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High-yield synthesis of transglycosylated mogrosides improves the flavor profile of monk fruit extract sweeteners

Journal:	<i>Journal of Agricultural and Food Chemistry</i>
Manuscript ID	jf-2020-072674.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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High-yield Synthesis of Transglycosylated Mogrosides Improves the Flavor Profile of Monk Fruit Extract Sweeteners

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ABSTRACT: Luo-Han-Guo fruit extract (*Siraitia grosvenorii*), mainly composed of mogroside V (50%) could be considered a suitable alternative to free sugars, however, its commercial applications are limited by its unpleasant off-notes. In the present work, a central composite design method was employed to optimize the transglycosylation of a mogroside extract by using cyclodextrin glucosyltransferases (CGTases) from three different bacteriological sources (*Paenibacillus macerans*, *Geobacillus* sp and *Thermoanaerobacter* sp) considering various experimental parameters such as maltodextrin and mogroside concentration, temperature, time of reaction, enzymatic activity and pH. Product structures were determined by liquid chromatography coupled to diode-array detector (LC-DAD), liquid chromatography-mass spectrometry (LC-ESI-MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Sensory analysis of glucosylated mogrosides showed an improvement in flavor attributes relevant to licorice flavor and aftereffect. Consequently, an optimum methodology was developed in order to produce new modified mogrosides more suitable when formulating food products as free sugar substitutes.

KEYWORDS: sweetener, CGTase, glycosylation, sensorial profile, mogroside V.

INTRODUCTION

According to the World Health Organization (WHO) non-communicable diseases (NCDs) caused an estimated 73% of deaths in 2017 with obesity being one of the major risk factors for some NCDs such as cardiovascular disease and type 2 diabetes.¹ One of the main factors leading to a high risk of obesity, and in consequence NCDs, is a high intake of free sugars.

An effective alternative to substitute the sweetness of free sugars in food is the use of high-intensity sweeteners (HIS).² There are several HIS on the market that are generally recognized as safe (GRAS) including aspartame, acesulfame-K, neotame, sucralose, rebaudioside A, stevioside and, more recently, an extract of the Luo Han Guo fruit or monk fruit.³

Monk fruit extract is formed by several compounds classified as cucurbitane-type triterpenoid glycosides, which are formed by a mogrol aglycone glycosylated with glucose units.⁴ The main compound found is Mogroside V followed by Mogrosides IV and VI⁵ (Figure 1), which are known to exert a sweetness intensity 250-300 times higher than sucrose.^{3,6} On the contrary, mogrosides with fewer than three glycoside units are not sweet.⁷ Several works have reported the potential pharmacological properties and bioactivities of various naturally occurring mogrosides, such as antioxidant and anti-inflammatory effects,⁸ antiproliferative activity, attenuation of liver fibrosis,⁹ and hypoglycaemic effects by inhibition of the intestinal maltase-glucoamylase, among others.¹⁰ However, most of these studies have been carried out using *in vitro* systems or animal trials, still, human interventions are needed to confirm all of these potentially beneficial properties. Despite this, mogrosides could make a valuable substitute to free sugars with potential added bioactivities.¹¹

Despite the use of mogrosides as HIS and the reported bioactive properties, there are few comprehensive studies on their flavor profiles, commonly identifying licorice aftertaste.¹² Based on this, an improvement of flavor might be feasible by the glycosylation of mogrosides as previously reported by Yoshikawa et al.¹³. These authors used various cyclodextrin glycosyltransferases (CGTases, E.C 2.4.1.19) to glucosylate mogroside V, obtaining an addition of up to three units of glucose to the mogroside V moiety and reporting an improvement in the taste. CGTases are hexosyltransferases produced by different bacterial species and are widely used to catalyze the synthesis of cyclodextrins from starch and other related compounds. There are also other compounds used as acceptors in CGTase transglycosylation reactions with different purposes, such as polyphenols,¹⁴⁻¹⁶ vitamins, sugar alcohols¹⁷, and other natural sweeteners based on entkaurene derivative from *Stevia rebaudiana*.¹⁸

Considering that CGTases can recognize a number of acceptors with different structural features, including terpenoid compounds as steviol glycosides^{18,19}, and that mogrosides are naturally occurring high-intensity sweeteners largely unexplored as substrates for enzymatic transglycosylation modification, this work addresses the potential improvement of the flavor profile of a mogroside extract (Mogroside V 50% w/w) attained by enzymatic transglycosylation. Response surface methodology has been used to assess the optimization of the experimental conditions. Unmodified and glucosylated mogrosides were subjected to a comprehensive structural analysis by MALDI-TOF MS and LC-MS analyses together with a detailed flavor study by determining 16 flavor characteristics.

