Tampere University

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Taste of Nordic Forests – Analysis of the Taste of Mushrooms and Taste Modification Properties of Nanocellulose

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ACADEMIC DISSERTATION
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ABSTRACT

The assessment of taste eliciting compound concentrations and interactions is a vital part of food sciences. Even though many interactions are known and widely studied, both the complexity of food and the unique nature of human taste perceptions make it more difficult to draw conclusions about the impact of these interactions on actual perception solely based on instrumental studies. In this thesis, the taste properties of two different matrices were studied with sensory and instrumental methods, the emphasis being on instrumental analysis. First, the taste of mushrooms was analyzed as a part of a larger research entity on the total flavor and hedonic liking of the Nordic mushrooms. Simultaneously, free amino acid and nucleotide/nucleoside contents of mushrooms were analyzed and combined with results from sensory analysis and data of sugar, sugar alcohol and organic acid composition. Secondly, to learn about possibilities to utilize nanocellulose in food applications, particularly in bitterness modification, the impact of nanofibrillar cellulose on selected taste compounds was analyzed with a novel high-throughput method based on fluorescent indicator displacement and further, with sensory methods.

In this thesis, new methods were developed to analyze the free amino acid and nucleotide/nucleoside contents in mushrooms and the binding strengths between nanocellulose and taste compounds. These results were compared with the results from sensory evaluations. According to the statistical analysis, umami taste of mushrooms was not correlated primarily with glutamic acid or sweetness with total sugar contents, but they correlated better with umami enhancing nucleotides/EUC values and with the sugar-acid ratio, respectively. Further, relatively high binding was found particularly between nanofibrillar cellulose and bitter tasting quinine while compounds like sucrose, glutamic acid or aspartame showed no binding letting us to believe that nanocellulose could have ability to suppress bitterness. However, according to sensory studies, the taste modification ability of nanocellulose was on the same level or poorer as with carboxymethyl cellulose that was used as a reference. Both research entities emphasize the complexity of the taste perception. Even though instrumental methods provide valuable guidelines to be followed, particularly when studying novel materials, such as nanocellulose in this dissertation, the effect of chemical composition on taste is difficult to predict without sensory evaluations.

TIIVISTELMÄ

Makuyhdisteiden pitoisuuksien ja niiden vuorovaikutuksien tutkiminen on tärkeä osa ruokatutkimusta. Koska ruoka on matriisina monimutkainen ja ihmisen aistimukset yksilöllisiä, ei yksittäisten yhdisteiden pitoisuuksien perusteella voida kuitenkaan muodostaa kokonaiskuvaa aistimuksesta. Tässä väitöskirjassa tutkittiin kahden erilaisen matriisin makuun vaikuttavia tekijöitä erityisesti instrumentaalisilla, mutta myös aistinvaraisilla menetelmillä. Ensimmäisessä osassa tutkittiin sienten makua osana laajempaa suomalaisten sienten kokonaisflavoria ja miellyttävyyttä tutkivaa kokonaisuutta. Vapaiden aminohappojen sekä nukleotidien/nukleosidien pitoisuudet yhdistettiin sokereiden, sokerialkoholien- ja orgaanisten happojen pitoisuuksiin sekä aistinvaraisen arvioinnin tuloksiin tilastoanalyysin avulla. Toisessa osassa tutkittiin nanofibrilloidun selluloosan vaikutusta ruoan makuun. Tarkoituksena oli arvioida, voitaisiinko nanoselluloosaa käyttää ruokasovelluksissa erityisesti ruoan karvauden vaimentamiseen. Makuyhdisteiden sitoutumista nanoselluloosamatriisiin tutkittiin instrumentaalisesti työssä kehitetyllä fluoresoivan merkkiaineen korvautumismenetelmällä sekä aistinvaraisella analyysillä.

Työssä kehitettiin uusia kemiallisia menetelmiä sekä vapaiden aminohappojen ja nukleotidien/nukleosidien analysoimiseen sienissä, että nanoselluloosan ja makuyhdisteiden välisten vuorovaikutuksien arvioimiseen. Tulokset osoittavat, että sienten umamimakuun eivät vaikuta ainoastaan umamiaminohapot eivätkä makeuteen sokerit, vaan niiden lisäksi umaminukleotidit ja umamiyhdisteiden kokonaispitoisuus korreloivat umamin ja sokeri/happo-suhde makeuden aistimuksen kanssa. Instrumentaalisissa analyyseissa korkein sitoutumisvakio löydettiin karvaalta maistuvan kiniinin ja nanoselluloosan väliltä, kun taas sakkaroosi, glutamiinihappo ja aspartaami eivät sitoutuneet, minkä vuoksi nanoselluloosan oletettiin voivan toimia maunmuokkaajana elintarvikkeissa. Aistinvaraisissa analyyseissa nanoselluloosa kuitenkin osoittautui saman tasoiseksi tai huonommaksi maunmuokkaajaksi kuin referenssinä käytetty karboksimetyyliselluloosa. Väitöskirja osoittaa siis osaltaan makuaistimuksen monimutkaisuuden. Instrumentaalisten tutkimusten avulla saadaan suuntaviivoja aistinvaraisiin tutkimuksiin, mutta yksittäisten yhdisteiden tai yhdisteryhmien pitoisuuksien avulla ei voida tehdä johtopäätöksiä ruoan kokonaisaistimuksesta.

CONTENTS

ABBREVIATIONS

AMP	adenosine monophosphate
BC	bacterial cellulose
CFW	calcofluor white
CMC	carboxymethyl cellulose
CMP	cytidine monophosphate
CNC	cellulose nanocrystals
CR	Congo red
EUC	equivalent umami concentration
FAA	free amino acids
FD	fluorescence detector
FI	fluorescent indicator
FMOC	fluorenylmethyloxycarbonyl
GMP	guanosine monophosphate
GPCR	G protein-coupled receptor
HPLC	high performance liquid chromatography
IMP	inosine monophosphate
ITC	isothermal titration calorimetry
MFC	microfibrillar cellulose
MSG	monosodium glutamate
NFC	nanofibrillar cellulose
OPA	o-phthalaldehyde
QHCl	quinine hydrochloride
PCA	principal component analysis
PLS	partial least squares
TRC	taste receptor cell
UMP	uridine monophosphate
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
XMP	xanthosine monophosphate

ORIGINAL PUBLICATIONS

Publication I Hanna Manninen, Minna Rotola-Pukkila, Heikki Aisala, Anu Hopia and Timo Laaksonen. Free amino acids and 5'-nucleotides in Finnish forest mushrooms. *Food Chemistry*, vol. 247, pp. 23 – 28, May 2018.

Publication II Heikki Aisala, Hanna Manninen, Timo Laaksonen, Takao Myoda, Kaisa Linderborg, Anu Hopia and Mari Sandell. Linking volatile and nonvolatile compounds to sensory profiles and consumer liking of wild edible Nordic mushrooms. *Food Chemistry*, vol. 304, pp. 125403, Jan 2020.

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AUTHOR´S CONTRIBUTION

Publication I H. Manninen planned the publication, carried out the experiments and is the corresponding author of the publication. M. Rotola-Pukkila and H. Manninen contributed in method development. M. Rotola-Pukkila, H. Aisala, A. Hopia and T. Laaksonen contributed in designing of the experiments and drafting the manuscript. All authors accepted the publication.

Publication II H. Manninen supplied the free amino acid data and 5'-nucleotide data, and created the multivariate models linking non-volatile compounds and sensory data together with M. Sandell and H. Aisala. H. Manninen and T. Laaksonen interpreted the NMR data for the unknown compound in curry milk cap. H. Manninen participated in interpreting the results and wrote the manuscript together with H. Aisala. H. Aisala performed the NMR experiments and the consumer test, processed and analyzed the data. All authors participated in writing of the manuscript and accepted the publication. The publication is included also in the dissertation by H. Aisala (Aisala, 2019), University of Turku.

Publication III H. Manninen planned and carried out the experiments as well as interpreted the results under the supervision of T. Laaksonen, A. Hopia and N. Durandin. H. Manninen drafted the manuscript and is the corresponding author. All authors participated in writing the manuscript and accepted the publication.

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1 INTRODUCTION

Taste compounds are a wide class of mainly water-soluble substances able to interact with the taste receptors inside the oral cavity (Bachmanov & Beauchamp, 2007). Taste has a major influence on the food choice (Drewnowski, 1997). Even though the links between taste perceptions, preferences and food choices are complex as taste responses are impacted by different factors starting from genetic variances, there are some universal guidelines on preferred taste qualities (Drewnowski, 1997). As an example, sweet foods are preferred whereas bitter tastes are often disliked as aversive bitterness might indicate the toxicity of the food (Bachmanov & Beauchamp, 2007; Drewnowski, 1997). Taste is a part of flavour perception consisting of tastes such as sweet, bitter, salty, sour, umami, odors such as beany or fruity, the chemesthesis such as burn from chilis or cooling from menthol and the mouthfeel (Lawless & Heymann, 2010). The exact definition of flavour according to International Standards Organization (ISO 5492, 1992) is that flavour is "Complex combination of the olfactory, gustatory and trigeminal perceptions perceived during tasting. The flavour may be influenced by tactile, thermal, painful and /or kinaesthetic effects" (Delwiche, 2004; ISO 5492, 1992).

In order to improve the palatability or other aspects such as the healthiness or cost-efficiency of food, it is important to know the chemical constitution of it. The aim in instrumental flavour analysis is often to find the most potent character-impact compounds, or to analyse the concentrations or changes in the concentrations of known flavour compounds. However, due to the complexity of human perception of flavour as well as the complexity of food as a matrix, sensory analyses are needed to form links between actual perception and measured physicochemical characteristics of a compound. These links can be complex, nonlinear, and/or strongly affected by other components in the food. Finally, to form links between instrumental measurements and sensory analysis, statistical methods are usually utilized. The data analysis and interpretation, together with planning of the studies, are the most crucial steps of flavor analysis (Yu, Low, & Zhou, 2018). In this thesis, both instrumental and sensory methods were applied in two case studies aiming to gather knowledge about the taste qualities and in order to improve the palatability of

foods: 1) Taste of Nordic mushroom samples is analyzed 2) The taste modification properties of nanocellulose are studied. In both studies, both methods of chemical and sensory analyzes were used.

Mushrooms are a good addition to the vegetable based and sustainable diet due to their palatable taste and good nutritional value (Longvah & Deosthale, 1998; Manzi, Aguzzi, & Pizzoferrato, 2001; Mattila et al., 2001; Mattila, Salo-Väänänen, Könkö, Aro, & Jalava, 2002; Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002). Concerning these health benefits, some reviews argue that the interest on edible mushrooms will grow in the coming years (Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017; Roncero-Ramos & Delgado-Andrade, 2017). Different groups of chemical substances such as sugars, sugar alcohols, amino acids, nucleotides and organic acids have been linked with mushroom taste (Sun et al., 2020). Especially palatable umami taste has often been associated with mushrooms and the compounds provoking this taste quality have been widely studied (Sun et al., 2020). However, most of these studies have been done with Asian (Mau, Lin, Chen, Wu, & Peng, 1998; Mau, Lin, Ma, & Song, 2001; Tsai, Tsai, & Mau, 2008; Yang, Lin, & Mau, 2001) or certain Southern-European species (Barros et al., 2007; Beluhan & Ranogajec, 2011) while knowledge about the taste properties of Nordic mushroom species is lacking. Further, there are only a few studies combining the chemical analysis of taste compounds in mushrooms with sensory analysis.

The demand for healthier food has also brought bitterness modification as an important question for food industry as many vegetables have bitter side tastes. Bitterness modification has already been of interest to the pharmaceutical industry for long time but nowadays it is also the interest of functional food and beverage developers due to the fortification of these products with healthy but often bitter tasting compounds (Ley, 2008). There are multiple methods for bitterness modification for food industry, which are shortly reviewed in the background section of this thesis.

As bitterness modification is a big question, new food additives that can modify the bitter taste would be valuable. One such candidate is nanocellulose. Nanocellulose materials are cellulose material with at least one nanoscale dimension (Klemm et al., 2011). Despite the fact that the use of nanocellulose as food additive was one of the first applications proposed for nanocellulose materials in the 1980s (Turbak, Snyder, & Sandberg, 1982) nanocellulose materials are not used in food industry because of the formerly high price of these materials as well as still partially lacking safety evaluations (Gómez et al., 2016). However, several interesting applications for the use of nanocellulose materials have been proposed as they have

many profitable material properties such as high surface area and aspect ratio, suitable rheological behaviour and the easiness of chemical modifications (Gómez et al., 2016; Klemm et al., 2011). For example, nanocellulose materials have been utilized as food stabilizers, functional food ingredients and particularly in food packaging applications (Gómez et al., 2016). These methodologies are shortly reviewed in Chapter 2.6. along with the studies on nanocelluloses safety aspects. Despite the various potential applications of nanocellulose in food industry there have been no published studies on the taste of nanocellulose materials or even further on the effect of nanocellulose materials on taste. Both are central aspects for the utilization of nanocellulose materials in food industry applications.

The core content of the first part of this thesis is the taste evaluation of Nordic wild mushrooms by means of both chemical and sensory analysis while the later studies concentrate on studying the taste modification properties of nanofibrillar cellulose by both chemical and sensory analysis. The aims of this thesis were firstly, to analyze the taste compounds and taste characteristics of Nordic mushroom species, and the impact of them in total flavor profile and liking of mushrooms, and secondly, to evaluate the taste-modification properties of nanofibrillar cellulose both in terms of analyzing the chemical interactions by instrumental methods and actual taste perception by sensory studies. The thesis is divided into six chapters. In this chapter, the motives of the work were introduced. In Chapter 2, the theoretical background of this work is described in more details. The molecular background behind the taste of food is described as well as the methods of studying the taste. Also, the basic mechanisms of taste, such as the impact of different taste compounds on each other are shortly reviewed. The literature concerning the taste of mushrooms is discussed. Further, methods for modification of taste properties are introduced as well as previous studies of the use of nanocellulose in food applications are discussed.

In Chapter 3 the research questions of this thesis are introduced. The methodologies to answer the questions are described in Chapter 4. The method developed for the analysis of amino acid and nucleotide concentration in **Publication I** is described as well as the sensory methods used in **Publications II** and **IV**. Finally, the method based on fluorescence indicator displacement in the **Publication III** is described. The main results from the **Publications I-IV** are summarized at Chapter 5. Chapter 6 concludes the thesis, indicates the main conclusions to be drawn, and provides future perspectives.

2 BACKGROUND

2.1 Chemistry of taste

Five major taste modalities, sweet, bitter, sour, salty and umami, are often referred as 'basic tastes'. The definition and the term to use for 'basic taste' is a subject of ongoing scientific discussion. In this thesis, the term 'taste modalities' is used instead elsewhere but in this chapter reviewing the background of the term. The term 'basic taste' itself was introduced by Aristotle in 384-322 B.C. who included seven tastes in this category, namely sweet, sour, salty, bitter, astringent, pungent and harsh (Hartley, Liem, & Keast, 2019). During the 1800s the list was shortened to include only tastes as knowledge about different characteristics of tactile perceptions was gathered (Hartley et al., 2019). After this, sweet, salty, bitter and sour taste have been widely accepted as four basic tastes (Hartley et al., 2019). In the 2000s the list expanded to include also umami, as the taste receptors of it were found (Chandrashekar, Hoon, Zuker, & Ryba, 2006; Kurihara, 2015; Nelson et al., 2001).

The definition of 'basic taste' as well as the need for the whole concept has been under scientific discussion for many years (Beauchamp, 2019; Delwiche, 1996; Hartley et al., 2019). Delwiche (1996) criticized the concept as it oversimplifies and directs the design of scientific research of taste. As the definition of the term 'basic taste' is not precise itself, the role of umami as one has also been under the discussion. For example, Kurihara and Kashiwayanagi (2000) proposed three criteria for basic taste. Firstly, it must be different from any other basic tastes. Secondly, it cannot be replicated by combining other basic tastes and finally it has to be commonly consumed and induced as a food component (Kurihara & Kashiwayanagi, 2000). Further, it is often required that the basic taste has an identified receptor (Kurihara, 2015). According to these criteria, umami is a basic taste. However, for example Hartley et al. (2019) used more comprehensive criteria for the definition of basic taste with seven points to be fulfilled: The class of effective stimuli causing the taste perception must be distinct from other compounds (1), and the detection of stimuli must be evolutionary beneficial (2). Further, there must be a transduction mechanism to change the chemical code to electric signal (3) and neurotransmission to move this signal to the taste processing regions of the brain

(4). The perceptual quality must be independent of other tastes (5) and the stimuli must cause both hedonic (6) and physiological and/or behavioral (7) responses. According to Hartley et al. (2019) from these criteria, umami fulfils all but criteria five, as the umami taste has perceptual associations with salty and sweet tastes as well as putative taste (kokumi). Furthermore, recently Beauchamp (2019) made another proposal for the definition proposing that basic tastes should be defined as those taste qualities that historical and anthropological data have consistently shown to constitute the human taste world. According to this criterion, the role of umami as basic taste is ambiguous. The ongoing discussion about the basic taste definition also includes discussion about possible new basic tastes, especially fat or fatty acids (Keast & Costanzo, 2015) but also calcium (Tordoff, 2001), metallic (Lawless, Stevens, Chapman, & Kurtz, 2005), complex carbohydrates (Low, Lacy, McBride, & Keast, 2018) and kokumi (Bachmanov & Beauchamp, 2007). Fat is predominantly perceived due to its effect on texture, but mechanisms for generating taste sensation has also been suggested (Bachmanov & Beauchamp, 2007). The mechanisms proposed involve the inhibition of the delayed rectifying potassium channels by certain fatty acids (Gilbertson, Fontenot, Liu, Zhang, & Monroe, 1997), the involvement of fatty acid transporter CD36 in taste bud cells (Laugerette et al., 2005) or fatty acid receptors GPR40 and GPR120 (Cartoni et al., 2010; Matsumura et al., 2009).

2.2 Taste perception inducing chemical substances and taste receptors

The taste receptor cells (TRCs) are clustered in groups of 50 to 150 in the taste buds in the papillae of the tongue and palate epithelium (Chandrashekar et al., 2006). Bitter, sweet and umami taste receptors belong mainly to the group of G protein-coupled receptors (GPCR), particularly to families of taste receptor type 1 (T1R) and taste receptor type 2 (T2R) receptors, which are believed to function as part of TRC membranes (Bachmanov & Beauchamp, 2007). The T1R proteins comprise in about 850 amino acid and have a large N-terminus referred as a Venus flytrap domain whereas T2Rs have 300 – 330 amino acids and short N-terminus (Bachmanov & Beauchamp, 2007; Lawless & Heymann, 2010). T1R and T2R receptors are coupled with G-proteins which is an intracellular messenger in contact with receptors inside the cells (Lawless & Heymann, 2010). For salty and sour taste, many of the suggested

taste receptors are ion channels (Bachmanov & Beauchamp, 2007; DeSimone & Lyall, 2006). However, the mechanism particularly for salty taste remains controversial (Roper & Chaudhari, 2017). Both the substances eliciting the taste perceptions and the receptors are shortly reviewed in this chapter.

2.2.1 Bitterness and bitter receptors

The structural variety of bitter eliciting compounds is large and many of them are harmful (Ley, 2008; Meyerhof, Born, Brockhoff, & Behrens, 2011). In plants, important classes include alkaloids, terpenoids and flavonoids (Ley, 2008). As the variety of bitter molecules is wide, the structural qualities causing bitterness are difficult to point out. Some attempts to organize and classify and thus recognize structures to cause bitterness have been done. Rodgers, Busch, Peters and Christ-Hazelhof (2005) used the approach of a phytologenetic-like tree to analyze the bitter compounds found in databases to find out which structures are repeated in bitter tasting compounds. Further, the analysis of a database by Wiener, Shudler, Levit and Niv (2012) of over 500 bitter molecules revealed that most of the molecules were moderately hydrophobic and had the molecular weights of some hundreds of Daltons (Coupland & Hayes, 2014).

The bitter perception is induced by binding of a bitter molecule to a G protein-coupled T2R type receptor (Roper & Chaudhari, 2017). Approximately 25 different receptors have agreed to mediate bitter taste (Meyerhof et al., 2010). Most of the bitter taste receptors can detect multiple bitter substances as well as one bitter molecule can bind to many subtypes of T2R receptors (Meyerhof, 2005; Meyerhof et al., 2010). Thus, as both the variety of the bitter molecules and transduction pathways are complex, there are still knowledge gaps to be filled to form a more comprehensive picture about bitter sensation and its background.

2.2.2 Umami and umami receptors

The umami taste is mainly caused by the sodium salts of L-forms of glutamic acid and aspartic acid present in many ingredients such as kombu in Japanese cuisine and was first discovered in 1908 by K. Ikeda (Ikeda, 1908; Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012; Maga & Yamaguchi, 1983). This taste sensation is further enhanced by certain 5'-nucleotides such as 5'-inosinate, 5'-guanylate and 5'-adenylate (Yamaguchi, 1967; Yamaguchi, S. & Ninomiya, 2000). Furthermore, some

peptides have been demonstrated to have umami taste (Sun et al., 2020). The majority of the recognized umami peptides are either dipeptides or tripeptides which generally consist of hydrophilic amino acids such as umami amino acids glutamic and aspartic acid (Sun et al., 2020). For umami peptides with longer chains not only the amino acid composition but also the spatial structure effect the umami taste properties (Sun et al., 2020). These peptides and their interactions with receptors are further reviewed by Zhang, Sun-Waterhouse, Su and Zhao (2019a).

Receptors for sweet and umami taste were recognized as G protein-coupled T1R type receptors T1R2 and T1R3 for sweetness and T1R1 and T1R3 for L-amino acids (Li et al., 2002; Nelson et al., 2001). The simplified figure of T1R1+3 receptor and binding of monosodium glutamate (MSG) and nucleotides is presented in Fig. 1. The molecular mechanism for the umami taste synergism was proposed in 2008 by Zhang et al.. The mechanism proposed involves a cooperative ligand-binding model where glutamate binds to the hinge region of the Venus flytrap shaped T1R1 receptor and nucleotides further stabilize the closing of the flytrap by binding to an adjacent site of the receptor (Zhang et al., 2008). This has been proved by molecular dynamics simulations (Mouritsen & Khandelia, 2012). Besides T1R1/T1R3 receptor, also other receptors are suggested for umami taste including taste-specific variants metabotropic glutamate receptors mGluR1 and mGluR4 (Chaudhari, Landin, & Roper, 2000; San Gabriel, Maekawa, Uneyama, & Torii, 2009; Zhang et al., 2019a).

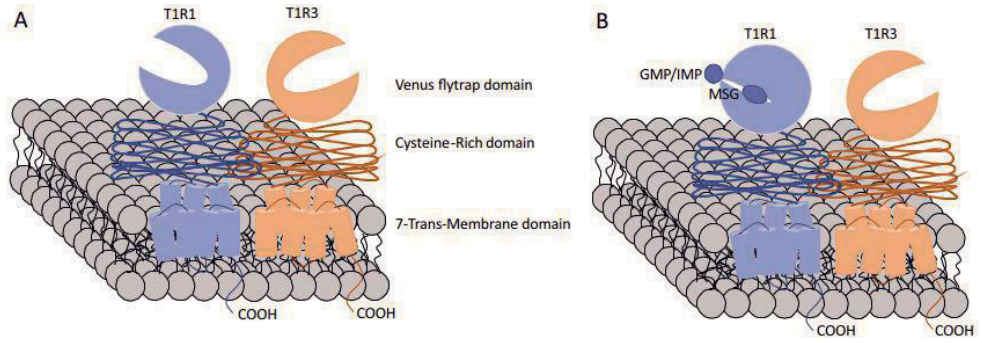


Figure 1. The simplified structure of T1R1/T1R3 taste receptors (A) and the mechanism of umami compound binding (B). Modified from (DuBois, 2016) based on (Mouritsen & Khandelia, 2012; Zhang, 2008).

2.2.3 Sweetness and sweet receptors

Sweetness detecting receptors, a heterodimer of T1R2 and T1R3, respond to a wide variety of different kinds of chemical substances including sugars, amino acids, glycosides, sweet proteins and some artificial compounds such as sucralose, aspartame, neotame, saccharin and acesulfame K (Chandrashekar et al., 2006; Masuda et al., 2012). The history of sweet taste is reviewed by Temussi (2006) who divides the research into two historical periods of before finding the sweet taste receptors in early the 2000s and after this. One early model attempting to explain the sweet taste of molecules is Shallenberger's and Acree's model (AH-B theory) that predicts that sweetness can be perceived if the distance of a hydrogen-bond donor (AH) to acceptor (B) is between 0.25 to 0.4 nm (Eggers, Acree, & Shallenberger, 2000; Schallenberger & Acree, 1967; Temussi, 2006). The same theory has been developed further mostly on the spatial geometry requirements of sweet molecules by different groups such as Kier (1972), Temussi et al. (1978; 1984), Kamphuis, Lelj, Tancredi, Toniolo and Temussi (1992) and Yamazaki, Benedetti, Kent and Goodman (1994). However, none of these models can explain the sweetness of some flexible structures or sweet-tasting proteins, such as brazzein (Temussi, 2006). The explanation is rather in different domains of the receptor that are used for the recognition of the different compounds (Chandrashekar et al., 2006). For example, Venus flytrap domain is needed for the recognizing aspartame and neotame (Xu et al., 2004) and Venus flytrap and cysteine-rich domain linking it to transmembrane domain for recognition of sweet proteins such as brazzein (Assadi-Porter et al., 2010; Jiang et al., 2004).

2.2.4 Sourness and saltiness

Sourness is evoked by acidic stimuli (DeSimone, Lyall, Heck, & Feldman, 2001) and saltiness is mainly induced by sodium salts (Bachmanov & Beauchamp, 2007). Sourness is not only depended on the concentration of hydrogen ions and thus pH, but it has been demonstrated in various publications that for example many organic acids are more sour than hydrochloric acid at the same pH (Da Conceicao Neta, Johanningsmeier, & McFeeters, 2007). For sour taste, there are several candidates as taste receptors (Bachmanov & Beauchamp, 2007). Ion channel PKD2L1 has been proposed for the primary taste receptor (Bachmanov & Beauchamp, 2007; Huang et al., 2006). These receptor cells response to the decrease in extracellular pH by triggering the action potential (Huang et al., 2006). However, as mice with the

PKD2L1 encoding genes knocked out retained at least partly their sensitivity to sour taste, it seems at least that this receptor is not the only receptor for sourness (Horio et al., 2011). Recently, Otopetrin-1 receptor was suggested as a mammalian sour receptor (Zhang et al, 2019b). Further, as organic acids, such as citric acid or acetic acid, can permeate to presynaptic type III cells in taste buds, the intracellular acidification of these cells and the blocking of the K+ channels have been suggested as proximate stimulus to sour taste (Huang, Maruyama, Stimac, & Roper, 2008; Lyall et al., 2001; Roper & Chaudhari, 2017; Ye et al., 2016).

For the salts, the reception mechanism is still unclear (Roper & Chaudhari, 2017). The amiloride-sensitive epithelial sodium channel (ENaC) has been proposed as a taste receptor based on studies with rodents (Heck, Mierson, & DeSimone, 1984; Oka, Butnaru, von Buchholtz, Ryba, & Zuker, 2013; Roper & Chaudhari, 2017). In some studies it is further suggested that in low or moderate concentrations, the responses are appetite generating and go through the epithelial amiloride-sensitive sodium channel (ENaC) pathways whereas in high concentration the sourness and bitterness sensing pathways are activated (Oka et al., 2013). However, the mechanism, receptors and further the role of ENaC in the salty taste perception of humans have not been clarified (Chandrashekar et al., 2006; Roper & Chaudhari, 2017).

2.2.5 Perceptions of taste in food

Concentration versus taste intensity curves are usually linear only in certain concentration ranges (Keast & Breslin, 2003). At high concentrations, taste perception becomes saturated whereas at subthreshold concentrations a small addition of a compound does not influence the perceived taste (Keast & Breslin, 2003). The psychophysical concentration-response function thus has roughly a sigmoidal shape (Keast & Breslin, 2003). Different parts of the curve can be modelled according to Stevens's power law (Stevens, 1960) stating that the intensity of sensation can be written as the power function of physical stimulus intensity (Keast & Breslin, 2003). Thus, for intensity of taste I can be written as

$$I = kC^n \tag{1}$$

where k is a constant, C concentration and n a variable associated with both the compound in question and its concentration (Breslin, 2001; Keast & Breslin, 2003).

In low concentrations, in the so-called expansive range, *n* gets values larger than one, which means that the perceived intensity grows faster than concentration. In linear range *n* equals one and in compressive range *n* is smaller than one, meaning that increasing the concentration results in a smaller increase in the perceived taste intensity. (Keast & Breslin, 2003)

Taste compounds are rarely tasted as solitary compounds. The rule of thumb is that when two taste compounds with different taste qualities are mixed, the intensity is less than the sum of individual taste intensities, particularly at high or moderate concentrations (Breslin, 2001; Keast & Breslin, 2003). This is commonly called mixture suppression (Lawless & Heymann, 2010). Suppression is seen as a rightward shift of the psychophysical concentration-response function, while the slope of the function stays unaltered (Breslin, 1996). Enhancement is the counterpart of suppression meaning the situation, where another component increases the taste intensity of another. In this situation, the psychophysical concentration-response function is shifted leftwards. Enhancement can occur particularly when to compounds of similar taste qualities are mixed. Both in suppression and enhancement, the slope of the concentration-response function remains the same. However, there are situations, where the slope is also altered. Term synergy is used for situation, where the concentration-response slope steepens and moves leftwards and masking for the counterpart situation. (Breslin, 2001) One common example of synergy is found between MSG and certain nucleotides (Lawless & Heymann, 2010; Yamaguchi, Shizuko, 1967). Masking is a rare process in food, but some examples can be found from the literature of bitter blocking or inhibiting agents (Breslin, 2001). In addition to the quality of the compounds the concentration of each of them affects the effect they have on each other. For example, according to review by Keast and Breslin (2003), salts enhance the sweetness at low concentrations and intensities, while in moderate concentrations the effect can vary. At high concentrations, salts either suppressed or had no effect on sweetness (Keast & Breslin, 2003).

The mechanisms where the taste compounds affect each other can be either chemical interactions, oral physiological interactions or cognitive interactions (Keast & Breslin, 2003). Chemical interactions can either result in completely new taste characteristics (such as salt formation in acid-base reactions) or change the intensity of taste via binding through weak forces such as hydrogen bonding (Keast & Breslin, 2003). Oral physiological interactions are interactions of mixture components altering the taste receptors or transduction mechanisms of the taste compound while cognitive interactions occur when the change in perceived taste due to other

component in the mixture is due to signal decoding and processing of the brain (Keast & Breslin, 2003; Lindemann, 2001). Kroeze and Bartoshuk (1985) studied the mechanism of the effects of sucrose and sodium chloride (NaCl) to the bitterness of quinine hydrochloride (QHCl) by utilizing a split-tongue taste stimulation test. In the test, either the whole tongue of the assessor was stimulated with a mixture of sweet/salty and bitter compounds at the same time or different sides of the tongue were exposed to different compounds (sweet or salty and bitter) (Kroeze & Bartoshuk, 1985). While with sucrose-QHCl no difference was seen in the suppression of bitterness between different tests, in NaCl-QHCl test more suppression was found in when the whole tongue was exposed to a mixture of compounds. Thus, the results suggest that the bitterness suppression by sucrose is due to cognitive interactions, while both oral physiological interactions and cognitive interactions influence on the suppression of bitterness by NaCl (Kroeze & Bartoshuk, 1985). The theory of oral physiological interaction is further supported by the ability of sodium salts (NaCl, sodium acetate, sodium gluconate) to decrease bitterness of various compounds similarly even as changing the anion decreases the perceived saltiness (Keast & Breslin, 2003).

It should be noted that not only taste compounds, but other sensory modalities influence the perception of taste (Lawless & Heymann, 2010). For example, certain salt-associated odors such as odor of sardines or bacon have been indicated to cause an increase in perceived of saltiness (Lawrence, Salles, Septier, Busch, & Thomas-Danguin, 2009). Furthermore, the burning sensation of capsaicin has been shown to reduce sweetness both of sucrose and tomato soup (Prescott, Allen, & Stephens, 1993) and temperature has been shown to influence the sweetness of sucrose in low concentrations (Bartoshuk, Rennert, Rodin, & Stevens, 1982). On top of these food matrix related variations, flavor release is impacted by mastication and saliva during eating, the adaptation of the senses as well as individual differences amongst people (Keast, Dalton, Breslin, & Taylor, 2004; Lawless & Heymann, 2010).

2.3 Measuring taste – comparing chemical and sensory methods

Flavor can be analyzed by either objective or subjective methods (Smyth & Cozzolino, 2013). Sensory evaluations based on human assessment are referred to as subjective measurements as the assessments of even highly trained panel might

vary due to physical and physiological factors (Huang, Lan, & Lacey, 2004; Smyth & Cozzolino, 2013). In addition, these methods are often time-consuming and might suffer from the adaptation or fatigue of the panel (Smyth & Cozzolino, 2013). Objective, instrumental methods are often cheaper as well as more accurate and precise (Ross, 2009). Further, knowing the constitution of the food helps, if the aim is to modify properties such as flavor or healthiness of the food. However, as flavor of food is complex phenomenon to which many factors influence, objective instrumental methods cannot fully mimic the human perception (Ross, 2009). The concentration of one compound analyzed from a food matrix does not tell much about actual flavor perception it causes, as eating is a dynamic process where the concentrations of compounds are affected by their solubilities, release from a matrix as well as rate of clearance by saliva (Linforth, 2000). Further, the flavor compounds have different thresholds and they interact with each other (Linforth, 2000). Thus, both instrumental and sensory measurements are needed to fully analyze and understand the flavor of food. For both sensory and instrumental analysis, a wide variety of methodologies can be found. Here, I will focus on the most central ones keeping in mind the topics of this thesis. Furthermore, the review of the instrumental analysis will focus on chemical analysis, of taste rather than flavor compounds. Besides traditional chemical analysis methods, different kinds of electronic tongues and noses have been developed consisting of different kinds of detectors in order to mimic the human perception (Huang et al., 2004). These methodologies are not included in this chapter but reviewed elsewhere, for example by Huang et al. (2004).

2.3.1 Instrumental methods on taste analysis

As the characteristics of flavor compounds as well as food matrices and the demands for the precision of the analysis vary greatly, the methodologies for both the extraction and instrumental analysis of flavor compounds have a wide spectrum. Depending on the sample of interest, sample handling and preparation methods are usually needed before actual analysis as foods are complex mixtures of many kinds of compounds which must be separated from each other prior to analysis. Different challenges are encountered depending on the properties (solubility, volatility, reactivity, interactions with matrix/other compounds) of the compound of interest. The sample handling and preparation usually involve homogenization (cutting, grinding, blending) and mixing particularly in case of heterogeneous samples (Fisher & Scott, 1997). Further, enzymes present in most of the animal or plant tissues

should be inactivated as they release when the cell walls are disrupted (Fisher & Scott, 1997). Often inactivation is reached with thermal processing or by drying the sample (Fisher & Scott, 1997; Raessler, 2011). Proper care should be taken, as for example for sugars caramelization occurs in high temperatures but efficient water removal is needed to stop enzymatic activity (Raessler, 2011). Further, in some cases both isolation and concentration of the compounds of interest is needed (Fisher & Scott, 1997). Different kinds of isolation and concentration methods, such as distillation for volatiles or evaporation of solvents can be used (Fisher & Scott, 1997). As non-volatile taste compounds are usually water soluble, water or mixtures of water with alcohols are often used, sometimes with the addition of formic acid to improve the extraction of ionic compounds (Reineccius & Peterson, 2013).

