Stevia y otros edulcorantes saludables en la formulación de golosinas funcionales: implicaciones tecnológicas y de calidad

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STEVIA Y OTROS EDULCORANTES SALUDABLES EN LA FORMULACIÓN DE GOLOSINAS FUNCIONALES: IMPLICACIONES TECNOLÓGICAS Y DE CALIDAD

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RESUMEN

Las golosinas, "manjar delicado que sirve más para el gusto que para el sustento", son un placer para niños y adultos. La industria del dulce, viene utilizando de forma tradicional para la elaboración de sus productos altas concentraciones de azúcares (fundamentalmente sacarosa y jarabes de glucosa), combinados con agentes gelificantes, ácidos, aromatizantes y colorantes. El consumo de dulces y golosinas debido a su elevado contenido en azúcares ha estado siempre unido al desarrollo de caries, a la subida del nivel de glucosa en sangre y al aumento de peso, entre otras alteraciones de la salud. Aunque las golosinas también son consumidas por adultos, su consumo está asociado mayoritariamente a la población infantil; por este motivo, los problemas relacionados con su ingesta son especialmente preocupantes. La sociedad está cada vez más concienciada de la importancia de la alimentación en la salud. Este hecho está influyendo de forma decisiva en las propuestas del sector de las golosinas en lo que respecta a la innovación y desarrollo de nuevos productos. Las principales tendencias del mercado, están orientadas a eliminar de las formulaciones ingredientes poco saludables, como los azúcares, e incluso a incorporar principios activos con propiedades funcionales, pero todo ello sin olvidar la satisfacción de los consumidores.

En la actualidad, como sustitutivo a los azúcares convencionales, la industria está utilizando edulcorantes artificiales intensos y de volumen. Estos aditivos presentan el inconveniente de estar sometidos a una constante controversia toxicológica. Además, desde el punto de vista industrial por si solos no satisfacen las necesidades tecnológicas propias de los azúcares tradicionales. Actualmente, la industria agroalimentaria tiene a su disposición nuevas alternativas de edulcorantes naturales, con el valor añadido de aportar ciertas ventajas saludables. Entre este tipo de edulcorantes ocupa un lugar destacado la stevia, la oligofructosa y la isomaltulosa.

Considerando lo anteriormente expuesto, la presente tesis doctoral se ha planteado con dos objetivos claramente diferenciados: 1. Profundizar en el conocimiento de los compuestos presentes en la hoja de stevia (glucósidos de steviol, antioxidantes, fenoles, flavonoides y volátiles), como posible fuente de principios activos con propiedades funcionales, evaluando en qué medida estos compuestos son afectados tanto por los métodos de secado (liofilización, secado en sombra y secado por aire caliente a 100 y a 180ºC) como por los procedimientos de obtención de extractos de sus hojas (infusión convencional, microondas y ultrasonidos); 2. Desarrollar golosinas (gomas y espumas dulces) saludables, no cariogénicas y con bajo índice glucémico utilizando ingredientes con propiedades funcionales (stevia, isomaltulosa y oligofructosa), y evaluar el efecto que estos ingredientes tienen sobre las propiedades físico-químicas, mecánicas, ópticas, antioxidantes y sensoriales de dichas golosinas.

De los resultados obtenidos del estudio se concluye que las condiciones óptimas de secado de hoja de stevia, así como las del proceso de obtención de sus extractos acuosos a partir de las hojas secas, se deben considerar teniendo en cuenta el posterior uso que se va a hacer, ya sea para endulzar, o para extracción de principios activos, principalmente antioxidantes.

Con relación a la hoja fresca, el secado (liofilizado, secado en sombra o aire caliente), ocasiona un aumento importante en los parámetros antioxidantes (fenoles totales, flavonoides y antioxidantes totales), especialmente con aire caliente a 180 °C, y una disminución en los principales glucósidos de steviol. Comparando los tres tratamientos de secado con relación a la concentración en compuestos volátiles y antioxidantes, se observa que ambos tipos de compuestos tienen un comportamiento opuesto. El secado en sombra es el que origina un mayor desarrollo de compuestos volátiles, de forma semejante a lo observado para los glucósidos de steviol, mientras que la mayoría de flavonoides y ácidos fenólicos tienen concentraciones mayores cuando se aplica el tratamiento de liofilización.

No existe un tratamiento único adecuado de extracción acuosa (convencional, ultrasonidos o microondas) que maximice simultáneamente el rendimiento en antioxidantes (fenoles totales, flavonoides y antioxidantes totales) y glucósidos de steviol ya que, con relación a la extracción de parámetros antioxidantes, el tratamiento convencional (infusión a presión atmosférica) es más conveniente que los otros dos, mientras que el rendimiento de glucósidos de steviol es mayor aplicando microondas a alta potencia. La mejor condición temperatura/tiempo del método convencional es la más similar al proceso culinario doméstico, agua muy caliente (ligeramente por debajo de ebullición) durante un corto tiempo. Concretamente, la extracción a 90ºC durante 5 minutos es la más apropiada para la extracción de antioxidantes y aminoácidos.

La isomaltulosa podría ser considerada por la industria de la confitería como un ingrediente apropiado para obtener gomas y espumas saludables, pudiéndose incorporar en la formulación de las gomas hasta un 30% y en la de las espumas hasta un 50%. En el caso de las espumas, los extractos de stevia en combinación con isomaltulosa y oligofructosa pueden llegar a sustituir totalmente a los azúcares tradicionales utilizados por la industria de las golosinas sin perder las propiedades físico-químicas, mecánicas, ópticas y sensoriales de las golosinas comerciales formuladas con sacarosa y jarabe de glucosa. Además, la incorporación de estos ingredientes aporta un valor añadido al producto por las propiedades funcionales de los mismos.

RESUM

Les llepolies, "menjar delicat que serveix més per al gust que per al manteniment", són un plaer per a xiquets i adults. La indústria del dolç, ve utilitzant de manera tradicional per l'elaboració dels seus productes altes concentracions de sucres (fonamentalment sacarosa i xarops de glucosa), combinats amb agents gelificants, àcids, aromatitzants i colorants. El consum de dolços i llepolies a causa del seu elevat contingut en sucres ha estat sempre unit al desenvolupament de càries, a la pujada del nivell de glucosa en sang i a l'augment de pes, entre altres alteracions de la salut. Encara que les llepolies també són consumides per adults, el seu consum està associat majoritàriament a la població infantil; per aquest motiu, els problemes relacionats amb la seua ingesta són especialment preocupants. La societat està cada vegada més conscienciada de la importància de l'alimentació en la salut. Aquest fet està influint de manera decisiva en les propostes del sector de les llepolies pel que fa a la innovació i desenvolupament de nous productes. Les principals tendències del mercat, estan orientades a eliminar de les formulacions ingredients poc saludables, com els sucres, i fins i tot a incorporar principis actius amb propietats funcionals, però tot això sense oblidar la satisfacció dels consumidors.

Actualment, com substitutiu als sucres convencionals, la indústria està utilitzant edulcorants artificials intensos i de volum. Aquests additius presenten l'inconvenient d'estar sotmesos a una constant controvèrsia toxicològica, a més que, des del punt de vista industrial per si sols, no satisfeien les necessitats tecnològiques pròpies dels sucres tradicionals. Actualment, la indústria agroalimentària té a la seua disposició noves alternatives d'edulcorants naturals, amb el valor afegit d'aportar certs avantatges saludables. Entre aquest tipus d'edulcorants ocupa un lloc destacat la stevia, l'oligofructosa i la isomaltulosa.

Atès tot això, la present tesi doctoral s'ha plantejat amb dos objectius clarament diferenciats: 1. Aprofundir en el coneixement dels compostos presents en la fulla de stevia (steviol glucòsids, antioxidants, fenols, flavonoides i volàtils), com a possible font de principis actius amb propietats funcionals, avaluant en quina mesura aquests compostos són afectats per diferents mètodes d' assecat (liofilització, assecat en ombra i assecat per aire calent a 100ºC i a 180ºC) i procediments d'obtenció d'extractes de les seues fulles (infusió convencional, microones i ultrasons) i 2 Desenvolupar llepolies (gomes i bromeres dolces) saludables, no cariogèniques i amb baix índex glucèmic utilitzant ingredients amb propietats funcionals (stevia, isomaltulosa i oligofructosa), i avaluar l'efecte que aquests ingredients tenen sobre les propietats fisicoquímiques, mecàniques, òptiques, antioxidants i sensorials d'aquestes llepolies.

Dels resultats obtinguts de l'estudi es conclou que les condicions òptimes d'assecat de fulla de stevia, així com les del procés d'obtenció dels seus extractes aquosos a partir de les fulles seques, s'han de considerar tenint en compte el posterior ús que es farà, ja siga per endolcir, o per extracció de principis actius, principalment antioxidants.

Amb relació a la fulla fresca, l'assecat (liofilitzat, assecat en ombra o aire calent), ocasiona un augment important en els paràmetres antioxidants (fenols totals, flavonoides i antioxidants totals), especialment amb aire calent a 180°C, i una disminució en els principals glucòsids de steviol. Comparant els tres tractaments d' assecat amb relació a la concentració en compostos volàtils i antioxidants, s'observa que el dos tipus de compostos tenen un comportament oposat. L'assecat en ombra es el que origina un major desenvolupament de compostos volàtils, de manera semblant al que s'ha observat per als glucòsids de steviol, mentre que la majoria de flavonoides i àcids fenòlics tenen concentracions més grans quan s'aplica el tractament de liofilització.

No existeix un tractament únic adequat d'extracció aquosa (convencional, ultrasons o microones) que maximitzen simultàniament el rendiment en antioxidants (fenols totals, flavonoides i antioxidants totals) i glucòsids de steviol ja que, amb relació a l'extracció de paràmetres antioxidants, el tractament convencional (infusió a pressió atmosfèrica) és més convenient que els altres dos, mentre que el rendiment de glucòsids de steviol és major aplicant microones a alta potència. La millor condició temperatura / temps del mètode convencional és la més semblant al procés culinari domèstic, aigua molt calenta (lleugerament per baix d'ebullició) durant un curt temps. Concretament, l'extracció a 90 º C durant 5 minuts és la més apropiada per a l'extracció d'antioxidants i aminoàcids.

La isomaltulosa podria ser considerada per la indústria de la confiteria com un ingredient apropiat per a obtindre gomes i bromeres saludables, podent incorporar en la formulació de les gomes fins a un 30% i en la de les bromeres fins a un 50%. En el cas de les bromeres, els extractes de stevia en combinació amb isomaltulosa i oligofructosa poden arribar a substituir totalment als sucres tradicionals utilitzats per la indústria de les llepolies sense perdre les propietats fisicoquímiques, mecàniques, òptiques i sensorials de les llepolies comercials formulades amb sacarosa i xarop de glucosa. A més, la incorporació d'aquests ingredients aporten un valor afegit al producte per les propietats funcionals dels mateixos.

ABSTRACT

Sweets, "delicacy that is consumed for pleasure rather than for nourishment," are loved by both children and adults. The confectionery industry has traditionally made their products using high sugar concentrations, mainly sucrose and glucose syrups, combined with gelling agents, acids, flavorings and colorings. Due to their high sugar content, eating sweets has always been linked to the development of caries, an increase in blood sugar levels and weight gain, among other health disorders. Although sweets are also consumed by adults, they are more associated with children; which is why the related health risks are particularly worrying. Society is becoming increasingly aware of the importance of nutrition in health, and this has a decisive impact on the proposals of the candy sector in terms of innovation and new product development. The main trends of the market are focused on eliminating the unhealthy ingredients in the formulations, such as sugars, and even incorporate active ingredients with functional properties, but without forgetting customer satisfaction.

At present, the industry is using both intense and volume artificial sweeteners as conventional sugar substitutes. These additives have the disadvantage of being under constant toxicological controversy, and also from an industrial point of view do not satisfy the technological requirements of traditional sugars. Currently, the food industry has the possibility of using alternative natural sweeteners, with the added value of providing certain healthy benefits. As examples of this type of sweetener, stevia, oligofructose and isomaltulose could play a leading role.

Taking all of this into account, two different objectives were proposed in this PhD thesis: 1. Extending knowledge about the compounds present in stevia leaves (steviol glycosides, antioxidants, phenols, flavonoids and volatiles) for use as a possible source of active ingredients with functional properties by assessing to what extent these compounds are affected by both drying methods (freeze drying, shade and hot air drying at 100 and 180°C) and procedures for obtaining extracts from the leaves (conventional infusion, microwave and ultrasound); 2. Developing healthy (gummy confections and marshmallows) non-cariogenic candies with a low glycemic index, using ingredients with functional properties (stevia, isomaltulose and oligofructose) and evaluate the effect that these ingredients have on the physical, chemical, mechanical, optical, antioxidant and sensory properties of this type of candy.

Considering the results obtained in this work it is possible to state that the optimum drying conditions for fresh stevia leaves, as well as the conditions used in the process applied to obtain aqueous extracts from dried leaves, are determined by whether they are used for sweetening or for the extraction of active components, mainly antioxidants.

With respect to the fresh leaves, drying (freeze drying, shade drying or air drying) produces a significant decrease in the principal steviol glycosides and a great increment in antioxidant parameters (total phenols, flavonoids and total antioxidants), especially with hot air at 180°C. Comparing the three drying treatments with respect to the concentration of volatile and antioxidant compounds, it was observed that the two types of compounds were affected differently. The content of volatile compounds was higher

with shade drying (similarly to what was observed for steviol glycosides), whereas most flavonoids and phenolic acids had higher concentrations when freeze drying was applied.

There is no one adequate aqueous extraction treatment (conventional, ultrasonic or microwave) which is satisfactory for the extraction of both antioxidant parameters (total phenols, total flavonoids and antioxidants) and steviol glycosides. In relation to the extraction of the antioxidants, the conventional treatment (infusion at atmospheric pressure) is more suitable than the others, while the yield of steviol glycosides is greater using high power microwaves. The best temperature/time conditions in the conventional method are the most similar to the domestic process, hot water (just below boiling point) for a short time. Specifically, the extraction at 90°C for 5 minutes is the most appropriate for the extraction of antioxidants and amino acids.

Isomaltulose should be considered by the confectionery industry as a suitable ingredient to obtain healthy gummy confections and marshmallows. It can be added to the formulation of gummy confections in proportions up to 30% and in marshmallows up to 50%. For marshmallows, stevia extracts in combination with isomaltulose and oligofructose can fully replace traditional sugars (used by the confectionery industry) without losing the physic-chemical, mechanical, optical and sensory properties of commercial confectionery formulated with sucrose and glucose syrup. Furthermore, the incorporation of these ingredients provided added value to the products due to their functional properties.

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I INTRODUCCIÓN

I. INTRODUCCIÓN

I.1. El sector de las golosinas

Las golosinas, consideradas "calorías vacías", aportan un elevado valor energético pero un valor nutritivo prácticamente nulo. La ingesta de golosinas en dosis moderadas es considerada como placentera y tiene un efecto positivo en el bienestar de las personas.

Una de estas primeras golosinas fue las pastillas de frutas Rowntree inventada en 1879 por Claud August Gaget, un famoso confitero inglés que carecía de conocimientos científicos. Sin embargo, históricamente, las golosinas tienen cierta conexión con otras industrias como la farmacéutica basada en "la ciencia". Ya en el siglo XVIII, los farmacéuticos fabricaban productos con actividad farmacológica pero con ciertas características de golosinas con el fin de conseguir que fueran apetecibles al enmascarar el sabor desagradable (Edwards, 2002). Hasta hoy, el campo de la confitería ha ido introduciendo "la ciencia" para obtener nuevos productos y mejorar los existentes, siendo el sector de los dulces, y en particular el de las golosinas los mayores beneficiados. Este tipo de productos está respaldado por un mercado con un gran volumen de ventas, en el que la innovación y el desarrollo son factores clave para marcar las diferencias en un sector cada vez más competitivo.

Las golosinas no son un producto de primera necesidad "manjar delicado que sirve más para el gusto que para el sustento" (R.A.E., 2014), sin embargo, la industria del dulce es un sector muy importante en España y en la Unión Europea. El consumo de este tipo de productos, tanto por la población infantil como adulta, y por tanto la producción no ha dejado de aumentar en estos últimos años, como se refleja en el balance de exportaciones e importaciones desde 2007 (2.985.563.000 euros) hasta 2012 (5.339.906.000 euros) y se prevé que no deje de hacerlo. En España, el consumo per cápita de golosinas es de 3,51 kg (Produlce, 2013), y representa un volumen de ventas considerable 690.862.000€ en 2012 (venta nacional 461.784.000€ y exportaciones 229.078.000€). La figura 1 muestra el porcentaje de consumo a nivel nacional, siendo el chicle sin azúcar y los caramelos duros consumidos fundamentalmente por la población adulta y los chicles con azúcar y golosinas blandas por la población infantil. En cuanto a la Unión Europea (27+Suiza y Noruega), su producción total fue de 1.700.455 toneladas

en 2012 y su consumo fue de 1.629.525 toneladas (Caobisco, 2013), siendo España el mayor productor de productos de confitería respecto a la producción total de dulces (27%), seguido de Alemania (23%), Francia (21%) y Reino Unido (20%) (Caobisco, 2013).

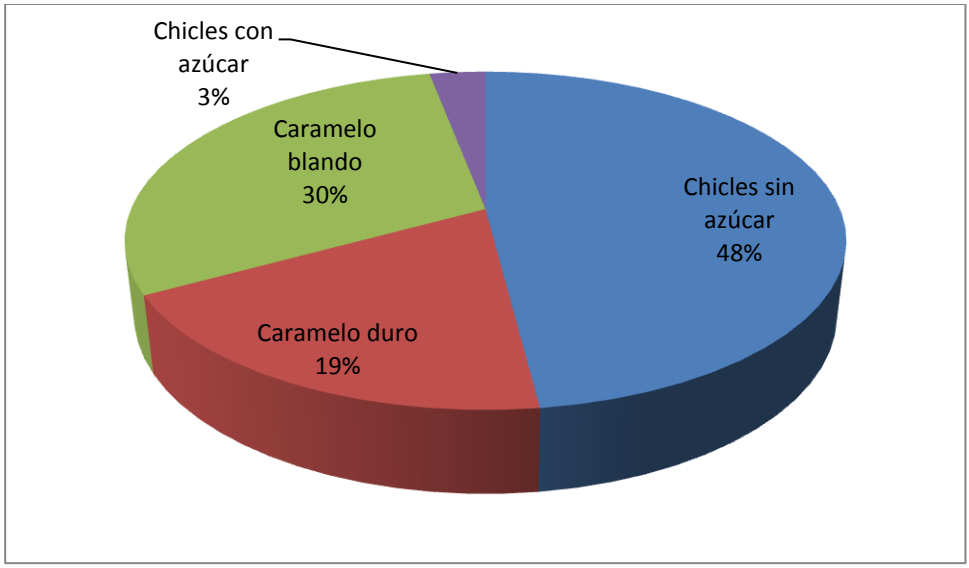


Figura I.1. Consumo nacional de caramelos y chicles en 2012 (Produlce, 2013).

I.2. Ingredientes utilizados tradicionalmente en la elaboración de golosinas

Según la legislación (Real Decreto 348/2011, B.O.E. 5394) las golosinas son productos alimenticios que están elaborados con azúcares o aditivos edulcorantes, a los que puede añadirse otros ingredientes aptos para el consumo humano, incluidos aromas y aditivos, siempre y cuando cumplan los requisitos y condiciones de utilización previstos en sus normas específicas. Los aditivos se regulan en toda la Unión Europea por el Reglamento (CE) nº 1333/2008 aprobado por el Parlamento Europeo y del Consejo, el 16 de diciembre de 2008. En este Reglamento, se recogen los que se pueden utilizar en el territorio de la Unión y se indican las dosis máximas y los alimentos en los que se pueden adicionar. Además los aditivos deberán cumplir con el Reglamento (CE) n.º 852/2004, relativos a la higiene de los productos alimenticios y con el Reglamento (CE) n.º 2073/2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios.

Los ingredientes o componentes principales para la elaboración de golosinas son: azúcares, sustitutos de azúcares (edulcorantes y polialcoholes), agentes gelificantes, colorantes y aromatizantes.

1.2.1. Azúcares

I.2.1.1. Sacarosa o azúcar común

Se designa exclusivamente al azúcar (sacarosa) como el "producto obtenido industrialmente de la caña de azúcar (*Saccharum officinarum*, L.), de la remolacha azucarera (*Beta vulgaris*, L., var. rapa) y de otras plantas sacarinas, en suficiente estado de pureza para la alimentación humana". La sacarosa o azúcar común es un disacárido no reductor constituido por dos monosacáridos reductores, glucosa y fructosa, unidos por el enlace glucosídico α-1,2, también denominado enlace O-glucosídico.

La sacarosa es el hidrato de carbono más utilizado en el mundo como edulcorante debido a sus propiedades físico-químicas y tecnológicas. Tiene un sabor especialmente agradable incluso a altas concentraciones y proporciona una textura única a los productos de confitería. Su solubilidad a temperatura ambiente es del 66%, la cual aumenta en función de la temperatura, aumentando cuando el azúcar se combina con otros azúcares en comparación con una disolución pura de sacarosa (Edwards, 2002).

I.2.1.2. Azúcar invertido

Mediante el proceso de inversión o hidrólisis, la molécula de sacarosa se rompe para dar glucosa y fructosa, obteniendo así el azúcar invertido (Edwards, 2002). El azúcar invertido solo se encuentra en forma de jarabe. Una de sus principales ventajas es que las soluciones de azúcar invertido pueden concentrarse hasta un 80%, con una actividad de agua lo suficientemente baja como para asegurar la estabilidad microbiológica, evitando además la cristalización (Edwards, 2002).

I.2.1.3. Jarabe de glucosa

La norma del Codex para los azúcares (CODEX STAN 212-1999) describe al jarabe de glucosa como una "solución acuosa concentrada y purificada de sacáridos nutritivos obtenidos del almidón y/o inulina". Se obtiene principalmente a partir de la hidrólisis acida del almidón de maíz, de patata o de trigo. El grado de concentración de monosacáridos presentes, expresados como dextrosa (glucosa), en el jarabe se mide en

términos de "dextrosa equivalente o equivalente en dextrosa" (DE), siendo la glucosa pura de DE=100. El jarabe de glucosa que más se utiliza en confitería es de DE 42 o similar. Éste es menos dulce que la sacarosa y afecta a la actividad de agua y a otras propiedades (Edwards, 2002).

I.2.1.4. Fructosa

La fructosa es un monosacárido presente de forma natural en las frutas y en la miel, aunque a escala industrial se extrae de la achicoria (*Cichorium intybus*) y de las alcachofas de Jerusalén (*Helianthus tuberosus*). La fructosa posee mayor poder edulcorante (120-180%) que la sacarosa (100%), por lo que es el azúcar natural más dulce, por ello se emplea para suavizar el sabor de los edulcorantes intensivos (Edwards, 2002). Sin embargo, altos niveles de fructosa en un producto proporciona un cierto sabor a quemado. Su metabolismo es independiente a la insulina, por lo que se utiliza como sustituto de la sacarosa en productos para diabéticos. Sin embargo, en los últimos años, se ha relacionado a la fructosa con el aumento de la obesidad, de la tasa de diabetes y de enfermedades del hígado. No obstante, no puede ser identificada como la única culpable de estos trastornos y se requiere mayores investigaciones para conocer en profundidad el efecto de la fructosa en nuestra dieta (Elliot *et al*., 2002; Bray, 2004).

I.2.2. Edulcorantes

Los edulcorantes se emplean para sustituir los azúcares convencionales con el fin de obtener un nuevo producto, similar al tradicional, apto para diabéticos, no cariogénico y saludable. Por ese motivo existe una lista positiva de aditivos en la que se encuentran los edulcorantes permitidos en alimentación (Reglamento (CE) Nº 1333/2008).

Los edulcorantes se pueden clasificar en: edulcorantes de volumen y edulcorantes intensivos. Los edulcorantes de volumen son polialcoholes, es decir, derivados de hidratos de carbono. Presentan bajo poder edulcorante, sin embargo, son utilizados por la industria para dar volumen al producto, es decir, para proporcionar una textura similar a la que aporta el azúcar común. Su principal inconveniente es que tienen un efecto laxante (Chattopadhyay et al., 2014), y esto debe reflejarse en el etiquetado de los productos. Los más importantes son: sorbitol (glucosa hidrogenada), maltitol (maltosa hidrogenada, tiene un poder edulcorante del 70-90%), lactitol (lactosa hidrogenada),

isomaltol (no es higroscópico), eritritol (tiene menor efecto laxante) y xilitol, entre otros (Edwards, 2002).

Los edulcorantes intensivos se emplean para compensar el déficit de dulzor de los edulcorantes de volumen. La sacarina es el primer edulcorante sintético de la historia. Fue obtenido por primera vez en 1879 por Remsem and Fahlberg (Weihrauch and Diehl, 2004) y el que mayor uso ha tenido en las sociedades hasta nuestros días. Su dulzor es 200 veces mayor que el azúcar común, pero los consumidores le encuentran un regusto amargo desagradable, por ello se combina con otros edulcorantes. La sacarina siempre ha sido motivo de controversia, ya que en ocasiones se la ha relacionado con el cáncer, aunque no hay nada concluyente (Weihrauch and Diehl, 2004).

En 1937 se descubrió el ciclamato, edulcorante no calórico utilizado desde 1950 en Estados Unidos. Es 30 veces más dulce que la sacarina y aunque tiene un pequeño regusto amargo cuando se consume sola, si se combina con la sacarina muestra un dulzor agradable (Chattopadhyay et al., 2014).

Más recientemente, en 1996 fue aprobado el aspartamo como edulcorante general en los alimentos y bebidas en Europa. Es un dipéptido de naturaleza edulcorante no calórico y a diferencia de la sacarina, tiene un sabor dulce, limpio, sin dejar ningún regusto, y es metabolizado por completo (Edwards, 2002). Según numerosos estudios, el consumo de aspartamo podría estar relacionado con la aparición de diversos efectos secundarios en el hombre, además de poder estar implicado en el aumento de la frecuencia de cánceres (Soffritti, 2007). En consecuencia, numerosos estudios científicos han tratado de poner fin a la controversia existente. En 2007, en la primera conferencia europea sobre el aspartamo se concluyó que no hay pruebas suficientes para afirmar claramente que el aspartamo es el causante del aumento en la incidencia de tumores (Renwick and Nordmann, 2007). La Comisión Europea solicitó a EFSA la reevaluación completa de aspartamo para el año 2012, concluyendo que era seguro para la salud en las dosis recomendadas.

Otro edulcorante ampliamente utilizado es el acesulfamo K, aproximadamente 200 veces más dulce que el azúcar. Posee una gran estabilidad ante los tratamientos tecnológicos y durante el almacenamiento. Suele emplearse junto con el aspartamo, ya que de este modo desaparece el regusto amargo y la mezcla de ambos es más dulce que

por ellos mismos. En el aspecto biológico, no parece ser metaboliza en el organismo humano, excretándose rápidamente sin cambios químicos, por lo que no tiende a acumularse. Sin embargo, algunos estudios han puesto de manifiesto que el acesulfamo K, es capaz de interactuar con el ADN y producir daño genético (Mukherjeea and Chakrabarti, 1997).

Al igual que ocurre con el aspartamo, la European Food Safety Authority (EFSA), ha puesto de manifiesto que todos los edulcorantes que se encuentran en la lista positiva de aditivos, son seguros para su consumo en las dosis recomendadas y no hay evidencias científicas de que sean perjudiciales para la salud. Sin embargo, en la actualidad existe un programa para la reevaluación de aditivos alimentarios que finalizará en 2020, por lo que estos aditivos están en continua evaluación por parte de la Comisión Europea.

I.2.3. Agentes gelificantes

Los agentes gelificantes, también denominados gomas, hidrocoloides, y agentes espesantes o estabilizantes, se utilizan principalmente para dar lugar a una estructura tridimensional al producto (Edwards, 2002). Los gelificantes más empleados en la industria confitera son: agar-agar, almidón, goma acacia o goma arábiga, goma guar, pectina, proteínas de leche y gelatina, entre otros.

La gelatina ha sido el agente gelificante utilizado en las formulaciones de golosinas saludables de esta tesis doctoral y por tanto se le va a dedicar una atención especial. Proviene de la hidrólisis (alcalina o ácida) del colágeno, proteína que se encuentra en los huesos y en las pieles de los animales, como el vacuno y el porcino. Este agente gelificante es uno de los ingredientes más versátiles utilizados en la confitería. Además, también se emplea como agente espumante ya que las proteínas tienden a estabilizar las espumas. Para poder utilizar la gelatina, se debe humedecer y disolver a 50-60ºC, evitando superar una temperatura de 80ºC debido al riesgo de que se hidrolice. En cuanto a su calidad gelificante, ésta se mide en grados Bloom, que equivalen a la fuerza del gel formado. El rango suele estar comprendido entre 60-260. La más empleada en confitería es la gelatina de 230º Bloom.

I.2.4. Colorantes

Los colorantes están regulados por el Reglamento CE nº 1333/2008 de Parlamento Europeo y del Consejo, de 16 de diciembre de 2008. Los colorantes se clasifican principalmente en naturales y sintéticos.

Los colorantes sintéticos son mucho más estables a la luz, al calor y a los extremos de pH que los naturales. Se pueden comercializar tanto en polvo como diluidos. Algunos colorantes sintéticos son: Ponceau 4R (E124), Tartrazina (E102), Índigo común (E132), Verde S (E142). Entre los colorantes sintéticos o artificiales se incluyen los azoicos o azocolorantes, que se han relacionado con reacciones alérgicas (sobre todo en niños) por consumo excesivo de golosinas coloreadas. En este sentido, desde el 20 de julio de 2010, el Reglamento CE 1333/2008 introduce la necesidad de incorporar en el etiquetado de alimentos que contengan estos colorantes artificiales la siguiente advertencia: "nombre o número E del/de los colorante(s): puede tener efectos negativos sobre la actividad y la atención de los niños". Esta situación también obliga a las industrias de la confitería a buscar alternativas más naturales para la obtención de golosinas, aunque existen en el mercado aditivos colorantes naturales que se utilizan en la formulación de golosinas.

Los colorantes naturales, en contraposición a los sintéticos, son menos estables al calor y a la luz, y proporcionan un color menos intenso. Los consumidores los prefieren por sus menores implicaciones toxicológicas. Los colorantes naturales, presentan un rango de color limitado, lo que intenta compensarse mediante purificación. De esta forma se consigue emplearlos en dosis muy bajas, incluso menores que los sintéticos. Algunos colorantes naturales son: E120-cochinilla (escarabajo mejicano), E140-Clorofila (Hojas verdes, alfalfa, hierba), E100-curcumina (raíz de la cúrcuma).

I.2.5. Aromatizantes

Los aromas son sustancias, no destinadas al consumo como tales, que se añaden a los alimentos para darles o modificar su olor o sabor. Es habitual que, durante su transformación, los alimentos pierdan en mayor o menor medida sus características organolépticas o, incluso, adquieran un sabor u olor distinto del esperado. En estos casos, la adición de un aroma puede aumentar, disminuir o modificar las características sensoriales de los mismos. Los aromas se regulan mediante el Reglamento (CE) nº

1334/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre los aromas y determinados ingredientes alimentarios con propiedades aromatizantes utilizados en los alimentos.

I.3. Definiciones y tipos de golosinas clasificados según el RD 348/2011

Las golosinas son productos alimenticios que están elaborados con azúcares o aditivos edulcorantes, a los que puede añadirse otros ingredientes. Entre los diferentes tipos de golosinas cabe destacar:

- Geles dulces: los obtenidos por gelificación de almidones o féculas que, como tales o formando parte de harinas, componen una mezcla de azúcares o aditivos edulcorantes y gelificantes.

- Dulces de regaliz: los elaborados con azúcares o aditivos edulcorantes, almidones o féculas, harinas y dextrinas a los que se incorpora extracto de regaliz.

- Espumas dulces: las obtenidas por la aireación de soluciones concentradas de azúcares o aditivos edulcorantes a las que se incorporan gelificantes, confiriéndoles esponjosidad y consistencia no elástica.

- Fondants: productos alimenticios obtenidos de soluciones concentradas de azúcares o aditivos edulcorantes a los que pueden incorporarse otros ingredientes, cuyo proceso de elaboración les confiere una estructura plástica.

- Golosina líquida para congelar: producto líquido o semilíquido obtenido con una mezcla de azúcares o aditivos edulcorantes y agua, al que se pueden incorporar otros ingredientes.

La Tabla 1 muestra la clasificación de golosinas realizada por Edwards (2002). En ella se especifican los ingredientes utilizados en cada tipo de golosina y la característica principal de cada una de ellas.

Tabla 1. Ingredientes y característica principal según el tipo de golosina, (adaptación de Edwards, 2002).

GRUPO	TIPO	COMPONENTES PROPIOS	CARACTERISTICAS
Caramelos	**Duros**	Azúcar refinado y jarabe de glucosa, saborizantes y colorantes (típicos)	Masa sobresaturada en azúcar, estado vítreo
	Blandos	Típicos, grasa y emulsionantes	Masa de alta viscosidad sin fase cristalina
	Toffees	Típicos, leche condensada, emulsionantes, grasa y mantequilla	Caramelos masticables sin cristalización con alto grado de pegajosidad
	Fugdes	Típicos, leche condensada, emulsionantes, grasa y mantequilla	Caramelos con pequeña cristalización de textura lisa, suave y no pegajosa
Gomas	**Gominolas**	Típicos y gelificantes	Productos gelificados y masticables muy dulces
	Regaliz	Típicos y harina de trigo, raíz de regaliz	Alta viscosidad y plasticidad
Aireados	**Espumas dulces**	Típicos, clara de huevo o gelatina y extracto de malvisco	Productos aireados con diferentes texturas
Goma de mascar	**Chicles**	Típicos, goma base, humectante	Goma masticable con sabor dulce
Grageados	**Duros**	Azúcar, saborizantes y colorantes	Productos recubiertos por capas de microcristales de sacarosa
	Blandos	Azúcar, jarabe de glucosa, saborizantes y colorantes	Productos recubiertos por capas no cristalizados y con relleno humedecido

Las gomas o productos gelificados, se elaboran a partir de una disolución altamente concentrada de azúcar y jarabe de glucosa y mediante la adición de una combinación de gelificantes convirtiendo esa mezcla en un gel viscoelástico. La gelatina es el agente gelificante primordial en las gomas. Les confiere el efecto rebote que hace que las gomas sean elásticas y vuelvan a su estado original y también la traslucidez típica de este tipo de golosinas. Por otro lado, también se pueden utilizar almidones modificados que una vez hidratados, hinchados y gelatinizados, tienen como función dar textura a las gomas, tendiendo a dar opacidad a ésta.

Las nubes o espumas dulces también llamadas malvaviscos (“marshmallows” en inglés, “guimauve” en francés) son golosinas aireadas. Tienen su origen en Francia donde en épocas antiguas se producían bajo el nombre de “pâte de guimauve” y se elaboraban con azúcar, clara de huevo, jarabe de glucosa y extracto de la raíz de la planta malvavisco, *Althaea officinalis*, que le otorgaba su aroma y actuaba como la gelatina de hoy en día (Edwards, 2002). La principal característica de las nubes es que son golosinas en las que se incorpora aire durante el batido, etapa principal en el

proceso de elaboración. Para conseguir el aireado se emplean agentes texturizantes, espumantes o de batido como pueden ser la gelatina de Alto Bloom 200-250 y el albumen de huevo en polvo. La gelatina de Alto Bloom (200-250) permite obtener una textura firme y elástica tipo caucho, y el albumen de huevo ofrece una textura corta y cremosa. La espuma de albumen de huevo proporciona un gel ligero y blando, mientras que una espuma de gelatina es más pesada, blanda y de textura gomosa. En algunos casos se pueden emplear en combinación proteínas de leche y soja modificadas para aumentar la cantidad de aire y estabilizar las espumas.

Para obtener la espuma característica se debe formar una emulsión. Se trata generalmente de un sistema disperso de dos líquidos inmiscibles: un líquido disperso en forma de gotitas extremadamente pequeñas (fase interna o dispersa, el aire en nuestro caso) y la matriz (fase externa o continua, la mezcla de azúcares y gelatina). Los agentes de batido son esencialmente materias proteicas, por lo que sus moléculas son hidrófilas y lipófilas. Durante el batido la gelatina ubicada en la mezcla se dispone de tal manera que forma una red proteica, la cual encierra el aire incorporado.

I.4. Proceso de elaboración de golosinas tipo gomas y espumas dulces

Las golosinas se obtienen mediante un proceso de disolución del azúcar en agua y la cocción del jarabe de azúcar con jarabe de glucosa con objeto de concentrar la mezcla así obtenida. Esta mezcla se calienta hasta ebullición para formar el jarabe inicial del producto. Una vez enfriada, se añaden aromas, colorantes y el agente gelificante que en el caso de la gelatina ha sido previamente disuelto en agua caliente. Tras la adición de todos los aditivos, la mezcla se agita y se calienta a una temperatura no superior de 60ºC, esta temperatura es importante porque si se supera se podría desnaturalizar la proteína de la gelatina. Después la mezcla se deposita en moldes, de goma o de almidón, como se hacía tradicionalmente. El depositado en moldes de almidón, también llamado depositado en mugol, se hace sobre bandejas con almidón que están marcadas con la forma que se quiera dar a la golosina. En caso de utilizar los moldes de goma se dejan en una cámara hasta la gelificación de las golosinas, y posteriormente se desmoldan.

Para la elaboración de espumas dulces, se realiza el mismo proceso con la única diferencia que una vez realizada la mezcla, se debe realizar una etapa de batido para incorporar el aire a la mezcla, ya que el aire es el principal responsable de la textura característica de este tipo de golosinas. Después de desmoldar las gominolas o las nubes

dulces se pone aceite, azúcar, almidón o azúcar glace para evitar la pegajosidad entre las muestras, y darle la textura externa característica de este tipo de golosinas

En al Figura 2 se muestra el diagrama de flujo seguido para la elaboración de gomas y espumas dulces.

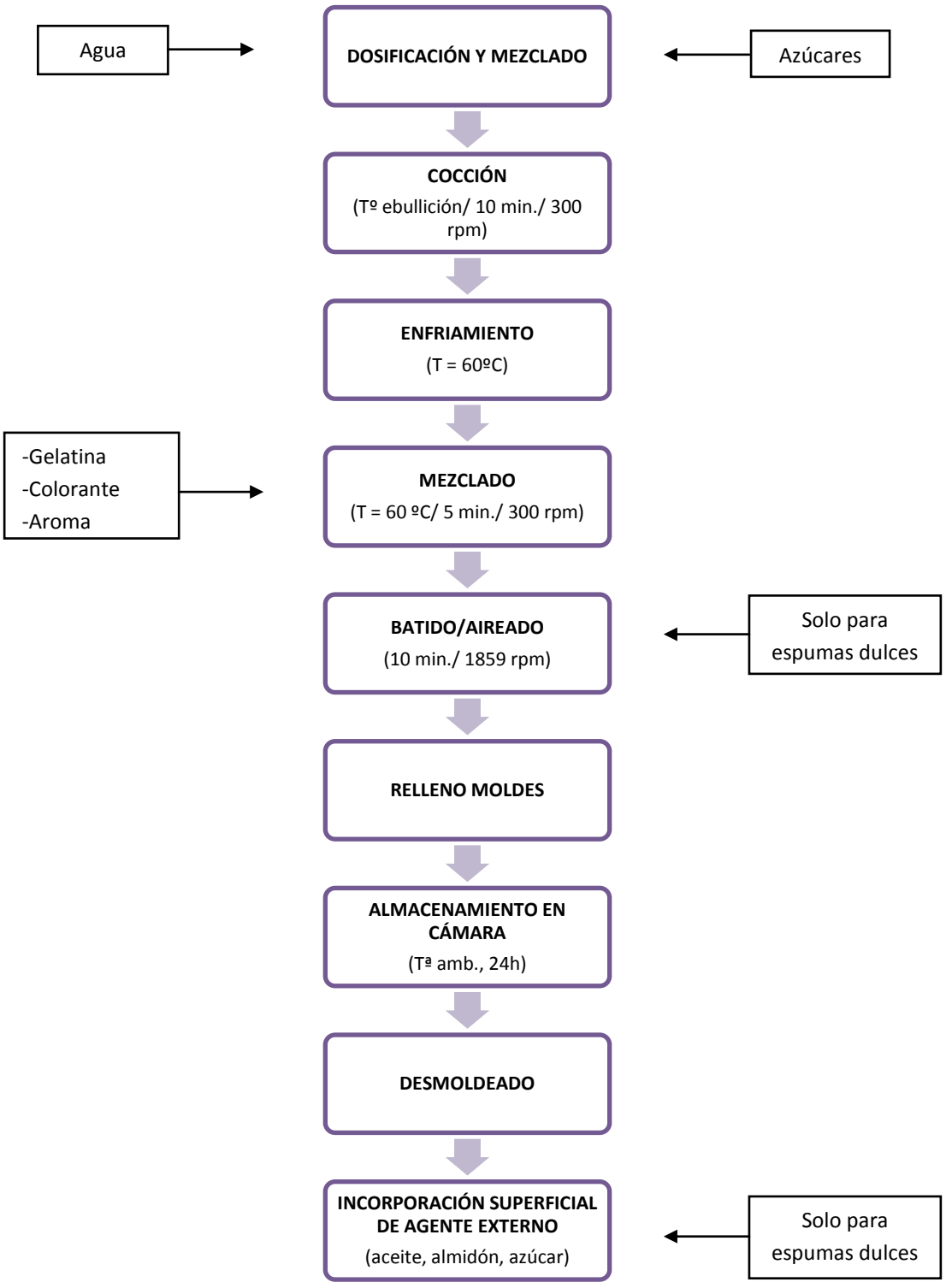


Figura I.2. Diagrama de flujo del proceso de elaboración de gomas y espumas dulces.

I.5. Implicación en la salud del consumo de golosinas fabricadas con azúcares convencionales

El consumo de dulces y golosinas debido a su elevado contenido en azúcares ha estado siempre unido a determinadas alteraciones de la salud entre las que destaca el desarrollo de caries, la subida del nivel de glucosa en sangre y al aumento de peso.

