UCAM

UNIVERSIDAD CATÓLICA
DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO
Programa de Doctorado en Ciencias de la Salud

Effect of polyphenol-rich beverages made with *Citrus* and maqui fruits added with low-caloric sweeteners in overweight subjects

Autor:
Hedyeh Masoodi

Directores:
Dr. Dña. Débora Villaño Valencia
Dr. Dña. Pilar Zafrilla Rentero

Murcia, Febrero de 2020

UCAM
UNIVERSIDAD CATÓLICA
DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO
Programa de Doctorado en Ciencias de la Salud

Effect of polyphenol-rich beverages made with *Citrus* and maqui fruits added with low-caloric sweeteners in overweight subjects

Autor:
Hedyeh Masoodi

Directores:
Dr. Dña. Débora Villaño Valencia
Dr. Dña. Pilar Zafrilla Rentero

Murcia, Febrero de 2020

UCAM

UNIVERSIDAD CATÓLICA
DE MURCIA

AUTORIZACIÓN DE LOS DIRECTORES DE LA TESIS
PARA SU PRESENTACIÓN

La Dra. Dña. Débora Villaño Valencia y la Dra. Dña. Pilar Zafrilla Rentero como Directoras de la Tesis Doctoral titulada "Effect of polyphenol-rich beverages made with *Citrus* and maqui fruits added with low-caloric sweeteners in overweight subjects" realizada por Dña. Hedyeh Masoodi en el Departamento de Farmacia, **autorizan su presentación a trámite** dado que reúne las condiciones necesarias para su defensa.

Lo que firmo, para dar cumplimiento al Real Decreto 99/2011, 1393/2007, 56/2005 Y 778/98, en Murcia a 20 de Febrero de 2020

Dra. Débora Villaño Valencia

Dra. Pilar Zafrilla Rentero

RESUMEN

Introducción

En las últimas décadas se ha observado una ingesta excesiva de azúcares en las sociedades industrializadas, principalmente a través de bebidas azucaradas. Grandes estudios epidemiológicos demuestran una relación positiva entre el consumo de este tipo de bebidas y el riesgo de obesidad, diabetes y enfermedades cardiovasculares. Con la finalidad de reducir el contenido en azúcares manteniendo el sabor dulce y la palatabilidad de los alimentos existe una tendencia a buscar nuevas opciones a través de edulcorantes. No obstante, hay una cierta polémica en torno a estos aditivos ya que parece ser que contribuyen a una mayor ingesta calórica y sus efectos a largo plazo no son claros. Paralelamente a la búsqueda de alternativas saludables al alto consumo de bebidas azucaradas, existe una necesidad importante de aumentar el consumo de frutas y hortalizas en la población y desarrollar nuevas formulaciones que aumenten la vida útil de las frutas frescas, preserven los nutrientes y reduzcan el contenido energético de los zumos de frutas. El limón, con su agradable aroma y alto valor nutritivo, es una apuesta importante en la elaboración de bebidas. Su combinación con el fruto rojo maqui contribuye a estabilizar el color y los compuestos (poli)fenólicos que ambos contienen (principalmente flavanonas y antocianos). Se ha descrito además que los compuestos polifénolicos presentes en estos vegetales pueden tener efectos beneficiosos en la mejora de la tolerancia a la glucosa.

Objetivos

Este estudio tiene por objetivo principal evaluar el efecto a largo plazo del consumo de diferentes bebidas a base de frutas añadidas con diferentes tipos de

edulcorantes sobre los biomarcadores de estrés oxidativo, inflamatorio y saciedad en sujetos con sobrepeso.

Métodos

Este estudio es un ensayo clínico de seguimiento, paralelo, aleatorio y triple ciego realizado en una población de sujetos con sobrepeso de la Región de Murcia, España. Se seleccionaron 138 voluntarios de ambos sexos de 35 a 55 años de edad, con sobrepeso, sanos y no fumadores. Se les proporcionó un cuestionario completo para evaluar su adhesión a la Dieta Mediterránea, así como sus hábitos de vida. Los participantes fueron estratificados por sexo, edad e IMC y asignados a un grupo de intervención. La intervención consistió en el consumo de una bebida (330 mL/día) durante 60 días, hecha con extracto de maqui y zumos de cítricos, adicionados con un tipo diferente de edulcorante (Stevia, Sucralosa, Sacarosa).

El estado antioxidante se evaluó por el método ORAC, así como los niveles de LDL-oxidado y de homocisteína. Se midió el perfil glucémico y lipídico, así como los marcadores inflamatorios (IL-6, IL-10, proteína C reactiva). También se evaluaron los niveles de las hormonas de la saciedad, incluidas la leptina y la grelina.

Resultados

Los participantes mostraron una disminución significativa de la masa de grasa corporal (8%) cuando consumieron la bebida que contenía la stevia. En cuanto al estado antioxidante, observamos un aumento significativo en los niveles de homocisteína con la sucralosa y las bebidas de sucralosa, a diferencia de la stevia, que no cambió significativamente.

Se observó un aumento significativo de la glucosa en ayunas con todas las bebidas, principalmente con el tratamiento de la Sucrosa, y un aumento en el

HOMA-IR, siendo significativo con la Sucralosa y la Sacarosa y no con el tratamiento de la Stevia. Los voluntarios con valores basales de glucosa más altos parecían ser menos susceptibles al efecto de la bebida.
Ni los triglicéridos ni el colesterol LDL cambiaron significativamente con ninguna bebida. El HDL aumentó significativamente con el tratamiento de la Sucralosa y en el caso del colesterol total, el aumento significativo se observó con la Sucrosa. La proteína C-reactiva aumentó significativamente con todos los tratamientos, principalmente con la Sucralosa (18% de Stevia, 29% de Sucralosa, 23% de Sacarosa), mientras que los cambios observados en la IL-6 no fueron significativos. La citoquina antiinflamatoria IL-10 aumentó, hasta 4 veces, después de la ingesta de la bebida añadida con la Stevia.
En cuanto a las hormonas relacionadas con el metabolismo energético, después de la ingesta de las tres bebidas los niveles de leptina disminuyeron significativamente (-9 % con la Stevia, -11 % con la Sucralosa) mientras que no hubo cambios significativos en las concentraciones de grelina.

Conclusiones

Teniendo en cuenta los resultados obtenidos en nuestro ensayo clínico, principalmente sobre la resistencia a la insulina y las condiciones inflamatorias después de la ingesta de las diferentes bebidas, las pruebas no apoyan la recomendación de la ingesta de bebidas con edulcorantes bajos en calorías como alternativa a las bebidas endulzadas con azúcar. La identificación del mecanismo subyacente ayudará a diseñar estrategias de nutrición para mejorar la salud general, incluido el estudio de los cambios en la composición de la microbiota intestinal.

Palabras clave: maqui, limón, edulcorantes, estevia, sucralosa, inflamación, saciedad, incretinas, estudio clínico

ABSTRACT

Introduction

Obesity is a major problem worldwide. Data from scientific literature has shown the progressively upward trend of obesity and consequently diabetes mellitus globally. The on-rise consumption of broad spectrum of sugar-sweetened products is among considerable causes of obesity, insulin resistance and metabolic syndrome. Food derived bioactive compounds have been spotlighted as regulators against various chronic diseases including diabetes due to their low toxicity as opposed to drugs that may induce severe side-effects. South American berries including maqui berry are recently more on international interest for their potential antioxidant benefits. Health properties of lemon have been associated to its content in vitamin C as well as flavanones. In the current study we evaluated fruit-based drinks formulated with different sweeteners as an alternative to the consumption of added sugar, as they might be able to counteract the post-prandial response associated with these added sugar beverages in overweight population, which present a chronic low-grade inflammatory status. Besides, the fruit-based drinks are expected to exert functions on physiological targets of obesity and its associated conditions (dyslipidemia, glycemic imbalance and inflammation), all of the relevant to the development of cardiovascular problems as well as diabetes mellitus.

Objectives

This research aims to evaluate the effect of consumption of different fruit based drinks added with different type of sweeteners in long term on oxidative stress biomarkers, inflammatory and satiety in overweight subjects.

Methods

This study is a follow-up clinical trial, parallel, randomized and triple blind performed in a population of overweight subjects from the Region of Murcia, Spain. 138 volunteers of both genders between 35-55 years of age, over-weight, healthy, non-smoker were selected. A complete questionnaire was provided to assess their adherence to the Mediterranean Diet as well as their life-style habits. Participants were stratified by sex, age and BMI rand andomly assigned to one intervention group. The intervention consisted of the consumption of a beverage (330 mL/day) for 60 days, made with maqui extract and Citrus juices, added with a different type of sweetener (Stevia, Sucralose, Sucrose).

Antioxidant status was assessed by ORAC method, as well as levels of LDL-oxidized and homocysteine. Glycemic and lipid profile were measured, as well as inflammatory markers (IL-6, IL-10, C-reactive protein). Levels of satiety hormones including leptin and ghrelin were also assessed.

Results

Participants showed a significant decrease in body fat mass (8 %) when consumed the beverage containing stevia. Concerning antioxidant status, we observed a significant increase in homocysteine levels with Sucralose and Sucrose drinks, different from Stevia, that didn't change significantly.

Regarding the glycemic profile, it showed a significant hike in fasting glucose with all drinks, mainly with Sucrose treatment, and an increase in HOMA-IR, being significant with Sucralose and Sucrose and not with Stevia treatment. Volunteer with higher baseline values of glucose seemed to be less susceptible to the effect of the drink.

Neither triglycerides nor LDL-cholesterol changed with any beverage. HDL increased significantly with Sucralose treatment and in case of total cholesterol, the significant increase was observed with Sucrose. C-reactive protein

significantly increased with all treatments, mainly with Sucralose (18 % Stevia, 29 % Sucralose, 23 % Sucrose), whilst the changes observed in IL-6 were no significant. The anti-inflammatory IL-10 increased, up to 4-fold, after the intake of the drink added with Stevia.
Regarding hormones related to energy metabolism, after the intake of the three drinks leptin levels decreased significantly (-9 % with Stevia, -11 % Sucralose) while there was no significant changes in ghrelin concentrations.

Conclusions

Considering the results obtained in our clinical trial, mainly on insulin resistance and inflammatory conditions after the intake of the different drinks, the evidence doesn’t support the recommendation of the intake of drinks with low-caloric sweeteners as alternative to sugar sweetened beverages. It is necessary to understand the mechanism of action behind, which include the effects on microbiota composition.

Keywords: maqui, lemon, sweeteners, stevia, sucralose, inflammation, satiety, incretins, clinical trial

FUNDING

La presente Tesis Doctoral ha sido financiada por el Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad (convocatoria 2016). Proyecto AGL2016-75332-C2-2-R.

This doctoral thesis has been financed by the State Programme for Research, Development and Innovation Oriented to the Challenges of Society (2016 call). Project AGL2016-75332-C2-2-R.

CONTRIBUTIONS DERIVED FROM SCIENTIFIC ACTIVITY DURING THE PERIOD OF THIS DOCTORAL THESIS

Presentation to Congresses

- Assesment of the adherence to the Mediterranean Diet in adult population with overweight and obesity. Masoodi H, Zafrilla P, García-Benzal JD, Cerdà B, Villaño D. V Congeso Internacional en Contextos Clínicos y de la Salud. 4-5 Abril 2019 Murcia (España).

- Natural and Artificial Non-caloric Sweeteners as Sugar Substitutes and Their Health Effects. Masoodi H, Zafrilla P, Marhuenda J, Bernardo V, Villaño D. XIII Congreso Internacional de Nutrición Alimentación y Dietética. XXIII Jornadas Internacionales de Nutrición Práctica. 3-4 Abril 2019 Madrid (España).

Scientific Publications

A comprehensive review on fruit Aristotelia chilensis (Maqui) for modern health: towards a better understanding. Masoodi H, Villaño D, Zafrilla P. Food & Function 2019, 10, 3057-3067.

ACKNOWLEDGMENTS

Present work is a result of help rendered to me by many authorities, professors and friends. It is my pleasant duty to express my sincere thanks to all of them. I wish to acknowledge and express my thanks to the following people who in a variety of ways have assisted me in the completion of this thesis.

The study no doubt benefited from the critical and sound judgment, continuous support and encouragement, unwavering dedication and availability of my supervisor, Dr. Debora Villaño Valencia and co-advisor Dr. Pilar Zafrilla Rentero, and my tutor Dr. Silvia Montoro Garcia. I have enjoyed working with my Professors. I appreciate the support, encouragement, throughout the 2.5 years period of this thesis. I would like to transfer my immense sincere heartedly gratitude for providing the general guidance, extreme support and for directing the course of this thesis, scientific discussions and above all for making me feel comfortable and welcomed at knocking on your door at any time without appointment.

Special appreciation goes to the Vice-Chancellor, the Dean Faculty of Science, the entire professors and staff of the Department of Pharmacy,specially (Alejandro Galindo, Begoña Cerdà, Victoria Bernardo, Javier Marhuenda, Alfonso Pérez, Marien Martínez, Ignacio Segarra, Pedro Matías Rodríguez), and the department of nursing, the director and staff of doctoral section, Andrés Hernández and Ana Baella, Office of International Exchange, Lonneke Meijer, at the University Catholica San Antonio de Murcia (UCAM), for their support and encouragement and providing me with a conductive atmosphere.

Many thanks to my assistants Carlos Iglesias Aguirre, José David García Benzal, and Farah Toutouh, for their contribution to the smooth progress of this thesis.

Last but not least I’m grateful to the research participants for their cooperation. And all people who made this research possible.

With love to:

My dear parents especially my dear mom whose heart, beauty and grace are unquestionably divinely inspired.

In eternal gratitude for tolerance, love, patience and the nest, the compass and the wings….

Dear Dr. Pilar Zafrilla Rentero and Dr. Debora Villaño Valencia

Without their great support this research would not have been possible….

INDEX

AUTHORIZATION OF DIRECTORS ………………………………............. 5
RESUMEN ………………………………………………………………… 7
ABSTRACT………………………………………………………………… 11
FUNDING ………………………………………………………………… 15
SCIENTIFIC CONTRIBUTIONS …………………………………….... 17
ACKNOWLEDGEMENTS……………………………………………… 19
GENERAL INDEX……………………………………………………..... 23
ABRREVIATIONS………………………………………………………. 27
INEDX OF FIGURES……………………………………………………. 31
INDEX OF TABLES…………………………………………………….. 33
INDEX OF ANNEX…………………………………………………….. 35
I. INTRODUCTION……………………………………………………… 37
1.1. OBESITY, OXIDATIVE STRESS AND INFLAMMATION……...... 39
1.2. SWEETENED BEVERAGES………………………………………… 40
1.2.1. Global consumption of sweetened beverages………………… 40
1.2.2. Sweetened beverages, insulin sensitivity, cardiovascular risk and obesity……………………………………………………………………… 44
1.3. DIETARY ANTIOXIDANTS: POLYPHENOLS……………………… 48
1.4. MAQUI BERRY…………………………………………….......... 54
1.4.1. Composition………………………………………………...... 54
1.4.2. Bioavailability of anthocyanins of maqui……………….......... 56
1.4.3. Antioxidant activity of maqui fruit…………………………… 57
1.4.4. Anti-inflammatory effect of maqui fruit……………………… 59
1.4.5. Effect on metabolic profile………………………………………… 60
1.4.6. Other biological activities of maqui……………………………… 62
1.5. LEMON………………………………………………………………. 69
1.5.1. Production and nutritional composition of lemon fruit……….. 69
1.5.2. Biological activities of lemon fruits………………………………. 71
1.6. CALORIC AND NON-CALORIC SWEETENERS…………………… 73
1.6.1. Caloric sweeteners………………………………………………… 74
1.6.1.1. Sucrose…………………………………………………….......... 74
1.6.1.2. Corn syrup…………………………………………………......... 74
1.6.2. Low-caloric sweeteners…………………………………………….. 75
1.6.2.1. Sucralose…………………………………………………………. 76
1.6.2.2. Stevia……………………………………………………………….. 77

1.6.2.3. Aspartame……………………………………………………………… 80
1.6.2.4. Saccharine……………………………………………………………… 81
1.6.2.5. Acesulfame-K…………………………………………………………... 82
1.6.2.6. Neotame………………………………………………………………… 83
1.6.2.7. Monk fruit (Luo han guo)……………………………….................... 84
1.6.3. Health effects of sweeteners………………………………………………… 84
II. OBJECTIVES……………………………………………………………………. 89
2.1. HYPOTHESIS…………………………………………………………………….. 91
2.2. GENERAL OBJECTIVE………………………………………………………… 91
2.3. SPECIFIC OBJECTIVES………………………………………………........ 91
III. MATERIAL AND METHODS…………………………………….............. 93
3.1 BEVERAGES USED FOR THE CLINICAL STUDY……….................. 95
3.2. DESIGN OF THE CLINICAL TRIAL……………………….................. 96
3.3. ETHICAL CONSIDERATIONS………………………………………………… 96
3.4. INCLUSION/EXCLUSION CRITERIA………………………………………… 97
3.5. WORKING PLAN……………………………………………………............. 97
3.6. EVALUATION OF DIETARY AND LIFESTYLE HABITS……............. 102
3.7. VARIABLES OF STUDY……………………………………………………… 103
3.7.1. Anthropometric measurements…………………………………………… 103
3.7.2. Cardiovascular markers……………………………………………………. 104
3.7.3. Antioxidant status…………………………………………………………... 104
3.7.3.1. Oxidized LDL………………………………………….................... 104
3.7.3.2. ORAC method…………………………………………………………… 105
3.7.3.3. Homocysteine……………………………………….................... 106
3.7.4. Biochemical analysis: Glycemic profile, Lipid profile, Safety parameters………………………………………………………............................ 107
3.7.4.1. HDL…………………………………………………………................ 107
3.7.4.2. LDL………………………………………………………………………. 108
3.7.4.3. Total Cholesterol…………………………………….................... 108
3.7.4.4. Triglyceride……………………………………………………….... 109
3.7.4.5. Alkaline Phosphatase (ALP)……………………………………….. 110
3.7.4.6. Alanine AminoTransferase (ALT)……………………................ 110
3.7.4.7. Aspartate AminoTransfrase (AST)………………….................. 111
3.7.4.8. Gama-Glutamyl-ransfrase (GGT)…………………................... 111
3.7.4.9. Total Bilirubin……………………………………………………….. 112
3.7.4.10 Glucose and insulin level, HOMA-IR……………………………….. 113
3.7.5. Inflammation markers……………………………………………………….. 113
3.7.6. C-Reactive Protein (CRP)………………………......................... 115
3.7.7. Satiety hormones…………………………………………………………… 116

3.7.7.1. Leptin………………………………………………………………. 116
3.7.7.2. Ghrelin…………………………………………………………….. 117
3.8. STATISTICAL ANALYSIS……………………………………................. 117
3.8.1. Effect of each drink (composition within the group)……………….. 118
3.8.2. Difference between drinks (comparson between groups)………… 118
IV.RESULTS………………………………………………………………… 119
4.1. BASAL CHARACTRISTICS OF STUDY POPULATION……………….. 121
4.2. DIETARY AND LIFE STYLE HABITS…………………………………….. 122
4.2.1. Life style habits…………………………………………………... 122
4.2.2. Adherence to the Mediterranean diet………………………………. 124
4.3. Knowledge and use of sweeteners………………………………………. 128
4.4. Acceptability of the test drinks by volunteers………………………….. 129
4.5. Composition of the tested beverages…………………………................ 132
4.6. Assessment of safety parameters………………………………................ 134
4.7. Anthropometric and cardiovascular markers……………………………… 135
4.8. Antioxidant status………………………………………………………… 135
4.9. Glycemic profile…………………………………………………………... 140
4.10. Lipid profile……………………………………………………………… 142
4.11. Inflammation markers…………………………………………………….. 143
4.12. Satiety hormones………………………………………………………… 146
V. DISCUSSION……………………………………………………................. 149
5.1. Adherence to Mediterranean diet………………………………………… 151
5.2. Knowledge and use of sweeteners……………………………………….. 152
5.3. Acceptability of the test drinks by volunteers………………….………. 153
5.4. Effect on anthropometric and cardiovascular markers.………………….. 153
5.5. Effect on antioxidant status ………………….…………………….………. 155
5.5.1. ORAC ………………….....................…………………….………. 155
5.5.2. Homocysteine…………......................……………………..……… 156
5.5.3. Oxidized LDL ……….....................………………….……….. 157
5.6. Effect on energy metabolism ……………………………………………... 159
5.6.1. Lipid profile ………….. 159
5.6.2. Glycemic profile …………………………………………………….. 159
5.7. Effect on inflammatory status ……………………………………………. 167
5.8. Effect on satiety hormones …………………..…………………………… 170
VI CONCLUSIONS …………………………….. 173
VII. STRENGHTS AND WEAKNESSES OF THE STUDY ……………………. 177
VII. REFERENCES……………………………………………………………. 183
VIII. ANNEX………………………………………………………………... 221

ACRONYMS AND ABBREVIATIONS

ABTS	3-ethylbenzothiazoline-6-sulfonic acid
AC	Antioxidant Capacity
AD	Alzheimer’s Disease
ADA	Adenosine DeAminase
ADI	Acceptable Daily Intake
ALP	Alkaline Phosphatase
ALT	Alanine Amino Transferase
ANIBES	Anthropometry, Intake, Energy Balance in Spain
ANOVA	Analysis of Variances
ASB	Artificially Sweetened Beverages
AST	Aspartate Amino Transferase
AUC	Area Under the Curve
BMI	Body Mass Index
CDC	Center for Disease Control
CEIC	Comité Ético de Investigación Clínica
COX-2	CycloOxigenase-2
CRP	C-Reactive Protein
CVD	Cardio Vascular Disease
D3S5G	Delphinidin-3-sambubioside-5-glucoside
DCFH-DA	2,7, dichlorodihydrofluorescin diacetate
DM	Diabetes Mellitus
DPPH	2,2,diphenyl-1-picrylhydrazyl
EDTA	Ethylene Diamine Tetraacetic Acid
EEC	Entero Endocrine Cells
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immuno-sorbent Assay
EPIC	European Prospective Investigation Committee
EU	European Union
FAO	Food and Agricultural Organization
FDA	Food and Drug Association
FIC	Ferrus Chelating Capacity
FRAP	Ferric Reducing Antioxidant Power
FSANS	Food Standards Australia New-Zealand
GLDH	Glutamate DeHydrogenase
GLUT-1	Glucose Transporter-1
GLUT-2	Glucose Transporter-2
GR	Glycemic Response

HbA1C	Glycosyated Hemoglobin
HDL	High Density Lipoprotein
HFCS	High Fructose Corn Syrope
HMTasa	Hcy S-Methyl-Transferase
IDF	International Diabetes Federation
IFCC	International Federation of Clinical Chemistry
IGT	Impaired Glucose Tolerance
IL-8	InterLeukin-8
iNOS	Inducible Nitric Oxide Synthesis
JECFA	WHO Expert Committee on Food Additives
LCS	Low Calorie Sweeteners
LDL	Low Density Lipoprotein
IL-1B1	InterLeukin-1B1
LNCS	Low or No Calorie Sweeteners
LPS	Lipo-Poly-Saccharide
MCP1	Monocyte Chemoattractant Protein1
Met	Methionine
MIC	Minimum Inhibitory Concentration
NAFLD	Non-Alcoholic Fatty Liver Disease
NAS	Non-caloric Artificial Sweeteners
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NNS	Non Nutritive Sweetener
NO	Nitric Oxide
NSS	Non-Sugar-Sweetener
OECD	Organization for Economic Cooperation and Development
OGTT	Oral Glucose Tolerance Test
ORAC	Oxygen Radical Absorbance Capacity
PPM	Particle Per Mililitre
RCT	Randomized Clinical Trial
ROS	Reactive Oxygen Species
RP-HPLC-DAD	Reverse Phase-High Performance Liquid Chromatography-with Diode Array Detector
SAH	S-Adenosyl-Homocysteine
SAHasa	SAH hydrolase
SAM	S-Adenosyl-Methionine
SSB	Sugar Sweetened Beverages
SSSD	Sucrose Sweetened Soft Drink
SSSD	Sugar Sweetened Soda Drink
STR	Sweet Taste Receptor

T2D/T2DM	Type 2 Diabetes Mellitus
TA	Total Anthocyanins
TBARS	Thiobarbituric acid reactive substances
TCEP	Tris (2-Carboxietil) Phosfina
TE	Total Energy
TMB	Tetra Methyl Benzidine
TNF-α	Tumor Necrotising Factor-alpha
TP	Total Phenols
UCAM	Universidad Católica San Antonio de Murcia
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WHR	Waist to Hip Ratio

INDEX OF FIGURES

Figure 1. Projected rates of obesity………………………………...	39
Figure2. UK criteria for sugar taxation……………………………	43
Figure 3. Sugar taxation around the world……………………….	43
Figure 4. Classification of common dietary phytochemicals……	49
Figure 5. Structure of the flavonoid skeleton……………………..	51
Figure 6. Biological activities of polyphenol compounds……….	53
Figure 7. Molecular structure of Delphinidin-3-sambubioside-5-glucoside……………………………………………………………	55
Figure 8. Chemical structure of Hesperetin……………………….	71
Figure 9. Type of sweeteners……………………………………….	73
Figure 10. Chemical structure of Sucrose………………………….	74
Figure 11. Chemical structure of Sucralose……………………….	77
Figure 12. Chemical structure of Steviol aglycone……………….	79
Figure 13. Stevia leaves……………………………………………..	79
Figure 14. Chemical structure of Aspartame……………………..	80
Figure 15.Chemical structure of Saccharin………………………..	82
Figure 16. Chemical structure of Acesulfame K………………….	83
Figure 17. Chemical structure of Neotame……………………….	83
Figure 18. Monk fruit………………………………………………..	84
Figure 19. Flow diagram for long-term interventional study…..	98
Figure 20. Bottles provided o the volunteers with the maqui-citrus beverage……………………………………………………………	100
Figure 21. Study design……………………………………………..	102
Figure 22. Coupled reactions to detect homocysteine levels in plasma………………………………………………………..........	106
Figure 23. Scheme simplified of ELISA test principle…………...	115
Figure 24. Frequency of consumption of sweetened products….	128
Figure 25. Opinion of participants on the taste of drink A (with Stevia)……………………………………………………………….	129
Figure 26. Hedonic scale of participants on drink A (with stevia)………………………………………………………………..	129
Figure 27. Opinion of participants on the taste of drink B (with Sucralose)………………………………………………………….	130

Figure 28. Hedonic scale of participants on drink B (with Sucralose)………………………………………………………….. 130
Figure 29. Opinion of participants on the taste of drink C (with Sucrose)…………………………………………………………….. 131
Figure 30. Hedonic scale of participants on drink C (with Sucrose)…………………………………………………………….. 131
Figure 31. Correlation of delta ORAC and initial ORAC level with the drink intervention……………………………………………….. 139
Figure 32. Correlation of delta and initial glucose level in drink A (stevia)……………………………………………………………… 141
Figure 33. Correlation of delta and initial glucose level in drink B (Sucralose)………………………………………………………….. 142
Figure 34. Correlation of delta and initial glucose level in drink C (Sucrose)……………………………………................................. 142
Figure 35. Gastrointestinal factors that influence glycemic control……………………………………………………………….. 167

INDEX OF TABLES

Table 1. Dietary intake of Spanish individual (free and intrinsic) sugar (g/day)………………………………………… 42
Table 2. Studies on the biological effects of maqui……………. 64
Table 3. FDA classification of high intensity, low-caloric sweeteners……………………………………………………….. 76
Table 4. Variable assessed in the long-term intervention trial... 101
Table 5. Reagents preparation for total bilirubin measurements…………………………………………………... 112
Table 6. Baseline characteristics of volunteers…………………. 121
Table 7. Demographic frequency and education level of participants……………………………………………………… 122
Table 8. Health history of participants………………………….. 123
Table 9. Result of Mediterranean diet questionnaire………….. 124
Table 11. Anthocyanin and Flavonone composition (mg/100mL) of the maqui-citrus juice ………………………….. 133
Table 12. Safety parameters: hepatic function………………….. 134
Table 13. Anthropometric and cardiovascular parameters…... 136
Table 14. Antioxidant status …………………………………….. 137
Table 15. Biochemical parameters: Glycemic profile………….. 140
Table 16. Biochemical parameters: Lipid profile………………. 144
Table 17. Inflammatory profile…………………………………... 145
Table 18. Satiety hormones…………………………………….... 147

INDEX OF ANNEX

ANNEX 1. Aproval from the Ethical Comittees ………………………… 223
ANNEX 2. Inclusion / Exclusion Criteria ………………………………... 225
ANNEX 3. Informed Consent …………………………………………….. 226
ANNEX 4. Written information for the volunteers …………………….. 228
ANNEX 5. Questionnaire of lifestyle habits and Mediterranean diet adherence ………………………………..………………………….. 231
ANNEX 6. Questionnaire of the use of sweeteners …………………….. 239
ANNEX 7. Organoleptic Questionnaire …………………………………. 241

I - INTRODUCTION

I - INTRODUCTION

1.1. OBESITY, OXIDATIVE STRESS AND INFLAMMATION

Obesity is a major problem worldwide. Data from scientific literature has shown the progressively upward trend of obesity and consequently diabetes mellitus globally, as stated by the Center for Disease Control (CDC), International Diabetes Federation (IDF), and World Health Organization (WHO) (Deraux et al., 2017). Based on the International Diabetes Federation (IDF), the estimated prevalence of diabetes in Europe in 2017 was approximately 58 million, which is likely to rise to 67 million by 2045, by 16% hike (Figure 1). In Spain it contributed to have around 3.5 million diabetes until 2017. Likewise, obesity as the main cause of later metabolic syndrome development worldwide prevalence is also nearly doubled.

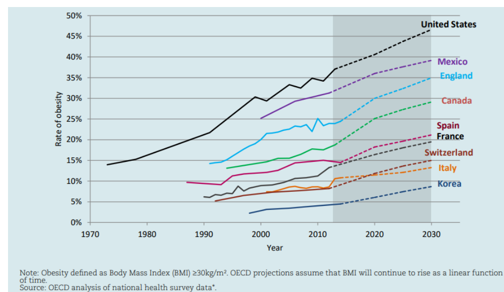


Figure 1. Projected rates of obesity (Deraux et al., 2017)

Obesity is the most common cause of dyslipidemia, and has complex pathways, characterized by an extra deposition of fat which leads to a chronic low grade inflammatory state associated with high levels of inflammatory markers in blood circulaion, such as cytokines generated by deposition of macrophages in adipose tissue. There is a local production of TNF-α that consequently generates hyperinsulinemia, with over stimulation of pancreatic β-cells and reduction of insulin receptors, all of which lead to insulin resistance (González-Castejón et al., 2011).

Insulin-resistance caused by obesity shows a chronic inflammatory state (Reyes-Farias et al., 2015, 2016). Consequently, metabolic syndrome manifests as overall and abdominal obesity, high blood pressure, insulin resistance, hyperglycemia, impaired glucose tolerance (IGT), low HDL cholesterol and elevated triglyceride level (Reaven, 2011). Chronic low-grade inflammation is implicated in the development and worsening effect of obesity and is associated to chronic diseases such as type II diabetes and cardiovascular disease.

1.2. SWEETENED BEVERAGES

1.2.1. Global consumption of sweetened beverages

The on-rise consumption of a broad spectrum of sugar-sweetened products is among the considerable causes for obesity, insulin resistance and metabolic syndrome. They include ready to drink, fruit based, carbonated and energy drinks.

Sugar-sweetened beverages (SSB), sweetened with either sucrose or high-fructose corn syrup, are the leading source of added sugars in the diets of American adults (Rosinger et al., 2017).

Sugar-sweetened carbonated and soft drinks are the most popular choices of snack over the last decades (Kerr et al., 2008). Most of them have high levels of sucrose, or high fructose corn syrup as ingredient (Malik et al., 2015). They represent a 16% of daily caloric intake of adults in the United States (Ervin et al., 2013) and 11% in Canada and Australia (Brisbois et al., 2014), high above levels recommended by the World Health Organization recommendation (10 % of daily caloric intake) (WHO Guidelines, 2015).

In the "Anthropometry, Intake, energy Balance in Spain" (ANIBES) study, performed on 2013 in a representative sample of Spanish population people (n= 2009) of 9-75 years of age, they reported a median total sugar consumption of 71.5 g/day (17 % total energy (TE) intake), following with intrinsic sugar consumption of 38.3 g/day (9.6 % TE) and free sugar of 28.8 g/day (7.3 % TE). The top source of consumption in adolescence and adult groups were in sugar soft drinks while sugar-bakery products and pastries, yoghurt/fermented milk and jam were more popular in old Spanish population (Ruiz et al., 2017). Only a moderate percentage followed the nutritional recommendations concerning the sugar intake (free sugar contributing less than 10 % total energy intake) and these findings point out to the need of urgent efforts to improve the quality of diet, especially in the youngest populations (Table 1).

Table 1. Dietary Intake of Spanish Individual (free and intrinsic) sugars (g/day) (Ruiz et al., 2017)

Age group (years)	Mono/Disaccharides (g/day)	
	Males	**Females**
9-12	9.9 ± 4.1	9.6 ± 3.8
13-17	9.6 ± 4.6	10.8 ± 4.2
18-64	7.0 ± 4.8	7.3 ± 4.5
65-75	4.8 ± 3.1	5.4 ± 3.7

These type of sugars are rapidly absorbed by the small intestine, so that glycemic levels increase sharply, what contributes to increased peripheral insulin resistance and increase of the risk of type 2 diabetes (Schulze et al., 2004).

The consumption of sugar-sweetened beverages has been associated with an increased risk of developing type 2 diabetes, and this effect was not related to the total energy intake or body mass (Malik et al., 2010 and 2015). Though these findings of epidemiological studies do not establish causality (Kahn et al., 2014; Rippe et al., 2016), due to the adverse health outcomes linked to high sugar consumption, changes in global health policy have been made to limit such intake.

In March 2016 the British government announced that a tax on sugary soft drinks would be introduced in the UK from 2018 to decrease the rates of obesity and type 2 diabetes, the so called "Soft Drinks Industry Levy" (Figure 2). Other countries have adopted similar actions (Figure 3).

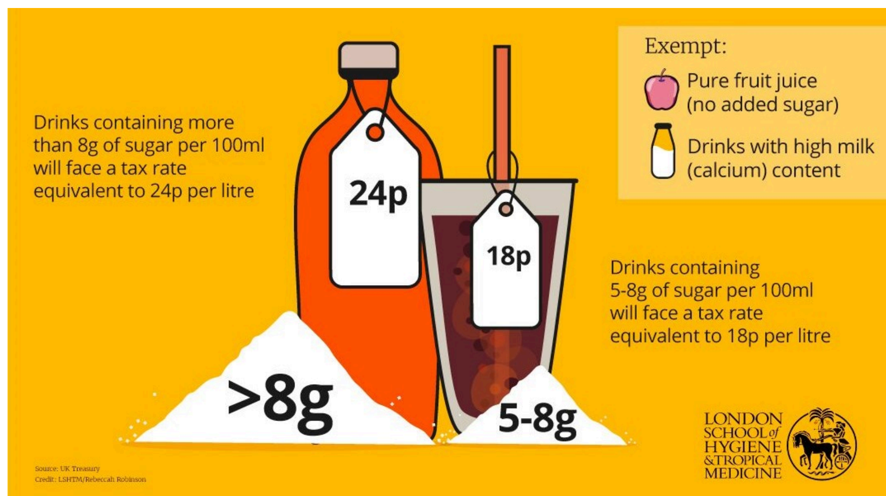


Figure 2. UK criteria for sugar taxation (London School of Hygiene & Tropical Medicine website)

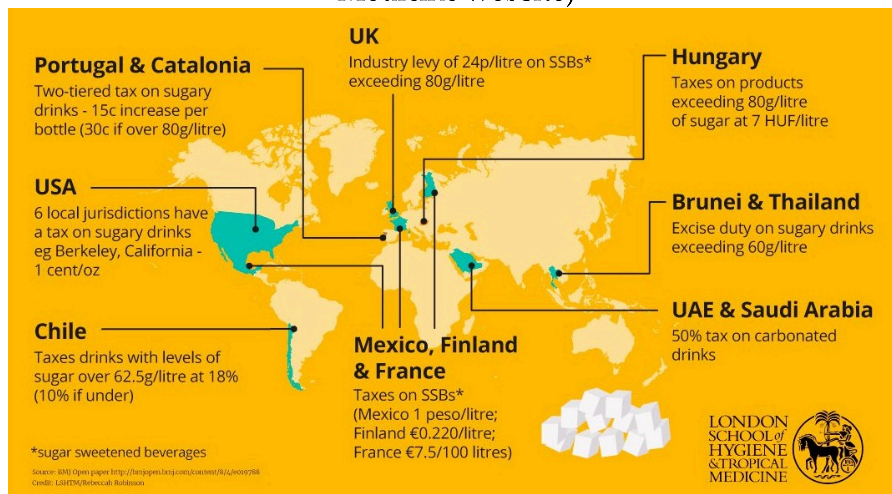


Figure 3. Sugar taxation around the world (London School of Hygiene & Tropical Medicine website)

Besides, the 2015-2020 Dietary Guidelines for Americans recommend to limit the dietary sugar to 10% of total energy (Clemens et al., 2016).

1.2.2. Sweetened beverages, insulin sensitivity, cardiovascular risk and obesity

Factors that influence sugar metabolism include dose-energy intake, food source, genetic, ethnicity, race and physical activity (Clemens et al., 2016).

Each type of sugar ingested determines the rise of blood glucose, as well as its pattern of elimination. The glycemic response (GR) is determined by the specific sugar, as well as by the food matrix in which it is included, the degree of processing suffered by the food product, the presence of fat, fiber, and protein or the concomitant ingestion of other foods (Wolever and Bolognesi, 1996).

Hepatic metabolism of sugars is related to the metabolism of each of the three absorbed monosaccharides (glucose, fructose, and galactose), whilst the metabolism occurring in other organs and tissues (extrahepatic) refers to glucose metabolism. Only minimal amounts of fructose and galactose reach the systemic circulation in healthy adults (Ning and Segal, 2000; Kawasaki et al., 2002).

The role of sugars in the development of cardio-metabolic disease is actively debated (Bray et al., 2014). Negative outcomes of sugar-sweetened beverages (SSB) including type 2 diabetes mellitus (DM), obesity and over-weight is well-known (Johnson et al., 2009; Malik et al., 2006; 2010; Temorenga et al., 2013; Van Back and Astrup, 2009). Observational studies have linked the consumption of sugar-sweetened beverages to an increase in the risk of type 2 diabetes and insulin resistance (Ludwig et al., 2001, Schulze et al., 2004; Palmer et al., 2008). Insulin resistance is a condition whereby the body’s sensitivity or responsiveness to the hormone insulin is decreased, leading to metabolic dysregulation. Insulin

resistance is a major cause of type 2 Diabetes Mellitus (T2DM) and is a key feature of many other cardiometabolic diseases (Laakso & Kuusisto, 2014).

It has been reported a significant positive association between soft drink consumption and energy intake (Rajeshwari et al., 2005, Berkey et al., 2004; Davy et al., 2004; Schulze et al., 2004). Soft drinks might stimulate appetite or suppress satiety due to high glycemic index and rapid rise in blood glucose (Ludwig et al., 2001). An epidemiological study performed to show that high sugar drinks might relate to other obesogenic dietary habits proved a deleterious relationship of sugar-sweetened beverages with BMI and impaired fasting glucose in a largely obese indigenous population (Lazzinnaro et al., 2012).

There was a positive significant association between soft drinks consumption and BMI (Kvaavik et al., 2005; Blum et al., 2005) as well as positive relation with body fat percentage (Giammattei et al., 2003), and people were in higher risk of being over-weight/obese (Strigel-Moore et al., 2006; Bes-Rastollo et al., 2006), though there was no relation in the study by Mc Gartland et al., (2003). Epidemiological studies have demonstrated an association between one or more soft drinks consumption per day and metabolic syndrome in a 4 years follow up study by Dhingra et al. (2007) as well as in the 9 years follow up by Lutsey et al. (2008).