The analysis methods are quite different depending on whether aroma, taste or chemesthesis is studied (Reineccius & Peterson, 2013). Gas chromatography is extensively used with odors but it cannot be used without derivatization with usually non-volatile taste compounds, which are instead often analyzed with liquid chromatography (Reineccius & Peterson, 2013). In addition, the accuracy needed dictates which method is chosen for the analysis. For example, refractometers or hydrometers can be used for measuring the amount of soluble solids and thus for the evaluation of sugar content of fruits and vegetables by assuming that the soluble solids mainly constitute of sugars, but in many cases this is rough or even not correct estimation (Magwaza & Opara, 2015). For the separation of carbohydrates, the most common methods in liquid chromatography are anion-exchange chromatography and reversed-phase chromatography with amino-bonded silica-based packing material (Raessler, 2011). For the detection, pulsed amperometry (PA), refractive index (RI), evaporate light scattering (ELS) and mass spectrometry (MS) are most used (Raessler, 2011). Similarly, for example amino acids responsible for multiple flavors are often analyzed with liquid chromatography, particularly with reversed phase liquid chromatography and pre-column derivatization with o-phthaldialdehyde or phenylisothiocyanate for fluorescence detection (Molnár-Perl, 2000). Further nucleotides have been analyzed most commonly with liquid chromatography, particularly with ion-pair reversed-phase chromatography (Ranogajec, Beluhan, & Šmit, 2010).

The aim of the study determines the methods used for flavor analysis. For example, as in **Publication III** of this thesis, when studying the effect of possible suppressing mechanisms on bitterness, quite different research protocols are needed than used with analysis of concentration. Bitter-suppressing compounds either can suppress the bitterness by performing as antagonist for receptors or by reversibly

binding to bitter compounds. In the latter case, knowing the strength of these interactions is important for the evaluation of the actual effect the bitter binding molecules might have. Different methodologies have been developed for this such as 1) isothermal titration calorimetry (ITC) for studying binding strengths between amino acids and quinine (Zhang, Zhu, Zhao, Wu, & Hu, 2016), 2) measuring the concentration of bitter compounds in filtrates after centrifugation with a binding agent in order to know the percentage of bound molecules (Tenney, Hayes, Euston, Elias, & Coupland, 2017) and 3) NMR (Linde et al., 2010) to name a few examples from the field of food sciences. Further, different technologies have been developed for studying the binding of macromolecules with drugs in the pharmaceutical field. Due to its applicability for different kinds of molecules as well as effectivity and even high-throughput character, one interesting methodology is fluorescent indicator displacement (FID) method which has been used before with macromolecules such as DNA, RNA and proteins (Asare-Okai & Chow, 2011; Ham, Winston, & Boger, 2003; Mock, Langford, Dubois, Criscimagna, & Horowitz, 1985; Zhang, Umemoto, & Nakatani, 2010). In short, this method is based on the competitive binding of the molecule of interest and fluorescence indicator molecule to macromolecule. When the interaction is stronger with the molecule of interest, the decrease of fluorescence can be detected. In **Publication III**, we utilized these methods for the assessment of nanocellulose-taste compound binding. To our knowledge, similar methods have not been used before in the taste compound interaction assessments or with nanocellulose matrices.

2.3.2 Sensory evaluations

Sensory evaluation is a scientific method which is used to evoke, measure, analyze and interpret the responses to products through different senses (sight, smell, touch, taste and hearing) (Lawless & Heymann, 2010; Tuorila & Monteleone, 2009). These methodologies include a wide variety of different kinds of tests, where assessors can be either highly trained panelists such as professional flavorists or untrained consumers or something between these two (Fisher & Scott, 1997). The methods for sensory evaluations can be classified to descriptive, discrimination methods or affective tests (Lawless & Heymann, 2010). Affective or hedonic tests utilize consumers to find out the preference or acceptance for the product of interest (Fisher & Scott, 1997). The discrimination tests are usually used when the aim is to find out whether two samples perceptibly differ from each other (Lawless &

Heymann, 2010). The discrimination tests are useful in investigating subtle differences in samples (Lawless & Heymann, 2010). The most recommended standard for scientific research is descriptive analysis with a trained panel (Lawless & Heymann, 2010). With this method the most important properties distinguishing the sample from the others can be found and thus samples can be qualitatively set apart based on their properties (Fisher & Scott, 1997; Lawless & Heymann, 2010; Murray, Delahunty, & Baxter, 2001). Further, the panel of sensory judges gives a quantitative assessment to the relevant properties (Murray et al., 2001). The registered methods of descriptive analysis of flavor include for example the Flavor Profile (FP) method, Quantitative Descriptive Analysis (QDA) and the Spectrum Method (Piggott, Simpson, & Williams, 1998). As these methodologies are registered and have strict protocols that need to be followed, in many cases there is a need to do some adaptations and thus the use of registered methods is not possible anymore (Lawless & Heymann, 2010). Thus, in many cases, generic descriptive analysis, combining aspects of the techniques described earlier, is used (Lawless & Heymann, 2010). Although generic descriptive analysis allows modifications to be made, general guidelines to conduct generic descriptive analysis should still be followed regarding the training of the judges, determining their reproducibility and consistency as well as the guidelines for sample evaluations (Lawless & Heymann, 2010).

2.3.3 Combining sensory and instrumental analysis

In order to find which compounds are responsible for the flavor of food, different approaches can be utilized. In the sensory-directed approach, the process involves the extraction of compounds, separation and fractioning of them and then analyzing them in sensory evaluation methods (Reineccius & Peterson, 2013). Another approach is to analyze the concentration of flavor compounds and compounds potentially contributing to flavor and correlate these to sensory analysis. The links between instrumental and sensory data are analyzed via data interpretation such as multivariate analysis methods (Reineccius & Peterson, 2013). Here I will focus on the methods of statistical analysis that are the most important in regard to this thesis. Full reviews on the topic of chemometrics in flavor research can be found elsewhere (Chambers & Koppel, 2013; MacFie & Hedderley, 1993; Seisonen, Vene, & Koppel, 2016; Yu et al., 2018; Zielinski et al., 2014).

Combining the results from sensory and instrumental analysis starts with data pre-processing of both instrumental and chemical data. Pre-processing data from instrumental analysis involves both removing the data artifacts meaning for example correcting the baseline and removing noise in chromatographical measurements and transforming or rescaling the data quite often by autoscaling combining both mean-centering and standardization. For sensory analysis data, pre-processing can mean for example removing outliers (both assessors with clearly different ratings and clear random mistakes). (Seisonen et al., 2016) This can be done by one-dimensional statistical methods as the analysis of variance (ANOVA) which provides information about trends and variations inside the data sets (Seisonen et al., 2016; Yu et al., 2018). Multivariate principal component analysis (PCA) seeks to reduce the dimensionality of the data to aid the interpretation of it to find for example samples that are clearly different from other samples (Seisonen et al., 2016). Partial least square regression method (PLSR) combines multiple linear regression with PCA and thus often provides a fuller picture of the samples as well as can address bigger data sets (Piggott et al., 1998; Yu et al., 2018). Two data matrices, for example from sensory and instrumental analysis can be related with PLSR (Seisonen et al., 2016). In PLSR instead of using independent variables for regression, new principal components are generated as the linear combinations of original variables (Yu et al., 2018). Besides PLSR, non-linear regression methods including fuzzy logic and artificial neural network can be utilized in the case where the relationships between data sets are nonlinear in the nature (Yu et al., 2018). Fuzzy logic models the human thought process to provide decision-making or classification protocol from imprecise information. Fuzzy logics enable analysis of data without absolute of definite values and with nonlinear relationships with variables and predictors (for example food constituents and perceptions) (Yu et al., 2018). Whereas fuzzy logic is based on the human thought process, artificial neural network (ANN) models the human brain nervous system with node network divided in three layers, input, output and hidden (Yu et al., 2018). However, even these newer methodologies may provide greater depths of analysis for complex situations, the traditional methods such as PLSR are still much more common (Yu et al., 2018).

2.4 Taste of mushrooms

Mushrooms are examples of macrofungi which are species belonging to the kingdom of Fungi with visible fruiting bodies (Boa, 2004). According to Boa (2004) there are

1069 species that are consumed as food globally. Most of the edible mushroom species are symbiotic forming mycorrhiza with plants and therefore are a vital part of growth of many plant species (Boa, 2004). Mushrooms are widely consumed and appreciated delicacy around the globe. The interest towards mushrooms both by consumers and scientists, as indicated in the literature survey by Sun et al. (2020), has increased in recent years. The pleasant taste of mushrooms is often associated with characteristic umami taste (Phat, Moon, & Lee, 2016). The taste of mushrooms is widely studied but there are some knowledge gaps still needed to be filled. As the information gathered about the taste properties of mushroom covers mainly species of importance to Asian or Southern European cuisine there is a need for studies covering the Nordic species. Furthermore, there are only a few sensory studies about the taste properties of mushrooms (Cho et al., 2007; Mittermeier, Dunkel, & Hofmann, 2018; Phat et al., 2016; Rotzoll, Dunkel, & Hofmann, 2006) and thus a few studies combining sensory and instrumental data (Mittermeier et al., 2018; Phat et al., 2016; Rotzoll et al., 2006). Flavor properties including both taste and aroma qualities of mushrooms have been reviewed by Zhang, Venkitasamy, Pan and Wang (2013) and recently by Aisala (2019) and Sun et al. (2020). Furthermore, the chemical composition of mushrooms including some flavor eliciting compounds has been reviewed by Kalač (2013).

2.4.1 Taste compounds in mushrooms

The studies of the taste eliciting compounds of mushrooms have focused on many different classes of substances such as sugars, polyols, organic acids and particularly on free amino acids and nucleotides. The literature and methods used in these studies are reviewed in this chapter. The major taste eliciting compound groups, examples of major compounds as well as examples of analysis methods are presented in the Table 1.

The most commonly reported free sugars and sugar alcohols in mushrooms are mannitol and trehalose found in many species such as in *Agaricus bisporus*, *Lentinula edodes* and *C. cibarius* as well as in *Pleurotus* and *Boletus* species (Beluhan & Ranogajec, 2011; Heleno et al., 2011; Reis, Barros, Martins, & Ferreira, 2012; Yang et al., 2001). Other commonly reported sugars are mannose, glucose, fructose and arabitol (Beluhan & Ranogajec, 2011; Tseng & Mau, 1999; Yang et al., 2001). In presented references, sugars and sugar alcohols are most commonly extracted from mushrooms with 80% ethanol and further quantified with HPLC and RI detection.

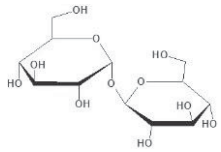
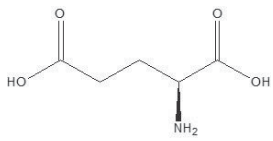
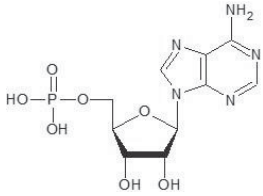
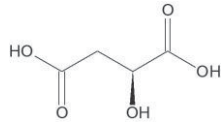
There are only a few examples, where other methods for quantification such as high-performance anion exchange chromatography with pulsed amperometric -detection (Chen et al., 2015; Mittermeier et al., 2018) have been used.

Similarly, liquid chromatography, particularly HPLC with UV-detection, is the most common method for analyzing the organic acids in mushrooms (Chen et al., 2015; Valentão et al., 2005a; Valentão et al., 2005b). Further, other methods such as enzymatic tests (Rotzoll et al., 2006) and LC-MS/MS (Mittermeier et al., 2018) have been utilized. Both water with an addition of acid and methanol have been used for the extraction of organic acids (Chen et al., 2015; Valentão et al., 2005a; Valentão et al., 2005b). Of organic acids, malic acid and citric acid have been found to be the most abundant organic acids in certain common species such as *B. edulis* and *C. cibarius* (Valentão et al., 2005a; Valentão et al., 2005b), while succinic acid was recognized as the most abundant in *L. edodes* (Chen et al., 2015).

The general acceptance is that the umami taste of mushrooms is caused by MSG-like free amino acids, aspartic acid and glutamic acid, as well as umami enhancing nucleotides (5'-GMP, 5'-IMP, 5'-AMP and 5'-XMP) (Phat et al., 2016). Furthermore, some peptides found in mushrooms have been proposed to have an umami taste such as Gly-Leu-Pro-Asp and Gly-His-Gly-Asp in *A. bisporus* (Feng et al., 2019). The analysis of free amino acids in mushrooms has been mainly performed using liquid chromatography (Phat et al., 2016; Rotzoll et al., 2006; Yang et al., 2001). Most commonly, water or dilute HCl have been used for extraction and o-phthaldehyde for the derivatization of primary amino acids sometimes accompanied by 9-fluorenylmethylchloroformate (FMOC-Cl) for secondary amino acid derivatization prior fluorescence detection (Beluhan & Ranogajec, 2011; Phat et al., 2016; Sun, Liu, Bao, & Fan, 2017; Yang et al., 2001). Further, other chromatographical methods such as GC-MS with derivatized amino acids (Dermiki, Phanphensophon, Mottram, & Methven, 2013) and LC-MS/MS (Mittermeier et al., 2018) have been used. Of the free amino acids, all proteinogenic amino acids have been found in mushroom species. Glutamic acid in particular has been found in abundance from many species as well as alanine, threonine, lysine and histidine (Beluhan & Ranogajec, 2011; Yang et al., 2001). In the literature, the free amino acids have been classified based on their taste characteristics (Belitz, Grosch, & Schieberle, 2009; Kawai et al., 2012; Mau et al., 2001; Solms, 1969; Yang et al., 2001). However, it should be noted, that these classifications are somewhat ambiguous as they are different depending on the reference cited and further, the divisions are formed based on pure solutions of amino acids. As with other taste compounds, the taste of a single compound is

dependent on the chemical environment and the concentration of a single amino acid may alter the taste characteristic it is inducing (Kawai et al., 2012).

Table 1. Major taste eliciting compounds in mushrooms and methods for their analysis

Compound group	Major compounds found	Example of compounds	Chemical analysis	Reference
Soluble sugars and sugar alcohols	Mannitol, trehalose	Trehalose 	HPLC-RI	Mau et al., 1998; Mau et al., 2001; Reis et al., 2012; Rotzoll et al., 2006; Tsai et al., 2008; Tseng & Mau, 1999; Yang et al., 2001
Free amino acids	Glutamic acid, alanine, histidine	Glutamic acid 	HPLC-FD; GC-MS; LC-MS/MS	Beluhan & Ranogajec, 2011; Dermiki et al., 2013; Mau et al., 1998; Mau et al., 2001; Mittermeier et al., 2018; Rotzoll et al., 2006; Tsai et al., 2008; Tseng & Mau, 1999; Yang et al., 2001
5'-nucleotides	AMP, XMP, GMP, IMP	AMP 	HPLC-UV; LC-MS/MS; capillary electrophoresis	Beluhan & Ranogajec, 2011; Dermiki et al., 2013; Mau et al., 1998; Mau et al., 2001; Mittermeier et al., 2018; Phat et al., 2016; Rotzoll et al., 2006; Tsai et al., 2008; Tseng & Mau, 1999; Yang et al., 2001
Organic acids	Malic acid, citric acid	Malic acid 	HPLC-UV; enzymatic test kit; LC-MS/MS	Mittermeier et al., 2018; Rotzoll et al., 2006; Valentão et al., 2005a; Valentão et al., 2005b

As the species studied as well as other factors such as cooking methods and maturity stage influence greatly on the amino acid compositions, the concentrations of MSG-like amino acids presented in the literature have a wide range (Zhang et al., 2013). For example, Beluhan and Ranogajec (2011) presented glutamic acid concentrations between 23.89 and 45.85 mg/g in dry weight for different fresh mushroom species while Tsai et al. (2008) presented the concentrations of 0.59 mg/g to 3.29 mg/g in dry weight for air-dried species. Due to these variations caused by both the differences in amino acid compositions of different species, samples of different origins, and pre-handling as well as detection methods, comparing the amino acid concentrations of mushrooms with other food products is difficult. Further, most probably similar variations can be seen in other food products. To provide some scale, a couple of examples are however presented in the Table 2. For example, high concentrations of glutamic acid have been found in matured cheeses such as from Cheddar where glutamic acid concentration increase from 17 mg/g to 289 mg/g during 8 months of ripening (the concentration is in dry weight approximate based on 37% water content (Chen, MacNaughtan, Jones, Yang, & Foster, 2020)) and in Parmigiano Reggiano 12 – 16 mg/g in wet weight (Ninomiya, 1998; Weaver, Kroger, & Thompson, 1978). According to a recent study by Mouritsen, Duelund, Petersen, Hartmann and Bom (2019), the glutamic acid concentrations of various species of brown seaweed varied approximately between 0.004 and 9 mg/g with a mean value of 1.7 mg/g in dry weight calculated according the instructions provided, while the mean value for glutamic acid concentration in 13 varieties of tomatoes of was 91.2 mg/g in pulp and 25.2 mg/g in flesh in dry weight (Oruna-Concha, Methven, Blumenthal, Young, & Mottram, 2007) calculated based on 95% moisture content estimated by Guil-Guerrero and Rebolloso-Fuentes (2009).

For the extraction of nucleotides, hot water with centrifugation and/or filtration has been utilized (Beluhan & Ranogajec, 2011; Ranogajec et al., 2010; Tseng & Mau, 1999; Yang et al., 2001). For the quantification, HPLC-UV has been the most used method (Beluhan & Ranogajec, 2011; Ranogajec et al., 2010; Rotzoll et al., 2006; Tseng & Mau, 1999; Yang et al., 2001), but also other methods such as LC-MS/MS (Mittermeier et al., 2018) and capillary electrophoresis (Dermiki et al., 2013) has been used. The distribution of nucleotides varies depending on the species studied as for example in the *B. edulis* 5'-adenosine monophosphate and 5'-uridine monophosphate are the major nucleotides (Beluhan & Ranogajec, 2011) while for *F. velutiples (yellow)* and *L. edodes* species 5'-cytidine monophosphate and 5'-xanthosine monophosphate were the major ones (Yang et al., 2001). Out of Croatian species studied in Beluhan & Ranogajec (2011) the highest concentration of flavor nucleotides (IMP, GMP and

XMP) was in *C. cornucopioides* (13.88 mg/g) while *C. cibarius* had only the concentration of 0.38 mg/g in dw. Most of the species presented in literature fit to this range (Zhang et al., 2013). In some publications, a classification according to Yang et al (Yang et al., 2001) has been used, dividing the flavor nucleotides into three ranges: low (<1 mg/g), middle (1–5 mg/g) and high (>5 mg/g). In comparison, in tomatoes, 5'-adenosine monophosphate is the major nucleotide with the concentration of 5.9 mg/g in pulp (Oruna-Concha et al., 2007) based on approximately 95% moisture content (Guil-Guerrero, & Rebolloso-Fuentes, 2009), while in crab meat the concentrations of AMP, IMP and GMP were 3.6, 1.6 and 0.11 mg/g (Chen & Zhang, 2007) on dry weight basis estimated based on 79% moisture (Chen, Zhang, and Shestra, 2007). The concentrations nucleotides in different foods are presented in the Table 3.

Table 2. Glutamic acid concentrations in wet and dry weight in different foods

	Sample	Concertation in mg/g wet weight	Concertation in mg/g dw	Reference
Glutamic acid	Mushroom species	–	23.89–45.85	Beluhan and Ranogajec, 2011
	Mushroom species (air-dried)	–	0.59–3.29	Tsai et al., 2008
	Cheddar cheese*	11–182	17–289[a]	Ninomiya, 1998; Weaver, Kroger, & Thompson, 1978
	Parmigiano cheese	12–16	–	Ninomiya, 1998
	Brown seaweed species	0.015–37 (mg/100ml)	0.0036–8.9[b]	Mouritsen et al., 2019
	Tomato species, flesh (pulp)	0.66–3.51 (2.03–16.5)	13.2–70.2[c] (40.6–330)[c]	Oruna-Concha et al., 2007

* Increase in concentration during 8 months of ripening

[a] Estimated based on 37% moisture content (Chen, MacNaughtan, Jones, Yang, & Foster, 2020)

[b] Estimated based on instructions given in Mouritsen et al. (2019)

[c] Estimated based on 95% moisture content (Guil-Guerrero and Rebolloso-Fuentes, 2009)

Table 3. Nucleotide concentrations in wet and dry weight in different foods

Sample	Nucleotide	Concentration in wet weight	Concentration in mg/g, dw	Reference
Mushroom species	AMP	–	0.35–6.57	Beluhan and Ranogajec, 2011
	CMP		0.09–5.13	
	GMP		0.12–2.88	
	IMP		0.00–3.97	
	UMP		0.59–4.19	
	XMP		0.14–7.03	
Mushroom species (air-dried)	AMP	–	0.00–4.37	Yang et al., 2001
	CMP		2.33–10.0	
	GMP		0.00–1.38	
	IMP		0.00–2.78	
	UMP		0.46–2.64	
	XMP		0.97–8.80	
Tomato species, flesh (pulp)	AMP	0.024–0.197 (0.213–0.561)	0.48–3.94[a] (4.26–11.22)[a]	Oruna-Concha et al., 2007
	CMP	0.003–0.014 (0.008–0.022)	0.05–0.28[a] (0.16–0.44)[a]	
	GMP	0.003–0.023 (0.013–0.038)	0.054–0.46[a] (0.256–0.76)[a]	
	UMP	0.028–0.100 (0.063–0.183)	0.56–2.00[a] (1.26–3.66)[a]	
Crab meat	AMP	0.753±0.004	3.586[b]	Chen & Zhang, 2007
	IMP	0.344±0.029	1.638[b]	
	GMP	0.023±0.002	0.110[b]	

[a] Estimated based on 95% moisture content (Guil-Guerrero and Rebolloso-Fuentes, 2009)

[b] Estimated based on 79% moisture content (Chen, Zhang, and Shestra, 2007)

The composition of taste compounds in mushrooms is affected by storage (Tseng & Mau, 1999) and cooking methods (Li et al., 2019; Li et al., 2011; Rotola-Pukkila, Yang, & Hopia, 2019) as well as the maturity stage (Tsai, Wu, Huang, & Mau, 2007) and the quality grade (Cho et al., 2007). These effects have been reviewed before by Zhang et al. (2013). Furthermore, concentration differences occur even inside a singular mushroom. For example, differences in MSG-like amino acid and

nucleotide concentrations in pileus and stipe of *Tricholoma matsutake* have been measured (Cho, Choi, & Kim, 2010). In pileus the concentrations of MSG-like amino acids were higher (i.e. 7.70 mg/g dw versus 3.87 mg/g dw with 1st quality grade mushroom samples) in all quality grades studied. A similar trend was detected with flavor 5'-nucleotides. Li et al. (2011) studied the effects of different cooking methods on MSG-like amino acids as well as on nucleotides and found that microwave cooking was the optimal method of preserving these compounds while autoclaving resulted in big losses in *Agaricus bisporus* soup. Rotola-Pukkila et al. (2019) studied the effect on cooking at 90 ºC on the free amino acid and nucleotide content concluding that the effect of the cooking is very much dependent on the species studied. For example, the percentage of MSG-like free amino acids (FAA) in *A. bisporus* was higher after cooking (24% in fresh mushrooms versus 42% in cooked) but the change in the case of some wild species such as with *C. cibarius* was much smaller (28% in fresh mushrooms, 22% in cooked ones). Recently, Li et al. (2019) studied the effect of boiling time for the flavor of *Lentinus edodes* revealing a decrease in sugar and sugar alcohol, organic acid, nucleotide and free amino acid contents compared with raw mushrooms during the first minutes of boiling.

2.4.2 Sensory studies of mushrooms taste

The amount of sensory studies concerning the flavor properties of mushrooms is limited and furthermore only a handful of these follow the recommended descriptive analysis protocols (Aisala, 2019). These limitations are further discussed in Aisala (2019). Furthermore, taste properties are studied in only a few studies as many of them are concentrated on odor properties. As the amount of the sensory studies of mushroom flavor is limited, the studies combining sensory evaluations with chemical analysis are even more scarce.

In a study by Rotzoll et al. (2006), the taste profile, including all five taste modalities, of *Morchella deliciosa* was studied with 14 assessors' panel by creating a taste reconstitute model for *Morchella deliciosa* samples based on 33 quantified non-volatiles. The flavor of morel extracts was associated with umami and sour tastes and mouth drying characteristic, astringency. They concluded that in morel mushrooms (S)-morelid was one of the key compounds contributing the taste with some other compounds such as L-glutamic acid, L-aspartic acid, malic acid, citric acid, acetic acid and γ-aminobutyric acid. Further, Mittermeier et al. (2018) studied the taste profiles of chanterelles from chanterelle extracts with 17 assessors panel and concluded that

the umami taste and bitterness were observed in the highest intensities (2.3 and 2.5 in a scale from 0 (not perceivable) to 5 (strongly perceivable)) in the samples while other studied taste and chemosensory qualities (sweet, sour, salty, astringent, pungent, kokumi) had scorings between 0.5 for sweet and 1.9 for kokumi. Furthermore, Mittenmeier et al. (2018) created recombinant mixtures that they compared with chanterelle samples discovering that certain octadecadien-12-unoic acids seemed to have kokumi enhancement activity. One of the studies combining sensory evaluations with instrumental analysis, is from Dijkstra and Wikén (1976), who created a mixture of different non-volatiles (FAAs, nucleotides, sugars and sugar alcohols) and some volatiles as well. Based on conducted omission experiments, they concluded that the glutamic acid, GMP, and AMP as well as sugars and sugar alcohols were important for the taste of *Agaricus bisporus* samples (Aisala, 2019; Dijkstra & Wikén, 1976). Dermiki et al. (2013) used a paired alternative forced choice method to find differences in umami intensity between two extraction methods with different extraction temperatures for the samples. The higher extraction temperatures yielded higher umami taste of the samples.

Umami taste has been also studied with sensory analyses by Phat et al. (2016) who utilized a sensory panel of 10 assessors to evaluate the umami intensity of 17 mushroom samples of different species as well as electronic tongue to evaluate taste properties. The strongest umami intensity based on human sensory evaluations was found in *A. bisporus* samples. Phat et al. (2016) calculated the equivalent umami concentrations (EUC) based on the measured glutamic and aspartic acid concentrations as well as 5'-nucleotide contents and linked this information with the information gathered with an electronic tongue with good correlation. The EUC equation can be written

$$Y = \sum a_i b_i + 1218(\sum a_i b_i)(\sum a_j b_j) \quad (2)$$

where Y is the EUC value of the sample (g MSG/100g), a_i (%) is the concentration of each umami amino acid, a_j (%) the concentration of each 5'-nucleotide and b_i and b_j the relative umami concentrations of each compound. This method has been used widely for evaluation of singular taste compounds on umami taste in literature (Beluhan & Ranogajec, 2011; Yang et al., 2001) since it was introduced (Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971) and is based on gathered experimental data. However, as the role of other umami compounds has been demonstrated in other recent studies the need to modify this equation has been discussed (Sun et al., 2020).

2.5 Bitter taste modification

Especially bitterness is often perceived as unpleasant taste modality. Thus, different strategies have been introduced to modify the bitterness of food products. These strategies include for example, the removal of unwanted components, physical barriers such as coatings or emulsions, the use of other flavors to suppress or mask the off-flavor and use of bitter inhibiting or reducing compounds (Gaudette & Pickering, 2013; Ley, 2008). This chapter will focus on the food related applications of bitterness modification which differ from pharmaceutical applications by their purposes: While in pharmaceutical applications the objective is to reach the palatable/acceptable level of bitterness, in food applications the goal is to modify the overall sensory profile to be acceptable for consumers to eat the food again (Gaudette & Pickering, 2013). Further, it should be noted that the pharmaceuticals are completely different matrices compared with foods. Foods usually have a large set of different flavor compounds whereas the number of compounds is more limited in pharmaceuticals. (Coupland & Hayes, 2014) As a large variety of different strategies for bitterness reduction exist, only a fraction of them are presented here. More complete reviews of the subject can be found in Sun-Waterhouse and Wadhwa (2013), Gaudette and Pickering (2013) and Coupland and Hayes (2014).

2.5.1 Suppressing bitterness with other tastes

In the literature multiple examples of suppressing bitterness can be found for sweet or salty tasting molecules, such as sucrose and NaCl. For example, Calvino, García-Medina and Cometto-Muniz (1990) studied the effect of sucrose on the taste of caffeine and on the flavor of coffee as well as the effect of bitterness of caffeine and coffee flavor on the sweetness of sucrose. The study revealed that both the caffeine bitterness and coffee flavor intensity decreased when sucrose intensity increased (Calvino et al., 1990). Further, the sweetness of sucrose was less affected by the bitterness of caffeine or flavor of coffee (Calvino et al., 1990). Besides sucrose, also other sweeteners, such as aspartame have been shown to reduce the bitterness of quinine and the bitter taste of Brussels sprouts (Nakamura et al., 2002; Wilkie, Phillips, & Wadhera, 2013). The ability of sodium salts to suppress the bitterness of substances such as QHCl, caffeine, magnesium sulfate and potassium chloride has been demonstrated in many studies (Breslin & Beauchamp, 1995; Keast, Breslin, & Beauchamp, 2001; Keast & Breslin, 2002). Keast, Breslin and Beauchamp (2001)

studied how different salts (NaCl, natriumacetate, natriumgluconate, LiCl, KCl) influence the taste of different bitter compounds (urea, QHCl, caffeine, amiloride-HCl, magnesium sulfate, KCl) and further the taste of mixtures with bitter and sweet compounds. The study revealed that the sodium salts and LiCl were able to suppress the bitterness while similar effect was not found with KCl (Keast et al., 2001). Further, sodium salts enhanced the sweetness and reduced the bitterness of the sweet/bitter mixtures (Keast et al., 2001).

Similar effects have been found with mildly salty sodium acetate (Breslin & Beauchamp, 1997). Besides sodium salts, magnesium sulfate and zinc salts, such as zinc sulfate have been studied for their ability to suppress the bitterness of QHCl (Keast, 2003). Further, Keast (2003) studied the effect of these salts on other taste qualities and concluded that while magnesium sulfate had no effect on other taste qualities, zinc sulfate decreased the sweetness of glucose. As zinc sulfate has no taste itself, and affinity to certain amino acids containing hydrogen groups, the mechanism for the suppression of taste can be the altering of extracellular portions of the taste receptors by forming complex with the suitable sequences of amino acids (Keast, 2003). Decrease in the bitterness of QHCl solutions has also been measured with the sodium salts of both umami amino acids, glutamic acid and aspartic acid (Keast & Breslin, 2002; Kemp & Beauchamp, 1994). Monosodium glutamate did suppress the bitterness at suprathreshold levels, but no difference was found at threshold concentrations (Kemp & Beauchamp, 1994).

2.5.2 Using matrix to suppress bitterness

One possible way to suppress the bitter sensation is to prevent the contact of bitter compounds with receptors by either retarding the release of these compounds during in-mouth processing or by reducing their possibilities to interact with receptors by either utilizing steric hindrances or increased viscosity (Sun-Waterhouse and Wadhwa, 2013). Considering the effect of viscosity on bitterness, there are many reports that this could indeed be an effective approach (Gaudette & Pickering, 2013). For instance, Moskowitz and Arabie (1970) studied the effect of viscosity on taste intensity using sodium carboxymethyl cellulose (CMC) in different concentrations. The studied compounds were glucose, citric acid, sodium chloride and quinine sulfate. They concluded that increased viscosity yielded lower taste intensities and even non-detectable taste in low concentrations of quinine sulfate (Moskowitz & Arabie, 1970). Pangborn, Trabue, and Szczesniak (1973) studied the capacity of

hydrocolloids (hydroxypropyl cellulose, sodium alginate and xanthan and CMC with low and medium viscosity) to reduce bitterness. The bitterness of caffeine was reduced the most with CMC in low viscosity, sodium alginate and xanthan (Pangborn et al., 1973). As CMC in medium viscosities did not have similar effect on the bitterness, it was suggested that the nature of used matrix affects its ability to suppress bitterness (Gaudette & Pickering, 2013; Pangborn et al., 1973). Furthermore, Pangborn et al. (1973) noticed that the added taste compounds effect on the viscosity. They concluded that particularly sucrose increased the viscosity of studied hydrocolloids. Smith, June and Noble (1996) studied the effect of CMC in medium viscosity on the bitterness of grape-seed tannins. The intensity of the bitterness was not decreased by the increase in CMC concentration whereas the intensity of astringency was (Smith, June, & Noble, 1996).

Another approach of a matrix utilizing bitterness reduction is the use of w/o/w or o/w/o type multiple emulsions, where the taste compound is dissolved in an internal phase (Sun-Waterhouse and Wadhwa, 2013). In pharmaceuticals, this approach has been used in the bitterness reduction of antimalaria drug chloroquine (Vaziri & Warburton, 1994). Further, lipids may act as a bitterness suppressing compounds by scavenging bitter molecules or by acting as surfactants (Sun-Waterhouse and Wadhwa, 2013). For example, oil-in-water emulsion of tuna oil has been demonstrated to decrease the bitterness and sourness as other taste modalities remained (Koriyama, Wongso, Watanabe, & Abe, 2002).

2.5.3 Bitterness inhibiting compounds

The bitter inhibiting compounds can reduce bitterness either via the interactions between bitter tasting molecules and bitter inhibiting molecules for example through complexation or encapsulation or by interfering in the binding of taste molecules with the receptors or the taste transduction mechanism (Gaudette & Pickering, 2013).

Cyclodextrins are cyclic oligosaccharides with a hydrophobic cavity and hydrophilic outer surface (Szejtli & Szente, 2005). The bitter compounds interact with the interior of cyclodextrins forming a complex that prevents the interactions between bitter compounds and taste receptors (Gaudette & Pickering, 2013; Szejtli & Szente, 2005). β-Cyclodextrins have been proved to decrease the bitterness of citrus juice containing naringin and limonin (Gaudette & Pickering, 2013; Konno, Misaki, Toda, Wada, & Yasumatsu, 1982) as well as the bitterness of caffeine and

some natural extracts when linked with chitosan (Binello, Cravotto, Nano, & Spagliardi, 2004).

Besides the widely studied cyclodextrins, different natural polymers from plant, animal or microbial origin can be used in bitterness inhibition in food systems (Coupland & Hayes, 2014). Riboflavin-binding proteins have been proved to decrease the bitterness intensity of various bitter compounds, such as caffeine and QHCl (Gaudette & Pickering, 2013; Maehashi, Matano, Nonaka, Udaka, & Yamamoto, 2008). This protein can bind with QHCl through hydrophobic interactions, but it might also inhibit some other taste qualities such as the sweetness of some sweet proteins (Gaudette & Pickering, 2013; Maehashi et al., 2008). Furthermore, phosphatidic acid–β-lactoglobulin lipoproteins have been demonstrated to suppress bitterness of some bitter compounds such as QHCl and caffeine (Katsuragi, Sugiura, Lee, Otsuji, & Kurihara, 1995; Katsuragi, Yasumasu, & Kurihara, 1996).