I.5.1. Caries dental

La caries dental es una enfermedad infecciosa de etiología multifactorial que afecta a las estructuras dentarias, destruyendo gradualmente el esmalte, la dentina y eventualmente la pulpa, hasta la posible pérdida del diente (Gómez-Álvarez, 2003). Se caracteriza por periodos de desmineralización alternados con periodos de remineralización de los tejidos duros del diente (Charland *et al*., 2001).Varios autores coinciden en el hecho de que la formación de la caries dental se da si coexisten a la vez tres factores: el factor sustrato (carbohidratos alimentarios), el factor microbiano (bacterias cariogénicas presentes en la placa dental) y el factor terreno (tejidos duros vulnerables) (Farge, 1998; Lewis and Ismail, 1995).

Las bacterias cariogénicas, principalmente *Streptococcus mutans* (implicado en la iniciación de la lesión cariogénica) pero también *Actinomyces* (implicado en la progresión de la lesión cariogénica) y *Lactobacillus* (implicado más particularmente en la caries radicular), poseen ciertas propiedades que les otorga el carácter de patógenos (Farge, 1998; Badet and Richard, 2004). Son bacterias fermentativas (producen ácido a partir de carbohidratos), adherentes (se adhieren a la superficie mediante la síntesis de glucanos y fructanos a partir de sacarosa) y acidogénicas (bajan el pH al producir ácidos). La infección por bacterias cariogénicas en la cavidad bucodental no es suficiente para que se desarrolle la enfermedad. También es necesaria la presencia de carbohidratos fermentables, por lo que existe una estrecha relación entre el consumo de azúcar y la formación de caries. Estas bacterias metabolizan cualquier carbohidrato fermentativo, como lo son la glucosa, fructosa, sacarosa o el almidón, convirtiéndolos en subproductos ácidos como el etanol o el ácido láctico causando la desmineralización superficial de los tejidos calcificados del diente, viéndose acentuada cuando el pH baja por debajo de 5,5 (Charland *et al*., 2001). Hay que tener en cuenta que no es la cantidad global de azúcar ingerido lo que determina el desarrollo de la caries, sino la frecuencia de su ingestión (Fioretti and Haïkel, 2010; Badet and Richard, 2004).

I.5.2. Índice glucémico

El índice glucémico (IG) cuantifica el aumento de los niveles de glucemia (concentración de glucosa en sangre) producido por la ingesta de un alimento en relación a la ingesta de glucosa. El índice glucémico fue definido por Jenkins *et al*. en 1981, como clasificación fisiológica de alimentos según su impacto en la glucemia postprandial para la planificación alimentaria de personas diabéticas (Romero *et al*., 2002).

Los valores de IG se pueden agrupar en tres categorías: IG altos: ≥ 70; IG intermedio: 56-69; IG bajo: ≤ 55. En la tabla 2 se muestran los índices glucémicos de algunos alimentos. Por consiguiente, un alimento con alto índice glucémico indica que su absorción en sangre es rápida, de manera que el páncreas tiene que producir rápidamente altas cantidades de insulina par su absorción intracelular, y por tanto una tarea inverosímil para un diabético, persona que produce bajas o nulas dosis de insulina por trastornos metabólicos (Artegada, 2006).

Tabla 2. Índices glucémicos y poder edulcorante en relación a la sacarosa de diferentes azúcares y edulcorantes (adaptación Godshall, 2007).

Azúcares/edulcorantes	Índice glucémico	Poder edulcorante
Glucosa	99-100	0.5
Sacarosa	61-65	1
Isomaltulosa	32	0.3-0.4
Fructosa	19-23	1.5-1.8
Xilitol	7-13	1
Sorbitol	9	0.6

I.5.3. Sobrepeso y obesidad

La obesidad infantil es uno de los problemas de salud pública más graves del siglo XXI. El problema es mundial y está afectando progresivamente a muchos países de bajos y medianos ingresos, sobre todo en el medio urbano. Se calcula que en 2010 hay 42 millones de niños con sobrepeso en todo el mundo, de los que cerca de 35 millones viven en países en desarrollo (WHO, 2010). En España, alrededor del 30% de los niños

tienen sobrepeso y de estos un 10% sufren obesidad (Instituto nacional de Estadística, 2014). La causa fundamental del sobrepeso y la obesidad infantiles es el desequilibrio entre la ingesta calórica y el gasto calórico. El mayor consumo de alimentos hipercalóricos (con alto contenido de grasas y azúcares) y la menor actividad física. Las dietas tradicionales han sido reemplazadas rápidamente por otras con una mayor densidad energética, lo que significa más grasa, principalmente de origen animal, y más azúcar añadido en los alimentos, unido a una disminución de la ingesta de carbohidratos complejos y de fibra. Estos cambios alimentarios se combinan con la tendencia a la disminución de la actividad física debido al aumento de la naturaleza sedentaria de muchas actividades recreativas, el cambio de los modos de transporte y la creciente urbanización.

I.6. Alternativa a los azúcares convencionales en la formulación de golosinas

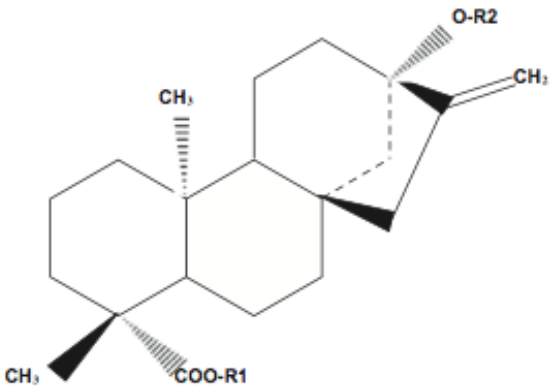
Debido a la problemática planteada anteriormente de los azúcares convencionales sobre la salud del consumidor y además de las posibles implicaciones toxicológicas de los edulcorantes artificiales intensivos y de no satisfacer las necesidades tecnológicas propias de los azúcares tradicionales. La industria agroalimentaria tiene a su disposición nuevas alternativas de azúcares y edulcorantes naturales, metabolizables por el organismo, que aportan además otras ventajas nutricionales. Entre éstos cabe destaca la stevia, isomaltulosa y la oligofructosa.

I.6.1. Stevia Rebaudiana

La *Stevia rebaudiana* es una planta perenne originaria de Paraguay perteneciente a la familia Asteraceae (Goyal et al., 2010). La Autoridad Europea de Seguridad Alimentaria reconoció la seguridad de los extractos de hoja de stevia para uso alimentario en noviembre de 2011 (EFSA, 2011). A pesar de que su uso estaba autorizado en diferentes países de Asia y América desde hace años.

La principal característica de la hoja de stevia es su elevado sabor dulce que se debe a la presencia de diterpenos tales como glucósidos de steviol que son entre 250-300 veces más dulces que la sacarosa (Ghanta et al., 2007). Los más importantes son el steviósido (4-13%), el rebaudiósido A (2-4%), el rebaudiósido C (1-2%) y el dulcósido A (0,4-0,7%) (Figura 4), aunque también se encuentran otros menos abundantes como

steviolmonoside, rubosido, steviolbiósido, rebaudiósido B y rebaudiósido F (Lemus-Moncada et al., 2012). Numerosos estudios toxicológicos ha demostrado que los steviosidos son seguros para el consumo humano (Carakostas et al, 2008).



Compound	R1	R2
Steviol	H	H
Steviolbioside	H	β-Glc-β-Glc(2→1)
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C (Dulcoside B)	β-Glc	β-Glc-α- Rha(2→1) | β-Glc(3→1)
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
Dulcoside A	β-Glc	β-Glc-α- Rha(2→1)

Figura I.3. Estructura de los glucósidos de steviol presentes en la hoja de stevia. (Geuns, 2003).

El uso más común de la hoja de stevia está dirigido a la extracción y purificación de los steviosidos con el fin de obtener un edulcorante natural acalórico. Japón fue el primer país en comercializar los steviósidos como edulcorante en fármacos y alimentos

en 1968 (Kroyer, 2010). La ingesta diaria admisible (IDA) de estos compuestos es de 4 mg por kg de peso corporal por día (JECFA, 2008). Aunque cada vez más, la stevia es consumida a modo de infusión o incorporada a distintas formulaciones alimentarias por las muchas propiedades terapéuticas que posee la hoja de stevia.

I.6.1.2. Beneficios de la stevia sobre la salud

Las hojas de stevia son ricas en compuestos con propiedades antiinflamatorias, diuréticas, antihipertensas, antihiperglicemicas, antidiarreicas y antitumorales (Chatsudthipong and Muanprasat, 2009), además de poseer propiedades antioxidantes (Tadhani et al., 2007; Muanda et al., 2011; Shukla et al., 2012) beneficiosas para la salud del consumidor. Por otro lado, también posee actividad antimicrobiana, muchos estudios afirman que extractos de hoja de stevia fermentados inhiben el crecimiento de microorganismos como *Salmonella typhimurum, B. subtilis* y *S. aureus* (Debnath, 2008; Ghosh et al., 2008). De ahí el creciente interés en el uso no solo de los steviosidos aislados sino de extractos acuosos de hoja deshidratada de stevia. Estos extractos podrían consumirse a modo de infusión o ser incorporados a diferentes formulaciones alimentarias como zumos, galletas, mermeladas, golosinas, etc.

I.6.2. Isomaltulosa

La isomaltulosa (6-O-α-D-glucopiranosil-D-fructosa) (Figura 3) es un disacárido reductor, isómero de la sacarosa, que se encuentra en pequeñas cantidades en la miel y en la caña de azúcar (Lina *et al*., 2002). Industrialmente es producida a partir de la sacarosa por la reorganización enzimática (*Protaminobacter rubrum*) del enlace glucosídico entre la glucosa y fructosa a partir del enlace α-1,2 de la sacarosa al enlace α-1,6 de la isomaltulosa seguido de una cristalización (Schiweck et al., 1990; Weidenhagen and Lorenz, 1957).

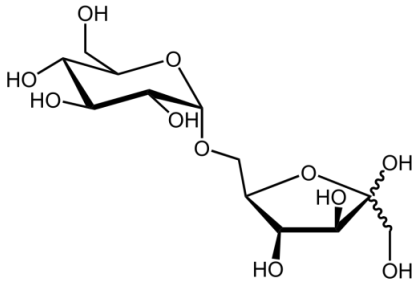


Figura I.4. Molécula de isomaltulosa

La isomaltulosa es un hidrato de carbono puro, blanco y cristalino, lo que le confiere una apariencia y sabor similar a la sacarosa con un dulzor natural sin ningún sabor residual, "aftertaste". Aunque, su poder edulcorante está comprendido entre un tercio y la mitad que el de la sacarosa (Lina *et al*., 2002; Beneo-Palatinit, 2010a,b). Por otro lado, la solubilidad (29%) de la isomaltulosa y su temperatura de fusión (120ºC-128ºC) son más bajas en comparación con la sacarosa (66%; 160ºC-185ºC), pero en una disolución acuosa la viscosidad de cada una es muy similar (Beneo-Palatinit, 2010b). Debido a que su estructura molecular es más estable que la sacarosa, no es higroscópica y también es más estable bajo condiciones ácidas. Una combinación de isomaltulosa con otros hidratos de carbono o edulcorantes muy intensos puede mejorar el dulzor, el sabor y la textura del producto final (Beneo-Palatinit, 2010a,b).

En relación a la situación legal del uso de la isomaltulosa, en julio 2005, la Comunidad Europea (Decisión C-2776) autorizó su comercialización como nuevo alimento o nuevo ingrediente alimentario con arreglo al Reglamento (CE) nº258/97. En 2009, la EFSA (European Food Safety Authority) reconoció positivamente la ventaja de utilizar isomaltulosa por sus características fisiológicas específicas al mejorar las propiedades relacionadas con la salud del producto final, para el beneficio del consumidor. Además, ha sido utilizada en varios productos alimentarios, como mermeladas, chocolate, galletas, etc. (Peinado et al. 2013).

I.6.2.1. Beneficios de la isomaltulosa sobre la salud

La isomaltulosa es completa y lentamente digerible, a diferencia de la sacarosa. Su lenta liberación en sangre es debida a que es hidrolizada y absorbida de cuatro a cinco veces más lentamente que la sacarosa gracias al enlace α-1,6 entre la glucosa y fructosa (Beneo-Palatinit, 2010 a,b). Durante su paso en el tracto gastrointestinal se hidroliza completamente y los monosacáridos resultantes, glucosa y fructosa, son absorbidos y metabolizados en el intestino delgado siguiendo la misma ruta clásica que la glucosa y fructosa provenientes de la sacarosa (Lina *et al*., 2002). De esta manera ofrece las mismas calorías que muchos otros azúcares (4 kcal/g). En consecuencia, muestra un aumento más lento y niveles máximos de glucosa e insulina en sangre inferiores a los de la sacarosa proporcionando al cuerpo energía por más tiempo, además de ser una característica que puede ser particularmente beneficioso para diabéticos. Por lo tanto, la isomaltulosa resulta ser de bajo índice glucémico (IG 32), por lo que la concentración de glucosa en sangre después de consumir isomaltulosa es de

aproximadamente el 50% comparado con la concentración después de consumir sacarosa (Beneo-Palatinit, 2010a; Kawai et al., 1989). Además, la tolerancia gastrointestinal de la isomaltulosa es comparable a la de la sacarosa incluso a altos niveles de ingesta, de hasta 50 g/día. A diferencia de otros edulcorantes como los polialcoholes, no tiene efectos laxantes (Beneo-Palatinit, 2010a; Lina *et al*., 2002).

Al contrario de la sacarosa, la isomaltulosa por su fuerte enlace molecular, es apenas fermentada por los microorganismos ambientales u orales ya que no la utilizan como sustrato, y en consecuencia no se producen ácidos nocivos capaces de atacar al esmalte dental, proporcionando ningún riesgo de formación de caries dental (Beneo-Palatinit, 2010b; Bucke and Cheetham, 1986). Aunque, algunos estudios indican que la placa dental contiene un número significante de bacterias, comunes en la cavidad oral humana, que tienen el potencial de degradar la isomaltulosa a ácidos. No obstante, la producción de ácidos a partir de la isomaltulosa es mínima (Matsuyama *et al*., 1997; Bucke and Cheetham, 1986).

La FDA (Food and Drugs Administration) permite el uso de declaraciones para la isomaltulosa tales como "no promueve caries dental" o "puede reducir el riesgo de caries dental" y por lo tanto, es una alternativa ideal a la sacarosa (Beneo-Palatinit, 2010a).

I.6.3. Oligofructosa

La oligofructosa es un oligosacárido lineal formado por entre 10 y 20 monómeros de fructosa, unidos por enlaces β(1→2) y que pueden contener una molécula inicial de glucosa, también es llamado fructooligosacárido o FOS en su forma abreviada. En la naturaleza se encuentra en una gran variedad de vegetales como el ajo, la cebolla y la achicoria (Van Loo et al., 1995). Industrialmente es obtenida de la raíz de la achicoria mediante una hidrolisis parcial enzimática, posteriormente se purifica evaporando o atomizando la muestra para obtener oligofructosa en jarabe o en polvo, respectivamente.

La oligofructosa tiene unas propiedades muy similares a las del azúcar y a las de jarabe de glucosa (Crittenden and Playne, 1996). Además, posee una alta solubilidad, alrededor del 80% a temperatura ambiente. Tiene un dulzor de un 40% en comparación con la sacarosa y sabor muy similar. Muestra una buena estabilidad durante el procesado de alimentos, como por ejemplo durante el tratamiento térmico. Además

contribuye a mejorar el sabor en boca, tiene propiedades humectantes y reduce la actividad de agua lo que asegura una alta estabilidad microbiológica (Franck, 2002).

En relación a la situación legal del uso de la oligofructosa, ha sido clasificado en la Unión Europea como alimento o ingrediente alimentario y no como aditivo. La evaluación positiva del Panel de Productos Dietéticos, Nutrición y Alergias (NDA) de la EFSA permitió la aprobación por parte de la Comisión, los Estados miembros y el Parlamento Europeo, del uso de la oligofructosa por sus propiedades nutricionales y saludables. En la industria alimentaria se ha utilizado con éxito en muchos productos como son los cereales, postres lácteos, chocolate, etc (Pimentel et al. 2014; Volpini-Rapina et al. 2012).

I.6.3.1. Beneficios de la oligofructosa sobre la salud

La oligofructosa es una fibra dietética, con los efecto fisiológicos atribuibles a este tipo de compuestos, como son la disminución de los niveles lipídicos y glucosa en sangre y la acción laxante (Camire et al., 2001). Otra propiedad ligada a la anterior es su función prebiótica que estimula el crecimiento de la flora intestinal (Coussement, 1996). Además posee un bajo valor calórico 1,5 kcal/g a diferencia de la sacarosa que su aporte calórico es de 4kcal/g. Además se ha comprobado que el uso de oligofructosa en la dieta mejora la absorción de calcio (Gibson et al.,1995).

I.7. Referencias

Artegada, A. (2006). EL índice glicémico. Una controversia actual. Nutrición Hospitalaria, 21(2): 55-60. ISSN 0212-1611

Badet C and Richard B. (2004). Étude clinique de la carie. EMC-Dentisterie, 1:40-48.

Bray, G.A., Nielsen, S.J., and Popkin, B.M. (2004). Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. American Society for Clinical Nutrition, 79 (4), 537-543.

Beneo-palatinit (2010a) Palatinose™. The only toothfriendly sugar for optimized energysupply.http://www.beneopalatinit.com/en/Food_Ingredients/Isomaltulose/What_is_Isomaltulose/Palatinose-Brochure_EN_1.pdf. Consultada 25 Junio 2014.

Beneo-palatinit (2010b). PALATINOSE™ – The functional carbohydrate providing better energy. Dirección URL: http://www.beneo-palatinit.com/en/Food_Ingredients/Isomaltulose/What_is_Isomaltulose/Palatinose-Brochure_EN_1.pdf . Consultada 25 Junio 2014.

Bucke C, Cheetham PS (1986) Method of reducing dental plaque formation with products for human or animal consumption using isomaltulose sucrose substitute. United States of America Patent No 4,587,119.

Camire M, Cho S, Craig S, Devrie J, Goron D, Jones J, Li B, Lineback F, Prosky L,Tungland B. (2001). The definition of dietary fiber. Cereal Foods World, 46,112-126.

Caobisco. (2013). Annual Report 2010. The Association of the Chocolate, Biscuit & Confectionery Industries of Europe. [en línea]. Fecha de consulta: 24 de junio de 2012. Dirección URL: http://caobisco.eu/public/images/page/caobisco-28052014144036-2013-Caobisco-Annual-Report-V6-WEB.pdf

Carakostas MC, Curry LL, Boileau AC, Brusick DJ, (2008). Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. Food and Chemical Toxicology 46, S1-S10.

Charland, R., Voyer R, Cudzinowski L, Salvall P, Abelardo L (2001). La carie dentaire. Journal dentaire de Québec, 38:409- 418.

Chatsudthipong V. and Muanprasat C., (2009). Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacol. Therapeut. 121, 41-54.

Chattopadhyay S, Raychaudhuri U and Chakraborty R (2014). Artificial sweeteners – a review. J Food Sci Technol 51(4): 611–621

Codex alimentarius. CODEX STAN 212-1999. (1999). Norma del codex para los Azúcares.

Coussement P, (1996). Pre and synbiotics with inulin and oligofructose. Food Technology Europe, January, 102-104.

Crittenden RG and Playne MJ , (1996) Production, properties and applications of food-grade oligosaccharides. Trends in Food science and Technology 7,353-361.

Debnath, M. (2008). Clonal propagation and antimicrobial activity of an endemic medicinal plant Stevia rebaudiana. Journal of Medicinal Plants Research, 2,45-51.

Decisión C-2776. (2005). Decisión de la Comisión, de 25 de julio 2005, por la que se autoriza la comercialización de la isomaltulosa como nuevo alimento o nuevo ingrediente alimentario con arreglo al Reglamento (CE) nº 258/97.

Edwards, W.P. (2002). La ciencia de las golosinas. Editorial Acribia, S.A., Zaragoza.

Elliot. S.S., Keim, N.L., Stern, J.S., Teff, K., and Havel, P.J. (2002). Fructose, weight gain, and the insulin resistance syndrome. The American Journal of Clinical Nutrition, 76(5), 911-922.

European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. EFSA Journal 2011; 9 (1):1972. doi:10.2903/j.efsa.2011.1972

Farge P (1998)Données récentes sur l'étiopathogénie de la carie. Arch Pédiatr, 5:1140-1144.

Fioretti F. and Haïkel, Y. (2010) Carie et sucres. Médecine des maladies métaboliques, vol.4, nº 5, 543-549. Doi: MMM-10-2010-4-5-1957-2557-101019-201004579

Franck A (2002).Technological functionality of inulin and oligofructose. British Journal of Nutrition 87, 287-291.

Geuns J. (2003). Stevioside. Phytochemistry, 64, 913-921.

Ghanta, S., Banerjee, A., Poddar, A., and Chattopadhyay, S. (2007). Oxidative DNA damage preventive activity and antioxidant potential of Stevia rebaudiana (Bertoni) Bertoni, a natural sweetener. *Journal of Agricultural and Food Chemistry*, 55, 10962-10967.

Ghosh, S., Subudhi, E. and Nayak, S (2008). Antimicrobial assay of Stevia rebaudiana Bertoni leaf extracts against 10 pathogens. International Journal of Integrative Biology, 2, 27-31.

Gibson, GR, Beatty ER, Wang X and Cummings, JH (1995). Selective stimulation of bifidobacteria in human colon by oligofructose and inulin. Gastroentrology 108,975-982.

Godshall MA (2007) The expanding world of nutritive and non-nutritive sweeteners. Sugar Journal , 69,12-20.

Gómez-Álvarez P. (2003) Nutrición y caries. Acción preventiva. Farmacia Profesional, 17:66-69.

Goyal, SK., SamsherR and Goyal, RK. (2010).Stevia (Stevia rebaudiana) a bio-sweetener. A review. International Journal of Food Sciences and Nutrition, 61,1-10.

Instituto nacional de estadística (2014) https://www.msssi.gob.es/estadEstudios/estadisticas/encuestaNacional/encuesta2011.htm. Consultada en Julio 2014.

JECFA. Joint FAO/WHO Expert Committee on Food Additives, Steviol glycosides. In Compendium of Food Additive Specifications, 69th Meeting, FAO/WHO Monographs 5, (2008) Rome, Italy.

Jenkins DJA, Wolever TM, Taylor RH, Barker H, Fielden H, Baldwin JM, Bowling AC, Newman HC, Jenkins AL y Goff DV. (1981). Glycemic index of foods: a physiological basis for carbohydrate exchange. American Journal of Clinical Nutrititon, 34(3): 362–366.

Kawai, K., Yoshikawa, H., Murayam, Y., Okuda, Y., & Yamashita, K. (1989). Usefulness of palatinose as a caloric sweetener for diabetic patients. Hormone and Metabolic Research, 21, 338–340.

Kroyer J. (2010). Stevioside and Stevia-sweeter in food: application, stability and interaction with food ingredients. J. Verbr. Lebensm. 5, 225–229.

Lina, B., Jonker, D., and Kozianowski, G. (2002). Isomaltulose (Palatinose®): a review of biological and toxicologycal studies. Food and Chemical Toxicology, 40, 1375-1381.

Lemus-Moncada R., Vega-Gálvez A., Zura-Bravo L., Ah-Hen K. (2012). *Stevia* rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects, Food Chem. 132, 1121-1132.

Lewis D.W. and Ismail, A.I. (1995). Prevention of dental caries. Canadian Medical Association Journal, 152: 836-846.

Matsuyama, J., Sato, T., and Hoshino, E. (1997). Acid production from palatinose, palatinit, erythritol and maltitol by bacteria isolated from dental plaque on human deciduous teeth. Japanese Association for Oral Biology, 39, 91-99.

Muanda F., Soulimani R., Diop B., Dicko A. (2011). Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT-Food Sci. Technol. 44, 1865-1872.

Mukherjeea,A, Chakrabarti J. (1997) In vivo cytogenetic studies on mice exposed to acesulfame-K—A non-nutritive sweetener Volume 35, Issue 12,1177–1179.

Peinado I, Rosa E, Heredia, I Escriche A, Andrés A.(2013) Influence of processing on the volatile profile of strawberry spreads made with isomaltulose. Food Chemistry, 138, 621-629.

Pimentel, T. C., Madrona GS, Prudencio SH (2014) Probiotic clarified apple juice with oligofructose or sucralose as sugar substitutes: Sensory profile and acceptability, LWT - Food Science and Technology, http://dx.doi.org/10.1016/j.lwt.2014.08.001

Produlce. (2013). Report Spanish Confectionary Association. Madrid, Spain.

R.A.E. (2014) Real Academia Española. El Diccionario de la lengua española. http://lema.rae.es/drae/?val=golosina. Consultada en Septiembre 2014.

Reglamento (CE) Nº 1334/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre los aromas y determinados ingredientes alimentarios con propiedades aromatizantes utilizados en los alimentos.

Reglamento (CE) Nº 1333/2008, 16 de diciembre de 2008 sobre aditivos alimentarios.

Reglamento (CE) n.º 852/2004, del Parlamento Europeo y del Consejo, de 29 de abril de 2004, relativos a la higiene de los productos alimenticios.

Reglamento (CE) n.º 2073/2005 de la Comisión, de 15 de noviembre, relativo a los criterios microbiológicos aplicables a los productos alimenticios.

Real Decreto 348/2011, B.O.E. A-2011-5394 por el que se aprueba la norma de calidad para caramelos, chicles, confites y golosinas.

Renwick AG, Nordmann H (2007) First European conference on aspartame: Putting safety and benefits into perspective. Synopsis of presentations and conclusions: Food Chem Toxicol 45 (7): 1308-1313.

Romero, LG., Charro AL, Calle-Pascual AL. (2002). Índice glucémico y tratamiento nutricional de las personas con diabetes mellitus. Endocrinología y Nutrición, 49(7): 232-239.

Schiweck, H., Munir, M., Rapp, K., Schenider, B., and Bogel, M. (1990). New developments in the use of sucrose as an industrial bulk chemical. Zuckerindustrie, 115, 555–565.

Shukla, S., Mehta, A., Menta, P., and Bajpai, V. (2012). Antioxidant ability and phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology* 64, 807-811.

Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M (2007) Life-Span Exposure to Low Doses of Aspartame Beginning during Prenatal Life Increases Cancer Effects in Rats. Environ Health Perspect 115(9): 1293-1297.

Tadhani, M., Patel, V., and Subhash, R. (2007). In vitro antioxidant activities of Stevia rebaudiana leaves and callus. *Journal of Food Composition and Analysis*, 20, 323-329.

Van Loo J., Coussement P, De Leen Heer L, Hoebregs H and Smits G (1995). On the presence of inulin and oligofructose as natural ingredientrs in the Western diet. Critical Reviews in Food Science and Nutrition 35,525-552.

Volpini-Rapina L.F., Sokei F.R., Conti-Silva A.C.(2012) Sensory profile and preference mapping of orange cakes with addition of prebiotics inulin and oligofructose. LWT - Food Science and Technology,48,37-42.

Weihrauch MR, Diehl V (2004) Artificial sweeteners-do they bear a carcinogenic risk? Ann Oncol 15(10): 1460-1465.

Weidenhagen, R. and Lorenz, S. (1957). Palatinose (6-alpha-glucopyranosido-fructofuranose), ein neues bakterielles Umwandlungsprodukt der Saccharose. Zeitschrift fur die Zuckerindustrie, 7, 533–534.

WHO, (2010). (http://www.who.int/dietphysicalactivity/childhood/es/) Consultada en Julio 2014.

II OBJETIVOS Y PLAN DE TRABAJO

II. OBJETIVOS Y PLAN DE TRABAJO

II.1. Objetivo general

La presente tesis doctoral plantea dos objetivos generales:

II.1.1. Caracterizar la hoja de stevia en relación a su contenido de diferentes compuestos (steviol glucósidos, antioxidantes, fenoles, flavonoides y volátiles) y evaluar en que medida estos compuestos son afectados por distintos métodos de secado y procedimientos de obtención de extractos de sus hojas.

II.1.2. Desarrollar golosinas (gomas y espumas dulces) saludables, no cariogénicas y con bajo índice glicémico utilizando ingredientes con propiedades funcionales (stevia, isomaltulosa y oligofructosa) y evaluar el efecto que estos ingredientes tienen sobre las propiedades físico-químicas, mecánicas, ópticas, antioxidantes y sensoriales de dichas golosinas.

II.2. Objetivos específicos

II.2.1. Analizar el contenido en glucósidos de steviol, compuestos volátiles, flavonoides, y ácidos fenólicos, así como la capacidad antioxidante, de hoja de stevia previamente secada aplicando diferentes métodos: liofilización, secado en sombra y secado por aire caliente a 100 y a 180ºC.

II.2.2. Evaluar en qué medida los métodos de secado afectan al contenido en glucósidos de steviol, compuestos volátiles, flavonoides, y ácidos fenólicos, así como a la capacidad antioxidante, de hoja de stevia.

II.2.3. Analizar el contenido en glucósidos de steviol, flavonoides y fenoles totales, así como la capacidad antioxidante, de extractos de hoja de stevia obtenidos aplicando diferentes métodos: infusión convencional, microondas y ultrasonidos.

II.2.4 Evaluar en qué medida los métodos de obtención de extractos de hoja de stevia afectan a la cantidad de glucósidos de steviol, flavonoides y fenoles totales así como a la capacidad antioxidante presente en dichos extractos.

II.2.5. Medir parámetros físico-químicos (aw, Brix, humedad, pH), propiedades mecánicas (dureza, gomosidad, elasticidad y cohesión), propiedades ópticas

(coordenadas CIEL*a*b*, croma y tono) y sensoriales en golosinas formuladas con stevia, isomaltulosa y oligofructosa.

II.2.6. Evaluar la influencia de la sustitución de los azúcares convencionales (sacarosa y jarabe de glucosa) por edulcorantes saludables (stevia, isomaltulosa y oligofructosa) en los parámetros físico-químicos, propiedades mecánicas, ópticas y sensoriales de las golosinas.

II.2.7. Correlacionar las propiedades mecánicas de las golosinas medidas instrumentalmente con las evaluadas sensorialmente mediante un panel de catadores.

II.2.8. Evaluar durante el almacenamiento la evolución de la estabilidad microbiológica, capacidad antioxidante, propiedades físico-químicos, propiedades mecánicas, ópticas y sensorial de golosinas formuladas con edulcorantes saludables en comparación con las formulas con azúcares convencionales.

II.3. Plan de trabajo

II.3.1. Revisión bibliográfica y de la legislación vigente, nacional e internacional.

II.3.2. Puesta a punto métodos fisicoquímicos, textura y color.

II.3.3. Puesta a punto y validación de los métodos cromatográficos:

- Flavonoides y ácidos fenólicos
- Steviol glucósidos
- Compuestos volátiles

II.3.4. Aplicación de tratamientos de secado en hojas de stevia: liofilización, secado en sombra y secado por aire caliente a 100 y a 180ºC

II.3.5. Aplicación de los procesos de obtención de extractos de hoja de stevia: infusión convencional, microondas y ultrasonidos.

II.3.6. Puesta a punto del procedimiento para elaborar golosinas saludables con edulcorantes naturales (stevia, isomaltulosa y oligofructosa).

II.3.7. Análisis de parámetros físico-químicos, propiedades mecánicas, ópticas y sensoriales en golosinas recién formuladas con stevia isomaltulosa, oligofructosa así como en golosinas formulas de forma convencional con sacarosa y jarabe de glucosa.

II.3.8. Evaluación durante el almacenamiento de la estabilidad microbiológica, capacidad antioxidante, parámetros físico-químicos, propiedades mecánicas, ópticas y sensoriales en golosinas formuladas con stevia isomaltulosa, oligofructosa así como en golosinas formulas de forma convencional con sacarosa y jarabe de glucosa.

II.3.9.Tratamiento de los datos aplicando técnicas estadísticas.

III. RESULTADOS

III.1. INFLUENCE OF DRYING METHOD ON STEVIOL GLYCOSIDES AND ANTIOXIDANTS IN *STEVIA REBAUDIANA* LEAVES

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ABSTRACT

The application of different drying conditions (hot air drying at 100ºC and 180ºC, freeze drying and shade drying) on steviol glycosides (stevioside, dulcoside A, rebaudioside A and rebaudioside C) and antioxidants in *Stevia* leaves was evaluated. Stevioside, the major glycoside found in fresh leaves (81.2 mg/g), suffered an important reduction in all cases, although shade drying was the least aggressive treatment. Considering the antioxidant parameters (total phenols, flavonoids and total antioxidants), the most suitable drying method was hot air at 180ºC, since it substantially increased all of them (76.8 mg gallic acid, 45.1 mg catechin and 126 mg Trolox, all equivalent/g *Stevia*, respectively), with respect to those present in fresh leaves (44.4, 2.5 and 52.9 mg equivalent/g). Therefore, the ideal method for drying *Stevia* leaves depends on their final use (sweetener or antioxidant), although, hot air at 180ºC is the most recommendable if only one treatment has to be chosen.

Keywords: steviol glycosides, antioxidants, total phenols, total flavonoids, freeze drying, shade drying, hot air drying.

1.Introduction

The food industry is increasingly interested in replacing artificial sweeteners with other natural sugars in order to offer the consumer a wider range of choice, and to satisfy the requirements of a segment of the population that does not want to or cannot eat sucrose. *Stevia* leaves (*Stevia rebaudiana*) have been used as a sweetener in South America for centuries, and nowadays its consumption all over the world. In fact, it is 300 times sweeter than sucrose, with the additional advantages of having: zero calories, zero carbohydrates, and not causing spikes in blood sugar levels. The sweetness of this plant is due to the presence of diterpenes such as steviol glycosides: stevioside (4-13%), rebaudioside A (2-4%), rebaudioside C (1-2%), dulcoside A (0.4-0.7%), and other less abundant types such as steviolmonoside, rubusoside, steviolbioside, rebaudioside B and rebaudioside F (Lemus-Moncada, Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012). The acceptable daily intake (ADI) for these compounds is 4 mg per kg bodyweight per day (JECFA 2008). The European Food Safety Authority recognized the safety of *Stevia* leaf extracts for alimentary use in November 2011(EFSA 2011).

Recently there has been an upsurge of interest in the therapeutic potential of plants, as antioxidants in reducing free radical induced tissue injury (Shukla, Mehta, Menta, & Bajpai, 2012). *Stevia* leaves are increasingly consumed as infusions due to their antioxidant properties, which stem from their high levels of flavonoids and phenolic compounds. Muanda, Soulimani, Diop and Dicko (2011) identified 18 phenolic compounds which demonstrated the high antioxidant capacity of *Stevia* leaves. Periche, Koutsidis, and Escriche (2014) found high levels of total phenols and flavonoids in *Stevia* infusions. Carbonell-Capella, Barba, Esteve and Frígola (2013) incorporated extracts of *Stevia* as a natural source of antioxidants to obtain low-calorie fruit extracts with antioxidant and antimicrobial activity.

Like other kinds of herbal teas, *Stevia* leaves need to be dried for conservation and consumption purposes. Thanks to the drying process two goals are reached, on one hand the growth of microorganisms is prevented and on the other hand storage and transportation is facilitated (Lin, Sung, & Chen, 2011). Dehydration of plants can be carried out using different methods. Capecka, Mareczek and Leja (2005) demonstrated the efficacy of shade drying (the simplest and cheapest method) for leaves of the Lamiaceae species. Chan et al. (2009) used hot air to accelerate the process of drying leaves for ginger species, while Pinela, Barros, Carvalho and Ferreira (2011) did the same for Fabaceae species. A newer technique using freeze drying (Lin et al. 2011) has been proved to better preserve the quality of medicinal plants (Abascal, Ganora, & Yarnell, 2005) although the cost is considerably higher than hot air drying.

It is important to highlight that the different drying techniques can influence the composition of some characteristic compounds present in different herbal teas. In this respect, Lin et al. (2011) obtained better results for the antioxidant capacity and total phenol values when the leaves of *Echinacea purpurea* were freeze dried, than when they were dehydrated with hot air. Pinela et al. (2011) also obtained larger amounts of antioxidants when leaves of the *Genista* sp. were freeze dried, in comparison with shade drying. On the contrary, Hossain, Barry-Ryan, Martin-Diana and Brunton (2010) obtained less antioxidants from leaves of the Lamiaceae family applying freeze drying than hot air drying.

Clearly, there is a great discrepancy about the extraction of active compounds from herbal teas according to the different drying techniques applied (Lewicki, 2006).

Moreover, as far as the authors know, there is no research related to the influence of different drying methods on the antioxidants and steviol glycosides of *Stevia* leaves. For this reason, the aim of this study was to evaluate how the drying method (shade drying, hot air drying and freeze drying) affects steviol glycosides and antioxidants (total phenols, flavonoids and antioxidant capacity) in *Stevia* leaves.

2.Material and Methods

2.1.Stevia samples and drying conditions

Organically produced *Stevia rebaudiana* leaves from Valencia (Spain) were used in this study. Four different drying conditions were used: shade drying at 20ºC for 30 days, hot air drying at 100ºC and 180ºC for 3 minutes in a convective drier, and freeze drying at a vacuum pressure of $9.5x10^{-1}$ mm Hg for 24 hours.

2.2.Steviol glycosides analysis

2.2.1.Steviol glycoside extraction procedure

The *Stevia* leaves (fresh or dried leaves) were ground in a grinding mill (A11 Basic, IKA, Germany), and 100 mg of *Stevia* leaves were shaken in 10 mL of ethanol/water (6:4 v/v) for 5 minutes. The mixture was sonicated for 10 minutes and then centrifuged at 5000 x g for 5 minutes. An aliquot of 0.5 mL of the alcoholic extract was diluted with water (2.5 mL). This solution was loaded on a 3 mL Strata SPE cartridge (500 mg, 55 μm, 70 Å, StrataC18-E Phenomenex, Torrance, CA) pre-activated with methanol (3 mL) and washed with water (3 mL). Then, the SPE cartridge was washed with 3 mL of water, followed by 3 mL of acetonitrile in water (2:8 v/v); and then air dried for 2 minutes. Finally, the steviol glycosides were eluted from the cartridge with 5 mL of 80% acetonitrile in water (Woelwer-Rieck, Lankes, Wawrzun, & Wüst 2010). The eluate was subjected to LC-MS-MS analysis.

2.2.2.Methodology

A LC-MS-MS method (HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer, Agilent Technologies Inc., CA, USA) was used in this study for the analysis of the steviol glycosides. Chromatographic separation was carried out in gradient mode by Zorbax SB-C18 column (50mm x 2.1mm, 1.8 μm). The temperature was maintained at 40ºC, with a mobile phase of 10 mM aqueous

ammonium acetate (A) and acetonitrile (B). Binary gradient conditions were used: starting with, 7% B, held for 0.2 min: linear gradient to 20% B at 0.3 min and then to 48% B at 5 min; increased to 100% B at 5.1 min and held until 7 min; followed by a linear gradient to initial conditions at 7.1 min and a final hold at this composition until 9 min. The flow-rate and injection volume were 0.4 mL/min. and 5 μL, respectively. The electrospray was in negative ion mode. Choi et al. (2002) stated that negative ion mode is 10 times more sensitive than positive ion mode. The ionization source conditions were: temperature of the drying gas (N_2) 325ºC to 11L/min, nebulizer pressure of 50 psi and capillary voltage of 4000 V. Identification and quantification of steviol glycosides in the samples and the standards were performed using the multiple reaction monitoring mode (MRM).

The stock standard solutions of steviol glycosides (stevioside, steviolbioside, rebaudioside A, rebaudioside C, dulcoside A standards (purity > 98%), Chromadex (CA, USA) were prepared by weighing the appropriate amount of the pure standard and diluting it with methanol to obtain a final concentration of 1 mg/mL. The working standard solution had a concentration of 0.01 mg/mL in water. The stock standard solution was stored at 20ºC and the working standard solution at 4ºC. Quantification was carried out by means of calibration curves obtained from standard solutions (0.5-10 μg/mL). Samples were spiked in order to verify the absence of a matrix effect in the analysis. To ensure the quality of the results and evaluate the stability of the proposed method, an internal quality control (a standard solution) was injected as a first step before each batch of the sample.

2.3.Validation of the steviol glycosides analysis method

The validation of the steviol glycosides analytical methodology was carried out according to the guidelines established by EU Commission Decision (2002). To this end, the parameters: linearity, accuracy and precision (repeatability and reproducibility) were studied. The accuracy of the method was established through recovery studies and the precision was verified by intraday precision or repeatability (RSD_r) and interday precision or reproducibility (RSD_R). Limit of detection (LOD) and limit of quantification (LOQ) were defined as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10 respectively.

2.4.Determination of total phenolic content

Total phenolic determination was realized with spectrophotometry (JASCO V-630) using the modified Folin-Ciocalteu method (Sakanaka, Tachibana, & Okada, 2004). Distilled water (0.5 mL), 0.125 mL of the infusion sample and 0.125 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) were mixed and shaking. After six minutes, 1.25 mL of a 7% sodium carbonate solution and 1 mL of distilled water were added. After 90 min, the absorbance was measured at 760 nm. A blank was considered in this analysis. The quantification was carried out considering a standard curve of gallic acid, expressing the results as mg of gallic acid equivalent per gram of dry matter. The fresh weight of the all fresh samples was converted into dry weight, on the basis of their respective moisture contents and then the dry weight was used for calculation.

2.5.Determination of total flavonoid content

Total flavonoid content was analyzed with colorimetry as described by Dewanto, Wu, Adom and Liu (2002). The infusion sample (0.25 mL), distilled water (1 mL) and sodium nitrite solution at 5% (0.075 mL) were mixed in a cuvette. After 6 min, a 10% aluminum chloride solution (0.15 mL) and 1M sodium hydroxide solution (0.5 mL) was mixed and left to settle for 5 min. Finally, distilled water (2 mL) was added and the absorbance was measured at 510 nm straightaway. A blank was considered in this analysis. The quantification was carried out considering a standard curve of (+)-catechin (Sigma-Aldrich, Germany) and the results were expressed as mg of (+)-catechin equivalent per gram of dry matter, as was explained above.

