	-110	-10	-22	-23	-20
palmitic acid	255,108	255	-120	-10	-16	-19	-19
	255,108	237	-120	-10	-28	-23	-19
	276,993	127,2	-110	-10	-30	-11	-20
linoleic acid	279,064	279	-175	-10	-14	-25	-18,5
	279,064	261,2	-175	-10	-26	-13	-18,5
stearic acid	283,097	283,1	-115	-10	-10	-17	-16
	283,097	265,1	-115	-10	-32	-23	-16
(9Z,11E)-13-oxooctadeca-9,11-dienoic acid	293,031	292,9	-85	-10	-8	-13	-7
	293,031	96,8	-85	-10	-36	-17	-7
	293,031	185,2	-85	-10	-28	-23	-7
(10E,12E)-9-hydroxyoctadeca-10,12-dienoic acid	295,063	295	-55	-10	-12	-21	-16,5
	295,063	277,1	-55	-10	-22	-25	-16,5
	295,063	171	-55	-10	-24	-19	-16,5
(10E,12Z)-9-hydroxyoctadeca-10,12-dienoic acid	295,011	295,2	-90	-10	-10	-23	-15,5
	295,011	277,1	-90	-10	-24	-21	-15,5
	295,011	171	-90	-10	-26	-9	-15,5
(9E,11E)-13-hydroxyoctadeca-9,11-dienoic acid	295,003	295,1	-105	-10	-14	-27	-19,5
	295,003	195,1	-105	-10	-24	-15	-19,5
	295,003	277,1	-105	-10	-22	-19	-19,5
(9Z,11E)-13-hydroxyoctadeca-9,11-dienoic acid	295,013	295	-40	-10	-8	-23	-17
	295,013	195,2	-40	-10	-24	-15	-17
	295,013	276,9	-40	-10	-24	-35	-17
9,10,11-trihydroxy-octadec-12-enoic acid	329,004	329	-55	-10	-12	-29	1,5
	329,004	200,9	-55	-10	-32	-21	1,5
	329,004	170,9	-55	-10	-30	-9	1,5
9,12,13-trihydroxy-octadec-10-enoic acid	329,041	329,2	-65	-10	-12	-27	-7,5
	329,041	211	-65	-10	-30	-13	-7,5
	329,041	229	-65	-10	-30	-15	-7,5
11,12,13-trihydroxy-octadec-9-enoic acid	329,027	329,2	-70	-10	-12	-27	-4
	329,027	199	-70	-10	-32	-15	-4
	329,027	211	-70	-10	-30	-13	-4

Supplementary Figures

Supplementary figures to Publication 4

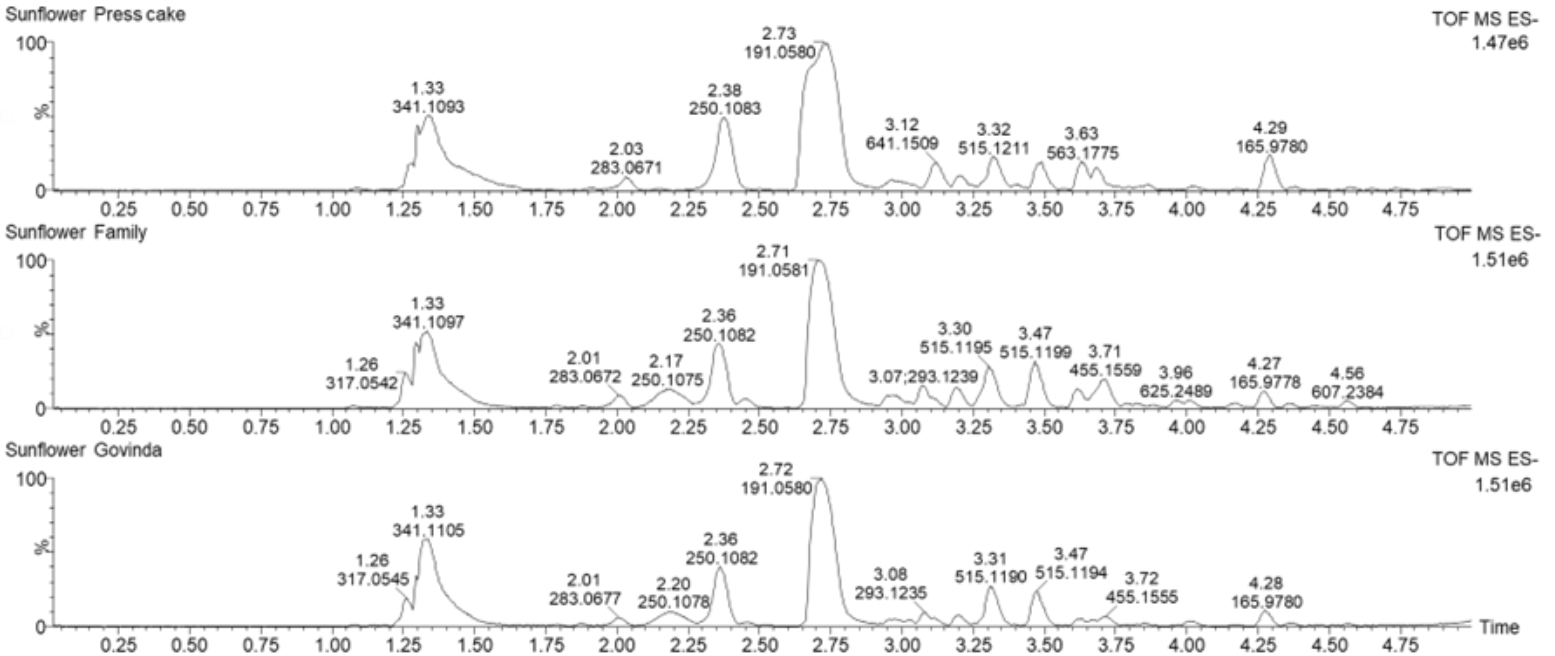

Figure S1. Untargeted screening by ultrahigh performance liquid chromatography–time-of-flight–mass spectrometry of sunflower press cake and two commercial sunflower proteins. *Sample 1 corresponds to the sunflower press cake, a by-product of oil extraction. Samples 2 (Sunflower Family) and 3 (Sunflower Govinda) represent two different commercially available sunflower proteins. The chromatograms indicate compositional similarity among all three samples.*

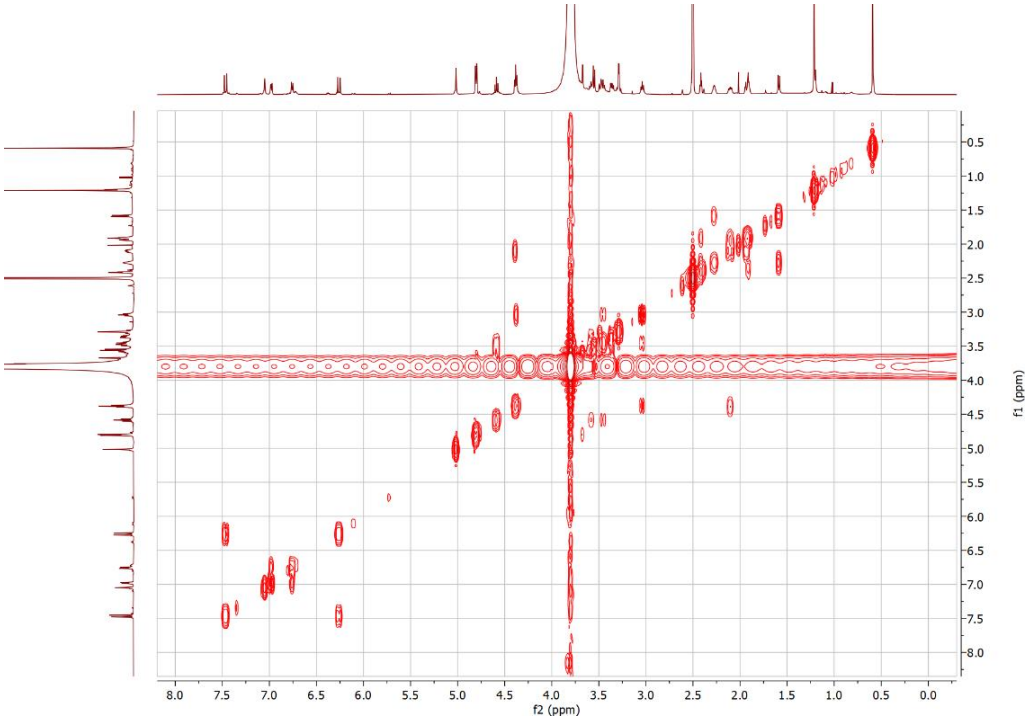

Figure S2. Two-dimensional $^{1}H–^{1}H$ COSY NMR spectrum of compound 15 (pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside) isolated from sunflower press cake.

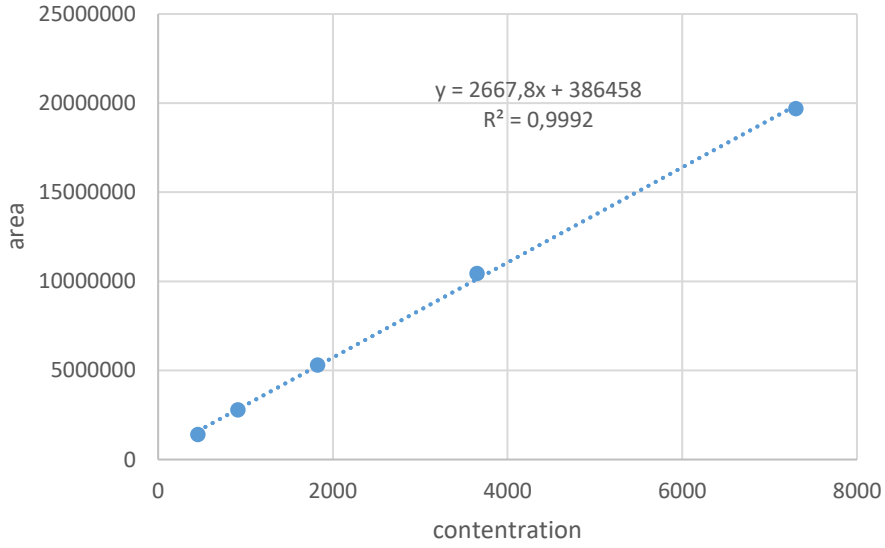

Figure S3. Calibration curve for the quantification of compound 15 (pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside) isolated from sunflower press cake.

Appendix 2

Publication I

Huseynli, L., Parviainen, T., Kyllönen, T., Aisala, H. & Vene, K. Exploring the protein content and odor-active compounds of black soldier fly larvae for future food applications. *Future Foods 7*, 100224 (2023).
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Exploring the protein content and odor-active compounds of black soldier fly larvae for future food applications

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ABSTRACT

Black soldier fly (*Hermetia illucens* L., BSF) larvae are a promising alternative for future sustainable nutrient sources both as feed and food. However, the scientific investigation of BSF larvae is still limited, especially on the flavor chemistry aspects of understanding consumer acceptance. This research examined the odor-active compounds and protein content of BSF larvae. Whole and partially defatted BSF larvae meals were compared to an anchovy fish meal. Gas chromatography/mass spectrometry and gas chromatography/olfactometry were employed to determine and identify the odor-active compounds of BSF larvae and observe the differences in volatile composition between fat-reduced samples. 95 volatile and more than 40 odor-active compounds were detected in the BSF larvae and anchovy fish meal. Overall BSF larvae meal odor profile was characterized by having more fishy, earthy, cheesy, and roasted notes. Trimethylamine, acetic acid, 3-methylbutanoic acid were considered by assessors as key off-flavor compounds contributing undesirable smell in BSF larvae meal. The defatting process slightly affected the overall odor profile; however, it did not change the volatile composition.

1. Introduction

The human and agricultural need for nutrients, especially proteins and lipids, is increasing rapidly. This can have significant destructive effects on many valuable ecosystems (Müller et al., 2017) . Thus, existing plant and animal production needs to be supplemented with new approaches to encounter future environmental and social needs. Insects offer promising feed and food solutions due to their nutritional content and environmentally friendly production compared to conventional livestock (Cutrignelli et al., 2018). Even though insects have been an essential source of nutrients for various animals including wild birds, fish and poultry, they have only recently been allowed for feed purposes in the EU. Among the insects, the black soldier fly (*Hermetia illucens* L.) is one of the most promising and widely studied species (Zotte et al., 2019). Black soldier fly larvae (BSFL) have emerged as the leading source of insect protein and are now used worldwide on a large scale in the feed industry (Barragan-Fonseca et al., 2017). This is due to their unique features. These include their potential for sourcing some essential nutrients, ability to decompose organic matter, use as animal feed, precursors to biofuel, and ability to resist and reduce the growth of some harmful bacteria (Barragan-Fonseca et al., 2017; Bessa et al., 2020; Müller et al., 2017; Smetana et al., 2019). Due to BSFL's high amylase, lipase, and protease activity, organic waste can be efficiently converted to protein and lipids, potentially lowering the amount of wasted food (de Souza-Vilela et al., 2019; Kim et al., 2011).

Adult black soldier flies rarely feed or do not feed at all in nature but can sometimes imbibe water. In order to provide females with enough resources to produce 500–1000 eggs and males with sufficient energy for sperm production and mating on the wing, the larvae need to accumulate enough protein and fat during the six larval stages (Ståhls et al., 2020). Therefore, BSF larvae are high in both protein and fat, with a protein content ranging from 30 to 53 g/100 g of dry matter (DM), a lipid content ranging from 20 to 41 g/100 g (DM), and chitin content ranging from 2 to 9 g/100 g (Bessa et al., 2020). Chitin acts primarily as a fiber in the human body. BSFL also contain a high amount of zinc and iron. This makes the micronutrient content especially valuable in some countries where the inadequate intake of these nutrients may cause dietary deficiencies in the community (Liland et al., 2017; World Health Organization, 2005). In the postmortem adult stage, the maximum value of crude protein is 57.6%, with a fat content of 21.6%. Due to this, larvae are collected at the 4th instar when they contain the maximum amount of protein and lipids. The nutritional composition of the BSF larvae can additionally be influenced by the composition of the feeding medium and the stage of their life cycle. This variation in the nutritional composition provides alternatives for the food and feed industry. By controlling the feed composition and the time of collection,

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products with optimized nutrition profiles can be developed for either the needs of the human diet, aquaculture, or animal feeds (Liland et al., 2017; Liu et al., 2017).

BSFL has been recommended as animal feed (Barragan-Fonseca et al., 2017; Cullere et al., 2018; Zotte et al., 2019) but legal restrictions limit its usage and efforts for human consumption are limited. Current regulation (EU) 2015/2283 requires novel foods to be evaluated and assessed before entering the European joint market area. In January 2021, products containing whole, dried mealworm became the first insect-based food approved by the EU Food Safety auditor (Turck et al., 2021). By January 2023, four different species (*Tenebrio molitor, Locusta migratoria, Acheta domesticus, and Alphitobius diaperinus*) have been authorized to be used as food. An application to place BSFL meal in the food market is ongoing. However, more research and legislation are required for its usage in food products. The use of insect-based products for human consumption is more restricted than the use as animal feed. Possible allergens, pathogen risks and overall safety concerns need to be addressed thoroughly. In this aspect, a recent study reviewed the applicability of yellow mealworms and black soldier fly larvae for feed and food production by providing valuable insight into the risks, obstacles and current state of the industry (Thrastardottir et al., 2021).

The feed uses and processing of industrial side streams and by-products is often a foreign concept to the average consumer. Such materials may be perceived as waste matter, which makes it harder to accept the derived food product that utilizes these side streams (Bessa et al., 2020). The so-called "yuck factor" is strong towards BSFL meal and it will require preparation and studies to change consumer attitudes (Bessa et al., 2020). According to studies, western consumers are much more likely to eat insects in products they are familiar with than adding insects to the market in entirely new or unknown products (Schäufele et al., 2019; Tan et al., 2015). According to Wang and Shelomi (2017), larvae could potentially be milled and processed into a textured protein with a strong flavor for consumer use. The BSF larvae meal possesses a strong, heavy smell which can be beneficial for the feed industry. However, this creates a challenge in the food industry, as the smell and flavor will affect the consumer perception and choice of food products. Even though previous research mentions the unappetizing and undesirable flavor (Bessa et al., 2020; Delicato et al., 2020; Wang and Shelomi, 2017), no further work has been done on volatile active compounds on BSF larvae. Therefore, the study aims to estimate and analyze the odor-active compounds in BSF larvae meal. The research was done in comparison with anchovy fish meal by hypothesizing similar volatile profiles to provide reference information. The anchovy meal is a good reference as they have an intense smell, complex lipids and is used both for feed and food purposes. This research aimed to collect data to provide reference information for future investigation regarding volatiles in BSFL. It is important to take one step forward by understanding the odor-active compounds in BSFL meal to determine whether it is suitable and will be accepted by consumers as a food ingredient.

2. Materials and methods

2.1. Materials

The black soldier fly larvae (*Hermetia illucens*) were reared in 60%RH moisture and 28 °C temperature using a diet consisting of rye bran and oat flakes. The larvae were reared for ten days until the 4th instar of the larvae development. Then the larvae were separated, washed with water, and dried for 48 h at 70 °C. The larvae were ground to pass a 2 mm sieve in a Wiley mill (Thomas Scientific, Inc. NJ, USA). The same procedure was followed with all samples. After this, the larva meal was either analyzed (called BSFL henceforth) or partially defatted first (pd_BSFL). The defatting of the larvae was done in two batches of 500 g with supercritical carbon dioxide ($SC–CO_2$) using 250 bar pressure in a Multi-Use SFE Plant with a pressure vessel of 10 L (Chematur Ecoplanning, Rauma, Finland). The extraction time was 40 min, and the flow rate of $SC–CO_2$ was 12.5 L/h. The temperature was maintained at 40 °C during the extraction process and 46 °C in the separation chamber. The control anchovy fish meal was acquired from Salmonfarm Oy produced by standard fish meal production process. The acquired meal was first pressed, and then cooked at 125 °C for 60 min, condensed and finally dried at 70 °C for 24 h, milled, and sieved to 2 mm.

2.2. Proximate composition

Samples were analyzed in triplicates. Nitrogen was analyzed using a Kjeldahl autoanalyzer (Foss Tacator Ab, Höganäs, Sweden), according to the American Association of Cereal Chemists (AACC) method 46–11.02 (AACC, 1999). Crude protein was calculated using a nitrogen-to-protein conversion factor of 4.76 (Janssen et al., 2017). For the determination of amino acids (histidine, serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine), the samples were oxidized with fresh performic acid solution and hydrolyzed with 6 N HCl. Amino acid derivation and UPLC analyses were performed according to the Waters AccQ·Tag method. Protein Digestibility Corrected Amino Acid Score (PDCAAS) was analyzed using the Megazyme K-PDCAAS kit. PD-CAAS method assesses the digestibility of the protein *in vitro* and has become a common alternative to *in vivo* rat models for amino acid digestibility and a general indication of the quality of the proteins. Crude fat quantification was done following the AACC Method 30–20.01, which has been modified from Soxhlet extraction. Ash content was analyzed by following the AACC Method 08–01.01, where the sample is heated to 550 °C for 24 h.

2.3. Chemicals and reagents

Reagent-grade chemicals were purchased from commercial suppliers and used without further purification. Internal standard 4-methyl-2-pentanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Linear retention indices were determined using a $C_8–C_{22}$ ($+C_5$, C_6, C_7) mix from Sigma–Aldrich.

2.4. Sample preparation for volatile extraction by solid-phase microextraction (SPME)

Extraction of volatiles was carried out using solid-phase microextraction (SPME). 0.3 g of BSFL sample was weighed into a 20-mL SPME vial with a glass-covered stirrer and capped with PTFE/silicone septum for gas chromatography/olfactometry (GC/O) analysis. The same preparation method was applied to both pd_BSFL and fish meal samples. They were placed in an autosampler tray at room temperature. For gas chromatography/mass spectrometry (GC/MS) analysis, 0.1 g of each sample was measured into a 10 mL sample vial. The vials were pre-incubated at 40 °C for 5 min for both analyses. SPME fiber (30/50 μm DVB/Car/PDMS Stableflex, length 2 cm; Supelco, Bellefonte, PA, USA) was used to adsorb/absorb the volatile compounds from the headspace (HS) for 20 min. The adsorbed/absorbed volatile compounds were subsequently desorbed into a GC injection port for 5 min.

2.5. Volatiles analysis by using HS-SPME-GC/TQMS and GC/O

Identification and quantification of volatile compounds were performed using a gas chromatograph system (2030; Shimadzu, Kyoto, Japan) equipped with a mass spectrometer (8050NX Triple Quadrupole; Shimadzu, Kyoto, Japan). A ZB5-MS column (30 m length × 0.25 mm i.d. × 1.0 μm film thickness; Phenomenex, Torrance, CA, USA) was used with helium as a carrier gas at a linear velocity of 35 cm sec^{-1}. The oven was programmed to ramp up from 40 °C at a rate of 7.5 °C/min to a final temperature of 280 °C with an additional holding time of 4 min (total run time 36 min). Mass spectra were obtained at the ionization energy

Table 1
Compositional analysis of the protein meals, amino acid composition and protein digestibility as mean values of three samples of each protein meal.

Description	Unit	BSFL meal	pd_BSFL meal	Anchovy meal
Protein	g/100 g	36.5	47.7	59.4
Lipids	g/100 g	26.0	4.0	12.0
Moisture	g/100 g	2.2	2.9	6.5
Ash	g/100 g	8.4	11.0	11.4
Total	g/100 g	73.1	65.6	89.4
Amino acid composition				
L-Cysteine	mg/g	3.01	3.76	5.03
L-Methionine	mg/g	7.68	9.99	17.39
L-Tryptophan	mg/g	9.58	12.75	14.40
L-HydroxyProline	mg/g	nd	nd	nd
L-Aspartic acid	mg/g	37.50	49.54	57.10
L-Threonine	mg/g	15.52	20.06	25.67
L-Serine	mg/g	16.37	21.01	26.34
L-Glutamic Acid	mg/g	45.22	58.53	81.39
L-Proline	mg/g	21.26	26.86	22.28
L-Glycine	mg/g	18.12	23.22	31.66
L-Alanine	mg/g	25.50	32.08	34.55
L-Valine	mg/g	22.46	28.24	28.57
L-Isoleucine	mg/g	26.57	34.32	43.23
L-Leucine	mg/g	16.81	21.73	23.97
L-Tyrosine	mg/g	21.37	26.63	15.92
L-Phenylalanine	mg/g	16.58	21.65	23.34
L-Lysine	mg/g	22.03	28.31	49.23
L-Histidine	mg/g	11.26	14.34	13.43
L-Arginine	mg/g	18.62	23.98	38.97
SUM OF AA	mg/g	355.44	457.00	552.47
Protein digestibility				
PD-CAAS				
In-vitro digestibility	%	92	96	95

of 70 eV with a mass-to-charge ratio scan range of 35 to 250. For each sample, three analytical replicates were made. Odorants were analyzed using a GC system (Agilent 7890A; Agilent Technologies Inc., Palo Alto, CA) equipped with a sniffing port (ODP-3; Gerstel Inc.). The column was a ZB5-MS (30 $m \times 0.25$ mm $\times$ 1.0 μm; Phenomenex, Torrance, CA, USA). The carrier gas was helium at a flow rate of 70 cm sec^{-1}. The injector (0.75 mm i.d.) was kept at 250 °C. The temperature program was as follows: from 35 °C at 45 °C/min up to 85 °C; from 85 °C at 9 °C/min up to 200 °C; from 200 °C at 45 °C/min up to 280 °C with an additional holding time of 1 min (total run time 16.67 min). Three assessors analyzed each sample in two replications. Odor description and linear retention indices were used for the identification of the odor-active compounds by correlating the results of GC–MS with GC–O. Standard deviation is calculated ($n = 3$).

Non-targeted identification of volatile compounds was carried out using GC/MS solution software (Shimadzu, Japan) and retention indices (RI). Experimental retention indices were calculated using the retention times of the eluting compounds normalized to the retention times of adjacent n-alkanes. The identification of the compounds was verified by comparing experimental retention indices to NIST17 and FFNSC libraries. A semi-quantitative approach (assuming a response factor of 1 for all compounds) against an internal standard (4-methyl-2-pentanol; 400 ppb) was used to quantify identified volatile compounds. Those relative concentrations allow per-compound comparison between samples. For odor descriptions, public databases available on the Internet were used (Acree and Arn; The Good Scents Company). The contents of volatile compounds were compared to published odor detection thresholds (Leffingwell and Associates, 2022).

2.6. Co-milling of the BSFL meal with citric acid (preliminary work)

A mechanochemical milling method was applied to the BSFL meal with citric acid in different combinations. The mechanochemical experiments were carried out in the FTS-1000 shaker mill at 30 Hz frequency using a 14 mL ZrO_2-coated grinding jar with a 10 mm ZrO_2 milling ball. The sensory odor intensity of samples was screened with three panelists on a 0–10-line scale.

2.7. Statistical analysis

GC–O data were processed using the modified frequency (MF) method, calculated with the formula, combining frequency and intensity values as proposed (Dravnieks, 1985):

$$MF = \sqrt{F(\%) \times I(\%)}$$

where F (%) represents the frequency of detecting an aromatic attribute expressed as a percentage of the maximum frequency of the panel, while I (%) represents the average intensity expressed as a percentage of the maximum intensity of the panel. The significance of difference was determined by using a one-way analysis of variance ANOVA.

3. Results and discussion

3.1. Proximate composition

Compositional analyses were performed on the samples, which revealed the major differences between the protein meals. Protein content was highest in the anchovy meal and lowest in the BSFL meal samples (Table 1). The major differences between the protein content of the BSFL meals were caused by differences in the lipid content between samples. The protein concentrations were similar to results reported in previous research (Shumo et al., 2019). The majority of the non-protein content of the BSFL is lipids and the larval exoskeleton, which consists of chitin polymer structures. The chitin content can be characterized by the composition of acid detergent fiber (ADF) and neutral detergent fiber (NDF). BSFL fiber composition is dependent on the type of feed the larvae are fed. ADF content varies between 12.6–15.0% DM and NDF content varies between 20.4–28.6%DM (Shumo et al., 2019). The protein is in a digestible form in all the samples as the protein digestibility is over 92% for all the samples. As a reference for plant proteins, soy protein concentrate has a protein digestibility of approximately 80%. Soy protein

Table 2
Identified volatile compounds and their relative contents in the three samples by HS-SPME/GC-TQMS.