Human intervention studies have also shown deleterious effects with the consumption of sugar-sweetened drinks. Njikeet et al. (2011), in a study of consumption of sugar-sweetened hot cocoa for six months on adults, found an increase in Body Mass Index (BMI) and waist circumference. In two different

years, Reid et al (2007) reported higher BMI and body weight with sugar sweetened beverages on adults of 20-55 years of age after 4 months consumption.

Studies in volunteers carried out with drinks added with caloric sweeteners show that postprandial glycemia and insulin response are proportional to the content of glucose, fructose and sucrose. Sucrose is easily converted into equimolar amounts of glucose and fructose at the intestinal level.

Fructose has been associated with a lower increase in glycemia and insulinemia compared to glucose or sucrose, due to the slower rate of absorption of fructose and the different metabolic pathways that follows in the liver (Feinman et al., 2013). Early considerations for the use of fructose as an alternative sweetener in people with diabetes, showed a potential to lower postprandial glycaemic excursions when compared with iso-caloric amounts of starch (Bantle et al., 1986). However, some concerns have been posed on the consumption of fructose in long term. Figlewicz et al. (2009) stated that the moderate consumption of fructose is related to negative profiles in plasma lipids. Increasing evidence has suggested that fructose could be particularly detrimental to metabolic health, more so than other sugars (Lustig, 2013). Uric acid is a byproduct of fructose metabolism when the normal pathways of metabolism are saturated and this compound is a known risk factor for hypertension (Khitan and Kim, 2013).

Ecological parallel studies have been drawn, to contrast the introduction of high fructose corn syrup (HFCS) as a popular sweetener during the 1970s and the global rises in obesity and diabetes prevalence (Gross et al., 2004).

Sugar sweetened beverages consumption is also known as a cause of Non-Alcoholic Fatty Liver Disease (NAFLD) without any other risk factor (Assy et al., 2008). In a six month randomized intervention study on four groups, comparing regular soda (Sugar Sweetened Soda Drink (SSSD)), iso-caloric semi-skimmed milk, aspartame-sweetened diet cola and water, the daily intake of SSSD led to increase the storage of fat in the liver, muscle and visceral (Maersk et al., 2012).

In the study of Te Morenga et al. (2014) it was observed worse patterns in blood pressure and serum lipids with the consumption of dietary sugars at high levels for prolonged periods.

Considering the data of both epidemiological as well as interventional studies, many health care scientists suggest the substitution with non-caloric high intensity sweeteners in food and beverages (Mattes and Popkin, 2009; Duffey et al., 2012; Fitch and Keim, 2012; Gardner et al., 2012). Diet beverages contain a single low caloric sweetener (LCS), or combinations of them, as aspartame (E951), sucralose (E955),and acesulfame-K (E950) (Sylvetsky et al., 2016).

Besides, it must be considered that, depending on their composition, these non-alcoholic beverages can be important sources of nutrients and bioactive compounds that may influence human health and decrease the risk of chronic diseases (Rothwell, 2018).

Juices made from fruit-extracts added with non-caloric sweeteners seem an interesting alternative for all these beverages, as they can provide vitamins, minerals, soluble fiber, and phytochemicals as phenolic compounds, in addition to sugars.

1.3. DIETARY ANTIOXIDANTS: POLYPHENOLS

Food derived bioactive compounds have been spotlighted as regulators against various chronic diseases including diabetes, due to their low toxicity as opposed to drugs that may induce severe side-effects (Lee et al., 2017). Rapidly increasing proportion of aging people in almost all parts of the world shows the need of natural solutions to delay age-related ailments.

Fruits and vegetables are the main source of antioxidants in the human diet, which includes polyphenols, carotenoids, vitamin C, vitamin E, zinc and selenium (Heis et al., 2010). Diet rich in polyphenol sources, has proved to be effective against non-communicable diseases such as cardiovascular disease, cancer and diabetes (Piljac-Zegrac et al., 2009).

However, there is limited literature properly describing requirements of various antioxidants and its potential benefits (Montonen et al., 2004). Since dietary phytochemicals may suppress growth of adipose tissue, stimulate lipolysis and induce apoptosis of existing adipocytes, they might be considered as anti-obesity agents (Gonzalez-Castejón and Rodriguez-Casado, 2011).

The more relevant family of bioactive phytochemicals with proven biological activities is the polyphenols. Common dietary polyphenols are categorized in different sub-groups (Figure 4).

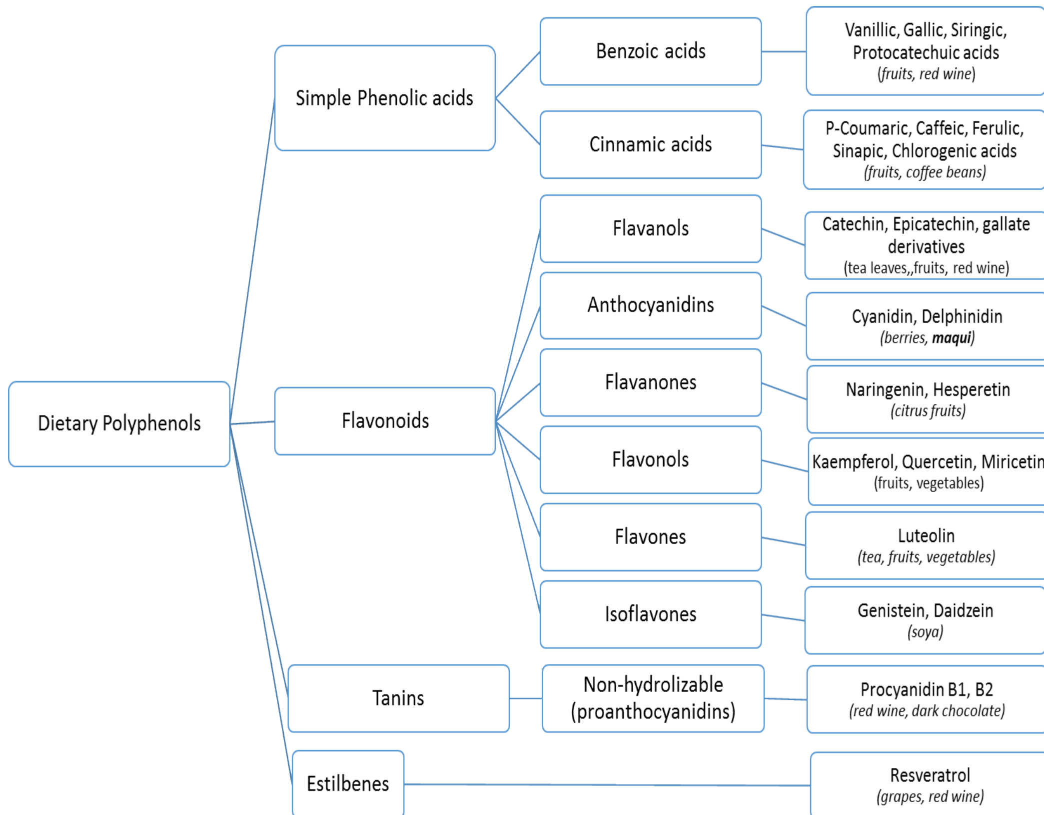


Figure 4. Classification of common dietary phytochemicals (modified from Gonzalez-Castejón and Rodriguez-Casado, 2011).

Phenolic compounds are products from secondary metabolic pathways with attributed defense properties against biotic and abiotic stress in the plant. They are widely distributed within the plant kingdom, especially in edible fruits and vegetables, and are commonly founded in conjugated from with mono-disaccharides and organic acids. They are generally classified depending on the

presence/absence of flavano skeleton in the categories of flavonoids and non-flavonoids (Parr & Bolwell, 2000).

The phenolic acids are included in the category of non-flavonoid compounds, which can be further subdivided in benzoic acid derivatives and hydroxycinnamic acid derivatives, being this last group the most common in nature (Manach et al., 2004).

The main subclasses are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins, depending on the level of oxidation of the C-ring. Other flavonoid groups are the chalcones, dihydrochalcones, dihydroflavonols, flavan3, 4-diols, and aurones (Crozier et al., 2009; Del Rio et al., 2013) (Figure 5).

Flavanones are the most abundant *Citrus* flavonoids, while Flavones are not distributed widely, and they have only been reported with significant occurrences in celery, parsley, some herbs and *Citrus* species (Crozier et al., 2009). On the other hand, the Flavonols are the most widespread subclass of the flavonoids, being dispersed throughout the plant kingdom with the exception of algae (Crozier et al., 2009). Among fruits, berries are the richest source of polyphenols (Nowak et.al, 2016).

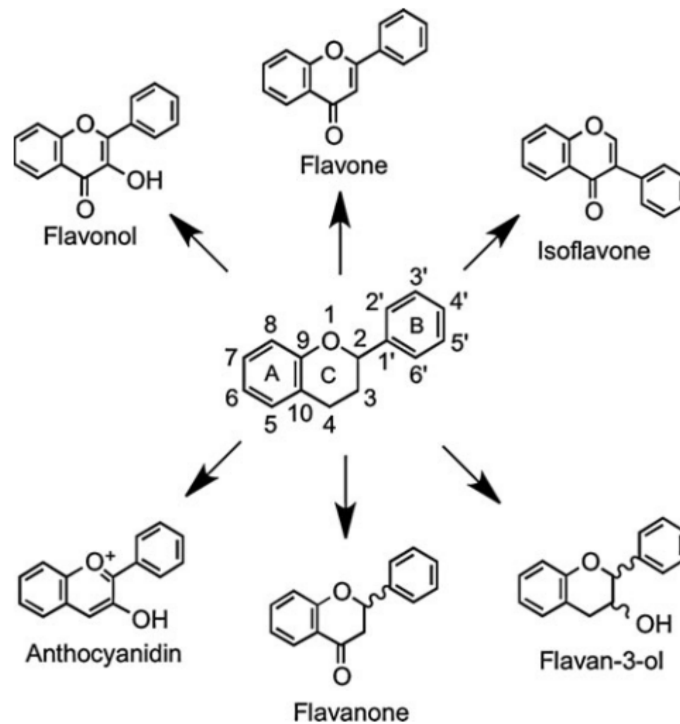


Figure 5. Structure of the flavonoid skeleton (Del Rio et.al, 2013)

Anthocyanins represent water-soluble flavonoid compounds, commonly found in berries and they are pigments responsible for the blue and red colors of certain kinds of grapes, berries, currants. It has been calculated a daily intake of anthocyanins of 1 g per day, in people who routinely eat berries and drink red wine (Del Rio et al., 2010).

Composition of polyphenols in the plant are varying depending on different species, and, within a plant species, is also based on their cultivation conditions and the time of harvesting. The total polyphenols content can be higher or lower depending on the ripeness, harvest time, climate, and genotype

of the fruit and storage conditions (Khoo et al., 2017; Cespedes et al., 2010; Escribano-Bailon, 2006), and it also depends on the origin, environmental factors, altitude and soil compositions (Romanucci et al., 2016).

The biological activity of flavonoids depends on their chemical structure, mainly on the number and position of hydroxyl groups and their substitutions (Kumar and Pandey, 2013).

Flavonoids execute the clearance of free radicals by transforming them into phenolic radicals after supplying the unpaired electron to the radicals from macromolecules (Ti et al., 2014). Flavonoids are also beneficial in treatment of vascular problems by decrease capillary permeability; phytotherapy products for the treatment of vascular disorders include plant extracts rich in flavonoids, as red vine, sweet clover, ruscus species (Miyake et al., 2007; Castillo and Solís, 2016). Also many flavonoids were shown to have anti-oxidant activity, free radical scavenging capacity, coronary heart disease prevention, hepato-protective effect, anti-inflammatory response, and anticancer activities, while some others have exhibited potential antiviral activities (Figure 6) (Cicerale et al., 2010; Kumar and Pandey, 2013). Also the cardio-protective effect of anthocyanins as a sub-group of flavonoids was proved by Schreckinger et al., (2010a), McGhie and Walton (2007), and Ojeda et al. (2011).

From the last decades there is enough evidence indicating that dietary intake of polyphenols reduce the risk of chronic diseases like cancer and CVD and are able to improve insulin sensitivity (Arts and Hollman, 2005; Stull et al., 2010). However, scarce information is available regarding their effect on obesity (as most important modifiable factor of metabolic syndrome). It appears that

anthocyanins or anthocyanin-rich food intake is related to the risk of type 2 diabetes, but there is no association for other polyphenol subclasses (Xiao and Hogger, 2015).

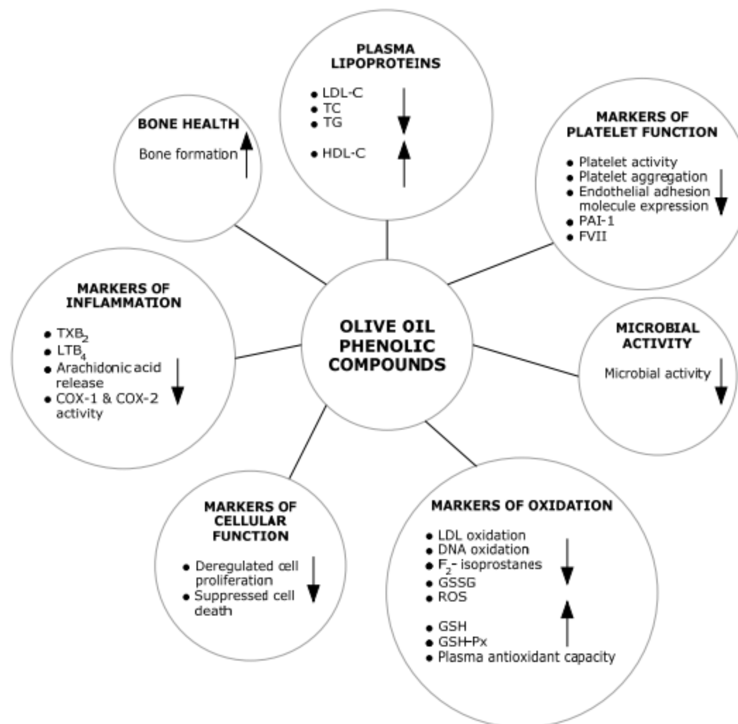


Figure 6. Biological activities of polyphenol compounds (Cicerale et al., 2010)

It has been shown in cell cultures and animal models that polyphenols can inhibit cytokines/chemokines production, reduce cell adhesion, molecule expression as well as inhibit neuro-inflammation (Del Rio et al., 2013). Polyphenolic compounds, such as flavanols, have become important potential chemo-preventive natural agents due to their proved benefits on health, with low toxicity and cost.

1.4. MAQUI BERRY

1.4.1. Composition

South American berries, including maqui berry (*Aristotelia Chilensis* (Mol.) stuntz, *Elaeocarpaceae*), are recently more on international interest for their potential antioxidant benefits (Fredes et al., 2014). *Aristotelia chilensis*, commonly known as Chilean wine-berry, is a native berry from Central and Southern Chile (Patagona region) and South West Argentina, also distributed in tropical temperate Asia, Australia and the Pacific area (Araos, 2015).

Maqui plant is a 4 to 6-meter-tall ever-green tree with pale yellow flowers that blossom in October and November, with 5-6 mm diameter, reddish stem, thin flexible branches, and edible black-purple colored fruit, containing three to four seeds (Muñoz et al., 2011) typically consumed fresh or used to making jam, tea, wine and juice. The fruit is harvested once per year from December to February (Braunch et al., 2017).

The chemical composition of the fruit is mainly predominated with anthocyanins, indole alkaloids and flavonoids (Braunch et al., 2017), coumarins (Damasco et al., 2008), caffeic and ferulic acid (Fredes et al., 2014). Considering other nutrients, it contains mostly vitamin C, Br, Zn, Co, Cr, Fe, Mo, and Na, K, Ca, Ba, Rb, Cs, and Sr (Fredes et al., 2012).

Maqui is exceptionally rich in phenolic acids and anthocyanins, mainly Delphinidin-3-glucoside-5-sambubioside and other flavonoids (Figure 7) (Gironés-Vilaplana et al., 2012 a; Mahdavi et al., 2014).

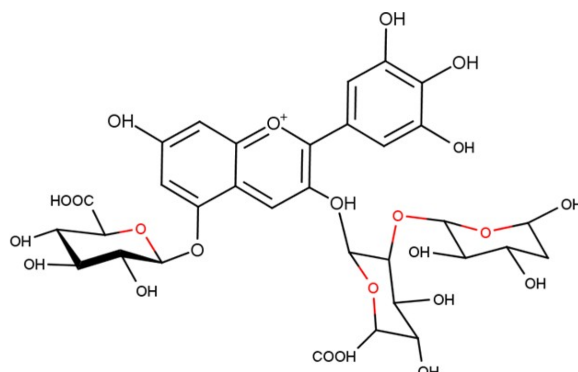


Figure 7. Molecular structure of Delphinidin-3-sambubioside-5-glucoside (Cespedes et al., 2018).

Escribano-Bailón et al. (2006) detected the following anthocyanins in maqui fruit: delphinidin-3-sambubioside-5-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-sambuioside, cyanidin-3-glucoside. The highest anthocyanin level in maqui (34% of total) comes from the main glycoside of the delphinidin aglycone, the delphinidin-3-sambubioside-5-glucoside (D3S5G) (Figure 7) (Cespedes-Acuña et al., 2018). On the other hand, Genskowsky et al. (2016) determined 19 polyphenolic compounds by HPLC analysis of maqui berry, including 8 derivatives of anthocyanins, 10 compounds of flavonols andellagic acid.

Concerning biological activities of maqui, some in vitro studies in cell cultures have been performed. The effect of maqui extracts on the growth of HT-29 and CaCo-2 colon cancer cells, as well as the activation of COX2, NF-kB pathways, Nitric Oxide (NO) formation and antioxidant activity has been evaluated. Fractions rich in anthocyanins were able to decrease the growth of

HT-29 and CaCo-2 colon cancer cells, as well as to suppress the production of NO, through the down regulation of inducible nitric oxide synthases (iNOS) (Reyes-Farias, 2016). In vitro studies performed in cell cultures showed that maqui berry extracts decreased oxidative stress and inflammation processes (Reyes-Farias et al., 2015, 2016).

1.4.2. Bioavailability of anthocyanins of maqui

Polyphenols can be absorbed by both stomach and intestine. Bioavailability studies proved that anthocyanins can be detected rapidly in the circulating system (Del Rio et al., 2013). However, they seem to possess limited bioavailability after oral intake (Wu et al., 2005). This limited bioavailability in terms of absorption and excretion of anthocyanins could be consequence of changes in their chemical structure due to pH fluctuations in the gastrointestinal tract, or influenced by the nature of sugar moiety (Woodward et al., 2009; Fredes et al., 2018).

Unlike other flavonoids that are absorbed and excreted, anthocyanins do not follow extensive phase 2 metabolic reactions and few amounts are found in glucuronide and sulfate derivative forms (Prior and Wu, 2006).

Anthocyanins are absorbed in their glycosidic forms in the stomach approximately 30 min after ingestion (Mueller et al., 2017) and the fraction not absorbed is rapidly absorbed at intestinal level, probably by glucose transporters as GLUT-1, GLUT-2 (Faria et al., 2009). Thereafter, they are quickly metabolized as glucuronidated, sulfated or methylated derivatives, but in a lesser extent than

other flavonoids. Those non-absorbed reach the colon and suffer structural modifications by microbiota, including ring escissions and further degradation to simple phenolic acids (Kay et al., 2017; de Ferrars et al., 2014).

1.4.3. Antioxidant activity of maqui fruit

Maqui berry has been identified to have the highest ORAC value (Oxygen Radical Absorbance Capacity) among different fruits. The antioxidant capacity of maqui is 3.5 times stronger than black-current and 2.9 times stronger than blueberry (Rojo et al., 2012).

While all anthocyanins present antioxidant activity, delphinidins represent the most potent antioxidant anthocyanin species due to largest number of hydroxyl groups in the B-ring. Maqui is mostly rich in delphinidin, which carries three hydroxyl groups, and hence due to this chemical structure acts as a great antioxidant and possesses high radical scavenging activity in the DPPH test (Miranda-Rottmann et al., 2012).

Among anthocyanins, delphinidin-3-glucoside-5-sambubioside (Figure 7), has shown superior anti-diabetic effect in vivo (Araneda et al., 2014; Alvarado et al., 2016).

In studies comparing the polyphenol content and antioxidant potency of different berries including blueberries, cranberries, raspberries, black berries and maqui fruit, using the unprocessed juice from these berries, maqui fruit greatly outperformed all of them (Schreckinger et al., 2010; Araneda et al., 2014), with high antioxidant capacity (Cespedes-Acuña et al., 2018). There is also evidence

that the combination of maqui juice with lemon juice has antioxidant effect on DPPH radical, SOD activity and hydroxyl radical (Cespedes et al., 2008).

Wu et al., (2004) determined the antioxidant activity measured with ORAC method in over 100 types of foods, including fruits, vegetables, nuts, dried fruits, species, cereals, showing that berries possessed the highest values and are outstanding sources of antioxidants.

In another study by Miranda-Rottman et al (2002), maqui juice had antioxidant, atherogenic effect on human endothelial cell cultures, showing high total radical-trapping potential and inhibition of copper-induced LDL oxidation. In this study, they developed a maqui juice with minimal processing, with a polyphenol content for unsweetened juice of 993.2 mg 100 mL $^{-1}$ gallic acid equivalents and juice with sugar of 829.208 mg 100 mL $^{-1}$ gallic acid equivalents. Juice from minimal processing presented high concentration of polyphenols.

Guerrero et al. (2010), determined Antioxidant Capacity (AC), Total Anthocyanins (TA), and Total Phenols (TP) in different locations in Chile. The extract was standardized in gallic acid equivalents through the Folin-Ciocalteu method. Maqui berry showed a significantly higher TA content (between 2240.2 and 1445.3 mg L^{-1} cyanidin 3-glucoside equivalents) than other berries. There was a strong correlation between anthocyanin content and total polyphenol content.

Genskowsky et al (2016) showed the antioxidant activity of maqui berry measured with DPPH, ABTS, FRAP and FIC methods of 28.18, 18.66, 25.22 g Trolox equivalent kg $^{-1}$and 0.12 g EDTA equivalent kg $^{-1}$, respectively.

The effect of hydro-ethanolic extract of maqui berry on blood vessels exposed to oxidative stress (by either glucose or pyrogallol) in isolated rat aortas of male Wistar rats (in vivo) was examined. Attenuated maximum vaso-relaxation response and reduced nitric oxide (NO) bioavailability were suppressed by the incubation with the maqui berry extract. Results suggest that maqui berry extract and its flavonoids rutin and quercetin may protect against induced dysfunction by sugars via enhanced generation and bioavailability of NO (Fuentes et al, 2013).

In lipopolysaccharide (LPS) activated murine macrophage RAW-264 cells, anthocyanin and flavonoids in extracts of maqui berry fruit suppressed production of nitric oxide through the down regulation of iNOS (inducible-nitric oxide synthase), COX-Z (cyclo oxygenase-Z) and protein expression, showing potent antioxidant activity against ABTS, TBARS, ORAC, FRAP and DCFH methods (Molgaard et al., 2011).

1.4.4. Anti-inflammatory effect of maqui fruit

Obesity is characterized by an inflammatory condition and hence treatments based with nutraceuticals with proved anti-inflammatory activities including maqui berry extract can be an interesting approach.

Schreckinger et al. (2010 a) showed that maqui berries are able to slow down the effect of LDL cholesterol while protecting against oxidative stress, which affects cardiovascular function and reduce the levels of inflammatory mediators. They also showed that the phenolic extract of maqui had the ability to control on cholesterol and fat accumulation, inflammatory mediators and control

insulin resistance and diabetes by improving glucose metabolism in tissue. The mechanism of action was the increase of insulin-stimulated glucose uptake in muscle cells and insulin down regulation in liver cells. In a four-week double-blind placebo controlled clinical study with supplements of maqui berry extract, it resulted a significant reduction in oxidative stress markers, including oxidized LDL and urinary 8-iso-PGF2α levels (Davinelli, 2015).

The treatment of Caco-2 cells with *Aristotelia chilensis* diluted juice for 24 hours reduced the protein and m-RNA expression of the enzyme Cycloxigenase 2 (COX-2) (which is highly expressed in human colon cancer) as well as TNF-α induced NF-κB activity and activation of lymphocytes T. At concentrations that reduced COX-2 expressions, the juice did not affect Caco-2 cell viability, suggesting anti-carcinogenic and anti-inflammatory effect (Nile and Par, 2014).

On the other hand, anthocyanins inhibit the secretion of inflammatory cytokines such as IL-8, MCP1, LI1B1, in neutrophil chemotactic cytokine-induced and IL-6 in cells and animal models after an inflammation stimulus (Rojo et al., 2012). In a histological analysis by Toro (2009) on 44 male Sprague-Dawley rats of 150-200 g weight in four groups, which were subjected to sub-plantar acute inflammation induced by Carrageenan, they showed a decrease of edema and inflammatory cells infiltration by 100 mg/kg AC but not by 0.025 mg/kg, though immune-histological analysis did not show significant difference in any group.

1.4.5. Effect on metabolic profile

Meals rich in carbohydrates as a dietary habit lead to fast spike in plasma glucose in 30 minutes as well as an increase in insulin levels, subsequently

leading to fat deposition and rapid recurring appetite. Hence, in long term it is in favor of weight gain and development of insulin intolerance and metabolic syndrome. Maqui lowers glucose absorption by avoiding the pass of carbohydrates from the intestine into the blood stream (Alvarado et al., 2016). In this three month trail study on 31 pre-diabetic subjects, the effect of daily supplementation of 180 mg maqui extract was assessed on glycosylated hemoglobin (HbA1C) levels, glucose tolerance (OGTT), lipid profile (HDL, LDL, VLDL, triglycerides, total cholesterol) in monthly intervals. Average HbA1C reduced after first, second and third month, showing improvement of glucose metabolism. Significant decrease in LDL was observed after three months and in VLDL after one month. HDL increased significantly over baseline, whilst total cholesterol and triglycerides didn't change. The authors demonstrated that, though total blood glucose was unaffected, maqui berry extract significantly and dose-dependently lowered the levels of glycaemia, one-hour after glucose intake, compared to control. They showed that lower doses delayed post prandial glucose and insulinemic peaks, while higher doses reverted this tendency and they suggested as possible mechanism the inhibition of intestinal glucose transporter.

Hidalgo et al., (2014), investigated the effect of an extract of *Aristotelia chilensis* (standardized to ≥ 25% delphinidins and ≥ 35% of total anthocyanins) on post-prandial blood glucose and insulin level of ten volunteers in a double-blind placebo controlled crossover study. They suggested that natural control of post-prandial blood glucose by the extract of maqui reduced the glucose absorption in intestine by inhibiting sodium-glucose co-transporter SGLT-1 in small intestine.

Similarly, in a clinical follow up study on pre-diabetic volunteers they showed that maqui extract significantly lowered blood glucose thirty minutes after oral glucose challenge test.

It has been proved an inverse correlation between the consumption of polyphenol rich foods in a regular basis and glycemic index; they can exert beneficial effects on the treatment and prevention of chronic diseases, by modification of post-meal glycemic response (Hidalgo et al., 2014) and inhibiting glucose or digestion transport, ameliorated fasting blood glucose, improved pancreatic insulin secretion and sensitivity, as demonstrated in a mouse model by Rojo et al (2012). Specially two anthocyanins of maqui, delphinidin-3-glucoside and cyanidin-3-glucoside up to altered gene expression and signaling pathways (Cespedes et al., 2017). Maqui extracts inhibit α-glucosidase enzyme, which is essential for the metabolism of carbohydrates, it hence can be considered as a useful tool in diabetes management (Jayaprakasam et al., 2005). Other studies have demonstrated anti-diabetes and hypoglycemic effects of maqui fruit as well (Cespedes et al., 2009; Rubilar et al., 2011). Moreover, in-vitro studies on some phenolic concentrates of maqui extract show that they reduced adipogenesis and lipid accumulation in adipocytes (Schreckinger et al, 2010 b).

1.4.6. Other biological activities of maqui

Considering other health effects of maqui, different studies exhibit wide range of beneficial protective and therapeutic properties of this fruit, including:

Anticholinergic effects: In Alloxan-treated diabetic rats' vascular reactivity impairment, hyperglycemia and dyslipidemia were measured (Fuentes

et al., 2013). The chronic treatment with maqui berry significantly corrected all the above abnormalities compared to control rats, whereas increased body weight in those diabetic rats.

Anti-microbial activity: Forty extracts of different plant species including *Aristothelia chilensis* were tested against the wound infection fungi *Pensillium expansum*, *Candida albicans* and the bacteria *Bacillus subtilis*, *Pseudomonas aeruginosa Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*. *Aristothelia chilensis* was among 13 most noteworthy plant species on antimicrobial activity (Molgaard et al., 2011).

In another study on anti-bacterial effect of maqui berry (Genskowsky et al., 2016), all strains tested were affected. *Aeromonas hydrophila* and *Liseria innocua* showed the highest sensitivity to the extract, with Minimum Inhibitory Concentration (MIC) values of 40 and 50 mg ml^{-1} respectively.

Neuroprotection: In a study with hippocampal cultures from rats, in which Alzheimer's Disease (AD) was induced by soluble oligomers of Aβ1-40, neuro-protection was observed when the neurons were co-incubated with amyloid-β protein (Aβ) (0.5 μM) plus maqui berry for 24 hours (Fuentealba et al., 2012). This co-incubation recovered the frequency of Ca^{2+} transient oscillations when compared to neurons treated with maqui berry alone, correlating with the changes observed in spontaneous synaptic activity. They also showed that in the presence of maqui berry, the toxic effect of Aβ is prevented, by preservation of the dendritic tree. They proposed a new complex mechanism of protecting the neural network, in which maqui induces a potent neuroprotective effect

including antioxidant properties, modulation of cell survival pathways and/or direct interaction with the kinetics of Aβ aggregates, producing variations in nucleation phase and altering ThioFlavin T insertion in β-sheets, and hence generating far less toxic species.

Delphinidins from maqui berry may also inhibit UV-induced expression of matrix metalloproteinase in fibroblasts (Addor et al., 2018). Some authors have proposed a recommended daily dosage of Maqui berry extract of 30-60 mg for the prevention of eye disease (Hitoe et al., 2014).

Table 2 summarizes the main findings of the biological activities of maqui fruit.

Table 2. Studies on the biological effects of Maqui

IN VITRO / CELL CULTURES ASSAYS		
Ref.	**Assay performed**	**Outcomes**
Cespedes et al., 2009	Maqui fruit extract in DPPH, crocin and TBARS assay	Strong antioxidant activity in DPPH, crocin and TBARS assay
Cespedes-Acuña et al, 2018	Effects on growth of HT-29 and CaCo-2 cancer cells, COX2 activity, NF-kB activation, Nitric Oxide (NO) formation and antioxidant activity by DPPH, TBARS, FRAP and ORAC	Potent decrease in the growth of HT-29 and CaCo-2 colon cancer cells Reduced production of NO by down regulation of inducible nitric oxide synthases (iNOS)

Table 2. Studies on the biological effects of Maqui *(continued)*

Ref.	Assay performed	Outcomes
Mahdavi et al., 2014	Antioxidant activity measured with DPPH, ABTS, FRAP Anti-bacterial effect	Anti-oxidant activities with DPPH, ABTS, FRAP and FIC method were of 28.18, 18.66, 25.22 g Trolox equivalent kg $^{-}$, respectively. All strains tested were affected by maqui berry, mainly *Aeromonas hydrophila* and *Listeria innocua*. In these strains, inhibitory concentration (MIC) values of maqui extract were 40 and 50 mg.ml $^{-1}$ respectively.
Hou et al., 2005	Maqui berry extract on colon cancer cells	Genomic DNA integrity, by reducing the protein and mRNA expression of COX-2 and TNF-α after 24 hours. Four hours after administration, AC reduced the cytoplasmic Iκ$B\alpha$ levels and increase ERK1/2 and Akt phosphorylation as well as C-fos expression. Caco-2 cell viability was not affected.
Miranda-Rottman et al., 2002	Maqui fruit juice on human endothelial cell cultures	Anti-atherogenic effect, inhibition of copper- induced LDL oxidation, protection from hydrogen peroxide-induced intracellular oxidative stress

Table 2. Studies on the biological effects of Maqui *(continued)*

ANIMAL AND HUMAN STUDIES		
References	**Assay performed**	**Outcomes**
Genskowsky et al., 2016	Maqui berry extract applied in lipopolysaccharide (LPS) activated murine macrophage RAW-264 cells	Reduced production of nitric oxide and down regulation of iNO), as well as cyclo oxygenase-Z (COXZ) Potent antioxidant activity against SOD, ABTS, TBARS, ORAC, FRAP and DCFH.
Schreckinger et al., 2010	Maqui berry extract on 3T3- L1 adipocytes and raw 264.7 macrophages	Cardio-protective effect by lowering LDL cholesterol, reduced adipogenesis and lipid accumulation in 3T3-L1adipocytes. Protecting against oxidative stress and anti- inflammation effect. Decreased production of nitric oxide and prostaglandin and the expression of inducible nitric oxide synthase (9.8-61.8%) and cyclooxygenase-2 (16.6-62.0%)
Molgaard et al., 2011	Forty plant species extract including Maqui extract on especially wound infection fungi (*Pencillium expansum, Candida albicans*) and bacterial cell cultures (*Bacillus subtilis, Pseudomonos aeuroginosa, Staphylococcus aureus, Escherichia coli, Streptoccoccus pneumonia*	Inhibition activity on *Staphyllococcus aureus, Pseudomonas aeuroginosa, Enterobacter aeorogenes*, and *Candida albicans*

Table 2. Studies on the biological effects of Maqui *(continued)*

References	**Assay performed**	**Outcomes**
Leal, 2009	Maqui fruit on 44 male Sprague Dawley rats of 150 to 200 g in four groups, subjected to subplantar acute inflammation induced by carrageenan	Anti-inflammatory effect: The histological analysis showed a decrease of edema and inflammatory cells infiltration by 100 mg/kg AC but not by 0.025 mg/kg. Immune-histo-chemical analysis did not show significant difference in any group
Rojo et al., 2012	A murine model of type 2 diabetes and different cell strains. Hyperglycemic obese C57BL/6J mice fed a high fat diet	In H411E liver cells, decreased glucose production and enhanced the insulin-stimulated down regulation of glucose-6-phosphatase. Improved fasting blood sugar and glucose tolerance
Fuentealba et al., 2012	Primary hippocampal cultures from rats (E18), Alzheimer's disease (AD) induced by soluble oligomers of A-β 1- 40.	Potent neural network protection effect by direct interaction with amyloid-β aggregates, by generating far less toxic species and altering Thioflavin T insertion in β-sheets. Better protection when neurons were co- incubated with amyloid-β (Aβ) (0.5 μM) plus AC for 24 hours, by recovering the frequency of Ca^{2+} transient oscillation and preservation of the dendritic tree

Table 2. Studies on the biological effects of Maqui *(continued)*

References	**Assay performed**	**Outcomes**
Fuentes et al, 2013	Chronic maqui berry extract on Alloxan (ALx) diabetic rats	Maqui reduced plasma levels of cholesterol, LDL, triglyceride and increased body weight of diabetic rats. Also improved nitric oxide bioavailability.
Kim et al., 2016	STZ diabetic rats, 4 months daily application of delphinol	Reduction of fasting blood sugar
Cespedes et al., 2008	The methanol extract from mature fruit of maqui in rat heart in vivo	The extract showed antioxidant activity and cardio-protective effect by diminishing lipid oxidation and on acute ischemia by incidence of reperfusion dysrhythmias and the non-recovery of the sinus rhythm.
Fuentes et al., 2013	Hydro-ethanolic extract of maqui berry on responsivity of blood vessels exposed to oxidative stress (by either glucose or pyrogallol) in isolated rat aortic rings	Preincubation with maqui berry extract suppressed the attenuated maximum vaso-relaxation response and the reduced nitric oxide (NO) bioavailability
Davinelli et al., 2015	Delphinol supplementation in RCT double blind, placebo controlled design, 42 healthy, overweight, smoker participants	Reduction of ox-LDL Reduction of urinary F2-Isoprostane

Table 2. Studies on the biological effects of Maqui *(continued)*

References	Assay performed	Outcomes
Alvarado et al., 2016a	Delphinol on pre-diabetic patients Oral glucose tolerance test	Reduced post-prandial glucose plasma levels
Kim et al., 2016	Double blind, cross-over trial, placebo in olunteers with moderate glucose intolerance	Reduction of post prandial glucose, due to inhibition of gut sodium glucose transporter
Hidalgo et al., 2014	In pre-diabetic humans underwent different doses of maqui extract one hour before OGTT, four times, in four weeks, with at least one week washout period	Insulinemia peaks were inversely and glycemia peaks were directly dose dependent, though total blood glucose remained unaffected.

1.5. LEMON

1.5.1. Production and nutritional composition of lemon fruit

World's leading citrus fruit producers are China, Brazil, U.S.A, India, Mexico, and Spain (Liu et al., 2012). Although the exact genetic origin of cultivated *Citrus* lemon is not clear, this fruit is original from Asia, being reported for the first time at third or fourth century A.C.

Spain is the leader producer of lemons in Europe and Murcia is the leading region with 50% of national production, followed by Alicante (30%), Malaga (13%) and Almería (5%) (Pérez-Pérez et al., 2005) constituting 95% of

national production. In the Region of Murcia, the production of lemon is the most important among *Citrus*, only approached by the peach production (Acosta et al., 2011). Lemon is a rich source of nutrients including vitamins, minerals, dietary fiber, essential oils and carotenoids. It contains ascorbic acid, citric acid, minerals, flavonoids including flavonones (400-600 mg/dl) and flavones and lesser amount of flavonols and hydroxyl cinnamic acids (González-Molina et al., 2010).

Lemon is rich in vitamin C and phytochemicals as flavonoids, all of which have been reported benefitial effects on health (Proteggente et al., 2002). Most flavonoids from *Citrus* species can be classified into three groups: flavanones, flavones and flavonols (Abad-García et al., 2012). Hesperetin is a flavanone particularly abundant in lemon fruits (Figure 8). Carotenoids are also present in lemon fruits, especially in mature stage, when it accumulates β-cryptoxanthin in the juice sacs (Kato et al., 2004).

Other vitamins are available in minor quantities including vitamin A and B group (B1, B2, B3, B6 and B9). Besides, the minerals present are mostly potassium (K), and in lesser amount calcium (Ca), magnesium (Mg) and phosphorus (P) and traces of copper (Cu), iron (Fe), manganese (Mn), selenium (Se), sodium (Na) and zinc (Zn) (González-Molina et al., 2010; Penniston et al., 2008).

Lemon fruit is rich in essential oil in the mesocarp and the major component is D-limonene (45–75%) (Russo et al., 1998). Citric acid is the most representative organic acid in lemon (Kefford and Chandler, 1970), comprising as much as 8 % in the dry weight and represents 5–6 g/100 mL (Penniston et al., 2008).

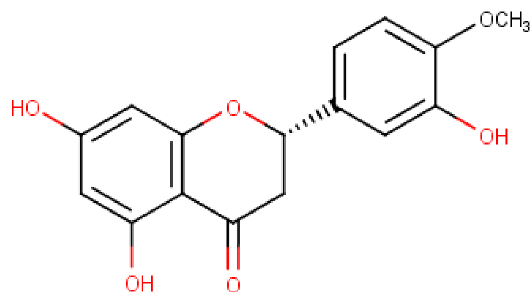


Figure 8: Chemical structure of Hesperetin (own source)

1.5.2. Biological activities of lemon fruits

Lemon is an important source of vitamin C, minerals, fiber, essential oils and phytochemicals as carotenoids and flavonoids. It has been reported that lemon phytochemicals can improve conditions of diabetes and consequently cardiovascular health (Aruoma et al., 2012; Bahorun et al., 2012; Adibelli et al., 2009; Benavente-García et al., 2008; González-Molina et al., 2010; Miyake et al., 2007).