MATERIALS AND METHODS

Chemicals

An extract from Luo-Han-Guo fruit (*Siraitia grosvenorii*) mainly formed by 50.33% Mogroside V and other cucurbitane triterpene glycosides, 0.34% ash content and 4.27% moisture (as declared by the supplier) was purchased from MonkFruit Corp. (Libertyville, USA). CGTases from *Paenibacillus macerans*, *Geobacillus* sp and *Thermoanaerobacter* sp were used. Maltodextrin from maize starch (20 dextrose equivalent), acetonitrile (MS grade), and formic acid (MS grade) were obtained from Sigma-Aldrich (St. Louis, USA). Mogroside V standard was purchased from Biosynth Carbosynth (Reading, UK). White granulated sugar (Tate and Lyle, London, UK) and water (Harrogate Spa mineral water) for sensory analysis were purchased in local supermarkets in Reading (UK). All other reagents were obtained from Sigma-Aldrich (St Louis, USA) and ThermoFisher Scientific (San Jose, USA).

Statistical experimental design for transglucosylation of mogrosides

Optimization of transglucosylation reactions with the three CGTases was carried out by multivariate analysis (Software Design Expert 10.1, StatEase, USA), considering the effect of 6 different parameters that were concentration of maltodextrin (g/L), mogroside V (g/L), enzymatic activity (U/g mogroside V), temperature (°C), reaction time (h) and pH. First, the most influential parameters were established using a fractional factorial design (2^{6-2}), and then a Central Composite Design (CCD) was used to obtain the optimum conditions for the glucosylation reactions. The assay precision was evaluated by

the relative percentage error (RE) and the appropriate relative standard deviation (RSD) from the theoretical concentrations of mogroside V by external calibration.

Liquid chromatography coupled to diode-array detector (LC-DAD) analysis

Concentration of glucosylated mogrosides was determined by LC-DAD using a reversed-phase Poroshell 120 C18 column (250 mm × 4.6 mm, 4 µm particle size, 120 Å pore size, Agilent Technologies, Palo Alto, USA) in an LC system (Agilent 1200 series, equipped with an autosampler, quaternary pump, column oven and DAD detector). The separation was performed at a flow rate of 0.7 mL/min at 30 °C. The elution gradient using deionized water and acetonitrile changed from 90:10 (v:v) to 10:90 (v:v) over 50 min, then to initial conditions over 2 min and maintained for 10 min for conditioning.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

The molecular weight distribution of mogroside mixtures was determined by MALDI-TOF MS. Samples were diluted 1:100 in water (Milli-Q water, Millipore, Bedford, USA), and 1 µL of the diluted solutions were mixed with 3 µL of the matrix consisting of 2,5-dihydroxybenzoic acid (>98%, Fluka, Buchs, Switzerland) at a concentration of 10 mg/mL in water. Then, 1 µL of this mixture was spotted onto a flat stainless-steel sample plate and dried in air before analysis.

A Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, USA), equipped with a nitrogen laser emitting at 337 nm and a delayed extraction ion source, was used for MALDI-TOF MS measurements. Ions generated by laser desorption of

unmodified and glucosylated mogroside mixtures were introduced into the time of flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, 94% grid voltage, 0.075% ion guide wire voltage, and a delay time of 400 ns. Mass spectra were obtained over the m/z range 500-4000 in the linear positive ion mode as $[M+Na]^+$. External mass calibration was applied using the average $[M+H]^+$ values of the components of the Calibration Mixtures 1 and 2 of Sequazyme Peptide Mass Standards Kits (Applied Biosystems).