2.6 Nanocellulose in food applications

Cellulose materials with widths in nanoscale dimension are referred to as nanocellulose materials (Klemm et al., 2011). These materials are produced either with enzymatic, chemical or physical methods from cellulose fibers and can be separated from different origins, such as from wood pulp or vegetables (so called top-down methods), or they can be synthesized by bacteria from glucose (the bottom-up method) (Klemm et al., 2011). Three groups of nanocellulose materials, nano-fibrillated cellulose (NFC, also referred as cellulose nanofibrils CNF or microfibrillated cellulose), cellulose nanocrystals (CNC, also called nanocrystalline cellulose) and bacterial cellulose (BC, also referred as bacterial nanocellulose, microbial cellulose or biocellulose), can be formed based on the production methods causing differences in their structures and properties (Klemm et al., 2011; Klemm et al., 2018). Due to the differences in the production methods, these materials differ from each other by their morphology and physical properties. Bacterial cellulose, which is produced by fermentation of sugars and carbohydrates using bacterial strains, forms typically nanofiber networks where the diameter of nanofibers is between 20 and 100 nm. (Klemm et al., 2018) This production method offers some advantages over plant derived nanocellulose species, including high purity from lignin and other contaminants (Shi, Zhang, Phillips, & Yang, 2014). Nanofibrillar cellulose on the other hand has cross-section of about 5–60 nm and length of 0.1–2

micrometers while cellulose nanocrystals have cross-section of 5–70 nm and length variating depending on the source used from 100 nm to several micrometers (Klemm et al., 2018). The most commonly used method for the preparation of NFC is high-pressure homogenization, in which the cellulose dispersion is pressed through the flow channel with high pressure (Yi et al., 2020). Due to the high shear forces involved in the process, the cellulose fibers entangle with each other causing gel formation in aqueous solutions (Yi et al., 2020). High-pressure homogenization is the oldest method for the preparation of NFC and has some disadvantages such as high energy consumption and clogging of the homogenizers. Thus, pre-treatment methods as well as other methods for production including ball milling, ultrasonic treatment, low temperature pressing, steam explosion and electrospinning have been developed (Yi et al., 2020; Klemm et al., 2018). Cellulose nanocrystals are usually produced from cellulose fibers with strong acid treatments hydrolysing the para-crystalline or disordered regions of cellulose and thus causing rod-like nanocrystals (Habibi et al., 2010). Many acids have been used in cellulose nanocrystal preparation most common ones being sulfuric acid and hydrochloric acid (Habibi et al., 2010).

Nanocellulose materials offer some profitable characteristics of nanomaterials, such as reactivity and good binding capability but they also have many useful characteristics of cellulose, such that they are abundant, renewable, biodegradable, non-toxic, hydrophilic and have wide chemical-modification capacities (Kangas, 2014). The utilization of nanocellulose as food additive was one of the first applications proposed for nanocellulose materials in the early 1980s as first few publications of manufacturing microfibrillar cellulose were published (Herrick, Casebier, Hamilton, & Sandberg, 1983; Turbak, Snyder, & Sandberg, 1982; Turbak, Snyder, & Sandberg, 1983a), but has yielded in surprisingly few applications.

2.6.1 The use of cellulose-based hydrocolloids in food industry

Cellulose-based hydrocolloids used in food industry include both non-substituted materials such as microcrystalline cellulose (MCC) and substituted materials such as carboxymethyl cellulose (CMC), hydroxypropyl methyl cellulose (HPMC) and methylcellulose (MC) (He et al., 2020). Bacterial cellulose is also utilized, particularly as traditional dessert originating from Philippines called Nata (Phisalaphong & Chiaoprakobkij, 2012). Of the cellulose derivatives, CMC is the most used one, mainly as viscosity modifier in many different applications such as ice creams, sauces and dressings and soft drinks (Murray, 2009). As indicated in chapter 2.5.2, the

utilization of hydrocolloids, including CMC, as taste-modifiers has also been previously studied. The cellulose-based hydrocolloids differ from the nanocellulose materials by their production method, dimensions and properties. For example, nanocellulose materials are insoluble in water and form nano- or microscale colloidal dispersions while CMC dissolves in water (Chu, Sun, Wu, & Xiao, 2020; Yaginume & Kijima, 2006; He et al., 2020). MCC with rod-like particles is most often prepared by acid hydrolysis from pure cellulose, followed by neutralization and washing (He et al., 2020; Trache et al., 2016). The yielded MCC material is crystalline, hydrophilic and has slightly negative charge (He et al., 2020; Trache et al., 2016). The dimensions of MCC are in microscale being between 50 to 200 micrometers for length and 50 to 100 micrometers for width (He et al., 2020). Cellulose-based derivatives, including CMC, HPMC and MC, are prepared from cellulose by chemical reactions, such as etherification (He et al., 2020). The properties of the materials are dependent on the degree of polymerization of cellulose molecules, the substituent group and the degree of substitution (Murray, 2009). CMC, in which the carboxymethyl groups are bound to the hydroxyl groups of cellulose, is hydrophilic and anionic, and the negative charge of it is dependent on the degree of substitution (He et al., 2020).

Nanocellulose materials have high surface area and surface functionality; thus they are easily chemically modified and have suitable rheological behavior for the applications in food industry (Gómez et al., 2016; Lee, Sundaram, & Mani, 2017). Larger specific surface area of NFC compared to other cellulose derivatives can favor binding as seen before for example with cholesterol adsorption to cellulose and NFC (Liu & Kong, 2019). The present knowledge is, that nanocellulose materials have no or have low toxicity but further research is still needed in this area (Gómez et al., 2016; Lee et al., 2017). According to reviews by Gómez et al. (2016) and Lee et al. (2017) there is still a gap in knowledge on the behavior of nanocellulose materials in complex matrices such as food. For example, to my knowledge the possible effects of nanocellulose materials on the taste of food have not been studied before.

2.6.2 Nanocellulose as a food additive

In reports about possible food applications, nanocellulose materials have been used as a food additive serving either as a stabilizing agent for emulsions or functional food ingredients, in food coatings, and in food packaging (Gómez et al., 2016; Lee et al., 2017). Various food applications have been proposed to utilize nanocellulose

materials as stabilizing agents such as products containing fats and oils (gravies, salad dressings, fillings, whipped toppings) (Turbak et al., 1982; Turbak et al., 1983a; Turbak, Snyder, & Sandberg, 1983b; Turbak, Snyder, & Sandberg, 1983c), cookie fillings (Kleinschmidt, Roberts, Fuqua, & Melchion, 1988), and ice cream (Velásquez-Cock et al., 2019). Further, Mikulcová, Bordes. & Kašpárková (2016) utilized nanocellulose materials for preparation of emulsions with often otherwise fragile essential oils. High contents (even 40 wt%) of antimicrobial oils in emulsion with low cellulose concentrations (0.1 wt% at the lowest) were reached (Mikulcová et al., 2016).The mechanism for the stabilization of emulsions with nanocellulose materials is proposed to be the formations of Pickering emulsions where the solid particles, in this case nanocellulose materials, are absorbed irreversibly at the interface of the oil-water surfaces (Winuprasith & Suphantharika, 2013). With microfibrillated cellulose, better stabilization effect was achieved with more homogenized and thus more degraded cellulose fibers (Winuprasith and Suphantharika, 2013).

The applications to use nanocellulose as functional food products are either low-calorie products or functioning as a dietary fiber (Gómez et al., 2016). The effect of nanocellulose addition has been studied in products with high energy content such as hamburgers (Ström, Öhgren, & Ankerfors, 2013) and sausages (Marchetti, Muzzio, Cerrutti, Andrés, & Califano, 2017; Qi et al., 2020; Wang et al., 2018). Ström at al. (2013) noticed favorable effects of adding microfibrillated cellulose (MFC) to the texture of the hamburgers, as they lost less water during frying. The addition to the bun caused increase in the softness and caused smoother, bigger and more even appearance compared with the buns without the addition (Ström et al., 2013). Further, the fat content of Chinese meatballs was successfully lowered while retaining the sensory properties as well as shelf stability time with addition of bacterial cellulose (10%) (Lin & Lin, 2004). The fermentation of bacterial cellulose with naturally red pigmented *Monascus* strains caused a complex with meat-like flavor (Ng & Shyu, 2004). Thus, the complex can be used as meat and fish replacement in vegetarian diet (Ng & Shyu, 2004). Besides replacing the fats and thus lowering the energy content, it has been suggested that purified bacterial cellulose might even reduce the amount of cholesterol in the digestive tract (Stephens, Westland, & Neogi, 1990). The applications for utilizing nanocellulose materials as stabilizing agents or as functional foods are summarized in the Table 4.

Table 4. Examples of food related applications for nanocellulose materials presented in the scientific journal or as patents

Application	Material	Reference
Edible suspensions, emulsion stabilizer, fat replacement in hamburgers	MFC*	Turbak et al., 1983c
Stabilizing of various food products such as dips and toppings	MFC	Turbak et al., 1982
Cookie fillings	MFC	Kleinschmidt et al., 1988
Shape retention of ice cream	NFC*	Velásquez-Cock et al., 2019
Emulsion stabilizer	CNC*/MFC	Mikulcová et al., 2016
Fat replacer in hamburgers	MCC*	Ström et al., 2013
Fat replacer in meatballs	BC*	Lin & Lin, 2004
Meat replacer by complexation with *Monascus*	BC	Ng & Shyu, 2004
Fat replacement in sausages	BC	Marchetti et al., 2017
	NFC/CNC	Qi et al., 2020
	NFC	Wang et al., 2018

*MFC = microfibrillar cellulose, NFC = nanofibrillar cellulose, CNC = cellulose nanocrystals, MCC = microcrystalline cellulose, BC = bacterial cellulose

Nanocellulose materials, especially bacterial cellulose, are a nature-friendly option to widely used fossil fuel-based materials in food packaging (Azeredo, Rosa, & Mattoso, 2017). High elastic modulus, transparency, ability to form strong, thin, smooth and dense films as well as good temperature resistance are all interesting properties for food packaging technologies (Kangas, 2014; Lee et al., 2017). Further, high air and oxygen barrier properties are important properties to extend the product shelf life by retaining the quality and safety (Aulin, Gällstedt, & Lindström, 2010; Azeredo et al., 2017; Gómez et al., 2016; Nair, Zhu, Deng, & Ragauskas, 2014; Syverud & Stenius, 2009). Detailed discussion of the applications of nanocellulose in food packaging is not included in this thesis but described elsewhere (Azeredo et al., 2017; Lee et al., 2017).

2.6.3 Safety evaluations of nanocellulose materials

Cellulose (microcrystalline cellulose and cellulose powder) and some derivatives of it are accepted as food additives with E-numbers E460–E466 and E468–E469 (EUR-Lex, 2012). Bacterial cellulose is also generally recognized as safe (Shi, Zhang,

Phillips, & Yang, 2014). There are no features in the chemical structures of cellulose material, which would make them harmful but because of their small particle sizes, nanocellulose materials should be considered separately (Kangas, 2014). The amount of research on nanocellulose safety on food products is still limited. The safety of microfibrillated cellulose was studied using mouse and human macrophages by Vartiainen et al. (2011). The study concluded no cytotoxicity or effects on the inflammatory systems in macrophages during exposure time of 6 and 24 hours (Vartiainen et al., 2011). Short-term toxicity and sub-lethal effects of cellulose nanofibrils from birch pulp as well as the ability of it to damage DNA has further been studied with human and animal cells (Pitkänen et al., 2010). In this study, no DNA damage or any cyto- or genotoxic effects were observed. In another publication, Pitkänen et al. (2014) studied sublethal effects, genotoxicity, systemic effects and cytotoxic effects of cellulose nanofibrils. Except for the test of cytotoxicity with the highest sample concentration, all these tests shoved negligible toxic indications. Andrade, Mendonça, Helm, Magalhães, Bonzon de Muniz and Kestur (2015) conducted *in vivo* research studying the effect of adding nanocellulose to the diet of mice and found no harmful effects. The *in vitro* cytotoxicity assessment of NFC by Pereira et al. (2013) showed a decrease in cell viability and effect on the expression of stress- and apoptosis-associated molecular markers. This effect was found however only in extremely high concentrations of NFC (2000 – 5000 μg/ml) while lower concentrations (0.02 – 100 μg/ml) did not have any cytotoxicity (Pereira et al., 2013). Tibolla et al. (2019) studied the cytotoxic effects of NFC *in vitro* using Caco-2 cell line and did not find cytotoxic effects on concentrations between 50-2000 μg/ml but the cell viability was decreased in extremely high concentrations above 2000 μg/ml. Further, recently both *in vitro* (gastrointestinal tract simulator) and *in vivo* (rats) studies were conducted by DeLoid et al. (2019) using both NCC and NFC with concentrations of 1.5% w/w and 0.75% w/w. They concluded that the studied materials are likely non-hazardous when consumed in small quantities. The safety issues of nanocellulose materials particularly in food applications are reviewed in Gómez et al. (2016) and Lee et al. (2017). In conclusion both reviews suggest that nanocellulose materials have no or low toxicity but to ensure this, there is a need for further studies (Gómez et al., 2016; Lee et al., 2017). Because of the lack of safety evaluations also common worldwide regulations for the use of nanocellulose materials as food additives are still missing, which prevents wider utilization of these materials (Gómez et al., 2016; Franco and de Muniz, 2018; Mu et al., 2019).

3 RESEARCH OBJECTIVES AND QUESTIONS

In this thesis, the taste and the chemical profile of compounds responsible for the taste of Nordic mushrooms as well as taste-modification properties of nanofibrillar cellulose were studied. **Publications I** and **II** concentrate on the analysis of taste and flavor properties of mushrooms with both instrumental and sensory methods. As the information about Nordic mushroom species and their taste properties is scarce, the aim was to form a fuller picture of them for the applications of food industry and for the basis of future studies. In **Publications III** and **IV** instrumental and sensory methods are developed and utilized to evaluate the taste-modification properties of nanofibrillar cellulose. Nanocellulose materials are known to have large surface area and thus binding capacity, suitable rheological behavior and emulsion stabilization effects for food applications, but yet, the literature covering the taste or taste-modification properties of them is scarce. Due to its large surface area and thus binding capacity, it was hypnotized that nanofibrillar cellulose might have ability to form bonds with some compounds eliciting taste. The interactions were studied with a fluorescence indicator displacement method in **Publication III**. A set of bitter, sweet and umami eliciting compounds were studied while salts and acids known to cause swelling were left out. Further, in **Publication IV**, a sensory study was conducted to evaluate whether nanocellulose has more pronounced effect to the taste than carboxymethyl cellulose already used in food industry.

The aims of this study were:

1. To measure what kind of taste compounds and taste characteristics can be found in Nordic mushroom species and further analyze the impact of them in total flavor profile and liking of mushrooms (**Publications I** and **II**)
2. To develop instrumental methods to evaluate the taste compound concentrations and their interactions with food matrices (**Publications I** and **III**)
3. To evaluate the potential of nanocellulose as a taste modifier for bitter, umami and sweet tasting samples (**Publications III** and **IV**)

4 MATERIALS AND METHODS

4.1 The taste of mushrooms

4.1.1 Mushroom sample material and prehandling

The mushroom species selected for the studies were *Cantharellus cibarius* (chanterelle, collected 8/2016, Salo/Finland), *Craterellus tubaeformis* (trumpet chanterelle, collected 9/2016, Salo/Finland or Kainuu/Finland), *Boletus edulis* (porchini, collected 9/2016, Salo/Köyliö/Finland) and *Lactarius camphoratus* (curry milk cap, collected 9/2016, Salo/Finland or 8/2016 Tampere/Finland). All these species except curry milk caps are popular wild mushroom species to pick and consume in Finland. Curry milk caps was chosen because of its peculiar flavor properties. Cultivated button mushrooms (*Agaricus bisborus*) were used as references in **Publication II**.

The fresh mushroom samples were kept in +4 °C until processing. Processing was conducted within 36 hours after picking. Based on the preliminary studies, a *sous vide* method was chosen for the sample handling. The fresh mushrooms were cut to 1-2 cm pieces and vacuum packed in plastic bags. Vacuum packed samples were heated at 80 °C for ten minutes after which the bags were immediately cooled in < 20 °C water for two minutes and icy water (5–9 °C) for 5 minutes. After cooling, the samples were moved to a freezer (-20 °C). After 1–12 weeks of storing, the frozen samples were cut to 1-2 cm^3 cubes in 4 °C. The samples were pooled, packed back to plastic bags and stored in a freezer while –20 °C waiting the analysis. Before sensory evaluations, the mushroom samples were thawed in *sous vide* bags in 70 °C water baths for 5 minutes.

Due to the poor availability of curry milk cap samples, curry milk cap samples used for the descriptive analysis of mushrooms described in detail in Aisala et al. (2018) were first dried in 36–37 °C for 7–8 hours with Evermat food dehydrator (Evermat AB, Bjurholm, Sweden) and stored in room temperature in a glass jar until analysis (up to 10 months). Before analysis, the curry milk cap samples were rehydrated by adding 700 g of active-carbon filtered water to 100 grams of dried sample. The samples were incubated for 15 minutes at ambient temperature. The

rehydrated samples were *sous vide* processed as described earlier for other samples. This species was not included in the samples chosen for hedonic testing.

Before amino acid/nucleotide analysis the samples were freeze-dried in a vacuum at -40 °C for 27–29 hours and ground to fine, uniform powder with mortar and pestle. The samples were weighted before and after freeze-drying to estimate the dry-matter content of mushrooms. Amino acid/nucleotide/nucleoside extraction method was modified from Ranogajec, Beluhan, and Šmit (2010) and Rotola-Pukkila et al. (2015). The method is based on the triple extraction of freeze-dried and ground mushroom powder with boiling water and described in detail in **Publication I**. Altogether 26 amino acids and 10 nucleosides/nucleotides were quantified with an UHPLC method. The reference materials described in detail in **Publication I** were either from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma Aldrich (St. Louis, Missouri, USA).

4.1.2 Sensory analysis of mushrooms

The sensory evaluations were performed in the ISO 8589 standardized sensory laboratory of Functional Foods Forum (University of Turku). All data was collected using Compusense Cloud 8.4 (Compusense Inc., Guelph, Ontario, Canada). Informed consent was obtained from each subject before their participation in the study. The descriptive analysis of mushrooms is described in detail in Aisala et al. (2018) and referred to in **Publication II**. In short, descriptive analysis of mushrooms was performed with 11 members panel of voluntary and experienced assessors. The mushroom samples (10–15 g) in 50–60 °C temperature consisted of both solid mushroom and dissociated liquids in ratios comparable to the mass ratios of freshly cooked ones. Four training sessions of 1.5 hours each and additional blind training session simulating the actual sensory evaluations were conducted. In the first session assessors were asked to describe the appearance, odor, taste, flavor, texture and chemesthetic properties of the samples. In the later session, the lexicon was clarified and agreed on and the reference samples and their intensities were agreed. The final profile of 18 attributes was formed. Of these 8 were odor descriptors, 3 taste descriptors, 3 chemesthetic and 4 texture descriptors. Three evaluation sessions were conducted. In these sessions all samples were evaluated in triplicates and served from the hotplate monadically (one sample served at a time). A line scale from 0 (none) to 10 (very strong) was utilized in the assessments.

Hedonic tests for mushroom samples were conducted with a total of 84 consumers between 20 and 74 years that regularly consumed mushrooms. The detailed description of the research can be found in **Publication II**. The participants were asked to evaluate the odor, appearance, flavor, texture and overall liking of each species studied using 9-point hedonic scale with number and descriptive anchors. The samples were presented monadically in a randomized order. The participants were asked to avoid strong perfumes on the day of evaluation as well as eating and drinking at least 30 minutes prior evaluation. Low-sodium crackers as well as active-carbon filtered water was served to clean the palate between evaluations. After evaluations, a set of background questions about usage of mushrooms and demographics as well as Food Choice Questionnaire from (Steptoe, Pollard, & Wardle, 1995) modified as (Pohjanheimo & Sandell, 2009) and Food Disgust Scale (Hartmann & Siegrist, 2018) was asked to fill.

4.1.3 UHPLC methodology

The nucleotide/nucleoside contents of mushroom samples were analyzed by a method modified from Ranogajec, Beluhan and Šmit (2010) and Rotola-Pukkila et al. (2015). For amino acid analysis, a method modified from the technical note of Shimadzu (Shimadzu Corporation) was used. For both analysis apparatus used was Shimadzu Nexera X2 quaternary pump (LC-30AD) combined with two degassers, autosampler, column oven and diode-array (SPD-M20A) and fluorescence detectors (RF-20AXS). The data was analyzed with Shimadzu LabSolutions-software. Synergi Hydro 4u column (Phenomenex, Torrace, California, USA) was used for nucleotide/nucleoside analysis and Kinetex 2.6 µm C18 (Phenomenex) for amino acid analysis. Diode-arrays detection with the wavelength of 254 nm and fluorescence detection with the excitation/emission wavelengths of 340/450 nm and 266/305 nm were used for nucleotide/nucleoside and amino acid detection, respectively. The solvents used for nucleotide/nucleoside analysis were 20 mM phosphate buffer (pH 5.9) and 100% MeOH and for amino acid analysis 20 mM phosphate buffer (pH 6.5) and 45/40/15 ACN/MeOH/H_2O solution. The gradient programs are presented in detail in **Publication I**.

All together ten nucleotides and nucleosides were analyzed simultaneously. The calibration was conducted using seven concentrations between 0.5 and 20 mg/l. For amino acid analysis, the amino acids were derivatized using a protocol from the technical note of Shimadzu (Shimadzu Corporation). The chemicals used for

derivatization were o-phatalaldehyde and 3-mercaptopropionic acid in 0.1 M borate buffer solution and 9-fluorenyl methyl chloroformate in acetonitrile. Phosphate buffer in pH 2.1 was added during derivatization. 26 amino acids were analyzed and calibration was conducted using nine standard solutions of each amino acid in concentrations between 1 and 125 or 250 μmol/l. Both methods used were validated using spiking experiments and residual extraction described in detail in **Publication I**. The limit of detection and the limit of quantification for both amino acid and nucleotide/nucleoside analysis were estimated based on signal-to-noise ratio and calculated with coefficients 3 and 10.

As a large peak in curry milk cap samples was detected at the same retention time as L-theanine in the UHPLC analysis and further also with qNMR (described in detail in **Publication II**), the sample was further analyzed with LC-MS to verify whether the peak actually corresponded to L-theanine. The underivatized sample of curry milk cap was measured with a Waters Acquity UHPLC instrument (Waters, Milford, MA) with Waters Acquity HSS T3 (2.1 × 100 mm, 1.8 μm) column connected to a Waters Xevo Q–TOF MS. Data analyses were performed with Waters MassLynx V4.1 software. The gradient program of 5 minutes in total consisted of a change from 95% of 0.1% formic acid in H_2O to 100% of acetonitrile and back to 95% 0.1% formic acid in H_2O. The injection volume used was 5 μL. Full scan mode (50 – 1000 m/z range) and electrospray ionization on positive mode was used with 2.5 kV capillary voltage.

4.1.4 Statistical analysis used in analysis of the mushrooms

The statistical tests for taste compound concentrations in mushrooms were performed using IBM SPSS Statistics 24.0 (IBM, NY, USA). The one-way analysis of variance (ANOVA) or Brown-Forsythe and Tukey's or Tamhane's T2 test for post hoc analysis were used to analyze the differences between mushroom samples. For ANOVA, the data was either square- or cube-transformed if necessary, and non-parametric Kruskal-Wallis and Mann-Whitney U tests with Bonferroni corrections were used if the data was not normally distributed. The choice of post hoc test was done based on the homogeneity of variances. The level of significance was $p < .05$ in all tests.

PCA analyses were conducted both for the non-volatile compounds and the sensory evaluations with The Unscrambler version 10.4.1 (Camo Process AS, Oslo, Norway) with auto-scaled data. EUC values were calculated from free amino acid and 5'-nucleotide contents using either data from **Publication I** or for the amino acids of button mushrooms from the NMR studies of **Publication II** and for nucleotides from Li et al. (2011). Also, total sugars, total acids and sugar-acid ratios were calculated as well as the total sugars in glucose equivalents, total acids in malic acid equivalents and equivalent sugar-acid ratios based on relative sweetness and sourness of these compounds (Moskowitz, 1971a, 1971b). Partial least squared regression (PLS) was conducted with chemical attributes as *X*-variables (predictors) and sensory properties as *Y*-variables (responses). Data was mean-centered and predictors autoscaled.

The consumer clustering was performed based on liking scores on mushrooms on five hedonic modalities. These modalities of each of four mushrooms species studied were first analyzed using PCA with FactoMineR package (Lê, Josse, & Husson, 2008) RStudio 1.2. with mean centered data without standardization in order to find the main sources of variation. This dataframe was used for hierarchical cluster analysis (HCA) with HCPC function of FactorMineR package. Ward's method and Euclidean distances were used. The number of clusters suggested by algorithm was three. The differences with hedonic modalities were analyzed with a 2-way ANOVA in SPSS. Appropriate data transformations were made in the case the data was not normally distributed. Eta squared values were calculated according to Levine and Hullet (2002) and post-hoc tests were built for cluster differences in each mushroom. One-way ANOVA or Kruskal-Wallis test were used for analyzing the effect of background variables on the cluster membership. Post-hoc tests were analyzed either Tukey's HSD or the Mann-Whitney U with Bonferroni corrections. PLS was used to measure the sensory drivers of liking by using sensory properties as the X-variables and average liking scores of each consumer clusters for liking modalities as the Y-variables with mean-centered and not scaled data.

4.2 The taste-modification properties of nanocellulose

4.2.1 Sample materials for nanocellulose studies

For the fluorescence indicator displacement study, 1.5% nano fibrillated cellulose (NFC) from UPM Biomedicals (Finland) was used. 1.5 wt% suspension of microfibrillated cellulose (MCC, Avicel®, Sigma-Aldrich) was prepared with water purified with a Milli-Q system (Millipore, Burlington, Massachusetts, USA). MCC was chosen for the study as it is similar to NFC by its chemical structure other than the dimensions. The studied taste compounds were caffeine (99%), naringin and aspartame (98%) from ThermoFisher GmbH (Kandel, Germany) and glutamic acid (99%), stevioside, sucrose (> 99%) and quinine (99%) from Acros Organics (Geel, Belgium). The compounds were chosen as they elicit both taste characteristics usually perceived as unpalatable (bitterness) and palatable (sweetness, umami). Salts and strongly acidic compounds were not included as they are known to cause swelling of the studied material which interferes the fluorescence indicator displacement method (Grignon & Scallan, 1980).

The NFC material used in the sensory evaluation studies was 1.5% Growdex® that is isolated from *Pinus Sylvestris* and *Picea abies* or *Betula sp.* and purchased from UPM Biomedicals (Finland). Similar material has been used before for example in Paukkonen et al. (2017), Valo et al., (2011) and in Kolakovic et al. (2012). Carboxymethyl cellulose (CMC) sodium salt (Cekol® 4000, CP Kelco/Finland) was chosen as a reference sample for the assessment of the taste modification properties of nanocellulose materials. CMC was chosen here as a reference since it is widely used in food industry as thickening agent. Four taste compounds, quinine hydrochloride (QHCl), caffeine, steviol glycoside and sucrose, were chosen for this study. The taste compounds were chosen so that possible changes in both bitterness and sweetness were studied. From these categories, four compounds were chosen based on the results from fluorescence indicator displacement study so that clear differences could be seen in the binding constants of compounds. QHCl was used instead of quinine and steviol glycoside mixture instead of stevioside as these are more commonly used in food industry. From these materials altogether 10 samples were produced (Table 5.). Each sample contained either 0.5% of NFC or CMC diluted in water and possible addition of taste compound in concentrations indicated in the Table 5. The concentration of NFC was chosen so, that the formed sample was clearly thicker than water, but the concentration level was on as low as possible

to minimize the exposure of assessors to NFC. CMC concentration was adjusted so that the viscosity was on a suitable approximate level to mimic the mouth shear rates estimated to range from 10 1/s to 1000 1/s. The viscosity measurements are described in detail in **Publication IV**. QHCl in the concentration of 0.0005% and sucrose in 2% and 4% were used as reference materials.

Table 5. The samples prepared for sensory evaluations of taste modification properties of nanofibrillar cellulose

	References	Matrix	Conc. taste compound (%)	Taste
	Sucrose	Water	4	Sweet
	Sucrose	Water	2	Sweet
	Quinine hydrochloride	Water	0.0005	Bitter
Sample number	**Taste compound**			
1.	-	NFC	-	Blank
2.	-	CMC	-	Blank
3.	Sucrose	NFC	4	Sweet
4.	Sucrose	CMC	4	Sweet
5.	Quinine hydrochloride	NFC	0.0005	Bitter
6.	Quinine hydrochloride	CMC	0.0005	Bitter
7.	Caffeine	NFC	0.04	Bitter
8.	Caffeine	CMC	0.04	Bitter
9.	Steviol glycoside	NFC	0.008	Sweet
10.	Steviol glycoside	CMC	0.008	Sweet

4.2.2 Sensory analysis

For the sensory evaluation of NFC and CMC samples in **Publication IV** altogether 10 voluntary assessors with former background of sensory profiling and known ability to identify and rank taste compounds participated. The evaluations were performed in the standardized sensory laboratory of Functional Foods Forum (University of Turku, ISO 8589). The ethical statement for the sensory evaluation study of NFC and CMC was applied from the University of Turku Ethics committee (statement 56/2019) and informed consent was received from every assessor prior the study. Participants were trained for analysis in two training sessions of approximately an hour each where the samples were introduced to assessors as well as the references were agreed on. The reference samples were agreed to represent

values 4 (sweetness 1 = sucrose 2%), 8 (sweetness 2 = sucrose 4%) and 5 (bitterness = QHCl 0.0005%). In the actual evaluations, the samples of 3.5 grams were served to assessors in white plastic spoons that were placed to lean against the side of the tray in a way that it was easy for assessors to lift them. The assessors were instructed to spit out the samples after tasting them. Reference taste solutions (approximately 40 ml each) were served with samples. The set up for research is presented in the Fig. 2. To minimize the effects of slight differences in appearance of the samples, samples were presented for assessors in white plastic spoons and red lightning was used in evaluation sessions. Wheat crackers and water were served for the assessors to clean their palates. Replacement samples were provided for the assessors when needed. The assessors were guided to rate the intensity of the bitterness and sweetness of each sample on a scale from 0 (not sensation at all) to 10 (extremely strong). In addition, the assessors were instructed to describe the samples with extra comments when needed. The assessments for each sample were repeated three times in different sessions. Compusense Cloud 8.4 was used for data collection.

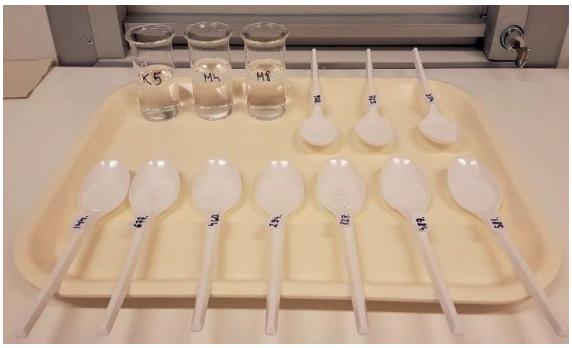


Figure 2. The sample presentation for the sensory evaluation of NFC and CMC samples

4.2.3 Fluorescence indicator displacement method

Fluorescence indicator displacement methods have previously been utilized for example studying interactions between drug and macromolecules such as DNA, RNA and proteins (Asare-Okai & Chow, 2011; Ham et al., 2003; Mock et al., 1985; Zhang, 2010). The idea in short is to utilize the competitive binding of a well-known fluorescent indicator (FI) molecule and the molecule in interest in studied macromolecule. With strong enough interaction, the pre-formed FI-macromolecule complex will be broken as FI is replaced by the molecule of interest. The decrease

of fluorescence intensity can be measured as a FI-macromolecule complex is deforming. The method is presented in the Fig. 3.

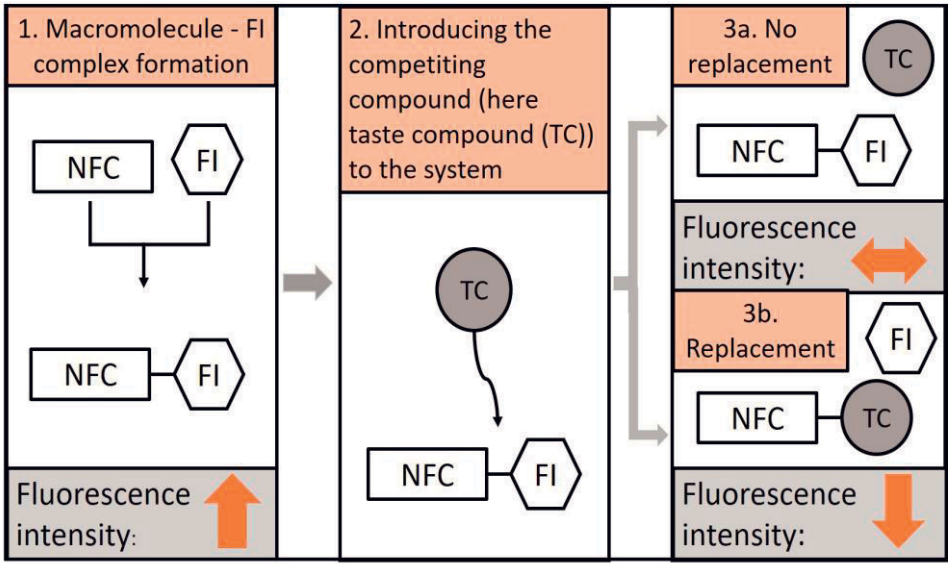


Figure 3. The schematic illustration for fluorescence indicator displacement method. In the first phase, the macromolecule (nano fibrillated cellulose, NFC) and the fluorescence indicator are mixed in order to initialize complex formation. The complex formation causes an increase in the fluorescence intensity compared to the fluorescence intensity of the free FI. In the second phase, taste compound is introduced to the system. Depending on the binding strength, the taste compound either cannot replace the FI and thus cause no change in the fluorescence intensity (3a) or if the binding strength is strong enough, the taste compound replaces the FI resulting in decreasing fluorescence intensity (3b).

In **Publication III**, we utilized a FI displacement method in order to study interactions between taste molecules and nanofibrillar cellulose. Two fluorescent dyes, Calcofluor white (CFW, Fluorescent brightener 28, Sigma Aldrich) and Congo red (CR, > 98%, Tokyo Chemical Industry CO., Tokyo, Japan) were chosen for the study. Two FIs were chosen to avoid the situation where the compound studied absorbs light in the wavelength used for excitation of FI. The changes in the fluorescence intensity were measured in triplicates by spectrofluorometer Fluorolog-3® (Jobin Yvon) or by using a conventional plate reader (Fluoroskan Ascent FL (Thermo Labsystems)) with 96 well-plates. The emission/excitation pairs chosen were 355/460 nm for CFW and 485/590 nm for CR. Studied compounds, caffeine, naringin, aspartame, glutamic acid, stevioside, sucrose and quinine, were chosen to provide different taste characteristics (bitter, sweet, umami). To estimate the molar

extinction coefficients of FIs absorption spectra of FIs in water was measured with UV-Vis-NIR spectrophotometer (UV-3600, Shimadzu) in 1 cm^2 standard quartz cuvettes in wavelengths from 250 to 600 nm. Absorption maxima for CFW was detected at 349 nm and for CR at 499 nm. Based on these measurements the concentrations of CFW and CR were chosen to be 6 and 2.5 μM respectively to avoid inner filter effects on the fluorescence of FIs.