2.6.Determination of total antioxidant capacity

The antioxidant activity (AA) was measured based on of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Germany) free radical as described by Shahidi, Liyana-Pathirana and Wall (2006), with some modifications. Accordingly, 0.1 mL of the infusion sample (diluted in methanol:water (80:20)) was mixed with 3.9 mL of a methanolic solution of DPPH (0.025mg/mL, prepared in methanol:water (80:20)). The solution was shaken, after 30 min the absorbance of the samples were measured at 515 nm using methanol as a blank. The quantification was calculated with a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-

carboxylic acid). The results were expressed as mg of Trolox equivalent per gram of dry matter, as explained previously.

2.7.Statistical analysis

An ANOVA (Statgraphics Centurion) was used to study the influence of the treatments on the steviol glycosides, antioxidants, phenols and flavonoids. In this analysis, the homogenous groups indicate statistical differences between types of treatment (α=99%). A Principal Component Analysis (PCA) was also performed using the software Unscrambler X.10 to describe the relationships between the treatments and the variables analysed.

3.Results and Discussion

3.1.Validation of the steviol glycosides analytical methodology.

An external standard calibration curve was made using standard solutions with final concentration levels of: 0.5, 1, 2, 5, 7 and 10 μg/mL, with the aim of obtaining the linearity value. For each level, six replicates were made. The linearity response from 0.5 to 10 μg/mL was $R^2 \geq 0.995$.

The recovery studies were carried out by adding known quantities of steviol glycosides to a sample (1, 5 and 10 μg/g). Six replicates of all the spiked sample levels were analyzed. The method used permitted recovery of steviol glycosides between 70.5% (for steviolbioside at 10 μg/g level) and 105.6 % (for rebaudioside A at 5 μg/g level) for the concentration range studied. The standard deviation corresponding to recovery values was less than 20% in all cases (ranging from 4.0 to 18), proving that the analytical method was accurate.

Repeatability or Intra-day precision (RSD_r) (carried out by the same operator on the same day) was evaluated by performing the assay (on six replicates of fortified *Stevia* samples) at three levels: 1, 5 and 10 μg/g. These values ranged from 1.7% for dulcoside A to 14.6% for steviolvioside. Reproducibility or inter-day precision (RSD_R) (carried out by 2 different operators on 3 consecutive days) ranged from 5.2% for dulcoside A to 16.5% for steviolbioside. These RSD values are in total agreement with EU Commission Decision (2002) requirements, since they were always lower than 20% for all the concentration levels assayed.

The limits of detection (LOD) were: 0.05 µg/g (dulcoside A), 0.11 µg/g (rebaudioside A), 0.09 µg/g (rebaudioside C), 0.04 µg/g (stevioside) and 0.14 µg/g (steviolbioside); and the limits of quantification (LOQ) were: 0.15 µg/g (dulcoside A), 0.32 µg/g (rebaudioside A), 0.31 µg/g (rebaudioside C), 0.15 µg/g (stevioside) and 0.49 µg/g (steviolbioside). From the results of these validation parameters, it can be concluded that the methodology applied in this work is appropriate to guarantee the quantitative values of steviol glycosides obtained in the *Stevia* leaves analyzed.

3.2.Influence of drying method on the steviol glycosides.

Figure 1 shows the average values and the standard deviation of the 4 steviol glycosides (dulcoside A, rebaudioside A, rebaudioside C and stevioside) identified and quantified in fresh, and dried *Stevia* leaves obtained applying different drying conditions (hot air drying at 100ºC and 180ºC, freeze drying and shade drying). All values are expressed in mg of compounds per gram of dry matter. Additionally, this figure shows the homogenous groups of the ANOVA carried out for the factor "drying method" for every compound. The F-ratio values were: 49.84, 5.31, 7.22 and 87.52 for dulcoside A, rebaudioside A, rebaudioside C and stevioside, respectively. These values reflect the greater influence of the drying method on dulcoside A and stevioside than the other two compounds.

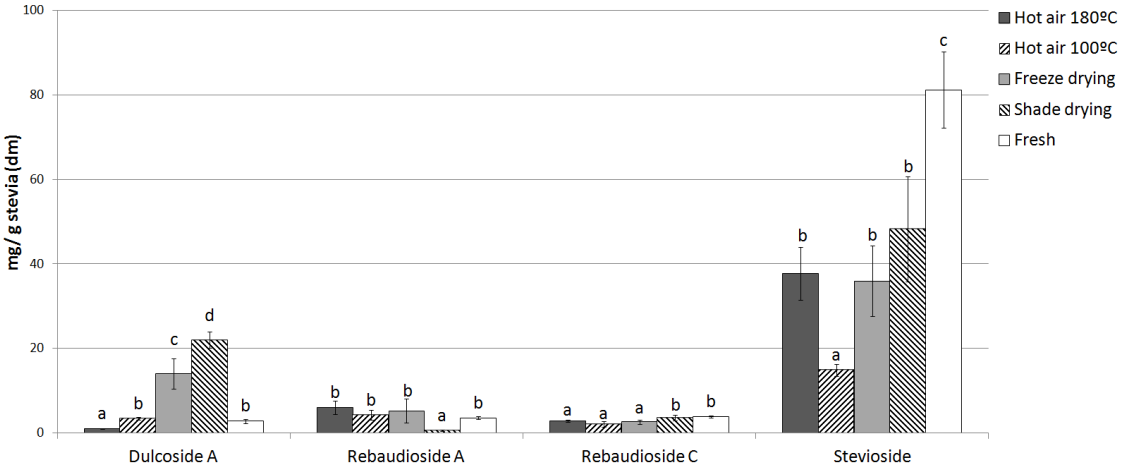

Figure III.1.1. Average values and the standard deviation of the 4 steviol glycosides (dulcoside A, rebaudioside A, rebaudioside C and stevioside) in fresh and dried *Stevia* leaves obtained applying different drying conditions (hot air drying at 100ºC and 180ºC, freeze drying and shade drying). The ANOVA homogenous groups are indicated by letters.

In contrast to other studies (Cacciola, Delmonte, Jaworska, Dugo, Mondello & Rader, 2011), steviolbioside was not found in any sample in this work. In fact, this is logical since this compound, like rebaudioside B, is not a native constituent of *Stevia rebaudiana*, however, in some cases they may appear as artifacts during the extraction process (Kennelly 2002; Prakash, Dubois, Clos, Wilkens & Fosdick, 2008).

By far the most abundant steviol glycoside in fresh leaves was stevioside (81.2± 9.3 mg/g), followed by rebaudioside C (3.8± 0.3 mg/g), dulcoside A (2.8 ± 0.5 mg/g) and rebaudioside A (3.5± 0.3 mg/g) (Fig. 1).

With respect to the results obtained when the leaves were dehydrated, it can be observed that rebaudioside A and rebaudioside C showed very low concentration values in all the conditions applied, ranging from 0.5 ± 0.14 mg/g (in shade drying) to 6.1 ± 1.6 mg/g (in hot air to 180ºC drying), and from 2.1 ± 0.6 mg/g (hot air to 100ºC drying) to 3.6 ± 0.7 mg/g (in shade drying), respectively. For these compounds, as Figure 1 shows, there were practically no differences between fresh and dehydrated leaves, even though the ANOVA analyses found different homogeneous groups. However, different behavior was observed in the case of stevioside and dulcoside A, for dehydrated samples. For both compounds, the highest values in the treated samples were obtained for shade drying. In the case of stevioside an important decrease occurred as a consequence of all the drying treatments applied, in comparison to the levels obtained in the fresh samples. For this compound there were no significant differences between shade drying (48±12 mg/g), hot air drying at 180ºC (37±6 mg/g) and freeze drying (35±8 mg/g). There is no information in the literature relating the behavior of steviosides and the air drying temperature. However, some authors reported that an increase in extraction temperature in combination with solvents results a higher yield of this compound. Specifically, Pól et al (2007) found that a temperature of 160ºC resulted in a 20% increase compared to 110ºC. Meanwhile, the behavior of dulcoside A was very different to the other three compounds showing a significant increase in yield as a consequence of the shade drying and the freeze drying treatments in comparison to the fresh sample, reaching 22.3±1.9 mg/g and 14.1±3.5 mg/g, respectively. The increase in the concentration as a consequence of using freeze drying and shade drying is not surprising as this is seen with other compounds such as phenols and flavonoids. This was observed in this study (section 3.2) and also by other authors (Chan et al. 2009;

Hossain et al. 2010; Hamrouni-Sellami, Rahali, Rebey, Bourgou, Limam, & Marzouk, 2013).

The research data reported by other authors about the concentration of the different steviol glycosides in dried *Stevia* leaves vary greatly, and in some occasions do not provide information about the drying method applied. One of the most recent works is by Woelver-Rieck et al. (2010) who obtained 79±2.9 mg/g and 77.8±6.1 mg/g of stevioside and 49.3±4.4 mg/g and 42.8±2.9 mg/g of rebaudioside A, in *Stevia* dried leaves grown in two different types of soil, fertile sandy loam and light loamy soil, respectively. The values for stevioside are similar to those obtained in this work, however for rebaudioside A they are much higher.

Moreover, Shafii, Vismeh, Beaudry, Warner and Jones (2012) found from 2 to 125 mg/g of stevioside, from 2.5 to 164 mg/g of rebaudioside A and from 1.5 to 125 mg/g of rebaudioside C in 1,100 *Stevia* leaf extracts. Gardana, Scaglianti and Simonetti (2010) reported 5.8 g of stevioside, 1.8 g of rebaudioside A, 1.3 g of rebaudioside C and 0.7 g of dulcoside A in 100g of *Stevia*.

3.3.Influence of drying method on the antioxidants.

The average values and the standard deviation of total phenols (mg gallic acid equivalent/g *Stevia*), flavonoids (mg of catechin equivalent/g *Stevia*) and total antioxidants (mg Trolox equivalent/g *Stevia*) quantified in fresh, and dried *Stevia* leaves obtained applying the different drying methods, are shown in Fig. 2. The ANOVA homogenous groups are indicated by letters in this figure.

In fresh leaves the amount of phenols, flavonoids and antioxidants were: 44.40±1.04 mg gallic acid equivalent/g *Stevia*, 2.52±0.24 mg catechin equivalent/g *Stevia* and 52.92±0.84 mg Trolox equivalent/g *Stevia*, respectively. It is noteworthy that drying treatments caused an increase in the content of flavonoids and antioxidants when compared with fresh leaves.

In contrast to the steviol glycosides, phenols, flavonoids and antioxidants exhibited similar behaviour as a consequence of the application of the different drying conditions. The highest values for the three parameters (total phenols, flavonoids and antioxidants) were found for hot air drying at 180ºC (76.8, 45.1 and 126 mg equivalent/g), followed by shade drying (39.1, 20.3, 75.9 mg equivalent/g), hot air

drying at 100ºC (31.5, 17.2, 64.9 mg equivalent/g), and finally freeze drying (26.2, 9.9, 48.5 mg equivalent/g), respectively. This last treatment showed the lowest values, thus being the least suitable treatment for the extraction of antioxidants.

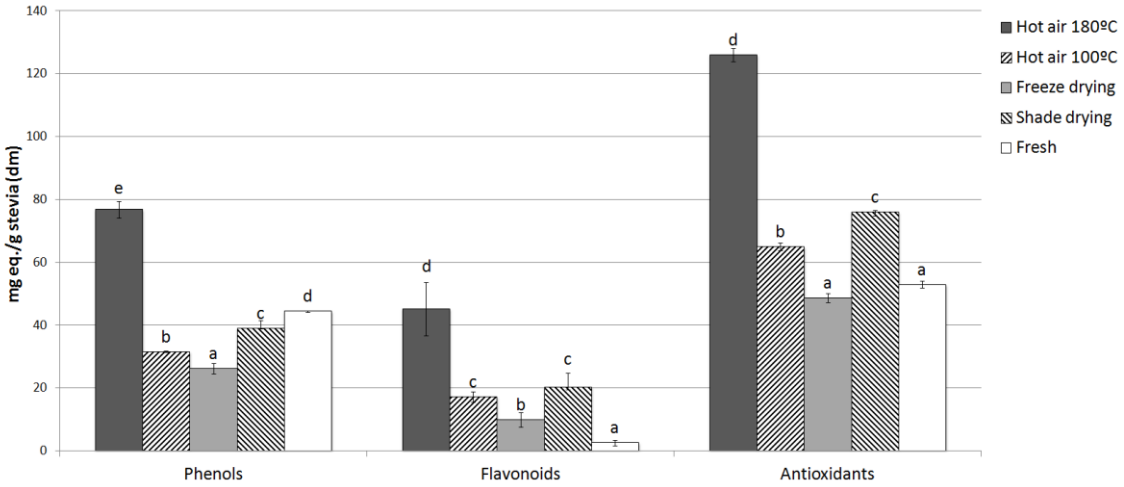

Figure III.1.2. Average values and the standard deviation of total phenols (mg gallic acid equivalent/g *Stevia*), flavonoids (mg of catechin equivalent/g *Stevia*) and total antioxidants (mg Trolox equivalent/g *Stevia*) in fresh and dried *Stevia* leaves obtained applying the different drying methods (hot air drying at 100ºC and 180ºC, freeze drying and shade drying). The ANOVA homogenous groups are indicated by letters.

The high content of flavonoids is due to the presence of flavonols and flavones in Stevia leaves. Ghanta, Banerjee, Poddar and Chattopadhyay (2007) isolated 6 flavonoids (quercetin-3-O-β-D-arabinoside, quercetin-3-O-β-D-rhamnoside, kaempherol-3-O-rhamnoside, apigenin, apigenin-4-O-β-D-glycoside, luteolin) and Cacciola et al. (2011) 4 different ones (quercetin-3-O-glucoside, quercetin-3-O-rutinoside, apigenin-7-O-β-D-glycoside, luteolin-7-O-β-D-glycoside). In this work, the flavonoid content was higher for all drying methods applied in comparison to fresh leaves. This result could be related to an increase in the extractability of such compounds as a consequence of the matrix changes during the drying process. As observed in the present work, Hamrouni-Sellami et al. (2013) also obtained higher values of total flavonoids in dried leaves of *S. Officinalis* than in fresh plants. However, in contrast to the present study, Ferreira and Luthria (2010), obtained lower levels of antioxidant capacity (in dried *Artemisia annua* L. leaves) for shade drying than hot air drying. In the case of phenols, in this study, hot air drying at 180ºC and fresh leaves showed the highest values, respectively. Capecka et al. (2005) also obtained lower levels of phenols for shade dried leaves (in Lemon balm leaves) than the fresh ones.

There are some works in the literature regarding the levels of total phenol, flavonoids and antioxidant activity in dried *Stevia* leaves, however, very few studies specify the drying method. For instance, in the case of phenols: 25.18 mg gallic acid/g (Tadhani, Patel, & Subhash, 2007); 56.74 mg gallic acid/g, obtained with air drying (Shukla et al. 2012); 0.86 mg gallic/mg with shade drying (Ghanta et al. 2007) and 130.67 mg catechin/g, air drying at 40ºC for 12h (Kim, Yang, Lee & Kang, 2011). In the case of total flavonoids: 21.73 mg gallic acid/g (Tadhani et al. 2007); 0.83 mg quercetin/mg (Ghanta et al. 2007); 15.64 mg quercetin/g (Kim et al. 2011) and 20.68 mg catechin/g drying room temperature (Muanda et al. 2011), and finally, for antioxidant activity: 38.24 mg trolox/g (Tadhani et al. 2007) and 8.72mg gallic acid/g (Abou-Arab, Abou-Arab, & Abu-Salem, 2010).

3.4.Global behavior of antioxidants and steviol glycosides.

A PCA was applied in order to appreciate the overall effect that the drying method had on steviol glycosides and antioxidants together. The corresponding bi-plot obtained (scores "treatments" and loading "variables") is shown in Fig. 3 (PC1 explained 46 % of the total variance and PC2, 25 %).

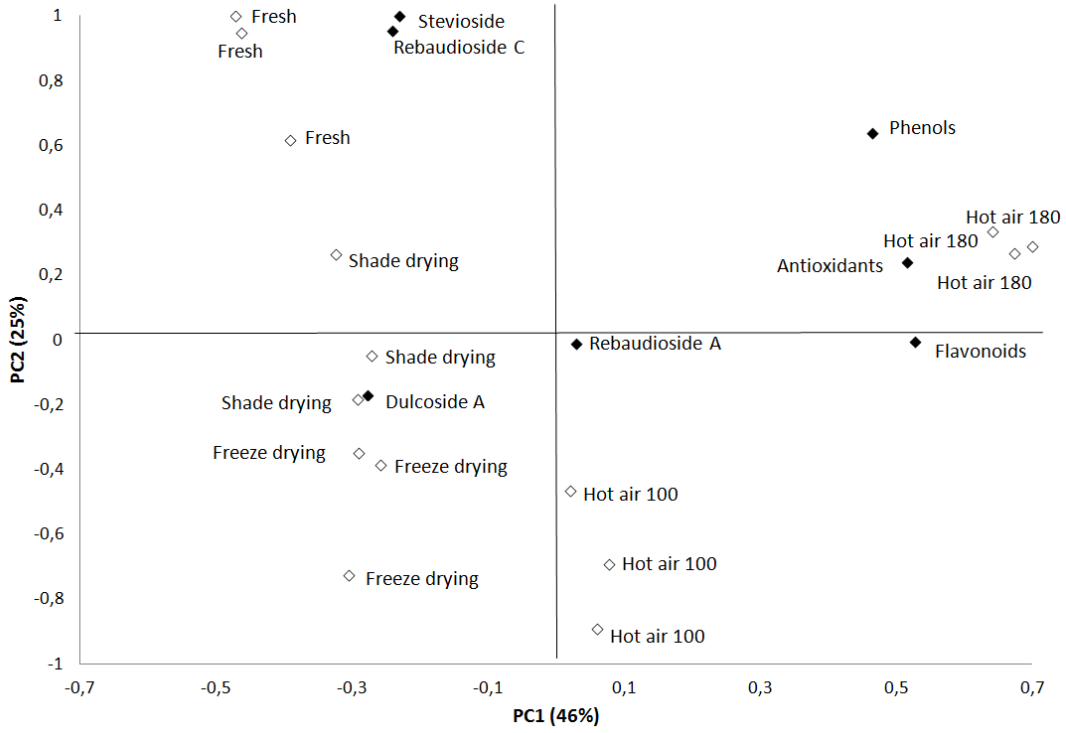

Figure III.1.3. Bi-plot of Principal Components Analysis for the drying treatments (white diamond ◊) and the analysed variables: steviol glycosides and antioxidant parameters (total phenols, flavonoids and antioxidant activity) (black diamond ♦).

The proximity between variables indicates the correlation between them, and in the case of drying treatments similar behavior. This figure shows more clearly that the two groups of variables (antioxidants and glycosides of steviol) show in general opposing behavior with respect to the effect of the drying treatments applied. That is to say, the hot air drying treatment at 180ºC is placed at the far end of the right axis in the figure, which corresponds to the highest values of the three antioxidant parameters (total phenols, flavonoids and total antioxidants) and the lowest of the steviol glycosides. On the contrary, fresh and shade drying are placed on the opposite side (left axis), which corresponds to the highest content of steviol glycosides (especially dulcoside A, rebaudioside C and stevioside) and the lowest level of all the antioxidant parameters. As it can been observed, not a single drying treatment permits the maximum extraction of all the compounds together.

4.Conclusions

The drying conditions applied in fresh *Stevia* leaves have a great impact on the extraction of steviol glycosides and antioxidants. In general, the yield of these compounds was affected in different ways according to the drying conditions (hot air drying at 100ºC and 180ºC, freeze drying and shade drying). The drying conditions produced an important increase in antioxidant capacity but an important decrease in the principal steviol glycoside (stevioside) which diminished with all treatments, especially with hot air at 100ºC. For this compound, there were no significant differences between the other treatments, although shade drying produced the highest values of this compound. Dulcoside A increased only with the shade and freeze drying treatments. On the other hand, the levels of the less abundant glycosides (rebaudioside A and rebaudioside C) changed very little when comparing fresh and dehydrated leaves. Considering all the steviol glycosides, the least aggressive treatment was shade drying.

With respect to the antioxidant parameters (total phenols, flavonoids and total antioxidants), the most suitable drying method was hot air at 180ºC, since it was able to substantially increase the level of all of them compared to the fresh *Stevia* leaves. Therefore, the optimum drying conditions for fresh *Stevia* leaves is determined by whether they are used for sweetening or for their antioxidant properties. Although, if one treatment had to be chosen, hot air drying at 180ºC is the most recommendable overall. As drying methods are known to be highly effective in the extraction of

antioxidants, the profile of specific antioxidant compounds should be studied in greater depth.

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References

Abascal, K., Ganora, L., & Yarnell, E. (2005). The effect of freeze drying and its implications for botanical medicines: a review. *Phytotherapy Research,* 19,655-660.

Abou-Arab, A.E., Abou-Arab, A.A., & Abu-Salem, M.F. (2010). Physicochemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana Bertoni plant. *African Journal of Food Science,* 4, 269-281. ISSN:1996-0794.

Cacciola, F., Delmonte, P., Jaworska, K., Dugo, P., Mondello, L., & Rader, J. (2011). Employing ultra-high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of Stevia rebaudiana extracts. *Journal of Chromatography A*, 1218, 2012-2018.

Capecka, E., Mareczek, A., & Leja, M. (2005). Antioxidant activity of fresh and dry herbs of some Lamiaceae species. *Food Chemistry,* 93, 223-226.

Carbonell-Capella, J.M., Barba, F.J., Esteve, M.J., & Frígola, A. (2013) High pressure processing of fruit juice mixture sweetened with Stevia rebaudiana Bertoni: Optimal retention of physical and nutritional quality. *Innovative Food Science and Emerging Technologies*, 18, 48-56.

Chan, E.W.C., Lim, Y.Y., Wong, K.K., Lim, K.K., Tan, S.P., Lianto, F.S., & Yong, M.Y. (2009). Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species. *Food Chemistry,* 113, 166-172.

Choi, Y.H., Kim, I., Yoon, K., Lee, S.J., Kim, C.Y., Yoo, K.P., Choi, Y.H., & Kim, J. (2002). Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from Stevia rebaudiana leaves. *Chromatographia,* 55, 617-620.

Commission Decision 2002/657/EC of 12 August (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJEC L221, 8-36. Brusels, Belgium.

Dewanto, V., Wu, X., Adom, K.K., & Liu, R.H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, 50, 3010-3014.

EFSA. European Food Safety Authority (2011). Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. *EFSA Journal* 9,1,1972. doi:10.2903/j.efsa.2011.1972

Ferreira, J.F. & Luthria, D.L. (2010). Drying Affects Artemisinin, Dihydroartemisinic Acid, Artemisinic Acid, and the Antioxidant Capacity of Artemisia annua L. Leaves. *Journal of Agricultural and Food Chemistry*, 58, 1691-1698.

Gardana, C., Scaglianti, M., & Simonetti, P. (2010). Evaluation of steviol and its glycosides in Stevia rebaudiana leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 1217, 1463-1470.

Ghanta, S., Banerjee, A., Poddar, A., & Chattopadhyay, S. (2007). Oxidative DNA damage preventive activity and antioxidant potential of Stevia rebaudiana (Bertoni) Bertoni, a natural sweetener. *Journal of Agricultural and Food Chemistry*, 55, 10962-10967.

Hamrouni-Sellami, I., Rahali, F.Z., Rebey, I.B., Bourgou, S., Limam, F., & Marzouk, B. (2013). Total Phenolics, Flavonoids, and Antioxidant Activity of Sage (Salvia officinalis L.) Plants as Affected by Different Drying Methods. *Food and Bioprocess Technology*, 6, 806-817.

Hossain, M.B., Barry-Ryan, C., Martin-Diana, A.B., & Brunton, N.P. (2010) Effect of drying method on the antioxidant capacity of six Lamiaceae herbs. *Food Chemistry,* 123, 85-91.

JECFA. Joint FAO/WHO Expert Committee on Food Additives (2008) Steviol glycosides. In Compendium of Food Additive Specifications, 69th Meeting, FAO/WHO Monographs 5, Rome, Italy.

Kennelly, E.J., 2002. Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni) Bertoni. In Kinghorn, A.D. (Ed.), Stevia, the genus Stevia. Medicinal and Aromatic Plants – Industrial Profiles, vol. 19 (pp. 68-85). London, UK: Taylor and Francis.

Kim, I., Yang, M., Lee, O., & Kang, S. (2011). The antioxidant activity and the bioactive compound content of Stevia rebaudiana water extracts. *LWT-Food Science and Technology*, 44, 1328-1332.

Lemus-Moncada, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia* rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, 132, 1121-1132.

Lewicki, P.P. (2006). Design of hot air drying for better foods. *Trends in Food Science and Technology* 17, 153-163.

Lin, S.D., Sung, J.M., & Chen, C.L. (2011). Effect of drying and storage conditions on caffeic acid derivatives and total phenolics of Echinacea Purpurea grown in Taiwan. *Food Chemistry*, 125, 226-231.

Muanda, F., Soulimani, R., Diop, B., & Dicko, A. (2011). Study on chemical composition and biological activities of essential oil and extracts from Stevia rebaudiana Bertoni leaves. *LWT-Food Science and Technology* 44, 1865-1872.

Periche, A., Koutsidis, G., & Escriche, I. (2014). Composition of antioxidants and amino acids in Stevia leaf infusions. *Plant Foods for Human Nutrition*, 69, 1-7.

Pinela, J., Barros, L., Carvalho, A.M., & Ferreira, I. (2011). Influence of the drying method in the antioxidant potential and chemical composition of four shrubby flowering plants from the tribe Genisteae (Fabaceae). *Food and Chemical Toxicology*, 49, 2983-2989.

Pól, J., Varadová-Ostrá, E., Karáse, P., Roth, M., Benesová, K., Kotlariková, P., & Cáslavsky, J. (2007). Comparison of two different solvents employed for pressurized fluid extraction of stevioside from *Stevia rebaudiana*: methanol versus water. *Analytical and Bioanalytical Chemistry*, 388, 1847-1857.

Prakash, I., Dubois, G.E., Clos, J.F., Wilkens, K.L., & Fosdick, L.E. (2008). Development of rebaudiana, a natural, non-caloric sweetener. *Food and Chemical Toxicology* 46, 75-82.

Sakanaka, S., Tachibana, Y., & Okada, Y. (2004). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha- cha). *Food Chemistry*, 89, 569-575.

Shafii, B., Vismeh, R., Beaudry, R., Warner, R., & Jones, A.D. (2012). Large-scale profiling of diterpenoid glycosides from Stevia rebaudiana using ultrahigh performance liquid chromatography/tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 403, 2683-2690.

Shahidi, F., Liyana-Pathirana, C.M., & Wall, D.S. (2006), Antioxidant activity of white and black sesame seeds and their hull fractions. *Food Chemistry*, 99, 478-483.

Shukla, S., Mehta, A., Menta, P., & Bajpai, V. (2012). Antioxidant ability and phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology* 64, 807-811.

Tadhani, M., Patel, V., & Subhash, R. (2007). In vitro antioxidant activities of Stevia rebaudiana leaves and callus. *Journal of Food Composition and Analysis*, 20, 323-329.

Woelwer-Rieck, U., Lankes, C., Wawrzun, A., & Wüst, M. (2010). Improved HPLC method for the evaluation of the major steviol glycosides in leaves of Stevia rebaudiana. *European Food Research and Technology*, 231, 581-588.

III.2. EFFECT OF DIFFERENT DRYING METHODS ON THE PHENOLIC, FLAVONOID AND VOLATILE COMPOUNDS OF *STEVIA REBAUDIANA* LEAVES

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Food Chemistry (En revisión)

ABSTRACT

Different drying methods (hot air drying, freeze drying and shade drying) were evaluated to discern the optimal conditions for the preservation of flavonoid, phenolic and volatile compounds in stevia leaves. All the methods applied affected the antioxidant and volatile compounds in dried stevia leaves differently. 2-hexenal, hexanal and α-pinene were the most abundant volatile compounds produced by shade drying and freeze drying; and hexane, furan tetrahydro and α-pinene by air drying. While chlorogenic acid, coumaric acid and sinapic acid were the most abundant phenolic compounds produced by all the drying treatments. The content of volatile compounds was higher with shade drying, whereas most flavonoids and phenolic acids had higher concentrations following freeze drying, although some flavonoids and phenolic acids exhibited a higher increment with air drying. There is no one best drying treatment, however, freeze drying results in an extract with satisfactory antioxidant properties and good aromatic characteristics.

Keywords: volatile compounds, freeze-drying, shade-drying, polyphenols, flavonoids, HPLC-DAD, GC-MS.

1.Introduction

Stevia rebaudiana is a perennial herb, native to Paraguay, which has economic value due to its high content in sweeteners (Kinghorn, 2002). In fact, its dried leaves have been used as a sweetener in South America for centuries, and nowadays extracts of steviol glycosides are consumed all over the world (Wölwer-Rieck, 2012). These extracts are 300 times sweeter than sucrose, with the advantage of having: zero calories, zero carbohydrates, and not causing spikes in blood sugar levels (Lemus-Moncada, et al., 2012). The European Food Safety Authority (EFSA 2011) recognized the safety of *Stevia* leaf extracts for alimentary use. *Stevia* leaves are more and more consumed as infusions due to their antioxidant properties, which stem from their high content in flavonoid and phenolic compounds (Periche et al., 2014; Carbonell-Capella, et al. 2013; Shukla et al., 2012; Muanda et al., 2011). In addition, their leaves have important therapeutic properties, are rich in compounds with anti-inflammatory, diuretic, anti-hypertensive, antihyperglycemic, antidiarrehal, antitumor and immunomodulatory effects (Chatsudthipong and Muanprasat 2009).

Stevia leaves, like other herbal teas or medicinal plants, need to be dried for conservation and consumption purposes. The drying process has two principal effects: preventing the growth of microorganisms and facilitating storage and transportation (Lin, et al., 2011). At the same time, drying herbs can give rise to other alterations which affect herb quality, such as changes in appearance and alterations in aroma caused by losses in volatiles or the formation of new volatiles as a result of oxidation reactions or esterification reactions (Hossain et al. 2010). Different methods can be applied to dehydrate plants. The simpler, cheaper ones include letting the leaves dry in the shade (Capecka et al., 2005) or using hot air to accelerate the process (Chan et al. 2009; Pinela et al., 2011). An innovative technique using freeze drying (Lin et al. 2011) has been proven to better preserve the quality of medicinal plants (Abascal et al. 2005). It should be noted that different drying techniques influence the characteristic of the different compounds present in herbal teas. There is a great discrepancy about the extraction of active compounds from herbal teas according to the different drying techniques applied (Lewicki, 2006). Different studies have reported changes in the antioxidant capacity of some herbal teas according to the drying method used (Lin et al., 2011; Pinela et al., 2011; Hossain et al., 2010). In this line, Di Cesare et al, 2003; Diaz-Maroto et al., 2002, observed changes in colour and volatile compounds of the aromatic herbs as a consequence of drying.

As far as the authors know, there is no research related to the influence of different drying methods on phenolic and volatile compounds of *Stevia* leaves. For this reason, the aim of this study was to evaluate how the drying method (shade drying, hot air drying and freeze drying) affects phenolic and volatile compounds in *Stevia* leaves, in order to optimize the drying method which maximizes the presence of these compounds.

2. Material and Methods

2.1.Stevia samples and drying conditions

Organically produced *Stevia rebaudiana* leaves from Valencia (Spain) were used in this study. Three different drying conditions were used: shade drying at 20ºC for 30 days, hot air drying at 180ºC for 3 minutes in a convective drier, and freeze drying at a vacuum pressure of $9.5x10^{-1}$ mm Hg for 24 hours.

2.2 Standard compounds and reagents

HPLC-grade acetonitrile and methanol were purchased from VWR (Fontenay-sous-Bois, France), and analytical grade ethanol and ammonium acetate were purchased from Scharlab (Barcelona, Spain). The standards of apigenin, caffeic acid, catequin, chlorogenic acid, cinnamic acid, coumaric acid, 4-methoxybenzoic, 4-methylcatechol, quercetin, rutin and sinapic acid (purity > 98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). De-ionized water from MilliQ (Millipore Corp., Bedford, MA) was used throughout the procedure.

2.3. Volatile compounds analysis

2.3.1. Extraction

Volatile compounds were extracted with purge and trap thermal desorption. 100 μL of the internal standard 2-pentanol (10 μg/mL) and 200 mg of samples were placed in a purging vessel flask in a water bath at 45°C for 20 min. Purified nitrogen (100 mL min^{-1}) was forced through a porous frit placed at the bottom of the vessel. The volatile compounds were collected by the stream of bubbles which passed through the sample and were then trapped in a 100 mg porous polymer (Tenax TA, 20–35 mesh) packed into a glass tube. A direct thermal desorber (TurboMatrix TD, Perkin ElmerTM, CT-USA) was used to thermally desorbed the volatile compounds. Desorption was carried out using a 10 mL min^{-1} helium flow at 220 °C for 10 min. The volatiles were then cryofocused in a cold trap at −30 °C and transferred directly onto the head of the capillary column by heating the cold trap to 250 °C (at a rate of 99 °C/s).

2.3.2. GC–MS analysis

Finnigan TRACETM MS (TermoQuest, Austin, USA) was used to carry out the GC–MS analyses. Volatile compounds were separated using a DB-WAX capillary column (SGE, Australia) (60 m length, 0.32 mm i.d., 1.0 μm film thickness). Helium at a constant flow rate of 1 mL min^{-1} was used as a carrier gas. The temperature was programmed to increase from 40 °C (2-minute hold time) to 190 °C at 4 °C min^{-1} (11-minute hold time) and finally to 220 °C at 8 °C min^{-1} (8-minute hold time). The MS interface and source temperatures were 250 °C and 200 °C, respectively. Electron

impact mass spectra were recorded using impact ionization mode at 70 eV and a mass range of m/z 33–433. A total of 3 extracts were obtained for each sample.

The identification of isolated volatile compounds was tentatively carried out by comparing their mass spectra (m/z values of the most important ions) with spectral data from the National Institute of Standards and Technology 2002 library as well as retention indices and spectral data published in the literature. A solution of the homogenous series of normal alkanes (C8–C20 by Fluka Buchs, Schwiez, Switzerland) was used to determine the Kovats retention indices.

Given that the purpose of this work was to determine whether the contribution of the volatile fraction can be used to differentiate between the drying methods used. It was considered more appropriate to use the values from the semiquantification of all the identified compounds. These data were calculated (μg/g stevia leaf) using the amount of internal standard and the relative area between the peak areas of each compound and the peak area of the internal standard, assuming a response factor equal to one.

2.4. Flavonoids and phenolic acids analysis

2.4.1. Extraction

The stevia leaves were ground in a grinding mill (A11 Basic, IKA, Germany), and 200 mg of the dried powder were shaken in 30 mL of methanol/water (1:1 v/v) for 5 minutes. The mixture was sonicated for 10 minutes and then centrifuged at 3000 x g for 5 minutes. An aliquot of the extract was injected in the HPLC, after being filtered through filter paper (0.45 μm pore size).

2.4.2. HPLC analysis

Analyses of the extracts were carried out using HPLC-Alliance 2695, with a 2996 photodiode array detector (Waters, USA). Flavonoids and phenolic compounds were separated on a Brisa LC2, C18 column (250 x 4.6mm x 5 μm) (Teknokroma, Spain). The binary mobile phase consisted of solvent A (ACN) and solvent B (water and formic acid, 99:1). Binary gradient conditions were used: initial, 90% B, linear gradient to 40% B at 25 min and then to 20% B at 26 min; holding until 30 min; followed by a linear gradient to initial condition at 35 min and a final hold at this composition until 40 min.

The column was maintained at 30ºC. The flow-rate and the injection volume were 0.5 mL/min. and 10 μL, respectively.

Chromatograms were recorded at three wavelengths (290, 320 and 360 nm). Flavonoids and phenolic acids were identified by comparison of chromatographic retention times and UV spectral characteristics of unknown analytes with authentic standards. Calibration curves were constructed via least squares linear regression analyses of the ratio of the peak area of each representative compound versus the respective concentration. Quantitative results were expressed as mg of component per 100g of stevia.

The pure standard of flavonoids and phenolic acids were diluted with methanol to obtain a final concentration of 1 mg/mL for the stock standard solution. The working standard solution was obtained at a concentration of 100 ng/mL in water. The stock standard solution was stored at -20ºC and the working standard solution at +4ºC.

Calibration curves obtained from standard solutions (0.5-10 ng/mL) were used to perform the quantification. Samples were spiked to verify the absence of a matrix effect in the analysis. An internal quality control (a standard solution) was injected into the equipment as a first step, before each batch of the sample, in order to ensure the quality of the results and evaluate the stability of the proposed method.

2.5. Validation of polyphenols analysis method.

The guidelines established by the EU Commission Decision (2002) were followed in order to validate the analytical methodology employed to analyse the flavonoids and phenolic acids. For this purpose, several parameters were studied: linearity, accuracy and precision (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the precision was verified by repeatability (intraday precision) and reproducibility (interday precision).

2.6.Statistical analysis

An analysis of variance (ANOVA) ($\alpha = 0.05$) with least significant difference (LSD) test using Statgraphics Plus 5.1 was performed on the data from flavonoids and phenolic acids as well as the volatile compounds. In addition to this, the data were

analyzed using multivariate techniques, applying the software Unscrambler version 9.7 (CAMO, 2005). The variables were weighted with the inverse of the standard deviation of all objects in order to compensate for the different scales of the variables. A Principal Components Analysis (PCA) was applied to describe the relationship between the flavonoids and phenolic compounds together with the volatile profile.

3. Results and Discussion

3.1. Influence of drying method on the phenolic and flavonoid compounds.

The average value of phenolic compounds (mg /100g stevia) quantified in the stevia leaves obtained using different drying methods (shade drying, freeze drying and air drying), as well as the ANOVA F-ratio and homogenous groups for each of the analyzed compounds are shown in Table 1. Eleven compounds were identified in all samples: apigenin, caffeic acid, catequin, chlorogenic acid, cinnamic acid, coumaric acid, 4-methoxybenzoic, 4-methylcatechol, quercetin, rutin and sinapic acid.

With regard to the validation parameters, good linearity was obtained, with R^2 values ranging from 0.991 for 4-methoxybenzoic to 0.999 for quercetin, catequin and 4-methylcatechol. The range of the average recoveries varied from 90% for caffeic acid to 117% for sinapic acid. The repeatability for all compounds was less than 9% and the reproducibility was always less than 13%.

The highest F-ratio in Table 1 shows that coumaric and sinapic acid were most influenced by the drying method. The concentrations of other compounds such as apigenin, quercetin and cinnamic acid showed practically no differences as a result of applying the three treatments.

The majority of the compounds analyzed reached their maximum values with the freeze drying method. For instance, compounds such as chlorogenic acid, coumaric acid and sinapic acid exhibited a higher concentration after freeze drying (191.84, 91.35 and 178.56 mg/100g stevia leaf, respectively) and air drying (167.56, 70.36 and 165.14 mg/100g stevia leaf, respectively) than shade drying (88.60, 41.71 and 33.21 mg/100g stevia leaf, respectively). However, the values obtained for 4-metoxybenzoic following freeze drying (7.48 mg/100g stevia leaf) were lower than those for the other treatments (air drying-26.28 mg/100g stevia leaf and shade drying-15.39 mg/100g stevia leaf).

Many antioxidant compounds have been identified in stevia leaves by different authors, but their conclusions with respect to both the specific compounds and the concentration levels are very different and even contradictory. This can be explained by the fact that the drying methods employed were different in each case. However, in some papers it was not even mentioned. Different flavonoids (flavonols and flavones) have been identified: quercetin and its derivatives, apigenin and its derivatives, kaempferol-3-O-rhamnoside, luteolin and their derivatives (Ghanta et al., 2007; Li et al., 2009; Cacciola et al., 2011) in stevia dried leaves. Karaköse et al. (2011) identified 24 chlorogenic acids using LC-ESI-MS. Muanda et al. 2011 identified (at room temperature) the same phenolic and flavonoid compounds in stevia dried leaves as in the present work, with the exception of 4-methoxybenzoic acid, 4-methylcatechol and sinapic acid. Kim et al. (2011) identified (at 40ºC for 12h) 6 phenolic acids: pyrogallol, 4-methoxybenzoic acid, 4- methylcatechol, sinapic acid, coumaric acid and cinnamic acid. All of them were identified in the present study, with the exception of pyrogallol. It is important to highlight that the values obtained by Kim et al. were lower than those reported by Muanda et al..

Table 1. Mean and standard deviation of flavonoid and phenolic compounds quantified in the three drying methods (mg/100 g of stevia leaf).

mg/100g stevia leaf	Freeze drying	Air drying	Shade drying	Anova F-ratio
apigenin	0.24(0.04)^{a}	0.25(0.02)^{a}	0.39(0.02)^{a}	1^{ns}
caffeic acid	1.22(0.02)^{b}	0.71(0.04)^{a}	0.75(0.03)^{a}	350***
catequin	8.35(0.38)^{c}	6.18(0.33)^{b}	4.38(0.42)^{a}	55**
chlorogenic acid	191.84(0.7)^{c}	167.56(0.12)^{b}	88.60(3.19)^{a}	1621***
cinnamic acid	0.27(0.07)^{ab}	0.34(0.02)^{b}	0.19(0.02)^{a}	7^{ns}
coumaric acid	91.35(0.16)^{c}	70.36(0.30)^{b}	41.71(0.48)^{a}	10616***
4-methoxybenzoic	7.48(0.39)^{a}	26.28(0.43)^{c}	15.39(0.2)^{b}	1394***
4-methylcatechol	2.49(0.02)^{b}	2.99(0.56)^{b}	0.73(0.07)^{a}	26*
quercetin	0.33(0.03)^{a}	0.28(0.02)^{a}	0.39(0.06)^{a}	5^{ns}
rutin	20.07(0.13)^{c}	15.08(0.22)^{b}	7.05(0.02)^{a}	4174***
sinapic acid	178.56(0.7)^{c}	165.14(1.53)^{b}	33.21(0.23)^{a}	13544***

* $p<0.05$, **$p<0.01$, *** $p<0.001$, ns: non significant

Considering other medicinal herbal teas, Lin et al (2011) claimed that freeze drying was the best method for preserving the higher contents of caffeic acid derivatives and total phenolics in *Echinacea Purpurea* leaves. Ferreira and Luthria (2010) obtained lower levels of antioxidant capacity for shade drying than hot air drying in *Artemisia annua* L. leaves.