Liquid chromatography coupled to mass spectrometry (LC-MS) analysis

LC-MS experiments were carried out on an Agilent 1100 Series LC system (Agilent Technologies, Palo Alto, USA) equipped with a quaternary pump, an autosampler, a column oven, and coupled to an HTC-Ultra ETD II ion trap mass spectrometer (Bruker Daltonics, Fremont, USA) using an ESI interface working in the negative-ion mode.

Unmodified and glucosylated mogroside mixtures were diluted 1:100 with water, and their separation (5 μ L) was performed at 35 °C on a C18 HyPURITY™ column (100 mm x 2.1 mm, 3 μ m particle size, Thermo Fisher Scientific, San José, USA). A gradient consisting of 0.1% (v:v) formic acid (analytical grade; Merck, Darmstadt, Germany) in water (eluent A) and acetonitrile (OPTIMA®LC/MS grade, ThermoFisher Scientific) containing 0.1% of formic acid (v:v) (eluent B) was applied at a flow rate of 0.2 mL/min as follows: 10% B for 1 min, 10-50% B linear in 45 min, 50% B for 5 min, 50-90% B linear from 50 to 51 min, 90% B isocratic from 51 to 60 min, ramped to original composition in 1 min, then re-equilibration of the column for 10 min.

The mass spectrometer spray voltage was set at 3.5 kV, the drying gas temperature at 350 °C, and the skimmer voltage at -40 V. Nitrogen (99.5% purity) was used as drying

(10 L/min) and nebulizer (40 psi) gas. Full scan mass spectra were recorded between m/z 100 and 2000. Bruker Compass 1.2 software was used for LC-MS data acquisition and processing.

Mogroside fraction isolation for sensory analysis

The reaction mixture (100 mL) was placed into a Diaion HP-20 column (2.2 × 50 cm) and washed with deionized water (1500 mL water). Both unmodified mogrosides and those resulting from the enzymatic transglycosylation were eluted with ethanol (95%) and subsequently dried using a rotary evaporator (40 °C). Samples were kept at 4 °C until analysis. Removal of free carbohydrates was efficiently carried out and monitored by gas chromatography with flame-ionization detection (GC-FID).

Sensory test procedure

The sweetness intensity of the glucosylated mogrosides obtained under optimal synthesis conditions together with the unmodified mogrosides was evaluated using 10 experienced sensory evaluation panelists. The panelists were trained at the Sensory Science Centre (Department of Food and Nutritional Sciences, University of Reading, UK). The study was approved by the University of Reading Research Ethics Committee (UREC study number 16_19). Sensory analysis was performed in an air-conditioned (23–24 °C, room temperature) sensory laboratory with individual booths and artificial daylight.

The panel used 16 attributes to define the samples (sweet, overall strength of off-taste/ flavor, bitter taste, licorice flavor, sour taste, cooked sugar flavor, cooling sensation, cardboard/stale, metallic, salty taste, crusty bread flavor, perfume flavor, sweet aftertaste,

bitter aftertaste, licorice aftereffect, and cooling aftertaste), followed by training focused on ensuring each panelist could reliably score sweetness relative to four sucrose standards (20, 30, 60 and 80 g/L). The average panel ratings for these standards were 10, 35, 75, and 100 respectively on a 0–100 line scale, and so these four positions were used as anchors to provide a structured scale on which to rate the sweetness of all samples. All other attributes were scored as relative values using unstructured line scales (0–100). Due to the limited sample availability, each panelist was presented with only 0.5 mL of sample for each scoring session. Therefore, training additionally focused on ensuring panelists were able to sip this small sample volume from a 30 mL transparent polystyrene cup and allow it to flow over the top of their tongue before swallowing and scoring sweetness accurately. Palate cleansing before and between sample scoring was done using filtered water and low salt crackers (Carr's water crackers, United Biscuits Ltd., Hayes, UK).

Samples were prepared in mineral water (Harrogate Spa mineral water) and labeled with random 3-digit codes. Sample order presentation was done in a balanced monadic sequential manner. An initial screening was carried out to choose the most appropriate mogroside concentration, this was concluded as 0.24 g/L.

The sucrose standards were presented at the start of each panel rating session for re-familiarisation in order to enable the panelists to score the sweetness of the samples accurately against the standard anchors.

