The contribution of phenolics to the bitter taste of honeybush (*Cyclopia genistoides*) herbal tea

Lara Alexander

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Supervisor: Prof Elizabeth Joubert

Co-supervisors: Ms Magdalena Muller and Prof Dalene de Beer

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes one original paper accepted for publication in a peer-reviewed journal and two unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Lara Alexander

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Summary

The occurrence of bitter taste in some production batches of *Cyclopia genistoides* herbal tea not only challenges efforts of the honeybush industry to achieve consistent product quality, but also adversely affects consumer purchase intent. Previous studies have attempted to understand this phenomenon by determining associations between the bitter intensity of honeybush infusions and their individual phenolic concentrations. Despite some significant correlations between specific compounds and bitter intensity, the data did not give conclusive evidence of the cause of bitterness. The current investigation thus aimed to provide decisive proof of the role of phenolic compounds in the bitterness of *C. genistoides* herbal tea. To achieve this, the first phase of the study utilised a hot water extract of unfermented *C. genistoides* plant material (yielding an infusion with a bitter intensity of ~45 on a 100-point scale), separated by column chromatography into three fractions rich in benzophenones, xanthones and flavanones, respectively. The bitter taste of the fractions was determined by descriptive sensory analysis (DSA) and discrimination tests, and their individual phenolic content was quantified by high-performance liquid chromatography. The benzophenone-rich fraction was not bitter (< 5), the flavanone-rich fraction was somewhat bitter (~13) and the xanthone-rich fraction was considered distinctly bitter (~31). Further investigation of the bitter xanthone-rich fraction included a focussed DSA comparison of the major xanthones and regio-isomers, mangiferin and isomangiferin. This comparison revealed that isomangiferin was only somewhat bitter (~15) and modulated the distinct bitter taste of mangiferin (~30) by suppressing it (~22). The second phase of the study focussed on possible bitter taste modulation by the benzophenone- and flavanone-rich fractions, as well as their major individual phenolic compounds using DSA. The results indicated that modulation is dose-dependent, and identified 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG) and naringenin-*O*-hexose-*O*-deoxyhexoside B (NHDB) as novel bitter modulators for their respective bitter suppressing and enhancing activities. In addition, a mixture of NHDB and its isomer, NHDA, formed upon heating of NHDB (to simulate the effect of fermentation), did not have any modulatory effect on bitter intensity and should be investigated further. For the third and final phase of the study, a large data set was utilised to produce a robust statistical model for the prediction of bitter intensity of infusions from their individual phenolic concentrations. Fermented and unfermented samples of several genotypes of *C. genistoides* and *C. longifolia* in the Agricultural Research Council's honeybush plant breeding

programme were analysed. Both species contain high xanthone and benzophenone levels and have been found to produce bitter infusions. The data also allowed the investigation of the effects of fermentation on bitter intensity and individual phenolic concentrations of the infusions. The final independent validated stepwise linear regression model was able to predict bitter taste of the infusion ($R^2 = 0.859$) using the concentration of only five phenolic compounds (IDG, hesperidin, 3-β-D-glucopyranosylmaclurin, mangiferin and isomangiferin) and soluble solids content, common to both *C. genistoides* and *C. longifolia*.

Uittreksel

Die bitter smaak van sommige produksielotte van *Cyclopia genistoides* kruitetee beperk nie alleen die heuningbosbedryf se doelwit om konstante gehalte te verseker nie, maar het ook ‘n negatiewe impak op verbruikersaankope. Vorige studies het probeer om hierdie verskynsel te verstaan deur assosiasies tussen bitter smaak intensiteit en die konsentrasie van ‘n aantal fenoliese verbindings te bepaal. Ten spyte van 'n aantal betekenisvolle korrelasies tussen spesifieke verbindings en bitter intensiteit, was afdoende bewys van die oorsaak van bitterheid nie moontlik nie. Die huidige ondersoek was dus daarop gemik om beslissende bewys te lewer van die bydrae van fenoliese verbindings tot die bitterheid van *C. genistoides*. Om hierdie doel te bereik het die eerste fase van die studie behels dat 'n warm water ekstrak van groen *C. genistoides* plantmateriaal (infusie bitter intensiteit van ~45 op ‘n 100-punt skaal) d.m.v. kolom-chromatografie in drie fraksies geskei is, onderskeidelik ryk aan bensofenone, xantone en flavanone. Die bitter smaak van die onderskeie fraksies is bepaal deur beskrywende sensoriese analise (BSA), asook diskriminasietoetse. Die individuele fenoliese verbindings in elke fraksie is d.m.v. hoë-druk vloeistofchromatografie gekwantifiseer. Die bensofenoon-ryke fraksie was nie bitter nie (< 5), die flavanoon-ryke fraksie was effens bitter (~13) en die xantoon-ryke fraksie was duidelik bitter (~31). Verdere ondersoek van die bitter xantoon-ryke fraksie het vergelyking van die hoof heuningbos xantoon verbindings en regio-isomere, mangiferien en isomangiferien, deur middel van BSA ingesluit. Hierdie vergelyking het getoon dat isomangiferien (~15) effens bitter is en die bitter smaak van mangiferien (~30) onderdruk het (~22). Die tweede fase van die projek het gefokus op die moontlike vermoë van die bensofenoon- en flavanoon-ryke fraksies, sowel as hul belangrikste individuele fenoliese verbindings, om die intensiteit van bitter smaak te moduleer. BSA is ook hiervoor aangewend. Die resultate het aangetoon dat die modulerende effek dosis-afhanklik is. Dit is bevestig dat 3-β-D-glukopiranosiel-4-β-D-glukopiranosieloksiriflofenoon (IDG) and naringenien-*O*-heksose-*O*-deoksiheksosied B (NHDB) bitter smaak moduleer weens hul vermoë om onderskeidelik die intensiteit van bitter smaak te onderdruk en te versterk. Daarbenewens is dit ook bevestig dat ‘n mengsel van NHDB en sy isomeer, NHDA (gevorm gedurende gesimuleerde fermentasie van NHDB), geen modulatoriese effek op bitter intensiteit het nie. Hierdie resultaat regverdig verdere ondersoek. Vir die derde en finale fase van die studie is ‘n groot datastel gebruik om ‘n robuuste statistiese voorspellingsmodel vir die intensiteit van bitter smaak op grond van fenoliese

samestelling te ontwikkel. Monsters van fermenteerde en ongefermenteerde plantmateriaal van verskeie genotipes van *C. genistoides* en *C. longifolia,* tans deel van die Landbounavorsingsraad se heuningbos plantverbeteringsprogram, is ontleed. Beide spesies bevat hoë xantoon- en bensofenoonvlakke en kan bitter infusies lewer. Die data het ook ondersoek na die effek van fermentasie op bitter smaak en fenoliese saamestelling moontlik gemaak. Die finale onafhanklike gevalideerde stapsgewyse lineêre regressiemodel kon die infusie se bitter smaak voorspel ($R^2 = 0.859$) deur slegs van vyf fenoliese verbindings (IDG, hesperidien, 3-β-D-glukopiranosielmaklurien, mangiferien and isomangiferien) en inhoud van oplosbare vastestowwe, wat in beide *C. genistoides* en *C. longifolia* voorkom, gebruik te maak.

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"*Live as if you were to die tomorrow. Learn as if you were to live forever*." – Mahatma Ghandi

Notes

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion, recommendations and conclusions. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

Please take note of the following:

- The language, style and referencing format of research chapters that have been published have been changed according to the requirements of the *International Journal of Food Science and Technology*.
- Minor formatting changes have been made throughout the thesis to ensure consistency.
- With regard to the nomenclature: in cases where the structure of a compound was not elucidated in full, the *O* refers to a hexosyloxy moiety, and the *C* to a hexosyl moiety.

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Chapter 1
General introduction

Cyclopia genistoides, traditionally known as bush tea or Cape tea, is currently one of the *Cyclopia* species commercially cultivated for production of honeybush tea. It has several attributes attractive to farmers and processors, such as a high conversion ratio from fresh to processed leaf product and production of a fine leaf product. Furthermore, this species is adapted to grow in the sandy, coastal areas of the fynbos biome, expanding the production area of honeybush from mountainous areas to the coast (Joubert *et al*., 2011). Consumers generally perceive conventional, "fermented" (processed by high-temperature oxidation) honeybush to have a sweet, honey-like taste (Vermeulen, 2015). Some honeybush tea brokers have indicated that the inherent bitter taste of fermented *C. genistoides* limits its acceptance by consumers and thus has a negative impact on sales. The bitter intensity of its infusions can vary from barely perceptible for some production batches to distinct for others (Moelich, 2018). A factor affecting bitter intensity of the infusions is the extent of fermentation of the plant material, with higher temperatures and longer times resulting in less bitter infusions (Erasmus *et al*., 2017). Inherent variation in composition due to genotype, production area and harvesting time (Joubert *et al*., 2014) may play a role in the sensory quality of the final product, yet no data are available.

A strategy to eliminate bitter tasting production batches would be to use only selected genotypes for propagation. The current honeybush plant breeding programme of the Agricultural Research Council (ARC), launched to respond to the demand for planting stock with genetically improved material, considers sensory quality of the infusions and phenolic content of the plant material as advanced selection criteria (Bester *et al*., 2016; Robertson *et al*., 2018). Included in the honeybush plant breeding programme are *C. genistoides* and *C. longifolia*, another species prone to bitter taste when under-fermented (Erasmus *et al*., 2017).

The perception of bitterness in food products could be due to the presence of bitter taste-active phenolic compounds. For example, catechins in *Camellia sinensis* tea are known to impart the typical bitter taste of the infusion, as well as providing antioxidant and health-related benefits to consumers (Tounekti *et al*., 2013; Kallithraka *et al.*, 1997; Takeo, 1992). Thus, while high levels of some compounds may be beneficial in terms of health-promoting properties, an enhanced bitter taste would be detrimental to consumer acceptance. Indeed, several honeybush compounds have been found to impart health-related benefits. For example,

mangiferin, the major common honeybush polyphenol, has been found to possess antidiabetic properties, amongst others (as reviewed by Vyas *et al*., 2012). Several honeybush benzophenones have α-glucosidase inhibitory (Beelders *et al*., 2014a; Feng *et al*., 2011), pro-apoptotic (Kokotkiewicz *et al*., 2013) and antioxidant (Malherbe *et al*., 2014) activities. Both *C. genistoides* and *C. longifolia* are exceptionally rich in both xanthones and benzophenones, making their herbal teas good dietary sources of these health-promoting polyphenols (Schulze *et al*., 2015). A compromise must thus be reached between the desirable bioactive contribution and the undesirable bitter taste of the phenolic compounds.

Researchers have attempted to find a degree of structural commonality between known bitter compounds, yet no definitive structural parameters have been established to identify compounds as bitter taste-active (Huang *et al*., 2015; Wiener *et al*., 2012; Ley, 2008; Rodgers *et al*., 2006). Specifically, no information is available on the taste activity of the major phenolic compounds in honeybush infusions. This lack of knowledge prevents honeybush producers and plant breeders from establishing acceptable levels for individual phenolic content in honeybush plant material for the production of high quality honeybush tea with no or barely perceptible bitter taste. In practice, blending of the processed plant material of different species has been applied to curb bitterness of honeybush (Moelich, 2018), although the root of the problem is still not understood.

Previous studies have established associations between bitter intensity and individual phenolic content of honeybush infusions, although these associations are not evidence of a cause-and-effect scenario (Moelich, 2018; Erasmus, 2015; Theron, 2012). Nevertheless, several common observations were documented in the associations between phenolic compounds, specifically the xanthones (mangiferin and isomangiferin) and several benzophenones, and bitter taste (Moelich, 2018; Erasmus, 2015; Theron, 2012). The associations observed in these studies, however, are not sufficient to prove this relationship, thus no definitive understanding has been established for explaining the cause of bitterness in honeybush. Theron (2012) used principal component analysis (PCA) with Pearson's correlation coefficients of descriptive sensory analysis (DSA) and high-performance liquid chromatography data to observe a significant association between bitter intensity and mangiferin ($r = 0.740$), as well as isomangiferin ($r = 0.623$) in honeybush infusions. Erasmus (2015) and Moelich (2018) attempted to develop statistical models to predict bitter intensity based on phenolic composition of the infusions, using several statistical methods, including PCA, stepwise linear regression and partial least squares regression. Even though phenolic and bitter intensity variation was introduced by

investigating several species (*C. genistoides*, *C. maculata*, *C. subternata* and *C. longifolia*), only fermented plant material was used to prepare infusions. Moelich (2018) used an "extended" sensory scale for bitter intensity in an effort to improve prediction, however, despite the expansion of the bitter intensity scale, variation within the sample set was not effectively increased and prediction was subsequently not explicit. As fermentation causes several phenolic changes during the production of fermented honeybush tea (Beelders *et al*., 2017; 2015; Erasmus, 2015), greater variation in phenolic content of the sample set may be achieved, if both fermented and unfermented samples are included. Beelders *et al*. (2017; 2015) observed that individual honeybush phenolic compounds demonstrated different rates and routes of degradation during simulated fermentation. Interesting examples are the major benzophenones, 3-β-D-glucopyranosyliriflophenone (IMG) and its di-glucoside, 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG). IMG undergoes severe degradation during fermentation, while IDG showed negligible degradation (Beelders *et al*., 2017). Mangiferin was also more susceptible to degradation than isomangiferin (Beelders *et al*., 2017).

The suggestion that bitter taste modulation impacts bitter taste in honeybush infusions was made by Erasmus (2015) and Moelich (2018). Indeed, the known bitter masking compound, eriodictyol (Ley *et al*., 2005), has been identified in *C. intermedia* extracts and several eriodictyol derivatives are present in various honeybush infusions (Schulze *et al*., 2015; Beelders *et al*., 2014b). In addition, the sweet taste-modulating flavanone aglycone, hesperetin (Reichelt *et al*., 2010a,b; Ley *et al*., 2005), has also been identified at low concentrations in fermented honeybush extracts, along with its glycoside, hesperidin, a flavanone common to *Cyclopia* species (Schulze *et al*., 2015).

This study represents the first targeted investigation to elucidate the role of specific compounds in the bitter taste of honeybush infusions. The knowledge gained will be applicable to the processing of several plant-based products, including honeybush tea for food, beverage, or nutraceutical applications, as well as the selection of genotypes for propagation as part of the second tier evaluation criteria of the ARC honeybush plant breeding programme (Bester *et al*., 2016). The aim of the present study was thus to investigate the possible contributions of phenolic compounds to the bitter taste of honeybush infusions prepared from *C. genistoides*. Firstly, the contributions of three fractions enriched in benzophenones, xanthones and flavanones, respectively, prepared from a bitter hot water extract of unfermented *C. genistoides*, to the bitter taste of the infusion was determined using DSA and sensory discrimination tests. Secondly, possible modulation of bitter intensity by the major honeybush compounds was determined by combining individual

compounds at various concentrations with the phenolic-rich fractions and assessing these combinations using DSA. Thirdly, the effect of fermentation on individual phenolic content and bitter intensity of the infusions of a large sample set comprised of both unfermented and fermented samples of *C. genistoides* and *C. longifolia* was determined and used to develop a statistical model to predict bitter intensity of the herbal tea infusions.

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Chapter 2
Literature review

1. Introduction

The perception of bitterness in food products is due to bitter taste-active compounds, including various phenolic compounds. Bitterness in honeybush has previously been linked to its phenolic profile, especially its xanthone content (Erasmus, 2015; Theron, 2012). Bitterness is not acceptable in honeybush tea, known and marketed for its characteristic and pleasant sweet taste (Theron *et al*., 2014). Yet, there is great variation in the bitterness of honeybush infusions and compounds responsible for this taste deviation have not yet been identified.

This chapter explores the current understanding of the perception of bitterness, including bitterness of honeybush, as well as the physiological functions and mechanisms of bitterness. Strategic approaches for understanding bitterness in food products are discussed. Finally, a short overview of the methods and approaches available for the analysis and understanding of bitterness in honeybush is provided.

2. *Cyclopia* and the honeybush industry

The tradition and medicinal benefits of drinking honeybush tea, combined with the growing health-related interest in herbal teas, has served as a driver to commercialise *Cyclopia* spp. These fynbos plants are endemic to the Western and Eastern Cape regions of South Africa (Joubert *et al*., 2011). To date 23 *Cyclopia* spp. have been identified, with several traditionally used as honeybush herbal tea. The different species occur localised in nature, indicating that they are adapted to thrive under specific environmental conditions. Colloquial names for the commercialised species, such as "*bergtee*" (mountain tea; *C. intermedia*), "*vleitee*" (marshland tea; *C. subternata*) and "*kustee*" (coastal tea; *C. genistoides*) refer to natural habitat (Joubert *et al*., 2008a). Two additional species, *C. maculata* and *C. longifolia*, are under evaluation for commercialisation (Joubert *et al*., 2011). Leaf shapes range from small, thin and pubescent, to larger, broad, flat leaves (Joubert *et al*., 2011). Recent studies focussing on the species of commercial importance have also demonstrated differences in phenolic composition (Schulze *et al*., 2015; Erasmus, 2015) as well as sensory profiles (Erasmus *et al.*, 2017; Bergh *et al*., 2017; Moelich *et al*., 2017).

At present, traditional fermented honeybush represents the major product of the industry. Use of the term "fermentation" is a misnomer, as the fermentation process represents a high-temperature chemical oxidation process. This allows the development of the dark brown colour and fragrant aroma and flavour characteristic of honeybush. Although the term "fermentation" is misleading, it is still the commonly used term in the global tea industry relating to the oxidation of various kinds of tea. This process will thus be here forth referred to as "fermentation".

Besides fermented honeybush tea, the honeybush industry has, in recent years, grown to incorporate several additional products. These include unfermented honeybush tea commonly flavoured or blended with herbs or plant extracts, iced honeybush tea formulations and instant honeybush tea powders. Furthermore, the production of extracts from honeybush material has gained application in food, cosmetics and, potentially, nutraceutical products. The economic potential of this product is thus clear and has been highlighted by the success of the related rooibos (*Aspalathus linearis*) tea industry.

Nevertheless, several factors hinder the rapid development and growth of the honeybush industry. Apart from challenges related to cultivation and biomass production, the inherent variation between species has resulted in major deviations in product quality. Processing has evolved from traditional practices to modern production techniques to aid in product consistency. Optimal fermentation processing conditions have been shown to vary amongst the four commonly used species, resulting in diverse sensory profiles (Bergh *et al*., 2017; Erasmus *et al*., 2017; Theron *et al*., 2014). An additional challenge contributing to variation in product quality is the limited cultivation of honeybush and subsequent dependence on wild harvested plant material to increase supply (Joubert *et al*., 2011). It is thus that much more important that product losses due to unacceptable quality be minimised. Genotype selection of several *Cyclopia* species is underway at the Agricultural Research Council of South Africa (ARC) to optimise biomass yield. Sensory quality and phenolic composition comprise secondary tier selection criteria for propagation (Bester *et al*., 2016). Without these focussed measures for honeybush production, the unfeasibility of wild harvesting may cripple the still developing industry. Finally, the bitter taste of *C. genistoides* (Erasmus *et al*., 2017; Theron *et al*., 2014) has led to a preference for other species by some marketers despite its established cultivation and availability. The bitter intensity of *C. genistoides* infusions is not consistent, however, it has been shown to depend to some extent on processing conditions affecting phenolic composition (Erasmus, 2015; Theron *et al*., 2014). There is

thus an urgent need to identify compounds responsible for the bitter taste of honeybush infusions in order to form a strategy to manage bitterness before end production.

2.1 *Cyclopia genistoides*

Cyclopia genistoides is currently one of the most commercially important honeybush species, together with *C. intermedia, C. subternata, C. longifolia* and *C. maculata*. The following section will be focussed specifically on *C. genistoides*, as the occurrence of bitterness in production batches of herbal tea from this species poses a problem to the honeybush industry. Natural occurrence of *C. genistoides* includes mainly coastal areas, distributed over a wide area, spanning from the West coast to the Southern Cape (**Fig. 2.1**). It grows well in sandy soils and produces the first harvest 24 to 36 months after planting, followed by annual harvesting (Joubert *et al*., 2011).

The species has thin needle-like leaves (**Fig. 2.2**), and commonly contains significantly higher amounts of the honeybush xanthones, mangiferin and isomangiferin, than other species (Schulze *et al*., 2015). In a recent study, comprehensive phenolic analysis resulted in the identification of ten, and tentative identification of 30 compounds (Beelders *et al*., 2014b). The presence of two compound subclasses, aromatic amino acids and glycosylated phenolic acids, was demonstrated for the first time in the *Cyclopia* genus. The major compounds present in hot water extracts of unfermented *C. genistoides* consist of several benzophenones, xanthones, flavanones and dihydrochalcones (**Table 2.1**). Several unidentified compounds present in minor or substantial quantities were detected, although structure elucidation has not yet been undertaken.

The sensory profile of fermented *C. genistoides* infusions presents pleasant and prominent “rose geranium” and “apricot jam” aromas (Erasmus *et al*., 2017). However, bitter taste taints are often present in batches of optimally fermented (90 °C/16 h) *C. genistoides* when prepared as an infusion, with an average intensity of about 9 on a 100-point scale (Erasmus *et al*., 2017). This is considerably more than the negligible (< 2) bitter taste intensities determined for other species (Erasmus *et al*., 2017). Under-fermented *C. genistoides* plant material produces even higher and unacceptable bitter taste intensities (> 20; Erasmus *et al*., 2017), suggesting the relationship between phenolic degradation and bitter taste reduction during fermentation.

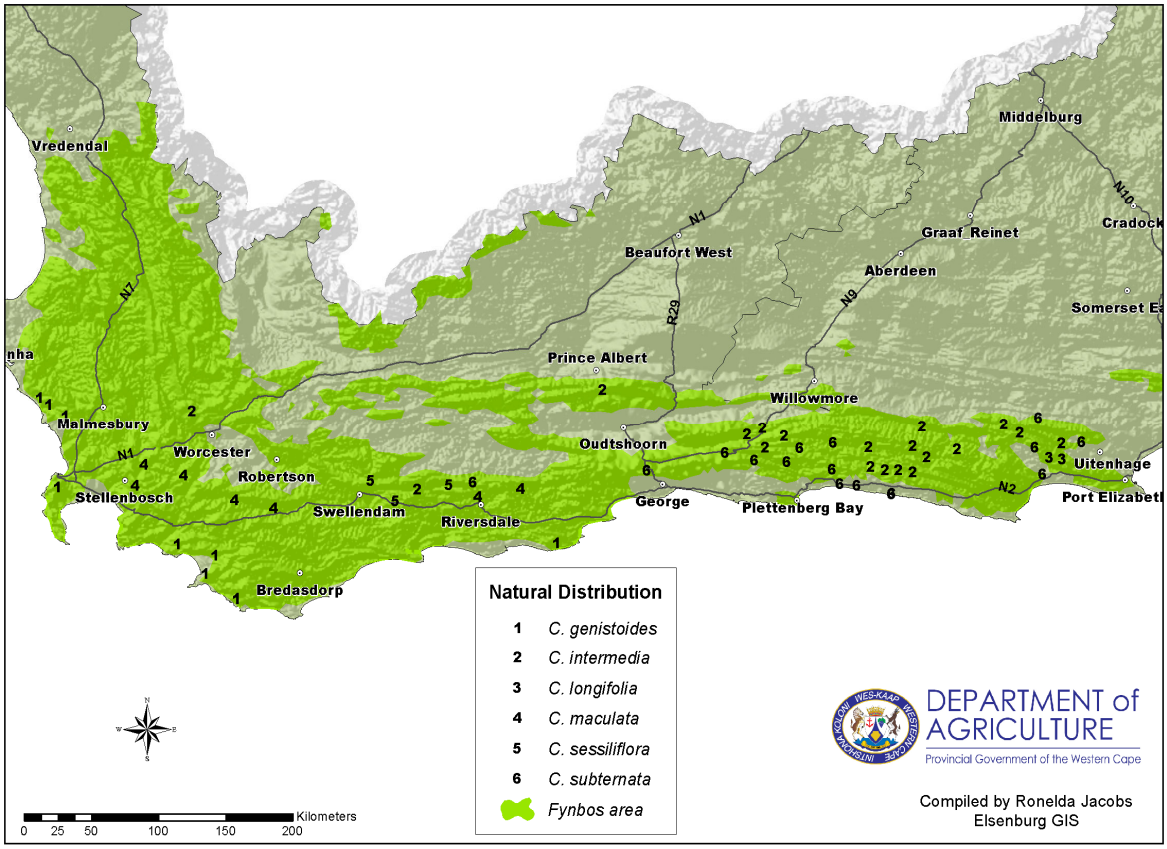

Figure 2.1 Natural distribution of several *Cyclopia* species (Joubert *et al*., 2011).

Figure 2.2 Leaf shape of *Cyclopia genistoides*.

Table 2.1 Major phenolic compounds present in a hot water extract of unfermented *Cyclopia genistoides*

Subclass	Compound[a]
Benzophenone	Maclurin-di-*O,C*-hexoside
Benzophenone	3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone [b]
Benzophenone	3-β-D-Glucopyranosylmaclurin
Benzophenone	3-β-D-Glucopyranosyliriflophenone
Xanthone	Tetrahydroxyxanthone-di-*O,C*-hexoside
Flavanone	Eriodictyol-*O*-hexose-*O*-deoxyhexoside
Xanthone	Mangiferin
Xanthone	Isomangiferin
Xanthone	Vicenin-2
Flavanone	Naringenin-*O*-hexose-*O*-deoxyhexoside A
Flavanone	Naringenin-*O*-hexose-*O*-deoxyhexoside B
Flavanone	Eriocitrin
Dihydrochalcone	3-Hydroxyphloretin-3′,5′-di-*C*-hexoside
Xanthone	Tetrahydroxyxanthone-*C*-hexoside isomer
Dihydrochalcone	3',5'-di-β-D-glucopyranosylphloretin
Flavanone	Hesperidin

[a]Compounds listed in order of elution using the species specific validated reversed phase high-performance liquid chromatography method (Beelders *et al*., 2014b). [b]Structure elucidation according to Beelders *et al*. (2014a).

2.2 Compounds in honeybush associated with bitterness

Several phenolic compounds in honeybush have been suspected to contribute to bitter taste. These include various xanthones, flavanones and benzophenones. Mangiferin is the major xanthone common to *Cyclopia* species and was the first to be implicated in the contribution to bitter taste (Theron, 2012). Infusions of fermented plant material from six *Cyclopia* species (*C. sessiliflora, C. longifolia, C. genistoides, C. intermedia, C. subternata,* and *C. maculata*) were analysed sensorially and by high-performance liquid chromatography (HPLC) for individual phenolic compound quantification. The pooled data indicated a strong positive correlation ($r = 0.74$) between mangiferin and bitter taste.

Subsequent investigation, however, suggested that mangiferin is not solely responsible for bitter taste in fermented and unfermented honeybush (Alexander, 2015; Erasmus, 2015). Fermented plant material from four *Cyclopia* species, *C. genistoides, C. longifolia, C. subternata* and *C. maculata* were investigated and analysed using Pearson's correlation analysis, partial least squares (PLS) regression, and stepwise linear regression to attempt the development of a prediction model for bitter intensity, based on phenolic contribution (Erasmus, 2015). This study indicated contributions from several additional compounds. Significant ($p < 0.05$) strong correlations ($r > 0.7$) were observed between bitter intensity and the benzophenones, maclurin-di-*O,C*-hexoside (MDH) and 3-β-D-glucopyranosylmaclurin (MMG), as well as mangiferin (Erasmus, 2015). Significant ($p < 0.05$) moderate positive correlations ($0.4 < r < 0.7$) were also observed for 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG), 3-β-D-glucopyranosyliriflophenone (IMG), naringenin-*O*-hexose-*O*-deoxyhexoside A (NHDA), naringenin-*O*-hexose-*O*-deoxyhexoside B (NHDB),

isomangiferin, tetrahydroxyxanthone-*C*-hexoside isomer A (THXA), and tetrahydroxyxanthone-*C*-hexoside isomer B (THXB). Stepwise linear regression, however, used mangiferin (partial $R^2 = 0.5698$), MDH and THXB to predict bitter taste.

The study also considered the species independently and observed a deviation with regard to the predictive ability of the respective phenolic compounds. For example, correlation data from *C. genistoides* indicated significant ($p < 0.05$) positive correlations ($r < 0.4$) between bitter taste and MDH and vicenin-2, but not mangiferin ($r = 0.132$). In contrast, the data from *C. longifolia* indicated significant positive correlations between bitter taste and soluble solids (SS) content, total phenolic (TP) content, and all quantified individual phenolics, except scolymoside, with mangiferin having the strongest correlation ($r = 0.800$). PLS regression and stepwise linear regression analysis were also conducted. The negative contribution of hesperidin and NHDA and the positive contribution of SS and mangiferin explained 74.22% of the variance in bitter intensity in the PLS model. The PLS prediction model developed for *C. longifolia*, however, explained 73.05% of the variation in bitter intensity when mangiferin, eriocitrin (negative contributions) and IMG (positive contribution) were included in the model. This indicates that the expression of bitter taste and its intensity most probably depends on qualitative and quantitative differences in composition between species.

Contributions from non-phenolic bitter compounds such as specific amino acids may also be critical to the bitter taste of honeybush, as the role of amino acids in the taste of traditional *Camellia sinensis* teas has been demonstrated (Yu *et al*., 2014; Ekborg-Ott *et al*., 1997). Indeed, two aromatic amino acids, tyrosine and phenylalanine, have recently been tentatively identified in *C. genistoides* (Beelders *et al.*, 2014b). Both of these amino acids are known to have a bitter taste in their L-enantiomer state (Solms, 1969).

The results from the study by Erasmus (2015) also suggested the possibility of taste modulation by compounds. For example, data from *C. genistoides* indicated moderate ($-0.7 < r < -0.4$) significant ($p < 0.05$) negative correlations between bitter taste and 3-hydroxyphloretin-3′-5′-di-*C*-hexoside, and the flavanones, NHDA, NHDB and hesperidin. This indicates that samples with higher concentrations of these compounds had a lower bitter intensity. Although this information does not imply causation, it is possible that these compounds may have modulatory effects. Indeed, some potentially bitter masking or modulating compounds, especially from the flavanone or related phenolic subclasses, are known to occur in honeybush extracts. For example, eriodictyol from herba santa (*Eriodictyon californicum*) extracts has been shown to mask bitter taste of caffeine (Ley *et al*., 2005). Derivatives of this compound are present in some honeybush extracts (Beelders

et al., 2014b; Schulze *et al*., 2014) and it has been successfully produced by acid hydrolysis, removing the glycoside moiety from eriocitrin in honeybush (Du Preez, 2014). Furthermore, the sweet-enhancing effect of hesperetin, present in *C. intermedia* extracts, has been observed by Reichelt *et al*. (2010b,c).

2.3 Factors affecting phenolic content

Since phenolic compounds are secondary plant metabolites, the phenolic content of plants (and thus tea) are initially dependent on a variety of factors including biotic and abiotic stress (Verma & Shukla, 2015; Tounekti *et al*., 2013). These factors have not yet been studied with regard to honeybush, but climate and cultivation areas have been implicated in the great variation of phenolic content in *C. maculata* and *C. longifolia* (Alexander, 2015). Apart from species (Schulze *et al*., 2015; De Beer & Joubert, 2010) and genotype (unpublished results), other known factors include maturity of the shoots, harvest date and leaf-to-stem ratio (Joubert *et al*., 2014; 2003). For example, *C. subternata* and *C. maculata* leaves contain higher levels of xanthones and eriocitrin than stems, while the opposite was found for hesperidin (Du Preez, 2014; De Beer *et al*., 2012).

Conditions during post-harvest processing usually favour quantitative and qualitative changes in phenolic composition (Beelders *et al*., 2015; Beelders *et al*., 2014b; Schulze *et al*., 2014). Fermentation leads to a loss of individual phenolic constituents and reduces the TP content of extracts (Beelders *et al.*, 2015; Joubert *et al*., 2008b). Joubert *et al*. (2008b) observed that the fermentation of *C. genistoides* reduced the TP content of a hot water extract by 23%, the smallest reduction among four species investigated. Beelders *et al*. (2015) demonstrated that fermentation of *C. genistoides* results in a loss of approximately 48% mangiferin as well as significant losses of most other quantified phenolics. The loss in phenolic content also leads to a loss of bioactivity (Beelders *et al*., 2015; Joubert *et al*., 2010; 2008b) and possibly bitterness. Bitter intensity is prominent in under-fermented *C. genistoides* and *C. longifolia* (Erasmus *et al*., 2017) containing the highest levels of polyphenols. Beelders *et al*. (2015) studied the thermal degradation kinetics of some of the major honeybush compounds during simulated fermentation. Mangiferin, isomangiferin and IMG were observed to follow a first order degradation reaction (Beelders *et al*., 2017; 2015). Although significant degradation took place, isomangiferin resulted in limited losses compared to its more susceptible regio-isomer, mangiferin. IMG also underwent considerable degradation. IDG, however, was found to be much more stable during fermentation, with negligible degradation under mild fermentation conditions (80 °C/24 h), and only slight

degradation under severe fermentation conditions (90 °C/16 h). This indicates that not all compounds follow the same rate of degradation or effect of fermentation. Indeed, although most compounds did show a decrease in concentration after fermentation, some remained stable, or even seemed to increase after fermentation (Beelders *et al*., 2017).

3. The perception of bitterness

Bitterness is considered an aversive taste perception and thus has a negative connotation associated with food product quality (Drewnoswki, 2001; Drewnoswki & Gomez-Carneros, 2000). There are some exceptions, however. A slight bitter taste is considered characteristic and even positive in products such as tonic water, black and green tea, dark chocolate and coffee (Ley, 2008).

3.1 Physiology of bitter taste transduction

Current understanding of the definitive and complete mechanisms of bitter taste transduction is still overwhelmingly speculatory (as reviewed by Riedel *et al*., 2017). It has been established that bitter taste is perceived through the activation of G protein-coupled receptors (GPCRs) mediated by an α-, β-, and γ-gustducin protein heterotrimer (α-gustducin/Gβ1/Gγ13; Gilbertson & Boughter, 2003; Huang *et al*., 1999; Wong *et al*., 1996). Bitter-perceiving receptors are comprised of the T2R group of protein sub-units characterised by a short extracellular N terminus (Alder *et al*., 2000; Chandrashekar *et al*., 2000). Receptors are located on taste receptor cells arranged in groups to form taste buds on the tongue (Behrens & Meyerhof, 2006).

Activation of the receptors is thought to follow binding of the bitter stimuli (agonist) to the receptor binding pocket. The receptor is co-expressed with α-gustducin within taste receptor cells, activating phosphodiesterase (PDE) and reducing cyclic nucleotide levels (like cAMP; Ming *et al*., 1999; Wong *et al*., 1996). This may activate transmitter release to signal the bitter perception. Concommitently, β-gustducin is activated, increasing the b2 isoform of phospholipase C (PLC; Huang *et al*., 1999). This prompts the release of Ca^{2+} from intracellular stores by engaging an inositol triphosphate (IP_3)- and diacylglycerol (DAG)-dependent pathway. The increase in intracellular Ca^{2+} elicits a transmembrane bitter perception response.

Other proposed activation mechanisms suggest direct activation of GCPRs by certain amphipathic lipophilic bitter compounds permeating into the taste cells (Peri *et al*., 2000). This effect may be related to slow taste onset or lingering aftertastes by the inhibition of signal termination-related kinases, affecting the

general quenching mechanisms of G protein-coupled receptors (Zubare-Samuelov *et al*., 2005; Peri *et al*., 2000). This could mimic receptor activities, inducing cellular responses by receptor-independent pathways (Hagelueken *et al*., 1994; Mousli *et al*., 1990). Finally, it has been suggested that some bitter stimuli may interact directly with ion channels within the cell membrane or with secondary messenger components, activating bitter perception signals by affecting voltage-dependent currents (Chen & Herness, 1997).

The T2R group of protein sub-units is much more diverse than the sweet and umami perceiving receptor protein sub-units (T1R and T3R groups), with 25 identified sub-unit varieties. This is a relatively limited number considering the vast variety of structurally diverse bitter tasting compounds humans are able to detect (Brockhoff *et al.*, 2010). This phenomenon may be partially accounted for by the vast receptive ranges of some bitter receptors. The TAS2R group of receptors show individual, unique agonist spectra, with overlapping between many bitter compounds, and differing in dose threshold sensitivities (Kohl *et al*., 2012). Some receptors have yet to be de-orphaned and some "specialist" receptors (*e.g.* TAS2R3, TAS2R5, TAS2R8, TAS2R9, TAS2R13, TAS2R20 and TAS2R50) detect only a few known bitter compounds (Meyerhof *et al.*, 2010). The most broadly tuned receptors, namely, TAS2R10, TAS2R14 and TAS2R46, have been found to respond to approximately 50% of the 104 chemically distinct bitter chemicals tested by Meyerhof *et al.* (2010). Several receptors, including TAS2R1, TAS2R4, TAS2R7, TAS2R31, TAS2R39, TAS2R40, TAS2R43 and TAS2R47, have been found to respond to many additional bitter tasting compounds, although these appear to be less broadly tuned than the previous group (Kohl *et al*., 2012; Meyerhof *et al.*, 2010). Structural categorisation according to recognised classes of bitter chemicals has not been successful in the classification of most receptors. However, two receptors, TAS2R16 and TAS2R38, have been found to be sensitive to a wide range of β-glycopyranosides or thioamides, carbamides and isothiocyanates (Bufe *et al*., 2005; 2002). TAS2R16, in particular, has been suggested to possess a strict ligand binding site for the β-D-glucopyranoside sugar moiety (Sakurai *et al*., 2009).

The diversity of receptor ranges thus indicates that the common ability of TAS2Rs to respond to many structurally different compounds may be facilitated by different mechanisms. Brockhoff *et al.* (2010) suggests three possibilities: Firstly, TAS2Rs may possess multiple structural agonist-subgroup-specific binding pockets. Secondly, it is possible that TAS2Rs do have a single binding pocket able to provide access to multiple agonists by adapting after establishing contact with critical receptor residues (Brockhoff *et al*., 2010). Thirdly and finally, receptor oligomerisation suggests that combinations of TAS2Rs might act as agonist binding units

(Kuhn *et al*., 2010; Terrillon & Bouvier, 2004). Indeed, the expression of multiple T2R protein sub-units in single receptor cells has been reported (Alder *et al.*, 2000). This may allow for a broader range of detection from a greater variety of bitter tasting compounds (Kuhn *et al*., 2010).

3.2 Bitter compounds (structure-activity relationships)

Similar to the activation of olfactory receptors by aroma-active volatile compounds, non-volatile taste active compounds are responsible for activating taste receptors. As opposed to the other taste modalities, there exists a great many and variety of bitter tastants, with every chemical class potentially containing molecules capable of eliciting a bitter taste (Ley *et al*., 2008a). Although little data is available on the taste-activities of specific and individual honeybush phenolics, similar groups of compounds have been associated with bitter taste or a lack thereof.

Although many bitter compounds have been identified, with great variation in class and structure, a selection of compounds have gained interest by the food and pharmaceutical industries. Among these, some polyphenols have been identified as bitter and are relevant in the context of the present study. Six polyphenolic compounds commonly found in polyphenol-rich foods such as red wine, beer, tea and chocolate have been demonstrated to activate different combinations of human bitter taste receptors (Soares *et al*., 2013).

Physical properties of the bitter molecule such as molecular size and hydrophobicity have been shown to affect bitterness intensity. Considering peptides, for example: higher hydrophobicity of the terminal amino acids of the peptide chain results in a more intense bitter response (Asao *et al*., 1987). However, larger peptides with more than three or four amino acid residues are not bitter (Asao *et al*., 1987). Similar results have been observed for flavanol (Peleg *et al*., 1999) and polymeric tannic acids (Robichaud & Noble, 1990), eliciting a more intense bitter response than their higher polymers. This loss of bitterness experienced by larger molecules may be as a result of a greater degree of steric hindrance, inhibiting tastant-receptor interaction (Peleg *et al*., 1999).

Additionally, bitterness can be significantly altered by very small structural changes to the bitter molecule. Amino acids are a good example of this phenomenon. The L-enantiomer of tryptophan and phenylalanine elicit a bitter taste and even a cellular TAS2R response (Kohl *et al*., 2012). The D-enantiomers, however, are not bitter and have a distinct sweet taste (Solms, 1969). Another example is hesperidin, a tasteless flavanone glycoside, but its positional isomer, neohesperidin, is intensely bitter (Steglich *et al*., 1997, as

referenced by Ley, 2008; Konishi *et al*., 1983). In addition, the dihydrochalcone derivative of neohesperidin (neohesperidin dihydrochalcone) is extremely sweet (Konishi *et al*., 1983). It has been suggested that the bitter taste of flavanone glycosides is directly affected by the rhamnose point of attachment. For example, flavanone-7-*O*-neohesperidosides (*e.g.* naringin) possess a bitter taste, whereas flavanone-7-*O*-rutinosides (*e.g.* narirutin) are tasteless (Rousseff *et al*., 1987, as referenced by Frydman *et al*., 2004). Similarly, the weakly astringent flavonol, quercetin, exhibits a strong bitterness after isomerisation to its 3-hydroxyl derivative, taxifolin (Roland *et al*., 2013; Ley, 2008).

The wide structural variation among bitter tasting compounds presents a great challenge when trying to generalise molecular requirements of bitter tastants. Attempts have been made to develop a reliable bitterness classification and prediction model based on structure-activity correlations with known bitter compounds. Most of these attempts, however, have utilised relatively small sample sets or specific selections of compounds, limiting the application of their findings. The searchable BitterDB database includes over 550 compounds reported to taste bitter to humans (Wiener *et al*., 2012). Belitz and Wieser (1985) suggested that in order for a molecule to be bitter, it must possess both a polar group and a hydrophobic moiety (monopolar-hydrophobic concept). However, studies would suggest that this is not always the case, with greater importance attributed to the spatial distribution of the two structural features (Ley, 2008).

Rodgers *et al*. (2006) developed a general classification model using MOLPRINT 2D circular finger prints based on the molecular structure of 649 bitter and 13530 randomly selected molecules from the MDL Drug Data Repository. The model achieved a successful bitterness identification of 72% of bitter compounds (Rodgers *et al*., 2006). However, these efforts have not led to decisive parameters for bitter prediction and the molecular mechanisms of bitterness are still poorly understood.

Roland *et al.* (2013) used 2D-fingerprint and 3D-pharmacophore models to map TAS2R14 and TAS2R39 receptor structures based on the structural requirements for bitter taste transduction by 97 flavonoid and isoflavonoid tastants. Based on this modelling, the structural requirements for activation of these receptors included that tastants possess two hydrogen bond donor sites, one hydrogen bond acceptor site and two aromatic ring structures, of which one needs to be hydrophobic. The predictions led to an understanding of 88% and 94% of assessed TAS2R14 and TAS2R39 tastants, respectively. Furthermore, the 3D-pharmacophore model indicated that the TAS2R39 differs from that of TAS2R14 with a possible additional hydrogen acceptor

site in the binding pocket. This accounted for the deviation in bitter transduction between the two receptors with regard to hydroxyl-rich compounds.

Levit *et al*. (2014) undertook an analysis of known TAS2R14 agonists using 1D properties, 2D chemical connectivity and 3D models. By combining both pharmacophore- and shape-based screens, the method could be used to identify previously unknown TAS2R14 ligands with novel scaffolds. The analysis indicated that agonists tend to have a lower number of aromatic rings than ligands that do not activate the receptor. Similar to findings by Roland *et al*. (2013), some of the ligands were found to be hydrophobic, while others were negatively charged.

More recently, Huang *et al*. (2015) developed an open-access tool called BitterX, to predict TAS2R activation from small molecule structures. It is based on 260 positive and 2379 negative available agonist-TAS2R interactions from literature. The model was verified to yield prediction accuracy, specificity and precision, and sensitivity of < 75%. The model output includes a percentage confidence for an interaction between agonist and specific TAS2Rs which may be useful for preliminary screening of potential bitter agonist compounds.