Considering the effect of lemon on oxidative stress, it is well known that Vitamin C (as a major micronutrient in the lemon) is a very important and powerful antioxidant that works in aqueous environments of the body. Recommended intakes are between 60 and 90 mg (Food and Nutrition Board, 2013) and lemon juice contains 40 mg/100 mL approximately. *Citrus* flavonoids have been also suggested as antioxidants due to the combined ability of vitamin C and phenolics, such as chelation of ions, ferric reducing ability, etc. (Al-Juoaimi et al., 2013; Masuoka et al., 2012). However, concerning to dietetic recommendations of the increase consumption of this particular fruit, EFSA

(European Food Safety Authority) presented one health claim concluding that a cause and effect relationship could be not established between the consumption of the food(s)/food constituent(s) evaluated and a beneficial physiological effect related to their antioxidant activity, antioxidant content, or antioxidant properties (EFSA, 2010).

Concerning the studies performed to evaluate the beneficial effects of lemon consumption, in a study in northern Turkey among 156 hypertensive patients, 40% were drinking lemon juice as effective alternative therapy to control blood pressure (Adibelli et al., 2009; Al-Johaimi, 2013). Other-study reported that after six months of citrus-based juice consumption, there were significant improvements in lipid profile and inflammation markers including C-reactive Protein (CRP), Homocystein, and LDL-Oxidized in metabolic syndrome patients (Mulero et al., 2012).

Lemon juice enriched with berries could be of potential interest in the design of new drinks with a nutritive related function on health for chronic diseases (Gironés-Vilaplana et al., 2012 b). Besides, there is an important need to increase the consumption of fruits and vegetables in the population in a popular acceptable way, so healthy drinks made with fruit extracts (as lemon and other citrus based juices), with safe and natural sugar substitutes could be considered an interesting healthy option.

1.6. CALORIC AND NON-CALORIC SWEETENERS

Sweeteners are food additive substances that provide sweet taste. High-intensity sweeteners (also called non-nutritive, low-calorie, high-potency, artificial or intense sweeteners) are an important class of food additives. The use of these sugar substitutes in food production is an easy way of giving food a sweet taste without increasing its calorie contents since they provide sweetness that is hundreds of times stronger than that of sucrose and only very small amounts of sweetener are needed to contribute the same level of sweetness as sucrose (Mortensen, 2006). Figure 9 classifies the type of sweeteners depending on their nature.

The intensity, quality, and temporal profile (defined as the changes in intensity over time) of the sweetness varies with the type of sugar (Godshall, 2007).

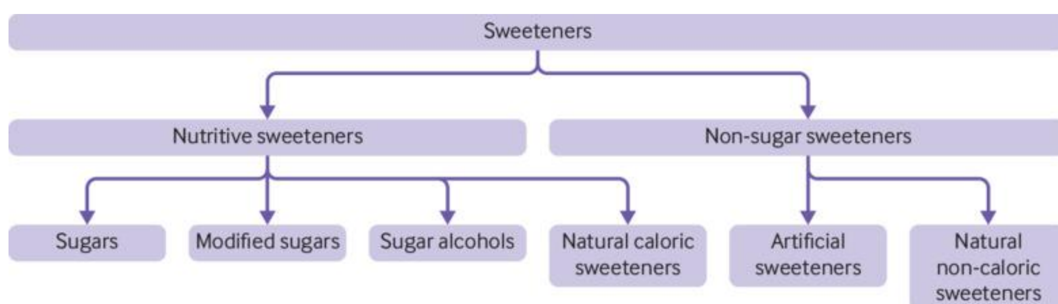


Figure 9. Type of sweeteners (Toews et al., 2019)

1.6.1. Caloric sweeteners

1.6.1.1. Sucrose

Sucrose, Sucrose or cane sugar (also known as table sugar) is a disaccharide formed by glucose and fructose units. It is as white odorless, sweet crystalline or powdery solid. Each gram contain 4 kcalorie energy (Figure 10).

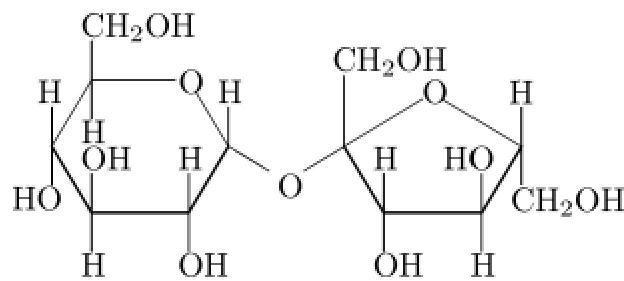


Figure 10. Chemical structure of Sucrose (source: Wikimedia Commons)

1.6.1.2. Corn syrup

High fructose corn syrup (HFCS) derives from corn starch, which breaks down into individual glucose molecules and the end product is corn syrup which is 100% glucose (Simple sugar) (FDA 2013). For this purpose, enzymes are added to corn syrup to convert some of the glucose to another simple sugar, the fructose (called Fruit sugar). HFCS is high in fructose (its different formulation contains different amount of fructose) while corn syrup is high in glucose and is a purified, concentrated aqueous solution of nutritive saccharides also obtained from edible starch. The only current guidelines available for it is 2010 dietary guidelines for American (FDA) which recommend that everyone limit

consumption of all added sugars including HFCS (Caballero et al., 2012; FDA, 2013).

1.6.2. Low- caloric sweeteners

Low-caloric sweeteners (LCS), discovered in the late 1800s, are defined as no-calorie sugar substitutes that provide a sweet taste without the energy density of sugar. This term includes a wide range of artificial sweeteners including chemically synthesized products like aspartame, cyclamate, sucralose, as well as natural sweeteners, as the plant-derived sugar alcohols such as xylitol, sorbitol, mannitol, and the stevia leaves or monk-fruit extracts (Sharma et al., 2016).

U.S. Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) approved six artificial sweeteners for use as per acceptable daily intake (ADI) value, including saccharine, sucralose, aspartame, acesulfame-K, neotame and advantame, and two natural ones, the steviol glycoside Stevia rebaudioside and Luo han guo (Monk fruit) extract (Chattopadhyay et al., 2014; Mooradian et.al 2017). Table 3 shows the FDA classification based on average daily intake.

Table 3. FDA classification of high intensity, low-caloric sweeteners

Name	**Sweetness**	**ADI** (Average daily intake)
Acesulfame k	200	15
Advantame	20,000	32.8
Aspartame	200	50
Monk fruit	300	Any
Neotame	8000	0.3
Saccharin	300	15
Stevia Rebaudioside	300	4
Sucralose	600	5

1.6.2.1. Sucralose

Sucralose is a compound derived from sucrose and is 600 times sweeter than sugar. Its acceptable daily intake is 5mg/kg of body weight. As it is resistant to high temperatures, it is possible to be added into hot or cold drinks for added sweetness or use as a sugar substitute in baking/cooking for recipes such as muffins, cookies, jams and granola, non-alcoholic beverages, (Henkel, 1999; Grice and Goldsmith, 2000) (Figure 11).

As the latest international non-calorie sugar substitute, it is the only one made from chlorinated sugar and 600 times more sweet than sugar. It was discovered by British researchers in 1976 and it can be replaced in almost all kind

of foods, beverages, chewing gum, frozen desserts, baked goods, etc. due to its stable nature to heat. Being insoluble in fat, it cannot be accumulated in body fat and it does not break down in regular digestion (Sims et al., 2000).

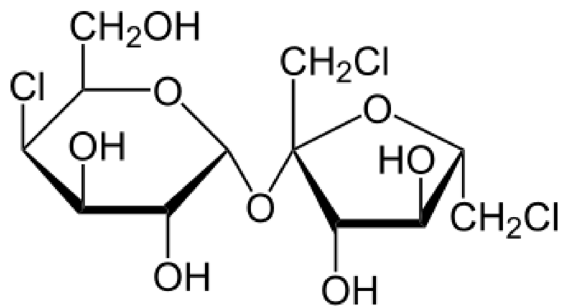


Figure 11. Chemical structure of Sucralose (source: Wikimedia Commons)

1.6.2.2. Stevia

Steviol glycosides are the sweet components isolated and purified from stevia leaves (EFSA, 2019) (Figures 12, 13). It is 10-15 times sweeter than sucrose and it is used as a natural sweetener (The Sugar Association, 2019; EFSA, 2019).

Body cannot metabolize steviol glycosides, hence they provide no calories. It does not break down with heat, therefore, it is perfect for cooking and baking. It can provide bitter after-taste, which sometimes is a desired effect depending on the food product, and it can be added into hot or cold drinks for added sweetness or use as substitute of baking/cooking for recipes such as muffins, cookies, jams and granola (The Sugar Association, 2019).

Round estimation of nutritional composition of stevia leaves on dry basis are including protein (20 %), fat (4 %), carbohydrates (35 %) as well as minerals

as potassium (2.5 %), calcium (1.55 %), magnesium (0.5 %), phosphorus (0.35 %). It also includes phytochemicals including tannins the highest and anthraquinones the lowest. The following phytochemicals are available in stevia leaves almost in the same average: alkaloids, saponins, sterols and triterpenes (Tadhani and Subhash, 2006). The composition of the leaf reflected a high nutritive value and polyphenol concentration averaging 4-25% by weight of dried leaf (Kaushik et al., 2010).

Steviol glycosides are neither genotoxic nor carcinogenic and in 2010 EFSA's Scientific Panel on additives established an Acceptable Daily Intake (ADI) for stevia as low-caloric sweetener. The Panel recommended an ADI of 4 mg/kg body weight/day which was in line with the recommendation of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2009.

In 2019, EFSA reviewed its 2010 assessment of steviol glycosides and concluded that adults and children who are high consumers of foods containing steviol glycosides, could still exceed the ADI established in 2010 if the sweetener is used at the maximum levels proposed. Later as of 2017, high-purity stevia glycosides were considered safe and allowable and its ingredients in food products sold in the United States. The food additive is included in the official EU list of authorized food additives with 'E 960' number (EFSA, 2019 a). The Standing Committee of the European Commission (Directorate-General Health and Consumers Protection) voted to approve stevia extracts (steviol glycosides) for use in the European Union (EU) at its meeting held in July 2019 (EFSA, 2019 a). EFSA held recently an open consultation on a scientific protocol for assessing consumer exposure to sweeteners, up to 22 November 2019. In the

U.S., steviol glycosides are used as sweeteners in foods and beverages as well as in tabletop sweeteners.

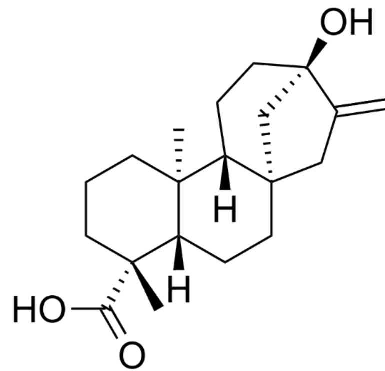


Figure 12. Chemical structure of Steviol aglycone (Source: Wikimedia Commons)

Figure 13. Stevia leaves (Source: Wikimedia Commons)

Steviol glycosides are required at low doses to sweeten foods. It has demonstrated a strong radical scavenging capacity, what suggests that, besides acting as a sweetener, it could also act as antioxidant. When added to food, it could delay the degradation of vitamin C hence it may partially replace the use of additional vitamin C and/or delay its degradation, acting as an antioxidant natural additive (Kroyer, 2010).

Wozniak et al. (2014) demonstrated that adding glycosides from *Stevia rebaudiana* in water acidic solutions, can increase the stability of Vitamin C (both ascorbic acid and dehydroascorbic acid) (but not anthocyanin) to a greater extent than sucrose in equivalent amount (50, 125, 200 mg/L). Besides, adding steviol glycosides in the same solution enhance the protective effect of sucrose on anthocyanins. The effect was related to radical oxygen species scavenging activity and was intensified by higher concentration of the sweeteners and higher acidity solutions. All above, suggest a practical application of steviol glycosides from the protection of other food compounds, some of them with biological activities (as vitamin C and anthocyanins).

1.6.2.3. Aspartame

Aspartame is a low calorie sweetener, sugar like taste, discovered in 1965, and it is 200 times sweeter than sucrose (Prodolliet and Bruellhant, 1993) (Figure 14). It is metabolized and completely broken down by the body to amino acids as aspartic acid, phenylalanine and a small amount of ethanol (Butchko et al, 2002). It has been approved to be used in soft drinks, yoghurt and confectionary (Food Standards Australia –New Zealand Food Standards Australia, (FSANS) (Magnuson et al.,, 2007).

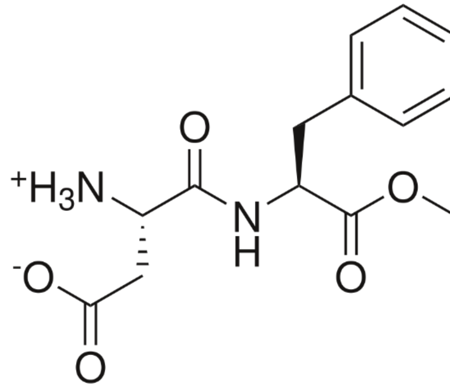


Figure 14. Chemical structure of Aspartame (Source: Wikimedia Commons)

1.6.2.4. Saccharine

It is a synthetic white crystal, its purest form is 550 times sweeter than sugar (Figure 15). It was just banned for a period of time in 1977 due to probability of carcinogenic nature (Okoduwa et al., 2013). Saccharin is 300-500 times as sweet as sucrose based on its concentration and the type of food matrix in which it is used. In contrast with sucrose, it has a slow onset of sweetness that increases to maximum and then remains. Its high concentration has bitter taste. In most countries, it is the only synthetic non-nutritive sweetener in soft drinks.

It has been shown that the combination of saccharin, aspartame and cyclamate were successful in many applications. Sweetness of 20 mg aspartame with 4 mg saccharine is equal to sweetness of 45 mg aspartame or 35 mg saccharine alone, for a cup of coffee (Caballero et al., 2012).

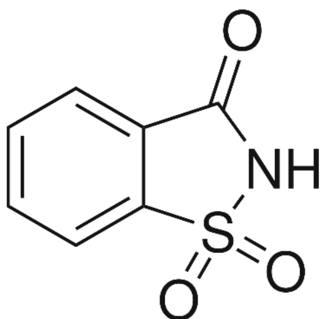


Figure 15. Chemical structure of Saccharin (Caballero et al., 2012)

1.6.2.5. Acesulfame-K

This sweetener was discovered in 1967 and is a solid white crystal, odorless, slight soluble in ethanol and soluble in water (Figure 16). This compound is metabolized in the organism and follows renal excretion. It is present in carbonated drinks and the maximum quantity allowed is 600 mg/kg. When the quantity used is high, it leaves a residual taste in the beverage (Calzada-León et al., 2013).

Its characteristics include stability to heat and good solubility, providing quickly perceptible sweet taste. Its general use is approved by FDA from 2003 onwards. It is one of the most common sweeteners used in food and beverage products, due to its resistance to thermal degradation, its good solubility, as well as its marked sweetness. It provides quickly perceptible sweet taste.

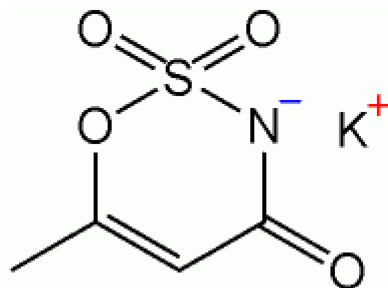


Figure 16. Chemical structure of Acesulfame K (source: Wikimedia Commons)

1.6.2.6. Neotame

Though still rarely used, this low calorie sweetener was approved by FDA in 2002. It is 8000 times sweeter than sugar and 40 times sweeter than aspartame, but more stable than them. It can be used in baked foods. The allowed consumption amount by EFSA and FDA is 0.05 mg/kg bw/ day for Europe and 0.02-0.05 mg/kg bw/day for U.S, respectively (Figure 17).

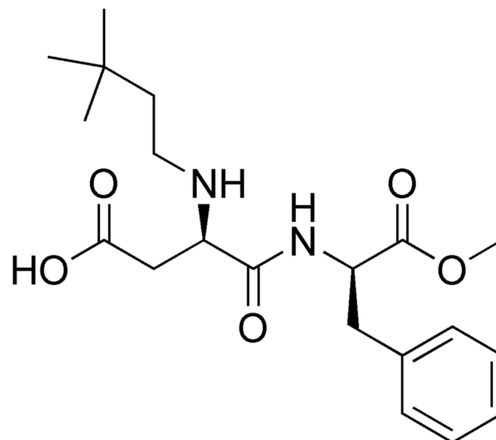


Figure 17. Chemical structure of Neotame (source: Wikimedia Commons)

1.6.2.7. Monk fruit (Luo han guo)

Monk fruit extract is derived from the fruit of *Siraitia grosvenorii*, a perennial vine native to Southern China (Figure 18). It has a more sugar-like taste than stevia and received regulatory approval in U.S, Asia and some Latin American countries and in Australia it was recently approved by Food Standards Australia –New Zealand (FSANS) in 2018, as well as by EFSA (2019 b). Blend of monk fruit and stevia allows benefit of combination of stevia's lower cost and monk fruit's better taste to counteract bitter-flavor notes in stevia-derived sweeteners.

Figure 18. Monk fruit (Luo han guo) (Source: American Botanical Council)

1.6.3. Health effects of sweeteners

A century ago non-caloric artificial sweeteners (NAS) were introduced for providing sweet taste foods with low energy content (Suez et al., 2014) and soon became popular in diet sodas, cereals, sugar-free desserts, due to their low cost

and low caloric intake. Today we include natural non-caloric sweeteners, so they are currently englobed in the term low (or non-caloric) sweeteners (LCS).

They are food additives that substitute other caloric sweeteners in food products, as well as in health care products (toothpaste, food supplements) (Serra-Majem et al., 2018). LCS are 200 to 13,000 times sweeter than sucrose by weight. As they do not provide extra energy, they were expected to be beneficial against obesity and type 2 diabetes and contribute to weight reduction in diabetes and glucose intolerance (Gardner et al., 2012; Kreuch et al., 2018).

At the beginning of this decade, though scarce information was available from long-term interventional studies on evidence regarding artificial sweeteners and metabolic health effects (Brown et al., 2010), some authors pointed out the beneficial effect of artificial sweeteners on energy intake, body weight, liver fat, fasting and post prandial glycemia and insulinemia and/or lipidemia compared with sugar (Raben and Richelsen, 2012). In this sense, a previous study showed that the 25 weeks of substitution of sugar drinks with diet ones among adolescents lead BMI reduction, but only among the heaviest ones (Ebbeling et al., 2006).

In contrast, others have shown that LCS lead to weight gain and increase the risk of type 2 diabetes (T2DM) (Nettleton et al., 2009). Association of development of T2DM and metabolic syndrome with consumption of diet soda per day was proved. However, after adiposity adjustment, significance was lost.

Though it is evident that substitution of caloric sweeteners with non-caloric ones reduce the energy density of foods and beverages, frequent consumption of non-caloric high-intensity sweeteners like aspartame, sucralose

and saccharine may have the counter-intuitive effect of inducing metabolic derangements (Swithers, 2013). In fact, consumers of LCS have shown higher risk of weight gain (Fowler et al., 2008), metabolic syndrome (Duffy et al., 2012; Dhingra et al., 2007) and type 2 diabetes (Fagherazzi et al., 2013). LCS and progression of metabolic syndrome and T2DM was proved by long term follow-up large cohort studies in women and men though the association was lost after adjustment for BMI (de Koning et al., 2011).

Moreover, the European Prospective Investigation into Cancer and Nutrition (EPIC) study performed in eight countries (InterAct Consortium et al., 2013) confirmed this association (Fagherazzi et al., 2013; Sakurai et al., 2014). Though again by adjustment for confounders the significance was lost.

A study by Van Wymelbeke et al. (2004) on consumption of beverages containing either sucrose or intense sweeteners (null energy) showed an induction of positive energy balance though continuous exposure to the extra-energy with sucrose beverages and there were no changes on food intake or hunger. On the other hand, Bellisle and Drewnowski (2007) concluded that intense sweeteners increase appetite for sweet foods, promote over eating, and may even lead to weight gain.

Animal models have demonstrated that LCS consumption in adults Sprague Dawley rats can disrupt the animal's ability to predict the metabolic results of sweet taste and hence the animal is unable to respond properly to sweetened foods (Swithers and Davison, 2008; Swithers, 2013). In a study by Abou-Donia et al. (2008), after ten days' consumption of small calorically sweetened snack with sucrose, glucose and Splenda® as predictive group and

saccharin and glucose sweetened ones as non-predictive group, sweet taste did not reliably predict caloric intake. De Matos Feijo et al. (2013) on a study on rats with aspartame reached to the same conclusion.

The long-term impact of their consumption is uncertain. The evidence from randomized controlled trials (RCT) does not clearly support the benefits of LCS for weight management (Azad et al., 2017).

In a review by Fowler (2016) of a series of conducted human long-scale, long-term prospective observational studies, an increase in weight, abdominal and incidence of adiposity, over-weight and obesity have been reported in participants who were using diet sodas and low calorie sweeteners (LCS)-beverages daily (often in dose-response manner) and consequently frequent consumption was associated with increased risk of hypertension-mediated syndrome and diabetes and heart attack.

Considering obesity as confounder in observational studies, LCS consumption and development of metabolic disease as well as the effect of LCS on glucose metabolism is not clear (Romo-Romo et al., 2016). The overall use of LCS remains controversial. There is only one recent RCT in healthy adults with/without obesity to compare NNS consumption (Taljaard et al., 2013) which showed that energy intake was lower in sucralose group than sucrose in their study.

In contrast with the investigations mentioned, in a study by Anton et al. (2010), stevia preload significantly reduced postprandial glucose levels compared to other sweeteners like the higher calorie sucrose pre-load.

In the 98th Annual Meeting of Endocrine Society in Boston, Kassi et al. (2016) reported that, after four months' intervention with a stevia snack, a reduction of BMI, total cholesterol, ox-LDL, Waist-Hip-Ratio (WHR) and leptin occurred.

In a meta-analysis of RCTs by Miller and Perez (2014) they showed modest but significant reduction in body weight, BMI, fat mass, waist circumference. While in contrast, among prospective cohort studies, LCS were associated with a slight higher BMI.

Considering the contradictory results observed with the consumption of LCS, and that their effects after long-term consumption have not been fully unraveled, there is a need of further studies on their biological effects after their ingestion, when included within in a food product and in a regular dosage. Parallel to that, there is a need of new alternatives for increasing consumption of fruits and vegetables. The development of products that preserve the nutrients contained in fruits and that reduce the energy content of fruit juices should be pursued. In this sense, the combination of lemon juice, with a distinctive and attractive aroma and high nutritive value, with maqui berry extracts rich in bioactive anthocyanins can be a proposal of worth value.

II - OBJECTIVES

II - OBJECTIVES

2.1. HYPOTHESIS

We hypothesize that fruit-based drinks formulated with different sweeteners are an alternative to the consumption of added-sugar beverages, as they are able to counteract the post-prandial response associated with these added-sugar beverages, in overweight population characterized by chronic inflammatory status.

2.2. GENERAL OBJECTIVE

The aim of the present study is to investigate the effect of long term consumption of different fruit based drinks added with different type of sweeteners on markers of oxidative stress, inflammation and satiety in overweight subjects.

2.3. SPECIFIC OBJECTIVES

- To describe the adherence to Mediterranean diet and lifestyle habits of a population of overweight subjects.
- To predict the effect of long-term consumption of the test beverages on anthropometric parameters.
- To predict the effect of test beverages on antioxidant status.
- To evaluate the effect of the test beverages on insulin resistance and glycemic response.

- To evaluate the effect of long-term consumption of the test beverages on the lipid profile.
- To estimate the effect of the test beverages on inflammatory status.
- To evaluate the effect of long-term consumption of the test beverages on satiety hormones

III - MATERIAL AND METHODS

III - MATERIAL AND METHODS

3.1. BEVERAGES USED FOR THE CLINICAL STUDY

Beverages were formulated with a base of lemon and maqui extracts, added with different types of sweeteners:

- Beverage A: Stevia
- Beverage B: Sucralose
- Beverage C: Sucrose

Drinks were provided by our partner in BEBESANO project, the research group of Dr. Viguera from CEBAS-CSIC that made the production in semi-industrial scale, in a pilot plant, following all legislation for beverages production. The exact composition of the drinks and quantity of ingredients used are protected under industrial confidenciality. Quality and safety tests were performed, as well as shelf-life tests. Fresh dry organic maqui powder was provided by Maqui New Life S.A. (Santiago de Chile, Chile). Cítricos de Murcia S.L. (Ceutí, Spain) and AMC Grupo Alimentación Fresco y Zumos S.A. (Espinardo, Spain) provided the citrus juices. Sucrose was provided by AB Azucarera Iberia S.L. (Madrid, Spain), Stevia by AgriStevia S.L. (Murcia, Spain), and Sucralose by Zukan (Murcia, Spain). For the maqui-citrus juices performance, maqui powder was mixed with of citrus juices to obtain the base beverage. Then, the three selected sweeteners were added to obtain the different beverages with an acceptable taste. The beverages underwent a pasteurization treatment by applying 85 °C during 58 seconds. Afterwards, the mixtures were bottled and stored at 5 °C until being consumed by the volunteers. Anthocyanin

and flavanone composition of the maqui-citrus juices was characterized by RP-HPLC-DAD.

3.2. DESIGN OF THE CLINICAL TRIAL

The study performed was a clinical trial, parallel, randomized and triple blind, in a population of overweight subjects from the Region of Murcia (Spain).

The study was performed with 138 volunteers, women and men, between 30-60 years of age, over-weight, healthy, non-smokers. They were recruited by advertisements in local public centers located nearby UCAM, as well as relatives of the staff of the University.

3.3. ETHICAL CONSIDERATIONS

The protocol of the study was approved by the Bioethical Committee of the UCAM before the start of the experiments (Annex). The study has been authorized by one of the Ethical Committee of Clinical Research (CEIC) of the Regional Government of Murcia (CEIC Hospital General Universitario José María Morales Meseguer) (Annex). The study followed the current valid Spanish regulation that regulates clinical research in humans (Real Decreto 561/1993 de Ensayos Clínicos con Medicamentos). This Clinical trial was registered in clinicaltrials.gov as NCT 040116337. Volunteer were provided with written information about the study and all of them signed the informed consent (Annex).

3.4. INCLUSION/EXCLUSION CRITERIA

Inclusion criteria were: healthy, non-smokers, aged 35-55 years, over-weight adults with BMI range 24-29.9 kg/m^2 (according to WHO criteria), non-dyslipidemic and normotense, without any chronic disease and taking no medication.

Exclusion criteria were: obese, smoker, age out of range of 35-55 years, having any chronic diseases including diabetes, cardiovascular, hepatic or renal disease, etc, taking any chronic medication.

In order to select volunteer according these inclusion/exclusion criteria, a questionnaire was used for interview before starting the intervention trial (Annex). Besides, the first day of intervention, a screening was performed to ultimately discard those people who didn't match the inclusion criteria.

3.5. WORKING PLAN

The clinical trial with the volunteers was performed between April and July 2018. After interviewing 196 individuals to assess their eligibility, 58 were not included in the study, forty of which did not fulfil the inclusion/exclusion criteria and 18 were excluded for other reasons (mainly for logistic-operational reasons). A total number of 138 volunteer were included in the study. They were randomly assigned to one group of drink (n=46) each, as can be seen in Figure 19, according to CONSORT 2010 Statement (Welch et al., 2015). In the follow-up period of the study, there were 2 drop outs, one from group B (due to influenza) and one from group C (as he failed to come to the visits and delivery of drinks). There were no exclusions from the analysis and we had a final sample size of n = 46 for beverage A, n= 45 for beverage B and n = 45 for beverage C.

Participants were randomized with the SPSS statistical program, considering their BMI, gender, and age, so that the three groups were homogenous in these three potential confounding factors.

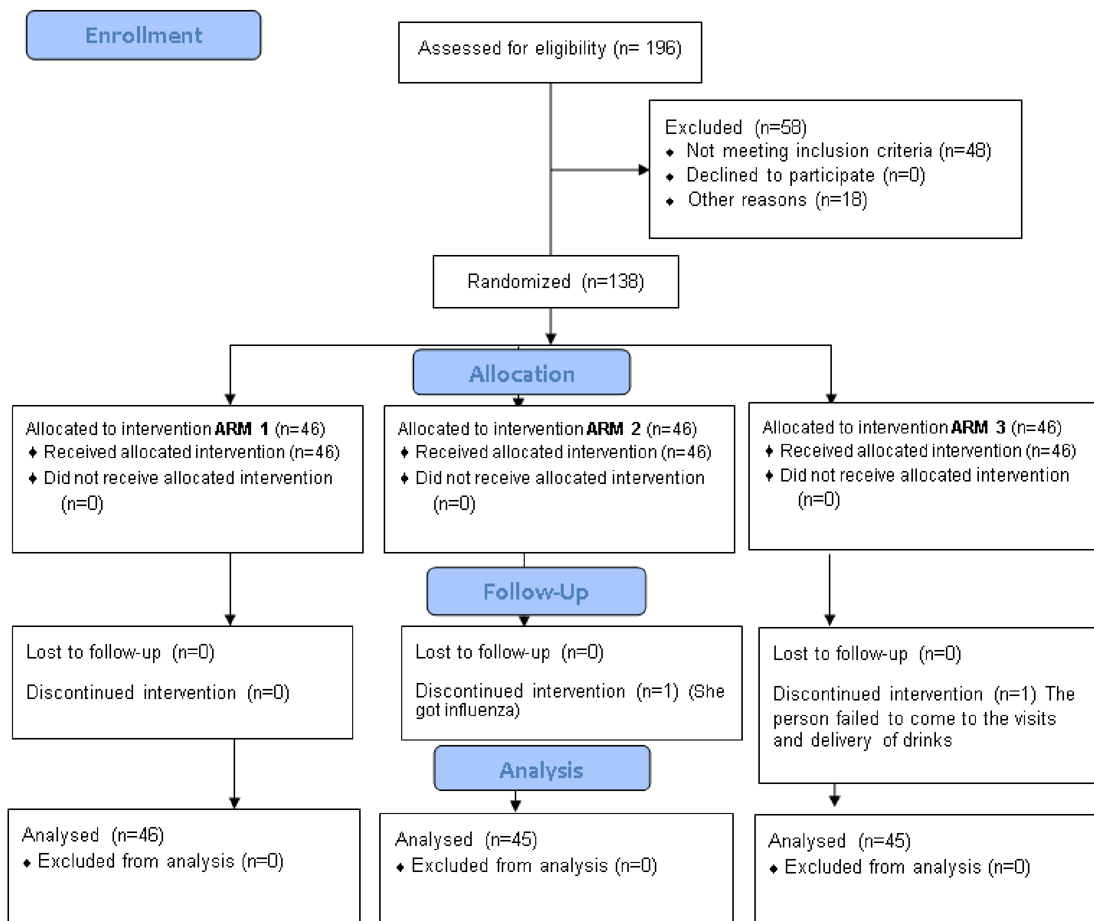


Figure 19: Flow Diagram for the long-term interventional study

All participants were recommended to follow the traditional Mediterranean diet low in cholesterol, saturated and total fat, to reduce inter-individual variability, and to avoid the used of added sweeteners.

They were provided with a detailed questionnaire to reply, including demographics, physical activity, and food history information, that was based on a questionnaire validated by the research team of the Universidad Catholica San Antonio de Murcia (UCAM) (Annex). A questionnaire about their knowledge and use of sweeteners was also provided (Annex).

Each group of volunteer was given one of the test drinks, for its consumption, a bottle of 330 mL per day, over 60 days (Figure 20). Bottles were codified as A, B or C by the manufacturer. The type of sweeteners were unknown for volunteers, as well as for researchers that provided the drinks and collected and processed the samples, as well as for researchers that did statistical analyses, hence the study is considered a triple-blind study. We knew the exact composition of the beverages only at the end of the study and after reporting the results and statistical data analysis.

We provided the drinks in lots of 15 bottles, for their intake in the following 15 days, with instructions of preserving them in the fridge. In this way, it was preserved the freshness of the ready-to-drink beverages. For that, we made some appointments with the volunteers during the whole period of intervention, for collecting the lots of drinks as well as for controlling compliance of intervention and dietary instructions.

Figure 20. Bottles provided to the volunteers with the maqui-*Citrus* beverages

Blood samples were taken and anthropometric and blood pressure measurements were recorded at initial (day 0) and final (day 60) day of intervention. Figure 21 shows the study design.

Urine samples were collected for analysis of bioavailability of metabolites in CEBAS-CSIC (not included in this Thesis). Plasma was aliquoted and kept in freezer at -80 °C, for further analysis.

Table 4 describes the variables that we assessed in each time point. Analysis were performed once the intervention period was finished and in the same batch to minimize analytical variations.

Table 4: Variables assessed in the long-term intervention trial

Category	Variables
Anthropometric measurements	BMI
	% Fat mass
Cardiovascular Markers	Systolic blood pressure
	Diastolic blood pressure
	Heart rate
Oxidative Status	ORAC
	Homocysteine
	LDL-oxidized
Glycaemic profile	Glucose
	Insulin
	HOMA index
Lipid profile	Total Cholesterol
	LDL-Cholesterol
	HDL-Cholesterol
	Triglycerides
Markers of Inflammation	IL-6
	C-reactive protein
	TNF-α
	IL-10
Satiety hormones	Ghrelin
	Leptin
Safety parameters	ALP
	AST
	ALT
	GGT
	Total Bilirubin

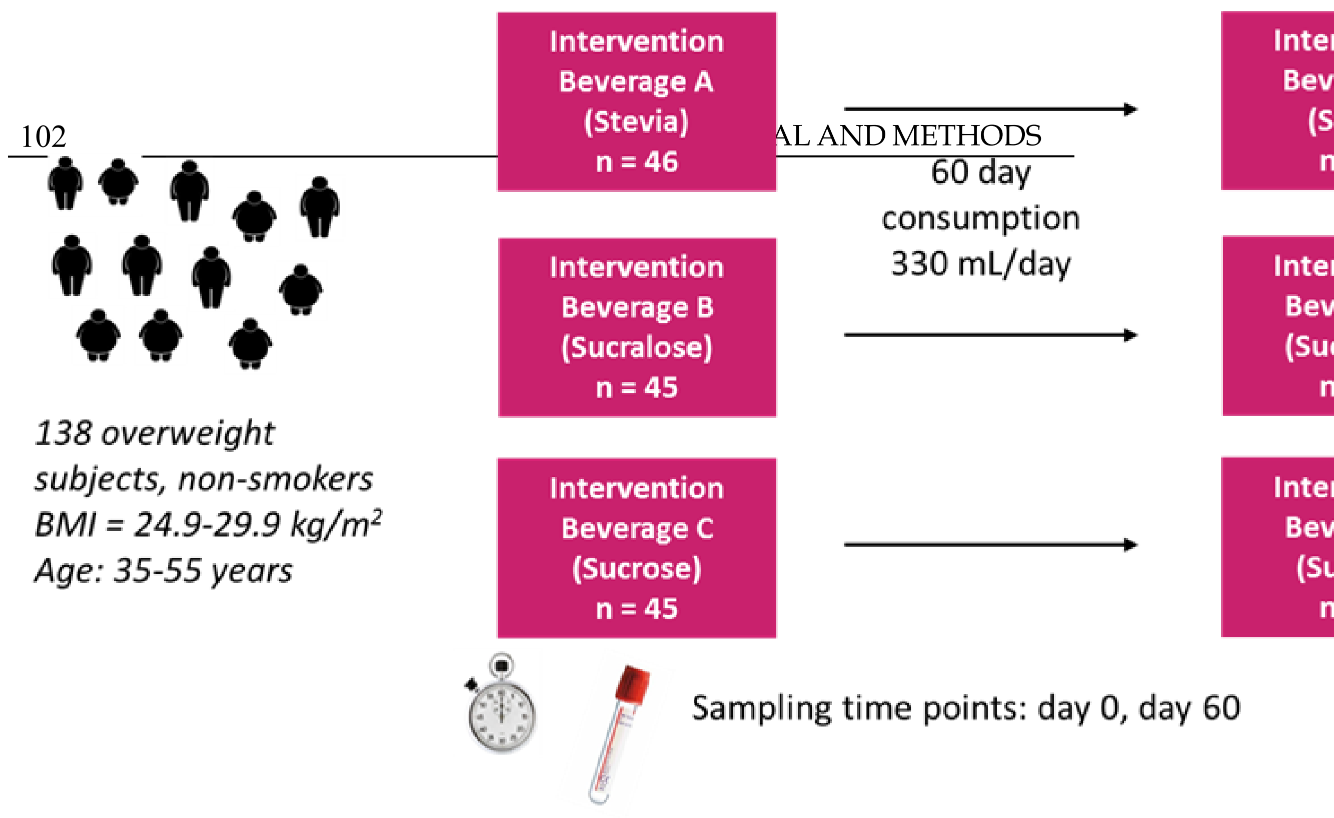


Figure 21: Study design

3.6. EVALUATION OF DIETARY AND LIFESTYLE HABITS

A complete questionnaire was provided to each participant, to assess its dietary and lifestyle habits (Annex). It contained different sections including demographic information and a cardiovascular risk assessment section (family history of diabetes, smoking habits, self-reported lipid profile, frequency and type of physical activity).

The questionnaire also included a section about adherence to the Mediterranean diet. The questions for this section were based on 4 point Likert scale, from daily to never and were comprising of: Frequency of consumption of cereals (rice, pasta, bread, breakfast cereals, bakery products), fruits, vegetables, legumes, fish specifically blue (in comparison with white), different types of meat rather than fish, low fat dairy products, type of oil, olive oil consumption, dried

fruits, eggs, red wine, beer, other alcoholic beverages, green leafy vegetables, tomato, red pepper, functional foods, ratio of consumption of fish to other types of meat, any other type of diet.

Mediterranean diet adherence score was calculated based on a mathematical algorithm.

Score less than 5: You must improve your eating habits.

Score between 5-7: It would be convenient to make some changes in your diet

Score between 7-10: You meet the recommendations of the Mediterranean diet

We assessed the intake of sweeteners by a self-made questionnaire on four point Likert scale ranging from never to daily. This questionnaire was based on literature (Myers et al., 2018). There were eight questions in the questionnaire for this purpose, including the consumption of: added sweeteners (Stevia, Saccharin), no-sugar drinks, no-sugar marmalades, no sugar desserts, no sugar candies and gums, no sugar or light yoghurt, no sugar or light biscuits and pastries, no sugar or light deserts. The frequency of consumption of sugar substitute sweeteners in cooking was also evaluated (Annex).

3.7. VARIABLES OF STUDY

3.7.1. Anthropometric measurements

We determined height and weight at the time of first visit, to calculate Body Mass Index (BMI) as (weight (kg)/ [height (m)] ^{2}). Weight was further recorded on the following visit days, as well as the body fat percentage, which was measured by the body-composition monitor Tanita®, based on impedance.

3.7.2. Cardiovascular markers

Systolic and diastolic blood pressure, as well as heart rate, were measured with the Omron® pressure monitor, as routine analysis.

3.7.3. Antioxidant status

3.7.3.1. Oxidized LDL

Oxidized LDL was analyzed with ELISA kits from ElabScience®. The assay procedure is based on a Sandwich-ELISA principle (Ab-Ag-Ab). The 96-wells plate provided is coated with an antibody specific to human oxidized LDL (LDL-ox). Samples added containing human LDL, combine with the specific antibody. Then a biotinylated detection antibody specific for the human LDL-ox and avidin-horseradish peroxidase are added to form a complex. Successive washing steps are performed to wash away free components. The substrate solution is added to each well and only those wells that contain the complex antibody-LDL-ox-biotinylated detection antibody and avidin-HRP conjugate will react with the substrate solution and give blue-green color to the well. The reaction finish by addition of the stop solution and the color turns yellow. The Optical Density (OD) is then measured at 450 nm wavelength. The absorbance is proportional to the concentration of LDL-ox and the concentration is calculate with appropriate standard curves. Samples were analyzed by duplicate. For this purpose a four parameter logistic curve on log-log graph paper with standard concentration on the x-axis and OD on the y-axis was plotted. Since the samples were diluted, the concentration was calculated from the standard curve, must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, re-test was done with appropriate dilution. Hence the

actual concentration was the calculated concentration multiplied by the dilution factor.