The mean sweetness ratings of the four sucrose standards were used to plot a dose-response curve, the linear regression for which was $\text{Perceived Sweetness} = (1.55 \times \text{Sucrose Concentration (g/L)}) - 22.5$ ($r^2 = 0.99$). The mean sweetness ratings for each sample were converted to equivalent sweetness (ES) values using this equation.

The sensory profile data were analyzed using a mixed model ANOVA where panelists were treated as random effects and samples as fixed effects. The main effects were tested against the sample by assessor interaction. Multiple pairwise comparisons were carried out using Fisher's LSD and a significant difference was declared at an alpha risk of 5% ($p \leq 0.05$). Data analysis was carried out using Senpaq software (Qi Statistics, Reading, UK).

RESULTS

Optimization of mogroside glucosylation parameters

A screening fractional factorial design $2^{(6-2)}$ was first used to study the synthesis of glucosylated mogrosides with the three different CGTases employed. This screening allowed us to evaluate the effect of 6 experimental factors (concentration of maltodextrin, concentration of unmodified mogrosides, enzymatic activity, temperature, time, and pH), using the concentration of glucosylated mogrosides (mg/mL) as a response variable. Table 1 shows the runs carried out and the responses obtained for each enzyme, calculated from LC-DAD analyses. The average RE of mogroside V was 2.3% (RSD = 1.8%, $n=4$) suggesting an acceptable accuracy and precision for the quantitation of the samples.

The highest concentration (14.0 mg/mL) of glucosylated mogrosides was obtained using the CGTase from *Thermoanaerobacter* sp (Table 1), which was chosen to continue with synthesis optimization.

Figure 2 shows the Pareto chart illustrating the analysis of variance (ANOVA) and p-value, which were used to evaluate the significance of the effect. Positive values (green bars) denote a directly proportional relationship of the variable with the response; whereas negative values (red bars) reflect an inverse relationship. The horizontal line corresponds to the t-value at a significance level of 5%. The concentration of unmodified mogrosides and the enzyme activity had a significantly positive effect on the response (glucosylated mogrosides concentration). Consequently, these two factors (concentration of unmodified mogrosides and the enzyme activity) were chosen to carry out the Central Composite Design (CCD) optimization.

Maltodextrin concentration, temperature, and time were fixed at their lowest value, 5 g/L, 50 °C, and 1 h respectively, and the pH value was adjusted to 5. The CCD was composed of 11 experimental points with 3 replicates in the central point. The experimental data provided by CCD design were analyzed by regression analysis. The relationship between the response evaluated and the variables were fitted into the polynomial Equation 1.

$$\text{Synthesized glucosylated mogrosides (mg/mL)} = 1.64 - 0.03 * \text{Enzymatic activity (U/g unmodified mogrosides)} + 0.43 * \text{unmodified mogrosides (mg/mL)} \quad \text{Equation 1}$$

Analysis of variance (ANOVA) was carried out to justify the significance and adequacy of the regression model fit. Statistical significance of the model was established at $p \leq 0.05$. The obtained model F-values < 0.03 for the response indicate that the model was highly adequate and significant. Likewise, the determination coefficient (R-squared) of the model was 0.98 for synthesized glucosylated mogrosides. The coefficient of variation (CV %) had lower values than 10% showing that the variation was acceptable and satisfactory.

The optimum concentration of unmodified mogrosides and enzymatic activity were determined to maximize the yield of glucosylated mogrosides (mg/mL). The response surface obtained for the global desirability function (D) is presented in Figure S1. The coordinates producing the maximum desirability value ($D=1$) were 56.7 g/L for the concentration of unmodified mogrosides and 6.5 U/g of mogroside V for the enzymatic activity.

The individual response values and their respective confidence intervals are depicted in Table 2. In order to validate this predictive model, optimal conditions were experimentally assessed through three replicates which showed no significant differences with the theoretical results. Finally, these conditions were selected to produce higher quantities of synthesized glucosylated mogrosides to be structurally characterized and for the sensory analysis.