3.2. Influence of drying method on the volatile compounds.

Thirty-two volatile compounds were identified. Table 2 shows the mean concentration values of the quantified volatile compounds (expressed as μg/g stevia leaf) as well as their standard deviations (SD) for the three drying methods.

The most abundant compounds produced by shade drying and freeze drying were 2-hexenal (21.09 and 19.78 μg/g), hexanal (14.23 and 10.02 μg/g) and α-pinene (19.40 and 5.04 μg/g), respectively. The most abundant compounds produced by air drying, were hexane (10.90 μg/g), furan tetrahydro (3.25 μg/g) and α-pinene (3.14 μg/g).

In contrast to the phenolic and flavonoid compounds, shade drying better preserves the volatile fraction of stevia leaves in comparison with freeze drying and air drying.

There are a few studies about the volatile fraction of stevia leaves and all of them analyzed the volatile compounds in the essential oils in stevia. Muanda et al. (2011) identified 34 volatile compounds, Moussa et al. (2005) found 22 compounds and Turko et al. (2007) reported 23 compounds, only 5 of them (α-pinene, hexanal, limonene, 1-octen-3-ol, caryophyllene) were identified in this study, which is logical because in the present work the analysis was performed directly on the stevia dried leaves and not on the essential oil.

Table 2. Semiquantification of volatile compounds (μg/g assuming a response factor equal to 1) in stevia dried leaves (n = 3).

Volatile compounds	Shade drying	Air drying	Freeze drying	Anova F-ratio
Alcohols				
1-penten-3-ol	0.60^{a}	0.15^{a}	1.96^{b}	14.8*
1-pentanol	0.42^{a}	0.63^{a}	0.33^{a}	2.43^{ns}
1-octen-3-ol	5.85^{c}	0.68^{a}	2.37^{b}	149***
3,7dimethyl-1,3 octadien-3-ol	5.50^{c}	0.93^{a}	2.35^{b}	99***
2 ethyl-1-hexanol,	0.88^{b}	0.18^{a}	0.27^{a}	46**
Aldehides				
2-ethyl-butanal	2.64^{b}	0.29^{a}	0.33^{a}	23.7**
3-methyl-butanal	3.01^{b}	0.11^{a}	0.78^{a}	10.87*
pentanal	2.25^{b}	1.26^{a}	1.22^{a}	2.03*
hexanal	14.23^{b}	0.86^{a}	10.02^{b}	27.4**
heptanal	0.41^{b}	0.11^{a}	0.08^{a}	14.3*
2-hexenal	21.09^{b}	0.83^{a}	19.78^{b}	24.9**
2-4 heptadienal	3.29^{c}	0.36^{a}	1.69^{b}	75***
octanal	0.34^{a}	0.20^{a}	0.29^{a}	3.7^{ns}
2-heptenal	2.63^{b}	0.28^{a}	0.64^{a}	121***
nonanal	2.34^{a}	1.28^{a}	1.73^{a}	3.5^{ns}
decanal	0.68^{a}	0.74^{a}	1.06^{a}	1.9^{ns}
Hydrocarbon				
2-methyl-pentane (isohexane)	0.39^{a}	0.44^{a}	0.12^{a}	3.24^{ns}
hexane	9.90^{b}	10.90^{b}	2.89^{a}	18.4**
2-methoxy-2-methyl-propane	1.29^{a}	1.19^{a}	0.76^{a}	1.18^{ns}
2-hydroxy-2-methyl-propanenitrile	3.41^{b}	0.56^{a}	0.53^{a}	19.5**
Ketones				
3-buten-2-one	0.50^{a}	0.69^{a}	0.49^{a}	0.2^{ns}
4-hydroxy-2-butanone	0.74^{a}	0.58^{a}	0.48^{a}	0.4^{ns}
6 methyl-5-hepten-2-one	0.29^{a}	0.22^{a}	0.29^{a}	0.4^{ns}
Terpenes				
1-heptene	1.92^{b}	0.05^{a}	0.81^{a}	21.08**
α-pinene	19.40^{b}	3.14^{a}	5.04^{a}	25.7**
limonene	0.72^{a}	0.54^{a}	0.45^{a}	2.2^{ns}
caryophyllene	8.24^{b}	1.68^{a}	2.36^{a}	47**
benzene	0.44^{a}	0.19^{a}	0.12^{a}	1.15^{ns}
1-octene	2.37^{b}	0.11^{a}	1.06^{ab}	11.12*
oxide trimethylene	1.04^{c}	0.05^{a}	0.57^{b}	30.9**
Furanes				
tetrahydro furan	2.61^{a}	3.25^{a}	1.34^{b}	1.26*
Sulfur compounds				
dimethyl sulfide	1.02^{b}	0.18^{a}	0.39^{a}	41.36**

* $p<0.05$, **$p<0.01$, *** $p<0.001$, ns: non significant

3.3. Global behavior of phenolic and volatile compounds.

A PCA was applied in order to appreciate the overall effect that the drying method has on phenolic and volatile compounds together. The corresponding bi-plot obtained (scores "treatments" and loading "variables") is shown in Figure 1 (PC1 explained 59 % of the total variance and PC2, 20 %). The proximity between variables indicates the correlation between them, and in the case of drying treatments similar behavior. In general, this figure shows opposing behavior between the two groups of variables (phenols and volatiles) with respect to the effect of the drying treatments applied.

The shade drying treatment is placed at the far end of the right axis in the figure, which corresponds to the highest values of the volatile compounds and the lowest of the phenolic compounds. On the contrary, freeze drying and air drying are placed on the opposite side (left axis), which corresponds to the highest content of phenolic compounds. The only exceptions to this general pattern are apigenin and quercetin which are placed with the volatile compounds even though they are antioxidant compounds.

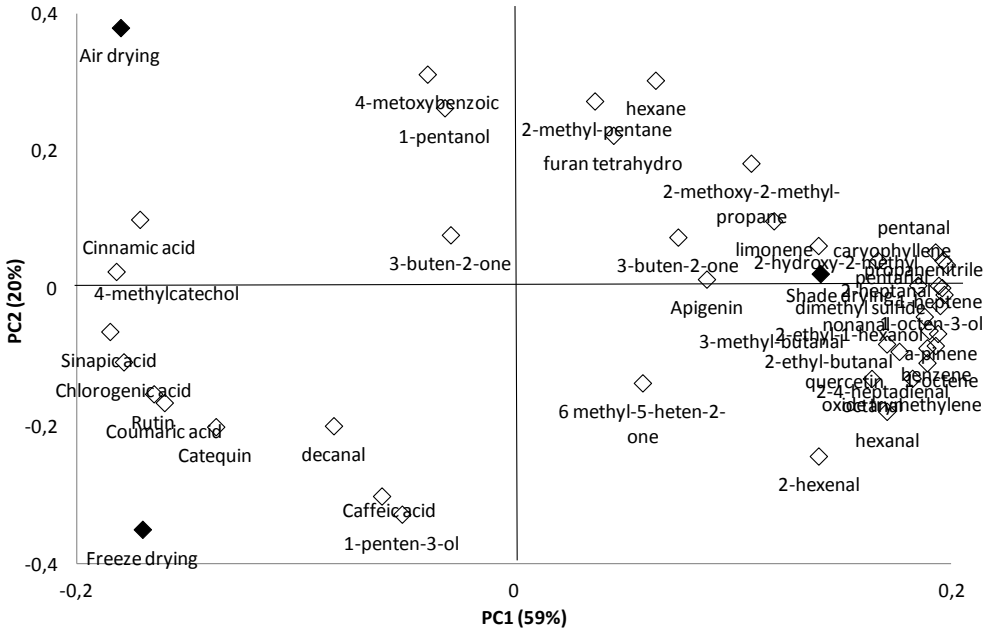

Figure. III.2.1 Bi-plot of Principal Components Analysis for the drying treatments (black diamond ♦) and the analysed variables: phenolic, flavonoid and volatile compounds (white diamond ◊).

4.Conclusions

All the drying methods applied (freeze drying, shade drying and air drying) affected the antioxidant and volatile compounds in the dried stevia leaves. The two types of compounds reacted differently; the content of volatile compounds was higher with shade drying whereas most flavonoids and phenolic acids had higher concentrations when freeze drying was applied. However, some flavonoids and phenolic acids exhibited a higher increment with air drying. Therefore there is no ideal drying treatment which can be chosen, although freeze drying is the most recommendable if an extract with sufficient antioxidant properties and satisfactory aromatic characteristics is desired.

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References

Abascal, K., Ganora, L., & Yarnell, E. (2005). The effect of freeze drying and its implications for botanical medicines: a review. *Phytotherapy Research*, 19,655-660.

Cacciola, F., Delmonte, P., Jaworska, K., Dugo, P., Mondello, L., & Rader, J. (2011). Employing ultra-high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of Stevia rebaudiana extracts. *Journal of Chromatography A*, 1218, 2012-2018.

Capecka, E., Mareczek, A., & Leja, M. (2005). Antioxidant activity of fresh and dry herbs of some Lamiaceae species. *Food Chemistry*, 93, 223-226.

Carbonell-Capella, J.M., Barba, F.J., Esteve, M.J., & Frígola, A. (2013) High pressure processing of fruit juice mixture sweetened with Stevia rebaudiana Bertoni: Optimal retention of physical and nutritional quality. *Innovative Food Science and Emerging Technologies*, 18, 48-56.

Chatsudthipong V., Muanprasat C., (2009) Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacol. Therapeut. 121 41-54.

Chan, E.W.C., Lim, Y.Y., Wong, K.K., Lim, K.K., Tan, S.P., Lianto, F.S., & Yong, M.Y. (2009). Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species. *Food Chemistry*, 113, 166-172.

Commission Decision 2002/657/EC of 12 August (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJEC L221, 8-36. Brusels, Belgium.

Di Cesare, LF, Forni, E, viscardi, D, and Nani RC (2003) Changes in the chemical composition of basil caused by different drying procedures. Journal Of Agricultural and food Chemistry, 51, 3575-3581.

Diaz- Maroto, MC, Perez Coello MS and Cabezudo MD (2002). Effect of different drying methods on the volatile components of parsley (Petroselinum crispum L.) European Food Research Technology, 215, 227-230.

EFSA. European Food Safety Authority (2011). Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. *EFSA Journal* 9,1,1972. doi:10.2903/j.efsa.2011.1972

Ferreira, J.F. & Luthria, D.L. (2010). Drying Affects Artemisinin, Dihydroartemisinic Acid, Artemisinic Acid, and the Antioxidant Capacity of Artemisia annua L. Leaves. *Journal of Agricultural and Food Chemistry*, 58, 1691-1698.

Ghanta, S., Banerjee, A., Poddar, A., & Chattopadhyay, S. (2007). Oxidative DNA damage preventive activity and antioxidant potential of Stevia rebaudiana (Bertoni) Bertoni, a natural sweetener. *Journal of Agricultural and Food Chemistry*, 55, 10962-10967.

Hossain, M.B., Barry-Ryan, C., Martin-Diana, A.B., & Brunton, N.P. (2010) Effect of drying method on the antioxidant capacity of six Lamiaceae herbs. *Food Chemistry*, 123, 85-91.

Kim, I., Yang, M., Lee, O., & Kang, S. (2011). The antioxidant activity and the bioactive compound content of Stevia rebaudiana water extracts. *LWT-Food Science and Technology*, 44, 1328-1332.

Kinghorn, AD. Overview. In: Stevia, the Genus of Stevia, Medicinal and Aromatic Plants-Industrial Profiles; Kinghorn, AD. Ed. Taylor and Frances: London, 2002; ISBN 0-415-26830-3,pp 1-17.

Lemus-Moncada, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia* rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, 132, 1121-1132.

Lewicki, P.P. (2006). Design of hot air drying for better foods. *Trends in Food Science and Technology* 17, 153-163.

Li J, Jiang H, Shi, R (2009). A new acetylated quercetin glycoside from the leaves of stevia rebaudiana Bertoni Nat. Prod. Res. 23, 1378-1383.

Lin, S.D., Sung, J.M., & Chen, C.L. (2011). Effect of drying and storage conditions on caffeic acid derivatives and total phenolics of Echinacea Purpurea grown in Taiwan. *Food Chemistry*, 125, 226-231.

Moussa, MM, Massoud-Mona I and Zeitoun, MA. (2005) Identification of some volatile compounds extracted from certain medical plants. Alexandria Journal Food Science and Technology, 2, 9-18.

Muanda, F., Soulimani, R., Diop, B., & Dicko, A. (2011). Study on chemical composition and biological activities of essential oil and extracts from Stevia rebaudiana Bertoni leaves. *LWT-Food Science and Technology* 44, 1865-1872.

Periche, A., Koutsidis, G., & Escriche, I. (2014). Composition of antioxidants and amino acids in Stevia leaf infusions. *Plant Foods for Human Nutrition*, 69, 1-7.

Pinela, J., Barros, L., Carvalho, A.M., & Ferreira, I. (2011). Influence of the drying method in the antioxidant potential and chemical composition of four shrubby flowering plants from the tribe Genisteae (Fabaceae). *Food and Chemical Toxicology*, 49, 2983-2989.

Shukla, S., Mehta, A., Menta, P., & Bajpai, V. (2012). Antioxidant ability and phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology* 64, 807-811.

Turko YA, Korobko NV, Shokun VV, Chernyak EN, Vyalkov AI, Stepankina ON, Kerimzhanova BF, and Baltaev UA (2007) GC—MS Research I. Essential oil from *Stevia rebaudiana. Chemistry of Natural Compounds, 43, 6.*

Wölwer-Rieck, U. (2012) The leaves of Stevia rebaudiana (Bertoni), their constituents and the analyses thereof: A review. Journal of agricultural and food chemistry, 60,886-895.

III.3. INFLUENCE OF EXTRACTION METHODS ON THE YIELD OF STEVIOL GLYCOSIDES AND ANTIOXIDANTS IN *STEVIA REBAUDIANA* INFUSIONS

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Plant Foods for Human Nutrition (En revisión)

ABSTRACT

This study evaluated the application of ultrasound technique and microwave energy, in comparison with conventional extraction methods (high temperatures at atmospheric pressure), in the solid-liquid extraction of steviol glycosides (sweeteners) and antioxidants (total phenols, flavonoids and antioxidant capacity) from dehydrated stevia leaves. Different temperatures (from 50 to 100 ºC), times (from 1 to 40 min) and microwave powers (1.98 and 3.30 W/g infusion) were used. There was a great difference in the resulting yields depending on the treatments applied. Steviol glycosides and antioxidants were negatively correlated; therefore there is no single treatment suitable for obtaining the highest yield in both groups of compounds simultaneously. The best conditions for steviol glycosides was microwave (3.30 W/g infusion, 2 min), whereas, the conventional method (90ºC, 1 min) was the most suitable for antioxidants. Consequently, the best process depends on the subsequent use (sweetener or antioxidant) of the aqueous extraction of stevia leaves.

Keywords: microwave energy, ultrasound technique, phenols, flavonoids, steviol glycosides.

1. Introduction

The *Stevia rebaudiana* Bertoni is a perennial herb of the family Asteraceae, from Brazil and Paraguay. Their high sweetness (250-300 higher than sucrose) is the main characteristic of the *Stevia* leaves due to the presence of diterpenes, specifically steviol glycosides [1]. The Joint Expert Committee on Food Additives (JECFA) has established regulations on the conditions of extraction and purification of steviol glycosides (steviolmonoside, rubusoside, steviolbioside, dulcoside A, stevioside, rebaudioside B, rebaudioside A, rebaudioside C, rebaudioside F) and their maximum daily intake. The purified extract of steviol glycosides must be at least 95% and the acceptable daily intake (ADI) is 4 mg per kg bodyweight and day [2]. Stevioside was reported to be the most abundant steviol glycoside (4-13%) found in the plant leaves, followed by rebaudioside A (2-4%), rebaudioside C (1-2%) and dulcoside A (0.4-0.7%). Steviolbioside, rebaudioside B, D, E, F were also identified in the leaf extracts, but as minor constituents [1].

Stevia leaves are most commonly used for the purpose of extracting and purifying steviol glycosides in order to obtain a non-caloric natural sweetener as a sugar substitute or as an alternative to artificial sweeteners [3]. The sweetening properties of those glycosides, however, differ from one another. Whereas stevioside exhibits a significant bitter aftertaste, Rebaudioside A has a sweet taste, which has been attributed to the presence of an extra glucose moiety in the Rebaudioside A structure [4].

Apart from the sweetening power of *Stevia*, their leaves have important therapeutic properties which are responsible for the increasing interest in the consumption of this aqueous extract. *Stevia* leaves are rich in compounds with anti-inflammatory, diuretic antihypertensive, antihyperglycemic, antidiarrehic, antitumor and antioxidant properties [5]. Flavonoids and phenolic compounds present in the *Stevia* leaves, are responsible for the high antioxidant capacity [6, 7]. Therefore the direct intake of dried *Stevia* leaf infusions or their addition in different food formulations such as juices, biscuits, jams, confectionery products, etc. will enhance the functional properties of these products. The EFSA (European Food Safety Authority) recognized the safety of *Stevia* leaf extracts for use in food and beverages in November 2011[8], although its use was authorized in different Asian and American countries decades ago. Japan was the first country to commercialize steviol glycosides as a sweetener in food and drugs in 1968 [9].

The active principles of fresh or dehydrated leaves are traditionally extracted by means of an aqueous extraction at high temperatures and atmospheric pressure (conventional method). However, some authors have shown that other techniques such as ultrasound or microwave energy can maximize or improve the extraction of active compounds from herbs [10, 11].

The application of ultrasound could be a good choice because this technique induces greater penetration by the solvent into the cellular matrix, an alteration of the structure and therefore an improvement in the mass transfer [12]. In fact, the ultrasound technique has been used successfully to extract steviol glycosides from *Stevia* leaves [13, 14]. Additionally, it has been used to extract antioxidant compounds from other plants: polyphenols and antioxidant capacity from olive leaves [15], polyphenols from grape seeds [16] and flavonoids from *Citrus aurantium* [17]. Another possible extraction technique is microwave energy. The friction resulting from this molecular

movement contributes to the rapid heating of the vegetable matrix, with the advantage of the great reduction in the time required for extraction compared to the conventional method. This technique has been widely used in the extraction of organic compounds, developing rapidly in the last decade. Wang et al. [18] successfully applied microwave energy in the extraction of phenolic compounds from Chinese herbs. Jaitak et al. [13] and Teo et al. [19] also applied microwave energy in the extraction of steviol glycosides.

This study aimed to evaluate the effect of the application of ultrasound and microwave energy in comparison with the conventional method in the solid-liquid extraction of antioxidants (total phenolic content, flavonoids and antioxidant capacity) and steviol glycosides from dehydrated *Stevia* leaves. The chromatographic procedure used to identify and quantify the steviol glycosides compounds was validated in order to ensure the suitability of the method.

2. Material and Methods

2.1. Stevia samples and extraction procedure

Organically produced dried leaves of *Stevia rebaudiana* (Raab, Vitalfood, Rohrbach, Germany) were used in this study. One gram of dried stevia leaf powder (ground in a grinding mill, A11 Basic, IKA, Germany) was dispersed in 100 mL of water. Aqueous extracts of dried stevia leaves were obtained at atmospheric pressure (conventional method) using a thermostatic bath (JP Selecta Precisdig, Spain) heated to different temperatures (50, 70, 90 and 100˚C) for different times (1, 5, 20 and 40 minutes); ultrasonic energy (US) in a thermostat bath (Ultrasounds-H, JPSelecta, Spain) at different temperatures (50, 70 and 90°C) and for different times (1, 5 and 20 minutes) and applying microwave energy (MW) (Samsung, GW72N) at a relative power of 1.98 W/g infusion for 1, 2, 3 and 5 minutes and 3.30 W/g infusion for 1 to 2 minutes. When this last power was applied, it was not possible to longer than 2 minutes because it caused the boiling and overflow of the sample. Subsequently, the aqueous extracts were filtered through filter paper and cooled before the analytical determinations were made. All the analyses were performed in triplicate.

2.2 Standard compounds and reagents

HPLC-grade acetonitrile and methanol were purchased from VWR (Fontenay-sous-Bois, France) and analytical grade ethanol and ammonium acetate were purchased from Scharlab (Barcelona, Spain). The standards Rebaudioside A, Rebaudioside C, Dulcoside A, Stevioside and Steviolbioside (purity > 98%) were obtained from Chromadex (CA, USA). De-ionized water from MilliQ (Millipore Corp., Bedford, MA) was used throughout the procedure. Solid-phase extraction (SPE) was carried out on a vacuum manifold system (Lichrolut, Merck, Darmstadt, Germany) using StrataC18-E cartridges (500 mg, 3 mL, 55 μm, 70 Å) from Phenomenex (Torrance, CA) for the determination of steviol glycosides. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) were used to determine the total antioxidant activity. Sodium nitrite, (+)-catechin, sodium hydroxide (Sigma-Aldrich, Germany) and aluminum chloride hexahydrate (Fluka, Germany) were used to analyse the flavonoids. Sodium carbonate, gallic acid and Folin-Ciocalteu reagent (all purchased from Sigma-Aldrich, Germany) were utilized for phenolic determination.

2.3. Steviol glycosides analysis

2.3.1. Steviol glycosides extraction procedure

The aqueous extract (0.5 mL) was diluted with water (2.5 mL) and this solution was used for SPE. The resulting solution was loaded on a 3 mL Strata SPE cartridge pre-activated with methanol (3 mL) and then washed with water (3 mL). The SPE cartridge was then sequentially washed with 3 mL each of water and 20% acetonitrile in water (2:8 v/v); and then air dried for 2 minutes; the steviol-glycosides were eluted from the cartridge using 5 mL of 80% acetonitrile in water [20]. The eluate was subjected to LC-MS-MS analysis.

2.3.2. Methodology

The chromatographic analysis was performed on an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA) with an ionization source electrospray type.

A LC-MS-MS method was used in the present study for the analysis of the steviol glycosides. Chromatographic separation was carried out in gradient mode by Zorbax SB-C18 column (50mm x 2.1mm, 1.8 μm) maintained at 40ºC, with a mobile phase consisting of 10 mM aqueous ammonium acetate (A) and acetonitrile (B). Binary gradient conditions were used: initial, 7% B, held for 0.2 min: linear gradient to 20% B at 0.3 min and then to 48% B at 5 min; sudden increase to 100% B at 5.1 min and hold until 7 min; followed by a linear gradient to initial condition at 7.1 min and a final hold at this composition until 9 min. The flow-rate and the injection volume were 0.4 mL/min. and 5 □L, respectively. The electrospray was operated in negative ion mode. Choi et al. [21] stated that negative ion mode is 10 times more sensitive than positive ion mode. The conditions used in the ionization source were: temperature of the drying gas (N_2) 325ºC to 11L/min, nebulizer pressure of 50 psi and the capillary voltage of 4000 V. Identification and quantification of steviol glycosides in the samples and the standards was performed using the multiple reaction monitoring (MRM) mode.

The stock standard solution of steviol glycosides was prepared by weighing the appropriate amount of the pure standard and diluting it with methanol to obtain a final concentration of 1 mg/mL. The working standard solution was obtained at a concentration of 0.01 mg/mL in water. The stock standard solution was stored at -20ºC and the working standard solution was at +4ºC.

Quantification was performed by means of calibration curves obtained from standard solutions (0.5-10 μg/mL). Samples were spiked to verify the absence of a matrix effect in the analysis. In order to ensure the quality of the results and evaluate the stability of the proposed method, an internal quality control (a standard solution) was injected in the equipment as a first step before each batch of the sample.

2.4. Validation of the steviol glycosides analysis method

The guidelines established by EU Commission Decision [22] were followed in order to validate the steviol glycosides analytical methodology. For this purpose, several parameters were studied: linearity, accuracy and precision (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the precision was verified by repeatability or intraday precision (RSD_r) and reproducibility or interday precision (RSD_R). LODs (limit of detection) and LOQs (limit of quantification) were determined through the analysis of standard solutions. These

values were defined as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10 respectively.

2.5. Determination of total phenolic content

The total phenolic content was determined spectrophotometrically using the modified Folin-Ciocalteu method [23]. Distilled water (0.5 mL), 0.125 mL of the infusion sample (appropriately diluted) and 0.125 mL of Folin-Ciocalteu reagent were introduced in a cuvette. After shaking and waiting six minutes, 1.25 mL of a 7% sodium carbonate solution and 1 mL of distilled water were added. After 90 min, the absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). A blank was considered in this analysis. The quantification was made considering a standard curve of gallic acid and the results were expressed as mg of gallic acid equivalent per gram.

2.6. Determination of total flavonoid content

Total flavonoid content was determined using the modified colorimetric method described by Dewanto et al. [24]. 0.25 mL of the infusion sample (appropriately diluted), 1 mL of distilled water and 0.075 mL of a 5% sodium nitrite solution were mixed in a cuvette. After 6 min, a 10% aluminum chloride solution (0.15 mL) and 1M sodium hydroxide solution (0.5 mL) was mixed and left to settle for 5 min. Finally, 2 mL of distilled water was added and the absorbance was immediately measured at 510 nm. A blank was considered in this analysis. The quantification was made considering a standard curve of (+)-catechin and the results were expressed as mg of (+)-catechin equivalent per gram of stevia.

2.7. Determination of total antioxidant capacity

The antioxidant activity (AA) of the extract was analyzed on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl free radical as described by Shahidi et al. [25], with some modifications. Accordingly, 0.1 mL of the infusion sample (previously diluted in methanol:water (80:20)) was mixed with 3.9 mL of a methanolic solution of DPPH (0.025mg/mL, prepared in methanol:water (80:20)). The solution was shaken and after 30 min the absorbance of the sample was measured at 515 nm using methanol as a blank. The quantification was made considering a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the results were expressed as mg of Trolox equivalent per gram of stevia.

2.8. Statistical analysis

A multifactor analysis of variance (ANOVA) (with Statgraphics Centurion) was used to study the influence of method, temperature and time required during the infusion treatments on the steviol glycosides, antioxidant, phenols and flavonoids. Furthermore, a Principal Component Analysis (PCA) was also performed using the software Unscrambler X.10 to describe the relationships between the treatments and the variables analysed.

3. Results and Discussion

3.1. Validation of the steviol glycosides analytical methodology

Table 1 shows the results from the steviol glycosides validation procedure. In order to obtain the linearity value an external standard calibration curve was made using standard solutions with final concentration levels of: 0.5, 1, 2, 5, 7 and 10 mg/L (ppm). Six replicates were made for each level. The calibration curves were obtained by plotting the peak area of the compound at each level versus the concentration. The linearity response observed from 0.5 to 10 mg/L was good because the correlation coefficient between peak areas and injected nominal concentrations was $R^2 \geq 0.995$.

The recovery studies were performed by adding known quantities of steviol glycosides to a sample (1, 5 and 10 mg/L). Six replicates of all the spiked sample levels were analyzed using the HPLC method. The method used permitted to recovery steviol glycosides between 70.5 and 105.6 % for the concentration range studied (Table 1). The relative standard deviation (RSD) corresponding to recovery values was less than 20% in all cases (ranging from 4.0 to 18), confirming that the analytical method was accurate.

Repeatability (RSD_r) was evaluated by performing the assay on six replicates of fortified stevia samples, at the same levels (1, 5 and 10 mg/kg), and was carried out by the same operator on the same day. In order to evaluate reproducibility (RSD_R) the experiment was performed by 2 different operators on 3 consecutive days. The results were expressed as the percentage of relative standard deviation.

Table 1. Validation parameters (accuracy and precision) of steviol glycosides. The numbers in brackets are the relative standard deviation.

Compound	LOD (µg/g)	LOQ (µg/g)	Level (µg/g)	%recovery (D.S.%)	RSD_r%	RSD_R %
Dulcoside A	0.05	0.15	1	75.3(11.9)	1.7	6.9
			5	78.5(4.0)	10.1	7.6
			10	70.9(6.2)	5.1	5.2
Rebaudioside A	0.11	0.32	1	86.0(15.8)	1.6	6.1
			5	105.6(12.7)	8.3	9.9
			10	88.2(15.9)	6.5	7.1
Rebaudioside C	0.09	0.31	1	99.9(18.0)	1.6	7.9
			5	83.7(10.9)	8.6	7.6
			10	73.9(9.5)	3.3	5.8
Stevioside	0.04	0.15	1	92.3(15.3)	14.1	9.9
			5	98.8(19.0)	11.1	9.6
			10	95.3(4.9)	5.2	6.1
Steviolbioside	0.14	0.49	1	73.8(9.3)	11.8	11.7
			5	71.4(14.2)	13.3	16.5
			10	70.5(8.4)	14.6	12.1

Intra-day precision (RSDr) ranged from 1.7% to 14.6%, inter-day precision (RSD_R) from 5.2% to 16.5% (Table 1). These RSD values are in complete agreement with EU Commission Decision [22] requirements since they were always lower than 20% for all the concentration levels assayed. The LOD (limit of detection) ranged between 0.04 and 0.14 and the LOQ (limit of quantification) ranged between 0.15 and 0.49. Therefore, it can be concluded that the method used in this work has good precision. The results of the validation prove that the analytical procedure carried out appropriately guarantees the quantitative values of steviol glycosides obtained in the samples analyzed.

3.2. Steviol glycosides in stevia infusions: Influence of extraction treatment, time and temperature

Figure 1 is presented in order to facilitate the comparison of variability patterns between the different conditions applied to obtain the infusions. It shows the average values and the standard deviation of the 4 steviol glycosides (Dulcoside A, Rebaudioside A, Rebaudioside C and Stevioside) identified and quantified in the infusions obtained using different methods: conventional (CV), ultrasound (US), and microwave (MW), at different temperatures: 50, 70 and 90ºC and times: (1, 2, 3, 5, 20 and 40 minutes).

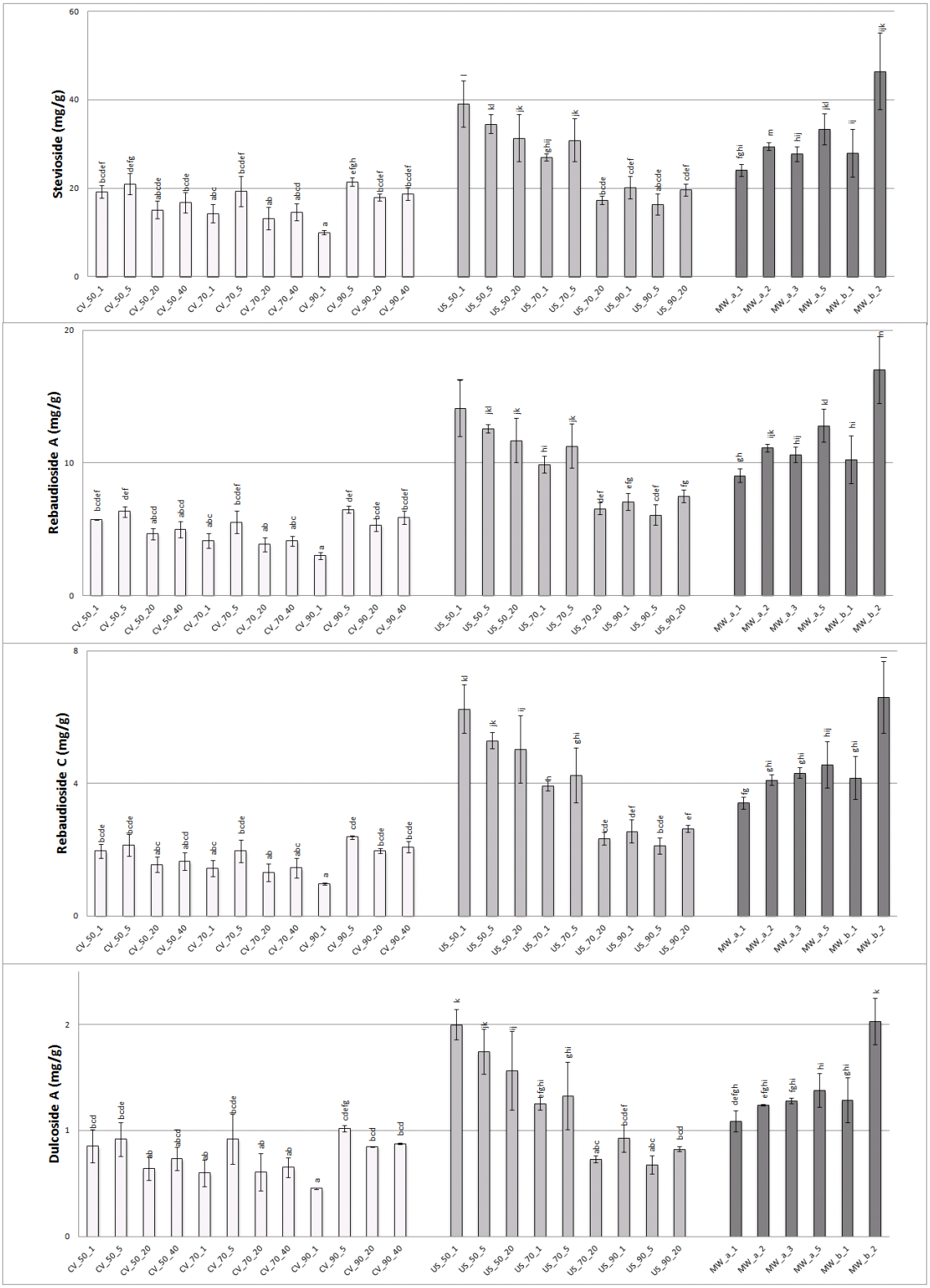

Figure III.3.1. Average values of Dulcoside A, Rebaudioside A, Rebaudioside C and Stevioside in the infusions of Stevia leaves obtained applying different methods: conventional (CV), ultrasound (US), and microwave (MW), at different temperatures: 50, 70 and 90ºC and times: 1, 2, 3, 5, 20 and 40 minutes. Letters in bars indicate homogenous groups. In legend of MW: a: 1.98 W/g infusion and b: 3.30 W/g infusion.

Additionally, this figure shows the homogenous groups of the ANOVA carried out for a single factor "treatment" (method-temperature-time), which means a total of 27 treatments. The F-ratio values ranged between 11.58 and 26.86 in all cases. Unlike the results found by other authors [26], in this study steviolbioside was not found in any sample. This finding is not considered to be surprising because there is evidence that rebaudioside B and steviolbioside are not native constituents of *Stevia rebaudiana*, but rather can be formed by partial hydrolysis during the extraction process, and are consequently artifacts of the extraction procedure [27, 28].

It can be observed that conventional infusion treatment (CV) had a lower yield than the other two treatments (US y MW). The higher the treatment time, the lower the level of extraction of different compounds. A maximum extraction occurred at 5 minutes, at all temperatures studied.

With respect to the ultrasound treatment (US), the highest extraction of steviol glycosides was observed at the lowest temperature (50°C) and shortest time (1 min) (2 mg Dulcoside A/g, 14.12 mg Rebaudioside A/g, 6.25 mg Rebaudioside C/g, 39.06 mg Stevioside/g). These results are in agreement with the findings of Liu et al. [14] who reported that extraction assisted by ultrasound increased the yield 1.5 times in comparison with the classical extraction method as long as low temperature and short times were applied.

Microwave treatment (MW) led to the extraction of the greatest amount of steviol glycosides, similar to US at 50ºC, 1 min. The highest yield (2.03 mg Dulcoside A/g, 17.03 mg Rebaudioside A/g, 6.6 mg Rebaudioside C/g, 46.48 mg Stevioside/g) being reached when applying the highest power (3.3 W/g) for 2 minutes of extraction. Teo et al. [19] obtained values of Stevioside (14.07-21.37 mg/g) lower than in this study using microwave extraction and confirmed the improved efficacy of this method as compared to extraction with hot pressurized water. Likewise, Jaitak et al. [13] obtained an increase in the yield of the Rebaudioside A and Stevioside by means of microwave extraction, in comparison with conventional cold extraction and ultrasound.

3.3. Total phenols, flavonoids and antioxidant capacity in stevia infusions: Influence of extraction treatment, time and temperature

Figure 2 shows the average values and the standard deviation of total phenols (mg gallic acid equivalent/g *Stevia*), flavonoids (mg of catequin equivalent/g *Stevia*) and total antioxidants (mg Trolox equivalent/g *Stevia*) quantified in the infusions obtained using different methods (conventional, ultrasound and microwave), temperatures (50, 70, 90 and 100ºC) and times (1, 2, 3, 5, 20 and 40 minutes). Furthermore, homogenous groups obtained in the ANOVA are also represented by letters in the figure, showing statistical differences among components in the treatments studied (α=99%) with the following F-ratios: 18.77 (phenols), 52.15 (flavonoids) and 24.72 (antioxidants).

Conventional extraction method achieved the highest efficiency in all cases in comparison with the results obtained with extraction by means of ultrasound technique and microwave energy. However, previous studies reported that ultrasound [15] and microwave [19] improved phenol extraction in olive leaves and Chinese herbs, respectively. Zhang et al. [29] also obtained successful results using ultrasonic and microwave techniques for the extraction of flavonoids in medicinal plants.

In this work efficiency was especially important when conventional infusions were carried out at 90 °C for phenols (93.41 mg gallic acid/g) and total antioxidants (131 mg trolox/g *Stevia*). An increase of temperature beyond 90°C did not improve extraction; on the contrary it had a negative effect on the phenolic compounds and total antioxidants, probably due to their degradation at boiling point. Liazid et al. [30] observed that some phenolic compounds were no longer stable at 100ºC and Inglett et al. [31] reported the instability of antioxidant compounds at these temperatures.

However, in the case of flavonoids the highest yield in this work was achieved when conventional extraction was carried out at 100 ºC (52.92-69.18 mg catechin/g). No significant differences were observed for phenol compounds between treatment times at 90ºC, 1 min being as effective as 40 min. In the case of flavonoids, from 5 min there were no significant differences at 100 ºC, the maximum yield temperature. However, for total antioxidants the greatest effectivity was achieved at 20 min of conventional treatment, without significant differences with 40 min.

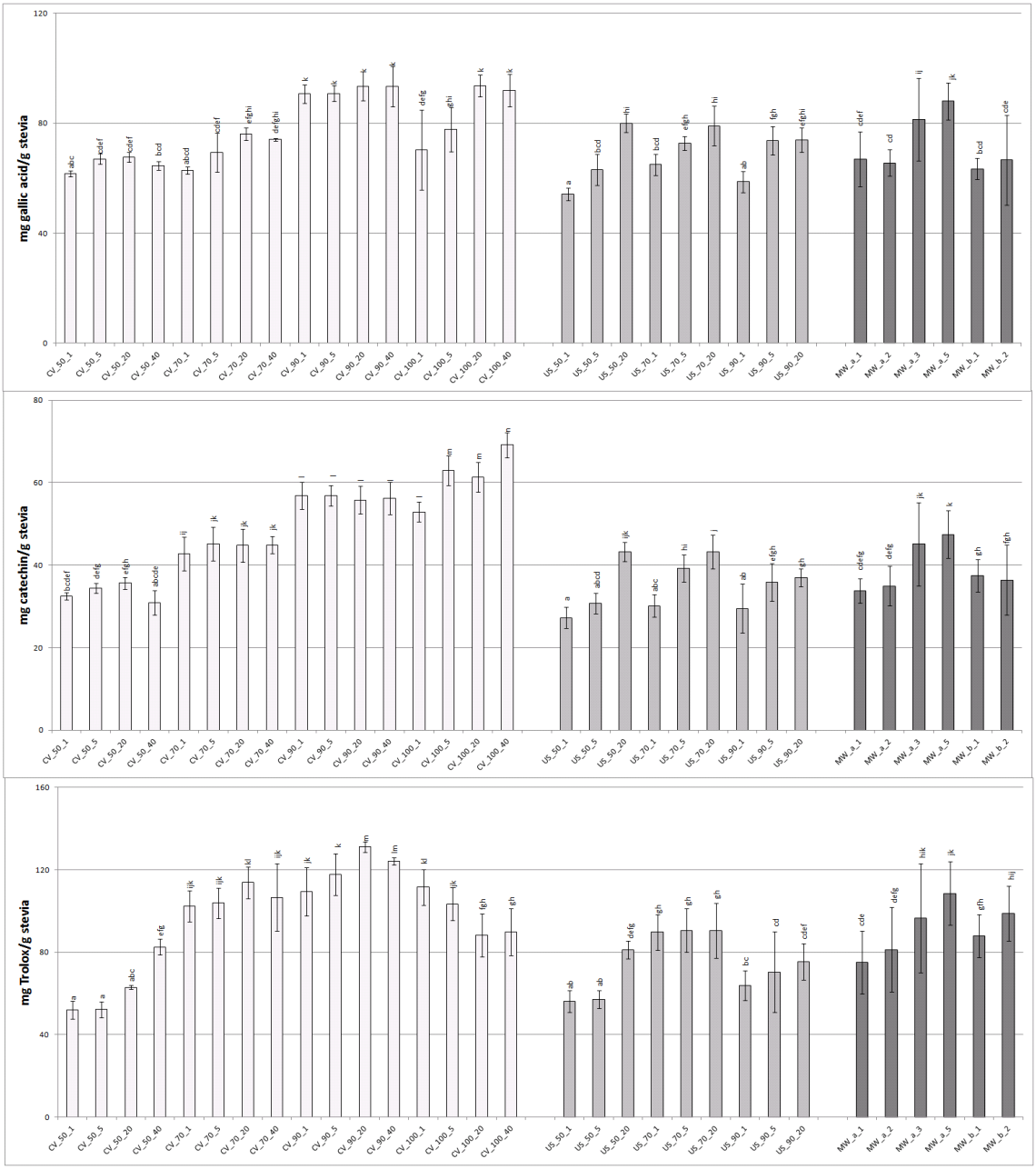

Figure III.3.2. Total phenols (mg galic acid equivalent/g stevia), flavonoids (mg of catequin equivalent/g stevia) and total antioxidants (mg Trolox equivalent/g stevia) quantified in the infusions obtained at different methods: conventional (CV), ultrasound (US) and microwave (MW); temperatures (50, 70, 90 and 100 ºC) and times (1, 2, 3, 5, 20 and 40 minutes). Letters in bars indicate homogenous groups. In legend of MW: a: 1.98 W/g infusion and b: 3.30 W/g infusion.