3.7.3.2. ORAC method

Antioxidant effect was measured by Oxygen Radical Absorbance Capacity (ORAC) assay. The ORAC method consists of measuring the decrease in fluorescence of the protein fluorescein, because of the loss of its conformation when it suffers oxidative damage caused by a source of peroxide radicals, the ADPH. The method measures the ability of antioxidants in the sample to protect the protein from oxidative damage, and hence the delay in the decay of fluorescence (Ou et al, 2001).

Phosphate buffer 75 mM, pH 7.4 is used to prepare the ADPH and fluorescein solutions, at concentrations of 0.125 M and 5.96 pM, respectively. The reaction is performed in a 96-wells plate, each well containing 100 µl of fluorescein + 20 µl of plasma diluted sample + 50 µl of buffer. After incubation for 30 minutes in darkness at 37 °C, 30 µl ADPH is added. Readings are performed in 5 min-cycles, for 2 h, in a plate reader with excitation and emission fluorescence wavelengths set at 485 and 520 nm. The plasma samples were getting diluted 1:1000 in phosphate buffer. Different concentrations of Trolox were used as the antioxidant standard curve. The Area under the Curve (AUC) was calculated for the decay of fluorescence of the protein and compared with that of Trolox, to calculate the ORAC values, expressed as µmol Trolox equivalent/L. Samples were analyzed by duplicate.

3.7.3.3. Homocysteine

Homocysteine levels were measured with the autoanalyzer Byosystems® A-15. The principle of the method is that the oxidized form of homocysteine (Hcy) is reduced to free Hcy by the reducing agent tris (2-carboxietil) fosfina (TCEP). The Hcy reacts with the co-substrate S-AdenosylMethionine (SAM), and the reaction is catalyzed by Hcy S-methyltransferase (HMTasa) to form methionine (Met) and S-AdenosylhoMocysteine (SAH). The SAH is assessed by coupled enzymatic reactions, and hydrolyzed by SAH hydrolase (SAHasa) to Adenosine deaminase (Ado) and Hcy. The synthesized Hcy reacts again with the co-substrate SAM and with Hcy S-methyltransferase to obtain SAH forming a cyclic reaction that amplifies the detection signal. Adenosine deaminase (ADA) catalyzes the deamination of adenosine (Ado) to inosine and ammonium. The Hcy concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the GLutamate Dehydrogenase (GLDH) coupled reaction (Dou et al., 2005). Calibration was performed every 7 days. Quality control tests, with a homocystein control serum level 1, (physiological concentrations, non-pathological level), as well as reagent blank were used every day, to verify the performance of the measurement procedure (Figure 22).

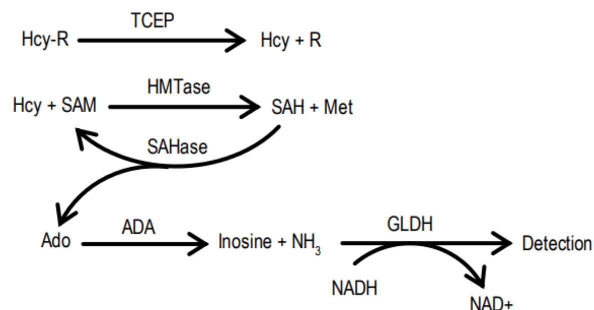


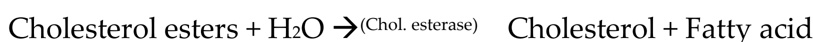
Figure 22: Coupled reactions to detect homocysteine levels in plasma (Source: Biosystems® A-15 homocysteine protocol)

3.7.4. Biochemical analysis: Glycaemic profile, Lipid profile, Safety parameters

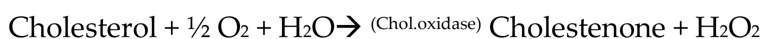
Current biochemical analyses were performed in the autoanalyzer Byosystems® A-15. For this purpose each time the device was calibrated. Sample preparation was performed based on the each kit instructions. Protocols were set up for biomarkers of lipid profile, glycemic profile, as well as safety parameters (hepatic enzymes and products).

3.7.4.1. HDL

The cholesterol from low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons, is broken down by the cholesterol oxidase in an enzymatic accelerated non-color forming reaction. The detergent present in the reagent B, solubilizes cholesterol from high density lipoproteins (HDL) in the sample. The HDL cholesterol is then measured spectrophotometrically by means of the coupled reactions described below (Warnick et al., 2001).



Cholesterol esters + H_2O → (Chol. esterase) Cholesterol + Fatty acid



Cholesterol + ½ O_2 + H_2O → (Chol.oxidase) Cholestenone + H_2O_2

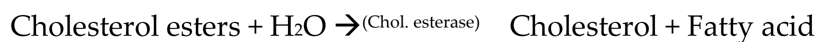
2 H_2O_2 + 4-Aminoantipyrine + DSBmT → (Peroxidase) Quinoneimine + 4 H_2O

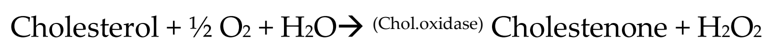
A reagent blank should be done every day and a calibration at least every 2 months, after reagent lot change or as required by quality control procedures. HDL cholesterol concentrations vary considerably with age and sex and also based on laboratory reference range.

Interferences are with bilirubin (up to 20 mg/dL), hemolysis (hemoglobin up to 1000 mg/dL) and lipemia (triglycerides up to 1800 mg/dL) do not interfere. Other drugs and substances may interfere too (Young 2000).

3.7.4.2. LDL

LDL is the main lipoprotein transporting cholesterol from liver to tissues. A specific detergent solubilizes the cholesterol from high density lipoproteins (HDL), very low density lipoproteins (VLDL) and chylomicrons. The cholesterol esters are broken down by cholesterol esterase and cholesterol oxidase in a non-color forming reaction. The second detergent, present in the reagent B, solubilizes cholesterol from low density lipoproteins (LDL) in the sample. The LDL cholesterol is then measured spectrophotometrically by means of the coupled reactions described below (Nauk et al., 2002). The rest of the procedure is the same as HDL-cholesterol, which is explained above.

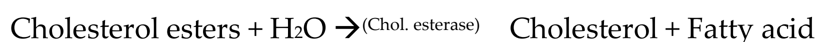

Cholesterol esters + H_2O $\rightarrow^{(Chol.\ esterase)}$ Cholesterol + Fatty acid

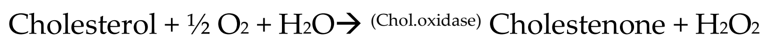

Cholesterol + ½ O_2 + H_2O $\rightarrow^{(Chol.oxidase)}$ Cholestenone + H_2O_2

2 H_2O_2 + 4-Aminoantipyrine + DSBmT $\rightarrow^{(Peroxidase)}$ Quinoneimine + 4 H_2O

3.7.4.3. Total Cholesterol

Free and esterified cholesterol in the sample originates, by means of the coupled reactions described below, a colored complex that can be measured by spectrophotometry (Meiattini et al., 1978). The rest of the procedure is the same as HDL-cholesterol, which explained above.


Cholesterol esters + H_2O $\rightarrow^{(Chol.\ esterase)}$ Cholesterol + Fatty acid

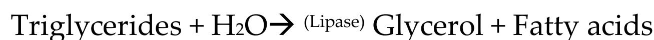


$$\text{Cholesterol} + \tfrac{1}{2}\,O_2 + H_2O \xrightarrow{\text{(Chol.oxidase)}} \text{Cholestenone} + H_2O_2$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{DSBmT} \xrightarrow{\text{(Peroxidase)}} \text{Quinoneimine} + 4\,H_2O$$

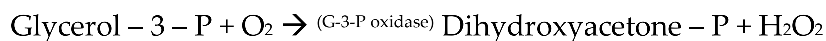
3.7.4.4. Triglycerides

Triglycerides in the sample originates, by means of the coupled reactions described below, a colored complex that can be measured by spectrophotometry (Bucolo et al., 1973; Fossati and Prensipe, 1982).



$$\text{Triglycerides} + H_2O \xrightarrow{\text{(Lipase)}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{(Glycerl kinase)}} \text{Glycerol} - 3 - \text{P} + \text{ADP}$$



$$\text{Glycerol} - 3 - \text{P} + O_2 \xrightarrow{\text{(G-3-P oxidase)}} \text{Dihydroxyacetone} - \text{P} + H_2O_2$$

$$2\,H_2O_2 + \text{4–Aminoantipyrine} + \text{4-Chlorophenol} \xrightarrow{\text{(peroxidase)}} \text{Quinoneimine} + 4\,H_2O$$

Reagent is provided ready to use. Expected interferences were hemolysis (hemoglobin up to 1000 mg/dL), though bilirubin (up to 2.5 mg/dL) do not interfere. Ascorbic acid (up to 5 mg/dL) does not interfere too. Other drugs and substances may interfere (Young 2000).

As safety parameters we evaluated the hepatic toxicity by means of bilirubin levels and hepatic enzymes concentrations in plasma. It included: ALP, ALT, AST, GGT and total bilirubin.

3.7.4.5. Alkaline phosphatase (ALP)

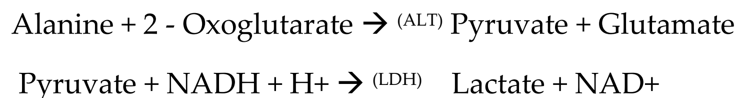
Alkaline phosphatase (ALP) catalyzes in alkaline medium the transfer of the phosphate group from 4-nitrophenylphosphate to di-ethanolamine (DEA), liberating 4-nitrophenol. The catalytic concentration is determined from the rate of 4-nitrophenol formation, measured at 405 nm.

4 – Nitrophenyl phosphate + DEA $\xrightarrow{(ALP)}$ DEA – phosphate + 4 - Nitrophenol

Limitations of the procedure are interferences with bilirubin (up to 20 mg/dL), though hemolysis (hemoglobin up to 500 mg/dL) and lipemia (triglycerides up to 1000 mg/dL) do not interfere. Other drugs and substances may interfere too (Rosalki et al., 1993).

3.7.4.6.Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT or GPT) catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the lactate dehydrogenase (LDH) coupled reactions.



Alanine + 2 - Oxoglutarate $\xrightarrow{(ALT)}$ Pyruvate + Glutamate

Pyruvate + NADH + H+ $\xrightarrow{(LDH)}$ Lactate + NAD+

Limitations of the procedure are interferences with bilirubin (up to 20 mg/dL), though hemolysis (hemoglobin up to 1000 mg/dL) and lipemia (triglycerides up to 200 mg/dL) do not interfere. Other drugs and substances may interfere too (Young, 2000).

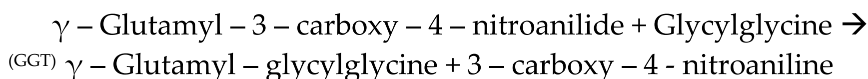
3.7.4.7.Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST or GOT) catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxalacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the malate dehydrogenase (MDH) coupled reactions.

Limitations of the procedure is Interferences with lipemia (triglycerides 2 g/L) while Bilirubin (20 mg/dL) and Hemolysis (hemoglobin 10 g/L) do not interfere. Other drugs and substances may interfere too (Young, 2000).

3.4.7.8. Gamma-glutamyl-transferase (GGT)

Gamma-glutamyl-transferase (γ-GT) catalyzes the transfer of the γ-glutamyl group from γ-glutamyl-3carboxy-4-nitroanilide to glycylglycine, liberating 3-carboxy-4-nitroaniline. The catalytic concentration is determined from the rate of 3-carboxy-4-nitroaniline formation.



The predicted interferences are with Hemoglobin (> 5 g/L), bilirubin (> 10 g/L) and lipemia (triglycerides > 4 g/L) may affect the results. Other drugs and substances may interfere too (Young, 2000).

3.7.4.9. Total Bilirubin

Direct bilirubin in the sample reacts with diazotized sulfanilic acid forming a colored complex that can be measured by spectrophotometry. Both direct and indirect bilirubin couple with diazo in the presence of cetrimide (Pearlman and Lee, 1974; Zoppi et al., 1976). The terms “direct” and “total” refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing (accelerating) reagents. The “direct” and “indirect” bilirubin are only approximately equivalent to the conjugated and unconjugated fractions.

For total bilirubin measurements, BT (sodium nitrate 11.6 mmol/L) was mixed into AT (sulfanilic acid 29mmol/L) in ratio of 4:1 and kept two minutes in room temperature. The samples were read by spectrophotometer in 540 ± 20nm. Reference value for adult total bilirubin was 2mg/dL or 34 μmol/L.

Table 5. Reagent preparation for total bilirubin measurements (Biosystem A-15 protocol)

	Reagent blank	Sample Blank	Sample	Standard
Distilled water	100μL	-	-	-
Sample	-	100μL	100μL	-
Standard	-	-	-	100μL
Reagent (AT)	-	1.0mL	-	-
Working reagent	1.0mL	-	1.0mL	1.0mL

For measurement of total bilirubin, the blank was read against distilled water and the samples and standard against reagent blank (Table 5). The bilirubin concentration in the sample is calculated using the following general formula:

((Abs sample - Abs blank)/Abs standard)* C standard= C sample

3.7.4.10. Glucose and Insulin levels – HOMA IR

Glucose and insulin levels were kindly assessed in the Laboratory for Clinical Analyses of Hospital Quirón (Murcia).

HOMA-IR index, as a measure of insulin resistance, based on the homeostatic model assessment, was calculated based on glucose and insulin level as:

HOMA-IR = $\left(fasting\ insulin\left(\mu\frac{U}{mL}\right) x\ fasting\ glucose\left(\frac{mmol}{L}\right)\right) / 22{,}5$

Values higher than 3.2 indicate insulin resistance (Matthews et al., 1985).

3.7.5. Inflammation markers

All the inflammation markers, IL-6, IL-10 and TNF-α were measured by high sensitivity ELISA kits provided by IBL International®.

To obtain optimal test performance, the exact kit manual procedure of washing steps, chronology of/and preparation of solutions and incubation time was followed. Absorbance values for each set of duplicate standards and samples were measured.

The principles of these tests is that an anti-human IL-6 (or IL-10 or TNF-α) coating antibody is adsorbed on the surface of the wells. Human interleukin present in the sample binds to the antibody. A biotin-conjugated anti-human IL-6 (or IL-10 or TNF-α) is added and binds to the interleukin captured by the first antibody.

Streptavidin-HRP is added and binds to the biotin-conjugated anti-interleukin antibody. Afterwards, Amplification Reagents are added to the wells, to increase the signal obtained. Each step of addition is followed by incubation and washing steps to remove the unbound reagent (Figure 23). The colored product is formed in proportion to the amount of human IL-6 (or IL-10 or TNF-α) present in the sample. The reaction finishes by addition of a stop acid solution and the absorbance is measured at 450 nm.

A standard curve was created by plotting the mean absorbance for each standard concentration on the ordinate against the human IL-6 concentration on the abscissa. A five-parameter curve fit was drawn and the absorbance values of the samples were interpolated in the standard curve to determine the concentration of circulating human IL-6 (or IL-10 or TNF-α) for each sample. All measurements of samples and controls were in duplicate.

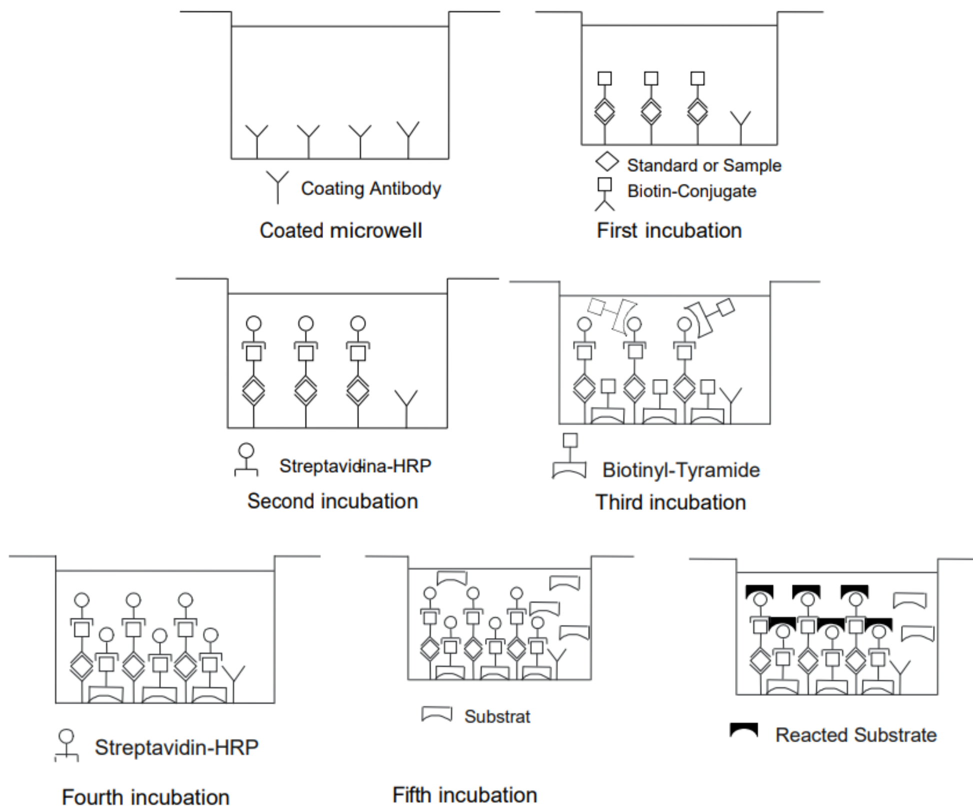


Figure 23: Scheme simplified of ELISA test principle (Based on protocol of IBL International kits)

3.7.6. C-Reactive Protein (CRP)

C-reactive protein (CRP) was measured with the ichroma™ reader, a portable fluorescent scanner that analyses plasma samples and displays measurement results on the screen.

The test consists of test cards that contain a monoclonal anti-mouse antibody and rabbit IgG immobilized. The detector buffer contains fluorescent anti-CRP and

fluorescent rabbit IgG. The capillary collection tubes are used to collect the plasma sample (10 μL).

The test uses a sandwich immune-detection method; the detector antibody in buffer binds to antigen in sample, forming antigen-antibody complexes. The more antigen in sample forms the more antigen-antibody complex and lead to stronger intensity of fluorescence signal on detector antibody, which is processed to show CRP concentration in sample (Oh et al., 2005). The instrument displays CRP concentration of the sample in terms of mg/L. The cut-off (reference value) wase 10 mg/L and the working range 2.5 - 3.00 mg/L.

3.7.7. **Satiety hormones**

3.7.7.1.Leptin

Leptin was measured by an ELISA kit provided by IBL International®. The assay is a solid-phase Sandwich ELISA based on the same principle as described above. An anti-human leptin coating antibody is adsorbed on the surface of the wells, to bind the leptin present in the sample.

Then, reactions are performed with biotin-conjugated anti-human leptin and streptavidin-HRP. No amplification reagents were used, but the substrate tetramethyl benzidine (TMB) as a chromogen. The intensity of the color obtained is proportional to the quantities of leptin present in plasma samples. All measurements of samples and controls were in duplicate.

3.7.7.2. Ghrelin

Ghrelin was measured by an ELISA kit provided by Merck®. The assay is a Sandwich ELISA based on the capture of human ghrelin in the sample by anti-human ghrelin IgG that is immobilized in the wells of a microtiter plate. Then, a second biotinylated antibody binds to the ghrelin, followed by incubation with horseradish peroxidase and quantification of immobilized antibody-enzyme conjugates by reaction with its substrate, the 3-tetramethylbenzidine. Washing steps were performed to wash away the unbound reactive.

The enzyme activity is measured at 450 nm and it is proportional to the amount of human ghrelin captured in the sample. Concentrations were calculated by interpolation in the standard curve made with different known concentrations of human ghrelin standard. The appropriate range of this assay was 50 pg/ml to 5000 pg/ml. All measurements of samples and controls were in duplicate.

3.8. STATISTICAL ANALYSIS

Age, sex and BMI can influence the results obtained. Hence, it is necessary that intervention groups are homogeneous in the parameters at time zero. Stratified randomization was performed based on se-age-BMI. They were verified, by means of one-factor ANOVA test, to make sure that there are no significant differences between the groups at time zero, in these parameters.

The normality test (Shapiro-wilks) was performed on the study variables, to choose the appropriate statistical test (parametric or non-parametric). The normality test was applied to the change observed, (that is, on the difference

between the final value - initial value at zero time), to compare data of the same individual within each group (intra-group) and to compare the different groups with each other (inter-group).

All variables followed a normal distribution, except for TNF-α, IL-10, IL-6 and C-reactive protein (CRP) because the deviations were quite large. In all these variables, non-parametric statistics were performed. In some cases concentrations were very low, near the limit of detection, hence no conclusions could be drawn (see Results Section).

3.8.1. Effect of each drink (Comparison within the group)

To evaluate if there were significant differences before and after drink consumption in each variable, and within each treatment, the t-test of paired samples has been applied in the variables that follow normal distribution. In the variables that do not follow a normal distribution, the Wilcoxon of paired samples has been applied.

3.8.2. Differences between drinks (Comparison between groups)

To compare the treatments, in the variables that follow a normal distribution, the variations before and after the treatment were compared, as independent samples, with the one-factor ANOVA test, being the factor the treatment, with Bonferroni post-hoc analysis. Data were also checked with Tukey post-hoc analysis and we obtained the same results.

For variables that do not follow a normal distribution, the Kruskal-Wallis test has been carried out. In case of significant differences, the post-hoc analysis performed was U of Mann-Whitney.

All statistical analyses were performed with SPSS version 24.0 software (IBM®).

IV - RESULTS

IV - RESULTS

4.1. BASAL CHARACTERISTICS OF POPULATION STUDIED

As can be seen in Table 6, the three intervention groups were homogeneous at the initial time point. One-way ANOVA test did not show any significant difference between the groups at time zero.

Table 6. Baseline characteristics of volunteers

Parameter	Stevia Beverage A (n=46)	Sucralose Beverage B (n=45)	Sucrose Beverage C (n=45)
Age (years)	44 ± 7	42 ± 8	42 ± 7
Sex	27 M; 19 F	27 M; 18 F	26 M; 19 F
Weight (kg)	82.9 ± 10.8	82.4 ± 10.8	84.8 ± 12.4
Height (m)	1.72 ± 0.09	1.72 ± 0.09	1.71 ± 0.09
BMI (kg/m^2)	27.9 ± 2.9	27.8 ± 2.3	29.1 ± 3.0
% Fat mass	32.6 ± 7.4	30.6 ± 7.7	32.7 ± 8.8

Data are expressed as mean ± SD
M: Male; F: Female

The majority of participants were male between 40-50 years old with post-graduate degrees (Table 7).

Table 7. Demographic frequency and Education Level of participants

	Variables	Valid percentage (%)
Age (years)	20-30	0.8
	30-40	36.4
	40-50	45.5
	50-60	17.4
Gender	Male	58.8
	Female	41.2
Education Level	Elementary	17.3
	BSc	26.0
	Msc	18.1
	Ph.D.	38.6

4.2. DIETARY AND LIFESTYLE HABITS

4.2.1. Lifestyle habits

After completion of the questionnaires, we observe that 11.9% of participants were past-smoker (more than 5 years ago), and the majority of them had physical activity plan for two to three times per week (44.5%) and were consuming different types of supplements (78.4%). The history of hypercholesterolemia and hyperglyceridemia were 24.8 and 4.9 % respectively (Table 8). Almost 80 % of volunteer had taken supplements or vitamins for short periods of time in the past.

Table 8. Health history of participants

Variables (Questions)		Valid percentage (%)
Past smoker	Yes	11.9
	No	65.9
Physical activity (weekly)	Every day	20.3
	4-5 times per week	17.2
	2-3 times per week	44.5
	No	18
	Missing	11
Physical activity (Hours per day)	More than 2 hours	2.7
	2 hours	7.3
	1.5 hours	43.6
	Half an hour	28.2
	Less than half an hour	18.2
Past history of Hypercholestrolemia (22.4 % missing)	Yes	24.8
	No	52.8
Past history of Hypertriglyceridemia (32.3% missing)	Yes	4.9
	No	61.8
Past history of Consumption of supplement or vitamin (13.6% missing)	Yes	78.4
	No	8.0

4.2.2. Adherence to Mediterranean diet

The results obtained with the questionnaire of the adherence to Mediterranean diet questionnaire is shown in Table 9. Concerning the daily consumption, we can observed that the following items were mainly consumed in one to two portions per day: cereals (66.1%), fruits (68.1%), vegetables (48%), milk and dairy products (78.1%), in which 36.7% were always using no fat dairy products (Table 10).

Concerning the weekly consumption of food products, the following items were more popular just one or two times per week including fish (67.5%, which mostly was blue fish (50.8%), poultry (57.5%), red meat (34.4%), sausage (38.6%), egg (48.4%), other types of meat (68.0%), dried fruits (35.9). In general meat were more popular than fish (69.5%) (Table 10).

Red wine and industrial pastries were consumed less than one time per week, by 44.1 and 38.6% respectively. Olive oil was the most popular among other types of oil and was used for cooking by 77.8% of participants (Table 9).

Table 9. Results of Mediterranean diet questionnaire

Variables	Frequency of consumption	Valid percentage (%)
Cereal	More than 7 portion per day	2.4
	5-7 portion per day	3.9
	3-4 portion per day	25.2
	1-2 portion per day	66.1
	None	2.4
Fruit	6 portions per day	0.8
	3-4 portion per day	15.7
	1-2 portion per day	68.5
	None	15.0

Table 9. Results of Mediterranean diet questionnaire (continued)

Variables	Frequency of consumption	Valid percentage (%)
Vegetables	More than 2 times per day	4.8
	2 times per day	29.6
	One time per day	48.0
	Less than one time per day	17.6
Legumes	4 times per day	0.8
	3-4 times per week	19.5
	1-2 times per week	65.6
	Less than one time per week	12.5
	None	1.6
Fish	More than 4 times per week	2.4
	3-4 times per weeks	11.9
	1-2 times per week	67.5
	Less than one time per week	17.5
	None	0.8
White fish or blue	Blue	62.1
	White	30.6
	None	4.0
	Both	3.2
Blue fish	More than four times per week	1.6
	3-4 times per week	4.8
	1-2 times per week	50.8
	Less than one time per week	38.9
	None	4.0
Poultry	More than 4 times per week	7.1
	3-4 times per week	29.9
	1-2 times per week	57.5
	Less than one tim per week	3.9
	None	1.6
Red meat	3-4 times per week	10.9
	1-2 times per week	52.3
	Less than one time per week	34.4
	None	2.3
Other types of meat	More than 4 times per week	1.6
	3-4 times per week	10.9
	1-2 times per week	68.0
	Less than one time per week	16.4
	None	3.1

Table 9. Results of Mediterranean diet questionnaire (continued)

More fish than meat	Yes	16.4
	No	69.5
	The same	14.1
Eggs	More than five eggs per week	5.6
	3-5 eggs per week	39.7
	1-2 eggs per week	48.4
	Less than one egg per week	4.8
	None	1.6
Milk and dairy products	More than 4 portion per day	2.3
	3-4 portion per day	15.6
	1-2 portion per day	78.1
	None	3.9
Low fat dairy products	No	14.1
	One time	28.1
	Always	36.7
	Low fat	21.1
Type of oil consumed	Olive	87.5
	Seeds	3.1
	Olive + seeds	9.4
Olive oil for cooking	Daily	77.8
	3-5 times per week	10.3
	1-2 times per week	7.1
	Less than one time per week	0.8
	Non	4.0
Dried fruit	More than 5 times per week	16.4
	3-4 times per week	16.4
	1-2 times per week	35.9
	Less than one times per week	27.3
	None	3.9
Sausages	Every day	5.5
	3-5 times per week	32.3
	1-2 times per week	38.6
	Less than one times per week	20.5
	None	3.1

Table 9. Results of Mediterranean diet questionnaire (continued)

Indusrial pastries	Everyday	3.1
	3-4 times per week	8.7
	1-2 times per week	20.5
	Less than one time per week	44.1
	None	23.6
Red wine	More than seven glasses per week	3.9
	4-7 glasses per week	3.1
	1-3 glasses per week	18.9
	Less than once per week	38.6
	None	35.4

We calculated the score of adherence to Mediterranean diet, based on our previous studies, giving one point to the questions that were answered with the highest value of consumption of fruits, vegetables, legumes or poultry meat and the lowest consumption of factory-baked products, as described in Abellán et al, (2016).

Score less than 5 = poor MD adherence, need of improvement of dietary habits.

Score between 5 - 7 = average MD adherence, which could be improved with some changes in the diet.

Score between 7 - 10 = good MD adherence, the recommendations of the Mediterranean diet has been met.

Considering Mediterranean score, 50 % of the volunteers were located in a score higher than 7, while 40% in scores between 5-7. Only 10% needed to improve their eating habits based on this score. The mean of Mediterranean score in study population was 6.5 ± 2.3 and 7.0 ± 1.4 in male and female groups,

respectively. We observed that 50% of the population sample should change their eating habits to improve their adherence to the Mediterranean diet.

4.3. KNOWLEDGE AND USE OF SWEETENERS

We provided a questionnaire about the knowledge and use of sweeteners (Annex). Among products added with low-caloric sweeteners, the sweetened yoghurt and sweetened drinks were on higher demand by 13 and 9 % of volunteers, respectively, consuming more than five times per week (Figure 25). The least popular sweetened products on this category which never been used were marmalade (83%), chewing gum (82%), pastry and biscuits (67%), desserts (70%). Consumption of LCS was majorly one to two time per day (41%) or never (47 %) (Figure 24).

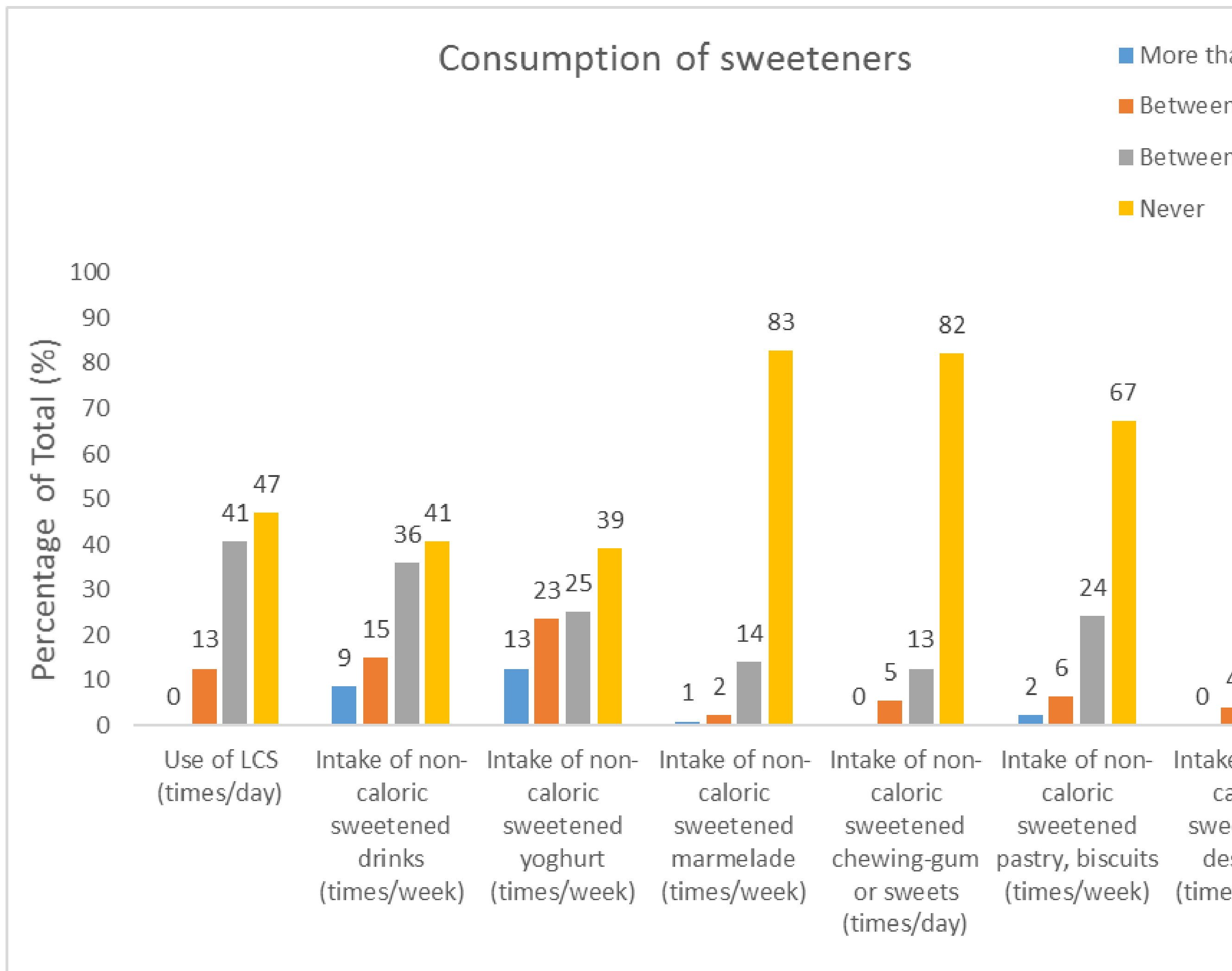


Figure 24. Frequency of consumption of sweetened products

4.4. ACCEPTABILITY OF THE TEST DRINKS BY VOLUNTEERS

We assessed the opinion and acceptability of the tested beverages by using a self-made questionnaire based on a Likert scale (Annex).

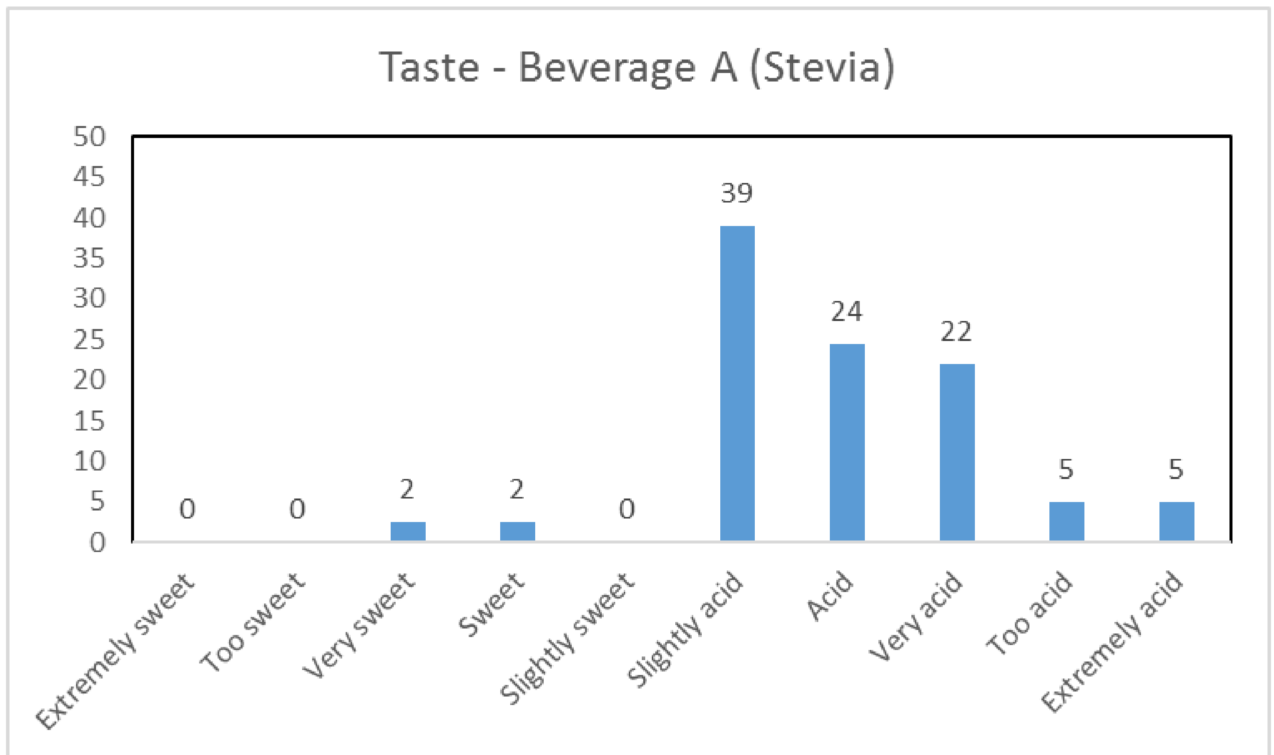


Figure 25. Opinion of participants on the taste of drink A (with Stevia)

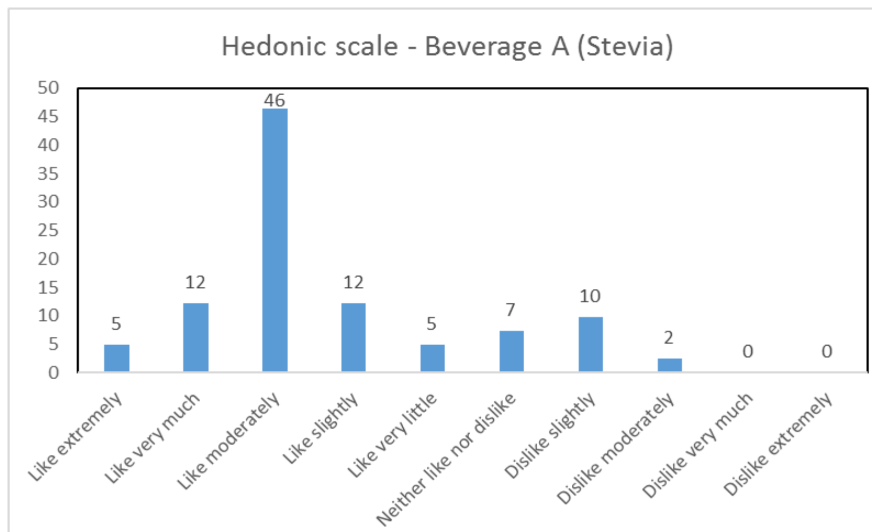


Figure 26. Hedonic scale of participants on drink A (with Stevia)

Drink A containing stevia, was majorly categorized as slightly acidic (39%) and a 46 % of participants liked it moderately. It was equally ranked as “like very much” and “like slightly”, each 12% (Figures 25, 26).