Structural characterization by mass spectrometry

According to Table 1, the CGTase from *Thermoanaerobacter* sp. generated the highest concentration of synthesized glucosylated mogrosides determined by LC-DAD. In order to qualitatively evaluate the polymerization activity of the three CGTases used in this study, MALDI-TOF MS analyses were carried out and compared with that of the unmodified mogrosides (Figure 3). The three CGTases showed a high glucosylation activity of up to 12 units of glucose. Deglucosylation activity was not detected and significant amounts of mogrosides IV or III ($[M+Na]^+$ 1148.3, 986.2, respectively) were not produced.

The initial mixture of unmodified mogrosides was properly characterized. using LC-DAD and LC-MS, and was composed of 50.2% mogroside V, 31.9% mogroside VI (and

other unknown minor mogrosides), 10.2% mogroside IV, and 7.7% mogroside III, which were identified by the retention times and their corresponding molecular ions (Figure S2). Due to the lack of structural information generated by LC-DAD and the impossibility of identifying if mogroside V and/or the other minor mogrosides present in the mixture are glucosylated, an LC-MS analysis was carried out on the enzymatically modified sample (Figure 4). Since the MALDI-TOF MS analysis showed that the three CGTases produce a similar glucosylation pattern (up to 12 glucose units), the LC-MS analysis was carried out on the sample obtained using optimized conditions (CGTase from *Thermoanaerobacter* sp).

The results shown in Figure 4 confirmed the glucosylation of the mogroside structures by the identification of new peaks with lower retention times (Figure 4B) when compared with the initial mogroside mixture (Figure 4A), and by the $[M-H]^-$ ions corresponding to the newly synthesized mogrosides with up to 4 new glucose units. Due to the low sensitivity of LC-MS to higher molecular weight compounds, mogrosides with more than 9 glycosides units were not identified by this technique. However, the LC-MS profiles revealed seven potential mogroside IV isomers (25.8, 26.3, 26.7, 27.0, 27.2, 27.6, and 27.9 min) in the unmodified mogroside mixture (m/z 1123.6; Figure 5). To the best of our knowledge, only two mogroside IV structures have been described, mogroside IVe and mogroside IVa. This is the first time that five additional different potential mogrosides IV have been described.

LC-MS data allowed us to determine the ratio of mogroside V and the mogroside IV in the unmodified and glucosylated mogroside samples. The most abundant mogroside was mogroside V (m/z 1285.5) followed by the mogroside IV isomers (m/z 1123.0, Figure 5). The area ratio of mogroside IV / mogroside V was similar in two of the mogroside IV

isomers, indicating that the rate of glucosylation is similar between mogroside V and these mogroside IV isomers. However, four of the mogroside IV isomers were more readily glucosylated by the CGTase than mogroside V. Also, after the enzymatic reaction, a new mogroside IV (Figure 4; 30.02 min) was synthesized. This new mogroside IV could be a glucosylated form of the minor mogroside III isomers presented in the initial mixture. Interestingly, Figure 5 also shows similar behaviour in mogroside III compared with mogroside IV. The main two mogroside III isomers decreased and one of mogroside III isomers increased (Figure 5; 29.70 min).

Sensory profiling

Mogroside V is reported to have a sweetness potency approximately 250 times that of sucrose.¹² Previous literature²⁰ has shown that bitterness and other off-flavors exist in mogroside extract (50% mogroside V). In this study, we decided to test solutions at equivalent sweetness levels above 2% sucrose to ensure that we were able to evaluate both sweetness and bitterness in levels suitable for potential food application. The glucosylated mogroside samples were purified using HP-20 resin, resulting in a purity higher than 98% w/w. This purification technique allowed to eliminate the free carbohydrates, i.e. glucose, maltose and maltodextrins, present after the synthesis (as monitored by GC-FID) and the resulting product was only based on mogrosides (unmodified and enzymatically glucosylated forms).

For the initial tasting, sucrose standards were prepared at 4, 6, 8, and 10% (w:v). Aiming for 8% sucrose, equivalent unmodified mogrosides and the glucosylated sample were prepared at 0.32 g/L. From the initial tasting we concluded that we needed to reduce the sucrose standard range to 2, 4, 6, and 8% as the 10% sample was too extreme. The

unmodified mogroside sample and the respective enzymatically modified sample were considered too sweet relative to the sucrose standards, therefore they were reduced in concentration to 0.24 g/L.