The titration of FIs with NFC from 0 to 0.04 M resulted in increasing fluorescence intensity as the FI-NFC complex was formed. The NFC concentration was estimated based on monomeric cellulose (162.14 g/mol) units per liter, reflecting the possible number of binding sites. Microcrystalline cellulose was studied in similar manner with concentration range of 0.002 to 0.088 M. The binding constants for FIs to macromolecules were calculated using the Benesi-Hildebrand method (Benesi & Hildebrand, 1949) as follows

$$\frac{I_{max}-I_{free}}{I_n-I_{free}} = 1 + \frac{1}{K_{bind}[NFC]} \quad (3)$$

where [NFC] is the estimated concentration of NFC, I_{max} is the fluorescence intensity of FI at the maximum when needed amount of NFC is added to reach the saturation, I_{free} is the fluorescence intensity of free FI, I_n is the fluorescence intensity of FI in the presence of NFC in concentration *n* and K_{bind} is the binding constant for the FI. K_{bind} values were obtained by plotting $\frac{I_{max}-I_{free}}{I_n-I_{free}}$ versus 1/[NFC].

Further, by titrating the preformed NFC-FI-complex with varying concentrations of taste compounds (chosen based on their solubilities in water), a decreasing fluorescence intensity was detected if an interaction between NFC and the taste compound occurred. Based on the measured saturation curves for NFC-FI complex formations, the concentration of NFC was set to 0.04 M with CFW and 0.025 M with CR as the FIs were 6 μM for CFW and 2.5 μM for CR. To calculate the binding constants, the Benesi-Hildebrand method was modified as

$$\frac{I_0-I_{free}}{I_0-I_n} = 1 + \frac{1}{K_{bind}[TC]} \quad (4)$$

where [TC] is the taste compound concentrations, K_{bind} is the binding constant of the taste compound, I_0 is the fluorescence intensity of FI-NFC mixture, I_{free} is the fluorescence intensity of free FI, I_n is the fluorescence intensity of FI-NFC mixture in the presence of the taste compounds in concentration *n*.

To ensure that both dyes behave with the materials in a similar manner, the binding of caffeine was measured with both FIs. Also, cross-validation for the method was performed with isothermal titration calorimetry which is described in detail in **Publication III**.

4.2.4 Statistical analysis used in analyzing the taste-modification properties of nanocellulose

The statistical analyses for nanocellulose taste assessment studies were performed using IBM SPSS Statistics 25. The normality of variances was tested with Shapiro-Wilk test. As the majority of the variances were not normally distributed ($p < .05$), non-parametric version, Mann-Whitney U-test was used for comparing each pair of thickening agents (CMC or NFC) with different addition of taste compounds. The panel reproducibility and consensus were studied using one-way analysis of variance (ANOVA) and post hoc tests (Tamhane's or Tukey's) or Kruskal-Wallis and Mann-Whitney U-tests with Bonferroni corrections For panel reproducibility, three replicates of the same sample and for the panel consensus the assessments of each participant were compared. The significance level was $p < .05$ in all tests.

5 RESULTS AND DISCUSSION

5.1 Taste of mushrooms

5.1.1 Free amino acids and nucleotides in Nordic mushroom species

FAA as well as nucleotide and nucleoside contents of each mushroom species was measured utilizing liquid chromatography in order to evaluate the possible differences between species and further to evaluate their importance for the taste of mushrooms. The FAA concentrations of each mushroom species in mg/g in dry weight were calculated based on dry matter percentages presented in detail in **Publication I**. The highest dry matter concentration was measured in curry milk cap (14.5%) and lowest in trumpet chanterelles (7.8%). Based on statistical analysis, the studied mushrooms were different in their free amino acid contents. Altogether, 26 free amino acids were quantified. The average free amino acid concentrations with standard deviations are provided in the Table 6.

The FAA content varied between 14.93 to 29.54 mg/g of dry weight in all mushroom samples being the highest in porcinis and lowest in trumpet chanterelles Glutamic acid and aspartic acid as well as arginine, histidine and glutamine were found at relatively high concentrations from all samples. Umami amino acids were amongst the five most abundant free amino acids in all five species. The highest concentration of glutamic acid was measured from curry milk caps (4.69 mg/g dw) and the lowest from trumpet chanterelles (1.92 mg/g). The residual content after extractions was calculated to be 3.7% on average for the studied FAAs whereas recovery in spiking experiments was 86.1% indicating good performance of the extraction.

The averaged nucleotide contents measured are provided in the Table 7 with standard deviations. The residual contents for nucleotides and nucleosides measured was on average 3.4% and the recovery of added standard was after extraction on average 97.3%.

Table 6. FAA contents of studied mushrooms in dry weight (mg/g) and standard deviations (n=3). The concentrations marked with same letter a-d in the same row have no statistical difference. Modified from (Manninen, Rotola-Pukkila, Aisala, Hopia, & Laaksonen, 2018)

	Chanterelle	Trumpet chanterelle	Porcini	Curry milk cap
Umami				
Aspartic acid[1]	1.29 ± 0.08 (a)	1.65 ± 0.15 (a)	2.85 ± 0.44 (b)	3.35 ± 0.26 (b)
Glutamic acid[1]	3.78 ± 0.24 (a)	1.92 ± 0.021 (b)	2.62 ± 0.40 (b)	4.69 ± 0.36 (c)
Sweet				
Alanine[1]	0.65 ± 0.02 (a)	0.66 ± 0.11 (a)	6.67 ± 0.92 (b)	0.98 ± 0.11 (c)
Glycine[1]	0.21 ± 0.02 (a)	0.36 ± 0.03 (b)	1.29 ± 0.21 (c)	0.26 ± 0.01 (d)
Serine[1]	0.53 ± 0.03 (a)	0.39 ± 0.03 (b)	1.53 ± 0.23 (c)	0.62 ± 0.04 (d)
Threonine[3]	0.47 ± 0.02 (a)	0.28 ± 0.02 (b)	0.90 ± 0.12 (c)	0.58 ± 0.03 (d)
Bitter				
Arginine[1]	4.47 ± 0.46 (a)	1.15 ± 0.13 (bc)	1.41 ± 0.20 (c)	0.92 ± 0.08 (b)
Histidine[2]	1.13 ± 0.08 (a)	1.07 ± 0.10 (a)	0.77 ± 0.14 (b)	1.73 ± 0.12 (c)
Isoleucine[3]	0.23 ± 0.01 (a)	0.32 ± 0.03 (b)	0.31 ± 0.03 (bc)	0.27 ± 0.01 (c)
Leucine[2]	0.43 ± 0.02 (a)	0.42 ± 0.03 (ab)	0.37 ± 0.05 (b)	0.53 ± 0.03 (c)
Methionine[2]	0.14 ± 0.00 (a)	0.14 ± 0.00 (a)	0.23 ± 0.03 (b)	0.26 ± 0.01 (b)
Phenylalanine[2]	0.25 ± 0.01 (a)	0.40 ± 0.03 (b)	0.30 ± 0.03 (c)	0.60 ± 0.03 (d)
Trypthophan[2]	0.32 ± 0.04 (a)	0.46 ± 0.05 (b)	0.48 ± 0.09 (b)	0.09 ± 0.03 (c)
Tyrosine[1]	0.43 ± 0.03 (a)	1.98 ± 0.19 (b)	0.69 ± 0.09 (c)	0.35 ± 0.01 (d)
Valine[3]	0.31 ± 0.02 (a)	0.32 ± 0.03 (ac)	0.54 ± 0.07 (b)	0.37 ± 0.02 (c)
Others				
β-Alanine[2]	0.04 ± 0.01 (a)	0.14 ± 0.03 (b)	0.19 ± 0.03 (b)	0.04 ± 0.04 (a)
GABA[3]	0.31 ± 0.02 (a)	0.26 ± 0.05 (a)	0.67 ± 0.11 (b)	0.05 ± 0.02 (c)
Aspargine[3]	0.29 ± 0.02 (a)	0.89 ± 0.10 (b)	0.69 ± 0.11 (c)	0.32 ± 0.04 (a)
Citrulline[1]	0.04 ± 0.01 (a)	0.04 ± 0.00 (a)	0.28 ± 0.04 (b)	0.07 ± 0.02 (a)
Glutamine*[2]	4.74 ± 0.39 (a)	0.69 ± 0.08 (b)	3.87 ± 0.60 (c)	5.10 ± 0.44 (a)
Lysine*[3]	1.00 ± 0.10 (a)	0.53 ± 0.05 (b)	1.03 ± 0.15 (a)	0.63 ± 0.04 (b)
Ornithine[1]	0.84 ± 0.14 (a)	0.63 ± 0.10 (a)	1.82 ± 0.28 (b)	0.10 ± 0.01 (c)
Taurine[1]	n.d.	0.21 ± 0.02 (a)	0.04 ± 0.00 (b)	0.03 ± 0.00 (c)
Grand total	**21.94**	**14.93**	**29.54**	**21.91**

[1] Statistical analysis with Brown-Forsythe and Tamhane's T2, [2] Statistical analysis with F-values and Tukey's, [3] Non-parametric tests*Sweet according to Belitz et al. (2009), Not detected concentrations are marked with n.d. and concentrations which could not be quantified with -.

Table 7. Nucleotide contents of studied mushrooms in dry weight (mg/g) and standard deviations (n=3). The concentrations which are marked with the same letter a-d in the same row have no statistical difference. Modified from (Manninen et al., 2018)

	Chanterelle	Trumpet chanterelle	Porcini	Curry milk cap
AMP[3]	0.38 ± 0.03 (a)	0.70 ± 0.08 (b)	1.39 ± 0.09 (c)	0.08 ± 0.00 (d)
CMP[1]	0.37 ± 0.01 (a)	0.57 ± 0.07 (b)	1.87 ± 0.11 (c)	0.86 ± 0.03 (d)
GMP[2]	0.19 ± 0.03 (a)	0.11 ± 0.01 (b)	0.60 ± 0.04 (c)	-
IMP[1]	0.22 ± 0.01 (a)	0.13 ± 0.01 (b)	0.35 ± 0.02 (c)	n.d.
UMP[1]	0.28 ± 0.02 (a)	0.29 ± 0.07 (a)	-	-
Flavor nucleotides (GMP+IMP)/ (GMP+IMP+AMP)	0.41/0.79	0.25/0.95	0.95/2.34	n.d./0.08
Total nucleotides	1.43	1.81	4.21	0.94

[1] Statistical analysis with Brown-Forsythe and Tamhane's T2, [2] Statistical analysis with F-values and Tukey's, [3] Non-parametric tests, Not detected concentrations are marked with n.d. and concentrations which could not be quantified with -.

Flavor nucleotide contents in studied mushroom species were low (< 1 mg/g) in all species according to classifications proposed by Yang et al. (2001) considering the concentrations of IMP, GMP and XMP (not quantified here). When also AMP was included in total amount of flavor nucleotides the concentrations were between 0.08 mg/g and 2.34 mg/g. The highest concentration of umami nucleotides was measured in porcini samples and lowest in curry milk cap with which GMP and UMP could not be quantified.

5.1.2 Linking concentrations of taste compounds with sensory characteristics

In order to estimate the role of each compound measured on taste, statistical analysis was conducted. The PLS model combining the data of non-volatiles with the sensory attributes of taste and chemosensory attributes is presented in the Fig. 4. Further, the PLS model combining the instrumental data for non-volatiles and volatiles with the whole sensory profile is presented and interpreted in **Publication II**. The NMR spectroscopy method of measuring sugars, sugar alcohols, organic acids and amino

acids as well as the main results are described in **Publication II**. Further, methods and results for sensory evaluations are described further in Aisala et al. (2018) and for the analysis of volatiles of mushrooms in Aisala, Sola, Hopia, Linderborg, & Sandell (2019). In qNMR studies, three sugars and sugar alcohols, four organic acids and 17 amino acids were identified as well as seven unidentified compounds. The major unknown peak was in the curry milk cap and was tentatively identified as an unknown pentose structure with the molecular mass of 147.

From the Fig. 4 the taste attributes sweet, umami and bitter as well as chemesthetic attributes pungent, metallic and astringent are mostly explained by factor 1 (x-axis). All chemesthetic attributes as well as bitterness have negative loadings whereas umami and sweet have highly positive loadings. Of studied mushrooms porcini correlates the best with umami and sweet whereas curry milk cap has negative loadings correlating with pungent, bitter, astringent and metallic.

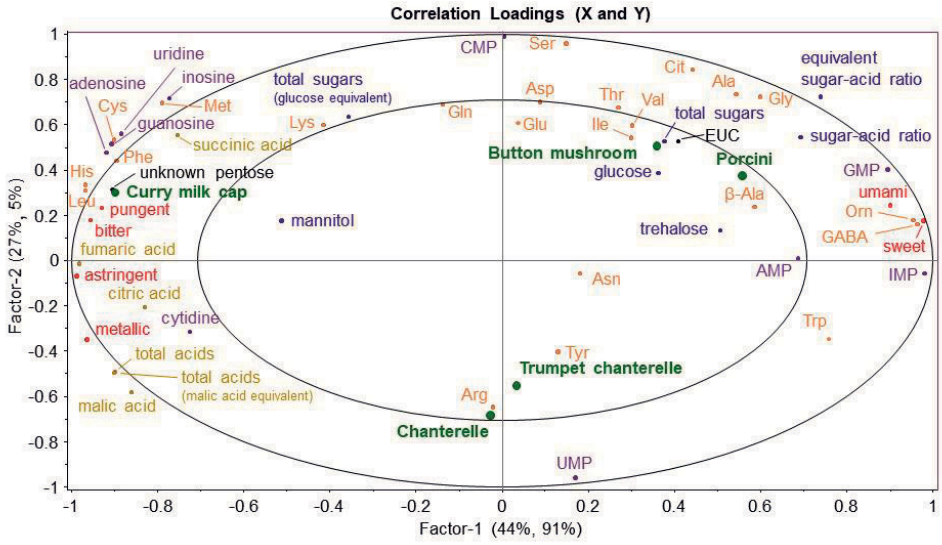


Figure 4. PLS analysis of taste and chemosensory attributes and measured sugars, sugar alcohols, amino acids and nucleotides and nucleosides. The colors in the picture are green for the mushroom species and red for the sensory properties. Amino acids are marked with orange, nucleotides with purple, organic acids with yellow and sugars with blue. (Aisala et al.,2020) Used with permission from the publisher.

Of studied compounds, all organic acids, all studied nucleosides, many amino acids with bitter taste (histidine, leucine, phenylalanine, methionine), cysteine and major unknown peak in curry milk cap correlated with bitter, pungent, astringent and metallic attributes and curry milk cap in factor 1.

The correlation between umami amino acids and umami taste based on factor 1 is not strong, but on the other hand the umami enhancing nucleotides GMP, IMP and AMP are very close of the umami taste attribute. In **Publication II**, even more pronounced trend was seen, when the volatile compounds, total flavor, texture and appearance are considered, as the umami amino acids had small or even negative correlations (factor 1) to umami taste, umami nucleotides, and EUC values. In Fig. 4 EUC value also correlates better with umami than umami amino acids, and particularly well with porcini and button mushroom. According to our results, EUC value and particularly GMP, IMP and AMP concentrations are more important predictors of umami taste than the levels of glutamic acid and aspartic acid. This is somewhat dissimilar to the results of Phat et al. (2016), who found good correlation between sensory studies and both EUC values and umami amino acid concentrations. As it can be seen from the Table 6, curry milk cap has the highest umami amino acid concentration of studied mushrooms. However, this species is particularly strongly correlated with strong flavor attributes such as pungent, bitter and astringent, which might suppress or mask other taste qualities. Based on the Fig. 4, the sugar-acid ratio has better correlation with sweetness in factor 1 than total sugars alone. Further, γ-aminobutyric acid (GABA) as well as ornithine are correlated with umami and sweetness.

In order to evaluate whether there are consumers with similar liking profiles towards mushrooms, cluster analysis was conducted. Three hierarchical clusters were formed with different liking profiles on mushroom species. The background variables for consumers are described in detail in **Publication II**. In short, the backgrounds of individuals in clusters were heterogeneous and did not provide an explanation for the liking profiles. The PLS model in the Fig. 5 as well as Table 8 give an overview of the liking profiles. The most different cluster was cluster 3, which evaluated all attributes but the appearance of button mushrooms with scores higher than 7, while cluster 2 gave systematically >1 units lower scores to all mushroom species but button mushrooms. Cluster 1 was statistically different from cluster 3 in all attributes. Cluster 1 gave lower scores than other two clusters to all mushrooms but trumpet chanterelles in taste, texture and overall liking and porcinis in odor and texture. Especially, button mushrooms were given low scores by cluster 1 which differed from cluster 2 and can be further seen in PLS model.

Table 8. Liking scores as averages with standard deviations for each consumer cluster and liking score two-way ANOVA p-values and effect sizes expressed as η^2 by factors. Significant differences between clusters are marked with letters a–c. Modified from (Aisala et al.,2020).

	Samples	Averages (STD)				ANOVA p values (η^2)		
		1 (n=20)	2 (n=38)	3 (n=26)	Overall	Species	Clusters	Interaction
Odor	Button mushroom	4.7 (1.3) c	6.1 (1.5) a	7.1 (1.1) a	6.1 (1.6)	0.001 (0.047)	0.001 (0.181)	0.228 (0.019)
	Chanterelle	6.5 (1.2) b	6.7 (1.6) b	7.8 (1.1) a	7.0 (1.5)			
	Trumpet chanterelle	5.7 (1.9) b	6.6 (1.4) b	7.9 (1.3) a	6.8 (1.7)			
	Porcini	6.1 (1.5) b	6.1 (1.7) b	7.5 (1.1) a	6.5 (1.6)			
Appearance	Button mushroom	3.9 (1.3) b	5.8 (1.5) a	6.4 (1.4) a	5.5 (1.7)	0.001 (0.062)	0.001 (0.227)	0.033 (0.029)
	Chanterelle	5.7 (1.6) b	6.5 (1.5) ab	7.2 (1.6) a	6.5 (1.6)			
	Trumpet chanterelle	5.3 (1.4) b	5.8 (1.4) b	7.3 (1.1) a	6.1 (1.5)			
	Porcini	4.4 (1.3) b	5.3 (1.7) b	7.1 (1.1) a	5.6 (1.8)			
Texture	Button mushroom	3.6 (0.9) b	6.1 (1.5) a	7.2 (1.5) a	5.8 (1.9)	0.09 (0.013)	0.001 (0.30)	0.001 (0.059)
	Chanterelle	4.6 (1.4) c	6.2 (1.4) b	7.5 (0.9) a	6.2 (1.7)			
	Trumpet chanterelle	5.6 (1.7) b	5.6 (1.5) b	7.2 (1.2) a	6.1 (1.6)			
	Porcini	5.4 (1.3) b	5.4 (1.3) b	7.5 (1.4) a	6.1 (1.8)			
Taste	Button mushroom	3.85 (1.6) b	6.7 (1.1) a	7.4 (1.3) a	6.2 (1.9)	0.33 (0.007)	0.001 (0.29)	0.001 (0.065)
	Chanterelle	4.85 (1.6) c	6.0 (1.5) b	7.8 (0.8) a	6.3 (1.8)			
	Trumpet chanterelle	5.65 (2.0) b	5.6 (1.4) b	7.3 (1.3) a	6.2 (1.7)			
	Porcini	5.75 (1.5) b	6.1 (1.5) b	7.5 (1.3) a	6.4 (1.6)			
Overall liking	Button mushroom	3.8 (1.1) b	6.7 (1.0) a	7.4 (1.2) a	6.2 (1.8)	0.04 (0.015)	0.001 (0.333)	0.001 (0.096)
	Chanterelle	5.2 (1.4) c	6.4 (0.9) b	7.7 (0.7) a	6.5 (1.4)			
	Trumpet chanterelle	5.9 (1.5) b	5.7 (1.4) b	7.4 (1.1) a	6.3 (1.5)			
	Porcini	5.7 (1.4) b	5.8 (1.4) b	7.7 (1.1) a	6.3 (1.6)			

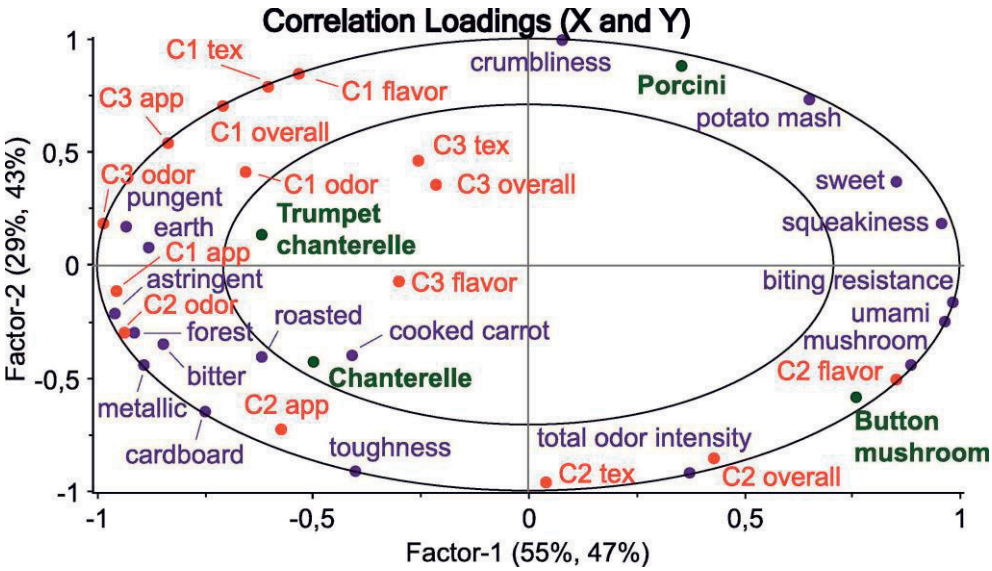


Figure 5. PLS model build based on mushroom sensory attributes (blue), mushroom samples (green), and liking scores for the consumer clusters (red). (Aisala et al.,2020) Used with permission from the publisher.

5.1.3 Discussion

The free amino acid and nucleotide/nucleoside profiles of four mushroom species were successfully measured with an UHPLC method. Good recoveries as well as low residual concentrations were approached in validation studies. Further, compared with the results of quantitative NMR studies presented in **Publication II**, good correlation was found among amino acids measured with both methodologies. All the studied mushrooms were different based on their free amino acid and nucleotide profiles. Of studied mushrooms, curry milk cap had the highest umami amino acid concentration. Based on classification introduced in the literature (Yang et al., 2001), all studied mushroom species except for trumpet chanterelle had medium concentrations of umami amino acids. However, compared with many species reported by Beluhan and Ranogajec (2011) the measured umami amino acid concentrations were on lower level. This might be explained by the differences in pre-processing methods as well as differences in original samples. Flavor nucleotides were found in low concentrations from all species studied. Compared with literature, the levels of measured nucleotides in porcinis are higher than measured before by

Tsai et al. (2008) and on the same level as measured by Beluhan and Ranogajec (2011) except for 5'-IMP which was found at higher levels from our samples. Similarly, for chanterelle, the levels of nucleotides were somewhat equal to ones measured by Beluhan and Ranogajec (2011), except for 5'-CMP and 5'-IMP concentrations which were on higher level and 5'-UMP found at lower levels in our samples.

PLS analysis correlating data from sensory evaluations of mushrooms, NMR studies and the free amino acid and nucleotide evaluations revealed that nucleotides are particularly important in the umami taste of mushrooms. However, it is unclear if the better correlation of umami perception with umami nucleotides than umami amino acids is due to the greater impact of nucleotides on umami taste or whether the umami taste is either suppressed or masked by other flavor attributes. For example, curry milk cap with the highest umami amino acid concentration is also correlated with pungent, astringent and metallic perceptions. As reviewed in the background section, the relationship between the concentration and taste perception is not linear and is further impacted by different flavors by different mechanisms.

Based on the hierarchical clustering of consumers in hedonic testing, three clusters were formed. These clusters had clearly different liking profiles on mushrooms. For example, cluster 2 appreciated particularly cultivated button mushrooms more than cluster 1 rating button mushrooms with low scores in all attributes. On the other hand, cluster 3 gave high scores for all the samples studied. In all, variations in liking were greater between clusters than between species studied. This indicates that individual differences between people are more important in the liking of mushrooms than flavor, appearance, or texture driven differences among samples.

5.2 Taste modification using nanocellulose

5.2.1 Nanocellulose – taste compound interactions

The interactions between nanofibrillar cellulose and taste compounds were assessed with a novel fluorescent indicator displacement method in order to evaluate the possible effects of nanocellulose matrix to the taste of food. Examples of the saturation curves for CFW and CR are presented in the Fig. 6 and reciprocal plots drawn after Eq. 3 are presented as insets. The binding constants for fluorescent indicators were estimated as 27 ± 7 M^{-1} for CFW and 58 ± 12 M^{-1} for CR. For MCC,

the binding constant of CR was estimated to be roughly 4 M^{-1} as the saturation was not reached in used concentration area with MCC. The stronger binding of CR on the NFC is most probably because of the smaller particle size of NFC.

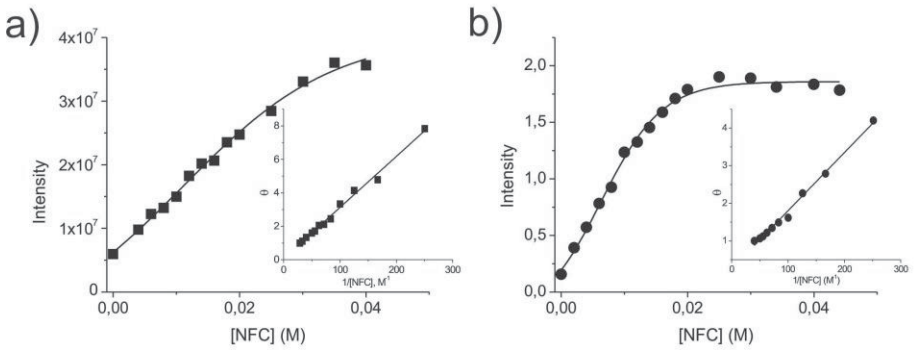


Figure 6. Examples of the saturation curves for CFW (a) and CR (b). The reciprocal plots according to Eq. 3 are presented in the insets. (Manninen, Durandin, Hopia, Vuorimaa-Laukkanen, & Laaksonen, 2020) Used with permission from the publisher.

Examples of the saturation curves of each compound binding to NFC are presented in the Fig. 7. Reciprocal plots drawn after Eq. 4 are presented as insets. From the Fig. 7 a–d, clear trends can be detected for caffeine, naringin, stevioside and quinine even though especially for stevioside the experimental fluctuations can clearly be seen. Clear differences can be detected between binding compounds. For example, 20% displacement of FI is achieved with approximately 5 mM concentration of caffeine, but only 0.025 mM concentration of quinine is needed to achieve the same displacement.

For the binding taste compounds, the magnitudes of calculated binding constants varied between 70 M-1 and 14300 M-1. Further, with sucrose, aspartame and glutamic acid change in fluorescence was not detected thus indicating negatable interactions between these molecules and NFC. The highest binding constant was achieved with quinine and the lowest with caffeine. Binding constants as well as solubilities to water are presented in the Table 9.

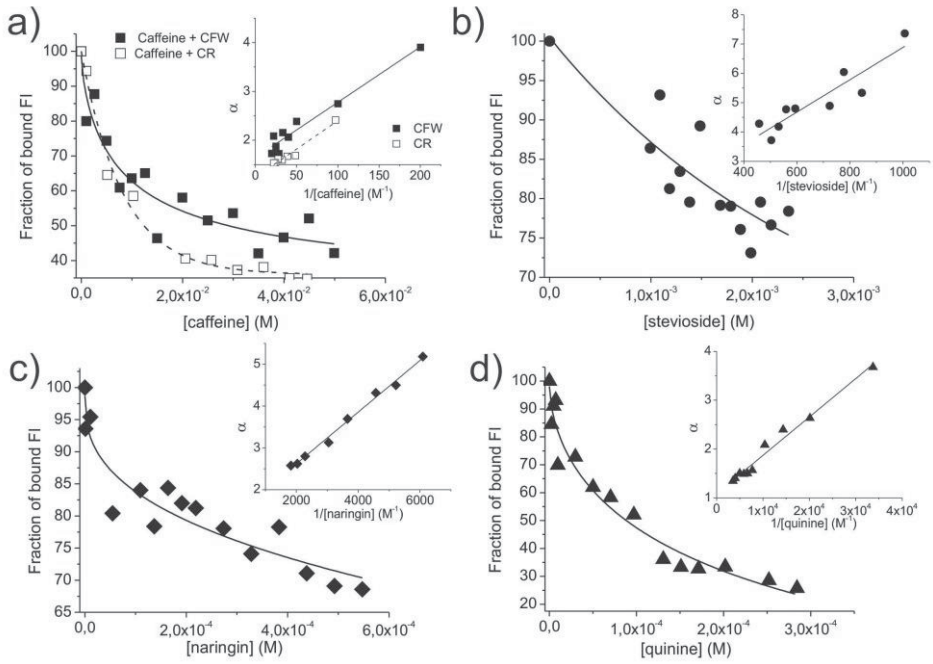


Figure 7. Examples of the saturation curves for caffeine (a), stevioside (b), naringin (c) and quinine (d). The reciprocal plots are presented in the insets. (Manninen et al., 2020). Used with permission from the publisher.

Table 9. Calculated binding constants with standard deviations (n=3) as well as the water solubilities of the compounds (Manninen et al., 2020).[1](Windholz, Budavari, Blumetti, & Otterbein, 1983)[2](Furia, 1980)

Compound	Taste	K_{bind} (M^{-1})	Solubility to water (mg/ml)
Sucrose	Sweet	Negl.	2 000[1]
Stevioside		146 ± 34	1.25[1]
Aspartame		Negl.	10.20[2]
Quinine	Bitter	14300 ± 1500	0.53[1]
Caffeine		70 ± 25	21.74[1]
Naringin		1251 ± 385	1.00[1]
Glutamic acid	Umami	Negl.	8.64[1]

In order to evaluate the possible differences in the functioning of FIs, the binding constant was measured for caffeine with both FIs, CFW and CR. With CR, the binding constant was estimated to be 86 M^{-1}. Thus, taking account the standard deviations, the FI used has only a little or no effect on the measured binding

constants. Further, binding constant for quinine was measured with isothermal titration calorimetry (ITC) described in detail in **Publication III**. The binding constant of quinine with NFC measured with ITC was 19 000 ± 5790 M-1. When considering the uncertainty of measurements causing big standard deviations, it can be concluded that the binding constants measured with different methods are close to each other.

Based on the binding constants provided in the Table 9, taste compounds can be divided into four groups: non-binding molecules (sucrose, aspartame, glutamic acid), molecules with weak interactions (caffeine and stevioside), molecules with moderate interactions (naringin) and molecules with distinct interactions (quinine). Quinine was the strongest binder to NFC and the least water-soluble of the compounds studied. From the Table 9 it can be seen that the interactions seem to partly correlate with the aqueous solubility of the compounds. This might be explained by hydrophobic interactions of aqueous NFC with poorly soluble compounds. Similar mechanism based on hydrophobic interactions have been proposed before in the case of lysosome and NFC (Kolakovic et al., 2013). However, other factors such as negative charges of binding molecules at the aqueous conditions might lower the probability of binding to NFC surface which contains negatively charged hemicellulose on its surface. This might explain the weak binding of molecules such as glutamic acid and aspartame. Furthermore, other effects such as amine groups present in quinine and caffeine might have an effect on the binding. As the structures of studied molecules varies greatly, exact mechanisms behind the differences in interactions between NFC and taste compounds cannot be verified based on this study but should be further examined in future.

5.2.2 Sensory evaluations

To evaluate the effect of found interactions on taste of food products, sensory evaluations were conducted. The averaged results from three sensory evaluation sessions as well as standard deviations of NFC and CMC samples with added taste compounds are presented in the Table 10. Further, status of statistical difference for each pair with the same taste compounds but different matrix is provided.

No statistical differences with Mann-Whitney U tests were found between CMC and NFC matrices with the addition of sweet tasting compounds (sucrose or steviol glycoside). The only statistically significant difference in the sweetness of CMC and NFC matrices with different taste compounds was found with the addition of

generally bitter tasting QHCl, in which case the CMC containing sample was assessed as slightly sweeter. However, the level of sweetness was close to 0 (no sensation at all). Sweetness of both CMC and NFC with sucrose were smaller than the used sweet standard with the same concentration of sucrose with the set sweetness value of 8 in the scale from 0 to 10. While this is not an exact comparison, it does give an indication that both CMC and NFC alter taste perception. In this case, they suppressed sweet taste.

Table 10. Means, standard deviations and statistical differences for the intensities of the bitterness and sweetness of studied samples (1.–10.) in different matrixes and additions of taste compounds perceived by ten assessors in three replicates.

Sample	Matrix	Taste compound	Conc. taste compound (%)	Bitterness		Sweetness	
				Mean	SD	Mean	SD
1.	NFC	None	-	1.65	1.71	0.15	0.29
2.	CMC			1.97	1.87	0.48	0.78
3.	NFC	Sucrose	4	0.31	0.62	6.74	1.46
4.	CMC			0.40	1.23	6.46	1.76
5.	NFC	Quinine hydrochloride	0.0005	6.17***	2.08	0.04*	0.10
6.	CMC			3.73***	2.12	0.54*	1.03
7.	NFC	Caffeine	0.04	5.88*	2.32	0.03	0.08
8.	CMC			4.35*	2.70	0.18	0.47
9.	NFC	Steviol glycoside	0.008	1.22	1.37	3.85	1.60
10.	CMC			1.21	2.03	4.46	1.68

Statistical differences between samples in different matrix but with the same added compound are according to non-parametric Mann-Whitney U-test either with * if $p < 0.05$, ** if $p < 0.01$ and *** if $p < 0.001$

Based on Mann-Whitney U test, blank CMC and NFC matrices were found similar on their bitterness. CMC had the mean value of 1.97 as NFC had the mean value of 1.67. CMC matrices were assessed as less bitter than NFC matrices with both added bitter tasting compounds (QHCl and caffeine). According to statistical analysis, these differences were statistically significant, however, only barely significant with caffeine ($p < 0.05$). NFC matrix with added QHCl was perceived as more bitter than standard with the same concentration of QHCl in water with set value of 5. As the intensity value of standard was set, this however cannot be statistically proven here.

The differences of assessments in bitterness and sweetness of the samples between different repetitions were determined with one-way ANOVA/Brown-Forsythe and Post-Hoc tests or Kruskal-Wallis and Mann-Whitney U-tests in order to evaluate the repeatability of the panel. No statistical differences were found in the perceived bitterness of the samples between sample sets. The sweetness of the CMC with added QHCl was evaluated differently in two sample sets but no other statistical differences were found between sample sets. Further, the agreement of assessors on sweetness and bitterness was evaluated with non-parametric Kruskal-Wallis and Mann-Whitney U-test. Kruskal-Wallis test shoved some differences between assessors, especially in bitterness the perceptions of CMC samples, but as the differences were not found with the Mann-Whitney U-test with Bonferroni corrections, it can be concluded that these differences were minor.