Compound name	RI_{exp}	RI_{lit}	Literature description	Content in IS equivalents (ppb)		
				BSFL meal	pd_BSFL meal	Anchovy meal
1. Ethylamine	458	NA	Fishy	51.09	50.36	579.8
2. Methanethiol	461	464	Sulfur, cabbage, garlic	1.18	1.67	0.21
3. Trimethylamine	462	479	Fishy	1196	1189	2125
4. 1-Propanol	548	555	Fermented	0.66	0.98	2.01
5. Propanal, 2-methyl-	553	552	Floral	22.79	31.89	1.63
6. Acetic acid	588	600	Pungent, vinegar	4587	4057	1051
7. Ethyl Acetate	607	610	Fruity, green	0.00	0.00	26.99
8. Butanal, 3-methyl-	645	655	Fruity, peach	39.33	79.85	7.92
9. Butanal, 2-methyl-	653	661	Cocoa, nutty	45.81	81.31	4.10
10. Propanoic acid	665	700	Cheesy	48.01	27.60	46.90
11. 2-Pentanone	670	680	Fruity, banana, winey	4.99	7.71	5.62
12. 2,3-Pentanedione	679	680	Buttery, creamy, nutty	3.46	5.91	7.04
13. 3-Pentanone	680	694	Acetone	3.55	7.92	2.47
14. Pentanal	683	696	Fermented, bready, nutty	13.37	26.41	5.73
15. Acetoin	698	715	Buttery	0.48	1.62	0.00
16. Butanoic acid, methyl ester	710	717	Fermented, banana, pineapple	1.37	1.67	0.65
17. Propanoic acid, 2-methyl-	736	785	Cheesy	254.8	134.5	1015
18. 2-Pentenal	738	744	Pungent, green	0.00	3.45	0.00
19. Disulfide, dimethyl	744	742	Sulfur, cabbage, garlic	2.21	3.77	0.00
20. 1-Pentanol	757	756	Fermented, bready	90.22	107.6	28.40
21. Butanoic acid	763	818	Pungent, cheesy	33.76	17.15	144.4
22. Isobutyl acetate	765	764	Fruity, banana	21.67	32.99	13.06
23. Toluene	767	767	Sweet	2.54	3.61	1.32
24. 2-Hexanol	783	786	Winey, fruity	4.39	3.22	0.00
25. 3-Hexanol	791	802	Medicinal	1.42	2.79	2.75
26. Hexanal	792	798	Green	116.7	223.0	10.98
27. Butanoic acid, ethyl ester	793	799	Fruity, pineapple	6.93	15.67	0.00
28. Ethyl lactate	805	815	Fruity, creamy	8.13	12.61	0.00
29. Butanoic acid, 3-methyl-	817	839	Cheesy	505.6	230.9	103.0
30. Butanoic acid, 2-methyl-	826	843	Cheesy	192.5	93.68	36.16
31. 2-Hexenal	844	832	Green	0.45	1.50	0.00
32. 1-Hexanol	857	865	Green	16.83	16.43	4.09
33. Pentanoic acid	858	887	Cheesy	52.14	18.18	2.88
34. Ethylbenzene	868	864	Chemical	0.73	1.78	1.78
35. 2-Heptanone	879	890	Sweet, herbal, coconut, wood	10.07	15.16	2.57
36. Heptanal	893	903	Green, herbal	4.58	12.34	0.53
37. p-Xylene	895	884	Sweet	0.89	2.19	1.83
38. γ-Butyrolactone	904	915	Creamy	15.00	16.86	20.66
39. Pyrazine, 2,5-dimethyl-	908	915	Roasted, nutty	31.56	61.93	2.49
40. Hexanoic acid, methyl ester	915	924	Banana, apple, apricot	19.94	17.29	0.38
41. α-Pinene	939	939	Earthy, woody	0.31	0.36	0.84
42. γ-Pentalactone	947	965	Herbal, cocoa, woody	2.34	2.83	0.41
43. Hexanoic acid	951	981	Cheesy, musty	252.9	65.71	10.93
44. Benzaldehyde	967	978	Almond	24.51	42.69	9.26
45. 1-Octen-3-ol	972	979	Mushroom	13.09	24.13	5.73
46. Hept-5-en-2-one, 6-methyl-	978	988	Green, musty, lemongrass	2.98	5.82	4.57
47. 2-Octanone	982	992	Earthy, woody, herbal	7.67	9.34	1.49
48. Furan, 2-pentyl-	987	993	Earthy, vegetative, metallic	10.54	16.95	4.56
49. Pyrazine, 2-ethyl-6-methyl-	997	997	Roasted potato	1.94	5.35	0.16
50. Octanal	999	1001	Citrus, orange	1.63	4.51	0.24
51. Pyrazine, trimethyl-	1001	1005	Nutty, cocoa	4.76	9.77	0.65
52. 1-Hexanol, 2-ethyl-	1023	1032	Rose, green	1.05	3.69	2.72
53. d-Limonene	1035	1039	Citrus	41.38	36.86	31.69
54. Heptanoic acid	1052	1073	Rancid, cheesy	6.66	1.44	0.41
55. γ-Hexalactone	1055	1056	Herbal, coconut, tobacco	18.20	25.10	7.97
56. Ethanone, 1-(1H-pyrrol-2-yl)-	1062	1060	Nutty, musty, cherry	5.49	6.25	0.00
57. Acetophenone	1076	1078	Almond, cherry	1.42	2.17	1.08
58. Pyrazine,3-ethyl-2,5-dimethyl-	1077	1078	Potato, roasted, nutty	1.36	5.71	0.25
59. Benzaldehyde, 4-methyl-	1079	1079	Cherry, phenolic	0.62	0.58	0.48
60. Pyrazine, tetramethyl-	1086	1087	Nutty, burnt	1.69	3.21	1.95
61. 2-Nonanone	1087	1093	Earthy, herbal	2.17	3.89	1.57
62. Nonanal	1103	1102	Rose, orange	1.65	7.44	1.14
63. Octanoic acid, methyl ester	1118	1128	Vegetative	2.49	4.08	0.00
64. 2-Nonenal	1133	1130	Green, cucumber	0.00	0.15	0.00
65. Non-3-en-2-one	1138	1136	Berry, spicy	0.07	0.00	0.00
66. Limonene oxide	1145	1139	Citrus, minty, herbal	0.00	0.00	0.23
67. Benzoic acid	1148	1158	Balsamic	3.17	2.70	2.44
68. Octanoic acid	1150	1158	Cheesy	1.89	1.98	0.43
69. γ-Heptalactone	1157	1163	Sweet, coconut, caramel	2.78	4.66	1.21
70. p-Menthan-3-one	1170	1166	Minty	0.66	1.05	0.63
71. Benzaldehyde, 4-ethyl-	1177	1181	Almond, cherry	0.65	0.73	0.80
72. Decanal	1208	1200	Citrus, orange	0.00	0.45	0.00
73. Nonanoic acid, methyl ester	1219	1227	Winey, tropical	2.71	3.04	0.00
74. 2-Decenal	1236	1250	Floral, citrus	0.43	1.03	0.12

(*continued on next page*)

Table 2 (*continued*)

Compound name	RI $_{exp}$	RI $_{lit}$	Literature description	Content in IS equivalents (ppb)		
				BSFL meal	pd_BSFL meal	Anchovy meal
75. Nonanoic acid	1251	1273	Cheesy	0.83	0.75	0.36
76. Carvone	1259	1252	Herbal, minty	3.20	5.29	6.15
77. γ-Octalactone	1265	1260	Coconut, creamy	0.83	1.89	0.80
78. 1-Decanol	1269	1275	Citrus, orange	0.00	0.00	0.12
79. 2-Decenol	1271	1273	Floral, citrus	0.00	0.00	0.64
80. 10-Undecenal	1277	1279	Citrus, soapy	0.00	0.08	0.00
81. 2-Undecanone	1292	1291	Floral, creamy, fruity	1.66	2.89	2.41
82. Indole	1311	1320	Animalic	1.31	1.43	0.60
83. Decanoic acid, methyl ester	1319	1323	Winey	13.19	11.18	0.00
84. Decanoic acid	1349	1380	Rancid, fatty	1.50	0.22	0.18
85. γ-Nonalactone	1371	1363	Coconut	1.78	3.46	1.73
86. Decanoic acid, ethyl ester	1390	1391	Fruity, apple, grape	7.77	10.89	5.05
87. Geranylacetone	1450	1455	Floral, green, fruity	0.44	0.60	0.54
88. 1-Dodecanol	1459	1466	Earthy, soapy	0.40	0.53	0.62
89. Dodecanoic acid, methyl ester	1520	1527	Soapy, coconut	32.74	59.83	0.00
90. Calamenene	1549	1557	Herbal, spicy	0.26	0.46	0.60
91. Dodecanoic acid	1549	1559	Fatty, coconut	1.21	0.21	0.00
92. Dodecanoic acid, ethyl ester	1589	1596	Floral, soapy	34.77	53.41	27.06
93. γ-Dodecalactone	1692	1695	Coconut, peach	1.01	0.88	0.44
94. Tetradecanoicacid, methyl ester	1720	1726	Waxy	0.20	0.39	0.00
95. Tetradecanoic acid, ethyl ester	1787	1794	Sweet, waxy, violet	0.25	0.30	0.08

RI- retention indices (ZB-5), Exp – experiment, Lib-libraries (NIST17 and FFNSC).

is used in PD-CAAS as one of the standard comparisons for protein digestibility. The higher protein concentration of anchovy meal compared to the BSFL meals may be the result of the high NDF and ADF concentrations in insects. However, the protein quality in terms of digestibility remains high. PD-CAAS results can only be considered indicative as *in vivo* trial results may differ from *in vitro* trials. Traksele et al. (2021) studied BSFL *in vivo* and *in vitro* protein digestibility. *In vitro* trials reached 75% digestibility and *in vivo* trials in rats, the BSFL had even higher 84% protein digestibility. *In vitro* protein digestibility study results in Traksele et al. (2021) are lower than in this study, which may be due to the different processing methods of the protein meal. As a nutritional substance, palatable, highly concentrated and digestible protein is in high demand.

3.2. Volatile compounds

In total, 95 volatile compounds were identified in the BSFL and anchovy fish meal (Table 2), including 14 acids, 21 esters, 12 ketones, 10 alcohols, 17 aldehydes, 2 sulfur compounds, 2 nitrogen compounds, 7 terpenes/terpenoids and 10 aromatic/cyclic compounds. Most of the volatile compounds were present in all three sample types. Therefore, the overall volatile compound profiles were similar.

More than 40 odor-active compounds were detected as a result of GC/O. However, the identification of all odor-active compounds was not possible due to the co-elution of certain compounds with differing odor thresholds. The pungent, dried, and unappetizing fishy aroma of BSFL (Wang and Shelomi, 2017) could be caused by the key volatiles that were identified, such as trimethylamine (fishy), acetic acid (sour, vinegar), and 3-methylbutanoic acid (cheesy, fishy, rancid). These compounds were likely to contribute to the perceived unpleasant odor that characterized the dried BSFL meal. It is important to mention that the flavor compounds like pyrazines, esters and etc., that are valuable in food applications were also present in both BSFL samples.

According to the identified volatile compounds, BSFL meal contained more ester compounds than anchovy meal. The pd_BSFL sample had more aldehydes and pyrazines than the other two samples, whereas the BSFL sample (26%) contained more certain volatile acids such as pentanoic, hexanoic and propanoic acids than the other sample types (Table 2). While the volatile compound composition was similar between the BSFL and anchovy meals, some of the odor-active compounds responsible for "sweet" and “pleasant” notes were not detected in the anchovy meal samples by GC/olfactometry. The odor profile of both BSFL meals was very similar, but the intensity of most odor-active compounds decreased in the partially defatted samples (Table 3).

Studies have revealed that lower ratios of monounsaturated to polyunsaturated fatty acids, above a certain level, are correlated with a negative odor, texture, and flavor profile in BSFL (Wang and Shelomi, 2017). The review by Thrastardottir et al. (2021) mentions that BSFL fatty acid composition varies highly depending on their diet. According to identified volatiles in BSFL meal 2-pentylfuran indicates the presence of polyunsaturated fatty acids which is an autoxidation product of linoleic acid (Condurso et al., 2018). Heptanal and hexanal are other examples of volatiles that are formed through the oxidation of polyunsaturated fatty acids (Klensporf Dorota and Jeleń, 2005). Aldehydes can also be derived from branched-chain amino acids. Through Strecker degradation, they can produce 3-methylbutanal from leucine, which is abundant in all samples (26% BSFL-16.58 mg/g; 4% BSFL-21.65 mg/g; Anchovy-23,34 mg/g) (Smit et al., 2009). 3-methylbutanal and 2-methylbutanal are two Strecker aldehydes that have been suggested to be increasingly formed during drying (Kröncke et al., 2019). Those compounds correlated with pleasant roasted aroma on BSFL samples according to assessors, but those aldehydes were absent in anchovy meal (Tables 2 & 3).

The pyrazines are a result of the Maillard reaction where sugars react with aqueous ammonia to form nitrogen-containing heterocyclic compounds that contribute to roasted aroma characteristics and Strecker aldehydes. The lipid degradation produces compounds that are responsible for specific aroma characteristics (alcohol, aldehyde, ketones, and furans) (Seo et al., 2020) of BSFL meal. Intermediates of both lipid oxidation (2-heptanone; 2-pentylfuran; heptanal; hexanal; hexanoic acid; octanal; pentanal; 2-pentylfuran) and Maillard reaction (3-methylbutanal; 3-methylbutanoic acid; 2-methylbutanal; 2,5-dimethylpyrazine; 2-methylbutanoic acid; 2-methylpropanoic acid) were identified in the BSFL meal. The same intermediates were also detected on yellow mealworms (Kröncke et al., 2019).

Some bacteria commonly found in foods (*e.g. Acetobacter* spp. and *Clostridium acetobutylicum*) produce acetic acid (Sengun and Karabiyikli, 2011). Acetic acid was associated with a sour pungent odor and according to the MF value, acetic acid was one of the key odor active compounds that contributed to the pungent odor of BSFL meal. Acetic acid (threshold 10 ppb) had a lower content on the partially defatted sample ($p \leq 0.05$).

Table 3
Identified odor-active compounds by HS-SPME/GC/O.

Compound name	Odor description *	RI	Identification method	Modified Frequency%		
				BSFL meal	pd_BSFL meal	Anchovy meal
Trimethylamine	Fishy	<500	TGSC, F, NIST, GC/MS	93	93	90
Acetic acid	Vinegar, sour	568	TGSC, F, NIST, GC/MS	73	63	47
Ethyl Acetate	Buttery, caramel	602	TGSC, NIST, GC/MS	–	–	45
Butanal, 3-methyl-	Pleasant	644	TGSC, F, NIST, GC/MS	27	10	–
Butanal, 2 methyl-	Roasted, pleasant	657	TGSC, F, NIST, GC/MS	18	44	–
Propanoic acid, 2-methyl-	Whiskey	733	TGSC, NIST, GC/MS	21	7	34
Butanoic acid	Cheese, urine	768	TGSC, NIST, GC/MS	25	–	75
Hexanal	Green	792	TGSC, F, NIST, GC/MS	83	77	44
Butanoic acid, 3-methyl-	Cheesy	817	TGSC, F, NIST, GC/MS	96	81	90
1-Hexanol	Fruity, green	837	TGSC, F, NIST, GC/MS	57	30	38
2-Heptanone	Sweet, pleasant	860	TGSC, NIST, GC/MS	27	10	–
γ-Butyrolactone	Roast, caramel, potato	907	TGSC, NIST, GC/MS	80	82	66
Pyrazine, 2,5-dimethyl-	Roasted, pleasant	917	TGSC, F, NIST, GC/MS	60	57	69
UNKNOWN	Pleasant, roast	933	–	87	87	80
Hexanoic acid	Metallic, fishy, herbal	981	TGSC, NIST, GC/MS	41	26	13
1-Octen-3-ol	Mushroom	985	TGSC, NIST, GC/MS	41	26	13
Furan, 2-pentyl-	Metallic, dried	988	TGSC, NIST, GC/MS	43	47	28
UNKNOWN	Sulfur	1001	–	45	39	28
UNKNOWN	Bad fish, cheese onion	1003	–	34	68	45
1-Hexanol, 2-ethyl-	Rose, sweet	1012	TGSC, NIST, GC/MS	32	32	21
UNKNOWN	Pleasant, roast	1035	–	27	51	42
γ-Hexalactone	Caramel, melted butter,	1057	TGSC, NIST, GC/MS	56	40	13
Pyrazine, tetramethyl-	Roast, rubbery	1095	TGSC, NIST, GC/MS	25	45	29
2-Nonanone	Earthy	1098	TGSC, NIST, GC/MS	57	21	10
UNKNOWN	Roasted	1106	–	77	27	52
UNKNOWN	Pleasant, roast	1127	–	37	38	22
Benzoic acid	Roasted	1163	TGSC, NIST, GC/MS	53	25	25
p-Menthan-3-one	Cucumber	1167	GC/MS	–	–	73
Phenylethylthiol	Rubber	1173	TGSC, NIST	54	26	40
UNKNOWN	Smoky, roasted, bouillon	1196	–	77	60	60
o-cresol	Grain, earthy, dust, soapy	1211	TGSC, NIST,	66	42	58
Nonanoic acid, methyl ester	Sweet, green, peppermint	1215	TGSC, NIST, GC/MS	34	44	–
UNKNOWN	Dusty, chalk, flower	1243	–	56	69	51
Carvone	Fresh, grassy	1251	TGSC, NIST	34	–	55
2-Undecanone	Sweet, bouillon, soap	1284	TGSC, NIST, GC/MS	28	53	44
Decanoic acid, methyl ester	Chalky, flowery	1288	TGSC, NIST, GC/MS	21	21	31
UNKNOWN	Coconut	1311	–	–	–	34
UNKNOWN	Soapy, dusty, nutshell	1320	–	54	29	49
UNKNOWN	Peppermint	1330	–	–	–	29
γ-Nonalactone	Coconut, woody	1361	TGSC, NIST, GC/MS	7	7	–
UNKNOWN	Sweet, soapy, fresh, mint	1381	–	62	66	63
Decanoic acid, ethyl ester	Herbs	1396	TGSC, NIST, GC/MS	36	21	–
UNKNOWN	Rubber, burnt	1403	–	51	13	13
Dodecanoic acid, methyl ester	Coconut	1491	TGSC, NIST, GC/MS	13	23	–
Dodecanoic acid, ethyl ester	Coconut, milky	1561	TGSC, NIST, GC/MS	23	–	13
γ-Dodecalactone	Flower, sweet, soap	1685	TGSC, NIST, GC/MS	42	–	40

* Descriptions are given by the GC–O panelists, TGSC- The Good Scent Company, F-Flavornet, NIST- National Institute of Standards and Technology.

Linear alcohols are formed by fatty acids (FA) catabolism, while branched alcohols are produced by branched amino acid metabolism (Gonda et al., 2010). One of those linear alcohols is 1-hexanol which has a sweet, grassy, marzipan-like fragrance and acetic acid was identified in all three samples. The same compounds have also been identified in olive oil, sunflower, sesame, pumpkin, and rapeseed seeds (Cecchi and Alfei, 2013; Ivanova-Petropulos et al., 2015).

Sulfur compounds are considered to have a significant influence on flavor. They can have a negative impact with only low threshold values and can cause off-flavor production in food. Non-volatile precursors to volatile sulfur compounds in food include sulfur-containing amino acids (methionine, cysteine, cystine), thiamine and reduced sugars (Mussinan and Keelan, 1994). In BSFL meal methanethiol and dimethyl disulfide are two sulfur-containing compounds that were not detected by the assessors.

The relative volatile content of aldehydes (3-methylbutanal, 2-methylbutanal, hexanal, etc.) ketones (2-heptanone, 2-nonanone 2-octanone, etc.) and pyrazines (2,5-dimethylpyrazine, trimethyl pyrazine, etc.) which are mostly correlated as pleasant odor by assessors in BSFL samples (Table 2) was significantly higher in the pd_BSFL samples ($p \leq 0.05$). The possible reason might be the additional heat applied during the fat extraction process. In addition, heat treatment is known to increase the levels of methyl ketones due to oxidation of saturated FA, followed by decarboxylation, or by -ketoacid decarboxylation (Shahidi and Abad, 2019).

Trimethylamine behaved differently than the compounds mentioned above, as there was no statistically significant change ($p \geq 0.05$) via the defatting process. The high MF value in all three samples (≥ 90%) points to trimethylamine as the main compound for fishy notes on the BSFL and anchovy meal. Despite the similar MF value, the anchovy meal contains a much higher trimethylamine content than the BSFL meal (Tables 2 & 3). In the literature threshold of trimethylamine is given as 260 ppb. Trimethylamine with a strong fishy smell will be one of the most potential undesirable compounds in BSFL when utilized in the food industry. According to Purnomo et al. (2003), citric acid and sodium chloride can decrease the fishy flavor and lowering the water activity can avoid deterioration. Following the information on how to neutralize trimethylamine and mask the fishy notes, a pilot study was conducted by us to

gather data to provide further hypotheses for future research. Sensory analyses were performed on the co-milled BSFL meal with citric acid. According to the panel, the fishy odor intensity of the untreated sample was on average 8.3 ± 1.3, whereas the intensity of the treated sample was 5.0 ± 0.8. Therefore, the citric acid treatment of BSFL meal seemed to significantly decrease the fishy odor by neutralizing trimethylamine. These preliminary results should be confirmed in the future by a larger panel. The process could also be further optimized with different concentrations of citric acid and processing parameters to decrease the undesirable off-notes in the BSFL meal.

Based on the MF values of 3-methylbutanoic acid, responsible for cheesy, pungent rancid flavor, it is the other key compound in all three samples for the unpleasant smell. The 3-methylbutanoic acid content was decreased in the pd_BSFL samples ($p \leq 0.05$), which is also reflected in MF values.

The volatile profile of the anchovy meal was different from the BSFL; however, the compounds that were detectable in high content were the same, such as trimethylamine, acetic acid, propionic acid, 2-methylpropanoic acid, 3-methylbutanoic acid, etc. (Table 2). The BSFL meal made from dried, whole larvae and the pd_BSFL meal sample's odor-active compounds were similar; however, the relative compound contents of those compounds differed. The recent study (Tejedor-Calvo et al., 2021) shows that using CO_2 extraction could potentially alter the aroma profile of a substance by selectively affecting non-polar compounds over polar compounds. This can cause a slight difference in overall flavor perception (Table 3) and improve the overall quality of the final product. The presence of same odor-active molecules alone is insufficient to maintain the same odor profile, as even minor changes in the ratios of a few odor-active compounds can result in a significant shift in the perceived odor. According to Nagy (2017) the taste of a BSFL meal is better than its odor with chocolate/malt, earthy flavor, and far lower fishy notes.

Unknown odor-active compounds were detected only by GC/O but not by GC/MS. According to the assessors' response, the majority of the unknown odor active compounds were assessed as having a roasted, burnt smell (Table 3). Volatiles of the BSFL meal had some similar compounds that have been identified in various insects. For example, hexanal and acetic acid were reported in yellow mealworms; 2-decenal and nonanoic acid in lesser mealworms; 1-octen-3-ol, 2-nonanone, acetic acid, 2-heptanone, and butanoic acid in crickets, and propanoic acid, butanoic acid, and isovaleric acid in cockroaches. These were considered undesirable compounds (Tzompa-Sosa et al., 2019).

4. Conclusions

The volatile composition of BSFL meal and anchovy fish meal were similar. Overall odor-active compounds of BSFL meal are classified as fishy, earthy, cheesy, and roasted aromas. According to the relative volatile compound contents and sensory intensities by GC-olfactometry: trimethylamine, acetic acid, 3-methylbutanoic acid were considered as key off-flavor compounds in BSFL meal. The partially defatted BSFL sample by CO_2 extraction can reduce but not remove volatile compounds in the BSFL meal, which causes a difference in overall odor perception. Further research will continue diminishing or masking unpleasant off-flavors in BSFL meals while utilizing it in food application.

Ethical statement - Studies in humans and animals

This article does not contain any studies on human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix 3

Publication II

Vene, K.; Lumi, E.; Alas, M.; **Huseynli, L**. Integrated Sensory, Nutritional, and Consumer Analysis of Sunflower Seed Butter: A Comparative Study of Commercial and Prototype Samples. *Foods* 2025, *14*, 1815.
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Article

Integrated Sensory, Nutritional, and Consumer Analysis of Sunflower Seed Butter: A Comparative Study of Commercial and Prototype Samples

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Abstract: Sunflowers (*Helianthus annuus* L.), traditionally cultivated for their oil, are increasingly valued for their nutritional and functional properties across a range of food applications. Sunflower seed butter is a nutritious, allergen-free alternative to traditional nut butter. Nevertheless, comprehensive information on its sensory properties and consumer acceptance is limited. This study aimed to evaluate the sensory characteristics, nutritional composition, and consumer preferences of sunflower seed butter, including commercial products and laboratory-developed prototypes. A total of 13 samples (11 commercial, 2 prototypes) were evaluated for protein, fat content (Kjeldahl, Soxhlet methods), and texture attributes, including hardness, stickiness, and spreadability. Descriptive sensory analysis was conducted by a trained panel (n = 10), and consumer acceptance was evaluated by 98 participants using a 9-point hedonic scale. The results indicated that consumer liking was primarily driven by flavor, particularly a roasted flavor profile with brown color and creamy texture. No significant correlations were found between consumer liking and protein or fat content. These findings underscore the dominant role of sensory attributes in shaping consumer perception and provide a basis for optimizing product formulation and marketing strategies in sunflower seed butter development.

Keywords: sunflower butter; consumer preference study; descriptive sensory analysis; sensory profiling

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1. Introduction

The sunflower crop, scientifically known as *Helianthus annuus* L., is one of the oldest oil plant species from North America, with evidence of cultivation dating back to 3000 BC [1]. Nowadays, sunflowers are primarily grown for oil production, with global output reaching approximately 22.2 million metric tons in the 2023/2024 marketing year, making it one of the major vegetable oils worldwide [2]. In addition to oil production, sunflower seeds are used as food, animal feed, for ornamental purposes, pharmaceuticals, cosmetics, biofuel, etc. The widespread popularity and extensive usage of sunflower seeds are due to their rich array of nutrients, including protein, unsaturated fats, fiber, vitamins, selenium, copper, zinc, folate, and iron [3]. According to studies, sunflower seeds consist of an average of 33.85% proteins, 65.42% lipids, and 2.73% ash, with the majority of these components concentrated in the kernels [4].

Beyond their traditional uses, sunflower seeds have also gained attention for their use in plant-based butter and spreads. The growing consumer demand for plant-derived alternatives has led to the development of nut and seed-based butter, which are recognized for their high nutritional value, including dietary fiber, proteins, and essential fatty acids. These products align with the increasing shift toward vegetarian and vegan diets, offering functional and health-promoting benefits [5,6]. Among these butter alternatives, sunflower butter provides a creamy and nutritious spread made from roasted sunflower seeds and additional ingredients such as sugars, salts, and oils [7]. Sunflower butter's popularity is also driven by a growing number of individuals with tree nut allergies seeking a delectable and safe alternative [8,9]. The nutritional attributes closely resemble peanut butter, featuring eight times more vitamin E and four times more iron [10]. The choice to compare sunflower butter to peanut butter is strategic, as peanut butter serves as a familiar benchmark for most consumers. This comparison helps to highlight the differences and potential advantages of sunflower butter, particularly for those with allergies to nuts. Therefore, premium sunflower seed butter shows great promise as a potential ingredient for cookies, candies (similar to Reese's), dairy-free spreads, high-protein snack bars, salad dressings, pestos, etc. Rich in antioxidants such as tocopherols and phenolic compounds, sunflower seeds processed into butter may enhance oxidative stability and protein content in food products, supporting shelf life, health, and protein demand [11–13]. However, the potential of sunflower butter remains underexplored in recent sensory studies, offering a compelling opportunity for further research. The most recent analysis of sunflower butter's sensory profile was completed in 2005 [14], with a previous examination conducted in 1983 [15] in the United States, and no comparable research has been performed within the European Union. In the study conducted by Dreher et al. [15], sunflower butter was found to receive lower ratings when compared to peanut butter. Considering the recent technological advancements that have led to the production of superior-quality sunflower products, previous preferences might be influenced.

Given the limited research on sunflower butter, our objective in this study was to explore the attributes and consumer preferences of sunflower seed butter as a potentially valued product. The research was conducted with the help of a trained panel to perform sensory descriptions and comparisons among commercially available and lab-developed sunflower butter. Furthermore, a consumer preference study was carried out. Additionally, certain physical-chemical analyses were conducted. Considering that protein and fat content influence texture and mouthfeel in butter-like products [16], these components were also examined to better understand their potential contribution to sensory perception. This research aims to fill the gap in existing knowledge and provide insight into the sensory attributes and consumer preferences of sunflower seed butter [17].