Of the 16 attributes rated, 5 of them differed significantly from sample to sample (Figure 6). In general, licorice and metallic flavors were significantly decreased by the enzymatic modification, although so was the sweet taste. All other specifically defined taste and flavor attributes were rated at low levels. The lowering power of glucosylation on sweetness has been observed previously.¹³ This decrease is in inverse proportion to the amount of glucose monomer attached to the aglycone. However, in the case of the mogrosides obtained after the glucosylation reaction, the sweetness potency is only 1.2 times lower than in the unmodified mixture (Table 3). Consequently, the glucosylated mogroside may still be considered a high-intensity sweetener.

DISCUSSION

It has been noted that glycosylation of natural sweeteners such as steviol glycosides^{18,19} and mogrosides¹³ can improve their flavor profile. In the case of mogrosides, very few reports have analyzed the off-flavors of these compounds. Tan et al.²⁰ reported a high level of bitterness and metallic flavor in mogroside extract (mogroside V, 50%), similar to that found in rebaudioside A, one of the major steviol glycosides present in commercial mixtures. On the contrary, Yoshikawa et al.¹³ and Kim et al.²¹ reported a relatively low bitter taste, but a high intensity of licorice flavor and general aftertaste. These discrepancies could be due to the different proportion of natural mogrosides and their concentrations, or as a result of the sensory analysis performed.

However, it is evident there is an undesirable aftertaste in the tested mogroside mixtures. Also, it is important to consider that the work of Yoshikawa et al. obtained transglycosylated products with only up to 1 to 3 additional glucose residues on the mogroside V structure.¹³

The improved taste profile of triterpenoid saponins derived from mogrosides using a biosynthetic pathway based on enzymes from microbial sources would provide a promising and green approach for supplying new consumer-friendly products to the market.²² Several attempts have used enzymes such as β -glucosidases, pectinases, cellulases, and uridine diphosphate glycosyltransferases (UGTs) in order to produce specific mogrosides.^{11,22,23} Cyclodextrin glycosyltransferases (EC 2.4.1.19) are an excellent approach to the modification of mogrosides due to their use of readily available glycosyl donors (starch or maltodextrin), food-grade status, and commercial availability. CGTases are mainly used in the food industry to catalyze the conversion of starch in cyclodextrins. These enzymes can also act as glucosyltransferases with different acceptors, such as steviol glycosides.¹⁹

Due to the nature of the catalytic activity of the CGTases, glucose units are transferred to an acceptor, mainly low molecular weight carbohydrates via α -(1-4) linkages. The mogroside V (CAS 88901-36-4) has five glucose units attached to the aglycone (mogrol). The elongation could hypothetically occur either on the glucose chain attached to the C-3 of the mogrol structure, or on the glucose chain attached to the C-24 chain of the mogrol structure (Figure 1), based on the additional glucose of other natural mogrosides such as mogrosides VI, VIa1, and VIa, respect to the mogrosides V.²⁴ Moreover, Wang et al.²⁵ carried out a transglycosylation method for which they formed new mogroside compounds and other known natural mogrosides. Based on these findings

and on the mechanisms of transglycosylation activity of bacterial CGTases, the transglycosylation of the present samples could take place through the transfer of glucose residues in an α -orientation to the accessible (terminal) glucoses attached in either the C-3 or the C-24 of the mogrol core. Whereas the preferable regioselectivity of bacterial CGTases could point out through the formation of α -(1-4)-linkages, other possible glycosidic bonds, such as α -(1-6) and α -(1-2)-linkages should not be fully dismissed²⁵. According to Figure 4B, three peaks corresponded to mogroside VI. These could be consequences of the glucosylation of two different C-4 on the glucoses presented in the mogroside V, however, the high abundance of one mogroside VI isomer over the other one is indicative that one C-4 is more prone to being glucosylated.