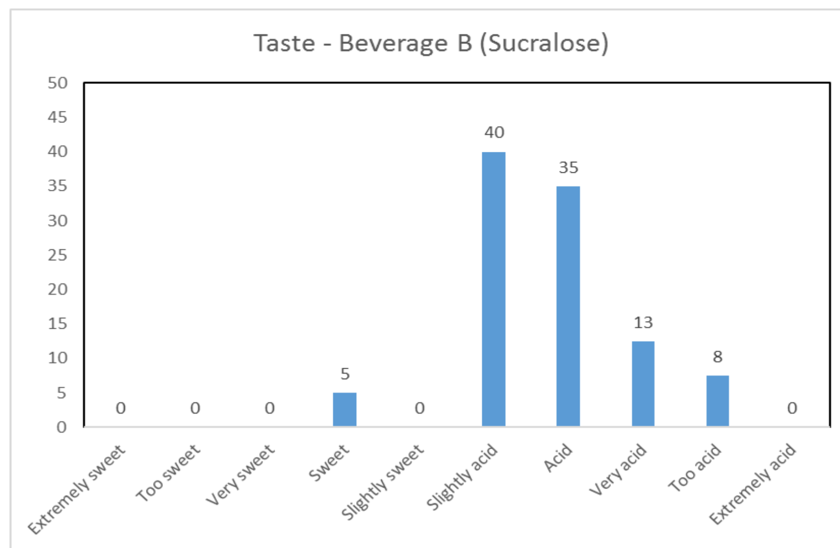


Figure 27. Opinion of participants on the taste of drink B (with Sucralose)

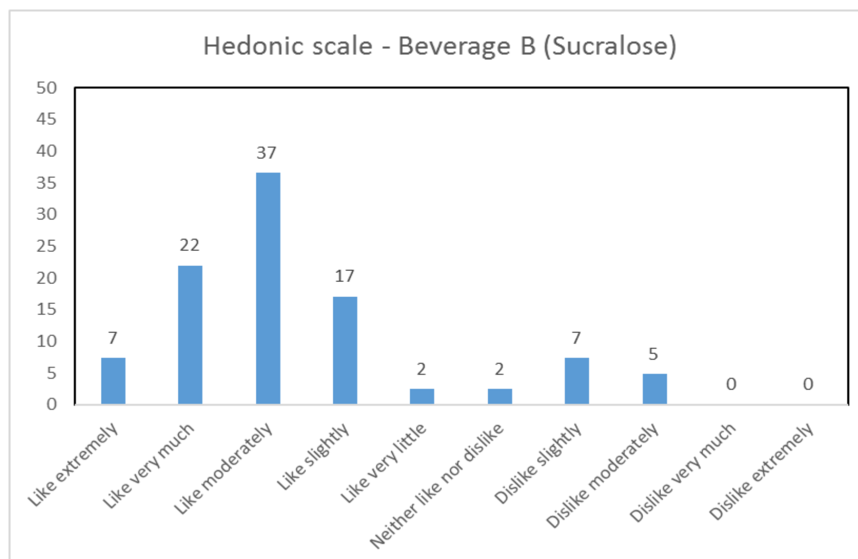


Figure 28. Hedonic scale of participants on drink B (with Sucralose)

Drink B containing Sucralose, was also mostly perceived as slightly acidic (40%) and acidic (35%), with moderate popularity (37% liked moderately), following 22% who liked it very much (Figures 27, 28).

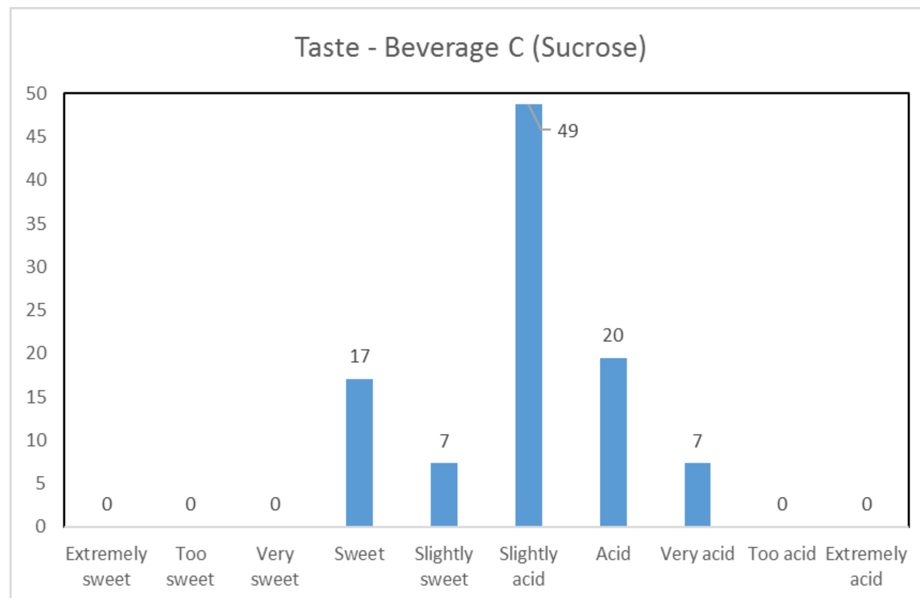


Figure 29. Opinion of participants on the taste of drink C (with Sucrose)

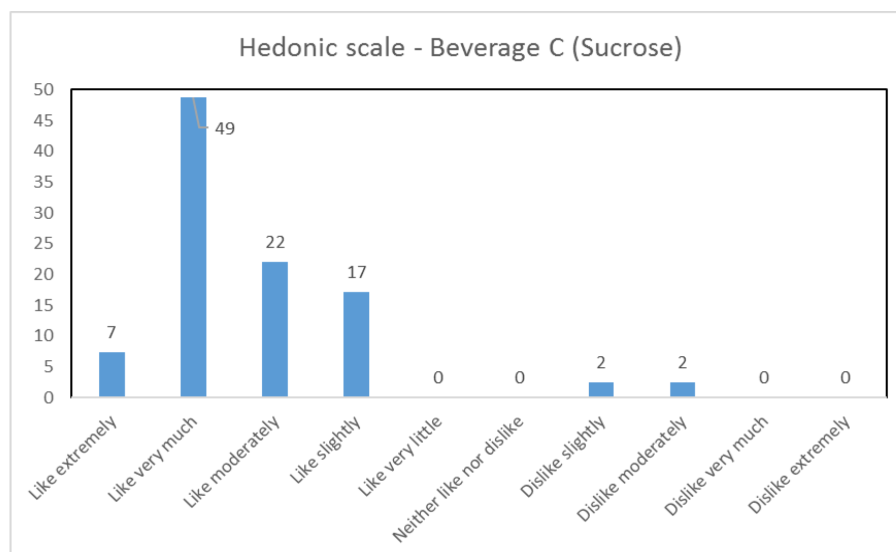


Figure 30. Hedonic scale of participants on drink C (with Sucrose)

Drink C containing Sucrose, was the most popular among three drinks by 49% of participants, who liked it very much. The test ranked slightly acidic (49%) followed by acidic 20% (Figures 29, 30).

4.5. COMPOSITION OF THE TESTED BEVERAGES

The flavanone and anthocyanin composition of the maqui-citrus juices was characterized previously (Agulló et al, 2020a, 2020b). As can be seen in Table 11, four flavanones were observed, with the higher concentration being of hesperetin-rutinoside (4.97 mg/100 mL). In case of anthocyanins, it was found 8 compounds, being the most abundant the delphinidin derivatives: Dp-3-*O*-sambubioside-5-*O*-diglucoside, and Dp-3,5-*O*-diglucoside, with mean concentrations of 3.15, 3.49 mg/100 mL, respectively. No significant differences were observed between the three drinks, neither on the flavanone nor on anthocyanin composition, considering both individual as well as total content.

Table 11. Anthocyanin and flavanone composition (mg/100 mL) of the maqui-citrus juices

	Stevia Beverage A	Sucralose Beverage B	Sucrose Beverage C	*P*-value
Dp 3-*O*-sam-5-*O*-glc	3.06 ± 0.12 [Y]	3.19 ± 0.05	3.19 ± 0.01	>0.05 [N.s.]
Dp 3,5-*O*-diglc	3.59 ± 0.02	3.51 ± 0.01	3.36 ± 0.09	>0.05 [N.s.]
Cy 3-O-sam-5-O-glc + Cy 3,5-*O*-diglc	1.54 ± 0.02	1.51 ± 0.01	1.38 ± 0.01	>0.05 [N.s.]
Dp 3-*O*-sam	1.09 ± 0.01	1.11 ± 0.01	1.09 ± 0.01	>0.05 [N.s.]
Dp 3-*O*-glc	2.87 ± 0.02	3.02 ± 0.01	2.90 ± 0.01	>0.05 [N.s.]
Cy 3-O-sam	0.40 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	>0.05 [N.s.]
Cy 3-O-glc	0.54 ± 0.01	0.57 ± 0.01	0.55 ± 0.01	>0.05 [N.s.]
TOTAL Anthocyanins	13.1 ± 0.2	13.3 ± 0.1	12.9 ± 0.2	> 0.05 [N.s.]
N-hexoside derivated	0.15 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	> 0.05 [N.s.]
E-rutinoside	0.32 ± 0.04	0.32 ± 0.01	0.31 ± 0.03	>0.05 N.s.
N-rutinoside	1.30 ± 0.01	1.31 ± 0.01	1.31 ± 0.01	>0.05 N.s.
H-rutinoside	4.87 ± 0.01	4.86 ± 0.01	4.88 ± 0.01	>0.05 N.s.
TOTAL Flavanones	6.64 ± 0.2	6.63 ± 0.1	6.64 ± 0.1	> 0.05 [N.s.]

Concentrations are presented as mean ± SD (n = 3).
Cy, cyanidin; Dp, delphinidin; Glc, glucose; Sam, sambubioside. N, naringenin; E, eriodyctiol; H, hesperetin. The quantification of anthocyanins was done on UV chromatograms recorded as cyanidin-3-*O*-glucoside at 520 nm.

4.6. ASSESSMENT OF SAFETY PARAMETERS

We determined the levels of hepatic enzymes, as well as total bilirubin, to assess live function and control for a possible, unexpected, toxicity with the nutritional intervention. Liver enzymes and bilirubin were within normal values, both before and after the intervention, indicating no hepatic toxicity. A significant decrease (of 9 %) in the enzyme alkaline phosphatase (ALP) was observed with the Stevia (p=0.001) and Sucralose (non-significant), whilst there is an increase with sucrose intervention, non-significant. Transaminases ALT and AST were significantly reduced with Stevia beverage (p=0.012 and 0.001, respectively), while they increase significantly with drinks Sucralose (p=0.001 and 0.009, respectively) and Sucrose (p=0.038 and 0.003, respectively), always within normal limits (Table 12). No significant changes were observed neither in GGT nor total bilirubin after any intervention.

Table 12. Safety parameters: hepatic function

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 60	Day 60	Day 60
ALP (U/L)	178 ± 31	161 ± 28 * [a]	168 ± 29	159 ± 38 [a]	172 ± 33	183 ± 33 [b]
ALT (U/L)	22 ±7	21 ± 7 * [b]	20 ± 7	24 ± 9 * [a]	25 ± 7	28 ± 8 * [a]
AST (U/L)	26 ± 6	24 ± 5 * [b]	24 ± 6	27 ± 7 * [a]	26 ± 7	28 ± 6 * [a]
GGT (U/L)	28 ± 16	27 ± 16	27 ± 13	27 ± 13	34 ± 21	35 ± 17
Total bilirubin (mg/dL)	0.59 ± 0.18	0.58 ± 0.17	0.57 ± 0.17	0.62 ± 0.13	0.61 ± 0.18	0.66 ± 0.20

Data are expressed as mean ± SD

Significant differences before and after are indicated by an asterisk *.

The letters indicate the groups that are different from each other, "a" and "b".

4.7. ANTHROPOMETRIC AND CARDIOVASCULAR MARKERS

As can be seen on Table 13, volunteers of the drink added with Stevia showed a non-significant decrease in body weight, whilst it increased significantly with Sucralose (0.5 %, p=0.043), as well as with Sucrose (0.4 %, non-significant).

The percentage of body fat decreased significantly with Stevia (- 8 %, p=0.001) and Sucrose treatment (-3.8 %, non-significant). In contrast, with Sucralose it increased (1.7 %), though not significant. No significant differences were observed in BMI before and after each intervention with the beverages.

There was a significant reduction in diastolic pressure with Sucrose treatment (p=0.009), and in systolic pressure with Stevia treatment (p=0.038). However, there were no significant differences between the drinks (Table 13).

4.8. ANTIOXIDANT STATUS

Regarding oxidative stress markers, we observed an increase in ORAC values with Stevia (9.8 %) and Sucralose treatments (2 %), as well as a reduction on the levels of oxidized LDL, although they were non-significant (Table 14). It must be pointed out the high inter-individual variability observed in the data; although they followed normal distribution demonstrated with Shapiro-Wilks test, the standard deviation observed was high. Concerning homocysteine levels, there was a significant increase both with Sucralose (27 %, p=0.001) and Sucrose (40 %, p=0.006) drinks, significantly different from Stevia, that didn’t change.

Table 13. Anthropometric and cardiovascular parameters

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
Weight (Kg)	82.9 ± 10.8	82.4 ± 10.7 [a]	82.4 ± 10.8	82.8 ± 10.7 * [b]	84.8 ± 12.4	85.1 ± 12.5 [b]
BMI (Kg/m^2)	27.9 ± 2.9	27.6 ± 2.6	27.8 ± 2.3	28.0 ± 2.3	29.1 ± 3.0	29.3 ± 3.2
% Fat mass	32.6 ± 7.4	30.2 ± 8.4 * [a]	30.6 ± 7.7	31.0 ± 8.3 [b]	32.7 ± 8.8	31.4 ± 8.7 * [a]
Diastolic pressure (mm Hg)	82 ± 11	81 ± 12	83 ± 12	83 ± 9	84 ± 9	81 ± 10 *
Sistolic pressure (mm Hg)	127 ± 15	123 ± 16 *	123 ± 16	122 ± 15	125 ± 13	123 ± 13
Cardiac-rate (pulses/min)	69 ± 11	71 ± 12	71 ± 10	72 ± 9	70 ± 11	68 ± 12

Data are expressed as mean ± SD
Significant differences before and after are indicated by an asterisk *.
The letters indicate the groups that are different from each other, "a" and "b".

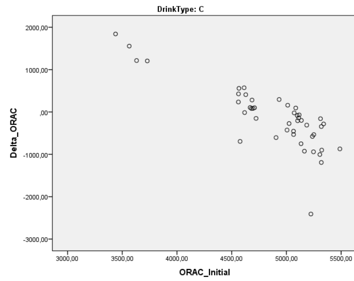
Table 14: Antioxidant status

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 0	Day 60	Day 0
Homocisteine (µmol/L)	12.7 ± 3.3	12.6 ± 3.5 [a]	12.4 ± 4.7	15.8 ± 5.6 * [b]	14.8 ± 6.9	18.4 ± 7.0 * [b]
ORAC (µmol Trolox/L)	4664 ± 835	4842 ± 693	4799 ± 405	4866 ± 264	4871 ± 482	4741 ± 437
LDL oxidized (U/L)	566 ± 168	541 ± 203	969 ± 684	859 ± 575	1226 ± 370	1322 ± 439

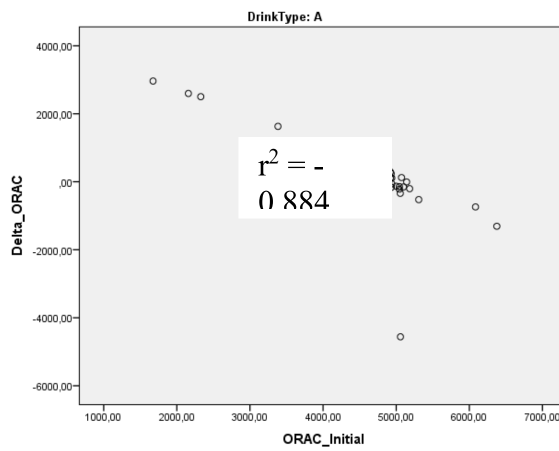
Data are expressed as mean ± SD
Significant differences before and after are indicated by an asterisk *.
The letters indicate the groups that are different from each other, "a" and "b".

In order to check if the ORAC level is related to the basal antioxidant status of the volunteer, we compared, for each volunteer and tested drink, the changes observed (ORAC values at day 60 – ORAC values at day 0) with the ORAC initial level at day 0. As can be seen in Figure 31, the three test drinks behave the same manner.

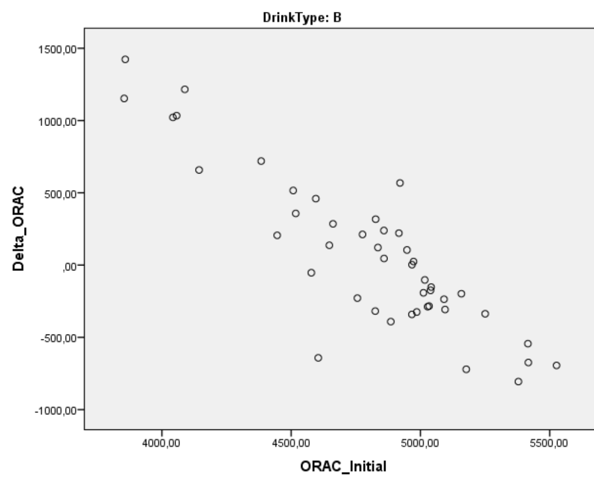
The changes (increases) were higher for the people with lower antioxidant status (lower ORAC basal value), irrespectively of the tested drink.



r^2 = - 0.764



r^2 = - 0.884



r^2 = - 0.824

Figure 31. Correlation of delta ORAC and initial ORAC level with the drink intervention

4.9. GLYCEMIC PROFILE

Regarding the glycemic profile, there is a significant increase in fasting glucose after consumption of the three drinks, mainly with Sucrose treatment (11%, 20%, 26 % with Stevia, Sucralose and Sucrose, respectively p=0.001). A parallel increase in insulin values was observed, but non-significant, and it results in an increase in HOMA-IR values with the three treatments, being significant with drinks added with Sucralose (p = 0.002) and Sucrose (p=0.001), and not with Stevia one (Table 15).

Table 15: Biochemical parameters: Glycemic profile

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 0	Day 0	Day 60
Glucose (mg/dL)	90.9±11.5	99.6 ± 8.8 * a	83.3±12.4	98.8 ± 12.1 * a,b	75.3±12.8	93.4 ± 11.2 * b
Insulin (μUI/mL)	8.4 ± 4.2	8.4 ± 4.0	8.1 ± 3.7	9.3 ± 5.3	7.5 ± 3.4	8.9 ± 4.9
HOMA-IR	1.89±1.03	2.07 ± 1.04	1.66±0.76	2.27 ± 1.37 *	1.4 ± 0.7	2.06 ± 1.1 *

Data are expressed as mean ± SD
Significant differences before and after are indicated by an asterisk *.
The letters indicate the groups that are different from each other, "a" and "b".

In order to check if the glycemic response was related to the basal glycemic status of the volunteer, we compared, for each volunteer and tested drink, the changes observed (glucose level at day 60 – glucose initial level at day 0) with the glucose initial level at day 0. As can be seen in Figures 32-34, the three test drinks behave the same manner.

There was an inverse correlation (mainly observed with Stevia drink) between the changes in glucose levels and the basal glucose at day 0. That means that volunteers with larger baseline values were less susceptible to the effect of the drink (their changes in glucose were smaller, and even in some cases the final value was smaller than the initial one).

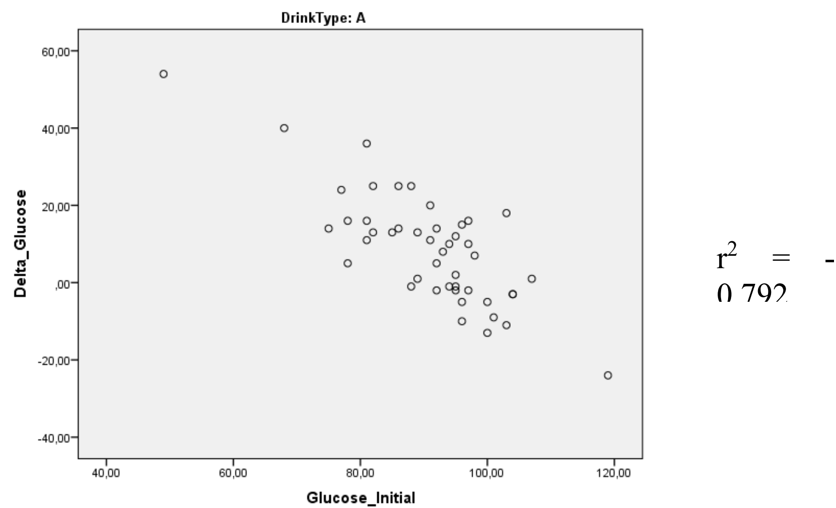


Figure 32. Correlation of delta and initial glucose level in drink A (Stevia)

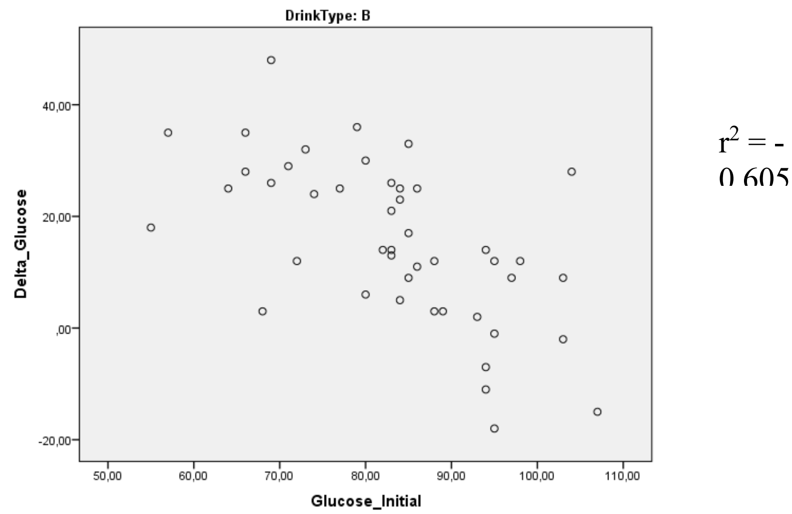


Figure 33. Correlation of delta and initial glucose level in drink B (Sucralose)

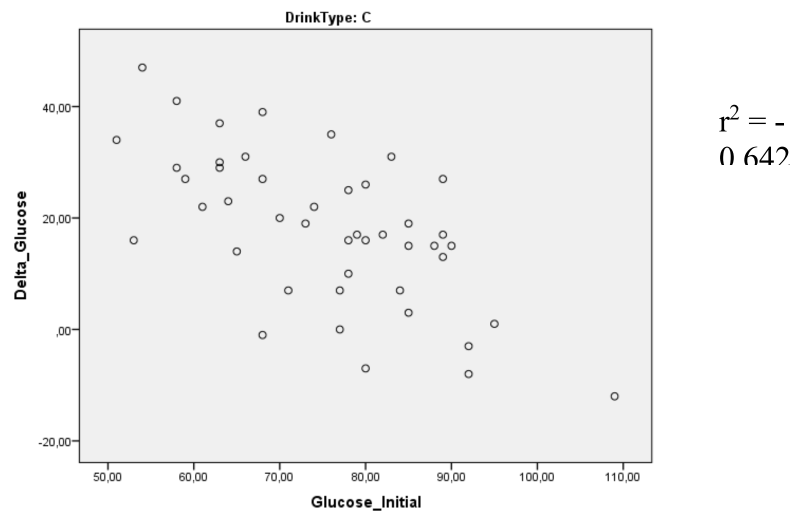


Figure 34. Correlation of delta and initial glucose level in drink C (Sucrose)

4.10. LIPID PROFILE

HDL levels increased significantly with Sucralose ($p=0.039$), and Sucrose (non-significant), whilst they decreased with Stevia (non-significant). No

significant changes were observed with LDL concentrations in any of the tested drinks. Total cholesterol levels increased significantly with Sucrose (p=0.001). Triglycerides did not vary with any beverage (Table 16).

4.11. INFLAMMATION MARKERS

In case of inflammatory markers, they didn't follow a normal distribution. Hence, for TNF-α, IL-10, C-Reactive-Protein and IL-6, nonparametric statistics were performed. The levels of TNF-α were, in all samples analyzed and in all treatments and times, close to the limit of quantification of 1.66 pg/mL. Therefore, we didn't perform statistical analysis on those data.

There was a significant increase, up to 4-fold, in IL-10 after the intake of drink added with Stevia, in the samples where it was detected (not all volunteer) (p=0.021). IL-10 also increased with Sucrose (non-significant) while Sucralose decreased its levels (non-significant) (Table 17). In the case of IL-6, there were changes within each group but not significant, but the different behavior of the groups showed significant differences between them, since IL-6 in the case of Stevia slightly increased, whilst in Sucralose and Sucrose it decreased.

C-reactive protein increased significantly with all treatments, mainly with Sucralose (29% p = 0.023), as well as with Stevia (18% p=0.007) and Sucrose (23% p = 0.031), without differences between them.

Table 16: Biochemical parameters: Lipid profile

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 0	Day 0	Day 60
HDL (mg/dL)	60.6 ± 13.7	58.3 ± 12.0	61.8 ± 15.8	67.1 ± 18.0 *	70.0±20.2	75.7 ± 24.3
LDL (mg/dL)	115.4 ± 20.2	115.4 ± 24.5	100.8 ± 26.4	101.3 ± 25.2	78.5 ± 29.7	78.9 ± 33.1
Total cholesterol (mg/dL)	192.4 ± 19.3	189.2 ± 27.0 [a]	180.5 ± 28.2	187.0 ± 24.8 [b]	166.8 ± 22.8	172.3± 21.4 * [a,b]
Triglycerides (mg/dL)	82.2 ± 31.3	77.8 ± 32.4	89.4 ± 28.1	92.9 ± 31.6	91.5 ± 25.7	89.0 ± 27.8

Data are expressed as mean ± SD

Significant differences before and after are indicated by an asterisk *.

The letters indicate the groups that are different from each other, "a" and "b".

Table 17: Inflammatory profile

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=45)		Sucrose Beverage C (n=45)	
	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
IL-10 (pg/mL)	0.206 (0.019; 0.473)	0.811 (0.126; 1.496) * [a]	0.320 (0.138; 0.502)	0.164 (0.001; 0.362) [b]	0.191 (0.001; 0.383)	0.210 (0.001; 0.423) [b]
IL-6 (pg/mL)	1.010 (0.565; 1.456)	1.925 (1.250; 2.599) [a]	1.172 (0.924; 1.419)	0.579 (0.326; 0.831) [b]	1.541 (1.057; 2.025)	1.107 (0.414; 1.801) [a,b]
C-reactive protein (mg/dL)	3.51 (1.59; 5.42)	4.64 (2.52; 6.76) *	3.30 (2.04; 4.56)	4.04 (2.39; 5.69) *	2.56 (2.43; 2.68)	2.54 (2.44; 2.65) *

Data are expressed as mean (interquartile range)

Significant differences before and after are indicated by an asterisk *.

The letters indicate the groups that are different from each other, "a" and "b".

4.12. SATIETY HORMONES

Regarding hormones related to energy metabolism, there was a significant decrease in leptin levels after the intake of the three drinks, which was of 9 % with Stevia ($p=0.039$) and 11 % with Sucralose ($p=0.001$). No significant changes were observed in ghrelin concentrations (Table 18).

Table 18: Satiety hormones

	Stevia Beverage A (n=46)		Sucralose Beverage B (n=46)		Sucrose Beverage C (n=46)	
	Day 0	Day 60	Day 0	Day 0	Day 60	Day 0
Leptin (pg/mL)	503.94 ± 380.27	440.37 ± 313.68 *	466.69 ± 297.27	392.34 ± 246.94 *	687.35 ± 198.66	673.58 ± 235.13
Ghrelin (pg/mL)	867.96 ± 473.29	915.63 ± 447.44	1023.51 ± 400.20	999.12 ± 339.16	971.48 ± 504.28	1087.62 ± 612.62

Data are expressed as mean ± SD
Significant differences before and after are indicated by an asterisk *.
The letters indicate the groups that are different from each other, "a" and "b".

V - DISCUSSION

V - DISCUSSION

We performed a parallel, randomized and triple blind clinical study with three different beverages made with the same base of *Citrus* and maqui extracts, added with different sweeteners. Hepatic enzymes (transaminases) and total bilirubin levels showed that the drinks were safe and they did not produce any hepatotoxic effect. In the following subsections, the changes observed and the possible mechanisms involved are discussed with the recent literature.

5.1. ADHERENCE TO MEDITERRANEAN DIET

The adherence to Mediterranean diet was evaluated by means of a questionnaire previously validated (Abellán et al., 2016).

The term 'Mediterranean diet' (MD) is representing the dietary habits of populations living near the Mediterranean Sea (de Lorgeril 2013), including Greece, Italy, Spain and Lebanon and hence based on geography, history, tradition and nationality, diet varies (Trichopulou, et al., 2014). Traditional MD is generally characterized by high consumption of vegetables, fruits and nuts, legumes, unprocessed cereal, low consumption of meat and meat products and moderate wine consumption.

MD has been known for a long time as optimal diet in preserving good health and preventing non-communicable diseases (Martínez-González et al., 2009). Epidemiological and clinical studies suggest an inverse correlation between MD and prevention or treatment of metabolic syndrome (Finicelli et al., 2019). Lower risk of metabolic syndrome has been shown in a cohort population

that strictly adhered to MD (Tortosa et al, 2007). ATTICA and PREDIMED studies have described the protective effect of the adherence to MD in Greek and Spanish populations, respectively (Panagiotakos et al., 2004; Chiva-Blanch and Badimon, 2017).

In a study by Agnoli et.al, (2018), in a long term (five years) assessment of basic anthropometric measurements with Mediterranean diet adherence, they found a reduction of weight in healthy weight subjects, but not in over-weights. Besides, the diet reduced the risk of becoming over-weight, suggesting that Mediterranean diet could prevent weight gain, but not helping weight reduction in obese subjects. Dietary Mediterranean pattern in our current study on over-weight Spanish adults, showed that the majority of participants needed to abide to their Mediterranean diet since the score in almost 50% were less than 7 (10% less than five, 40% between 5-7). We observed a significant relationship between gender and higher consumption of MD, with more adherence of women (mean value 7.4 vs 6.2).

5.2. KNOWLEDGE AND USE OF SWEETNERS

There are very few studies reporting the knowledge and use of sweeteners by population. In a cross-sectional study by Sadiq and Salih in Iraq (2018) on 440 diabetes patients, they showed that 79% had good knowledge in the FDA approved tablet sweeteners and consumed 5-10 tablets of different sweeteners per day. In contrast, an insufficient knowledge about Non-Nutritive-Sweeteners (NNS) was reported among University students by Wilson (2019). Similarly, in a cross-sectional study on type two diabetes patients, Nayakan and Ritwik (2018) showed awareness about the knowledge of how long they should

be consumed, sweeteners' types and content, and their health benefits and hazards.

In the current study, the knowledge about type, content and effect of NNS were not assessed, but we assessed the consumption of sweetened food products. The most frequent consumption of sweeteners were in the form of yoghurt and drinks and the least popular were in chewing gums, marmalade, pastry, biscuits, and desserts.

5.3. ACCEPTABILITY OF THE TEST DRINKS BY VOLUNTEERS

The drinks containing Stevia and Sucralose were moderately appreciated by the volunteers of the current study but the drinks with Sucrose were the most popular, above the other two. All three drinks were highly ranked as slightly acidic in taste. In our knowledge there was no other recent studies that evaluate the taste popularity of drinks made with the same food matrix and added with different sweeteners.

5.4. EFFECT ON ANTHROPOMETRIC AND CARDIOVASCULAR MARKERS

There are epidemiological and clinical data on the effect of different sweeteners on anthropometric parameters, as previously described in Introduction Section. An epidemiological study performed about the intake of sugar-sweetened beverages and physical activity levels in adolescents (Bremer et al., 2009) showed that these products were each independently associated with anthropometric measurements (waist circumference and BMI) as well as insulin resistance (HOMA-IR) and associated metabolic parameters (HDL, LDL, TG).

Prospective cohorts on LCS beverages in adults also reported increases in BMI, fat mass and weight (Fowler et al., 2008), or only body weight (Nettleton et al., 2009).

Njike et al., (2011) found the same result with the intake of sugar-sweetened hot cocoa for six months on 52 year old adults. Moreover, they reported an increase in Body Mass Index (BMI) and waist circumference. In two different years (2007 and 2010) Reid et al. performed a study with sugar sweetened beverages (aspartame and acesulfame K -sweetened soda) on adults of 20-55 years, and reported higher BMI and body weight respectively after four weeks consumption of the drinks.

We didn't observe high relevant changes in the anthropometric parameters in our clinical trial. The participants who consumed Sucralose and Sucrose treatments increased significantly their weight, but just a 1 %. The changes on weight were not followed by a change in BMI. The decrease of fat mass percentage was of more relevance, being of 8 % with Stevia. Considering the different results found on the changes in anthropometric parameters, both in the literature as well as in our study, it might be necessary larger randomized clinical trials with longer duration as well as the use of other anthropometric markers to confirm the long term effect of these sweeteners. It is possible that changes on these markers require longer periods to be stable and of relevance.

Besides, some randomized studies have been performed in overweight populations that followed diet restrictions that could have minimized the negative impact of the exposure to LCS. Diet prescription or increase in physical exercise might protect from the adverse effects observed in some observational studies (Serra-Majem et al, 2018).

The systolic blood pressure decreased by participants who were consuming Stevia drink and diastolic blood pressure was reduced by Sucrose drink. Scarce information is available on the effect of sweeteners on blood pressure. Baird et al., (2000) found no change in electrocardiogram pattern of healthy subjects after consumption of sucralose. In another study in northern Turkey of 156 hypertensive participants, blood pressure was controlled by consumption of lemon juice (Adibelli et al., 2009). It should be considered that individual measurements of blood pressure on the days of the intervention may be influenced by the moment (before blood sampling) and a better approach for the assessment of vascular status (although it was not in the principal aims of our study) might be the continuous measurement of blood pressure 24 h with by Holter-monitoring.

5.5. EFFECT ON ANTIOXIDANT STATUS

5.5.1. ORAC

Studies on antioxidant activity of maqui and *Citrus* fruits have shown that they possess high potency both in vitro and in vivo, and some of them are summarized in Introduction Section. Miranda-Rottman et al. (2002), showed that *Aristotelia chilensis* is more successful for total radical-trapping potential in comparison with other berries. Concerning clinical trials, in a four week, double-blind placebo controlled clinical study with supplements of maqui berry extract, it resulted a significant reduction in oxidative stress markers, including oxidized LDL and urinary 8-iso-PGF2α levels (Davinelli et al., 2015). Bioactive compounds from *Citrus* fruits, as naringenin, have been isolated and tested as nutraceuticals

in several clinical trials (Salehi et al., 2019). No studies up to date have been reported on the combination of both maqui and *Citrus* extracts on in vivo antioxidant activity, and much less if they contain low-caloric sweeteners.

In our study, there was a non-significant increase in ORAC values after the ingestion of the fruit beverages added with Stevia and Sucralose and a decrease with Sucrose treatment. The non-significance may be due to the inter-individual variability observed in ORAC values (as can be pointed out by the standard deviations, although all of them followed normal distribution).

The higher increases in ORAC values were observed in people with lower basal values, irrespectively of the tested drink. This effect could be in line with a homeostatic approach, in which the exogenous antioxidant source helps to endogenous antioxidant system to maintain the redox status within its normal limits; hence the benefits of an antioxidant food product might be more relevant in people with worse health conditions (Serafini et al., 2006). A similar difference in the grade of antioxidant response was observed after consumption of orange juice for 8 weeks, being more positive in individuals with higher weight (Dourado and Cesar, 2015).

5.5.2. Homocysteine

Elevated levels of homocysteine have been set as an independent risk factor for severe cardiovascular events, including thrombosis, myocardial infarction or stroke (Hortin, 2006; Sachder, 2004). Other diseases in which homocysteine levels are higher than normal limits include dementia and Alzheimer, and it has been related to a higher prevalence of chronic heart disease,

higher incidence of bone fracture, pre-eclampsia and neural tube defects during pregnancy (Selhub, 2008).

Decrease in homocysteine level was seen in metabolic syndrome patients by six month citrus-based juice consumption (Mulero, et al., 2012) . Similar reductions were observed with maqui extract supplements in a double blind randomized clinical trial, placebo-controlled design on 42 healthy, overweight smokers of 45-65 years of age (Davinelli et al., 2015). In a cross-over study performed on 26 healthy subjects, with the consumption of two capsules of fruits and two of vegetable powder per day for one month, followed by one month as control phase, the homocysteine level significantly decreased (Panunzio et al., 2003).

In contrast, in our current study there was a significant increase in homocysteine levels after the 60-day treatment with beverages added of Sucralose or Sucrose, but not with Stevia. To our knowledge, no human studies have been performed on the effect of these sweeteners on this marker of oxidative stress. The increase might be related to the increases observed in glucose levels (see next subsection). In fact, some authors have found positive correlations between high levels of homocysteine and high fasting glucose levels and insulin resistance, in obese patients (Vayá et al., 2012), but further studies are needed to confirm this association.

5.5.3. Oxidized LDL

The biological action of the oxidized LDL as atherogenic factor is mediated via its receptors on smooth muscle cells, monocytes and macrophages (Li and Mehta, 2005). In the early steps of atherosclerosis, a crucial step is the

migration of monocytes and T-lymphocytes into the vascular intima layer. The oxidized LDL plays a role in the activation of these cells and it works as an antigen, causing a secretion of cytokines by T-cells, that further activate the macrophages and alter the endothelium. Hence, it leads to monocyte involvement into the vessel wall, resulting in the dysfunction of vascular endothelial cells by generation of free radicals (Cominacini et al., 2000). Endothelial cells also secrets inflammatory cytokines as TNF-α, perpetuating the process (Rafian-Kopaie et al., 2014).

Polyphenol rich beverages have shown to reduce levels of oxidized LDL. Fifteen patients with coronary artery disease with fourteen days of ingestion of 7 mL/kg/day of purple grape juice showed reduction of LDL susceptibility to oxidation (Stein et al., 1999). Similar results were observed by Naissides et al., (2005) in hyper-cholesterolemic postmenopausal women with red wine supplementation. In contrast with the above, the LDL oxidation was not modified by neither the consumption of red wine (De Rijke et al., 1996) nor the consumption or onions and green tea (O'Reilly et al., 2001). Moreover, Zern et al., (2005) determined that the intake of lyophilized grape powder by postmenopausal women during 4 weeks did not reduce the LDL oxidation, even if plasma triglycerides, plasma LDL-cholesterol and apolipoproteins B and E concentrations are lowered by the treatment. It has been hypothesized that the effect of polyphenols on LDL oxidation may vary depending on their structure, the type of natural diet they were originated from and the dose used (Curin and Andrianntsitohaina, 2005). In the current study, the effect of the drinks added with Stevia and Sucralose on the reduction of LDL-oxidized was not significant, maybe due to the inter-individual variability. In contrast, significant decreases in LDL-oxidized level were seen in patients with metabolic syndrome by six-month

citrus-based juice consumption (Mulero, et al., 2012). It is possible that our intervention was not long enough to produce significant changes on the oxidative grade of this apolipoprotein, or that our volunteer did not show a high initial level of LDL oxidation, as they were not patients with metabolic syndrome, and hence they were less susceptible to decrease this marker by the treatment.

5.6. EFFECT ON ENERGY METABOLISM: GLYCEMIC AND LIPID PROFILE

5.6.1. Lipid profile

Concerning the effects on lipid profile, some improvements, with decreases in plasma total cholesterol and LDL cholesterol, were reported in metabolic syndrome patients by six-month consumption of a citrus-based juice(300 ml/day) (Mulero, et al., 2012). The results of lipid profile in our current study were not significant, may be due to small number of participants in each group, though HDL and total cholesterol level decreased with Stevia drink and increased with Sucralose and Sucrose drinks. The effects on lipid profile are not conclusive and need to be confirmed in other clinical trials with larger sample size.

5.6.2. Glycemic profile

Results from prospective cohort studies have suggested inverse associations between the intake of total flavonoids and specific flavonoid subclasses and the risk of T2D (Guasch-Ferré, 2017, Zamora, et al., 2013, 2014; Ding et al., 2016).

It has been observed, in acute studies, that the consumption of maqui

berry significantly and dose dependently decreased glycaemia one hour after glucose intake, compared to control (Alvarado et al., 2016a). The same result concluded by Hidalgo et al (2014), 30 minutes after and oral glucose test performed on pre-diabetic volunteers. Long-term studies have also demonstrated a better glucose and lipid profile after three-month consumption of maqui berry extracts (Alvarado et al., 2016b).

We observed a significantly higher fasting blood glucose levels with all three drinks, and higher insulin resistance (expressed as HOMA-IR index) with Sucralose and Sucrose. It seems that the positive effect of glycemic control due to the flavonoids from the maqui-citrus extracts is counteracted by the presence of the different sweeteners.