2. Materials and Methods

2.1. Sample Preparation

Eleven commercially available sunflower seed butters were purchased from Amazon and retail shops (Table 1). For recipe development, raw sunflower seeds were provided by Letofin AS, originating from Ukraine. Additives (Sodium ascorbate, emulsifiers from Kerry Group (Ireland) and Puratos Group (Belgium), unrefined cane sugar, and sea salt) were selected based on their properties to mimic the sweetness, saltiness, and smoothness of butter spreads.

Seven hundred grams of raw sunflower seeds were soaked in a 2% sodium ascorbate solution for 30 min, stirring and keeping the temperature at 75–80 °C. After rinsing with distilled water, the seeds were roasted at 180 °C for 40 min, stirring occasionally for the roasting to be even. The roasted seeds were weighed, 8% sugar, 0.8% salt, and 1.5%

emulsifier Purato (for "N42") or 2% emulsifier Admul (for "N44") were added based on weight. The mixture was stirred for another half hour after the addition of the emulsifier.

Table 1. Ingredients of sunflower butter samples, including commercial and lab-formulated (N42 and N44) sunflower butter samples.

Sunflower Butter Sample	Ingredients
SunButter creamy (Sun Butter LLC, Fargo, ND, USA)	Roasted sunflower seeds, mono- and di-glycerides, sugar, and salt.
Nature's Promise Organic Sunflower (Foodhold USA, LLC, Landover, MD, USA)	Dried organically grown sunflower seeds, organic sugar, organic sunflower oil, and salt.
SunButter, no sugar added (SunButter LLC, Fargo, ND, USA)	Roasted sunflower seeds and salt.
Wild Friends organic honey sunflower butter (Wild Friends, Portland, OR, USA)	Organic roasted sunflower seeds, organic sunflower oil, organic clover honey, and sea salt.
Monki creme de tournesol (Monki, Horizon Natuurvoeding, IJsselstein, The Netherlands)	Roasted sunflower seeds from controlled organic farming and sea salt.
SunButter Natural (SunButter LLC, Fargo, ND, USA)	Roasted sunflower seeds, sugar, and salt.
Once again, sunflower seed butter (Once Again Nut Butter, Nunda, NY, USA)	Organic sunflower seeds and organic sunflower oil.
88 Acres sunflower (88 Acres, Canton, MA, USA)	Organic sunflower seeds, organic maple sugar, organic pressed sunflower oil, and sea salt.
Dattelmann sunflower seed butter organic (Dattelmann, Berlin, Germany)	100% roasted sunflower seeds from certified organic farming.
Biona Organic Sunflower Smooth (Biona, London, UK)	Freshly roasted seeds.
Dastony Sprouted sunflower seed butter (Windy City Organic, LLC, Northbrook, IL, USA)	100% certified organic sprouted sunflower seeds.
N42 (Letofin AS, Tallinn, Estonia)	Roasted sunflower seeds, emulsifier, sugar, and salt.
N44 (Letofin AS, Tallinn, Estonia)	Roasted sunflower seeds, emulsifier, sugar, and salt.

All samples were purchased in 2022.

2.2. Protein Content

For the determination of protein content, the Kjeldahl method was employed in this study, using Velp Scientifica UDK 127 (Usmate, Italy) [18]. Three replicates were made for each sample.

2.3. Fat Content

The determination of crude fat content was conducted utilizing the Soxhlet method, exemplifying a thorough methodology for precise fat quantification [19]. The extractor used was a Velp Scientifica SER 148 (Usmate, Italy), and the solvent for the extraction was hexane. For each sample, three replicates were made.

2.4. Texture Analysis

The TA-XT2i Stable Micro Systems texture profile analyzer was used for the measurement of hardness, stickiness, and spreadability. For texture analysis, an average of 3 replicates was obtained. The sunflower butter was placed in a plastic container (35 mm diameter, 40 mm depth) to the top. Cone probe P/45C traveled downward through the sample at 1 mm/s, penetrating to a 20 mm depth and returning upward at the same speed, to the initial position. Hardness was evaluated by determining the maximum force of penetration, expressed in Newtons (N), which corresponds to the height of the positive

peak on the force-deformation curve. The results were analyzed using the Texture Expert Exceed program.

2.5. Sensory Analysis

Consumer Analysis. The panelists (n = 98) were from Tallinn (Estonia), aged between 15 and 70 (68 were women, 30 were men). The panel represented a diverse range of dietary habits, including vegan. Five participants reported lactose intolerance, and two had hazelnut allergies. Most participants had limited prior exposure to sunflower seed butter; however, many were familiar with other plant-based spreads. Their overall consumption of nut and seed butters varied: 19% regularly consumed nut and seed butters (e.g., peanut, cashew, almond butter, and tahini) at least once a week, 36% consumed them 1–3 times per month, 37% reported intake between 1–6 times per year, and 8% consumed them less than once annually. This range of experience provided a balanced perspective on the sensory attributes of the product. Ten grams of the sunflower butter samples were placed into plastic cups coded with 3-digit random numbers for sample evaluation to ensure anonymity during the assessment process. The samples were presented to the panelists in a randomized order to mitigate order bias. Samples were served with water and crackers on a plastic tray to maintain consistency in presentation. Multiple sensory evaluation sessions were conducted to ensure comprehensive sensory feedback. Panelists were asked to rinse their mouths with water between samples to prevent flavor carry-over and ensure an accurate assessment of each sample. Panelists were instructed not to compare and rate the samples but to express their liking or disliking of each sample independently. It was emphasized that two completely different samples could be equally liked. A 9-point hedonic scale ranging from 1 dislike extremely to 9 like extremely was used to evaluate preference for the attributes of appearance, color, odor (smell), flavor, texture, spreadability, stickiness, and overall liking.

Descriptive Analysis. The descriptive sensory analysis was carried out by the sensory panel of the Center of Food and Fermentation Technologies. The analysis took place in a controlled environment, free from any disruptive odors, adhering to the guidelines outlined in ISO standard 8589:2007. Ten assessors (10 females, aged between 22 and 51 years) with a background in evaluating plant-based products participated in the sensory evaluation. All panelists were trained, experienced, and undergo regular monitoring. Before qualifying, each panelist successfully participated in a taste sensitivity test and demonstrated the ability to identify common food flavors.

Odor and flavor attributes were selected based on [14,20]. Following comprehensive discussion and training sessions with the panelists, only pertinent attributes were selected for the evaluations, as listed in Table 2. Additionally, an optional comment box was provided, enabling assessors to offer detailed descriptions of odor and flavor characteristics.

The sensory analysis sessions were conducted on separate days, during which the samples were assessed in two parallel sessions. Each session comprised the evaluation of three to four samples, completed within a half-hour timeframe. To ensure the panelists' palates remained neutral between samples, they were provided with spring water and crackers for cleansing purposes.

The samples were served in 10 g portions and were assigned unique codes, with randomized presentation order using Williams' Latin Square design. A numerical scale ranging from 0 to 9 was employed, with "0" indicating undetectable, "1" as low, "5" as medium, and "9" indicating high intensity. Sensory data was collected using the RedJade software (https://redjade.net/) developed by RedJade Sensory Solutions LLC, based in Martinez, CA, USA.

Table 2. The selected attributes for descriptive analysis are used in this study.

Sensory Modalities	Descriptors	Definition	Reference Values
Appearance	Brown color	Intensity of brown hue in the product.	Light (0), Dark (9)
	Additional comments	Any additional visual characteristics or observations.	
Odor	Overall intensity	Strength or potency of the aroma.	Weak (0), Strong (9)
	Raw material	Natural scent of the plant-based raw materials.	Weak (0), Strong (9)
	Roasted	Aroma resulting from the roasting process.	Weak (0), Strong (9)
	Rancid	Perception of a stale or off-odor.	Weak (0), Strong (9)
	Additional comments	Any additional olfactory notes or observations.	
Flavor	Overall intensity	Strength or intensity of the overall flavor.	Weak (0), Strong (9)
	Raw material (seeds)	Natural flavor of plant-based seeds.	Weak (0), Strong (9)
	Roasted	Flavor profile resulting from the roasting process.	Weak (0), Strong (9)
	Sweet	Perception of sweetness.	Weak (0), Strong (9)
	Salty	Perception of saltiness.	Weak (0), Strong (9)
	Sour	Perception of acidity.	Weak (0), Strong (9)
	Bitter	Perception of bitterness.	Weak (0), Strong (9)
	Astringent	Perception of a puckering or drying sensation.	Weak (0), Strong (9)
	Rancid	Perception of a stale or off-flavor.	Weak (0), Strong (9)
	Additional comments	Any additional taste-related observations.	
Texture	Graininess	Presence of coarse particles or granules.	Weak (0), Strong (9)
	Hardness	Firmness or resistance to pressure.	Weak (0), Strong (9)
	Density	Compactness or thickness of the product.	Weak (0), Strong (9)
	Adhesiveness	The degree to which the product sticks to the surface.	Weak (0), Strong (9)
	Cohesiveness	The degree to which the product holds together.	Weak (0), Strong (9)
	Oiliness	Perception of oil or grease in the mouth.	Weak (0), Strong (9)
	Smooth	Absence of roughness or irregularities.	Weak (0), Strong (9)
	Ease of swallow	The effort required to swallow the product.	Easy (0), Hard (9)
	Mouthcoating	The degree to which the product coats the mouth.	Weak (0), Strong (9)
	Spreadability	Ease with which the product spreads.	Weak (0), Strong (9)
	Additional comments	Any additional textural observations.	

2.6. Statistical Analysis

The correlation analysis of consumer liking and physical-chemical analysis was done using MS Excel (version 2503). The correlation coefficient ranges from −1 to +1. The significance of differences was determined using one-way analysis of variance (ANOVA). Analyses were performed in SPSS version 26 ($p < 0.05$).

3. Results and Discussion

The research results indicate significant ($p < 0.05$) variations in both protein and fat content among the sunflower seed butter products. These diverse nutritional profiles among brands are likely influenced by differences in sunflower raw material quality and processing conditions. For instance, sample "Monki creme de tournesol" contains 25.7 g protein, in contrast to 4.8 g protein in "Wild Friends organic honey sunflower butter". Similarly, fat content was 58.9 g in the "Dattelmann sunflower seed butter organic" sample compared to 14.2 g in "Dastony Sprouted sunflower seed butter". Table 3 illustrates the protein and fat content of various sunflower butter samples.

While the protein and fat content provide insight into the nutritional value, these components also influence the texture attributes of the sunflower butter. Texture analysis of sunflower butter samples on hardness, stickiness, and spreadability is shown in Table 4. One of the key features of nut and seed butter is its spreadability. It is essential that the product maintains a soft consistency to prevent ripping bread or shattering crackers [16].

Stabilizers or emulsifiers are incorporated during product development to preserve the desired qualities of sunflower butter, as they influence the structural properties of the butter. Stabilizers create a structure that prevents oil from separating, ensuring uniform butter consistency. Studies show that hydrogenated oils and wax-based stabilizers, such as hydrogenated cottonseed oil, enhance oil binding, improve structural integrity, and can effectively maintain peanut butter consistency over time [21,22]. However, at times, these stabilizers can cause a repelling effect, resulting in a harder texture and reduced spreadability [23]. Grind size is another crucial factor that affects the texture, and study shows that grind size directly correlates with textural properties, such as decreased grind size increases spreadability, hardness, and stickiness [24]. This aligns with findings in sesame butter, where finer grinding led to increased viscosity, firmness, and consistency, with the smallest particle sizes producing the most stable and texturally desirable product [25].

Table 3. Protein and fat content of sunflower butter samples analyzed in the laboratory. Different letters in columns indicate statistically significant differences ($p < 0.05$).

Sunflower Butter Samples	Protein Content, g/100 g	Fat Content g/100 g
SunButter creamy	8.9 ± 0.14^{a}	$29.5 \pm 1.70^{l,m}$
Nature's Promise Organic Sunflower	6.2 ± 0.21^{b}	33.2 ± 0.78^{m}
SunButter, no sugar added	11.4 ± 0.2^{c}	49.6 ± 2.26^{n}
Wild Friends organic honey sunflower butter	4.8 ± 0.14^{d}	$31.7 \pm 1.27^{l,m}$
Monki creme de tournesol	25.7 ± 0.42^{e}	53.6 ± 0.64^{o}
SunButter natural	13.0 ± 0.14^{f}	18.5 ± 0.42^{p}
Once again, sunflower seed butter	11.4 ± 0.49^{c}	21.6 ± 0.57^{q}
88 Acres sunflower	12.0 ± 0.78^{c}	17.2 ± 1.13^{p}
Dattelmann sunflower seed butter organic	19.6 ± 0.35^{h}	58.9 ± 0.07^{r}
Biona Organic smooth	18.5 ± 0.28^{i}	45.4 ± 0.64^{s}
Dastony Sprouted sunflower seed butter	6.80 ± 0.28^{b}	14.2 ± 0.57^{p}
N42	$19.8 \pm 0.28^{h,k}$	28.8 ± 1.70^{l}
N44	20.5 ± 0.28^{k}	$31.3 \pm 1.56^{l,m}$

Low hardness values indicate increased fluidity or less toughness, leading to better spreadability of sunflower butter [10]. These lower values may signify an optimal use of stabilizers or a different ratio in the product formulation. However, the texture characteristics of the "Monki creme de tournesol" sunflower sample stand out distinctly when compared to other samples. Its high hardness could be attributed to its high protein content. The samples' spreadability might be influenced by a higher fat content, the particular processing techniques employed, as well as the specific emulsifiers or stabilizers used during the manufacturing process.

Sample "N42", with its moderate hardness and spreadability, seems to align more closely with most of the commercial samples. However, "N44", with its pronounced hardness and spreadability, was similar to the "SunButter creamy" sample. The distinct differences in texture profiles between "N42" and "N44" might be attributed to variations in the emulsifiers used in their formulations. Such differences further emphasize the diversity of formulation strategies among commercially available products.

Table 4. Texture analysis of sunflower butter samples. Different letters in columns indicate statistically significant differences ($p < 0.05$).

Sunflower Butter Samples	Hardness, N	Stickiness, N	Spreadability, N·S
SunButter creamy	1.35 ± 0.10 [b]	0.35 ± 0.02 [d]	7.01 ± 0.99 [g]
Nature's Promise Organic Sunflower	0.26 ± 0.12 [a]	0.09 ± 0.03 [e]	1.06 ± 0.38 [h]
SunButter, no sugar added	0.19 ± 0.04 [a]	0.09 ± 0.02 [e]	0.88 ± 0.17 [h]
Wild Friends organic honey sunflower butter	0.07 ± 0.02 [a]	0.02 ± 0.01 [e]	0.37 ± 0.09 [h]
Monki creme de tournesol	20,73 ± 1.60 [c]	1,69± 0.26 [f]	120.65 ± 9.86 [i]
SunButter natural	0.12 ± 0.02 [a]	0.04 ± 0.01 [e]	0.56 ± 0.07 [h]
Once again, sunflower seed butter	0.34 ± 0.06 [a]	0.15 ± 0.03 [e]	1.65 ± 0.21 [h]
88 Acres sunflower	0.18 ± 0.03 [a]	0.05 ± 0.02 [e]	0.71 ± 0.11 [h]
Dattelmann sunflower seed butter organic	0.19 ± 0.01 [a]	0.09 ± 0.01 [e]	0.84 ± 0.01 [h]
Biona Organic smooth	0.14 ± 0.01 [a]	0.05 ± 0.01 [e]	0.60 ± 0.01 [h]
Dastony Sprouted sunflower seed butter	0.08 ± 0.01 [a]	0.02 ± 0.00 [e]	0.34 ± 0.02 [h]
N42	0.22 ± 0.06 [a]	0.08 ± 0.03 [e]	0.96 ± 0.22 [h]
N44	1.50 ± 0.17 [b]	0.33 ± 0.04 [d]	6.99 ± 1.41 [g]

The categorization of high, medium, or low values is based on relative comparisons among the tested samples.

The results of a consumer study assessing various factors, such as appearance, color, texture, smell, flavor, stickiness, spreadability, and overall liking, are illustrated in Figure 1. Overall, consumer preferences varied across the samples, with attributes such as appearance, texture, and flavor playing significant roles in determining the liking of sunflower butter products. "SunButter Creamy" and sample "N42" were the top-rated sunflower butter options in the consumer study, followed by sample "N44" and "SunButter Natural" according to their overall liking. While "Dastony" and "Dattelmann" received the lowest ratings, suggesting that they were less preferred by the participants. Those sunflower samples received an especially low ranking on color and flavor attributes. Those variations in color and flavor can be due to the qualities of raw materials as well as the roasting [26]. Sunflower butter is generally accepted within a darker color range compared to most commercially available peanut butter [27].

The correlation between the extent of overall liking and the protein, lipid content, and texture attributes of the product is presented in Table 5. Texture parameters showed a strong correlation (1.0), meaning that harder textures were also stickier and less spreadable. However, there was no direct correlation between texture attributes and consumer liking. Spreadability (consumer) showed a negative correlation with hardness (−0.7) and stickiness (−0.6). Appearance (consumer) attribute showed a lack of correlation with protein and fat content but demonstrated stronger positive correlations with color, texture, smell, and flavor, suggesting that visually appealing samples were generally rated more positively across other sensory domains. Protein and fat content varied a lot between samples, and there was minimal to no correlation between overall liking. The lack of correlation between liking and nutritional content suggests that while consumers may value nutritional aspects, these are not the primary drivers of sensory acceptance [28]. This aligns with the well-established understanding that sensory attributes dominate consumer purchase decisions and satisfaction, often outweighing nutritional considerations [29,30].

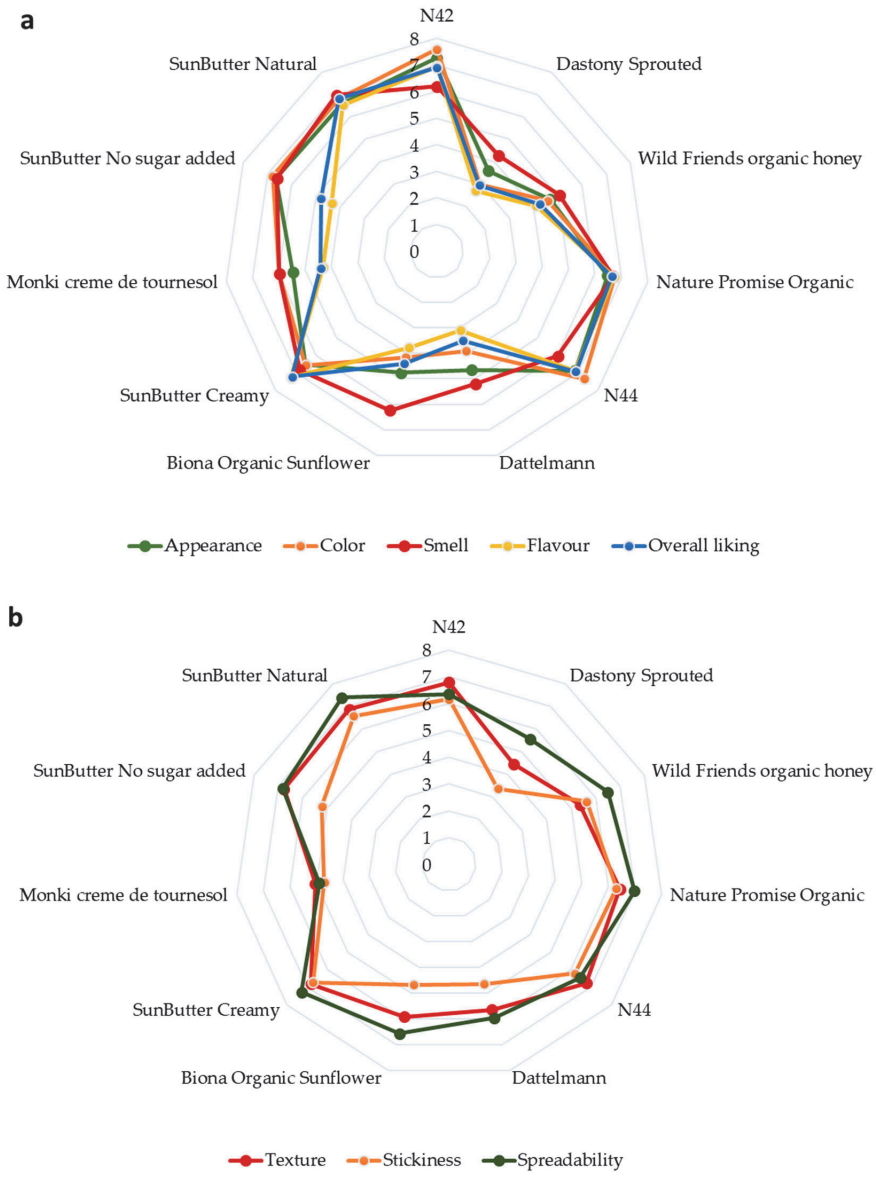

Figure 1. Spider web graph illustrating consumer evaluation of sunflower butter samples based on sensory attributes: (**a**) appearance, color, odor, flavor, and overall liking; (**b**) texture, spreadability, and stickiness (expressed 0–8 scale).

The possible explanation for this lack of correlation may stem from protein-flavor and lipid-flavor interactions. The protein content can enhance flavor binding, potentially delaying volatile release and modifying flavor perception, while lipids may modulate flavor volatility, influencing the overall aroma and taste profile [31]. This suggests that, rather than a true lack of correlation, the absence of a direct link between overall liking and protein/lipid content may result from complex interactions involving flavor, texture, and sensory perception, warranting further investigation.

Table 5. Correlation analysis of consumer liking in relation to composition and texture characteristics of the product.

	Hardness	**Stickiness**	**Spreadability**	**Appearance (Consumer)**	**Color (Consumer)**	**Texture (Consumer)**	**Smell (Consumer)**	**Flavor (Consumer)**	**Stickiness (Consumer)**	**Spreadability (Consumer)**	**Overall Liking**	**Fat**	**Protein**
Hardness	1.0												
Stickiness	1.0	1.0											
Spreadability	1.0	1.0	1.0										
Appearance (consumer)	−0.2	−0.1	−0.2	1.0									
Color (consumer)	0.1	0.2	0.1	0.9	1.0								
Texture (consumer)	−0.3	−0.2	−0.3	0.9	0.8	1.0							
Smell (consumer)	0.0	0.1	0.0	0.8	0.8	0.8	1.0						
Flavor (consumer)	−0.2	−0.1	−0.2	0.8	0.8	0.7	0.7	1.0					
Stickiness (consumer)	−0.1	−0.1	−0.1	0.7	0.7	0.7	0.7	0.8	1.0				
Spreadability (consumer)	−0.7	−0.6	−0.7	0.4	0.2	0.5	0.6	0.4	0.4	1.0			
Overall liking	−0.2	−0.1	−0.2	0.9	0.9	0.8	0.7	1.0	0.8	0.4	1.0		
Fat	0.4	0.4	0.4	−0.1	−0.1	0.1	0.1	−0.5	−0.1	−0.2	−0.3	1.0	
Protein	0.6	0.6	0.6	0.1	0.1	0.2	0.0	0.0	0.0	−0.5	0.0	0.5	1.0

The high correlation between flavor and overall liking (correlation 1.0) following appearance and color (correlations both 0.9) underscores the important role of flavor in consumer satisfaction. This aligns with previous studies on walnut butter, where appearance, flavor, and consistency played a significant role in product acceptance [32].

A descriptive sensory analysis was conducted using a trained panel to understand further the sensory characteristics driving consumer preferences. This method provides valuable insights into the key sensory attributes [33], enabling the optimization of the product to meet the needs and expectations of the consumers [34]. Flavor, appearance, odor, and texture attributes showed variation across the samples, according to panel scores. However, some of the samples showed similarity in their attributes and aligned closely. Such as "N42" and "N44" shared common attributes, including denser texture, higher cohesiveness, and a notable presence of sweetness, saltiness, and strong overall flavor intensity.

Moving on to "SunButter Creamy" and "SunButter Natural", both samples exhibit dense and cohesive textures with minimal graininess. "SunButter Creamy" leans toward a less sweet flavor profile compared to "SunButter Natural". Among the samples, "Wild Friends Organic Honey" was distinctly different with its unique flavor profile, characterized by a pronounced sweetness and a prominent honey flavor. "Nature's Promise Organic" was marked by "oiliness" and "lower density" compared to other samples, setting it apart in terms of texture. "Biona Organic Sunflower Seed Butter", on the other hand, differs from the lab samples in terms of sweetness, saltiness, and texture attributes (Figure 2).

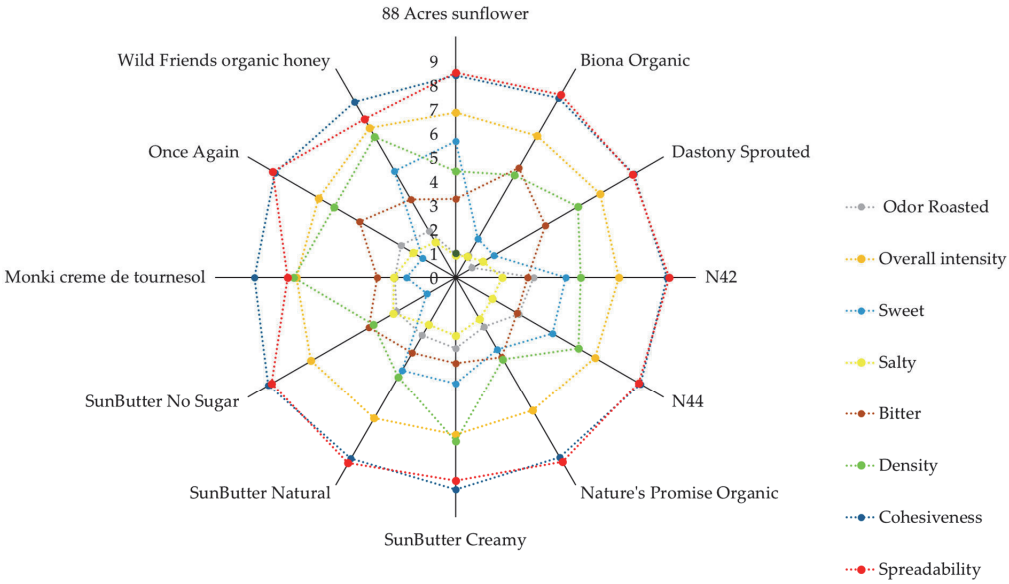

Figure 2. Descriptive sensory analysis (0–9 scale) of sunflower butter samples, visualized as a spider web graph. Part of the full dataset is presented to ensure clarity; complete data can be found in the Supplementary Material.

This sample also featured flavor descriptors such as "chemical", "green", and "oily", which may indicate the presence of unusual or off-putting flavor notes, potentially resulting from lipid oxidation. Similar off-flavors, particularly "grassy" and "beany" notes, have also been reported in soy butter products, which were associated with lipoxygenase activity and oxidation of unsaturated fatty acids [5,35]. In the case of peanut butter, the study ob-

served that prolonged storage leads to the development of additional off-flavors, including "cardboard", "painty", and "rancid" [36].

"Dastony Sprouted" emerges as a unique sample with a characteristic unpleasant taste. It distinguishes itself with a crunchier texture and an unpalatable flavor described as "fungus" and "halva". The sample differed by the fact that the seeds were sprouted before the spread production. In soy butter, sprouting before roasting has been shown to improve flavor by reducing off-notes [35]. However, in this sample, the development of undesirable flavors may be the result of other processing factors or raw materials.