3.3 Dose-response and detection thresholds

The relationship between bitter intensity and tastant concentration has shown itself to be far more complicated than expected. Although taste detection (minimum) and suprathreshold (maximum) intensities are popularly considered to be part of a linear function of concentration, this is not necessarily the case (Keast & Roper, 2007). The kinetics of taste transduction have been compared to the Michaelis-Menten model of enzyme kinetics, where the rate of taste transduction, or receptor-tastant activation varies with tastant concentration (Keast & Breslin, 2002a). As indicated in **Fig. 2.3**, once a tastant concentration reaches detection level, receptor activation takes place to elicit a taste-perception response to a degree where the tastant solution is discriminated against a blank solution. If the tastant concentration is increased, recognition threshold is reached, where the taste-perception response intensity is sufficient for recognising the tastant. As tastant concentration is then further increased through the dynamic phase, perceived taste intensity increases. Theoretically, once the maximum perceivable taste intensity, or the terminal threshold (suprathreshold), is reached, the taste receptor system is saturated and perceived taste intensity cannot increase, even with an increase in tastant concentration (Keast & Roper, 2007; Keast & Breslin, 2002a).

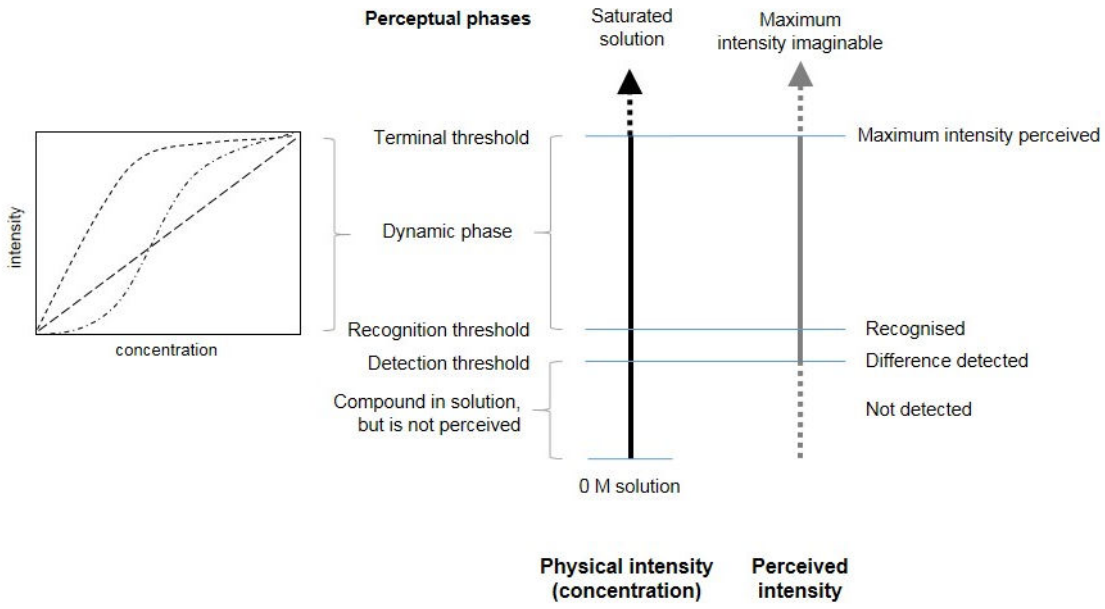

Figure 2.3 Schematic indicating the relationship between physical intensity (molecular concentration) and perceived intensity (Adapted from Keast & Roper, 2007).

Great variation exists in the human perception and individual bitter threshold concentrations of bitter stimuli. This has been attributed to several factors including environmental (as reviewed by Duffy, 2007) and genetic factors such as age, gender and hormonal status (as reviewed by Reed *et al*., 2006). Individual saliva characteristics such as salivary flow or composition may also influence perceived astringency and taste (Dinella *et al*., 2009; 2011). Variation in the human genome, however, is considered a major reason for variation in bitter perception between individuals. The ability to experience bitter taste seems to be dependent on the required receptor genes and number of receptor cells an individual possesses. So-called non-, medium- and super-tasters represent the categories of individuals based on the threshold at which bitter taste induced by specific bitter compounds, phenylthiocarbamine (PTC) and 6-*n*-propylthiouracil (PROP), can be perceived (Bartoshuk *et al*., 1994). Taster status has been found to influence an individual's liking or preference for bitter food or beverage products (Dinehart *et al*., 2006; Duffy & Bartoshuk, 2000; as reviewed by Feeny, 2011).

3.4 Additional factors affecting bitter taste perception

In addition to molecular characteristics and concentration of bitter compounds, several external factors can affect perceived bitter taste. Certain taste qualities including bitterness and astringency tend to have a carry-over effect (Lesschaeve & Noble, 2005; Noble, 2002). This problem with bitterness and astringency build-up

is encountered especially in the analysis of wine. For this reason, time delays are often implemented between analyses of individual samples to prevent overestimation of the intensity of these taste sensations. These intervals vary between 1 min (Peleg *et al*., 1999) and about 10 min (Lopez *et al*., 2007) and allow panellists to rinse their palate to remove any residual bitter or astringent substances in the mouth. Even when water and biscuits are used as palate cleansers, carry-over effects can still cause substantial problems with data collection. Vidal *et al.* (2016) evaluated several potential palate cleansers for the prevention of astringency during red wine evaluation. These included water, biscuits, skimmed milk, plain sweetened yogurt and a 2 $g.L^{-1}$ pectin solution. None were found to effectively prevent carry-over effects.

Temperature can also affect taste intensity (Kadohisa *et al*., 2004; McBurney *et al*., 1973; Moskowitz, 1973). Increasing red wine temperatures decreased the intensity of perceived bitterness and astringency (Ross *et al*., 2012). This could especially affect astringency in food products: lower temperatures may favour the aggregation and subsequent precipitation of salivary protein-binding tannins. In addition, higher serving temperatures may increase volatility of aroma compounds likely to influence taste perception. In addition, low temperatures of 0 to 5 °C have been observed to decrease the bitter taste of a nutritional product, Aminoleban®EN, when compared to its unpalatable bitter taste at room temperature (25 - 30 °C; Haraguchi *et al*., 2011).

Bitter taste perception can also be influenced by acidity. Acidic di-peptides have been observed to reversibly inhibit TAS2R16 activation by salicin (Sakurai *et al*., 2009). It was suggested that an acidic pH (~4) may be crucially responsible for bitter masking by affecting a reduction of binding affinity between the agonist bitterant and the receptor binding pocket.

3.5 Other functions and distribution of bitter receptors

The evolutionary function of bitter taste perception is thought to relate to the avoidance of poisonous or dangerous foodstuffs such as toxic plant metabolites which often taste bitter (Brockhoff *et al.,* 2010). In a similar way, sweetness is thought to signal energy rich food sources and salty foods indicate mineral sources. However, some compounds known to produce a bitter taste include many beneficial dietary compounds such as flavonoids, isoflavones, terpenes and glucosinolates (Drewnowski & Gomez-Carneros, 2000).

Recently, non taste-related functions of bitter receptor expressing genes have been suggested. This follows the identification of non-gustatory (extra-oral) TAS2R gene expressing tissues. These include, but are

not limited to, the respiratory epithelium of the nasal cavity chemosensory cells, where bitter activation affects respiratory rates (Finger *et al*., 2003), and human airways (Deshpande *et al*., 2010; Shah *et al*., 2009). Dotson *et al*. (2008) and Wu *et al*. (2002; 2005) reported the presence and influence of TAS2R-type taste receptors in the gastrointestinal tract affecting glucose and insulin homeostasis. Furthermore, the presence of TAS2Rs in human gastric smooth muscle cells have been identified as well as their *in vitro* effects on gastric mobility and satiation (Avau *et al*., 2015). Clark *et al*. (2015) also established that TAS2R receptors expressed in human and mouse thyrocytes negatively regulate thyroid stimulating hormones when activated by bitter substances. Interestingly, these receptors have also been identified in brain tissue (Chen *et al*., 2011; Singh *et al*., 2011). All this evidence indicates that the activation of gustatory and non-gustatory TAS2R receptors by bitter compounds may have an underestimated, yet invaluable evolutionary functionality beyond food preference or avoidance.

4. Bitter taste modulation

Interactions between taste qualities in food products, and taste-altering contributions by tasteless or aroma compounds have been observed. Bennett *et al*. (2012) summarised the modes of bitter suppression as (i) inhibition of tastant binding to TAS2Rs receptor extracellular polypeptide chains at the peripheral level (Maehashi *et al*., 2008), (ii) modulation by olfaction on the central cognitive level (Köster, 2005; Köster *et al*., 2004), or (iii) physically blocking access of the tastant to the receptors by encapsulating the bitter compound (Funasaki *et al*., 2006). The implication of this taste modulation or interaction is of great importance in the food industry. These principles may be applied to mask the bitter taste of products or enhance the sweetness of products without the addition of sugars (Ley, 2008). The exact mechanisms of each of these taste modulating phenomena are not definitively understood at present, although many modulation studies have been undertaken. The following discussion will focus mainly on the first mode of bitter suppression: peripheral inhibition of tastant-receptor interactions.

With the great variety of individual compounds producing similar taste qualities, various unique tastant-specific taste transduction pathways may be employed to elicit a single taste quality, *e.g.* bitter taste (Nelson *et al*., 2002; Alder *et al*., 2000; Chandrashekar *et al*., 2000). It may thus be possible for a number of interactions to occur between similar tasting compounds via peripheral intracellular mechanisms (Ley, 2008; Keast & Breslin, 2002b).

According to Bartoshuk and Cleveland (1977), mixtures of some bitter compounds have a suppressive effect, with the mixture producing a less bitter overall taste than the theoretical sum of individual intensities. However, in the investigation of binary bitter taste interactions, Keast *et al*. (2003) observed a linear additive effect between bitter compounds, with the exception of some suppressive activity at weak intensities. Urea has been identified as a bitter taste suppressor of some bitter tasting compounds (Keast *et al*., 2003). According to a Japanese patent, the bitter aftertaste of high potency sweeteners can reportedly be suppressed by some amino acids, including the slightly bitter L-methionine, L-leucine, or L-proline (Takahishi & Kawai, 2000). In contrast, the extremely bitter denatonium benzoate has been observed to synergistically enhance the bitterness of some bitter compounds (Keast *et al*., 2003).

Taste interactions between tastants of different taste qualities are also known and appear to be highly variable. For example, the suppression of bitterness by salty taste has been established, although salty taste is not affected by bitterness (Breslin & Beauchamp, 1997; 1995). Moderate or high concentration mixtures of bitter and sweet taste qualities are mutually suppressive, whereas sour taste and bitterness are enhanced in low concentration mixtures, but bitterness is suppressed in medium concentration mixtures (as reviewed by Keast & Breslin, 2002a).

Several low molecular weight compounds are known to affect taste qualities, even when present below taste threshold concentrations or exhibiting no specific or prominent taste of their own. The sodium salt, lactisol (500 ppm), for example, is able to completely mask the bitterness of a 95% KCl solution, although it also suppresses sweet taste (Kurtz & Fuller, 1997). According to a review by Ley (2008), similar bitter modulating effects are elicited by some orotic acids (Fuller & Kurtz, 1997b), aspartame derivatives (Fuller & Kurtz, 1997c), phenolic acids (Fuller & Kurtz, 1997d) and flavonoids, including flavone itself (Kurtz & Fuller, 1997a), without aversive taste consequences. In addition, the flavonol-3-glycoside, rutin, has been demonstrated to enhance the bitter taste of caffeine in *C. sinensis* black tea (Hofmann *et al*., 2006; Scharbert & Hofmann, 2005).

Sensory studies have identified the flavanones, homoeriodictyol, its sodium salt (sterubin), as well as eriodictyol, isolated from the North American indigenous herb, herba santa (*Eriodictyon californicum* (H. & A.) Torr.), as bitter inhibitors (Ley *et al*., 2005). The bitter taste of several different bitter compounds, including salicin, amarogentin, paracetamol and quinine, were decreased by between 10% and 40%, and that of caffeine

by ~60%. However, some bitter tastants, including potassium salts, linoleic acid emulsions and the peptide, L-leucyl-L-tryptophan, were not inhibited (Ley *et al*., 2005).

Subsequent studies were undertaken to develop or identify structurally related bitter taste inhibitors with a common vanillyl functional group. This led to the demonstration of strong bitter masking activity of several compounds, including hydroxybenzoic acid vanillylamides (Ley *et al*., 2006a), hydroxylated deoxybenzoins (Ley *et al*., 2006b), short chain gingerdiones (Ley *et al.*, 2008a) and the dihydrochalcone, phloretin (Ley *et al*, 2008b). The activity of these mostly tasteless compounds appear similar and likely follow similar mechanisms of bitter blocking.

One of the proposed mechanisms of taste inhibition is the steric blocking of receptor membranes by the relevant modulating compound (Maehashi *et al*., 2008; Katsuragi *et al*., 1997). Indeed, modulation has been observed to take place by inhibiting bitter receptor activity. TAS2R antagonists, γ-aminobutryic acid (GABA) and *N*α,*N*α-bis(carboxymethyl)-L-lysine (BCML), have been identified as competitive inhibitors of TAS2R4 by cellular studies (Pydi *et al*., 2014). Ley *et al*. (2005) suggested that bitter modulators such as sodium salts bind to secondary allosteric sites on bitter receptors without activation of the activator tastant binding pocket. This would disable the activator tastant binding pocket, possibly blocking taste transduction by preventing tastant binding. Brockhoff *et al*. (2007) observed the activation of various TAS2Rs by the sesquiterpene lactones, absinthin and artabin. However, these same bitter compounds inhibited the activity of TAS2R46 in the presence of TAS2R46 bitter activating compounds, strychnine and denatonium benzoate (Brockhoff *et al*., 2011). Single compounds can thus act both as agonists and antagonists of bitter taste. The possibility of molecular interaction between the tastant and modulating agent have also been considered. Binding of quinine to riboflavin-binding protein (bitter inhibitor) has been observed, although this was not found to be the main mechanism of inhibition (Maehashi *et al*., 2008).

Pharmacophore models have also been used to identify possible bitter modulating compounds, based on their receptor interactions. Such a model was developed from the available structure-activity relationships between the bitter modulator, homoeriodictyol, and its related bitter blocking compounds, and the TAS2R10 receptor as activated by caffeine (Ley *et al*., 2012). This model successfully identified two non-obvious related compounds, enterodiol and enterolactone, as novel bitter modulators. Additional verification using sensory tests, confirmed the moderate (~30%) bitter reduction capacity of enterodiol (25 $mg.L^{-1}$) added to a 500 $mg.L^{-1}$ caffeine solution. The enterolactone, however, showed a slight bitter enhancing capacity.

Similarly, there exists the possibility of taste modulation by olfaction, where taste/aroma associations may influence the perceived attributes of a product (Köster, 2005; Köster *et al*., 2004). This odour-induced modulation is reportedly a neural regulated perceptual process based on the individual's personal associations made during previous food and beverage experiences (Small & Prescott, 2005; Delwiche, 2004). The addition of sweet-associated aromas such as vanilla or fruity odours to a sweet tasting solution has been found to increase perceived sweet taste intensity (Labbe *et al*., 2008; Lavin & Lawless, 1998; Bonnans & Noble, 1993; Cliff & Noble, 1990). Similarly, the addition of bitter-associated flavours such as cocoa results in an enhanced bitter taste (Labbe *et al*., 2008). For this reason, many sensorial taste analyses are carried out with the use of nose clips to prevent interaction between perceived aroma and taste. Flavoured teas are a common product category in European markets, with vanilla-flavoured rooibos, for instance, gaining great market popularity. Locally, green ("unfermented") honeybush is often marketed as a flavoured tea with added mint or berry flavours. Many consumers may, however, prefer more "natural" products without added flavours. Indeed, enhanced floral and fruity aromas have been attained without the addition of foreign flavours with the appropriate time/temperature combinations for the crucial high-temperature oxidation process of fermented honeybush (Bergh *et al*., 2017; Erasmus *et al*., 2017; Theron *et al*., 2014). Furthermore, expanding market opportunities have allowed the development of ready-to-drink honeybush beverages such as iced teas. These products commonly contain additives such as sweeteners or flavours to increase product acceptability.

Although bitter modulation may be effective in many products and model solutions, complex mixtures with multiple sensory components do not necessarily yield comparable results. For example, Gaudette *et al*. (2015) observed the (+)-catechin bitter blocking capacity of cyclodextrin and sterubin only in the presence of sweeteners. Solutions lacking sweeteners were not affected by the addition of the bitter blocking compounds. Intensive investigation is thus required to determine the outcome of the addition of bitter blockers to complex solutions.

5. Strategies for controlling bitterness

The following section provides a brief discussion of several of these strategies relevant to the honeybush industry. Ley (2008) presented a summary of strategies by which bitterness may be managed: firstly, unpleasant tasting components could be removed. However, if bitter or unpalatable compounds are still desirable for their bioactivity, other avenues must be explored. Secondly, Ley (2008) suggested the addition

of physical barriers by encapsulation, coatings, emulsions or suspensions. These methods are only feasible for highly processed products, requiring additional cost and time. Thirdly, the addition of scavengers or complexing agents, stronger flavours and tastants, or masking flavours are suggested. The addition of additives, however, poses a further challenge to products. Consumers desire "cleaner" labels for food products, especially when health related claims are made. Addressing bitterness on a molecular level is finally presented as a viable method to control bitterness. This method requires an in-depth understanding of compounds responsible for bitter tastes and modulation.

Selected cultivation or breeding in tea (*C. sinensis*) production is an approach currently applied to ensure better yields and higher quality products (Tounekti *et al*., 2013). By selective breeding of genetic lines adhering to the relevant compositional parameters, the yield of good and acceptable quality material is increased and losses minimised. The phenolic content of *C. sinensis* tea has been shown to provide vital information to tea producers regarding the sensory quality of the product. A great deal of research has focussed on defining the contribution of phenolics to the taste of tea. Clones are selected for specific quality outcome-based attributes based on phenolic and catechin content (Takeo, 1992; Gerats & Martin, 1992). For example, for the production of green tea, plants are propagated that are known to have low enzymatic polyphenol oxidase activity as to prevent excessive oxidation, detrimental to the desired green colour (Chu, 1997; Takeo, 1992). Similarly, since flavonol glycosides are responsible for astringency in tea infusions (Scharbert *et al*., 2004), levels of these phenolics are monitored at the point of plant selection in order to control taste and mouthfeel in the final product. Catechins are known to contribute largely to the bitter and astringent properties of tea with (+)-catechin the least bitter and astringent of the catechins (Kallithraka *et al.*, 1997). This selection thus avoids the production of an excessively bitter tasting product while providing the necessary slight astringent mouthfeel (Tounekti *et al*., 2013; Takeo, 1992). The umami flavour of green tea has been linked to the amino acid threanine that accounts for more than 60% of the total amino acids in green tea (Juneja *et al.*, 1999; Takeo, 1992).

A similar approach may aid in quality optimisation of honeybush, where genotypes may be selected with the desired ratio of phenolic compounds to minimise bitterness and/or enhance modulation of bitterness. The honeybush plant breeding programme of the ARC only recently included sensory quality and phenolic composition as second tier selection parameters (Robertson *et al*., 2018; Bester *et al*., 2016).

6. Identification of bitter tasting or modulating compounds from food and plant extracts

6.1 Correlation studies of instrumental measurements and quantified bitter intensity

The time-consuming and expensive nature of sensory analysis has prompted the development of rapid instrumental methods related to the quantification of known bitter compounds to determine bitterness through statistical modelling. Although intensive, broad-based research is required to mathematically predict bitter taste based on instrumental measurements, this modelling, or multivariate calibration, represents a valuable quality control tool to industry to minimise product analysis and ensure acceptable quality.

Although there is agreement that specific links or correlations exist between chemical levels or characteristics and physical characteristics like bitter taste, establishing definitive predictability is a challenging procedure. Multiple and multivariate statistical methods are employed to determine and assess correlations between, or to predict a specific characteristic based on, several measured variables (**Table 2.2**).

Multiple regression involves the regression of one dependent variable on two or more independent variables. Often, only a subset of independent variables may be selected to best predict the values of the dependent variable. Classic selection procedures include adjusted R^2, partial correlation, Mallows' Cp, the Akaike information criterion (AIC), the Bayesian information criterion (BIC), cross validation-based criteria, forward selection, backward elimination and stepwise regression.

A fundamental problem with the involvement of multiple potential predictors is that some may become redundant when combined, resulting in multi-collinearity (Yeniay & Göktaş, 2002). Principal component analysis (PCA) exposes this multi-collinearity by revealing uncorrelated variables, including those that are linear combinations of the original predictors, and which account for maximum possible variance. If the redundancy is abundant, only a few principal components might be required.

Partial least squares regression (PLSR) and principal components regression (PCR) both model a response variable when there are several highly correlated/collinear predictor variables to be considered. Both methods construct components as linear combinations of the original predictor variables, but in different ways, as explained by Wentzell and Montoto (2003). Where PCR does not consider the response variable when creating components to explain the observed variability in the predictor variables, PLSR does take the response variable into account, allowing the model to fit the response variable with fewer components. Both methods

have an advantage over classical regression, in that they have the ability to simply depict the relationship among the variables as well as between explanatory and dependent variables.

In PCR, PCA is first performed to reduce the number of dimensions using cross-validation or test set error. Finally, regression is conducted using the selected principal components. However, this method can easily mislead, as dimension reduction via PCA does not necessarily produce new predictors.

PLS uses both the variation of the independent and dependent variables to construct new factors that will play the role of explanatory variables. The intension of PLS is to form new components to describe the information in the independent variables that is useful for predicting the dependent variable values while using a fewer number of variables. PLS appears to be favoured among chemists. This may be due to a number of perceived advantages when compared to PCR, although theoretical analysis indicates that one does not necessarily predict better than the other (Wentzell & Montoto, 2003).

Table 2.2 Statistical multivariate analyses used for predictive model building

Analytical method	Abbrev.	Subject	Reference
Principal component analysis	PCA	epinephrine; pharmaceutical sweeteners; wine polysaccharides; known taste samples; honey; mango; honeybush	Moelich (2018); Juan-Borrás *et al*. (2017); Erasmus (2015); Nassur *et al*. (2015); Choi *et al*. (2014); Bagnasco *et al*. (2014); Altan *et al*. (2014); Rachid *et al*. (2010)
Principal component regression	PCR	beer	Corzo and Bracho (2004)
Partial least squares regression	PLSR	wine; epinephrine; dairy protein hydrolysates; wine polysaccharides; honeybush; coffee	Moelich (2018); Erasmus (2015); Bagnasco *et al*. (2014); Newman *et al*. (2014); Ribeiro *et al*. (2011); Rudnitskaya *et al*. (2010); Rachid *et al*. (2010)
Multilinear regression	MLR	wine; honey; olive	Juan-Borrás *et al*. (2017); Marx *et al*. (2017); Rudnitskaya *et al*. (2010)
Stepwise linear regression		honeybush	Erasmus (2015)

In combination with sophisticated modelling programmes, much progress and potential have been seen in recent decades with the development of the electronic tongue, designed to simulate the human sense of taste. These sensory array systems are based on non-selective electerochemical, optical or mass spectrometry-based sensors coupled with data processing by chemometric pattern recognition methods (Bagnasco *et al*., 2014; Vlasov *et al*., 2002). Electronic tongues are calibrated to determine single or complex mixtures of various substances and make use of potentiometric, voltammetric or amperometric sensors (Maniruzzaman & Douroumis, 2014). Sufficient accuracy has been found with electronic tongues when compared to sensory tests for taste attributes in various food substances including amino acids, peptides and ribonucleotides (Bagnasco

et al., 2014), green tea catechins (Hayashi *et al*., 2010), black and oolong tea (Hayashi *et al*., 2013) and dairy protein hydrolysates (Newman *et al*., 2014).

Application of this kind of technique to complex mixtures, such as wine, has proven challenging. For example, Rudnitskaya *et al*. (2010) noted the crucial impact of pH on electronic tongue measurements in pinotage red wines. Calibration models were developed to predict bitter taste on the presence of phenolic compounds, catechin, epicatechin, gallic and caffeic acids and quercetin. Nevertheless, these kinds of methods require extensive chemometric investigation and modelling to be applied to specific food products.

As discussed, the taste profile (bitter, sweet, sour and astringent) of honeybush tea was not sufficiently explained by correlations between quantified phenolic compounds (Moelich, 2018; Erasmus, 2015). Without a definitive understanding of bitter compounds within the food matrix, and interactions such as modulatory effects between compounds, successful prediction is hard to establish. It is thus proposed that a model incorporating various factors, such as phenolic bitter taste profiles, phenolic chemical profiles, receptor activation and compound stability during fermentation may provide an effective model for the understanding of bitter taste of honeybush herbal tea infusions.

6.2 Sensory-guided fractionation

In order to identify bitter compounds responsible to the bitter taste characteristics of food products, deconstructive strategies have been developed to methodically isolate, analyse and identify bitter tastants in food products. Sensory analysis remains the most reliable and simple method of analysis and has been integrated with separation and isolation techniques through **sensory-guided fractionation** to identify bitter compounds in complex mixtures.

Sensory-guided fractionation has led to the discovery of previously unknown taste active compounds. Pungent contributors in olive oil (Andrewes *et al*., 2003), key astringent compounds in spinach (Brock & Hofmann, 2008), thermally generated bitter compounds (Soldo & Hofmann, 2005; Frank *et al.*, 2003; 2001), bitter tastants in coffee (Frank *et al*., 2006) and cooling compounds in dark malt (Ottinger *et al*., 2003) have all been identified using this method. In addition, astringent and bitter compounds in black tea infusions (Hofmann *et al*., 2006; Scharbert & Hofmann, 2005; Scharbert *et al*., 2004) and sake (Hashizume *et al*., 2012) have also been elucidated.

Based on the concepts of bioresponse-guided fractionation, this approach relies on separating the food matrix into several fractions to be analysed individually using sensory methods (**Fig. 2.4**). The sample is typically extracted in solvent and the extract fractionated, often repeatedly, until individual compounds can be isolated and identified for their taste activity. Crude initial fractionation is often undertaken and may be based either on molecular size using ultrafiltration (Meyer *et al*., 2016; Stark *et al*., 2005; Scharbert *et al*., 2004), or on polarity, either by sequential solvent extraction (Brock & Hofmann, 2008; Frank *et al*., 2006), classical column, or even 2D liquid chromatography (Pickrhan *et al*., 2014; Reichelt *et al*., 2010a; Frank *et al*., 2003). These approaches are commonly combined to deconstruct the extract. Ideally, final fractionation is conducted using preparative HPLC in order to separate individual peaks on the chromatogram. The organic solvent is removed by evaporation and repeated freeze-drying, reconstituted to relevant concentrations in water (or an EtOH-water mixture), and analysed for the relevant activity, in this case, sensory analysis for bitter taste.

Once peak-wise fractionation has been achieved, the fractions are typically evaluated using **taste dilution analysis** (TDA; Hofmann *et al*., 2006; Frank *et al*., 2006; 2001). Fractions are prepared as serial stepwise dilutions ranging above and below the expected taste threshold concentration. These dilutions are presented to a trained sensory panel in order of increasing concentrations to determine minimum detection threshold concentrations. The 3-alternative forced choice (3-AFC) test is commonly used to determine the minimum concentration at which panellists can differentiate between two blanks and the diluted sample (discussed in **section 8.3**). Alternatively, the *taste dilution (TD) factor* of a food extract fraction can be determined by presenting serial dilutions of decreasing concentrations to a trained panel (Reichelt *et al*., 2010a,b,c). The TD factor is defined as the maximum number of dilutions at which a taste difference is detected between the diluted fraction (sample) and two blanks (water; Frank *et al*., 2001). By determining relative *response threshold concentrations* for each peak or fraction, *dose over threshold (DoT)* factors are often calculated by comparing response thresholds to the actual concentrations in the food extract to determine taste contributions. This gives a clear representation of the taste activities or taste impact of the food matrix components.

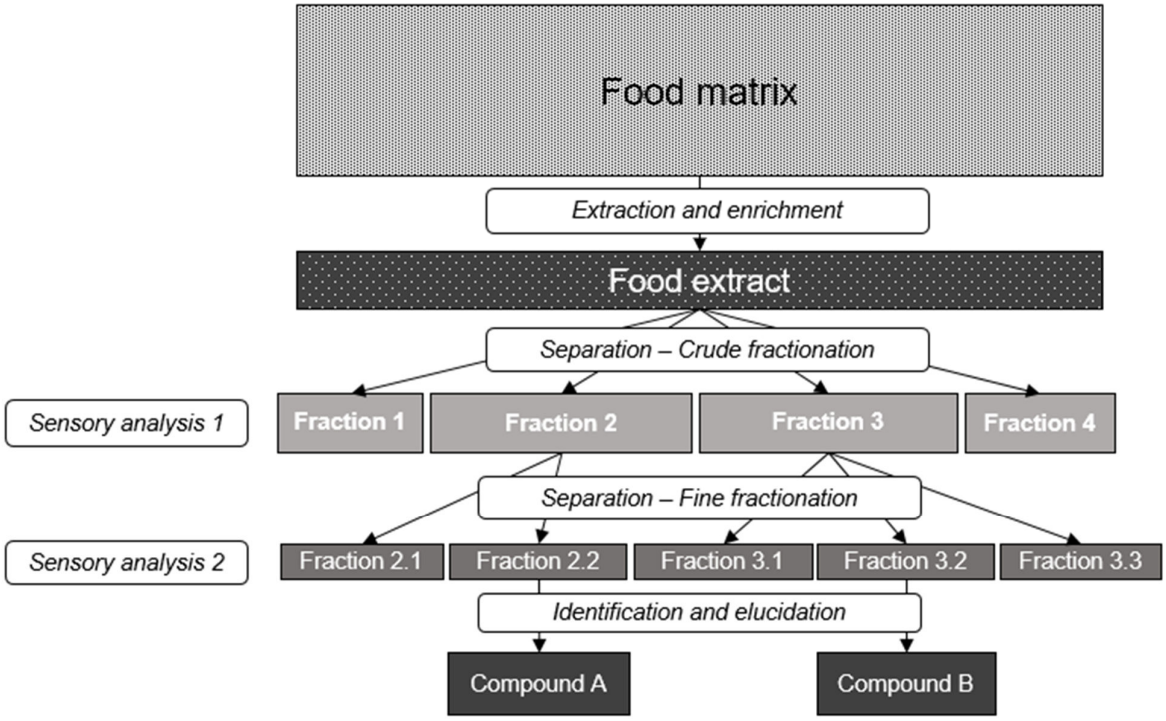

Figure 2.4 Schematic summary of sensory-guided fractionation strategy.

6.3 Taste modulation and reconstruction studies

The incidence of taste modulation in food extract systems presents a great challenge to the analysis of the contribution of specific compounds to a taste modality. With the taste contributions from compounds not necessarily taste active in isolation, identifying and investigating the effects of these compounds can be very extensive. Strategies involving comparative sensory analysis have been applied for addressing this complicated matter.

Similar to the principles of TDA to identify taste-active compounds present in fractions from food extracts, **comparative TDA** (cTDA) was developed to identify fractions or compounds eliciting modulating effects (Soldo & Hofmann, 2005; Ottinger *et al*., 2003). In essence, cTDA involves dissolving the extract fraction or possible modulating compounds in a base tastant solution (*e.g.* 50 mmol.L^{-1} sucrose). Increasing stepwise dilutions of the fraction or compound in the constant concentration of base tastant solution are then subjected to comparative sensory analysis against a blank sample (standard tastant solution). TD factors are once again calculated for each fraction, indicating the likelihood of taste contribution by modulation.

Extract reconstruction is a method that has been used to successfully identify compounds responsible for specific taste characteristics in a variety of products (Meyer *et al*., 2016; Hofmann *et al*., 2006; Scharbert & Hofmann, 2005). Typically, taste thresholds are determined for each fraction by sensory analysis and DoT factors calculated. In the study by Meyer *et al*. (2016), fractions present in the extract at DoT > 0.1 were considered to contribute to the typical taste and added to a solution at their extract concentrations. Although the reconstructed extract was similar to the original extract, a deviation in sweet taste quality was observed. Comparative (c)TDA of the low molecular weight fractions was thus undertaken to identify sweet modulating compounds. For cTDA, the reconstructed extract was used as the base tastant solution, with tasteless (DoT < 0.1) fractions added to this extract for comparative evaluation. Fractions contributing to sweet taste were further investigated to identify and isolate the sweet taste modulating compound, betaine. Re-engineering of the extract with tastants and modulating compounds at their natural extract concentrations was thus able to simulate the original extract taste.

In a similar experiment, Scharbert and Hofmann (2005) investigated the taste contributions of flavonol-3-glycopyranosides, catechins, theaflavins and caffeine as key tastants in a Darjeeling black tea infusion. By blending 51 of these compounds at natural concentrations in the infusion, a complete reconstructed infusion was prepared to match the black tea infusion. **Taste omission experiments** were then conducted to determine the taste contributions of specific compounds or groups of compounds. Omission of the flavonol-3-glycosides resulted in a significant (~50%) reduction in bitter intensity, despite lacking any bitter taste of their own. The modulation effect of a natural concentration (0.011 mmol.L^{-1}) of rutin on various caffeine solution concentrations were verified by sensory analysis.

The determination of the taste modulating ability of compounds can be a tedious procedure. These methods require the isolation of sufficient volumes of compounds or fractions for intensive sensory analysis. The recent development of the **LC taste®** procedure (Reichelt *et al*., 2010b,c), however, has provided a simplified screening method for determining the taste modulating activity of extract components. By using high-temperature liquid chromatography (HTLC), peak-wise fractionation of serial dilutions of a food extract is possible using only water as solvent. The fractions may be analysed by descriptive and cTDA sensory methods without laborious removal of toxic solvents. Since the added fractions are not standardised in terms of concentration and the modulation effects observed cannot definitively be attributed to the activity of a single compound, the *taste modulation probability (TMP) factor* was introduced. The TMP factor is applied as the

number of panellists experiencing a modulation effect compared to chance, for a particular fraction (Reichelt *et al*., 2010c):

$$\text{TMP} = \frac{(n_{higher} - n_{lower})}{n_{total}} \times 100$$

The higher the TMP factor the higher the probability of an enhancing effect, whereas strongly negative TMP factors indicate a high probability of a masking effect by a specific fraction. As a probability-based factor, the TMP only provides indicative information regarding the strength or intensity of the effect and cannot directly describe the maximum activity of a single compound. Validation of observed modulating effects of fractions can be performed by compound isolation and further pure-compound comparative tests in basic tastant solutions.

7. Preparation and instrumental analysis of phenolic compounds

The most common and reliable method for compound separation in complex matrices is liquid chromatography. Liquid chromatography is based on principles of interaction between the liquid mobile phase and a solid stationary phase. The affinity of a compound to the liquid phase will determine the speed of elution; a sample with high affinity for the stationary phase will elute later with a greater retention period. Manipulation of retention times of different compounds, and thus separation, is achieved though changes in phase composition and temperature, and can result in reproducible compound-specific retention times (t_R) for specific conditions (Snyder *et al*., 2010). Following the development of an appropriate method for the separation of specific food extracts, method validation is required to ensure the reliability of the method for quantification. Validation is conducted in terms of accuracy, specificity, precision, detection and quantification limits, linearity, range and robustness (Snyder *et al*., 2010). It is also necessary to verify that the intended compounds have been identified and selected for separation. This is commonly performed using one or two dimensional mass spectrometry (MS or MS/MS) or nuclear magnetic resonance (NMR) detectors to accurately identify the structures of unknown compounds, or to confirm peak identity (Snyder *et al*., 2010).

Classical column chromatography is a useful technique for crude fractionation. Although batch processing is required, fractions can be obtained with this method on a relatively large scale. Resin beads are packed in the separation column which is then filled with solvent before sample loading. Drawbacks for this kind of chromatography includes large solvent volumes and time-intensive methodology with low flow rates.

However, large volumes can be fractionated to obtain several fractions of differing polarities and possible taste qualities. Without a detector, quantification is undertaken separately. This technique of crude fractionation has been used for *C. intermedia* extracts (Richards, 2003). Fractionation was performed using XAD-1180 polymeric beads in an open glass column with MeOH solvent (15%, 30%, 50%, 80% and 100%) as mobile phase. Successful separation was performed in six fractions, quantitatively differing in mangiferin, isomangiferin, hesperidin, eriodictyol and luteolin content. The individual fractions were assessed for antioxidant and antimutagenic activity.

Finer methods of fractionation can be applied to fractions with known bitter activities. For example, **preparative HPLC**. HPLC, the most common and well defined of liquid chromatography methods, is commonly used in food laboratories for routine separation, isolation and quantification. Indeed, several *Cyclopia* species-specific analytical methods have been developed and validated for the routine separation and quantification of various phenolic compounds in honeybush extracts (Schulze *et al*., 2015; 2014; Beelders *et al*., 2014b; De Beer *et al*., 2012). With a solid stationary phase, compound retention is manipulated by a solvent polarity gradient of the mobile phase (Snyders *et al*., 2010). Solvent systems are used to strategically and gradually decrease the polarity of the mobile phase, allowing the selection of highly polar molecules at short retention times, and more non-polar molecules with greater retention times. Reversed-phase HPLC allows manipulation of the mobile phase with an increase in polarity. This method of preparative separation is more precise than classical column chromatography and can accurately isolate individual compounds. The complex nature of food extracts, however, limit the direct application of preparative HPLC due to the difficulty in achieving effective separation. In addition, the very small amounts of individual compounds in complex mixtures also result in a small yield, requiring multiple separation runs. This method is thus ideal as a secondary fractionation step following enrichment or primary fractionation.

8. Sensory analysis of bitter taste

Bitter taste is most commonly quantified or determined by a trained sensory panel. In this way bitter intensity of a solution, foodstuff or pharmaceutical may simply be determined. There are, however, various factors to consider including panellist selection, experimental designs with trained or untrained panellists, and scales or measurements to be employed.

8.1 Panel selection

Bearing in mind the inherent genetic variation in the human population with regard to bitter perception, it is necessary to ensure that panel participants are indeed able to discriminate bitter taste to the required degree. Various approaches to panel selection have been taken. The most common is panel screening following basic taste training. The panel is trained by the 3-AFC presentation (discussed in **section 8.3**) of solutions adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol.L^{-1}): sucrose (50 mmol.L^{-1}) for sweet taste, lactic acid (20 mmol.L^{-1}) for sour taste, NaCl (12 mmol.L^{-1}) for salty taste, caffeine (1 mmol.L^{-1}) for bitter taste, monosodium glutamate (8 mmol.L^{-1}, pH 5.7) for umami taste and tannin (gallustannic acid; 0.001%) or quercetin-3-*O*-β-D-galactopyranoside for the astringent/rough or the velvety, mouth-drying oral sensation, respectively (Brock & Hofmann, 2008; Schwarz & Hofmann, 2007; Stark *et al*., 2005; Scharbert *et al.*, 2004). Furthermore, panellists may be asked to complete a bitterness ranking task (Andrewes *et al*., 2003). Additional panel testing has also included assessment of panellist salivary flow, which has been found to significantly affect the perceptions of bitterness and astringency (Dinella *et al*., 2011; 2009; Peleg *et al*., 1999).

Furthermore, the determination of taster PROP status may be useful to indicate panellists' exposure and response to bitterness (Kobue-Lekalake *et al*., 2012; Drewnowski *et al*., 1997; Thorngate & Noble, 1995). Using the Tepper's test (Tepper *et al*., 2001) for determination of PROP status, panellists are categorised as non-, medium- and super-tasters based on their compared perception of the bitter intensity of solutions of PROP and sodium chloride (NaCl).

8.2 Descriptive and quantitative tests

Traditional descriptive sensory analysis (DSA) or sensory profiling provides the most extensive insight into the sensory characteristics of a product (Lawless & Heymann, 2010). It is, however, also an expensive and time-intensive technique, as consensus training with a large panel is required. The analysis of multiple attribute intensities is undertaken, making it a lengthy assignment. If used for screening, however, it may be a valuable tool to gain supplementary information and insight into fraction contributions. This consensus-based screening method was used by Reichelt *et al*. (2010a,b,c) to describe fractions as pungent, sweet, fishy, *etc*.

In addition, DSA measures specific attributes on an intensity scale, providing quantitative intensity data. This is essential for the comparison of multiple samples, as is the case with the present study. Repeated analyses with multiple panellists, however, has drawbacks both in terms of sample availability and panellist

fatigue related to carry-over effects. Experimental design models, however, may be adapted to limit sample exposure and prevent excessive carry-over or large sample volumes.

By using a quantitative indication of bitter intensity, *dose-response functions* can be produced to determine at which concentration bitterness is not only present, but unacceptably high. This can also give an indication of taste interactions between bitter tasting fractions, whether an additive, linear, or diminutive effect is observed when compared to the bitter intensity of recombinants, or that of the original extract.

8.3 Taste threshold determination

Tastants not only differ in taste intensity, but also in taste threshold concentrations. Where some tastants are detectable in very dilute solutions, others may only be detected at considerable concentrations. Since the focus of many projects in recent years has aimed at food fortification and addition of health promoting or active ingredients with aversive taste characteristics, the determination of *taste threshold concentrations* has been of interest in the food and pharmaceutical industries.

Although individual thresholds vary, methods have been contrived to determine representative "panel" threshold values for sensory studies. Although inter-laboratory comparison is not always appropriate due to differences in analysis methodology and panel sensitivity, standardised methods have been developed and aid in producing more comparable results. Since the aim is not to quantify and compare bitter taste intensities, discriminant sensory analysis is appropriate. The basic test used for this method is the 3-AFC presentation as a derivation of the triangle test (ASTM E 679-04 rapid method; ASTM E 1432-04; ASTM International, 2007a,b). Samples are presented to panellists in increasing concentrations as sets of two blanks and one sample. A dilution series is selected in consistent intervals above and below the suspected threshold. Panellists must choose "the odd one out", even if it by guessing, with between 20 and 40 sample sets presented to each panellist (in a panel of 5 - 15 members; ASTM E 1432-04; ASTM International, 2007b). This method can be used for detection or recognition thresholds. For the latter, panellists need to be familiar with the compound or substance at hand. Panel training is thus recommended. For the rapid method of threshold estimation (ASTM E 679-04; ASTM International, 2007a), analysis for each panellist is terminated once two or more correct discriminations have been made, indicating the threshold has been exceeded. The *best estimated threshold (BET)*, or the closest measure to the actual threshold for each panellist is determined as the geometric mean between the concentration of the last miss and the concentration of the first of the consistent correctly

indicated samples. Panel BET can then be determined as the geometric mean of all individual panellist BET values. Geometric means overcome the non-linear dilution factor.

9. Cell-based assays to determine bitterness

The recent discovery of the group of human bitter receptors has led to the development of heterologous expression experimental methodologies to successfully measure TAS2R activation by bitter tastants *in vitro* (as reviewed by Riedel *et al*., 2017). Transfected cells expressing the selected TAS2R receptors are induced and activated by exposure to the bitter tastants in solution. Measurement of intracellular Ca^{2+} release during transduction is quantified as intensity of fluorescence to indicate receptor activity. By doing so, and measuring activation by different concentrations of tastants, dose-response curves are generated indicating maximum activity and concentration required to achieve a significant response, or the half-maximal effective concentration (EC_{50}). This analysis thereby indicates whether a compound may be bitter, as well as what concentration is required for a bitter taste response. Modulatory potential may also be assessed by comparing the signals of compounds in combination and in isolation. According to Riedel *et al*. (2017), cell-based models described up to now have been expressed either by recombinant taste receptors in surrogate (non-human) non-taste tissue cell lines, taste receptors in non-taste (extra-oral) mammalian cell lines, or taste cell cultures and immortal taste cell lines derived from human tongue tissue. The context of the receptors within the cell lines will thus affect the relevance of the bitter taste activation or signal resulting from these types of experiments (Riedel *et al*., 2017). It must, however, be considered that these methods are in their infancy, as pointed out by Riedel *et al*. (2017), and that other factors may play a role in sensory bitter taste perception *in vivo*, as discussed previously (**section 3.4**). It is thus necessary that cell-based assays are validated by sensory analysis to confirm taste or modulatory effects when compounds or extracts are screened for taste activities.

Nevertheless, purified bitter chemicals present in some food or beverage matrices, for example beer hops (Intermann *et al*., 2009), synthetic and natural sweeteners (Acevedo *et al*., 2016; Kuhn *et al*., 2004) and soy products (Roland *et al*., 2011), have been found to activate specific bitter receptors. Although few studies use the full known array of bitter receptors (25 receptors), some studies have undertaken this task. Screening for bitterness activation by six polyphenols commonly present in red wine, beer, tea and chocolate was conducted by Soares *et al.* (2013). Notably, malvidin-3-glucoside and β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (PPG) were found to induce bitter transduction at very low concentrations. Meyerhof *et al*.

(2010) also investigated a large number (104) of natural or synthetic known bitter compounds for bitter receptor activity (25 receptors). Both these studies, amongst others, have indicated that polyphenols activate different combinations of the TAS2R receptors, with great variation in receptor response as measured by EC_{50} values.