When the extractions were carried out with ultrasound technique, the best results were obtained for moderate temperatures: 50 and 70ºC. In the case of 50ºC the highest yield in all cases occurred at 20 min of treatment: total phenols (80 mg gallic acid equivalent/g *Stevia*), flavonoids (43 mg of catequin equivalent/g *Stevia*) and total antioxidants (81 mg Trolox equivalent/g *Stevia*). No significant differences were

observed between the treatments of maximum yield at 50 ºC (50ºC y 20 min) and the treatments of maximum yield at a 70 ºC.

In the case of microwave energy extraction a power of 1.98 W/g led to a slight increase in all the compounds analyzed when the time of extraction reached 3 min. Therefore, the highest yield would be obtained using a microwave power of 1.98 W/g infusion for 3 minutes: total phenols (81 mg gallic acid equivalent/g *Stevia*), flavonoids (45mg of catequin equivalent/g *Stevia*) and total antioxidants (96 mg Trolox equivalent/g *Stevia*).

The worst results observed for antioxidant activity when ultrasonic and microwave extraction was applied (around 100 mg Trolox eq/g *Stevia* in the most favorable conditions) differ from those reported by Ahmad-Qasem et al. [15] in olive leaves. These authors registered higher antioxidant levels with ultrasound methods in these leaves. However, the total antioxidant capacity values for microwave extraction were consistent with other studies. Inglett et al. [31] obtained results showing lower antioxidant activity by extraction with microwave energy, because this technique could degrade some antioxidant compounds.

Ya-Quin et al. [32] determined that temperatures over 40ºC in extraction assisted by ultrasound did not improve the extraction of some phenols in citrus peel due to high temperature induced instability of phenolic compounds.

3.4 Global behavior of antioxidant properties and steviol glycosides

Once the individual behaviour of steviol glycosides and antioxidant compounds were analyzed, a PCA was used to assess the overall effect of the conditions (method, time and temperature) used to obtain the stevia infusions. Figure 3 shows the PCA biplot (scores "treatments" and loading "variables") obtained. The first two components explained 93 % of the total variance (PC1, 65 % and PC2, 28 %). The proximity between treatments of infusions implies similar behaviour, while the proximity between variables denotes the degree of correlation between them. Taking this into consideration, the treatment of ultrasound for 1 min. (US_50_1) and microwave for 2 min (MW_b_2), placed at the far end of the left axis in the figure, had the most steviol glycosides. On the contrary, the samples at 90ºC situated on the opposite side on the top (right axis), had the highest level of the antioxidant properties analyzed. Moreover, the

steviol glycosides were negatively correlated with the antioxidants properties. Therefore the treatments with low values of antioxidants showed high values of steviol glycosides.

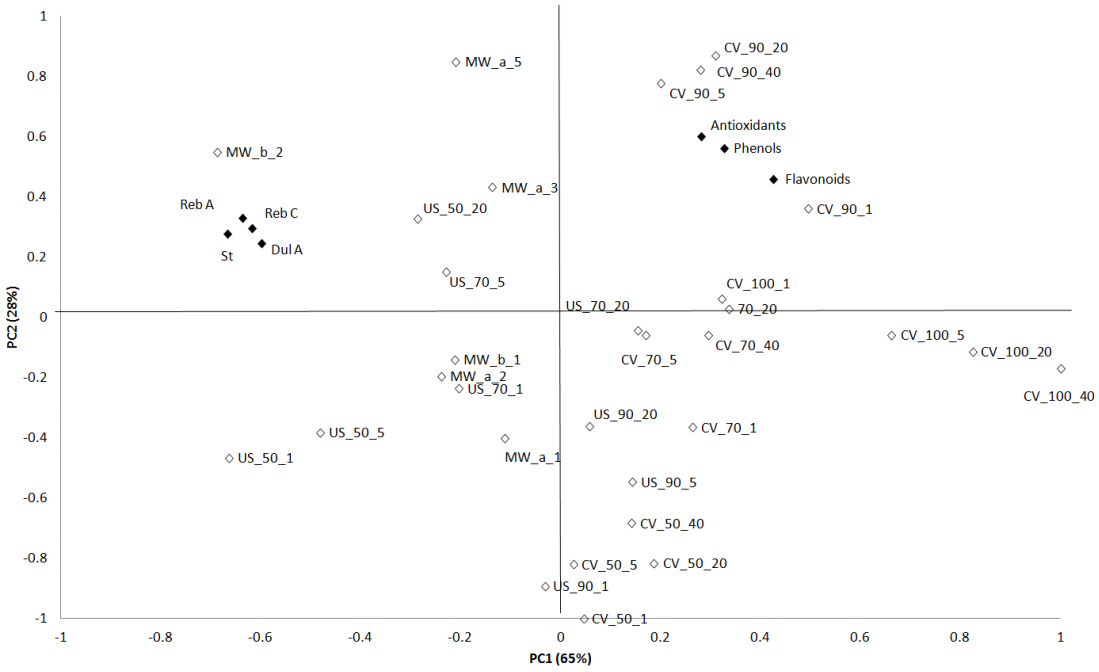

Figure III.3.3. Bi-plot of Principal Components Analysis for the treatments (white rhombus ◊) and the variables (black rhombus ♦).

4.Conclusions

The extraction method used to obtain aqueous extracts of stevia dehydrated leaves (conventional at atmospheric pressure, ultrasound and microwave), has a great effect on the yield of steviol glycosides and antioxidants. Due to the fact that both groups of compounds are negatively correlated there is no single treatment suitable for obtaining the best yield in both groups of compounds simultaneously. High microwave power (3.30 W/g infusion) and long microwave treatment time (2 min) was found to be most recommendable for obtaining the maximum amount of steviol glycosides, followed by ultrasound at a low temperature (50ºC) and short treatment time (1 min). On the contrary, ultrasound and microwave energy degraded the antioxidant compounds of aqueous extracts of stevia, the conventional treatment being the most suitable for obtaining the greatest amount of phenols, flavonoids and total antioxidants. In this case, 90ºC and short treatment times (1 min) maximized the yield of these compounds.

Therefore, the optimum solid-liquid extraction conditions would depend on whether the aqueous extraction of stevia leaves is used for sweetening or for antioxidant purposes.

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References

[1] R. Lemus-Moncada, A. Vega-Gálvez, L. Zura-Bravo, K. Ah-Hen, *Stevia* rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects, Food Chem. 132 (2012) 1121-1132.

[2] JECFA. Joint FAO/WHO Expert Committee on Food Additives, Steviol glycosides. In Compendium of Food Additive Specifications, 69th Meeting, FAO/WHO Monographs 5, (2008) Rome, Italy.

[3] J.M. Carbonell-Capella, F.J. Barba, M.J. Esteve, A. Frígola, High pressure processing of fruit juice mixture sweetened with Stevia rebaudiana Bertoni: Optimal retention of physical and nutritional quality, Innov. Food Sci. Emerg. 18 (2013) 48–56.

[4] B.H. De Oliveira, J.F. Packer, M. Chimelli, D.A. De Jesus, Enzymatic modification of stevioside by cell-free extract of Gibberella fujikuroi. J. Biotechnol. 131 (2007) 92-96.

[5] V. Chatsudthipong, C. Muanprasat, Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacol. Therapeut. 121 (2009) 41-54.

[6] F. Muanda, R. Soulimani, B. Diop, A. Dicko. Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT-Food Sci. Technol. 44 (2011) 1865-1872.

[7] A Periche, G Koutsidis, I Escriche. Composition of antioxidants and amino acids in Stevia leaf infusions, Plant Foods Hum. Nutr. 69 (2014) 1-7.

[8] European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. EFSA Journal 2011; 9 (1):1972. doi:10.2903/j.efsa.2011.1972

[9] J. Kroyer (2010) Stevioside and Stevia-sweeter in food: application, stability and interaction with food ingredients. J. Verbr. Lebensm. 5 (2010) 225–229.

[10] M. Sališova, S. Toma, T.J. Mason, Comparison of conventional and ultrasonically assisted extractions of pharmaceutically active compounds from Salvia officinalis. Ultrason. Sonochem. 4 (1997) 131-134.

[11] H. Feng, G. Barbosa-Canovas, J.Weiss, Ultrasound Technologies for Food and Bioprocessing. Food Engineering Series, Springer, New York, 2011.

[12] M. Toma, M. Vinatoru, L. Paniwnyk, T.J. Mason, Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. Ultrason. Sonochem. 8 (2001) 137-142.

[13] V. Jaitak, B.S. Bandna, V.K Kaul, An efficient microwave-assisted extraction process of stevioside and rebaudioside-A from *Stevia* rebaudiana (Bertoni). Phytochem. Anal. 20 (2009) 240-245.

[14] J. Liu, J.W. Li, J. Tang. Ultrasonically assisted extraction of total carbohydrates from *Stevia* rebaudiana Bertoni and identification of extracts. Food Bioprod. Process. 88 (2010) 215-221.

[15] M.H. Ahmad-Qasem, J. Cánovas, E. Barrajón-Catalán, V. Micol, J.A. Cárcel, J.V. García-Pérez, Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. Innov. Food Sci. Emerg. 17 (2013) 120-129.

[16] C. Da Porto, E. Porretto, D. Decorti, Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (Vitis vinifera L.) sedes. Ultrason. Sonochem. 20 (2013) 1076-1080.

[17] L. Yang, Y.L. Cao, J.G. Jiang, Q.S. Lin, J. Chen, L. Zhu, Response surface optimization of ultrasound-assisted flavonoids extraction from the flower of Citrus aurantium L. var. amara. Engl. J. Sep. Sci. 33 (2010) 1349-1355.

[18] J.X. Wang, X.H. Xiao, G.K. Li, Study of vacuum microwave-assisted extraction of polyphenolic compounds and pigment from Chinese herbs. J. Chromatogr. A, 1198-1199 (2008) 45-53.

[19] C.C. Teo, J.W.H. Yong, S.N. Tan, E.S. Ong, Validation of green-solvent extraction combined with chromatographic chemical fingerprint to evaluate quality of *Stevia* rebaudiana Bertoni. J. Sep. Sci. 32 (2009) 613-622.

[20] U. Woelwer-Rieck, C. Lankes, A. Wawrzun, M. Wüst, Improved HPLC method for the evaluation of the major steviol glycosides in leaves of Stevia rebaudiana. Eur. Food Res. Technol. 231 (2010) 581-588.

[21] Y.H. Choi, I. Kim, K. Yoon, S.J. Lee, C.Y. Kim, K.P. Yoo, Y.H. Choi, J. Kim, Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from Stevia rebaudiana leaves. Chromatographia, 55 (2002) 617-620.

[22] Commission Decision 2002/657/EC of 12 August (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJEC L221, 8-36. Brusels, Belgium.

[23] S. Sakanaka, Y. Tachibana, Y. Okada, Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chem. 89 (2004) 569-575.

[24] V. Dewanto, X. Wu, K.K. Adom, R.H. Liu, Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agri. Food Chem. 50 (2002) 3010-3014.

[25] F. Shahidi, C.M. Liyana-Pathirana, D.S. Wall, Antioxidant activity of white and black sesame seeds and their hull fractions. Food Chem. 99 (2006) 478-483.

[26] F. Cacciola, P. Delmonte, K. Jaworska, P. Dugo, L. Mondello, J. Rader. Employing ultra-high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of Stevia rebaudiana extracts. J. Chromatogr. A 1218 (2011) 2012-2018.

[27] E. Kennelly, Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni) Bertoni. Stevia, the Genus of Stevia, Medicinal and aromatic plants- industrial profiles. Taylor and Francis, London, 2002.

[28] I. Prakash, G.E. Dubois, J.F. Clos, K.L. Wilkens, L.E. Fosdick, Development of rebaudiana, a natural, non-caloric sweetener. Food Chem. Toxicol. 46 (2008) 75−82.

[29] G. Zhang, L. He, M. Hu, Optimized ultrasonic-assisted extraction of flavonoids from Prunella vulgaris L. and evaluation of antioxidant activities in vitro. Innov. Food Sci. Emerg. 12 (2011) 18-25.

[30] A. Liazid, M. Palma, J. Brigui, C.G. Barroso, Investigation on phenolic compounds stability during microwave-assisted extraction. J. Chromatogr. A, 1140 (2007) 29–34.

[31] G.E. Inglett, D.J. Rose, D. Chen, D.G. Stevenson, A. Biswas, Phenolic content and antioxidant activity of extracts from whole buckwheat (Fagopyrum esculentum Möench) with or without microwave irradiation. Food Chem. 119 (2010) 1216-1219.

[32] M. Ya-Qin, C. Jian-Chu, L. Dong-Hong, Y. Xing-Qian, Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. Ultrason. Sonochem. 16 (2009) 57-62.

III. 4.COMPOSITION OF ANTIOXIDANTS AND AMINO ACIDS IN STEVIA LEAF INFUSIONS

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ABSTRACT

Stevia, a non-caloric natural sweetener with beneficial properties and considerable antioxidants and amino acids, is increasingly consumed as an infusion. This work evaluates the influence of the conditions (temperature: 50, 70 or 90ºC and time: 1, 5, 20 or 40 min) applied to obtain *Stevia* infusions, on antioxidants (total phenols, flavonoids and antioxidant activity) and amino acids. The total concentration of the eleven amino acids found was 11.70 mg/g in dried leaves and from 6.84 to 9.11 mg/g per gram of Stevia in infusions. However, infusions showed higher levels of certain amino acids (alanine, asparagine, leucine and proline), and greater values of the three antioxidant parameters in comparison with dry leaves. Temperature had more influence (minimum values at 50 °C and maximum at 90 °C) than time in the case of antioxidants. At 90ºC there were no important increases in the extraction of antioxidant compounds after 5 min; each gram of *Stevia* having117 mg trolox (Total antioxidant activity), 90 mg gallic acid (total phenols) and 56 mg catechin equivalents (flavonoids). Varying the temperature and time conditions no notable differences were observed in the concentrations of the majority of amino acids. However, the infusion treatment 90ºC for 5 minutes was the best, as it gave the highest yield of 8 of the 11 amino acids. Therefore, with respect to the compounds analyzed in this study, the best way to obtain *Stevia* leaf infusions is the same as the domestic process, almost boiling water for a short time.

Keywords: antioxidant activity, total phenols, flavonoids, amino acids, *Stevia*.

1.Introduction

Stevia rebaudiana Bertoni, (Asteraceae family) is a perennial plant from Brazil and Paraguay [1]. The main characteristic of *Stevia* leaves is high sweetness (250-300 times sweeter than sucrose) due to the diterpene compounds, called steviol glycosides [2]. The most common use of *Stevia* leaves is the extraction and purification of steviosides to obtain a non-caloric natural sweetener, as a sugar substitute, or as an alternative to artificial sweeteners [3]. Other authors have demonstrated that *Stevia* leaves also have beneficial properties, showing them to be: anti-inflammatory, diuretic, antihypertensive, antihyperglycemic, antidiarrehic, antitumoral and antioxidant [4]. These antioxidant effects, as in other plants, are in part due to the presence of flavonoids and phenolic compounds [5, 6]. Although several authors have studied the antioxidant

capacity of extracts from different plant leaves such as tea [7], mate [8] or mint [9], there are fewer works related specifically to infusions, except for the results given by Atuoi et al. [10] and Gorjanovic et al. [11] for tea and herbal teas [12,13], and by Samaniego et al. [14] for green tea.

In addition to the before mentioned properties, *Stevia* leaves, like other herbs such as Chinese tea [15] and black tea [16], have considerable amino acid content. In fact, Rafiq et al. [17] and Abou-Arab et al. [18] identified seventeen amino acids in *Stevia* leaves (glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine, arginine, histidine, methionine, phenylalanine, leucine, valine, threonine, glycine, cystine). *Stevia* leaves contain all the indispensable amino acids [19] with the exception of tryptophan.

Due to these therapeutic properties, *Stevia* leaves are consumed more and more as an aqueous extract of dried leaves. These extracts are drunk as a simple infusions or incorporated in different food formulations: juices, biscuits, jams, sweets, etc. This has become an option for the European industry as EFSA (European Food Safety Authority) recognized the safety of *Stevia* leaf extracts for alimentary use in November 2011 [20]. However, the use of *Stevia* was authorized in different Asian and American countries one decade ago.

As far as the authors know, there is no research related to the antioxidant properties and the free amino acid content of *Stevia* leaf infusions. For this reason, the aim of this study was to evaluate how the conditions (time and temperature) used to obtain infusions (from dehydrated *Stevia* leaves), affect amino acids, antioxidant capacity, total phenolic content, and total flavonoid content.

2.Material and Methods

2.1.Plant material and infusion preparation

Organically produced (based on minimizing the use of external inputs, avoiding the use of synthetic fertilizers and pesticides) dried leaves of *Stevia rebaudiana* (Raab, Vitalfood, Rohrbach, Germany) were used in this study. Aqueous extracts of dried *Stevia* leaves were obtained at atmospheric pressure and different temperatures using a thermostatic bath (JPSelecta Precisdig, Spain). 1g of dried *Stevia* leaf powder (ground in a grinding mill, A11 Basic, IKA, Germany) was dispersed in 100 mL of water.

Different temperatures (50, 70 and 90 ˚C) and times (1, 5, 20 and 40 minutes) were applied to obtain the infusions. It was decided to use these combinations of time and temperature in order to cover a wide range of possible treatment conditions, from less aggressive (low temperatures and short time) to more aggressive (high temperatures and long time). Subsequently, the aqueous extracts were filtered through filter paper and cooled before the analytical determinations. Although treatment at 50ºC and 70ºC is not really infusion, in this work in order to facilitate the terminology, all the thermal treatments are called "infusions". All the analyses were performed in triplicate.

2.2.Standard compounds and Reagents

A EZ-Faast amino acid kit (Phenomenex, Torrance, CA, USA) was used to carry out the amino acid analyses. This kit, in addition to chloroform, hydrochloric acid, isooctane, n-propanol, sodium carbonate and sodium hydroxide, contains the following amino acid standards: alanine (Ala), asparagine (Asn), aspartic acid (Asp), cistine (C-C), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val) and norvaline (Nor) ; all 99+% purity. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) were used to determine the total antioxidant activity. Sodium nitrite, (+)-catechin, sodium hydroxide (Sigma-Aldrich) and aluminum chloride hexahydrate (Fluka) were used for flavonoid analysis. Sodium carbonate, gallic acid and Folin-Ciocalteu reagent (all purchased from Sigma-Aldrich) were utilized for phenolic determination.

2.3.Determination of Free Amino Acids.

The free amino acid content of the *Stevia* infusion was measured using the derivatization technique for GC-MS [21] with the before mentioned EZ-Faast amino acid kit. The derivatized amino acids were extracted with isooctane/chloroform (100 μL) and analyzed using the 6890 GC-MS Agilent system. An aliquot of the derivatized amino acid solution (10 μL) was injected into a 10 m x 0.25 mm Zebron ZB-AAA capillary column (250 ºC in split mode, 5:1).The oven temperature was 110 ºC for 1 min, then increased at 30 ºC/min to 320 ºC, and held at 320 ºC for 2 min. The transfer line was held at 320 ºC, and the carrier gas flow rate was kept constant throughout the

run at 1.1 mL/min. The ion source was maintained at 220 ºC and the electron impact mode was 70 eV.

In order to calculate the amount of each amino acid in the infusions, a calibration curve (50, 100, 200, 350, 500, 700 nmol/mL) was plotted for each amino acid using the amino acid standard mixtures solution (200 nmol/mL). The area of each amino acid was measured relative to the area of internal standard, norvaline (m/z 158 ion).

2.4.Determination of total phenolic content

The total phenolic content was determined spectrophotometrically by the modified Folin-Ciocalteu method [22]. Absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The quantification was made considering a standard curve of gallic acid and the results were expressed as mg of gallic acid equivalent per gram of *Stevia* (dry matter).

2.5.Determination of total flavonoid content

Total flavonoid content was determined using the modified colorimetric method described by Dewanto et al. [23]. Absorbance was measured at 510 nm. The quantification was made considering a standard curve of of (+)-catechin and the results were expressed as mg of (+)-catechin equivalent per gram of *Stevia* (dry matter).

2.6.Determination of total antioxidant activity

The antioxidant activity (AA) of the extract was analyzed on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl free radical as described by Shahidi et al. [24] with some modifications. Absorbance of the sample was measured at 515 nm using methanol as a blank. The quantification was made considering a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the results were expressed as mg of Trolox equivalent per gram of *Stevia* (dry matter).

2.7.Statistical analysis

A multifactor ANOVA (with LSD test and $\alpha = 0.05$), using the Statgraphics Centurion program, was applied to study the effect of temperature and time on the amino acids, total phenols, flavonoids and antioxidant activity. The interaction between both factors was also considered. A Principal Component Analysis (PCA), with the

software Unscrambler X.10., was also applied to describe the relationships between the treatments and the variables analysed.

3.Results and Discussion

3.1.Free amino acids in Stevia infusions: Influence of time and temperature conditions.

The average values and the standard deviations of the eleven free amino acids (Ala, Asn, Asp, Glu, Ile, Leu, Phe, Pro, Ser, Tyr and Val) quantified in dried *Stevia* leaves are shown in Table 1. The data corresponding to these compounds in the infusions (obtained at different temperatures: 50, 70, 90ºC and times: 1, 5, 20, 40 minutes) are available in Online Resource 1. In addition, Table 1 shows the multifactor ANOVA results (homogenous groups, F-ratios for time and temperature factors and the interactions of both factors) for each of the analyzed compounds.

Of the eleven amino acids found in this study, four of them (Ile, Leu, Phe and Val) are recognized by the FAO as indispensable. The total amino acid concentration, considering the sum of all the compounds, was higher in the dried leaves (11.70 mg/g) than in infusions; in which the maximum values for the majority of the compounds analyzed was achieved with the treatment 90 °C, 5 minutes. Compounds such as Glu, Phe, Ser and Tyr showed greater concentration in the dried leaves (0.95, 0.30, 8.10 and 0.12 mg/g, respectively) than in infusions, as the average values obtained for these compounds considering all treatments were: 0.38, 0.06, 4.43 and 0.05 mg/g, respectively.

ESM 1: Means and standard deviations of amino acids (AA) in stevia leaf infusions, expressed as mg/g of stevia.

	INFUSIONS											
	50ºC				70ºC				90ºC			
AA	**1 min**	**5 min**	**20 min**	**40 min**	**1 min**	**5 min**	**20 min**	**40 min**	**1 min**	**5 min**	**20 min**	**40 min**
Ala	0.49(0.04)	0.499(0.009)	0.639(0.112)	0.55(0.04)	0.54(0.03)	0.58(0.02)	0.56(0.05)	0.535(0.014)	0.57(0.02)	0.62(0.03)	0.55(0.02)	0.55(0.04)
Asn	0.32(0.08)	0.361(0.014)	0.40(0.03)	0.31(0.03)	0.32(0.04)	0.41(0.03)	0.38(0.04)	0.31(0.03)	0.372(0.005)	0.42(0.02)	0.35(0.03)	0.39(0.03)
Asp	0.48(0.5)	0.43(0.04)	0.36(0.06)	0.41(0.07)	0.39(0.03)	0.44(0.02)	0.49(0.08)	0.45(0.06)	0.47(0.04)	0.44(0.05)	0.38(0.09)	0.41(0.14)
Glu	0.34(0.08)	0.27(0.04)	0.32(0.04)	0.41(0.06)	0.41(0.07)	0.31(0.02)	0.42(0.09)	0.40(0.12)	0.44(0.12)	0.46(0.15)	0.40(0.16)	0.38(0.19)
Ile	0.195(0.017)	0.179(0.007)	0.221(0.013)	0.20(0.03)	0.21(0.03)	0.206(0.005)	0.23(0.04)	0.211(0.015)	0.218(0.005)	0.240(0.012)	0.210(0.012)	0.226(0.016)
Leu	0.079(0.003)	0.088(0.006)	0.158(0.004)	0.155(0.012)	0.108(0.008)	0.110(0.004)	0.112(0.012)	0.114(0.009)	0.094(0.005)	0.113(0.008)	0.102(0.009)	0.095(0.012)
Phe	0.046(0.012)	0.051(0.005)	0.077(0.012)	0.082(0.012)	0.064(0.009)	0.067(0.008)	0.066(0.02)	0.069(0.017)	0.07(0.04)	0.071(0.005)	0.066(0.008)	0.07(0.03)
Pro	0.9 (0.2)	0.93(0.14)	1.1(0.3)	0.85(0.08)	0.91(0.02)	1.1(0.1)	0.995(0.07)	0.89(0.06)	0.94(0.12)	1.1(0.2)	0.97(0.17)	1.1(0.2)
Ser	4.2(0.8)	5.04(0.06)	4.5(0.3)	4.1(0.9)	3.5(0.7)	5.6(0.3)	4.6(0.9)	3.9(0.5)	4.6(0.7)	4.5(0.8)	4.43(1.14)	3.8(1.2)
Tyr	0.055(0.018)	0.054(0.003)	0.068(0.009)	0.067(0.017)	0.053(0.003)	0.059(0.002)	0.055(0.013)	0.053(0.004)	0.052(0.006)	0.053(0.004)	0.052(0.012)	0.055(0.009)
Val	0.225(0.012)	0.227(0.012)	0.278(0.013)	0.27(0.02)	0.263(0.014)	0.266(0.017)	0.279(0.018)	0.266(0.007)	0.265(0.009)	0.286(0.015)	0.260(0.006)	0.269(0.018)
Total	7.43	8.14	8.26	7.41	6.84	9.11	8.26	7.23	8.21	8.35	7.79	7.24

Table 1. Amino acids (mg /g stevia) in dried leaves (mean and standard deviation) and multifactor ANOVA results for these amino acids in stevia infusions obtained at different temperatures and treatment times. F-ratios for each of the two factors (temperature and time) and their interactions are included.

	Dried Leaves	ANOVA INFUSIONS									
		Temperature (T)				Time (t)					Interaction
Amino acids	**mean(SD)**	**50**	**70**	**90**	**F-ratio**	**1**	**5**	**20**	**40**	**F-ratio**	**T*t**
Ala	0.34(0.02)	0.54^{a}	0.55^{a}	0.57^{a}	1.21^{ns}	0.53^{a}	0.56^{ab}	0.58^{b}	0.54^{ab}	1.81^{ns}	3.49^{*}
Asn	0.36(0.14)	0.35^{a}	0.35^{ab}	0.38^{b}	3.05^{ns}	0.33^{a}	0.39^{b}	0.38^{b}	0.33^{a}	5.7^{**}	2.19^{ns}
Asp	0.38(0.04)	0.42^{a}	0.44^{a}	0.42^{a}	0.49^{ns}	0.44^{a}	0.44^{a}	0.41^{a}	0.42^{a}	0.51^{ns}	1.52^{ns}
Glu	0.95(0.12)	0.34^{a}	0.38^{a}	0.42^{a}	1.59^{ns}	0.40^{a}	0.34^{a}	0.38^{a}	0.39^{a}	0.41^{ns}	0.73^{ns}
Ile	0.26(0.03)	0.20^{a}	0.21^{ab}	0.22^{b}	3.72^{*}	0.20^{a}	0.20^{a}	0.22^{a}	0.21^{a}	0.89^{ns}	1.75^{ns}
Leu	0.082(0.006)	0.11^{b}	0.11^{b}	0.10^{a}	17.51^{**}	0.09^{a}	0.10^{b}	0.12^{c}	0.12^{c}	27.92^{**}	28.47^{**}
Phe	0.30(0.06)	0.06^{a}	0.06^{a}	0.07^{a}	0.56^{ns}	0.06^{a}	0.06^{a}	0.06^{a}	0.07^{a}	0.89^{ns}	0.9^{ns}
Pro	0.55(0.04)	0.96^{a}	0.95^{a}	1.00^{a}	0.26^{ns}	0.92^{a}	1.00^{a}	1.03^{a}	0.91^{a}	0.98^{ns}	0.52^{ns}
Ser	8.1(0.7)	4.48^{a}	4.44^{a}	4.36^{a}	0.06^{ns}	4.17^{a}	5.08^{b}	4.55^{ab}	3.92^{a}	3.3^{*}	0.93^{ns}
Tyr	0.12(0.08)	0.05^{a}	0.05^{a}	0.06^{a}	2.3^{ns}	0.05^{a}	0.05^{a}	0.05^{a}	0.05^{a}	0.7^{ns}	0.9^{ns}
Val	0.29(0.02)	0.25^{a}	0.26^{b}	0.26^{b}	5.57^{*}	0.25^{a}	0.25^{ab}	0.27^{b}	0.27^{b}	4.47^{*}	5.69^{**}
Total	11.7	7.79	7.85	7.80	-	7.49	8.53	7.97	7.28	-	-

For each factor. different letters in each row indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test). ns=Not significant; * *p<0.05;* ** *p<0.01*

The amount of Ala, Asp, Leu and Pro in dried leaves (0.34, 0.38, 0.08 and 0.55 mg/g, respectively) was lower than in infusions (average values considering all treatments: 0.56, 0.43, 0.11 and 0.97 mg/g, respectively). However, the values obtained for Leu in the less aggressive treatments (50°C during 1 and 5 minutes) were almost identical to those for the dried leaves. Other compounds such as Asn, Ile and Val showed practically no differences between the concentration obtained for the dried leaves (0.36, 0.26 and 0.29, respectively) and for the infusions (average values: 0.37, 0.22 and 0.26, respectively).

Considering the abundance of the different compounds, Serine on its own accounts for 68.8% of the total amino acids quantified in dry leaves and 56.4 % of the total amino acids quantified in infusions (average value of those obtained in all the treatments). In addition to Serine, other amino acids were also quite abundant in dried leaves: Asp (3.2%), Glu (8.1%) and Pro (4.7%), which represented 16.1% of the total. Likewise, with respect to the infusions, after Serine the next three most abundant compounds were Ala (7.1%), Asp (5.5%) and Pro (12.4%), accounting for 25.1% of total.

With respect to the influence of temperature and time on the evolution of the different compounds, the ANOVA showed that both factors had practically no influence on the concentration of the compounds. Of the 11 compounds quantified, only three of them presented significant differences for temperature (Val, Leu and Ile) and four of them for time (Val, Leu, Ser and Asn). However, it is important to note that although the statistical analysis revealed significant differences, the observation of the values shows that actually these were very small.

There is little information relating to free amino acids in *Stevia*. Rafiq et al. [17] only identified eight of them in *Stevia* leaves (Ala, Ile, Ser, Pro, Asp, Glu, Tyr and Lys). All of these amino acids were found in this study, with the exception of Lys. However, Val, Leu, Phe, and Asn, found in this study, were not found by the above authors. Li et al. [25] and Abou-Arab et al. [18] identified 15 amino acids (Glu, Asp, Lys, Ser, Ile, Ala, Pro, Tyr, Arg, His, Phe, Leu, Val, Thr and Gly) in *Stevia* leaves previously subjected to protein hydrolysis. Besides these, the latter authors found Met and Cys, as well. All the amino acids present in *Stevia* in this study (with the exception of Val), are also found in green and black tea [15] with the total quantity ranging between 1.19 and 6.98 mg/g, depending on the tea variety. These data were somewhat lower than those

found in this work for *Stevia* infusions (6.84 and 9.11 mg/g). However, for other varieties of green tea, Ding et al. [26] reported higher values, between 24.70 and 33.50 mg/g.

3.2. Antioxidant activity, total phenols and flavonoids in Stevia infusions: Influence of time and temperature conditions.

Table 2 shows the average values and the standard deviations of total phenols, flavonoids and antioxidant activity found in dried *Stevia* leaves, as well as the multifactor ANOVA results (homogenous groups, F-ratios for the factors time and temperature, and the interactions of both factors). Higher concentrations of the three parameters were found in the infusions than in the dried leaves.

The time/temperature interaction (95% LSD interval), obtained from the ANOVA, is included in Fig. 1 in order to facilitate the comparison of variability patterns between factors. Fig. 1 shows that the evolution of these parameters depends on the temperature and the time conditions applied to obtain the infusions. For the three parameters analyzed, the higher the temperature the greater the aqueous extraction. Although both factors had a significant influence, temperature had greater impact than time, which is reflected by the F-ratio values (F ratio of temperature ranged from 323.74 to 490.57 and F ratio of time ranged from 2.52 to 21.97) on the three parameters analyzed.

Considering treatment time, it can be stated that, practically, it does not influence the result of the total phenol content and flavonoids. However, time had a greater influence in the case of total antioxidant activity when compared to the other two parameters. For antioxidant activity, there was a significant increase in concentration from minute 5 (52 mg trolox/g) to minute 40 (82 mg trolox/g) at 50 ºC. For the other two temperatures (70 and 90ºC), the behavior with time was similar, with a maximum value at 20 min, and without significant differences with respect to 40 min. The difference with time for total antioxidant activity with respect to phenols and flavonoids could be due to the presence of other compounds with antioxidant capacity, which can contribute to total antioxidant activity [31].

Table 1. Total phenols (mg gallic acid equivalent/g stevia), flavonoids (mg catechin equivalent/g stevia) and antioxidant activity (mg Trolox equivalent/g stevia) in dried leaves (mean and standard deviation) and multifactor ANOVA results for these compounds in stevia infusions obtained at different temperatures and treatment times. F-ratios for each of the two factors (temperature and time) and their interactions are included.

Antioxidant parameters	**Dried Leaves mean (SD)**	**ANOVA INFUSIONS**									
		Temperature (T)				**Time (t)**					**Interaction**
		50	**70**	**90**	**F-ratio**	**1**	**5**	**20**	**40**	**F-ratio**	**T*t**
Phenols	63.8(1.3)	65.20^{a}	71.02^{b}	92.07^{c}	323.74^{***}	71.70^{a}	75.71^{b}	77.34^{c}	79.63^{c}	9.38^{**}	2.16^{ns}
Flavonoids	22.2(0.9)	32.47^{a}	47.53^{b}	56.72^{c}	490.57^{***}	44.64^{a}	45.93^{b}	45.77^{ab}	44.26^{a}	2.52^{*}	1.74^{ns}
Antioxidants	48(2)	62.45^{a}	106.68^{b}	119.12^{c}	344.71^{***}	87.96^{a}	89.26^{a}	102.64^{b}	104.48^{b}	21.97^{**}	5.67^{**}

For each factor, different letters in each row indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test). ns=Not significant; * *p<0.05;* ** *p<0.01;* ****p<0.001*

Regarding the total phenol content, Tadhani et al. [27] reported lower average values (25.18 mg gallic acid/g) in dried *Stevia* leaf extracts (obtained with HCl 0.3N and methanol) than those obtained in this paper (63.80 mg gallic acid/g). Shukla et al. [5, 27] reported a total phenol content of 56.74 and 61.50 mg of gallic acid/g in ethanolic and aqueous *Stevia* extracts respectively (obtained by a maceration process at room temperature). These values are similar to those obtained in this paper at 50 °C (average values: 65.20 mg gallic acid/g *Stevia*) but below those obtained at 70ºC and 90ºC (average values considering all treatments: 71.02 and 92.07 mg gallic acid/g), respectively.

With respect to the total flavonoids (Table 2), a level of 22.20 mg catechin/g dried leaves of *Stevia* was found in this study. Muanda et al. [6] obtained similar values (20.68 mg catechin/g) in dried *Stevia* leaf aqueous extracts, as well. Other authors reported values for the total flavonoids in this matrix but expressed in terms of other compounds: 21.73 mg gallic acid/g [27], and 0.83 mg quercetin/g [29]. In addition, the extracts were obtained using different solvents: HCl 0.3N and methanol by the first authors and ethyl acetate extract and methanol by the second ones. Kim et al. [30] obtained values of 15.64 mg quercetin per gram of *Stevia* in infusions (3 hour at 100 °C).

In relation to antioxidant activity, the average value obtained in this study was 48.17 mg trolox/g of dried *Stevia* leaves (aqueous extraction). This value was higher than reported by Tadhani et al. [27] (38.24 mg trolox/g).

The evolution of antioxidant activity, with temperature and time, observed in this study was similar to that described by other authors for tea infusions. Specifically, Samaniego et al. [14] reported that at 90 ºC, the extraction of polyphenol was faster and more effective than at lower temperatures, as long as time does not exceed 5 minutes. These authors highlight that higher times at this temperature may cause the loss of polyphenol compounds and, consequently, of antioxidant capacity.

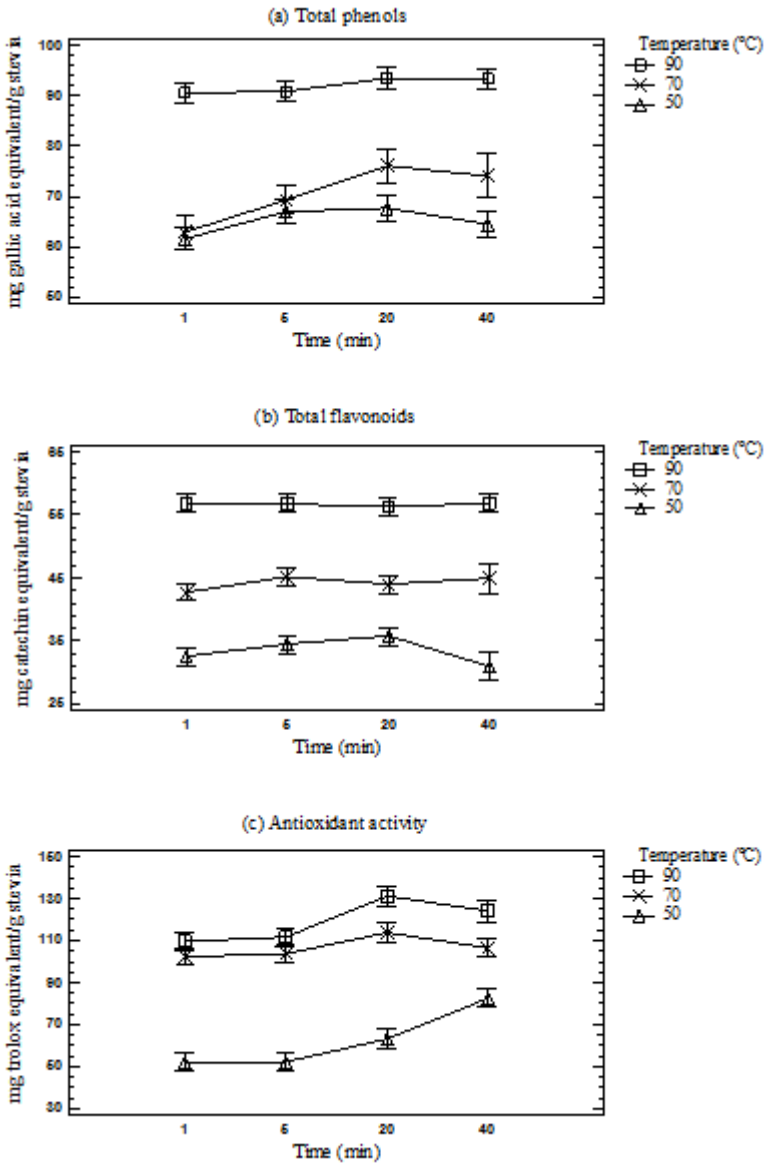

Figure III.4.1. Time-temperature interaction (95% LSD interval) of the antioxidant parameters: total phenols (a), total flavonoids (b) and antioxidant activity (c), obtained from the ANOVA.

3.3.Global behavior of antioxidant properties and amino acid composition

Once the individual behavior of amino acids and antioxidant compounds were analyzed, a PCA was used to assess the overall effect of the conditions (time and temperature) used to obtain the *Stevia* infusions. Fig. 2 shows the PCA biplot (scores "treatments" and loading "variables") obtained. The first two components explained 65 % of the total variance (PC1, 39 % and PC2, 26 %). The proximity between infusion treatments implies similar behaviour, while the proximity between variables implies the

degree of correlation between them. Taking this into consideration, the infusion "90ºC for 5 minutes" (90_5) placed at the far end of the right axis had the most amino acids (except Asp, Ser and Tyr) and antioxidants. On the contrary, the samples (50_1 and 50_5) situated on the opposite side (left axis), had the lowest level of the majority of the analyzed variables.

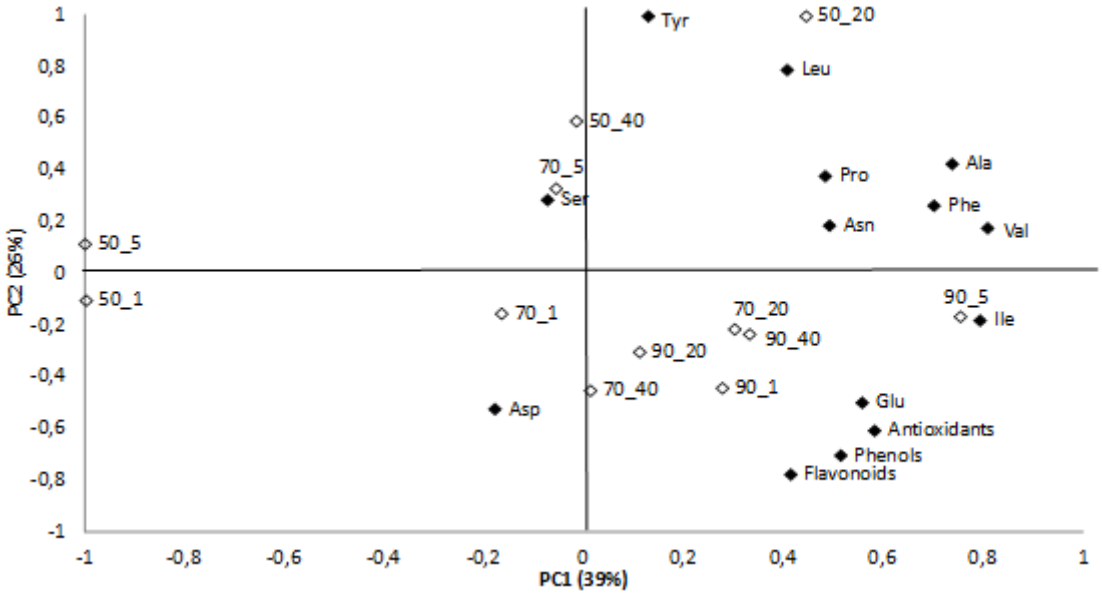

Figure III.4.2. Bi-plot of Principal Component Analysis for the infusion treatments (white diamond ◊) and the analysed variables: amino acids and antioxidant parameters (total phenols, total flavonoids and antioxidant activity) (black diamond ♦).