Overall, the evaluations suggest that butter made from roasted sunflower seeds was preferred, which was characterized by a roasty aroma, brown color, balanced taste, and creamy texture. These samples exhibited overall high aroma intensity, along with the characteristic sunflower seed flavor. "SunButter Creamy", "SunButter Natural", "N42", and "N44" are good examples of this preference. Similarly, in the cashew nut butter study, consumers preferred samples with a roasted aroma and smooth texture [37]. In contrast, products that were characterized by strong bitter, green, astringent, and rancid flavors were generally disliked. According to studies, to improve these kinds of butter off-flavors, methods such as optimized roasting, sprouting, selecting suitable kernel varieties, and maintaining proper storage conditions have been widely used [25,35,36]. These findings emphasize that the role of processing methods plays a crucial role in shaping the sensory properties of sunflower butter, influencing both flavor development and overall consumer acceptance.

With growing health awareness, the demand for lower-fat, lower-sugar nut and seed butter products is expected to increase as consumers seek healthier alternatives without compromising sensory appeal. In this context, studies on pistachio butter have explored the use of fat and sweet replacers to develop healthier, lower-calorie formulations [38,39]; however, similar research has not yet been conducted for sunflower butter. This gap presents an opportunity to leverage sensory research in optimizing formulations that achieve nutritional improvements while delivering a positive sensory experience.

4. Conclusions

This study highlights the diverse sensory profiles and consumer preferences associated with sunflower butter products. The findings demonstrate that while texture parameters such as hardness, stickiness, and spreadability are crucial, they do not directly correlate with overall consumer liking. Instead, flavor, appearance, and color emerged as the primary drivers of preference. The variation in protein and lipid content among samples suggests the influence of different processing techniques and raw material quality. This research provides valuable insights for manufacturers aiming to align their products with consumer expectations, emphasizing the need for continued innovation and adaptation to evolving market trends. Future studies should focus on exploring the impact of specific ingredients and processing methods on sensory properties and consumer acceptance of sunflower butter.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods14101815/s1.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Appendix 4

Publication III

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Review

Toward a Comprehensive Understanding of Flavor of Sunflower Products: A Review of Confirmed and Prospective Aroma- and Taste-Active Compounds

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Abstract: The global demand for sustainable protein sources has led to a growing interest in plant-based alternatives, with sunflower products emerging as a promising yet underutilized option. This review provides a comprehensive overview and critical evaluation of current knowledge on the flavor and off-flavor profiles and codes of sunflower seeds and their by-products, with a focus on both volatile and non-volatile low-molecular-weight compounds. It can highlight the importance of the sensomics approach and the knowledge on key food odorants and tastants. Furthermore, this review underscores the importance of advanced analytical methodologies for linking chemical composition to sensory outcomes. While volatile compounds that activate human olfactory receptors, such as aldehydes, terpenes, and pyrazines, are well described in sunflower products, using the sensomics approach the key odor-active stimuli are just verified in sunflower oil. In addition, the roles of non-volatile components including lipids, proteins, carbohydrates, and secondary metabolites such as polyphenols require further investigation and experimental validation to confirm their role as key tastants and their effect on sensory perception. By compiling existing data, this review establishes a foundational database of known and potential flavor-relevant compounds in different sunflower products, providing a valuable resource to directly or indirectly guide sensory (sensomics) studies and promote sunflower-based product innovation. Identifying the key flavor contributors in the different sunflower-based products and raw materials would facilitate precise approaches in processing and product formulation to enhance sensory quality while mitigating off-flavors. Addressing these challenges will support the development of sunflower-based food products with optimized flavor and nutritional profiles, consistent with global sustainability goals and consumer acceptance.

Keywords: sunflower; taste; metabolites; polyphenols; aroma; *Helianthus annuus* L.

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1. Introduction

The sunflower (*Helianthus annuus* L.) belongs to the family Asteraceae [1] and has its origins in North America (Figure 1). Sunflowers are cultivated worldwide for their fruits, for human consumption as they provide essential nutrients and as a livestock

feed resource [2]. In the context of sunflower, the term "seed" describes the whole fruit, whereas the term "kernel" describes the dehulled seed [3]. According to the United States Department of Agriculture (USDA) [4], sunflower kernels contain approximately 51% fat, 21% protein, and 20% carbohydrates (Table 1).

Table 1. Nutrient composition of dried sunflower kernels according to the United States Department of Agriculture [4].

Parameter	Content (g/100 g Kernels)
Moisture	4.73
Protein	20.8
Fat	51.5
Carbohydrates	20.0
Fiber	8.6
Ash	3.02

The sunflower hull accounts for 25–30% of the fruit [2] and often remains unused. It contains 50.0% cellulose and lignin, 25.7% reducing sugars, 4.0% proteins, and 5.17% lipids and wax [5]. The hull has a chemical composition similar to that of sunflower kernels but has a 100-fold lower polyphenol content [6].

Sunflowers are categorized into two main types on the basis of their seed composition. Oilseed sunflower seeds, which contain at least 40% lipids, are used to produce different types of oil such as mid-oleic, high-oleic, high-stearic, and high-palmitic sunflower oil, which have distinct applications. Non-oilseed sunflowers are used for human consumption (in confectionery) or as livestock feed [2]. Factors such as growth conditions, abiotic and biotic stress conditions, and storage practices cause variations in the chemical composition of sunflower seeds, even within the same plant species or genotype [7]. The fatty acid content varies according to the climate and seasonal conditions. For example, higher total lipid content and oleic acid concentrations are observed under warmer conditions. Moreover, processing conditions such as high temperature and high pressure significantly affect the nutritive value of sunflower seeds [8].

Additionally, germination induces changes in the seed chemical composition. For example, sunflower sprouts contain a higher concentration of phenolic acids and flavonoids than ungerminated seeds [7]. Sunflower seeds have a high number of tocopherols and polyphenols, contributing to their significant antioxidant potential, which surpasses that of many other commonly consumed seeds, such as flax, chia, and sesame [9,10]. Tocopherols have a significant anti-inflammatory effect, which alleviates asthma, osteoarthritis, and rheumatoid arthritis. Sunflower seeds also contain several healthy unsaturated fatty acids, proteins, fiber, vitamins, and minerals, which are beneficial for nerves, muscles, bones, and blood in humans. In traditional medicine, sunflower seeds are used to treat cancer owing potentially to their antioxidant effects and rich selenium content, which induce apoptosis of cancerous cells [8,11].

Sunflower sprouts not only have a higher nutritive value but also contain fewer anti-nutritional factors than fresh seeds. Previous research suggests that metabolic changes occur during sunflower seed germination at different temperatures [12]. The findings showed that the concentrations of fatty acid methyl esters and free fatty acids decrease during germination, whereas the concentrations of sterols, α-tocopherols, amino acids, and carbohydrates increase. This metabolic shift occurs as triacylglycerides are broken down and converted to sugars through the glyoxylate cycle, serving as the main energy source for embryonic development. Additionally, a study reported a 22% increase in total polyphenol content, attributed to the activation of endogenous enzymes [12].

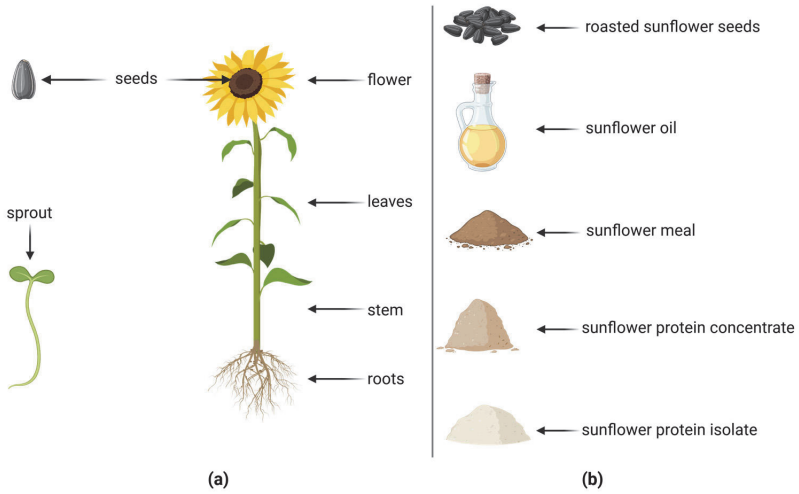

Figure 1. (**a**) Parts of a sunflower plant according to Puttha et al. [13] and (**b**) products derived from sunflower seeds: whole seeds, oil, meal, protein concentrate, and protein isolate. Created with BioRender. Huseynli, L. (2025) https://BioRender.com/okkej50 (accessed on 22 May 2025).

The sunflower crop is cultivated mainly to produce oil for human consumption, with the annual global sunflower production reaching 45 million tons, yielding 19 million tons of sunflower oil annually as of 2017/18 [14,15]. The largest producers include Ukraine, Russia, and the European Union, which together dominate the global supply [14]. After palm, soybean, and rapeseed oil, sunflower oil is the fourth most important commercial vegetable oil [1]. Over the years, sunflower yield, oil content, and disease resistance have been optimized through hybridization. These advancements have enhanced not only its agricultural value but also its health benefits. Sunflower oil is reported to reduce blood cholesterol levels because of its low saturated fatty acid content and high levels of oleic acid. Apart from its dietary benefits, the oil is also valued for external use in skin treatments and rheumatism [16].

During oil production, a large quantity of sunflower meal is generated, which consists of proteins, cellulosic fibers, lignins, other polyphenols, and minerals [17]. In 2019, approximately 21.85 million tons of sunflower meal was produced worldwide [18]. Sunflower meal is an inexpensive and rich source of nitrogen and carbon and can be used as a functional food or as an antioxidant supplement [19]. It is mainly used as animal feed and occasionally as fertilizer.

With the rapidly growing population, the requirement for nutrients, especially proteins, is also increasing rapidly; however, the cultivable acreage remains the same. This imbalance between nutrient needs and agricultural capacity threatens several valuable ecosystems [20]. To address this challenge, it may be necessary to adopt novel approaches that optimize the use of by-products from existing agricultural production practices. Sunflower meal, a by-product of oil production, is a rich source of protein (30–34%) [21,22]. However, its protein quality is limited by its amino acid composition, with lysine as the primary limiting amino acid [23–27]. The protein digestibility-corrected amino acid score (PDCAAS) of sunflower protein sources varies across studies but is generally reported as ∼0.6, indicating moderate protein quality [28–33]. It can be complemented with lysine-rich proteins such as pea and rapeseed proteins to improve its nutritional value, which enhances its overall amino acid profile [32,34]. A previous study has reported that a 1:1

blend of sunflower and pea protein compensates for limiting amino acids, resulting in a more complete and nutritionally balanced protein profile [34].

A few studies suggest that sunflower proteins require fewer odor-masking agents than pea proteins, making them a more favorable choice in formulations [35]. Additionally, sunflower flour is considered blander than soybean flour, further enhancing its appeal [36]. However, sunflower flour can impart a bitter taste in some food products [37]. Despite its advantages in certain aspects, understanding the taste profile of sunflower products remains challenging.

To promote the use of sunflower as a protein source, it is crucial to identify the off-flavor compounds and pleasant-smelling and pleasant-tasting molecules and to facilitate the broader use of sunflower seeds as a food ingredient by improving their sensory acceptability. The present study aimed to achieve this objective by compiling a database of both volatiles and non-volatiles that have the potential to be aroma- and taste-active compounds in sunflower seeds. In addition, this review highlights methodological and knowledge gaps in flavor research in relation to sunflower-based products.

2. Integrated Physiology of Aroma and Taste Perception

The perception of complex food matrices arises from the interplay of gustatory and olfactory stimuli. The human gustatory sense comprises five taste qualities, namely sweet, umami, bitter, salty, and sour, whereas the olfactory cells can detect approximately 10,000 distinct odors [38]. Odor-active volatile compounds are detected by olfactory receptors, whereas taste-active non- or semi-volatile compounds stimulate chemosensory receptors and nerve ends located on the tongue and throughout the oral cavity. Together, these sensory inputs and synergistic interactions contribute to flavor, which is defined as the combination of taste, texture, and odor [39].

The odor compounds play an essential role in assessing the freshness, ripeness, and overall desirability of food. Volatile aroma compounds emitted by ripe fruits signal high energy and nutrient availability, complementing the sweetness detected by taste receptors. Conversely, unpleasant odors from spoiled or fermenting food often indicate microbial activity or toxicity, discouraging consumption [40].

Aroma perception begins when volatile molecules enter the nasal cavity and bind to the ~390 odorant receptor proteins on sensory neurons located in the *regio olfactoria*. These neurons detect aroma stimuli that enter the nasal cavity during inhalation (orthonasal) or are released from food during consumption (retronasal). The binding and interaction of these molecules to specific olfactory receptors are determined by their molecular structure and concentration, triggering neural signals that are processed in the olfactory bulb and higher brain centers [41]. These signals, in combination with gustatory inputs, modulate taste perception by enhancing, suppressing, or complementing specific taste attributes, which significantly shapes the overall flavor experience (Figure 2).

The identification of volatile aroma-active compounds involves specialized techniques that differ from those used to identify taste-active compounds. One such method is gas chromatography–olfactometry (GC-O), which combines gas chromatographic separation with human sensory detection to identify and quantify aroma-active compounds. In GC-O, volatile compounds are separated chromatographically and then assessed by trained panelists who sniff the effluent to detect odor-active components [42]. Although GC-O facilitates the detection of odor-active volatiles, it does not determine which compounds are truly essential for flavor perception. Many of the detected compounds may be present in concentrations too low to influence the sensory experience, whereas others may interact in ways that amplify or suppress their effects. The sensomics approach may be used to identify the key aroma-active and taste-active compounds responsible for sensory

characteristics. Unlike conventional profiling methods that simply list the detected compounds, sensomics combines sensory-guided fractionation with analytical techniques to identify the key flavor-active compounds in complex food matrices [43]. By directly linking sensory perception to chemical composition and to quantitative data via reconstitution and omission experiments, this approach characterizes compounds responsible for specific taste and odor attributes, offering crucial insights into the molecular interactions that define flavor [43,44]. The sensomics approach has been successfully applied to various foods in previous studies [45–50].

Taste perception helps animals, especially omnivores with a broad food spectrum, to assess and choose their food [51]. Sweetness, mainly evoked by sugars and some amino acids, indicates a high-energy food source [51]. Similarly, the umami taste serves as a key indicator of protein-rich foods, guiding the selection of nutrient-dense meals [52]. In contrast, bitterness often acts as a warning sign, suggesting the presence of potentially harmful substances such as cyanides and alkaloids, leading to food rejection [51]. However, not all bitter compounds are avoided; some bitter compounds, such as those found in coffee, beer, and red wine, are not only tolerated but even preferred [51,53,54]. This acceptance varies among individuals owing to genetic differences in bitter taste perception and among compounds on the basis of receptor activation affinities [55–57]. Salty taste plays a crucial role in maintaining the ion balance in the body, making salty foods necessary for physiological function, whereas sour taste is often associated with unripe fruits, signaling a lower energy content and leading to avoidance in many cases [51].

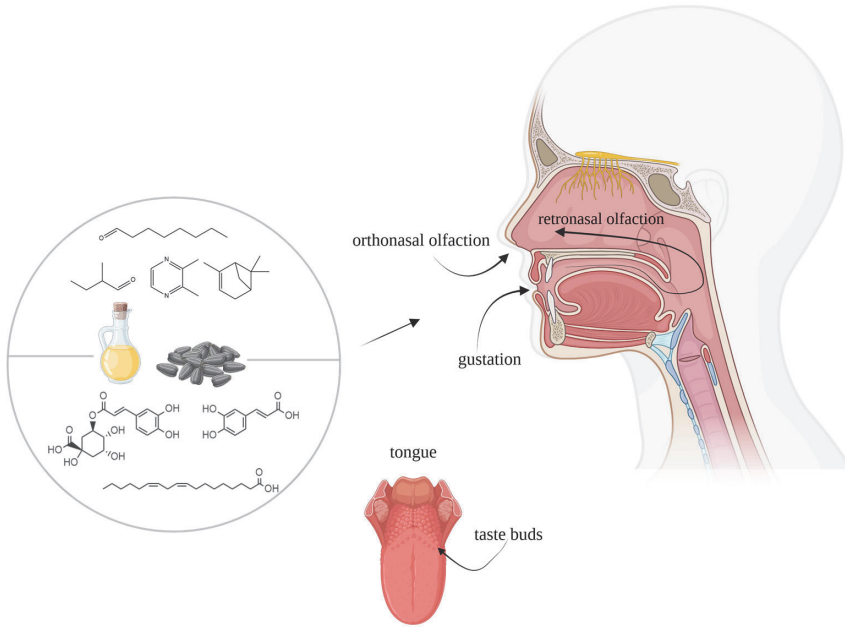

Figure 2. Overview of taste and smell pathways [58] involved in the flavor perception of sunflower-derived products. Created with BioRender. Huseynli, L. (2025) https://BioRender.com/u8c0013 (accessed on 6 May 2025).

In general, taste perception is induced by the interaction of taste-active compounds with ~40 taste receptor proteins on the tongue. These receptors are located in specific cells called taste receptor cells. A taste receptor cell expresses receptors specific for only one taste quality. Approximately 50–100 of these taste receptor cells are arranged in taste buds, which are located in taste papillae on the tongue, the soft palate, and the throat [59–62]. The bind-

ing of taste-active molecules to these receptors triggers the activation of specific receptor families, primarily G-protein-coupled receptors (GPCRs) and ion channels, which initiate signaling cascades leading to neurotransmitter release. Once activated, these receptors mediate distinct taste qualities. Sweet taste perception is facilitated by the T1R2/T1R3 GPCR complex, which belongs to the class C GPCR family. These receptors detect a broad range of sweet stimuli, including sugars, artificial sweeteners, and certain amino acids. Upon ligand binding, the receptor undergoes a conformational change, activating intracellular signaling pathways that result in neurotransmitter release and subsequent taste perception [60,63]. In addition to T1R2/T1R3, other mechanisms have been reported to be involved in sugar detection, providing an alternative pathway for sweet taste transduction [63–65]. Similarly, the umami taste, which signals the presence of amino acids and nucleotides, is primarily mediated by the T1R1/T1R3 receptor complex. This receptor is specifically responsive to L-glutamate and synergistically activated by 5′-ribonucleotides such as inosine monophosphate (IMP) and guanosine monophosphate (GMP). Additionally, metabotropic glutamate receptors (mGluR1 and mGluR4) are also involved in umami perception, particularly for detecting free glutamate at dietary concentrations [63,65,66]. These receptors activate G-protein-mediated signaling pathways, leading to intracellular calcium mobilization and neurotransmitter release. Bitter taste perception, in contrast, is facilitated by a diverse family of T2R GPCRs, with approximately 25 functional receptors in humans. Each T2R can recognize multiple structurally diverse bitter compounds, and conversely, many bitter compounds can activate multiple T2Rs [59,60,63,67,68]. Upon activation, the T2Rs initiate intracellular signaling cascades via the G-protein gustducin, which ultimately leads to neurotransmitter release and aversive taste perception [66,69]. Notably, genetic variations in T2Rs contribute to individual differences in bitter taste sensitivity [59]. Remarkably, sour taste is primarily mediated by intracellular acidification rather than extracellular proton concentration. Weak organic acids, such as citric acid and acetic acid, penetrate taste cells and lower the intracellular pH, leading to inhibition of potassium leak channels and subsequent depolarization of the cell membrane [63]. Type III taste cells have been identified as the primary detectors of sour stimuli, and recent evidence suggests that inwardly rectifying potassium channels play a key role in mediating the sour taste response [66]. Finally, salty taste transduction remains less well understood, but it is known that epithelial sodium channels are crucial in detecting low concentrations of sodium. These amiloride-sensitive channels allow sodium ions to enter the cell, leading to membrane depolarization and neurotransmitter release [60]. However, high concentrations of sodium are detected via an amiloride-insensitive pathway, likely involving other ion channels [63]. Unlike the other taste modalities, which rely on GPCRs, salty taste perception is mediated by direct ion flux through channels [66].

In general, the activation of taste receptor cells leads to neurotransmitter release, conveying sensory information via afferent nerve fibers to the brain, where it is processed and integrated with other sensory inputs to produce the perception of taste [59–61,63,66].

Four attributes are used to describe taste perception: (i) quality, which is the most important sensation for defining taste; (ii) intensity, which describes the magnitude of the sensation; (iii) temporal pattern, which characterizes how long the taste perception lasts; and (iv) spatial topography, which explains the location of taste perception [70]. Similarly, odor perception includes attributes such as intensity, hedonic tone, and aroma character. An important indicator is the recognition threshold of a taste stimulus, which describes the lowest concentration of a taste-active compound that can be perceived as a specific taste [71]. This threshold is determined using three-alternative forced choice test or a half-tongue test by a qualified sensory panel [72]. To further assess a compound's contribution to sensory perception, the dose-over-threshold (DoT) factor is commonly used.

This factor is calculated as the ratio between a compound's actual concentration in a food matrix and its sensory detection threshold, where a DoT value above one indicates that the compound contributes to taste perception [73–77].

Furthermore, somatosensory perceptions modify taste perception and contribute to flavor. These include sensations such as pungency, which arises from the activation of the transient receptor potential vanilloid receptor, as well as chemical-induced cooling and astringency, both of which influence flavor perception. The sensory perception of a prickle is characterized by a mild tingling sensation, often experienced as a light electric-like stimulation on the tongue, which may be associated with mild irritation or discomfort. Similarly, orosensory detection of CO_2 and the somatosensory perception of fats through specific receptors and nerve endings shape flavor perception. Finally, the concept of kokumi perception, which is exhibited by compounds modulating and intensifying the perception of all flavors, is important for a food's flavor profile [78].

3. Characterization of Volatile Compounds in Sunflower Products and Their Potential Role in Aroma Formation

The aroma profile of sunflower products is shaped by a complex interplay of volatile compounds, influenced by processing methods, chemical transformation, and differences in species or cultivars. Understanding these factors is essential for optimizing the sensory quality and addressing potential off-flavors. Numerous studies have explored the volatile and aroma profiles of sunflower products, but only one has used a molecular sensory science approach to identify key odor-active compounds in sunflower oil [79]. However, this comprehensive methodology has not yet been extended to other sunflower-derived products, limiting the precise identification of their key odor-active compounds.

According to previous studies [80,81], raw sunflower seeds contain dominant volatiles such as terpenes, aldehydes, lipid oxidation products; among these, α-pinene is prominent for its distinct pine-like aroma, contributing to the overall sensory profile. Other major volatiles in raw seeds include hexanal, furfural, octane, and γ-butyrolactone, which impart grassy, caramel, and other nuanced notes [82]. However, the volatile profile of sunflower seeds undergoes significant changes during roasting, primarily through the Maillard reaction and enhanced lipid oxidation. α-pinene, a major volatile in raw sunflower seeds, remains dominant in roasted seeds, exceeding its odor threshold and potentially contributing to a pine-like aroma [79,80]. Similarly, several volatiles, including 2-methylbutanal, 3-methylbutanal, and 1-octen-3-ol, are present in both raw and roasted seeds; however, their concentrations often increase with roasting. Pyrazines, such as 2-ethyl-3-methylpyrazine, 2,5-dimethylpyrazine, 2,3-dimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine, dominate the roasted aroma, contributing nutty and roasted notes. During roasting, furfural, a furan derivative known for its caramel-like aroma, increases alongside other lipid oxidation products such as pentanal and nonanal. These compounds enhance the roasted profile, whereas higher concentrations of hexanal, nonanal, and pyridine lead to undesirable off-flavors, particularly at high roasting temperatures and extended heating periods [79]. This characterization of sunflower seeds is based on the flavoromics approach, which uses headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC-MS) and principal component analysis (PCA) [81,82]. These studies provide valuable insights into changes in volatile composition; however, they do not establish a direct correlation between volatiles and sensory perception. At the same time, certain volatiles have been reported to exceed their odor thresholds, suggesting they contribute to the aroma profile, but no recombination or omission tests were conducted to confirm their actual impact on perception. Without such validation, the role of these key odorants remains speculative. Especially highlighted by the groups of Thomas Hofmann and Peter

Schieberle, key food odorants have to be defined via reconstitution and omission tests to verify the importance of single flavor stimuli in a complex food system [83]. Additionally, the methods used for volatile extraction may not fully capture all aroma-active compounds; instead, using solvent-assisted flavor evaporation (SAFE) [43] could offer a broader and more representative volatile profile by leading to a more comprehensive understanding of sunflower seed aroma.

Regional practices and seed varieties influence the volatile profile, as seen in Chinese traditional aromatic sunflower seeds, which feature unique compounds such as eugenol, *E*-anethole, and *E*-cinnamaldehyde, known for their spicy and floral notes [84]. A previous study identified volatile compounds in Chinese traditional aromatic sunflower seeds using static headspace and simultaneous distillation and extraction methods, followed by GC-MS analysis [84]. Although odor activity values (OAVs) were calculated to assess aroma contribution, the absence of a recombination test limits the confirmation of the role of these compounds in aroma perception [43].

Although sunflower oil is extracted from sunflower seeds, its volatile profile evolves uniquely through extraction, processing, and storage, reflecting both similarities with and differences from the raw material. These changes lead to a diverse array of aroma-active compounds that define the sensory identity of sunflower oil.

Cold-pressed sunflower oil is widely recognized for its distinctive volatile profile, with α-pinene consistently identified as the dominant compound. Bocci et al. (1996) [85] highlight that α-pinene accounts for most of the volatile fraction, accompanied by other terpenes such as limonene, sabinene, β-pinene, and 1,2,6,6-tetramethyl-1,3-cyclohexadiene. Additionally, minor quantities of hexanal and traces of unidentified volatiles, likely other terpenic hydrocarbons, have been reported using dynamic headspace sampling with GC-MS. Considering the study's focus on volatile composition rather than aroma perception, the absence of sensory validation methods reflect methodological limitations during that period [85].

According to Bendini et al. [86], sunflower seed-like and nutty aromas in virgin or cold-pressed sunflower oils are linked to α-pinene and 3-methyl-1-butanol, identified as volatile markers through solid phase microextraction–gas chromatography (SPME-GC) and quantitative descriptive analysis (QDA) [86]. In contrast, samples exhibiting defects, such as rancid or fried oil off-notes, showed elevated levels of E-2-heptenal, a volatile aldehyde indicative of lipid oxidation. However, the absence of direct sensory validation techniques, such as GC-olfactometry (GC-O) analysis and aroma recombination experiments, limits the ability to confirm the aroma contribution of these volatiles.

Yin et al. [79] provide further insight into the key odor-active compounds in cold-pressed sunflower oil using molecular sensory science which offers comprehensive analysis through techniques such as SAFE and aroma extract dilution analysis and emphasizing the role of volatiles with high OAVs. The researchers were able to rebuild recombination models with no significant differences from the original samples (Figure 3). This analysis identified α-pinene, β-pinene, linalool, hexanal, octanal, α-phellandrene, and (*E*)-2-octenal as the most significant contributors to the sensory profile of sunflower oil. These compounds collectively define the raw sunflower seed, woody, green, earthy, and sweet aromas characteristic of cold-pressed oils. In contrast to cold-pressed oil, roasted sunflower oil undergoes significant changes during heat processing, resulting in a more complex and intense aroma profile. Thermal degradation of terpenes and the promotion of Maillard reaction pathways lead to the formation of compounds such as pyrazines, furans, and aldehydes, which dominate the aroma of roasted oils. As a result of these reactions [78], the key contributors to the roasted, smoky, and burnt aromas include 2-

and 3-methylbutanal, 2,6-dimethylpyrazine, 2,5-dimethylpyrazine, 2,3-dimethylpyrazine, 2,3-pentanedione, 2-pentylfuran, 2,3-dimethyl-5-ethylpyrazine, and 1-pentanol [79].