10. Conclusions

The identification and analysis of bitter tasting or bitter modulating compounds requires a multi-disciplinary approach, incorporating both food chemistry and sensory science. Applications of such data ranges from food to pharmaceutical approaches and are of great importance in these industries. The identification and relation between bitter-causing compounds in honeybush will be a valuable addition in the development of the growing industry, and may facilitate the informed compromise between health-related and bitter-causing phenolics during herbal tea production and processing. In addition, the identification of bitter modulating compounds in honeybush may have application in other food or pharmaceutical products containing related phenolics.

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Chapter 3
Bitter profiling of phenolic fractions of green *Cyclopia genistoides* herbal tea

Abstract

No data is available on the bitter taste activity of honeybush phenolic compounds and their quantitative contributions to the bitter taste of honeybush infusions. The contribution of phenolic compounds to the bitter taste of an unfermented *Cyclopia genistoides* herbal tea infusion was therefore investigated by descriptive sensory analysis (dose-response) and discriminant sensory analysis (taste threshold) of a hot water extract and fractions enriched in benzophenones, xanthones and flavanones. The bitterness of the hot water extract and the xanthone-rich fraction were predicted as 45 and 31 (on a 100-point scale), respectively, when tasted at their honeybush infusion equivalent concentrations (IEC's). The benzophenone-rich fraction did not have a bitter taste (< 5), while the flavanone-rich fraction had a moderate bitter taste (~13) when tasted at their IEC's. Mangiferin elicited a more intense bitter taste than its regio-isomer, isomangiferin (*ca* 30 vs 15), when tasted at the same concentration in a model solution. A combination of these major honeybush xanthones at concentrations expected in an infusion (IEC in the xanthone-rich fraction, 118 $mg.L^{-1}$ mangiferin and 34 $mg.L^{-1}$ isomangiferin), resulted in a significantly lower bitter intensity (~22) than mangiferin in isolation. This indicates that isomangiferin exerted a bitter intensity suppressing effect on the bitter taste of mangiferin, suggesting the importance of taste modulation in the bitter taste of honeybush tea infusions.

1. Introduction

Consumers associate honeybush herbal tea with a sweet taste (Vermeulen, 2015), so that the bitter taste of *Cyclopia genistoides* impacts negatively on its acceptance as a herbal tea. Bitter taste has long been considered an adverse taste perception, especially in food products which are not commonly associated with bitter taste. Phenolic compounds that offer bioactive benefits often contribute to bitter taste, presenting the consumer with competing demands of taste and health (Barratt-Fornell & Drewnoski, 2002). Plant material from *C. genistoides* produces honeybush herbal tea that is a good dietary source of bioactive xanthones and benzophenones (Schulze *et al*., 2015), however, many production batches are perceived as uncharacteristically bitter (Erasmus *et al*., 2017). Compounds belonging to the benzophenone, xanthone and flavanone phenolic sub-classes present in *C. genistoides* have gained attention for the potential beneficial health effects they may impart to *Cyclopia* extracts (Jack *et al*., 2017; Beelders *et al*., 2014a; Chellan *et al*., 2014). Whether these phenolic compounds contribute to the bitter taste of honeybush tea needs to be elucidated.

Moelich (2018), applying partial least squares (PLS) regression analysis to quantitative phenolic and sensory data from fermented *C. genistoides* and *C. longifolia* samples, identified the major benzophenones and xanthones as candidate predictors of bitter intensity of honeybush infusions. Their contribution to the bitter taste of *C. genistoides* infusions, however, has not yet been confirmed. Their role could be two-fold, *i.e.* a direct contribution to bitter taste or an indirect contribution by amplifying or diminishing the bitter taste of other compounds (taste modulation). This indirect taste contribution can have a substantial effect on the bitter intensity of honeybush tea. Indeed, Reichelt *et al.* (2010c) found that the addition of honeybush fractions to a caffeine solution resulted in several cases of bitter suppression and bitter amplification.

The current study is a first step in the investigation to confirm the contribution of bitter-tasting compounds in unfermented (green) *C. genistoides*. Descriptive sensory analysis (DSA) and discriminant sensory analysis were applied for the determination of dose-response curves and taste threshold values of the benzophenone-, xanthone- and flavanone-rich fractions. DSA of the major xanthones, mangiferin and isomangiferin (pure compounds), were also conducted to investigate their contribution to the bitter taste of honeybush herbal tea.

2. Materials and methods

2.1 Chemicals

Analytical-grade EtOH, dimethyl sulfoxide (DMSO), L-ascorbic acid and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, USA). Analytical-grade formic acid and HPLC-grade MeOH (98 - 100%) were sourced from Merck Millipore (Darmstadt, Germany). Authentic phenolic reference standards (purity > 95%) were sourced from Sigma-Aldrich (mangiferin, isomangiferin, hesperidin and 3-β-D-glucopyranosyliriflophenone (IMG)), Phytolab (Vestenbergsgreuth, Germany; vicenin-2 and eriocitrin) and Extrasynthese (Genay, France; narirutin). Reference standards for sensory panel training were sourced from Sigma-Aldrich (caffeine and citric acid), Huletts (Tongaat Hulett, Durban, South Africa; sucrose) and Pinnacle Pharmaceuticals (Cape Town, South Africa; alum). XAD porous resin (Amberlite, 20 - 60 mesh) for open column chromatography was sourced from Sigma-Aldrich.

Deionised water was prepared using an Elix water purification system (Merck Millipore). The deionised water was purified to HPLC-grade, using a Milli-Q Reference A+ water purification system (Merck Millipore).

2.2 Plant material

Cyclopia genistoides (genotype GK5) from the honeybush plant breeding programme of the Agricultural Research Council (ARC) was selected for experimental work, based on preliminary bitterness screening of eight genotypes. Shoots (20 kg) were harvested in April 2016 at the farm, Toekomst (GPS coordinates −34.24052, 20.47272), situated in the Bredasdorp district of the Western Cape Province of South Africa. Leafless stems were trimmed and the remaining shoots processed to obtain the unfermented (green) herbal tea: shoots were shredded to 2 - 3 mm pieces using a mechanised fodder cutter, followed by thin layer drying at 40 °C in a laboratory cross-flow drying tunnel to a moisture content < 10%. The dried material was sieved and the tea-bag fraction (< 1.68 mm, > 0.42 mm) collected.

2.3 Extraction and fractionation

2.3.1 *Hot water extraction of plant material*

Freshly boiled deionised water was added to the sieved plant material (150 g) in a 1:10 ratio ($m.v^{-1}$), after which the mixture was placed in a water bath at 93 °C for 30 min. The mixture was swirled at *ca* 10 min intervals. After extraction the hot mixture was strained (74 µm steel filter) and vacuum-filtered using Whatman No. 4 filter paper and a Buchner funnel to collect *ca* 1200 mL filtrate. This procedure was repeated several

times before the filtrate was pooled and freeze-dried (VirTis Genesis, Model 35ES, SP Scientific, Gardiner, NY, USA).

2.3.2 *Preparation of EtOH-soluble fraction of hot water extract*

The freeze-dried hot water extract and EtOH were combined in a 1:10 ratio ($m.v^{-1}$) and the mixture sonicated (Branson Ultrasonics Corporation, Danbury, USA) for 60 min. The precipitate (EtOH-insoluble) and supernatant (EtOH-soluble) were then separated by vacuum filtration (using Whatman No. 4 filter paper and a Buchner funnel). EtOH was removed at 40 °C under vacuum by rotary evaporation and the residue suspended in deionised water for freeze-drying.

2.3.3 *Open column fractionation of EtOH-soluble solids*

Fractionation of the EtOH-soluble solids was performed on an open column (68 × 500 mm) fitted with a peristaltic pump (Model 505U, Watson Marlow Ltd, Falmouth, England) operated at a suction flow rate of *ca* 38 $mL.min^{-1}$. The column was loaded with dechlorinated XAD suspended in water and conditioned by flushing with six column volumes (4.5 L) of HPLC-grade water. The EtOH-soluble solids (15 g solids suspended in 50 mL HPLC-grade water) was added to the column and several 750 mL sub-fractions (SF) collected at each solvent gradient interval, representing 0% (6 SF; 1 - 6), 5% (6 SF; 7 - 12), 10% (8 SF; 13 - 20), 20% (6 SF; 21 - 26), 30% (6 SF; 27 - 32) and 100% (6 SF; 33 - 38) EtOH. A summary of the achieved separation is provided in **Fig. A.1** (Supplementary material). The sub-fractions were analysed by HPLC (**section 2.4**) and pooled according to phenolic composition to produce four crude fractions, with one fraction containing unidentified polar compounds and three fractions rich in benzophenones, xanthones and flavanones, respectively (**Table 3.1**). The solvent was removed by vacuum evaporation at 40 °C, the residue suspended in deionised water and freeze-dried. Mass-based yields ($m.m^{-1}$) were calculated as a percentage of the plant material ($g.100\ g^{-1}$ plant material). The quantified phenolic content of each phenolic sub-class was also presented as a percentage of the mass of each crude fraction (**Table 3.1**; Supplementary material, **Fig. A.2**).

2.4 HPLC analysis

HPLC with diode-array detection (DAD) was used to qualitatively screen and quantitatively analyse extracts and fractions, according to the validated species-specific method of Beelders *et al*. (2014b). Samples were dissolved in 10% aq. DMSO (900 µL) with the addition of 100 µL 10% ascorbic acid to prevent degradation.

These solutions were filtered (0.45 µm syringe filter; 33 mm diameter, Merck Millipore) into wide-necked amber HPLC vials for analysis.

The authentic phenolic standards for HPLC quantification were prepared as stock solutions of *ca* 1 mg.mL^{-1} in DMSO and aliquots frozen (-20 °C) until analysis. Where reference standards were unavailable, compounds were quantified as reference equivalents, or calculated by pre-determined response factors (*i.e.* the response of an authentic standard relative to another compound using the same method). Gradient separation at 30 °C was achieved on a Kinetex C_{18} column (150 × 4.6 mm ID, 2.6 µm dp; Phenomenex, Torrance, USA), using a Agilent 1200 HPLC-DAD system consisting of an autosampler, column thermostat and detector equipped with an Agilent 1260 Infinity II quaternary pump, controlled by OpenLAB Chemstation software (Agilent, Santa Clara, USA). The mobile phase consisted of 1% formic acid (A), MeOH (B) and acetonitrile (C). The composition of the solvent gradient (1.0 mL.min^{-1} flow rate) was as follows: 0 - 5 min (2.5% B; 2.5% C), 5 - 45 min (2.5% B; 2.5% C - 12.5% B; 12.5% C), 45 - 55 min (12.5% B; 12.5% C - 25% B; 25% C), 55 - 56 min (25% B; 25% C), 56 - 57 min (25% B; 25% C - 2.5% B; 2.5% C) and 57 - 65 min (2.5% B; 2.5% C). For quantification of the major compounds, mangiferin and isomangiferin, the gradient was adapted after elution of the xanthones (*ca* 25 min) to shorten separation time: 0 - 5 min (2.5% B; 2.5% C), 5 - 29 min (2.5% B; 2.5% C - 8.5% B; 8.5% C), 29 - 31 min (8.5% B; 8.5% C - 25% B; 25% C), 31 - 32 min (25% B; 25% C), 32 - 33 min (25% B; 25% C - 2.5% B; 2.5% C) and 33 - 41 min (2.5% B; 2.5% C). The injection volume was adjusted between 10 and 100 µL, depending on the concentration of the compounds in the samples.

2.5 LC-DAD-ESI-MS analysis

LC-DAD analysis coupled to electrospray ionisation mass spectrometry (ESI-MS) detection was conducted as described by Beelders *et al*. (2014b) to confirm the identification of individual phenolic compounds in the extract and fractions. An Acquity ultra-performance liquid chromatography (UPLC) system (binary solvent manager, sample manager, column heating compartment and photodiode-array detector) was coupled to a Synapt G2 Q-TOF system with an electrospray ionisation source (Waters, Milford, USA). The HPLC method used for quantification (**section 2.4**) was also used for front end separation, but with premixed MeOH and acetonitrile (45:55, v.v^{-1}) to accommodate the binary solvent manager. The mass spectrometer was operated using both positive and negative ionisation in MS^E mode and calibrated using a sodium formate solution.

Leucine enkephalin was used for lockspray and MS parameters set to capillary voltage 2.5 kV, sampling cone voltage 15.0 V, source temperature 120 °C, desolvation temperature 275 °C, desolvation gas flow (N_2) 650 $L.h^{-1}$, cone gas flow (N_2) 50 $L.h^{-1}$ and collision energy ramp from 20 to 60 V. An injection volume of 10 µL was used and UV-Vis spectra acquired over 220 - 400 nm at 20 Hz. MS data were acquired using resolution mode (scanning from 150 - 1500 amu) and processed using MassLynx v.4.1 software (Waters).

2.6 Panel selection and training

A panel of 13 judges with previous experience in sensory analysis of honeybush tea was selected and subjected to intensive training to ensure panel reliability with regard to bitter taste recognition and intensity rating. Firstly, the panel was trained using solutions representing basic tastes and mouthfeel (sweet, sour, bitter and astringent; Haseleu *et al*., 2009; Scharbert *et al*., 2004). All combinations of these basic taste sensations were also presented to ensure panellists could discriminate between mixtures of taste sensations. Details of these test solutions used for training are provided as Supplementary material (**Table A.1**).

Secondly, in order to determine an appropriate caffeine concentration to serve as reference for a suitable level of bitter intensity during analysis, a series of 10 caffeine concentrations, ranging from 0.0 to 0.9 $g.L^{-1}$, was presented to the panel during duplicate sessions. The hot water unfermented *C. genistoides* extract was also presented to panellists at a concentration equivalent to the soluble solids (SS) concentration of its infusion (termed infusion equivalent concentration, IEC). The IEC (2 $g.L^{-1}$) was determined by preparing triplicate infusions from the plant material, according to the standard procedure (Erasmus *et al*., 2017). Consensus was reached to select 0.4 $g.L^{-1}$ caffeine as a bitterness reference with an intensity resembling that of the hot water extract at its IEC, with a score of 45 on a scale of 0 (not bitter) to 100 (extremely bitter).

Finally, the caffeine taste threshold for individual panellists and the entire panel was determined to familiarise panel members with the protocol. According to the prescribed method (ASTM E679-04; ASTM International, 2007) described in **section 2.8**, eight solutions were presented to the panel as randomised 3-alternative forced choice (3-AFC) tests in increasing concentration (3:1 serial dilution) from 0.07 to 0.52 $g.L^{-1}$. This sample set was presented four times to each panellist.

2.7 Dose-response sensory analysis of hot water extract and crude phenolic fractions

Descriptive sensory analysis (DSA; Lawless & Heymann, 2010) was performed for the quantification of bitter intensity of the hot water extract and crude phenolic fractions. Samples were prepared in 2.5% EtOH ($v.v^{-1}$) to improve solubility and presented in 30 mL transparent plastic serving cups at room temperature (~21 °C). Panel training and sample familiarisation were guided by an experienced panel leader over two days with four 30 min sessions and 10 min breaks scheduled between consecutive sessions. The panel was introduced to the bitterness scale (0 = not bitter; 100 = extremely bitter) using the pre-determined bitter reference (0.4 $g.L^{-1}$ caffeine = 45 bitter intensity). A blank reference (2.5% EtOH = 0 bitter intensity) was also provided. During training the hot water extract and crude fractions were each presented to the panel at serial dilutions of seven concentrations (2:1 serial dilutions), including the blank sample reference (2.5% EtOH) during 30 min sessions (Supplementary material, **Table A.2**). The range of concentrations for each crude fraction or extract sample included the IEC (relative to each fraction's fractional contribution to the SS content of the hot water extract prepared at its IEC of 2 $g.L^{-1}$), as well as the typical "cup-of-tea" concentration of major compounds in fermented honeybush tea infusions (Schulze *et al*., 2015). Each sample was considered in terms of overall aroma impression, as well as overall taste impression before consensus was reached on bitter intensity.

Bitter intensity was determined by presenting each diluted sample to each panellist during triplicate 20 min sessions on each of four days. Once again, 10 min breaks were scheduled between sessions to prevent panel fatigue. Marked reference samples were presented to assist scoring of bitter intensity on the defined bitter intensity scale. Following each sample, a 2.5 min delay was implemented to minimise bitter carry-over between samples. As per standardised practice, samples were blind-coded and randomised. A separate booth was allocated to each panellist during analysis and *Compusense® five* software (Compusense, Guelph, Canada) was used for data collection on an anchored (0 = not bitter, 100 = extremely bitter), unstructured line scale. Booths were fitted with red light bulbs to mask any colour differences between samples and the analysis room was controlled at 21 °C. Panellists were provided with distilled water, dried apple pieces and water biscuits as palate cleansers.

Panel performance was monitored using *Panelcheck* software (Version 1.4.2; Nofima, Ås, Norway). Sensory data were pre-processed according to the model suggested by Næs *et al*. (2010) that includes panellist, replicate, sample and interaction effects. Outliers were removed where necessary, after normality of the standardised residuals was assessed using the Shapiro-Wilk test. Statistical analyses of quantitative sensory data were performed using *SAS* software (Version 9.2; SAS Institute Inc., Cary, USA). Analysis of variance

(ANOVA) was performed on the collected data (n = 7 samples) for each fraction and the hot water extract over triplicate analyses and judges. Fisher's LSD was calculated with a confidence interval of 95%. Regression was conducted on the dose-response bitter intensity profile of the fractions and hot water extract at different concentrations.

2.8 Taste threshold determination of crude phenolic fractions

The crude benzophenone-, xanthone-, and flavanone-rich fractions obtained after open column chromatography of the EtOH-soluble solids of the hot water *C. genistoides* extract were subject to taste threshold determination (ASTM E679-04; ASTM International, 2007) by a trained panel. Each fraction, dissolved and diluted in 2.5% EtOH to a range of eight predetermined concentrations (2:1 serial dilutions), was presented to each panellist in 30 mL plastic serving cups. Employing the 3-AFC test, one sample and two reference blanks (2.5% EtOH) were presented simultaneously for each of the eight concentrations. The diluted samples were presented in order of increasing concentration (Supplementary material, **Table A.3**). Analysis was conducted as per standardised practice, described in **section 2.7**. A total of four sample sets were presented to each panellist over two days, with two sessions per day separated by a 10 min break.

Data analysis was conducted according to the prescribed ASTM E679-04 method (ASTM International, 2007). The first of the initial two sequential concentrations each panellist recognised correctly was considered as the first incidence of detection. The best estimated threshold (BET) for each panellist and replicate was determined as the geometric mean of the concentration of the first incidence of detection and the last incorrectly identified concentration. The detection threshold was thus calculated as the geometric mean of the BET values over all judges and replicates, with a 95% confidence interval.

2.9 Bitter taste contribution of mangiferin and isomangiferin

The bitter taste of mangiferin and isomangiferin were investigated to determine their individual contribution to the bitter taste of the xanthone- and flavanone-rich fractions. The flavanone-rich fraction also contained small amounts of these xanthones, in addition to several flavanones. The analyses were performed by a panel of eight members, selected based on superior performance during previous tests, necessitated by the small amount of sample available. Additionally, hot deionised water was used for sample dissolution to more accurately simulate the contribution of these xanthones to honeybush herbal tea infusions normally consumed hot. Samples were dissolved in water at 80 °C for *ca* 40 min in a jacketed glass flask, heated by circulated

water and stirred to aid dissolution (Supplementary material, **Fig. A.3**). Dilutions were prepared using only deionised water and the solutions (10 mL per panellist) served in 40 mL screw-cap amber vials placed in metal racks in water baths at 60 °C to prevent precipitation of the sample (Supplementary material, **Fig. A.4**). Preliminary testing confirmed that phenolic degradation of samples during preparation and testing as a result of this heating and setup procedure was negligible.

Bitter intensity of mangiferin was determined using the dose-response method (**section 2.7**) at a maximum concentration of 300 $mg.L^{-1}$ and a range of seven dilutions (2:1 serial dilutions) including a blank sample (deionised water). This range included the calculated concentration of mangiferin in the xanthone- and flavanone-rich fractions, as well the hot water extract, at their IEC's (118, 9 and 178 $mg.L^{-1}$, respectively).

As the availability of pure isomangiferin was limited, a dose-response test was not performed, but the two regio-isomers, mangiferin and isomangiferin, were compared by the trained sensory panel at the calculated concentration of mangiferin (118 $mg.L^{-1}$) and isomangiferin (34 $mg.L^{-1}$) in the xanthone-rich fraction at its IEC (258 $mg.L^{-1}$). The combination of the isomers was also tested at their respective calculated concentration in the xanthone-rich fraction at IEC (118 and 34 $mg.L^{-1}$, respectively). As a means of control, a blank sample (warm deionised water) and the xanthone-rich fraction at IEC (258 $mg.L^{-1}$) were also included. A summary of mangiferin and isomangiferin IEC's in the extract and fractions is provided in **Fig. 3.1**. All samples were analysed in triplicate for bitter intensity on an anchored line scale according to the DSA method described in **section 2.7**.

3. Results and discussion

Plant material (*C. genistoides*) was selected and processed as green herbal tea for the present investigation, based on the intense bitter taste of the infusion. The polyphenols were extracted with hot water instead of an organic solvent to prevent extraction of compounds not normally present in the herbal tea infusion. Subsequent preparation of the EtOH-soluble fraction and column fractionation were performed to obtain fractions enriched in benzophenones, xanthones and flavanones, respectively. The relative yields of the extract and various fractions (expressed in terms of the $m.m^{-1}$ plant material) are indicated in **Table 3.1**. A summary of the quantified individual phenolic compounds in the hot water extract and fractions is provided in **Table 3.2**. HPLC-DAD chromatograms of the extract and fractions are provided in **Fig. 3.2** and total ion chromatograms obtained in negative ionisation mode, as well as LC-MS data, retention times and maximum wavelength of the

phenolic compounds present in the extract and fractions are provided as Supplementary material (**Fig. A.5**, **Table A.4** and **Table A.5**).

Hot water extraction yielded *ca* 20% SS with the quantified phenolics contributing only 17% of the overall SS, and mangiferin and isomangiferin contributing a combined 11.9% of the extract (**Table 3.1**). This is typical of hot water extracts of unfermented *C. genistoides* (Beelders *et al*., 2014b). The benzophenones were also prominent, representing 3.7% of the extract (**Table 3.2**). Unlike IMG, 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG) is resistant to degradation during fermentation (Beelders *et al*., 2017) and was present at levels similar to that found in the typical infusion of fermented plant material (1.5%; Schulze *et al*., 2015). The flavanone, naringenin-*O*-hexose-*O*-deoxyhexoside B (NHDB), is typically present in higher concentrations in unfermented extracts, whereas the A isomer (NHDA) is present at higher concentrations in fermented extracts (Beelders *et al*., 2015). The high concentration (1.2%) of NHDB in the hot water extract is thus to be expected (**Table 3.2**). The plant material produced a hot water extract with a very low hesperidin content (0.08%), even when compared to infusions of fermented *C. genistoides* (0.5%; Schulze *et al*., 2015). Trimming of leafless stems would have contributed to this lower hesperidin content, as hesperidin is predominant in *Cyclopia* stems as found for *C. subternata* and *C. maculata* (De Beer *et al*., 2012; Du Preez *et al*., 2016).

In order to improve loading and separation on the macroporous XAD resin, non-phenolic EtOH-insoluble components such as polysaccharides were removed from the hot water extract. The EtOH-soluble fraction was subsequently separated by open column low pressure chromatography into three crude fractions, enriched in benzophenones, xanthones and flavanones, respectively. LC-MS of the fractions (Supplementary material, **Tables A.4** and **A.5**) indicated the presence of several previously tentatively identified compounds in addition to some positively identified compounds (Beelders *et al*., 2014b), as well as unidentified compounds, some producing considerably large peaks on the chromatogram that were not quantified (**Fig. 3.2**; Supplementary material, **Fig. A.5**). For example, 41.3% of the benzophenone-rich fraction consisted mostly of benzophenones: maclurin-di-*O,C*-hexoside, IDG, 3-β-D-glucopyranosylmaclurin and IMG, but also some other phenolic compounds (**Fig. 3.2b**, **Table 3.2**). The remaining 58.7% is unaccounted for and includes unquantified xanthone derivatives and other unidentified compounds (Supplementary material, **Fig. A.5**; **Tables A.4** and **A.5**).

The xanthone-rich fraction contained 45.4% mangiferin and 13.3% isomangiferin (**Table 3.2**). However, this fraction also contained a small amount of IMG (2.5%), as well as various flavanones, including 1.2% NHDB, 0.3% NHDA, 0.8% eriodictyol-*O*-hexose-*O*-deoxyhexoside isomer A (EHDA) and an unquantified amount of the tentatively identified eriodictyol-*O*-hexose-*O*-deoxyhexoside isomer B (EHDB) (**Table 3.2**; Supplementary material, **Tables A.4** and **A.5**).

The flavanone-rich fraction only contained 13% quantified phenolics, including 7.8% quantified flavanones (**Tables 3.1 - 3.2**). The quantified flavanones consisted of 0.05% EHDA, 0.7% NHDA, 6.3% NHDB and 0.7% hesperidin. The xanthones, mangiferin and isomangiferin, were the main quantified "impurities" contributing 3.7% and 0.5% of the fraction, respectively (**Table 3.2**, **Fig. 3.2d**). Other non-flavanone impurities include two tetrahydroxyxanthone-*C*-hexoside isomers, the flavone, vicenin-2, and the dihydrochalcones, 3-hydroxyphloretin-3′,5′-di-*C*-hexoside and 3′,5′-di-β-D-glucopyranosylphloretin. Although only four flavanones have been conclusively quantified in *C. genistoides* extracts, eight additional flavanone compounds have previously been tentatively identified by LC-MS and MS/MS (Beelders *et al*., 2014b). In the current study, LC-MS analysis revealed several additional flavanone compounds, namely EHDB, narirutin, an additional naringenin derivative and hesperetin, in the current flavanone-rich fraction (Supplementary material, **Table A.5**). Additional unidentified peaks may also represent flavanone compounds (**Fig. 3.2d**; Supplementary material, **Fig. A.5**). Quantification of the exact flavanone content of this fraction was thus not possible.

As indicated by preliminary bitterness screenings, the hot water extract of the unfermented plant material diluted to its IEC (2 $g.L^{-1}$) was scored very bitter (45; **Fig. 3.3a**), similar to a caffeine solution of 0.4 $g.L^{-1}$. Regression of the dose-response data for bitter intensity of the extract indicated a prominent bitter taste (> 20 intensity score) even at ~1 $g.L^{-1}$ (**Fig. 3.3a**). Extreme bitter taste (> 60) was detected when the concentration was increased by more than 50%, and approached a plateau at the highest concentration tested (8 $g.L^{-1}$). It is, however, unlikely that such a high intensity will be encountered in real tea infusions.

Dose-response curves and bitter taste threshold concentrations for the fractions (**Fig. 3.3b-d**; Supplementary material, **Table A.6**) provide insight into their respective bitter taste contributions. The benzophenone-rich fraction did not have a distinctly bitter taste at any of the analysed concentrations (~5; **Fig. 3.3b**). Even at a concentration more than four times the IEC, bitter intensity was still negligible. Considering the previously observed associations between these benzophenone compounds and bitter taste

(Moelich, 2018; Erasmus, 2015) it is surprising that the fraction does not present a bitter taste on its own. Despite limited information relating to the taste activity of benzophenone compounds, the possibility of taste modulation caused by the benzophenone compounds, or the unidentified compounds in the benzophenone-rich fraction, should not be disregarded.

Limited solubility prevented testing of high concentrations of the xanthone-rich fraction so that the maximum concentration tested did not greatly exceed its IEC. Nevertheless, the bitter intensity for the xanthone-rich fraction at its IEC was by far the highest, scoring ~31 on a 100-point scale (**Fig. 3.3c**) and was thus most likely to contribute to the overall bitter taste of the infusion. In fact, the bitter intensity was still notable (> 10) when diluted to a third of its IEC (with approx. 40 $mg.L^{-1}$ mangiferin and 11 $mg.L^{-1}$ isomangiferin). Although the data might suggest that the xanthones (mangiferin and isomangiferin) contribute greatly to the bitter intensity of honeybush infusions, no literature were available on the taste activity of these individual xanthones. The bitter intensity of both mangiferin and isomangiferin was therefore determined. Mangiferin was also investigated by means of a dose-response test (**Fig. 3.4**). Its bitter intensity at its IEC in the hot water extract (178 $mg.L^{-1}$) scored a predicted 29 on the 100-point scale. According to the linear model (R^2 = 0.946), concentrations below 120 $mg.L^{-1}$ elicited only mild bitter taste (< 20), whereas higher concentrations (above 180 $mg.L^{-1}$) could result in bitter intensity of 30 and above.

Isomangiferin was less bitter than mangiferin when compared at a similar concentration (**Fig. 3.5**). This compound elicited a bitter intensity of only ~15 when tasted at both its IEC (34 $mg.L^{-1}$) and at that of mangiferin (118 $mg.L^{-1}$) in the xanthone-rich fraction. This indicates that isomangiferin had already reached maximum bitter intensity at a lower concentration than mangiferin. Tasted in combination with mangiferin at their IEC in the xanthone-rich fraction (34 $mg.L^{-1}$ isomangiferin and 118 $mg.L^{-1}$ mangiferin), the bitter intensity of the solution decreased to ~22. In contrast, mangiferin on its own (118 $mg.L^{-1}$) had a bitter taste of ~30. This bitter-supressing activity of isomangiferin illustrated the complex nature of bitter tastants in food matrices. The present study is the first to our knowledge to quantify bitter intensity of the important xanthones, mangiferin and isomangiferin.

Other food products known to be rich sources of xanthones, such as mangosteen (Wittenauer *et al*., 2012) and mango (Berardini *et al*., 2005) fruit, are not associated with a bitter taste. This may be as a result of the comparably large sweet taste contributions or taste modulation by saccharides and the cellulose matrix, or modulation by other compounds naturally present in the fruit. Alternatively, it is also possible that xanthones

in these foodstuffs are consumed at a comparably lower concentration than that found in honeybush, especially unfermented honeybush herbal tea.

The flavanone-rich fraction elicited a detectable bitter taste (~13) at its IEC, indicating that it probably contributes to the bitter taste of *C. genistoides* infusions (**Fig. 3.3d**). This is supported by the low threshold intensity at less than half its IEC (**Fig. 3.3d**). Higher concentrations resulted in a significant ($p < 0.05$) increase in bitter intensity, reaching ~34 at about three times its IEC (**Fig. 3.3d**). This fraction, however, contains a considerable amount of the major xanthones, mangiferin and isomangiferin, at its IEC (9 and 1 $mg.L^{-1}$, respectively; **Fig. 3.2d**, **Table 3.2**). Based on the linear model for mangiferin, its IEC in the flavanone-rich fraction (9 $mg.L^{-1}$) should result in a bitter intensity of < 10. Given the complexity of the fraction, however, as well as the modulatory interaction between mangiferin and isomangiferin, it is difficult to judge the exact contribution of flavanone compounds to the bitter taste of the flavanone-rich fraction.

The presence of similar flavanone compounds in citrus fruits has inspired research on the taste activity of several flavanones. Indeed, Horowitz and Gentili (1961) investigated the bitter taste of several citrus flavanone glycoside derivatives. The 7-*O*-rutinoside of naringenin, narirutin, had no taste activity, whereas the 7-*O*-neohesperidoside, naringin, was bitter. The structure of the sugar moiety is therefore important. The flavanone-rich fraction of honeybush contained flavanones conjugated with rutinose at position 7 (narirutin and hesperidin). In addition, several flavanones present in honeybush are structurally related to the known bitter taste modulator, eriodictyol (Reichelt *et al*., 2010a,b; Ley, 2008; Ley *et al*., 2005). This includes hesperidin (a 7-*O*-rutinoside), as well as its aglycone, hesperetin, present in small quantities in the current flavanone-rich fraction. The confirmed modulatory capacity of hesperetin from *C. intermedia* extracts (Reichelt *et al*., 2010b,c) also implies its effect on the taste of honeybush infusions.

As the sensory analysis of the fractions was not conducted in a completely aqueous solution, it is not possible to draw absolute conclusions comparable to that of the compounds in the infusion. In fact, it has been found that EtOH may increase bitter taste in wine (Fontoin *et al*., 2008), although these results have been contradicted (Rudnitskaya *et al*., 2010). Furthermore, the fractions were also tasted at room temperature whereas honeybush infusions are typically tasted hot (60 - 65 °C). Temperature has previously been implicated in modifying the taste profile of wines, including increasing bitter taste at lower temperatures (Ross *et al*., 2012). Tasting of pure mangiferin and isomangiferin in water at 60 °C, however, unequivocally confirmed that the two xanthones will contribute to bitter intensity of honeybush infusions when consumed. Bearing in mind

the limitations of the study, the results indicate the possibilities of bitter taste contributions and modulation within honeybush tea infusions.

4. Conclusions

Contrary to previous findings where benzophenone compounds were indicative in predicting bitter taste, the benzophenone-rich fraction did not itself have a bitter taste. However, these compounds may cause bitter taste amplification. The bitter taste of the herbal tea infusion was attributed largely to the direct bitter taste contributions of the flavanone- and xanthone-rich fractions. The xanthone-rich fraction, in particular, had a severely bitter taste due to the presence of mangiferin. The taste activity of mangiferin and isomangiferin that was reported here for the first time suggested a suppressive bitter taste interaction between the two regio-isomers. The presence of a small quantity of mangiferin may contribute to the bitter taste of the flavanone-rich fraction, but the concentration present is not sufficient to account for the bitter taste of this fraction. These findings support the possibility of taste modulation between honeybush phenolics, which should be considered in further investigations.

Addendum A. Supplementary material

Supplementary material associated with this chapter can be found in **Addendum A** (p 149).

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Table 3.1 Overall extraction yield and phenolic sub-class summary for each fraction

Material	Extract	EtOH-soluble and -insoluble fractions	XAD fractions	XAD sub-fractions	% Yield	% B	% X	% F	IEC
					g.100 g^{-1} plant material	g.100 g^{-1} hot water extract or XAD sub-fractions			$g.L^{-1}$
Plant material					100				
	Hot water extract				20	3.7	11.9	1.6	2
		EtOH-insoluble			10				
		EtOH-soluble			8.6	5.6	19.7	2.7	
			Polar	1 - 7	2.7				
			B	8 - 21	0.9	41.4	0.1	0.0	0.085
			X	22 - 28	2.6	2.5	60.7	2.3	0.258
			F	29 - 38	2.4	0.0	4.8	7.8	0.236

Benzophenone- (B), xanthone- (X) and flavanone-rich (F) fraction yields calculated from 13 quantified phenolics according to the method by Beelders *et al.* (2014b). IEC = infusion equivalent concentration as determined by % yield of solids from plant material and infusion soluble solids content of 2 $g.L^{-1}$.

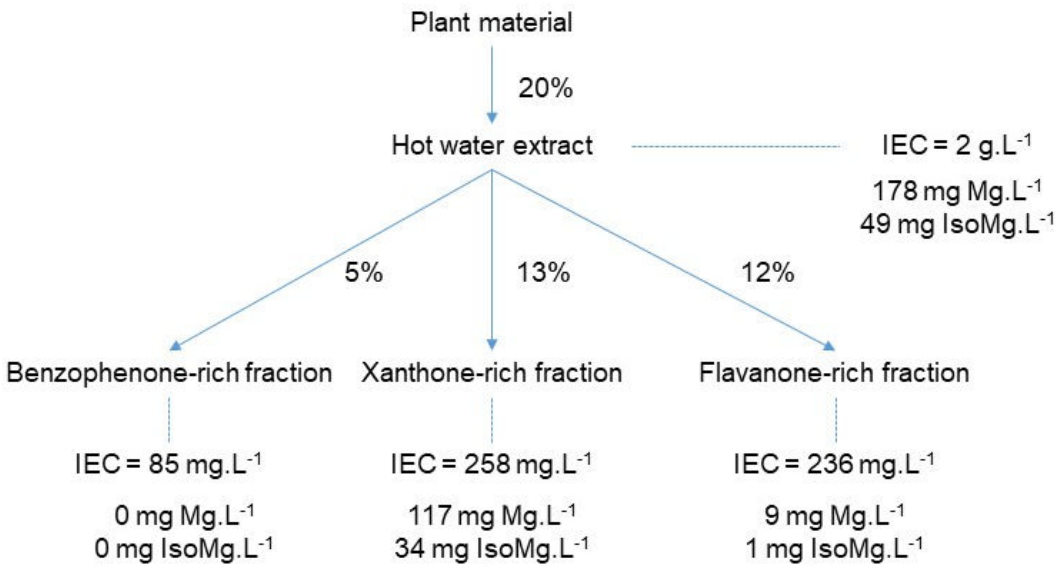

Figure 3.1 Mangiferin (Mg) and isomangiferin (IsoMg) infusion equivalent concentrations (IEC) relative to the hot water extract and crude phenolic fractions.

Table 3.2 Phenolics quantified in *Cyclopia genistoides* hot water extracts and fractions

	Compound (g.100 g^{-1} extract or fraction)													
	B1[a]	**B2**	**B3**	**B4**	**X1**	**X2**	**X3[b]**	**X4[b]**	**Fl1**	**F1[c]**	**F2**	**F3**	**F4**	**Total (% of SS)**
Hot water extract	0.058	1.534	0.505	1.597	8.885	2.433	0.023	0.033	0.490	0.152	0.153	1.208	0.084	17
Benzophenone-rich fraction	0.683	21.855	6.362	12.454	0.029	0.038	nd	nd	nd	nd	nd	nd	nd	41
Xanthone-rich fraction	nd	nd	nd	2.486	45.406	13.272	0.038	nd	1.930	0.814	0.280	1.187	nd	65
Flavanone-rich fraction	nd	nd	nd	0.018	3.691	0.511	0.148	0.174	0.229	0.045	0.737	6.264	0.742	13

B1 = maclurin-di-*O,C*-hexoside; B2 = 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone; B3 = 3-β-D-glucopyranosylmaclurin; B4 = 3-β-D-glucopyranosyliriflophenone; X1 = mangiferin; X2 = isomangiferin; X3 = Tetrahydroxyxanthone (THX)-*C*-hexoside isomer A; X4 = THX-*C*-hexoside isomer B; Fl1 = vicenin-2; F1 = eriodictyol-*O*-hexose-*O*-deoxyhexoside isomer A; F2 = naringerin-*O*-hexose-*O*-deoxyhexoside isomer A; F3 = naringenin-*O*-hexose-*O*-deoxyhexoside isomer B; F4 = hesperidin; SS = soluble solids; nd = not detected.

[a] Expressed as 3-β-D-glucopyranosylmaclurin equivalents.

[b] Expressed as mangiferin equivalents.

[c] Expressed as eriocitrin equivalents.

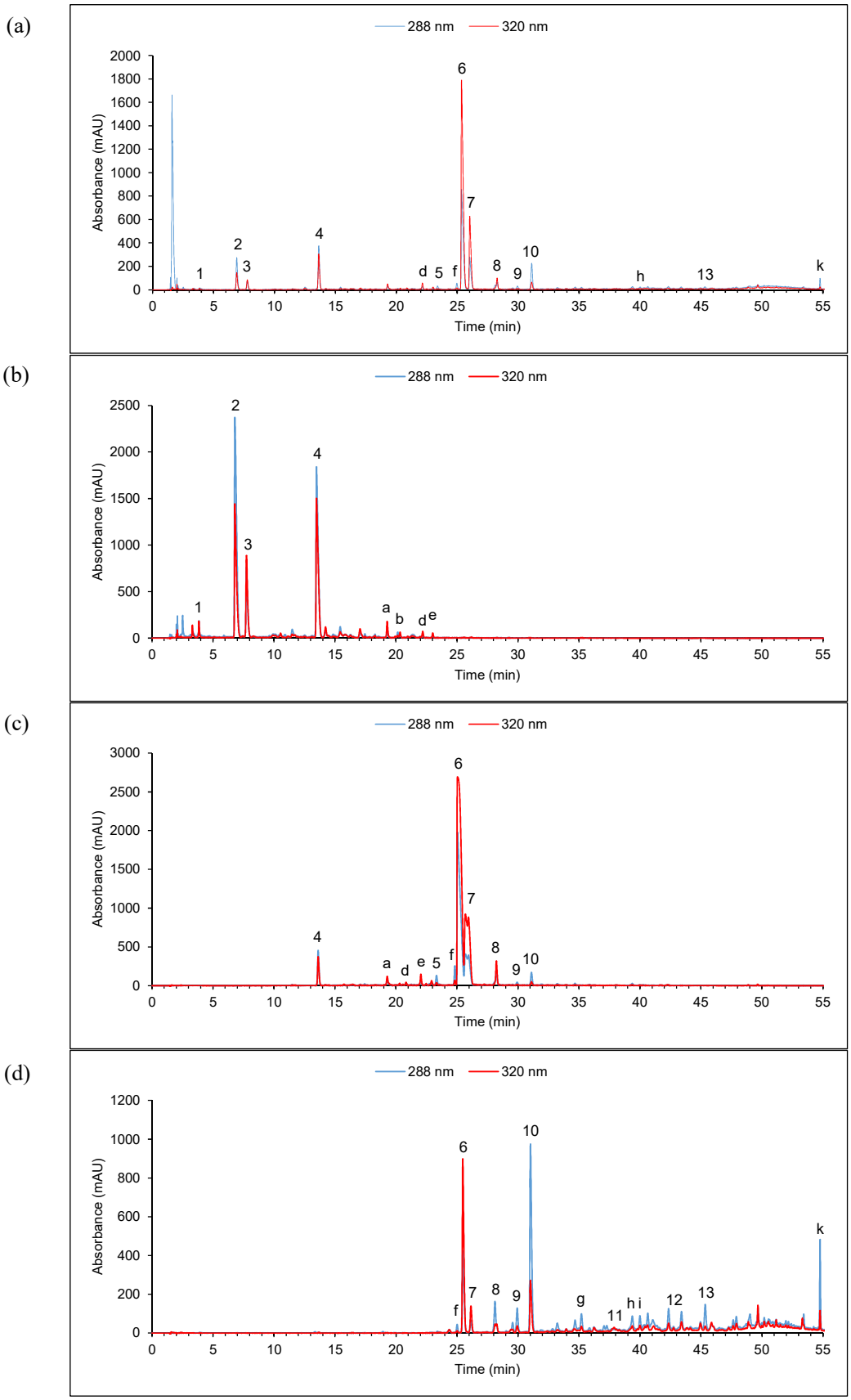

Figure 3.2 HPLC-DAD chromatogram of (a) hot water extract, (b) benzophenone-, (c) xanthone- and (d) flavanone-rich fractions. Peak numbers correspond to those in **Tables A.4** and **A.5** (Addendum A).