In this study, for the first time, was reported a glucosylation of the mogrosides in a commercial sweetener mixture (Mogroside V 50% w/w) by up to 12 glucose units, resulting in an aglycone (mogrol) glucosylated by up to 17 glucose units. Interestingly, it was found that the isomers of mogrosides present in the initial mixture are glucosylated in different ratios. Glucosylation significantly improved the sensory profile. However, to ensure that this new product could suppose an optimal alternative to sugar, future research dealing with metabolic studies following oral ingestion, including the potential impact on gut microbiota composition and activity, should be considered.

Furthermore, the use of response surface methodology approach allowed for optimization of conditions for the cost-effectiveness of the new modified mogroside mixture production, which could be easily scaled-up to pilot or industrial level.²³

In summary, this study provides a new optimized synthesis methodology, based on experimental design methodologies, which is reported to produce highly glucosylated mogroside V, using a commercial mixture of mogrosides (50% mogroside V),

maltodextrin and CGTases that are widely used in the food industry as starting materials. This modification allows the production of a natural sweetener with improved flavor, formed from a mixture of mogrol with up to 17 glucose units. This optimized mogroside mixture has an improved flavor with lower licorice and metallic flavors, which are characteristic of the unmodified mogroside mixtures currently found on the market.

Funding: This research was funded by Optibiotix Health plc (York, UK), by the Spanish Ministry of Science, Innovation and Universities (project AGL2017-84614-C2-1-R) and by FEDER-Spanish Ministry of Science and Innovation (project RTI2018-101273-J-I00).

Conflicts of Interest: The authors declare the following competing financial interest(s): S.K is the R&D Director of Optibiotix Health plc.

SUPPORTING INFORMATION

This material is available free of charge via the Internet at <http://pubs.acs.org>: Three-dimensional plot showing the effect of initial unmodified mogrosides and the enzymatic activity on the desirability to maximize the concentration of synthesized glucosylated mogrosides (Figure S1); LC-DAD chromatogram of unmodified Luo-Han-Guo fruit extract. The identified compounds correspond to Mogrosides V, VI*, IV, and III. *Other minor non-identified mogrosides (Figure S2A); LC-MS spectrum corresponding to the cucurbitane triterpene glycosides found in the unmodified Luo-Han-Guo fruit extract: 1285.6 m/z – Mogroside V, 1447.6 m/z – Mogroside VI, 1123.6 m/z – Mogroside IV, and 965.4 m/z – Mogroside III (Figure S2B).

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FIGURE CAPTIONS

Figure 1. Structures of triterpenoid compounds isolated from *S. grosvenorii*.

Figure 2. Pareto chart showing individual and interaction effects of the response evaluated (synthesized glucosylated mogrosides using CGTase from *Thermoanaerobacter* sp. (A) maltodextrin concentration, (B) initial mogrosides concentration, (C) enzymatic activity, (D) temperature, (E) time and (F) pH. Using a confidence value of $p = 0.05$, based on a null hypothesis test, values exceeding this limit (horizontal line) are considered as significant to the response values.

Figure 3. MALDI-TOF MS profiles of unmodified mogrosides (a) and after the glucosylation under optimized conditions, using CGTases from *Thermoanaerobacter* sp (b), *Paenibacillus macerans* (c) and *Geobacillus* sp (d). Mog: Mogrol; Glc:glucose.

Figure 4. LC-MS ion traces profiles of the unmodified mogrosides mixture (A) and the glucosylated mixture obtained under optimized conditions using CGTases from *Thermoanaerobacter* sp (B).

Figure 5. LC-MS ion traces profiles of the initial unmodified mogrosides mixture (-, **black line**) and the glucosylated mixture (-, **red line**) obtained under optimized conditions using CGTases from *Thermoanaerobacter* sp. Figure 6. Mean panel ratings of attributes that either differed significantly between samples or where mean rating > 10 . Attributes that were not significantly different between samples or rated < 10 were Bitter taste, Sour taste, Salty taste, Cooling sensation, Stale flavor, Crusty Bread flavor, Perfume, Bitter aftertaste, and Cooling aftereffect.

Figure 6. Mean panel ratings of attributes that either differed significantly between samples or where mean rating > 10 . Attributes that were not significantly different between samples or rated < 10 were Bitter taste, Sour taste, Salty taste, Cooling sensation, Stale flavor, Crusty Bread flavor, Perfume, Bitter aftertaste, and Cooling aftereffect.