The results from our clinical trial are in line with the effects observed in epidemiological observational studies. In an early study of Yoshida et al., (2007), sugar-sweetened drink consumption, assessed by food-frequency questionnaire in adult population, was significantly correlated with fasting insulin and not with fasting glucose, while the contrary was observed with fruit juice consumption. The authors conclude that sugar-sweetened drink consumption might unfavorably affect markers related with hepatic insulin sensitivity.

In a retrospective study on a subgroup of middle-aged adults of Framingham Heart Study, the regular SSB intake was associated with a greater increase in insulin resistance and a higher risk of developing prediabetes (Ma et.al, 2016). Mean values of HOMA-IR were also higher in adolescents consuming sugar-sweetened beverages (SSB) more than 5 times/week compared with those consuming less frequently, although a statistically significant difference was

detected between those consuming SSB 5–6 times/week and 2–4 times/week (Kondaki et al., 2013).

Other observational studies that have associated the consumption of artificial sweetened beverages (ASB) with metabolic syndrome and type two diabetes include: ARIC follow up study of nine years in U.S.A (Atherosclerosis Risk In Communities) (Lutsey et al., 2008), MESA (Multi Ethnic Study of Atherosclerosis) (Nethleton et al., 2009), HPFS (the Health Professional Follow up Study) (de Koning et al., 2011), EPIC (the European Prospective Investigation into Cancer and nutrition study) (The InterAct consortium 2013) and the Epidemiologic study of French female teacher (E3N in EPIC study) (Fagherazzi et al., 2013). Similar results were observed in the EPIC Norfolk study in UK (O'Connor et al., 2015). In the SAHS (The Saint Antonio Heart Study), the consumption of ASB was associated with obesity (Fowler et al., 2008). Duffey et al. (2012), in a 20-years follow up study, showed that the non-consumers of diet beverages had lower risk of developing metabolic syndrome compared to consumers. In contrast, in BWHS (Black Women's Health Study) they found no relationship between the intakes of one or more diet soft drink per day and the risk of type two diabetes (Palmet et al., 2008). Over a seven years period determination of *diabetes mellitus* incidence in Japanese men, the consumption of diet soda, though it is zero-calorie drink, was significantly associated with an increased risk for diabetes (Sakurai, et al., 2014).

These findings from observational studies are in favor of the negative outcomes of our current study, with higher levels of HOMA-IR observed with sucrose drink.

In contrast, results from clinical trials with sweeteners give controversial results. In cross-over studies with healthy obese subjects, the consumption of

sucralose (Wu et al., 2012, Brown et al., 2011) or aspartame (Bryant et al, 2014; Maersk et al., 2012 a) had no additional effect on glucose and insulin levels, when administered concomitant to an oral glucose test or a breakfast meal. Similarly, the sucralose consumption (667 mg/day) for 13 weeks in type-2 diabetes subjects had no effect on glucose and insulin (Grotz et al., 2003).

Previously, Ma et al., (2009) had performed intra-gastric infusions of water solutions of sucralose (800 mg) and demonstrated that sucralose didn’t stimulate insulin release.

In contrast, Temizkan et al., (2015), showed lower glucose AUC with sucralose when ingested together with carbohydrates, compared to control, in healthy subjects. Pepino et al., (2013) and Suez et al., (2014) showed blood glucose increments after seven days exposure to sucralose and saccharine respectively. Besides, in the first one it was accompanied by decrease in insulin sensitivity and increase in insulin concentration. This in line with the results of current study, in which the participants experienced a significant increase in fasting glucose in all three groups, HOMA-IR level increments in the groups of sucralose and sucrose, showing higher insulin resistance.

Concerning stevia intake, in a cross-over study of 18-49 years old population, lower glucose and insulin concentrations were reported with stevia compared to sucrose (Anton et al., 2010).

Gregersen et al., (2004) in a clinical trial of 12 subjects with T2DM and BMI 25-32 kg/m^2 and HbA1C less than 10%, demonstrated that stevioside reduced the glycemic response and increased insulinogenic index by 40%, with no effects on insulin levels, while in another study by Barriocanal et al., (2008) steviol glycosides made no changes in glucose, insulin and HbA1C in healthy subjects.

The controversy of the results of the different clinical trials seems to be due to inconsistencies in type of studied population, type of drinks including drink mixtures, duration of study, sample size, timing and the number of times for blood collection, different analytical methods for biomarkers determination and different geographical areas, all of which should be considered. The investigation with prolonged exposure, considering different aspects of dietary, anthropometric and individual differences is required to better understanding of the hormonal effects in the context of human consumption (Rother et al., 2018; Romo-Romo et al, 2016).

A plausible mechanism for the metabolic activity of LCS includes activation of sweet taste receptors in oral and extra-oral tissues (e.g., intestine, pancreatic β cells, and brain), what is linked to stimulation of insulin and incretin secretion. Other mechanisms are the altered nutrient sensing by the brain, as well as alterations of the gut microbiome (Rother et al., 2018). All of them may have a synergistic effect.

The sweet perception of low-caloric sweeteners is due to their activation of sweet taste receptor (STR). It is a heterodimer of the class C, G-protein coupled receptor family and comprises two subunits, T1R2, and T1R3 (Nelson et.al, 2001). It has been described the presence of STRs not only in tongue and oral mucosal areas but also on enteroendocrine cells in the proximal small intestine, including K-cells, L-cells and enterochromaffin cells in humans (Jang et.al, 2007, Young et.al, 2013).

The role of these STRs has been majorly described in animal models. The sweet-taste receptors have been linked to the increase of glucose absorption, by means of augmenting the function of the sodium-dependent glucose co-

transporter (SGLT-1) (Kreuch et.al, 2018). Mace et al., (2007) showed that sweet-taste receptors stimulate glucose absorption through activation of GLUT-2 transporter in small intestine in rats, increase glucose absorption by signal to a functional taste reception system within few minutes, being in the order of potency Acesulfame potassium > Sucralose. They showed that brush cells might participate in sugar sensing by a mechanism analogous to the taste buds and their receptors (T2R bitter and T11 sweet taste). Low-caloric sweeteners are able to upregulate GLUT-2 as well as SGLT-1 expression and function (Stearns et al., 2010, Moran et al., 2010). The dysregulation of intestinal sweet taste receptors can exacerbate postprandial glycemic excursions, mainly in type 2 diabetes. Furthermore, given that this type of patients are 3-fold more likely to consume beverages sweetened with LCS than healthy individuals (Mackenzie et.al 2006), it is possible that high dietary LCS consumption contributes to, rather than alleviates, postprandial glycemic dysregulation.

The activation of STR in gastrointestinal tract leads to carbohydrate absorption, together with an increase in the secretion of incretin hormones (Tuckar and Tan, 2017). Incretin hormones, as Glucagon like Peptide (GLP-1), accelerate glucose-dependent insulin secretion. Low caloric sweeteners, by interacting with small intestine sweet taste receptors, are able to induce the secretion of GLP-1. This effect has been proved in in vivo studies, as that of Wölnerhanssen et al., (2016), on a study on ten lean and ten obese volunteers provided with 75 g glucose, 50 g xylitol and 75 g Erythritol in 300 ml water or just water for placebo group both by naso-gastric tubes. Both sweeteners were able to increase GLP-1 levels. Similarly, though with different sweetener, Temizkan et al., (2015) showed significant GLP-1 increase by sucralose consumption compared to placebo (water) group. Brown et al., (2009), on 22 healthy young

volunteers, they consumed soda or carbonated water ten minutes before an oral load glucose test (OGTT). They showed an increase of GLP-1 in diet soda consumers, suggesting a possible stimulation of sweet-taste receptors on L-cells by artificial sweeteners.

In vitro studies with stevia main glycoside, the rebaudioside-A proved an increased secretion of GLP-1 on small intestine of mice (Van der Wielen et al., 2016).

Another possible mechanism that might explain the effects of sweeteners on energy metabolism is the alterations of the gut microbiome. Though yet there are scarce information available by human studies.

The oral microbiome possesses the capacity to metabolize sugars, with differences between types of sugars (Clemens, et al., 2016). Passing through the gastrointestinal tract, LCS directly encounter the intestinal microbiota involving in profound multiple physiology process of regulation. It is said that these non-nutritive sweeteners alter the composition of gut microbiota with vast microorganisms responsible for digesting foods, releasing metabolites and synthesizing vitamins. There is myriad of controversies in this area.

In one study by Frankenfeld et al., (2015), they showed no effect on the gut composition in a dietary high intensity sweetener. Volunteer completed a four-day food record on consumption of aspartame or acesulfame-K or both. Their fecal samples were collected and no differences were observed on bacterial abundance, but on overall bacterial diversity.

Sucralose has low oral bioavailability (around 15%) and it is excreted largely in unchanged form in feces; the low percentage absorbed suffer type 2 glucuronidation reactions and further renal excretion (Schiffman et al., 2013).

Hence, the exposure of the non-absorbed sucralose to the gut microbiota may occur.

In an eight week study on diet-induced obese male Sprague Dawley rats, the low dose of aspartame (5-7 mg/kg body w/day) showed elevation of short chain fatty acid as bacterial end product, which could be related to negative effects on insulin tolerance (Palmnäs et al., 2014). Similarly, Bian et al. (2017), on C57BL/6 male mice proved that the intake of sucralose in their drinking water for six months lead to disruption in fecal metabolites and alteration in amino acid derivatives, bile acids and proinflammatory gene expression in liver.

On the other hand, Daly et al., (2014) proved an induction of beneficial imbalance on microbiota composition, by showing an increase in the number of *Lactobacillus* in intestinal microbiota of piglets with saccharine and neohesperidin dihydrochalcone, rather than with lactose or natural sugar.

Suez et al, (2014) published an interesting study on *Nature*, about the effect on microbiota of the addition of saccharine to the drinking water in mice, for 5 weeks, observing that the consumption of saccharin was related to higher production of short chain fatty acids and an increase in *Bacteroidetes* filum and a decrease of *Firmicutes* filum. This was related to an impaired glucose tolerance. Faecal transplantation from mice consuming saccharine to normal diet mice showed the replication of the glucose phenotype intolerance. These results in animal models were also reproduced in a human clinical study with healthy individuals, which, after consumption of 5 mg saccharin/day for 7 days, showed poorer glycemic responses. They showed dysbiosis in microbiota as well

Figure 35 summarizes the factors related to sweeteners that influence glycemic control, including the stimulus of STR, the effect on glucose transport, the release of incretin hormones and the action on gut microbiome. All these

factors are necessary to be considered to adequately interpret the *in vivo* effects of LCS (Kreuch et al., 2018).

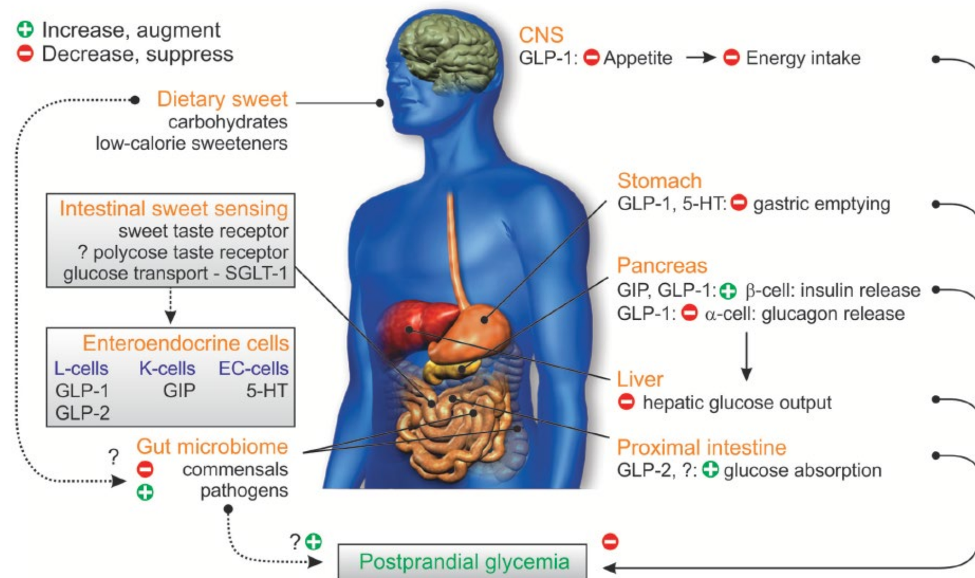


Figure 35: Gastrointestinal factors that influence glycemic control (Kreuch et al., 2018)

5.7. EFFECT ON INFLAMMATORY STATUS

Overweight people, especially those with an important percentage of body fat mass, show an inflammatory status higher than lean subjects. Festa et al., (2000) showed a significant relationship between CRP and fibrinogen level with BMI, waist circumference, and adipose body mass, in 1559 both healthy and diabetes subjects (mostly in women) from three ethnic population of non-Hispanic white, African-American, and Mexican-American, suggesting chronic, subclinical non-infectious inflammation as the pathophysiological mechanism for higher risk of atherosclerosis. Having higher regional fat distribution in 197 End

stage Renal Disease (ESRD) patients of almost 52 years of age, before their dialysis session, with higher IL-6 and higher CRP, Axelsson et al., (2004) proved with these findings of the relationship between fat mass (especially visceral and abdominal fat) and non-infectious inflammation.

Likewise, Fontana et al., (2007) showed that levels of IL-6 were fifty percent higher from normal ranges in 25 extremely obese subjects who were candidate for gastric bypass surgery, and there was a significant association with CRP level, proving the relationship of visceral fat and abdominal obesity with systematic inflammation. Similarly, in 295 patients with moderate to severe Chronic Obstructive Pulmonary Disease (COPD), positive correlations were found between CRP levels and abdominal obesity (Rutten et al., 2010).

You et al., (2004), in a study on 58 women of around 59 years of age with BMI average of 33 kg/m^2, showed that the group with higher visceral fat who had metabolic syndrome had higher level of TNF-α Receptor 1 (TNFR-1), confirming the inflammatory effect of fat deposition. On the other hand, in a different study by Maffeis et al, (2007) on 28 children (13 boys and 15 girls) with different degrees of overweight, the adipocyte diameter was significantly associated with IL-6 and TNF-α and insulin sensitivity rather than fat mass and BMI.

This basal chronic low-grade inflammatory status is linked to the development of atherosclerosis and pathologies as cardiovascular diseases, insulin resistance or metabolic disruptions, as well as neurological disorders. Hence, an interesting approach is to reduce their inflammatory status by food products with proven anti-inflammatory activities.

Matching NHANS (National Health and Nutrition examination Survey) food consumption with USDA flavonol database, in US adults the CRP levels showed inverse association with dietary flavonoids (Chun et al., 2008).

In vitro and cell culture studies have demonstrated the ability of maqui berry extracts to block inflammation processes, as well as the development of oxidative stress and insulin resistance (Reyes-Farias, et al., 2016). *Citrus* fruit juices have demonstrated anti-inflammatory actions, as well. Anthocyanins inhibit the secretion of inflammatory cytokines such as IL-8 or MCP1 in neutrophil chemotactic cytokine-induced and IL-6 in cells and animal models after an inflammation stimulus (Davinelli, 2015; Rojo et al., 2012). Concerning clinical trials, a decrease in CRP level was seen in metabolic syndrome patients by six month citrus-based juice consumption (Mulero, et al., 2012). Similar reductions in CRP, as well as stimulation of immune response with IL-12 increase was observed after orange juice consumption in normal and overweight subjects (Dourado and Cesar, 2015).

However, the effects reported by other authors are in contrast with ours, as the inflammatory C-reactive protein increased significantly in the participants of all three drinks. In the current study these metabolically healthy but overweight participants presented non-favorable inflammatory profile by showing significant increase in CRP after consumption of all three drinks as well as an increase in homocysteine levels with drinks added with sucralose and sucrose. The presence of the sweetener may counteract the anti-inflammatory effect associated to the flavonoids present in the fruit extracts. Of notice is our finding of a significant increase in IL-10 level with Stevia drink, non-significant changes with the other drinks. IL-10 is known as anti-inflammatory cytokine and its release may counteract the inflammation response observed with the drinks, although specific studies are needed to confirm this hypothesis.

5.8. EFFECT ON SATIETY HORMONES

Satiety may be defined as the suppression of ongoing eating (Geraedts et al., 2011). It is a complex process affected by both qualities of the food ingested as well as the physiological conditions of the subject (as gender or age).

Generally to maintain energy homeostasis, sweet taste evoke numerous central and peripheral physiological responses, by signaling the arrival of nutrients in the gut and by facilitating the absorption and utilization of energy contained in food (Smeets et al., 2010). Sweet taste associated to a nutritive compound includes an insulin response and the storage of blood glucose in tissue.

Artificial sweeteners are able to activate the same signaling pathways of satiety-hunger nervous centers. However, since there is no a parallel increase in sugar absorption, there would be hypoglycemia, activation of satiety-hunger nervous centers and hence an increase in the food intake (Swithers and Davidson 2008, Hampton 2008).

It seems that LCS that provide sweet taste without providing energy may confound the regulatory mechanisms of satiety and hunger in the brain. This effect has been proved in animal studies. The uncoupling of sweet taste with caloric load, by LCS, disrupt the animal's ability to respond appropriately to sweet tasting foods, as the conditioning principle of Pavlov (Fowler 2016, Swithers 2008). Rats that received dietary supplements together with LCS exhibited higher weight gain compared to those that received the same diets with sucrose or glucose, following Pavlovian principles (Davidson et al., 2011, 2014). In a study on rats by Polyák et al. (2010), with the maximum amount of

saccharine, cyclamate, acesulfame-K and aspartame intake, it showed significantly increased body weight, though no changes in food intake.

Mitsutomi et al., (2014) showed that sucrose supplementation increased significantly hyperglycemia and leptin in white adipose tissue in mice, opposed to the control and LCS groups. In another study by Cong et al., (2013), forty weeks exposure to Acesulfame-K in normal mice altered the levels of fasting insulin and leptin.

Concerning human studies, Anton et al., (2010) in a cross over study in population of 18-49 years old showed that energy intake did not increase with LCS consumption and no effects were found on appetite parameters.

On the other hand, preload experiments generally have found that sweet taste either by sugar or artificial sweeteners enhance human appetite (Liem and de Graaf 2004, Yang 2010). Green et al., (2012) explained altered reward processing of sweet tastes in young adults by sucrose (nutritive sweetener) and saccharine (non-nutritive sweetener). Regular diet soda drinkers showed greater activation of brain regions involved with food rewards than non-drinkers, measured by neuroimaging scanning.

The consumption of artificial sweeteners could exacerbate the negative effects of sugars by altering the cognitive processes that lead to overconsumption. Some authors have pointed out that LCS could be perceived as "healthy" grants, which permit to over consume "non-healthy" foods, with the consequence of an increase in energy intake (Swithers, 2013).

On the other hand, no difference in appetite or food taking has been reported by consumption of sucralose by Ford et al., (2011) and Wu et al., (2012). Brown et al., (2011, 2012) in two consecutive years in two cross-over studies on healthy subjects with sucralose consumption showed no differences in glucose,

insulin, hunger, ghrelin and triglycerides and no differences in glucose and C peptide.

Leptin levels are correlated with adipose tissues and are decreased during periods of starvation. This satiety hormone regulates the appetite and energy intake, by activation of vagus nerve and hypothalamus centers to estimulate the anorexigenic neurons and decrease food ingestion (Bruen et al, 2012). In our current study, we observed a significant lower level of leptin after consumption of drinks containing Stevia and Sucralose. Body fat mass was also significantly decreased with Stevia treatment. Similar results were observed by Beck et al., (2002) on consumption of aspartame for 14 weeks, in which plasma leptin level significantly reduced parallel to the body weight and the fat mass.

Though yet, the association between LCS consumption with development of metabolic disease and changes in hormones which regulate appetite is not clear and further studies are needed to confirm the association.

VI - CONCLUSIONS

VI CONCLUSIONS

1. The dietary and lifestyle habits of our population of overweight subjects indicate that 50 % of participants have an average adherence to Mediterranean Diet, which could be improved with some changes in the diet, showing the need of education on dietary habits to increase this adherence.

2. The intake of a beverage made with Citrus and maqui extracts and added with stevia showed a decrease in weight and body fat percentage, as expected from a natural non-caloric sweetener. Surprisingly, there was an increase in body weight with the consumption of Sucralose sweetened beverage, a non-caloric artificial sweetener, and also a more predictable increase with Sucrose treatment, what highlights the implication of pathways of energy saving other than just caloric intake.

3. Beverages made with Stevia and Sucralose as sweeteners had a non-significant improvement in antioxidant status, with increases in ORAC values and decreases in the level of LDL-oxidized. Sucrose addition negatively affected the antioxidant status, as it significantly increase homocysteine levels, in a higher degree than Sucralose.

4. The consumption of all beverages increased the insulin resistance, with a rise on fasting glucose levels not followed by a parallel increase in insulin, being more significant with Sucralose and Sucrose, possibly reflecting the

activation of sweet sensing receptors and their signaling pathways involved in glycemic control.

5. Changes on apoliprotein profile didn't follow a clear pattern and no changes were observed in triglycerides levels, with any of the tested drinks.

6. Regardless of type of drinks, inflammatory state worsened with the intake of the three drinks, as shown by the increase in C-reactive protein levels. The anti-inflammatory interleukin IL-10 also raised after Stevia-added drink consumption, possibly as a response reaction against this inflammatory insult, but further studies are needed to confirm this aspect.

7. A decrease in long-term satiety hormones as leptin was observed with the intake of the three drinks, mainly with Stevia and Sucralose. This change is no clearly correlated with the changes on fat mass with both sweeteners and further studies are needed.

VII. STRENGTHS AND WEAKNESSES OF THE STUDY – GENERALIZABILITY AND FUTURE DIRECTION

VII –STRENGHTS AND WEAKNESSES OF THE STUDY – GENERALIZABILITY AND FUTURE DIRECTION

To our knowledge this is the first study to examine the effect of a polyphenols-rich beverages added with different sweeteners on a combination of health factors in humans. Comparisons of different sweeteners added to the same food matrix with the doses commonly used by food industry have not been performed up to date.

There are many systematic reviews and meta-analyses concerning the effects of sweeteners on health, but few clinical intervention studies, and those performed just measure few parameters or present failures. Several failures are attributed to clinical trials conducted with sweeteners: small sample size, cross-over studies conducted with short resting periods that may have a residual effect or carry-over between treatments, short duration of treatment. We randomized the intervention groups stratified by sex, gender and BMI, to avoid biased results. We performed a parallel study so that the drinks were taken in the same period of year, avoiding changes in food habits with the seasons of the year. Sample size was calculated by the Statistical Unit of UCAM, so that to detect the significant effects, non- biased. The fact of knowing that a sweetener is being taken that is perceived as "healthy", can make the volunteer allow himself to consume more of other unhealthy foods. This is not our case because our study was performed as triple blind, which involved a major logistical task.

Concerning the weaknesses of the study, assessing inflammation markers in human samples is challenging due to their unstable nature. On the other hand,

the anthropometric parameters were assessed with multiple measurements (Body Mass Index (BMI), average weight, body fat percentage, etc.). We adjusted the groups by BMI but we didn't control covariates such as the grade of abdominal adiposity that could influence the results obtained. The level of adiposity can be a confounding factor.

One limitation of the study could be the exposure time that might be longer to detect higher variations in some markers. However, the increases on inflammatory conditions observed don't recommend this line.

Generalizability and Future direction

Obesity is the main reason of non-communicable diseases such as diabetes and CVD, showing the necessity of identifying the strategies that helps regulate body weight by reducing energy intake, considering preserving the palatability of beverages and foods with lower calories.

An interesting option is the combination of maqui extract with lemon juice, which has proven high antioxidant activity, for the design of new drinks with a nutritive related function on health for chronic disease.

Since Murcia is a center of citrus fruit cultivation in Europe, lemon is widely available. Moreover, the project can bring the opportunity of technology transfer to the beverage industries in the region. Besides both fruits selected are of high nutritional value including vitamin C, citric acid, minerals, and flavonoids (flavanones and flavones).

The results obtained in our trial link the consumption of low-caloric sweeteners with metabolic abnormalities, mainly insulin resistance and pro-inflammatory conditions, hence calling for a reassessment in a larger study.

Intervention trials of larger sample size and longer duration are recommended. The role of gut microbiome must be properly assessed as it can help to clarify the effects observed.

VIII - REFERENCES

VIII – REFERENCES

Abad-García, B., Berrueta, L. A., Garmón-Lobato, S., Urkaregi, A., Gallo, B., & Vicente, F. (2012). Chemometric characterization of fruit juices from Spanish cultivars according to their phenolic compound contents: I. citrus fruits. *Journal of Agricultural and Food Chemistry*, *60*(14), 3635-3644.

Abellán Alemán, J., Zafrilla Rentero, M. P., Montoro-García, S., Mulero, J., Pérez Garrido, A., Leal, M., ... & Ruilope, L. M. (2016). Adherence to the "Mediterranean diet" in Spain and its relationship with cardiovascular risk (DIMERICA study). *Nutrients*, *8*(11), 680.

Abou-Donia, M. B., El-Masry, E. M., Abdel-Rahman, A. A., McLendon, R. E., & Schiffman, S. S. (2008). Splenda alters gut microflora and increases intestinal p-glycoprotein and cytochrome p-450 in male rats. *Journal of Toxicology and Environmental Health, Part A*, *71*(21), 1415-1429.

Acosta, J. A., Faz, A., Jansen, B., Kalbitz, K., & Martínez-Martínez, S. (2011). Assessment of salinity status in intensively cultivated soils under semiarid climate, Murcia, SE Spain. *Journal of Arid Environments*, 75, 1056-1066.

Adibelli, Z., Dilek, M., & Akpolat, T. (2009). Lemon juice as an alternative therapy in hypertension in Turkey. *International Journal of Cardiology*, *135*(2), e58-e59.

Addor, F. A. S. A., Vieira, J. C., & Melo, C. S. A. (2018). Improvement of dermal parameters in aged skin after oral use of a nutrient supplement. *Clinical, cosmetic and investigational dermatology*, 11, 195.

Agnoli, C., Sieri, S., Ricceri, F., Giraudo, M. T., Masala, G., Assedi, M., ... & Krogh, V. (2018). Adherence to a Mediterranean diet and long-term changes in weight and waist circumference in the EPIC-Italy cohort. *Nutrition & Diabetes*, 8(1), 1-10.

Agulló, V., Domínguez-Perles, R., Moreno, D. A., Zafrilla, P., & García-Viguera, C. (2020a). Alternative Sweeteners Modify the Urinary Excretion of Flavanones Metabolites Ingested through a New Maqui-Berry Beverage. *Foods*, *9*(1), 41.

Agulló, V., Villaño, D., García-Viguera, C., & Domínguez-Perles, R. (2020b). Anthocyanin Metabolites in Human Urine after the Intake of New Functional Beverages. *Molecules*, *25*(2), 371.

Al-Juhaimi, F. Y., & Ghafoor, K. A. S. H. I. F. (2013). Bioactive compounds, antioxidant and physico-chemical properties of juice from lemon, mandarin and orange fruits cultivated in Saudi Arabia. *Pak. J. Bot*, *45*(4), 1193-1196.

Allain, C. C., Poon, L. S., Chan, C. S., Richmond, W. F. P. C., & Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, *20*(4), 470-475.

Alvarado, J. L., Leschot, A., Olivera-Nappa, Á., Salgado, A. M., Rioseco, H., Lyon, C., & Vigil, P. (2016 a). Delphinidin-rich maqui berry extract (Delphinol®) lowers fasting and postprandial glycemia and insulinemia in prediabetic individuals during oral glucose tolerance tests. *BioMed Research International*, *2016*.

Alvarado, J., Schoenlau, F., Leschot, A., Salgad, A. M., & Portales, P. V. (2016 b). Delphinol (R) standardized maqui berry extract significantly lowers blood glucose and improves blood lipid profile in prediabetic individuals in three-month clinical trial. *Panminerva Med*, *58*(3 Suppl 1), 1-6.

Anton, S. D., Martin, C. K., Han, H., Coulon, S., Cefalu, W. T., Geiselman, P., & Williamson, D. A. (2010). Effects of stevia, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. *Appetite*, 55(1), 37-43.

Araneda, X., Quilamán, E., Martínez, M., & Morales, D. (2014). Elaboración y evaluación de jugo de maqui (Aristotelia chilensis (Mol.) Stuntz) por arrastre de vapor. *Scientia Agropecuaria*, *5*(3), 149-156.

Araos, J. P. (2015). Aristotelia chilensis: a possible nutraceutical or functional food. *Medicinal Chemistry*, *5*, 378-382.

Arts, I. C., & Hollman, P. C. (2005). Polyphenols and disease risk in epidemiologic studies. *The American Journal of Clinical Nutrition*, *81*(1), 317S-325S.

Aruoma, O. I., Landes, B., Ramful-Baboolall, D., Bourdon, E., Neergheen-Bhujun, V., Wagner, K. H., & Bahorun, T. (2012). Functional benefits of citrus fruits in the management of diabetes. *Preventive Medicine*, 54, S12-S16.

Assy, N., Nasser, G., Kamayse, I., Nseir, W., Beniashvili, Z., Djibre, A., & Grosovski, M. (2008). Soft drink consumption linked with fatty liver in the absence of traditional risk factors. *Canadian Journal of Gastroenterology and Hepatology*, *22*(10), 811-816.

Atalay, M., Gordillo, G., Roy, S., Rovin, B., Bagchi, D., Bagchi, M., & Sen, C. K. (2003). Anti-angiogenic property of edible berry in a model of hemangioma. *Febs Letters*, *544*(1-3), 252-257.

Axelsson, J., Rashid Qureshi, A., Suliman, M. E., Honda, H., Pecoits-Filho, R., Heimbürger, O., ... & Stenvinkel, P. (2004). Truncal fat mass as a contributor to inflammation in end-stage renal disease. *The American Journal of Clinical Nutrition*, 80(5), 1222-1229.

Azad, M. B., Abou-Setta, A. M., Chauhan, B. F., Rabbani, R., Lys, J., Copstein, L., ... & MacKay, D. S. (2017). Nonnutritive sweeteners and cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials and prospective cohort studies. *CMAJ*, *189*(28), E929-E939.

Bahorun, T., Ramful-Baboolall, D., Neergheen-Bhujun, V., Aruoma, O., Kumar, A., Verma, S., Tarnus, E., Silva, C., Rondeau, P., & Bourdon, E. (2012). Phytophenolic Nutrients in Citrus: Biochemical and Molecular Evidence. In A. K. Srivastava (Ed.), *Advances in Citrus Nutrition*, (pp. 25-40): Springer Netherlands.

Baird, I. M., Shephard, N. W., Merritt, R. J., & Hildick-Smith, G. (2000). Repeated dose study of sucralose tolerance in human subjects. *Food and Chemical Toxicology*, *38*, 123-129.

Bantle, J. P., Laine, D. C., & Thomas, J. W. (1986). Metabolic effects of dietary fructose and sucrose in types I and II diabetic subjects. *Jama*, *256*(23), 3241-3246.

Barriocanal, L. A., Palacios, M., Benitez, G., Benitez, S., Jimenez, J. T., Jimenez, N., & Rojas, V. (2008). Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and in Type 1 and Type 2 diabetics. *Regulatory Toxicology and Pharmacology*, 51(1), 37-41.

Bellisle, F., & Drewnowski, A. (2007). Intense sweeteners, energy intake and the control of body weight. *European Journal of Clinical Nutrition*, *61*(6), 691-700.

Benavente-García, O., & Castillo, J. (2008). Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti - inflammatory activity. *Journal of Agricultural and Food Chemistry*, 56, (15), 6185-6205.

Beck, B., Burlet, A., Max, J. P., & Stricker-Krongrad, A. (2002). Effects of long-term ingestion of aspartame on hypothalamic neuropeptide Y, plasma leptin and body weight gain and composition. *Physiology & Behavior*, 75(1-2), 41-47.

Berkey, C. S., Rockett, H. R., Field, A. E., Gillman, M. W., & Colditz, G. A. (2004). Sugar-added beverages and adolescent weight change. *Obesity Research*, *12*(5), 778-788.

Bes-Rastrollo, M., Sánchez-Villegas, A., Gómez-Gracia, E., Martínez, J. A., Pajares, R. M., & Martínez-González, M. A. (2006). Predictors of weight gain in a Mediterranean cohort: the Seguimiento Universidad de Navarra Study. *The American Journal of Clinical Nutrition*, *83*(2), 362-370.

Bian, X., Chi, L., Gao, B., Tu, P., Ru, H., & Lu, K. (2017). Gut microbiome response to sucralose and its potential role in inducing liver inflammation in mice. *Frontiers in Physiology*, 8, 487.

Blum, J. W., Jacobsen, D. J., & Donnelly, J. E. (2005). Beverage consumption patterns in elementary school aged children across a two-year period. *Journal of the American College of Nutrition*, 24(2), 93-98.

Brauch JE, Reuter L, Conrad J, Vogel H, et.al. (2017). Characterization of anthocyanins in novel Chilean maqui berry clones-by HPLC-DADA-ESI/MS and NMR. *Journal of Food Composition and Analysis*, 58, 16-22.

Bray, G. A., & Popkin, B. M. (2014). Dietary sugar and body weight: have we reached a crisis in the epidemic of obesity and diabetes?: health be damned! Pour on the sugar. *Diabetes Care*, *37*(4), 950-956.

Bremer, A. A., Auinger, P., & Byrd, R. S. (2009). Relationship between insulin resistance–associated metabolic parameters and anthropometric measurements

with sugar-sweetened beverage intake and physical activity levels in US adolescents: findings from the 1999-2004 National Health and nutrition examination survey. *Archives of Pediatrics & Adolescent Medicine*, *163*(4), 328-335.

Brisbois, T. D., Marsden, S. L., Anderson, G. H., & Sievenpiper, J. L. (2014). Estimated intakes and sources of total and added sugars in the Canadian diet. *Nutrients*, *6*(5), 1899-1912.

Brown, R. J., Walter, M., & Rother, K. I. (2009). Ingestion of diet soda before a glucose load augments glucagon-like peptide-1 secretion. *Diabetes Care*, 32(12), 2184-2186.

Brown, R. J., De Banate, M. A., & Rother, K. I. (2010). Artificial sweeteners: a systematic review of metabolic effects in youth. *International Journal of Pediatric Obesity*, *5*(4), 305-312.

Brown, A. W., Brown, M. M. B., Onken, K. L., & Beitz, D. C. (2011). Short-term consumption of sucralose, a nonnutritive sweetener, is similar to water with regard to select markers of hunger signaling and short-term glucose homeostasis in women. *Nutrition Research*, 31(12), 882-888.

Brown, R. J., & Rother, K. I. (2012). Non-nutritive sweeteners and their role in the gastrointestinal tract. *The Journal of Clinical Endocrinology & Metabolism*, 97(8), 2597-2605.

Bruen, C. M., O'Halloran, F., Cashman, K. D., & Giblin, L. (2012). The effects of food components on hormonal signalling in gastrointestinal enteroendocrine cells. *Food & Function*, 3(11), 1131-1143.

Bryant, C. E., Wasse, L. K., Astbury, N., Nandra, G., & McLaughlin, J. T. (2014). Non-nutritive sweeteners: no class effect on the glycaemic or appetite responses to ingested glucose. *European Journal of Clinical Nutrition*, 68(5), 629-631.

Bucolo, G., & David, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clinical Chemistry*, *19*(5), 476-482.

Butchko, H. H., Stargel, W. W., Comer, C. P., Mayhew, D. A., Benninger, C., Blackburn, G. L., ... & Leon, A. S. (2002). Aspartame: review of safety. *Regulatory Toxicology and Pharmacology*, 35(2), S1-S93.

Caballero, B. (2012). Dietary Sucrose and Disease. *Encyclopedia of Human Nutrition*, 231.

Calzada-León, R., Ruiz-Reyes, M. D. L. L., Altamirano-Bustamante, N., & Padrón-Martínez, M. M. (2013). Características de los edulcorantes no calóricos y su uso en niños. *Acta Pediátrica de México*, *34*(3), 141-153.

Céspedes, C. L., El-Hafidi, M., Pavon, N., & Alarcon, J. (2008). Antioxidant and cardioprotective activities of phenolic extracts from fruits of Chilean blackberry Aristotelia chilensis (Elaeocarpaceae), Maqui. *Food Chemistry*, *107*(2), 820-829.

Céspedes, C. L., Alarcon, J., Valdez-Morales, M., & Paredes-López, O. (2009). Antioxidant activity of an unusual 3-hydroxyindole derivative isolated from fruits of Aristotelia chilensis (Molina) Stuntz. *Zeitschrift für Naturforschung C*, *64*(9-10), 759-762.

Céspedes, C. L., Alarcon, J., Avila, J. G., & Nieto, A. (2010 a). Anti-inflammatory activity of Aristotelia chilensis mol. (Stuntz)(elaeocarpaceae). *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, *9*(2), 127-135.

Céspedes, C. L., Valdez-Morales, M., Avila, J. G., El-Hafidi, M., Alarcón, J., & Paredes-López, O. (2010 b). Phytochemical profile and the antioxidant activity of Chilean wild black-berry fruits, Aristotelia chilensis (Mol) Stuntz (Elaeocarpaceae). *Food Chemistry*, *119*(3), 886-895.

Cespedes, Carlos L., et al. (2017). The chilean superfruit black-berry Aristotelia chilensis (Elaeocarpaceae), Maqui as mediator in inflammation-associated disorders. *Food and Chemical Toxicology,* 108, 438-450.

Céspedes-Acuña, C. L., Xiao, J., Wei, Z. J., Chen, L., Bastias, J. M., Avila, J. G., ... & Kubo, I. (2018). Antioxidant and anti-inflammatory effects of extracts from Maqui berry Aristotelia chilensis in human colon cancer cells. *Journal of Berry Research*, *8*(4), 275-296.

Chattopadhyay, S., Raychaudhuri, U., & Chakraborty, R. (2014). Artificial sweeteners–a review. *Journal of Food Science and Technology*, *51*(4), 611-621.

Chiva-Blanch, G., & Badimon, L. (2017). Effects of polyphenol intake on metabolic syndrome: current evidences from human trials. *Oxidative Medicine and Cellular Longevity*, *2017*.

Chun, O. K., Chung, S. J., Claycombe, K. J., & Song, W. O. (2008). Serum C-reactive protein concentrations are inversely associated with dietary flavonoid intake in US adults. *The Journal of Nutrition*, 138(4), 753-760.

Cicerale, S., Lucas, L., & Keast, R. (2010). Biological activities of phenolic compounds present in virgin olive oil. *International Journal of Molecular Sciences*, *11*(2), 458-479.

Clarke, M. A., & Edye, L. A. (1996). Sugar beet and sugarcane as renewable resources.

Clemens, R. A., Jones, J. M., Kern, M., Lee, S. Y., Mayhew, E. J., Slavin, J. L., & Zivanovic, S. (2016). Functionality of sugars in foods and health. *Comprehensive Reviews in Food Science and Food Safety*, *15*(3), 433-470.

Cominacini, L., Pasini, A. F., Garbin, U., Davoli, A., Tosetti, M. L., Campagnola, M., ... & Sawamura, T. (2000). Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-κB through an increased production of intracellular reactive oxygen species. *Journal of Biological Chemistry*, *275*(17), 12633-12638.

Cong, W. N., Wang, R., Cai, H., Daimon, C. M., Scheibye-Knudsen, M., Bohr, V. A., ... & Maudsley, S. (2013). Long-term artificial sweetener acesulfame potassium treatment alters neurometabolic functions in C57BL/6J mice. *PLoS One*, 8(8).

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: Chemistry, bioavailability and effects on health. Natural Product Reports, 26, 1001-1043.

Curin, Y., & Andriantsitohaina, R. (2005). Polyphenols as potential therapeutical agents against cardiovascular diseases. *Pharmacological Reports*, *57*, 97.

Daly, K., Darby, A. C., Hall, N., Nau, A., Bravo, D., & Shirazi-Beechey, S. P. (2014). Dietary supplementation with lactose or artificial sweetener enhances swine gut Lactobacillus population abundance. *British Journal of Nutrition*, 111(S1), S30-S35.