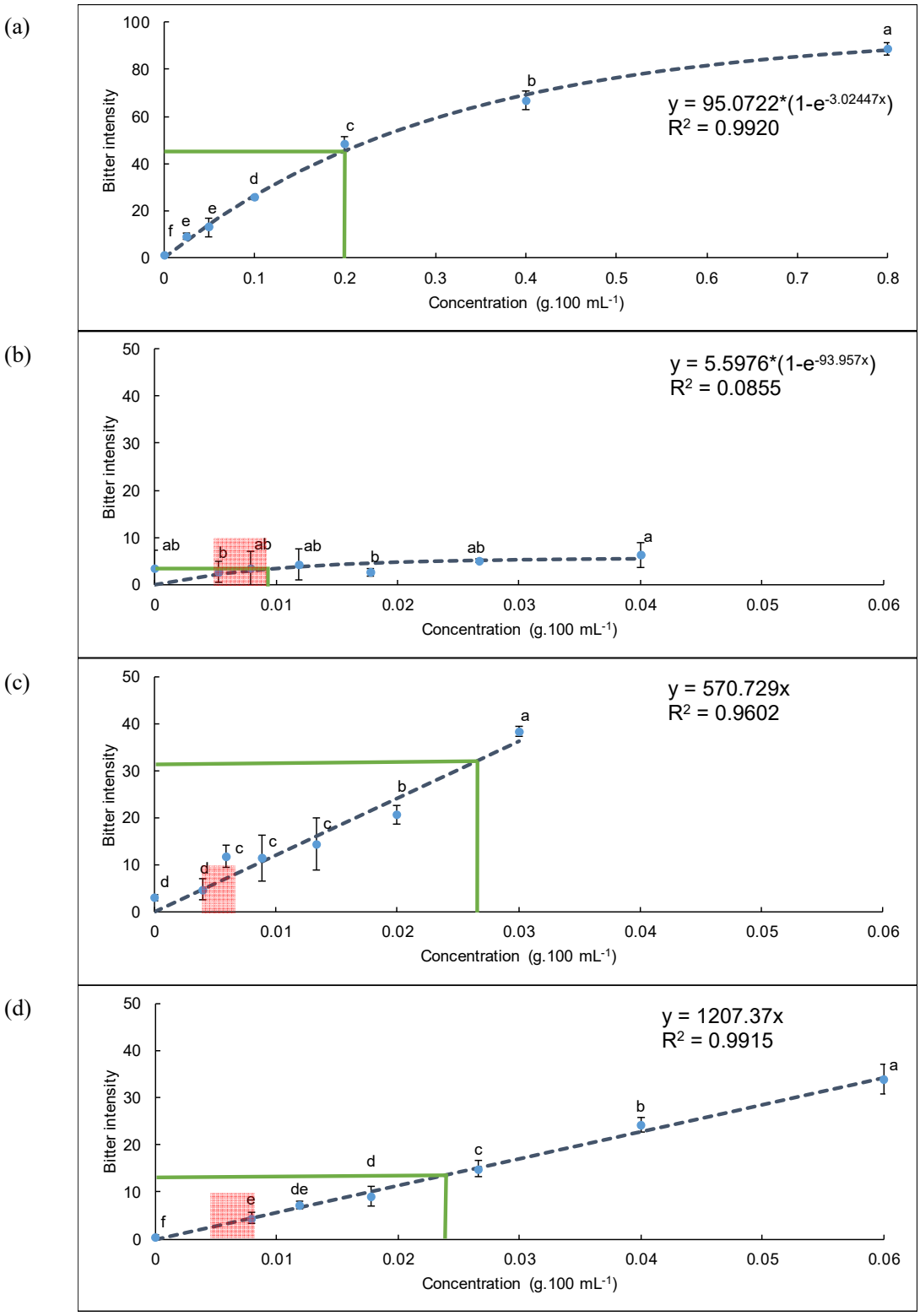

Figure 3.3 Dose-response bitter intensity of (a) hot water extract and dose-response bitter intensity and taste threshold concentrations of (b) benzophenone-, (c) xanthone- and (d) flavanone-rich fractions. The dotted line indicates the regression curve, the green line indicates infusion equivalent concentration. Pink range indicates sensory threshold concentration range. Different letters indicate significant differences ($p < 0.05$). Error bars indicate standard deviation.

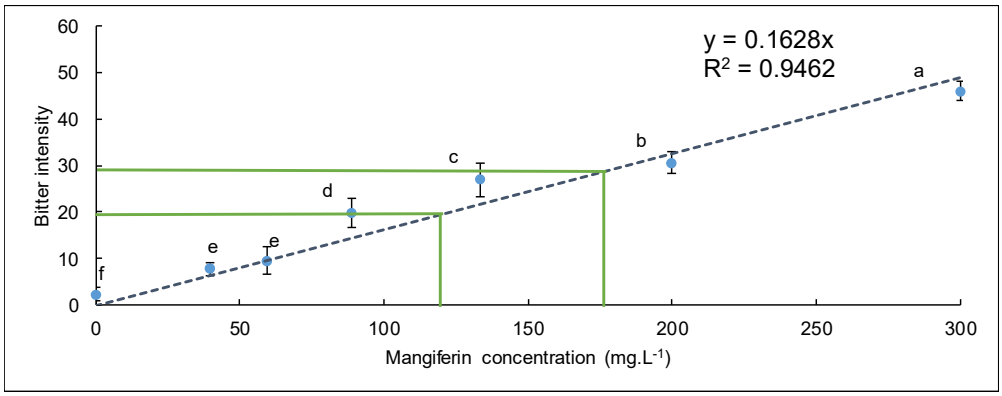

Figure 3.4 Dose-response bitter intensity analysis of mangiferin. The dotted line indicates the linear regression curve and the green lines indicate infusion equivalent concentration in the hot water extract (178 $mg.L^{-1}$) or the xanthone-rich fraction (118 $mg.L^{-1}$). Different letters indicate significant differences ($p < 0.05$). Error bars indicate standard deviation.

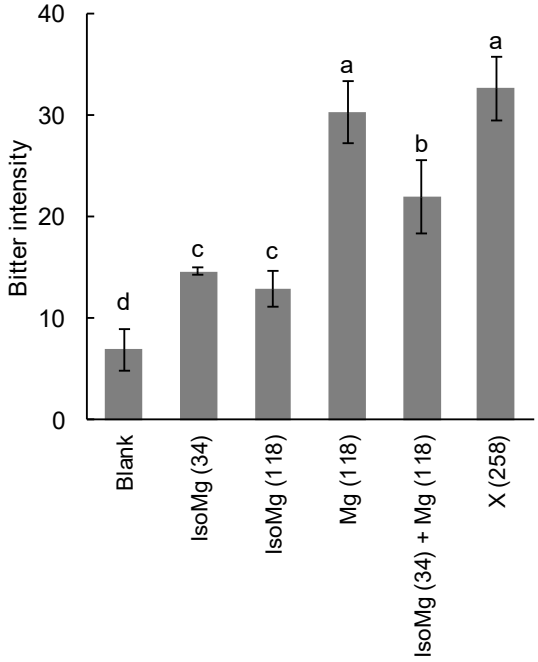

Figure 3.5 Comparative bitter intensities of mangiferin (Mg), isomangiferin (IsoMg) and the xanthone-rich fraction (X). Values in parenthesis indicate concentration as $mg.L^{-1}$. Different letters indicate significant differences ($p < 0.05$). Error bars indicate standard deviation.

PUBLICATION DECLARATION BY THE CANDIDATE

With regard to *Chapter 3 (Pp. 58-81)*, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Conducted all experimental work and data interpretation. Wrote the entire manuscript and edited the document in its entirety.	80%

The following co-authors have contributed to *Chapter 3 (Pp. 58-81)*:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Prof Dalene de Beer	DBeerD@arc.agric.za	Advice and assistance with chemical methods and analysis.	5%
Ms Magdalena Muller	MM7@sun.ac.za	Advice on sensory methods and analysis.	5%
Ms Marieta van der Rijst	VanDerRijstM@arc.agric.za	Advice and assistance with statistical analysis	5%
Prof Elizabeth Joubert	JoubertL@arc.agric.za	Advice and assistance with experimental approach and document structure.	5%

*Declaration with signature in possession of candidate and supervisor — Oct 2018

Signature of candidate — **Date**

PUBLICATION DECLARATION BY CO-AUTHORS

The undersigned hereby confirm that:

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to *Chapter 3 (Pp. 58-81)*,
2. no other authors contributed to *Chapter 3 (Pp. 58-81)*, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in *Chapter 3 (Pp. 58-81)* of this dissertation.

Signature*	Institutional affiliation	Date
Prof Dalene de Beer	Plant Bioactives Group, Post-Harvest & Agro-Processing Technologies, Agricultural Research Council (ARC), Infruitec-Nietvoorbij	
Ms Magdalena Muller	Department of Food Science, Stellenbosch University	
Ms Marieta van der Rijst	Biometry Unit, Agricultural Research Council (ARC), Infruitec-Nietvoorbij	
Prof Elizabeth Joubert	Plant Bioactives Group, Post-Harvest & Agro-Processing Technologies, Agricultural Research Council (ARC), Infruitec-Nietvoorbij	

Chapter 4
Modulation of bitter intensity of *Cyclopia genistoides*

Abstract

Variation in the bitter taste of *Cyclopia genistoides* (honeybush) herbal tea prompted investigation on the potential modulatory effect of crude benzophenone-, xanthone- and flavanone-rich phenolic fractions and their major individual phenolic compounds by descriptive sensory analysis. The fractions were prepared from a hot water extract of green *C. genistoides*. The tasteless benzophenone-rich fraction enhanced the bitter intensity of the xanthone- and flavanone-rich fractions, although neither of the major individual benzophenones retained this activity. 3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone, however, decreased the bitter intensity of the xanthone-rich fraction when added at a low concentration. It is speculated that minor maclurin-glycosides present in the benzophenone-rich fraction may contribute to bitter taste enhancement, as these compounds possess a catechol moiety that can activate the bitter taste receptor (TAS2R5). The flavanone-rich fraction suppressed the bitter intensity of the xanthone-rich fraction. Naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (NHDB), a major compound of the flavanone-rich fraction, enhanced the bitter intensity of the xanthone-rich fraction when added at concentrations comparable to that in fermented honeybush tea infusions. The complex nature of these bitter taste modulation interactions are concentration-dependent and may be responsible for the variable bitter intensity of *C. genistoides* herbal tea. In addition, the heat-induced conversion of NHDB to its A isomer (NHDA) was demonstrated, converting ~50% of NHDB to NHDA and a minor naringenin derivative. As several phenolic changes affecting compound concentration take place during honeybush fermentation (high-temperature oxidation processing), care should be taken when the suitability of plant material genotypes to produce honeybush tea with low bitter intensity is evaluated based on phenolic content before fermentation as part of the Agricultural Research Council's honeybush plant breeding programme.

1. Introduction

In our previous research, crude xanthone- and flavanone-rich fractions, prepared from a hot water extract of green *Cyclopia genistoides*, were shown to contribute to bitter taste, while the crude benzophenone-rich fraction did not have a bitter taste (Alexander *et al*., 2018). Besides direct bitter taste contributions, modulation of the bitter intensity, either by amplification or suppression, has been observed for various naturally occurring phenolic compounds (Ley, 2008). This modulation may be distinct from commonly perceived additive effects, as the responsible compounds often elicit no taste sensation in isolation. Reichelt *et al*. (2010c) reported that fractions of a *C. intermedia* extract may have both bitter and sweet taste modulating capacity. Indeed, previous research indicated that isomangiferin, a major xanthone present in *Cyclopia* species, suppresses the bitter intensity of its regio-isomer, mangiferin (Alexander *et al*., 2018).

Several phenolic compounds present in *Cyclopia* herbal teas (Schulze *et al*., 2015) could potentially have sensory modulatory properties. Eriodictyol, present in *C. intermedia* (Ferreira *et al*., 1998), and several related phenolic compounds have also been demonstrated to modulate bitter taste (Reichelt *et al*., 2010a,b,c; Ley *et al*., 2008a,b; 2005). The presence of related eriodictyol-derivatives in *C. genistoides* alludes to the possibility of bitter modulation by honeybush flavanones. In addition, several honeybush benzophenones were included in statistical models for prediction of the bitter intensity of honeybush infusions (Moelich, 2018; Erasmus, 2015). This contradicts our previous finding that a crude benzophenone-rich fraction containing ~41% quantifiable benzophenones had no bitter taste, even at concentrations higher than that typically found in the herbal tea infusion (Alexander *et al*., 2018), suggesting complex interactions between compounds.

The aim of the current study was to determine the effect of interactions between the phenolic fractions and individual phenolic compounds from a hot water extract of green *C. genistoides* on the bitter taste perception of infusions. In particular, the non-bitter benzophenone-rich fraction was investigated to determine the effects of this fraction, as well as individual benzophenones, 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG) and 3-β-D-glucopyranosyliriflophenone (IMG), on the bitter intensities of xanthone- and flavanone-rich fractions and the major bitter xanthone, mangiferin (**Chapter 3**). The flavanone-rich fraction and main flavanones, hesperidin and naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (NHDB) present in this fraction, were also investigated for their taste modulating effects.

2. Materials and methods

2.1 Chemicals

Details of chemicals, phenolic standards, XAD resin and water preparation are described in **Chapter 3**. Isomangiferin authentic phenolic reference standard (purity > 95%) was sourced from Phytolab (Vestenbergsgreuth, Germany) and IDG was isolated from *C. genistoides* (Beelders *et al*., 2014a).

2.1.1 *Preparation of phenolic-rich fractions*

A hot water extract of unfermented *C. genistoides* plant material was fractionated to prepare benzophenone-, xanthone- and flavanone-rich fractions as described in **Chapter 3**. Briefly, multiple separations were conducted on an open column loaded with XAD porous resin, using an EtOH-water gradient. Column fractions, monitored by high-performance liquid chromatography with diode array detection (HPLC-DAD), were pooled according to phenolic composition and solvents removed under vacuum (40 °C) before freeze-drying (VirTis genesis, Model 35ES, SP Scientific, Gardiner, NY, USA) to yield the three fractions of interest.

2.1.2 *Isolation of naringenin-O-hexose-O-deoxyhexoside isomer B*

Preparative HPLC was employed for the isolation of NHDB from the flavanone-rich fraction. Equipment consisted of a Waters preparative LC equipped with autosampler, variable UV-visible detector and fraction collector (Waters, Milford, USA). Solvents were degassed using helium sparging and a water bath was used to control the column temperature (35 °C). The flavanone-rich fraction was dissolved (5 $mg.mL^{-1}$) in a 23% MeOH-water solution containing 10% DMSO ($v.v^{-1}$) and filtered through a 0.45 µm hydrophilic PVDF syringe filter (Merck-Millipore) directly into a 10 mL autosampler vial. The sample was injected (5000 µL injection volume) on a Gemini-NX C_{18} preparative column (150 × 21.2 mm, 100 Å, 5 µm; Phenomenex, Torrance, USA) and separated at a flow rate of 21.2 $mL.min^{-1}$ for the collection of the target peak. The following gradient solvent programme (solvent A: 0.1% formic acid; solvent B: 100% MeOH) was employed: 0 - 2 min (23% B), 2 - 15 min (23 - 24% B), 15 - 16 min (24 - 90% B), 16 - 17 min (90% B), 17 - 18 min (90 - 23% B) and 18 - 25 min (23% B). Repeated injections were necessary to obtain an adequate quantity of purified compound (97% purity as confirmed by liquid chromatography with mass spectrometery (LC-MS), **section 2.2.3**). The collected fractions were pooled and the solvent evaporated at 40 °C under vacuum. The residue was suspended in HPLC-grade water and freeze-dried. Further drying was carried out at 40 °C under vacuum for 24 h in the presence of P_2O_5.

2.1.3 *Phenolic quantification and identification*

Phenolic quantification was conducted by HPLC-DAD using the species-specific method for *C. genistoides* (Beelders *et al*., 2014). Qualitative analysis was conducted using LC-MS, as described in **Chapter 3** (Beelders *et al*., 2014).

2.2 Bitter taste modulation

2.2.1 *Phenolic-rich fractions*

A series of experiments was conducted to determine the effect of the respective phenolic-rich fractions on the bitter intensity of the xanthone- and flavanone-rich fractions. Each fraction was evaluated at concentrations similar to their infusion equivalent concentration (IEC, as defined in **Chapter 3**), by dissolving the sample in 2.5% EtOH-water ($v.v^{-1}$). A specific fraction (IEC's simplified as 300 $mg.L^{-1}$ for both the xanthone- and flavanone-rich fractions and 100 $mg.L^{-1}$ for the benzophenone-rich fraction) was combined with each of the other phenolic-rich fractions at three concentration levels (100, 200 and 300 $mg.L^{-1}$) for sensory analysis.

2.2.2 *Phenolic compounds*

The ability of individual phenolic compounds to modulate the bitter intensity of the crude xanthone-rich fraction, as well as the effect of the benzophenone- and flavanone-rich fractions on the bitter intensity of mangiferin, was investigated by performing several experiments. A dose range employed a blank sample in each case. The respective IEC of each compound was used as a guideline for dose determination, with the actual dose range varying slightly depending on sample availability and compound solubility. The IEC of hesperidin was based only on its infusion concentration according to Schulze *et al*. (2015) without taking into account its fractional contribution to the soluble solids of the hot water extract. A summary of the combinations and their intended IEC values is presented in **Tables B.1** and **B.2** (Supplementary material).

The effect of the isomerisation of NHDB to NHDA on its taste was also determined. The isomer mixture was prepared by heating an aqueous solution of NHDB at its IEC for 16 h at 90 °C, a temperature-time regime used to produce optimum aroma development during fermentation of *C. genistoides* plant material (Erasmus *et al*., 2017). The HPLC-DAD chromatogram of the solution before and after heating is presented as Supplementary material (**Fig. B.1**).

For sensory analysis of individual compounds and fraction-compound combinations, the samples were solubilised in hot water by heating for *ca* 30 min in jacketed flasks connected to a circulation bath at 80 °C. The solution was continuously stirred with the assistance of a magnetic stirrer bar.

2.3 Descriptive sensory analysis

Descriptive sensory analysis was conducted as described in **Chapter 3**. For comparison of fractions, samples solubilised in 2.5% EtOH ($v.v^{-1}$) were presented to each panellist (10 mL servings) in clear 30 mL plastic cups at ambient temperature (21 °C), as described in **Chapter 3**. A summary of all combinations tested is presented in Supplementary material (**Table B.1**). For combinations of fractions and pure compounds, samples were served (10 mL servings) in 40 mL screw-cap amber vials. Samples were presented in metal racks placed in water baths to be kept warm (60 °C) during analysis to prevent precipitation during cooling and taking into account the typical serving temperature of honeybush infusions. Preliminary testing confirmed that phenolic degradation of samples during preparation and testing as a result of exposure to heat was negligible. A summary of combinations tested is presented in Supplementary material (**Table B.2**).

Panel training and sample familiarisation were guided by an experienced panel leader during 30 min sessions with 10 min breaks scheduled between consecutive sessions. Two training sessions were conducted per day. Bitter intensity of the samples was determined by presenting each sample set (n = 5 - 6 samples) to each panellist during triplicate 20 min sessions. Once again, 10 min breaks were scheduled between sessions and no more than three sessions conducted per day to prevent panel fatigue. Marked reference samples were presented to assist scoring of bitter intensity on the defined bitter intensity scale (0 = 2.5% EtOH at room temperature for fraction combinations and hot water for phenolic compound combinations; 45 = 2 $g.L^{-1}$ hot water extract equal in bitter taste to a 0.4 $g.L^{-1}$ caffeine solution). Following tasting of each sample, a 2.5 min delay was implemented to minimise bitter carry-over between samples. As per standardised practice, samples were blind-coded and randomised. Separate booths fitted with red lighting were allocated to panellists during analysis and *Compusense® five* software (Compusense, Guelph, Canada) was used for data capture on an anchored (0 = not bitter, 100 = extremely bitter), unstructured line scale. During analysis, ambient temperature was controlled at 21 °C. Panellists were provided with distilled water, dried apple pieces and water biscuits as palate cleansers between samples.

2.4 Statistical analysis

Panel performance was monitored, sensory data were pre-processed, outliers were removed and statistical analyses of quantitative sensory data were performed as described in **Chapter 3**.

3. Results

3.1 Benzophenone-rich fraction and benzophenones

The potential of the benzophenone-rich fraction as a bitter taste modulator was investigated by addition to both the xanthone- and flavanone-rich fractions, as well as the bitter compound, mangiferin. The bitter intensities observed at room temperature for the individual crude phenolic fractions and the combination of the benzophenone-fraction with xanthone- and the flavanone-rich fractions, respectively, are depicted in **Fig. 4.1**. Samples were dissolved and analysed in 2.5% EtOH. The xanthone- and flavanone-rich fractions were both fixed at 300 mg.L^{-1}, while the benzophenone concentration was varied (100, 200 and 300 mg.L^{-1}). As previously demonstrated (**Chapter 3**), the benzophenone-rich fraction was not bitter at a concentration approximating its IEC (100 mg.L^{-1}). Bitter intensity was increased when increasing concentrations of the benzophenone-rich fraction were added to the fixed concentration of the xanthone- and flavanone-rich fractions. The xanthone-fraction (300 mg.L^{-1}), spiked with 200 or 300 mg.L^{-1} benzophenone-fraction was significantly ($p < 0.05$) more bitter than the xanthone-fraction alone (**Fig. 4.1a**). A similar trend was observed for the flavanone-rich fraction when combined with the benzophenone-fraction at 200 or 300 mg.L^{-1} (**Fig. 4.1b**). The bitter intensity of mangiferin at its IEC (178 mg.L^{-1}) was, however, not significantly ($p \geq 0.05$) affected by the benzophenone-rich fraction (50, 100 and 200 mg.L^{-1}; data not shown) when prepared in water (60 °C).

For further elucidation of the role of benzophenones, the two major benzophenones, IMG and IDG, were investigated for their potential modulation of the bitter intensity of the xanthone-rich fraction (300 mg.L^{-1}), prepared in water and served hot (60 °C). Only two dose concentrations of IMG were tested, *i.e.* its IEC (32 mg.L^{-1}) and half of this concentration (16 mg.L^{-1}; **Fig. 4.2a**), because of a limited quantity of material available. The three dose concentrations of IDG tested included its IEC (31 mg.L^{-1}), 0.5 IEC (16 mg.L^{-1}) and 2 IEC (62 mg.L^{-1}; **Fig. 4.2b**). The mono-glucoside, IMG, did not have a significant effect ($p \geq 0.05$) on the bitter intensity of the xanthone-rich fraction (**Fig. 4.2a**), nor did it elicit a notable bitter taste (< 10 on a 100-point scale) at its IEC. The di-glucoside, IDG, also had a negligible bitter taste at its IEC (**Fig. 4.2b**), but it decreased the bitter intensity of the xanthone-rich fraction significantly ($p < 0.05$) when

added at 0.5 IEC and IEC (16 and 31 mg.L^{-1}, respectively). When IDG was added at double its IEC (62 mg.L^{-1}), the bitter intensity of the sample did not differ from that of the xanthone-rich fraction on its own.

3.2 Flavanone-rich fraction and flavanones

For investigation of possible bitter modulation when the xanthone- and flavanone-rich fractions are combined, different concentrations of the one fraction (100, 200 and 300 mg.L^{-1}) was added to the other fraction fixed at 300 mg.L^{-1} (**Fig. 4.3**). These samples were also prepared in 2.5% EtOH and served at ambient temperature (21 °C). In both cases, bitter intensity increased with increasing dose concentrations of the other fraction. This was not unexpected, as both the xanthone- and flavanone-rich fractions were bitter without the presence of the other fraction. The results confirmed that the xanthone-rich fraction was more bitter than the flavanone-rich fraction when compared at the same concentration. The lowest dose of the flavanone-rich fraction (100 mg.L^{-1}) did not have a notable bitter taste (< 10; **Fig. 4.3a**), but was moderately bitter (~25) at 300 mg.L^{-1} (**Fig. 4.3b**). The xanthone-rich fraction at 300 mg.L^{-1} was very bitter (~45; **Fig. 4.3a**) and still notably bitter (~20) at its lowest dose concentration (100 mg.L^{-1}; **Fig. 4.3b**). The combinations of these two fractions indicated the possibility of bitter taste suppression. Specifically, a combination of the flavanone-rich fraction at 300 mg.L^{-1} and the xanthone-rich fraction at its lowest dose (100 mg.L^{-1}) resulted in a bitter intensity of ~30 (**Fig. 4.3b**), compared to a theoretical bitter intensity of ~45, when an additive effect is assumed. Furthermore, the bitter score of ~30 for the combined sample was not significantly higher than that of the flavanone-rich fraction alone, despite the considerable bitter taste of the added xanthone-rich fraction (bitter intensity ~20). This effect was not as notable with the addition of different doses of the flavanone-rich fraction to the xanthone-rich fraction (**Fig. 4.3a**), possibly as a result of the high bitter intensity of the xanthone-rich fraction at this concentration (~45). As demonstrated in **Chapter 3** (**Fig. 3.3a**), higher bitter intensities do not always necessitate a proportionally higher response at a higher concentration, as the bitter intensity may reach a plateau region in the dose-response curve (suprathreshold region).

A similar result was observed between combinations of the flavanone-rich fraction and mangiferin at its IEC in the hot water extract (178 mg.L^{-1}; **Fig. 4.4**). Although both the flavanone-rich fraction (~30) and mangiferin (~25) were bitter, the combination resulted in a bitter intensity of only ~45, less than a theoretical ~55 if the effect was additive.

The two major flavanones, hesperidin and NHDB, were added at different dose concentrations to the xanthone-rich fraction at 300 mg.L^{-1} (prepared and served in hot water). As the hesperidin content of the hot water extract was less than that representative of typical honeybush infusions, its typical concentration in an infusion (11 mg.L^{-1}; Schulze *et al*., 2015) and its limited solubility guided the selection of its dose concentration range (7 and 15 mg.L^{-1}). Hesperidin at these concentrations did not have a significant effect ($p \geq 0.05$) on the bitter intensity of the xanthone-rich fraction (**Fig. 4.5a**). The bitter taste of hesperidin was low (~10), although panellists did note during training sessions that the bitter taste of this compound is immediate and intense, but disappears almost immediately.

NHDB was added at two dose concentrations to the xanthone-rich fraction (**Fig. 4.5b**). Due to limited availability of NHDB, the concentrations equalled 0.5 IEC (12 mg.L^{-1}) and IEC (24 mg.L^{-1}). The purified compound did not have a notable bitter taste (< 10) at IEC (24 mg.L^{-1}), but the lower concentration (12 mg.L^{-1}) elicited a significant ($p < 0.05$) increase in the bitter intensity of the xanthone-rich fraction.

The heated NHDB solution (IEC, 24 mg.L^{-1}), partially converted to isomer A (~45% conversion), was compared to pure isomer B (24 mg.L^{-1}) and the xanthone fraction (300 mg.L^{-1}; Supplementary material, **Fig. B.2**). Neither NHDB, nor the isomerised mixture had a prominent bitter taste (*ca* 10). NHDB elicited no significant effect ($p \geq 0.05$) on the bitter intensity of the xanthone-rich fraction, and neither did the isomerised mixture.

4. Discussion

In our previous investigation (**Chapter 3**), we demonstrated modulation of the bitter taste of mangiferin by isomangiferin, both present predominantly in the xanthone-rich fraction. We also demonstrated that the intensity of the bitter taste elicited by the xanthone-rich fraction was not sufficient to explain the bitter intensity of the complete extract at its IEC. The IEC values of the fractions were used as starting point to ensure that their sensory analyses were carried out at realistic concentrations, given inherent variation in phenolic composition of infusions (Schulze *et al*., 2015) and the typically higher phenolic content of unfermented plant material than processed, fermented material (Beelders *et al*., 2015). Potential bitter taste modulation was investigated to better understand factors contributing to the bitter intensity of some honeybush infusions.

In order to facilitate identification of potential bitter compounds, databases based on chemical structure and experimental data have been developed to predict the bitter taste responses of various compounds (Huang

et al., 2015; Wiener *et al*., 2011). One of these databases, BitterX, has been developed to predict the probability of bitter receptor (TAS2R) activation using a model based on documented interactions between a range of phenolic compounds and the known TAS2R structures *in vitro* (Huang *et al*., 2015). The model utilises similarities of structural features of the phenolic compounds to predict the probability of activation for each receptor. These predictions may aid in explaining bitter agonist/antagonist receptor interactions by relating structural information to observed bitter supressing/enhancing effects in the current study (Supplementary material, **Table B.3**).

4.1 Potential of benzophenone-rich fraction and benzophenone compounds as bitter taste modulators

The results emphasise complex interactions that may be involved between benzophenones and the bitter xanthones in honeybush infusions. The benzophenone-rich fraction significantly ($p < 0.05$) enhanced the bitter taste of the xanthone-rich fraction (**Fig. 4.1a**), although it had no significant effect ($p \geq 0.05$) on the bitter taste of mangiferin. The individual benzophenone compounds, when added to the xanthone-rich fraction, however, did not appear to retain the same bitter enhancing activity. Furthermore, the mono-glucoside, IMG, did not seem to affect the bitter intensity of the xanthone-rich fraction (**Fig. 4.2a**), while the di-glucoside, IDG, decreased the bitter intensity of the xanthone-rich fraction, especially at lower doses (16 and 31 $mg.L^{-1}$; **Fig. 4.2b**).

Structural similarities between xanthones and benzophenones may translate into common bitter taste receptor (TAS2R) binding. Glycosolation has been shown to be a significant factor in structure-activity relationships between phenolic compounds and bitter taste receptors (Soares *et al*., 2013; Bufe *et al*., 2002). For both the benzophenones and xanthones, the hydrophobic residue is attached to glucose by a β-glycosidic bond, a structural feature activating TAS2R16, although the hydrophobicity and size of the aglycone retain a high relevance (Bufe *et al*., 2002). Soares *et al*. (2013) also suggested that glucose residues may be important for activation of the bitter receptor, TAS2R7. They observed bitter receptor activation for the glycosylated anthocyanin, 3-β-D-glucopyranosyloxymalvidin, although a previous study (Vidal *et al*., 2004) showed that a fraction containing five anthocyanidin-glucosides (with 3-β-D-glycopyranosyloxymalvidin as the major component) was not bitter. This suggests that although receptors may be activated by phenolic compounds, the interaction between bitter a taste receptor and a compound does not necessarily translate into *in vivo* bitter taste perception.

The BitterX programme (Huang *et al*., 2015), developed to predict interactions between the different TAS2Rs and bitter compounds, including phenolic compounds, predicts similar binding receptors for both IMG and IDG, and the major xanthones, mangiferin and isomangiferin (Supplementary material, **Table B.3**). The probability for the binding of IDG, isomangiferin and mangiferin to TAS2R5 is particularly high (71%, 67% and 69%, respectively), whereas IMG has a lower chance of interaction (54%) with the same bitter receptor. Given that IDG lowered the bitter intensity of the xanthone-rich fraction, it is plausible that this compound may act as an antagonist of the bitter taste receptors activated by compounds in the xanthone-rich fraction. This relatively large compound (2 sugar moieties) may also sterically block the bitter xanthones from binding to the bitter taste receptors. A confounding result, however, was that the highest dose of IDG tested (62 $mg.L^{-1}$) did not elicit a modulatory response at a fixed xanthone concentration (300 $mg.L^{-1}$), challenging this argument relating to antagonism and invites further investigation.

Considering the fermentation (high-temperature oxidation) processing step (90 °C/16 h or 80 °C/24 h) of traditional honeybush tea manufacture, IMG, mangiferin and isomangiferin are extensively degraded, whereas IDG is very stable under these conditions (Beelders *et al*., 2017; 2015), changing the relative ratios of the compounds in the infusion. In addition, IDG is very prominent in *C. genistoides* and *C. longifolia* (Schulze *et al*., 2015). Some samples of both species were previously shown to produce bitter herbal teas (Moelich, 2018; Erasmus *et al*., 2017). The stability of IDG during processing would mean that its concentration would remain high even after the fermentation step, thereby possibly exceeding the necessary concentration threshold at which a bitter reducing effect may be observed. This may explain the variation in bitter intensity of some batches of plant material where the xanthone content of the batches is similar, but their IDG content differ.

Nevertheless, the above-mentioned argument does not explain the bitter taste amplification afforded by the benzophenone-rich fraction as a whole. This may be related to some of the other compounds (not UV-absorbing) in this fraction, or the minor benzophenones, maclurin-di-*O*,*C*-hexoside (MDG) and 3-β-D-glucopyranosylmaclurin (MMG). Both these benzophenones are structurally even more similar to the major xanthones, because of the presence of the catechol group, a structural feature of phenolic compounds associated with activation of TAS2R5 (Soares *et al*., 2013). Although the presence of the catechol group is not sufficient evidence for ligand binding, it results in a higher activation response (Soares *et al*., 2013). Other evidence for the role of hydroxyl groups is the finding that TAS2R39 responds with higher activation to the presence of

three hydroxyl groups in the ligand as opposed to the presence of two or less hydroxyl groups as demonstrated for isoflavonoids (Roland *et al*., 2013). The possible synergistic effect of the maclurin derivatives on xanthone bitter intensity may stem from an agonist activity following interaction with the relevant bitter taste receptors.

Both MMG and MDG are highly susceptible to degradation (Beelders *et al*., 2017; 2015) and are often not present at detectible levels in fermented honeybush tea infusions (Schulze *et al*., 2015). Their presence in and subsequent possible bitter taste enhancement of infusions prepared from the unfermented tea material may thus be irrelevant for fermented tea material, however, MMG is converted to mangiferin and isomangiferin during heating (Beelders *et al*., 2017), which will impact on bitter taste. Elucidation of their potential role in bitter taste modulation of xanthones merits investigation in future.

4.2 Potential of flavanone-rich fraction and flavanone compounds as bitter taste modulators

Several common flavanones have been identified as bitter modulators (as reviewed by Ley, 2008). These include flavanone aglycones, some either present in honeybush as aglycones or glycosides. Indeed, hesperetin, the aglycone of the common *Cyclopia* compound, hesperidin, is a known sweet taste enhancer (Reichelt *et al*., 2010b). Its presence at a low quantity in the current flavanone-rich fraction has been confirmed (**Chapter 3**). Eriodictyol, also a known honeybush flavanone (*C. intermedia*; Ferreira *et al*., 1998), has been shown to act as an effective bitter masking compound (Ley *et al*., 2005), together with several of its derivatives: homoeriodictyol, its sodium salt and sterubin (Ley *et al*., 2005; Reichelt *et al*., 2010b).

Results obtained for combinations of the xanthone- and flavanone-rich fractions support the possibility of bitter taste suppression. Both fractions, tested on their own, elicited a bitter response, although to different degrees. The bitter suppressing effect was most notable for the combination of 300 $mg.L^{-1}$ flavanone-rich fraction and 100 $mg.L^{-1}$ xanthone-rich fraction (**Fig. 4.3b**). The bitter taste of the combination was less intense than a theoretical summation of their individual bitter taste intensities. This effect was also observed for the combination of the flavanone-rich fraction with mangiferin (**Fig. 4.4**).

Two individual flavanone compounds were subsequently also tested for bitter taste contribution in combination with the xanthone-rich fraction. Hesperidin, as the major flavanone compound in *Cyclopia* species, is usually present at varying levels (4.2 - 15.9 $mg.L^{-1}$) in infusions of fermented *C. genistoides* (Schulze *et al*., 2015). The plant material selected for the current study had a notably low hesperidin content, probably due to a low stem content, as leafless stems were removed before cutting and drying (**Chapter 3**). De Beer *et*

al. (2012) and Du Preez *et al*. (2016) showed that the stems are the major source of hesperidin in herbal tea prepared from *C. subternata* and *C. maculata*, respectively. The IEC of hesperidin, based on its concentration in the hot water extract was therefore not used in the tests, as this value was very low (2 $mg.L^{-1}$) and therefore did not represent a typical honeybush infusion. For this reason, hesperidin was tested at the IEC of fermented *C. genistoides* (Schulze *et al.*, 2015). Hesperidin on its own was somewhat bitter, with panellists noting during preliminary training sessions that the solution elicited an immediate bitter response that also disappeared rapidly. Flavanone-7-*O*-rutinosides such as hesperidin are considered to have no bitter taste of their own (Rousseff *et al*., 1987, as referenced by Frydman *et al*., 2004). The BitterX model, however, still predicts possible TAS2R interaction (Supplementary material, **Table B.3**). Although our results indicated no significant difference ($p \geq 0.05$) between the xanthone-rich fraction and the samples spiked with hesperidin (**Fig. 4.5a**), it is still possible that a modulatory interaction could take place. It is notable that all scores were slightly higher than in previous tests. Indeed, preliminary training indicated that hesperidin may increase the bitter taste of the xanthone fraction. This may be due to a possible "carry-over" modulatory effect by hesperidin, causing a bitter enhancing effect even after the 3 min waiting period between the testing of samples.

NHDB significantly increased the bitter taste of the xanthone-rich fraction, although it has a negligible bitter intensity in isolation (**Fig. 4.5b**). This lack of a bitter response for the pure compound is surprising due to its structural similarity to the bitter flavanone, naringin, responsible for the bitter taste of grapefruit (Guadagni *et al*., 1974; 1973). Furthermore, the bitter enhancing effect elicited by NHDB was more pronounced at the lower concentration (12 $mg.L^{-1}$), equalling approximately 50% of its concentration in the hot water extract at its IEC. This result may relate to the degradation of this compound during honeybush herbal tea processing. As this compound is converted into NHDA during processing (Supplementary material, **Fig. B.1**), species such as *C. genistoides* with high initial concentrations of NHDB in the unprocessed plant material will subsequently produce fermented plant material and infusions with lower NHDB concentrations. Such an interplay between concentration of different compounds and bitter response may result in infusions with an unacceptably bitter intensity, despite reduced mangiferin levels as a result of fermentation. It should also be considered whether the mixture of NHDB and NHDA, formed upon heating (Supplementary material, **Fig. B.2**), could result in a different taste modulating effect when compared to the modulating effect observed for the lower concentration of NHDB (12 $mg.L^{-1}$), at concentrations typically present in fermented *C. genistoides* infusions (7 and 8 $mg.L^{-1}$ for NHDA and NHDB, respectively; Schulze *et al*., 2015). The current

investigation, however, revealed no taste modulating effect when the combination of NHDA and NHDB (10 and 13 mg.L^{-1}, respectively; Supplementary material, **Table B.3**) was added to the xanthone fraction (Supplementary material, **Fig. B.2**). Given that 24 mg.L^{-1} NHDB has no bitter enhancing effect on the xanthone-rich fraction and the combined concentration of NHDB and NHDA was ~24 mg.L^{-1}, it can be inferred that NHDA either elicits a similar response to NHDB, or that it hinders the bitter modulation of NHDB observed at low concentrations (12 mg.L^{-1}). Further investigation into the changes affected by fermentation and their effects on bitter taste would thus be warranted.

5. Conclusions

Both bitter enhancing and bitter reducing effects were observed. A novel bitter agonist (NHDB) and antagonist (IDG) were thus identified, although the extent of modulation depends on the dose concentration of the modulatory compounds. The capacity of these modulators was typically more pronounced at lower concentrations. Lowering of the phenolic content of *C. genistoides* plant material though fermentation will thus translate into changes to the bitter intensity of its infusions. Although this study represents only a first step in identifying possible modulatory compounds in honeybush, the findings emphasise the complex interactions between phenolic components of honeybush infusions. This will be further explored by investigating the feasibility of a bitterness prediction model based on the concentration of selected compounds.

Addendum B. Supplementary material

Supplementary material associated with this chapter can be found in **Addendum B** (p 157).

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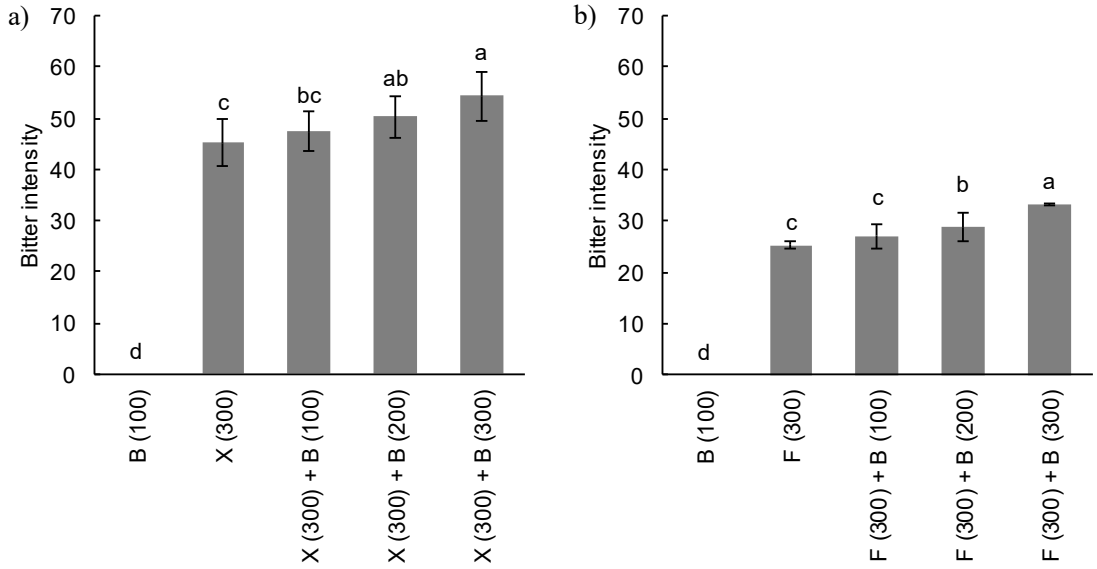

Figure 4.1 Bitter intensity of (a) X and (b) F in combination with B at three dose concentrations. Samples were prepared in 2.5% EtOH and analysed at ambient temperature (21 °C). B = benzophenone-rich fraction, X = xanthone-rich fraction, F = flavanone-rich fraction. Values in parentheses indicate concentration as $mg.L^{-1}$. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

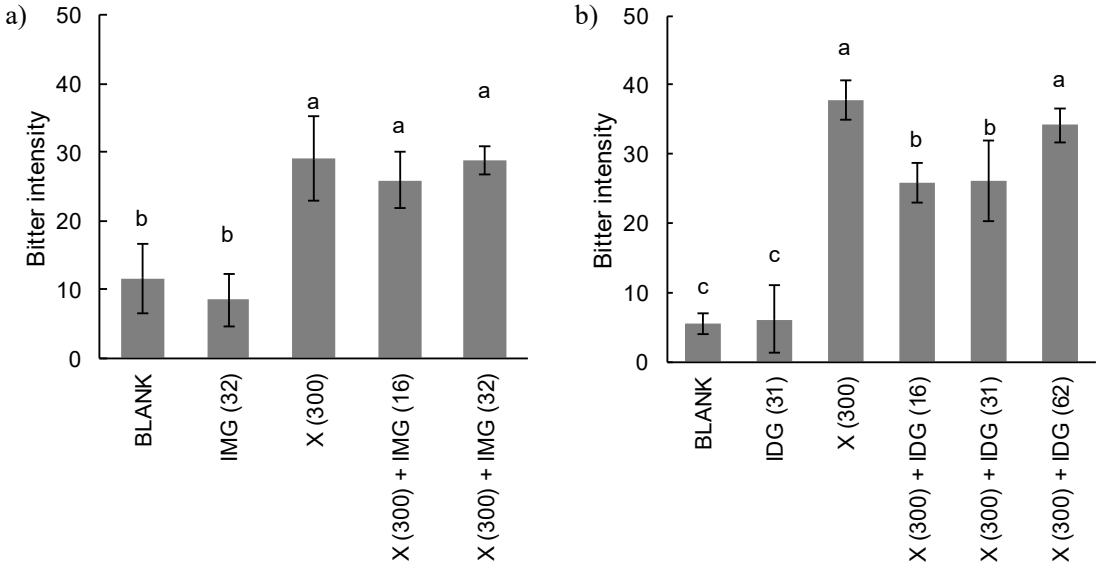

Figure 4.2 Bitter intensity of X in combination with (a) IMG at two dose concentrations and (b) IDG at three dose concentrations. Samples were prepared in hot water and analysed at 60 °C. BLANK = water, IMG = 3-β-D-glucopyranosyliriflophenone, IDG = 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone, X = xanthone-rich fraction. Values in parentheses indicate concentration as $mg.L^{-1}$. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

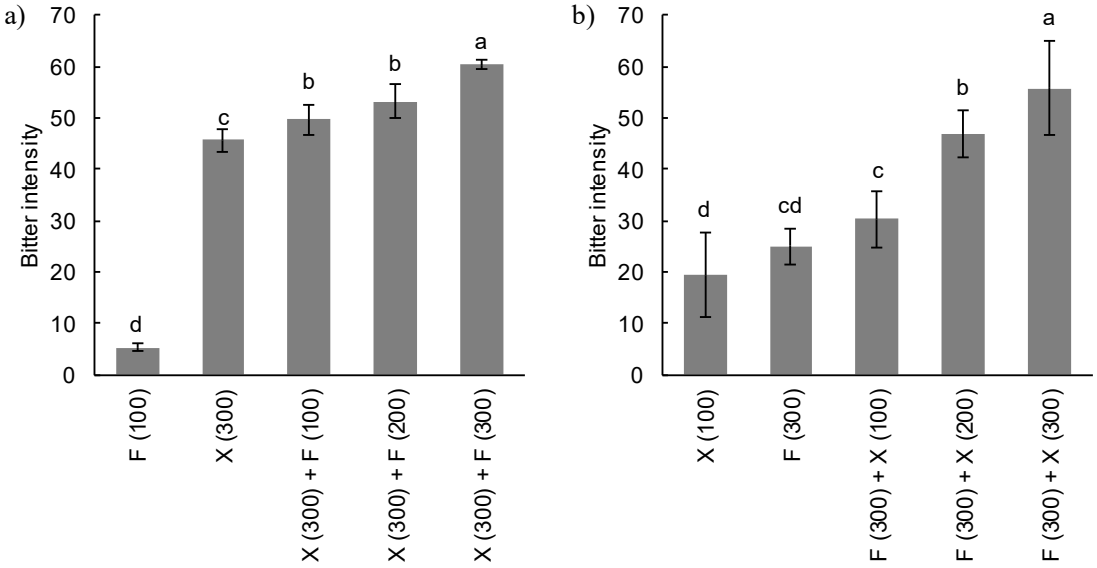

Figure 4.3 Bitter intensity of (a) X in combination with F at three dose concentrations and (b) F in combination with X at three dose concentrations. Samples were prepared in 2.5% EtOH and analysed at ambient temperature (21 °C). F = flavanone-rich fraction, X = xanthone-rich fraction. Values in parentheses indicate concentration as $mg.L^{-1}$. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

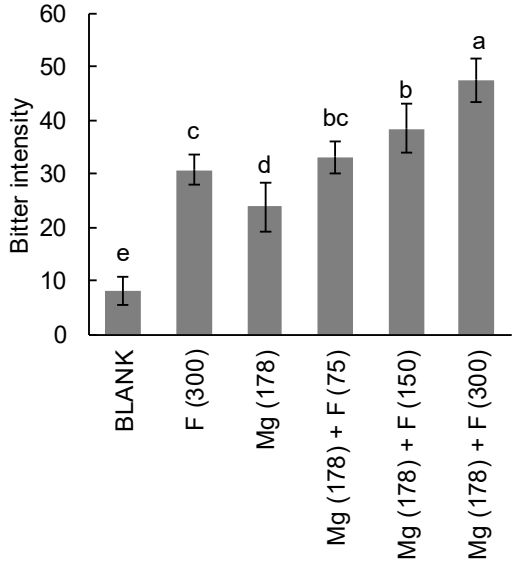

Figure 4.4 Bitter intensity of Mg in combination with F at three dose concentrations. Samples were prepared in hot water and analysed at 60 °C. BLANK = water, Mg = mangiferin, F = flavanone-rich fraction. Values in parentheses indicate concentration as $mg.L^{-1}$. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

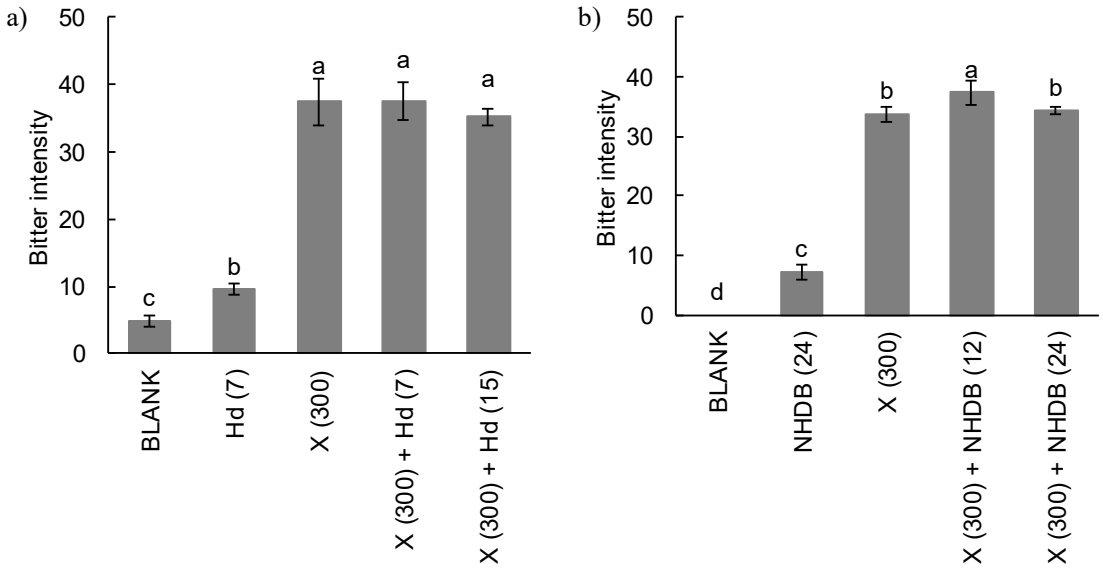

Figure 4.5 Bitter intensity of X in combination with (a) Hd at two dose concentrations and (b) NHDB at two dose concentrations. Samples were prepared in hot water and analysed at 60 °C. BLANK = water, Hd = hesperidin, NHDB = naringenin-*O*-hexose-*O*-deoxyhexoside isomer B, X = xanthone-rich fraction. Values in parentheses indicate concentration as $mg.L^{-1}$. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

Chapter 5
A realistic bitter intensity prediction model for honeybush herbal tea

Abstract

High-temperature oxidation ("fermentation") develops the typical colour and aroma of honeybush tea, as well as reducing bitter intensity of the infusions. Despite this, infusions of *Cyclopia longifolia* and particularly *C. genistoides* may remain bitter in taste. This is the first study to investigate the reduction of bitter intensity of infusions due to honeybush fermentation, as well as the relationships between phenolic profiles and bitter intensity of infusions prepared from fermented and unfermented *Cyclopia* material. Several genotypes of *C. genistoides* and *C. longifolia* from the Agricultural Research Council's honeybush plant breeding programme were sourced and both unfermented and fermented herbal tea were produced. Their infusions were profiled according to phenolic content and bitter intensity using high-performance liquid chromatography and descriptive sensory analysis, respectively. Fermentation reduced phenolic content, although variation was observed between species and compounds varied in susceptibility to oxidative degradation. It also lowered the bitter intensity of infusions, especially from *C. longifolia*. Several *C. genistoides* samples remained exceptionally bitter after fermentation, often in spite of a substantial reduction in xanthone content. Although mangiferin was previously demonstrated to be bitter, a high mangiferin content did not necessarily result in an unacceptably bitter infusion. Finally, a validated stepwise linear regression model ($R^2 = 0.859$) was developed to predict the bitter intensity of an infusion based on five phenolic compounds and soluble solids content common to the infusions of both species. The results confirmed previous observations regarding bitter taste and bitter modulating contributions of several phenolic compounds. This robust model predicted the contribution of mangiferin to bitter taste, as well as the bitter suppressing effect of isomangiferin. The different benzophenones were also associated with bitter taste. Levels of selected phenolic compounds in plant material may thus indicate suitability of a genotype for herbal tea production and the prediction model could be employed to screen and select genotypes for propagation.