Compound name	R ₁	R ₂	R ₃
Mogrol	H	H	α -OH
Mogroside IA	H	Glc(β 1)	H ₂
Mogroside IE1	Glc(β 1)	H	α -OH
Mogroside IIA1	H	Glc(β 1-6)Glc(β 1)	α -OH
Mogroside IIA2	Glc(β 1-6)Glc(β 1)	H	α -OH
Mogroside IIE	Glc(β 1)	Glc(β 1)	α -OH
Mogroside IIIA1	H	Glc(β 1-6)[Glc(β 1-2)]Glc(β 1)	α -OH
Mogroside IIIA2	Glc(β 1-6)Glc(β 1)	Glc(β 1)	α -OH
Mogroside IIIE	Glc(β 1)	Glc(β 1-2)Glc(β 1)	α -OH
Mogroside IVA	Glc(β 1-6)Glc(β 1)	Glc(β 1-6)Glc(β 1)	α -OH
Mogroside IVE	Glc(β 1-6)Glc(β 1)	Glc(β 1-2)Glc(β 1)	α -OH
Mogroside V	Glc(β 1-6)Glc(β 1)	Glc(β 1-6)[Glc(β 1-2)]Glc(β 1)	α -OH
Mogroside VI	Glc(β 1-6)[Glc(β 1-2)]Glc(β 1)	Glc(β 1-6)[Glc(β 1-2)]Glc(β 1)	α -OH

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Table 1. Experimental design by using a fractional factorial design $2^{(6-2)}$ and the corresponding responses per enzyme.

Run	Factors						Responses		
	1	2	3	4	5	6	Synthesized glucosylated mogrosides (mg/mL)		
	Maltodextrin (mg/mL)	Unmodified mogrosides mixture (mg/mL)	Enzyme activity (U/g mogrosides) ^a	Temperature (°C)	Time (h)	pH	CGTase <i>Paenibacillus macerans</i>	CGTase <i>Geobacillus</i> sp	CGTase <i>Thermoanaerobacter</i> sp
1	5	50	5	70	6	5	2.9	7.8	1.4
2	5	5	5	70	1	7	2.0	1.5	1.5
3	5	50	25	50	1	5	2.9	5.3	8.9
4	5	50	5	50	6	7	6.0	8.2	13.7
5	50	5	5	50	6	5	1.9	1.6	2.6
6	5	50	25	70	1	7	2.5	8.3	14.0
7	50	50	25	70	6	7	4.5	7.4	12.5
8	50	5	5	70	6	7	1.0	1.3	2.1
9	50	50	25	50	6	5	5.3	7.9	13.2
10	50	50	5	50	1	7	7.8	4.8	8.0
11	5	5	25	50	6	7	1.7	1.7	2.9
12	50	5	25	70	1	5	1.4	2.0	3.3
13	5	5	5	50	1	5	1.8	0.2	0.0
14	50	5	25	50	1	7	1.3	0.8	1.3
15	5	5	25	70	6	5	2.3	2.7	4.4
16	50	50	5	70	1	5	7.8	6.6	6.6

^aEnzyme activity (U) was declared by the supplier and determined by methods described elsewhere based on cyclization activity²⁶

Table 2. Experimental value obtained from the model equation 1 by maximizing the response.

Response	Theoretical result ^a	Experimental result ^{bc}	Confidence interval ^d	
			(-)	(+)
Synthesized glucosylated mogrosides (mg/mL)	26.2	25.2 ± 1.6 (6.4%)	24.2	28.2

^aObtained from model prediction at the optimal settings.

^bObtained from an average of additional three runs conducted at the optimal settings.

^cStandard deviation and relative standard deviation (n = 4) of experimental result are also represented.

^dLower (-) and upper (+) confidence interval values calculated to a confidence level of 95%.

Table 3. Equivalent sucrose and potency for unmodified and modified mogrosides by CGTase from *Thermoanaerobacter* sp.

	Equivalent sucrose (%)	Potency
Mogrosides	4.5	188
Modified mogrosides	3.7	156

TOC GRAPHIC