4.Conclusions

Infusions of Stevia leaves have higher levels of both antioxidants (total phenols, flavonoids and antioxidant activity) and certain amino acids such as Ala, Asp, Leu and Pro, in comparison with dry Stevia leaves. Temperature has a greater effect than time in the case of the three antioxidant parameters, so the higher the temperature, the greater the aqueous extraction. Minimum values for these parameters were obtained at 50 °C and maximum at 90 °C. At this last temperature there were no important improvements in the extraction of these compounds after 5 min. With respect to the majority of amino acids, no important differences were observed in their concentrations as a consequence of varying the temperature and time conditions. However, the infusion treatment 90ºC for 5 min can be considered the most appropriate since it promoted a small increment in the concentration of 8 of the 11 compounds. Therefore, with respect to the compounds

analyzed in this study, the best conditions for obtaining Stevia leaf infusions are the most similar to the domestic culinary process, very hot water (slightly below boiling) for a short time. Given that infusions have been shown to be efficacious in the extraction of antioxidants, it would be interesting to investigate the profile of specific antioxidants further.

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References

1. Goyal SK, Samsher R, Goyal RK (2010) Stevia (*Stevia rebaudiana*) a bio-sweetener: A review. Int J Food Sci Nutr 61: 1–10.
2. Hanson JR, De Oliveira BH (1993) Stevioside and related sweet diterpenoid glycosides. Nat Prod Res 10: 301-309.
3. Anton S, Martin C, Han H, Coulon S, Cefalu W, Geiselman P et al. (2010) Effects of *Stevia*, aspartame, and sucrose on food intake, satiety and postprandial glucose and insulin levels. Appetite 55: 37–43.
4. Chatsudthipong V, Muanprasat C (2009) Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacol Therapeut 121:41–54.
5. Shukla S, Mehta A, Bajpai V (2009) In vitro antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food Chem Toxicol 47: 2338-2343.
6. Muanda F, Soulimani R, Diop B, Dicko A (2011) Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT-Food Sci Technol 44:1865-1872.
7. Yao L, Jiang Y, Datta N, Singanusong R, Liu X, Duan J, Raymont K, Lisle A, Xu Y(2004) HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (Camellia sinenesis) grown in Australia. Food Chem 84: 253-263.
8. Dugo P, Cacciola F, Donato P, Assis R, Bastos E, Mondello L (2009) High efficiency liquid chromatography techniques coupled to mass spectrometry for the characterization of mate extracts. J Chromatogr A 43:7213-7221.
9. Biswas AK, Chatli MK, Sahoo J (2012) Antioxidant potential of curry (Murraya koenigii L.) and mint (Mentha spicata) leaf extracts and their effect on colour and oxidative stability of raw ground pork meat during refrigeration storage. Food Chem 133:467-472.

10. Atoui K, Mansouri A, Boskou G, Kefalas P (2005) Tea and herbal infusions: Their antioxidant activity and phenolic profile. Food Chem 89, 27-36.
11. Gorjanovic S, Komes D, Pastor F, Belscak A, Pezo L, Hecimovic I, Suznjevic D (2012) Antioxidant Capacity of Teas and Herbal Infusions: Polarographic Assessment. J Agric Food Chem 60: 9573-9580.
12. Quispe C, Viveros-Valdez E, Schmeda-Hirschmann G (2012) Phenolic Constituents of the Chilean Herbal Tea *Fabiana imbricata* R. et P. Plant Food Hum Nutr 67:242–246.
13. Büyükbalci A, Nehir El S (2008) Determination of In Vitro Antidiabetic Effects, Antioxidant Activities and Phenol Contents of Some Herbal Teas. Plant Food Hum Nutr 63:27–33.
14. Samaniego C, Inurreta Y, Quesada JJ, Blanca R, Villalón M, López García H, López Martínez MC (2011) The influence of domestic culinary processes on the Trolox Equivalent Antioxidant Capacity of green tea infusions. J Food Compos Anal 24:79-86.
15. Wang L, Xu R, Hu B, Li W, Sun Y, Tu Y, Zeng X (2010) Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction. Food Chem 123: 1259–1266.
16. Gökmen V, Serpen A, Mogol BA (2012) Rapid determination of amino acids in foods by hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry. Anal Bioanal Chem 403: 2915–2922.
17. Rafiq M, Dahot U, Mangrio SM, Naqvi HA, Qarshi IA (2007) In vitro clonal propagation and biochemical analysis of field established Steva rebaudiana Bertoni. Pak J Bot 39: 2467-2474. ISSN:2070-3368.
18. Abou-Arab AE, Abou-Arab AA, Abu-Salem MF (2010) Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana* Bertoni plant. AJFS 4: 269–281. ISSN:1996-0794.
19. WHO (2007). Report of a Joint WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition (2002, Geneva, Switzerland). WHO Technical Report Series; No. 935.
20. European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. EFSA Journal 2011; 9 (1):1972. doi:10.2903/j.efsa.2011.1972
21. Husek P (2000) Method of preparing sample for amino acid analysis and kit for analyzing the same. Eur Patent Appl EP 1033576.
22. Sakanaka S; Tachibana Y; Okada Y (2004) Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chem 89: 569-575.
23. Dewanto V, Wu X, Adom KK, Liu RH (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agri Food Chem 50:3010-3014.

24. Shahidi F, Liyana-Pathirana CM, Wall DS (2006) Antioxidant activity of white and black sesame seeds and their hull fractions. Food Chem 99: 478-483.

25. Li G, Wang R, Quampah A, Rong Z, Shi C, Wu J (2011) Calibration and Prediction of Amino Acids in *Stevia* Leaf Powder Using Near Infrared Reflectance Spectroscopy. J Agri Food Chem 59: 13065–13071.

26. Ding Y, Yu H, Mou S (2002) Direct determination of free amino acids and sugars in green tea by anio-exchange chromatography with integrated pulsed amperometric detection. J Chromatogr A 928:237-244.

27. Tadhani M, Patel V, Subhash R (2007) In vitro antioxidant activities of *Stevia rebaudiana* leaves and callus. J Food Compos Anal 20: 323–329.

28. Shukla S, Mehta A, Menta P, Bajpai V (2012) Antioxidant ability and phenolic content of aqueous leaf extract of *Stevia rebaudiana* Bert. Exp Toxicol Pathol 64:807-811.

29. Ghanta S, Banerjee A, Poddar A, Chattopadhyay S (2007) Oxidative DNA damage preventive activity and antioxidant potential of *Stevia rebaudiana* (Bertoni) Bertoni, a natural sweetener. J of Agri Food Chem 55:10962–10967.

30. Kim I, Yang M, Lee O, Kang S (2011) The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. LWT Food Sci Technol 44: 1328–1332.

31. Michiels JA, Kevers Cl, Pincemail J, Defraigne JO, Dommes J (2012) Extraction condicitons can greatly influence antioxidant capacity assays in plant food matrices. Food Chem 130:986-993.

III.5. OPTICAL, MECHANICAL AND SENSORY PROPERTIES OF BASED-ISOMALTULOSE GUMMY CONFECTIONS

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ABSTRACT

The replacement of traditional sugars by isomaltulose could be a revolution in the confectionery sector, since isomaltulose is a functional, digestible, non-cariogenic and low glycemic disaccharide. This study assesses the addition of isomaltulose (ranging between 30-70% in combination with fructose) with different percentages of gelatine (6-10%) in gummy confection by analyzing its effect on composition, water activity (a_w), pH, mechanical and optical properties, and sensory perception. Results show that the combination of 30% isomaltulose and 70% fructose in the total amount of sugars would be suitable for developing functional gummy confections. Besides its stability (a_w (0.79±0.02) and ºBrix (73.5±1.3)) and great similarity to commercial gummies in terms of optical and mechanical properties, it received high global acceptability and intention of buying scores. Additionally, the correlation between instrumental and sensory parameters leads to the conclusion that the instrumental texture could be suitable for evaluating consumer's global acceptability for this innovative product.

Keywords: gummy confections, isomaltulose, fructose, non-cariogenic, glycemic index and insulinemic index.

1.Introduction

Confectionery products are not exactly foods, but they are widely consumed by children and adults. According to the Spanish association of confectionary products, more than 50% of adults regularly consume candies and chewing gums (Martínez, 2012). Confectionery is a lucrative and continuously growing market in Europe. Between 2005 and 2009 the whole category of confectionery products increased by 19% and it is expected it to grow by 16% through 2014 (Moloughney, 2011). The growth in the consumption of confectionery products is related to the pleasurable effects and wellness they are capable of providing us when consumed in moderate quantities. In fact, O´Neil, Fulgoni and Nicklas (2011) reported a lower body fat index and precursors for type 2 diabetes development in subjects who consumed a moderate amount of confectionaries compared to those who do not eat these products. Nevertheless, excessive consumption has been associated with a high incidence of some health diseases such as obesity, tooth decay and hyperglycemia. Despite the positive effects of their consumption in moderation, the overconsumption of confectionery products by children continues to concern parents.

Among confectionery products, gummy confection is second in sales (Moloughney, 2011). Therefore, there is continual consumer demand for more exciting textures, flavors and appearances in gummy confections. In addition, consumer demand is turning away from traditional products to low-sugar or healthier products. Traditional gummy confection consists of high amounts of sucrose and glucose syrup combined with a gelling agent, commonly known as gelatine, along with acids, flavorings and colourings (Marfil *et al.,* 2012). The replacement of sucrose and glucose syrup with healthier natural sugars could lead to the production of added value gummy confections. In this context, isomaltulose has been pointed out as a suitable sucrose replacer in most food and beverages (Lina *et al.,* 2002). Isomaltulose is a reducing sugar occurring naturally, in little quantity, in honey, sugar cane juice and some molasses (Bárez *et al*., 2000). Commercial isomaltulose, also known as Palatinose®, is obtained from sucrose by enzymatic rearrangement of the glycosidic linkage from a (1,2)-fructoside to a (1,6)-fructoside followed by crystallization (Schiweck *et al.,* 1990). Isomaltulose is characterized as having a profile of color, texture and taste which is similar to sucrose (regular sugar) although there are some differences. It has only half the sweetening power of sucrose and its solubility is only 30% at 25ºC (Kaga and Mizutani, 1985; Schiweck *et al*., 1990). In terms of health, the linkage (1,6)-fructoside is hardly hydrolyzed by enzymes produced by oral bacteria, therefore isomaltulose preserves dental health due to prevention of tooth decay (Matsuyama *et al.,* 1997). It is also considered suitable for the formulation of foods for athletes and diabetics because of its low-glycemic and low-insulinemic indexes (Kawai *et al.,* 1989; Lina *et al*., 2002), since it provides the same amount of energy as common sugar, but for a significantly longer period. Unlike artificial sweeteners such as sodium cyclamate, saccharin, aspartame, polyols (sorbitol), isomaltulose has not laxative effect. In fact, only bifidobacteria, no enterobacteria, are able to ferment isomaltulose, which limits the growth of microorganisms of putrefaction to cause diarrhea (Weidenhagen and Lorenz, 1957).

As mentioned before, the main technological handicap for the successful replacement of sucrose and glucose syrup with isomaltulose in gummy confections could be its lower solubility and sweetening power than common sugar. Therefore, the mixture of isomaltulose with other natural healthier sugar, such as fructose (common sugar in the formulation of sweet foods) could be an alternative, which solves these problems. Fructose is one of the sugars found in plants, fruits and especially in honey.

Industrially, the hydrolysis of sugar cane leads to an equal amount of glucose and fructose. The most important properties of this sugar are its sweetening power, which is nearly twice that of sucrose, and its high hygroscopicity (ideal for syrups) and insulin-independent metabolism, which has led it to become the quintessential substitute for sucrose. Although recent studies have refuted this property since they show that fructose is ultimately metabolized as glucose, and is therefore not recommended for diabetics (Elliot *et al.,* 2002), fructose is safe for healthy individuals as long as it is consumed in moderate quantities (Mann *et al*., 2004).

Any substitution of one ingredient by another, or by a combination of ingredients, can affect the physical and chemical properties of the food matrix, and therefore sensory acceptability. In this context, the aim of this study was to evaluate the possible replacement of sucrose and glucose syrup with isomaltulose and fructose, by analyzing their effect on physicochemical, textural and optical properties in different gummy confection formulations. Additionally, a sensory acceptance study was carried out and a correlation between instrumental measures and sensory attributes was made for the formulation, which most resembled (from the point of view of instrumental parameters) commercial gummy confections.

2. Materials and methods

2.1. Materials

Isomaltulose (Beneo-Palatinit; Germany), sucrose (Azucarera Ebro S.L.; Spain), fructose (Gabot Biochemical Industries; Israel), glucose syrup 43 DE (Emilio Peña, S.A., Spain), corn starch (Roquette, France), gelatine A 220 Bloom (Junca Gelatines S.L.; Spain), strawberry flavouring (Flavorix Aromáticos S.A.; Spain), natural red liquid colour (Roha Europe S.L.; Spain) and sunflower oil (Koipesol, Spain) were used as ingredients in the formulation of gummy confections.

2.2. Experimental Procedure

The gummy confections prepared consisted of 6-10 % of gelatine, 40% of water and 50-54% of sugars as recommended for gummy confections (Edwards, 2002). Also, 0.2 ppm of red coloring and 0.5 ppm of strawberry flavoring were added in all cases. Six different mixtures of sugars were studied. The control sample (code: S) was prepared with 40% of sucrose and 60% of glucose syrup (40:60 (w/w)) of the total sugar

content. Other samples were obtained combining different sugars (isomaltulose, glucose syrup or fructose). In order to simplify the description of each sample, the percentage of the total amount of sugars replaced is shown between brackets along with the code used: isomaltulose: glucose syrup (40:60, w/w), (code: I), fructose:glucose syrup (40:60, w/w) (code: F); isomaltulose:fructose (30:70, w/w) (code: I30) and isomaltulose:fructose (50:50, w/w) (code: I50). In this study, the gelatine percentage (6, 8 or 10%) was always shown next to these codes. In addition to the control sample, a total of 14 different formulations were studied.

A thermal blender (Thermomix, TM31, Vorwerk, Germany) was used to blend the sugars and water until they reached boiling temperature at 300 rpm for 10 minutes. This mixture was shaken until reaching 60ºC following which pH and ºBrix were measured. The gelatine was then dissolved in water in a gelling agent: water ratio of 1:2 (w/w) to obtain a homogeneous mix and subsequently added to the syrup with the flavoring and coloring agents. All the ingredients were blended for 5 minutes at 60ºC and 300 rpm. The final mixture was poured into silicone moulds with a thin layer of sunflower oil. The silicone moulds have cylindrical shape with a diameter of 28 mm and a height of 20 mm. Then, the moulds were placed in a chamber at 20 °C for 24 hours. The samples were removed from their mould and analyses of texture, color, water activity and moisture performed.

2.3. Analytical determinations

2.3.1 Physicochemical Analyses

Soluble solid content (ºBrix) (measured with a refractometer at 20ºC, ATAGO 3 T), and pH (by a pH-meter, SevenEasy, Mettler Toledo) were evaluated in the initial syrup. Moisture content (obtained gravimetrically by drying to a constant weight in a vacuum oven at 60ºC (AOAC, 20.103, 2000) and water activity (by dew point hygrometer, Aqualab, 4TE) were measured on the final products. All analyses were carried out in triplicate.

2.3.2 Color

Instrumental measurements of color were conducted at room temperature in a Minolta spectrophotometer (model CM-3600d) by placing the gummy confections on the diaphragm aperture (8 mm). CIEL*a*b* coordinates were obtained using illuminant

D65 and standard observer (10° visual field) as references. The parameters registered were: L* (brightness), a* (red component), b* (yellow component), chrome ($C^*=[(a^*)^2+(b^*)^2]^{1/2}$) and hue ($h^*=arctg(b^*/a^*)$). The samples were previously measured with both black and white calibration tiles in order to study the possible translucency of the samples, since different spectrum was obtained with the black and white tiles. The results were analyzed using the Kubelka Munk theory (Kubelka and Munk, 1931).

2.3.3 Texture

The samples which have the same shape and dimensions as the silicone moulds were subjected to an instrumental texture profile analysis (TPA) test using a TA.XT plus Texture Analyzer (Stable Micro Systems, U.K.) equipped with a load cell of 50 kg and a 45 mm diameter cylindrical probe. The test conditions involved two consecutive cycles of 50% compression with 15 s between cycles. The test speed was 1 mm/s. From the resulting force-time curve the following parameters were quantified, and are defined by Bourne (1978) as: hardness (N) (maximum peak force during the first compression cycle), springiness (the ratio between the time of the beginning of the second cycle and the time of the end of the first cycle), cohesiveness (the ratio of the positive force area during the second compression and the first compression), gumminess (N) (hardness x cohesiveness).

2.4. Sensory Evaluation

An acceptance test using a 9-point hedonic scale (ISO 4121:2003) was used to evaluate the following attributes: appearance, color, strawberry flavor, sweetness, texture, hardness, gumminess, springiness, cohesiveness and global acceptability (ISO 5492:2008). Moreover, intention of buying was considered. The panel consisted of 17 trained panelists who are regular consumers of this kind of sweet. Two samples (control and I3010) were given to each panelist following a randomized design. For every formulation tested, the panelists evaluated three units independently. Testing sessions were conducted in a sensory evaluation laboratory built according to the international standards for test rooms.

2.5. Statistical Analyses

Statgraphics Centurion was used to perform the multifactor Analyses of Variance (ANOVA) in order to discern whether the effect of the process variables (kind of sugar and percentage of gelatine) on the final product was significant. The interactions between factors were also considered. Furthermore, Principal Component Analysis (PCA) and Partial Least Square regression (PLS2) were applied to describe the relationships between the sensory and the instrumental texture measurements. These analyses were performed using the Unscrambler version.10X (CAMO Process AS, Oslo, Norway).

3. Results and discussion

3.1. Compositional characteristics, pH and water activity

Table 1 shows the resulting ºBrix and pH of syrup for each formulation in addition to moisture content (%), water activity and the theoretical sweetening power of the gummy confections depending on the degree to which conventional sugars were replaced and the percentage of sugar used.

The content of soluble solids in the syrup was higher in the case of samples confected with isomaltulose and fructose in granulated form than when glucose syrup was used, given the amount of water in this syrup. These results are coherent with those established in other studies on gummy confections (Edwards, 2002). Furthermore, pH was higher in samples confected with glucose syrup than in the other cases. Specifically, one point less of pH was registered in samples confected with isomaltulose-fructose in comparison to the other samples, meaning that there could be an increase in their shelf life.

All formulations showed lower than the recommended moisture content values (24%) for this type of product (Edwards, 2002), except in the case of the samples confected with glucose syrup and fructose (samples F), which exceeded this limit. Additionally, the statistical analysis showed that the interaction between the sugar and the percentage of gelatine used had a significant effect, the control samples (S) and samples with glucose syrup and fructose (F) were responsible for these significant differences.

Water activity indicates the fraction of the total humidity of a product which is free and consequently subject to the growth of microorganisms and to different chemical reactions which might affect stability of these products. In this regard, samples made up of 30 % of isomaltulose and 70 % of fructose (with respect to the total amount of sugars) and with the lowest content of gelatine (I306) had the least water activity, which might imply higher stability than in the other cases. In contrast, the formulation with fructose and glucose syrup and with 8% of gelatine (F8) had the most water activity and hence was the most likely to be spoilt. On the other hand, samples formulated with isomaltulose-fructose in granulated form (I30 and I50) had less water activity than samples formulated with isomaltulose and glucose syrup (I), showing the increased ability of this combination of sugars to retain water.

Table 1. Mean and standard desviation of ºBrix and pH initial syrup and moisture content (%), water activity and sweetness of the gummy confections.

	Initial syrup		**Product: gummy confection**		
Formulation	ºBrix	pH	Moisture content (%)	a_w	Sweetness (SP)*
S6			16.167 (0.303)^{a}	0.822(0.003)^{c}	38
S8	69.7 (1.3)^{a}	6.1 (0.4)^{a}	17.90 (0.14)^{b}	0.837(0.015)^{c}	36
S10			21.047 (1.005)^{c}	0.844(0.007)^{c}	35
F6			24.5 (0.6)^{e}	0.843(0.014)^{c}	49
F8	70 (2)^{a}	5.7 (0.6)^{b}	31.5 (0.3)^{f}	0.908(0.006)^{a}	47
F10			31.6 (0.4)^{f}	0.868(0.018)^{b}	45
I6			21.9 (1.3)^{c}	0.859(0.004)^{b}	23
I8	70.9 (0.5)^{a}	5.81 (0.07)^{b}	23.8 (0.7)^{e}	0.867(0.003)^{b}	22
I10			24.3 (1.7)^{e}	0.851(0.012)^{b}	21
I306			23.2 (0.3)^{d}	0.721(0.007)^{e}	62
I308	73.5 (1.3)^{b}	5.10 (0.04)^{c}	22.9 (1.4)^{de}	0.788(0.005)^{d}	59
I3010			22.9 (0.2)^{d}	0.792(0.013)^{d}	57
I506			21.99 (0.16)^{cd}	0.796(0.005)^{d}	49
I508	72.4 (2.3)^{b}	5.12 (0.07)^{c}	22.5 (0.5)^{d}	0.812(0.003)^{d}	47
I5010			22.8 (0.7)^{d}	0.831(0.012)^{c}	45

*Theoretical Sweetness Power of the gummy confections: $SP=\Sigma m_i \cdot SP_i/\Sigma m_i$ (m_i: grams of each compound; SP_i: Sweetness Power of each component (individual sugar)) (González et al., 1989).

According to the above results, mixtures of isomaltulose-fructose with the lowest level of gelatine (6%) would be recommendable for gummy confections in terms of composition (moisture content and soluble solids), pH and water activity.

3.2. Instrumental mechanical and optical properties

Texture is the result of the interaction and arrangement of various constituents and structural elements at both macroscopic and microscopic levels (Ibañez *et al.*, 1998). Table 2 shows the mean values, and standard deviation, of the mechanical parameters from TPA (springiness, hardness (N), gumminess (N) and cohesiveness) of the gummy confections formulated with the different combination of sugars and percentage of gelatine studied. The statistical effect (F-ratio and level of significance from ANOVA multifactor) of the percentage of gelatine and combination of sugars on the mechanical parameters studied is also shown in Table 2. Regarding texture, the replacement of sucrose and glucose syrup by isomaltulose and/or fructose (F, I, I30 and I50) led to gummy confections with lower hardness and gumminess than the control samples (S) with the same percentage of gelatine. The effect of the percentage of gelatine was the variable in the formulation with the most influence (higher values of F-ratio) on both hardness and gumminess, although the combination of sugars also had a significant effect on these mechanical parameters. The difference in terms of hardness and gumminess between the samples formulated with isomaltulose and/or fructose and the control samples was noteworthy for the samples I30 or I50 and 10% of gelatine, but not at lower percentages of gelatine.

Cohesiveness results from the interaction of structural forces acting at a molecular level. The results of this study indicated that the new formulations exhibited higher cohesiveness than the control sample (S). Therefore, the incorporation of isomaltulose in the formulation of gummy confections enhanced the structural stability of the samples. A statistical significant effect of both individual parameters (percentage of gelatine and combination of sugars) was also found for cohesiveness. Nevertheless, the combination of sugars used in the formulation had more of an influence than the percentage of gelatine on this parameter unlike in the case of hardness and gumminess. Moreover, the formulation (combination of sugars and percentage of gelatine) had more of an influence on hardness and gumminess (higher F-ratios and level of significance) than on the cohesiveness and springiness of the samples (Table 2).

Lastly, the samples exhibited high springiness (values above 0.95) which was similar to control samples (S). Consequently, the presence of isomaltulose in the gum structure had a positive effect on the elastic properties of the samples.

Table 2. Mean and standard deviation of hardness (N), gumminess (N), springiness and cohesiveness. F-ratio and level of significance from ANOVA multifactor of the factors: percentage of gelatin and combination of sugars.

Formulation	Hardness (N)	Gumminess (N)	Springiness	Cohesiveness
S6	27.1(1.6)^{h}	24.4(1.6)^{g}	0.95(0.02)^{b}	0.90(0.03)^{d}
S8	44.3(0.5)^{d}	40.4(0.4)^{cd}	0.965(0.002)^{b}	0.912(0.002)^{d}
S10	62.1(0.5)^{a}	56.2(0.3)^{a}	0.969(0.002)^{ab}	0.905(0.002)^{d}
F6	21.3(0.3)^{i}	20.14(0.12)^{h}	0.972(0.006)^{ab}	0.94(0.02)^{abc}
F8	39(2)^{ef}	38(2)^{d}	0.97(0.02)^{ab}	0.961(0.003)^{ab}
F10	41.1(1.2)^{e}	39.2(0.5)^{d}	0.984(0.004)^{a}	0.953(0.015)^{abc}
I6	21.2(0.7)^{i}	19.3(0.5)^{h}	0.95(0.02)^{b}	0.91(0.02)^{d}
I8	44.2(0.6)^{d}	41.7(0.6)^{c}	0.983(0.005)^{a}	0.943(0.002)^{abc}
I10	59.4(1.6)^{b}	56.3(1.2)^{a}	0.962(0.012)^{b}	0.948(0.006)^{abc}
I306	22.3(0.8)^{i}	20.7(0.9)^{h}	0.960(0.013)^{b}	0.931(0.016)^{bcd}
I308	34.1(0.2)^{g}	32.2(0.3)^{f}	0.977(0.007)^{ab}	0.947(0.004)^{abc}
I3010	37.1(0.5)^{f}	35.2(0.7)^{e}	0.98(0.01)^{ab}	0.951(0.006)^{abc}
I506	22.8(0.2)^{i}	21.40(0.08)^{h}	0.980(0.006)^{ab}	0.935(0.008)^{b}
I508	37.6(0.8)^{f}	35.3(0.7)^{e}	0.975(0.006)^{ab}	0.939(0.003)^{bc}
I5010	48.2(1.9)^{c}	45.4(1.5)^{b}	0.977(0.005)^{ab}	0.943(0.007)^{abc}
ANOVA (F-Ratio)				
Sugars combination	232.21**	217.16**	2.45	14.77**
% Gelatine	2352.63**	2825.34**	3.6*	5.77**
Interaction	72.01**	82.60**	1.74	0.88

** Statistical significance≥99% (p-value≤ 0.01)
* Statistical significance≥95% (p-value≤ 0.05)

Figure 1 shows the color planes L*-a* and b*-a* of control samples and confected gummies with isomaltulose and/or fructose. The results obtained indicated that values of luminosity were very similar in all formulations. However, it is noteworthy that it was not possible to replace the overall percentage of sugars with more than 50% of isomaltulose due to the crystallization of the sugars and the appearance of a whitish instead of a translucent color according to some previous trials (data not shown). In fact, samples formulated with isomaltulose and glucose syrup with 8 and 10% of gelatine (I8 and I10) and samples with 50% of isomaltulose and 50% of fructose in the weight of sugars (I50) in this study, showed values of luminosity which were slightly higher than in the other cases. This behavior might be related to the lower solubility of isomaltulose

at room temperature, which could lead to crystallization (Schiweck *et al.,* 1990; Kaga and Mizutani, 1985).

Statistical analysis (ANOVA multifactor) showed that the effect of the interaction between the blend of sugars and the percentage of gelatine and their interactions on luminosity and coordinates a* and b* (data not shown) was significant.

It is also noteworthy that the samples I50 showed greater values of both a* and b* coordinates, with a tendency towards an orange color, although not perceivable visually. On the whole, the increase in the percentage of gelatine used led to an increase in both coordinates, except for the control samples and the sample confected with a mixture of 70% fructose and 30 % isomaltulose in the weight of sugars (I30). In coherence with these results, the values of chrome (data not shown) were greater in samples I50, followed by samples confected with 60% of glucose syrup and 40% of isomaltulose in the weight of sugars with 10% of gelatine (I10). In this regard, isomaltulose might improve the purity of the gummies' color. Nevertheless, the samples I30 were the most similar to control samples, so this increase in purity associated with high concentrations of isomaltulose might considerably modify the color of samples. In terms of hue (data not shown), samples were placed very close in quadrants I and IV of the chromatic diagram. This suggests that the samples were very similar.

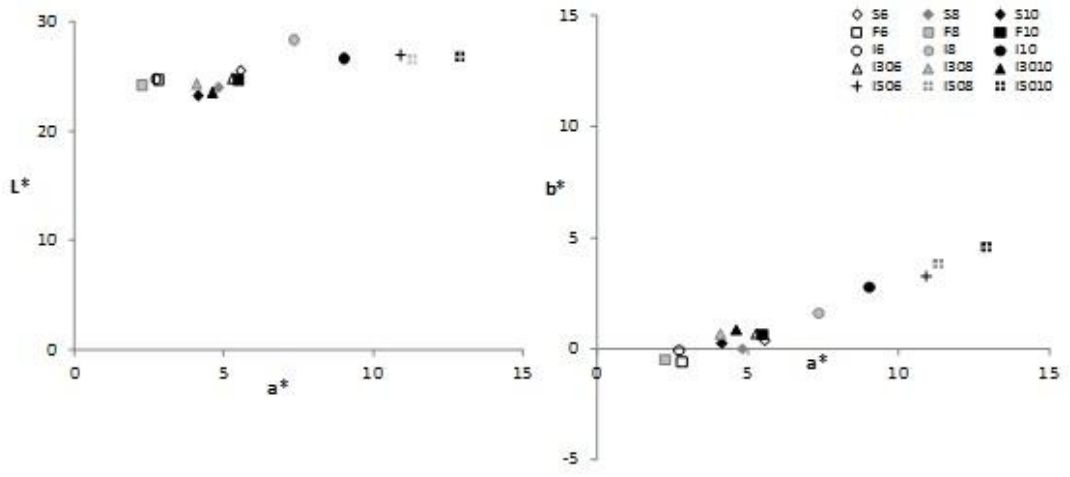

Figure III.5.1. Colour planes L*-a* and b*-a* of control samples and confected gummies with isomaltulose and/or fructose.

According to the results for colour, the recommended formulation would be 30% of isomaltulose and 70% of fructose in the weight of sugars (I30) since it showed an appearance similar to control samples (S), regardless of the percentage of gelatine used.

3.3. Sensory Evaluation

An acceptance test (using a 9-point hedonic scale) was carried out for the formulation which most resembled (from the point of view of the instrumental parameters) the sample that was prepared with a composition of sugars equivalent to the commercial gummies coded as S8 (40% sucrose and 60% of glucose syrup with 8% of gelatine). This control sample was also considered in the acceptance test.

To this end, a principal component analysis (PCA), of the instrumental parameters (hardness, gumminess, cohesiveness and elasticity) of 12 formulations was performed. The formulations I506, I508, I5010 were not considered due to the fact that they crystallized in the rest stage.

Figure 2 shows the PCA biplot (score "formulations" and loading "instrumental variables") obtained. The first two components accounted for 82 % of the total variance (PC1, 52 % and PC2, 30 %). The proximity between formulations implies similar texture profiles, while the proximity between variables shows the degree of correlation between these formulations.

Taking the above consideration, the samples I10 (40% of isomaltulose and 60% of glucose syrup in the total sugar content with 10% of gelatine) and S10 (40% of sucrose and 60% of glucose syrup in the total sugar content and 10% of gelatine) placed in the figure at the right end of the right axis of the figure are more rubbery and hard than the samples situated on the opposite site (left axis). The cohesiveness and springiness had less influence on the PC1 because they were situated near the center of this axis.

Table 3. Mean of score, standard deviation and the F-ratio of each attribute evaluated by means of sensory analysis using a 9-point hedonic scale.

Attribute	S8	I3010	F-ratio
Appearance	7.2 (1.5)	7.88 (1.12)	2.41
Color	6.9 (1.7)	8.0 (0.8)	5.55*
Aroma	6.5 (1.8)	6.5 (1.9)	0.00
Sweetness	6.47 (1.007)	6.23 (1.09)	0.43
Texture	7 (1)	6.6 (1.5)	1.47
Springiness	6.2 (1.8)	5.7 (1.5)	0.52
Hardness	5.9 (1.8)	5.3 (1.5)	1.05
Gumminess	6.3 (1.6)	5.7 (1.5)	1.51
Cohesiveness	6.3 (1.7)	6.6 (1.5)	0.29
Global acceptability	6.1 (1.6)	6.35 (1.06)	0.26
Intention of buying	6.6 (1.5)	6.9 (1.4)	0.48

* Statistical significance≥95% (p-value≤ 0.05).

As observed in Figure 2, the I3010 formulation (30% of isomaltulose and 70% of fructose in the total sugar content with 10% gelatine) was the nearest to the control sample S8 (40% of sucrose and 60% of glucose syrup in the total sugar content and 8% of gelatine), so it was chosen for the sensory analysis.

An ANOVA analysis was carried out for every one of these attributes (appearance, colour, strawberry flavour, sweetness, texture, hardness, gumminess, springiness, cohesiveness, global acceptability and intention of buying) considering "formulation" as a factor. Table 3 shows the average score, the standard deviation and the F-ratio obtained for each attribute evaluated in both the selected (I3010) and the control formulation (S8).

Color was the only attribute where significant differences (P-value=0.025) were shown between the selected formulation and the control sample. Sample I3010 was scored better in terms of visual appearance, color, and sweetness, than the control sample. On the contrary, the texture attributes (elasticity, hardness, gumminess, cohesiveness) obtained slightly higher scores in the sample S8. The same score was obtained by both samples for aroma, which proves that the type of sugars and the percentage of gelatine had no influence on the aroma evaluation. Finally, sample I3010 had the best average score for global acceptability (6.4) and intention of buying (6.9), although no significant differences were observed with respect to the other formulation (averages scores of 6.1 and 6.5, respectively).

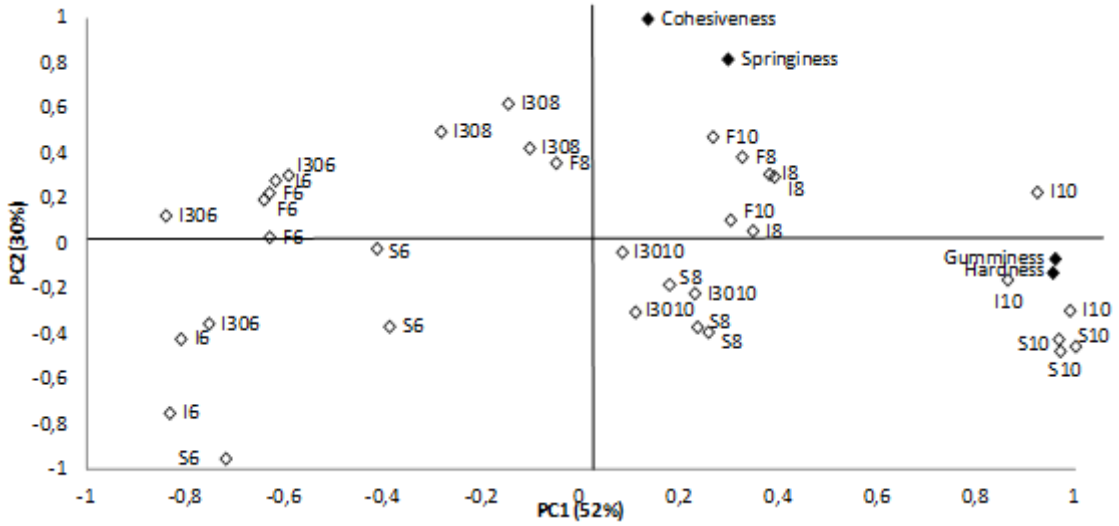

Figure III.5.2. Bi-plot of Principal Components Analysis for the samples (white rhombus ◊) and the texture parameters (black rhombus ♦).

3.4. Correlation between sensory and instrumental variables

Texture is the characteristic that decisively influences the consumer when eating gummy confection. For this reason identifying the consumer's preference with regard to this attribute is essential for the industry, both from the point of view of quality control and the design of new products. The use of textural instrumental equipment which gives information equivalent to that provided by a sensory panel could be a great aid. However, first it is essential to assess whether the sensory information about this type of product correlates with the instrumental variables. With this aim in mind, Figure 3 shows the results from PLS2 regression analysis, which describes the relationship between the instrumental variables (X-matrix) and the acceptability score for the sensory attributes (Y-matrix). The sensory parameters placed in the outer ellipse are correlated with the instrumental variables, with the exception of sensory cohesiveness, which being placed in the inner ellipse is not correlated (r^2=50%). Sensory gumminess and hardness were negatively correlated with respect to instrumental gumminess and hardness, and positively correlated with instrumental springiness and cohesiveness. That is to say, the lower the instrumental values for gumminess and hardness and the higher the values for springiness and cohesiveness, the higher the sensory acceptance scores and the global acceptability. In other words, the panelists preferred samples with low hardness and gumminess and high springiness and cohesiveness.

In summary, the correlation between instrumental and sensory parameters leads to the conclusion that instrumental texture measurement could be suitable for evaluating consumer opinion about gummy confections without the need to use a trained panel.

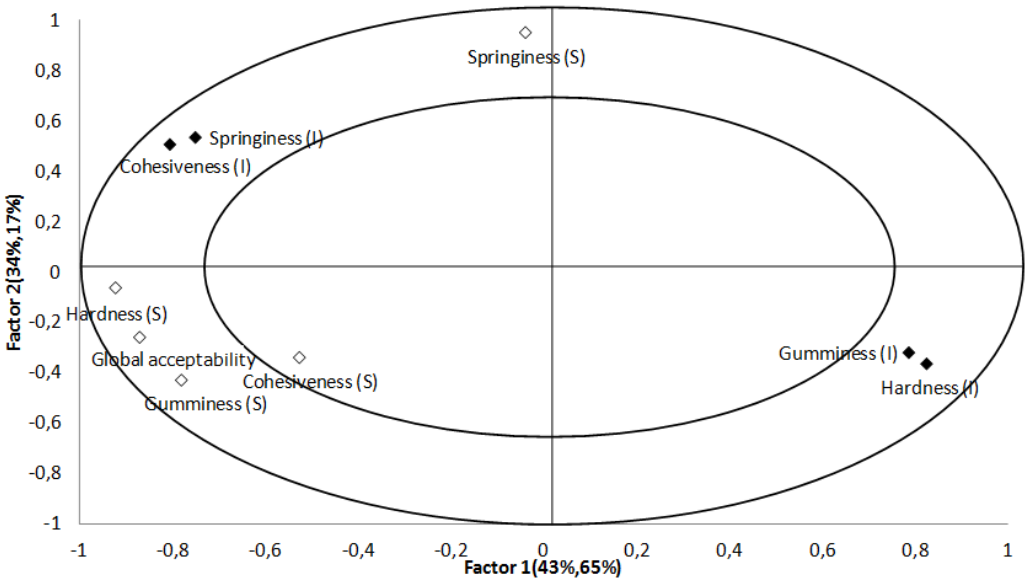

Figure III.5.3. Correlation loadings (X and Y) between instrumental and sensory texture variables. Black rhombus (♦) instrumental and white rhombus (◊) sensory texture variables.

4. Conclusions

The replacement of traditional sugars by isomaltulose and fructose in gummy confections is possible. More specifically, the combination of 30% of isomaltulose and 70% of fructose in the total amount of sugars would be recommendable to develop healthier gummy confections in terms of low cariogenicity and glycemic index. These gummies showed instrumental color and texture characteristics which were similar to commercial gummies. Additionally, a trained panel rated it with good global acceptability and intention of buying scores. Finally, a high correlation between instrumental and sensory parameters was found. Therefore, it could be concluded that instrumental texture parameters are adequate tools for estimating the global acceptabilityof consumers for this kind of gummy confections.

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Conflict of Interest Statement

The authors disclose that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

References

AOAC (2000). Official Methods of Analysis of AOAC International (17th edition). Gaithersburg, MD, USA.

Barez, J., Villanova, R., Garcia, S., Pala, T., Paramas, A., & Sanchez, J. (2000). Geographical discrimination of honeys through the employment of sugar patterns and common chemical quality parameters. *European Food Research and Technology*, 210, 437–444.

Bourne, M.C. (1978). Texture profile analysis. *Food technology*, 32, 62-66.

Bray, G.A., Nielsen, S.J., & Popkin, B.M. (2004). Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *American Society for Clinical Nutrition*, 79 (4), 537-543.

Edwards, W.P. (2002). La ciencia de las golosinas. Editorial Acribia, S.A., Zaragoza.

Elliot. S.S., Keim, N.L., Stern, J.S., Teff, K., & Havel, P.J. (2002). Fructose, weight gain, and the insulin resistance syndrome. *The American Journal of Clinical Nutrition*, 76(5), 911-922.

González, P., Camacho, F., & Robles, A. (1989). Hidrolizados enzimáticos de interés en la industria agroalimentaria. I. Hidrolizados de cereales. *Alimentación, equipos y tecnología*, 8(3), 201-207.

Ibañez, F.C., Loygorri, S., Ordoñez, A., & Torre, I.P. (1998). Evaluación instrumental y sensorial de la textura en quesos de oveja con Denominación de Origen. *Alimentaria*, 292, 49 -53.

ISO 5492 (2008). Sensory analysis. Vocabulary. International Organization for Standardization.

ISO 4121 (2003). Sensory analysis. Guidelines for the use of quantitative response scales. International Organization for Standardization.

Kaga, T., & Mizutani, T. (1985). Verwendung von Palatinose fur Nahrungsmittel. *Seito Gijutsu Kenkyukaishi*, 34, 45-57.

Kawai, K., Yoshikawa, H., Murayam, Y., Okuda, Y., & Yamashita, K. (1989). Usefulness of palatinose as a caloric sweetener for diabetic patients. *Hormone and Metabolic Research*, 21, 338–340.