Damascos, M. A., Arribere, M., Svriz, M., & Bran, D. (2008). Fruit mineral contents of six wild species of the North Andean Patagonia, Argentina. *Biological Trace Element Research*, *125*(1), 72-80.

Davidson, T. L., Martin, A. A., Clark, K., & Swithers, S. E. (2011). Intake of high-intensity sweeteners alters the ability of sweet taste to signal caloric consequences: implications for the learned control of energy and body weight regulation. *The Quarterly Journal of Experimental Psychology*, 64(7), 1430-1441.

Davidson, T. L., Sample, C. H., & Swithers, S. E. (2014). An application of Pavlovian principles to the problems of obesity and cognitive decline. *Neurobiology of Learning and Memory*, 108, 172-184.

Davinelli, S., Bertoglio, J. C., Zarrelli, A., Pina, R., & Scapagnini, G. (2015). A randomized clinical trial evaluating the efficacy of an anthocyanin–maqui berry extract (Delphinol®) on oxidative stress biomarkers. *Journal of the American College of Nutrition*, *34*(sup1), 28-33.

Davy, B. M., Harrell, K., Stewart, J., & King, D. S. (2004). Body weight status, dietary habits, and physical activity levels of middle school-aged children in rural Mississippi. *Southern Medical Journal*, *97*(6), 571-578.

De Ferrars, R. M., Czank, C., Zhang, Q., Botting, N. P., Kroon, P. A., Cassidy, A., & Kay, C. D. (2014). The pharmacokinetics of anthocyanins and their metabolites in humans. *British Journal of Pharmacology*, *171*(13), 3268-3282.

De Koning, L., Malik, V. S., Rimm, E. B., Willett, W. C., & Hu, F. B. (2011). Sugar-sweetened and artificially sweetened beverage consumption and risk of type 2 diabetes in men. *The American Journal of Clinical Nutrition*, *93*(6), 1321-1327.

de Lorgeril, M. (2013). Mediterranean diet and cardiovascular disease: historical perspective and latest evidence. *Current Atherosclerosis Reports*, *15*(12), 370.

de Matos Feijó, F., Ballard, C. R., Foletto, K. C., Batista, B. A. M., Neves, A. M., Ribeiro, M. F. M., & Bertoluci, M. C. (2013). Saccharin and aspartame, compared with sucrose, induce greater weight gain in adult Wistar rats, at similar total caloric intake levels. *Appetite*, *60*, 203-207.

de Rijke, Y. B., Demacker, P. N., Assen, N. A., Sloots, L. M., Katan, M. B., & Stalenhoef, A. F. (1996). Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *The American Journal of Clinical Nutrition*, *63*(3), 329-334.

Del Rio, D., Costa, L. G., Lean, M. E. J., & Crozier, A. (2010). Polyphenols and health: what compounds are involved?. *Nutrition, Metabolism and Cardiovascular Diseases*, *20*(1), 1-6.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*, *18*(14), 1818-1892.

Devaux, M. G. S., Goryakin, Y., Cecchini, M., Huber, H., & Colombo, F. (2017). OECD Obesity Update 2017.

Dhingra, R., Sullivan, L., Jacques, P. F., Wang, T. J., Fox, C. S., Meigs, J. B., ... & Vasan, R. S. (2007). Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. *Circulation*, *116*(5), 480-488.

Ding, M., Pan, A., Manson, J. E., Willett, W. C., Malik, V., Rosner, B., ... & Sun, Q. (2016). Consumption of soy foods and isoflavones and risk of type 2 diabetes: a pooled analysis of three US cohorts. *European Journal of Clinical Nutrition*, *70*(12), 1381-1387.

Dou, C., Xia, D., Zhang, L., Chen, X., Flores, P., Datta, A., & Yuan, C. (2005). Development of a novel enzymatic cycling assay for total homocysteine. *Clinical Chemistry*, *51*(10), 1987-1989.

Dourado, G. K., & Cesar, T. B. (2015). Investigation of cytokines, oxidative stress, metabolic, and inflammatory biomarkers after orange juice consumption by normal and overweight subjects. *Food & Nutrition Research*, *59*(1), 28147.

Duffey, K. J., Steffen, L. M., Van Horn, L., Jacobs Jr, D. R., & Popkin, B. M. (2012). Dietary patterns matter: diet beverages and cardiometabolic risks in the longitudinal Coronary Artery Risk Development in Young Adults (CARDIA) Study. *The American Journal of Clinical Nutrition*, *95*(4), 909-915.

Ebbeling, C. B., Feldman, H. A., Osganian, S. K., Chomitz, V. R., Ellenbogen, S. J., & Ludwig, D. S. (2006). Effects of decreasing sugar-sweetened beverage consumption on body weight in adolescents: a randomized, controlled pilot study. *Pediatrics*, 117(3), 673-680.

EFSA. (2010). Scientific Opinion on the substantiation of health claims related to various food(s)/food constituent(s) and protection of cells from premature aging, antioxidant activity, antioxidant content and antioxidant properties, and protection of DNA, proteins and lipids from oxidative damage pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal*, 8, 1489.

EFSA Panel on Food Additives and Flavourings (FAF), Younes, M., Aquilina, G., Engel, K. H., Fowler, P., Frutos Fernandez, M. J., ... & Mennes, W. (2019 a). Safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive: Rebaudioside M produced via enzyme-catalysed bioconversion of purified stevia leaf extract. *EFSA Journal*, 17(10), e05867.

EFSA Panel on Food Additives and Flavourings (FAF), Younes, M., Aquilina, G., Engel, K. H., Fowler, P., Frutos Fernandez, M. J., ... & Mennes, W. (2019 b). Safety of use of Monk fruit extract as a food additive in different food categories. *EFSA Journal*, 17(12), e05921.

Ervin, R. B., & Ogden, C. L. (2013). Consumption of added sugars among US adults, 2005-2010 (No. 122). US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics.

Escribano-Bailón, M. T., Alcalde-Eon, C., Muñoz, O., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (2006). Anthocyanins in berries of maqui [Aristotelia chilensis (Mol.) Stuntz]. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *17*(1), 8-14.

Fagherazzi, G., Vilier, A., Saes Sartorelli, D., Lajous, M., Balkau, B., & Clavel-Chapelon, F. (2013). Consumption of artificially and sugar-sweetened beverages and incident type 2 diabetes in the Etude Epidémiologique auprès des femmes de la Mutuelle Générale de l'Education Nationale–European Prospective Investigation into Cancer and Nutrition cohort. *The American Journal of Clinical Nutrition*, *97*(3), 517-523.

Fang J. (2014). Bioavailability of anthocyanins. *Drug Metabolism Reviews*, 46 (4): 508-520.

Faria, A., Pestana, D., Azevedo, J., Martel, F., de Freitas, V., Azevedo, I., ... & Calhau, C. (2009). Absorption of anthocyanins through intestinal epithelial cells–Putative involvement of GLUT2. *Molecular Nutrition & Food Research*, *53*(11), 1430-1437.

Feinman, R. D., & Fine, E. J. (2013). Fructose in perspective. *Nutrition & Metabolism*, 10(1), 45.

Festa, A., D'Agostino Jr, R., Howard, G., Mykkanen, L., Tracy, R. P., & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Stud (IRAS). *Circulation*, 102(1), 42-47.

Figlewicz, D. P., Ioannou, G., Jay, J. B., Kittleson, S., Savard, C., & Roth, C. L. (2009). Effect of moderate intake of sweeteners on metabolic health in the rat. *Physiology & Behavior*, 98(5), 618-624.

Finicelli, M., Squillaro, T., Di Cristo, F., Di Salle, A., Melone, M. A. B., Galderisi, U., & Peluso, G. (2019). Metabolic syndrome, Mediterranean diet, and polyphenols: Evidence and perspectives. *Journal of Cellular Physiology*, 234(5), 5807-5826.

Fitch, C., & Keim, K. S. (2012). Position of the Academy of Nutrition and Dietetics: use of nutritive and nonnutritive sweeteners. *Journal of the Academy of Nutrition and Dietetics*, *112*(5), 739-758.

Food and Nutrition Board. (2013). Dietary Reference Intakes (DRIs): Recommended Intakes for Individuals, Vitamins. Institute of Medicine, National Academies.

Fontana, L., Eagon, J. C., Trujillo, M. E., Scherer, P. E., & Klein, S. (2007). Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes*, 56(4), 1010-1013.

Ford, H. E., Peters, V., Martin, N. M., Sleeth, M. L., Ghatei, M. A., Frost, G. S., & Bloom, S. R. (2011). Effects of oral ingestion of sucralose on gut hormone response

and appetite in healthy normal-weight subjects. *European Journal of Clinical Nutrition*, 65(4), 508-513.

Fossati, P., & Prencipe, L. (1982). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clinical Chemistry*, *28*(10), 2077-2080.

Fowler, S. P., Williams, K., Resendez, R. G., Hunt, K. J., Hazuda, H. P., & Stern, M. P. (2008). Fueling the obesity epidemic? Artificially sweetened beverage use and long-term weight gain. *Obesity*, *16*(8), 1894-1900.

Fowler, S. P. (2016). Low-calorie sweetener use and energy balance: Results from experimental studies in animals, and large-scale prospective studies in humans. *Physiology & Behavior*, 164, 517-523.

Frankenfeld, C. L., Sikaroodi, M., Lamb, E., Shoemaker, S., & Gillevet, P. M. (2015). High-intensity sweetener consumption and gut microbiome content and predicted gene function in a cross-sectional study of adults in the United States. *Annals of Epidemiology*, 25(10), 736-742.

Fredes, C., Montenegro, G., Zoffoli, J. P., Gómez, M., & Robert, P. (2012). Polyphenol content and antioxidant activity of maqui (Aristotelia chilensis Molina Stuntz) during fruit development and maturation in Central Chile. *Chilean Journal of Agricultural Research*, *72*, 582-589.

Fredes, C., Yousef, G. G., Robert, P., Grace, M. H., Lila, M. A., Gómez, M., ... & Montenegro, G. (2014). Anthocyanin profiling of wild maqui berries (Aristotelia chilensis [Mol.] Stuntz) from different geographical regions in Chile. *Journal of the Science of Food and Agriculture*, *94*(13), 2639-2648.

Fredes, C., Osorio, M. J., Parada, J., & Robert, P. (2018). Stability and bioaccessibility of anthocyanins from maqui (Aristotelia chilensis [Mol.] Stuntz) juice microparticles. *LWT*, *91*, 549-556.

Fuentealba, J., Dibarrart, A., Saez-Orellana, F., Fuentes-Fuentes, M. C., Oyanedel, C. N., Guzmán, J., ... & Aguayo, L. G. (2012). Synaptic silencing and plasma membrane dyshomeostasis induced by amyloid-β peptide are prevented by Aristotelia chilensis enriched extract. *Journal of Alzheimer's Disease*, *31*(4), 879-889.

Fuentes, O., Fuentes, M., Badilla, S., & Troncoso, F. (2013). Maqui (Aristotelia chilensis) and rutin (quercetin-3-O-rutinoside) protects against the functional impairment of the endothelium-dependent vasorelaxation caused by a reduction of nitric oxide availability in diabetes. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, *12*(3), 220-229.

García, E. C., & Solís, I. M. (2016). *Manual de Fitoterapia*. 2nd Ed. Elsevier Doyma.

Gardner, C., Wylie-Rosett, J., Gidding, S. S., Steffen, L. M., Johnson, R. K., Reader, D., & Lichtenstein, A. H. (2012). Nonnutritive sweeteners: current use and health perspectives: a scientific statement from the American Heart Association and the American Diabetes Association. *Circulation*, *126*(4), 509-519.

Genskowsky, E., Puente, L. A., Pérez-Álvarez, J. A., Fernández-López, J., Muñoz, L. A., & Viuda-Martos, M. (2016). Determination of polyphenolic profile, antioxidant activity and antibacterial properties of maqui [Aristotelia chilensi s (Molina) Stuntz] a Chilean blackberry. *Journal of the Science of Food and Agriculture*, *96*(12), 4235-4242.

Geraedts, M. C. P., Troost, F. J., & Saris, W. H. M. (2011). Gastrointestinal targets to modulate satiety and food intake. *Obesity Reviews*, 12(6), 470-477.

Giammattei, J., Blix, G., Marshak, H. H., Wollitzer, A. O., & Pettitt, D. J. (2003). Television watching and soft drink consumption: associations with obesity in 11- to 13-year-old schoolchildren. *Archives of Pediatrics & Adolescent Medicine*, *157*(9), 882-886.

Gironés-Vilaplana, A., Mena, P., García-Viguera, C., & Moreno, D. A. (2012 a). A novel beverage rich in antioxidant phenolics: Maqui berry (Aristotelia chilensis) and lemon juice. *LWT*, *47*(2), 279-286.

Gironés-Vilaplana, A., Valentão, P., Moreno, D. A., Ferreres, F., García-Viguera, C., & Andrade, P. B. (2012 b). New beverages of lemon juice enriched with the exotic berries maqui, açaı, and blackthorn: Bioactive components and in vitro biological properties. *Journal of Agricultural and Food Chemistry*, *60*(26), 6571-6580.

Godshall, M. A. (2007). Sugar and other sweeteners. In *Kent and Riegel's Handbook of Industrial Chemistry and Biotechnology* (pp. 1657-1693). Springer, Boston, MA.

González-Castejón, M., & Rodriguez-Casado, A. (2011). Dietary phytochemicals and their potential effects on obesity: a review. *Pharmacological Research*, *64*(5), 438-455.

González-Molina, E., Domínguez-Perles, R., Moreno, D. A., & García-Viguera, C. (2010). Natural bioactive compounds of Citrus limon for food and health. *Journal of Pharmaceutical and Biomedical Analysis*, 51, 327-345.

Green, E., & Murphy, C. (2012). Altered processing of sweet taste in the brain of diet soda drinkers. *Physiology & Behavior*, 107(4), 560-567.

Gregersen, S., Jeppesen, P. B., Holst, J. J., & Hermansen, K. (2004). Antihyperglycemic effects of stevioside in type 2 diabetic subjects. *Metabolism*, 53(1), 73-76.

Grice, H. C., & Goldsmith, L. A. (2000). Sucralose-an overview of the toxicity data. *Food and Chemical Toxicology*, 38(suppl 2).

Gross, L. S., Li, L., Ford, E. S., & Liu, S. (2004). Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes in the United States: an ecologic assessment. *The American Journal of Clinical Nutrition*, 79(5), 774-779.

Grotz, V. L., Henry, R. R., McGill, J. B., Prince, M. J., Shamoon, H., Trout, J. R., & Pi-Sunyer, F. X. (2003). Lack of effect of sucralose on glucose homeostasis in subjects with type 2 diabetes. *Journal of the American Dietetic Association*, 103(12), 1607-1612.

Guerrero, J., Ciampi, L., Castilla, A., Medel, F., Schalchli, H., Hormazabal, E., ... & Alberdi, M. (2010). Antioxidant capacity, anthocyanins, and total phenols of wild and cultivated berries in Chile. *Chilean Journal of Agricultural Research*, *70*(4), 537-544.

Guasch-Ferré, M., Merino, J., Sun, Q., Fitó, M., & Salas-Salvadó, J. (2017). Dietary polyphenols, Mediterranean diet, prediabetes, and Type 2 diabetes: A narrative review of the evidence. *Oxidative Medicine and Cellular Longevity*, *2017*.

Hampton, T. (2008). Sugar substitutes linked to weight gain. *Jama*, 299(18), 2137-2138.

Hanekamp, J. C., Bast, A., & Calabrese, E. J. (2015). Nutrition and health–transforming research traditions. *Critical Reviews in Food Science and Nutrition*, 55(8), 1074-1080.

Heiss, C., Keen, C. L., & Kelm, M. (2010). Flavanols and cardiovascular disease prevention. *European Heart Journal*, *31*(21), 2583-2592.

Henkel, J. (1999). Sugar substitutes: Americans opt for sweetness and lite. *FDA Consumer Magazine*, *33*(6), 12-6.

Hidalgo, J., Flores, C., Hidalgo, M. A., Perez, M., Yanez, A., Quinones, L., ... & Burgos, R. A. (2014). Delphinol® standardized maqui berry extract reduces postprandial blood glucose increase in individuals with impaired glucose regulation by novel mechanism of sodium glucose cotransporter inhibition. *Panminerva Med*, *56*(2 Suppl 3), 1-7.

Hitoe, S., Tanaka, J., & Shimoda, H. (2014). MaquiBright™ standardized maqui berry extract significantly increases tear fluid production and ameliorates dry eye-related symptoms in a clinical pilot trial. *Panminerva medica*, 56(3 Suppl 1), 1-6.

Hortin, G. L. (2006). Homocysteine: Clinical significance and laboratory measurement. *Laboratory Medicine*, 37(9), 551-553.

Hou, D. X., Yanagita, T., Uto, T., Masuzaki, S., & Fujii, M. (2005). Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: structure–activity relationship and molecular mechanisms involved. *Biochemical Pharmacology*, *70*(3), 417-425.

InterAct Consortium. (2013). Consumption of sweet beverages and type 2 diabetes incidence in European adults: results from EPIC-InterAct. *Diabetologia*, 56(7), 1520-1530.

Jang, H. J., Kokrashvili, Z., Theodorakis, M. J., Carlson, O. D., Kim, B. J., Zhou, J., ... & Bernier, M. (2007). Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. *Proceedings of the National Academy of Sciences*, 104(38), 15069-15074.

Jara, E., Hidalgo, J., Flores, C., Pérez, M., Yáñez, A., Hidalgo, A., ... & Burgos, R. (2012). Estudio de un extracto estandarizado de maqui rico en delfinidinas en el mantenimiento del balance de glucosa. *Revista de Farmacología de Chile*, *5*(2), 27-39.

Jayaprakasam, B., Vareed, S. K., Olson, L. K., & Nair, M. G. (2005). Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. *Journal of Agricultural and Food Chemistry*, *53*(1), 28-31.

Johnson, R. K., Appel, L. J., Brands, M., Howard, B. V., Lefevre, M., Lustig, R. H., ... & Wylie-Rosett, J. (2009). Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association. *Circulation*, *120*(11), 1011-1020.

Joint, F. A. O., WHO Expert Committee on Food Additives, & World Health Organization. (2009). Evaluation of certain food additives and contaminants: sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization

Kahn, R., & Sievenpiper, J. L. (2014). Dietary sugar and body weight: have we reached a crisis in the epidemic of obesity and diabetes?: we have, but the pox on sugar is overwrought and overworked. *Diabetes Care*, *37*(4), 957-962.

Kassi, E., Landis, G., Pavlaki, A., Lambrou, G., Mantzou, A., Androulakis, I., ... & Chrousos, G. (2016, May). Long-term effects of Stevia rebaduiana on glucose and lipid profile, adipocytokines, markers of inflammation and oxidation status in patients with metabolic syndrome. In 18th European Congress of Endocrinology (Vol. 41). BioScientifica.

Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H., & Yano, M. (2004). Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiology*, 134(2), 824-837.

Kaushik, R., Narayanan, P., Vasudevan, V., Muthukumaran, G., & Usha, A. (2010). Nutrient composition of cultivated stevia leaves and the influence of polyphenols and plant pigments on sensory and antioxidant properties of leaf extracts. *Journal of Food Science and Technology*, *47*(1), 27-33.

Kvaavik, E., Andersen, L. F., & Klepp, K. I. (2005). The stability of soft drinks intake from adolescence to adult age and the association between long-term

consumption of soft drinks and lifestyle factors and body weight. *Public Health Nutrition*, 8(2), 149-157.

Kawasaki, T., Akanuma, H., & Yamanouchi, T. (2002). Increased fructose concentrations in blood and urine in patients with diabetes. *Diabetes Care*, *25*(2), 353-357.

Kay, C. D., Pereira-Caro, G., Ludwig, I. A., Clifford, M. N., & Crozier, A. (2017). Anthocyanins and flavanones are more bioavailable than previously perceived: A review of recent evidence. *Annual Reviews in Food Science and Technology*, 28(8): 155-180.

Kefford, J. F., & Chandler, B. V. (1970). The Chemical Constitutents of Citrus Fruits. New York: Academic Press.

Kerr, M. A., Rennie, K. L., McCaffrey, T. A., Wallace, J. M., Hannon-Fletcher, M. P., & Livingstone, M. B. E. (2008). Snacking patterns among adolescents: a comparison of type, frequency and portion size between Britain in 1997 and Northern Ireland in 2005. *British Journal of Nutrition*, *101*(1), 122-131.

Khitan, Z., & Kim, D. H. (2013). Fructose: a key factor in the development of metabolic syndrome and hypertension. *Journal of Nutrition and Metabolism*, 2013.

Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research*, *61*(1), 1361779.

Kim, Y., Keogh, J. B., & Clifton, P. M. (2016). Polyphenols and glycemic control. *Nutrients*, *8*(1), 17.

Kondaki, K., Grammatikaki, E., Jiménez-Pavón, D., De Henauw, S., Gonzalez-Gross, M., Sjöstrom, M., ... & Gilbert, C. (2013). Daily sugar-sweetened beverage consumption and insulin resistance in European adolescents: the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) Study. *Public Health Nutrition*, *16*(3), 479-486.

Kreuch, D., Keating, D. J., Wu, T., Horowitz, M., Rayner, C. K., & Young, R. L. (2018). Gut mechanisms linking intestinal sweet sensing to glycemic control. *Frontiers in Endocrinology*, *9*, 741.

Kroyer, G. (2010). Stevioside and Stevia-sweetener in food: application, stability and interaction with food ingredients. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *5*(2), 225-229.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *The Scientific World Journal*, *2013*.

Laakso, M., & Kuusisto, J. (2014). Insulin resistance and hyperglycaemia in cardiovascular disease development. *Nature Reviews Endocrinology*, 10(5), 293-302.

Lazzinnaro, R., Plourde, H., Johnson-Down, L., Dewailly, E., & Egeland, G. M. (2012). High-sugar drink consumption: an associate of obesity and impaired fasting glucose in Canadian Indigenous (Cree) communities. *Nutrition, Metabolism and Cardiovascular Diseases*, *22*(8), e17-e19.

Lee, Y. M., Yoon, Y., Yoon, H., Park, H. M., Song, S., & Yeum, K. J. (2017). Dietary anthocyanins against obesity and inflammation. *Nutrients*, *9*(10), 1089.

Li, D., & Mehta, J. L. (2005). Oxidized LDL, a critical factor in atherogenesis. *Cardiovascular Research*, 68(3), 353-354.

Liem, D. G., & De Graaf, C. (2004). Sweet and sour preferences in young children and adults: role of repeated exposure. *Physiology & behavior*, 83(3), 421-429.

Liu, Y., Heying, E., & Tanumihardjo, S. A. (2012). History, global distribution, and nutritional importance of citrus fruits. *Comprehensive reviews in Food Science and Food safety*, *11*(6), 530-545.

Ludwig, D. S., Peterson, K. E., & Gortmaker, S. L. (2001). Relation between consumption of sugar-sweetened drinks and childhood obesity: a prospective, observational analysis. *The Lancet*, *357*(9255), 505-508.

Lustig, R. H. (2013). Fructose: it's “alcohol without the buzz”. *Advances in Nutrition*, 4(2), 226-235.

Lutsey, P. L., Steffen, L. M., & Stevens, J. (2008). Dietary intake and the development of the metabolic syndrome. *Circulation*, *117*(6), 754-761.

Ma, J., Bellon, M., Wishart, J. M., Young, R., Blackshaw, L. A., Jones, K. L., ... & Rayner, C. K. (2009). Effect of the artificial sweetener, sucralose, on gastric emptying and incretin hormone release in healthy subjects. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 296(4), G735-G739.

Mace, O. J., Affleck, J., Patel, N., & Kellett, G. L. (2007). Sweet taste receptors in rat small intestine stimulate glucose absorption through apical GLUT2. *The Journal of Physiology*, 582(1), 379-392.

Maersk, M., Belza, A., Stødkilde-Jørgensen, H., Ringgaard, S., Chabanova, E., Thomsen, H., ... & Richelsen, B. (2012 a). Sucrose-sweetened beverages increase fat storage in the liver, muscle, and visceral fat depot: a 6-mo randomized intervention study. *The American Journal of Clinical Nutrition*, *95*(2), 283-289.

Maersk, M., Belza, A., Holst, J. J., Fenger-Grøn, M., Pedersen, S. B., Astrup, A., & Richelsen, B. (2012 b). Satiety scores and satiety hormone response after sucrose-sweetened soft drink compared with isocaloric semi-skimmed milk and with non-caloric soft drink: a controlled trial. *European Journal of Clinical Nutrition*, *66*(4), 523-529.

Maffeis, C., Silvagni, D., Bonadonna, R., Grezzani, A., Banzato, C., & Tatò, L. (2007). Fat cell size, insulin sensitivity, and inflammation in obese children. *The Journal of Pediatrics*, 151(6), 647-652.

Magnuson, B. A., Burdock, G., Doull, J., Kroes, R. M., Marsh, G. M., Pariza, M. W., ... & Williams, G. M. (2007). Aspartame: a safety evaluation based on current use levels, regulations, and toxicological and epidemiological studies. *Critical Reviews in Toxicology*, 37(8), 629-727.

Mahdavi, S. A., Jafari, S. M., Ghorbani, M., & Assadpoor, E. (2014). Spray-drying microencapsulation of anthocyanins by natural biopolymers: a review. *Drying Technology*, 32(5), 509-518.

Malik, V. S., Schulze, M. B., & Hu, F. B. (2006). Intake of sugar-sweetened beverages and weight gain: a systematic review–. *The American journal of clinical nutrition*, *84*(2), 274-288.

Malik, V. S., Popkin, B. M., Bray, G. A., Després, J. P., Willett, W. C., & Hu, F. B. (2010). Sugar-sweetened beverages and risk of metabolic syndrome and type 2 diabetes: a meta-analysis. *Diabetes care*, *33*(11), 2477-2483.

Malik, V. S., & Hu, F. B. (2015). Fructose and cardiometabolic health: what the evidence from sugar-sweetened beverages tells us. *Journal of the American College of Cardiology*, *66*(14), 1615-1624.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727-747.

Martinez-Gonzalez, M. A., Bes-Rastrollo, M., Serra-Majem, L., Lairon, D., Estruch, R., & Trichopoulou, A. (2009). Mediterranean food pattern and the primary prevention of chronic disease: recent developments. *Nutrition Reviews*, *67*(suppl_1), S111-S116.

Masuoka, N., Matsuda, M., & Kubo, I. (2012). Characterisation of the antioxidant activity of flavonoids. *Food Chemistry*, *131*(2), 541-545.

Mattes, R. D., & Popkin, B. M. (2009). Nonnutritive sweetener consumption in humans: effects on appetite and food intake and their putative mechanisms. *The American Journal of Clinical Nutrition*, *89*(1), 1-14.

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*, *28*(7), 412-419.

Mackenzie, T., Brooks, B., & O'Connor, G. (2006). Beverage intake, diabetes, and glucose control of adults in America. *Annals of Epidemiology*, 16(9), 688-691.

McGartland, C., Robson, P. J., Murray, L., Cran, G., Savage, M. J., Watkins, D., ... & Boreham, C. (2003). Carbonated soft drink consumption and bone mineral density in adolescence: the Northern Ireland Young Hearts project. *Journal of Bone and Mineral Research*, *18*(9), 1563-1569.

Mc Ghie TK, Walton MC. (2007). The bioavailability and absorption of anthocyanins: Towards a better understanding. (Review). *Molecular Nutrition and Food Research*, 51(6), 702-713.

Meiattini, F., Prencipe, L., Bardelli, F., Giannini, G., & Tarli, P. (1978). The 4-hydroxybenzoate/4-aminophenazone chromogenic system used in the enzymic determination of serum cholesterol. *Clinical Chemistry*, *24*(12), 2161-2165.

Miller, P. E., & Perez, V. (2014). Low-calorie sweeteners and body weight and composition: a meta-analysis of randomized controlled trials and prospective cohort studies. *The American Journal of Clinical Nutrition*, *100*(3), 765-777.

Miranda-Rottmann, S., Aspillaga, A. A., Pérez, D. D., Vasquez, L., Martinez, A. L., & Leighton, F. (2002). Juice and phenolic fractions of the berry Aristotelia chilensis inhibit LDL oxidation in vitro and protect human endothelial cells against oxidative stress. *Journal of Agricultural and Food Chemistry*, *50*(26), 7542-7547.

Mitsutomi, K., Masaki, T., Shimasaki, T., Gotoh, K., Chiba, S., Kakuma, T., & Shibata, H. (2014). Effects of a nonnutritive sweetener on body adiposity and energy metabolism in mice with diet-induced obesity. *Metabolism*, 63(1), 69-78.

Miyake, Y., Mochizuki, M., Okada, M., Hiramitsu, M., Morimitsu, Y., & Osawa, T. (2007). Isolation of antioxidative phenolic glucosides from lemon juice and their suppressive effect on the expression of blood adhesion molecules. *Bioscience, Biotechnology and Biochemistry*, 71, 1911-1919.

Mølgaard, P., Holler, J. G., Asar, B., Liberna, I., Rosenbæk, L. B., Jebjerg, C. P., ... & Simonsen, H. T. (2011). Antimicrobial evaluation of Huilliche plant medicine used to treat wounds. *Journal of Ethnopharmacology*, *138*(1), 219-227.

Montonen, J., Knekt, P., Järvinen, R., & Reunanen, A. (2004). Dietary antioxidant intake and risk of type 2 diabetes. *Diabetes Care*, *27*(2), 362-366.

Mooradian, A. D., Smith, M., & Tokuda, M. (2017). The role of artificial and natural sweeteners in reducing the consumption of table sugar: A narrative review. *Clinical Nutrition eSPen*, *18*, 1-8.

Moran, A. W., Al-Rammahi, M. A., Arora, D. K., Batchelor, D. J., Coulter, E. A., Daly, K., ... & Shirazi-Beechey, S. P. (2010). Expression of Na+/glucose co-transporter 1 (SGLT1) is enhanced by supplementation of the diet of weaning piglets with artificial sweeteners. *British Journal of Nutrition*, 104(5), 637-646.

Mortensen, A. (2006). Sweeteners permitted in the European Union: safety aspects. *Scandinavian Journal of Food and Nutrition*, *50*(3), 104-116.

Mueller, D., Jung, K., Winter, M., Rogoll, D., Melcher, R., & Richling, E. (2017). Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries. *Food Chemistry*, *231*, 275-286.

Mulero, J., Bernabé, J., Cerdá, B., García-Viguera, C., Moreno, D. A., Albaladejo, M. D., Avilés, F., Parra, S., Abellán, J., & Zafrilla, P. (2012). Variations on cardiovascular risk factors in metabolic syndrome after consume of a citrus-based juice. *Clinical Nutrition*, 31(3): 372-377.

Muñoz O, Christen P, Cretton S, Backhouse N, et. al. (2011). Chemical study and anti-inflammatoy, analgesic and antioxidant activities of the leaves of Aristotelia chilensis (Mol.) stuntz, Elaeocarpaceae. *Journal of Pharmacy and Pharmacology*, 63, 849-859.

Myers, E. A., Passaro, E. M., & Hedrick, V. E. (2018). The Reproducibility and Comparative Validity of a Non-Nutritive Sweetener Food Frequency Questionnaire. *Nutrients*, 10(3), 334.

Naissides, M., Mamo, J. C., James, A. P., & Pal, S. (2006). The effect of chronic consumption of red wine on cardiovascular disease risk factors in postmenopausal women. *Atherosclerosis*, *185*(2), 438-445.

Nauck, M., Warnick, G. R., & Rifai, N. (2002). Methods for measurement of LDL-cholesterol: a critical assessment of direct measurement by homogeneous assays versus calculation. *Clinical Chemistry*, *48*(2), 236-254.

Nayaka R., Ritwik J. (2018). Knowledge, Attitude And Practice Of Diabetic Patients Regarding The Use Of Artificial Sweetners As Substitute To Sugar. *National Journal of Integrated Research in Medicine*, 9 (6),

Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J., & Zuker, C. S. (2001). Mammalian sweet taste receptors. *Cell*, 106(3), 381-390.

Nettleton, J. A., Lutsey, P. L., Wang, Y., Lima, J. A., Michos, E. D., & Jacobs, D. R. (2009 a). Diet soda intake and risk of incident metabolic syndrome and type 2 diabetes in the Multi-Ethnic Study of Atherosclerosis (MESA). *Diabetes care*, *32*(4), 688-694.

Nettleton, J. A., Polak, J. F., Tracy, R., Burke, G. L., & Jacobs Jr, D. R. (2009 b). Dietary patterns and incident cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *The American journal of clinical nutrition*, *90*(3), 647-654.

Nile, S. H., & Park, S. W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition*, *30*(2), 134-144.

Njike, V. Y., Faridi, Z., Shuval, K., Dutta, S., Kay, C. D., West, S. G., ... & Katz, D. L. (2011). Effects of sugar-sweetened and sugar-free cocoa on endothelial function in overweight adults. *International Journal of Cardiology*, *149*(1), 83-88.

Ning, C., & Segal, S. (2000). Plasma galactose and galactitol concentration in patients with galactose-1-phosphate uridyltransferase deficiency galactosemia: determination by gas chromatography/mass spectrometry. *Metabolism-Clinical and Experimental*, *49*(11), 1460-1466.

Nowak, D., Gośliński, M., & Wojtowicz, E. (2016). Comparative analysis of the antioxidant capacity of selected fruit juices and nectars: Chokeberry juice as a rich source of polyphenols. *International Journal of Food Properties*, *19*(6), 1317-1324.

Oh, S. W., Moon, J. D., Park, S. Y., Jang, H. J., Kim, J. H., Nahm, K. B., & Choi, E. Y. (2005). Evaluation of fluorescence hs-CRP immunoassay for point-of-care testing. *Clinica Chimica Acta*, 356(1-2), 172-177.

Ojeda, J., Evelyn, J. A. R. A., Molina, L., Parada, F., Burgos, R. A., Hidalgo, M. A., & Hancke, J. L. (2011). Effects of Aristotelia chilensis berry juice on cyclooxygenase 2 expression, NF-kB, NFAT, ERK1/2 and PI3K/Akt activation in colon cancer cells. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, *10*(6), 543-552.

Okoduwa, S. I. R., Ebiloma, G. U., Baba, J., & Ajide, S. (2013). The metabolism and toxicology of saccharin. *Infohealth Aware. Artic*, *1*, 14-19.

O'Reilly, J. D., Mallet, A. I., McAnlis, G. T., Young, I. S., Halliwell, B., Sanders, T. A., & Wiseman, H. (2001). Consumption of flavonoids in onions and black tea: lack of effect on F2-isoprostanes and autoantibodies to oxidized LDL in healthy humans. *The American Journal of Clinical Nutrition*, 73(6), 1040-1044.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Laboratories, B.; Lane, T. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry*, 49, 4619-4626.

Palmer, J. R., Boggs, D. A., Krishnan, S., Hu, F. B., Singer, M., & Rosenberg, L. (2008). Sugar-sweetened beverages and incidence of type 2 diabetes mellitus in African American women. *Archives of internal medicine*, *168*(14), 1487-1492.

Palmnäs, M. S., Cowan, T. E., Bomhof, M. R., Su, J., Reimer, R. A., Vogel, H. J., ... & Shearer, J. (2014). Low-dose aspartame consumption differentially affects gut microbiota-host metabolic interactions in the diet-induced obese rat. *PloS one*, 9(10).

Panagiotakos, D. B., Pitsavos, C., Chrysohoou, C., Skoumas, J., Tousoulis, D., Toutouza, M., ... & Stefanadis, C. (2004). Impact of lifestyle habits on the prevalence of the metabolic syndrome among Greek adults from the ATTICA study. *American Heart Journal*, 147(1), 106-112.

Panunzio, M. F., Pisano, A., Antoniciello, A., Di Martino, V., Frisoli, L., Cipriani, V., ... & Bronzetti, G. (2003). Supplementation with fruit and vegetable concentrate decreases plasma homocysteine levels in a dietary controlled trial. *Nutrition Research*, 23(9), 1221-1228.

Parr, A. J., & Bolwell, G. P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*, *80*(7), 985-1012.

Pearlman, F. C., & Lee, R. T. (1974). Detection and measurement of total bilirubin in serum, with use of surfactants as solubilizing agents. *Clinical Chemistry*, *20*(4), 447-453.

Penniston, K. L., Nakada, S. Y., Holmes, R. P., & Assimos, D. G. (2008). Quantitative assessment of citric acid in lemon juice, lime juice, and commercially-available fruit juice products. *Journal of Endourology*, 22, 567570.

Pepino, M. Y., Tiemann, C. D., Patterson, B. W., Wice, B. M., & Klein, S. (2013). Sucralose affects glycemic and hormonal responses to an oral glucose load. *Diabetes Care*, 36(9), 2530-2535.

Perez-Perez, J. G., Castillo, I. P., Garcia-Lidon, A., Botia, P., & Garcia-Sanchez, F. (2005). Fino lemon clones compared with the lemon varieties Eureka and Lisbon on two rootstocks in Murcia (Spain). *Scientia Horticulturae*, 106, 530538.

Piljac-Žegarac, J., Valek, L., Martinez, S., & Belščak, A. (2009). Fluctuations in the phenolic content and antioxidant capacity of dark fruit juices in refrigerated storage. *Food Chemistry*, *113*(2), 394-400.

Polyák, É., Gombos, K., Hajnal, B., Bonyár-Müller, K., Szabó, S., Gubicskó-Kisbenedek, A., ... & Ember, I. (2010). Effects of artificial sweeteners on body weight, food and drink intake. *Acta Physiologica Hungarica*, 97(4), 401-407.

Prior RL, Wu X. (2006).Anthocyanins: structural charactristics thatresult in unique metabolic patterns and biological activities. *Free Radical Research* 40, 1014-1028.

Prodolliet, J., & Bruelhart, M. (1993). Determination of aspartame and its major decomposition products in foods. *Journal of AOAC International*, *76*(2), 275-282.

Proteggente, A. R., Pannala, A. S., Paganga, G., Buren, L. V., Wagner, E., Wiseman, S., ... & Rice-Evans, C. A. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Research*, 36(2), 217-233.

Raben, A., & Richelsen, B. (2012). Artificial sweeteners: a place in the field of functional foods? Focus on obesity and related metabolic disorders. *Current Opinion in Clinical Nutrition & Metabolic Care*, *15*(6), 597-604.

Rafieian-Kopaei, M., Setorki, M., Doudi, M., Baradaran, A., & Nasri, H. (2014). Atherosclerosis: process, indicators, risk factors and new hopes. *International Journal of Preventive Medicine*, *5*(8), 927.

Rajeshwari, R., Yang, S. J., Nicklas, T. A., & Berenson, G. S. (2005). Secular trends in children's sweetened-beverage consumption (1973 to 1994): the Bogalusa Heart Study. *Journal of the American Dietetic Association*, *105*(2), 208-214.

Rastogi, S., Zakrzewski, M., & Suryanarayanan, R. (2001). Investigation of solid-state reactions using variable temperature X-ray powder diffractrometry. I. Aspartame hemihydrate. *Pharmaceutical Research*, *18*(3), 267-273.

Reaven, G. M. (2011). The metabolic syndrome: time to get off the merry-go-round?. *Journal of Internal Medicine*, *269*(2), 127-136.

Reid, M., Hammersley, R., Hill, A. J., & Skidmore, P. (2007). Long-term dietary compensation for added sugar: effects of supplementary sucrose drinks over a 4-week period. *British Journal of Nutrition*, *97*(1), 193-203.

Reid, M., Hammersley, R., & Duffy, M. (2010). Effects of sucrose drinks on macronutrient intake, body weight, and mood state in overweight women over 4 weeks. *Appetite*, 55(1), 130-136.

Reyes-Farias, M., Vasquez, K., Ovalle-Marin, A., Fuentes, F., Parra, C., Quitral, V., ... & Garcia-Diaz, D. F. (2015). Chilean native fruit extracts inhibit inflammation linked to the pathogenic interaction between adipocytes and macrophages. *Journal of Medicinal Food*, *18*(5), 601-608.