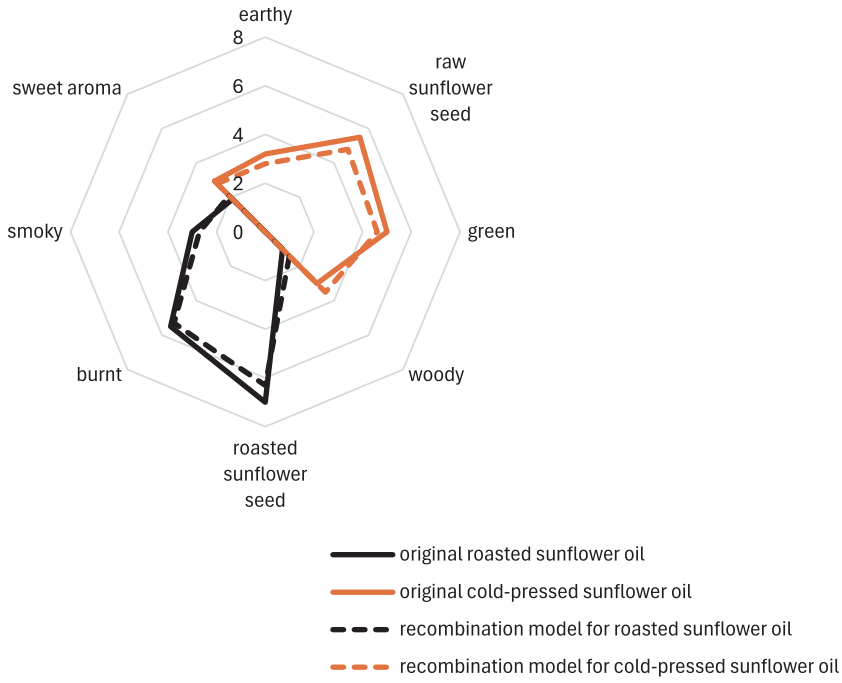

Figure 3. Aroma profiles of cold-pressed and roasted sunflower oils and their corresponding aroma recombination models (adapted from [79]).

Processing methods such as microwave treatment enhance the roasted and smoky aromas while reducing the raw sunflower seed and woody notes, as demonstrated in a previous study using SAFE, HS-SPME, and GC-O-MS [87]. This study highlights changes in aroma-active compounds; however, it does not include OAVs, which are essential for understanding the impact of these compounds on the overall aroma profile [43]. Study [87] also mentions that enzymatic treatments promote the release of aroma precursors, leading to the formation of compounds such as 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, benzeneacetaldehyde, furaneol, and acetic acid. In addition to enhancing sensory richness, these treatments improve oxidative stability by lowering the acid and peroxide values, offering dual benefits [87].

Analysis of Chinese sunflower oil using HS-SPME-GC-Quadrupole-MS, along with QDA and partial least squares regression, revealed that specific volatiles are positively correlated with sensory notes. For example, the "ripe sunflower seed" note was linked to 1-octen-3-ol, *n*-heptaldehyde, and dimethyl sulfone; the "sunflower seed" attributes were associated with γ-terpinene, octanal, and (±)-linalool; and "sweet" notes were derived from aldehydes such as *n*-nonanal and 2,3-butanediol [88]. However, although these correlations provide valuable insights, the study relies on statistical associations and literature descriptors rather than direct sensory validation methods, which strengthen the confirmation of aroma-active compounds (Table 2).

Table 2. The main volatile compounds in sunflower products with odor thresholds and sensory attributes.

	Compound(s)	Reported Concentrations (μg/kg)	Odor Threshold (μg/kg)	Odor Description	Method [a,b,d,e]
Raw and roasted seeds	α-pinene	7570 [f], 21,850 [r]	6 [a,e]	pine	HS-SPME-GC-MS, multivariate statistical analysis
	β-pinene	760 [f], 3000 [r]	140 [a]	woody, pine-like	
	octane	2670 [f]	/	/	
	furfural	950 [f], 8180 [r]	3000 [e]	almond, sweet	
	hexanal	1350 [f], 8490 [r]	479 [e]	green, fatty	
	γ-butyrolactone	1150 [f], 3190 [r]	1000 [e]	creamy	
	2-methylbutanal	540 [f], 2110 [r]	23 [e]	malty, almond	
	2,5-dimeththylpyrazine	200 [f], 10,190 [r]	800 [e]	roasty, cocoa	
	2,3-dimethylpyrazine	550–1300 [r]	100 [e]	nut, peanut, cocoa,	
	2-ethyl-3- methylpyrazine	140 [f] 3890 [r]	20 [c]	nutty, cereal like	
	2-ethyl-3,5-dimethylpyrazine	2090 [r]	7.5 [e]	nutty	
Cold-pressed oil	α-pinene	11,145 [a]–94,890 [b]	6 [a]	woody, pine-like	Dynamic HS-GC-MS; SPME-GC-MS, QDA; Molecular sensory science/Sensomics
	β-pinene	4068 [a]	140 [a]	woody, pine-like	Dynamic HS-GC-MS; Molecular sensory science
	sabinene	/	980 [c]	woody, citrus-like	Dynamic HS-GC-MS
	limonene	/	2100 [c]	lemon, citrus	
	hexanal	541 [a]	73 [a]	green, grassy	Dynamic HS-GC-MS; Molecular sensory science/Sensomics
	3-methyl-1-butanol	200–480 [b]	100 [b]	nutty, fruity	SPME-GC-MS, QDA
	linalool	56 [a]	6 [a]	citrus, fruity	Molecular sensory science/Sensomics (HS-SPME GC-O-MS, SAFE, AEDA, OAV, GC-O, recombination model)
	octanal	125 [a]	56 [a]	fruity, green	
	α-phellandrene	36 [a]	40 [a]	citrus, sweet, peel	
	(*E*)-2-octenal	69 [a]	61 [a]	fatty, floral	
Roasted oil	2-methylbutanal	6726 [a]	34 [a]	roasted, malty	Molecular sensory science/Sensomics (HS-SPME GC-O-MS, SAFE, AEDA, OAV, GC-O, recombination model)
	3-methylbutanal	714 [a]	15 [a]	fatty, almond	
	2,6-dimethylpyrazine	2329 [a]	20 [a]	nutty, roasted, coffee	
	2,5-dimethylpyrazine	12,228 [a]	200 [a]	nutty, potato	
	2,3-dimethylpyrazine	238 [a]	8 [a]	nutty, popcorn	
	2,3-dimethyl-5-ethylpyrazine	213 [a]	100 [a]	roasted, nutty, sweet	
	2,3-pentanedione	1456 [a]	50 [a]	buttery, sweet, spicy	
	2-pentylfuran	1332 [a]	130 [a]	buttery, caramel	
	1-pentanol	693 [a]	470 [a]	bread-like, sweet	

[a]—Yin et al. [79], [b]—Bendini et al. [86], [c]—Li et al. [89], [d]—Bocci et al. [85], [e]—Guo et al. [81]. [f]—Concentration in raw sunflower seeds, [r]—highest concentration in roasted sunflower seeds. Relative concentration values for [f] and [r] are taken from Guo et al. [81]. Odor descriptions are taken from corresponding literature where the data were reported. Threshold values vary between studies, which may be due to differences in matrix, methodology, or the population used for sensory determination.

In conclusion, a systematic sensomics approach for a more comprehensive analysis of sunflower seeds remains lacking. Implementing a sensomics approach step by step plays a critical role: dilution analyses help narrow down potent odorants; quantification allows concentration-to-threshold comparisons; and omission/recombination tests validate whether these compounds truly shape perception. Without applying this approach, findings remain partial and risk misidentifying or overlooking key contributors to flavor. As a result, conclusions about sensory quality and potential improvements may be speculative or misleading.

Additionally, the understanding of key aroma compounds in sunflower-derived protein sources such as meal, concentrates, and isolates remains limited. Addressing their sensory challenges is essential as these ingredients receive increasing attention for food applications.

4. Mapping Non-Volatiles and Taste-Associated Metabolites in Sunflower

Although it is critical to study the volatile and potentially aroma-active compounds that contribute to the characteristic sensory profile of sunflower products [79,81,90], it is equally important to consider the potential role of non-volatile compounds in taste perception. Unlike volatiles, non-volatile and semi-volatile compounds influence taste directly by

interacting with ~40 taste receptors, contributing to taste attributes such as bitterness or sweetness [60,63,66]. However, the specific contribution of these compounds to the taste of sunflower seeds has not yet been experimentally validated using sensomics approaches.

Plant phytochemicals, the sources of many sensory attributes, are broadly categorized into primary and secondary metabolites. Primary metabolites such as sugars, organic acids, amino acids, and nucleic acids are essential for plant growth and metabolic functions. In contrast, secondary or special metabolites, which are classified on the basis of their chemical structure, play a non-essential yet critical role in plant survival as defense-related compounds. These compounds protect against environmental stressors such as microbes, herbivores, and UV radiation and contribute to sensory properties such as bitterness and astringency. Additionally, they aid in attracting pollinators and influence human sensory perception such as bitterness and astringency, which stem from their interaction with taste receptors [91].

The sensory profile of freshly roasted sunflower kernels is defined by nutty, roasted, buttery, and sweet attributes. However, over time, storage-related changes result in the development of off-flavors, such as unpleasent off-notes. This dynamic sensory evolution, as documented in a study from 1988, is captured in Figure 4 [92]. More recent studies have explored sensory evaluation in terms of overall liking or acceptance, but they often lack the depth required to identify and analyze specific sensory attributes [93–95]. This gap underscores the need for updated research.

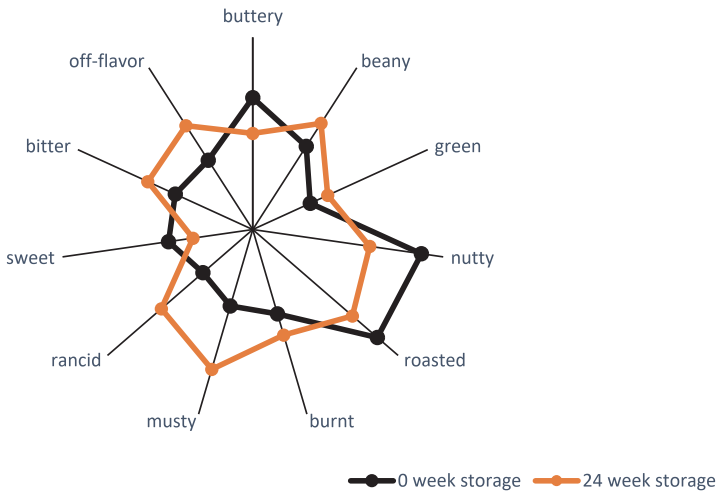

Figure 4. Taste profiles of roasted sunflower kernels at 0 and 24 weeks of storage adapted from Robertson et al. [92].

To further understand the potential contributors to sunflower seed flavor, it is essential to consider the role of macronutrients and their potential effects on taste perception through interactions with taste receptors and other sensory-active compounds. The following section explores the macronutrient and micronutrient composition of sunflower products, highlighting their presence, distribution, and relevance.

Among the reported non-volatile compounds, the approaches used for their identification and quantification differ widely across studies. These methodological differences should be taken into account when interpreting the values presented in the upcoming tables. Detailed analytical procedures can be found in the corresponding original publications referenced in each table.

4.1. Macronutrients

4.1.1. Lipids

The main fraction of sunflower seeds corresponds to lipids (35–42%), primarily in the form of triacylglycerides, which show high variability in their fatty acid composition owing to varietal differences; nevertheless, triacylglycerides such as 50:2, 52:3, 52:4, 54:5, and 54:6 have been reported to be the most abundant in sunflower seeds [96,97]. A characteristic feature of sunflower seeds is the presence of very-long-chain fatty acids, such as arachidic acid and behenic acid. Although the seeds contain only small quantities of free fatty acids, these compounds significantly influence specific flavors and promote fat oxidation. The free fatty acid content depends on the enzymatic activity of lipase, which hydrolyzes triacylglycerides into free fatty acids. This activity is further accelerated by wet harvesting and moisture during storage [98]. Furthermore, as mentioned earlier, sunflower seeds are broadly classified into non-oilseed and oilseed varieties, with oilseed varieties being highly diverse in their fatty acid profiles, suited for distinct industrial and sensory applications [99–101]. These differences in the composition of fatty acids may significantly affect their flavor contribution and the characteristics of their oxidation products. The oxidation reactions contribute to off-flavors and reduce the nutritional value of the seeds. Table 3 [102,103] shows the fatty acid composition of sunflower seeds. In the referenced study, fatty acids were extracted using the Bligh and Dyer method, methylated with acid catalysis, and analyzed by GC-FID using nonadecanoic acid (C19:0) as the internal standard [103]. Please note that different studies may use varying extraction and analytical methods, which can lead to differences in reported values.

Table 3. Fatty acids in sunflower seeds and their potential contribution to taste.

Name	Content (µmol/kg) $^{R}_{1}$	Taste	Taste Threshold (µmol/kg) $^{R}_{2}$	DoT
α-Linolenic acid	~539–1077	Scratchy, bitter	~189 [a], ~277 [b]	~1.9–3.9
Linoleic acid	~17,508–26,422	Scratchy, bitter	~270 [a], ~1810 [b]	~9.7–14.6
Oleic acid	~9983–29,631	Scratchy, bitter	~203 [a], ~2180 [b]	~4.6–13.6
Palmitic acid	~2067–3003	Scratchy, bitter	~1002 [a], ~1546 [b]	~1.3–1.9
Stearic acid	~949–1582	Scratchy, bitter	~645 [a], ~726 [b]	~1.3–2.2

[a]—threshold for scratchy perception, [b]—threshold for bitter taste. $^{R}_{1}$—data from Thepthanee et al. [103]; $^{R}_{2}$—data from Günther-Jordanland et al. [102]. DoT—dose-over-threshold factor.

Roasted sunflower seeds contain palmitoleic acid (16:1), eicosanoid acid (20:0), and gadoleic acid (20:1) [104]. Typically, fresh, intact sunflower seeds contain low concentrations of linoleic acid, α-linolenic acid, oleic acid, palmitic acid, and stearic acid; however, variations in composition may influence their potential contribution to taste perception [12,103]. However, during storage or processing methods such as soaking, the fatty acid composition of germinated seeds changes. These alterations influence the sensory perception of sunflower seeds by modifying sensory attributes, potentially leading to rancid, musty, bitter, and beany flavors [92].

Sunflower oil is rich in unsaturated fatty acids, particularly the essential omega-6 fatty acid linoleic acid, which comprises 55–70% of the lipid fraction [1]. The oil contains only 1.19% –1.35% (*w*/*w*) free fatty acids [98]. These free fatty acids, despite their low concentrations, are associated with a bitter taste, which is generally disliked [51]. To mitigate oxidation and maintain nutritional value, the presence of antioxidants such as phenolic acids is crucial. However, sunflower oil contains phenolic acids only in trace amounts owing to their poor solubility in oil [6,105].

A previous study reported DoT values of >1 for certain fatty acids (Table 3), indicating that they contribute to taste perception. Of these fatty acids, linoleic and oleic acids exhibited higher DoT values, suggesting a potentially more significant role in taste modulation. This is consistent with their predominance among fatty acids in sunflower oil [106,107]. Notably, these values vary with processing conditions and seed fatty acid composition.

4.1.2. Proteins

Sunflower seed protein is increasingly being explored for its role in plant-based formulations. In particular, sunflower meal and press cake represent a promising alternative protein resource in food applications, consistent with efforts to diversify protein sources [108]. Sunflower seeds contain approximately 20% proteins, primarily the seed storage proteins 11S globulins and 2S albumins. The seed amino acid composition is balanced, with a high proportion of sulfur-rich amino acids, but a notable drawback is the low level of the essential amino acid lysine [1]. The quantity of free amino acids is important for sensory analysis because they impart intrinsic taste activity. For example, histidine, isoleucine, leucine, phenylalanine, and valine, in sufficiently high concentrations, impart a bitter taste [109–113]. In contrast, alanine, glycine, serine, threonine, and proline are sweet-tasting amino acids, whereas glutamic acid and aspartic acid are associated with an umami note when present in concentrations above their threshold [110–114].

Data on the amino acid content of sunflower seeds are limited, and when presented, they are often shown in charts, making it difficult to determine the exact numerical values [12,115]. However, several studies have investigated the amino acid composition of sunflower meal, isolate, hydrolysates, and press cake [26,116–119]. This is particularly important because these products are protein alternatives, and understanding their composition is crucial for assessing their potential applications and addressing flavor challenges. A previous study [117] reported specific numerical data for certain amino acids in sunflower seeds (Table 4). Although it does not provide data for all amino acids, it offers valuable insights into their composition. Study [117] demonstrated that sunflower oilcakes have a higher total amino acid content than seeds. In descending order, alanine, glycine, glutamic acid, leucine, and aspartic acid were reported to be the most abundant amino acids in seeds. Asparagine and glutamine were likely not detected because they are more likely converted into aspartic acid and glutamic acid, respectively, during acidic hydrolysis. Seeds contain a total amino acid content of 5790.26 nmol/g, with 6.63% being essential and 93.37% non-essential amino acids. However, seeds exhibit fewer detectable amino acids than meal products, such as valine, isoleucine, methionine, and tryptophan, among others, are absent because of the protective role of hulls, which reduce enzymatic digestibility [12,115,117,120]. The presence of tryptophan, asparagine, and glutamine was also not reported by Chen et al. [115].

Table 4. Amino acids in sunflower seeds with potential taste activity.

Name	Content (μmol/kg) ^{R_1}	Taste	Taste Threshold (μmol/kg) ^{R_2}	DoT
Alanine	~2110.4	Sweet	~12,000	<1
Arginine	/	Bitter	~75,000	
Aspartic acid	~255.53	Umami	~600	<1
Glutamic acid	~1229.56	Umami	~1100	1.1
Glycine	~1810.93	Sweet	~25,000	<1
Histidine	/	Bitter	~45,000	
Isoleucine	/	Bitter	~10,000	
Leucine	~383.84	Bitter	~11,000	<1

Table 4. *Cont.*

Name	Content (μmol/kg) $^{R}{}_{1}$	Taste	Taste Threshold (μmol/kg) $^{R}{}_{2}$	DoT
Lysine	/	Bitter	~80,000	
Methionine	/	Sweet	~5000	
Phenylalanine	/	Bitter	~45,000	
Proline	/	Sweet	~25,000	
Serine	/	Sweet	~25,000	
Threonine	/	Sweet	~35,000	
Tyrosine	/	Bitter	~4000	
Valine	/	Bitter	~30,000	

$^{R}{}_{1}$—data from Petraru et al. [117]; $^{R}{}_{2}$—data from Hillmann et al. [120]. DoT—dose-over-threshold factor. In the referenced study, amino acids were extracted, derivatized, and analyzed using GC-MS. Analytical conditions and derivatization protocols may vary across studies, potentially affecting the reported amino acid content.

According to Bao et al. [121], enzymatic hydrolysis of sunflower seed proteins with Flavourzyme generated umami-tasting peptides, with the highest umami intensity observed after 480 min of hydrolysis performed using an electronic tongue. At this point, peptides such as DVNNPANQLD, NNENQLDEYQR, and EFEGGSIEH were identified as contributors to the umami taste. However, prolonged hydrolysis increased bitterness, likely owing to the release of bitter-tasting amino acids and hydrophobic peptides, highlighting the importance of regulating the degree of hydrolysis to optimize the flavor balance. These findings suggest potential applications of sunflower seed hydrolysates as natural flavor enhancers in plant-based or low-sodium food products. Among the reported amino acids in Table 4, only glutamic acid exhibited a DoT value >1, signifying its contribution to the umami taste. Furthermore, products of Maillard reaction, whereby the carbonyl group of reducing sugars reacts with an amino group, affect the flavor, color, and stability of sunflower seeds [122]. A previous study reported that the Maillard reaction promoted by heating and addition of xylose and cysteine to sunflower peptides intensifies the meat-like flavor and umami taste [123]. The Maillard reaction may occur in sunflower seeds naturally or during processing.

4.1.3. Carbohydrates

In sunflower kernels, the predominant carbohydrates are complex polysaccharides and fiber, with their reported content ranging from 2.86 to 3.88 g/100 g. The total carbohydrate content in sunflower seeds varies across studies, reported between 14.72 and 27.36 g/100 g, influenced by the variety and analytical methods used [124]. According to Muttagi et al. [124], the total sugar content of sunflower seeds ranges from 2.36 to 3.04 g/100 g, consistent with the values reported by the USDA [124–126].

Similarly, Laemont et al. [80] reported that the total sugar content in sunflower seeds is relatively low, comprising approximately 3%, with sucrose being the predominant sugar at 3.2 g per 100 g. During roasting, sucrose gradually breaks down into fructose and glucose, and significant changes occur in carbohydrate composition, potentially influencing flavor development. The sugar content of sunflower seeds fluctuates during germination as well [12,80].

The starch content of sunflower seeds is notably low (0.42%) [2]. In contrast, sunflower hulls are primarily composed of structural carbohydrates, with cellulose and lignin together constituting nearly 50% of the hull weight. Reducing sugars represents the second largest component, comprising 25.7% of the total hull weight. This compositional distinction underscores the role of sunflower kernels as a nutrient-rich storage unit, whereas the hull provides essential structural support to the seed [5].

In sunflower seeds, free monosaccharides such as fructose, glucose, galactose, and mannitol are found in low concentrations below their threshold values. Fresh sunflower seeds also contain small quantities of arabinose and trace amounts of rhamnose [127].

Given that the reported sucrose content in sunflower seeds is 3.2 g/100 g (93,485 µmol/kg) [80], surpassing its taste detection threshold of 12,500 µmol/kg [128], (DoT~7) suggests that sucrose is likely to contribute to the overall taste perception. However, the slight sweetness of sunflower seeds likely arises from the combined effect of multiple sweet-tasting molecules.

4.2. Micronutrients

4.2.1. Minerals and Vitamins

Sunflower seeds contain valuable nutrients, vitamins, and minerals [1]. Among these, sodium and potassium ions are particularly associated with salty tastes, with intrinsic taste thresholds of 3900 µmol/kg and 13,000 µmol/kg, respectively [46]. In some foodstuffs, minerals such as magnesium and calcium potentially contribute to bitter and astringent taste perception, in addition to salty taste [46]. A recent study [125] provided a detailed analysis of the mineral content of dehulled sunflower seeds, revealing higher levels of minerals in dehulled seeds than in whole seeds owing to the removal of mineral-poor hulls. For instance, dehulled seeds contained iron (6.4–10.19 mg/100 g), zinc (9.533–17.855 mg/100 g), magnesium (8.21–18.06 mg/100 g), and potassium (5.14–7.0 mg/100 g). These findings differ from the USDA-reported average values for raw sunflower seeds: 302 mg/100 g for magnesium and 657 mg/100 g for potassium [126]. The study attributed these discrepancies to differences in sunflower hybrids, climatic conditions, soil quality, and the USDA's reliance on averaged data across multiple sources. These differences can potentially alter the flavor profile.

To provide context, the mineral levels shown in Table 5 [2,46,126] reflect USDA values for raw sunflower seeds. DoT values calculated on the basis of these values suggest that minerals such as magnesium and potassium play a notable role in sunflower seed taste perception.

Table 5. Minerals with potential taste effects in sunflower seeds.

Name	Content (µmol/kg) $^{R}_{1}$	Taste	Taste Threshold (µmol/kg) $^{R}_{2}$	DoT
Calcium	~19,461–28,942	Bitter, astringent	~6200	~3.1–4.7
Magnesium	~124,254	Bitter, astringent	~6400	~19.4
Potassium	~168,030	Salty	~13,000	~12.9
Sodium	<~1087	Salty	~3900	<1

$^{R}_{1}$—data from USDA report [126]; $^{R}_{2}$—data from Dirndorfer et al. [46]. DoT—dose-over-threshold factor.

In addition, sunflower seeds are an excellent source of B vitamins and the antioxidant vitamin E. The high concentration of vitamin E (87,762 µmol/kg) helps to inhibit photooxidation [1]. Most vitamins are taste-active compounds, and sunflower seeds contain approximately 6.96 mg of niacin, 0.84 mg of pantothenic acid, 0.76 mg of pyridoxine, 0.507 mg of riboflavin, and 0.473 mg of thiamine per 100 g [4,110].

Thiamine (a common flavor precursor), pyridoxine, and pantothenic acid impart an intrinsic bitter taste, whereas niacin and riboflavin are particularly bitter. Thiamine and vitamin C activate sour taste receptors, whereas vitamin K (phylloquinone) exhibits a moderately sweet taste. However, the taste thresholds for these vitamins have not yet been determined [110]. The fat-soluble vitamins A, D, and E are abundant in sunflower seeds; they have traditionally not been associated with significant taste activity [4,129]. Nevertheless, a recent study [130] has shown that vitamins A and D can activate specific

bitter taste receptors in vitro, indicating potential taste activity despite earlier assumptions. The same study also reported, for the first time, human bitter taste detection thresholds for vitamin B1 (as thiamine hydrochloride), B2 (as riboflavin phosphate), B3 (as niacinamide), and B6 (as pyridoxine hydrochloride) [130]. As these thresholds were determined using specific supplemental forms of the vitamins, no further calculations were performed to avoid a potentially misleading interpretation.

4.2.2. Phenols

Phenols are compounds characterized by an aromatic ring with at least one hydroxyl group attached to the benzene ring [131]. Approximately 463 phenolic compounds have been identified in sunflower, flaxseed, poppy, pumpkin, and sesame seeds [132]. In sunflower seeds, the total phenolic content in defatted non-oilseed kernels (3291–3611 mg/100 g dry weight) is similar to that in oilseed kernels (3938–4175 mg/100 g); however, the latter exhibit slightly but not significantly higher levels [6]. Chlorogenic acid (5-*O*-caffeoylquinic acid) is the predominant phenol in sunflower oilseeds, with a concentration of 69,628.29 µmol/kg dry weight. Additionally, 3-*O*- and 4-*O*-caffeoylquinic acids are also present [2]. These compounds themselves do not taste bitter, but bitter-tasting derivatives such as chlorogenic acid lactones and caffeoyl quinides may be produced during processes such as roasting [133]. Moreover, these phenolic compounds interact with proteins, potentially lowering the nutritional quality by altering the organoleptic properties and reducing protein digestibility [134].

Furthermore, phenols may undergo browning reactions, during which chlorogenic acid is oxidized to form a highly reactive *o*-quinone intermediate. This intermediate reacts with the amino group of lysine or the thiol group of cysteine or methionine, leading to the development of green or brown discoloration. Such reactions produce toxic compounds, destroy essential amino acids, and reduce the nutritional quality, as the condensation products generated cannot be metabolized. This is one of the primary reasons sunflower protein isolates are not yet widely used in industrial food products [135]. Studies have attempted to address these challenges; for example, dephenolization of sunflower meal by washing the dried meal with 60% methanol for 8 h has been shown to reduce the adverse effects of phenols [22].

Polyphenols exhibit antiviral, antimicrobial, and antioxidant activity, which explains why the total phenolic content in plants increases under mechanical stress [136]. The flavonoid myricetin, found in sunflower seeds, is an even stronger antioxidant than α-tocopherol [137]. In addition to these benefits, phenols are reported to have anti-inflammatory, anticancer, and antimutagenic effects in humans, reducing the risk of coronary heart disease [136]. Phenols are also present in other parts of the sunflower plant; for example, flavonoids found in trichomes act as a natural defense mechanism against insects [138].

Phenolic acids are present in high concentrations in sunflower seeds and are associated mainly with bitterness or astringency. Astringency is described as a puckering and drying sensation on the whole tongue surface, which is linked to phenolic compounds with at least two hydroxyl groups in 1,2-dihydroxy or 1,2,3-trihydroxy configurations. These configurations enhance the binding of phenols to salivary proteins, forming phenol–protein complexes that precipitate when sufficiently hydrophobic, resulting in the characteristic astringent sensation [136]. Table 6 lists the phenols reported in sunflower seeds [2,7,102,137,139–143]. Sunflower oil contains only small amounts of vanillic and p-hydroxybenzoic acids owing to the poor solubility of phenolic compounds in oil [6,142], with reported concentrations of 6.9 µg and 1.5 µg per 100 g, respectively [142].