Kubelka, P., & Munk, F. (1931). Ein Beitrag zur Optik der Farbanstriche. Zeitschrift für technische Physik (Leipzing),12, 593-620.

Lina, B., Jonker, D., & Kozianowski, G. (2002). Isomaltulose (Palatinose®): a review of biological and toxicologycal studies. *Food and Chemical Toxicology*, 40, 1375-1381.

Mann, J.I., De Leeuw, I., Hermansen, K., Karamanos, B., Karlström, B., Katsilambros, N., Riccardi, G., Rivellese, A.A., Rizkalla, S., Slama, G., Toeller, M., Uusitupa, M., & Vessby, B. (2004). Evidence based nutritional approaches to treatment and prevention of Diabetes mellitus. Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD). *Nutrition Metabolism and Cardiovascular Diseases*, 14: 373-394.

Marfil, P., Anhê, A., & Telis, V. (2012). Texture and Microstructure of Gelatine/Corn Starch Based Gummy Confections. *Food Biophysics*, 7, 236-243.

Martínez, O. (2012). Report Spanish Confectionary Association. Produlce. Madrid, Spain.

Matsuyama, J., Sato, T., & Hoshino, E. (1997). Acid production from palatinose, palatinit, erythritol and maltitol by bacteria isolated from dental plaque on human deciduous teeth. *Japanese Association for Oral Biology*, 39, 91-99.

Moloughney, S. (2011). Is there a place for healthiness in the indulgent world of candy, chocolate and gum? URL: http://www.nutraceuticalsworld.com/issues/2011-03/view_features/functional-confectionery-finding-the-sweet-spot/

O'Neil, C., Fulgoni, V., & Nicklas, T. (2011). Association of candy consumption with body weight measures, other health risk factors for cardiovascular disease, and diet quality in US children and adolescents: NHANES 1999_2004. *Food and Nutrition Research*, 55 5794.

Schiweck, H., Munir, M., Rapp, K., Schenider, B., & Bogel, M. (1990). New developments in the use of sucrose as an industrial bulk chemical. *Zuckerindustrie*, 115, 555–565.

Weidenhagen, R., & Lorenz, S. (1957). Palatinose (6-alpha-glucopyranosido-fructofuranose), ein neues bakterielles *Umwandlungsprodukt der Saccharose. Zeitschrift fur die Zuckerindustrie*, 7, 533–534; *und Angewandte Chemie*, 69, 641.

III.6. ISOMALTULOSE AS A SUGAR REPLACER IN MARSHMALLOWS: INFLUENCE ON COLOUR, TEXTURE AND CONSUMER RESPONSE

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LWT-Food Science and Technology (En revisión)

ABSTRACT

The aim of this study was to evaluate the potential of isomaltulose as a substitute for traditional sugars in marshmallows. 18 formulations combining different amounts of sugars (sucrose, glucose syrup, fructose and isomaltulose) and different percentages of gelatine (4, 5 and 6%) were studied. Composition (ºBrix and moisture content), pH, water activity (a_w), instrumental colour and texture as well as a sensorial analysis were evaluated. Marshmallows with 30% and 50% isomaltulose (in combination with fructose) had the longest shelf-life, since pH and a_w had the lowest values (5.13-5.14 and 0.653-0.691, respectively). These formulations had lower instrumental hardness, higher cohesiveness and springiness, and the best sensory acceptance. In summary, sucrose and glucose can be totally replaced by up to 50% isomaltulose. In addition, a multivariate analysis (PLS2) showed a good correlation between sensorial acceptance and instrumental texture, indicating that it is suitable for discerning consumer acceptability and preference, considering marshmallows.

Keywords: marshmallows, isomaltulose, texture, colour, hedonic scale.

1.Introduction

Sweets are attractive due to their intense colours, shapes and characteristic aroma and taste, both for children and adults. Eating sweets in moderate amounts is pleasing and has a positive effect on well-being attributable to the sugar and flavourings present. However, these food products are not basic necessities and they are actually considered to be quite unhealthy since they are related to different diseases such as tooth decay, a sharp increase in the glycemic index and obesity. Despite these effects, they account for a significant volume of sales, candy consumption in Europe being valued at €710,291,000 in 2011 (Martínez, 2012).

In confectionery products, it is usual to find a combination of different sugars such as sucrose, fructose, and glucose with inverted sugar or glucose syrups which are meant to increase their solubility, decreasing the water activity of the final product and hence improving their physicochemical stability. The structure of gummy confectionery products is related to the combination of sugars and proteins, resulting in the typical gel texture of jelly babies and similar products. Gelatin is the preferred gelling agent in sugar confectionery, gelatine gels melt at relatively low temperature (melt-in mouth),

and they are slow-setting. Marshmallow, an aerated gelled confectionery, uses gelatin as gelling agent for its elasticity (Saha and Bhattacharya, 2010). It has a foam-structure formed by the addition of air into the protein-sugar combination through fast stirring. The presence of air not only adds volume to the mixture but also contributes greatly to a soft and light texture (Tan and Lim, 2008).

The candy industry is a sector which is continually innovating in order to please consumers and develop new sugar free products meeting the demand for low calorie goods. The artificial sweeteners used as sugar substitutes show different disadvantages. For instance, polyalcohols have a laxative effect (Franz et al., 1994; Edwards, 2002) and intensive sweeteners such as aspartame have been related to the development of cancer and other health issues (AFSSA, 2002; Weihrauch and Diehl, 2004; Soffritti et al., 2007; Renwick and Nordmann, 2007).

An interesting natural sugar that can be used to revise the formula of candies is isomaltulose which is found in small amounts in honey and sugarcane juice (Barez, et al., 2000) but can be obtained massively from sucrose by means of an enzymatic process (Schiweck et al., 1990). Isomaltulose is a reducing disaccharide composed of glucose and fructose, just like sucrose, but joined by a stronger α-(1-6) type glycoside bond (Weidenhagen and Lorenz, 1957), which is why it cannot be broken down by the bacteria responsible for tooth decay (Matsuyama et al., 1997). Therefore, it is noncariogenic, and is also slowly released in the bloodstream (Bucke and Cheetham 1986; Beneo-palatinit, 2010). It supplies the same amount of energy as sucrose but this energy lasts significantly longer. It has only a third of the sweetening power of sucrose. Furthermore, it has only a slight effect on sugar and insulin levels and thus, it is totally digestible (Hawai et al., 1989; Lina et al., 2002).

Due to the fact that, like sucrose, isomaltulose also provides consistency to the food matrix, it is a good option as it affords the protein-sugar structure typical of candies. However, the modification of the food ingredients could affect the interactions between them resulting in changes in texture, colour and even in the perceived flavour profile and off-flavours (Helstad, 2006). Therefore, the selection of appropriate processing conditions in the reformulation of new healthier marshmallows with isomaltulose is essential to evaluate its viability as an ingredient. That is to say, to assess whether the marshmallow reformulated with this sugar-substitute has the properties that the consumer expects from the traditional product.

In consideration of all the above, the objective of this study was to evaluate the potential use of isomaltulose as sugar replacer in soft marshmallow type candies. For this purpose, the effects of the interaction of isomaltulose (on the composition, colour, texture and sensory acceptability) with other ingredients (gelatine and sugars) were analysed.

2.Materials and methods

2.1.Materials

The ingredients used in the formulation of marshmallows were: sucrose (Azucarera Ebro S.L.; Spain), fructose (Gabot Biochemical Industrie; Israel), isomaltulose (Beneo-Palatinit; Germany), glucose syrup 43 DE (Emilio Peña, S.A., Spain), gelatin A 220 Bloom (Junca Gelatines S.L.; Spain), corn starch (Roquette, France), natural red liquid colour (Roha Europe S.L.; Spain), strawberry flavouring (Flavorix Aromáticos S.A.; Spain) and sunflower oil (Koipesol, Spain).

2.2.Experimental Methodology

The marshmallows were produced with 36% of water, 58-60% of sugars and 4-6 % of gelatin. The percentage of sugars depended on the amount of gelatin used. Furthermore, 0.5 ppm of strawberry flavouring and 0.2 ppm of red colouring were added. Six different mixtures of sugars were studied. In the case of the control samples, the total sugar content was composed of 40% sucrose and 60% of glucose syrup (40:60 (w/w)) and the code was (S). The new samples were obtained by replacing the sugars with different combinations of isomaltulose, glucose syrup or fructose. Isomaltulose was combined with the other sugars to balance its low sweetening power. In order to simplify the description of each sample, the percentage of the total amount of sugars replaced is shown between brackets along with the code used: isomaltulose:glucose syrup (I: 40:60 (w/w)), fructose:glucose syrup (F: 40:60 (w/w)), isomaltulose:fructose (I30: 30:70 (w/w)), isomaltulose:fructose (I50: 50:50 (w/w)), isomaltulose:fructose (I70: 70:30 (w/w)). Besides, the level of gelatin (4, 5 or 6%) was subsequently noted by including the percentage of gelatine used (S4, S5, S6, I4, I5, I6, etc.) after the code, a total of 17 different formulations being studied plus the control. Commercial marshmallows were also analysed to compare them to the new ones only in terms of

composition, water activity and mechanical properties, but not colour since they did not have similar optical properties.

Each formulation was made in a thermal blender (Thermomix, TM31, Vorwerk, Germany) by blending the sugars and water until they reached boiling temperature at 300 rpm for 10 minutes. This mixture was shaken until reaching 60ºC and pH and ºBrix were measured. The gelatine was then dissolved in water in a gelling agent: water ratio of 1:2 (w/w) to obtain a homogeneous mix and subsequently added to the syrup with the flavouring and colouring agents. All the ingredients were blended for 5 minutes at 60ºC and 300 rpm. Then, the syrup was shaken for 10 minutes at 1859 rpm to add air to the mixture, which is what mainly accounts for the texture of the marshmallows. For molding purposes, the final mixture was poured into silicone molds with a thin layer of sunflower oil. The silicone moulds have cylindrical shape with a diameter of 28 mm and a height of 20 mm. Finally, the molds were placed in a chamber at 20 °C for 24 hours. The samples were then removed from their mould and covered with starch to prevent the samples from sticking together. After an additional 24 hours, analyses of texture, colour, water activity and moisture performed.

2.3.Physicochemical Analyses

Moisture content and water activity analyses were carried out on the final products. Moisture content was determined gravimetrically by drying to a constant weight in a vacuum oven at 60 ºC (AOAC, 2000). Water activity (a_w) was determined with a dew point hygrometer (FA-st lab, GBX). Soluble solid content (ºBrix) was measured with a refractometer at 20 ºC (ATAGO 3 T) and pH was determined with a pH-meter (SevenEasy, Mettler Toledo) in the initial syrup. All measurements were carried out in triplicate.

2.4.Colour

Instrumental measurements of colour were conducted at room temperature in a Minolta spectrophotometer (model CM-3600d) by placing the marshmallow on the diaphragm aperture (8mm). CIEL*a*b* coordinates were obtained using illuminant D65 and standard observer (10° visual field) as references. Registered parameters were: L* (brightness), a* (red component), b* (yellow component), chroma ($C^*=[(a^*)^2+(b^*)^2]^{1/2}$) and hue ($h^*=arctg(b^*/a^*)$). The samples were previously measured with both black and white calibration tiles in order to study the possible translucency of the samples. Since

the same spectrum was obtained with the black and white tiles, the opacity of the samples was confirmed. The analysis was carried out in triplicate.

2.5.Texture

The samples were examined with Texture Profile Analysis test (TPA) using a TA.XT plus Texture Analyzer (Stable Micro Systems, U.K.). Instruments were equipped with a load cell of 50 kg and a 45 mm diameter cylindrical probe. The test conditions involved two consecutive cycles of 50% compression with 15 s between cycles. The test speed was 1 mm/s. From the resulting force-time curve the following parameters were quantified, and are defined by Bourne (1978) as: hardness (N) (maximum peak force during the first compression cycle), springiness (the height that the sample recovers during the time that elapses between the end of the first cycle and the beginning of the second cycle), cohesiveness (the ratio of the positive force area during the second compression and the first compression), gumminess (N) (hardness x cohesiveness). The analysis was carried out in triplicate.

2.6.Consumer test

An acceptance test using a 9-point hedonic scale (Sanz et al., 2009) was used to evaluate the following attributes: appearance, colour, strawberry flavour, sweetness, texture, hardness, gumminess, springiness, cohesiveness, global preference and intention of buying (ISO 5492, 2008). A total of 50 consumers (half female and half male, aged 21–59) who are regular consumers of this kind of sweet (employees and students of the Institute of Food Engineering for Development, Spain), were used for this study. The acceptance test took place in 2 sessions on separate days. The consumers evaluated 3 formulations (S4, I504, I505) on the first day and 2 formulations (I506 and S4-Ar [control which had double the added aroma]) on the second day; each of the different formulations (3 units) were presented independently. The consumers were informed about the advantages of the new ingredients (isomaltulose and fructose). Testing was conducted in a sensory evaluation laboratory built according to the international standards for test rooms.

2.7. Statistical Analyses

Statgraphics Centurion was used to perform the statistical analyses. Analyses of variance (multifactor ANOVA) were carried out to discern whether the effect of the process variables (kind of sugar and percentage of gelatine) on the final product was

significant. The interactions between factors were considered. Furthermore, Principal Component Analysis (PCA) and Partial Least Square regression (PLS2) were applied to describe the relationships between the sensory and the instrumental texture measurements. These analyses were performed using the Unscrambler version.10X (CAMO Process AS, Oslo, Norway).

3.Results and discussion

3.1.Compositional characteristics, pH and water activity

Table 1 shows the ºBrix and pH of the initial syrups for each formulation as well as the moisture content (g/100g) and water activity of the marshmallows obtained. In addition, this table shows the theoretical sweetening power of each formulation. These values were calculated as described by González et al. (1989). As expected, significant differences were observed in the theoretical sweetness depending on the sucrose replacer used; when sucrose is replaced by fructose a significant increase in this parameter is obtained while the contrary is observed when isomaltulose is used to replace sucrose. According to this parameter, it could be said that the optimal ratio to obtain similar sweetness to the reference sample would be that corresponding to the I70 formulation. However, the choice of the optimal formulation has to be based on the assessment of all the studied parameters (texture, colour, composition, acceptability, etc.).

Samples confected with isomaltulose and fructose (I30, I50, I70) had similar values of ºBrix (72.9, 73.7, 74.4, respectively) to the control ones (75.4). As regards pH values, no significant differences were observed as a consequence of replacing sucrose with fructose or isomaltulose separately (S, F, I), but a significant decrease in the pH is observed in those formulations where fructose and isomaltulose were combined, especially in I70 with the greatest amount of isomaltulose. These results could affect the shelf-life of the final product since fructose and other reducing sugars are affected by pH differently. They are stable in modestly acidic environments but become unstable as the pH approaches neutral, and enters the alkaline range. As the pH of the system rises, the sugars become more chemically active and reactive, breaking down into colour bodies and flavour compounds, and reacting with proteins. This pH instability is marked

by accelerated colour degradation, going from colourless to yellow to brown (Helstad, 2006).

With respect to moisture content, most of the formulations were in the recommended range for this kind of product (15-22 g/100g) (Edwards, 2002). With respect to the percentage of gelatin and the moisture content no clear behavior was observed in the samples (Table 1).

Table 1. Mean and standard deviation of ºBrix and pH of the initial syrup and moisture content (%), water activity and sweetness of the marshmallows. The ANOVA homogenous groups are indicated by letters.

		Initial syrup		**Product: marshmallow**		
Formulation	% Gelatin	ºBrix	pH	Moisture content (g/100g)	a_w	Sweetness*
	4			18.8(0.3)^{ad}	0.816(0.002)^{b}	0.42
S	5	75.4(1.3)^{d}	6.57(0.09)^{c}	16.0(1.4)^{b}	0.736(0.003)^{c}	0.41
	6			12.8(0.8)^{c}	0.687(0.005)^{d}	0.41
	4			20.8(0.3)^{ad}	0.797(0.005)^{b}	0.54
F	5	71(4)^{b}	6.68(0.18)^{c}	19.1(0.5)^{ad}	0.721(0.002)^{c}	0.53
	6			18.1(0.8)^{ab}	0.739(0.003)^{c}	0.52
	4			23.9(1.9)^{e}	0.873(0.004)^{a}	0.25
I	5	65.0(0.6)^{a}	6.69(0.13)^{c}	19.0(0.5)^{ad}	0.785(0.004)^{b}	0.25
	6			22(5)^{de}	0.786(0.005)^{b}	0.25
	4			17.2(1.3)^{ab}	0.683(0.002)^{d}	0.68
I30	5	72.9(0.4)^{c}	5.13(0.09)^{b}	17.31(1.06)^{ab}	0.653(0.004)^{d}	0.67
	6			18.4(1.7)^{ad}	0.671(0.007)^{d}	0.66
	4			16.2(0.8)^{b}	0.691(0.006)^{d}	0.54
I50	5	73.7(1.2)^{c}	5.14(0.15)^{b}	18.32(0.97)^{abd}	0.689(0.004)^{d}	0.53
	6			17.27(1.15)^{ab}	0.678(0.003)^{d}	0.52
	4			13.9(1.9)^{bc}	0.762(0.003)^{b}	0.40
I70	5	73.4(1.2)^{c}	4.992(0.108)^{a}	14.0(0.7)^{bc}	0.679(0.002)^{d}	0.39
	6			17.5(1.3)^{ab}	0.709(0.004)^{c}	0.38

*Theoretical Sweetness Power (SP) of the marshmallows: $SP=\sum m_i \cdot SP_i / \sum m_i$ (m_i: grams of each compound; SP_i: Sweetness Power of each component (individual sugar))

The a_w values were lowest in all the formulations with isomaltulose and fructose, especially I30 and I50, and the percentage of gelatine had no significant influence. This is logical due to the fact that fructose is the sugar which most depresses the a_w. Therefore, the product obtained with isomaltulose and fructose, especially I30 and I50,

would have a greater shelf-life than that obtained with conventional sugars and consequently is the most recommendable for the replacement of these sugars.

3.2.Mechanical and optical instrumental properties

Fig. 1 shows the instrumental TPA parameters (springiness, hardness, cohesiveness and gumminess) of the marshmallows obtained using the different formulations. The springiness values for all samples were higher than 0.85, even the control ones. The inclusion of gelatine in the formulation provided a visco-elastic texture and stable foam that led to the high springiness desirable in this kind of product (Hamann et al., 2006; PB Gelatins, 2012).

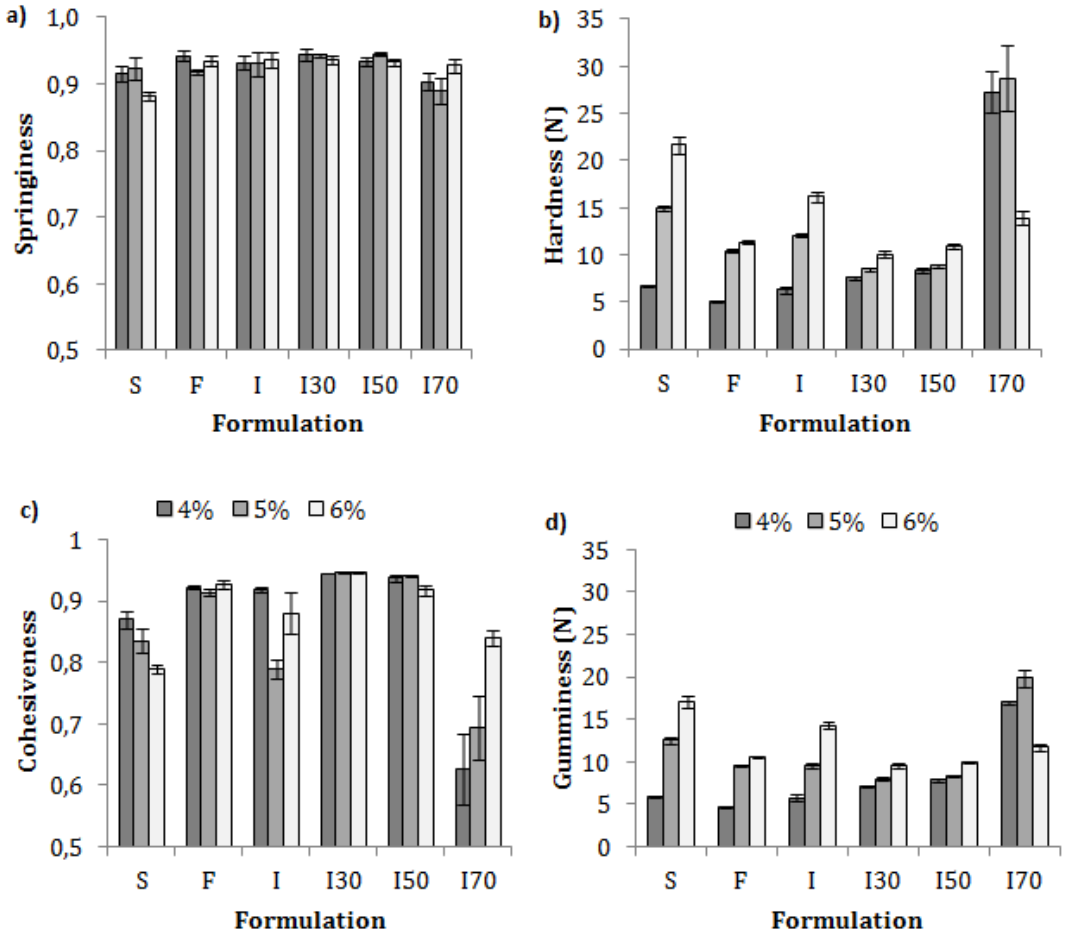

Figure III.6.1. Mean values (n=3) and standard deviation of springiness (a), hardness (b), cohesiveness (c) and gumminess (d) of the marshmallows.

With respect to hardness, the effect of gelatine also depended on the blend of sugars used (Fig. 1). In general, the higher the percentage of gelatine, the harder the

samples, with the exception of marshmallows confected with the maximum quantity of isomaltulose (I70). The highest value of hardness observed in the I70 samples (with 4 and 5% gelatine) could be related to the crystallization of isomaltulose during the cooling step due to the low solubility of this sugar at room temperature (Mitchell, 2006). An increase in the level of jellification to 6% gelatine seemed to limit this phenomenon (PB Gelatins, 2012). This result was a consequence of the low structural cohesiveness exhibited by these samples (Fig. 1) which permits isomaltulose molecules to achieve enough mobility to form crystals. In turn, the intermolecular interaction or cohesiveness of the I, F, I30 and I50 samples, and therefore their structural integrity, was higher than in the control samples (S). With respect to gumminess, all samples had the same behaviour as hardness since gumminess, is the interaction between the hardness and the cohesive forces taking place at a structural level.

Table 2. Mean and standard deviation of Luminosity, coordinates a*, b*, chrome and hue. The F-ratio values from the ANOVA for the factors "formulation" and "percentage of gelatine", and their interaction.

Formulation	L*	a*	b*	C*	h
S4	85.5 (0.7)	10.9(1.2)	1.5(0.4)	11.03(1.07)	8(3)
S5	86.4(0.3)	10.7(0.3)	2.009(0.005)	10.8(0.2)	10.6(0.3)
S6	86.9(0.1)	10.25(0.12)	1.70(0.04)	10.3(0.2)	9.4(0.3)
F4	82.8(0.1)	14.3(0.3)	0.4(0.4)	14.4(0.2)	1.6(1.6)
F5	87.7(0.6)	9.4(0.6)	1.4(0.2)	9.5(0.6)	8.9(0.8)
F6	85.5(0.8)	11.7(0.8)	0.26(0.09)	11.7(0.8)	1.2(0.4)
I4	81.7(0.4)	16.2(0.6)	-0.98(0.05)	16.2(0.6)	356.51(0.12)
I5	86.6(0.4)	10.1(0.3)	2.3(0.2)	10.3(0.3)	12.9(0.7)
I6	84.2(0.9)	13.08(1.02)	0.6(0.6)	13.10(1.02)	2.8(0.7)
I304	84.1(0.2)	12.7(0.3)	1.52(0.13)	12.8(0.3)	6.8(0.8)
I305	87.4(0.9)	10.15(1.17)	2.1(0.2)	10.37(1.12)	11.9(0.8)
I306	87.78(0.09)	9.65(0.08)	2.21(0.08)	9.90(0.08)	12.9(0.5)
I504	84.8(0.3)	11.95(0.14)	1.786(0.105)	12.09(0.15)	8.5(0.4)
I505	88.2(0.3)	9.3(0.2)	1.93(0.04)	9.5(0.2)	11.7(0.3)
I506	87.7(0.6)	10.1(0.7)	1.91(0.03)	10.2(0.6)	10.7(0.5)
I704	86.6(0.5)	11.1(0.2)	1.41(0.09)	11.1(0.2)	7.2(0.5)
I705	87.33(0.05)	10.4(0.3)	1.79(0.15)	10.5(0.3)	9.7(0.8)
I706	88.7(0.2)	9.1(0.2)	1.93(0.09)	9.21(0.17)	12.1(0.8)
ANOVA F-ratio					
Formulation	52.1*	32.9*	95.6*	31.8*	18,732*
% Gelatine	194.1*	119.3*	134.9*	113.9*	17,191*
Interaction	12.7*	13.59*	36.1*	13.2*	19,369*

* Statistical significance≥99% (p-value≤ 0.01)

Table 2 shows the values of luminosity, coordinates a* and b*, and the chroma and hue of marshmallows depending on the type of sugar and the percentage of gelatine, as well as the F-ratio values for these optical parameters obtained from the ANOVA for the factors "formulation" and "percentage of gelatine", and their interaction. As can be observed, the percentage of gelatine was the factor with the greatest influence on luminosity, a*, b* and C*. The lower the level of gelatine, the lower the value of luminosity and the higher the value of a* and C*, and therefore the redder the colour. Neither the factors nor their interactions showed a clear influence (similar F-ratios) on hue. Despite the significant differences in colour parameters obtained by the ANOVA, no visual differences were appreciated between samples.

3.3.Consumer test

As was described previously, the instrumental texture measurements were made for the 18 possible marshmallows formulations (6 combinations of sugars and 3 levels of gelatine). However, due to the complexity of the sensory studies, only a few of these formulations were selected. To this end, the information given by a principal component analysis (PCA) of the instrumental parameters (hardness, gumminess, cohesiveness and elasticity) obtained from the 18 formulations as well as a commercial sample (C), was taken into account. The latter was also included to facilitate selection of the formulations. The first two components of this PCA explained 83 % of the total variance (PC1, 63 % and PC2, 20 %) (data not shown). The formulations were selected due to their proximity to the commercial sample, which means they had a similar texture profile. S4 (with 4% gelatine) was selected from those exclusively made with sucrose (sugar used in commercial sweets). The differences between the formulations made with a mixture of isomaltulose and fructose, (I30 and I50), were practically non-existent. Therefore, samples which had the highest levels of isomaltulose (I504, I505, I506) were chosen because of the advantages of this sugar to consumer health.

The result of the ANOVA (using "formulation" as a factor), carried out for the different attributes evaluated by the untrained panellists, is shown in a radial chart (Fig. 2). This figure shows the average score for each attribute evaluated by the panellists, and the F-ratio of each attribute in brackets. There are no significant differences between the samples evaluated by the panellists for any of the attributes. However, considering the average values, some differences between the samples can be seen.

Sample S4 scored lowest on all the attributes except hardness. This low score was reflected in global appreciation and intention of buying.

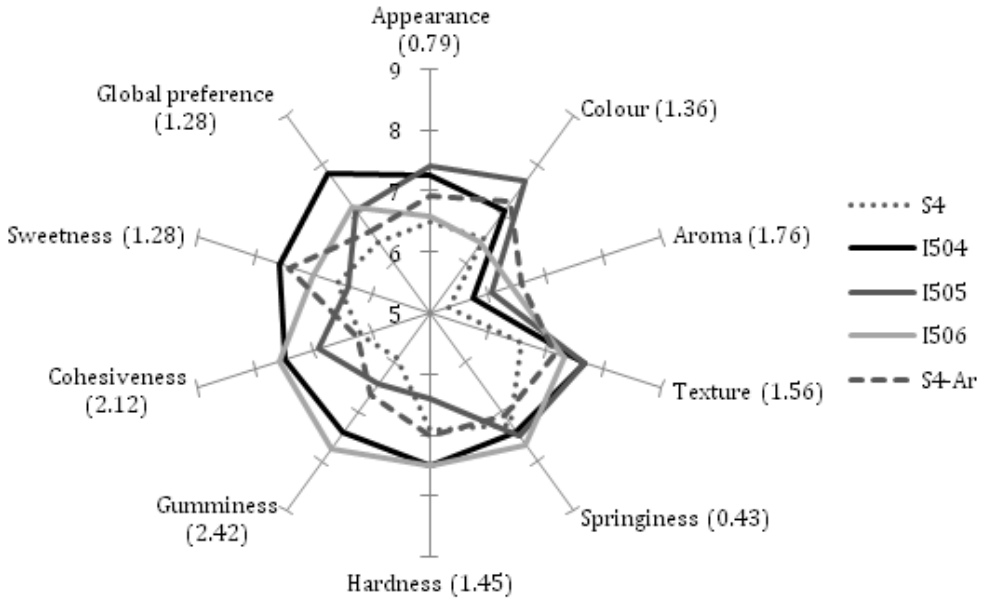

Figure III.6.2. Radial chart of the average scores for each attribute and the F-ratio of each attribute in brackets.

In relation to global appearance and colour, sample I505 was the most appreciated. Regarding texture attributes (hardness, gumminess, cohesiveness, springiness), samples I504 and I506 had similar scores; on the contrary, sample I505 obtained slightly lower scores. As regards the aroma attribute, sample S4-Ar was the best. As this formulation had double the added aroma, it is clear that the panellists liked a more intense aroma in this kind of product. Finally, sample I504 had the best score for global preference and intention of buying.

In order to ascertain the possible linear dependence between the sensory attributes, and especially to know which attribute has more influence on global preference and intention of buying, Pearson correlation coefficients (95.0% confidence level) were calculated for each pair of variables. Table 3 shows the correlation matrix obtained. The best positive correlations are shown for intention of buying and global preference (0.959) and for intention of buying and texture (0.942).

Table 3. Pearson correlation of different attributes.

	Appearance	Colour	Aroma	Texture	Springiness	Hardness	Gumminess	Cohesiveness	Sweetness	Global preference	Intention of buying
Appearance	1.000										
Colour	**0.908***	1.000									
Aroma	0.116	0.258	1.000								
Texture	0.828	0.617	0.393	1.000							
Springiness	0.085	-0.197	0.073	0.497	1.000						
Hardness	-0.360	-0.620	0.086	0.063	0.248	1.000					
Gumminess	0.052	-0.222	0.475	0.578	0.600	0.790	1.000				
Cohesiveness	0.268	-0.096	0.184	0.719	0.845	0.576	**0.878***	1.000			
Sweetness	0.094	-0.026	0.318	0.268	-0.259	0.724	0.593	0.277	1.000		
Overall preference	0.579	0.210	0.052	0.832	0.538	0.497	0.739	0.871	0.493	1.000	
Intention of buying	0.777	0.469	0.141	**0.942***	0.486	0.253	0.617	0.786	0.389	**0.959****	1.000

** Statistical significance≥99% (p-value≤ 0.01)

* Statistical significance≥95% (p-value≤ 0.05)

Global preference is fairly well correlated with cohesiveness, gumminess and texture with values of 0.871, 0.739 and 0.832 respectively, although these values were not significant. Colour is positively correlated to the overall appearance (0.908), however, it is texture that defines acceptability and intention of buying the product.

A PCA analysis was conducted to better understand the relationship between the samples and the evaluated attributes from a descriptive point of view. Fig. 3 shows the biplot of the sample scores and the attribute loadings obtained by means of this analysis. The first two dimensions explained 77 % of the total variance (PC1, 50% and PC2, 27%). Samples with isomaltulose (I504, I505, I506) are placed at the right side next to the highest values of the sensory variables analysed and hence the most preferred, especially for I504 (with 4% of gelatine). On the other hand S4 and S4-Ar are situated on the opposite side, which implies the lowest values of these variables for these two last samples, especially for S4.

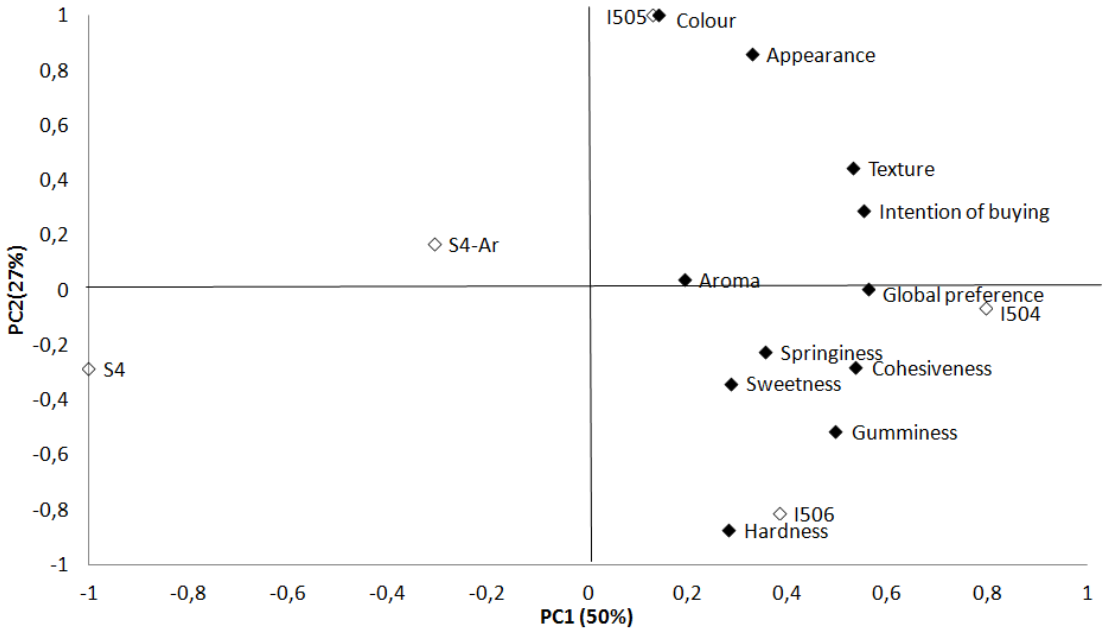

Figure III.6.3. Bi-plot Principal Components Analysis for the samples (◊) and the attributes (♦).

3.4.Correlation between sensory and instrumental variables

As explained before, texture is the characteristic that decisively influences the consumer when buying this type of product. For this reason, it was decided to assess whether texture sensory variables, as well as global preference, are correlated with the instrumental variables. With this aim, Figure 4 shows the results from PLS2 regression analysis, which describes the relationship between the instrumental variables (X-matrix) and the acceptability score for the sensory attributes (Y-matrix). The sensory parameters placed in the outer ellipse are correlated with the instrumental variables, with the exception of sensorial hardness, which being placed in the inner ellipse is not correlated (r^2=50%). In summary, it could be asserted that the instrumental texture analyses are suitable and can be used to discern the overall preference for marshmallows without using trained panellists.

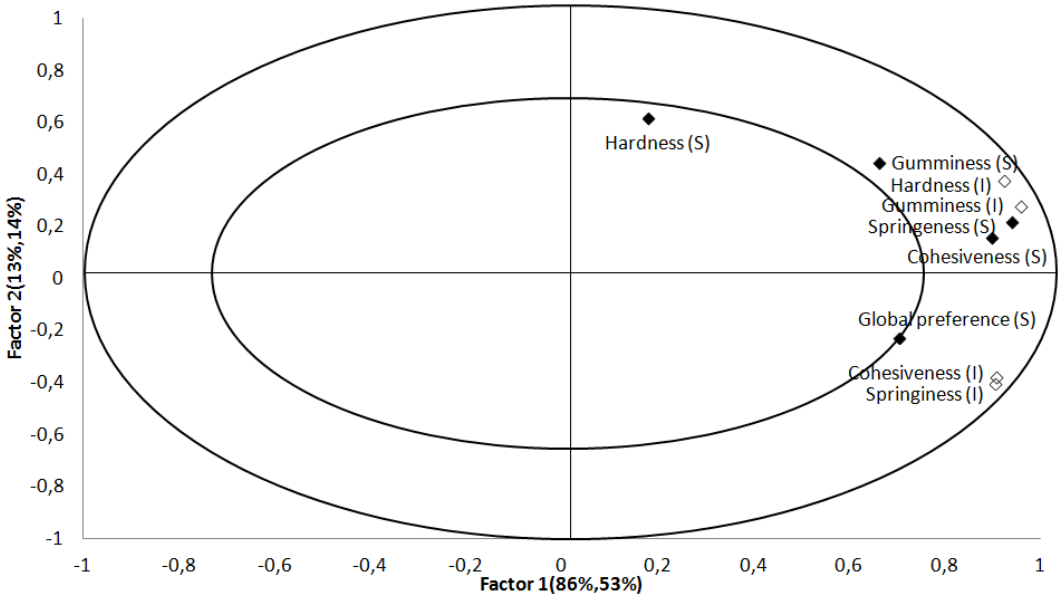

Figure III.6.4. Correlation loadings (X and Y) between instrumental (◊) and sensory (♦) texture variables.

4.Conclusions

According to the instrumental textural and colour results, traditional sugars in commercial marshmallows could be replaced by a mixture of up to 50:50 (w/w) isomaltulose and fructose. Marshmallows prepared with these conditions obtained a better sensory evaluation than those confected with sucrose and glucose syrup. Additionally, a good correlation was found between the instrumental parameters and the acceptance sensory attributes, and global preference, indicating that texture measurements can be used for quality assessment purposes. To sum up, isomaltulose could be considered by the confectionary industry to obtain healthier candies.

Acknowledgments

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References

AFSSA (2002) Rapport de l'Agence Française de Sécurité Sanitaire des Aliments sur la question d'un éventuel lien entre exposition à l'aspartame et tumeurs du cerveau. Maisons-Alfort, France.

AOAC (2000) Official Methods of Analyisis of AOAC International (17th). Gaithersburg, MD, USA.

Barez J, Villanova R, Garcia S, Pala T, Paramas A, Sanchez J (2000) Geographical discrimination of honeys through the employment of sugar patterns and common chemical quality parameters. Eur Food Res Technol 210: 437-444.

Beneo-palatinit (2010) Palatinose™. The only toothfriendly sugar for optimized energy supply.http://www.beneopalatinit.com/en/Food_Ingredients/Isomaltulose/What_is_Isomaltulose/Palatinose-Brochure_EN_1.pdf. Accessed 25 Juny 2012.

Bourne MC (1978) Texture profile analysis. Food Technology 32: 62-66.

Bucke C, Cheetham PS (1986) Method of reducing dental plaque formation with products for human or animal consumption using isomaltulose sucrose substitute. United States of America Patent No 4,587,119.

Edwards WP (2002) La ciencia de las golosinas. Acribia, S.A., Zaragoza.

Franz MJ, Horton ES, Bantle JP, Beebe CA, Brunzell JD, Coulston AM, Henry RR, Hoogwerf BJ, Stacpool PW (1994) Nutrition Principles for the Management of Diabetes and Related Complications. Diabetes Care 17(5): 490-518.

González P, Camacho F, Robles A (1989) Hidrolizados enzimáticos de interés en la industria agroalimentaria. I. Hidrolizados de cereales. Alimentación, equipos y tecnología 8(3): 201-207.

Hamann DD, Zhang J, Daubert CR, Foegeding EA, Diehl KC (2006) Analysis of compression, tension and torsion for testing food gel fracture properties. J Texture Stud 37(6): 620-639.

Hawai K, Yoshikawa H, Murayam Y, Okuda Y, Yamashita K (1989) Usefulness of palatinose as a caloric sweetener for diabetic patients. Horm Metab Res 21: 338-340.

Helstad S (2006) Ingredients interactions: Sweeteners. In: McPherson A and Gaonkar AG (ed) Ingredient interactions: Effects on Food Quality. Taylor & Francis. New York, pp.167-194

ISO 5492 (2008) Sensory analysis. Vocabulary. International Organization for Standardization.

Lina BAR, Jonker D, Kozianowski G (2002) Isomaltulose (Palatinose®): a review of biological and toxicologycal studies. Food Chem Toxicol 40: 1375-1381.

Martínez O (2012). Report Spanish Confectionary Association. Produlce. Madrid, Spain.

Matsuyama J, Sato T, Hoshino E (1997) Acid production from palatinose, palatinit, erythritol and maltitol by bacteria isolated from dental plaque on human deciduous teeth. J Oral Biosci, 39: 91-99.

Mitchell HL (2006) Sweeteners and Sugar Alternatives in Food Technology. Blackwell Publishing Ltd, Oxford, UK.

PB Gelatins (2012) Propiedades estabilizantes del batido y la espuma. www.pbgelatins.com/es/applications/food-applications/confectionary. Accessed 27 juny 2012.

Renwick AG, Nordmann H (2007) First European conference on aspartame: Putting safety and benefits into perspective. Synopsis of presentations and conclusions: Food Chem Toxicol 45 (7): 1308-1313.

Saha D, Bhattacharya S (2010) Hydrocolloids as thickening and gelling agents in food: a critical review. J Food Sci Technol 47(6):587–597.

Sanz T, Salvador A, Baixauli R, Fiszman SM (2009) Evaluation of four types of resistant starch in muffins. II. Effects in texture, colour and consumer response. Eur Food Res Technol 229:197-204.

Schiweck H, Munir M, Rapp K, Schenider B, Bogel M (1990) New developments in the use of sucrose as an industrial bulk chemical. Zuckerindustrie 115: 555-565.

Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M (2007) Life-Span Exposure to Low Doses of Aspartame Beginning during Prenatal Life Increases Cancer Effects in Rats. Environ Health Perspect 115(9): 1293-1297.

Tan JM, Lim MH (2008) Effects of gelatine type and concentration on the shelf-life stability and quality of marshmallows. Int J Food Sci Technol 43, 1699-1704.

Weidenhagen R, Lorenz S (1957) Palatinose (6-alpha-glucopyranosido-fructofuranose), ein neues bakterielles Umwandlungsprodukt der Saccharose. Zeitschrift fur die Zuckerindustrie, 7, 533-534; und Angewandte Chemie, 69, 641.