1. Introduction

Predictive modelling for the development of quality control tools is a concept which has been applied in many industries. The application of such models or quality control tools can minimise post-production product losses by screening raw material before end-point production has taken place. A near infrared model was developed for prediction of mangiferin content of unfermented *Cyclopia genistoides* plant material (Joubert *et al*., 2006). Integration of such a type of method with a bitterness prediction model could be useful to screen plant material, especially in plant breeding when large numbers of genotypes are initially included for evaluation.

High-temperature oxidation ("fermentation") of *Cyclopia* is essential for the formation of the sought-after dark brown colour and the typical woody, fruity, sweet and floral aroma profile associated with traditional honeybush herbal tea (Bergh *et al*., 2017; Erasmus *et al*., 2017; Theron *et al*., 2014). This processing step leads to a reduction in the phenolic content of the plant material and a decrease in bitter intensity of the infusions (Erasmus, 2015). Beelders *et al*. (2015) demonstrated a substantial reduction in the mangiferin, isomangiferin and 3-β-D-glucopyranosyliriflophenone (IMG) content of a hot water extract of *C. genistoides* following simulated fermentation of the plant material. Interestingly, 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG) was much more stable, with little change in its content. Erasmus (2015) applied multivariate statistical techniques, including principal component analysis (PCA), partial least squares regression (PLS) and stepwise linear regression to create a model to predict bitter intensity from the individual phenolic content of a large set of infusions prepared from fermented plant material of several *Cyclopia* species. The study was unsuccessful in conclusively identifying specific predictors of bitter taste, partly due to limited variation in bitter intensity and phenolic content within the sample set. Moelich (2018) attempted a similar feat by applying an extended bitter intensity scale to the fermented honeybush infusions. However, this still did not increase variation and was once again limited by the extent of variation in the phenolic content of the infusions.

Our previous investigations have yielded conclusive evidence that mangiferin does taste perceptibly bitter (**Chapter 3**), while the honeybush benzophenones are not bitter (**Chapter 4**). A study to investigate the possibility of bitter taste modulation by compounds in the infusion confirmed this phenomenon (**Chapter 4**). Specifically, a phenolic fraction rich in benzophenones was demonstrated to enhance the bitter taste of a xanthone-rich fraction, although neither of the major individual iriflophenone derivatives (IMG and IDG) retained this bitter enhancing activity. IDG was found to suppress the bitter taste of a xanthone-rich fraction

when added at low concentrations. The flavanone, naringenin-*O*-hexose-*O*-deoxyhexoside B (NHDB), was also demonstrated to enhance the bitter intensity of the xanthone-rich fraction when added at a low concentration, although the flavanone-rich fraction had a bitter supressing effect. Some of these results contradict associations observed by Erasmus (2015) and Moelich (2018), although this may be a result of simple co-linearity or statistical over-fitting of the models to increase predictability.

The aim of the present study was to further the current understanding of the association between specific phenolic compounds and the bitter intensity of honeybush, and the role of fermentation in the reduction of bitter taste, especially with the view of developing a bitter intensity prediction model based on phenolic content. Additionally, *C. genistoides* and *C. longifolia*, both known for inherently high levels of xanthones shown to contribute to bitter taste, were selected for investigation. Plant material from different genotypes, including both the unfermented and fermented product, were evaluated to maximise variation in bitter intensity and phenolic content of infusions. Data were generated, using descriptive sensory analysis (DSA) and high-performance liquid chromatography (HPLC), and were analysed using multivariate statistical tools to determine potential individual phenolics as predictors of bitter intensity of infusions.

2. Materials and methods

2.1 Chemicals

Chemicals were sourced as described in **Chapter 3**.

2.2 Plant material

Cyclopia genistoides and *C. longifolia* genotypes from the Agricultural Research Council (ARC)'s honeybush plant breeding programme were harvested in 2015 and 2017. Some clones were planted at several locations in the Western Cape Province of South Africa (**Table 5.1**).

Leafless stems were trimmed and the shoots shredded to 2 - 3 mm pieces with a mechanised fodder cutter. Shredded plant material was then processed according to three possible processing schemes, using a standard protocol. Fermentation was either carried out at 80 °C for 24 h (first processing scheme) or 90 °C for 16 h (second processing scheme) after wetting the plant material. Following fermentation, samples were spread out on drying trays (39.5 × 56.5 cm, 30 mesh, Polymon; Swiss Silk Bolting Cloth Mfg. Co. Ltd., Switzerland) and dried in a laboratory cross-flow drying tunnel at 40 °C for 6 h, to a moisture content < 10% (Erasmus *et al*., 2017). The third processing scheme produced unfermented (green) samples, involving drying of the plant

material directly after shredding to retain green colour and phenolic content. The dried material of all samples was then sieved to obtain the typical tea-bag fraction (> 12 mesh, 1.68 mm; < 40 mesh, 0.42 mm) for analysis. A summary of the processed samples are provided in **Table 5.1**.

Table 5.1 Summary of processed samples (n = 126 samples)

Species	Harvest year	Location	Processing scheme			TOTAL
			fermented		unfermented	
			80 °C/24 h	90 °C/16 h		
Cyclopia genistoides	2015	Toekomst (T), Bredasdorp	8	8	8	24
	2015	Elsenburg (E), Stellenbosch	9	5		14
	2017	Riviersonderend (R)			6	6
	2017	Elsenburg (E), Stellenbosch		2	10	12
	2017	Elsenburg and Riviersonderend (pooled; E+R)		3	3	6
			17	18	27	62[a]
Species	**Harvest year**	**Location**	**Processing scheme**			**TOTAL**
			fermented		**unfermented**	
			80 °C/24 h	**90 °C/16 h**		
Cyclopia longifolia	2017	Toekomst (T), Bredasdorp		4	20	24
	2017	Nietvoorbij (N), Stellenbosch		18	18	36
	2017	Toekomst and Donkerhoek (pooled; T+D)		2	2	4
			0	24	40	64[b]

[a] Total of 13 batches with corresponding fermented (90 °C/16 h) and unfermented samples, and 8 batches with corresponding fermented (80 °C/24 h) and unfermented samples.
[b] Total of 24 batches with corresponding fermented (90 °C/16 h) and unfermented samples.

2.1 Preparation of infusions

Infusions were prepared in triplicate according to a standard procedure (Erasmus *et al*., 2017). Boiling distilled water (1000 g) was added to 12.5 g plant material in a glass beaker and stirred. The beaker was covered with aluminium foil and the plant material infused for 5 min. The infusion was poured through a stainless steel tea sieve into a pre-heated 1 L insulated flask. Furthermore, 100 mL of each infusion was filtered (Whatman No. 4 filter paper) for soluble solids (SS) content determination and phenolic quantification by HPLC. Multiple aliquots of 1 mL were stored at -18 °C for HPLC analysis at a later stage.

2.2 Sensory analysis of infusions

Descriptive sensory analysis (DSA) was used to determine the bitter intensity of all samples. A sensory panel of 8 - 13 members experienced in bitter taste analysis was trained by a panel expert during several sessions to familiarise the panel with the samples and to reach consensus for scoring bitter intensity. Measures were taken

to ensure the samples remain warm before and during analysis. Flasks and porcelain serving mugs were pre-heated at 70 °C. Samples were also presented in heated water baths (60 °C; Theron *et al*., 2014; Koch *et al*., 2012). Panellists were provided with individual booths for analysis and ambient temperature controlled at 21 °C. Water biscuits, distilled water and dried apple pieces were provided as palate cleansers. For analysis, panellists were required to stir the infusion with a soup spoon and sip from the spoon to taste the infusion. Analysis was conducted in triplicate across separate sessions, with up to eight samples presented in a single session. Samples were blind-coded and randomised per panellist. Samples were scored on an anchored 100-point line scale (0 = not bitter, 100 = extremely bitter) using *Compusense® five* software (Compusense; Guelph, Canada). In order to ensure comparable results, a marked reference sample (an unfermented infusion with a bitter intensity score of 30) was also presented with each set.

2.3 Soluble solids (SS)

Gravimetric determination of the SS content of each infusion was performed in triplicate. An aliquot (15 mL) of the filtered and cooled infusion was pipetted into a nickel dish, pre-weighed to four decimals, and evaporated on a heated water bath. Dishes were transferred to a heated laboratory oven (100 °C) for 1 h and then placed in a desiccator to cool to room temperature. Dishes were weighed again and the total SS content calculated (expressed as g $SS.L^{-1}$ infusion).

2.4 High-performance liquid chromatography (HPLC)

Each infusion was analysed by HPLC at two injection volumes (80 µL and 10 µL; duplicate injections per volume) to accommodate large differences in peak areas of individual phenolic compounds. A frozen aliquot of each infusion was defrosted directly before analysis. Ascorbic acid (10% $v.v^{-1}$; 100 µL) was added to 1 mL of infusion prior to filtration (0.45 µm pore size for *C. genistoides*, 0.22 µm pore size for *C. longifolia*; 33 mm diameter hydrophilic PVDF syringe filter devices, Merck Millipore; Darmstadt, Germany) to prevent phenolic degradation during analysis.

Species-specific HPLC methods for *C. genistoides* and *C. longifolia* were applied (Schulze *et al*., 2015; Beelders *et al*., 2014) to quantify 11 specific phenolic compounds for each species (only seven phenolic compounds correspond to both species). Authentic reference standards were used to prepare seven-point calibration curves for accurate quantification. Where authentic reference standards were not available,

compounds were quantified in terms of reference compounds of similar type (results expressed as equivalents), or calculated according to a pre-determined response factor relative to an authentic standard.

2.5 Statistical procedures

For sensory data, panel performance was monitored, data were pre-processed, outliers were removed and statistical analysis was conducted according to the experimental design on means over judges for each sample or sample category, as described in **Chapter 3**.

Instrumental data were subjected to ANOVA to test for sample differences, using *SAS* software (Version 9.2; SAS Institute Inc., Cary, USA). The Shapiro-Wilk test was performed to test for normality. Fisher's least significant difference was calculated at the 5% level to compare sample means.

Regression and multivariate analysis were performed using *XLStat* (Version 2016.1.01, Addinsoft, New York, USA). These included PCA employing the Pearson's correlation matrix, stepwise linear regression and partial least squares (PLS) regression. Individual infusions (3 infusions per sample = 3 × 126 samples = 378 infusions) were considered as individual data points. Due to the qualitative differences in the phenolic profiles of *C. genistoides* and *C. longifolia*, their data sets were first considered separately, and then as a combined data set, which included only the seven phenolic compounds common to both species. The relationship between measured variables of fermented (90 °C/16 h) and unfermented samples were determined only when both types of plant material originated from the same batch. Where linear regression of variables before and after fermentation were conducted, the data set included eight additional *C. genistoides* batches fermented at 80 °C/24 h. To determine which phenolic compounds contributed to the prediction of bitter intensity, the final stepwise linear regression model was developed from the complete total data set (including both species, fermented and unfermented samples). External validation of the model was performed using a subset of the data (n = 50 randomly selected infusions), excluded from the model-building set.

3. Results and discussion

3.1 Species variation

Both *C. genistoides* and *C. longifolia* have been identified as species that could potentially produce bitter tasting fermented honeybush tea (Erasmus *et al*., 2017; Erasmus, 2015). *Cyclopia maculata,* a typically non-bitter species, can also produce a bitter tea infusion from unfermented plant material (Alexander, 2015). *Cyclopia* species do not only vary morphologically (*e.g.* leaf shape), but also in terms of sensory and phenolic

profiles (Erasmus *et al*., 2017; Schulze *et al*., 2015; Beelders *et al*., 2014; Joubert *et al*., 2011). Furthermore, phenolic content may be affected by harvest season, plant maturity, climate and processing (North *et al*., 2017; Joubert *et al*., 2014; 2003). For the current study, several genetic lines ("genotypes") of two species, *C. genistoides* and *C. longifolia*, were selected for processing and analysis to ensure large variation in phenolic content (and potentially bitter intensity), required for the development of a robust prediction model.

Although variation was detected between genotypes (results not shown), variation due to species and processing were most notable (**Tables 5.2** and **5.3**). No significant differences for the various parameters (bitter intensity, SS content and individual phenolic content; $p \geq 0.05$) were found between *C. genistoides* samples fermented at 80 °C/24 h and 90 °C/16 h (data not shown), justifying pooling of the data. Classification based on processing was thus limited to fermented and unfermented plant material for the rest of the study.

Bitter taste, along with most individual phenolic compounds, was found to be substantially more prominent for infusions prepared from the unfermented plant material compared to those of the fermented plant material, irrespective of species (**Tables 5.2 - 5.3**). It is notable that fermented *C. longifolia* samples never exceeded a bitter intensity of 25 on a 100-point scale (mean intensity of 11). This mean bitter intensity is well below 20, the point at which the sensory panel considered bitter taste to become definitively notable. Fermented *C. genistoides* samples, however, commonly exceeded this threshold. The mean bitter intensity of the fermented *C. genistoides* samples was nearly twice that of *C. longifolia* (19 vs 11). The mean bitter intensities for the unfermented samples of each species did not differ as greatly, *i.e.* 46 for *C. longifolia* and 51 for *C. genistoides*, although the range of bitter intensity was greater for *C. longifolia* samples (54 vs 40, for *C. longifolia* and *C. genistoides*, respectively).

Infusions of the fermented plant material of both *C. genistoides* and *C. longifolia* were previously demonstrated to contain high levels of the major xanthones, mangiferin and isomangiferin, as well as benzophenones, especially IDG (Schulze *et al*., 2015). Infusions of *C. genistoides* in the present sample set contained the benzophenone, maclurin-di-*O*,*C*-hexoside (MDH; **Table 5.2**), which was absent or not present at a quantifiable level in the *C. longifolia* infusions (**Table 5.3**). Several flavanones were quantified in the *C. genistoides* samples, including hesperidin, eriodictyol-*O*-hexose-*O*-deoxyhexoside and two naringenin-*O*-hexose-*O*-deoxyhexoside isomers. Isomer A (NHDA) was more prominent in fermented samples, whereas isomer B (NHDB) was more prominent in unfermented samples. This will be further discussed in **section 3.2**. The mangiferin content of the unfermented *C. genistoides* infusions ranged from 105 - 286 $mg.L^{-1}$ (**Table 5.2**)

compared to 26 - 483 mg.L^{-1} for *C. longifolia* (**Table 5.3**). The latter species also contained quantifiable levels of two tetrahydroxyxanthone-di-*O*,*C*-hexoside isomers, the flavanone, eriocitrin and the flavone, scolymoside.

When considering the phenolic sub-classes (**Fig. 5.1**), unfermented *C. genistoides* infusions contained a higher number of flavanone compounds (NHDA, NHDB, eriodictyol-*O*-hexose-*O*-deoxyhexoside and hesperidin, total 45.22 mg.L^{-1}) than *C. longifolia*, (eriocitrin and hesperidin, total 13.15 mg.L^{-1}). In terms of benzophenone compounds, unfermented *C. genistoides* produced infusions with higher levels than unfermented *C. longifolia* (81.57 vs 55.09 mg.L^{-1}). Despite the previous implication that the xanthones (including the major compound, mangiferin) cause bitter taste (Moelich, 2018; Erasmus, 2015), *C. longifolia*, being less bitter than *C. genistoides*, also contained higher levels of xanthones (300.18 vs 228.96 mg.L^{-1}). This is mainly due to the contribution of the major honeybush compound, mangiferin. It should also be considered that the second-most prominent xanthone, isomangiferin, may suppress the bitter intensity of mangiferin (**Chapter 3**); this will be discussed further in **section 3.2**. The higher flavone content of unfermented *C. longifolia* (18.16 mg.L^{-1}) compared to *C. genistoides* (9.98 mg.L^{-1}) is mainly as a result of the presence of considerable scolymoside levels in the former species.

The SS content of the infusions of the two species also differed, as previously observed (Schulze *et al*., 2015), with *C. longifolia* having higher values than *C. genistoides*. It could be speculated that the differences in drought resistance of the species, as evidenced by the leaf shape (flat, elongated vs needle-like, respectively) and natural habitats, would affect the composition of the hot water soluble matter, however, at this stage no information is available.

3.2 Phenolic degradation and bitter taste reduction due to fermentation

Phenolic degradation and bitter taste reduction due to fermentation were investigated for individual genotypes of *C. genistoides* and *C. longifolia* to gain greater insight into the variation in bitter intensity of different production batches. In this case only a sub-set of *C. genistoides* batches (n = 13 batches) were available, of which several clones were planted to produce enough material to allow processing of both unfermented and fermented (90 °C/16 h) samples. For *C. longifolia* a larger set of batches were available (n = 24 batches). The bitter intensities of their infusions prepared from fermented and unfermented plant material are presented in **Figs. 5.2** and **5.3**, and the mean quantified variables are presented in **Tables 5.4** and **5.5**, for *C. genistoides* and *C. longifolia*, respectively. The relative percentage change values for each parameter as a result of fermentation

were calculated per species and are presented in **Fig. 5.4**. As expected, **bitter taste** of both species was greatly influenced by fermentation.

The two species not only differed in mean bitter intensity (**Tables 5.4** and **5.5**), but also in the extent to which fermentation reduced bitter intensity (**Fig. 5.4**). Reduction in the bitter intensity of *C. genistoides* infusions varied between 34% and 68%, with a mean reduction of 55% (**Fig. 5.2**; **Fig. 5.4a**). Notably, five *C. genistoides* samples (GG31/T, GK1/E, GK4/T, GK7/E+R and GT1/E) had bitter intensities > 20 after fermentation, with GK3/T and GT2/T having intensities of ~20. This relates to the problem often met in industry where fermentation does not adequately reduce the bitter taste of *C. genistoides*. On the contrary, *C. longifolia* was much more susceptible, varying between 55% and 86% reduction in the bitter intensity, with a mean reduction of 73% (**Fig. 5.3**; **Fig. 5.4b**). Although unfermented *C. longifolia* samples less frequently displayed extreme bitter intensities (> 40), the unfermented plant material of one batch (LGR1 from Toekomst) produced an infusion which was scored exceptionally bitter (71; **Fig. 5.3**). Fermentation reduced the bitter intensity of this batch, as well as four others (LHK23/T+D, LHK47/T, LMD11/T and LMD37/T+D), from > 50 to ≤ 22. This indicates that fermentation was successful in reducing bitter taste of *C. longifolia* to acceptable levels.

The individual phenolic content of *C. genistoides* and *C. longifolia* infusions decreased with fermentation as indicated by sample comparisons where both unfermented and fermented plant material originated from the same batches (**Tables 5.4** and **5.5**). The benzophenone di-glucoside, IDG, remained resistant to degradation ($p \geq 0.05$; **Tables 5.4** and **5.5**) and was present in both *C. genistoides* and *C. longifolia*. The mono-glucoside, IMG, however, degraded by 66% and 41% in *C. genistoides* and *C. longifolia*, respectively (**Fig. 5.4**). A kinetic study of the thermal degradation of these compounds during simulated fermentation of *C. genistoides* (Beelders *et al*., 2015) demonstrated similar results. The thermal stability of IDG was attributed to glucosylation at C4, inhibiting oxygen-radical formation (Beelders *et al*., 2017). In agreement with Beelders *et al*. (2015), MMG was found to be extremely labile (**Fig. 5.4**) and present at much higher levels in *C. genistoides*. Absence of this compound in the infusions may thus serve as an indicator of minor oxidative changes in unfermented material. As a benzophenone precursor to xanthones, this compound forms several xanthone dimer degradation products, as well as mangiferin and isomangiferin, as major products when heated (Beelders *et al*., 2017). Overall, the extent of degradation of the benzophenones as a

group was greater in *C. genistoides* than in *C. longifolia*. The mean benzophenone content of *C. genistoides* (79.53 $mg.L^{-1}$) and *C. longifolia* (30.80 $mg.L^{-1}$) was reduced by 51% and 35%, respectively.

It may be noted that NHDA was once again found in higher concentrations in fermented *C. genistoides* samples than unfermented ones, with a relative mean increase of 113% (**Table 5.4**; **Fig. 5.4a**). Beelders *et al*. (2015) previously observed the increase in this compound during simulated fermentation of *C. genistoides.* In **Chapter 4**, conclusive evidence was provided that NHDB is converted to NHDA during heating. The decrease in NHDB content in the infusion (66%; **Fig. 5.4**) was higher than expected considering the study by Beelders *et al.* (2015).

The extent of hesperidin degradation due to fermentation was much more pronounced in *C. genistoides* (32%) than *C. longifolia* (5%). It is also notable that infusions of unfermented *C. longifolia* contained less hesperidin than those of unfermented *C. genistoides*. It is possible that the matrix effects of the two different leaf structures have an effect on the relative degradation or extraction of the individual phenolic compounds in each species. The relative stem-to-leaf ratio would also be important. Du Preez *et al*. (2016) and De Beer *et al*. (2012) have both found hesperidin to be higher in extracts from stems than those from leaves for *C. maculata* and *C. subternata*, respectively. *Cyclopia longifolia* produces bushes with much thicker stems than *C. genistoides* (Supplementary material, **Fig. C.1**), forming larger pieces upon cutting. These are readily removed through sieving, which could result in a lower stem-to-leaf ratio in the final tea-bag fraction of *C. longifolia* than of *C. genistoides* with its thinner smaller stems.

Mangiferin and isomangiferin were found to be affected by fermentation to a large degree, more so for *C. longifolia* (75% and 52%, respectively) than for *C. genistoides* (61% and 41%, respectively; **Fig. 5.4**). The kinetic degradation study by Beelders *et al*. (2017) predicted the degradation of mangiferin and isomangiferin (90 °C/16 h) to be slightly lower, at 57% and 37%, respectively. Mean mangiferin and isomangiferin content of the two species was very similar for unfermented samples (181 and 49 $mg.L^{-1}$ in *C. genistoides*, and 183 and 50 $mg.L^{-1}$ in *C. longifolia*, respectively). The higher MMG content of *C. genistoides* and its conversion to mangiferin (Beelders *et al*., 2017) would, to some extent, mask mangiferin degradation. After fermentation, the mean mangiferin content for *C. genistoides* was substantially higher than for *C. longifolia* (72 vs 46 $mg.L^{-1}$), but differences in isomangiferin were less pronounced (mean values of 29 and 24 $mg.L^{-1}$, respectively). The difference in stability between the two xanthone regio-isomers thus resulted in a change in the mangiferin:isomangiferin ratio of the two species. The ratio was consistent between samples

of each category, and is fairly comparable when unfermented infusions of *C. genistoides* (3.66) and *C. longifolia* (3.64) are considered, however, after fermentation the ratio decreased to a greater extent for *C. longifolia* (1.90) than for *C. genistoides* (2.47). This may be a contributing factor for the lower bitter intensity of *C. longifolia* infusions after fermentation of the plant material despite high mangiferin levels given that isomangiferin suppresses the bitter intensity of mangiferin (**Chapter 3**).

3.3 Associations between measured parameters before and after fermentation

Associations between individual phenolic content, bitter intensity and SS content measured for the corresponding infusions of unfermented and fermented plant material were first calculated for the individual species, and then for the combined data set. The *C. genistoides* data set included the data of eight additional batches (*C. genistoides* seedlings fermented at 80 °C/24 h) in order to increase the sample set for the establishment of more reliable associations (n = 13 + 8 = 21 batches). Linear associations between individual phenolic content of corresponding infusions of unfermented and fermented samples were generally stronger for *C. genistoides* (Supplementary material, **Fig. C.2**) than for *C. longifolia* (Supplementary material, **Fig. C.3**), indicating more consistent changes in the variables as a result of processing ($R^2 > 0.7$, $p < 0.05$) for all variables (*C. genistoides*), except for hesperidin and bitter taste ($R^2 < 0.7$, $p < 0.05$; Supplementary material, **Fig. C.2**). Despite the moderate to good linear association of the phenolic parameters, association between bitter intensity of infusions before and after fermentation of the plant material was very low ($R^2 = 0.350$, $p = 0.005$; Supplementary material, **Fig. C.2**), indicating that the effect of fermentation on bitter intensity was not consistent. Better association was observed for the bitter intensity of *C. longifolia* ($R^2 = 0.760$, $p < 0.0001$), although $R^2 < 0.7$ for the other variables except scolymoside (Supplementary material, **Fig. C.3**). By combining the sample sets (n = 45 genotypes), the linear association for all variables as a result of processing became moderate ($R^2 > 0.5$, $p < 0.05$; **Fig. 5.5**). The individual benzophenones retained a high coefficient of determination ($R^2 > 0.8$) in the combined data set (**Fig. 5.5**). Mangiferin and isomangiferin, however, showed poor association before and after fermentation, attributed to the differing extent of degradation for the respective *Cyclopia* species (**Fig. 5.5**). *Cyclopia longifolia* batches typically underwent a greater loss of mangiferin during fermentation than *C. genistoides*.

Although the behaviour of certain individual parameters such as SS and phenolic content may be fairly consistent, especially for *C. genistoides*, the response of bitter taste to processing still remains difficult to

predict and interpret. The data indicate the inconsistent response between the two species, challenging the predictability of bitter taste after fermentation based on measured parameters before fermentation.

3.4 Associations of phenolic compounds with bitter taste

Considering previous results for individual compounds and fractions (**Chapters 3** and **4**), individual phenolic compounds were evaluated as predictors of bitter taste. Although other studies have also attempted to determine associations between individual phenolic content and bitter intensity of honeybush infusions (Moelich, 2018; Erasmus, 2015), it is important to note that these studies made use of only fermented samples (80 and 90 °C for 8, 16, 24 and 32 h). A consequence of the fermented sample set is limited variation in both bitter intensity and phenolic content, reducing the overall robustness of the subsequent model. The present study made use of both unfermented and fermented samples, greatly increasing the extent of variation in the parameters. By doing so, the current study is able to benefit from a more robust data set and subsequent prediction model. PCA was conducted on the separate data sets of *C. genistoides* and *C. longifolia*, as well as the combined set to elucidate correlations between bitter intensity and individual phenolic content and SS content of the infusions. The PCA loadings plot for each species (Supplementary material, **Figs. C.4** and **C.5**) indicated similar differentiation between fermented and unfermented samples along the first principal component (F1), as also evident for the combined sample set (60.31%; **Fig. 5.6**). The second principal component (F2), differentiated between the two species (15.36%; **Fig. 5.6**). Bitter taste and mangiferin seemed to be the primary drivers of separation of samples along F1, as indicated by the high squared cosines of the variables > 0.8. All the phenolic parameters are, however, grouped together to the right of the plot showing close correlations between all the individual phenolic compounds, with significant ($p < 0.05$) correlations between bitter intensity and individual phenolic content. This is not surprising, considering the major effect of fermentation (as represented by F1) on phenolic content and bitter intensity.

Subsequently, individual linear associations were considered using linear regression plots for bitter intensity and individual phenolic content. Coefficients of determination (R^2) for the seven compounds common to both data sets were evaluated (**Fig. 5.7**). All associations were deemed significant ($p < 0.05$). The plots indicate a progressive increase in bitter intensity with increasing compound concentration, although the only moderate to good coefficients of determination ($R^2 \geq 0.7$) were observed between bitter taste and the xanthones, mangiferin and isomangiferin**.** Apart from these, the benzophenone, MMG, showed the strongest linear

association with bitter intensity (R^2 = 0.505). The other benzophenones, IMG and IDG, as well as the flavonoids, hesperidin and vicenin-2, associated poorly with bitter intensity (R^2 < 0.5).

The strong linear association between isomangiferin and bitter intensity (R^2 = 0.702) is probably a result of co-linearity, as it is usually present in concentrations related to mangiferin. This is related to their shared xanthone biosynthetic pathway, whereby the synthesis of mangiferin is favoured over its regio-isomer (Joubert *et al*., 2014). Mangiferin has been shown to be bitter (**Chapter 3**), although its contribution to the bitterness of the infusions cannot simply be calculated. Despite its strong association, a concentration of 200 mg.L^{-1} in an infusion can still, according to the linear regression model (**Fig. 5.7**), result in a variable bitterness of between < 20 and 60.

Given that direct linear associations are not sufficient to represent the bitter taste contribution of the phenolic compounds, PLS regression was performed on the individual (Supplementary material, **Figs. C.6** and **C.7**) and combined (**Fig. 5.8**) data sets in order to determine bitter taste from the measured parameters of the infusions. The model developed for the combined data set was simpler than for either of the species separately. This may be expected, as all 11 individual phenolic compounds were considered for the individual models, but only seven individual phenolics common to both species were considered for the combined model. Nevertheless, good linear association was obtained (R^2 = 0.841) for the combined model (**Fig. 5.8**), although association for the separate *C. genistoides* and *C. longifolia* models were slightly better (R^2 = 0.866 and 0.844, respectively; Supplementary material, **Fig. C.6** and **C.7**). There was some discrepancy between the two individual models. For example, IDG and hesperidin were both negative contributors to bitterness in the *C. longifolia* model, whereas the same compounds had a positive contribution in the *C. genistoides* model. The opposite was also seen for SS content in the two models. The combined model, however, takes into account all the data points (infusions) to provide a model with similar predictability, but with fewer variables, which is desirable. For the combined and individual models, mangiferin, isomangiferin and MMG contributed the most to the prediction of bitter intensity (variable importance in the projection (VIP) > 1). This may be related to the extremely low levels of MMG in fermented samples due to the labile nature of the compound. According to PLS, both mangiferin and isomangiferin are positive contributors to bitter taste, despite the bitter supressing effect of isomangiferin (**Chapter 4**), highlighting the limitations of this approach. The co-linearity of their occurrence in honeybush infusions may be responsible for this result, however, the progressive change in their ratio due to a difference in the extent of degradation may also contribute. In addition, the slight negative

contribution of IDG may stem from its resistance to degradation, although it does not represent the bitter suppressing effect observed for this compound (**Chapter 4**). The contribution by hesperidin may imply taste modulation. Hesperidin, however, did not have a significant effect on the bitter taste of the xanthone-rich fraction (**Chapter 4**). Indeed, it is possible that some other flavanone compounds may amplify bitter taste, such as was found for the flavonol, rutin, that increased caffeine bitterness in model solutions (Scharbert & Hofmann, 2005). Bitter taste amplification of caffeine solutions have also been observed by the addition of fractionated honeybush extracts (Reichelt *et al*., 2010).

Overall, the PLS models provided high coefficients of determination ($R^2 > 0.8$), although it is clear that these models do not represent the bitter and modulatory contributions observed previously (**Chapters 3** and **4**). The simple associations are thus an oversimplification and are not effective in explaining phenolic compounds as predictors of bitter taste.

The next step was to apply stepwise linear regression. Previous studies showed that it is not a useful statistical technique for the effective prediction of bitter taste (Moelich, 2018; Erasmus, 2015). However, in the present study it was successfully employed for each species (Supplementary material, **Figs. C.8** and **C.9**). Finally, an independently validated (n = 50 infusions) stepwise linear regression model of the combined data set (n = 328 infusions) to predict bitter taste based on individual measured parameters was developed (**Fig. 5.9**; $R^2 = 0.859$). The models all concurred with previous results in this study to various degrees. The major contribution to bitter taste was indeed consistently assigned to mangiferin, in agreement with results of a previous experiment that demonstrated a dose-response for bitter taste of this xanthone (**Chapter 3**). Its regio-isomer, isomangiferin, contributed negatively to bitter taste indicating a bitter taste suppression. This was observed previously (**Chapter 3**), where a combination of mangiferin (178 $mg.L^{-1}$) and isomangiferin (34 $mg.L^{-1}$) at infusion equivalent concentrations resulted in a reduction in bitterness from ~35 (mangiferin) to ~22 (combination of mangiferin and isomangiferin).

The effect of the benzophenones, IDG and MMG, were also taken into account. The constant association of MMG to bitter taste implicates it as a potential bitter taste enhancer. We previously speculated that MMG may have a bitter enhancing modulatory effect, as the benzophenone fraction from which it originates has no bitter taste (**Chapters 3** and **4**). The presence of a catechol moiety in its structure and its potential to activate TAS2R5 (Soares *et al*., 2013) supports this speculation. Indeed, both the validated model from the combined data set (**Fig. 5.9**), as well as the model for *C. longifolia* (Supplementary material, **Fig. C.9**)

includes MMG as a positive contributor to bitter taste. The model for *C. genistoides* (Supplementary material, **Fig. C.8**), however, assigned a negative bitter taste contribution to MMG. IDG did not show significant bitter taste enhancement. Its modulatory effects have been shown to be dose-dependent, supressing bitter taste at low concentrations (**Chapter 4**). Although the validated model for the combined data set (**Fig. 5.9**) assigned a small positive contribution to bitter taste by IDG, the model for *C. longifolia* assigned a small negative contribution to this compound (Supplementary material, **Fig. C.9**).

Hesperidin may possesses a bitter taste as panellists noted a sudden bitter taste response that disappeared rapidly (**Chapter 4**). It may also have the ability to enhance bitterness, although it did not significantly affect bitter taste of a xanthone-rich fraction (**Chapter 4**). As mentioned in **Chapter 4**, this may have been as a result of carry-over effects of this taste modality during sensory analysis. According to the *C. longifolia* model, the flavanone, eriocitrin contributed to bitter taste, whereas hesperidin did not (Supplementary material, **Fig. C.9**). Also in agreement with previous observations, the flavanone, NHDB, shown to have bitter supressing properties at low concentrations (**Chapter 4**), made a negative contribution to the bitter taste in the model for *C. genistoides* (Supplementary material, **Fig. C.8**). Stepwise linear regression models for the two species and for the combined data set thus reflect the results seen previously and support our previous findings (**Chapters 3** and **4**).

Mangiferin is consistently associated with bitter taste, irrespective of prediction model, which is not surprising, given the dose-response demonstrated in **Chapter 3**. Mangiferin content of the infusions (**Tables 5.2** and **5.3**), was therefore used to calculate theoretical values for bitterness. The linear regression models for individual and combined data sets for species showed good correlation ($R^2 > 0.78$; **Fig. 5.10**). In all cases, the predicted bitter intensity was less than the actual bitter intensity. Under-estimation of bitter intensity was more evident for *C. genistoides*, confirming the contribution of other compounds and/or effects. Despite the under-estimation of bitter intensity of infusions when only their mangiferin content is used, the results confirmed that the compound is a major driver of the bitter taste of honeybush.

4. Conclusions

The two species, *C. genistoides* and *C. longifolia*, differed in terms of bitter taste, phenolic content and response to fermentation. Different genotypes of the same species also produced infusions varying both in phenolic composition and bitter intensity. *Cyclopia genistoides* persistently produced bitter tasting infusions

even after fermentation, whereas the bitter taste of *C. longifolia* infusions was adequately reduced by fermentation. Correlation between bitter taste of infusions before and after fermentation of the plant material was weak, although degradation was predictable for several phenolics, especially for *C. genistoides*. The ratio between mangiferin and isomangiferin changed during fermentation, resulting in a lower ratio for *C. longifolia* than for *C. genistoides* after fermentation, which could impact on bitter intensity of the final product. The consequences of this observation should be further investigated.

Several predictive models were developed for predicting bitter taste based on individual phenolic content of a large sample set of fermented and unfermented samples of two *Cyclopia* species, with varying results. The developed multivariable models consistently implicated the role of benzophenones, especially MMG. Similarly, the xanthones, mangiferin and isomangiferin, were also associated with bitter taste, although the bitter suppressing effect of isomangiferin was not evident from the PLS models. Prediction of bitter intensity of infusions based solely on their mangiferin content under-estimated bitter intensity, but confirmed mangiferin as a major contributor to bitter taste of honeybush. Stepwise linear regression models confirmed several previous observations regarding bitter contribution and modulation by the phenolic compounds in honeybush infusions. Finally, a robust external validated stepwise linear regression model to predict bitter taste, based on five compounds and SS content common to both *C. genistoides* and *C. longifolia*, was developed. This model may aid in a simple screening of plant material for herbal tea production, or in selecting genotypes for propagation and cultivation at the first levels of production.

Addendum C. Supplementary material

Supplementary material associated with this chapter can be found in **Addendum C** (p 163).

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Table 5.2 Bitter intensity, individual phenolic content (mg.L^{-1}) and soluble solids content (g.L^{-1}) of *Cyclopia genistoides* infusions prepared from unfermented and fermented plant material

	Abbrev.	Unfermented (n = 26)*					Fermented (n = 35)*				
		Min.	**Max.**	**Mean**	**SD[a]**	**%RSD[b]**	**Min.**	**Max.**	**Mean**	**SD[a]**	**%RSD[b]**
BITTER		32.4	72.7	**51.1**	8.2	16.1	9.9	38.6	**18.8**	5.7	30.2
Maclurin-di-*O*,*C*-hexoside (***a***) [c]	MDG	0.60	3.20	**1.87**	0.71	37.78	0.37	2.46	**1.04**	0.45	43.16
3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (***b***)	IDG	4.84	85.18	**29.87**	18.14	60.72	4.33	47.76	**13.64**	10.73	78.71
3-β-D-Glucopyranosylmaclurin (***c***)	MMG	5.39	29.35	**11.88**	5.17	43.53	0.18	7.39	**1.56**	1.46	93.38
3-β-D-Glucopyranosyliriflophenone (**3**)	IMG	14.49	90.22	**37.95**	19.72	51.97	1.85	33.29	**11.41**	8.58	75.15
Eriodictyol-*O*-hexose-*O*-deoxyhexoside (***f***)[d]	EHD	1.42	8.03	**4.41**	1.67	37.83	0.37	4.75	**2.37**	1.27	53.62
Mangiferin (**4**)	Mg	104.52	285.50	**180.03**	40.65	22.58	21.33	141.23	**55.08**	29.15	52.93
Isomangiferin (**5**)	IsoMg	26.81	83.51	**48.93**	11.25	22.99	10.76	49.04	**23.01**	9.32	40.52
Vicenin-2 (**6**)	Vic-2	5.61	14.58	**9.98**	1.89	18.91	3.91	12.19	**7.54**	1.89	25.07
Naringenin-*O*-hexose-*O*-deoxyhexoside isomer A (***v***)	NHDA	0.27	5.49	**2.42**	1.31	54.04	0.86	14.34	**5.01**	3.29	65.69
Naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (***w***)	NHDB	2.64	49.61	**19.04**	10.89	57.21	0.89	16.97	**6.02**	4.27	70.94
Hesperidin (**9**)	Hd	10.44	28.83	**19.34**	3.89	20.11	2.82	16.78	**9.70**	2.81	29.02
Soluble solids	SS	1.37	2.52	**2.00**	0.23	11.54	1.56	2.76	**1.99**	0.26	12.91

*21 batches of plant material were processed as both fermented and unfermented samples.
Bold letters or numbers in brackets correspond to compound identification according to Beelders *et al*., 2014.
[a] Standard deviation.
[b] Percentage relative standard deviation.
[c] Expressed as 3-β-D-glucopyranosylmaclurin equivalents.
[d] Expressed as eriocitrin equivalents.