The open-ended question answers were grouped to two groups, chemesthesis or taste related and texture related descriptors. The most frequent answers to the open-ended questions of taste or chemesthesis are provided in the Table 11 accompanied by the number of answers including the descriptor and number of assessors mentioning the descriptor.

Table 11. The descriptors found the most frequently from open-ended questions related to chemesthesis or taste of the samples. #Answers is the number of answers including the keyword (n=30 for each sample) and #Assessors marks the number of assessors that included the keyword in their open answers at least at one repetition (n=10).

	Taste compound	Astringent		Saltiness		Burning/tingly		Cooling	
		#Answers	#Assessors	#Answers	#Assessors	#Answers	#Assessors	#Answers	#Assessors
NFC	None	14	7	1	1	3	3	2	2
CMC	None	1	1	8	3	5	2	2	2
NFC	Sucrose	9	6	0	0	3	3	2	1
CMC	Sucrose	0	0	4	2	3	2	0	0
NFC	QHCl	16	7	0	0	2	2	3	2
CMC	QHCl	3	2	5	3	7	4	5	3
NFC	Caffeine	15	8	0	0	2	1	1	1
CMC	Caffeine	4	3	4	2	4	3	6	3
NFC	Steviol glycoside	13	7	0	0	1	1	3	2
CMC	Steviol glycoside	4	2	5	3	4	3	3	3

The most used description in open-ended question answers was astringency, which was repeated in all NFC containing samples. It was included in about 45% of all 150 open-ended answers for NFC containing samples while in the descriptions for CMC containing samples the word occurred only in 8% of the answers. Out of 10

assessors, 6 to 8 mentioned astringency at least in one repetition when evaluating NFC containing samples. Furthermore, common descriptors were tingly/burning sensations, which were found in both NFC (7%) and from CMC (15%), cooling sensation (NFC 7%, CMC 13%) and saltiness which was more commonly associated with CMC (NFC 1%, CMC 17%). Texture related descriptors did not reveal any clear conclusions. Some words, such as thickness, was repeated one to three times in open-question answers for all samples. With some samples mentioned texture related descriptions were somewhat contradictory. For example, the samples of NFC with added QHCl were described both as thick and as runny.

5.2.3 Discussion

The nanocellulose-taste compound interactions were studied with a newly developed high-throughput screening method utilizing a plate reader. Binding constants between 70 M^{-1} for caffeine and 14 300 M^{-1} for quinine were measured with good accuracy. Of the studied compounds, glutamic acid, aspartame and sucrose showed no binding, caffeine and stevioside weak binding, naringin moderate binding and quinine strong binding.

The ability of CMC to suppress the bitterness of caffeine and quinine sulfate as well as astringency of phenolic compounds has been demonstrated before (Moskowitz & Arabie, 1970; Pangborn et al., 1973; Troszyńska et al., 2010). Based on the results from the fluorescent indicator displacement studies of the binding of taste compounds to nanocellulose, it was expected that NFC might have effect on bitterness intensity of QHCl. Further, as caffeine had significantly lower binding constant, we were interested to see if the difference in binding capacity could be seen as differences in bitterness suppression abilities in sensory evaluations.

Surprisingly, based on sensory analysis, the effect was not found. On the contrary, it was noticed that QHCl samples containing nanofibrillar cellulose had more bitter taste than samples containing QHCl in the same concentration in CMC. Also, with caffeine similar effect was seen, but the magnitude of it was slighter. Whereas NFC with added QHCl had slightly higher bitterness intensity as used reference with the same concentration of QHCl in water, the QHCl in CMC had lower intensity of bitterness. Further, sweetness of the samples with sucrose addition did not differ from each other but was on the lower level than the reference sample with the same concentration of sucrose in water indicating possible suppression of sweetness with both samples. This is in line with previous data as the viscosity of sodium CMC has

been demonstrated to suppress the sweetness and bitterness intensities of sucrose and quinine sulfate before (Moskowitz, 1970). However, as statistical analysis could not be done when comparing the samples to the set intensity of the reference, and the literature surveyed at the background section indicates that the both the nature and concentration of hydrocolloids have effect on the suppression of the taste intensities, these effects need to be further studied in more comprehensive study involving different concentrations of both CMC and NFC and taste compounds. Furthermore, fluorescent indicator displacement studying the binding between CMC and taste compounds would enlighten the possible mechanism of bitterness suppression.

The lack of ability of NFC compared to CMC to suppress the bitterness of QHCl is somewhat surprising as the same order of magnitude interaction have been seen to suppress bitterness of quinine with L-lysine and L-arginine (Zhang et al., 2016). Based on our studies, in this case other factors than only binding explain the materials ability to suppress bitterness. Firstly, from the open-ended questions of sensory evaluations it can be seen, that NFC was perceived as astringent by several assessors. This might affect the perceived bitterness when the differences between the samples are otherwise small as it has been demonstrated before, that astringency can enhance the bitterness at food samples (Scharbert & Hofmann, 2005). Further, saltiness perceived in CMC samples is a known suppressant for bitterness (Keast, Breslin & Beauchamp, 2001). As the viscosity of the sample materials was somewhat difficult to match due to the differences in the properties of the matrices, it is also possible, that the small differences of perceived viscosity might affect the taste. However, the differences in viscosities can be expected to be small as the assessors did not point out dissimilarities in open end questions. Further, it can be that the interactions seen in fluorescent indicator studies are too small to have effect on taste and thus to be noticed with sensory studies. QHCl activates taste receptors even in low concentration. The high enough concentrations of QHCl could prevent nanocellulose from fully binding it. More precise knowledge about the interactions could be provided by time-intensity evaluations as it might be that the interactions suppress the taste first, but then soon break and release the taste.

6 CONCLUSIONS, LIMITATIONS AND FUTURE PERSPECTIVES

The taste properties of four different Nordic mushroom species were measured both with instrumental methods and sensory evaluations. The results were linked using PLS regression analysis. The study provides new information about taste properties of Nordic mushroom species. The species studied had different profiles based on their free amino acid and nucleotide profiles. Of studied species, curry milk caps had the highest concentration of MSG-like amino acids while porcinis contained the highest concentration of umami enhancing nucleotides. The sensory evaluations and PLS regression analysis of taste revealed that the concentration of umami enhancing nucleotides was more important predictor of umami taste than the MSG-like amino acids. Also, EUC value had better correlation with umami taste than umami amino acids alone. Similarly, sugar/organic acid ratio had better prediction power to sweetness than total sugars alone. This indicates the complexity of taste perception and indicates the importance of taking account the interactions between different taste compounds when evaluating the taste of mushrooms and food in general. In consumer testing of liking on mushrooms, consumers were successfully divided into three clusters with different likings on mushrooms. These results emphasize the meaningfulness of personal variations on the likings of the food matrices, as different clusters were the main source of variation in this test rather than different species.

As the literature survey indicated that the concentration of taste compounds such as free amino acids and nucleotides are on different levels in different publications, it is likely that the origin of the mushrooms (location including climate and amount of light) as well as type of growing area (forest, swamp or open field for example), harvesting season, and maturity affect the concentrations of taste compounds. In order to form a complete picture of the taste properties of Nordic mushrooms, a more systematic and wider study should be conducted with samples collected systematically in different locations and different seasons. As indicated in the **Publication II** both volatile and non-volatile factors effect are of importance on the palatability of mushroom species and thus both compounds should be considered.

A novel method for the evaluation of interactions between taste compounds and nanocellulose utilizing a fluorescent indicator displacement system was developed.

As the developed method is a high-throughput method utilizing a plate reader, it can be used both for purposes of food and pharmaceutical studies. The fluorescent indicator studies indicated rather surprisingly that some taste compounds such as quinine form bonds with nanofibrillar cellulose. Out of seven studied compounds, four showed interactions, whose strength varied between 70 M^{-1} and 14300 M^{-1}. The highest binding constant was found between bitter tasting quinine and NFC and thus it was expected that nanocellulose might have potential as a taste modifier as well. However, taste assessments proved otherwise. The taste evaluations with a trained panel concluded that CMC and NFC had similar taste modifying properties with most of the compound studied. With quinine hydrochloride, CMC showed better ability to suppress bitterness. There might be different reasons for this. Firstly, based on open questionnaires it was concluded that nanocellulose material studied had an astringent property as well. As described in background section of this thesis, the taste is not only affected by all taste compounds, but also by odors as well as other properties such as chemesthetic properties and viscosity. Each of these properties might have an impact on the perceived taste separately or synergistically. Secondly, it is difficult to say whether the observed interactions are powerful enough to prevent the interaction between taste compounds and taste receptors. Based on this study, it is not possible to say whether the interactions between NFC and taste compounds could change the taste if different concentrations had been used. However, it is probable that with higher concentrations, also the astringency of NFC would be more pronounced.

As the taste of nanofibrillar cellulose was studied with sensory methods for the first time, the study provides important information of the utilization of it in the future applications. Even though sensory studies indicate that the use of NFC in the bitterness suppression of food might have limited potential, the clear interactions found in **Publication III** indicate that interactions are indeed possible and could have applications in the pharmaceutical field. Thus, in the future, larger sets of compounds should be studied to learn about possible structures causing the interactions. Possible causes for the astringency found in nanocellulose in sensory evaluations should be studied to enable the use of it in food applications. Further, as stated in the background section, the safety aspects of nanocellulose materials should be studied to fully utilize the potential of this material.

As a conclusion from the studies of this thesis, it can be emphasized how complicated a concept taste really is. From **Publications I** and **II** it can be seen how no single compound alone is responsible for the taste of real food samples. Further, as can be seen from the **Publications III** and **IV**, when studying relatively simple

matrices the chemically measurable interactions are not always as meaningful as other aspects, such as possible off flavors and interactions with saliva in the mouth.

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PUBLICATIONS

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Mushrooms

ABSTRACT

Edible mushrooms are valued because of their umami taste and good nutritional values. Free amino acids, 5′-nucleotides and nucleosides were analyzed from four Nordic forest mushroom species (*Lactarius camphoratus*, *Boletus edulis*, *Cantharellus cibarius, Craterellus tubaeformis*) using high precision liquid chromatography analysis. To our knowledge, these taste components were studied for the first time from *Craterellus tubaeformis* and *Lactarius camphoratus*. The focus was on the umami amino acids and 5′-nucleotides. The free amino acid and 5′-nucleotide/nucleoside contents of studied species differed from each other. In all studied samples, umami amino acids were among five major free amino acids. The highest concentration of umami amino acids was on *L. camphoratus* whereas *B. edulis* had the highest content of sweet amino acids and *C. cibarius* had the highest content of bitter amino acids. The content of umami enhancing 5′-nucleotides were low in all studied species.

1. Introduction

Edible wild mushrooms are a highly valued food because of their pleasant taste properties. Furthermore, mushrooms are low in energy and fat contents and have high amounts of dietary fibers (Longvah & Deosthale, 1998; Manzi, Aguzzi, & Pizzoferrato, 2001). They are also great supplements of protein and essential amino acids (Longvah & Deosthale, 1998; Mattila, Salo-Väänänen, Könkö, Aro, & Jalava, 2002) and good sources of certain vitamins (vitamin B_2, niacin and folates) and minerals (K, P, Zn, Cu) (Mattila et al., 2001). Moreover high contents of vitamin D_2 and ergosterol have been found in wild forest mushrooms (Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002). Thus, edible mushrooms are a healthy addition to a diet.

Volatile compounds, especially carbonyl compounds and alcohols, such as 1-octen-3-ol and 1-octen-3-one, contribute to the aroma of mushrooms (Pyysalo & Suihko, 1976) whereas non-volatile compounds, like free amino acids, 5′-nucleotides, sugars, polyols and organic acids contribute to the taste of edible mushrooms (Beluhan & Ranogajec, 2011; Mau, 2005). Edible mushrooms have an especially rich umami taste, which makes them palatable and a potential raw material for the food spice industry (Zhang, Venkitasamy, Pan, & Wang, 2013). Umami, which is described as savory, meaty or brothy taste, was named and originally identified as the salt of L-glutamic acid by Kikunae Ikeda in 1908 (Ikeda, 1909, 2002). Umami taste is caused by the salts of two amino acids, L-glutamic acid (L-Glu) and L-aspartic acid (L-Asp), binding to umami taste receptors T1R1 + T1R3 (Nelson et al., 2002) and mGluR4 (Chaudhari, Landin, & Roper, 2000). L-glutamic acid has a much stronger umami taste than L-aspartic acid (Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971). Also 5′-nucleotides 5′-ionosine monophosphate (5′-IMP), 5′-guanosine monophosphate (5′-GMP), 5′-xanthosine monophosphate (5′-XMP) and 5′-adenosine monophosphate (5′-AMP) attribute to the umami taste. 5′-nucleotides enhance the umami flavor in order 5′-GMP > 5′-IMP > 5′-XPM > 5′-AMP (Yamaguchi et al., 1971). They work in synergy with amino acids by intensifying the taste sensation by binding to the same T1R1 + T1R3 receptor as glutamate (Mouritsen & Khandelia, 2012; Zhang et al., 2013).

Taste properties of mushrooms have been studied from East Asian (Mau, Lin, Chen, Wu, & Peng, 1998; Mau, Lin, Ma, & Song, 2001; Tsai, Tsai, & Mau, 2008; Yang, Lin, & Mau, 2001), East African (Mdachi, Nkunya, Nyigo, & Urasa, 2004) and Southern European species (Beluhan & Ranogajec, 2011), but there is a gap in knowledge in taste properties of northern mushroom species. Umami taste of mushrooms is affected by different factors such as maturity stage and quality, storage time and conditions, species type and also the sub-strains of different species (Zhang et al., 2013). Different climate and thus different flora of

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northern countries gives a unique breeding ground for mushroom species. Specific knowledge of their taste properties could promote industrial utilization of this great natural resource and increase common interest towards conservation of their distribution areas in northern woodlands. The annual crop of edible Finnish mushrooms is about 1200 million kilos (Salo & Lindroos, 2008). Only a fraction of it is picked mainly for home use and only a small part of it is sold (Turtiainen, Saastamoinen, Kangas, & Vaara, 2012). In a survey executed in 2011 (Turtiainen et al., 2012) it was found that chanterelles (*C. cibarius*) and milkcaps formed each about 20% of annual crop picked in Finland (23 and 21%, respectively), whereas ceps (*B. edulis)* and other boletus species formed 14% and russulas 2%. Other mushroom species, such as false morels (*Gyromitra esculenta*) and funnel chanterelles (*C. tubaeformis*), composed 40% of annual crop picked. To our knowledge, the taste properties of even some of the most common Nordic mushroom species, such as milkcaps and funnel chanterelles have not been investigated before.

In this study free amino acids and nucleotides and their corresponding nucleosides were measured from four edible Finnish forest mushroom species. 26 amino acids and 5 nucleosides were measured. The studied species were chosen so that a comparison with literature could be done (*C. cibarius* and *B. edulis*). Also, species (*L. camphoratus* and *C. tubaeformis*) were chosen, the taste properties of which have not been measured before.

2. Materials and methods

2.1. Solvents and reagents

Amino acid standards used were either 2500 μmol/l standard solutions in 0.1 M HCl (Amino acid mixture standard solution, Type H, Wako Pure Chemical Industries, Ltd. (Osaka, Japan)) or dissolved solid standards (L-asparagine monohydrate (≥99%), L-glutamine (≥99%), L-tryptophan (≥99%) and L-theanine (≥97%) from Wako pure chemicals, 4-aminobutyric acid (≥99%), beta-alanine (≥99%), L-citrulline (≥98%), L-ornitihine monohydrochloride (≥99%) and taurine (≥99%) from Sigma Aldrich, St. Louis, Missouri, USA). For spiking experiments, corresponding liquid amino acid mix from Honeywell Fluka chemicals (Morris Plains, New Jersey, USA) and solid standards of L-glutamic acid (≥99.5%) and L-aspartic acid (≥99%) from Sigma Aldrich were used. Nucleotides and nucleosides (adenosine 5′-monophosphate sodium salt (≥99%), uridine 5′-monophosphate disodium salt (≥99%), cytidine 5′-monophosphate disodium salt (≥99%), guanosine 5′-monophosphate disodium salt hydrate (≥99%), inosine 5′-monophosphate disodium salt (≥98%), inosine (≥99%), guanosine (≥98%), cytidine (≥99%), uridine (≥99%) and adenosine (≥99%)) used in this study were purchased from Sigma Aldrich. Because adenosine 5′-monophosphate sodium salt, inosine 5′-monophosphate disodium salt and guanosine 5′-monophosphate disodium salt hydrate contain an unspecified amount of water (under 20, 27 and 26% relatively) and adenosine 5′-monophosphate sodium salt also a maximum 8% of sodium, the results slightly overestimate the concentrations of these substances.

Sodium hydroxide (≥99%), boric acid (≥99.5%) and potassium dihydrogen phosphate (≥99%) used in the analysis were purchased from Merc KGaA (Darmstadt, Germany). Potassium phosphate dibasic (≥98%) and 3-mercaptopropionic acid (≥99%) were from Sigma Aldrich, 35% HCl (35–38%), methanol (HiPerSolv CHROMANORM® gradient for HPLC) and acetonitrile (HiPerSolv CHROMANORM® Super gradient for HPLC) were from VWR Chemicals (Radnor, Pennsylvania, USA), ethanol anhydr. from Yliopiston Apteekki (Helsinki, Finland) and 85% orthophosphoric acid (85–90%), o-phthalaldehyde (≥98%) and 9-fluorenylmethyl chloroformate from MP Biomedicals (Santa Ana, California, USA).

2.2. Samples

Four species of Nordic forest mushrooms, chanterelle (*Cantharellus cibarius*), funnel chanterelle (*Craterellus tubaeformis*), porcini (*Boletus edulis*) and curry milkcap (*Lactarius camphoratus*), were studied. The chanterelles (3.3 kgs) were collected during mid-August of 2016 from the south-western coast of Finland and bought from a local market. Porcinis (3.4 kgs), curry milkcaps (0.4 kgs) and a quarter of funnel chanterelles (1.0 kgs) were collected during early or mid-September of 2016 from the south-west coast of Finland. The rest of the funnel chanterelles (2.7 kgs) were bought during early September of 2016 from mushroom pickers in the Kainuu region in eastern Finland. The samples were cleaned with a brush and cut to pieces (width 1 *cm*) within 36 h of collection. The samples were vacuum packed and cooked at 80 °C for 10 min. The samples were cooled in water (room temperature) for 2 min and in ice water (5–9 °C) for 5 min and then frozen at −20 °C. Frozen samples were cut to 0.5 cm pieces, pooled, and put back in a freezer.

The samples were kept in a freezer at −20 °C for 5–6 months. Samples were moved to −40 °C a day before freeze-drying. The samples were weighed in small plastic containers in batches of about 30 g and freeze-dried in vacuum at −40 °C for 27–29 h. 8–9 batches of 30 g were freeze-dried at the same time. Freeze-dried mushroom samples were ground using a mortar and pestle until a fine powder was reached. The samples were weighed before and after freeze-drying and dry matter content was calculated based on the lost weight to ensure the operation of the freeze-drying method. Dry matter contents of the mushroom species are presented in Table 1. The dry matter content of mushroom species varied between 77.7 and 145.2 g/kg. In a review by Kalač (2013) dry matter content in mushrooms in general was estimated to be between 60 and 140 g/kg. Thus, the species in this study fit to these margins except for *L. camphoratus*, which had a dry matter content of 145.2 g/kg on average, slightly above the range given by Kalač. The samples of *L. camphoratus* were slightly dehydrated when picked, which could explain this difference. Additionally, the samples in our study were vacuum cooked and kept in the freezer before analysis.

2.3. Instrumentation

The samples were analyzed with UHPLC (Nexera X2, Shimadzu, Kyoto, Japan). The apparatus used consisted of Shimadzu Nexera X2 quaternary pump (LC-30AD) combined with two degassers (DGU-20A3R, DGU-20A5R), autosampler (SIL-30AC), column oven (CTL-20AC) and detectors (diode array (SPD-M20A) and fluorescence (RF-20AXS)) connected to a computer equipped with Shimadzus LabSolutions-software (LC/GC).

2.4. Extraction

The same extraction method was used for the extraction of FAAs (free amino acids) and nucleotides/nucleosides. The method was modified from Ranogajec, Beluhan, and Šmit (2010). Freeze-dried and ground samples (ca. 0.5 g) were weighed in centrifuge tubes. 20 ml of ultrapure water was added, and the samples were carefully shaken until fully mixed. Samples were heated for 1 min in boiling water (100 °C) and kept in an ultrasound bath for 10 min (23 °C in the beginning).

Table 1
Dry matter content of mushroom species. n = number of freeze-dried samples.

	n	Dry matter ± STD [g/kg]
C. cibarius	6	80.4 ± 5.6
C. tubaeformis	10	77.7 ± 5.6
B. edulis	7	102.4 ± 4.6
L. camphoratus	5	145.2 ± 5.1

Samples were centrifuged at 2525*g* with Heraeus (Hanau, Germany) Biofuge primo centrifuge for 10 min and the supernatant was collected in 50 ml measuring flasks. The treatment was repeated three times for each sample. During the second repetition, 15 ml of water was added, and during the third repetition 10 ml. The collected supernatants were mixed and the measuring flask was filled with water to a volume of 50 ml. For the amino acid and nucleotide/nucleoside analyses, the sample solutions were diluted with water in ratios of 1:5 or 1:4 respectively. The solutions were finally filtered with a 0.20-μm RC syringe filter. Five repetitions of each mushroom species were prepared for both FAA and nucleotide/nucleoside analyses.

The validation of the extraction method was studied by spiking experiments and residual extraction for both amino acids and nucleotides/nucleosides. Spiking was carried out by adding standard solution to the sample before the first repetition of the extraction method. In the case of the nucleotides/nucleosides, 1 ml of each standard stock solution (500 mg/l) was added to reach a final concentration of 2.5 mg/l. In the case of the amino acids, the final added standard concentration was 5 μmol/l (for amino acids originating from liquid standard) or 10 μmol/l (solid amino acid standards and glutamic and aspartic acid). The spiking was carried out using samples of *C. tubaeformis* and replicated three times for both compound groups. To calculate recovery, three samples without standard addition were prepared. The recovery percent was calculated by subtracting the FAA/nucleotide/nucleoside contents of samples without standard addition from concentrations of samples with spiking and dividing it then with the concentration of added standard and multiplying it by 100.

The residual extraction was carried out by adding 10 ml of water to the precipitate after three extraction rounds. The extraction routine (heating, ultrasound bath, centrifuging) was done once and the collected extract was diluted to 50 ml. The residual extraction was carried out using *B. edulis* samples and replicated three times. Residue percent was calculated by dividing the content of compounds measured after residual extraction with content of compounds measured before residual extraction and multiplying it by 100.

2.5. 5′-Nucleotide and nucleoside analysis

The nucleotide/nucleoside contents were analyzed by the method modified from Ranogajec et al. (2010). The nucleotide/nucleoside composition of samples was analyzed using HPLC with a diode array detector at wavelength 254 nm. The column used was Synergi Hydro 4u Hydro-RP 80 Å 150 * 3.0 mm (Phenomenex, Torrace, California, USA) with Security Cartidges AQ C18 4 * 2.0 mm pre-column. The solvents used were A: 20 mM phosphate buffer (pH 5.9) and 100% MeOH. The gradient program was: 3–12 min, 0 → 30% B; 12–13.50 min, 30% B; 13.50–16 min, 30 → 0% B; 16 – 25 min, 0% B with a total time of 25 min. The injection volume was 5 μl and the needle was washed after injection with water and 20% ACN. The calibration curve was collected using seven different concentrations (20, 10, 5, 2.5, 2, 1, and 0.5 mg/l). 10 nucleotide/nucleoside standards were used. Stock solutions were prepared by diluting 5 mg of solid standard in 10 ml of water.

2.6. Free amino acid analysis

The free amino acid content of the samples was analyzed with UHPLC with a method modified from the technical note of Shimadzu (Shimadzu Corporation). The fluorescence detector was used with excitation/emission wavelengths of 340/450 and 266/305 nm. The compounds were separated on a 100 * 4.6 mm Kinetex 2.6 μm C18 100 Å column (Phenomenex) with a SecurityGuard ULTRA cartridge UHPLC C18 pre-column for 4.6 columns (Phenomenex) on an AJO-9000 holder.

The samples were derivatized as described on the technical note of Shimadzu. In short, the samples were derivatized with o-phthalaldehyde and 3-mercaptopropionic acid in 0.1 M borate buffer solution and 9-fluorenyl methyl chloroformate in acetonitrile. Acidic phosphate buffer (pH 2.1) was added to the solutions during derivatization. Solvents used in the gradient program were A: 20 mM phosphate buffer with pH 6.5 and B: 45/40/15 ACN/MeOH/H_2O. The gradient program used was 0–2 min, 11% B; 2–4 min, 11 → 17% B; 4–5.5 min, 17 → 31% B; 5.5–10 min, 31 → 32.5% B; 10–12 min, 32.5 → 46.5% B; 12–15.5 min, 46.5 → 55% B; 15.5–16 min, 55 → 100% B; 16–19.5 min, 100% B; 19.5–20 min, 100 → 11% B; 20–25 min, 11% B. The needle was washed from outside after every injection with 80% MeOH and 20% ACN. The temperature of the column oven was 35° and injection volume was 1 μl. Calibration curves were constructed using nine different concentrations (125, 50, 25, 20, 15, 10, 5, 2.5, 1 μmol/l). In total 26 standards were used. Nine amino acid standards were prepared using solid standards and the rest using the liquid standard solution. Solid standards were diluted in 0.1 M HCl to get a 5000 μmol/l stock solution. A diluted 125 μmol/l stock solution was prepared from these stock solutions by adding 0.125 ml of each stock solution from solid standards and 0.250 ml of liquid standard solution in a 5 ml measuring bottle and diluting them with water. In addition, a 250 μmol/l standard was prepared from the liquid standard solution and used in calibration. All dilutions were prepared using ultrapure water.

2.7. Statistical analysis

Differences between mushroom samples in each analyzed compound were tested with one-way analysis of variance (ANOVA) with square-, or cube-transformed data, if necessary. Tukey's HSD or Tamhane's T2 test was used for post hoc tests as directed by the tested homogeneity of variance. If the data was not normally distributed, nonparametric tests (Kruskal-Wallis and Mann-Whitney using Bonferroni correction) were used. Level of statistical significance was set to $p < .05$ in all tests. The tests were performed with IBM SPSS Statistics 24.0 (IBM, Corporation, Armonk, NY).

3. Results and discussion

3.1. Method validation

To validate the extraction method both the content of compounds after residual extraction and the conservation of standards during extraction routine was studied. The percentage of nucleotides/nucleosides remaining after residual extraction and the percentage of added standard remaining after the extraction method are presented in Table 2.

Table 2
Validation of the extraction and measurement method (nucleotides/nucleosides). The proportions of averages and standard deviations of the residual nucleotides/nucleosides compared to the original samples (n = 3, number of extractions and analyzes made from freeze-dried samples of each species) and the proportions of averages and standard deviations of the standard remaining after extraction method used (n = 3, number of extractions and analyzes made from freeze-dried samples of each species) are shown. Negligible stds are not shown. Limits of detection and quantification for nucleotides and nucleosides in milligrams per litre.

Compound	Residue %	Recovery %	LOD [mg/l]	LOQ [mg/l]
5′-AMP	2.5	89.7 ± 12.9	0.07	0.24
5′-CMP	1.8	88.4 ± 12.4	0.15	0.48
5′-GMP	1.2	93.1 ± 15.2	0.11	0.37
5′-IMP	n.d.	75.4 ± 5.9	0.17	0.55
5′-UMP	–	94.7 ± 10.9	0.13	0.44
Adenosine	8.2 ± 0.1	104.8 ± 3.9	0.04	0.15
Cytidine	n.d.	97.5 ± 9.0	0.12	0.39
Guanosine	n.d.	108.8 ± 4.3	0.03	0.11
Inosine	n.d.	108.4 ± 5.0	0.04	0.15
Uridine	n.d.	112.6 ± 7.9	0.05	0.16
Average	3.4	97.3	0.09	0.30

n.d. not detected, – not possible to quantify.

Table 3
Validation of the extraction method (free amino acids). The proportions of averages and standard deviations of the residual FAAs compared to the original samples (n = 3, extractions and analyzes made from freeze-dried samples of each species) and the proportions of averages and standard deviations of the standard remaining after extraction method used (n = 3, extractions and analyzes made from freeze-dried samples of each species) are given. Negligible stds are not shown. Limits of detection and quantification for amino acids in micromoles per litre.

Compound	Residue (%)	Recovery (%)	LOD [μmol/l]	LOQ [μmol/l]
β-Alanine	1.3 ± 0.3	87.3 ± 2.3	0.07	0.22
L-Alanine	1.5 ± 0.3	66.3 ± 5.7	0.11	0.38
γ-Aminobutyric acid	1.7 ± 0.4	95.9 ± 5.9	0.07	0.23
L-Arginine	3.2 ± 0.1	96.7 ± 6.4	0.19	0.63
L-Asparagine	1.7 ± 0.2	91.2 ± 3.4	0.07	0.24
L-Aspartic acid	1.9 ± 0.2	84.6 ± 8.5	0.10	0.34
L-Citrulline	2.4 ± 0.2	99.9 ± 3.7	0.08	0.26
L-Cystine	n.d.	26.3 ± 20.8	0.08	0.27
L-Glutamic acid	1.9 ± 0.2	73.1 ± 6.5	0.10	0.34
Glutamine	1.2 ± 0.3	94.2 ± 3.6	0.07	0.23
Glycine	2.5 ± 0.4	78.7 ± 15.3	0.19	0.63
L-Histidine	3.9 ± 0.4	151.4 ± 118.6	0.36	1.20
L-Isoleucine	6.9 ± 0.2	81.5 ± 1.9	0.17	0.56
L-Leucine	8.2 ± 0.8	81.0 ± 5.3	0.18	0.58
L-Lysine	3.8 ± 0.2	90.0 ± 2.3	0.37	1.23
L-Methionine	10.2 ± 0.8	82.7 ± 1.8	0.13	0.44
L-Ornithine	1.1 ± 0.2	100.3 ± 2.3	0.18	0.59
L-Phenylalanine	8.6 ± 0.8	83.2 ± 1.8	0.18	0.58
L-Proline	–	–	–	–
L-Serine	2.6 ± 0.3	73.3 ± 8.9	0.12	0.40
Taurine	8.0 ± 0.7	92.8 ± 2.8	0.05	0.18
L-Theanine	n.d.	99.6 ± 6.0	0.06	0.20
L-Threonine	3.4 ± 0.3	81.8 ± 3.1	0.18	0.60
L-Tryptophan	0.0 ± 0.5	91.1 ± 2.3	0.09	0.28
L-Tyrosine	4.9 ± 0.5	70.1 ± 4.9	0.10	0.33
L-Valine	4.3 ± 0.3	79.2 ± 1.4	0.12	0.39
Average	3.7	86.1	0.14	0.45

n.d. not detected, – not possible to quantify.

On average, the residual content was 3.4% and with many compounds, residual content was not detected at all. Added standards seem to have been well preserved. The average was 97.3% and the lowest recovery was detected with 5′-IMP (75.4%).

The percentage of amino acids remaining after residual extraction and percentage of added standard remaining after the extraction method are presented in Table 3. Based on these results, the extraction method seems to remove amino acids from the sample material efficiently. The percentage of amino acids remaining in the precipitate was 3.7% on average. In the majority (19/23) of the studied amino acids < 5% was left in the precipitate after three rounds of extraction. The percentage remaining was < 10% in all studied FAAs, except in L-methionine with a 10.2% percentage. The spiking experiments showed that the amino acids are well preserved during extraction. On average 86.1% preservation was measured. The only differing results are L-histidine and L-cystine with 151.4 and 26.3% recovery, respectively. To conclude it can be expressed that apart from a few exceptions in both groups of compounds, the extraction method effectively removes the FAAs and nucleotides/nucleosides from the starting material and the compounds are well retained. Thus the results collected with this method are reliable.

Limit of detection (LOD) and limit of quantification (LOQ) for both amino acids and nucleotides/nucleosides were estimated based on S/N-ratio and calculated with coefficients 3 and 10. LOD and LOQ values are presented in tables 2 and 3. For all nucleotides and nucleosides, the linear range was 0.5–20 mg/l and with values of $r^2 > 0.999$. For free amino acids, the linear range was 1–125 μmol/l or 1–250 μmol/l (FAAs from liquid standard) with values of $r^2 > 0.997$.

3.2. 5′-Nucleotide and nucleoside contents of the mushrooms

The nucleotide/nucleoside content of mushroom species are presented in Table 4. The contents of umami enhancing nucleotides have been divided into three ranges, low (< 1 mg/g), medium (1–5 mg/g) and high (> 5 mg/g), according to Yang et al. (2001). Accordingly, the contents of these nucleotides were low in all the studied species. Here, the contents of 5′-GMP and 5′-IMP were assumed to count towards this amount. Based on the results of statistical analysis, the mushroom species are distinctive from each other by their nucleotide/nucleoside contents.

In the literature reviewed by Zhang et al. (2013) contents of umami enhancing 5′-nucleotides (5′-GMP, 5′-IMP, 5′-XMP) of mushrooms varied between 0.38 and 13.88 mg/g (dw). For *B. edulis* umami enhancing nucleotide contents of 2.01 mg/g (dw) (Tsai et al., 2008) and 1.63 mg/g (dw) (Beluhan & Ranogajec, 2011) and for *C. cibarius* 0.38 mg/g (dw) (Beluhan & Ranogajec, 2011) have been reported. Based on the review by Zhang et al. (2013), both *B. edulis* and *C. cibarius* have a relatively low concentration of these 5′-nucleotides compared to other mushroom species presented. Thus it can be hypothesized that also *L. camphoratus* and *C. tubaeformis* have relatively low concentration

Table 4
The nucleoside/nucleotide content of the studied mushroom species in mg/g (dry weight) and standard deviation (n = 5, extractions and analyzes made from freeze-dried samples of each species) are shown. Three major nucleotides/nucleosides are in bold.

	C. cibarius	*C. tubaeformis*	*B. edulis*	*L. camphoratus*
5′-AMP[d]	**0.38 ± 0.03 (A)**	**0.70 ± 0.08 (B)**	**1.39 ± 0.09 (C)**	0.08* ± 0.00 (D)
5′-CMP[c]	**0.37 ± 0.01 (A)**	**0.57 ± 0.07 (B)**	**1.87 ± 0.11 (C)**	**0.86 ± 0.03 (D)**
5′-GMP[a,b]	0.19 ± 0.03 (A)	0.11* ± 0.01 (B)	**0.60 ± 0.04 (C)**	–
5′-IMP[a,c]	0.22* ± 0.01 (A)	0.13* ± 0.01 (B)	0.35 ± 0.02 (C)	n.d.
5′-UMP[c]	0.28 ± 0.02 (A)	**0.29 ± 0.07 (A)**	–	–
Adenosine[d]	**0.37 ± 0.01 (A)**	0.10 ± 0.01 (B)	0.16 ± 0.02 (C)	**1.08 ± 0.03 (D)**
Cytidine[d]	0.05* ± 0.03 (A)	0.09* ± 0.04 (A)	n.d.	0.06* ± 0.00 (A)
Guanosine[d]	0.16 ± 0.02 (A)	0.02* ± 0.00 (B)	0.07 ± 0.05 (A)	**0.96 ± 0.06 (C)**
Inosine[c]	0.08 ± 0.01 (A)	0.05* ± 0.00 (B)	0.20 ± 0.09 (AB)	0.31 ± 0.01 (C)
Uridine[c]	0.09 ± 0.00 (A)	0.06* ± 0.00 (B)	0.13 ± 0.01 (C)	0.62 ± 0.02 (D)
Umami 5′-nucleotides	0.41	0.25	0.95	n.d.
Total nucleotides	1.43	1.81	4.21	0.94
Total nucleosides	0.75	0.32	0.56	3.06

– not possible to quantify.
* Results smaller than LOQ, n.d. not detected (smaller than LOD).
[a] Umami enhancing nucleotides.
[b] Statistical analysis with F-values and Tukey's.
[c] Statistical analysis with Brown-Forsythe and Tamhane's T2.
[d] Non-parametric tests, mushrooms that are not statistically different in one row are marked with the same letter A–D.