Weihrauch MR, Diehl V (2004) Artificial sweeteners-do they bear a carcinogenic risk? Ann Oncol 15(10): 1460-1465.

III.7. STEVIA REBAUDIANA, OLIGOFRUCTOSE AND ISOMALTULOSE AS SUGAR REPLACERS IN MARSHMALLOWS - STABILITY AND ANTIOXIDANT PROPERTIES

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Journal of Food Engineering (En revisión)

ABSTRACT

Consumers are currently demanding products with natural ingredients and functional properties. The replacement of conventional sugars with recently available sugars/sweeteners could result in the perception of candies as healthier products. Therefore, the objective of this work was to evaluate the influence of isomaltulose, oligofructose and stevia extracts on the physico-chemical, mechanical, optical and antioxidant properties as well as the shelf-life of marshmallows. A sensory test was carried out in order to evaluate the influence of these ingredients on the acceptance of this product. The instrumental and sensorial textural results indicate that the sucrose and glucose syrup in commercial marshmallows could be totally replaced by a mixture of isomaltulose, oligofructose and stevia. The evaluation of these marshmallows by adults, in comparison to children, was very similar to the traditional product. These new marshmallows, besides being more microbiologically stable, have added value due to their antioxidant properties.

Keywords: marshmallows, isomaltulose, oligofructose, stevia, texture, colour, antioxidants.

1. Introduction

The confectionery industry is a sector which is continually innovating in order to satisfy consumers and develop new sugar free products. The consumption of sweets and candies, due to their high sugar content, has always been linked to the development of tooth decay, an increase in the glycaemic index and obesity. These problems can be reduced by replacing the conventional sugars with alternative healthy sweeteners, which have recently become available on the market as isomaltulose, oligofructose and stevia.

Marshmallow is a sweet, soft solid foam made primarily of aerated sugar and gelatin (Kaletunc et al., 1992). The typical structure of marshmallows is formed by the addition of air into the protein-sugar combination through fast stirring. The presence of air not only adds volume to the mixture but also contributes greatly to a soft and light texture (Groves, 1995). The properties that bubbles or air impart to foods are: a reduction of product density, a change in rheology and texture, modification of appearance and mouthfeel, increased surface area, alteration of digestibility and shelf-

life due to increased porosity, and modulated flavour intensity (Campbell and Mougeot 1999).

It is well-known that marshmallows produced with conventional sugars have a limited shelf-life due to changes in texture which occur with time, mainly hardening and the loss of elasticity of the foam. Moisture loss, sugar crystallization, foam collapse, gel network formation and the ingredients, could contribute to the hardening process (Lees, 1991; Groves, 1995; Tan and Lim, 2008). In this line, Lim et al. (2006) reported an inhibitory effect of the combination of glucose syrup and high dextrose equivalent on sugar crystallization.

Marshmallows could be produced with healthy sugars/sweeteners in order to reduce the negative effects of conventional sugars such as oligofructose, isomaltulose and stevia, which possess technological properties which are closely related to those of sugar and glucose syrup.

Oligofructose shows good stability during the usual food processes (e.g. heat treatment), contributes to improved mouthfeel, shows humectant properties and reduces water activity ensuring high microbiological stability (Frank, 2002). However, in the pure form oligofructose only has a sweetness of about 35% in comparison with sucrose.

Isomaltulose only has a slight effect on sugar and insulin levels and therefore is totally digestible (Hawai et al., 1989; Lina et al., 2002), in addition, it supplies the same amount of energy as sucrose but this energy lasts significantly longer. On the other hand, it only has a third of the sweetening power of sucrose.

Stevia is a perennial plant from Brazil and Paraguay which is extremely sweet (250-300 times more than sucrose) due to the presence of diterpenes, specifically steviol glycosides (Lemus-Moncada et al., 2012). Thus, the use of stevia could compensate the lack of sweetness that the aforementioned sugars have and provide many other functional properties to the products. In fact, the European Food Safety Authority recognized the safety of stevia leaf extracts for alimentary use in November 2011(EFSA, 2011). The food industry has shown increased interest in plant extracts from stevia as it is another alternative to sucrose, due to its high content in non-caloric sweeteners (Carbonell-Capella et al. 2013). Moreover, stevia leaves are increasingly consumed as infusions due to their antioxidant properties (Periche et al. 2014; Shukla et al., 2012; Muanda et al., 2011) and its beneficial effects on human health as anti-

hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory effects (Chatsudthipong and Muanprasat, 2009). However, it is not well known how the replacement of conventional sugars by these new ones affects the shelf life, texture or colour of this kind of product. Therefore, the aims of the present study were to investigate the influence of isomaltulose, oligofructose and stevia extracts on physico-chemical, mechanical, optical and antioxidant properties as well as shelf-life of marshmallows during storage. Additionally, a consumer test was carried out in order to evaluate the influence of these ingredients on the sensory acceptance of this product.

2.Material and methods

2.1. Materials

The ingredients used in the formulation of marshmallows were: oligofructose (Frutalose OFP, Sensus, Netherlands), isomaltulose (Beneo-Palatinit; Germany), sucrose (Azucarera, Spain), glucose syrup 43 DE (Emilio Peña, S.A., Spain), gelatin A 220 Bloom (Junca Gelatines S.L.; Spain), corn starch (Roquette, France), natural red liquid colour (Roha Europe S.L.; Spain), strawberry flavouring (Flavorix Aromáticos S.A.; Spain) and sunflower oil (Koipesol, Spain). In addition, *Stevia Rebaudiana* leaves (Raab, Vitalfood, Rohrbach, Germany) were used in order to prepare the aqueous stevia extract incorporated in the formulations. The leaves were previously treated with UV radiation in order to minimize the microbial load.

2.2. Experimental Methodology

The marshmallows prepared in this study consisted of 36% water, 59% sugars (isomaltulose and oligofructose in a ratio 2:3) and 5% gelatine (Edwards, 2002). In the formulations with stevia the water was replaced by an aqueous extract of stevia. This extract was prepared with 1 g of dried leaves in 100 mL of water at 90ºC for 5 minutes. Four different formulations were obtained depending on the amount of water replaced by stevia aqueous extract (%): A (0%), B (33%), C (66%) and D (100 In addition, a "control sample", prepared in the same way as the commercial ones with only glucose syrup and sucrose, was used in this study. Furthermore, 0.5 ppm of strawberry flavouring and 0.2 ppm of red colouring were added.

Each formulation was made in a thermal blender (Thermomix, TM31, Vorwerk, Germany) by blending the sugars and water until they reached boiling point at 300 rpm for 10 minutes. This mixture was stirred until it reached 60ºC when pH and ºBrix were measured. The gelatine was then dissolved in water at a ratio of 1:2 (w/w) to obtain a homogeneous mix and subsequently added to the syrup with the flavouring and colouring agents. All the ingredients were blended for 5 minutes at 60 ºC and 300 rpm. Then, the syrup was stirred for 10 minutes at 1859 rpm to add air to the mixture, which is what mainly accounts for the texture of the marshmallows. For moulding purposes, the final mixture was poured into silicone moulds with a thin coating of sunflower oil. The silicone moulds are cylindrical in shape with a diameter of 28 mm and a height of 20 mm. Finally, the moulds were placed in a chamber at 20°C for 24 hours. The samples were then removed from their mould and covered with starch to prevent the samples from sticking together. Samples were stored in plastic bags (multilayer polyethylene) at room temperature for 45 days.

2.3. Analytical determinations

2.3.1. Physicochemical Analyses

Moisture content and water activity analyses were carried out on the final products. Moisture content was determined gravimetrically by drying to a constant weight in a vacuum oven at 60 ºC (method 20.103 AOAC, 2000). Water activity (a_w) was determined with a dew point hygrometer (LabFerrer, Spain). Soluble solid content (ºBrix) was measured with a refractometer at 20 ºC (ATAGO 3 T) and pH was determined with a pH-meter (SevenEasy, Mettler Toledo) in the initial syrup. All measurements were carried out in triplicate.

2.3.2 Colour

Instrumental measurements of colour were conducted at room temperature in a Minolta spectrophotometer (model CM-3600d) by placing the marshmallow on the diaphragm aperture (8 mm). CIEL*a*b* coordinates were obtained using illuminant D65 and standard observer (10° visual field) as references. Registered parameters were: L^* (brightness), a^* (red component), b^* (yellow component), chroma ($C^*=[(a^*)^2+(b^*)^2]^{1/2}$) and hue ($h^*=\text{arctg}(b^*/a^*)$). Global colour differences, $\Delta E^*_{ab,}$ were calculated using the standard formula: $\Delta E^*_{ab}=((\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2)^{1/2}$. These differences were calculated

with respect to the control and the sample A. It is assumed that when ΔE^*ab is higher than 3 units, a colour variation can be perceived by an average observer (Francis and Clydesdale, 1975). The samples were previously measured with both black and white calibration tiles in order to study the possible translucency of the samples. Since the same spectrum was obtained with the black and white tiles, the opacity of the samples was confirmed.

2.3.3. Texture

The samples were examined with Texture Profile Analysis test (TPA) using a TA.XT plus Texture Analyzer (Stable Micro Systems, U.K.). Instruments were equipped with a load cell of 50 kg and a 45 mm diameter cylindrical probe. The test conditions involved two consecutive cycles of 50% compression with 15 s between cycles. The test speed was 1 mm/s. From the resulting force-time curve the following parameters were quantified: hardness (N) (maximum peak force during the first compression cycle), springiness (the height that the sample recovers during the time that elapses between the end of the first cycle and the beginning of the second cycle), cohesiveness (the ratio of the positive force area during the second compression and the first compression), gumminess (N) (hardness x cohesiveness) [20].

2.3.4. Determination of total antioxidant capacity

The antioxidant activity (AA) of the extract was analyzed on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl free radical as described by Shahidi, Liyana-Pathirana and Wall (2006), with some modifications. Marshmallows were freeze-drying at a vacuum pressure of $9.5x10^{-1}$ mm Hg for 24 hours. Then, they were ground in a grinding mill (A11 Basic, IKA, Germany) and diluted in methanol:water (80:20). 0.1 mL of sample was mixed with 3.9 mL of a methanolic solution of DPPH (0.025mg/mL, prepared in methanol:water (80:20)). The solution was shaken and after 30 min the absorbance of the sample was measured at 515 nm using methanol as a blank. The quantification was made considering a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the results were expressed as mg of Trolox equivalent per gram of marshmallow.

2.3.5. Microbiological analysis

Serial dilutions were prepared by homogenising 5 g of marshmallow with 45 mL of 1% sterile peptone water in a stomacher bag, using sterile techniques. Mesophilic aerobic populations were analysed in Plate Count Agar (Scharlau, Barcelona, Spain) incubating samples for 72 h at 31 ºC. Yeast and molds were determined on Sabouraud Chloramphenicol Agar (Scharlau, Barcelona, Spain) plates for 5 days at 31 ºC. Samples for analysis were taken on days 1, 7, 14, 21, 28, 35 and 45. Plates were inoculated, in duplicate, with 1 mL of the corresponding dilutions. After the incubation time, Petri dishes with a number of colonies between 30 and 300 for mesophilic aerobic and between 15 and 150 for molds and yeasts, were considered. Microbial counts were expressed as CFU/g.

2.4. Sensorial analysis

An acceptance test using a 9-point hedonic scale [22] was used to evaluate the following attributes: appearance, colour, aroma, sweetness, texture, hardness, gumminess, global preference and intention of buying [23]. This study was performance by 50 children aged 11-12 from the State School "Nou d'Octubre" (Tavernes Blanques, Valencia, Spain) and 30 adults (employees and students of the Institute of Food Engineering for Development, Spain). The consumers evaluated 3 formulations (control, A and D); each of the different formulations was presented independently. The consumers were informed about the advantages of the new ingredients (isomaltulose, oligofructose and stevia). The test was conducted in a sensory evaluation laboratory built according to the international standards for tasting rooms [24].

2.5. Statistical Analyses

Statgraphics Centurion was used to perform the statistical analyses. Analyses of variance (multifactor ANOVA) were carried out to discern whether the effect of the process variables (formulation and storage time) on the final product was significant. The interactions between factors were considered.

3. Results and discussion.

3.1. Compositional characteristics, pH and water activity

Table 1 shows the mean and standard deviation of ºBrix and pH of the initial syrup as well as the moisture content and water activity of the marshmallows during storage. As expected, ºBrix were very similar in all cases ranging from 68.65 to 71.6; whereas samples formulated with the healthier sugars (A, B, C and D) exhibited lower pH than the control. A slight increase in moisture content and water activity was found in the samples A, B and C during storage.

This could be attributed to the slight permeability of the package material. Water activity increased for samples A, B and C until day 7 and then remained constant. Sample D had a greater initial moisture content and water activity than the other formulations, but no evolution was found with storage time. For the control sample, the values of moisture content and water activity were higher than those values in samples A, B, C and D due to the additional quantity of water provided by the glucose syrup used in the formulation of the control.

Table 1. Mean and standard deviation of ºBrix and pH of the initial syrup, moisture content and water activity of the marshmallows. The ANOVA homogenous groups are indicated by letters. Samples codification: control, A (0%), B (33%) C (66%) and D (100%) (% stevia aqueous extract replacement).

		Initial syrup		**Product: marshmallow**	
Formulation	Time (days)	ºBrix	pH	Moisture content (g/100g)	a_w
Control	1	70.3(0.04)^{c}	6.9(0.07)^{c}	23.1(0.7)^{fg}	0.821(0.002)^{d}
	7			21.9(0.2)^{e}	0.810(0.008)^{d}
	14			19.3(0.3)^{c}	0.815(0.005)^{d}
	21			18.2(0.2)^{b}	0.801(0.004)^{c}
	28			19.9(0.3)^{c}	0.796(0.003)^{b}
	35			19.6(0.7)^{c}	0.759(0.002)^{a}
	42			20.1(0.5)^{d}	0.745(0.007)^{a}
A	1	70.45(0.07)^{c}	6.26(0.06)^{b}	17.4(0.9)^{a}	0.749(0.002)^{a}
	7			17.6(0.3)^{a}	0.785(0.003)^{b}
	14			18.2(0.3)^{b}	0.780(0.005)^{b}
	21			17.5(0.7)^{a}	0.778(0.005)^{b}
	28			18.7(0.3)^{b}	0.780(0.002)^{b}
	35			18.4(0.3)^{b}	0.794(0.003)^{b}
	42			20.8(0.7)^{d}	0.778(0.004)^{b}
B	1	71.60(0.09)^{d}	6(0.08)^{a}	17.4(1.7)^{a}	0.757(0.002)^{a}
	7			19.6(1.1)^{bc}	0.820(0.003)^{d}
	14			21.3(0.3)^{d}	0.815(0.005)^{d}
	21			21.0(0.5)^{d}	0.818(0.005)^{d}
	28			22.6(0.3)^{ef}	0.809(0.002)^{d}
	35			21.9(0.9)^{e}	0.820(0.003)^{d}
	42			22.1(1.2)^{ef}	0.814(0.004)^{d}
C	1	68.85(0.07)^{b}	6.20(0.12)^{ab}	18.3(0.3)^{b}	0.786(0.002)^{b}
	7			16.7(0.6)^{a}	0.791(0.003)^{b}
	14			19.2(0.2)^{c}	0.781(0.005)^{b}
	21			18.1(0.6)^{b}	0.792(0.005)^{b}
	28			18.6(1.3)^{b}	0.803(0.002)^{c}
	35			18.9(0.2)^{b}	0.800(0.003)^{c}
	42			22.1(0.4)^{ef}	0.796(0.004)^{b}
D	1	68.65(0.07)^{a}	6.11(0.12)^{ab}	21.6(0.6)^{e}	0.817(0.002)^{d}
	7			20.8(0.5)^{d}	0.826(0.003)^{d}
	14			22.4(0.3)^{ef}	0.831(0.005)^{d}
	21			20.9(0.5)^{d}	0.822(0.005)^{d}
	28			21.7(1.2)^{e}	0.817(0.002)^{d}
	35			21.8(0.7)^{e}	0.826(0.003)^{d}
	42			22.9(1.0)^{ef}	0.820(0.004)^{d}

3.2. Instrumental mechanical and optical properties

Figure 1 shows the instrumental TPA parameters (springiness, hardness, cohesiveness and gumminess) of the formulated marshmallows. On one hand, the ANOVA found statistical differences in hardness and gumminess between the control and the other formulations. Therefore, the replacement of glucose syrup and sucrose by isomaltulose and oligofructose resulted in a decrease in hardness and gumminess. Nevertheless, the addition of stevia did not affect the mechanical properties.

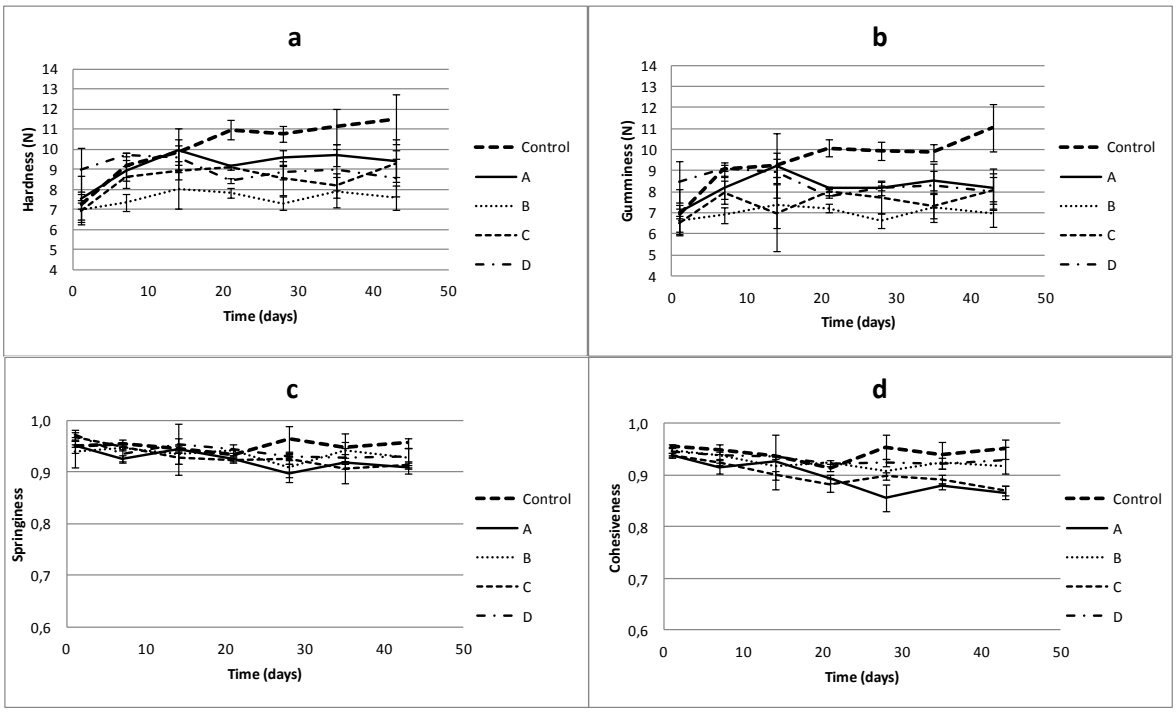


Figure III.7.1. Mean and standard deviation of texture parameters: a) hardness, b) gumminess, c) springiness, d) cohesiveness. Samples codification: control, A (0%), B (33%) C (66%) and D (100%) (% stevia aqueous extract replacement).

On the other hand, the statistical analysis established the significant influence of storage time on all TPA parameters. Specifically, a slight increase in hardness and gumminess was observed from 0 to 21 days together with a reduction in springiness and cohesiveness during the whole storage time.

Colour planes L*-a* and b*-a* of the samples are shown in Figure 2. The factor "formulation" had more influence than "time" on the colour of the marshmallows. Samples made with sucrose and glucose syrup showed higher a* and lower L* than the samples formulated with isomaltulose and oligofructose. The higher the replacement of water by stevia extract and the longer the storage time, the lower the luminosity but the higher the a* and b* coordinates of the samples. Table 2 shows the global colour differences ($\Delta E^*_{ab,}$) between samples: due to storage time ($\Delta E=S_{t42}-S_{t0}$); at the initial time with respect to sample A ($\Delta E= S_{t0}-A_{t0}$) and the control sample ($\Delta E= S_{t0}-Control_{t0}$); and at the end of storage ($\Delta E= S_{t42}-A_{t42}$ and $\Delta E= S_{t42}-Control_{t42}$).

Table 2. Global colour differences (ΔE*ab,) between samples: due to storage time ($\Delta E=S_{t42}-S_{t0}$); at the initial time with respect to sample A ($\Delta E= S_{t0}-A_{t0}$) and the control sample ($\Delta E= S_{t0}-Control_{t0}$); and at the end of storage ($\Delta E= S_{t42}-A_{t42}$ and $\Delta E= S_{t42}-Control_{t42}$).

Sample	$\Delta E=S_{t42}-S_{t0}$	$\Delta E= S_{t0}-Control_{t0}$	$\Delta E= S_{t42}-Control_{t42}$	$\Delta E= S_{t0}-A_{t0}$	$\Delta E= S_{t42}-A_{t42}$
Control	$5.2(0.2)^{c}$				
A	$2.1(0.3)^{ab}$	$13.8(0.3)^{b}$	$17.7(0.7)^{b}$		
B	$1.9(0.2)^{ab}$	$12.4(0.5)^{b}$	$16.0(0.5)^{b}$	$2.0(0.3)^{a}$	$2.0(0.7)^{a}$
C	$2.7(0.9)^{b}$	$10.1(0.9)^{a}$	$12.1(1.7)^{a}$	$4.6(1.3)^{b}$	$5.0(0.2)^{b}$
D	$1.7(0.3)^{a}$	$9.8(0.8)^{a}$	$13.5(1.2)^{a}$	$6.9(0.9)^{c}$	$6.1(1.3)^{b}$
Anova F-ratio	20.58^{***}	25.57^{***}	18.49^{***}	18.66^{**}	16.85^{**}

The colour differences in each sample during storage time ($\Delta E=S_{t42}-S_{t0}$) were lower than 3, except for the control samples. These differences cannot be perceived by the human eye (Francis and Clydesdale, 1975). The colour difference of A, B, C and D with respect to the control samples ($\Delta E= S_{t0}-Control_{t0}$) was also higher than 3, mainly due to the different sugars (sucrose and glucose syrup) used in the formulation of the control samples, and also due to the incorporation of stevia aqueous extract in the other samples (B, C and D). As expected, the colour difference of B, C, D with respect to sample A ($\Delta E= S_{t0}-A_{t0}$) was lower because the only change was the incorporation of stevia aqueous extract since oligofructose was present in all these samples.

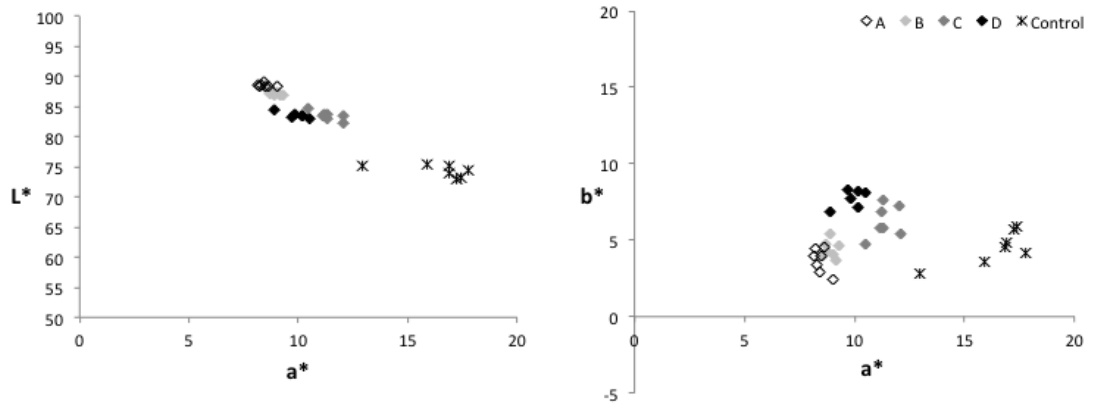


Figure III.7.2. Colour planes L*-a* and b*-a* of control samples and confected marshmallows with stevia aqueous extract during storage. Samples codification: control, A (0%), B (33%) C (66%) and D (100%) (% stevia aqueous extract replacement).

3.3. Antioxidant activity

In order to evaluate the contribution of stevia to marshmallows in terms of antioxidant properties, the antioxidant activity (mg trolox/ 100 g of marshmallow) of the formulations and its evolution with storage time is shown in Figure 3. Taking into account the antioxidant activity of stevia aqueous extract (117 mg trolox/100g stevia aqueous extract) added to the formulations and the values of antioxidant activity at the beginning of storage (1st day of storage) (figure 3), it can be affirmed that the processing of marshmallows did not affect the antioxidant activity of the stevia extract. A sharp decrease in antioxidant properties, however, occurred during the first 21 days of storage. From 21st day, antioxidant activity tended to stabilize. Specifically, an average loss of antioxidant activity of 77, 62 and 71 % was registered at the end of storage for the formulations B, C and D, respectively.

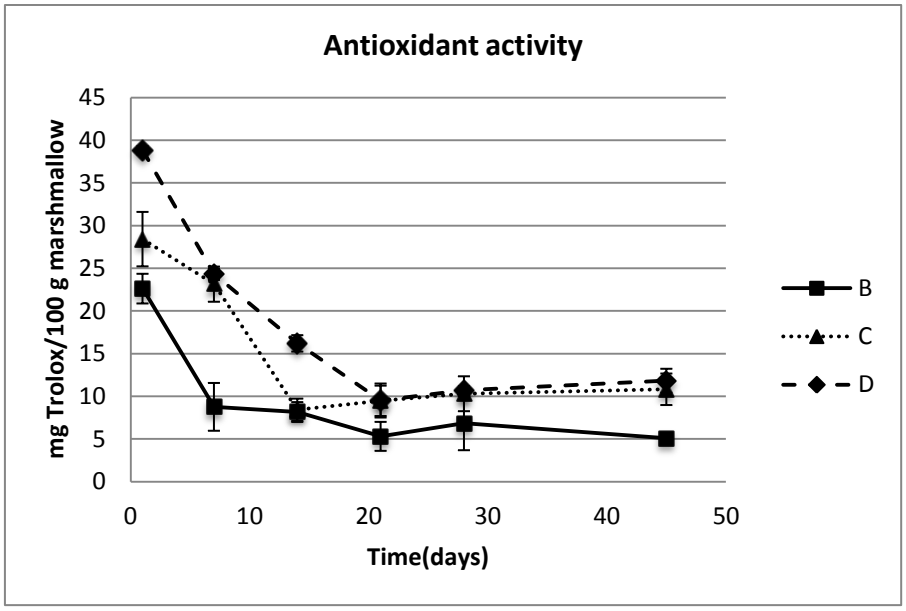


Figure III.7.3. Mean and standard deviation of the antioxidant activity of marshmallows during storage time. Samples codification: B (33%) C (66%) and D (100%) (% stevia aqueous extract replacement).

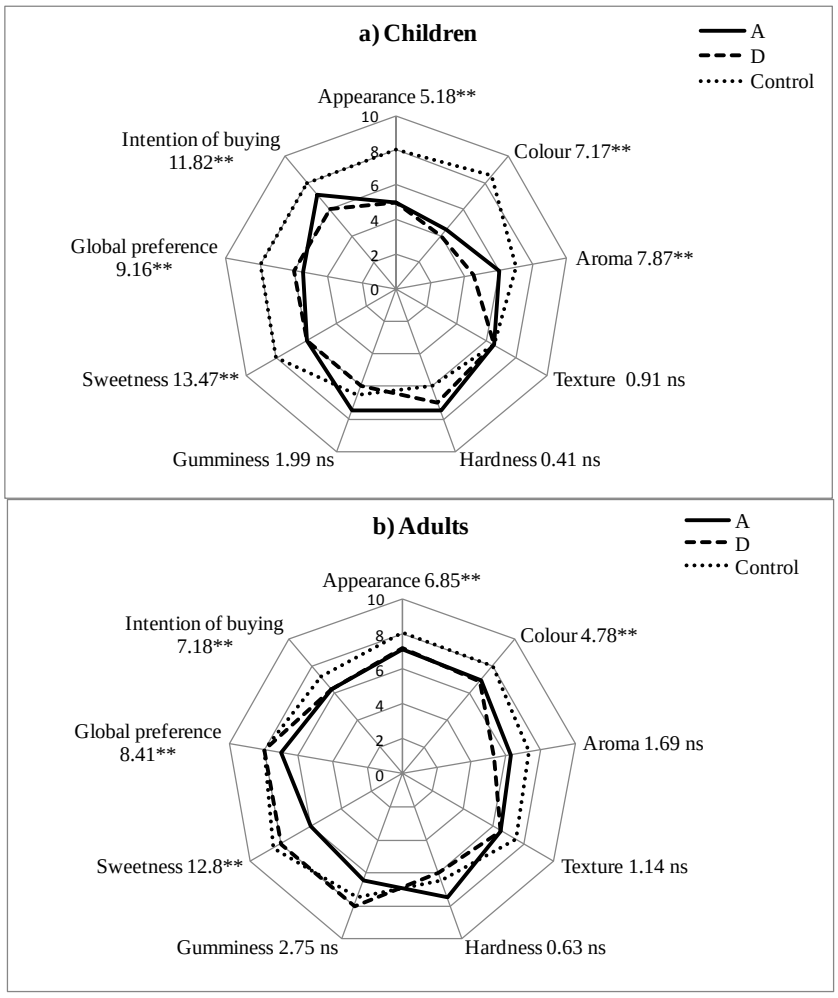
3.4. Microbiological analysis

Microbial counts of mesophilic aerobics, yeasts and moulds were not found in any of the marshmallows during storage, except the control at the end of storage, although the count was low ($3*10^1$ CFU/g mesophilic aerobics and $2*10^1$ CFU /g yeasts and moulds). These results make it clear that the product is microbiologically stable during the analysed period of time. The microbiological stability of the samples with stevia could be attributed to the antimicrobial properties of the leaves (Debnath, 2008; Seema, 2010; Sivaram and Mukundan, 2003; Tadhani et al., 2006). Barba et al. 2014 incorporated stevia infusions in other products like fruit extracts with good results in terms of stability. Belda et al. 2014 showed that an infusion of stevia leaves had the highest antimicrobial capability against *L. innocua* in comparison to a crude extract of this leaf, and a purified extract of steviol glycosides.

3.5. Sensorial analysis

Figure 5 shows a radial chart of the average scores for each attribute evaluated (appearance, colour, strawberry flavour, sweetness, texture, hardness, gumminess) besides the global preference and intention to buy. In addition, the F-ratio (of the ANOVA considering "formulation" as a factor) is shown next to each attribute. Two

groups of consumers (children and adults) evaluated three samples: the control (sucrose and glucose syrup), "A" (isomaltulose and oligofructose) and "D" (isomaltulose, oligofructose and stevia). Neither of the groups of consumers found significant differences between the samples for the texture parameters (global texture, hardness, gumminess). In relation to the aroma only children found significant differences between samples. Both groups found significant differences between samples for the remaining attributes (appearance, colour, sweetness) and global preference and intention of buying.



ns=not significant **Statistical significance ≥ 99% (p-value ≤ 0.01).

Figure III.7.4 Radial chart of the average scores for each attribute. a) children, b) adults. Samples codification: control, A (0% stevia aqueous extract) and D (100% stevia aqueous extract). Numbers refer to the F-ratio of the ANOVA considering "formulation" as a factor.

Considering that the higher the F-ratio, the greater the effect that a factor has on a variable, sweetness, global preference and intention of buying were most affected by the type of formulation for both types of consumer. As expected, both groups gave higher scores to the control sample (formulated with sucrose and glucose syrup) than samples A and D (formulated with isomaltulose, oligofructose and stevia). However, adults scored sample D similarly to the control sample, especially for sweetness, gumminess and global preference. The better evaluation by adults in comparison to children could be because they know the benefits of stevia and are more aware of the advantages of the product.

4.Conclusions

The instrumental and sensorial textural results, in particular, indicate that traditional sugars in commercial marshmallows could be totally replaced by a mixture of isomaltulose, oligofructose and stevia. The sensory evaluation of these marshmallows by adults, in comparison to children, was very similar to those confected with sucrose and glucose syrup, which could be because they know the benefits of stevia and are more aware of the advantages of the product. These new products, besides being more microbiologically stable, have added value due to their antioxidant properties. However, as the results show that there is a drastic decrease in antioxidants during the first 21 days. It is important to continue studying this topic in greater depth in order to maintain the functional properties of these new marshmallows.

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References

AOAC (2000) Official Methods of Analyisis of AOAC International (17th edition). Gaithersburg, MD, USA.

Barba JF, Criado MN, Belda-Galbis CM, Esteve MJ and Rodrigo D. (2014). Stevia rebaudiana Bertoni as a natural antioxidant/antimicrobial for high pressure processed fruit extract: Processing parameter optimization. *Food Chemistry*, 148, 261-267.

Belda-Galbis CM, Pina-Pérez MC, Espinosa J, Marco-Celdrán A, Martínez A, Rodrigo D. 2014. Use of the modified Gompertz equation to assess the Stevia rebaudiana Bertoni antilisterial kinetics. *Food Microbiology* 38, 56-61.

Bourne MC (1978) Texture profile analysis. *Food technology*, 32, 62-66.

Campbell GM and Mougeot E, (1999) Creation and characterization of aerated food products. *Trends Food Science Technology*. 10, 283-296.

Carbonell-Capella JM, Barba FJ, Esteve MJ, Frígola A, (2013) High pressure processing of fruit juice mixture sweetened with Stevia rebaudiana Bertoni: Optimal retention of physical and nutritional quality. *Innovative Food Science and Emerging Technologies,*18:48-56

Chatsudthipong V, and Muanprasat C, (2009) Stevioside and related compounds: Therapeutic benefits beyond sweetness. *Pharmacology and Therapeutics*. 121, 41-54.

Debnath M, (2008). Clonal propagation and antimicrobial activity of an endemic medicinal plant Stevia rebaudiana. *Journal of Medicinal Plants Research*. 2, 45-51.

Edwards WP (2002). La ciencia de las golosinas. Editorial Acribia, S.A., Zaragoza.

European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. EFSA Journal 2011; 9 (1):1972. doi:10.2903/j.efsa.2011.1972

Francis FJ, and Clydesdale FM (1975) Food colorimetry: theory and applications. AVI, Westport.

Frank A. (2002) Technological functionality of inulin and oligofructose. *British Journal of Nutrition*, 87, 2, 287-291.

Groves R, (1995) Technology and techniques in marshmallow production. *Candy Industry*, 160, 46-53.

Hawai K, Yoshikawa H, Murayam Y, Okuda Y, and Yamashita K (1989) Usefulness of palatinose as a caloric sweetener for diabetic patients. *Hormone and Metabolic Research*, 21, 338–340.

ISO 5492 (2008). Sensory analysis. Vocabulary. International Organization for Standardization.

ISO 8589 (2007). Sensory analysis. General guidance for the design of test rooms.

Kaletunc G, Normand MD, Johnson EA and Peleg M. (1992) Instrumental determination of elasticity of marshmallow. *Journal of Texture Studies* 23, 47-56.

Lees R. (1991) Fundamental principles in the production and characteristics of foam confectionery products. *Confectionery Production*, 57, 210-211.

Lim MH, Yin J, and Heenan S, (2006) The mistery of marshmallow hardening. In: Food Preservation Technology Series-Water properties of food, Pharmaceutical and biological

materials (edited by P. Buera, Jo. Welti-Chanes, P.J. Lillford and H.R. Corti) pp 325-342. New York, CRC (Taylor and Francis).

Lina BAR, Jonker D and Kozianowski G (2002) Isomaltulose (Palatinose®): a review of biological and toxicologycal studies. *Food and Chemical Toxicology*, 40, 1375-1381.

Lemus-Moncada R, Vega-Gálvez A., Zura-Bravo L and Ah-Hen K. (2012) Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects, *Food Chemistry*. 132, 1121-1132.

Muanda F, Soulimani R, Diop B, and Dicko A. (2011) Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. *LWT-Food Science Technology* 44, 1865-1872.

Periche A, Koutsidis G, and Escriche I. (2014) Composition of antioxidants and amino acids in Stevia leaf infusions, *Plant Foods for Human Nutrition* 69, 1-7.

Sanz T, Salvador A, Baixauli R, and Fiszman SM (2009) Evaluation of four types of resistant starch in muffins. II. Effects in texture, colour and consumer response. European Food Research and Technology 229:197-204.

Seema T, (2010) Stevia rebaudiana: a medicinal and nutraceutical plant and sweet gold for diabetic patients. *International Journal of Pharmaceutical and Life Sciences*, 1, 451-457.

Shahidi F, Liyana-Pathirana CM and Wall DS (2006) Antioxidant activity of white and black sesame seeds and their hull fractions. *Food Chemistry* 99, 478-483.

Shukla S, Mehta A, Menta P, and Bajpai V. (2012) Antioxidant ability and phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology* 64, 807-811.

Sivaram L and Mukundan U (2003). In vitro culture studies on Stevia rebaudiana. *In Vitro Cellular and Developmental Biology-Plant*, 39, 520-523.

Tadhani M, Patel V, and Subhash R. (2007) In vitro antioxidant activities of Stevia rebaudiana leaves and callus. *Journal of Food Composition and Analysis*, 20, 323-329.

Tan JM, and Lim MH (2008) Effects of gelatine type and concentration on the shelf-life stability and quality of marshmallows. *International Journal of Food Science and Technology*, 43, 1699-1704.

IV. CONCLUSIONES

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IV.1. Conclusiones del objetivo general 1. (Aportaciones: Food Chemistry (172:1-6 (2015); Plant Foods for Human Nutrition, 69, 1-7 (2014); Plant Foods for Human Nutrition (En revisión); Food Chemistry (En revisión))

IV.1.1. Las condiciones óptimas para secar hojas de stevia fresca se deben considerar teniendo en cuenta el posterior uso que se va a hacer de ellas, para endulzar o por sus propiedades antioxidantes. El secado, ocasiona un aumento importante en los parámetros antioxidantes (fenoles totales, flavonoides y antioxidantes totales), siendo el método más adecuado el aire caliente a 180 °C. Sin embargo, el secado también origina una disminución en los principales glucósidos de steviol, especialmente el steviosido, resultando para estos compuestos menos agresivo el secado a la sombra.

IV.1.2. Comparando los tres tratamientos de secado (liofilizado, secado en sombra y aire caliente) en relación a los compuestos volátiles y antioxidantes (ambos analizados por cromatografía), se observa que ambos tipos de compuestos tienen un comportamiento opuesto. Mientras que el secado en sombra origina un desarrollo de compuestos volátiles (de forma semejante a lo observado para los glúcosidos de steviol), la mayoría de los compuestos fenólicos aumentan su concentración con el liofilizado.

IV.1.3. Las condiciones óptimas para extracción acuosa de parámetros antioxidantes (fenoles totales, flavonoides y antioxidantes totales) y glucósidos de steviol a partir de hojas secas de stevia dependerá de que dichos extractos se utilicen para endulzar o por sus propiedades antioxidantes. No existe un tratamiento único adecuado para obtener simultáneamente el mejor rendimiento de ambos. Con relación a la extracción de parámetros antioxidantes, el tratamiento convencional (infusión a presión atmosférica), es más adecuado que el de ultrasonidos y microondas. Sin embargo, el rendimiento de glucósidos de steviol es mayor aplicando microondas a alta potencia.

IV.1.4. Considerando únicamente la obtención de extractos acuosos de stevia por el método de infusión a presión atmosférica, la condición de temperatura/tiempo que mayor rendimiento proporciona (en relación a fenoles totales, flavonoides, antioxidantes totales y al contenido en aminoácidos) es la más similar al proceso culinario doméstico, agua muy caliente (ligeramente por debajo de ebullición) durante un corto tiempo. Concretamente, el tratamiento de infusión a 90ºC durante 5 minutos puede ser

considerado el más adecuado. El incremento de la temperatura, de 50 a 90ºC, no influye de forma significativa en la extracción de aminoácidos de la hoja de stevia; aunque promovió un ligero aumento de la mayoría de ellos. Sin embargo, para los tres parámetros antioxidantes la elevación de la temperatura aumenta significativamente su extracción. Este hecho no se observa con el incremento del tiempo.

IV.2. Conclusiones del objetivo general 2. (Aportaciones: Food Bioscience 7: 37-44 (2014); LWT-Food Science and Technology (En revisión) y Journal of Food Engineering (En revisión)).

IV.2.1. La isomaltulosa puede ser considerada por la industria de la confitería como un ingrediente apropiado para obtener gomas y espumas saludables. En el caso de las gomas, la isomaltulosa se puede incorporar hasta en un 30% en la formulación (en combinación con la fructosa), aunque debido a problemas de cristalización este porcentaje no puede superarse. En relación a las espumas, se puede aumentar el porcentaje de incorporación de isomaltulosa hasta un 50%, ya que hasta esta proporción no se observó el problema de cristalización que mostraban las gomas. Tanto las gomas como las espumas mostraron características de textura, instrumentales y sensoriales, similares a las formuladas con azúcares convencionales. Se observó una alta correlación entre ambos tipos de parámetros (instrumentales y sensoriales) por lo que se puede concluir que la medida instrumental de la textura puede ser una herramienta adecuada para la estimación de la aceptabilidad global de los consumidores de este tipo de golosinas.

IV.2.2. Extractos de stevia, en combinación con la isomaltulosa y la oligofructosa pueden sustituir totalmente a los azúcares tradicionales utilizados por la industria de la confitería en la fabricación de espumas. Este nuevo producto, además de mostrar resultados similares al formulado según el procedimiento convencional (en relación a los parámetros instrumentales de textura y estabilidad microbiológica), tiene el valor añadido de las propiedades antioxidante aportadas por la stevia. La aceptación de las nuevas espumas, fue similar a las tradicionales, con valoraciones más elevadas en el caso de consumidores adultos, probablemente debido a las ventajas que aporta la incorporación de la stevia como ingrediente.

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