Table 5.3 Bitter intensity, individual phenolic content (mg.L^{-1}) and soluble solids content (g.L^{-1}) of *Cyclopia longifolia* infusions prepared from fermented and unfermented plant material

		Unfermented (n = 40)*					Fermented (n = 24)*				
	Abbrev.	**Min.**	**Max.**	**Mean**	**SD[a]**	**%RSD[b]**	**Min.**	**Max.**	**Mean**	**SD[a]**	**%RSD[b]**
BITTER		18.9	73.3	**46.1**	15.1	32.72	3.2	24.9	**10.6**	5.7	53.8
3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (**5**)	IDG	4.43	79.14	**23.22**	14.94	35.85	4.86	40.65	**16.10**	8.75	52.50
3-β-D-Glucopyranosylmaclurin (**6**)	MMG	nd[c]	17.66	**5.07**	5.01	98.89	nd[c]	1.97	**0.24**	0.55	224.08
3-β-D-Glucopyranosyliriflophenone (**13**)	IMG	1.15	98.02	**26.14**	27.58	105.52	0.88	20.21	**4.41**	5.15	116.98
Tetrahydroxyxanthone-di-*O*,*C*-hexoside isomer A (**16**)[d]	THXA	1.00	6.98	**3.47**	0.97	27.95	0.66	2.47	**1.49**	0.39	26.44
Tetrahydroxyxanthone-di-*O*,*C*-hexoside isomer B (**17**)[d]	THXB	0.93	5.59	**2.81**	0.98	34.75	0.95	3.22	**1.85**	0.56	30.10
Mangiferin (**22**)	Mg	25.77	483.48	**226.64**	103.62	45.72	13.13	138.26	**46.65**	30.12	64.58
Isomangiferin (**25**)	IsoMg	15.64	127.28	**61.21**	25.37	41.45	9.56	60.02	**24.52**	11.57	47.18
Vicenin-2 (**26**)	Vic-2	4.35	15.53	**8.82**	2.34	26.53	3.91	11.15	**6.74**	1.66	24.61
Eriocitrin (**32**)	ErioT	1.12	9.54	**4.53**	2.28	50.21	0.66	7.28	**2.89**	1.61	55.73
Scolymoside (**35**)	Scol	2.30	19.71	**9.34**	4.90	52.44	1.83	11.70	**6.19**	2.80	45.28
Hesperidin (**42**)	Hd	4.45	14.96	**8.55**	2.05	24.01	5.66	12.23	**8.50**	1.46	17.18
Soluble solids	SS	1.59	3.46	**2.45**	0.48	19.73	1.75	3.31	**2.27**	0.38	16.79

*24 batches of plant material were processed as both fermented and unfermented samples.
Bold numbers in brackets correspond to compound identification according to Schulze *et al.*, 2015.
[a] Standard deviation.
[b] Percentage relative standard deviation.
[c] nd, Not detected; limit of detection (LOD) = 0.0768 mg.L^{-1}.
[d] Expressed as mangiferin equivalents.

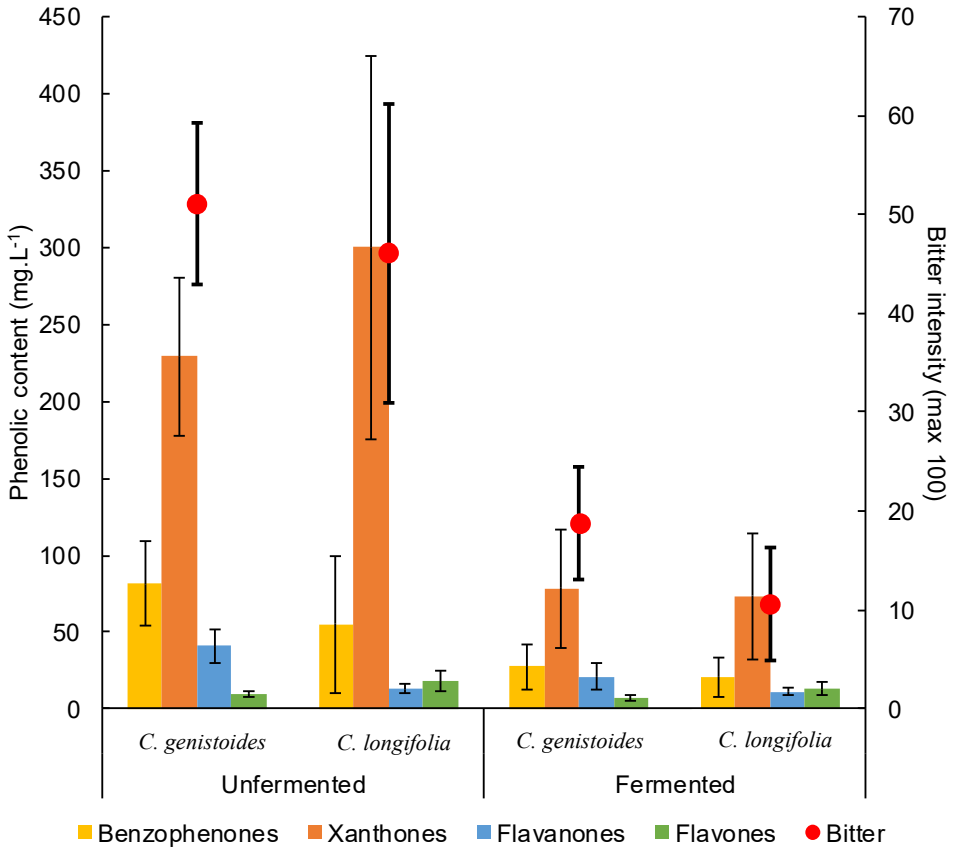


Figure 5.1 Summary of major phenolic sub-classes and bitter intensity of all samples showing both species and processing categories.

Table 5.4 Bitter intensity, individual phenolic content (mg.L^{-1}) and soluble solids content (g.L^{-1}) of *Cyclopia genistoides* infusions, prepared from fermented and unfermented plant material of corresponding batches (n = 13 batches)

	Abbrev.	Unfermented (n = 13)					Fermented (90 °C/16 h) (n = 13)				
		Min.	Max.	Mean		SD[a]	Min.	Max.	Mean		SD[a]
BITTER		34.3	64.4	**48.3**	a	8.5	13.8	36.3	**21.8**	b	6.8
Maclurin-di-*O*,*C*-hexoside (***a***)[c]	MDG	0.64	3.08	**1.64**	a	0.78	0.58	2.34	**1.24**	b	0.49
3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (***b***)	IDG	4.90	49.73	**20.18**	a	15.25	5.27	47.03	**21.73**	a	14.81
3-β-D-Glucopyranosylmaclurin (***c***)	MMG	6.19	28.62	**13.79**	a	6.03	0.18	7.27	**2.26**	b	1.79
3-β-D-Glucopyranosyliriflophenone (**3**)	IMG	15.52	89.10	**43.91**	a	21.96	6.49	33.12	**15.08**	b	8.67
Eriodictyol-*O*-hexose-*O*-deoxyhexoside (***f***)[d]	EHD	1.68	7.70	**4.77**	a	1.61	0.90	4.65	**3.22**	b	1.21
Mangiferin (**4**)	Mg	112.35	277.53	**180.76**	a	50.41	32.72	135.72	**72.00**	b	30.42
Isomangiferin (**5**)	IsoMg	29.96	81.47	**49.34**	a	14.40	17.40	48.61	**29.15**	b	9.45
Vicenin-2 (**6**)	Vic-2	6.36	12.78	**9.64**	a	1.66	5.05	11.99	**8.67**	b	2.01
Naringenin-*O*-hexose-*O*-deoxyhexoside A (***v***)	NHDA	0.54	5.49	**2.84**	b	1.39	0.86	14.34	**6.27**	a	3.75
Naringenin-*O*-hexose-*O*-deoxyhexoside B (***w***)	NHDB	4.61	49.61	**21.70**	a	11.42	2.38	14.93	**7.24**	b	3.83
Hesperidin (**9**)	Hd	10.77	22.06	**18.11**	a	3.33	8.50	16.59	**12.03**	b	2.22
Soluble solids	SS	1.57	2.50	**2.06**	a	0.24	1.74	2.50	**2.07**	a	0.20

Bold letters or numbers in brackets correspond to compound identification according to Beelders *et al*., 2014.
[a] Standard deviation.
[b] Percentage relative standard deviation.
[c] Expressed as 3-β-D-glucopyranosylmaclurin equivalents.
[d] Expressed as eriocitrin equivalents.

Table 5.5 Bitter intensity, individual phenolic content (mg.L^{-1}) and soluble solids content (g.L^{-1}) of *Cyclopia longifolia* infusions, prepared from fermented and unfermented plant material of corresponding batches (n = 24 batches)

		Unfermented (n = 24)					Fermented (90 °C/16 h) (n = 24)				
	Abbrev.	**Min.**	**Max.**	**Mean**		**SD[a]**	**Min.**	**Max.**	**Mean**		**SD[a]**
BITTER		22.4	71.2	**38.3**	a	13.8	4.4	21.7	**10.6**	b	5.6
3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (**5**)	IDG	4.59	34.31	**16.94**	a	9.09	4.99	40.35	**16.45**	a	8.79
3-β-D-Glucopyranosylmaclurin (**6**)	MMG	nd	10.98	**2.33**	a	3.31	nd[c]	1.93	**0.23**	b	0.55
3-β-D-Glucopyranosyliriflophenone (**13**)	IMG	1.23	57.46	**11.53**	a	15.62	0.89	20.06	**4.32**	b	5.17
Tetrahydroxyxanthone-di-*O*,*C*-hexoside isomer A (**16**)[d]	THXA	2.14	6.77	**3.55**	a	1.05	0.87	2.45	**1.47**	b	0.39
Tetrahydroxyxanthone-di-*O*,*C*-hexoside isomer B (**17**)[d]	THXB	1.79	5.44	**3.20**	a	0.88	1.15	3.17	**1.83**	b	0.55
Mangiferin (**22**)	Mg	101.79	390.08	**183.48**	a	79.31	13.95	135.13	**45.82**	b	30.35
Isomangiferin (**25**)	IsoMg	28.47	94.58	**50.38**	a	18.42	9.87	58.48	**24.18**	b	11.66
Vicenin-2 (**26**)	Vic-2	4.48	11.37	**8.12**	a	1.94	3.98	10.85	**6.72**	b	1.65
Eriocitrin (**32**)	ErioT	1.16	9.15	**3.68**	a	2.27	0.67	7.08	**2.90**	b	1.59
Scolymoside (**35**)	Scol	2.32	17.45	**8.71**	a	4.61	1.92	12.00	**6.43**	b	2.91
Hesperidin (**42**)	Hd	5.72	13.74	**9.13**	a	2.14	6.03	11.61	**8.45**	b	1.44
Soluble solids	SS	1.63	3.11	**2.18**	a	0.38	1.77	3.28	**2.25**	a	0.39

Bold numbers in brackets correspond to compound identification according to Schulze *et al.*, 2015.
[a] Standard deviation.
[b] Percentage relative standard deviation.
[c] nd, Not detected; limit of detection (LOD) = 0.0768 mg.L^{-1}.
[d] Expressed as mangiferin equivalents.

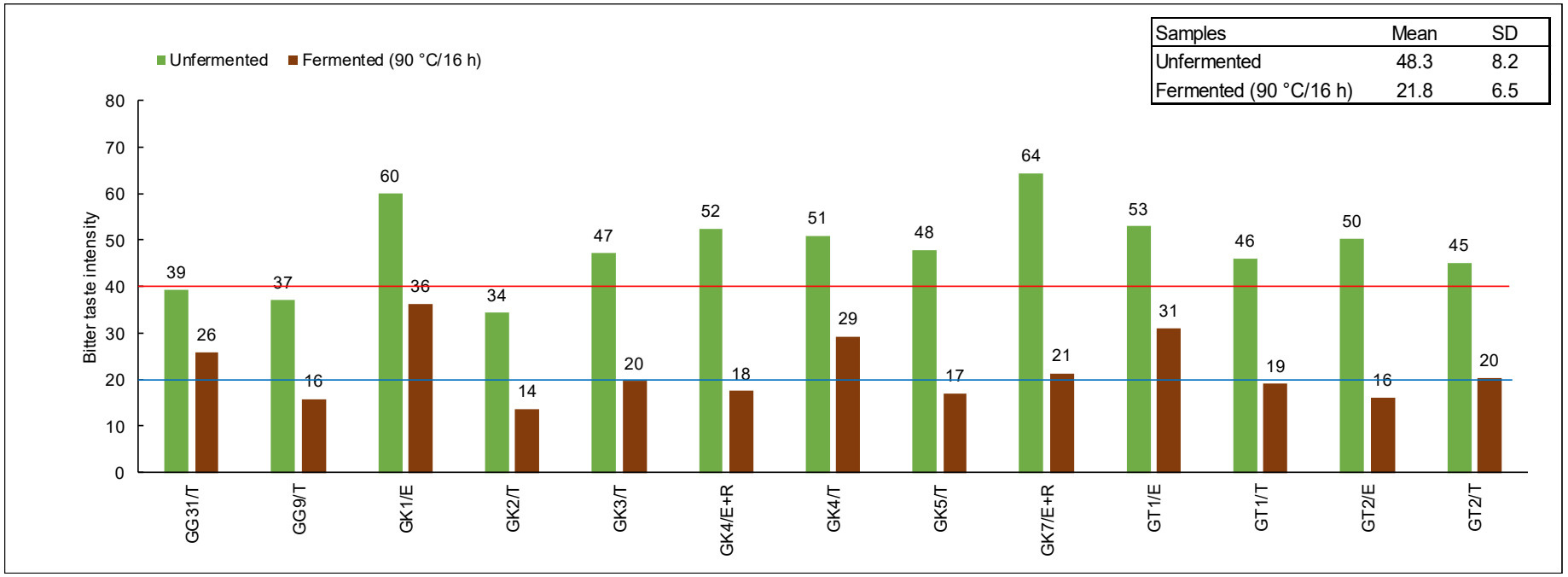


Figure 5.2 Effect of fermentation of *Cyclopia genistoides* on the bitter intensity of the infusions prepared from different batches of plant material (n = 13 batches). Samples are identified by genotype and locality (T = Toekomst, E = Elsenburg, E+R = Elsenburg and Riviersonderend, pooled). Blue line indicates mild bitter intensity. Red line indicates extreme bitter intensity.

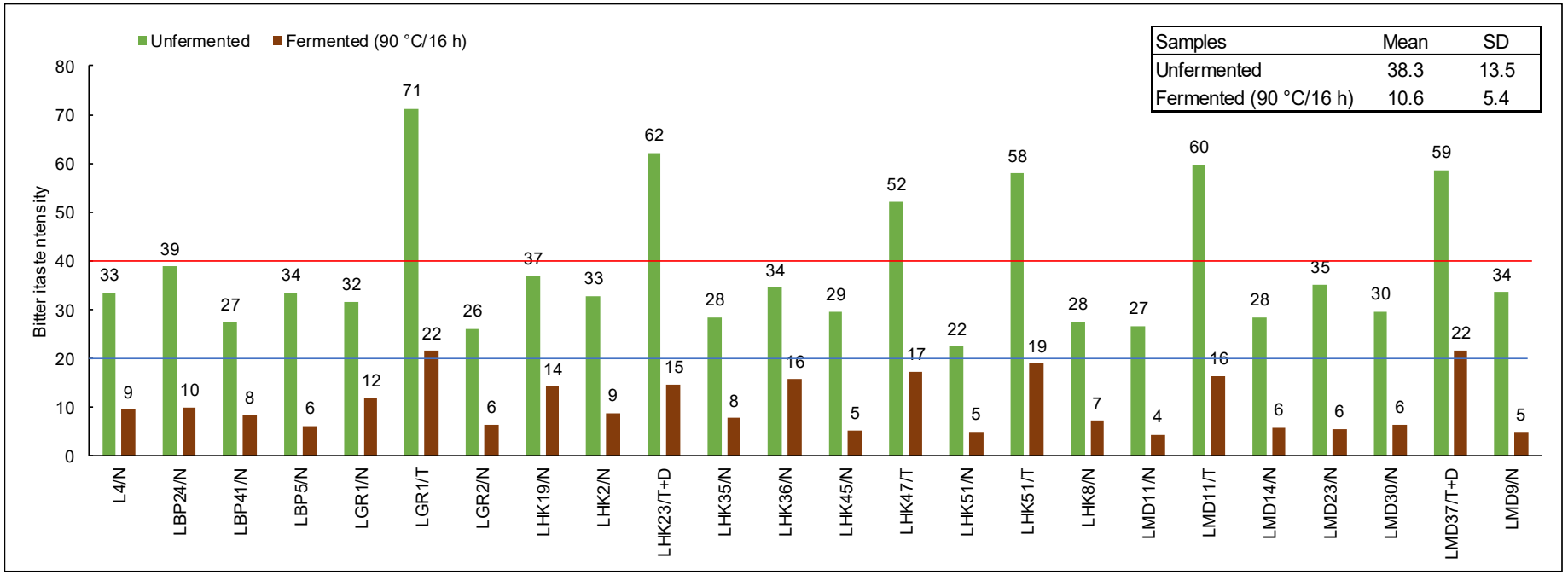


Figure 5.3 Effect of fermentation of *Cyclopia longifolia* on the bitter intensity of the infusions prepared from different batches of plant material (n = 24 batches). Samples are identified by genotype and locality (N = Nietvoorbij, T = Toekomst, T+D = Toekomst and Donkerhoek, pooled). Blue line indicates mild bitter intensity. Red line indicates extreme bitter intensity.

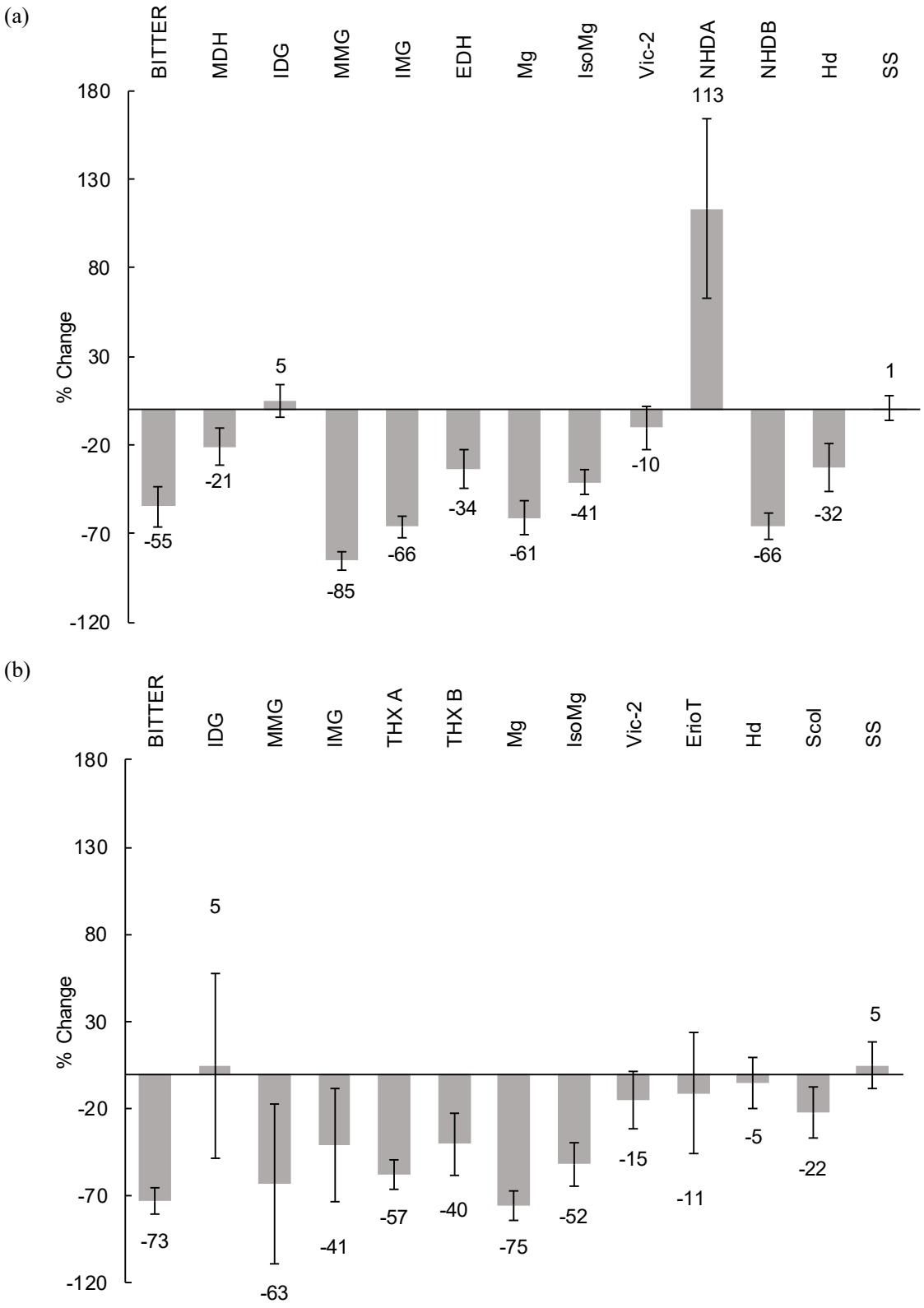
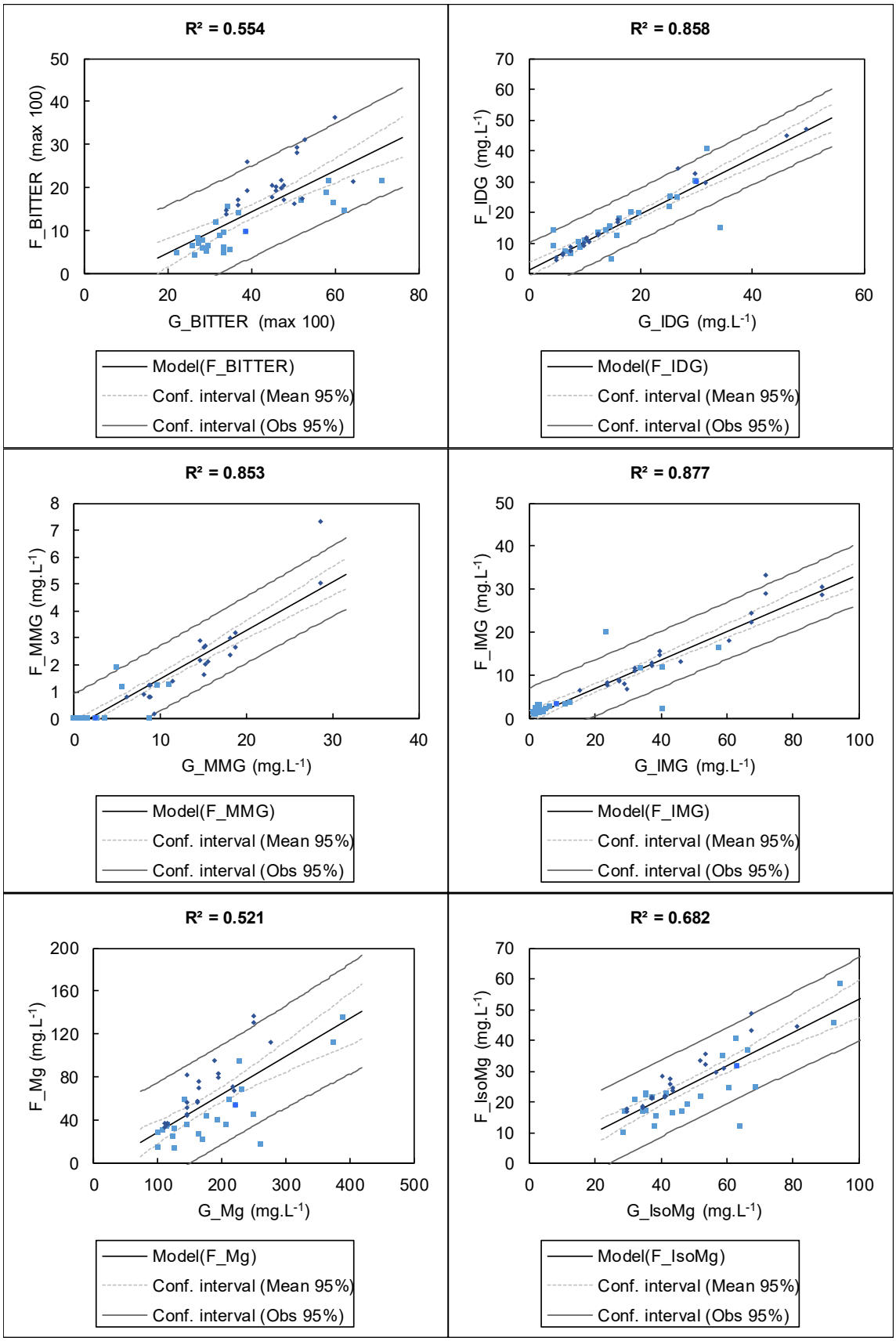


Figure 5.4 Mean percentage change in bitter intensity, individual phenolic content and soluble solids content of (a) *Cyclopia genistoides* (n = 13 batches) and (b) *C. longifolia* (n = 24 batches) infusions as a result of fermentation of the plant material. Abbreviations correspond to listed measurements in **Table 5.2** and **5.3**, respectively. Error bars indicate standard deviation.



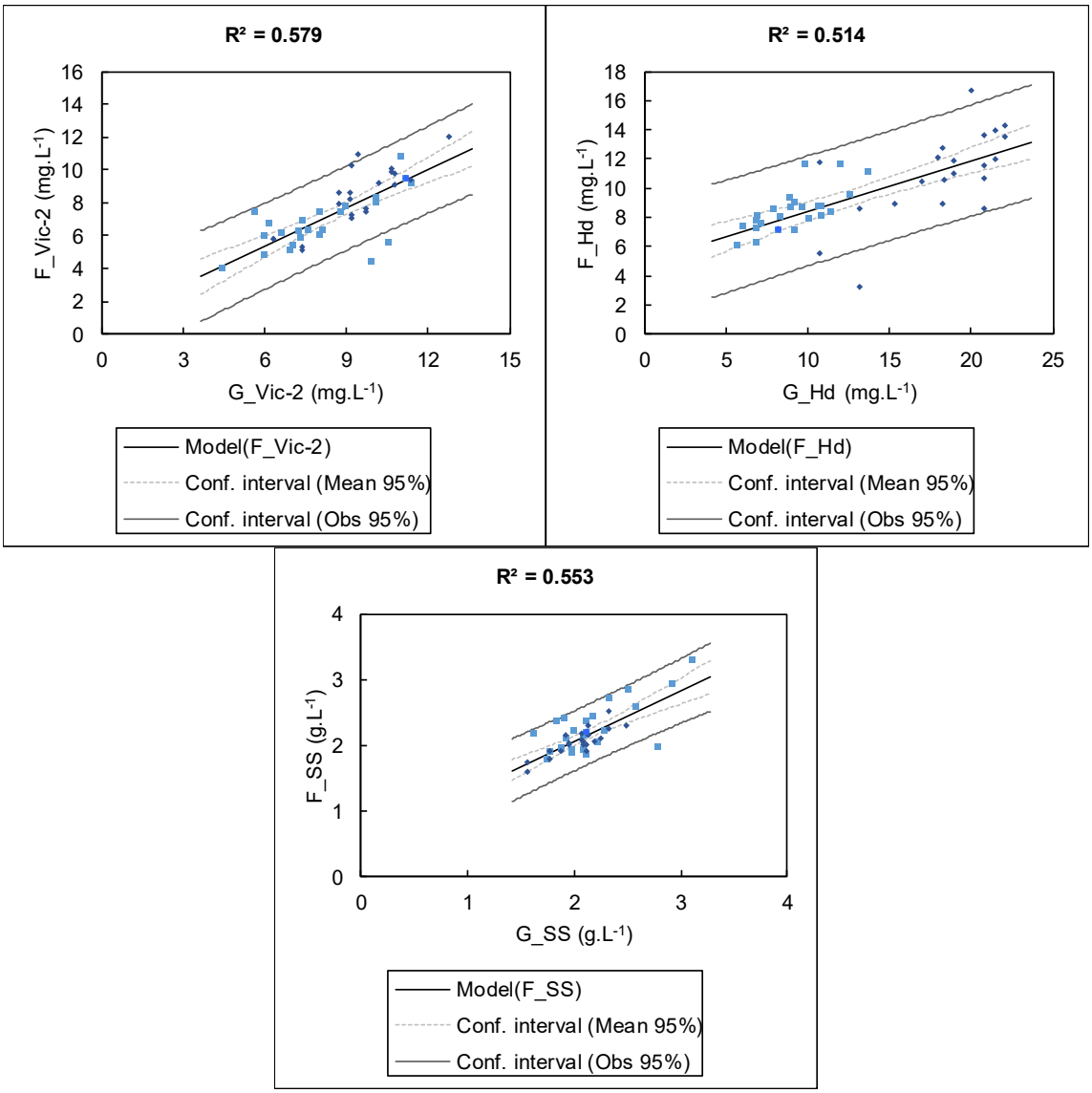


Figure 5.5 Correlations between mean parameters of infusions of corresponding fermented and unfermented plant material of the combined sample set (n = 45 batches). "F_" denotes fermented samples and "G_" denotes unfermented samples. Light blue squares show *Cyclopia longifolia* samples, dark blue diamonds show *C. genistoides* samples. Abbreviations explained in **Tables 5.2** and **5.3**.

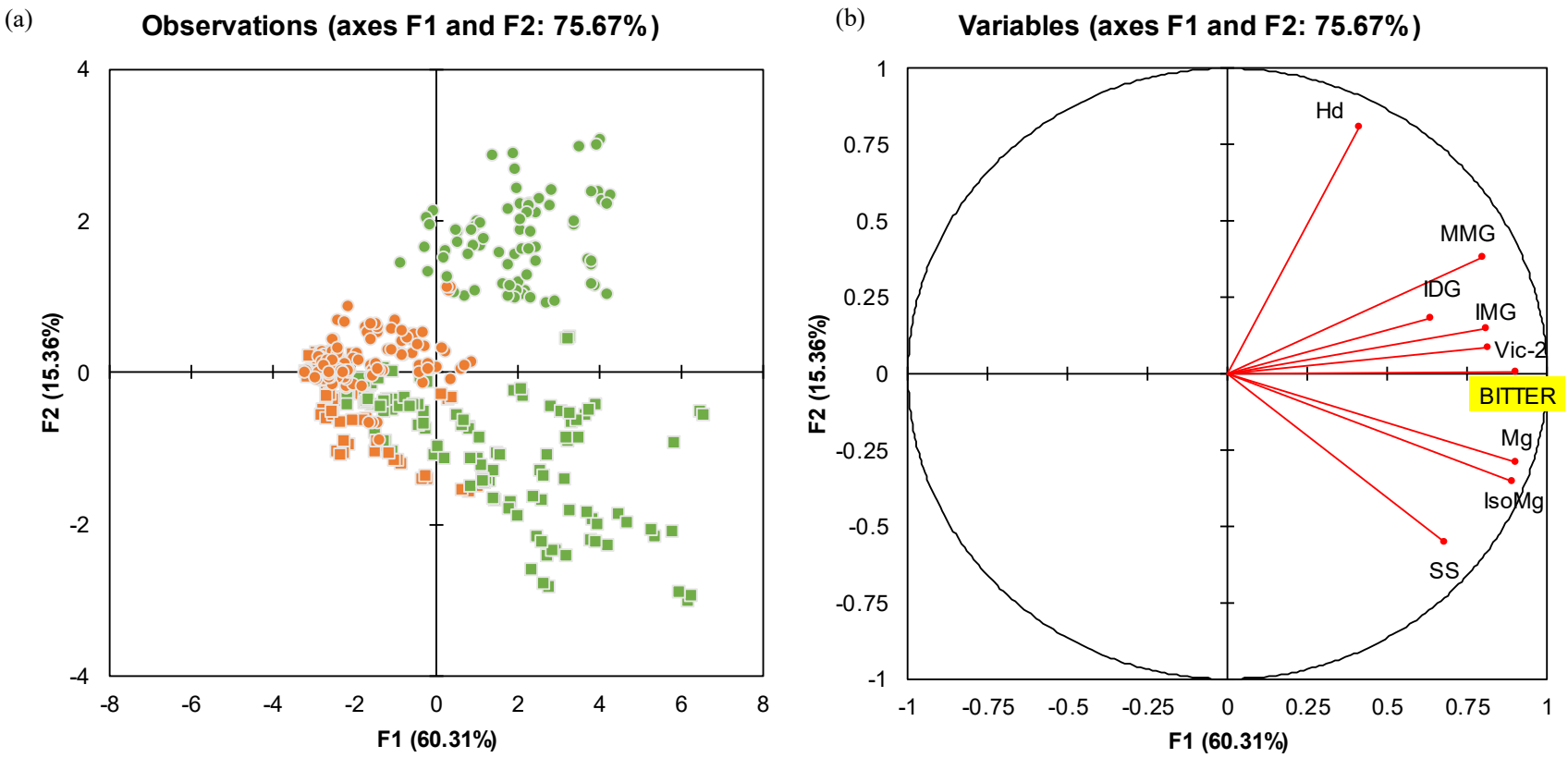
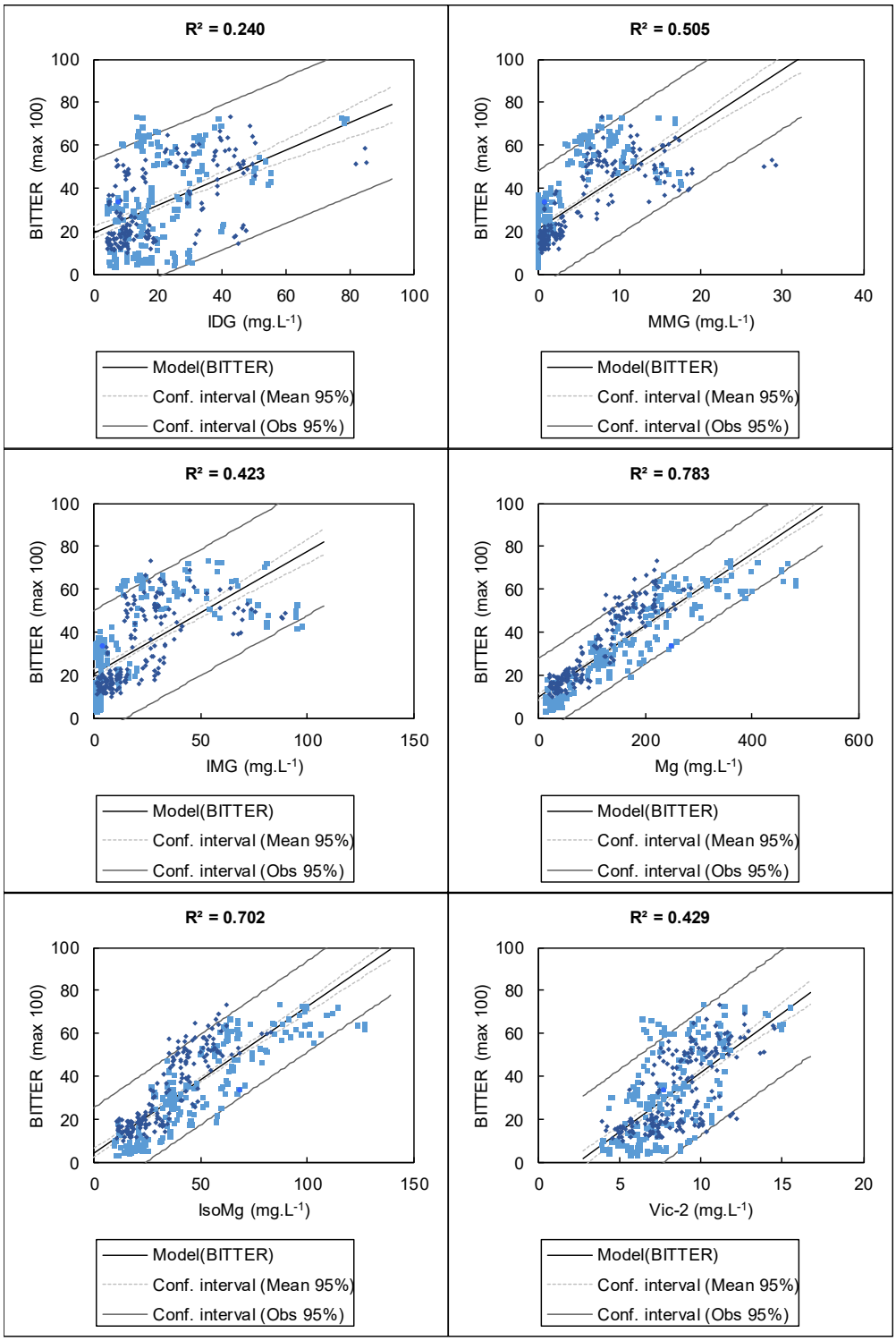


Figure 5.6 PCA scores (a) and loadings (b) plots of all analysed samples, considering bitter taste and all common HPLC quantified phenolic compounds (n = 378 infusions). Green markers indicate unfermented samples and orange markers indicate fermented samples. Circular markers indicate *Cyclopia genistoides*, and square markers indicate *C. longifolia* samples. Abbreviations explained in **Tables 5.2** and **5.3**.



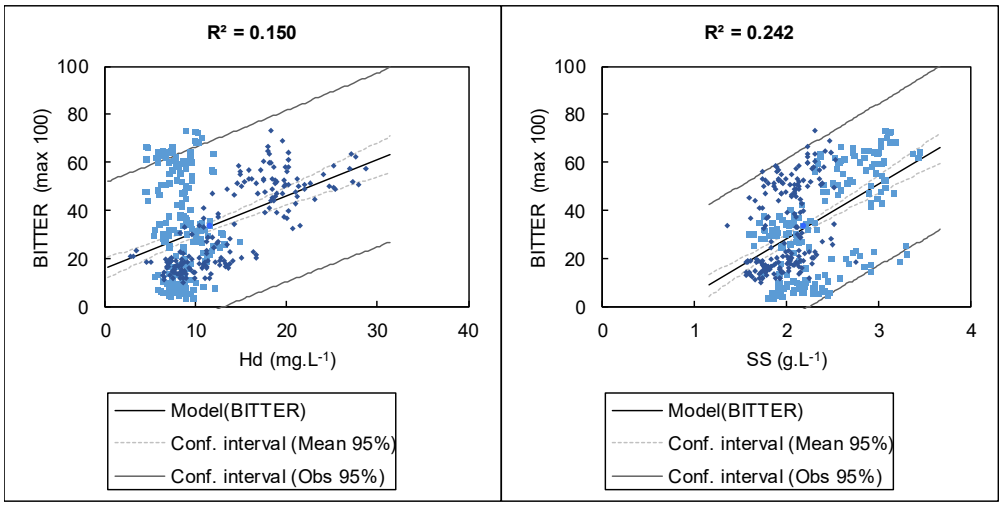


Figure 5.7 Linear regression of bitter intensity on all measured parameters for combined sample set (n = 378 infusions). Light blue squares show *Cyclopia longifolia* samples, dark blue diamonds show *C. genistoides* samples. Abbreviations explained in **Tables 5.2** and **5.3**.

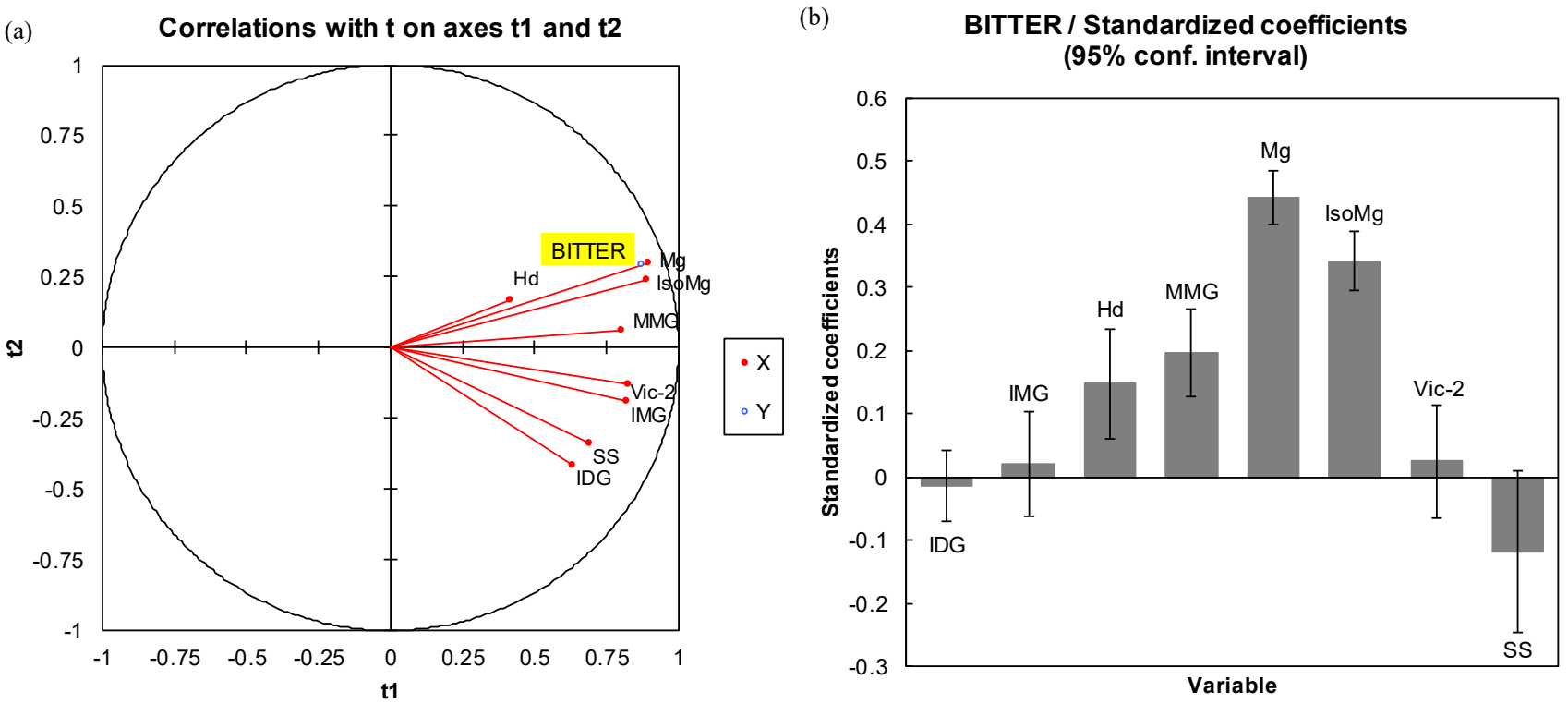


Figure 5.8 PLS regression (a) correlations and (b) standardised coefficients of bitter intensity on measured variables of all combined samples (n = 378 infusions). (R^2 = 0.841). Abbreviations explained in **Tables 5.2** and **5.3**. BITTER = 10.6 - 1.8E-02*IDG + 1.8E-02*IMG + 0.6*Hd + 0.7*MMG + 8.3E-02*Mg + 0.3*IsoMg + 0.2*Vic-2 - 5.4*SS.