Table 5
The amino acid content of the studied mushroom species in mg/g (dry weight) and standard deviation (n = 5, extractions and analyzes made from freeze-dried samples of each species) are shown. The amino acids are classified based on their taste properties. The five major amino acids are in bold.

	C. cibarius	*C. tubaeformis*	*B. edulis*	*L. camphoratus*
MSG-like				
L-Aspartic acid[c]	**1.29 ± 0.08 (A)**	**1.65 ± 0.15 (A)**	**2.85 ± 0.44 (B)**	**3.35 ± 0.26 (B)**
L-Glutamic acid[c]	**3.78 ± 0.24 (A)**	**1.92 ± 0.21 (B)**	**2.62 ± 0.40 (B)**	**4.69 ± 0.36 (C)**
Total	5.08	3.57	5.47	8.04
Sweet				
L-Alanine[c]	0.65 ± 0.02 (A)	0.66 ± 0.11 (A)	**6.67 ± 0.92 (B)**	**0.98 ± 0.11 (C)**
Glycine[c]	0.21 ± 0.02 (A)	0.36 ± 0.03 (B)	1.29 ± 0.21 (C)	0.26 ± 0.01 (D)
L-Serine[c]	0.53 ± 0.03 (A)	0.39 ± 0.03 (B)	1.53 ± 0.23 (C)	0.62 ± 0.04 (D)
L-Threonine[a, d]	0.47 ± 0.02 (A)	0.28 ± 0.02 (B)	0.90 ± 0.12 (C)	0.58 ± 0.03 (D)
Total	1.86	1.68	10.38	2.43
Bitter				
L-Arginine[c]	**4.47 ± 0.46 (A)**	**1.15 ± 0.13 (BC)**	1.41 ± 0.20 (C)	0.92 ± 0.08 (B)
L-Histidine[a, b]	**1.13 ± 0.08 (A)**	**1.07 ± 0.10 (A)**	0.77 ± 0.14 (B)	**1.73 ± 0.12 (C)**
L-Isoleucine[a, d]	0.23 ± 0.01 (A)	0.32 ± 0.03 (B)	0.31 ± 0.03 (BC)	0.27 ± 0.01 (C)
L-Leucine[a, b]	0.46 ± 0.02 (A)	0.42 ± 0.03 (AB)	0.37 ± 0.05 (B)	0.53 ± 0.03 (C)
L-Methionine[a, b]	0.14 ± 0.00 (A)	0.14 ± 0.00 (A)	0.23 ± 0.03 (B)	0.26 ± 0.01 (B)
L-Phenylalanine[a, b]	0.25 ± 0.01 (A)	0.40 ± 0.03 (B)	0.30 ± 0.03 (C)	0.60 ± 0.03 (D)
L-Tryptophan[a, b]	0.32 ± 0.04 (A)	0.46 ± 0.05 (B)	0.48 ± 0.09 (B)	0.09 ± 0.03 (C)
L-Tyrosine[c]	0.43 ± 0.03 (A)	**1.98 ± 0.19 (B)**	0.69 ± 0.09 (C)	0.35 ± 0.01 (D)
L-Valine[a, d]	0.31 ± 0.02 (A)	0.32 ± 0.03 (AC)	0.54 ± 0.07 (B)	0.37 ± 0.02 (C)
Total	7.75	6.28	5.09	5.11
Tasteless or no information found				
β-Alanine[b]	0.04 ± 0.01 (A)	0.14 ± 0.03 (B)	0.19 ± 0.03 (B)	0.04 ± 0.04 (A)
γ-Aminobutyric acid[d]	0.31 ± 0.02 (A)	0.26 ± 0.05 (A)	0.67 ± 0.11 (B)	0.05 ± 0.02 (C)
L-Asparagine[d]	0.29 ± 0.02 (A)	0.89 ± 0.10 (B)	0.69 ± 0.11 (C)	0.32 ± 0.04 (A)
L-Citrulline[c]	0.04 ± 0.01 (A)	0.04 ± 0.00 (A)	0.28 ± 0.04 (B)	0.07 ± 0.02 (A)
L-Cystine	n.d.	n.d.	n.d.	0.03 ± 0.02
L-Glutamine[b]	**4.74 ± 0.39 (A)**	0.69 ± 0.08 (B)	**3.87 ± 0.60 (C)**	**5.10 ± 0.44 (A)**
L-Lysine[a,d]	1.00 ± 0.10 (A)	0.53 ± 0.05 (B)	1.03 ± 0.15 (A)	0.63 ± 0.04 (B)
L- Ornithine[c]	0.84 ± 0.14 (A)	0.63 ± 0.10 (A)	**1.82 ± 0.28 (B)**	0.10 ± 0.01 (C)
L-Proline	–	–	–	–
Taurine[c]	n.d.	0.21 ± 0.02 (A)	0.04 ± 0.00 (B)	0.03 ± 0.00 (C)
L-Theanine	0.05 ± 0.00	n.d.	n.d.	–
Grand total	21.99	14.93	29.54	21.94

n.d. not detected (the results smaller than LOD), – not possible to quantify.
[a] Essential amino acids.
[b] Statistical analysis with F-values and Tukey's.
[c] Statistical analysis with Brown-Forsythe and Tamhane's T2.
[d] Non-parametric tests, mushrooms that are not statistically different in one row are marked with the same letter A–D.

of umami enhancing 5′-nucleotides in comparison to other species.

3.3. Free amino acids contents of the mushrooms

The amino acid contents of the studied mushroom species are presented in Table 5. The total free amino acid content varied between 14.93 and 29.54 mg/g and was the lowest in *C. tubaeformis* and the highest in *B. edulis* mushrooms. L-aspartic acid and L-glutamic acid were among the five major free amino acids in all studied species. Further L-arginine, L-glutamine and L-histidine were found in relatively high concentrations in all species. Based on the statistical analysis, mushrooms can be distinguished based on their amino acid profiles.

FAAs were classified into four groups, MSG-like, sweet, bitter and tasteless, based on their taste properties as described by previous publications (Beluhan & Ranogajec, 2011; Mau et al., 2001; Yang et al., 2001). Also FAAs with no reported taste properties were included to tasteless amino acids. *L. camphoratus* had the highest total umami amino acid content, whereas *B. edulis* had the highest content of sweet amino acids and *C. cibarius* the highest content of bitter amino acids.

The amount of umami amino acids was 3.57–8.04 mg/g in studied species. With the exception of *B. edulis*, all of the studied mushrooms had higher content of L-Glu than L-Asp. Based on the classification presented in the literature (Yang et al., 2001), all the studied species, except *C. tubaeformis,* had medium concentrations of MSG-like amino acids (5–20 mg/g). In the literature, Beluhan and Ranogajec (2011) reported L-glutamic acid contents of 39.09 mg/g for *B. edulis* and 29.99 mg/g for *C. cibarius* measured from freeze-dried fresh samples. Compared to other species studied in the literature reviewed by Zhang et al. (2013), both *C. cibarius* and *B. edulis* are relatively high in MSG-like amino acids. Based on our results *L. camphoratus* has a higher content of these amino acids than *C. cibarius* or *B. edulis*, thus it can be expected to have a high concentration in relation to other species too.

Measured concentrations are however in lower level compared to contents reported by Beluhan and Ranogajec (2011). This difference can be explained both by the differences in samples and by the differences in pre-processing methods. For example, Tsai et al. (2008) reported L-glutamic acid concentration as low as 0.59 mg/g from air-dried *B. edulis* samples. It has to be noted that our samples had been sous vide cooked and freeze-dried before analysis. In the literature it has been noted that cooking methods (Li et al., 2011), preservation methods (Liu et al., 2014) and post-harvest storage (Tseng & Mau, 1999) change the concentrations of amino acids and nucleotides/nucleosides. Also, the grade of the mushrooms have an effect on the amino acid and nucleotide/nucleoside contents (Cho, Choi, & Kim, 2006, 2010).

Therefore, the differences in pre-processing have an influence on our results and in the precision of comparison.

4. Conclusions

This study is to our knowledge the first one investigating the amino acid and 5′-nucleotide and nucleoside concentrations of Nordic wild edible mushrooms and the first one to measure these concentrations from *L. camphoratus* and *C. tubaeformis*. Thus it gives important information about these commonly picked northern mushroom species that might be interesting for scientific, industrial and household use.

Based on our results, it can be concluded that both amino acid and nucleotide/nucleoside profiles were distinctive from each other in our mushroom samples. They all contained significant concentrations of umami amino acids. Thus it can be predicted that umami is a significant component of the taste profile in the studied mushrooms. The concentration of umami amino acids was the highest in *L. camphoratus*. However, especially in *C. cibarius* and *C. tubaeformis* the content of bitter amino acids and in *B. edulis* sweet amino acids were high. Sensory profile of a food product is a complex phenomenon where concentration of different taste compounds are only one important factor. In addition, the interaction of different tastes and sensory factors, such as smell and texture, influence each other. Therefore, further sensory examinations are needed to ensure these predictions. Furthermore, there is still a need for more comprehensive study of taste differences caused by biological variations of Nordic mushroom species encompassing both geographical and seasonal variations.

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PUBLICATION 2

Linking volatile and non-volatile compounds to sensory profiles and consumer liking of wild edible Nordic mushrooms

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Linking volatile and non-volatile compounds to sensory profiles and consumer liking of wild edible Nordic mushrooms

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ABSTRACT

Current information on the links between the chemistry and hedonic liking of edible mushrooms is scarce. In this study, 84 consumers evaluated the appearance, odor, taste, texture and overall liking of samples of Nordic edible wild mushroom species. Subsequently, multivariate models on the effects of non-volatile compounds, odor-contributing volatile compounds, sensory attributes and hedonic likings were created. The non-volatile compounds were measured with quantitative NMR. The five studied mushroom species were different in their sugar and acid contents. Three consumer clusters were found with species*cluster interactions. Correlations with sensory attributes and chemical components were found, and the multivariate models indicated predictor attributes for each consumer cluster. The results indicate that the sensory properties could be correlated to both volatile and non-volatile compounds, there are consumer clusters with differing likings as regards mushrooms, and these clusters are heterogenic groups with no simple factors such as age explaining their liking scores.

1. Introduction

Edible mushrooms are a valued delicacy in many cultures. They are a wide group with a variety of different flavors as well as cooking and consumption methods. In our generic descriptive analysis of five cooked mushroom species (Aisala et al., 2018), the samples had characteristic odors, tastes, chemosensory and textural properties and were easily distinguishable. The flavor of mushrooms results from a vast variety of volatile and non-volatile compounds. Pyysalo (1976) studied the concentrations of volatile aroma compounds from seven Finnish edible mushroom species and concluded that the aroma of these species mainly originated from volatiles with eight carbon atoms, such as 1-octen-3-ol and 1-octen-3-one. Results from later studies have indicated that 1-octen-3-one is the major compound causing the mushroom-like odor, while the identifiable odors of different species are caused by other compounds, such as fatty acid degradation products, 3-(methylthio)propanal, terpenoids and *N*-heterocyclic compounds (Cho, Lee et al., 2007; Grosshauser & Schieberle, 2013; Zhang et al., 2018). In our gas chromatography–olfactometry measurements (Aisala, Sola, Hopia, Linderborg, & Sandell, 2019), the fatty acid degradation products especially contributed to the odor of wild edible mushrooms. As to the non-volatile components, the flavor of mushrooms is generated by free amino acids and nucleotides, as well as various other compounds, such as organic acids, soluble sugars and polyols (Beluhan & Ranogajec, 2011; Rotzoll, Dunkel, & Hofmann, 2006).

Several mushroom species are described as having an especially rich umami taste (Phat, Moon, & Lee, 2016). Umami is the fifth taste modality originating from the sodium salts of amino acids, mainly glutamic and aspartic acid, binding to T1R1 + T1R3 receptors (Nelson et al., 2002). This taste sensation is intensified by 5′-nucleotides, 5′-guanosine monophosphate, 5′-inosine monophosphate, 5′-xanthosine monophosphate and 5′-adenosine monophosphate (Yamaguchi, Yoshikawa, Ikeda, & Ninomiya, 1971). This synergy is typically calculated as an equivalent umami concentration value (EUC) which expresses the synergy in glutamic acid equivalents (Yamaguchi et al., 1971). Free amino acids comprise only 0.4–7% of the total dry matter content and 1–27% of the crude protein in mushrooms (Beluhan & Ranogajec, 2011; Tsai, Tsai, & Mau, 2008; Yang, Lin, & Mau, 2001). However, amino acids generate a great variety of taste perceptions and can be divided into classes based on which taste modality they induce. The taste properties of some amino acids are ambiguous, thus multiple classifications have been used (Kawai, Sekine-Hayakawa, Okiyama, &

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Ninomiya, 2012; Yang et al., 2001). In the literature (Beluhan & Ranogajec, 2011; Mau, Lin, Ma, & Song, 2001; Yang et al., 2001), amino acids are classified based on their pure tastes: umami-like (glutamic acid and aspartic acid), sweet (serine, glycine, threonine, alanine) and bitter (histidine, arginine, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine) and tasteless. In our measurements of the free amino acids and 5′-nucleotides in wild edible mushrooms (Manninen, Rotola-Pukkila, Aisala, Hopia, & Laaksonen, 2018), the contents of each amino acid class and nucleotides varied between species. Organic acids are usually described as sour (Moskowitz, 1971a) but also astringent (Thomas & Lawless, 1995). In contrast, sugars and polyols are described as sweet (Moskowitz, 1971b). The non-volatile compounds are typically measured by liquid chromatography (Ajlouni, Beelman, Thompson, & Mau, 1995; Heleno et al., 2011; Li et al., 2011), but it has been demonstrated that methods based on NMR can determine the organic acid, sugar and amino acid composition of the sample in a single measurement (Aisala et al., 2016; Cho, Kim, & Choi, 2007).

Although extensive research has been conducted on both volatile and non-volatile compounds of mushrooms, there have only been a few studies comparing the chemical data of flavor compounds with the results from sensory analysis. Cho, Lee et al. (2007) performed a 15-attribute descriptive sensory analysis with a trained panel as well as gas chromatography–olfactometry on matsutake mushrooms and correlated these datasets with a multivariate model. Rotzoll et al. (2006) created a taste profile of a morel extract, and compared the profile with a taste recombinant mixture which was synthesized *via* the results of a series of liquid chromatography-based measurements. The recombinant mixture was further refined by a series of omission experiments. Phat et al. (2016) compared the umami taste compound contents in mushroom extracts with the results collected by sensory analysis and electronic tongue system.

To our knowledge, there are no studies combining chemical data of both volatile and non-volatile compounds with sensory profiles of mushroom species. Furthermore, the knowledge on the flavor profile of Nordic mushroom species is scarce. Additionally, the hedonic studies for mushrooms have only been done thus far with cultivated species (Hiraide, Yokoyama, & Miyazaki, 2005; Ren, Pan, Li, Chen, & Duan, 2018). In the hedonic studies, there has been no consumer clustering apart from age groups (Ren et al., 2018).

The objective of this study was to analyze the relationship between the sensory perception attributes of wild edible Nordic mushrooms and volatile and non-volatile flavor components. Partial least squares regression (PLS) was used in order to find correlations with the results of chemical and sensory analysis. A nuclear magnetic resonance (NMR) spectroscopy dataset on mushroom non-volatile compounds was collected. This was combined with the previously collected data of the free amino acids and 5′-nucleotides (Manninen et al., 2018) and odor-contributing volatile compounds (Aisala et al., 2019) to explain the sensory properties of mushrooms (Aisala et al., 2018). Moreover, a hedonic liking study was conducted on studied mushrooms in order to evaluate correlations between liking and descriptive sensory profiles.

2. Materials and methods

2.1. Samples

Four species of Nordic edible wild mushrooms, chanterelle (*Cantharellus cibarius* Fr.), trumpet chanterelle (*Craterellus tubaeformis* (Fr.) Quél.), porcini (*Boletus edulis* Bull.) and curry milk cap (*Lactarius camphoratus* (Bull.) Fr.) as well as cultivated button mushrooms (*Agaricus bisporus* (J.E. Lange) Imbach), were studied. Samples from the same batch were used in previous studies (Aisala et al., 2018; Manninen et al., 2018) and prepared in the same way. In brief, fresh mushrooms were cooked with a *sous vide* process (80 °C) for 10 min, frozen at −20 °C, cut to 1–2 cm^3 cubes and pooled while frozen and stored at −20 °C until analysis.

2.2. Chemicals

Altogether 31 reference sugars, organic acids and amino acids were used in the nuclear magnetic resonance (NMR) measurements: D-glucose, D-fructose, sucrose, trehalose dihydrate, citric acid, DL-malic acid, formic acid, fumaric acid, maleic acid, γ-aminobutyric acid, L-alanine, L-arginine, L-asparagine monohydrate, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine and choline chloride. These were all bought from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) except D-fructose, which was bought from Kanto Chemical Co, Inc. (Tokyo, Japan).

For the phosphate buffer, K_2HPO_4 from Kanto Chemical Co, Inc. and KH_2PO_4 from Chameleon reagent (Osaka, Japan) were diluted in D_2O (99,8% D) from Acros Organics (Geel, Belgium). 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) was used as the internal standard.

2.3. Sample preparation for NMR spectroscopy

Frozen samples were weighed in 50-mL Falcon tubes in batches of approximately 10 g and freeze-dried under vacuum at −30 °C for 46 h. Before the NMR measurements, the samples were freeze-dried a second time for 70 h to eliminate any build-up of moisture. Dry matter content was determined based on the weighed masses before and after freeze-drying. The average dry matter contents (and standard deviations) were 81.1 (0.4) g kg^{-1} for button mushrooms, 79.3 (1.1) g kg^{-1} for chanterelle, 83.4 (2.2) g kg^{-1} for trumpet chanterelle, 100.1 (0.5) g kg^{-1} for porcini, and 142.3 (8.2) g kg^{-1} for curry milk cap. These values were in good agreement with our previous measurements of the same batch (Manninen et al., 2018). The freeze-dried samples were ground to a fine powder with a mortar and pestle, pooled by species and stored at −18 °C for one week until extractions.

Sixty milligrams of mushroom powder were measured into 2-mL centrifuge tubes in quadruplicate; 600 μL of 0.1 M phosphate buffer in D_2O (pH 7.0) were added to the powder. The mixture was vortexed for 30 s, sonicated for 10 min at room temperature, incubated for 15 min in a sample shaker and centrifuged (10,000*g*, 15 min). The supernatant was removed and the procedure repeated once. The supernatants were combined, centrifuged once more and 600 μL of the combined extract supernatant were used for NMR measurements. Finally, 100 μL of 5 mM DSS in the D_2O-phosphate buffer were added to this aliquot for chemical shift referencing and quantification.

2.4. NMR spectroscopy

There were two main factors that advocated the use of NMR spectroscopy in the analysis of the mushroom samples. While NMR as a measurement technique is less sensitive than liquid or gas chromatography for the analysis of sugars, organic acids and amino acids, it provides a broad overview of the sample across several compound groups (Aisala et al., 2016; Cho, Kim, et al., 2007). Another benefit is that, in contrast to chromatographic methods, no derivatization steps are needed. ^{1}H qNMR spectra were measured with an Agilent 400-MR DD2 spectrometer (Agilent Technologies, Santa Clara, CA) operating at proton frequency 399.79 MHz. The spectrometer was equipped with a OneNMR Protune probe and was controlled with VnmrJ 3.2 Revision A. Spectra were recorded at 295 K with sample spinning at 16 Hz in a 5-mm NMR tube (Type S; Wako Pure Chemical Industries, Osaka, Japan) and locked to D_2O. Samples were shimmed to a DSS signal width at half height < 0.7 Hz. The NMR parameters were 30° pulse angle, 16 ppm spectral width and 64 k data points (acquisition time 5.11 s), 5 s recycle delay, and 128 scans. The receiver gain was set to 30. The free induction decays were Fourier transformed with zero-filling to 128 k and with LB = 0.3 Hz in MestReNova version 12.0.3 (Mestrelab Research

S.L, Santiago de Compostela, Spain).

2.5. NMR spectroscopy validation

Light validation of the extraction method and NMR linearity was performed with two calibration curves, residual extraction and spiking experiments. The first calibration curve contained 0–60 mM glucose, sucrose, citric acid, malic acid, L-alanine, L-glutamic acid, and L-arginine in six levels. The second curve was created after the sample measurements also in six levels. It contained trehalose (0–50 mM), fumaric acid (0–16 mM), malic acid (0–32 mM), L-glutamine (0–16 mM), L-alanine (0–20 mM), and L-isoleucine (0–16 mM); the ranges were selected based on the sample results. Spiking was carried out by adding known amounts (16–140 mg) of crystalline trehalose, malic acid, fumaric acid, alanine, glutamine and isoleucine to 1800 mg of chanterelle powder before weighing and performing the extraction method as above in triplicate. The recovery coefficient was calculated as the quotient of the experimentally determined and weighed added standard contents. The residual extraction was carried out by continuing with three of the porcini sample precipitates after the main extraction. Three additional extraction rounds were performed and the pooled residual extract measured as above. The residue coefficient was calculated as the quotient of residual extraction and main extraction contents.

2.6. NMR compound identifications and quantitation

Each NMR signal was first tentatively assigned by comparing the ^{1}H 1D spectra to published data and reference spectra in the Human Metabolome Database (Aisala et al., 2016; Cho, Kim, et al., 2007; Wishart, Feunang, Marcu, Guo, Liang, Vázquez-Fresno, & Scalbert, 2018). Additional composite sample extracts containing all mushroom species were spiked consecutively with all reference compounds listed in the *Chemicals* section to confirm peak shapes and *J* values. Chemical shift drift due to pH changes was employed in identification of organic acids. Finally, metabolite identification was confirmed by using gCOSY, HSQCAD and gHMBCAD 2D measurements of the composite samples.

The non-overlapping proton signal areas that were above the limit of quantification were determined. The data analysis protocol of Malz and Jancke (2005) was followed and all concentrations were calculated for fresh weight. Quantification focused on sugars, sugar alcohols and organic acids as the amino acids and 5′-nucleotides had been determined previously (Manninen et al., 2018). However, L-alanine, L-glutamine, L-isoleucine, L-valine, L-glutamic acid, and L-aspartic contents were determined for between-method comparison.

2.7. LC–MS analysis of the unknown compound in curry milk cap

Curry milk cap samples without derivatization were prepared as previously reported (Manninen et al., 2018) and measured with a Waters Acquity UHPLC instrument (Waters, Milford, MA) connected to a Waters Xevo Q–TOF MS. The column was a Waters Acquity HSS T3 (2.1 × 100 mm, 1.8 μm). Mobile phases were 0.1% formic acid in H_2O (**A**) and acetonitrile (**B**). The column flow was 0.3 mL/min and the eluant changed from 95% of **A** to 100% of **B** and back to 95% of **A** over a period of 5 min. The injection volume was 5 μL. Electrospray ionization on positive mode was used with a 2.5 kV capillary voltage. Full scan mode was used with an m/z 50–1000 range. Data were analyzed with Waters MassLynx V4.1 software.

2.8. Sample preparation for the hedonic test

On the sensory evaluation day, frozen samples were thawed in 20–60 g aliquots in *sous vide* bags in a 70 °C water bath for 5 min. However, curry milk cap was not included because of poor sample availability and because this mushroom is typically used as a spice instead of a food ingredient. Representative samples (7–8 g) containing both solid mushroom and dissociated liquid were served in 70-mL transparent glass bowls covered with glass plates. The samples were tempered on a hotplate to 50–60 °C for at least 15 min before evaluation. Sample cups were coded with three-digit numbers.

2.9. Hedonic test

A total of 84 consumers between 20 and 74 years old (median age 47 years) who used mushrooms or mushroom products at least sometimes participated in the hedonic testing. Volunteer consumers were recruited mainly from the Turku region in Finland. The hedonic test was conducted in a sensory laboratory (ISO 8589, University of Turku). The consumers evaluated the odor, appearance, flavor, texture and overall liking of each of the four mushroom samples. Liking was evaluated using the 9-point hedonic scale labeled with numeric and descriptive anchors in Finnish. Samples were presented monadically and the sample presentation order was randomized among the subjects. The participants were asked to refrain from using strong perfumes on the evaluation day and to refrain from eating or drinking anything aside from water at least 30 min before the evaluation. They were instructed to clean their palate with active-carbon filtered water and a piece of low-sodium cracker between the samples.

After the end of the hedonic test, the consumers answered a set of background questions related to consumer demographics and mushroom usage. The questionnaire also included the Food Choice Questionnaire (Steptoe, Pollard, & Wardle, 1995) as modified previously (Pohjanheimo & Sandell, 2009) and the 8-question version of the Food Disgust Scale (Hartmann & Siegrist, 2018), both translated into Finnish. The typical completion time for the whole test was about 30 min. Data were collected with the Compusense Cloud version 8.4 (Compusense Inc., Guelph, Ontario, Canada).

2.10. Statistical analysis

2.10.1. Metabolomics approach for NMR data

An unsupervised, initial overview of the NMR data was performed with the ChemoSpec package version 4.4.97 (Hanson, 2017) in RStudio 1.2 running R 3.6.0. First, a correction factor based on dry matter contents and extraction masses was applied to the spectra from 0.3 ppm onwards. Then, the whole spectra were normalized based on the 0.00 ppm DSS signal and binned to 0.02 ppm/point data buckets. Water and DSS peaks as well as redundant spectral regions at < 0.7 ppm and > 10 ppm were removed. A principal component analysis (PCA) was performed with mean centering and Pareto scaling. Classical 95% confidence ellipses were used in determining the sample populations in the scores plots. In the loadings plot, the binned curry milk cap spectra were used as a reference.

2.10.2. Differences between mushrooms in quantified NMR data

Differences between mushroom samples in each analyzed compound were tested with one-way analysis of variance (ANOVA) or the Brown-Forsythe test with either Tukey's HSD or Tamhane's T2 post-hoc test as directed by the tested homogeneity of variance. The tests were performed with IBM SPSS Statistics 24.0 (IBM Corporation, Armonk, NY).

2.10.3. Consumer clustering

The consumers were clustered based on their mushroom liking scores. First, the main sources of variation in the 20 hedonic variables (5 hedonic modalities in the 4 mushrooms) were determined with principal component analysis (PCA) using the PCA function of the FactoMineR package (Lê, Josse, & Husson, 2008) in RStudio 1.2. The data were mean centered and no standardization was used. The resulting dataframe was then used as source data for hierarchical cluster analysis (HCA) using the HCPC function of the FactoMineR package. HCA was performed with Ward's method and Euclidean distances, with

the algorithm automatically deciding the number of clusters. The algorithm suggested three 20–38 member clusters, and this solution was retained.

Differences in each hedonic modality were examined with a 2-way ANOVA (general linear model, univariate with mushroom species and cluster membership as fixed factors; model included main effects and the interaction term) in SPSS after appropriate data transformations, in order to conform to normality. Eta squared values (η^2) were calculated with the recommended procedure (Levine & Hullett, 2002). Post-hoc tests were built for cluster differences in each mushroom using simple contrasts with the LMATRIX subcommand and Bonferroni corrections.

The effect of cluster membership on the background variables was studied with either a one-way ANOVA (age, Food Choice Questionnaire variables, Food Disgust Scale results, number of known mushroom species) or with the Kruskal-Wallis test (sample familiarity, mushroom usage frequency). Tukey's HSD or the Mann-Whitney U with Bonferroni corrections were used as post-hoc tests. The effects of gender, education and diet were not examined with statistical tests due to the imbalanced sample.

2.10.4. Combinatory multivariate models

The data retrieved from non-volatile compounds using liquid chromatography (Manninen et al., 2018), odor-contributing volatile compounds using headspace solid-phase microextraction–gas chromatography–olfactometry (Aisala et al., 2019) and sensory properties using generic descriptive analysis (Aisala et al., 2018) including the replicate analyses were first analyzed separately with PCA using The Unscrambler version 10.4.1 (Camo Process AS, Oslo, Norway) with auto-scaled data (readers are referred to these source publications for the methods related to these datasets). EUC values were calculated from free amino acid and 5′-nucleotide contents (Yamaguchi et al., 1971). NMR amino acid data for button mushrooms were used and 5′-nucleotide values from Li et al. (2011) were used to calculate the EUC for button mushrooms. Total sugar, total acid and sugar-acid ratios were calculated. Additionally, the total sugars in glucose equivalents, total acids in malic acid equivalents, and equivalent sugar-acid ratios were calculated based on reported relative sweetness and sourness of these compounds (Moskowitz, 1971a, 1971b). After confirming over 75% of the explained variations in both the calibration and validation models in each dataset, the data were averaged over the replicates.

The analysis was continued with a partial least squares regression (PLS) analysis (Unscrambler), using chemical attributes as *X*-variables (predictors) and sensory properties as *Y*-variables (responses). The predictors were autoscaled and all data was mean-centered. Separate models for non-volatile and odor-contributing volatile compounds were made before making the final composite model. Sensory drivers of liking were measured with PLS following Guinard et al. (2016). The sensory properties were used as *X*-variables, and the average liking scores for each liking modality and consumer cluster as *Y*-variables. All data were mean-centered but no scaling was used. The limit for statistical significance for all statistical tests was $p < 0.05$.

3. Results and discussion

3.1. NMR spectroscopy

3.1.1. Identified compounds

In total, three sugars and sugar alcohols, four organic acids and 17 amino acids were identified in the qNMR samples (Supplementary material, Table S1). Additionally, seven major unidentified compounds were present in the samples. These features resulted in unique NMR spectra for each species (Fig. S1). The major unknown peak in curry milk cap was tentatively identified as a dimethylsubstituted pentose with a molecular mass of 147, but more research is needed to unequivocally designate the compound. Therefore, we will only refer to this compound in the following text as "unknown pentose", with the knowledge that the structure is not fully identified.

3.1.2. Light method validation

The NMR signals in both the first and second calibration curves were linear ($R^2 > 0.999$) with the 95% confidence intervals for the slope based on DSS content being 0.96–1.02 and 0.96–1.03, respectively. Residual contents in the porcini extract (Table S2) were on average 6.2% and ranged from 5% (L-valine) to 9% (mannitol). However, several compounds were below the limit of quantification in the residual extract, which limited the accuracy of these determinations. Recovery was on average 103.5% (Table S2) and ranged from 85% (L-alanine) to 190% (L-isoleucine). The largest sources of variation in the recovery experiments were the small absolute reference compound additions. This made the additions more representative of the typical contents in the mushrooms, but resulted in large variations, especially in the case of L-alanine, as only a handful of crystal particles comprised the whole standard addition. The free amino acid levels measured with NMR were generally 1–20% higher than the previous UHPLC measurements from samples in the same batch (Table S3).

3.1.3. Separation of species with the metabolomics approach

In the PCA model created with the metabolomics approach, principal component 1 explained 50% of the variation and principal component 2 explained 32% of the variation (Supplementary material, Fig. S2). Each sample species separated clearly into their own group in the PCA model (no overlapping 95% confidence ellipses). The loadings plot (Fig. S3) indicates that the main regions driving the separation are the sugar regions at 5.2 ppm and 3.6–3.9 ppm, as well as the saturated alkane region at 0.9–1.3 ppm.

3.1.4. Content of sugars and organic acids in mushrooms

The measured sugars, sugar alcohols and organic acids are presented in Table 1, with the main compounds in bold. For cooked button mushroom, we found a lower amount of sugars and no fructose compared to that previously reported (Li et al., 2011). The mannitol content was well in agreement with that of fresh button mushrooms (Ajlouni et al., 1995; Tseng & Mau, 1999). On the other hand, the trehalose content was slightly higher than that reported by Reis, Barros, Martins, and Ferreira (2012) in fresh mushrooms but lower than that reported by Ajlouni et al. (1995). In porcinis, we found smaller amounts of glucose and mannitol, and no mannose, but higher amounts of trehalose compared to the literature (Beluhan & Ranogajec, 2011; Heleno et al., 2011; Tsai et al., 2008). We measured higher concentrations of fumaric and malic acid than previously measured from fresh porcini samples in the literature (Ribeiro et al., 2006; Valentão, Andrade et al., 2005) but did not, in contrast to the literature, detect citric acid (Ribeiro et al., 2006; Valentão, Lopes et al., 2005). In chanterelles, we found less glucose and mannitol but more trehalose than reported (Beluhan & Ranogajec, 2011). Concentrations of measured organic acids were higher than reported in the literature for dried chanterelles (Valentão, Andrade et al., 2005).

3.2. Hedonic liking of mushrooms

3.2.1. Consumer clusters based on mushroom liking scores

The individual liking scores for each liking modality and mushroom species were used to segment the consumers into clusters. This was done to see whether there are groups of consumers with similar liking profiles. The hierarchical cluster analysis indicated that among the participants of this study, there were three different consumer clusters (Fig. S4). On average, all mushrooms, except button mushroom, were at least slightly liked in all liking modalities. However, different consumer clusters had differing liking profiles and the cluster effect size was 3–40 times larger than that of the mushroom species (Table 2).

The overview of mushroom preferences and drivers of liking related to each cluster can be seen in the PLS model in Fig. 1. The most different cluster (in terms of Euclidean distance, Fig. S4) was cluster 3 ($n = 26$, 31% of consumers), which had high liking scores for all

Table 1
Measured contents (with standard deviations, $n = 4$) of sugars and organic acids in the studied mushroom species expressed in mg g^{-1} fresh mushroom. Significant differences between species are based on one-way ANOVA and Tukey's or the Tamhane T2 post-hoc test and are marked with different letters A–E. Major compounds are in bold.