Table 6. Phenolic acids in sunflower seeds and their taste qualities and potential flavor contribution.

Name	Content (μmol/kg) $^{R}_{1}$	Taste	Taste Threshold (μmol/kg) $^{R}_{2}$	DoT
Caffeic acid	~142–1482 [a]	Astringent	~72	~2–20
p-Coumaric acid	~15.2 [b]	Astringent	~139	<1
Ferulic acid	~87–639	Astringent	~67	~1.2–9.5
Gallic acid	~65.8 [b]	Astringent	~292	<1
p-Hydroxybenzoic acid	/	Astringent	~665	
Protocatechuic acid	~329.6 [b]	Astringent	~206	~1.6
Rosmarinic acid	~233–391	Bitter	~102.6	~2–3.8
Sinapic acid	~69.6 [b]	Astringent	~693	<1
Syringic acid	/	Astringent	~263	
Vanillic acid	/	Astringent	~315	

[a]—dry matter, [b]—free acids. $^{R}_{1}$—data from Khurana et al. [2], Pająk et al. [7], Žilic et al. [137] and Pedrosa et al. [135]. $^{R}_{2}$—data from Günther-Jordanland et al. [102], Hofmann et al. [141] and Gracia et al. [140]. DoT—dose-over-threshold factor. Quantification methods, analytical conditions, and sample matrices in the referenced studies vary. Such variation may affect the reported concentrations, thresholds, and DoT values.

The phenolic composition of sunflower seeds varies depending on factors such as extraction method, plant species, and growth environment. This has been observed in studies showing that drought stress can lead to higher total phenolic content and antioxidant activity in sunflower seeds [144]. Genotype and cultivation location have also been shown to contribute to significant variations in the phenolic profiles across sunflower varieties [145]. For example, one study [2] reported 638.58 μmol/kg ferulic acid, whereas Žilić et al. [137] reported 272.94–308.99 μmol/kg ferulic acid. Such variability in reported phenolic compound concentrations may alter their DoT value, consequently modulating their sensory impact and direct role in taste perception. Furthermore, synergistic interactions between phenols in sunflower seeds influence taste perception. For instance, ferulic acid (90 ppm) combined with p-coumaric acid (40 ppm) has a bitter taste threshold of 25 ppm [146]. Remarkably, ferulic acid alone has been reported to evoke a sour taste with a threshold of 90 ppm [147].

Furthermore, organic acids such as hydroxyacetic acid, 4-hydroxybutyric acid, phosphoric acid, and 2-piperidinecarboyxlic acid have been detected in fresh and roasted sunflower seeds, but their concentrations have not been reported [104]. Among these, malic acid is characterized by its sour taste, with a threshold of 3700 μmol/kg, whereas ß-aminoisobutyric and γ-aminobutyric acids contribute exclusively to an astringent sensation, with thresholds of 120 μmol/kg and 20 μmol/kg, respectively [104,128,148].

The taste thresholds of flavonoids, a subgroup of polyphenols, in sunflower seeds are presented in Table 7 [7,71,128,137,148–151]. Among flavonoids, apigenin, kaempferol, and quercetin are present only in small quantities [7]. The quantity of myricetin (131.98–194.82 μmol/kg) in some samples has been reported to exceed the threshold and is more likely to contribute to an astringent and bitter taste. The human tongue is highly sensitive to rutin, a glycoside of quercetin with glucose, which imparts astringency at a detection threshold of only 0.00115 μmol/kg [131,152].

Table 7. Flavonoids in sunflower seeds and their potential roles in taste perception.

Name	Content (μmol/kg) $^{R}_{1}$	Taste	Taste Threshold (μmol/kg) $^{R}_{2}$	DoT
Apigenin	~11	Bitter	/	
Catechin	/	Bitter, astringent	~800, 410	
Coumestrol	~0.004 [a]	Bitter	~250″	<1

Table 7. *Cont.*

Name	Content (μmol/kg) $^{R}_{1}$	Taste	Taste Threshold (μmol/kg) $^{R}_{2}$	DoT
Daidzein	~0.094 [a]	Bitter	~500″	<1
Epicatechin	/	Bitter, astingent	~1000, 930	
Epicatechin gallate	/	Astringent	~260	
Epigallocatechin	/	Astringent	~520	
Epigallocatechin gallate	/	Astringent	~190	
Formononetin	~0.026 [a]	Bitter	~500″	<1
Genistein	~0.074 [a]	Bitter	~4–8″	<1
Glycetein	~0.018 [a]	Bitter	~500″	<1
Kaempferol	~1.75	Bitter, astringent	~69.87	<1
Luteolin	/	Bitter	/	
Myricetin	~131.9–194.8	Bitter, astringent	~31.42	~4.2–6.2
Quercetin	~19 [b]	Bitter, astringent	~33.09	<1
Rutin	/	Astringent	~0.00115	

[a]—in wet weight; ″—measured in vitro, [b]—free and bonded $^{R}_{1}$—data from Pająk, P. et al. [7], Žilić, S. et al. [137], Thompson et al. [149]. $^{R}_{2}$—data from Scharbert et al. [71], Stark, et al. [128], Roland et al. [150], Gonzalo-Diago et al. [151]. DoT—dose-over-threshold factor. Quantification methods, analytical conditions, and sample matrices in the referenced studies vary. Such variation may affect the reported concentrations, thresholds, and DoT values.

Additionally, sunflower seeds contain other flavonoids such as heliannone and luteolin. The total isoflavone content in sunflower seeds has been reported to be 534 mg/100 g [1]. Isoflavones such as formononetin, daidzein, glycitein, coumestrol, and genistein are also present [149]. Although these compounds are taste-active, their concentrations in sunflower seeds are low. For example, genistein activates the bitter receptors hTAS2R14 and hTAS2R39 at concentrations of 4 μmol and 8 μmol, respectively [150]. In addition, minor lignan components such as matairesinol, lariciresinol, pinoresinol, and secoisolariciresinol are also found in sunflower seeds [149].

Catechins are derived from flavan-3-ol, and some of these elicit a bitter and puckering sensation. (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, and (−)-epicatechin gallate are known to be present in sunflower seeds, but exact concentrations have not been reported [148]. The catechins constitute 0.7×10^{-3}% of the flavonoids in crude sunflower oil.

The DoT values of most flavonoid compounds based on their reported concentrations in sunflower seeds were below one, suggesting that they do not directly contribute to taste perception, except myricetin, the DoT value (>1) of which indicated a potential impact on taste. These values may vary across sunflower cultivars, influencing overall sensory characteristics.

4.2.3. Other Secondary Metabolites

Bioactive phytosterols are present in higher amounts in sunflower kernels (131.9–511.9 mg /100 g) than in several other nuts and seeds [2]. However, the taste activity of compounds such as β-sitosterol, campesterol, stigmasterol, and 7-stigmastenol has not been reported. Additionally, sunflower seeds contain various lignans, including 1-acetoxypinoresinol, 7-hydroxymatairesinol, 7-hydroxysecoisolariciresinol, arctigenin, conidendrin, coumestrol, dimethylmatairesinol, lariciresinol, lariciresinol-sesquilignan, medioresinol, pinoresinol/matairesinol, secoisolariciresinol-sesquilignan, secoisolariciresinol, todolactol A, sesamin, sesaminol, sesamol, sesamolinol, and syringaresinol [132,153]. The total lignan content in sunflower seeds is reported to be 0.4 mg/100 g [154]. Lignans, produced by the dimerization of two phenylpropanoids, represent a wide-ranging group of com-

pounds. Some lignans, such as (+)- lyoniresinol in wine and spirits are known to impart bitterness [155], but the taste activity of lignans in sunflower seeds remains unknown.

In addition to phytosterols and lignans, sunflower seeds contain secondary metabolites such as saponin and cynarin, which exhibit anti-inflammatory and cholesterol-lowering effects [1]. Other bioactive compounds include sesquiterpene lactones such as costunolide, dehydrocostus lactone, 8-epixanthatin, and tomentosin [156] and the triterpene squalene, detected at a concentration of 0.51 μmol/kg [12]. Diterpenoids such as grandiflorolic acid, kaurenoic acid, and trachylobanoic acid have demonstrated significant anti-inflammatory activity in cell cultures and mouse models [16]. Crude sunflower oil contains 0.277 mg/100 g of carotenoids, mainly carotene, cryptoxanthin, lutein, and zeaxanthin as well as chlorophyll; however, these compounds are not known to be taste active [157,158]. Amakura et al. [158] identified novel compounds in sunflower seeds, including benzyl alcohol β-D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside, methyl caffeate, methyl chlorogenate, and eriodictyol 5-O-β-D-glucoside. Furthermore, Jan et al. [159] reported the presence of caftaric acid; rosmanol; proanthocyanidin B1; and derivatives of sinapic acid, caffeic acid, gallic acid, caffeoylmalic acid, and quercetin.

Furthermore, sunflower seeds contain a small number of anti-nutritional compounds, including cyanide (4.026–4.175 mg/100 g), tannins (623–651 mg/100 g), and oxalates (98–113 mg/100 g). Although phytate reduces the bioavailability of minerals, it also exhibits antioxidative and cholesterol-lowering properties [2]. Tannins, which are known to impart bitterness and astringency to products such as red wine, are present in sunflower seeds; however, their contribution to the taste profile of sunflower products remains to be determined [160]. Many other phenols, such as various quinic acid derivatives [2], isoferulic acid, and 4-hydroxybenzoic acid [17], have been identified in sunflower seeds. However, not all are included in Table 6, as their taste activity remains largely unknown.

5. Discussion

Evidence suggests that flavor remains the primary determinant influencing food purchases [161]. Meeting these flavor expectations while addressing the global demand for sustainable protein sources poses a significant challenge, particularly considering the growing global population [162]. The exploration of plant-based protein sources and their side-stream products is therefore critical as potential solutions.

Sunflower-based food products offer a low-cost, plant-based alternative to animal-derived proteins, reducing the environmental footprint of protein production [13,163], which is consistent with global sustainability goals. Sunflowers, predominantly cultivated as oil crops and utilized as animal feed in the form of press cake after oil extraction, represent an underexplored opportunity as a protein alternative. The major limitation to their broader application in human nutrition is their undesirable flavor profile, a challenge commonly associated with plant proteins. The recent interest in utilizing sunflower meal/cake and protein concentrates as alternatives in plant-based food applications has further underscored the significance of addressing associated flavor challenges. These ingredients have been explored in a variety of products, such as cookies, snack bars, pasta, breads, and meat analogues [27,31,37,163–166].

To date, no comprehensive study has provided a systematic framework for mapping the complete flavor profile of sunflower seeds and their by-products. To address this gap, the present review consolidates the current knowledge on both volatile and non-volatile compounds in sunflower seeds that potentially influence their flavor. Volatile odor-active compounds are already well established as significant contributors to flavor perception [86]. Notably, although considerable research has focused on odor-active volatiles in sunflower oil, research on volatiles contributing to the aroma of sunflower seeds remains relatively

limited. This focus on sunflower oil is logical, considering that sunflower is one of the most cultivated oil crops worldwide [167]. However, non-volatile taste-active compounds remain largely underexplored despite their critical role in determining sensory characteristics.

The flavor profile of sunflower seeds is shaped by a combination of macronutrients, including lipids, proteins, and carbohydrates as well as secondary metabolites such as polyphenols, minerals, and vitamins. Unique interactions among these components influence the overall flavor experience by contributing to different sensory characteristics. Lipids, primarily present as triacylglycerides, are the predominant components of sunflower seeds. Fresh lipids contribute minimal flavor, but their oxidation during storage or processing may lead to the release of free fatty acids, resulting in rancid and bitter off-notes. Such oxidation products are a well-documented source of undesirable tastes in plant-based protein isolates [168]. In contrast, proteins may impart desirable as well as undesirable flavors, as enzymatic hydrolysis leads to the release of umami-enhancing peptides and production of bitter-tasting hydrophobic peptides. However, their exact potential taste activity in sunflower-based products remains unknown. Carbohydrates, although less abundant in sunflower seeds, play a crucial role in flavor enhancement. They contribute sweetness and serve as the precursor for aroma-active compounds formed via the Maillard reaction during roasting, resulting in roasted and nutty flavor notes. To better understand complex interactions, it is also important to examine how macronutrients can modulate flavor perception at a molecular level. For example, proteins can bind aroma compounds through covalent as well as non-covalent (e.g., π,π, hydrophobic, and hydrogen) bonding interactions, while lipids primarily influence aroma retention via hydrophobic partitioning; both mechanisms can significantly affect flavor release and perception [169–171]. Additionally, carbohydrates may impact volatility by increasing matrix viscosity or through weak interactions such as hydrogen bonding and inclusion complex formation [171,172]. Within real food matrices, macronutrients coexist and may influence not only the behavior of flavor compounds but also one another's functional and sensory contribution. These interactions may be governed by macronutrient structure and physicochemical properties, ultimately shaping the availability and perception of flavor compounds in the food matrix [171,172].

In addition to the contribution of macronutrients, the role of secondary metabolites that are abundant in sunflower also warrants attention. These compounds occur in both free and bound forms (e.g., esters, glycosides, or complexes), which significantly affects taste perception [1]. Moreover, it is well known that polyphenols interact at specific stages of various reaction cascades, further complicating their contribution to taste. Several phenolic compounds such as phenolic acids and flavonoids, which are abundant in sunflower seeds, are typically associated with a bitter and astringent taste. The intensity of the astringent sensation varies depending on the presence of other taste stimuli, highlighting the complexity of these interactions [173]. Moreover, minerals such as sodium and potassium contribute to saltiness, whereas calcium and magnesium impart bitterness or astringency. However, the extent of their contribution to sunflower flavor requires experimental validation. Similarly, the flavor impact of vitamins present in sunflower seeds remains unexplored, as the taste thresholds of vitamins have not yet been determined.

The intricate complexity of sunflower flavor arises from molecular interactions that influence both perceptual and cognitive processes. For instance, the intensity of taste perception from individual components may be modulated by the presence of others; for example, several bitter-tasting compounds at low concentrations may collectively activate various bitter receptors, leading to a bitter taste even if individual taste thresholds are not met [146]. This phenomenon is particularly noteworthy in plant-based proteins, as processing methods such as hydrolysis convert non-proteinogenic off-taste compounds into bitter derivatives, whereas proteinogenic compounds also contribute to the bitterness

of protein hydrolysates [108]. Furthermore, cognitive processes, such as the integration and interpretation of multiple taste stimuli in the brain, add another layer of complexity. For example, "mixture suppression" reduces the perceived intensity of individual tastes in compound mixtures, as described by Keast and Breslin [70]. These dynamics may have synergistic effects, where compounds below their individual taste thresholds collectively enhance flavor perception, or suppressive effects, where certain compounds diminish the impact of other compounds.

A major limitation in existing research is the absence of integrated sensory and sensomics analyses. Although data on sunflower seed composition are available, the lack of systematic studies linking these compounds to actual sensory perception remains a bottleneck for advancing product development. By addressing these challenges, this review provides a foundational framework by consolidating existing data on known non-volatile compounds in sunflower seeds that may influence taste. However, experimental studies using advanced tools such as the sensomics approach are required to conclusively determine their direct contribution to sensory perception [43,44,174].

Furthermore, the effects of environmental factors, cultivar-specific differences, and stress conditions on the flavor profile of sunflower seeds need to be taken into consideration. Identifying and leveraging these variations is instrumental for optimizing sunflower products to meet both sensory and nutritional demands. Moreover, the choice of processing techniques, such as fermentation or enzymatic hydrolysis, should be guided by a deeper understanding of sunflower flavor dynamics. Identifying off-flavors is essential to selecting appropriate interventions for their mitigation while preserving nutritional quality. Therefore, selecting appropriate treatment methods on the basis of the intended application is critical for optimizing the sensory outcomes.

6. Conclusions

Sunflower has long been cultivated primarily as an oilseed crop, and scientific attention toward its flavor has largely focused on oil-based products. Of all sunflower-derived matrices, only cold-pressed and roasted sunflower oils have undergone systematic investigation using molecular sensory science (sensomics), allowing for the identification of key odorants through validated techniques. Recently, however, sunflower has also gained attention as a promising protein source in the development of plant-based foods and alternatives. This shift brings new opportunities but also significant flavor-related challenges, particularly in protein-rich matrices such as meal, press cake, concentrates, and isolates which remain largely uncharacterized from a sensory perspective. Although a few studies have noted the presence of off-putting aromas in these products, no systematic work has been conducted to identify their key odorants using the sensomics approach.

The knowledge gap is even more pronounced when it comes to taste. To date, no studies have been conducted to identify or validate taste-active compounds in sunflower protein products. As a consequence, the taste profile of sunflower protein remains poorly understood.

To fully utilize sunflower's potential as a sustainable and nutritionally valuable protein source, it is critical to extend sensory research beyond oil and apply integrated sensomics methodologies to protein-based products. Identifying and confirming both odor- and taste-active compounds through reconstitution experiments, omission tests, and in vitro receptor assays while also considering matrix effects that modulate flavor perception will provide mechanistic understanding.

Once the key taste-active compounds are confirmed through these methods and their sensory contributions are well understood, it becomes possible to implement precise interventions to improve flavor. As demonstrated by Mittermeier-Kleßinger et al. [108],

strategies such as controlled fermentation, selection of clean protein isolates, and genotype optimization can be employed to minimize undesirable taste impressions and enhance overall acceptability. These approaches, framed within the concept of delivering a full flavor experience, enable the development of targeted, scalable solutions that can address the sensory complexity and technological constraints of sunflower-based ingredients while unlocking their full potential in modern plant-based foods to match consumer acceptance.

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Appendix 5

Publication IV

Huseynli, L., Gigl, M., Müller, J., Walser, C., Frank, O., Vene, K., & Dawid, C. Characterization of bitter off-taste stimuli in sunflower press cake using the sensomics approach.
Under review

Characterization of Bitter Off-Taste Stimuli in Sunflower Press Cake Using the Sensomics Approach

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ABSTRACT

The Sensomics approach, including activity-guided fractionation and taste dilution analysis, was employed to identify the key compounds responsible for the bitter off-taste of sunflower press cake. A combination of liquid chromatography−tandem mass spectrometry, liquid chromatography−time-of-flight–mass spectrometry, one-/two-dimensional nuclear magnetic resonance spectroscopy, and dose-over-threshold factor calculation led to the identification of 9,12,13-trihydroxyoctadec-10-enoic acid, 9,10,11-trihydroxyoctadec-12-enoic acid, 11,12,13-trihydroxyoctadec-9-enoic acid, (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid, α-linolenic acid, linoleic acid, oleic acid, 2-hydroxyoleic acid, palmitic acid, stearic acid, and novel pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside as contributors to the bitterness of sunflower press cake. The findings provide valuable insights into the sensory challenges associated with using sunflower press cake in food applications and offer pathways to enhance its palatability and potential as a sustainable protein alternative to meet future protein demands.

KEYWORDS: sunflower, bitter taste, off-flavor, fatty acid oxidation products, liquid chromatography−tandem mass spectrometry

Introduction

The sunflower (*Helianthus annuus* L.), a member of the Asteraceae family, is cultivated as a crop worldwide for human and livestock consumption.[1,2] Sunflower is primarily used for oil production, and it is the third most widely cultivated oilseed crop in the world, with a production volume of 57 million tons per year, after soybeans (364 million tons) and rapeseed (71 million tons).[3] Furthermore, sunflower oil is the fourth most popular vegetable oil worldwide and is often valued for its monounsaturated fatty acid content.[1,4] Sunflower meal or cake, a by-product of oil extraction, contains proteins, cellulosic fibers, lignins, phenols, minerals[5] and is primarily used as fertilizer and animal feed. In 2017, approximately 19 million tons of sunflower press cake was generated worldwide.[6] The sunflower press cake contains 19.9%–44.9% of protein,[7] which makes it a valuable potential source to meet the growing global protein demand.[8] This is especially relevant as food industries increasingly prioritize the development of sustainable protein sources in response to growing population needs.[8]

In light of this, plant-based proteins, used as alternatives to traditional protein sources, are promising not only for their nutritional benefits but also for their potential to contribute to food security. However, the consumer acceptance of these proteins is often compromised by off-flavors, particularly bitter taste.[9–11] Addressing the factors that contribute to these bitter off-flavors is crucial for optimizing the use of emerging protein sources. Recently, there has been increasing interest in valorizing sunflower meal/cake as a sustainable protein source for food applications.[12–14] However, despite the high protein content of sunflower meal/cake, research on its sensory properties, particularly taste, remains limited. A recent review has drawn attention to the sensory challenges arising from the limited research on sunflower-derived materials, which may

complicate their integration into food applications, suggesting the importance of further exploration of their taste-active compounds.[4]

To understand these sensory challenges more effectively and on a molecular level, the sensomics approach can be used to identify the key taste and off-flavor compounds in food products.[15] Previous studies have used the sensomics approach to identify taste-active compounds in linseed oil,[16] hazelnuts,[17] poppy seeds,[18] asparagus,[19] rapeseed protein isolates,[20,21] and pea protein isolates.[22] Pickardt et al. (2015) highlighted the presence of a bitter taste in sunflower meal, which may pose a challenge to consumer acceptance.[23] Therefore, the present study aimed to identify the key compounds contributing to the bitter off-taste of sunflower press cake by means of activity-guided fractionation, taste dilution analysis, and dose-activity calculation.

Materials and Methods

Chemicals. The following compounds were obtained commercially: acetonitrile (ACN), methanol (MeOH) (J.T. Baker, Deventer, The Netherlands); acetone, ethyl acetate, n-pentane (BDH Prolabo, Briare, France); formic acid (Merck, Darmstadt, Germany), dimethyl sulfoxide-d_6 (DMSO)–deuterium oxide (D_2O), linoleic acid, ricinoleic acid, [$^{13}C_{18}$]-linoleic acid, α-linolenic acid, hydrochloric acid, anhydrous pyridine, L-cysteine methyl ester hydrochloride, phenylethyl isothiocyanate, D-glucose, D-galactose, D-mannose, D-xylose, D-ribose, D-apiose (Sigma-Aldrich, Steinheim, Germany); isopropyl alcohol (Honeywell, Seelze, Germany); (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*Z*, 11*E*)-13-oxooctadeca-9,11-dienoic acid, 2-hydroxyoleic acid, and 18-hydroxyoleic acid (Larodan AB, Solna, Sweden). The acetonitrile used for high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) analysis was liquid chromatography−mass spectrometry (LC−MS)

grade (Honeywell, Seelze, Germany); acetone, ethyl acetate, and n-pentane were distilled before use, and all other solvents were HPLC grade. The water used for chromatographic separation was purified using an Advantage A10 water System (Millipore, Molsheim, France). Bottled water (Evian) was adjusted to pH 5.9 using formic acid and used for sensory analyses. Sunflower press cake was obtained from SUNFLY OU in Estonia.

Sequential Solvent Extraction. A total of 300 g of sunflower press cake was subjected to solid–liquid extraction with methanol/water (70:30, v/v, 1500 mL) three times by stirring for 30 min at room temperature, followed by centrifugation (4 min, 5000 rpm) and filtration. The filtrates were collected and combined, separated from the solvent by vacuum evaporation at 40 °C, and lyophilized to obtain the MeOH/H_2O extractables (fraction F1). The residue was extracted further with MeOH (1500 mL, fraction F2), followed by ethyl acetate (1500 mL, fraction F3) and n-pentane (1500 mL, fraction F4). The extracted solvent fractions F1−F4 were freeze-dried twice to remove trace amounts of solvents and stored at -20 °C until they were used for a comparative taste profile analysis.

Fractionation of F1 by Solid-Phase Extraction. For solid-phase extraction fractionation, an aliquot (1 g) of fraction F1 was dissolved in water (40 mL) and sonicated at room temperature (10 min). This solution was separated on a Chromabond C18 endcapped cartridge (45 µm, 70 mL/10 g, Macherey-Nagel, Düren, Germany), which was preconditioned with methanol (2 × 70 mL), followed by water (2 × 70 mL). Elution was performed with water (2 × 70 mL) to obtain fraction F1-1, methanol/water (30:70, v/v, 2 × 70 mL) to obtain fraction F1-2, methanol/water (50:50, v/v, 2 × 70 mL) to obtain fraction F1-3, methanol/water (70:30, v/v, 2 × 70 mL) to obtain fraction F1-4, and methanol (2 × 70 mL) to obtain fraction F1-5. The collected fractions were separated from

the solvent by vacuum evaporation at 40 °C, lyophilized twice, and stored at -20 °C until subsequent use for chemical and sensory analyses.

Separation of Fraction F1-4 by Preparative High-Performance Liquid Chromatography (HPLC). Fraction F1-4 was dissolved in H_2O/ACN (80:20, v/v; 320 mg in 6 mL) with ultrasonication at room temperature (10 min). The sample was membrane filtered and injected (300 µL) into a Nucleodur C18 Pyramid column (250 × 21 mm, 5 µm, 110Å, Macherey-Nagel, Düren, Germany) equipped with a guard column of the same type. The separation was performed at a flow rate of 20 mL/min using 0.1% aqueous formic acid as solvent A and acetonitrile as solvent B. The effluent was monitored using a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) at Gain 12. The gradient flow was as follows: 0 min, 15% B; 3 min, 15% B; 28 min, 60% B; 30 min, 100% B; 32 min, 100% B; 35 min, 15% B; 40 min, 15% B. In total, 17 fractions were collected (F1-4-1 to F1-4-17), separated from the solvent (vacuum evaporation at 40 °C), lyophilized twice, and then stored at -20 °C until further use.

Identification of Fatty Acids and Fatty Acid Oxidation Products as Key Bitter Compounds in Fractions F1-4-15 and F1-4-16. Fractions were subjected to untargeted screening using ultrahigh performance liquid chromatography–time-of-flight–mass spectrometry (UPLC-TOF-MS) to facilitate the identification of compounds within the fractions. To verify these identifications, the samples were analyzed alongside reference compounds using a previously established method.[18,22] The retention times and mass spectral data obtained in the present analysis were consistent with those of the reference compounds, confirming that the identified compounds aligned with those reported in previous research.[18,22]

Identification of the Bitter Compound in Fraction F1-4-12. Fraction F1-4-12 was dissolved in H_2O/ACN (80:20, v/v; 10 mg/mL) and, after membrane filtration, fractionated by semipreparative

HPLC using a Luna Phenyl-Hexyl column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), equipped with a guard column of the same type, with a binary gradient using 0.1% formic acid in H_2O as solvent A and 0.1% formic acid in ACN as solvent B (flow rate 4.7 mL/min): 0 min, 30% B; 3 min, 30% B; 25 min, 45% B; 28 min, 100% B; 30 min, 100% B; 32 min, 30% B; 35 min, 30% B. For detection DAD detector model MD-2010 Plus (Jasco, Groß-Umstadt, Germany) was used. Fraction F1-4-12-5 contained the bitter target compounds and was collected in multiple HPLC runs, combined, separated from the solvent (vacuum evaporation at 40 °C), and then lyophilized. For further purification fraction F1-4-12-5 was dissolved in ACN/H_2O (30/70, v/v; 1mg/mL) and fractionation was performed with an analytical Luna Phenyl-Hexyl column (250 × 4.60 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) with a flow rate of 1 mL/min and using 0.1% formic acid in H_2O as solvent A and 0.1% formic acid in MeOH as solvent B. DAD detector model MD-2010 Plus (Jasco, Groß-Umstadt, Germany) was used. Separation was performed using the following gradient: 0 min, 50% B; 2 min, 50% B; 29 min, 62% B; 32 min, 100% B; 34 min, 100% B; 36 min, 50% B, 40 min, 50% B. The fraction containing the bitter compound (**15**) was collected in multiple HPLC runs, separated from the solvent, and lyophilized; subsequently, structural analysis was conducted using MS/MS following hydrolysis, TOF-MS, and nuclear magnetic resonance spectroscopy (NMR), as well as sensory threshold analysis.