Reyes-Farias, M., Vasquez, K., Fuentes, F., Ovalle-Marin, A., Parra-Ruiz, C., Zamora, O., ... & Garcia-Diaz, D. F. (2016). Extracts of Chilean native fruits inhibit oxidative stress, inflammation and insulin-resistance linked to the pathogenic interaction between adipocytes and macrophages. *Journal of Functional Foods*, *27*, 69-83.

Rippe, J. M., & Angelopoulos, T. J. (2016). Added sugars and risk factors for obesity, diabetes and heart disease. *International Journal of Obesity*, *40*(S1), S22.

Rojo, L. E., Ribnicky, D., Logendra, S., Poulev, A., Rojas-Silva, P., Kuhn, P., ... & Raskin, I. (2012). In vitro and in vivo anti-diabetic effects of anthocyanins from Maqui Berry (Aristotelia chilensis). *Food Chemistry*, *131*(2), 387-396.

Romanucci, V., D'Alonzo, D., Guaragna, A., Di Marino, C., Davinelli, S., Scapagnini, G., ... & Zarrelli, A. (2016). Bioactive compounds of Aristotelia chilensis Stuntz and their pharmacological effects. *Current Pharmaceutical Biotechnology*, *17*(6), 513-523.

Romo-Romo, A., Aguilar-Salinas, C. A., Brito-Córdova, G. X., Díaz, R. A. G., Valentín, D. V., & Almeda-Valdes, P. (2016). Effects of the non-nutritive sweeteners on glucose metabolism and appetite regulating hormones: systematic review of observational prospective studies and clinical trials. *PloS One*, *11*(8), e0161264.

Rosalki, S. B., Foo, A. Y., Burlina, A., Prellwitz, W., Stieber, P., Neumeier, D., ... & Bodenmüller, H. (1993). Multicenter evaluation of Iso-ALP test kit for measurement of bone alkaline phosphatase activity in serum and plasma. *Clinical Chemistry*, *39*(4), 648-652.

Rosinger, A., Herrick, K. A., Gahche, J. J., & Park, S. (2017). Sugar-sweetened beverage consumption among US youth, 2011-2014.

Rother, K. I., Conway, E. M., & Sylvetsky, A. C. (2018). How non-nutritive sweeteners influence hormones and health. *Trends in Endocrinology & Metabolism*, 29(7), 455-467.

Rothwell, J. A., Madrid-Gambin, F., Garcia-Aloy, M., Andres-Lacueva, C., Logue, C., Gallagher, A. M., ... & Dragsted, L. O. (2018). Biomarkers of intake for coffee, tea, and sweetened beverages. *Genes & Nutrition*, *13*(1), 15.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., & Shene, C. (2011). Extracts of Maqui (Aristotelia chilensis) and Murta (Ugni molinae Turcz.): sources of antioxidant compounds and α-Glucosidase/α-Amylase inhibitors. *Journal of Agricultural and Food Chemistry*, *59*(5), 1630-1637.

Ruiz, E., Rodriguez, P., Valero, T., Ávila, J. M., Aranceta-Bartrina, J., Gil, Á., ... & Varela-Moreiras, G. (2017). Dietary intake of individual (free and intrinsic) sugars

and food sources in the Spanish population: Findings from the ANIBES study. *Nutrients*, *9*(3), 275.

Russo, M., Galletti, G. C., Bocchini, P., & Carnacini, A. (1998). Essential Oil Chemical Composition of Wild Populations of Italian Oregano Spice (Origanum vulgare ssp. hirtum (Link) Ietswaart): A Preliminary Evaluation of Their Use in Chemotaxonomy by Cluster Analysis. 1. Inflorescences. *Journal of Agricultural and Food Chemistry*, 46, 3741-3746.

Sachdev, P. (2004). Homocysteine, cerebrovascular disease and brain atrophy. *Journal of the Neurological Sciences*, *226*(1-2), 25-29.

Sadiq, M. A., & Salih, A. A. (2018). Knowledge and Attitude About Sweeteners Among Type 2 Diabetic Patients. *Archives of Internal Medicine Research*, *1*, 1-5.

Salehi, B., Fokou, P. V. T., Sharifi-Rad, M., Zucca, P., Pezzani, R., Martins, N., & Sharifi-Rad, J. (2019). The therapeutic potential of naringenin: a review of clinical trials. *Pharmaceuticals*, 12(1), 11.

Sakurai, M., Nakamura, K., Miura, K., Takamura, T., Yoshita, K., Nagasawa, S. Y., ... & Suwazono, Y. (2014). Sugar-sweetened beverage and diet soda consumption and the 7-year risk for type 2 diabetes mellitus in middle-aged Japanese men. *European Journal of Nutrition*, *53*(1), 251-258.

Schreckinger, M. E., Wang, J., Yousef, G., Lila, M. A., & Gonzalez de Mejia, E. (2010 a). Antioxidant capacity and in vitro inhibition of adipogenesis and inflammation by phenolic extracts of Vaccinium floribundum and Aristotelia chilensis. *Journal of Agricultural and Food Chemistry*, *58*(16), 8966-8976.

Schreckinger, M. E., Lotton, J., Lila, M. A., & de Mejia, E. G. (2010 b). Berries from South America: a comprehensive review on chemistry, health potential, and commercialization. *Journal of Medicinal Food*, *13*(2), 233-246.

Schiffman, S. S., & Rother, K. I. (2013). Sucralose, a synthetic organochlorine sweetener: overview of biological issues. *Journal of Toxicology and Environmental Health*, Part B, 16(7), 399-451.

Schulze, M. B., Liu, S., Rimm, E. B., Manson, J. E., Willett, W. C., & Hu, F. B. (2004a). Glycemic index, glycemic load, and dietary fiber intake and incidence of

type 2 diabetes in younger and middle-aged women. *The American journal of clinical nutrition*, *80*(2), 348-356.

Schulze, M. B., Manson, J. E., Ludwig, D. S., Colditz, G. A., Stampfer, M. J., Willett, W. C., & Hu, F. B. (2004 b). Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *Jama*, *292*(8), 927-934.

Selhub, J. (2008). Public health significance of elevated homocysteine. *Food and nutrition bulletin*, *29*(2_suppl1), S116-S125.

Serafini, M. (2006). Back to the origin of the 'antioxidant hypothesis': the lost role of the antioxidant network in disease prevention. *Journal of the Science of Food and Agriculture*, *86*(13), 1989-1991.

Serra-Majem, L., Raposo, A., Aranceta-Bartrina, J., Varela-Moreiras, G., Logue, C., Laviada, H., ... & López-García, R. (2018). Ibero–American Consensus on Low- and No-Calorie Sweeteners: Safety, Nutritional Aspects and Benefits in Food and Beverages. *Nutrients*, *10*(7), 818.

Sharma, A., Amarnath, S., Thulasimani, M., & Ramaswamy, S. (2016). Artificial sweeteners as a sugar substitute: Are they really safe?. *Indian Journal of Pharmacology*, 48(3), 237.

Silveira, J. Q., Dourado, G. K., & Cesar, T. B. (2015). Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. *International Journal of Food Sciences and Nutrition*, *66*(7), 830-836.

Sims, J., Roberts, A., Daniel, J. W., & Renwick, A. G. (2000). The metabolic fate of sucralose in rats. *Food and Chemical Toxicology*, *38*, 115-121.

Smeets, P. A., Erkner, A., & De Graaf, C. (2010). Cephalic phase responses and appetite. *Nutrition Reviews*, 68(11), 643-655.

Stearns, A. T., Balakrishnan, A., Rhoads, D. B., & Tavakkolizadeh, A. (2010). Rapid upregulation of sodium-glucose transporter SGLT1 in response to intestinal sweet taste stimulation. *Annals of Surgery*, 251(5), 865.

Stein, J. H., Keevil, J. G., Wiebe, D. A., Aeschlimann, S., & Folts, J. D. (1999). Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation*, 100(10), 1050-1055.

Striegel-Moore, R. H., Thompson, D., Affenito, S. G., Franko, D. L., Obarzanek, E., Barton, B. A., ... & Crawford, P. B. (2006). Correlates of beverage intake in adolescent girls: the National Heart, Lung, and Blood Institute Growth and Health Study. *The Journal of Pediatrics*, *148*(2), 183-187.

Stull, A. J., Cash, K. C., Johnson, W. D., Champagne, C. M., & Cefalu, W. T. (2010). Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *The Journal of nutrition*, *140*(10), 1764-1768.

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C. A., Maza, O., ... & Kuperman, Y. (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*, 514(7521), 181-186.

Swithers, S. E., & Davidson, T. L. (2008). A role for sweet taste: calorie predictive relations in energy regulation by rats. *Behavioral Neuroscience*, *122*(1), 161.

Swithers, S. E. (2013). Artificial sweeteners produce the counterintuitive effect of inducing metabolic derangements. *Trends in Endocrinology & Metabolism*, *24*(9), 431-441.

Sylvetsky, A. C., & Rother, K. I. (2016). Trends in the consumption of low-calorie sweeteners. *Physiology & Behavior*, *164*, 446-450.

Tadhani, M., & Subhash, R. (2006). Preliminary studies on Stevia rebaudiana leaves: proximal composition, mineral analysis and phytochemical screening. *Journal of Medicinal Sciences*, *6*(3), 321-326.

Taljaard, C., Covic, N. M., Van Graan, A. E., Kruger, H. S., Smuts, C. M., Baumgartner, J., ... & Jerling, J. C. (2013). Effects of a multi-micronutrient-fortified beverage, with and without sugar, on growth and cognition in South African schoolchildren: a randomised, double-blind, controlled intervention. *British Journal of Nutrition*, *110*(12), 2271-2284.

Temizkan, S., Deyneli, O., Yasar, M., Arpa, M., Gunes, M., Yazici, D., ... & Yavuz, D. G. (2015). Sucralose enhances GLP-1 release and lowers blood glucose in the presence of carbohydrate in healthy subjects but not in patients with type 2 diabetes. *European Journal of Clinical Nutrition*, 69(2), 162-166.

Te Morenga, L., Mallard, S., & Mann, J. (2013). Dietary sugars and body weight: systematic review and meta-analyses of randomised controlled trials and cohort studies. *British Medical Journal*, *346*, e7492.

Te Morenga, L. A., Howatson, A. J., Jones, R. M., & Mann, J. (2014). Dietary sugars and cardiometabolic risk: systematic review and meta-analyses of randomized controlled trials of the effects on blood pressure and lipids. *The American Journal of Clinical Nutrition*, 100(1), 65-79.

The Sugar Association, https://www.sugar.org/resources/health/ accessed November 2019.

Ti, H., Zhang, R., Zhang, M., Li, Q., Wei, Z., Zhang, Y., ... & Ma, Y. (2014). Dynamic changes in the free and bound phenolic compounds and antioxidant activity of brown rice at different germination stages. *Food chemistry*, *161*, 337-344.

Toro MAL. Evaluation del efecto antiinflamatorio de un con cenrado de futos de Aristotelia chilensis en un modelo de inflamacion aguda subplanter inducida por carragenina en ratas. Universidad Austral de Chile. Memoria para optar al tiul de medico veterinorio, Valdivia, Chile, 2009.

Tortosa, A., Bes-Rastrollo, M., Sanchez-Villegas, A., Basterra-Gortari, F. J., Nuñez-Cordoba, J. M., & Martinez-Gonzalez, M. A. (2007). Mediterranean diet inversely associated with the incidence of metabolic syndrome: the SUN prospective cohort. *Diabetes Care*, *30*(11), 2957-2959.

Toews, I., Lohner, S., de Gaudry, D. K., Sommer, H., & Meerpohl, J. J. (2019). Association between intake of non-sugar sweeteners and health outcomes: systematic review and meta-analyses of randomised and non-randomised controlled trials and observational studies. *British Medical Journal*, 364.

Trichopoulou, A., Martínez-González, M. A., Tong, T. Y., Forouhi, N. G., Khandelwal, S., Prabhakaran, D., ... & de Lorgeril, M. (2014). Definitions and

potential health benefits of the Mediterranean diet: views from experts around the world. *BMC medicine*, 12(1), 112.

Tucker, R. M., & Tan, S. Y. (2017). Do non-nutritive sweeteners influence acute glucose homeostasis in humans? A systematic review. *Physiology & behavior*, 182, 17-26.

US Food and Drug Administration. (2013). High fructose corn syrup: Questions and answers.

Van Baak, M. A., & Astrup, A. (2009). Consumption of sugars and body weight. *Obesity Reviews*, *10*, 9-23.

van der Wielen, N., Ten Klooster, J. P., Muckenschnabl, S., Pieters, R., Hendriks, H. F., Witkamp, R. F., & Meijerink, J. (2016). The noncaloric sweetener rebaudioside a stimulates glucagon-like peptide 1 release and increases enteroendocrine cell numbers in 2-dimensional mouse organoids derived from different locations of the intestine. *The Journal of Nutrition*, 146(12), 2429-2435.

Van Wymelbeke, V., Beridot-Therond, M. E., de La Gueronniere, V., & Fantino, M. (2004). Influence of repeated consumption of beverages containing sucrose or intense sweeteners on food intake. *European journal of clinical nutrition*, *58*(1), 154-161.

Vayá, A., Rivera, L., Hernández-Mijares, A., de la Fuente, M., Solá, E., Romagnoli, M., ... & Laiz, B. (2012). Homocysteine levels in morbidly obese patients. Its association with waist circumference and insulin resistance. *Clinical hemorheology and microcirculation*, 52(1), 49-56.

Wallace, T. C. (2011). Anthocyanins in cardiovascular disease. *Advances in Nutrition*, *2*(1), 1-7.

Warnick, G. R., Nauck, M., & Rifai, N. (2001). Evolution of methods for measurement of HDL-cholesterol: from ultracentrifugation to homogeneous assays. *Clinical Chemistry*, *47*(9), 1579-1596.

Welch, V., Jull, J., Petkovic, J., Armstrong, R., Boyer, Y., Cuervo, L. G., ... & Kristjansson, E. (2015). Protocol for the development of a CONSORT-equity

guideline to improve reporting of health equity in randomized trials. *Implementation Science*, 10(1), 146.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., & Tataranni, P. A. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *The Journal of Clinical Endocrinology & Metabolism*, *86*(5), 1930-1935.

Wilson, T., Murray, B., Price, T., Atherton, D., & Hooks, T. (2019). Non-Nutritive (Artificial) Sweetener Knowledge among University Students. *Nutrients*, *11*(9), 2201.

Wolever, T. M., & Bolognesi, C. (1996). Source and amount of carbohydrate affect postprandial glucose and insulin in normal subjects. *The Journal of Nutrition*, *126*(11), 2798-2806.

Wölnerhanssen, B. K., Cajacob, L., Keller, N., Doody, A., Rehfeld, J. F., Drewe, J., ... & Meyer-Gerspach, A. C. (2016). Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. American *Journal of Physiology-Endocrinology and Metabolism*, 310(11), E1053-E1061.

Woodward, G., Kroon, P., Cassidy, A., & Kay, C. (2009). Anthocyanin stability and recovery: implications for the analysis of clinical and experimental samples. *Journal of Agricultural and Food Chemistry*, *57*(12), 5271-5278.

World Health Organization. (2015). *Guideline: sugars intake for adults and children*. World Health Organization.

Woźniak, Ł., Marszałek, K., & Skapska, S. (2014). Influence of steviol glycosides on the stability of vitamin C and anthocyanins. *Journal of Agricultural and Food Chemistry*, 62(46), 11264-11269.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry*, 52(12), 4026-4037.

Wu, X., Pittman III, H. E., Mckay, S., & Prior, R. L. (2005). Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *The Journal of Nutrition*, *135*(10), 2417-2424.

Wu, T., Zhao, B. R., Bound, M. J., Checklin, H. L., Bellon, M., Little, T. J., ... & Rayner, C. K. (2012). Effects of different sweet preloads on incretin hormone secretion, gastric emptying, and postprandial glycemia in healthy humans. *The American Journal of Clinical Nutrition*, 95(1), 78-83.

Xiao, J. B., & Hogger, P. (2015). Dietary polyphenols and type 2 diabetes: current insights and future perspectives. *Current medicinal chemistry*, *22*(1), 23-38.

Yang, Q. (2010). Gain weight by “going diet?” Artificial sweeteners and the neurobiology of sugar cravings: Neuroscience 2010. *The Yale Journal of Biology and Medicine*, 83(2), 101.

Yoshida, M., McKeown, N. M., Rogers, G., Meigs, J. B., Saltzman, E., D'agostino, R., & Jacques, P. F. (2007). Surrogate markers of insulin resistance are associated with consumption of sugar-sweetened drinks and fruit juice in middle and older-aged adults. *The Journal of Nutrition*, 137(9), 2121-2127.

Young, D. S. (2000). *Effects of Drugs on Clinical Laboratory Tests*. AACC press.

Young, R. L., Chia, B., Isaacs, N. J., Ma, J., Khoo, J., Wu, T., ... & Rayner, C. K. (2013). Disordered control of intestinal sweet taste receptor expression and glucose absorption in type 2 diabetes. *Diabetes*, 62(10), 3532-3541.

Zamora-Ros, R., Forouhi, N. G., Sharp, S. J., González, C. A., Buijsse, B., Guevara, M., ... & Clavel-Chapelon, F. (2013). The association between dietary flavonoid and lignan intakes and incident type 2 diabetes in European populations: the EPIC-InterAct study. *Diabetes Care*, *36*(12), 3961-3970.

Zamora-Ros, R., Forouhi, N. G., Sharp, S. J., González, C. A., Buijsse, B., Guevara, M., ... & Fagherazzi, G. (2014). Dietary intakes of individual flavanols and flavonols are inversely associated with incident type 2 diabetes in European populations. *The Journal of Nutrition*, *144*(3), 335-343.

Zern, T. L., Wood, R. J., Greene, C., West, K. L., Liu, Y., Aggarwal, D., ... & Fernandez, M. L. (2005). Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *The Journal of Nutrition*, 135(8), 1911-1917.

Zoppi, F., Peracino, A., Fenili, D., Marcovina, S., & Ramella, C. (1976). Metodo per la determinazione della bilirubina totale e coniugata. *Uso di un tensioattivo cationico come agente solubilizzante. Giorn It Chim Cl*, *1*, 343-359.

IX - ANNEX

ANNEX 1: Approval from the Ethical Committees

Informe Dictamen Protocolo Favorable
Otros Estudios
C.P. - C.I. EST: 52/16
19 de diciembre de 2016

CEIC Hospital General Universitario José María Morales Meseguer

Dra. María Dolores Nájera Pérez
Presidenta del CEIC Hospital General Universitario José María Morales Meseguer

CERTIFICA

1º. Que el CEIC Hospital General Universitario José María Morales Meseguer en su reunión del día 19/12/2016, Acta EXTRAORDINARIA ha evaluado la propuesta del promotor referida al estudio:

Título: Estudio: " Evaluación del efecto de bebidas ricas en compuestos bioactivos para modular el metabolismo energético en adultos con sobrepeso". (Proyecto de Investigación).

Código Interno: EST: 52/16 **Promotor:** UCAM.
Versión Protocolo Evaluada: Versión Noviembre 2016
Versión Hoja Información al Paciente Evaluada: GENERAL / Versión Noviembre 2016
Fecha Entrada ACLARACIONES: 18/12/2016
Investigador Principal: **Dra. Débora VILLAÑO VALENCIA. Farmacia de la UCAM.**

1º. Considera que :

- Se respetan los principios éticos básicos y es adecuado el procedimiento para obtener el consentimiento informado.

2º. Por lo que este CEIC emite un **DICTAMEN FAVORABLE**.

Lo que firmo en Murcia, a 19 de diciembre de 2016

Dra. María Dolores Nájera Pérez
Presidenta del CEIC Hospital General Universitario José María Morales Meseguer

---

Hospital General Universitario J.M. Morales Meseguer
Marqués de los Vélez s/n Murcia 30008 Murcia España
Tel. 968 36 52 02 Fax. 968 36 09 49 Correo electrónico: ceic.hmm@carm.es

COMITÉ DE ÉTICA DE LA UCAM

DATOS DEL PROYECTO

Título:	"Evaluación del efecto de bebidas ricas en compuestos bioactivos para modular el metabolismo energético en adultos con sobrepeso"	
Investigador Principal	**Nombre**	**Correo-e**
Dra.	Débora Villaño Valencia	dvillano@ucam.edu

INFORME DEL COMITÉ

Fecha	**03/06/2016**

Tipo de Experimentación

Investigación experimental clínica con seres humanos.	X
Utilización de tejidos humanos procedentes de pacientes, tejidos embrionarios o fetales.	
Utilización de tejidos humanos, tejidos embrionarios o fetales procedentes de bancos de muestras o tejidos.	
Investigación observacional con seres humanos, psicológica o comportamental en humanos.	
Uso de datos personales, información genética, etc.	X
Experimentación animal.	
Utilización de agentes biológicos de riesgo para la salud humana, animal o las plantas.	
Uso de organismos modificados genéticamente (OMGs).	

Comentarios Respecto al tipo de Experimentación
Nada Obsta

Comentarios Respecto a la metodología de experimentación
Nada Obsta

ANNEX 2: Inclusion/Exclusion Questionnaire

CUESTIONARIO - ESTUDIO BEBESANO

Nombre:
Apellidos:
Edad:
Altura:
Peso: IMC:

Fumador: SI NO Exfumador: desde ….. años

¿Sabe aproximadamente sus valores de tensión arterial?

¿Tiene el colesterol un poco elevado? ¿Sabe aproximadamente qué valores tiene?

¿Tiene los triglicéridos un poco elevados? ¿Sabe aproximadamente qué valores tiene?

¿Toma algún medicamento de forma crónica o habitualmente?

ANNEX 3: Informed consent

CONSENTIMIENTO INFORMADO

Yo, ..., con DNI:..............................

DECLARO:

Haber sido informado/a del estudio y procedimientos de la investigación del Proyecto titulado: Nueva Bebida, Rica en Compuestos Bioactivos, para Modular el Metabolismo Energético en Adultos con Sobrepeso.

Los investigadores que van a acceder a mis datos personales y a los resultados de las pruebas son: Débora Villaño Valencia , Pilar Zafrilla Rentero, Alejandro Galindo Tovar, Begoña Cerdá Martínez-Pujalte

Asimismo, he podido hacer preguntas del estudio, comprendiendo que me presto de forma voluntaria al mismo y que en cualquier momento puedo abandonarlo sin que me suponga perjuicio de ningún tipo.

CONSIENTO:

1.-) Someterme a las siguientes pruebas exploratorias (en su caso): medidas antropométricas (peso, talla, circunferencia abdominal y cadera), extracción de sangre y recogida de orina.

2.-) El uso de los datos obtenidos según lo indicado en el párrafo siguiente:

En cumplimiento de la Ley Orgánica 15/1999, de 13 de diciembre, de Protección

de Datos de Carácter Personal, le comunicamos que la información que ha facilitado y la obtenida como consecuencia de las exploraciones a las que se va a someter pasará a formar parte del fichero automatizado INVESALUD, cuyo titular es la FUNDACIÓN UNIVERSITARIA SAN ANTONIO, con la finalidad de INVESTIGACIÓN Y DOCENCIA EN LAS ÁREAS DE CONOCIMIENTO CIENCIAS
EXPERIMENTALES Y CIENCIAS DE LA SALUD. Tiene derecho a acceder a esta información y cancelarla o rectificarla, dirigiéndose al domicilio de la entidad, en Avda. de los Jerónimos de Guadalupe 30107 (Murcia). Esta entidad le garantiza la adopción de las medidas oportunas para asegurar el tratamiento confidencial de dichos datos.

En Guadalupe (Murcia) a .... de ....de 20

El investigador,

Fdo……………………….. fdo………………………..

ANNEX 4: Written information for the volunteer

ANEXO V

DOCUMENTO DE INFORMACIÓN PARA SUJETOS SOMETIDOS A ESTUDIO (HOJA INFORMATIVA)

Nueva Bebida, Rica en Compuestos Bioactivos, para Modular el Metabolismo Energético en Adultos con Sobrepeso

1. EN QUÉ CONSISTE Y PARA QUÉ SIRVE:

El proyecto estudiará los efectos beneficiosos del consumo de unas bebidas libres de azúcares añadidos y ricas en compuestos bioactivos de origen vegetal, que pueden ser una alternativa a las bebidas comerciales azucaradas.
Las bebidas se desarrollarán a base de limón y maqui que son ingredientes ricos en compuestos bioactivos beneficiosos para la salud. Añadiremos edulcorantes de tipo natural (stevia) o artificial (sucralosa) y los compararemos con una bebida elaborada con el edulcorante comúnmente empleado en la industria de las bebidas (sacarosa).

Estudiaremos la influencia de diversos ingredientes endulzantes en la biodisponibilidad y en los efectos biológicos de los compuestos procedentes de los extractos de frutas presentes en las bebidas.

Para ello realizaremos estudios de intervención nutricional en voluntarios sanos con sobrepeso. Gracias a estos estudios estableceremos los efectos de estas nuevas bebidas en el metabolismo lipídico y glicémico, estrés oxidativo, inflamación y regulación hormonal y determinaremos cuáles son los metabolitos en circulación responsables de los efectos observados.

Los resultados de este proyecto permitirán valorar el efecto de una nueva generación de bebidas saludables, que pueden ser una opción interesante frente a los problemas actuales de desarrollo de enfermedades cardiovasculares y diabetes de tipo II, con su consecuente impacto social y económico.

2. COMO SE REALIZA:

Realizaremos dos tipos de estudio, estudio en agudo y estudio crónico (a medio plazo). A continuación se resume brevemente sus características:

Estudio en agudo

Se seleccionará un grupo reducido, 20 personas sanas con sobrepeso. Los criterios de inclusión son:

- Edad: 35-55 años
- IMC: 24,9-29,9 kg/m^2
- No fumador
- No hipertensión arterial (No cifras superiores a 140/90 mm Hg)
- No colesterol muy elevado, posible en el límite (No superior a 220 mg/dL)
- No triglicéridos muy elevado, posible en el límite (No superiores a 200 mg/dL)
- No padecer ninguna de las siguientes patologías: enfermedad cardiovascular, diabetes, enfermedad renal, enfermedad hepática, enfermedades del sistema inmune
- No tomar medicamentos de forma crónica

Tras una fase inicial de 2 días en la que restringiremos los alimentos ricos en compuestos fenólicos así como alimentos ricos en azúcares añadidos, realizaremos un ensayo en la Ucam con el fin de evaluar la respuesta glicémica e insulinémica de las bebidas.
Recogeremos una muestra de orina de 24 h, del día anterior. Pondremos una vía y se tomará una muestra de sangre inicial (tiempo cero).
Daremos la bebida test (250 mL) y tomaremos muestras de sangre en diversos tiempos (15, 30, 60, 90, 120, 240 min). Recogeremos orina en este intervalo de 4 h.
Tras las 4 h finaliza el ensayo ese día. Daremos al voluntario unos bidones para recoger la orina en los siguientes intervalos: 4-12 h y de 12 a 24 h.
Al día siguiente el voluntario nos dará los bidones de orina y se llevará otro bidón para recoger la orina del siguiente día (24 a 48 h).
Al día siguiente el voluntario nos dará los bidones de orina recogidos.

Tras 15 días se repetirá de nuevo y el voluntario tomará otra bebida, hasta terminar con la ingestión de las 3 bebidas por parte de todos los voluntarios.

Estudio a medio plazo

Realizaremos el estudio con 180 voluntarios. La selección de voluntarios seguirá el mismo criterio que en el estudio en agudo.

El estudio incluirá las bebidas desarrolladas que ya habrán sido estudiadas en el estudio en agudo. Se recomendará a los participantes seguir una dieta equilibrada en nutrientes (Mediterránea tradicional) y pobre en colesterol y grasas saturadas, pero no habrá una dieta especial a seguir.

Dividiremos los 180 voluntarios en 3 grupos, al azar. El voluntario no sabrá qué bebida tomará (estudio placebo).
Daremos a cada voluntario la bebida test (250 mL) por un tiempo de 60 días. Se tomarán muestras de sangre y orina de 24 h a día 0, en una etapa intermedia (30 días) y al final del estudio (60 días) para realizar diversas valoraciones relacionadas con el metabolismo lipídico, glucídico y respuesta hormonal. Se determinarán diversos marcadores relacionados con el metabolismo lipídico e inflamatorio para comprobar los efectos de los edulcorantes, así como respuesta hormonal. Se evaluarán parámetros antropométricos como peso corporal, IMC y circunferencia abdominal. Durante el tiempo de estudio llevaremos a cabo un registro de los alimentos ingeridos y la actividad física desarrollada.

3. QUÉ EFECTOS LE PRODUCIRÁ:
Sólo efectos beneficiosos dado el carácter antioxidante y antiinflamatorio de los ingredientes incluidos en la formulación de las bebidas.

4. EN QUÉ LE BENEFICIARÁ:
Al ser un alimento con numerosas propiedades beneficiosas para la salud y que no presenta ningún riesgo. Su consumo se asocia a efectos positivos.

5. QUÉ RIESGOS TIENE:

5.1 LOS MÁS FRECUENTES: Ninguno
5.2 LOS MÁS GRAVES: Ninguno

6. SITUACIONES ESPECIALES QUE DEBEN SER TENIDAS EN CUENTA:
Ninguna
7. OTRAS INFORMACIONES DE INTERÉS (a considerar por el/la profesional)
Ninguna
8. OTRAS CUESTIONES PARA LAS QUE LE PEDIMOS SU CONSENTIMIENTO
Ninguna

ANEXO 5: Questionnaire of Life Style habits and Mediterranean Diet Adherence

ENCUESTA DE SALUD

Identificación: Sexo:

Peso: Talla: IMC:

Perímetro de la cintura:

Talla de pantalón:

FACTORES DE RIESGO CARDIOVASCULAR

Fumador A sí
B no
C Exfumador
Tiempo en años…………..

Medicamentos que habitualmente consume………

…….

Tiempo en años:……..

1. ¿Cuántos cigarrillos fuma al día??

A	Ninguno, Nunca fumo
B	Sólo fumo de forma ocasional
C	Menos de 5 cigarrillos al día
D	Entre 5-20 cigarrillos al día
E	Más de 20 cigarrillos al día

Tiempo en años…………

2. ¿Tiene el colesterol elevado?

A	Sí
B	No
C	No lo se

Tiempo en años……………

3. ¿Tiene elevados los triglicéridos?

A	Sí
B	No
C	No lo se

Tiempo en años……….

4. ¿Es diabético?

A	Sí
B	No
C	No lo se

Tiempo en años……….

5. ¿Tiene hipertensión?

A	Sí
B	No
C	No lo se

6. ¿Dedica un tiempo a realizar ejercicio físico, incluyendo actividades como caminar o subir y bajar escaleras?

A	Todos los días
B	4-5 veces por semana
C	2-3 veces por semana
D	No

7. Si realiza ejercicio físico ¿Cuántas horas al día hace ejercicio físico?

A	Más de 2 horas
B	2 horas
C	1 hora y media
D	Media hora
E	Menos de media hora

8. Cuanto tiempo al día dedica a ver la televisión

A	30 minutos o menos
B	Entre 1-2 horas
C	Entre 2-3 horas
D	Más de 3 horas

9. ¿Es vegetariano?

A	No
B	Si

10. ¿Consume suplementos nutricionales o vitamínicos?

A	No
B	Si
C	Ocasionalmente

11. ¿Qué estudios ha cursado?

A	Estudios primarios
B	Bachiller
C	Estudios universitarios de grado medio
D	Estudios universitarios de grado superior

ENCUESTA DE DIETA MEDITERRÁNEA

1. ¿Consume cereales (arroz, pasta, pan, galletas, cereales de desayuno, etc..…)?

A	Más de 7 raciones al día
B	5-7 raciones al día
C	3-4 raciones al día
D	1-2 raciones al día
E	Nunca

2. ¿Cuántas piezas de frutas consume al día?

A	Más de 6 raciones al día
B	5-6 raciones al día
C	3-4 raciones al día
D	1-2 raciones al día
E	Ninguna

3. ¿Cuántas veces a la semana consume legumbres?

A	Más de 4 veces a la semana
B	3-4 veces por semana
C	1-2 veces por semana
D	Menos de una vez a la semana

E	Nunca

4. ¿Cuántas veces consume verduras y hortalizas al día?

A	Más de 2 veces al día
B	2 veces al día
C	1 vez al día
D	Menos de una vez al día
E	Nunca

5. ¿Cuántas veces a la semana consume pescado?

A	Más de 4 veces a la semana
B	3-4 veces por semana
C	1-2 veces por semana
D	Menos de una vez a la semana
E	Nunca

6 ¿Cuántas veces a la semana consume carne de ave?

A	Más de 4 veces a la semana
B	3-4 veces por semana
C	1-2 veces por semana
D	Menos de una vez a la semana
E	Nunca

7. ¿Cuántas veces a la semana consume otro tipo de carne ?

A	Más de 4 veces a la semana
B	3-4 veces por semana
C	1-2 veces por semana
D	Menos de una vez a la semana
E	Nunca

8. ¿Cuántas veces al día consume leche y derivados lácteos?

A	Más de 4 raciones al día
B	3-4 raciones al día
C	1-2 raciones al día
D	Nunca

9. ¿Qué tipo de aceite consume habitualmente?

A	Aceite de oliva
B	Aceite de semillas
C	Aceite de oliva y de semillas

10. ¿Utiliza aceite de oliva para cocinar?

A	Diariamente
B	3-5 veces a la semana
C	1-2 veces a la semana
D	Menos de 1 vez a la semana
E	Nunca

11. ¿Consume normalmente frutos secos?

A	Más de 5 veces a la semana
B	3-4 veces a la semana Menos de una vez a la semana
C	1-2 veces a la semana
D	Menos de una vez a la semana
E	Nunca

12. ¿Cuántos huevos consume a la semana?

A	Más de 5 huevos a la semana
B	3-5 huevos a la semana
C	1-2 huevos a la semana
D	Menos de un huevo a la semana
E	Nunca

13. ¿Cuántas veces a la semana consume embutido?

A	Todos los días
B	3-5 veces por semana
C	1-2 veces por semana
D	Menos de una vez a la semana
E	Nunca

14. ¿ Consume normalmente vino tinto? (125 mL, un vasito de vino)

A	Más de 7 vasitos de vino a la semana
B	4-7 vasitos de vino a la semana
C	1-3 vasitos de vino a la semana

D	Menos de una vez a la semana
E	Nunca

15. ¿ Con que frecuencia consume productos de bollería industrial

A	Diariamente
B	3 o 4 veces a la semana
C	1-2 veces a la semana
D	Menos de una vez a la semana
E	Nunca

HÁBITOS ALIMENTARIOS

1. ¿Prefiere el pescado azul o el blanco?
   A El pescado azul
   B El pescado blanco
   C Ninguno

2. ¿Cuántas veces a la semana consume pescado azul?
   A Más de 4 veces a la semana
   B 3-4 veces por semana
   C 1-2 veces por semana
   D Menos de una vez a la semana
   E Nunca

3 ¿Cuántas veces a la semana consume carne roja?
   A Más de 4 veces a la semana
   B 3-4 veces por semana
   C 1-2 veces por semana
   D Menos de una vez a la semana
   E Nunca

4 ¿Consume más pescado que carne a lo largo de la semana?
   A Si
   B Igual
   C No

5. ¿Los derivados lácteos que consume son desnatados?
   A Nunca
   B A veces

C Siempre
D Son semidesnatados

6. ¿Consume normalmente café?
A Nunca
B Ocasionalmente
C 1-2 veces al día
D 3-4 veces al día
E Más de 4 veces al día

7. ¿Consumes normalmente cerveza?
A Nunca
B Menos de una vez a la semana
C 1-2 veces a la semana
D 3-4 veces a la semana
E Más de 4 veces al día

8. ¿Consume normalmente bebidas alcohólicas?
A Nunca
B Menos de una vez a la semana
C 1-2 veces a la semana
D 3-4 veces a la semana
E Más de 4 veces a la semana

9. ¿Cuántas veces a la semana consume verduras de hoja verde?
A Nunca
B Menos de una vez a la semana
C 1-2 veces por semana
D 3-4 veces por semana
E Más de 4 veces a la semana

10. ¿Cuántas veces a la semana consume tomate?
A Nunca
B Menos de una vez a la semana
C 1-2 veces por semana
D 3-4 veces por semana
E Más de 4 veces a la semana

11. ¿Cuántas veces a la semana consume pimiento rojo?
A Nunca
B Menos de una vez a la semana
C 1-2 veces por semana

D 3-4 veces por semana
E Más de 4 veces a la semana

12. ¿Ha realizado alguna vez alguna dieta para adelgazar?
A No
B Si

13. ¿Está realizando en este momento algún tipo de dieta?
A No
B Si

14. ¿Consume alimentos funcionales generalmente en su dieta?
A No
B Si
C Sólo de forma ocasional

ANNEX 6: Questionnaire of use of sweeteners

Cuestionario de Consumo de Edulcorantes No Calóricos

Sexo:
Edad:

1. ¿Sabe qué son los edulcorantes no calóricos como por ejemplo: aspartame, sucralosa, estevia, acesulfame K, sacarina o polialcoholes?
a. Si
b. No estoy muy seguro/a
c. No

2. ¿Utiliza edulcorantes no calóricos como sustituto de azúcar para endulzar sus alimentos o bebidas como café, té etc.?
a. Siempre
b. Con frecuencia
c. Ocasionalmente
d. Nunca

3. ¿Consume productos que contengan edulcorantes no calóricos, por ejemplo: bebidas "light", yogurts "light", mermeladas sin azúcar, chicles sin azúcar etc?
a. Siempre
b. Con frecuencia
c. Ocasionalmente
d. Nunca

4. ¿Cuántas veces al día consume edulcorantes como sustitutos del azúcar, para endulzar los alimentos?
a. Más de 6 veces al día
b. 3-4 veces al día
c. 1-2 veces al día
d. Nunca

5. ¿Cuántas veces a la semana consume bebidas light o edulcoradas?
a. Más de 4 veces a la semana
b. 3-4 veces por semana
c. 1-2 veces por semana
d. Nunca

6. ¿Cuántas veces a la semana consume yogures light o edulcorados?
 a. Más de 4 veces a la semana
 b. 3-4 veces por semana
 c. 1-2 veces por semana
 d. Nunca

7. ¿Cuántas veces a la semana consume mermeladas light o sin azúcar?
 a. Más de 4 veces a la semana
 b. 3-4 veces por semana
 c. 1-2 veces por semana
 d. Nunca

8. ¿Consume normalmente chicles o caramelos sin azúcar?
 a. Más de 4 veces al día
 b. 3-4 veces al día
 c. 1-2 veces al día
 d. Ocasionalmente

9. ¿Cuántas veces por semana consume galletas o bollería sin azúcar?
 a. Más de 4 veces a la semana
 b. 3-4 veces por semana
 c. 1-2 veces por semana
 d. Nunca

10. ¿Cuántas veces por semana consume postres edulcorados o sin azúcar?
 a. Más de 4 veces a la semana
 b. 3-4 veces por semana
 c. 1-2 veces por semana
 d. Nunca

11. ¿Consume productos para diabéticos generalmente en su dieta?

 a. Más de 4 veces a la semana
 b. 3-4 veces por semana
 c. 1-2 veces por semana
 d. Nunca

ANNEX 7: Organoleptic questionnaire

Fecha:

ENCUESTA ORGANOLÉPTICA

Indique cuál es su muestra y rodee la opción que considere más acorde en cada una de las dos escalas (p.e. "Extremadamente dulce" y "Me gusta muchísimo").

MUESTRA:

ESCALA DE SABOR	**ESCALA DE SATISFACIÓN**
Extremadamente dulce	Me gusta muchísimo
Demasiado dulce	Me gusta mucho
Muy dulce	Me gusta moderadamente
Dulce	Me gusta un poco
Ligeramente dulce	Me gusta muy poco
Ligeramente ácido	Me es indiferente
Ácido	Me disgusta un poco
Muy ácido	Me disgusta moderadamente
Demasiado ácido	Me disgusta mucho
Extremadamente ácido	Me disgusta muchísimo
COMENTARIOS:	