Compound	Trumpet chanterelle			Button mushroom			Curry milk cap			Chanterelle			Porcini		
Glucose	traces[a]		C	traces		C	0.206	(0.048)	B	0.122	(0.022)	B	0.881	(0.070)	A
Mannitol	**18.13**	**(0.17)**	**A**	**19.22**	**(0.23)**	**A**	**19.99**	**(1.43)**	**A**	**4.29**	**(0.18)**	**B**	**0.44**	**(0.17)**	**C**
Trehalose	**0.202**	**(0.008)**	**E**	**0.399**	**(0.010)**	**D**	**2.27**	**(0.032)**	**C**	**14.03**	**(0.181)**	**B**	**33.56**	**(0.455)**	**A**
Citric acid	nd[b]		C	nd		C	0.75	(0.01)	A	0.609	(0.025)	B	nd		C
Fumaric acid	0.284	(0.006)	B	0.25	(0.012)	C	0.563	(0.006)	A	0.294	(0.004)	B	0.108	(0.005)	D
Malic acid	**4.05**	**(0.07)**	**C**	**1.53**	**(0.03)**	**D**	**5.54**	**(0.06)**	**A**	**5.03**	**(0.06)**	**B**	**1.13**	**(0.03)**	**E**
Succinic acid	0.03	(0.001)	C	0.057	(0.002)	B	0.102	(0.002)	A	0.019	(0.000)	E	0.025	(0.001)	D
Unknown pentose	nd		B	nd		B	**5.48**	**(0.06)**	**A**	nd		B	nd		B
Total sugars	18.3	(0.2)	C	19.6	(0.2)	B	22.5	(1.4)	BC	18.5	(0.2)	C	34.9	(0.5)	A
Total acids	4.36	(0.08)	C	1.83	(0.02)	D	6.95	(0.06)	A	5.96	(0.07)	B	1.26	(0.03)	E
Sugar-acid ratio	4.21	(0.04)	C	10.6	(0.1)	B	3.23	(0.21)	D	3.10	(0.02)	D	27.61	(0.95)	A

[a] Detected, but under the limit of quantification.
[b] Not detected.

mushrooms and liking modalities, with all liking averages > 7 except for the appearance of button mushroom. There was little variation between the liking of different wild mushroom species for cluster 3 and therefore, all liking modalities are in the center part or left side of the PLS model. The largest ($n = 36$, 45% of all consumers) cluster 2 gave systematically > 1 liking units lower scores to all wild mushrooms compared to cluster 3. On the other hand, the difference was smaller in the case of button mushroom and not statistically significant. The sensory attributes associated with cluster 2 are total odor intensity, forest odor, lack of mushroom-type odor, umami and toughness.

Cluster 1 ($n = 20$, 24% of consumers) gave lower liking scores than the other two clusters to all mushrooms apart from taste, texture and overall liking of trumpet chanterelles. This rating difference was always statistically significant between clusters 1 and 3. Cluster 1 gave especially low scores to button mushroom, indicating that they disliked this cultivated species in contrast to the wild mushroom samples. This was the main line of statistical separation between clusters 1 and 2 and is displayed clearly with opposite configurations in the PLS model in relation to button mushroom. Cluster 1 is also negatively correlated with mushroom odor, sweet and umami attributes and squeakiness and biting resistance, but positively correlated with earthy, cardboard and forest odors. The consumer clustering was successful as different liking profiles for the mushroom species could be determined. These profiles indicated that, for example, the most distinct cluster 3 consisted of consumers with a general liking for all sample species.

3.2.2. Background variables for consumers

The volunteer consumers were predominantly female, represented multiple age groups, were predominantly highly trained and followed an omnivore diet. Fourteen males (17% of total participants) and 70 females (83%) completed the evaluation. There were 26 (31%) 20–35 year olds, 29 (35%) 36–50 year olds and 29 (35%) 50 year old or older participants. Thirteen (15%) had a high school diploma or equivalent, 18 (21%) had an undergraduate degree and 53 (63%) had a

Table 2
Liking score two-way ANOVA p-values and effect sizes expressed as η^2 by factors, and liking scores (average and standard deviations) for each cluster. Significant differences between clusters are based on Bonferroni-corrected simple contrasts in the two-way ANOVA ($p < 0.05$) and are marked with letters a–c.

Attributes and samples	ANOVA p values (η^2)			Averages (standard deviations)										
	Species	Consumer cluster	Interaction	Overall		1 ($n = 20$)			2 ($n = 38$)			3 ($n = 26$)		
Odor														
Button mushroom	0.001 (0.047)	0.001 (0.181)	0.228 (0.019)	6.1	(1.6)	4.7	(1.3)	c	6.1	(1.5)	a	7.1	(1.1)	a
Chanterelle				7.0	(1.5)	6.5	(1.2)	b	6.7	(1.6)	b	7.8	(1.1)	a
Trumpet chanterelle				6.8	(1.7)	5.7	(1.9)	b	6.6	(1.4)	b	7.9	(1.3)	a
Porcini				6.5	(1.6)	6.1	(1.5)	b	6.1	(1.7)	b	7.5	(1.1)	a
Appearance														
Button mushroom	0.001 (0.062)	0.001 (0.227)	0.033 (0.029)	5.5	(1.7)	3.9	(1.3)	b	5.8	(1.5)	a	6.4	(1.4)	a
Chanterelle				6.5	(1.6)	5.7	(1.6)	b	6.5	(1.5)	ab	7.2	(1.6)	a
Trumpet chanterelle				6.1	(1.5)	5.3	(1.4)	b	5.8	(1.4)	b	7.3	(1.1)	a
Porcini				5.6	(1.8)	4.4	(1.3)	b	5.3	(1.7)	b	7.1	(1.1)	a
Taste														
Button mushroom	0.33 (0.007)	0.001 (0.29)	0.001 (0.065)	6.2	(1.9)	3.85	(1.6)	b	6.7	(1.1)	a	7.4	(1.3)	a
Chanterelle				6.3	(1.8)	4.85	(1.6)	c	6.0	(1.5)	b	7.8	(0.8)	a
Trumpet chanterelle				6.2	(1.7)	5.65	(2.0)	b	5.6	(1.4)	b	7.3	(1.3)	a
Porcini				6.4	(1.6)	5.75	(1.5)	b	6.1	(1.5)	b	7.5	(1.3)	a
Texture														
Button mushroom	0.09 (0.013)	0.001 (0.30)	0.001 (0.059)	5.8	(1.9)	3.6	(0.9)	b	6.1	(1.5)	a	7.2	(1.5)	a
Chanterelle				6.2	(1.7)	4.6	(1.4)	c	6.2	(1.4)	b	7.5	(0.9)	a
Trumpet chanterelle				6.1	(1.6)	5.6	(1.7)	b	5.6	(1.5)	b	7.2	(1.2)	a
Porcini				6.1	(1.8)	5.4	(1.3)	b	5.4	(1.8)	b	7.5	(1.4)	a
Overall liking														
Button mushroom	0.04 (0.015)	0.001 (0.333)	0.001 (0.096)	6.2	(1.8)	3.8	(1.1)	b	6.7	(1.0)	a	7.4	(1.2)	a
Chanterelle				6.5	(1.4)	5.2	(1.4)	c	6.4	(0.9)	b	7.7	(0.7)	a
Trumpet chanterelle				6.3	(1.5)	5.9	(1.5)	b	5.7	(1.4)	b	7.4	(1.1)	a
Porcini				6.3	(1.6)	5.7	(1.4)	b	5.8	(1.4)	b	7.7	(1.1)	a

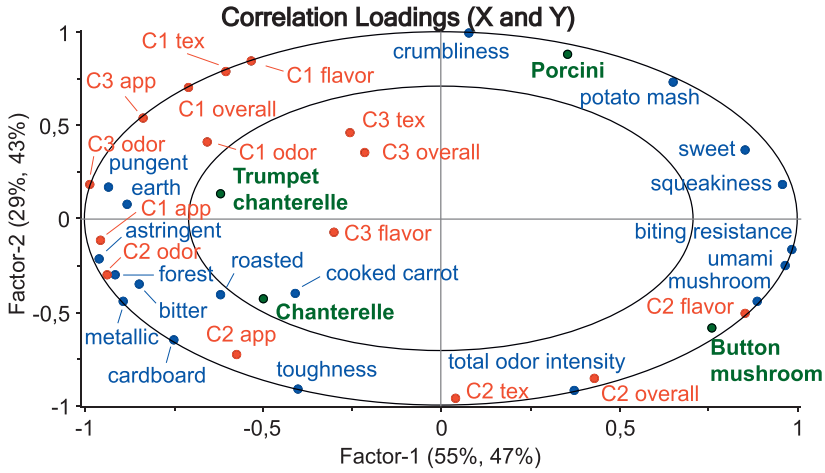

Fig. 1. Partial least squares model for the associations of mushroom sensory attributes (blue), mushroom samples (green), and liking scores for the three consumer clusters (red). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

higher university degree. Sixty consumers (71%) followed an omnivore diet, 18 (21%) a plant-based omnivore diet and 6 (7%) were vegetarians or vegans.

Of all the background variables, only one FCQ category (natural content) was statistically significantly different ($p < 0.05$, Table 3). There were additionally four variables with a non-statistically significantly different tendency (FCQ health, sensory appeal, ethical concern, and number of known mushroom species). Their η^2 was 6–11%, signifying that only a small part of the variation was explained by cluster membership. There were no statistically significant differences between clusters in other Food Choice Questionnaire categories (mood, convenience, price, weight control, familiarity), Food Disgust Scale, age, sample familiarity, or mushroom usage frequency. The consumers mostly (48% of consumers) used mushrooms two or three times a month. Thirty-seven percent of participants reported mostly using cultivated mushrooms while 54% mostly used self-picked wild mushrooms. The most common types of mushroom use were as a slightly seasoned ingredient in cooked dishes (selected by 80% of consumers in the top three usage scenarios) and cooked with onion and cream (85% of consumers).

3.3. The correlations between chemical data and the results from sensory analysis

In the PLS model (Fig. 2) 79% of the variation in the measured odor-contributing volatile compounds and non-volatile compounds explained 95% of the variation in the sensory profile. The curry milk cap has a major negative loading on factor 1, while porcini has a major positive loading on factor 1. Chanterelle has a negative loading on factor 2 and curry milk cap and porcini positive loadings on factor 2. Trumpet chanterelle has a small positive loading on factor 1 and small negative loading on factor 2. Similar configurations were found in the separate PLS models for odor-contributing volatile compounds and non-volatile compounds (Supplementary materials, Figs. S5–S6).

3.3.1. Odor-contributing volatile compounds explaining the odor attributes

Potato mash and mushroom attributes have very positive loadings on factor 1, while total odor intensity, earthy, cardboard and roasted attributes have highly negative loadings. Forest and cooked carrot attributes have mildly positive loadings on factor 1 and highly negative loadings on factor 2, while potato mash has a positive loading on factor 2. The odor activities of 1-octen-3-one and 1-octen-3-ol on the HP-Innowax column correlate well with the mushroom attribute. However, these two compounds coeluted on the nonpolar column, and the combinatory perception inversely correlates with the same attribute. This is explained by our previous publications: curry milk cap has high SNIF values (surface of nasal impact frequency, the area of the GC–olfactometry signal) for these compounds (Aisala et al., 2019), but low perceived mushroom-like odor in the descriptive analysis (Aisala et al., 2018). Total odor intensity correlates strongly with the sum of all SNIF values. Methional, which had high SNIF values in both curry milk cap and porcini, correlates with the potato mash attribute but also somewhat with the roasted attribute. Regarding the roasted attribute that is mostly present in curry milk cap, 2-acetyl-1-pyrroline likely contributes to this perception due to its popcorn-like and roasted odor quality (Grosshauser & Schieberle, 2013) even though the correlation is more via factor 2. Interestingly, while different pyrazines with earthy and roasted odor descriptions have been found from mushrooms (Grosshauser & Schieberle, 2013; Zhang et al., 2018), no compounds with matching retention indices or odor descriptions were found in

Table 3
The major background variables (averages and standard deviations) related to consumer clusters. Only variables with ANOVA p-values < 0.1 are included. Significant differences between clusters are based on Tukey's HSD post-hoc test in the one-way ANOVA ($p < 0.05$) and are marked with letters a–b. FCQ: Food Choice Questionnaire.

Cluster	ANOVA p-values (η^2)	1 (n = 20)			2 (n = 38)			3 (n = 26)		
FCQ health	0.055 (0.069)	30,8	(4,6)	a	28,3	(6,2)	a	31,6	(5,4)	a
FCQ sensory appeal	0.056 (0.069)	22,5	(3,6)	a	20,8	(5,0)	a	23,3	(3,0)	a
FCQ natural content	0.009 (0.11)	14,0	(4,0)	ab	12,9	(4,0)	b	15,8	(2,6)	a
FCQ ethical concern	0.060 (0.067)	14,3	(4,9)	ab	13,2	(4,2)	b	15,8	(3,4)	a
Known mushroom species	0.065 (0.065)	6,3	(3,6)	a	6,2	(4,0)	a	8,4	(3,9)	a

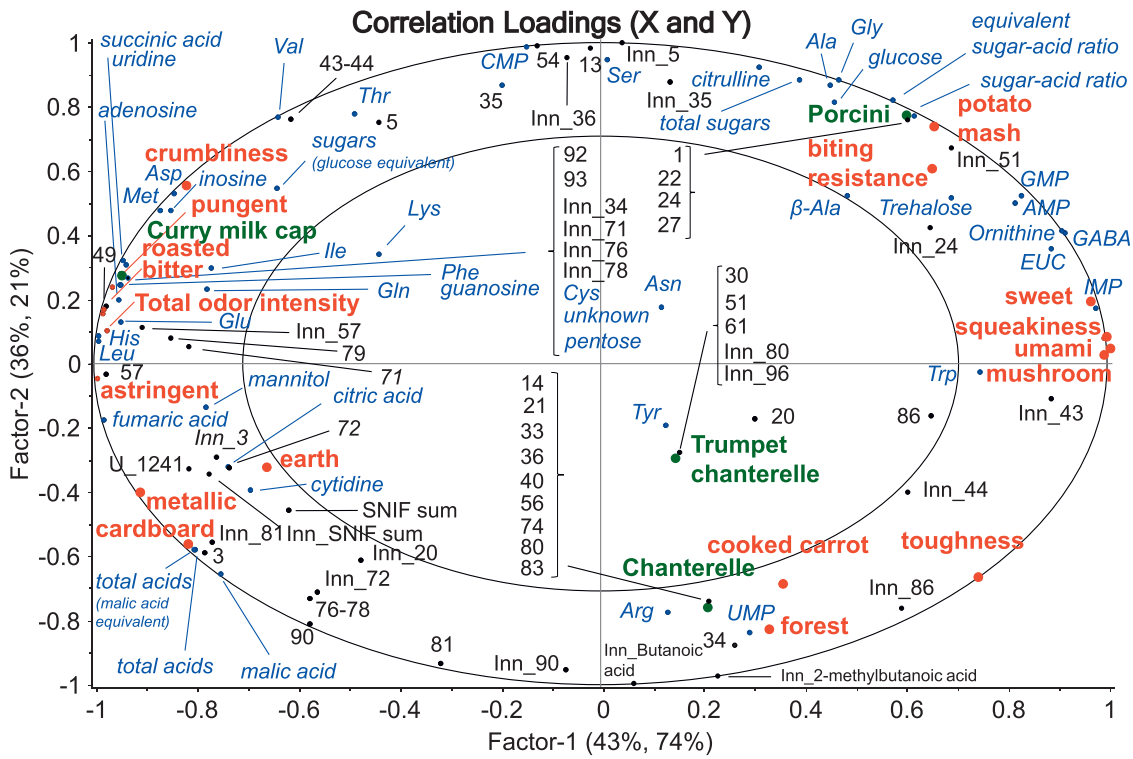

Fig. 2. PLS model of the instrumentally measured compounds (predictors: GC-O data marked in black, HPLC and NMR data marked in blue) explaining the whole sensory profile (responses, generic descriptive analysis, marked in red) in wild edible mushrooms (green). The numbering on the odor-contributing volatile compounds corresponds to the numbering in Table 3 of Aisala et al. (2019). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

curry milk cap samples. Compounds explaining the cooked carrot attribute include several fatty acid degradation products such as (*E,E*)-2,4-nonadienal, (*E,E*)-2,4-decadienal, heptanal and (*E*)-2-nonenal. While the forest attribute is also closely correlated with the above-mentioned compounds, their contribution to forest-like perception is less likely. Instead, compounds such as hexanal and several unidentified compounds might cause the odor perception as hypothesized earlier (Aisala et al., 2019). The cardboard odor attribute was also closely correlated with several fatty acid degradation products, mainly (*E*)-2-octenal and (*E*)-2-nonenal.

3.3.2. Non-volatile compounds explaining the taste and chemosensory attributes

The three taste modalities as well as the three chemosensory attributes are mainly explained with factor 1. Umami and sweet had highly positive loadings while bitter, pungent, astringent and metallic had negative loadings. Curry milk cap correlates with bitterness, astringency, pungency and metallic attributes. Porcini mostly correlates with sweetness and umami. Chanterelle had a secondary link to the metallic attribute, while trumpet chanterelle was not clearly linked to any taste or chemosensory attribute. The results of the PLS analysis are in line with the PCA presented earlier (Aisala et al., 2018). All the studied organic acids, including their total concentration, several of the bitter tasting amino acids (histidine, leucine, phenylalanine, methionine), four out of the five studied 5′-nucleosides (uridine, cytidine, adenosine, and guanosine), cysteine, and the major unknown compound in curry milk cap had negative loadings on factor 1. Thus, they correlated strongly with pungency, bitterness, astringency and metallicity.

Umami 5′-nucleotides (GMP, AMP, and IMP) had high positive loadings with factor 1, while surprisingly, glutamic and aspartic acid have negative loadings on factor 1. EUC, on the other hand, had positive loadings on both on factor 1 and factor 2. This result conforms well to the established theory of EUC values as a predictor of umami taste, taking into account both levels of umami amino acids and nucleotides. As reported previously, curry milk cap had the highest concentrations of Glu and Asp, but the lowest concentrations of GMP, AMP, and IMP (Manninen et al., 2018). On the other hand, porcini had moderate levels of Asp and Glu, but relatively high levels of GMP, AMP, and IMP among the four species studied (Manninen et al., 2018). The two chanterelles had intermediate levels of these compounds. Thus in our model, EUC was a stronger predictor of umami intensity than Asp and Glu levels alone. It is therefore similar in effectiveness to GMP, IMP and AMP levels, which is dissimilar to that reported by Phat et al. (2016). It is likely that the other sensory attributes in curry milk cap can mask or suppress the umami intensity.

Mannitol and total sugars have limited predictor values on the model. However, the trehalose content, low levels of individual and total acids and especially the sugar-acid ratios have good correlations with sweetness. Moreover, the sweet amino acids Ala and Gly correlate with sweetness, but it is not predicted by Thr and Ser. On the other hand, the unknown pentose-type compound in curry milk cap correlates mostly with bitterness rather than sweetness.

4. Conclusions

In this research, three consumer clusters were found based on the hedonic liking of 84 consumers for mushrooms. The previous data on

the odor-contributing volatile and non-volatile compounds of cooked mushroom samples were supplemented with quantitative NMR measurements and further projected on the sensory profile of mushrooms using multivariate statistical methods. To our best knowledge, this is the first time that all three aspects (chemical measurements, analytical sensory profiles and hedonic liking tests of consumers) of cooked mushrooms have been taken into account. Edible mushroom species could be separated based on both metabolomic approaches as well as targeted methods. They are different as regards both their non-volatile and odor contributing volatile profiles, as well as sensory properties. When taking the sensory properties, the non-volatile, and the volatile chemical compounds into account at the same time it is clear that trumpet chanterelle is more similar to chanterelle than to porcini and curry milk cap.

Surprisingly, it was the consumer cluster instead of the mushroom species which was the main source of variation as regards the liking of mushrooms for consumers. The consumer clusters were significantly different in their liking profiles for the selected mushroom species. The individuals in these clusters were heterogeneous in their background: neither age, mushroom usage frequency, nor differences in their food choice motives provided a good explanation for the liking profiles.

As a result of the experiments, it was possible to link chemical compounds of the studied mushrooms to different odor and taste attributes. The same mushroom samples could be used to divide consumers into groups of differing liking profiles. Therefore, this research brings new information as to why mushrooms are not pleasing to everyone. This information is also useful for product development and the individual marketing of mushroom products.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125403.

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Supplementary material

Linking volatile and non-volatile compounds to sensory profiles and consumer liking of wild edible Nordic mushrooms

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Table S1. Identified compounds and their signals in qNMR experiments. Presentation order: sugars and sugar alcohols, organic acids, amino acids, unknown compounds.

#	Compound	Position (IUPAC)	δ_H (ppm)	Multiplicity (a)	J (Hz)	# of H's	Identification methods (b)	13C correlations (HSQC, HMBC)
1	**Glucose**	H4	3.52	dd	9.8, 3.7	1	1H (shift, J),	
		β-D H1	4.64	d	8.0	1	standard,	98.6
		α-D H1	5.22	d	3.8	1	HSQC	94.8
2	**Mannitol**	H1, H6	3.66	dd	11.6, 5.9	2	1H (shift, J),	66.0
		H2, H5	3.75	m		2	13C, HSQC,	73.5
		H3, H4	3.79	d	8.6	2	HMBC,	72.0
		H1, H6	3.85	dd	11.7, 2.6	2	COSY	66.0
3	**Trehalose**	α-D H1, α-D H1'	3.44	t	9.4	2	1H (shift, J), standard,	72.4
		α-D H2, α-D H2'	3.64	dd	9.9, 3.9	2	HSQC	73.7
		α-D H4, α-D H4'	5.18	d	3.8	2		95.9
4	**Citric acid**	H1	2.54	d	14.9	2	1H (shift, J),	48.7
		H3	2.70	d	13.9	2	pH, standard, HSQC	48.7
5	**Fumaric acid**	H2, H3	6.51	s		2	1H (shift, J), pH, standard, HSQC	138.0
6	**Malic acid**	H3	2.35	dd	15.4, 10.1	1	1H (shift, J),	45.3
		H3	2.66	dd	15.4, 3.0	1	pH, standard,	45.3
		H2	4.30	dd	10.1, 3.0	1	HSQC	73.1

#	Compound	Position (IUPAC)	δ_H (ppm)	Multiplicity (a)	J (Hz)	# of H's	Identification methods (b)	13C correlations (HSQC, HMBC)
7	**Succinic acid**	H2, H3	2.39	s		4	1H (shift), pH, HSQC, HMBC	36.8
8	**γ-Aminobutyric acid**	H2	2.29	t	7.4	2	1H (shift, J), standard, HSQC	37.1
9	**Alanine**	H3	1.47	d	7.3	3	1H (shift, J), standard, HSQC	18.9
10	**Arginine**	H4	1.68	m		2	1H (shift), standard	
		H3	1.92	m		2		
11	**Asparagine**	H3	2.85	dd	16.9, 7.6	1	1H (shift, J), standard, HSQC	37.2
		H3	2.95	dd	16.9, 4.3	1		37.2
12	**Aspartic acid**	H3	2.67	dd	17.5, 8.7	1	1H (shift, J), standard, HSQC	39.3
		H3	2.80	dd	17.5, 3.8	1		39.3
13	**Glutamine**	H3	2.13	m		2	1H (shift), standard, HSQC, COSY	29.0
		H4	2.44	m		2		33.5
14	**Glutamic acid**	H3	2.07	m		2	1H (shift), standard, HSQC, HMBC, COSY	29.7
		H4	2.35	m		2		36.2
15	**Glycine**	H2	3.55	s		2	1H (shift), standard, HSQC	44.0
16	**Isoleucine**	H5	0.93	t	7.4	3	1H (shift, J), standard, HSQC	13.9
		H3'	1.00	d	7.1	3		17.4
17	**Leucine**	H5, H5'	0.95	t	5.9	6	1H (shift, J), standard, HSQC	23.6, 24.8
18	**Phenylalanine**	H5, H9	7.32	m		2	1H (shift, J), standard, HSQC	132.2
		H7	7.38	m		1		130.6
		H6, H8	7.42	m		2		
19	**Proline**	H3	2.06	m		1	1H (shift, J), standard	
		H5	3.33	m		1		
		H2	4.12	dd	8.8, 6.3	1		
20	**Serine**	H3	3.96	m		2	1H (shift), standard, HSQC	62.9
21	**Threonine**	H4	1.32	d	6.6	3	1H (shift, J), standard, HSQC, HMBC	22.2
		H3	4.24	m		1		68.6
22	**Tryptophan**	H7	7.31	s		1	1H (shift, J), standard	
		H7	7.52	d	8.2	1		
		H6	7.72	d	8.0	1		
23	**Tyrosine**	H2, H6	6.89	d	8.5	2	1H (shift), standard, HSQC, HMBC	118.7
		H3, H5	7.18	m		2		133.4

#	Compound	Position (IUPAC)	δ_H (ppm)	Multiplicity (a)	J (Hz)	# of H's	Identification methods (b)	13C correlations (HSQC, HMBC)
24	**Valine**	H4	0.98	d	7.1	3	1H (shift, J),	19.4
		H4'	1.03	d	7.1	3	standard,	20.7
		H3	2.26	m		1	HSQC	31.8
		H2	3.60	d	4.4	1		
25	**Unknown pentose**	CH3	0.95	d	7.1	3		14.8
	(curry milk cap)	CH3	1.24	d	6.3	3		23.4
		N/A	1.92	m		N/A		44.0
		N/A	3.90	d	4.4	N/A		59.6
		N/A				N/A		72.6
		N/A	3.88			N/A		
26	**Unknown** *(porcini)*	N/A	3.22	s		N/A		56.76
27	**Unknown** *(chanterelle)*	N/A	3.27	s	21.9	N/A		54.91
28	**Unknown** *(chanterelle)*	Anomeric sugar?	4.97	d	3.6	N/A		108.6
29	**Unknown**	Anomeric sugar?	5.24	d	3.3	N/A		
30	**Unknown** *(chanterelle, porcini)*	Anomeric sugar?	5.41	d	3.9	N/A		
31	**Unknown** *(porcini)*	N/A	6.79	s		N/A		135.4, 178.8

Table S2. Residual and recovery values for the qNMR measurements. The coefficients of averages and standard deviations of the residual compounds compared to the original samples (*Boletus edulis*) and the coefficients of recovery averages and standard deviations of the added standards to the original sample (*Cantharellus cibarius*) are shown (n=3, extractions and analyzes made from freeze-dried samples of each species).

Compound	Residue (%) [a]		Recovery (%)	
Glucose	traces			
Mannitol	8.9	(6.6)		
Trehalose	5.5	(0.3)	112.8	(27.8)
Citric acid	traces			
Fumaric acid	4.9	(1.1)	119.4	(16.3)
Malic acid	7.4	(1.3)	111.9	(17.3)
Succinic acid	traces			
L-alanine	5.6	(0.2)	85.4	(76.3)
L-glutamine	traces		105.2	(4.3)
L-isoleucine	traces		190.0	(65.5)
L-valine	4.8	(0.9)		
Average	6.2		103.5	

[a] traces = content below limit of quantification in the residual extract

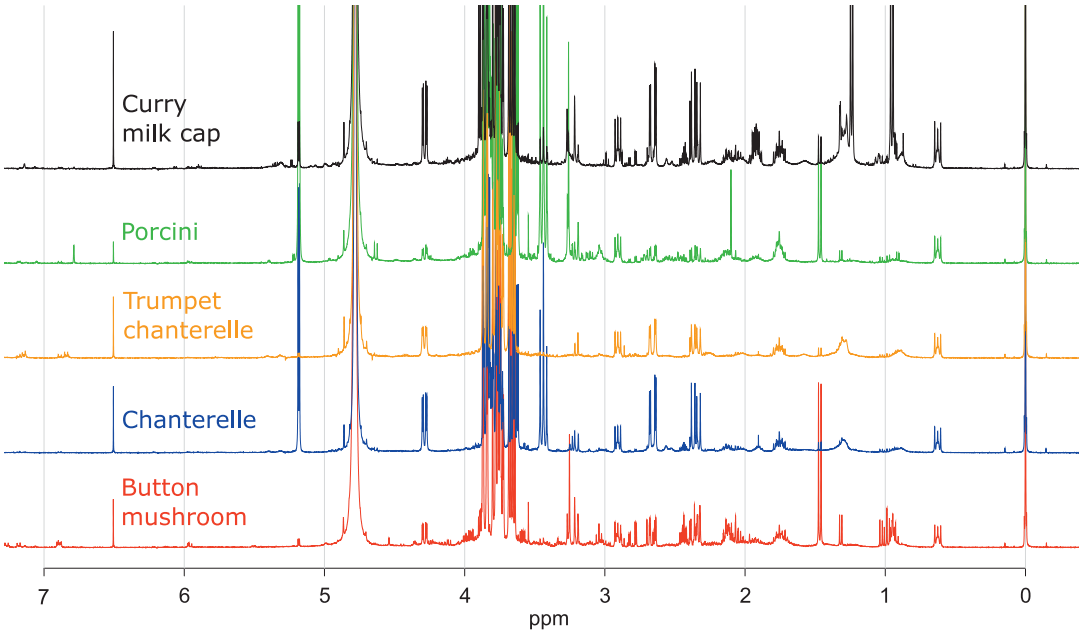


Figure S1. Representative NMR spectra of the five mushroom species. The NMR signals are normalized to extracted fresh mushroom mass and the internal standard DSS signal (0.0 ppm, 0.63 ppm, 1.75 ppm, 2.91 ppm).

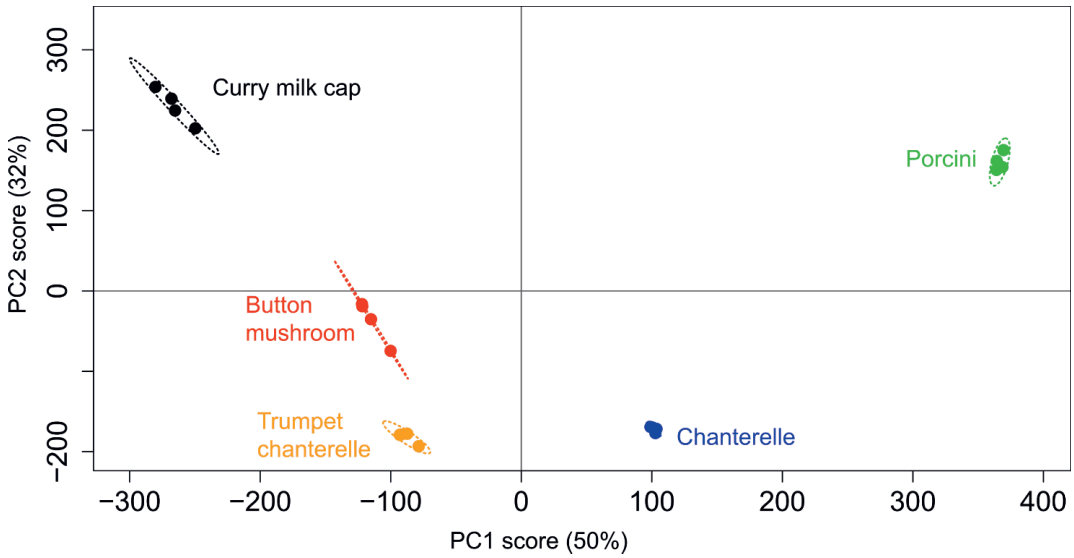


Figure S2. Principal component analysis Scores plots of PCs 1 and 2 built from ^{1}H 1D qNMR data. The Fourier transformed spectra were normalized to sample masses and DSS signal, binned to 0.02 ppm/point data buckets and redundant spectral regions were removed. The PCA model was created using Pareto scaling. Ellipses are 95% confidence limits for each mushroom species (n=4 for each species).

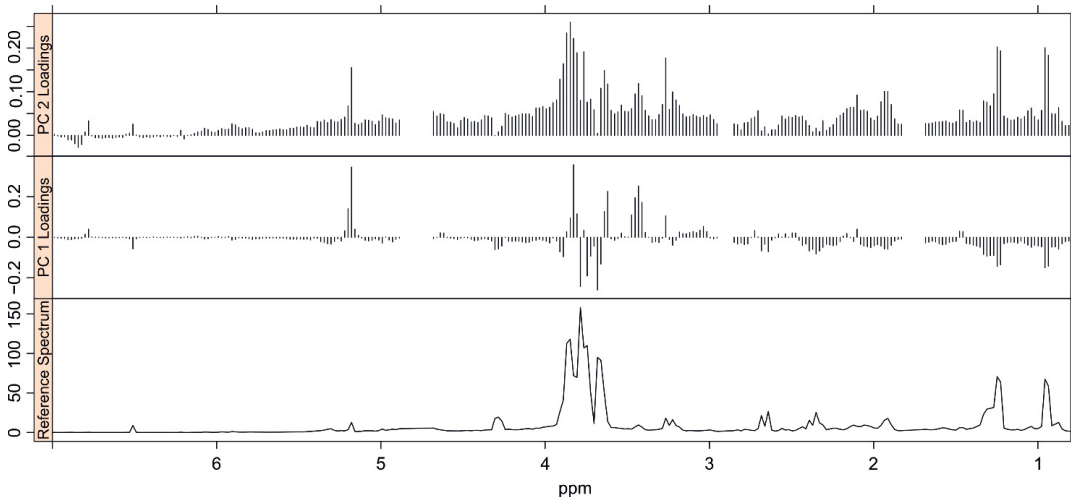
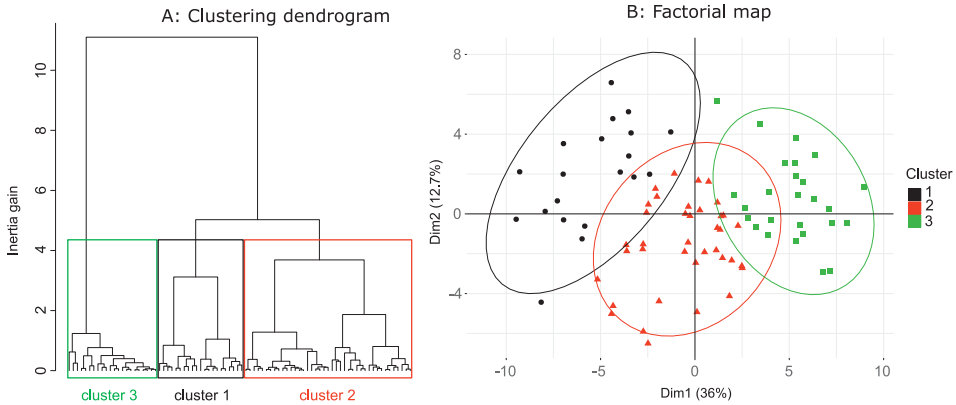


Figure S3. Principal component analysis Loadings plots of PCs 1 and 2 built from ^{1}H 1D qNMR data. The binned curry milk cap chromatogram is used as reference.

Table S3. Comparison of the measured amino acid contents (expressed as mg g^{-1} dry mass; averages with standard deviations in brackets) in the current qNMR experiments (left), previous measurements from the same batch of samples with UHPLC (Manninen et al. 2018) and literature values from button mushrooms determined with HPLC (Li et al. 2011).