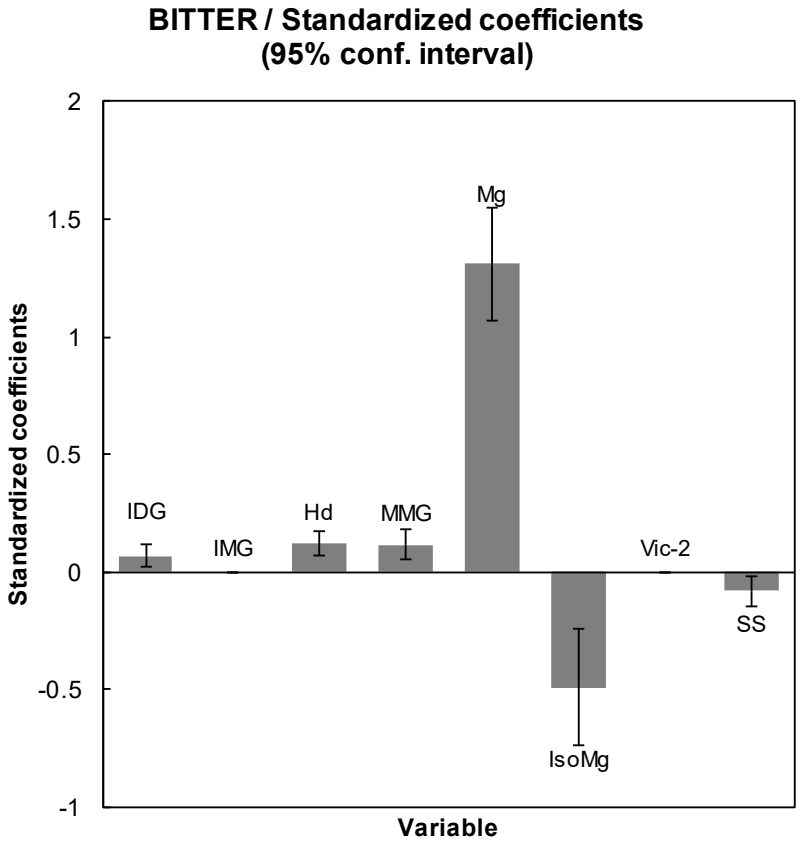


Figure 5.9 Validated stepwise linear regression standardised coefficient of the measured variables on bitter intensity of the complete data set (all *Cyclopia genistoides* and *C. longifolia* infusion samples, fermented and unfermented; training set, n = 328 infusions; independent validation set, n = 50 infusions). BITTER = 14.9 + 8.9E-0.2*IDG + 0.5*Hd + 0.4*MMG + 0.2*Mg - 0.4*IsoMg - 3.8*SS. (R^2 = 0.859). Abbreviations explained in **Tables 5.2** and **5.3**.

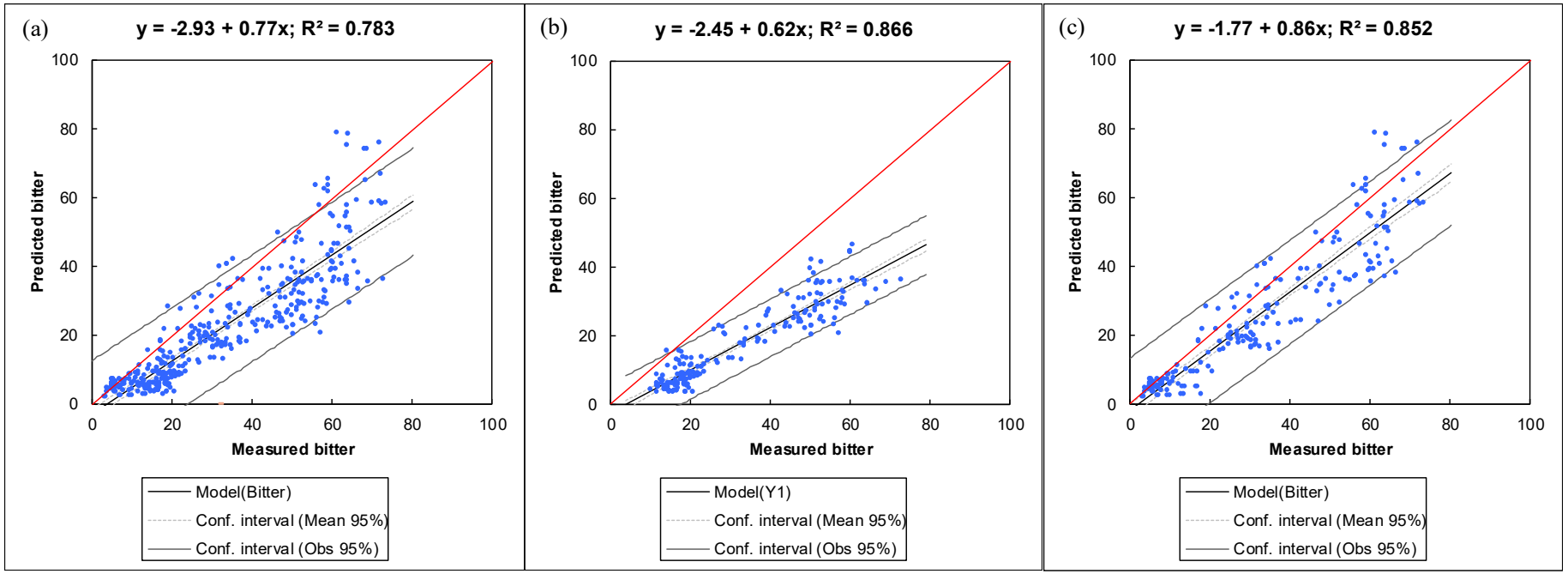


Figure 5.10 Linear regression of predicted and measured bitter intensity of (a) the total (n = 378 infusions), (b) the *Cyclopia genistoides* (n = 186 infusions) and (c) the *C. longifolia* (n = 192 infusions) data sets, according to the mangiferin dose-response model (**Chapter 3**, **Fig. 3.4**).

Chapter 6
General discussion, recommendations and conclusions

The growing honeybush tea industry is largely dependent on wild-harvesting and struggling to meet demand. Not only does it require expansion of commercial cultivation, but also cultivation of plant material delivering higher yields and good, consistent quality (Bester *et al*., 2016). The sensory quality of this herbal tea is of foremost importance as it determines consumer acceptance and loyalty, while high levels of bioactive phenolic compounds are important for the production of secondary value-added products, *i.e.* nutraceutical ingredients. High levels of polyphenols, however, may counter product acceptability due to their contribution to bitter taste of food and beverages (Drewnowski & Gomez-Carneros, 2000). Indeed, consumers expect honeybush tea to have a sweet taste and honey-like flavour (Vermeulen, 2015). This is relevant, as some *Cyclopia* species, especially *C. genistoides*, may produce a herbal tea with unacceptable bitter taste, detracting from their market value. *Cyclopia genistoides* is important for the growth of the honeybush industry as it has been successfully cultivated and grows well in sandy soils near the coast, as opposed to the mountainous areas of other species, subsequently expanding the cultivation area (Joubert *et al*., 2011).

The honeybush breeding programme of the Agricultural Research Council (ARC) is evaluating genetic lines of honeybush plants (genotypes) for improved product yield and quality (Bester *et al*., 2016). This entails the tedious process of sensory analysis of the processed product (Robertson *et al*., 2018). An alternative to sensory analysis to identify non-bitter genotypes and siblings, as well as other factors contributing to bitter taste, would help to stream-line the selection process.

In addition to good production potential, *C. genistoides* is also known for exceptionally high levels of specific phenolic compounds including xanthones and benzophenones (Schulze *et al*., 2015; Beelders *et al*., 2014a,b). Indeed, many xanthones and benzophenones, including those specific to honeybush, have been found to have desirable bioactive properties (Schulze *et al*., 2016; Lim *et al*., 2014; Wu *et al*., 2014; Zhang *et al*., 2013). Although the potential of phenolic compounds to contribute to the bitter taste of food products is considerable (Drewnowski & Gomez-Carneros, 2000), little to no information is available on the bitter taste

activity of the major honeybush phenolic compounds. Previously, several studies have attempted to predict the bitter intensity of infusions prepared from "fermented" honeybush (processed by high-temperature oxidation) from their phenolic content through associative multivariate statistical analyses (Moelich, 2018; Erasmus, 2015; Theron, 2012). Despite the obvious limitations of such an approach, where correlation does not necessarily indicate causation and co-linearity between parameters prevails, several general consistencies have been observed between the studies. These include common associations between bitter taste and the major xanthones (mangiferin and isomangiferin), as well as several benzophenones. Although these compounds are also the major phenolics in honeybush that are affected by fermentation (Beelders *et al*., 2015), the studies by Theron (2012), Erasmus (2015) and Moelich (2018) pointed the way forward for a closer investigation of the contribution of phenolic compounds to the bitter taste of honeybush infusions.

The present study therefore aimed to provide insight into the contribution of phenolic compounds to the bitter taste of honeybush infusions. The direct and indirect (modulation) contributions of crude phenolic fractions, as well as individual major phenolic compounds to the bitter taste of *C. genistoides* were determined. Further insight was gained by studying the effect of fermentation on bitter intensity and individual polyphenol content of hot water infusions prepared from several genotypes of two species (*C. genistoides* and *C. longifolia*). *Cyclopia longifolia* was included since this species also has a high xanthone and benzophenone content (Schulze *et al*., 2015) and produces an infusion with a detectable bitter taste when under-fermented (Erasmus *et al*., 2017). Multivariate statistical analyses of this data confirmed several observations regarding bitter taste contributions of individual compounds and provided a stepwise linear regression model to predict the bitter intensity of a honeybush infusion based on the levels of five compounds common to both *Cyclopia* species, along with soluble solids (SS) content.

The first step in the current investigation was the preparation of three crude phenolic fractions from a hot water extract of unfermented *C. genistoides* material specifically selected based on the distinct bitter taste of its hot water infusion. These fractions, rich in benzophenones, xanthones and flavanones, respectively, were analysed for bitter taste using dose-response and threshold sensory methodologies. Samples were prepared in an aqueous EtOH solution (2.5%) and served at room temperature. Considering the infusion equivalent concentration (IEC) of each fraction, the bitter taste of each fraction was analysed. The IEC of a fraction represents its fractional yield contribution from the hot water extract to 2 g $SS.L^{-1}$, which equals the SS concentration of a hot water infusion prepared from the same plant material. The results indicated that the

benzophenone-rich fraction was not bitter (< 5 on a 100-point intensity scale), the flavanone-rich fraction was somewhat bitter (~13) and the xanthone-rich fraction was distinctly bitter (~31). Closer investigation of the xanthone-rich fraction was undertaken by descriptive sensory analysis (DSA) of the major xanthones, mangiferin and isomangiferin. The pure compounds were dissolved in hot water and served at 60 °C to better represent the herbal tea infusion (sensory analysis of honeybush tea taste modalities is normally performed at 60 °C; Robertson *et al*., 2018; Bergh *et al*., 2017; Erasmus *et al*., 2017; Theron *et al*., 2014). Jacketed flasks and a magnetic stirrer were used to prepare the solutions. Mangiferin was indeed found to be a major contributor to bitter taste (~30), although its regio-isomer, isomangiferin, was only somewhat bitter (~15) when tested either at the same concentration as mangiferin, or at its IEC (lower than that of mangiferin). A combination of the two isomers at their IEC values in the xanthone-rich fraction (118 and 34 $mg.L^{-1}$, respectively) indicated that isomangiferin reduced the bitter intensity of mangiferin. This was the first conclusive evidence of bitter taste modulation of a major honeybush polyphenol contributing to the bitter taste of honeybush infusions and prompted further investigation into the possible modulatory capacity of honeybush crude phenolic fractions and major individual phenolic compounds.

For the second step of the current investigation, several combinations of either benzophenones or flavanones with the xanthone-rich fraction or mangiferin were tasted at concentrations taking into account their IEC, solubility and availability. One major challenge was the availability of pure compounds. Many of the compounds were extremely expensive when purchased commercially, or subject to time- and resource-intensive isolations. Indeed, the isolation of naringenin-*O*-hexose-*O*-deoxyhexoside isomer A (NHDA) was unsuccessful using available resources and will be pursued in future. Due to the lack of availability of the desired compounds, several experimental adaptations to the approach had to be made. An expert sensory panel of eight experienced panellists was selected to reduce the number of samples and quantity required whilst retaining the statistical integrity of the experiment. Although the aim was to use a realistic range of concentrations relative to the IEC for a dose-response effect, the target concentrations (IEC, half and double IEC) were often reduced to levels that were more attainable. The solubility of individual compounds also limited reachable concentrations in both the hot water medium and at the low EtOH concentration (2.5%), with a higher EtOH content not a feasible option because of a severe impact on taste perception. In addition, the small quantity of compound that had to be weighed off (often not more than 2 mg on a 5-decimal balance) posed problems for the accuracy of each sample and led to slight discrepancies in experimental concentration

between samples. Regarding the calculated phenolic concentrations, the hot water extract according to which these calculations were performed, was prepared with unfermented material which has a higher phenolic content than fermented material. The expected concentrations of individual phenolics in an infusion of fermented material will thus often be lower than the calculated IEC in the current study. Further investigations may focus on the lower (and possibly more attainable) expected "baseline" concentrations of these compounds expected in fermented infusions (Schulze *et al*., 2015) to determine their effect in a typical cup of honeybush tea from fermented material. Model solutions investigating the effect of modulatory compounds on the main bitter contributor, mangiferin, could also yield valuable insight.

Although the tasteless benzophenone-rich fraction increased the bitter intensity of the xanthone-rich fraction, the major individual benzophenones did not produce the same effect. It is thus speculated that minor benzophenone compounds, such as 3-β-D-glucopyranosylmaclurin (MMG) or maclurin-di-*O*,*C*-hexoside, may provide the benzophenone-rich fraction with its bitter enhancing capacity of the xanthone-rich fraction. Indeed, these compounds contain a catechol moiety which has been implicated in bitter taste receptor (TAS2R5) activation (Soares *et al*., 2013). This, however, would require further investigation.

The flavanone-rich fraction and its major flavanone compounds were also investigated for modulating capacity. The flavanone-rich fraction suppressed the bitter taste of the xanthone-rich fraction, as the bitter intensity of the combination was lower than the theoretical additive bitter intensity of the individual components. In addition, hesperidin was found to possess a slight bitter taste, contrary to literature (Rousseff *et al*., 1987, as referenced by Frydman *et al*., 2004), although this compound had no effect on the bitter intensity of the xanthone-rich fraction. The other major flavanone, naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (NHDB), is prominent in *C. genistoides* plant material, and was shown to convert to its isomer A during the fermentation process. NHDB enhanced the bitter intensity of the xanthone-rich fraction at lower concentrations (12 $mg.L^{-1}$; similar to concentrations typically found in fermented herbal tea infusions; Schulze *et al*., 2015). A combination of the two isomers prepared by heating NHDB at its IEC (24 $mg.L^{-1}$) for 16 h at 90 °C yielded a mixture (approx. 1:1) of NHDB and NHDA. Although the NHDB content was similar to the lower concentration at which bitter modulation was observed, the combination had no effect on the bitter intensity of the xanthone-rich fraction. This may suggest that NHDA restricts the bitter enhancing effect of NHDB, or that the combined concentration of both compounds (24 $mg.L^{-1}$) was the same as the higher concentration of NHDB which resulted in no modulatory effect when combined with the xanthone-rich fraction. It can therefore

indicate that the two isomers either elicit the same effect, whether in a mixture or in isolation, or that NHDA is able to avert the bitter enhancing activity of NHDB. This experiment presented several limitations in terms of solubility and sample availability and we were not able to pursue the matter further. There would, however, be value in understanding the contribution of these isomers to the bitter taste of *C. genistoides* infusions. Indeed, investigation is currently underway to determine the kinetics of NHDB degradation and formation of its isomer, NHDA, for greater insight into their behaviour during the production of fermented honeybush tea. Structure elucidation of the two isomers is also ongoing, but these investigations fall outside the scope of the current study.

As many of these compounds occur in honeybush infusions at low concentrations and are expensive to isolate, it may be prudent to apply techniques other than DSA. Specifically, the application of cell-based assays may be useful to determine bitter taste receptor activation and response (as reviewed by Riedel *et al*., 2017). It may be interesting to determine which TAS2R bitter taste receptors may be activated by bitter honeybush infusions. It may also aid in identifying possible additional minor compounds as modulators, and to confirm bitter taste contributions and bitter modulatory effects observed in this study.

Another hurdle in the analysis of honeybush bitter taste is the observation that not all bitter taste perceptions are the same. In the study it was a common occurrence for panellists to comment on the "late blooming" effect of the bitterness of some samples, whereas other samples may have had a more rapid bitter taste response, although they may have had the same "absolute" bitter intensity. Other observations were a "hard" bitterness and a more "rounded" bitter taste. Application of time intensity analysis in future studies may allow a more holistic approach to bitter taste analysis and aid in characterising the different "types" of bitterness while quantifying bitter intensity.

The results of this study also indicated the relevance of the concentrations of the phenolic compounds to their ability to affect the bitter intensity of the crude phenolic fractions. As fermentation is the major factor in reducing the phenolic content of honeybush plant material, the effects of this process on both bitter intensity and individual phenolic content were investigated to gain additional insight into the effect that different concentrations of phenolics will have on bitter intensity of the infusion. The final phase of the study thus included DSA and phenolic quantification of a large number of infusions prepared from fermented and unfermented material of several genotypes of the two potentially bitter-tasting species (*C. genistoides* and *C. longifolia*). Clones of some genotypes, planted at different locations, were included in the sample set. The

total sample set also included a subset for each species where the batch of plant material was divided and processed to produce both unfermented and fermented (90 °C/16 h) material (*C. genistoides* = 13 batches; *C. longifolia* = 24 batches). This allowed direct comparison of fermented and unfermented samples, without natural variation in composition confounding the effect of fermentation on phenolic composition. This was not possible for all samples, as plant material shortage due to drought was unavoidable. Despite this limitation, this study is the first to present data on the reduction of bitter intensity of infusions due to fermentation by comparing a large sample set containing both fermented and unfermented plant material. Differences were evident between the two *Cyclopia* species, both in terms of the extent of phenolic degradation and bitter taste reduction. Bitter taste reduction through fermentation was more effective for *C. longifolia* than for *C. genistoides*, highlighting the problem often faced in industry with inconsistent production batches. The relative degradation of the two major xanthones, mangiferin and isomangiferin, also differed, with infusions from the two species having different ratios of these two compounds in infusions from unfermented and fermented material. The effect of bitter modulation due to the interaction of these two compounds on bitter taste in the fermented infusions is not yet completely understood. Further investigations focussing on the impact that variation in their ratio will have on bitter intensity of the infusions may be interesting, although the cost of pure isomangiferin may be prohibitive. Nevertheless, correlation between mangiferin and bitter intensity was relatively strong (R^2 = 0.783 for the combined data set). By applying the dose-response linear regression model of mangiferin from the first phase of the study to the individual samples of the combined data set, representing unfermented and fermented *C. genistoides* and *C. longifolia* (R^2 = 0.7831), it was evident that the model consistently underestimated the true bitter intensity of the infusions. Although it is tempting to use of this sole compound as bitterness predictor, this may lead to a great variation in predicted bitterness which will not benefit the industry.

Multivariate statistical analysis of the complete sample set was the first to include both fermented and unfermented samples, increasing the variation in phenolic content and bitter intensity. Analysis was hindered by the divergence in phenolic profiles between the two species, allowing for the consideration of only seven phenolic compounds common to both species (IDG, MMG, 3-β-D-glucopyranosyliriflophenone, mangiferin, isomangiferin, vicenin-2 and hesperidin). Apart from the difference in phenolic stability and thus extent of their degradation in the two species during fermentation, the variables used in bitterness prediction models also differed between the two species. A factor that may play a role includes the physical differences in leaf

shape and thickness between the two species and the consequential difference in their response to applied heat during fermentation. This variation in the accuracy of model prediction between species has been seen before (Erasmus, 2015). Several multivariate methods were applied, including principal component analysis, partial least squares regression and stepwise linear regression, as used in previous studies (Moelich, 2018; Erasmus, 2015).

Interestingly, stepwise linear regression proved to be the most valuable method, whereas previous studies found it to be ineffective. The model confirmed several observations in the study and utilised only five phenolic compounds (IDG, MMG, mangiferin, isomangiferin and hesperidin) and SS content common to both species. This method addressed the modulation between mangiferin and isomangiferin, as well as the observed bitter enhancement by NHDB (in the *C. genistoides* model), and bitter suppression by IDG (in the *C. longifolia* model). Validation of the model for the combined dataset with an external dataset provided acceptable prediction ($R^2 > 0.8$).

Nevertheless, the developed model may not necessarily be applicable to all *Cyclopia* species, especially since not all contain high levels of benzophenones. A large-scale investigation comprising of the commercialised species and both fermented and unfermented material may yield interesting insights. Such data may also shed light on the significance of the mangiferin:isomangiferin ratio in bitter intensity. These species may contain additional minor components that could complicate the comparison significantly. This was potentially the case in the study by Erasmus (2015), where fermented samples from four species were used for the development of a similar bitterness prediction model.

The present study has identified several phenolic compounds that are relevant to bitter taste of honeybush infusions. Many of these compounds are also relevant in terms of bioactivity, confounding the objective of plant breeding and selection for plant material with a low bitterness potential. Recent studies, however, have revealed that bitter taste receptors are located in several extra-oral locations in the body whereby bitter compounds may bind to allow beneficial physiological responses such as increased gastric mobility and satiation, thyroid stimulation, as well as glucose and insulin homeostasis (Avau *et al*., 2015; Clark *et al*., 2015; Chen *et al*., 2011; Singh *et al*., 2011; Deshpande *et al*., 2010; Shah *et al*., 2009; Dotson *et al*., 2008; Wu *et al*., 2005; 2002). Bitter receptor activation studies utilising bioactive honeybush compounds, as mentioned before, may thus also provide clues as to the possible health benefits of honeybush and how these are delivered in the body. This may further support the use of honeybush extracts and compounds as nutraceuticals. Indeed, this

route of plant material utilisation is a viable alternative for plant material rejected for herbal tea production due to unacceptable bitter taste. *Cyclopia genistoides* thus remains a promising species for the development of the industry. It may also be useful to investigate the application of micro- or nano-encapsulation of the extract with a suitable polymer to mask bitter taste (Coupland & Hayes, 2014; Ley, 2008) of honeybush ready-to-drink iced tea beverages, prepared with extracts containing high levels of mangiferin.

Overall, the insights gained in this study will aid in understanding the potential bitter taste of this *Cyclopia* species, as well as others. The application of the bitter prediction model may aid in reducing product losses and improve product consistency. Selection of genotypes for propagation in the honeybush plant breeding programme may also be simplified by avoiding tedious sensory analysis and limiting HPLC analysis to the quantification of five phenolic compounds. Understanding the bitter taste contributions of honeybush phenolics may also be of interest regarding the global interest in food products and plant secondary metabolites as food additives or nutraceuticals. This study has served as an introductory investigation, laying the foundation for several interesting new routes of investigation to support the growth and development of the honeybush industry as a key driver of socioeconomic development in rural South Africa.

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benzophenone *C*-glucosides from leaves of *Mangifera indica* L. *Journal of Agricultural and Food Chemistry*, **61**, 1884-1895. DOI: 10.1021/jf305256w.

ADDENDUM A

Supplementary material

Bitter profiling of phenolic fractions of green *Cyclopia genistoides* herbal tea

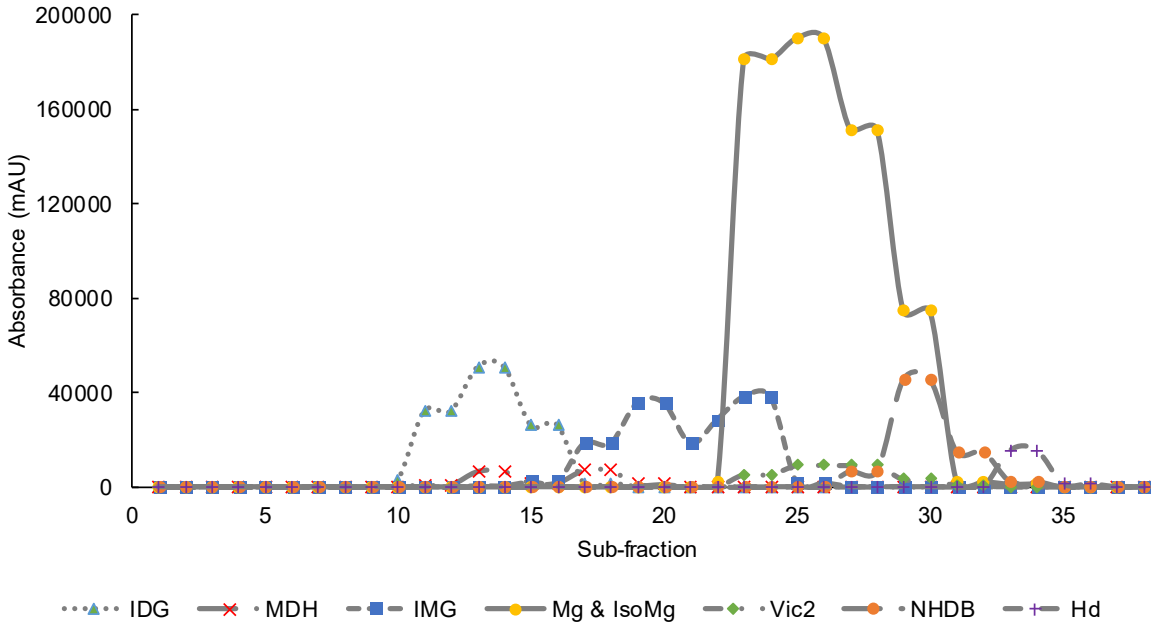


Figure A.1 Main phenolic composition of sub-fractions. IDG = 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone, MDH = maclurin-di-*O,C*-hexoside, IMG = 3-β-D-glucopyranosyliriflophenone, Mg = mangiferin, IsoMg = isomangiferin, Vic2 = vicenin-2, NHDB = naringenin-*O*-hexose-*O*-deoxyhexoside isomer B, Hd = hesperidin.

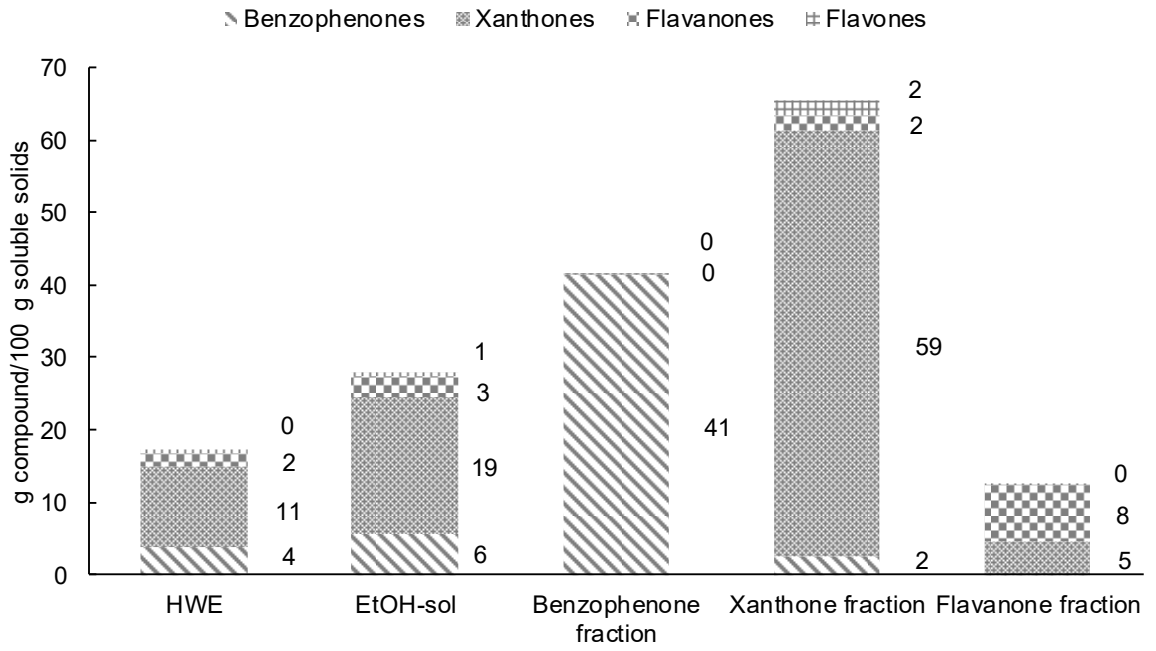


Figure A.2 Phenolic sub-class composition of original hot water extract (HWE), EtOH-soluble solids fraction of HWE (EtOH-sol) and crude phenolic fractions of EtOH-sol.

Table A.1 Basic tastes and taste combinations used in panel taste training

Taste sensation	Tastant	Concentration ($g.L^{-1}$)	Tastant	Concentration ($g.L^{-1}$)
Sweet	sucrose	20		
Sour	citric acid	0.7		
Bitter	caffeine	0.7		
Astringent	alum	1		
Sweet/Sour	sucrose	40	citric acid	1.4
Bitter/Astringent	caffeine	1.4	alum	1
Sour/Astringent	citric acid	1.4	alum	1
Bitter/Sour	caffeine	1.4	citric acid	1.4
Sweet/Astringent	sucrose	40	alum	1
Sweet/Bitter	sucrose	40	caffeine	1.4

Table A.2 Fraction and extract concentrations for the measurement of dose-response bitter taste profiles

Sample level	Concentration ($mg.L^{-1}$)			
	B	X	F	HWE
1	0	0	0	0
2	53	40	79	250
3	79	59	119	500
4	119	89	178	1000
5	178	133	267	2000
6	267	200	400	4000
7	400	300	600	8000

B = benzophenone-rich fraction, X = xanthone-rich fraction, F = flavanone-rich fraction, HWE = hot water extract.

Table A.3 Concentrations of phenolic fractions presented for sensory threshold analysis

Sample level	Concentration ($mg.L^{-1}$)		
	B	X	F
1	23	18	16
2	35	26	23
3	53	40	35
4	79	59	53
5	119	89	79
6	178	133	119
7	267	200	178
8	400	300	267

B = benzophenone-rich fraction, X = xanthone-rich fraction, F = flavanone-rich fraction.

Figure A.3 Jacketed flasks and circulation water bath for sample dissolution in hot water.

Figure A.4 Amber vials placed in metal racks for sample presentation.

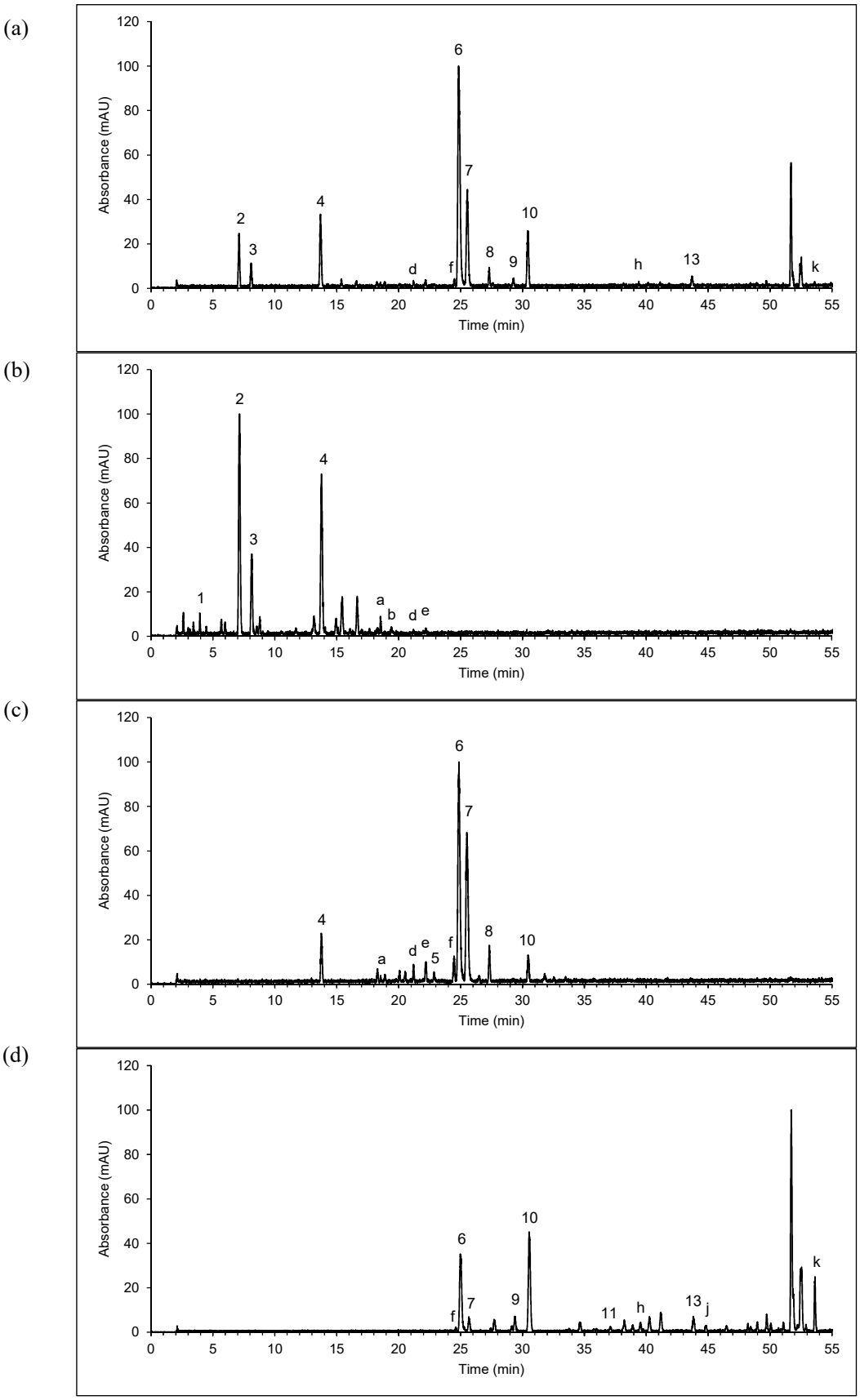


Figure A.5 LC-MS negative ionisation total ion chromatogram of (a) hot water extract, (b) benzophenone-, (c) xanthone- and (d) flavanone-rich fractions. Peak numbers correspond to those in **Tables A.4** and **A.5**.

Table A.4 Retention time (t_R), UV-Vis and LC-MS characteristics of quantified (Beelders *et al*., 2014b) phenolics in hot water extract (H), EtOH-soluble (E), benzophenone- (B), xanthone- (X) and flavanone-rich (F) fractions

#	t_R (min)	λ_{max} (nm)	Mode	H	E	B	X	F	$[M-H]^-$/ $[M+H]^+$ (*m/z*)	Molecular formula	Error	Fragment ions	Compound name
1	3.94	237, 315	-		x	x			585.1474	$C_{25}H_{29}O_{16}$	3.1	465, 385, 355, 341, 333, 329, 325, 303*, 223, 193	Maclurin-di-*O,C*-hexoside
			+	x	x	x			587.1619	$C_{25}H_{31}O_{16}$	1.2	407, 33, 329, 287, 275, 231, 219, 195, 177, 165, 149	
2	7.11	234, 294	-	x	x	x			569.1506	$C_{25}H_{29}O_{15}$	0.0	479, 449*, 317, 287, 193, 167	3-β-D-Glucopyranosyl-4-β-D-glucopyranosyl-iriflophenone
			+	x	x	x			571.1663	$C_{25}H_{31}O_{15}$	3.3	391, 373, 355, 343, 337, 327*, 313, 309, 297, 289, 271, 261, 243, 231, 219, 195, 177, 165, 121	
3	7.99	237, 319	-	x	x	x			423.0921	$C_{19}H_{19}O_{11}$	-1.4	303*, 223, 193	3-β-D-Glucopyranosyl-maclurin
			+	x	x	x			425.1084	$C_{19}H_{21}O_{11}$	-0.3	343, 341, 329, 313, 299, 287, 275, 261, 243, 231, 219, 195*, 177, 165, 149, 137, 121	
4	13.68	234, 296	-	x	x	x	x		407.0971	$C_{19}H_{19}O_{10}$	-1.7	317, 287*, 245, 193	3-β-D-Glucopyranosyl-iriflophenone
			+	x	x	x	x		409. 1135	$C_{19}H_{21}O_{10}$	-1.7	391, 357, 337, 327*, 313, 297, 285, 271, 243, 231, 219, 195, 177, 165, 149, 121	
5	22.91	236, 283	-	x	x				595.1681	$C_{27}H_{31}O_{15}$	3.0	459, 421, 325, 175, 161, 151, 137*, 135	Eriodictyol-*O*-hexose-*O*-deoxyhexoside isomer A
			+	x	x		x		597.1819	$C_{27}H_{33}O_{15}$	3.3	359, 427, 411*, 327, 299, 289, 241, 201, 195, 175, 163, 153, 131	
6	24.84	241, 258, 319, 366	-	x	x		x	x	421.0766	$C_{19}H_{17}O_{11}$	-1.2	331, 313, 301*, 271, 258	Mangiferin
			+	x	x		x		423.0927	$C_{19}H_{19}O_{11}$	0.2	405*, 387, 369, 357, 327, 303, 280, 273, 251, 240, 231	
7	25.55	256, 317, 366	-	x	x		x	x	421.0756	$C_{19}H_{17}O_{11}$	-3.6	331, 313, 301*, 271, 258	Isomangiferin
			+	x	x		x		423.0927	$C_{19}H_{19}O_{11}$	-4.2	405, 387, 345, 318, 299, 273, 264, 247, 239, 231*, 195	
8	27.19	234, 271, 334	-	x	x		x		593.1525	$C_{27}H_{29}O_{15}$	3.2	503, 473, 395, 383, 353*, 325, 297	Vicenin-2
			+	x	x		x		595.1663	$C_{27}H_{31}O_{15}$	0.0	577, 559, 541, 527, 511, 481, 457, 439, 427, 421, 409, 403, 391, 379, 363, 355, 349, 337, 325*, 307, 295	
9	29.3	233, 282	-	x	x			x	579.1706	$C_{27}H_{31}O_{14}$	-1.4	459, 433, 355, 271*, 151	Naringenin-*O*-hexose-*O*-deoxyhexoside isomer A
			+	x	x		x		581.1870	$C_{27}H_{33}O_{14}$	-1.4	561*, 415, 385, 369, 353, 326, 311, 299, 287, 273, 231, 219, 195, 189, 173, 165, 153, 147	
10	30.42	235, 281	-	x	x		x	x	579.1699	$C_{27}H_{31}O_{14}$	-2.6	459, 433, 355, 271*, 151, 145	Naringenin-*O*-hexose-*O*-deoxyhexoside isomer B
			+	x	x		x	x	581.1870	$C_{27}H_{33}O_{14}$	-1.9	561, 473, 353, 311, 199, 285, 273*, 231, 15, 189, 153, 147	
11	37.09	258, 318, 366	-	x	x			x	421.0768	$C_{19}H_{17}O_{11}$	-0.7	331, 301*, 271, 258	Tetrahydroxyxanthone-*C*-hexoside isomer A
			+	x				x	423.0920	$C_{19}H_{19}O_{11}$	-1.7	341, 303, 299, 273*, 257	
12	42.84	257, 318, 366	-	x	x			x	421.0745	$C_{19}H_{17}O_{11}$	-2.6	331, 301*, 271, 258	Tetrahydroxyxanthone-*C*-hexoside isomer B
			+	x				x	423.0941	$C_{19}H_{19}O_{11}$	1.4	303, 299, 273, 257*	
13	43.68	233, 285	-	x	x			x	609.1805	$C_{28}H_{33}O_{15}$	-2.3	323, 301*	Hesperidin
			+	x				x	611.1976	$C_{28}H_{35}O_{15}$	1.8	303*	

Table A.5 Retention time (t_R), UV-Vis and LC-MS characteristics of unquantified phenolics in hot water extract (H), EtOH-soluble (E), benzophenone- (B), xanthone- (X) and flavanone-rich (F) fractions

#	t_R (min)	λ_{max} (nm)	Mode	H	E	B	X	F	$[M-H]^-$/ $[M+H]^+$ (*m/z*)	Molecular formula	Error	Fragment ions	Compound name
a	18.54	241, 260, 316, 366	-	x	x	x	x		583.1283	$C_{25}H_{27}O_{16}$	-2.7	493, 463, 421, 331, 301*, 272, 175	Tetrahydroxyxanthone-di-*O,C*-hexoside isomer
			+	x	x	x	x		585.1452	$C_{25}H_{29}O_{16}$	-0.7	405, 387, 369, 327, 303, 299, 273*	
b	19.55	not clear	-		x	x			583.1292	$C_{25}H_{27}O_{16}$	-1.2	493, 481, 463, 457, 331, 301*, 272, 259, 175, 163	Tetrahydroxyxanthone-di-*O,C*-hexoside isomer
c	20.53	236, 263, 320, 374	-	x	x				871.1937	$C_{40}H_{39}O_{22}$	0.5	871, 691, 595, 557, 539, 421, 355, 331, 301*, 269, 175	Aspalathin derivative of (iso)mangiferin
d	21.19	241, 257, 317, 356	-	x	x	x	x		729.1869	$C_{31}H_{37}O_{20}$	-1.2	647, 639, 609, 421, 403, 331, 301, 175	Tetrahydroxyxanthone-di-*O,C*-hexose-*C*-deoxyhexoside
e	22.02	261, 315, 367	-		x	x	x		729.1898	$C_{31}H_{37}O_{20}$	2.7	421, 331, 301, 272, 259, 175, 131	Tetrahydroxyxanthone-di-*O,C*-hexose-*C*-deoxyhexoside
f	24.49	231, 283	-	x	x		x	x	595.1634	$C_{27}H_{31}O_{15}$	-4.9	459, 433, 421, 325, 287, 175, 161, 151*, 135, 125	Eriodictyol-*O*-hexose-*O*-deoxyhexoside isomer B
			+	x	x		x		597.1819	$C_{27}H_{33}O_{15}$	-0.7	543, 473, 425, 373, 355, 341, 331, 327, 299, 289, 261, 247,231, 201, 195, 189, 175, 163, 153*, 149, 135	
g	33.67	236, 286	-	x	x				613.1772	$C_{27}H_{33}O_{16}$	0.5	433, 403, 395, 373*, 305, 287, 209, 175, 151, 131	3-Hydroxyphloretin-3',5'-di-hexoside
			+	x	x			x	615.1925	$C_{27}H_{35}O_{16}$	6.0	597, 501, 487, 441, 425, 381, 327, 301*, 273, 247, 201, 157, 149, 123, 119	
h	39.4	238, 285	-	x	x			x	597.1823	$C_{27}H_{33}O_{15}$	0.7	579, 477, 447, 417, 387, 357*, 209, 185, 167	3',5'-di-β-D-glucopyranosylphloretin
i	39.63	238, 285	-	x	x				579.1727	$C_{27}H_{31}O_{14}$	2.2	271*	Narirutin
j	44.67	233, 320sh, 371	-	x	x			x	579.1688	$C_{27}H_{31}O_{14}$	-4.5	271*	Naringenin derivative
			+	x				x	581.1870	$C_{27}H_{33}O_{14}$	-1.4	311, 299, 273*, 257, 231, 201, 189, 153, 147	
k	53.63	232, 288, 335sh	-		x			x	301.0714	$C_{16}H_{13}O_6$	0.7	286, 242, 164, 151	Hesperetin
			+	x	x			x	303.0869	$C_{16}H_{15}O_6$	-0.7	658, 555, 339, 325, 303, 299, 283, 267, 224, 215, 201, 177, 153*, 149, 145, 137	

Compounds highlighted in blue were previously tentatively identified in *C. genistoides* extracts by Beelders *et al.* (2014b).

Table A.6 Sensory characteristics of analysed fractions

Fraction	IEC (g.L^{-1})	Taste threshold (g.100 mL^{-1})			Bitter intensity at IEC[a]
		Mean	Lower limit	Upper limit	
HWE	2				45
B	0.085	0.0066	0.0047	0.0091	< 5
X	0.258	0.0051	0.0039	0.0067	31
F	0.236	0.0060	0.0044	0.0081	13

[a] Based on regression analysis of the dose-response sensory data. IEC = infusion equivalent concentration.
B = benzophenone-rich fraction, X = xanthone-rich fraction, F = flavanone-rich fraction, HWE = hot water extract.