Determination of monosaccharide constituents via ultrahigh performance liquid chromatography–tandem mass spectrometry following acidic hydrolysis. Using a previously reported protocol,[24] the monosaccharide constituents of **15** were determined after acidic hydrolysis. Therefore, the isolated compound **15** (1 mg) was dissolved in a mixture of ACN/H_2O (3:7, v/v, 100 µL) and treated with hydrochloric acid (6 mol/L, 1 mL). The mixture was heated at

100 °C for 60 min. After heating, the mixture was evaporated to dryness under nitrogen. The resulting residue was resuspended in 2 mL of H_2O and extracted three times with 2 mL of ethyl acetate. The aqueous layer was then evaporated to dryness to obtain a monosaccharide-containing residue. This residue was dissolved in anhydrous pyridine (100 µL), and a solution of L-cysteine methyl ester hydrochloride (500 µL, 2 mg/mL) was added. The mixture was shaken at 60 °C at 1400 rpm in a thermo shaker (PHMT-PSC24N, Grant Bio, Cambridge, UK) for 60 min. Next, phenylethyl isothiocyanate (50 µL) was added to the solution, and the resulting mixture was shaken again at 60 °C for 60 min. The mixture was dried under a stream of nitrogen, reconstituted in a mixture of ACN/H_2O (1:1, v/v, 500 µL), and transferred to an autosampler vial; then, an aliquot (1 µL) was subjected to UHPLC-MS/MS analysis. Mass spectrometry was conducted using a QTRAP 6500 mass spectrometer (AB Sciex, Darmstadt, Germany) operated in ESI+ mode. The ion source parameters were as follows: ion spray voltage at 5500 V (ESI^+), curtain gas at 35 psi, nebulizer gas at 55 psi, heater gas at 65 psi, collision-activated dissociation high, and source temperature 500 °C. The MS system was coupled to a Shimadzu Nexera X2 UHPLC (Shimadzu, Duisburg, Germany). The system consisted of two pumps (LC-30AD), a degasser (DGU-20A5R), an autosampler (SIL-30AC), a column oven (CTO-30A), and a controller (CBM-20A). Data acquisition was performed using Analyst 1.6.3 (AB Sciex, Darmstadt, Germany). For all reference compounds, individual MS/MS parameters were first tuned and optimized on the UHPLC-MS/MS system for each compound after derivatization. After optimizing instrument settings with reference compounds, the derivatized monosaccharides were analyzed using the mass transitions Q1/Q3 of m/z 461.0/298.1 (DP = 86 V, CE = 17 V, CXP = 6 V) for D-glucose, Q1/Q3 of m/z 461.1/298.2 (DP = 71 V, CE = 17 V, CXP = 6 V) for D-galactose, Q1/Q3 of m/z 461.0/298.1 (DP = 71 V, CE = 17 V, CXP = 6 V) for D-mannose, Q1/Q3 of m/z 430.9.1/268.0 (DP = 76 V, CE = 17 V, CXP =

12 V) for D-xylose, Q1/Q3 of m/z 430.9.1/268.0 (DP = 71 V, CE = 29 V, CXP = 11 V) for D-ribose, and Q1/Q3 of m/z 430.9.1/268.0 (DP = 76 V, CE = 17 V, CXP = 9 V) for D-apiose. Chromatography was performed using a Phenomenex Kinetex F5 column (100 × 2.1 mm i.d., 100 Å, 1.7 µm, Phenomenex, Aschaffenburg, Germany) kept at 40 °C. Compound elution was performed with a flow rate of 0.4 mL/min, and the mobile phase contained (A) 1% aqueous formic acid and (B) ACN (1% formic acid) with the following gradient: 0 min, 5% B; at 3 min, 5% B; at 5 min, 20% B; at 25 min, 25% B; at 27 min, 100% B; at 30 min, 100% B; at 31 min, 5% B; at 35 min, 5% B. A comparison of the retention times and mass transitions of reference compounds allowed the identification of the monosaccharides D-glucose and D-apiose, present in the isolated bitter compound **15** from fraction F1-4-12-5.

Sensory Analysis. *Sensory Panel Training and Sample Preparation*. The 12 panelists (six females and six males, 22−30 years of age) participated in the sensory tests and provided informed consent to participate in the present study. The trained panelists had no history of known taste disorders. They were familiar with the sensory analysis methodologies used and were able to evaluate various chemosensory attributes. Panelists underwent weekly training sessions for at least two years to become proficient in taste terminology and sensory evaluation techniques. Sensory training utilized aqueous reference solutions (2.0 mL, pH 5.9), including sucrose (50 mmol/L) for sweet, L-lactic acid (20 mmol/L) for sour, NaCl (20 mmol/L) for salty, caffeine (1 mmol/L) for bitter, and monosodium L-glutamate (3 mmol/L) for umami taste perception.[18] All sensory analyses were performed at 22−25 °C in a sensory panel room using the sip-and-spit method. Nose clips were used during all sensory analyses to avoid cross-model interactions with odor-active compounds.

Taste Profile Analysis. An aliquot (6 g) of sunflower press cake was suspended in water (100 mL, pH 5.9) and presented to the trained panel. To prevent sedimentation, the suspension was stirred

during the sensory test. The trained panelists were asked to evaluate the taste attributes sweet, bitter, umami, salty, astringent, and sour on a scale from 0 (not detectable) to 5 (strongly detectable). Additionally, an aliquot of fraction F1 and subfractions F1-4 was dissolved in bottled water (25 mL, pH 5.9) in natural concentrations and evaluated by the trained sensory panelists regarding bitterness, sweetness, sourness, saltiness, umami, and astringency.

Taste Dilution Analysis. The HPLC subfractions F1-4-1−F1-4-17, isolated from an aliquot (320 mg) of fraction F1-4, were dissolved in bottled water (20 mL, pH 5.9) and sequentially diluted 1:1 (v/v) with bottled water. The dilution series was presented to the panel in ascending concentrations, and the taste dilution (TD) factor for bitterness was determined by asking the sensory panel to mark the first detectable difference between the sample and the control (bottled water, pH 5.9).

Human Taste Recognition Thresholds. The two-alternative forced choice test was used to determine threshold concentration, at which the bitter taste quality of the compound was just detectable. For this purpose, the purified substances were dissolved in bottled water at increasing concentrations. The individual recognition thresholds were determined by calculating the geometric mean of the first falsely and the last correctly identified concentrations. The taste threshold for the sensory panel was estimated by averaging the threshold values obtained from each panelist.

High-Performance Liquid Chromatography. The HPLC setup (Jasco, Groß-Umstadt, Germany) consisted of a binary pump system PU-2087 Plus, a DG-4400 degasser, and a Rheodyne injection valve, model Rh 2807i type (Rheodyne, Bensheim, Germany). The effluent was monitored using an MD-2010 Plus diode array detector (Jasco, Groß-Umstadt, Germany) operating within a wavelength range of 200−500 nm, along with a Sedex LT-ELSD detector Model 80 (Sedere, Alfortville, France). Chromatographic separation was performed on a preparative

Nucleodur C18 Pyramid column (250 × 21 mm, 5 µm, 80 Å, Macherey-Nagel, Düren, Germany), a semipreparative Luna Phenyl-Hexyl column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), and Luna Phenyl-Hexyl column (250 × 4.60 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) all equipped with a guard column of the same type. Data acquisition was managed using Galaxie Chromatography Software, version 1.10.0.5590.

Ultrahigh Performance Liquid Chromatography/Time-of-Flight Mass Spectrometry. High-resolution mass spectra were obtained by injecting 2 µL aliquots of all analytes in ACN/H_2O (80:20, v/v) into an Acquity UPLC core system (Waters, Manchester, UK). This system included a binary solvent manager, a sample manager, and a column oven. Chromatographic separation was performed on a BEH C18 column (150 × 2.1 mm, 1.7 µm, 130 Å; Waters, Manchester, UK) at a flow rate of 0.4 mL/min and a temperature of 40 °C with 0.1% formic acid in H_2O (v/v) as solvent A and 0.1% formic acid in ACN (v/v) as solvent B. For the initial screening of fractions, the gradient started at 5% B and increased to 100% B within 8 min and remained isocratic for 5 min. The methods used were previously reported in the literature.[18,22] High-resolution mass spectra were acquired on a Synapt G2-S HDMS (Waters, Manchester, UK) in positive and negative ESI resolution modes using a capillary voltage of 2.5 kV and -1.7 kV, respectively; 50 V sampling cone; 4.0 kV extraction cone; 150 °C source temperature; 450 °C desolvation temperature, 2 and 30 L/h cone gas, and 800 L/h desolvation gas. The mass spectrometer was calibrated across a range of m/z 50−1200 using a sodium formate solution (0.5 mmol/L) in isopropanol/H_2O (90:10, v/v). The data were lock mass corrected by infusing a solution of leucine enkephalin (1 ng/µL, m/z 556.2771, $[M+H]^+$ and m/z 554.2615, $[M-H]^-$) at 10 µL/min. Data processing was performed using MassLynx 4.2 (Waters, Manchester, UK).

Quantification of Fatty Acids and Fatty Acid Oxidation Products Using Liquid Chromatography–Differential Mobility Separation–Tandem Mass Spectrometry. The reference compounds were obtained commercially, and to ensure accurate quantification, two different commercially available internal standards structurally similar to the analytes were selected: [$^{13}C_{18}$]-linoleic acid (IS 1) was used as the internal standard for fatty acids, whereas 18-hydroxyoleic acid (IS 2) served as an internal standard for the oxylipins. MS/MS parameters for each analyte and internal standard were optimized individually in ESI negative ionization mode to monitor the fragmentation of pseudomolecular ions.[25]

Solvent Extraction for Quantification. To perform the quantification in triplicate, 3 × 500 mg of sunflower press cake, a mixture of MeOH/H_2O (50:50, v/v, 5 mL), and the following internal standard solutions were added to a cryogenic tube (10 mL, VWR Chemicals, Fontenay-sous-Bois, France): 25 µL of [$^{13}C_{18}$]-linoleic acid (IS1, 0.5 mM in MeOH) and 25 µL of 18-hydroxyoleic acid (IS 2, 0.5 mM in MeOH). The extraction was performed using an Analogue Orbital Shaker 3005 (GFL, Burgwedel, Germany) for 1 h at 300 U/min. The extracts were membrane filtered (Minisart RC 15, 0.45 µm, Sartorius AG, Göttingen, Germany) and subsequently injected into the liquid chromatography–differential mobility separation–tandem mass spectrometry (LC−DMS−MS/MS) system.[25]

Calibration Curve. The exact concentration of the analytes was verified using quantitative NMR (qNMR), and a stock solution (0.2 mM) was prepared in MeOH. This stock solution was diluted to 0.1, 0.05, 0.025, 0.0125, 0.0063, 0.0031, 0.0016, 0.0008, and 0.0004 mM. Next, 1 mL of each dilution was mixed with 10.1 µL of an internal standard solution mixture. The UHPLC−DMS−MS/MS analysis of each sample was performed in triplicate. Then, calibration curves were prepared by plotting the peak area ratio of the analyte to the internal standard versus

the concentration ratio of each analyte to the internal standard. Linear regression was used for quantitation using MultiQuant version 3.03 (Sciex, Darmstadt, Germany).[22]

Ultrahigh Performance Liquid Chromatography−Differential Mobility Separation–Tandem Mass Spectrometry System and Parameters. The MS/MS analysis was performed on a QTrap 6500+ mass spectrometer equipped with a SelexION + DMS cell (Sciex, Darmstadt, Germany) in the negative ionization mode, as reported in the literature.[25] The ion mobility parameters were as follows: isopropanol as the chemical modifier at a flow rate of 363.6 μL/min (low), a separation voltage (SV) of 3500 V, a DMS temperature of 225 °C (medium), and a DMS offset of 3 V. The declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were optimized using commercial references of methanolic solutions of the analytes and internal standards.[25]

The mass spectrometer was operated in the MRM full scan mode (ion spray voltage: –4500 V for ESI negative ionization) with the following parameters: temperature, 450 °C; gas 1, 55 psi; gas 2, 65 psi. The MS/MS system was coupled to a Shimadzu LC system Nexera X3 (Shimadzu, Duisburg, Germany) consisting of a Shimadzu LC-40D pump, a Shimadzu DGU-405 degasser, a Shimadzu SIL-40C autosampler, a Shimadzu CTO-40C column oven AC, and a Shimadzu SCL-40 control unit.

Sample injections (1 μL) were followed by chromatography on a Kinetex C18, (150 × 10 mm, 1.7 μm; Phenomenex, Aschaffenburg, Germany) with a binary gradient using 5 mM NH_4Ac in H_2O (pH 5) as solvent A and 5 mM NH_4Ac in H_2O (pH 5)/ACN/isopropanol (5:55:40, v/v/v) as solvent B (flow rate of 0.35 mL/min): 0 min, 15% B; 0.5 min, 15% B; 2 min, 30% B; 6 min, 50% B; 17 min, 71% B; 19 min, 100% B; 21 min, 100% B; 22 min, 15% B; 24 min, 15% B. The instrument was controlled using the Analyst 1.6.3 software (Sciex, Darmstadt, Germany). Data analysis was

performed using Microsoft Excel (Microsoft Office, 2016) and Multiquant (version 3.0.3, Sciex, Darmstadt, Germany).

Quantification of Bitter Compound 15 Using Ultrahigh Performance Liquid Chromatography−Tandem Mass Spectrometry.

Solvent Extraction for Quantification. The sunflower press cake (1 g, n = 3) was weighed into bead beater tubes (5 mL, CKMix, Bertin Technologies, Montigny-le-Bretonneux, France), and a mixture of methanol and water (50:50, v/v, 5 mL) was added. Extractive grinding was performed at 6000 rpm for 3×30 s with 30 s breaks in between using a bead beater (Precellys Homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France). The samples were centrifuged (10 min, 4800 × g), and the clear supernatant was separated. The residue was extracted using the same protocol a total of five times prior to quantification to assess the viability of external calibration. Therefore, the individual extraction steps were injected into the LC-MS system and the compound areas were compared. After three extractions, less than 5% of the initial compound area was detected, which remained consistent after two additional extractions (**Table S1**). In total, five extraction steps were deemed sufficient for quantification. The combined supernatants of all five extraction steps were evaporated to dryness under nitrogen and reconstituted in a mixture of methanol and water (50:50, v/v, 400 µL), transferred to autosampler vials, and stored at -20 °C until LC-MS analysis.

Ultrahigh Performance Liquid Chromatography–Tandem Mass Spectrometry System and Parameters. Mass spectrometry was conducted using the QTRAP 6500 system described earlier, operated in ESI negative mode with the following ion source parameters: ion spray voltage at -5500 V (ESI^-), curtain gas at 35, nebulizer gas at 55, heater gas at 65, collision-activated dissociation medium, and source temperature at 500 °C. The MS system was coupled to the

Shimadzu Nexera X2 UHPLC (Shimadzu, Duisburg, Germany) mentioned earlier. The MS/MS parameters of compound **15** were tuned and optimized, resulting in the characteristic Q1/Q3 transitions of m/z 607.2/161.0 (DP = -140 V, CE = -46 V, CXP = -17 V) as the quantifier, and m/z 607.2/178.9 (DP = -140 V, CE = -44 V, CXP = -21 V) as the qualifier. Chromatography was performed using a Kinetex Biphenyl column (100 × 2.1 mm, 1.7 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) maintained at 40 °C. Aliquots (1 µL) were injected into the system at a flow rate of 0.4 mL/min and using 0.1% formic acid in water and 0.1% formic acid in methanol as solvents A and B, respectively, with the following gradient: 5% B held for 1 min, increased in 2.5 min to 60 % B, held at 60% B for 2 min, increased in 1 min to 100% B, and held at 100% for 1 min, then decreased in 1.5 min to 5% B, and re-equilibrated for 1.5 min at 5% B.

Calibration Curve. For quantification, a stock solution of bitter tastant **15** was prepared in a mixture of acetonitrile and water (50:50, v/v). The concentration was determined using quantitative ^{1}H-NMR spectroscopy. The stock solution (10.96 mmol/L) was then diluted successively (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256), using the same solvent mixture. All dilutions were analyzed by means of UHPLC-MS/MS using a scheduled (20 s window) multiple reaction monitoring (MRM) method. Then, the peak area was plotted against the concentrations, and an external calibration curve was established with linear regression ($y = 2667.8x + 386458$, $R^2 = 0.9992$), which was used for quantification of **15** in the sunflower press cake.

Nuclear Magnetic Resonance Spectroscopy. NMR spectra were recorded using a Bruker Avance Neo 600 MHz system (Bruker, Rheinstetten, Germany) equipped with a cryo-TCI probe at 300 K. The samples were prepared using 100 × 3 mm NMR tubes (Hilgenberg, Münnerstadt, Germany). The data acquisition and processing were performed using TopSpin 4.1.1 (Bruker, Rheinstetten,

Germany) and MestReNova 11.0.4 (Mestrelab Research, La Coruña, Spain). Chemical shifts were referenced to the residual solvent signals of DMSO-d_6 or D_2O.

Quantitative NMR spectroscopy (qHNMR). For quantification, data were recorded on a Bruker AV III 400 MHz system, which was equipped with a Broadband Observe BBFOplus probe. The concentration of the target compounds was determined with the external reference L-tyrosine (5.21 mmol/L) via the ERETIC II procedure, as described by Frank et al. (2014).[26]

RESULTS AND DISCUSSION

This study aimed to identify and characterize key bitter compounds in sunflower press cake that have not yet been extensively researched in terms of their taste profile. The results offer novel insights into the sensory properties of sunflower press cake, thereby presenting opportunities to enhance its palatability and broaden its applicability within the food industry.

Sensory Analysis and Sequential Solvent Extraction of Sunflower Press Cake.

The sunflower press cake was first analyzed by a trained panel by applying a taste profile analysis to gain initial insight. On a scale from 0 (not detectable) to 5 (strongly detectable), the panelists were asked to rank the taste intensity of bitter, sweet, sour, umami, salty, and astringent. Bitterness and astringency exhibited the highest intensity ratings, with scores of 2.5 and 2.4, respectively, followed by sourness with a score of 0.8. In comparison, sweetness and saltiness were perceived with lower intensity, each scoring 0.4, whereas umami was perceived with the lowest intensity score of 0.3. Based on the prominent bitterness detected during the sensory analysis, the sensomics approach was subsequently applied to identify the key bitter compounds responsible for the off-taste in sunflower press cake. Therefore, sunflower press cake was extracted sequentially with a series of solvents, beginning with MeOH/H_2O (F1), followed by MeOH (F2), ethyl acetate (F3),

and n-pentane (F4). A rotary evaporator and freeze dryer were used to remove the solvent from each fraction. Subsequently, fractions were dissolved in their natural concentrations in water and analyzed using comparative taste profile analysis (**Table 1**). Fraction F1, with an intensity score of 2.5, showed the highest bitterness compared with fractions F2–F4. Due to its high bitterness intensity, fraction F1 was further fractionated to identify the key bitter molecules.

Activity-Guided Identification of the Key Bitter Compounds in Fraction F1

To identify the fraction with key bitter compounds, F1 was separated further with RP-18 solid-phase extraction into five subfractions (F1-1 to F1-5). Fractions F1-1–F1-5 were separated from the solvent and used for sensory analysis. Fraction F1-4 showed higher bitterness (3.0) than other subfractions (**Figure 1**). Fraction F1-4 was further separated using preparative RP18-HPLC, and 17 subfractions were collected (F1-4-1 to F1-4-17). Subfractions were separated from the solvent and dissolved in equal amounts of water for taste dilution analysis at ascending concentrations. According to the TD factor analysis of 17 subfractions, fractions F1-4-12, F1-4-15, and F1-4-16 showed high bitterness with TD factors of 32, 28, and 32, respectively (**Figure 2**). Following this analysis, the subfractions were screened using UPLC-TOF-MS to determine their compound complexity and to assess whether further sub-fractionation was necessary. This screening suggested that the bitter fractions F1-4-15 and F1-4-16 may contain fatty acids and fatty acid oxidation products; therefore, we focused on the fatty acid components in subsequent analyses. These compounds were further characterized using LC-TOF-MS (ESI^-) analysis, which revealed pseudomolecular ions ($[M-H]^-$) with m/z values of 329.2329, 329.2330, 329.2332, 295.2277, 295.2274, and 293.2113. On the basis of the elution times of those fractions and fragmentation patterns, it was hypothesized that these compounds are lipid oxidation products previously identified in pea protein and poppy seeds, which are known to cause bitterness.[18,22] Furthermore,

known taste-active compounds and free fatty acids were screened and analyzed against reference standards using LC-MS/MS and UPLC-TOF-MS. The results indicated that trihydroxyoctadecenoic acids and hydroxyoctadecenoic acids, found in various plant-based products, were also present in sunflower press cake.

The identified trihydroxyoctadecenoic acids included 9,12,13-trihydroxyoctadec-10-enoic acid (**1**) (*m/z* 329.2329, $[C_{18}H_{33}O_5]^-$), 9,10,11-trihydroxyoctadec-12-enoic acid (**2**) (*m/z* 329.2330, $[C_{18}H_{33}O_5]^-$), and 11,12,13-trihydroxyoctadec-9-enoic acid (**3**) (*m/z* 329.2332, $[C_{18}H_{33}O_5]^-$). Further lipid oxidation derivatives including (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**4**) (*m/z* 295.2277, $[C_{18}H_{31}O_3]^-$), (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**) (*m/z* 295.2273, $[C_{18}H_{31}O_3]^-$), (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**6**) (*m/z* 295.2274, $[C_{18}H_{31}O_3]^-$), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**) (*m/z* 295.2271, $[C_{18}H_{31}O_3]^-$) and (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**8**) (*m/z* 293.2113, $[C_{18}H_{29}O_3]^-$) was detected.

Free fatty acids affecting to the flavor profile of sunflower press cake included α-linolenic acid (**9**) (*m/z* 277.2180, $[C_{18}H_{29}O_2]^-$), linoleic acid (**10**) (*m/z* 279.2336, $[C_{18}H_{31}O_2]^-$), oleic acid (**11**) (*m/z* 281.2479, $[C_{18}H_{33}O_2]^-$), palmitic acid (**13**) (*m/z* 255.2324, $[C_{16}H_{31}O_2]^-$), and stearic acid (**14**) (*m/z* 283.2630, $[C_{18}H_{35}O_2]^-$). These compounds and 2-hydroxyoleic acid (**12**) (*m/z* 297.2433, $[C_{18}H_{33}O_3]^-$) are well-documented contributors to the sensory characteristics of some plant-based matrices, particularly bitterness and fatty taste attributes (**Figure 3**).

The E/Z isomer configurations were identified by comparing the retention times of the analytes to those of corresponding commercial reference compounds using LC-TOF-MS. Hydroxyoctadecadienoic acids (**4-7**) are recognized as metabolites in the lipoxygenase pathway, produced through the enzymatic activity of 9-/13-LOX and subsequent reduction by peroxygenases.[27–29] Their bitter activity has been previously reported.[18] Similarly,

trihydroxyoctadecenoic acids (**1-3**) have been previously identified in various plants and plant-based products, as well as their bitter activity.[18,22] The 2-hydroxy derivatives of the α-oxidation enzyme system of other plants[29–31] and the bitter taste threshold has been identified.[22] The (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid was previously identified as a product of the enzymatic pathway, where hydroperoxides are produced by 9- and 13-LOXs, followed by dehydration or dehydrogenation of fatty acid hydroxides.[22]

Fraction F1-4-12, displayed a significantly more complex composition, requiring further fractionation. Therefore, it was further fractionated by semi-preparative HPLC, and eight fractions were collected. The bitter target compound was contained in fraction F1-4-12-5, which was further purified with analytical HPLC for final structural analysis. After the isolation of the target compound by iterative HPLC fractionation, the structure of **15** (**Figure 4**) was determined using TOF-MS and 1D-/2D-NMR. First, high-resolution mass spectra were acquired, which showed a mass-to-charge (*m/z*) ratio of 607.2404, resulting in a predicted elemental composition of $C_{30}H_{39}O_{13}$ ($[M-H]^-$) in ESI negative mode. The calculated m/z ratio (607.2396 m/z for $C_{30}H_{39}O_{13}$, $[M-H]^-$) was in good agreement with the measured value, indicated by a mass error of 1.58 ppm. Additionally, MS^e spectra showed characteristic fragment ions, previously reported[32] with *m/z* ratios of 179.0348, 161.0240, and 135.0444, all of which suggested that caffeic acid (179.0349 *m/z*, $[M-H]^-$) is a putative constituent of **15** (**Figure 5, A**). To further elucidate the structure of **15**, NMR experiments were performed (**Table 2**). The ^{1}H- and ^{13}C-NMR spectra of compound **15** exhibited signals typical of a trans-caffeoyl moiety, which was identified via the AMX spin system consisting of the proton resonances H-C(9`), H-C(8`), and H-C(5`) at 6.76 ppm, 6.98 ppm, and 7.05 ppm. The characteristic coupling pattern as well as the coupling constants of the aromatic signals—8.2 Hz (doublet), 8.2/1.9 Hz (doublet of doublets), and 1.9 Hz (doublet)—were in

agreement with the values reported in the literature.[33] The distinctive trans-olefinic proton signals resonating at 6.26 ppm and 7.47 ppm, with a coupling constant of 15.8 Hz (doublet), supported by nine carbon signals C-1`–C-9` (δ 166.1, 114.2, 146.2, 124.7, 114.8, 145.9, 150.8, 122.2, 116.1) in the ^{13}C NMR spectrum, confirmed the presence of caffeic acid as a substructure of **15** (**Figure 5, B**). In addition, the ^{1}H-NMR spectrum displayed eight proton signals in the aliphatic region, integrating for twelve protons. The signals resonating at 0.59 ppm and 1.21 ppm could be assigned to methyl groups (H-(9), H-C(10)) integrating for three protons each. Two diastereotopic methylene groups resonating at 1.92–1.95 ppm/2.05–1.14 ppm (H-C(2)) and 1.58/2.24–2.30 ppm (H-C(8)) as well as three methine protons at 1.88–1.93 ppm (H-C(3)), 2.41 ppm (H-C(5)), and 4.39 ppm (H-C(1)) were assigned using the heteronuclear (C,H) single quantum coherence and homonuclear (H,H) correlated spectroscopy experiments. Moreover, the presence of an exocyclic double bond, resonating at 4.81/5.01 ppm (H-C(7)), was assigned using heteronuclear multiple bond correlation (HMBC) spectroscopy, optimized for $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings. The correlation of proton signals H-C(1), H-C(3), H-C(5), H-C(7), H-C(8), H-C(9), and H-C(10) with carbon C-5 (50.4 ppm) cumulatively indicated the presence of a pinocarveol moiety. This assignment was supported by the identification of the two quaternary carbon atoms C-4 at 40.1 ppm and C-6 at 151.2 ppm, which were determined using ^{13}C-NMR. The presence of a pinocarveol group was further supported by the respective carbon signals C-1–C-7 (δ 72.6, 32.2, 39.6, 40.1, 50.4, 151.2, 114.1, 27.2, 1.2, 0.6), which closely resembled previously reported values[34] (**Figure 5C**). In addition to caffeic acid and pinocarveol, the presence of two carbohydrate moieties in **15** was indicated by the presence of two anomeric proton signals at 4.37 ppm (H-C(1``)) and 4.79 ppm (H-C(1```)). It is hypothesized that these residues include a hexose and a branched-chain pentose, evidenced by the ^{13}C-NMR spectrum, which showed 11 aliphatic carbon signals, including one

quaternary carbon (C-3```) at 79.6 ppm and three methylene carbon resonance signals at 67.4 ppm, 63.6 ppm, and 73.8 ppm, respectively. The carbohydrates were ultimately identified as D-glucose and D-apiose after acidic hydrolysis, followed by chemical derivatization with L-cysteine methyl ester and phenyl isothiocyanate using LC-MS/MS, as described previously.[35] The β-glycosidic linkage of the glucose was indicated by the coupling constant of 7.9 Hz of H-C(1``), and the anomeric configuration of the apiose moiety was determined to be β on the basis of a comparison of the ^{13}C-NMR data for **15** with those of α- and β-D-apiofuranoside[36] and coupling constants of 2.8 Hz (H-C(1```)) consistent with the reported data for β-D-apiofuranoside (J = 2.6 Hz).[37–39] Finally, the connections of the individual substructures were determined via the HMBC correlations of H-C(1) of pinocarveol (4.39 ppm) with C-1`` (101.8 ppm) of glucose, the proton at position H-C(4``) of glucose (4.59 ppm) and C-1` of caffeic acid (169.1 ppm), as well as the methylene protons H-C-(6``) of glucose (3.36/3.49 ppm) with C(1```) of apiose (109.7 ppm), as highlighted in **Figure 5D**. Consequently, compound **15** was identified as pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside. To the best of our knowledge, compound **15** has not yet been described in the literature. Though similar phenolic glycosides like benzyl alcohol β-D-apiofranosyl-(1→6)-β-D-(4-O-caffeoyl) glucopyranoside were described as potent antioxidants found in sunflower seeds.[38] Additionally, multiple terpenoid glycosides, like Campholenol-10-O-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, Myrtenol-10-O-α-D-apiofuranosyl-(1→6)-β-d-glucopyranoside among others were shown to possess protective effects against H_2O_2-induced myocardial cell injury.[39] However, there are no reports indicating any taste activity.