	This research										Manninen et al. 2018						Li et al. 2011	
Amino acid	**Trumpet chanterelle**		**Curry milk cap**		**Chanterelle**		**Porcini**		**Button mushroom**		**Trumpet chanterelle**		**Curry milk cap**		**Chanterelle**		**Porcini**		**Button mushroom**, microwaved	
L-alanine	0.72	(0.09)	1.13	(0.06)	0.63	(0.03)	8.22	(0.14)	13.88	(0.23)	0.66	(0.11)	0.98	(0.11)	0.65	(0.02)	6.67	(0.92)	7.08	(0.09)
L-glutamine	traces		5.65	(0.06)	4.30	(0.28)	3.79	(0.39)	13.55	(0.16)	0.69	(0.08)	5.1	(0.44)	4.74	(0.39)	3.87	(0.60)	10.74	(0.07)
L-isoleucine	0.30	(0.02)	0.11	(0.02)	0.13	(0.01)	0.44	(0.03)	2.99	(0.42)	0.32	(0.03)	0.27	(0.01)	0.23	(0.01)	0.31	(0.03)	0.91	(0.01)
L-valine	0.30	(0.01)	0.46	(0.04)	0.30	(0.01)	0.56	(0.02)	3.19	(0.05)	0.32	(0.03)	0.37	(0.02)	0.31	(0.02)	0.54	(0.07)	1.20	(0.01)
L-Aspartic acid	traces		4.13	(0.07)	1.24	(0.08)	2.85	(0.06)	13.81	(0.33)	1.65	(0.15)	3.35	(0.26)	1.29	(0.08)	2.85	(0.44)	2.39	(0.01)
L-Glutamic acid	0.81	(0.57)	5.88	(0.56)	3.27	(0.79)	2.80	(0.13)	19.29	(0.51)	1.92	(0.21)	4.69	(0.36)	3.78	(0.24)	2.62	(0.40)	17.12	(0.08)

Figure S4. Results of the consumer clustering. A: clustering dendrogram of the individual consumers with clustering inertia gain as the abscissa; B: internal preference map of the consumers colored by the cluster membership. Multivariate t distribution-based 95% confidence intervals are drawn around the cluster center point.



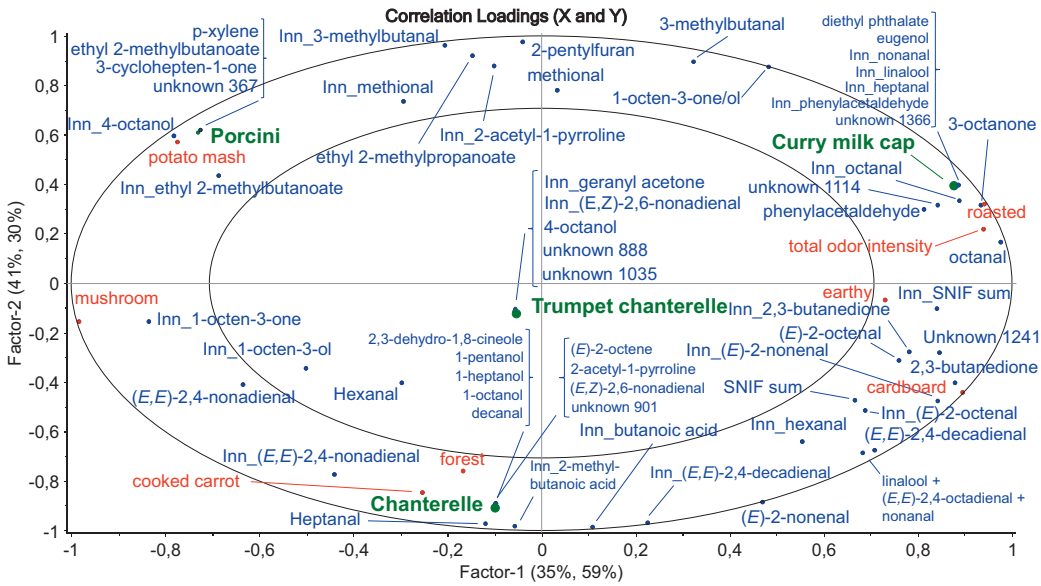


Figure S5. PLS model of the odor-contributing volatile compounds (blue) and odor attributes of the sensory profile (red) of the four wild edible mushrooms (green).

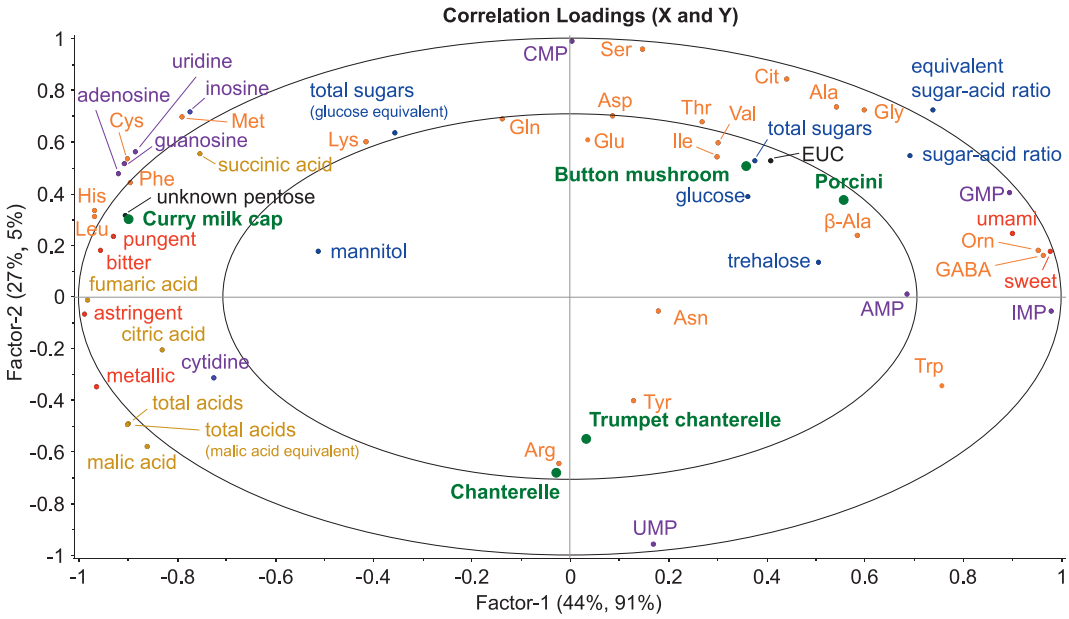


Figure S6. PLS model of non-volatile compounds explaining the taste and chemosensory properties. The mushroom samples are marked with green and the sensory properties with red. Among the non-volatile compounds, the amino acids are marked with orange, nucleotides with purple, organic acids with yellow and sugars with blue color.

PUBLICATION 3

Taste compound–nanocellulose interaction assessment by fluorescence indicator displacement assay

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Taste compound – Nanocellulose interaction assessment by fluorescence indicator displacement assay

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ABSTRACT

Interactions between taste compounds and nanofibrillar cellulose were studied. For this, a new fluorescent indicator displacement method was developed. Two fluorescent indicators, namely, Calcofluor white and Congo red, were chosen because of their specific binding to cellulose and intrinsic fluorescence. Seven taste compounds with different structures were successfully measured together with nanofibrillar cellulose (NFC) and ranked according to their binding constants. The most pronounced interactions were found between quinine and NFC ($1.4 \times 10^4\ M^{-1}$), whereas sucrose, aspartame and glutamic acid did not bind at all. Naringin showed moderate binding while stevioside and caffeine exhibited low binding. The comparison with microcrystalline cellulose indicates that the larger surface area of nanofibrillated cellulose enables stronger binding between the binder and macromolecules. The developed method can be further utilized to study interactions of different compound classes with nanocellulose materials in food, pharmaceutical and dye applications, using a conventional plate reader in a high-throughput manner.

1. Introduction

Nanocellulose materials represent a class of cellulose materials with at least one nanoscale dimension produced either with enzymatic, chemical or physical methods from natural cellulose fibers (Klemm et al., 2011). Nowadays they have many uses for example as bioabsorbents in wastewater treatment and in biomedical applications, drug delivery systems, tissue engineering and wound dressings (Ngwabebhoh & Yildiz, 2019). In particular, the utilization of nanocellulose as a food additive was one of the first applications proposed (Turbak, Snyder, & Sandberg, 1983a). High surface area and aspect ratio, suitable rheological behavior (high viscosity even at low concentrations) and the ease of chemical modifications are advantageous for applications in the food industry, particularly in food packaging (Gómez et al., 2016; Klemm et al., 2011).

Since the 1980s, many food related applications utilizing nanocellulose have been developed. In a review by Gómez et al. (2016) the applications in food science were divided into three groups: 1) as a food stabilizer, 2) as a functional food ingredient, and 3) in food packaging. As a stabilizing agent, nanocellulose materials have been used in various food products such as in fat and oil containing products (gravies, salad dressings, and whipped toppings) (Turbak, Snyder, & Sandberg, 1982, 1983a, 1983b). Furthermore, they have been used in this way to prevent the spreading of cookie fillings (Kleinschmidt, Roberts, Fuqua, & Melchion, 1988), to improve the shape retention of frozen desserts (Yano, Abe, Kase, Kikkawa, & Onishi, 2012) and most recently, in the shape retention of ice cream (Velásquez-Cock et al., 2019). In functional foods, nanocellulose materials have been used in low-calorie applications in products with high-energy content such as hamburgers (Ström, Öhgren, & Ankerfors, 2013) and to replace fats in food formulations and thus reduce their energy density (Cantiani, Knipper, & Vaslin, 2002). Furthermore, nanocellulose materials have showed promising characteristics as dietary fibers (Andrade et al., 2015).

In food packaging applications, nanocellulose materials offer a nature-friendly option to fossil fuel based and non-biodegradable materials (Azeredo, Rosa, & Mattoso, 2017). Nanocellulose materials can act as high air and oxygen barriers, which makes them competitive to other packaging materials (Aulin, Gällstedt, & Lindström, 2010; Gómez et al., 2016). They can also serve as carriers for active substances in

Abbreviations: NFC, nanofibrillar cellulose; FI, fluorescence indicator; CR, Congo red; CFW, calcofluor white; MQ, Milli-Q water

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food packaging applications (Huq et al., 2012). For example, Lavoine, Desloges, and Bras (2014) used a paper coated with microfibrillated cellulose for the controlled release of caffeine, whereas Jipa, Stoica-Guzun, and Stroescu (2012) studied controlled release of sorbic acid from bacterial cellulose films.

In this study, the aim was to evaluate the interactions between nanocellulose materials and taste compounds. Despite the many food related applications of nanocellulose materials, to our knowledge there are no systematic studies about the possible effects of nanocellulose to the taste of food. Troszyńska et al. (2010) studied the effect of food gums (i.e. guar, xanthan, arabic) and carboxymethylcellulose (CMC) on the astringency induced by phenolic compounds. According to their study, CMC was the best at masking astringency. Furthermore, the interactions between nanocellulose materials and drug molecules have been studied. Particularly, Kolakovic et al. (2013) used isothermal titration calorimetry (ITC) and an incubation method (incubation of drug molecules with NFC, centrifugation and quantification of an unbound drug from supernatant) to study the binding of drug compounds to nanofibrillated cellulose (NFC). In a similar manner, Jackson et al. (2011) studied the binding of drug molecules to nanocrystalline cellulose (NCC) by measuring the amount of unbound molecules by using a spectrophotometry method.

The methodologies presented above are accurate, but time consuming and molecule dependent. For each compound, a new or at least refined methodology is needed. In contrast, a more generic method based on fluorescent indicator (FI) displacement for nanocellulose–taste compound interaction assessment is developed in this study. With this method, it is possible to screen a wide spectrum of molecules with different characteristics with one method using a plate reader with, e.g., a 96-well plate. Thus, the developed method is both affordable and efficient. The method is based on the competitive binding of a well-known FI molecule and a second molecule, whose binding to a macromolecule, in this case to NFC, is investigated. If the interaction between molecule of interest and NFC occurs, a decrease of FI fluorescence intensity can be detected as it is displaced from the fiber surface. Similar methods have been used before, for example in the assessment interactions of different analytes to DNA, RNA and proteins (Asare-Okai & Chow, 2011; Ham, Winston, & Boger, 2003; Mock, Langford, Dubois, Criscimagna, & Horowitz, 1985; Zhang, Umemoto, & Nakatani, 2010). These methodologies have been reviewed by Nguyen and Anslyn (2006), and Tse and Boger (2004). Nevertheless, to our knowledge these methods have not been used before to assess macromolecule interactions with taste compounds. Two FIs were chosen based on their specific binding to cellulose (Wood, 1980) and different photophysical properties, to avoid a possible scenario where the molecule of interest absorbs light at the same wavelength that is used to excite the FI. Calcofluor white has its absorption maximum at around 350 nm while the absorption maximum of Congo red is at around 500 nm (Wood, 1980). With these indicators, a wide variety of taste compounds with different taste characteristics could be studied. Seven taste compounds, caffeine, aspartame, quinine, stevioside, sucrose, naringin and glutamic acid, with different taste characteristics (sweet, bitter, umami), were chosen for this study. Salts and strongly acidic compounds were excluded from the study as salts and extreme pH cause swelling of cellulose materials (Grignon & Scallan, 1980).

2. Materials and methods

2.1. Materials

Cellulose nanofibrils (dimeric unit presented in Fig. 1a) were obtained from UPM Corporation (Finland) as a 1.5 wt% hydrogel. Microfibrillated cellulose (MCC, Avicel®; Sigma-Aldrich) was used as a 1.5 wt% suspension prepared with water purified using a Milli-Q system (MQ; Millipore, Burlington, MA). The FIs used were Fluorescence brightener 28 (Calcofluor white M2R) (Fig. 1b) from Sigma-Aldrich (St. Louis, MO) and Congo red (> 98%) from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) (Fig. 1c).

The studied taste compounds (Fig. 1 d–j) were caffeine (99%), naringin and aspartame (98%) from ThermoFisher GmbH (Kandel, Germany) and glutamic acid (99%), stevioside, sucrose (> 99%) and quinine (99%) from Acros Organics (Geel, Belgium). The compounds were chosen based on their known taste properties to include compounds, which either create a pleasant taste (sweet and umami) or have related unpleasant characteristics (bitter).

2.2. Methods

2.2.1. UV–Vis characterizations

Water solutions of the fluorescent indicators i.e. calcofluor white (CFW) and Congo red (CR) were measured with UV–Vis-NIR spectrophotometer (UV-3600, Shimadzu) in 1 cm^2 standard quartz cuvettes. Absorption spectra were measured from 250 to 600 nm varying the concentration from 0 to 26 μM for both Calcofluor white and Congo red. MQ-water was used to adjust the samples concentrations. Absorption maxima were detected at 349 nm and 499 nm for CFW and CR, respectively. Molar extinction coefficients were calculated based on the absorption measurements.

2.2.2. Titration of the fluorescent indicator with nanofibrillar cellulose

Fluorescent indicators CFW and CR in concentrations of 6 μM and 2.5 μM, respectively, were titrated with an NFC hydrogel to a final NFC concentration of 0.04 M. The concentrations of FIs were chosen to avoid inner filter effects on the fluorescence of FIs. As the molecular weight of NFC macromolecules varies, the concentration of NFC is represented in moles of monomeric cellulose units per liter using 162.14 g/mol as the molar mass of the monomer. This is a common practice when the binding of a small molecule to a macromolecule with multiple binding sites is studied. The changes in the fluorescence intensity of CFW upon titration with NFC were measured in triplicates by spectrofluorometer Fluorolog-3® (Jobin Yvon) or plate reader Fluoroskan Ascent FL (Thermo Labsystems). The changes in the fluorescence intensity of CR upon titration with NFC were measured in triplicates by using a plate reader. The excitation/emission filter pairs for measurement with spectrofluorometric plate reader were chosen to be 355/460 nm for CFW and 485/590 nm with CR based on their absorption/emission spectra. The titration of MCC with CR was conducted in a similar manner as with NFC with concentration range from 0.002 to 0.088 M. MCC concentration was estimated in the same way as for NFC.

The binding constants (K_{bind}) for FIs with NFC were calculated using Benesi-Hildebrand method (Benesi & Hildebrand, 1949) as follows:

$$\frac{I_{max} - I_{free}}{I_n - I_{free}} = 1 + \frac{1}{K_{bind}[\text{NFC}]} \quad (1)$$

where [NFC] is the added NFC concentrations, I_{max} is the maximum fluorescence intensity of FI in the presence of NFC when the saturation is reached, I_{free} is the fluorescence intensity of FI in the absence of NFC, I_n is the fluorescence intensity of FI in the presence of NFC at an intermediate concentration and K_{bind} is the binding constant for the FI. By plotting $\frac{I_{max} - I_{free}}{I_n - I_{free}}$ *versus* $1/[\text{NFC}]$ the values of K_{bind} were obtained from the slope of the linear fit.

2.2.3. Titration of pre-formed fluorescent indicator–nanofibrillar cellulose complex with taste compounds

All the samples contained either 0.04 M of NFC with 6 μM CFW or 0.025 M of NFC with 2.5 μM CR and varying concentrations of the taste compounds (Table 1). The concentration ranges for the taste compounds were chosen based on their solubility in water. All solutions were mixed carefully to avoid bubbles. FI for each compound was chosen based on their photophysical characteristics, i.e. whether they would absorb light at the excitation wavelength of the FI or not. In

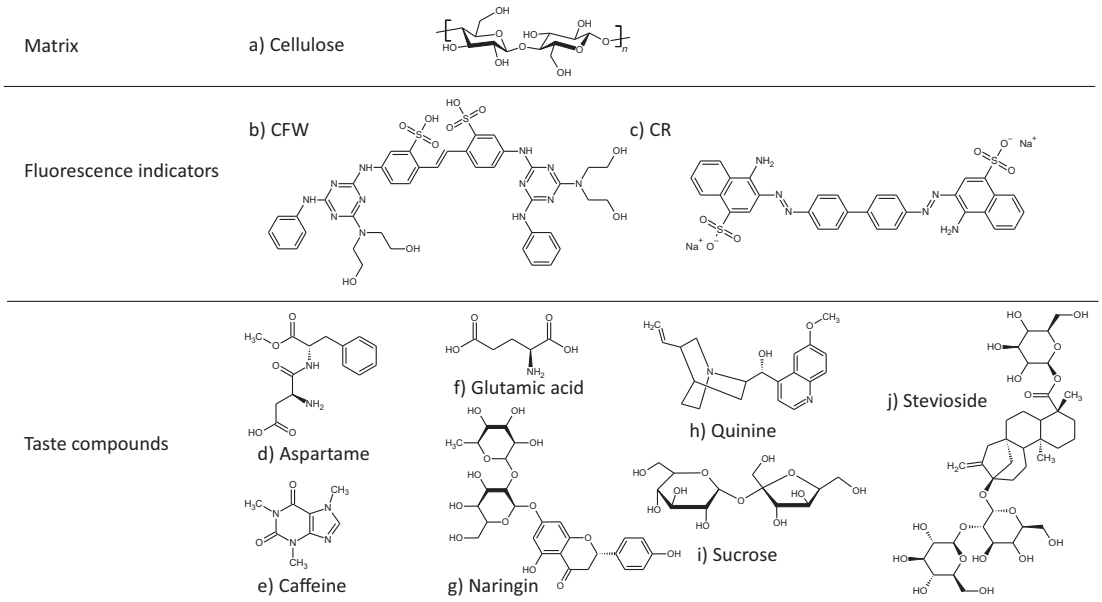

Fig. 1. Chemical structures of cellulose (a), the FIs Calcofluor white (b) and Congo red (c), and the studied taste compounds: aspartame (d), caffeine (e) glutamic acid (f), naringin (g), quinine (h), sucrose (i) and stevioside (j).

order to estimate possible errors by using different FIs, cross-validation of caffeine-NFC interaction was studied by using both CFW and CR. A150-μL aliquot of each sample solution was pipetted onto a well plate and measured with plate reader as above (*Section 2.2.2*). Each taste compound was studied as triplicates.

The binding constants were determined with Benesi-Hildebrand method as before. As the substitution of FI causes decreasing fluorescence intensity, equation (1) was modified as follows:

$$\frac{I_0 - I_{free}}{I_0 - I_n} = 1 + \frac{1}{K_{bind}[TC]} \quad (2)$$

[TC] is the added taste compound concentration, K_{bind} is the binding constant of the taste compound, I_0 is the fluorescence intensity of FI–NFC mixture in the absence of the taste compounds, I_{free} is the fluorescence intensity of FI in the absence of NFC, I_n is the fluorescence intensity of FI–NFC mixture in the presence of the taste compounds at an intermediate concentration.

2.2.4. Cross-validation with ITC

Isothermal titration calorimetry was performed using a Microcal VP-ITC (Malvern Panalytical Ltd, Malvern, UK) with VPViewer2000 version 1.4.11 (Microcal LLC) software. A sample cell was filled with quinine (0.39 mM). Experiments were carried out at 25 ^{0}C by injecting 20 μL of 15 mM NFC sample solution 15 times. As control measurements, MQ was titrated with 15 mM NFC and 0.39 mM quinine with MQ. The differential enthalpy curves of heat of titration of MQ with NFC and the averaged enthalpy of titration of quinine with MQ were then subtracted from the curves of binding of quinine to NFC. Data was were with Origin 7.0383 SR2 Microcal (Northampton, MA) software using one binding site model for curve fitting and enthalpy calculation.

3. Results and discussion

3.1. Binding constants of fluorescence indicators

Based on the results of spectrophotometry, the molar extinction coefficients in water for CFW and CR were calculated to be ε_{CFW} (349 nm) = 53.2 × 10^3 $M^{-1}cm^{-1}$ and ε_{CR}(499 nm) = 38.9 × 10^3 $M^{-1}cm^{-1}$. Thus, indicator concentrations of 6 μM and 2.5 μM for CFW and CR were used for the titration experiments with NFC to neglect the inner filter effect on the fluorescence. During the titrations of CFW and CR with NFC an increase in the fluorescence intensity of both dyes was observed, indicating that both FIs bind to NFC. The fluorescence intensities of CFW and CR against the added NFC concentration are

Table 1
Binding constants of taste compounds to NFC as mean values with standard deviations (n = 3). Compounds are grouped based on their taste characteristics. For each compound, molar mass, solubility in water and logP value is provided. negl. = negligible. [1]Windholz, 1983; [2]Furia, 1980; [3]Mazzobre, Román, Mourelle, & Corti, 2005; [4]Dreisewerd, Merz, & Schembecker, 2015, [5]Cargill, 2010; [6]Valko, Du, Bevan, Reynolds, & Abraham, 2000; [7]Yuan et al., 2019; [8]Rankovic, 2017; [9]Hansch, Leo, & Hoekman, 1995.

Taste	Compound	FI	MW [g/mol]	Solubility in water [mg/mL]	Log P	K_{bind}, M^{-1}
Sweet	Sucrose	CFW	342.30	2 000[1]	–3.3[3]	negl.
	Stevioside	CFW	804.88	1.25[1]	1.19[4]	146 ± 34
	Aspartame	CFW	294.31	10.20[2]	0.07[5]	negl.
Bitter	Caffeine	CFW (CR)	194.19	21.74[1]	–0.07[6]	70 ± 25 (86)
	Naringin	CFW	580.53	1.00[1]	−0.5[7]	1251 ± 385
	Quinine	CR	324.42	0.53[1]	2.51[8]	14300 ± 1500
Umami	Glutamic acid	CFW	147.13	8.64[1]	–3.69[9]	negl.

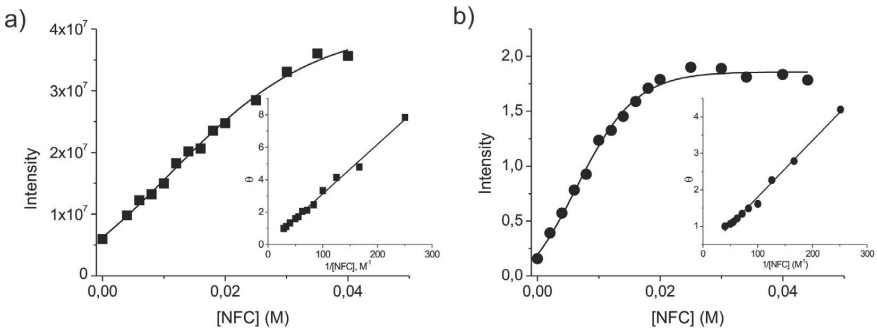

Fig. 2. Examples of the saturation curves for titration of the FIs with NFC, i.e. the fluorescence intensity of CFW (6 μM) (a) and CR (2.5 μM) (b) as a function of NFC concentration. The reciprocal plots (Eq. (1), $\theta = \frac{I_{max} - I_{free}}{I_n - I_{free}}$) are presented in insets.

presented in Fig. 2. The data are also presented according to Eq. (1) to calculate the binding constants (Fig. 2, insets).

The obtained binding constants were 27 ± 7 M^{-1} for CFW and 58 ± 12 M^{-1} for CR. Based on the saturation curves (Fig. 2), NFC concentrations of 0.04 M with CFW and 0.025 M with CR were chosen for taste compound titrations as the saturation and maximum intensity were reached at these concentrations. When MCC was titrated with CR, saturation was not reached within the studied concentration range and the binding constant was estimated to be approximately 4 M^{-1}. This is more than 10 times lower compared to NFC–CR interaction and is probably due to the considerably lower specific surface area of MCC (ca. 1.3 m^2/g for Avicel pH 102 (Ardizzone et al., 1999)) compared to NFC (50–70 m^2/g (Missoum, Belgacem, & Bras, 2013)). As the binding is surface area dependent, it is logical that the binding constants are considerably lower in the case of MCC. This further means that the possible effect of binding of the taste compounds on the taste of foods can be perceived with NFC even if this is not the case with MCC-containing formulations or products.

3.2. Binding constants of taste compounds

The interaction between the pre-formed NFC–FI complexes and taste compounds resulted in a clear decrease in the fluorescence of the FIs because of FI displacement from the NFC matrix. As the FI–NFC complexes have stronger fluorescence than the free FIs, the overall fluorescence intensity in the system will decrease if the dyes are released from the NFC surface. This happens when a taste compound binds to a cellulose surface that is initially fully covered by the FI. It is good to notice that the measured signal comes from the FI in all the measurements, and not from the taste compounds. FI displacement curves are presented in Fig. 3 for those taste compounds that showed clear complex formation with NFC. These curves can be used to evaluate the binding constants according to eq. (2).

Clear trends in the fluorescence intensity can be seen in Fig. 3 for caffeine, stevioside, naringin and quinine, even though the experimental fluctuations are considerable especially for stevioside and naringin. The binding isotherms can be used for ranking the taste compounds in the order of binding strength and for the evaluation of the binding constants. As an example, a 20% FI (CFW) displacement was achieved at ca. 5 mM concentration of caffeine, whilst for quinine the same percent was achieved at ca. 0.025 mM concentration. Roughly 1.8 mM and 0.2 mM concentrations for stevioside and naringin respectively were needed to reach the same displacement. Sucrose, aspartame and glutamic acid had negligible binding according to our measurements, as no clear fluorescence decrease was seen with these molecules. Finally, the binding constants of all the tested compounds calculated with Eq. (2) are presented in Table 1 as mean values of triplicates.

In order to verify the comparability of the results obtained by different FIs, the binding values for caffeine was estimated with both indicators. The slopes for caffeine-NFC interactions plotted according to Eq. (2) are close to each other. The calculated binding constant of caffeine to NFC obtained with CR was 86 M^{-1}, which is very close to the measured binding constant with CFW (70 M^{-1}, Table 1). Thus, the method can be used with either of the selected fluorescence indicators, and the indicator can be chosen based on whether the molecules have spectral overlap with the FI or not. As all seven taste compounds with different structures and properties were measured successfully using a plate reader and 96-well plates in a high-throughput manner, it can be concluded that a similar methodology can be also utilized in future for studying larger sets of compounds for applications in, e.g., food industry and pharmaceutical fields.

Isothermal titration calorimetry (ITC) was used for cross-validation of the method. As only relatively high enthalpy changes can be measured with this method, quinine with the highest binding constant to NFC was chosen for these studies. The enthalpy curve of quinine binding to NFC resulting from ITC is presented on Fig. 4. The estimated binding constant for quinine measured with ITC and calculated with one binding sites model was 19 000 ± 5790 M^{-1}. Based on these results it can be concluded that the binding constants achieved with fluorescence indicator displacement method are reasonably accurate and in line with results obtained with more established ITC methods. Furthermore, with this method weaker interactions can be measured than with traditional methods like ITC.

Based on estimated binding constants, taste compounds can be divided into four groups: non-binding molecules, molecules with weak interactions, molecules with moderate interactions and molecules with distinct interactions. Of the studied compounds, sucrose, aspartame and glutamic acid belong to the group of non-binding molecules, caffeine and stevioside have weak interactions, whereas naringin has moderate interactions. Quinine has clearly more pronounced interactions than the other studied molecules, with *ca.* 200 times higher binding constant than caffeine for example, making it the strongest binding molecule in our test set. The measured binding constant (14 000 M^{-1}) is of the same order of magnitude as was measured for hydrophobin proteins binding to NFC (Kolakovic et al., 2013), indicating strong binding between quinine and NFC.

From Table 1 it can be seen that the interactions seem to partly correlate with the aqueous solubility and octanol/water partition of the compounds. The highest binding constant was achieved with quinine, which is also the least water soluble of the studied molecules. This indicates that despite the hydrophilic nature of NFC, in aqueous solutions the nanofibrillar cellulose is acting as a slightly hydrophobic target as water molecules already occupy most of its surface. Also, all the non-binding molecules have relatively high solubilities and low logP values. However, caffeine with higher solubility has higher binding constant

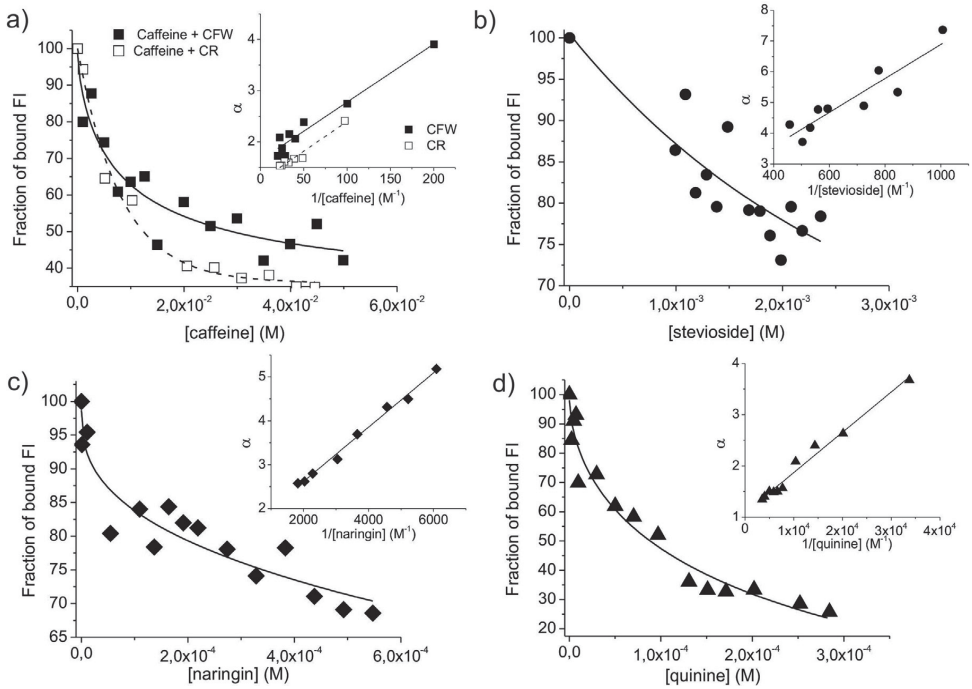

Fig. 3. Examples of the saturation curves for the binding of each taste compound to NFC i.e. the fraction of bound FI as a function of taste compound concentration with estimated trend-lines to help reading. For caffeine (a), both CFW and CR were used as FI. For stevioside (b) and naringin (c) CFW was used as FI and for quinine (d) CR was chosen as FI. Reciprocal plots (Eq. (2), $\alpha = \frac{I_0 - I_{free}}{I_0 - I_n}$) are presented as insets.

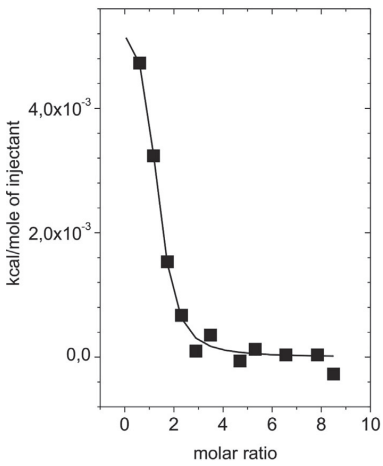

Fig. 4. Enthalpy curve of titration of quinine with NFC.

compared to aspartame and glutamic acid. This might be explained by the negative charge of aspartame as well as glutamic acid in aqueous solutions (near neutral or slightly acidic conditions) lowering the probability of binding to nanofibrillar cellulose, which contains a proportion of negatively charged hemicellulose on its surface (Kolakovic et al., 2013). Indeed, slightly negative zeta-potential values for NFC at pH 5 have been previously reported (Fall, Lindström, Sundman, Ödberg, & Wågberg, 2011). Furthermore, Kolakovic et al. (2013) have stressed the stronger interaction of NFC with positively charged drugs in comparison to neutral or anionic drug molecules, as the electrostatic interactions have a significant impact on complex formation. On the other hand, quinine with the highest binding constant has a positive charge at this pH favoring the binding. Also, the amine groups might increase the binding probability in the case of quinine and caffeine. Furthermore, it is probable that other effects, such as hydrogen bonding ability of the compounds, planarity and steric hindrances affect the binding.

Based on our results, the bitter tasting molecules are top ranked in terms of their NFC binding constants. This finding indicates that NFC might be used as a bitterness suppressing material in the future. It should be noted that it has been already shown that CMC is able to mask the astringent taste of phenolic compounds (Troszyńska et al., 2010). NFC can be expected to have similar or even more pronounced effect on these compounds due to its small particle size and large surface area. Thus, this study reveals a new promising characteristic of NFC in food applications as a taste modifier besides the known uses of nanofibrillar cellulose as an emulsion stabilizer and a functional food ingredient. Despite the foreseen applications of nanocellulose and the commercial use of bacterial cellulose as a food ingredient in Philippines (nata de coco), nanofibrillar cellulose has not yet been accepted as a food additive in EU or USA. This study indicates a further possibility for the utilization of this abundant biopolymer in future applications. However as stated in the literature (Gómez et al., 2016), there is still a need for rigorous safety evaluations of nanocellulose materials before their full potential can be realized.

4. Conclusions

A high-throughput screening method utilizing a plate-reader was developed for the estimation of binding constants of taste molecules

with NFC. In this study, binding constants between 70 M^{-1} and 14 000 M^{-1} were measured with good accuracy. The method seems promising for looking at the binding of taste compounds but also as a generic interaction assay. The studied taste compounds were divided into four groups based on their interaction strengths. Non-binding molecules were sucrose, aspartame and glutamic acid. Caffeine and stevioside were weak binders whereas naringin was a moderate NFC ligand. The bitter tasting quinine was the strongest binder in the set of molecules studied. The magnitudes of the binding strengths seem to be at least partly correlated to the hydrophobicity of compounds. As the bitter tasting compounds are among the best NFC binders in the set, the finding can be useful for the development of bitter suppressing or masking applications both in the food and pharmaceutical industries. This should be further studied with sensory analysis to evaluate the real effects of these interactions on perceived taste.

CRediT authorship contribution statement

Hanna Manninen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition. **Nikita Durandin:** Conceptualization, Methodology, Formal analysis, Validation, Writing - review & editing, Supervision. **Anu Hopia:** Conceptualization, Writing - review & editing, Supervision. **Elina Vuorimaa-Laukkanen:** Formal analysis, Writing - review & editing. **Timo Laaksonen:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision, Project administration.

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.

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PUBLICATION 4

Comparing the taste modifying properties of nanocellulose and carboxymethyl cellulose

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