ADDENDUM B

Supplementary material

Modulation of bitter intensity of *Cyclopia genistoides*

Table B.1 Combinations of crude *Cyclopia genistoides* fractions for the determination of modulatory capacity

Combination	Variable	mg.L^{-1}	Constant	mg.L^{-1}
	B	100		
			X	300
Benzophenone- (B) and xanthone (X)-rich fractions	B	100	X	300
	B	200	X	300
	B	300	X	300
	B	100		
			F	300
Benzophenone- (B) and flavanone (F)-rich fractions	B	100	F	300
	B	200	F	300
	B	300	F	300
	X	300		
			F	300
Xanthone- (X) and flavanone (F)-rich fractions	X	100	F	300
	X	200	F	300
	X	300	F	300
	F	300		
			X	300
Flavanone- (F) and xanthone (X)-rich fractions	F	100	X	300
	F	200	X	300
	F	300	X	300

Table B.2 Combinations of crude *Cyclopia genistoides* fractions and major individual compounds for the determination of modulatory capacity

	Constant	Tested conc.[a]	Intended conc.[b]	Variable	Tested conc.[a]	Intended conc.[b]	Variable relative conc.
		mg.L^{-1}	mg.L^{-1}		mg.L^{-1}	mg.L^{-1}	
Benzophenone (B)-rich fraction and mangiferin (Mg)	Mg	167	178				
	Mg	177	178	B	50	43	Half IEC
	Mg	180	178	B	100	85	IEC
	Mg	173	178	B	200	170	Double IEC
				B	100	85	IEC
Xanthone (X)-rich fraction and 3-β-D-glucopyranosyliriflophenone (IMG)	X	300	258				
	X	300	258	IMG	38	32	IEC
	X	300	258	IMG	19	16	Half IEC
				IMG	34	32	IEC
Xanthone (X)-rich fraction and 3-β-D-glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone (IDG)	X	300	258				
	X	300	258	IDG	31	31	IEC
	X	300	258	IDG	15	15	Half IEC
	X	300	258	IDG	61	62	Double IEC
				IDG	30	31	IEC
Flavanone (F)-rich fraction and mangiferin (Mg)	Mg	166	178				
	Mg	174	178	F	75	59	Quarter IEC
	Mg	176	178	F	150	118	Half IEC
	Mg	179	178	F	300	236	IEC
				F	300	236	IEC
Xanthone (X)-rich fraction and hesperidin (Hd)	X	300	258				
	X	300	258	Hd	15	22	Double IEC*
	X	300	258	Hd	7	11	IEC*
				Hd	6	11	IEC*
Xanthone (X)-rich fraction and naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (NHDB)	X	300	258				
	X	300	258	NHDB	19	24	IEC
	X	300	258	NHDB	11	12	Half IEC
				NHDB	17	24	IEC
Xanthone (X)-rich fraction and naringenin-*O*-hexose-*O*-deoxyhexoside isomer A & B (NHDA & NHDB; 1:1)	X	300	258				
				NHDB	18	24	IEC
				NHDA,B	7, 6	24	IEC
	X	300	258	NHDB	25	24	IEC
	X	300	258	NHDA,B	10, 13	24	IEC

IEC = infusion equivalent concentration. *Based on Schulze *et al*. (2015).
[a] As quantified by HPLC for individual compounds and weighed off for crude fractions. [b] As calculated from IEC.

Naringenin-*O*-hexose-*O*-deoxyhexoside isomer A and B component of flavanone-rich fraction

NHDB was successfully isolated from the crude flavanone-rich fraction by preparative HPLC. The conversion of isomer B to isomer A during "fermentation" conditions was confirmed, as shown in **Fig. B.1**, presenting the HPLC chromatograms of the compound before (a) and after (b) simulated fermentation at 90 °C for 16 h. This simulated fermentation led to a conversion of approx. 45% to isomer A and ~5% of a tentatively identified naringenin derivative (**Chapter 3**).

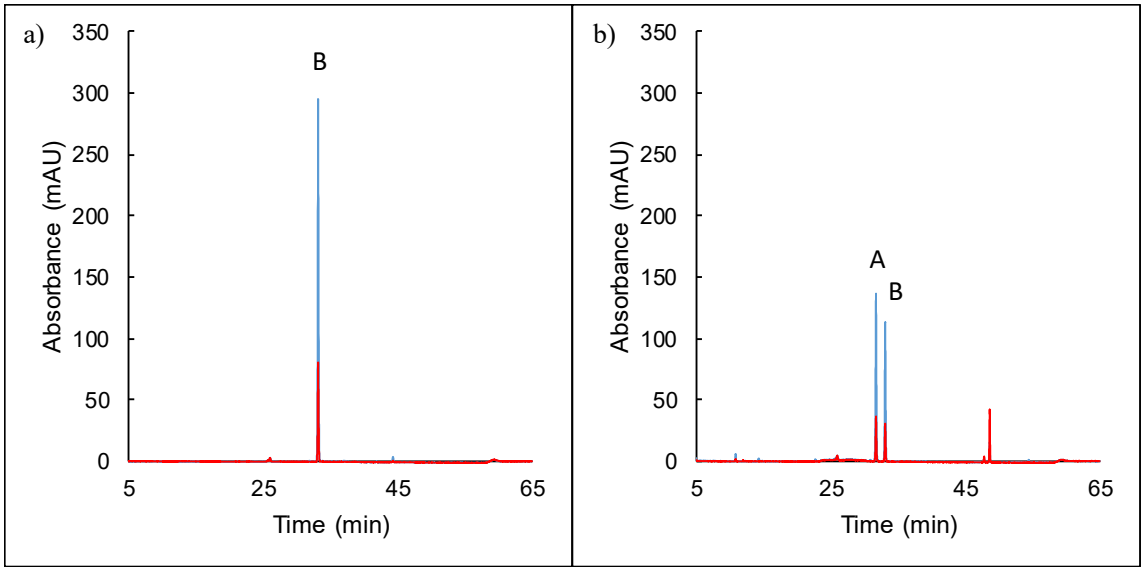


Figure B.1 HPLC-DAD chromatograms of the isolated naringenin-*O*-hexose-*O*-deoxyhexoside isomer B (a) before and (b) after heating of an aqueous solution at 90 °C for 16 h. B = isomer B, A = isomer A. Blue line indicates absorbance at 288 nm. Red line indicates absorbance at 320 nm.

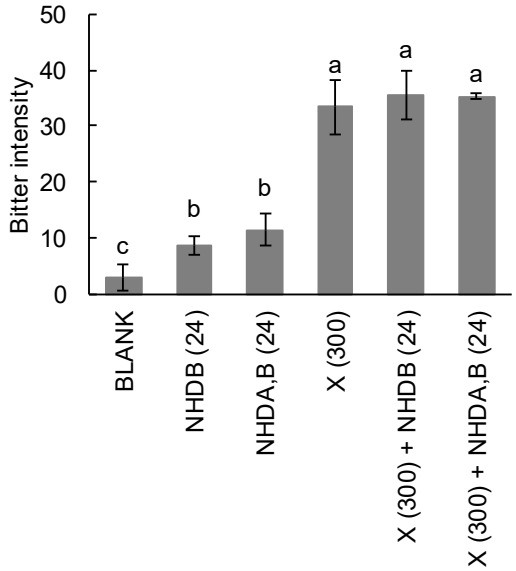


Figure B.2 Bitter intensity of X in combination with NHDB and the mixture of isomer A and B obtained after heating (90 °C/16 h) of NHDB. Samples were prepared in hot water and analysed at 60 °C. BLANK = water, NHDB = naringenin-*O*-hexose-*O*-deoxyhexose isomer B, NHDA,B = 1:1 mixture of isomer A and B, X = xanthone-rich fraction. Values in parentheses indicate concentration as mg.L^{-1}. Different letters indicate a significant difference ($p < 0.05$) in mean values. Error bars indicate standard deviation.

Table B.3 BitterX receptor activation predictions of honeybush compounds identified in *Cyclopia genistoides* extracts (Huang *et al*., 2015)

Compound class	Compound name	Bitter receptor (TAS2R)														
		1	**4**	**5**	**7**	**10**	**14**	**16**	**38**	**39**	**40**	**41**	**43**	**44**	**46**	**47**
Benzophenone	3-β-D-Glucopyranosyl-4-β-D-glucopyranosyloxyiriflophenone			71	64		63			65		55				
Benzophenone	3-β-D-Glucopyranosylmaclurin			62			59	59		58						
Benzophenone	3-β-D-Glucopyranosyliriflophenone	53		54			66			65		52				
Xanthone	Mangiferin			67			65			68		51				
Xanthone	Isomangiferin			69			65			69		52				
Flavone	Vicenin-2			74	70		63			70	54	55				
Flavanone	Hesperidin			71	71		66	58		68		57	55			53

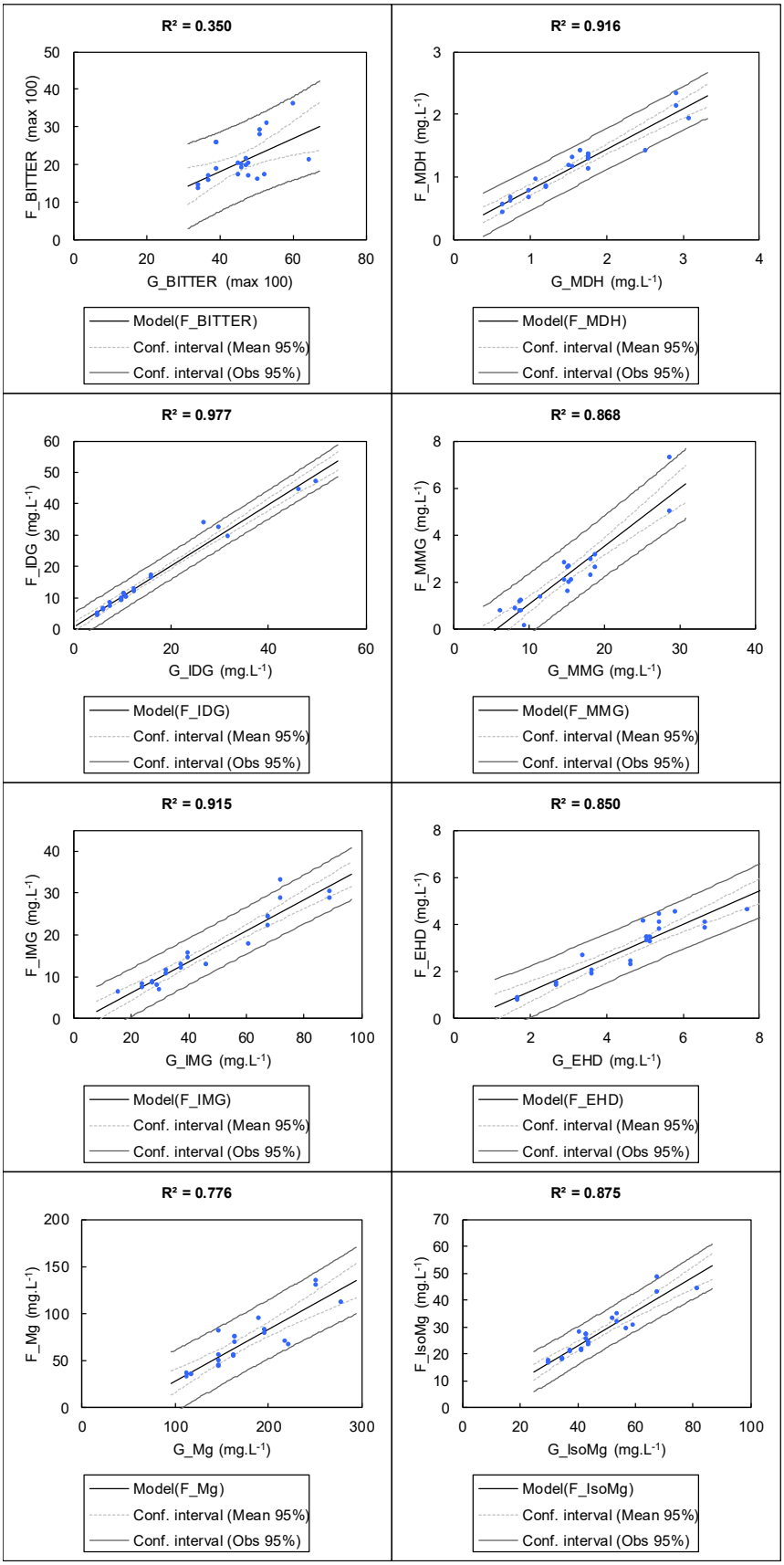
Receptor values for each compound indicates the likelihood (out of 100) that the receptor will be activated by the relevant compound.

ADDENDUM C

Supplementary material

A realistic bitter intensity prediction model for honeybush herbal tea

Figure C.1 Physiology of *Cyclopia genistoides* and *C. longifolia* to illustrate differences in stem thickness.



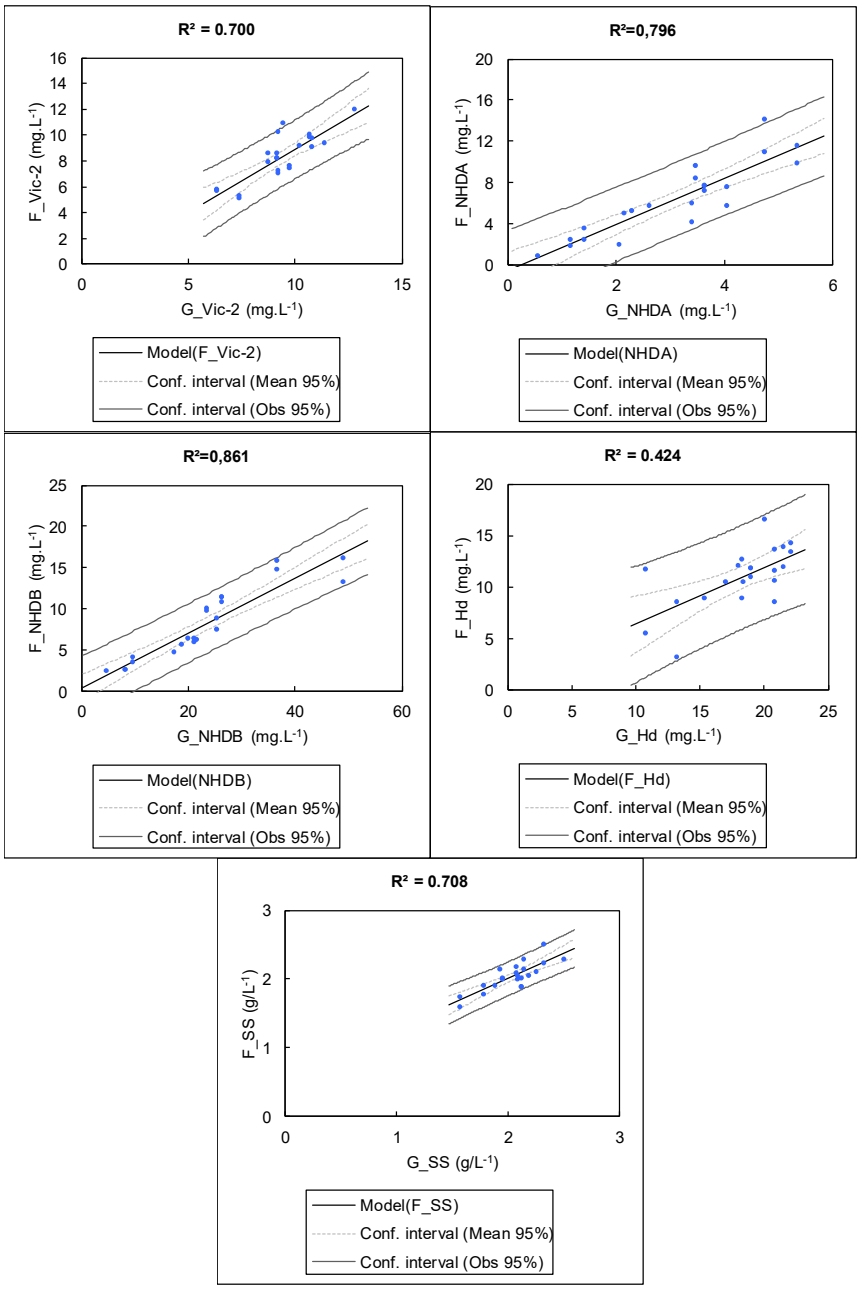
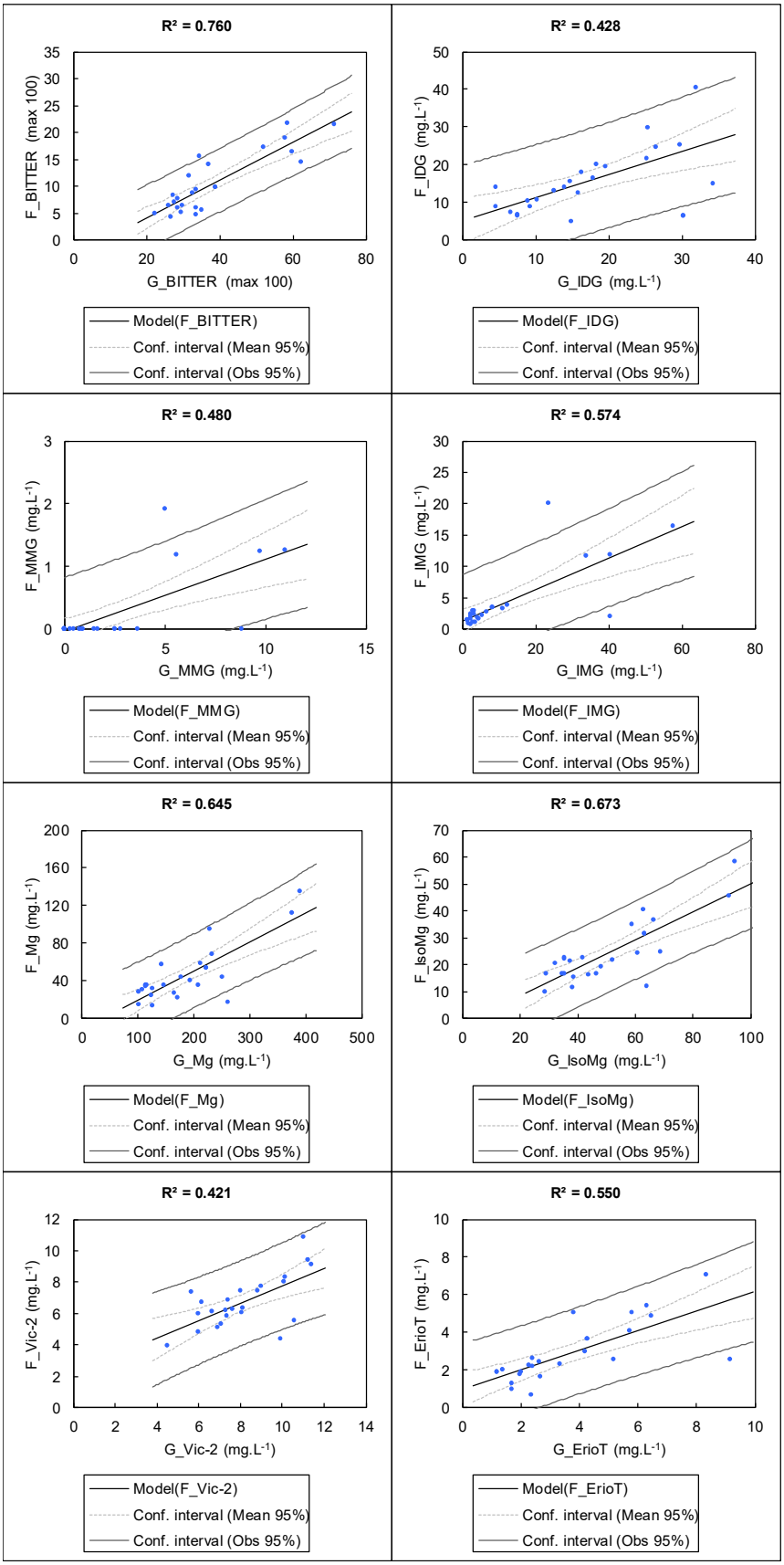


Figure C.2 Correlations between parameters of infusions from plant material of corresponding unfermented and fermented (80 °C/24 h or 90 °C/16 h) batches of *Cyclopia genistoides* (n = 21 batches). "F_" denotes fermented samples and "G_" denotes unfermented samples. Abbreviations explained in **Table 5.2**.



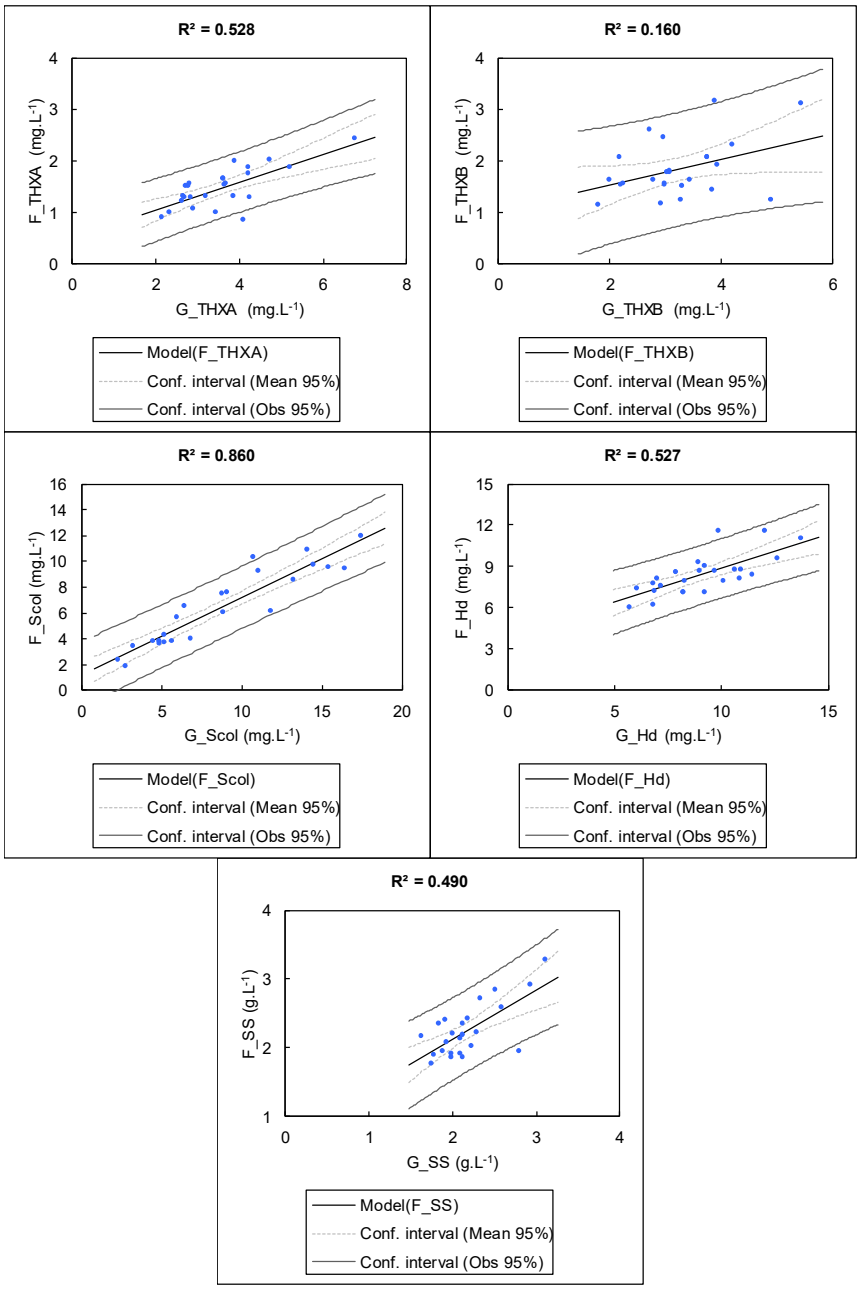


Figure C.3 Correlations between parameters of infusions of corresponding plant material of unfermented and fermented (90 °C/16 h) batches of *Cyclopia longifolia* (n = 24 batches). "F_" denotes fermented samples and "G_" denotes unfermented samples. Abbreviations explained in **Table 5.3**.

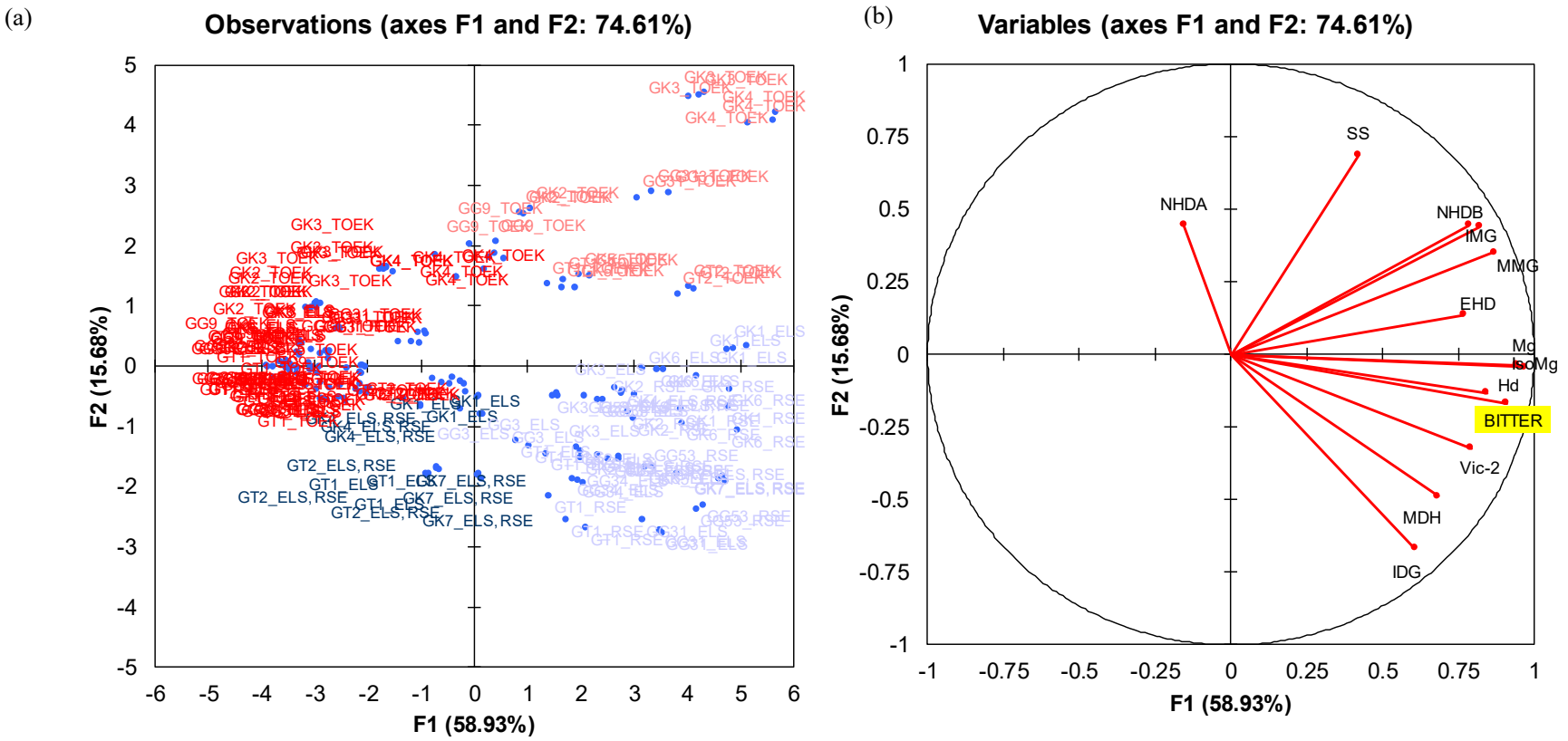


Figure C.4 PCA scores (a) and loadings (b) plots of all *Cyclopia genistoides* samples, considering bitter intensity and all HPLC quantified phenolic compounds. Blue and purple samples indicate 2015 harvest, red and pink samples indicate 2017 harvest. The darker colours (blue and red) indicate fermented samples and lighter colours (pink and purple) indicate unfermented samples. (n = 183 infusions). Abbreviations explained in **Table 5.2**.

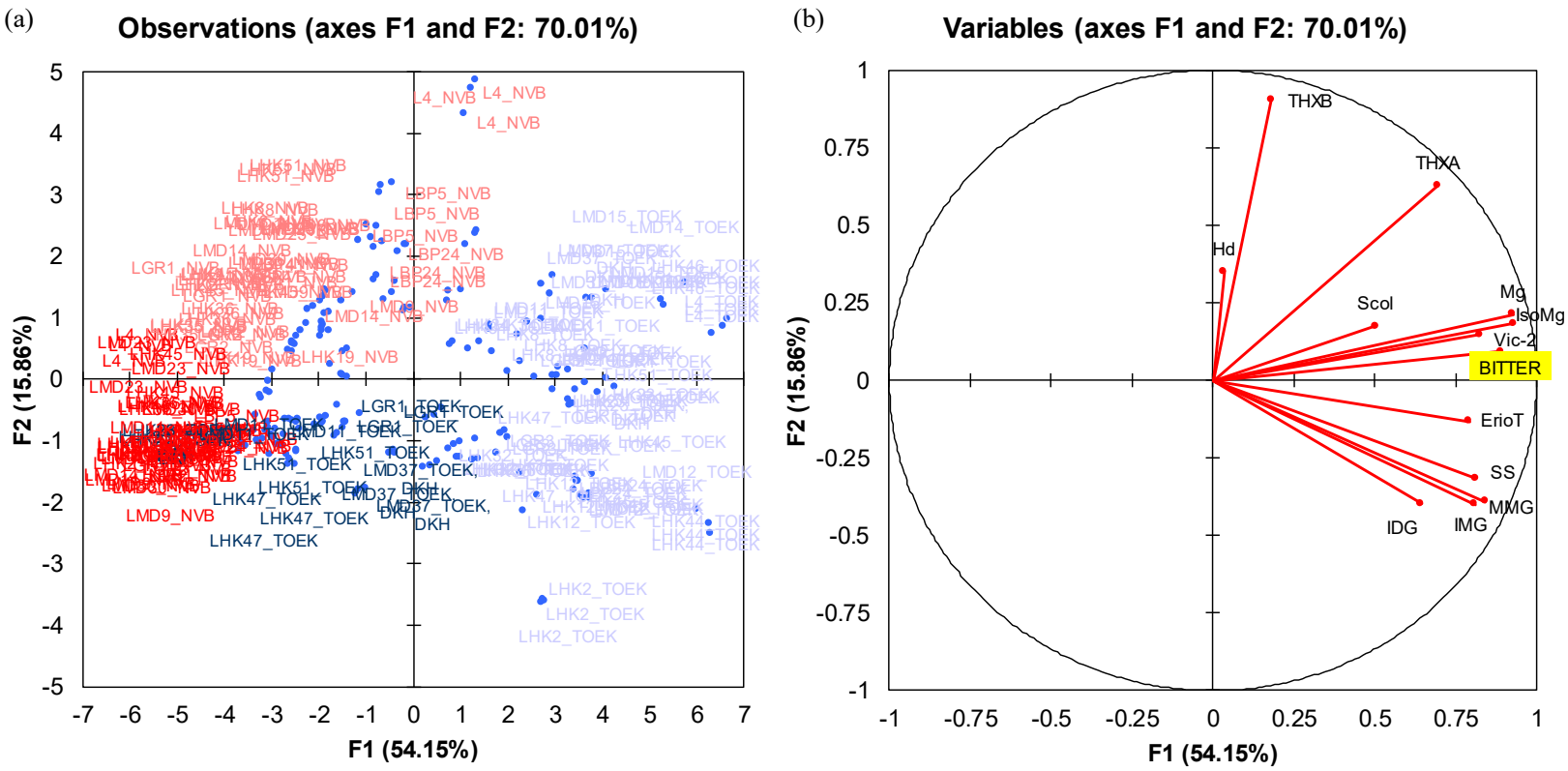


Figure C.5 PCA scores (a) and loadings (b) plots of all *Cyclopia longifolia* samples, considering bitter intensity and all HPLC quantified phenolic compounds. Blue and purple samples indicate harvest location Toekomst, red and pink samples indicate harvest location Nietvoorbij. The darker colours (blue and red) indicate fermented samples and lighter colours (pink and purple) indicate unfermented samples. (n = 195 infusions). Abbreviations explained in **Table 5.3**.

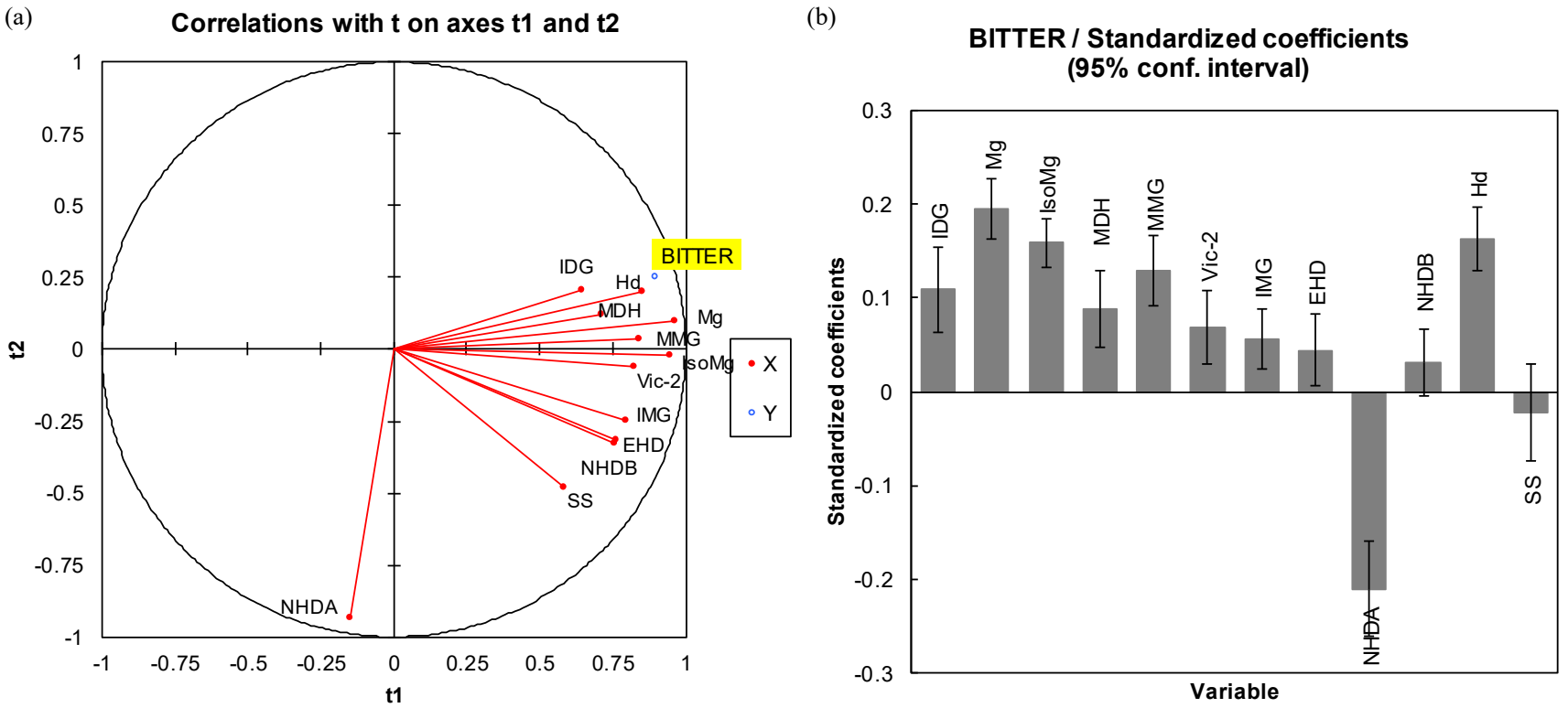


Figure C.6 PLS regression (a) correlations and (b) standardised coefficients of bitter intensity on measured variables of *Cyclopia genistoides* samples (n = 183 infusions). (R^2 = 0.866). BITTER = 7.6 + 0.1*IDG + 4.8E-02*Mg + 0.2*IsoMg + 2.2*MDH + 0.4*MMG + 0.5*Vic-2 + 5.0E-02*IMG + 0.4*EHD - 1.5*NHDA + 6.6E-02*NHDB + 0.5*Hd - 1.6*SS. Abbreviations explained in **Table 5.2**.

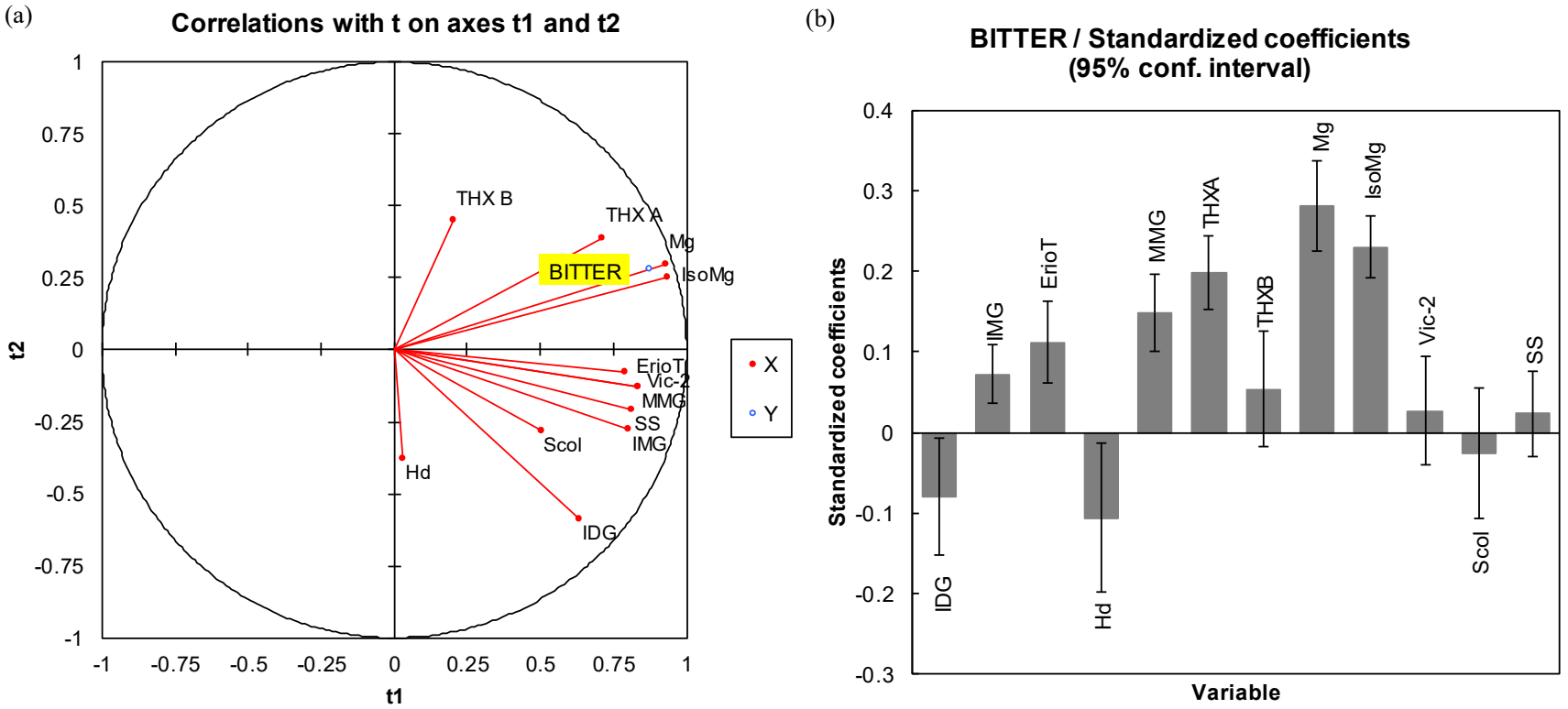


Figure C.7 PLS regression (a) correlations and (b) standardised coefficients of bitter intensity on measured variables of *Cyclopia longifolia* samples (n = 195 infusions). (R^2 = 0.844). BITTER = 5.8 - 0.1*IDG + 6.2E-02*IMG + 1.1*ErioT - 1.2*Hd + 0.7*MMG + 3.4*THXA + 1.2*THXB + 4.9 - 02*Mg + 0.2*IsoMg + 0.2*Vic-2 - 0.1*Scol + 1.1*SS. Abbreviations explained in **Table 5.3**.

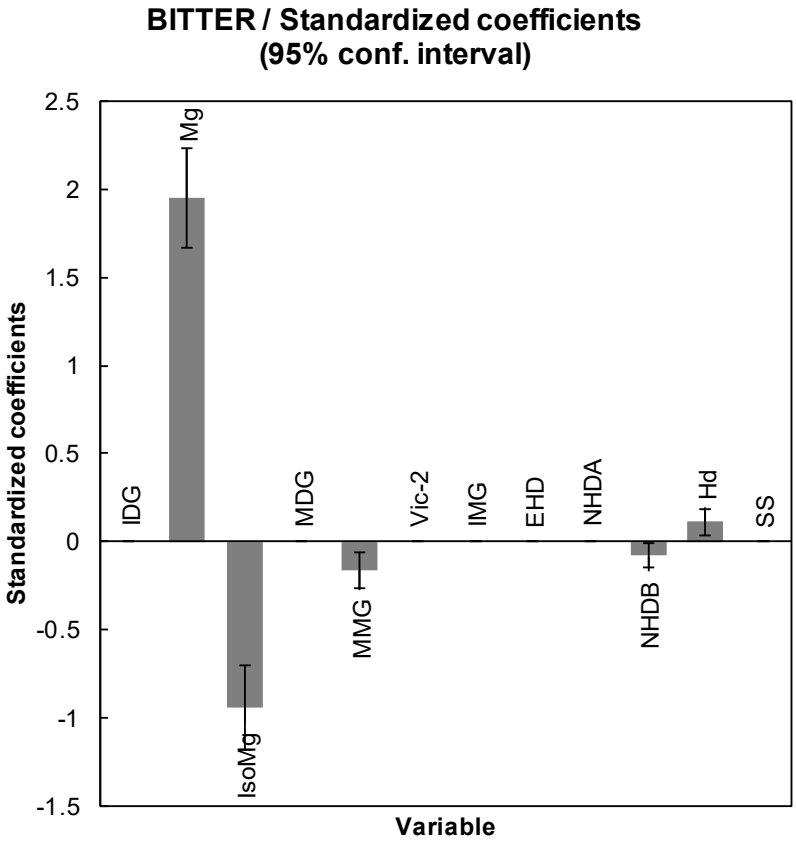


Figure C.8 Stepwise linear regression standardised coefficients of the measured variables on bitter intensity of *Cyclopia genistoides* infusion samples, (fermented and unfermented, n = 183 infusions). BITTER = 14.3 + 0.5*Mg - 1.0*IsoMg - 0.4*MMG - 0.2*NHDB + 0.3*Hd. (R^2 = 0.911). Abbreviations explained in **Table 5.2**.

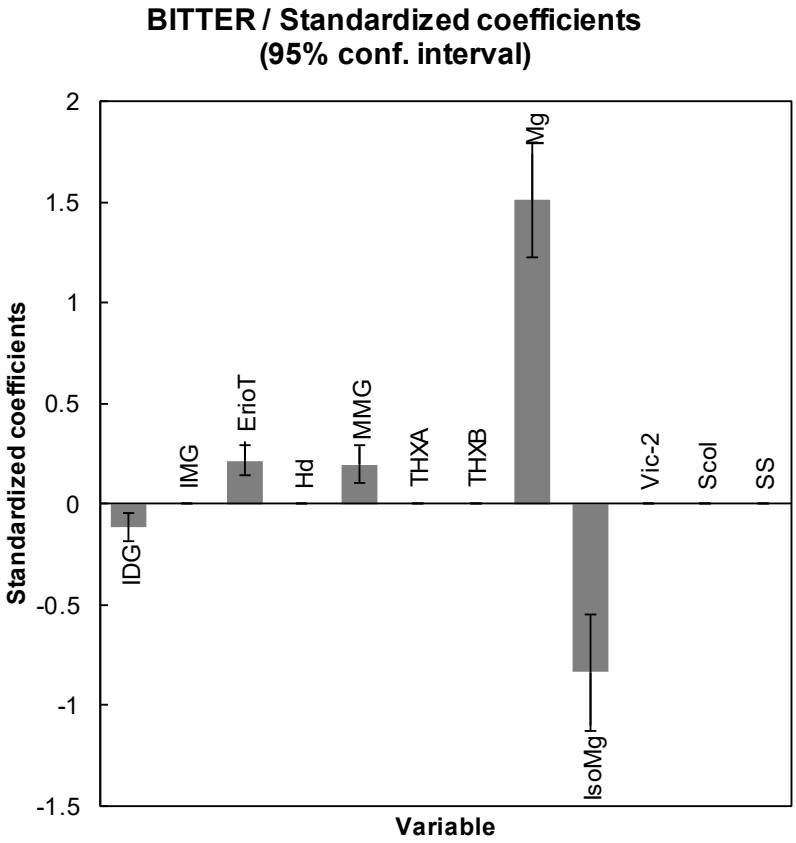


Figure C.9 Stepwise linear regression model of the measured variables on bitter intensity of *Cyclopia longifolia* infusion samples, (fermented and unfermented, n = 195 infusions). BITTER = 13.2 - 0.2*IDG + 2.1*ErioT + 0.9*MMG + 0.3*Mg - 0.6*IsoMg. (R^2 = 0.897). Abbreviations explained in **Table 5.3**.