Sensory Activity of Bitter Compounds.

To assess the bitter taste properties of compounds in sunflower press cake, we referred to the taste threshold concentrations reported in recent studies.[18,22] These thresholds were determined using a 3% aqueous ethanol solution in a two-alternative forced choice test to address the solubility challenges of hydrophobic compounds. The reported thresholds for the compounds listed in **Table 3** include a notably low bitter threshold for 2-hydroxyoleic acid and slightly higher thresholds for 9,12,13-trihydroxyoctadec-10-enoic acid and its isomeric counterparts (9,10,11-trihydroxyoctadec-12-enoic acid and 11,12,13-trihydroxyoctadec-9-enoic acid) at 0.13 mmol/L. Previously reported thresholds for free fatty acids, such as linoleic acid, oleic acid, palmitic acid, and stearic acid, indicate that these compounds display relatively higher bitter thresholds than α-linolenic acid. The newly identified pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside showed a bitter taste recognition threshold of 0.42 mmol/L.

Quantitation of Bitter Compounds in Sunflower Press Cake and Calculation of Dose-over-Threshold Factors.

The free fatty acids (FFAs) oleic acid, linoleic acid, palmitic acid, stearic acid, and α-linolenic were identified as the predominant compounds in the tested sunflower press cake, with concentrations of 96.64, 52.56, 47.31, 28.36, and 2.32 mmol/kg, respectively. Although no other comparative quantitative data for sunflower press cake are available, this distribution is in line with previous studies reporting that oleic and linoleic acids are consistently found in the highest concentrations among FFAs in various sunflower samples.[40–44] Traditional sunflower oil, which has been widely cultivated, contains moderate levels of oleic acid (14%–39%) and high levels of linoleic acid, typically over 50%, reaching up to 61% in some cases. However, the fatty acid composition of sunflower oil is highly variable depending on the breeding strategies implemented. For instance, high-oleic sunflower varieties have been bred to contain over 75% oleic acid, and

high-stearic–high-oleic sunflower varieties contain approximately 15%–20% stearic acid. Other fatty acids such as palmitic acid generally range between 4.6% and 7% across different sunflower oil types, whereas α-linolenic acid remains very low, typically below 0.1% in most varieties.[40,42–52] The concentrations of trihydroxy-octadecenoic acids (THOAs) and hydroxy-octadecadienoic acids were significantly lower, ranging from 0.07 to 0.33 mmol/kg and 0.03 to 0.61 mmol/kg, respectively.

In addition to fatty acids and their oxidation products, the second most intense bitter fraction, identified by the TDA, was fraction F1-4-12. This fraction was shown to contain pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (**15**) with a bitter threshold concentration of 0.42 mmol/L. To demonstrate the importance of this compound to explain the overall bitter off-taste of sunflower press cake, UHPLC-MS/MS quantification was performed. The analysis of sunflower press cake revealed a concentration of 1.41 mmol/kg of compound **15**. Since this compound was isolated and described for the first time, there are no reference concentration ranges available in the literature. However, isolating protein from plant sources often leads to an enrichment of secondary plant metabolites. These secondary metabolites frequently possess a bitter off-taste and are typically present in concentrations similar to that of compound **15** in the final protein isolates, as reported in the literature.[20,21]

To assess the bitter taste impact of compounds **1**−**15**, dose-over-threshold (DoT) factors were calculated as the ratio of the taste threshold concentration for each specific tastant.[17,53–56] The DoT signifies taste relevance, with values above 1 indicating a direct contribution to bitterness. The taste threshold values used in these calculations were previously reported in the literature.[18,22,57] The calculation of DoT factors revealed that oleic acid (DoT 98.6) had the highest bitter taste impact in the tested sunflower press cake, followed by linoleic acid (DoT 56.5), palmitic acid (DoT

58.4), stearic acid (DoT 35.0), and α-linolenic acid (DoT 8.3). The FFAs had DoT values over 1, indicating that this substance class contributes to the bitterness. Among the oxidized fatty acids, THOAs, particularly 9,12,13-trihydroxyoctadec-10-enoic acid (DoT 1.1) and 11,12,13-trihydroxyoctadec-9-enoic acid (DoT 2.5) exhibited the highest bitter impact.

To date, there has been no quantitative analysis of the key bitter compounds present in sunflower press cake. The high DoT values observed for FFAs such as oleic acid, linoleic acid, palmitic acid, stearic acid, and α-linolenic acid as well as the THOAs 9,12,13-trihydroxyoctadec-10-enoic acid and 11,12,13-trihydroxyoctadec-9-enoic acid are consistent with the significant amount of residual oil in sunflower press cake, ranging from 7% to 16.6% depending on the extraction process.[12,49,58,59] This residual oil contains free fatty acids and their oxidation products and consequently contributes significantly to the perceived bitterness of sunflower press cake. Additionally, this study identified a novel bitter tastant, representing a previously unrecognized class of bitter compounds. Pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside showed a DoT value of 3.4, indicating a direct contribution to the overall bitterness of sunflower press cake. Therefore, it can be concluded that, in addition to fatty acids and their oxidation products, compound **15** contributes to the bitter off-flavor in sunflower press cake. Detailed sensory reconstitution, as well as omission experiments, will be performed in a future study to elucidate the precise contribution of individual constituents to the overall off-flavor profile of sunflower press cake and investigate how these compounds are generated during the food processing of sunflower seeds. It is also important to recognize that the chemical composition may be influenced by both the genetic background of the sunflower cultivar and the processing conditions, as these factors can lead to differences in the types and concentrations of free fatty

acids and secondary metabolites, ultimately resulting in variations in their contribution to the overall sensory profile.[4]

In summary, the application of the sensomics approach provided detailed insights into the bitter off-taste profile of sunflower press cake, a promising by-product for sustainable protein sourcing. By identifying key bitter compounds, including free fatty acids and their oxidation products, as well as a novel terpenoid glycoside, this study has established the primary contributors to the off-flavor challenges that limit the broader acceptance of sunflower press cake in food applications. These findings offer a pathway to mitigating bitterness through targeted processing and formulation strategies, paving the way for sunflower press cake to become a more palatable and viable option in addressing the global protein demand. Strategies such as enzymatic hydrolysis, fermentation, selection of clean protein isolates, and flavor masking or texture adaptation have been proposed to mitigate off-flavors in plant proteins.[9] Future research should focus on refining processing techniques to reduce these compounds while preserving nutritional value, to further enhance the palatability and consumer acceptance of sunflower press cake.

Author Contributions

L.H. and M.G. contributed equally.

Notes

The authors declare no competing financial interest.

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Figure 1. Sensory analysis of solid-phase extraction fractions F1-1−F1-5 isolated from sunflower press cake. The panelists were asked to rate the bitterness and astringency on a scale from 0 (not detectable) to 5 (strongly detectable). Error bars represent the 95% confidence interval of the mean value.

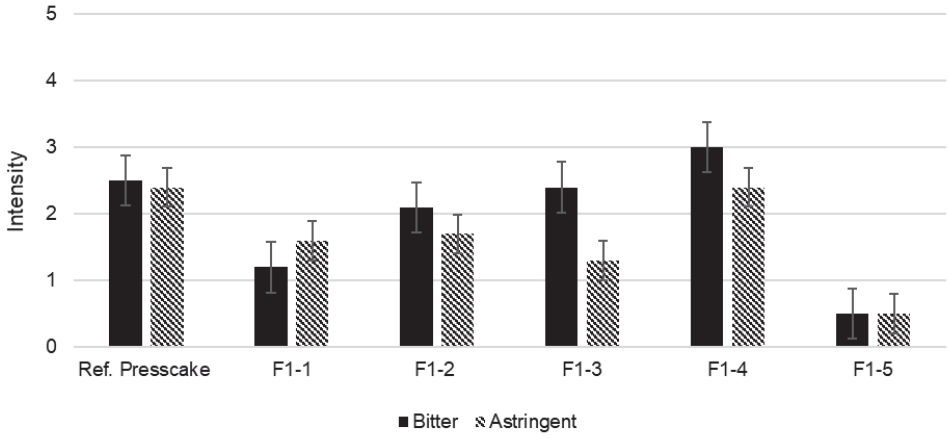
Figure 2. Reversed-Phase High-Performance Liquid Chromatography with Evaporative Light Scattering Detection (RP-HPLC ELSD) chromatogram of F1-4 along with taste dilution (TD) factors of collected subfractions F1-4-1 – F1-4-17.

Figure 3. Chemical structures of identified compounds from sunflower press cake: 9,12,13-trihydroxyoctadec-10-enoic acid (**1**), 9,10,11-trihydroxyoctadec-12-enoic (**2**), 11,12,13-trihydroxyoctadec-9-enoic acid (**3**), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**4**), (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**), (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**6**), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**8**), α-linolenic acid (**9**), linoleic acid (**10**), oleic acid (**11**), 2-hydroxyoleic acid (**12**), palmitic acid (**13**), stearic acid (**14**), and pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside (**15**) (shown in Figure 4).

Figure 4. Chemical structure of pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside. Arbitrary carbon numbering refers to nuclear magnetic resonance (NMR) assignments given in **Table 2**.

Figure 5. MS^e (20–40 eV, ESI^- mode) spectrum of bitter tastant **15** isolated from sunflower press cake (**A**); Chemical structure of **15** showing key correlations for structural elucidation via nuclear magnetic resonance (NMR) spectroscopy (**B**); Excerpts of heteronuclear multiple bond correlation (HMBC) spectrum (600/150 MHz, DMSO-d_6, 300 K) of **15** indicating the presence of a pinocarveol moiety (**C**) and the connection of individual substructures (**D**).

780 **Huseynli et al. Figure 1**



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782 **Huseynli et al. Figure 2**

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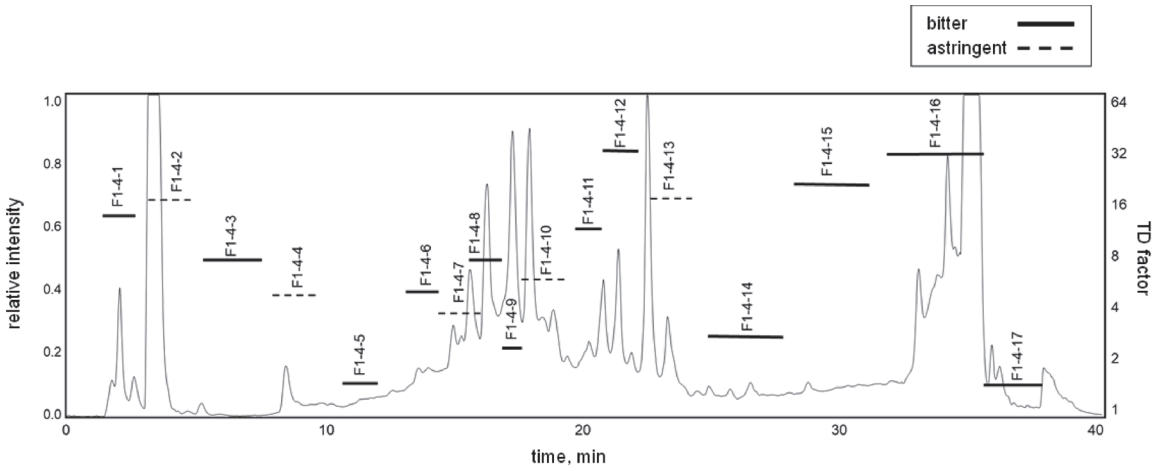

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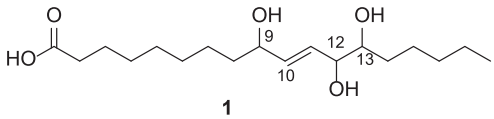
Huseynli et al. Figure 3



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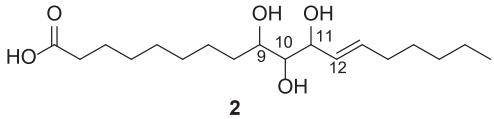

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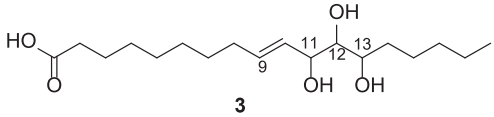

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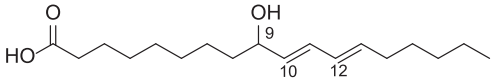

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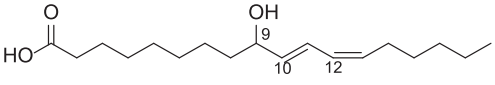

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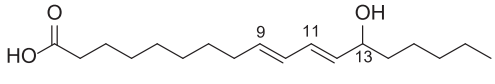

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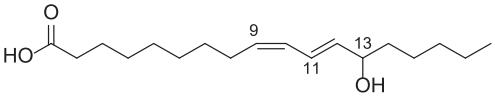

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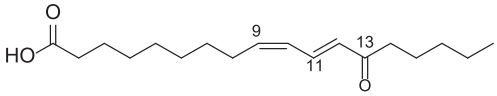

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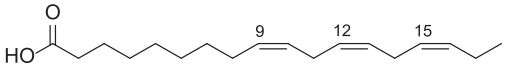

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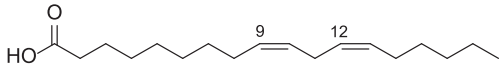

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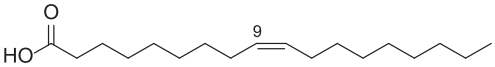

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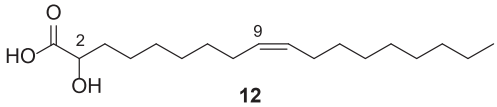

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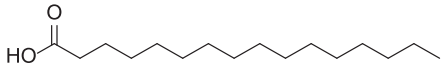

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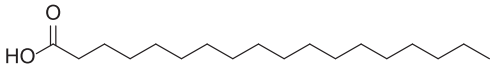

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788 **Huseynli et al. Figure 4**

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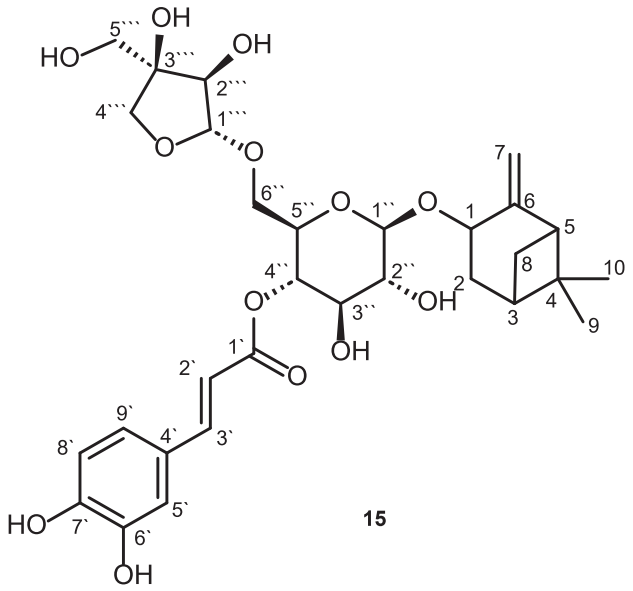

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791 **Huseynli et al. Figure 5**

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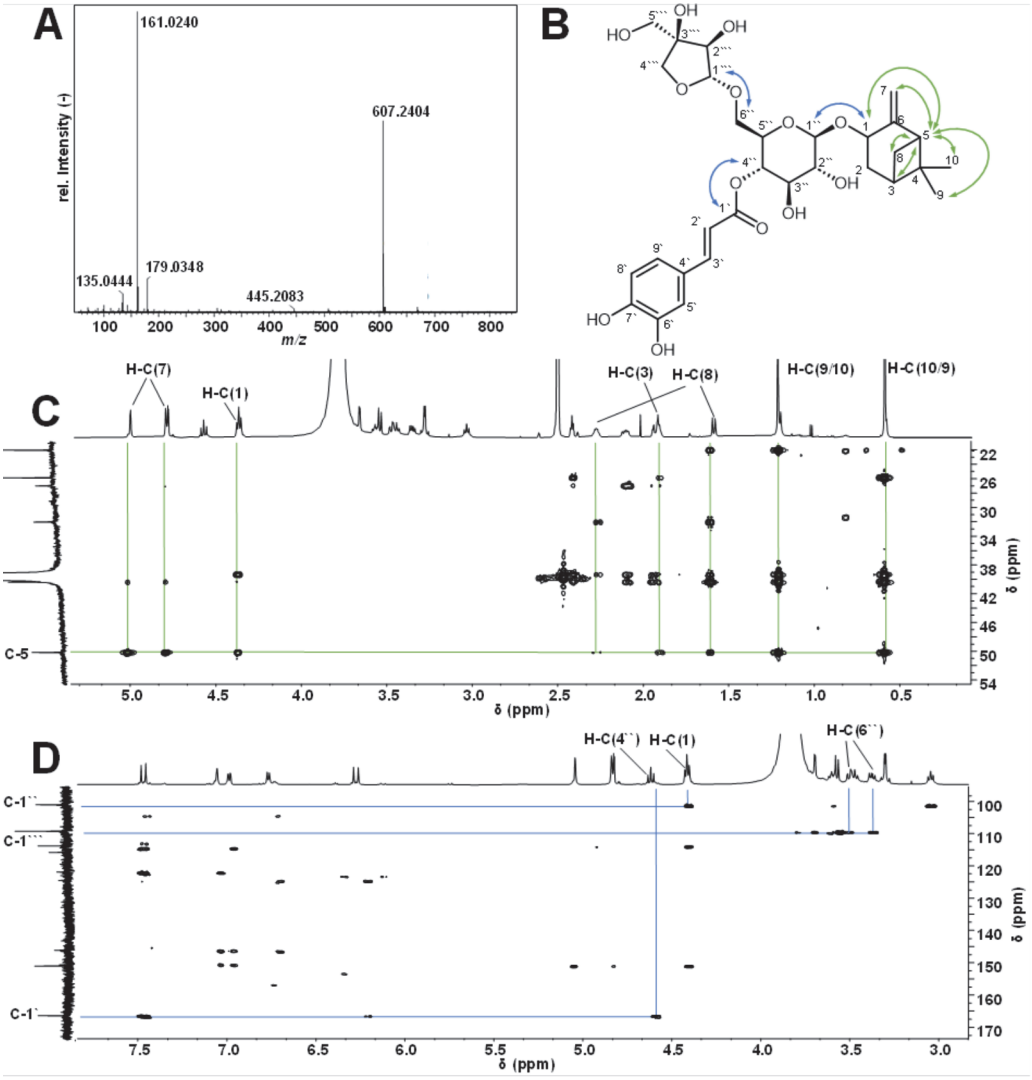

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TOC graphic

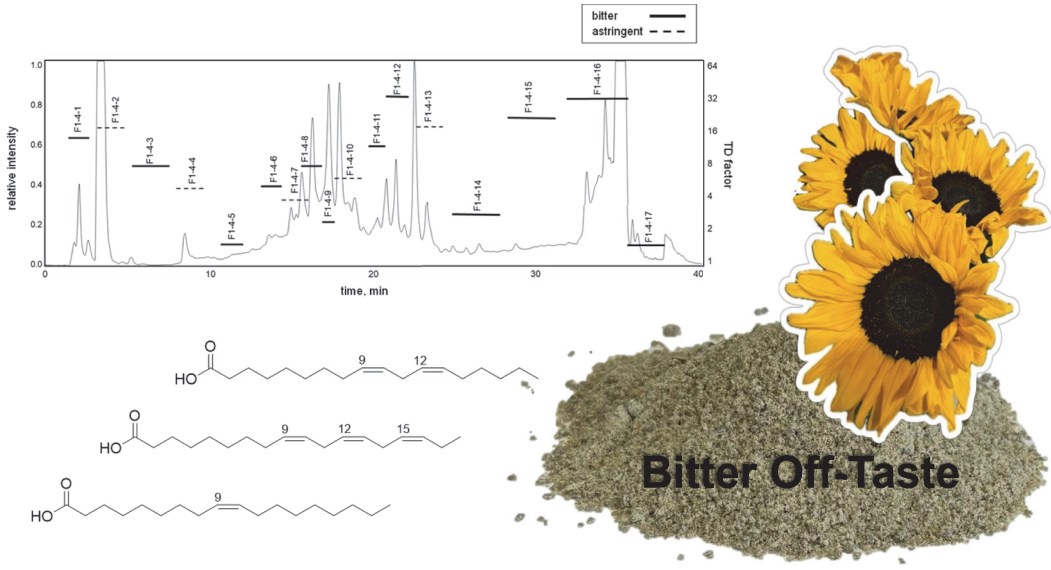

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Table 1. Sensory Evaluation of Sunflower press cake Isolated Fractions. The panelists were asked to rate aqueous solutions of the natural concentrations of the fractions F1−F4 and the residue.

	taste intensities for individual fractions[a]				
Taste attributes	**Press cake**	**F1**	**F2**	**F3**	**F4**
sweet	0.4	0.3	0.1	0.2	0.2
sour	0.8	0.6	0.2	0.5	0.3
umami	0.3	0.2	0.1	0.2	0.1
salty	0.4	0.2	0.1	0.2	0.3
bitter	2.5	2.5	1.0	1.1	0.9
astringent	2.4	1.8	1.0	1.1	1.1

[a]The intensity of the individual taste descriptors was rated by a trained panel on a scale from 0 (not detectable) to 5 (strongly detectable).

Table 2. ^{1}H- and ^{13}C-NMR assignments (600/150 MHz, DMSO-d_6, 300 K) of pinocarveol D-apiofuranosyl-(1→6)-β-D-(4-*O*-caffeoyl) glucopyranoside.

	Position	δ_C (ppm)	HSQC	δ_H (ppm)	M (*J*, Hz)
pinocarveol	1	72.6	[CH]	4.39	d (*J* = 7.3 Hz)
	2	32.2	[CH_2]	1.92–1.95 2.05–2.14	m m
	3	39.6	[CH]	1.88-1.93	m
	4	40.1	[C]	-	-
	5	50.4	[CH]	2.41	t (*J* = 5.4 Hz)
	6	151.2	[C]	-	-
	7	114.1	[CH_2]	4.81 5.01	s s
	8	27.2	[CH_2]	1.58 2.24–2.30	d (*J* = 9.5 Hz) m
	9	1.21	[CH_3]	25.9	s
	10	0.59	[CH_3]	22.3	s
caffeic acid	1`	166.1	[C]	-	-
	2`	114.1	[CH]	6.26	d (*J* = 15.8 Hz)
	3`	146.2	[CH]	7.47	d (*J* = 15.8 Hz)
	4`	124.7	[C]	-	-
	5`	114.8	[CH]	7.05	d (*J* = 2.2 Hz)
	6`	145.9	[C]	-	-
	7`	150.8	[C]	-	-
	8`	122.2	[CH]	6.98	dd (*J* = 1.9, 8.3 Hz)
	9`	116.1	[CH]	6.76	d (*J* = 8.2 Hz)
β-D-glucose	1``	101.8	[CH]	4.37	d (*J* = 7.8 Hz)
	2``	73.6	[CH]	3.04	t (*J* = 8.5 Hz)
	3``	74.1	[CH]	3.45	t (*J* = 9.5 Hz)
	4``	72.1	[CH]	4.59	t (*J* = 8.5 Hz)
	5``	73.2	[CH]	3.58–3.62	m
	6``	67.4	[CH_2]	3.36 3.49	dd (*J* = 6.3, 11.6 Hz) dd (*J* = 2.4, 11.6 Hz)

β-D-apiose	1```	109.7	[CH]	4.79	d (J = 2.8 Hz)
	2```	76.0	[CH]	3.66	d (J = 2.5 Hz)
	3```	79.6	[C]	-	-
	4```	63.6	[CH_2]	3.26–3.31	m
	5```	73.8	[CH_2]	3.55 3.76	d (J = 9.5 Hz) d (J = 9.5 Hz)

835 **Table 3.** Bitter Taste Threshold Concentrations and calculated DoT factors of Compounds found in

836 Sunflower press cake

compound no.	compound name	concentrations [mmol/kg]	bitter threshold concentration [mmol/L]	DoT factor
1	9,12,13-trihydroxyoctadec-10-enoic acid	0.14 ± 0.01	0.13 [b]	1.1
2	9,10,11-trihydroxyoctadec-12-enoic acid	0.07 ± 0.01	0.13 [b]	0.5
3	11,12,13-trihydroxyoctadec-9-enoic acid	0.33 ± 0.07	0.13 [b]	2.5
4	(10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid	0.03 ± 0.01	0.35 [a, b]	0.08
5	(10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid	0.11± 0.01	0.79 [a, b]	0.1
6	(9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.55 ± 0.15	0.97 [a]	0.6
7	(9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.61± 0.03	0.79 [a,b]	0.8
8	(9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid	0.65± 0.06	0.79 [a]	0.8
9	α-linolenic acid	2.32 ± 0.40	0.28 [a]	8.3
10	linoleic acid	52.56 ± 2.03	0.93 [a]	56.5
11	oleic acid	96.64 ± 4.20	0.98 [a]	98.6
12	2-hydroxyoleic acid	0.01 ± 0.01	0.06 [b]	0.2
13	palmitic acid	47.31 ± 2.45	0.81 [a]	58.4
14	stearic acid	28.36 ± 0.37	0.81 [a]	35.0
15	pinocarveol *β*-D-apiofuranosyl-(1→6)-*β*-D-(4-*O*-caffeoyl) glucopyranoside	1.41 ± 0.10	0.42	3.4

837 [a] Taste threshold taken from Lainer et al. (2020) [b] Taste thresholds are taken from Gläser et al. (2020)

Curriculum vitae

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Keelteoskus

Inglise keel	C1
Eesti keel	A2
Aserbaidžaani keel	emakeel
Türgi keel	C2
Vene keel	B2

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